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THE UNIVERSITY OF ALBERTA

QUANTIFICATION OF ALLELOPATHY USING ALFALFA TISSUE CULTURE BIOASSAYS

BY

M. ALISON KILVERT

A thesis

submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Genetics

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The undersigned certify that they have read, and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled Quantification of Allelopathy Using Alfalfa Tissue Culture Bioassays, submitted by M. Alison Kilvert in partial fulfillment of the requirements for the degree of Master of Science.

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This thesis is dedicated to Alan and Lillian Kilvert for their love and support and to Gary Adams for his continual patience, encouragement, and help throughout this long endeavour.

ABSTRACT

The standard bioassays for quantifying allelopathy utilise germination and seedling extension measurements. However, the germination bioassay is not very sensitive, whereas the seedling extension bioassay is not very fast. Thus, the feasibility of using tissue culture techniques in the development of a simple, sensitive, and fast bioassay for allelopathy was studied.

The allelopathic effects of ferulic acid, *p*-coumaric acid and umbelliferone on the growth of alfalfa tissue cultures were quantified by fresh and dry weight measurements of callus and cell suspension cultures, respectively. To evaluate the veracity of the tissue culture bioassays for allelopathy, comparisons between the results of these bioassays and other commonly used bioassays were required. For this reason, alfalfa germination and extension bioassays were performed.

Even though the cell suspension bioassays were not amenable to statistical analysis, the callus, germination, and extension bioassays all exhibited significant differences in the varietal responses to the phytotoxins. As well, significant differences existed in the potency of the phytotoxins in inhibiting alfalfa growth or germination. The relative order of sensitivity of the alfalfa calli to the phytotoxins proved interesting; the least sensitive varieties were the wild isolates, whereas the most sensitive varieties were the cultivars. This result suggests that breeding of alfalfa may inadvertently select against allelopathic resistance. In fact, no phytotoxin-resistant callus line was achieved in calli initiated from alfalfa cultivars by the sequential selection of healthy calli exposed to progressively higher concentrations of phytotoxin.

Finally, a comparison between the *in vitro* and *in vivo* bioassays in quantifying allelopathy revealed that no correlation existed between bioassays in the relative order of sensitivity of the alfalfa varieties. Therefore, the callus bioassay is not a good method for the quantification of allelopathy as it does not reflect these *in vivo* growth responses.

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2,4-D	2,4-dichlorophenoxyacetic acid
ddH_2O	double-distilled water
DMR	Duncan's Multiple Range test
F	ferulic acid
GN	Genelle alfalfa
MF	*Medicago falcata* L.-127 alfalfa
MS	*Medicago sativa* L.-503 alfalfa
MS media or plates	Murashige and Skoog media or plates
P	*p*-coumaric acid
RG	Regen-S alfalfa
RM	Roamer alfalfa
SP	Spredor-2 alfalfa
U	umbelliferone

INTRODUCTION

Definition of Allelopathy

The term *allelopathy* was coined by Molisch in 1937. It was derived from the Greek words *allelon* and *pathos* which mean *of each other* and *to suffer*, respectively. Hence, the term allelopathy literally means *mutual suffering*. However, Molisch (cited in Mandava, 1985) defined allelopathy as "both detrimental and beneficial biochemical interactions among all classes of plants, including microorganisms." This broader definition including the beneficial biochemical interactions is applied because some allelochemicals which have a detrimental effect on the growth of some species at a certain concentration, also have a beneficial effect on the same or different species at a lower concentration.

The term allelopathy should not be confused with the term *competition*. Allelopathy involves a plant's release of a chemical into the environment which affects the growth of another plant. Competition, on the other hand, does not involve the addition of a chemical to the environment. Competition is when a plant depletes a factor from the environment that was necessary for the growth of another plant, to the point that the second plant's growth is reduced. Some confusion between these two terms arises when the term competition is incorrectly used for the term *interference*. Interference is a broader term which is defined as "all forms of reaction by one plant that prove deleterious to another" (Muller, 1969). This definition includes both allelopathy and competition.

History of Allelopathy

The science of allelopathy is rather young, but the history of allelopathy dates back before Christ. About 300 B.C., Theophrastus noticed that one plant can affect the growth

of another plant. He stated that "a peculiarity of chick-pea [*Cicer arietinum*] as compared with other leguminous plants is that ... it does not reinvigorate the ground, since it exhausts it; but it destroys weeds, and above all and soonest caltrop [*Tribulus terrestris*]".

In the first century A.D., Pliny (Plinius Secundus, C.) claimed that "chick-pea [*Cicer arietinum*], ... barley [*Hordeum vulgare*], fenugreek [*Trigonella foenum-graecum*], bitter vetch [*Vicia ervilia*], - these all scorch up a cornland". He also noted that shade of different trees have special properties and declared "that of walnut [*Juglans regia*] is heavy, and even causes headache in man and injury to anything planted in its vicinity; and that of the pinetree also kills grass; .... the shadow of a walnut tree or a stone pine or a spruce or a silver fir to touch any plant whatever is undoubtedly poison." Pliny realized that plants can affect one another by mechanisms other than competition. This is indicated in his statement that "the nature of some plants though not actually deadly is injurious owing to its blend of scents or of juice - for instance the radish and the laurel are harmful to the vine; for the vine can be inferred to possess a sense of smell, and to be affected by odours in a marvellous degree".

During the seventeenth century, Sir Thomas Browne reported that odours from one plant can affect another plant. He wrote in The Garden of Cyrus (originally published in 1658) that "the good and bad effluviums of Vegetables promote or debilitate each other" (Keynes, 1964). Also, in the latter half of the seventeenth century, Banzan Kumazawa noticed that "rain water or dew which comes washing the leaves of red-pine is harmful to crops standing thereunder" (Lee and Monsi, 1963).

In 1804, Young claimed "that clover was extremely apt to fail in districts where it has been cultivated constantly because the soil became sick of clover" (cited in Rice, 1984). Later, DeCandolle (1832) also noted that certain plant species can inhibit the growth of the same or other species. For example, he perceived that *Euphorbia* inhibited the growth of flax and thistles inhibited the growth of oats. It was suggested that the inhibition could be due to the release of toxic substances into the soil by plant roots. With this in mind,

DeCandolle proposed that crop rotation may be a way around soil sickness if each crop in the rotation is not inhibited by the preceding crop (cited in Bonner, 1950; Börner, 1960). This rotation of crops proved to be successful for clover soil sickness. Ambaston in 1845 reported a vigorous cropping of clover when a seven or eight year interval between clover crops was employed in the crop rotations.

Another case of root exudates affecting a plant's growth was brought forward by Beobachter in 1845. He suggested that root excrements from the Heath plant formed "a hard black stratum a few inches below the surface, impervious to water, and equally impervious to the roots of trees." He recommended that this stratum be pierced through or else trees planted in this soil would not thrive.

In 1881, Stickney and Hoy supported Banzan Kumazawa's observations that toxic rain or dew washings from trees can hinder growth of crops underneath. They noticed that vegetation was very sparse under black walnut trees (*Juglans nigra*). Moreover, Hoy attributed this effect to the poisonous nature of walnut tree water drippings.

DeCandolle's theory that soil infertility could be due to toxic exudates was not widely accepted. It was not until more evidence was produced by Livingston in the early twentieth century that this toxic theory gained more acceptance. In 1904 and 1905, he published on the analysis of the physical properties of bog water. Previous recordings indicated that plants growing in these peat bogs exhibited similar structural characteristics to plants growing in very dry soils (xerophytes). Livingston designed an experiment to measure the growth of a small green alga (*Stigeoclonium*) in these bog waters. From this experiment, he concluded that there are chemical substances in some bog waters that affect *Stigeoclonium* growth. Furthermore, these substances have an affect similar to toxins and they also may be involved in the inhibition of non-xerophilous plants in peat bogs.

Next, Livingston (1905, 1907) studied the properties of unproductive soils. From a series of experiments involving the growth of wheat seedlings in unproductive soil extracts, Livingston concluded that toxic substances exist in these soils.

Further and still more convincing evidence supporting DeCandolle's toxic theory emerged during the experiments of Schreiner and Reed (1908). These scientists conducted experiments similar to those of Livingston (1907). The growth of wheat seedlings in water cultures containing compounds, which often occur naturally in vegetable tissues, was measured. These compounds were amino acids, lecithin-related compounds, urea derivatives, pyridin derivatives, phenols, oxybenzene derivatives and some miscellaneous compounds. These scientists determined that a number of compounds which occur naturally in vegetables are toxic to the growth of wheat seedlings.

Later, Schreiner and Lathrop (1911) examined the organic constituents in both poor and good soils which were collected in the United States. They found that 51% of the poor soils contained dihydroxystearic acid and only 4% of the good soils contained this acid. Furthermore, the acid-bearing good soils were barely fertile while all of the infertile poor soils showed appreciable amounts of this acid. From this evidence, they concluded that dihydroxystearic acid is either a direct or an in direct factor in the cause of soil infertility.

Pickering (1917) continued along this line of experimentation. He demonstrated that the leachings of one crop of mustard can have a deleterious effect on the growth of another crop of mustard. He accomplished this by setting up staircase experiments where the washings that percolated through one crop of mustard were allowed to water the second mustard crop. These experiments were directly associated with his earlier experiments involving the effect of grass on apple trees (Pickering, 1903).

Opposition to these early studies on soil toxicity arose because the postulated toxins had not been isolated, identified, and shown to be liberated from roots. Also, some scientists did not appreciate the use of living plants as an indirect method for indicating the physiological properties of the soil. This viewpoint was expressed by Loehwing in 1937. In his review of the literature up to that time, Loehwing concluded that soil sickness was probably due to disturbed nitrogen nutrition or other nutritional impoverishments rather than toxic root excretions.

In the same year Molisch published his paper concerning the allelopathic effect of ethylene on higher plants. This sparked more interest in allelopathy. With the advent of new chemical techniques, such as paper chromatography, more effort was given to the identification and determination of the phytotoxic activity of compounds liberated from higher plants.

Proof of Allelopathy

Much confusion exists in the literature because of the readiness of scientists to designate an interference effect as competition and also by using these terms synonymously. The problem arises from the fact that the visible symptoms resulting from competition and allelopathy are similar. This may be caused by both types of interference invoking a similar stress response in the target plant. In order to distinguish allelopathy from competition, competitive effects must be minimized or eliminated in the experimental design and analysis of the chemical involved in the allelopathic effect must be performed.

A proposed proof of allelopathic versus competitive interference was suggested by Fuerst and Putnam (1983). They suggested that in order to unequivocally prove that an interference is due to an allelochemical, this toxin should first be isolated, assayed and characterized. Synthesis of the toxin should be performed next so that it may be used to simulate the interference symptoms in the absence of the donor plant. This simulated toxin should be used at concentrations found in nature. Finally, the toxin should be quantified to determine if it is produced in sufficient concentrations to cause the interference symptoms. This should involve monitoring the release of the toxin from the donor plant into the environment. The subsequent toxin movement from the donor plant to the recipient plant should also be monitored along with the uptake of the toxin by the recipient plant.

These criteria are suitable to prove the allelopathic potential of a chemical. However, two important factors should also be considered in this evaluation, namely

osmotic potential and synergistic effects of the chemical. For example, if the chemical or extract being assayed is dissolved in water then the control should be adjusted to the same osmotic pressure with an nontoxic osmoticant like sucrose. This is to ensure that the allelopathic effect is actually due to the chemical and not to osmotic stress. Additionally, the synergistic effects of the assayed chemical with other chemicals present in the same environment need to be determined because these effects lower the physiological threshold concentration of the assayed chemical. This last factor is important in determining if a chemical is physiologically active.

It is immediately evident from the previously mentioned criteria, that numerous experiments need to be performed to prove that an interference is indeed allelopathic. Due to the complexities of these experiments, most, if not all scientists have not performed all the suggested experiments. The result is that for most cases of interference, allelopathy is postulated but not adequately proven to occur.

Allelopathic Effects and Examples

Allelopathy has been postulated to occur in a variety of situations. One of the most commonly recorded instances of alleged allelopathy occurs in agriculture. There are numerous reports on the effects weeds have on crop plants. For example, Schumacher, Thill, and Lee (1983) determined that wild oat (*Avena fatua*) root exudates suppress the growth of bread wheat (*Triticum aestivum*) plants. Growth inhibitions of soyabean (*Glycine max*) and cotton (*Gossypium hirsutum*) plants have also been shown to occur (Bhowmik & Doll, 1982) when these crops were watered with a solution containing velvetleaf (*Abutilon theophrasti*) residues.

Publications based on the alleged allelopathic effects crop plants have on weeds are not as numerous as the previously mentioned examples. Liebl and Worsham (1983) noticed that morning glory (*Ipomoea lacunosa* L.) populations were reduced in no-till

wheat fields. With the idea that allelopathy might be involved in reducing the morning glory population, these scientists measured the percent germination and root length of morning glory seedlings germinated in aqueous extracts of wheat plants. Both germination and root growth were inhibited.

Strong evidence of allelopathic activity is indicated in rye (*Secale cereale* L.). Barnes (cited in Barnes and Putnam, 1986) found that if winter rye is planted in the spring it can reduce the density of early season weeds such as common lambsquarters (*Chenopodium album* L.), large crabgrass (*Digitaria sanguinalis* L.), and common ragweed (*Ambrosia artemisiifolia* L.). Following up on this possibility of allelopathy, Barnes and Putnam conducted a series of experiments designed to measure the effect of rye on barnyardgrass (*Echinochloa crus-galli* L.) growth. In one of these experiments, barnyardgrass seeds were germinated in a petri dish that contained soil with killed rye residues. Although the germination of these seeds was not significantly inhibited, the root growth of the resulting seedlings was reduced significantly.

An equally important problem in agriculture, besides the inhibitory effect some weeds have on crop growth, is the inhibitory effect some crops have on the growth of succeeding crops. In the previously mentioned experiments (Barnes & Putnam, 1986), rye residues were also shown to inhibit the root growth of lettuce (*Lactuca sativa* L.) and cress (*Lepidium sativum* L.) seedlings. Varieties of sorghums have been known for quite some time (Conrad, 1927) to be inhibitory to the growth of succeeding small grain crops. Weston, Harmon, and Mueller (1989) demonstrated that tomato (*Lycopersicon esculentum*) and cress have a significantly smaller radicle length when grown in petri dishes containing soil and sudex (sorghum sudangrass hybrid) seedling shoot tissue residues.

One of the reasons that crop rotations are employed is that autotoxicity resulting from the accumulation of some crop exudates exists in some long term monocultures. An example of this is the soil sickness problem of clover crops. This form of allelopathy is also seen in replant problems of fruit trees. Peach trees do not grow vigorously in sites

where old peach trees have been removed (Koch, 1955). In a series of experiments conducted by Patrick (1955), it was demonstrated that toxins are produced from the degradation of peach root residues by certain microorganisms occurring in old peach orchard soils. These toxins inhibited respiration of excised peach root tips. Other effects were browning of root apical meristems, drying of leaves, and wilting of peach seedlings.

Allelopathy is also evident in the patterning of vegetation and plant succession. One of the most striking examples of this is the bare zones of grassland inhibition surrounding colonies of *Salvia leucophylla* and *Artemisia californica* in the California Chaparral (Muller, Muller, & Haines, 1964). This zone of inhibition usually extends 60 cm to 90 cm beyond the colonies. Beyond this zone, a differential inhibition zone of annual grasses and forbs occurs for 2 m to 9 m. This consists of some species of stunted grasses and forbs near the bare soil to unaffected grassland near the outer edge of the zone. Allelopathy, *via* volatile inhibitors, is suspected to play a role in this patterning. Muller (1966), used gas chromatography to identify volatile terpenes liberated from *Salvia.* These inhibited the germination of various grass seeds when only aerial contact was possible between the two plant species contained in a storage dish. Autotoxicity also appears to play a role in the old colonies of *Salvia* or *Artemisia* because there exists areas of bare, eroded soil in the interiors of some old colonies. Germination of *Salvia* or *Artemisia* seeds rarely occurs in these bare interiors but these seeds do germinate in the bare zone of inhibition surrounding the colonies. Hence, *Salvia* and *Artemisia* are gradually invading the grassland resulting in a plant succession. These shrubs will eventually succumb to autotoxicity in the interiors of the colonies. What is next in this line of succession is not known.

Aquatic ecosystems can also be affected by allelopathy. It has been suggested that the dominance and succession that occurs in algal blooms is due to allelopathy. Some evidence supporting this proposal resulted from studying the effects of dominant algal filtrates on their predecessors and successors in the bloom sequence *in vitro* and from monitoring the algal blooms *in situ* (Keating, 1977). The correlations between these two

studies indicated allelopathy as a major factor in controlling bloom sequence. *In vitro*, filtrates of dominant species were found to be inhibitory or neutral to the growth of their predecessors and neutral or stimulatory to the growth of their successors in the bloom sequence. Additionally, water from algal blooms *in situ* was used as a filtrate source in these bioassays. The results obtained from these bioassays correlated well with the previously mentioned *in vitro* results and with the observed bloom sequence *in situ*.

Another interesting plant pattern is that obtained from black mustard (*Brassica nigra*) invading the grasslands of coastal southern California. At the first rainfall of late autumn, the seeds of black mustard and the surrounding grassland simultaneously germinate. However, black mustard seedlings predominantly germinate in old black mustard sites and grass seeds germinate in old grass sites. This provides a pattern that can be seen year after year as the black mustard seedlings grow among the debris of the previous mustard growth. Bell and Muller (1973) studied this phenomenon. Based on their findings, they concluded that leachates from dead stalks and leaves of black mustard significantly inhibited the germination and radicle growth of a neighbouring annual grass (*Bromus rigidus*) when seeds were germinated on sponges containing the leachates. Concentrated black mustard stalk drip collected in the field also gave similar inhibitions in radicle growth of the grasses: *Bromus rigidus*, *Bromus mollis* and *Avena fatua*. Also the germination, radicle and epicotyl growth of *Avena fatua* was significantly inhibited when seeds were germinated in soil collected from the field after the first rainfall. This supported the theory of phytotoxic leachates from black mustard repressing invasion of grassland.

Allelopathy has also been implicated in forest regeneration failures. In the Allegheny Plateau in northwestern Pennsylvania, the virgin forest consisting of hemlock (*Tsuga canadensis* L.), beech (*Fagus grandifolia* Ehrh.), and beech-maple (*Acer saccharum* Marsh.) was almost completely clear-cut around 1900. A secondary succession consisting of black cherry (*Prunus serotina* Ehrh.), red and sugar maple, and white ash (*Fraxinus americana* L.) developed in some areas. However, other areas with poorly drained soils

failed to regenerate forests. Horsley (1977) attributed this to the allelopathic effect of the dense ground cover of bracken fern (*Pteridium aquilinum* L.), wild oat grass (*Danthonia compressa* Aust.), goldenrod (*Solidago rugosa* Ait.), and flat-topped aster (*Aster umbellatus* Mill.) on the tree seeds. In a series of experiments, Horsley showed that black cherry seed germination was significantly inhibited when these seeds were allowed to germinate on peat watered with 5% to 100% fern, goldenrod or aster foliage extracts. Aster foliage extracts (100%) also inhibited shoot growth and reduced the number of first-order lateral roots of black cherry seedlings grown for 17 days in sand. In addition, root washings of goldenrod and aster supplied in a staircase arrangement reduced black cherry seedling growth.

Allelopathy also can occur between different trees in a forest. On well drained soils red pine (*Pinus resinosa* Ait.) and white pine (*Pinus strobus* L.) often inhibited black walnut (*Juglans nigra* L.) growth. However, on poorly drained soils, the opposite effect was seen (Fisher, 1978). The percent survival, height, and diameter of 22- to 25-year old pine trees were significantly inhibited on poorly drained soils when adjacent walnut trees were present. These parameters were not significantly affected when the pine trees were growing alone in the poorly drained soils. Since juglone (5-hydroxy-1,4-naphthoquinone) is a principle phytotoxin produced by black walnut trees, its impact on the environment was studied. Juglone (50 ppm) had greater inhibiting effects on radicle extension of red pine seedlings growing in wet moisture regime soil than in dry moisture regime soil. Radicle extension was still inhibited at 90 days growing in the wet moisture regime soil. However, the activity of juglone did not last 45 days in the dry moisture regime soil.

The allelopathic effects of black walnut are not limited to trees; many plants are affected. Juglone at concentrations of 10^{-3} M have been shown (Kessler, 1989) to inhibit the growth of five species of chlorophytes *in vitro*. At 10^{-4} M, only three species of these freshwater algae were inhibited.

Even plant pathology has an allelopathic component (Bell, 1977). Development

and morphogenesis of pathogens is one area where allelopathy plays a role. For example, many parasitic fungal spores produce fungistatic inhibitory substances at the site of spore production. These substances inhibit the germination of the producing and adjacent spores. This mechanism of self-inhibition helps to ensure the dispersal of viable, ungerminated spores. It is of interest to note that these substances are structurally related to residues or leachates of plants that cause inhibition of seed germination.

Nonhost organisms can also produce allelochemicals that antagonize pathogens. For example, cotton produces strigol. This allelochemical stimulates the germination of parasitic witchweed (*Striga lutea*) seeds. However, since cotton is not a host for witchweed, the parasite dies.

A host plant often uses allelochemicals to obtain resistance to a disease. These allelochemicals can be produced constitutively or produced after an injury. If the latter wound-induced compounds are newly synthesised antibiotics, they are called phytoalexins. The effects of phytoalexins were demonstrated when potato slices were inoculated with avirulent races of *Phytophthora infestans*. Two days post inoculation, these potato slices showed resistance to virulent races.

A final involvement of allelopathy in plant pathology is in the development of disease symptoms. A pathogen can produce an allelochemical that may cause the development of disease symptoms in the target plant. This may take the form of chlorosis, necrosis, and wilting.

Different phases of the nitrogen cycle are also influenced by allelopathy. These phases include both nitrogen fixation and nitrification. Nitrogen fixation can be inhibited by allelochemicals affecting the growth of symbiotic or asymbiotic nitrogen fixers. Similarly, nitrification can also be inhibited by affecting the growth of the organisms involved. The two principle organisms are *Nitrosomonas*, which oxidizes ammonium to nitrite, and *Nitrobacter*, which further oxidizes nitrite to nitrate. Many plant extracts have been shown to affect the growth of these organisms. Furthermore, it has been suggested

that the slow succession in old fields may be due to pioneer plants inhibiting these nitrogen-fixing and nitrifying bacteria.

Rice, Penfound, and Rohrbaugh (1960) determined that the nitrogen requirement of triple awn grass (*Aristida oligantha*), little bluestem (*Andropogon scoparius*), and switch grass (*Panicum virgatum*) increased respectively. This correlated with the successive order of grasses occurring in abandoned fields in Oklahoma. Later, Rice (1964) studied the effects of plant extracts on the growth of *Azotobacter*, *Rhizobium*, *Nitrobacter*, and *Nitrosomonas*. Virtually all species of the pioneer weed stage inhibited the growth of the nitrogen-fixing and nitrifying bacteria. Extracts from the dominant plant of the second successional stage, gave similar results. However, extracts from plants of the climax and third successional stage only inhibited the nitrifying bacteria. It has been suggested (Rice, 1984) that inhibition of nitrification may help to conserve nitrogen by preventing the formation of nitrate which may be subsequently leached from the rhizosphere. Hence, these climax plants may be using ammonium as a nitrogen source.

Prevention of seed decay may also be influenced by allelopathy. Seeds may exude chemicals that inhibit the growth of microorganisms involved in decomposition. This form of prevention of seed decay could enable a seed to lie dormant for many years before the conditions are favorable for germination. Ferenczy (1956) demonstrated the existence of this exudation by using the agar diffusion method. When certain intact seeds were placed into the agar, a zone of bacterial growth inhibition was observed.

Allelochemicals and Their Production

Many different chemicals have been identified as allelopathic agents. Most of these chemicals are termed secondary compounds because they are not required for the normal growth and development that occurs in all plants. Rice (1984) has classified these allelochemicals into fifteen different categories. One example of each category is given in

Table 1.

These allelochemicals are unevenly distributed in virtually all tissues of higher plants, including roots, rhizomes, stems, leaves, flowers, fruits, and seeds (Putnam, 1985). They are synthesised primarily through the acetate or shikimate pathways. Some compounds are derived from both of these pathways; these include the flavonoids and their derivatives, the condensed tannins.

Many factors can alter the production of allelochemicals (Rice, 1984). Some of these factors can even act in an additive or synergistic manner. These factors include the quality, intensity, and length of light as well as changes in temperature, and the type and age of the allelochemical-producing plant organ. It has been noticed that many types of stresses can increase this production. Mineral deficiencies (boron, calcium, magnesium, nitrogen, phosphorous, potassium, and sulfur) cause the production of allelochemicals to increase in a plant growing in these infertile soils. An increase in production is also seen in plants exposed to water stress or stress imposed by commercial herbicidal agents. In fact, stress caused by an infection can increase allelochemical production. This may enhance resistance to the pathogen or predator. The last factor, but certainly not the least important, is the role genetics plays in allelochemical production. Genotypic variation exists in many crop accessions and cultivars. This results in a range of allelochemical production levels.

Transfer of Allelochemicals and Their Mechanisms of Action

Allelochemicals are usually stored in a nontoxic bound form in plant cells (Mandava, 1985). They may take the form of water-soluble glycosides, salts, and polymers such as tannins and lignins. Enzymatic cleavage of these bound forms or exposure to environmental stresses has been suggested (Einhellig, 1985) to initiate the release of these toxic chemicals into the environment. Once released, these allelochemicals must escape from the donor or producing plant and reach the target plant. Many modes of

escape are possible depending on the allelochemical involved (Rice, 1984). Some allelochemicals are volatilized while others are released from decomposing plant residues, exuded from roots, or leached from leaves, stems, and seeds by rain, dew, mist, or fog.

Most of the liberated chemicals (Table 1) are active allelochemicals. However, some compounds must be modified or degraded by microorganisms to become active. For example, when amygdalin is degraded, HCN and benzaldehyde are produced. Both of these products inhibit the growth of peach seedlings (Patrick, 1955). Modification of allelochemicals may also occur by other means. Apparently allelochemicals in *Eucalyptus* leaves may be modified in the guts of herbivorous insects (Silander, Trenbath, & Fox, 1983) and koalas (Eberhard, McNamara, Pearse, & Southwell, 1975) resulting in the enhancement of the allelopathic effects. It is thought that these modified allelochemicals may be partially responsible for the bare zones beneath *Eucalyptus* canopies.

When these allelochemicals reach the target plant, they must be of sufficient concentration or accumulate to this concentration in order to be physiologically active in the target plant. When these allelochemicals are taken up in a sufficient concentration, many factors at the cellular level may be affected (Einhellig, 1986). The regulation of growth by allelochemicals may be altered by affecting cell division, elongation, protein synthesis, phytohormones levels, and enzyme synthesis or function. Respiratory metabolism may be altered by interfering with mitochondrial functions including oxygen uptake, oxidative phosphorylation, and ATP production. Stomatal closure, chlorophyll loss, and inhibition of enzymes may directly or indirectly affect photosynthesis of the target plant. Another factor that may be affected is mineral ion uptake. For example, this may take the form of altered absorption of phosphate, potassium, calcium, nitrogen, and magnesium ions. Depolarization of the cell membranes or increased membrane permeability may cause this altered mineral ion uptake, which may in turn cause reductions in water potential. This reduction may indirectly reduce the growth of the target plant. Essentially all allelochemicals directly or indirectly affect seed germination or plant growth with many

allelochemicals affecting both of these processes.

Mechanisms of Action for Selected Allelochemicals

The allelochemicals (phytotoxins) selected for this study consisted of two cinnamic acid derivatives (ferulic acid and *p*-coumaric acid) and one coumarin (umbelliferone) according to Rice's classifications in Table 1 (Rice, 1984). These three phytotoxins are also complex phenolic compounds (Figure 1). Other major plant phenolic compounds include some flavonoids, quinones, tannins, and lignins. The biosynthesis and metabolism of phenolic compounds have been extensively studied (Harborne, 1980). However, the function of phenolic compounds in plants is unclear.

Generally, phenolics have an inhibitory effect on plant growth. Ferulic acid, *p*-coumaric acid, and umbelliferone display this effect. For example, these phytotoxins have been shown to inhibit seed germination, radicle elongation and seedling growth in a variety of plant species (Van Sumere, Cottenie, De Greef, & Kint, 1972; Jankay & Muller, 1976; Williams & Hoagland, 1982; Einhellig, Schon, & Rasmussen, 1982). Additionally, synergistic inhibitory effects on sorghum seed germination and growth have been noted between ferulic acid and *p*-coumaric acid (Rasmussen & Einhellig, 1977; Einhellig, Schon, & Rasmussen, 1982). However, cellular mechanisms contributing to these effects are poorly understood.

All three phytotoxins inhibit photosynthesis and respiration when exogenously applied but not necessarily by the same mechanisms. There is evidence indicating that they can affect photosynthesis by causing a reduction in the chlorophyll content (Einhellig & Rasmussen, 1979; Toro, Leather, & Einhellig, 1988). More specifically, ferulic acid and umbelliferone are inhibitors of the ATP-generating pathway and, to a lesser extent, the electron transport pathway in spinach chloroplasts. As well, both phytotoxins inhibited electron transport in mung bean mitochondria (Moreland & Novitzky, 1987). Inhibition of

respiration in mung bean mitochondria was also noted with *p*-coumaric acid (Demos, Woolwine, Wilson, & McMillan, 1975). This inhibition was accompanied by a reduction of hypocotyl growth, a release of respiratory control and a reduction of Ca^{2+} uptake.

Ferulic acid can also cause altered ion absorption. In excised soyabean roots, it has been shown to inhibit phosphate absorption (McClure, Gross, & Jackson, 1978). It also can affect the accumulation and incorporation of phenylalanine in barley and lettuce seeds or seed embryos (Van Sumere *et al.*, 1972). From the latter observation, it was proposed that ferulic acid may act as a germination inhibitor by restricting the transport of amino acids and the formation of proteins in seeds. Another proposed cause of growth inhibition by ferulic acid as well as *p*-coumaric acid is their stimulatory effect on the degradation of the phytohormone indole acetic acid. Both of these phytotoxins increase the rate of indole acetic acid decarboxylation, thereby inhibiting elongation by this auxin *in vitro* (Zenk & Müller, 1963; Lee, Starratt, & Jevnikar, 1982).

Allelopathic Exploitation and Bioassays

Considering that the majority of allelochemicals inhibit seed germination or growth of a target plant, there are many ways to potentially exploit these chemicals, particularly in agriculture (Putnam & Duke, 1978). Originally crop plants with allelopathic activity were avoided in crop rotations. Nevertheless, allelopathic crop plants can be exploited for their ability to inhibit weed seed germination and growth. An allelopathic crop may be used in a rotation or as a companion crop to inhibit weed growth, providing there are no deleterious effects to the other crops. Allelopathic plants may also be used as mulches. Another possible use of allelopathic plants is in the aquatic environment, for instance, in the management of aquatic weeds. Although the majority of allelopathic plants studied have an inhibitory effect on the target plant, some allelopathic plants have a stimulatory effect. This may be utilised in mixed cultures when grown as a companion crop.

Applying allelochemicals to plants or soil has been suggested as another method of exploiting allelopathy. This may be used as a mechanism for controlling weed seed germination, increasing weed seed decay and controlling plant diseases and pests. However, in order to exploit allelopathy, bioassays must be performed to evaluate the allelopathic potential of a chemical. Also the target specificity of this allelochemical must be determined.

Before a bioassay can be performed, the suspected allelopathic compounds must be extracted from the donor plant (Putnam & Duke, 1978). This may involve extraction from dried or live-diced plant material in cold or hot water or in organic solvents. Extraction of compounds from intact plant may also be performed. Volatile compounds can be collected in a gas trap and stem or leaf leachates can be collected after the simulation of rain or fog drip. Exudates from intact roots can also be collected after leaching these compounds out of the growth matrix.

After collection of the chemical, its allelopathic potential can be determined in a bioassay. There are many types of bioassays that can be performed (Leather & Einhellig, 1985). The most frequently used bioassay is to test the effect of the chemical on seed germination of the target plant. Other bioassays are designed to demonstrate the effect of a chemical on the target plant's growth and development. Measurements might include shoot or root extensions, radicle elongations, chlorophyll or anthocyanin content, and fresh or dry weight of specific plant tissues.

Another method for performing a bioassay without extracting the suspected allelochemical is to use both the donor and target plant in the same experiment. This may involve a staircase system where the root exudates of the donor plant are used to water the target plant. Intact plants may also be employed to study the allelopathic potential of volatile chemicals. In this situation a system can be arranged such that only aerial contact exists in a sealed container between the donor and target plant. In both systems involving intact plants, the allelopathic potential of the donor plant can be determined by the

previously mentioned bioassays.

Tissue Culture Bioassays and Variability

Tissue culture bioassays may provide an alternative to the conventional bioassays commonly employed. Conceivably, tissue cultures could be used to evaluate the allelopathic potential of a phytotoxin in a similar fashion to the preliminary evaluation of drugs using animal cell cultures.

Tissue culture offers some potential advantages over conventional bioassays (Zilkah & Gressel, 1977a). It is an axenic culture consisting of a large proportion of metabolically active cells. Hence, it should generate fairly rapid measurements of phytotoxicity. It is also thought (Zilkah, Bocion, & Gressel, 1977) that these measurements should reflect the sensitivity of the whole plant due to the totipotency of tissue culture cells. However, with the absence of a cuticle in tissue culture, allelochemical penetration and translocation problems are virtually nonexistent. Therefore, both *in vitro* and *in vivo* bioassays may be needed for identification of compounds which are active at a cellular level but not at the plant level.

Other differences exist between cells in tissue culture and cells *in vivo*. In a plant, callus formation is a wounding response. For example, if a branch is cut off a tree, callus tissue may form at the site of wounding. Eventually, the callus tissue may cover the exposed site, thereby protecting the tree from infection. This callus tissue is formed by the onset of meristematic activity in parenchyma cells adjacent to the wound site. The resulting callus is an amorphous mass consisting of large, loosely arranged parenchymatous cells.

In tissue culture, callus forms at the wound or cut surface of the explant with the aid of auxins and cytokinins. This callus will exhibit variations in morphology and metabolism. Some of this variation will be due to the heterogeneity of cell types within the explant. Additional heterogeneity can have a genetic basis. It is possible for some cells of

the explant to be polyploid. These kinds of genetic changes may be retained in the tissue culture from the explant. Another source of variation can arise in tissue culture. These variations when retained in regenerated plants have been labeled as somaclonal variations (Larkin & Scowcroft, 1981). Their presence in somatic cells demonstrates that mitosis is not as conservative a process as once thought.

Types of genetic changes that can occur in tissue culture include chromosome number changes (euploidy and aneuploidy), chromosome structure rearrangements (duplications, deletions, inversions and translocations), single gene mutations (point mutations), deamplification and amplification of genes, and mobilization of transposable elements (Gould, 1986; Larkin, 1987). Epigenetic changes may include cytokinin habituation and drug or stress resistance. However, this is a somewhat ambiguous category because "many changes whose genetic basis is unknown end up in the epigenetic category" (Karp & Bright, 1985).

Surprisingly, some of these changes only occur in tissue culture and several of these changes occur at a higher frequency than that expected based on classical mutagen data (Gavazzi *et al.*, 1987). However, if the change is not conducive to a rapid cell division as expressed by the calli or cell suspension cultures, the heterogeneity generated from these genetic and epigenetic changes may only constitute a small proportion of the total cell population.

Probably, a major source of variation expressed in tissue culture bioassays originates from the heterogeneity in the growth rates from individual callus samples. Calli naturally have nodules of high meristematic activity. Hence, calli do not naturally express a uniform growth rate. Alternatively, cell suspension cultures are more homogeneous in their growth rates than calli cultures. Suspension cultures consist of single cells and cell clusters. Depending on the species and the culture conditions, the suspensions can range from fine or relatively single-celled cultures to cultures having a large proportion of cell clusters. Therefore, fine cell suspensions would be more amenable for use in

allelochemical bioassays.

Research Project

The goal of this project was to determine if allelopathy could be quantified using tissue culture techniques. It was also of interest to document any variation in sensitivity between the different varieties of the target species to specific phytotoxins. If genetic variation exists, it may be possible to genetically manipulate chosen varieties to become resistant to certain allelochemicals.

This project was also intended to ascertain whether selection for alfalfa tissue cultures resistant to phytotoxins was feasible. Furthermore, it was of importance to determine if resistance obtained through somaclonal variation could be retained in the regenerated plants and their progeny. This could prove to be an important technique for obtaining plants resistant to allelochemicals. These regenerants may have a selective advantage over sensitive plants because weeds exuding these allelochemicals may not be able to inhibit the growth of these resistant plants. Finally, if resistance to one allelochemical is retained, it can be determined if cross-reactivity exists to the other allelochemicals in the resistant tissue culture, regenerants, or their progeny. This may give insight into the allelochemical metabolism, especially if these chemicals are degraded by a common pathway. This also may reveal whether additive or synergistic effects exist between these allelochemicals.

The allelochemicals or phytotoxins utilised in this research project were ferulic acid, *p*-coumaric acid and umbelliferone (Figure 1). These allelochemicals are synthesised (Figure 2) *via* the shikimate pathway (Floss, 1986; Brown, 1979) with ferulic acid and *p*-coumaric acid directly involved in lignin biosynthesis (Higuchi, 1985). They are also widely distributed in nature, commercially available, and fairly soluble in aqueous solutions. One other potentially useful allelochemical was esculetin (Figure 1), however,

its solubility in aqueous solutions is limited to low concentrations. For this reason this allelochemical was not included in this project. Scopoletin (Figure 1) was also disregarded because it is expensive.

Alfalfa was chosen as the target plant species for the bioassays. It is an important and widely studied forage crop which shows strong autotoxicity. Hence, it may already have genes involved in the detoxification of the allelochemicals produced. Unfortunately, the allelochemicals involved have not been identified. It is known (Hall & Henderlong, 1989), however, that the allelochemicals involved are water-soluble and have phenolic characteristics. Alfalfa was chosen as the target plant because of its previously mentioned characteristics and also because alfalfa grows well in tissue cultures. Additionally, these tissue culture procedures are well defined. From many different alfalfa varieties, six were chosen that were easily handled in tissue culture. Two of these six varieties were wild isolates. These were included to determine if any variation in sensitivity to the allelochemicals exist between the cultivars and the wild isolates.

Alfalfa tissue cultures were initiated for the measurement of the allelopathic effects on alfalfa growth. In calli cultures, fresh weight was the chosen measurement to quantify growth, whereas in cell suspension cultures dry weight was chosen. To evaluate the efficiency of the tissue culture bioassays for allelopathy, comparisons were necessary between the results of these bioassays and other commonly used bioassays. For this reason, alfalfa germination and extension bioassays were performed. The phytotoxins selected may not necessarily affect both of these *in vivo* processes. Thus, the final goal of this project was to determine if a correlation exists between the *in vitro* and *in vivo* bioassays and hence, to determine if tissue culture bioassays are a good method for the quantification of allelopathy.

Table 1 Classes of Allelopathic Agents

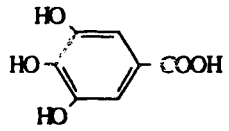
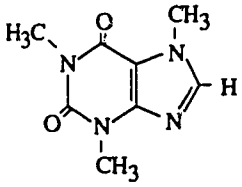
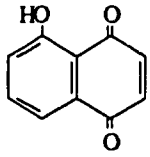
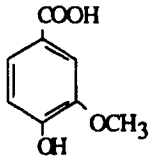
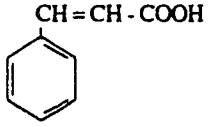
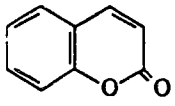
Class	Example		
	Structural Formula	Chemical Name	Natural Source
1. Simple water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, and ketones	$CH_3 - CH_2 - OH$	ethanol	Tomato plants (volatile)
2. Tannins		gallic acid	Oak trees (tannin hydrolysis product)
3. Purines and nucleosides		caffeine	Coffee plants
4. Naphthoquinones, anthraquinones, and complex quinones		juglone	Black walnut trees
5. Simple phenols, benzoic acid, and derivatives		vanillic acid	Corn plants
6. Cinnamic acid and derivatives		cinnamic acid	Guayule roots
7. Coumarins		coumarin	*Melilotus alba*

Table 1 Continued

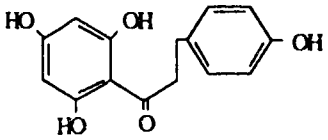
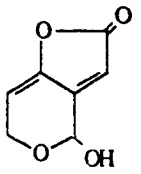
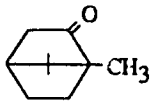
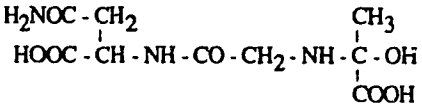
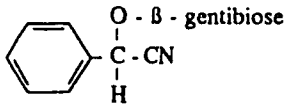
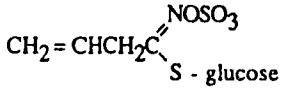
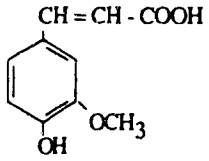
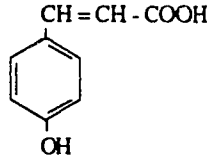
Class	Example		
	Structural Formula	Chemical Name	Natural Source
8. Flavonoids		phloretin	Decomposing apple roots
9. Simple unsaturated lactones		patulin	*Penicillium* fungus when grown on wheat straw
10. Terpenoids and steroids		camphor	*Salvia* shrubs (volatile)
11. Amino acids and polypeptides		lycomarasmin	*Fusarium oxysporum* f. *lycopersicum*
12. Alkaloids and cyanohydrins		amygdalin	Decomposing peach roots
13. Sulfides and mustard oil glycosides		sinigrin	Black mustard plants
14. Long-chain fatty acids and polyacetylenes	$CH_3(CH_2)_{16}COOH$	stearic acid	Decomposing *Polygonum aviculare*
15. Miscellaneous	$CH_2=CH_2$	ethylene	Apple fruits (volatile)

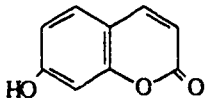
Figure 1 Allelopathic Agents



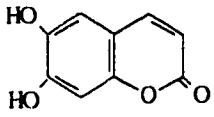
Ferulic Acid



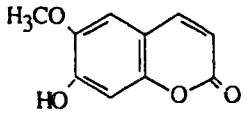
p-Coumaric Acid



Umbelliferone

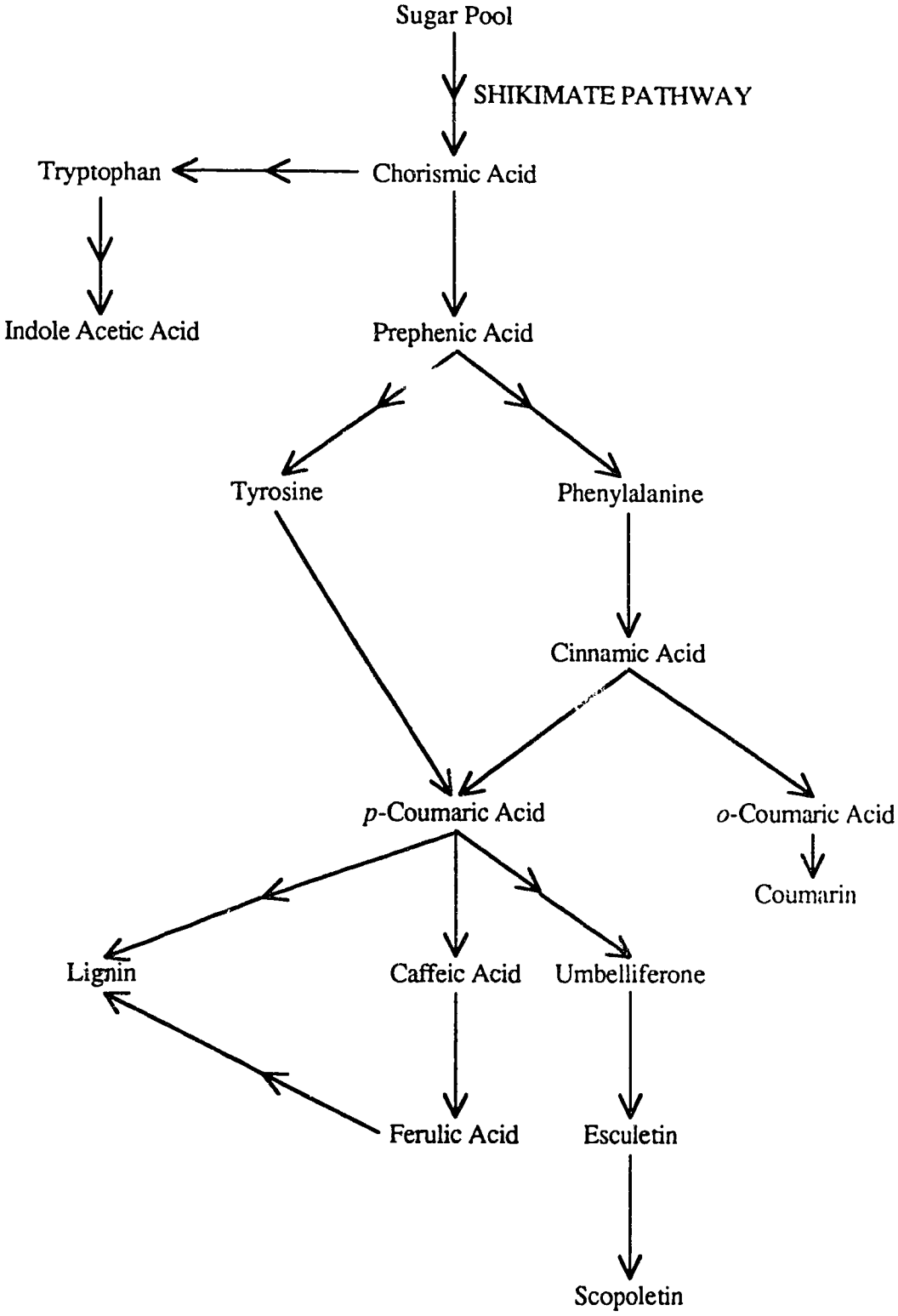


Esculetin



Scopoletin

Figure 2 Biosynthetic Pathway of Selected Allelochemicals



MATERIALS AND METHODS

Tissue Cultures

Tissue cultures were initiated from the six alfalfa varieties listed in Table 2. *Medicago falcata* L.-127 and *Medicago sativa* L.-503 are part of Lesins' collection (Lesins, 1976) maintained at the Devonian Botanical Gardens. Genelle, Regen-S, Roamer, and Spredor-2 are all cultivars. Their genetic parentage is listed in Table 3.

Media

The following media were prepared by dissolving the reagents in double-distilled water (ddH_2O). The resulting solutions were made up to the indicated volumes with ddH_2O and these solutions were stored at 4°C. Any deviations from this procedure are indicated in the subsequent solution protocols.

50x Stock Solution A

82.5 g NH_4NO_3

95.0 g KNO_3

9.04 g $MgSO_4$

8.50 g KH_2PO_4

This macroelement solution was made up to a final volume of one liter.

1000x Stock Solution B

22 g $CaCl_2 \cdot 2H_2O$

The final volume of this calcium chloride stock solution was 50 ml.

1000x Stock Solution C

1.39 g $FeSO_4 \cdot 7H_2O$

2.06 g $Na_2\text{-}EDTA \cdot 2H_2O$

These salts were dissolved in hot ddH_2O. After overnight aeration, this solution was made up to a final volume of 50 ml.

1000x Stock Solution D

620 mg H_3BO_3

1.69 g $MnSO_4 \cdot H_2O$

860 mg $ZnSO_4 \cdot 7H_2O$

83 mg KI

25 mg $Na_2MoO_4 \cdot 2H_2O$

2.5 mg $CuSO_4 \cdot 5H_2O$

2.5 mg $CoCl_2 \cdot 6H_2O$

This microelement stock solution had a final volume of 100 ml.

500x 2,4-Dichlorophenoxyacetic acid (2,4-D) Stock Solution (1 mg/ml in 35% ethanol)

100 mg 2,4-D

The 2,4-D was dissolved in 36.8 ml of 95% ethanol. The resulting solution was made up to a final volume of 100 ml with the addition of ddH_2O. This 2,4-D stock solution was stored at 4°C for up to one month.

500x Kinetin Stock Solution (1 mg/ml)

100 mg Kinetin

The kinetin was dissolved in 10 ml of ddH_2O with five to eight drops of 6 *N* HCl. After the kinetin was dissolved, ddH_2O was added until the volume of the soluti as 100 ml. This kinetin stock solution was stored at 4°C for up to one month.

1000x B5 Vitamin Stock Solution

0.10 g Nicotinic Acid

0.10 g Pyridoxine•HCl

1.05 g Thiamine•HCl•H_2O

10.0 g *myo*-Inositol

This vitamin solution was made up to a final volume of 100 ml and filter-sterilized (Nalgene Disposable Filterware, 0.2 μm). Aliquots of 4 ml were stored at -20°C.

1x Modified Murashige and Skoog Medium (Modified MS Medium)

20	ml	50x	Stock Solution A
1	ml	1000x	Stock Solution B
1	ml	1000x	Stock Solution C
1	ml	1000x	Stock Solution D
2	ml	500x	2,4-D Stock Solution
2	ml	500x	Kinetin Stock Solution

The aforementioned stock solutions were added sequentially to 800 ml of ddH_2O. Next, 30 g of sucrose was dissolved in this solution. With the addition of KOH, the pH was adjusted to 5.65 ± 0.05 for a solid medium or to 5.05 ± 0.05 for a liquid medium. The solution was made up to a final volume of one liter with ddH_2O. If solid medium was desired, 10 g of Difco Bacto-agar was added to this solution. This medium was autoclaved at 121°C for twenty-five minutes. After cooling for fifteen minutes, 1 ml of 1000x B5 Vitamin Stock Solution was aseptically added.

100x Ammonium Phosphate Stock Solution

5.75 g $NH_4H_2PO_4$

The ammonium phosphate solution was made up to a final volume of 500 ml.

100x Potassium Nitrate Stock Solution

30.33 g KNO_3

The final volume of this potassium nitrate solution was 500 ml.

100x Calcium Nitrate Stock Solution

47.23 g $Ca(NO_3)_2 \cdot 4H_2O$

The calcium nitrate solution had a final volume of 500 ml.

100x Magnesium Sulfate Stock Solution

24.65 g $MgSO_4 \cdot 7H_2O$

The magnesium sulfate solution was made up to a final volume of 500 ml.

1000x Micronutrient Stock Solution

143 mg H_3BO_3

90.5 mg $MnCl_2 \cdot 4H_2O$

11 mg $ZnSO_4 \cdot 7H_2O$

4.0 mg $CuSO_4 \cdot 5H_2O$

1.3 mg $Na_2MoO_4 \cdot 2H_2O$

Boric acid was dissolved in boiling ddH_2O before being added to the other dissolved salts. This micronutrient solution was made up to 50 ml.

500x Iron-EDTA Stock Solution

2.36 g $FeSO_4 \cdot 7H_2O$

3.54 g Na_2-EDTA$\cdot 2H_2O$

These salts were dissolved in hot ddH_2O. After overnight aeration, this solution was made up to a final volume of 100 ml.

1x Hoagland's Nutrient Solution

Hoagland's Nutrient Solution was prepared from the formula specified by Jones (1983).

10 ml	100x	Ammonium Phosphate Stock Solution
10 ml	100x	Potassium Nitrate Stock Solution
10 ml	100x	Calcium Nitrate Stock Solution
10 ml	100x	Magnesium Sulfate Stock Solution
1 ml	1000x	Micronutrient Stock Solution
2 ml	500x	Iron-EDTA Stock Solution

The above stock solutions were sequentially added to 800 ml ddH_2O. With the addition of HCl or KOH, the pH was adjusted to 5.65 ± 0.05. The final volume of this nutrient solution was one liter.

Preparation of Phytotoxic Media

The phytotoxins used in the following media were the three coumarin derivatives: ferulic acid, *p*-coumaric acid, and umbelliferone.

Concentrated phytotoxic solutions. The final concentrations of the ferulic acid and *p*-coumaric acid solutions ranged from 0 mM to 15 mM. Due to insolubility of the umbelliferone at higher concentrations, the umbelliferone solution had a lower final concentration range of 0 mM to 10 mM. All solutions were made by dissolving the phytotoxin in boiling ddH_2O. The volume of ddH_2O used was approximately 80% of the final volume of the phytotoxic solution. When the solution had cooled to between 70°C and 80°C, the pH was adjusted with the addition of HCl or KOH. A pH meter (Orion Research digital ionalyzer 501) with an automatic temperature compensation (ATC) probe was used. Unless otherwise specified, the pH was adjusted to 5.65 ± 0.05 for solid media or to 5.05 ± 0.05 for liquid media. The phytotoxic solution was then made up to the final volume with ddH_2O and immediately filter-sterilized (Nalgene Disposable Filterware,

0.2 μm).

When cool, all three concentrated phytotoxic solutions precipitated at their upper concentration limits, mentioned previously. For this reason, these phytotoxic solutions were incorporated into hot, sterile media immediately after they were filter-sterilized.

Nutritive phytotoxic solutions. For each concentration of phytotoxin, a different erlenmeyer flask was used. Each flask contained 1x Hoagland's Nutrient Solution at a pH of 5.65 ± 0.05. The volume of Hoagland's Nutrient Solution in each flask was one-half the final volume of the completed nutritive phytotoxic solution. For dilution of the phytotoxin to the appropriate concentration, different volumes of ddH_2O were added to each flask. These solutions were autoclaved at 121°C for twenty-five minutes. Before these solutions cooled, different amounts of a 10 mM filter-sterilized phytotoxic solution at a pH of 5.65 ± 0.05 were added to the flasks. The completed solutions consisted of a 0.5x Hoagland's Nutrient Solution with the phytotoxin concentration ranging from 0 mM to 5 mM (Table 4).

Phytotoxic MS plates. As in the previous medium, a separate erlenmeyer flask was used for each concentration of phytotoxic solution. Each flask contained a volume of 3x modified MS medium equal to one-third of the final volume of the phytotoxic MS medium. Different volumes of ddH_2O were added to each flask before these solutions were autoclaved at 121°C for twenty-five minutes. Before these solutions cooled, varying amounts of sterile 15 mM ferulic acid, 15 mM *p*-coumaric acid or 7.5 mM umbelliferone were added. The last reagent added was the vitamin component of the modified MS medium. The completed solutions consisted of a 1x modified MS medium with the concentration of ferulic acid and *p*-coumaric acid ranging from 0 mM to 10 mM and the concentration of umbelliferone ranging from 0 mM to 5 mM (Table 5).

Phytotoxic MS cell suspension media. Twenty-five 125 ml erlenmeyer flasks were required for the phytotoxic MS cell suspensions. There were five samples of each phytotoxin concentration. Each flask contained 15 ml of 2x modified MS medium along

with varying amounts of ddH_2O. After autoclaving these solutions at 121°C for twenty-five minutes, varying amounts of sterile 3 mM phytotoxin were added. The last reagent added was the vitamin component of the modified MS medium. The completed solutions consisted of 30 ml of a 1x modified MS medium. The phytotoxin concentration ranged from 0 mM to 1 mM after these solutions were inoculated with 15 ml of cell suspension (Table 6).

Culturing Conditions

Surface Sterilization of Alfalfa Seeds

The following procedure was performed in a laminar flow hood. Alfalfa seeds were stirred for two minutes in 70% ethanol, followed by two minutes in sterile ddH_2O. The seeds were then stirred twice in a solution of 20% Javex bleach (6% sodium hypochlorite) with 1% Tween 80 for twenty minutes each time. This was followed by three rinses with sterile ddH_2O. Each rinse lasted two minutes.

Germination Plates For Initiation of Tissue Cultures

4.0 g Sucrose

6.0 g Difco Bacto-agar

The sucrose, which was added to check for contamination, was dissolved and made up to one liter with ddH_2O. Bacto-agar was added and this mixture was autoclaved at 121°C for twenty minutes.

Initiation of Tissue Cultures

The Regen-S tissue culture was generously donated by P. Nagarajan. It was obtained in suspension form in a modified Blaydes' medium (Croughan, 1978) with 2 mg/liter each of kinetin and 2,4-D. To change the medium that Regen-S was grown in,

4 ml of cell suspension was layered over a 1x modified MS plate. After one month growth in the dark at 26 ± 1°C, Regen-S cells were scraped off the plate. These cells were used to initiate Regen-S cell suspension and callus cultures grown in the presence of the modified MS medium.

The Regen-S tissue culture was initiated from petioles. All other cultures were initiated from hypocotyls. This was accomplished by surface sterilizing alfalfa seeds. The sterile seeds were then transferred to germination plates and incubated in the dark at 26 ± 1°C. After incubation for five to six days, hypocotyl sections 4 mm to 6 mm long were aseptically removed. These hypocotyl sections were placed on modified MS plates for the initiation of callus cultures. To initiate cell suspension cultures, these hypocotyl sections were placed in a 50 ml erlenmeyer flask containing 10 ml to 20 ml modified MS liquid medium. Four to five hypocotyl sections were used to initiate a culture.

Occasionally, a new cell suspension culture was initiated from existing callus or vice versa. This was designated in the tissue culture name by a C (for callus) or an S (for suspension) in parentheses.

Maintenance of Tissue Cultures

Both callus and cell suspension cultures were grown in the dark at 26 ± 1°C. Following transfer of these cultures, their transfer number was increased by one. This number was indicated after the hyphen in the tissue culture name. If the tissue culture was initiated from existing callus or cell suspension culture, a transfer number was enclosed in the parentheses. This indicated the number of times the tissue culture was transferred in its former state.

Callus cultures. Every four to five weeks, vigorous calli approximately 1 cm in diameter were transferred to 100 x 15 mm standard petri dishes containing 25 ml fresh modified MS medium. After transfer, each plate contained four 1 cm callus clumps.

Cell suspension cultures. These cultures were maintained in one liter erlenmeyer

flasks on a rotary shaker at 115 rpm. Every three to four weeks, these suspensions were transferred to 250 ml fresh modified MS liquid medium. A one to five dilution ratio was used such that the total volume was approximately 300 ml.

Bioassays for Allelopathy

Callus Bioassay

Inoculation of calli cultures. The procedure followed was similar to that of Croughan, Stavarek, and Rains (1978). Callus clumps were aseptically mixed to form one homogeneous clump of callus. Next, 100 ± 3 mg of callus was aseptically transferred to each phytotoxic MS plate. Six numbered samples of each phytotoxin concentration were set up; each sample consisted of a separate phytotoxin plate. Occasionally, a sample was discarded due to contamination during inoculating or transferring the callus.

Growth measurements of calli cultures. The calli were grown in an incubator with no illumination at 26 ± 1°C and were transferred to fresh phytotoxic MS plates every five days. Starting on the tenth day, fresh weight measurements were aseptically recorded. Measurements were continued until the twentieth or twenty-fifth day. Two different methods were used to take these measurements.

The first method involved weighing the calli on a Mettler H20T analytical balance in a UV room. Before weighing commenced, this room and the balance were washed down with 95% ethanol and were irradiated with UV for thirty minutes.

The second method was more efficient; it was faster, and it generated less contamination than the first method. For this second method, the calli were weighed on a Sartorius toploading electronic balance in a laminar flow hood. This balance had approximately a 3 cm diameter circle cut out of the metal lid to the enclosure surrounding the weighing pan. This permitted use of the balance, without removing the lid, by dropping the callus clump onto a tared fresh phytotoxic MS plate. Without the lid, the air

flow in the laminar flow hood would disrupt the taring of the balance. Before weighing commenced, the laminar flow hood and the balance were washed down with 95% ethanol.

Cell Suspension Bioassay

Inoculation of cell suspension cultures. The following procedure was performed in a laminar flow hood. Approximately 425 ml to 450 ml of cell suspension was aseptically filtered through a fine sieve with a pore size of 1 mm to 2 mm. The filtrate was collected in a sterile 600 ml beaker and was continuously stirred to maintain a homogeneous suspension. Each of the twenty-five 125 ml erlenmeyer flasks containing 30 ml of phytotoxic MS cell suspension medium was inoculated with 15 ml of this homogeneous suspension. These cell suspension cultures were grown in an incubator with a rotary shaker set at 115 rpm. The temperature was maintained at 26 ± 1°C and there was no illumination.

Growth measurements of cell suspension cultures. Five 25 mm Whatman #41 filter paper disks were dried at 50°C to 60°C for two days and then were weighed. For a zero day measurement, one flask from each phytotoxin concentration was used. A 4 ml sample was aseptically taken from each one of these flasks and was vacuum filtered through the disks using a Millipore 1225 Sampling Manifold filtration unit. The primary filtrate was collected and was removed for pH measurements. The samples were then rinsed with 4 ml of ddH_2O and the secondary filtrate was discarded. The filter paper disks were dried and were weighed as above. Other measurements were taken every five days starting on the tenth day and proceeding until the twentieth or twenty-fifth day. For these measurements, samples were taken from all of the flasks. However, the primary filtrates were only collected from one flask of each phytotoxin concentration.

Germination Bioassay

Germination studies were performed on Genelle, Roamer and Spredor-2 alfalfa

seeds. The Genelle seeds were hard. For that reason, they needed to be scarified with 180 Grit diamond-cut sand paper for twenty minutes. Scarification was not needed on Roamer and Spredor-2 seeds. The germination bioassay was performed separately on each variety. After surface sterilization of seeds from one variety, twenty-five seeds were aseptically transferred to a 100 x 15 mm standard petri dish. This dish contained two sterile 9 cm Whatman #2 filter papers soaked with 5 ml of the nutritive phytotoxic solution. Four numbered samples or petri dishes consisting of twenty-five seeds per petri dish were set up for each phytotoxin concentration. These dishes were sealed with parafilm (American National Can) and incubated in the dark at 26 ± 1°C. For the first week, the germinated seeds were removed daily under sterile conditions and were counted. A twelve day germination measurement was also carried out. Seeds were considered germinated when 3 mm of the radicle protruded through the seed coat.

Shoot and Root Extension Bioassay

Roamer, Spredor-2 and the scarified Genelle seeds were used in this study. Following surface sterilization, approximately fifty seeds of each cultivar were aseptically transferred to a 100 x 15 mm standard petri dish. These plates contained two sterile 9 cm Whatman #2 filter papers soaked with 5 ml of sterile ddH_2O. Depending on the percent germination of the variety, seven to fifteen plates were made. These seeds were incubated for twenty-four to thirty-six hours in the dark at 26 ± 1°C. After this period of germination, seedlings with a radicle length of 3 mm to 10 mm were aseptically transferred to 18 x 150 mm pyrex (Corning Glass Works 9820) test tubes. These test tubes contained 7 ml to 10 ml of medium grade vermiculite soaked with 5 ml of the nutritive phytotoxic solution. To prevent the seedlings from drying out, 1 ml of vermiculite was used to cover each seedling. Altogether, twenty samples per phytotoxin concentration were initiated.

The seedlings were grown in a growth chamber with sixteen hours of light

(approximately 220 $\mu E/m^2/sec$) and eight hours of darkness per day. The temperature varied from 24 ± 2°C during the light period to 22 ± 2°C during the dark period. The relative humidity also varied. A relative humidity of about 60% was maintained during the light period. This increased to about 85% relative humidity during the dark period.

After three weeks in the growth chamber, the shoot and root extensions were measured. The shoot extension was measured from the bottom of the stem to the top of the uppermost stipule. The root extension was measured from the bottom of the stem to the tip of the tap-root.

Statistical Analyses of *In Vitro* and *In Vivo* Bioassays

The statistical test chosen for the analyses of the data from the *in vitro* and *in vivo* bioassays was the Duncan's Multiple Range Test (Duncan, 1955). This test consisted of ordering means from the lowest value to the highest value and then performing analysis of variance on these ordered means. The results of this analysis are presented either by assigning a letter to each mean such that means designated by the same letter were not significantly different or by underscoring the ordered means such that means underscored by the same line were not significantly different. The data obtained from these bioassays was sufficiently complex to warrant analysis on the main frame computer (Michigan Terminal System or MTS). The statistical package run on MTS was SPSSx. This is a general purpose statistical package containing many programs. The program chosen for the analyses was UANOVA. This is a multivariate analysis of covariance program developed at the University of Alberta by Terry Taerum (1985). It utilises the Cornfield-Tukey algorithm to establish the appropriate error term.

Table 2 Alfalfa Varieties

Name	Symbol	Chromosome Number	Source	Origin
Genelle	GN	2n=4x=32	P. J. Hastings Dept. of Genetics University of Alberta Edmonton, Alberta	K. Lesins Dept. of Genetics University of Alberta Edmonton, Alberta
Medicago falcata L.-127[a]	MF	2n=2x=16	Devonian Botanical Gardens Edmonton, Alberta	Bulgaria
Medicago sativa L.-503[a]	MS	2n=4x=32	Devonian Botanical Gardens Edmonton, Alberta	Ottawa, Ontario
Regen-S	RG	2n=4x=32	P. Nagarajan Alberta Research Council Edmonton, Alberta	E. T. Bingham Dept. of Agronomy University of Wisconsin Madison, Wisconsin
Roamer	RM	2n=4x=32	Saskatchewan Wheat Pool Regina, Saskatchewan	Experimental Farm Swift Current, Saskatchewan
Spredor-2	SP	2n=4x=32	Northrup King Company Edmonton, Alberta	Northrup King Company Minneapolis, Minnesota

Note.

[a]These numbers refer to the Devonian Botanical Gardens' accession numbers.

Table 3 Genetic Parentage of Alfalfa Cultivars

Cultivar	Genetic Parentage	Reference
Genelle	*Medicago falcata* L. Ferax[a]	(Lesins, 1975)
Regen-S	DuPuits[a] Saranac[a]	(Bingham, Hurley, Kaatz, & Saunders, 1975)
Roamer	*Medicago falcata* L. Cossack[a] Hardistan[a] Ladak[a] Rambler[a] Ranger[a] Rhizoma[a]	(Heinrichs, 1967)
Spredor-2	Cardinal[a] Drylander[a] Glacier[a] Kane[a] Kane-2[a] Rambler[a] Roamer[a] Roamer-2[a] Spredor[a] Travois[a] Vernal[a]	(Northrup King Company, 1980)

Note.

[a]These parents are commercial varieties.

Table 4 Nutritive Phytotoxic Solutions (per flask)

Ferulic Acid, *p*-Coumaric Acid, or Umbelliferone Concentration (mM)	Volume of 10 mM Phytotoxic Solution (ml)	Volume of 1x Hoagland's Nutrient Solution (ml)	Volume of ddH_2O (ml)	Total Volume (ml)
0	0	50	50	100
0.1	1	50	49	100
1	10	50	40	100
3	30	50	20	100
5	50	50	0	100
Total Volumes (ml)	91	250	159	500

Table 5 Phytotoxic MS Plates (per 6 plates)

Ferulic Acid or *p*-Coumaric Acid Concentration (mM)	Volume of 15 mM Phytotoxic Solution (ml)	Volume of 3x Modified MS Medium (ml)	Volume of ddH_2O (ml)	Total Volume (ml)
0	0	50	100	150
0.1	1	50	99	150
1	10	50	90	150
5	50	50	50	150
7	70	50	30	150
10	100	50	0	150
Total Volumes (ml)	231	300	369	900

Umbelliferone Concentration (mM)	Volume of 7.5 mM Phytotoxic Solution (ml)	Volume of 3x Modified MS Medium (ml)	Volume of ddH_2O (ml)	Total Volume (ml)
0	0	50	100	150
0.1	2	50	98	150
1	20	50	80	150
5	100	50	0	150
Total Volumes (ml)	122	200	278	600

Table 6 Phytotoxic MS Cell Suspension Media (per flask)

Ferulic Acid, *p*-Coumaric Acid, or Umbelliferone Concentration (mM)	Volume of 3 mM Phytotoxic Solution (ml)	Volume of 2x Modified MS Medium (ml)	Volume of ddH_2O (ml)	Total Volume	
				Before Inoculation (ml)	After Inoculation (ml)
0	0	15	15.0	30	45
0.1	1.5	15	13.5	30	45
0.3	4.5	15	10.5	30	45
0.5	7.5	15	7.5	30	45
1.0	15.0	15	0	30	45
Total Volumes (ml)	28.5	75	46.5	150	225

RESULTS

Alfalfa tissue culture bioassays were developed to quantify allelopathy. These *in vitro* bioassays were in the form of calli or cell suspension cultures. For quantitative comparisons of allelopathy between *in vitro* and *in vivo* bioassays, two *in vivo* bioassays were performed. These *in vivo* bioassays consisted of measurements of alfalfa germination and seedling extension. Additionally, alfalfa calli were utilised in the generation of tissue resistant to phytotoxins.

Callus Experiments

Callus Bioassay

Essentially, allelopathy is the release of an allelochemical into the environment resulting in a change in the growth of the target plant. Hence, growth measurements may be used to quantify allelopathy. However, to develop a suitable callus bioassay for the quantification of allelopathy, various preliminary experiments were required. When callus cultures are used as the target plant in a bioassay, growth may be quantified by fresh or dry weight measurements. Dry weight measurements are preferred because variations in weight due to fluctuations in water content are eliminated. However, dry weight measurements are more tedious and result in the loss of the sample while fresh weight measurements are rapid and preserve the sample. A comparison of both of these techniques is made in Table 7. Since both of these techniques produced approximately the same result, when expressed as percentage of control weight, fresh weight measurements were chosen to quantify allelopathy.

To further develop the bioassay for allelopathy, a standard fresh weight inoculum was needed. Similar experiments (Croughan, Stavarek, & Rains, 1978) involving alfalfa callus growth used 100 mg inocula. This quantity proved to be a suitable inoculation

weight for the callus bioassay because it had higher growth rates than those obtained from 200 mg or 300 mg inocula and equal or higher growth rates than those obtained from 50 mg inocula.

Another preliminary experiment involved the characterization of a growth pattern after each subculture. One such curve is depicted in Figure 3a. For this curve, measurements were recorded every five days when transferring the callus clumps to fresh modified MS plates. The mean growth was calculated as the average fresh weight of the six callus clumps per plate. These six values produced a variance from which one standard error could be generated (Equation 1). These standard error values were then used to construct error bars shown in Figure 3a and in subsequent graphs.

$$SE = \frac{s}{\sqrt{n}} \qquad (1)$$

where: SE = sample standard error of the mean
s = sample standard deviation
n = sample size

Some of the characteristics of the growth curve in Figure 3a were unequal variances and nonadditivity of the means. The variances were unequal because they increased as the mean fresh weight increased. Nonadditivity existed because the growth curve was not linear. Both of these characteristics proved problematic in statistical analyses of these data. In order for parametric analyses of variance to be valid, certain basic assumptions must be met. These include normality, additivity (linearity), and homoscedasticity (equal variances). To meet these assumptions, a natural logarithmic transformation was performed on the fresh weight measurements of the individual samples. The means of these logarithmic values are shown in Figure 3b. The variances for this curve were more homogeneous than the curve in Figure 3a. Also, the logarithmic graph was linear from the 10 to 20 day interval. This linearity reflected the exponential growth of the callus in the

logarithmic phase of the growth curve in Figure 3a.

Another important characteristic of these graphs was the existence of a lag phase between the 0 and 10 day interval. This generated a nonlinear interval on the logarithmic curve which was not easily applicable to statistical analyses. For this reason, the 5 day measurements were disregarded in subsequent experiments. A further alteration to the design of this bioassay was to inoculate only one callus clump per plate in subsequent experiments. With six samples per plate, there was a possibility of competition for nutrients occurring between the samples.

Figure 4a shows the six sample logarithmic growth curves generated according to the specifications in the Materials and Methods section. These exhibited some heterogeneity even though these samples were inoculated from one callus source. Calli derived from the same source but exposed to 5 mM ferulic acid are shown in Figure 4b. As expected, lower growth rates were observed. The mean growth curves of these six callus samples are shown in Figure 5. A lag phase was not apparent on these curves. In addition, these curves were linear within one standard error for the duration of the experiment. These characteristics were maintained when the ferulic acid concentration was varied from 0.1 mM to 10 mM (Figure 6). Furthermore, these characteristics were sustained throughout the varietal mean logarithmic growth curves.

In order to make comparisons between the callus growth responses to phytotoxins, a standardized system was required. One of the inconsistencies between experiments was the duration of the experiment. Fresh weight measurements were continued to either 20 or 25 days, depending on the growth rate of the callus. Calli with slow growth rates were measured until the twenty-fifth day while calli with fast growth rates were only measured until the twentieth day. Therefore, although the 25 day experiments generated linear growth curves from the 10 to 25 day interval for the entire phytotoxin concentration range, the 25 day measurement was not included in the determination of the growth rate (Equation 2). This produced a consistent time frame more conducive to statistical analyses.

$$GR = \frac{\ln (FW_{20}) - \ln (FW_{10})}{10} \qquad (2)$$

where:
- GR = logarithmic growth rate (units/day)
- $\ln(FW_{20})$ = logarithmic fresh weight at 20 days
- $\ln(FW_{10})$ = logarithmic fresh weight at 10 days
- 10 = growth rate interval (days)

The logarithmic growth rates generated from Equation 2 were both positive and negative measurements which represented growth and decay, respectively. Since only the growth response to the phytotoxin concentrations was of importance to this project, all negative sample logarithmic growth rates were set to zero. This absence of negative values also proved beneficial in statistical analyses.

The graph of the mean logarithmic growth rate as a function of ferulic acid concentration is presented in Figure 7a. This graph showed a slight stimulation of the growth rate at 0.1 mM ferulic acid. However, Duncan's Multiple Range Test or DMR (Duncan, 1955) comparing the mean logarithmic growth rate at 0.1 mM (0.943 units/day) to that at 0 mM ferulic acid (0.858 units/day) indicated no significant difference between these rates (DMR: α=0.05). In fact, only one (Figure 8) of the forty-two growth rate curves displayed a significant stimulation (DMR: α=0.05), although not highly significant (DMR: α=0.01), between the mean logarithmic growth rate at 0.1 mM *p*-coumaric acid (1.211 units/day) when compared to 0 mM *p*-coumaric acid (0.996 units/day).

As yet, only the mean logarithmic growth rates as a function of a specific phytotoxin concentration have been investigated. To compare growth rates of different calli or to compare one variety's growth rates between replications or from exposure to different toxins, each control mean logarithmic growth rate was set to 1.00 using Equation 3. This standardization of the logarithmic growth rates was required because the control mean logarithmic growth rates varied significantly for different calli as well as for different replications (transfer numbers) of the same calli (DMR: α=0.05). Figure 7b is the

standardized graph generated from Figure 7a. This standardization procedure maintained the relationships between the mean logarithmic growth rates as well as the standard error proportion of the means.

$$SGR = \frac{GR}{MGR_C} \qquad (3)$$

where: SGR = standardized growth rate
GR = logarithmic growth rate (units/day)
MGR_C = control mean logarithmic growth rate (units/day)

Since none of the logarithmic growth rates were negative, the standardized growth rates generated were all positive. If the mean standardized growth rate was zero, it implied that the mean logarithmic growth rate was either stationary or decreasing. This would indicate an average response of no growth or decomposition respectively, for calli growing on MS phytotoxic plates. A mean standardized growth rate between zero and one implied that the growth rate of the calli growing on MS phytotoxic plates was less than the control growth rate. For example, in Figure 7b a mean standardized growth rate of 0.54 was obtained when RM-14 calli were grown in 5 mM ferulic acid. This meant that the mean logarithmic growth rate was 54% of the control growth rate or in other words, these calli were experiencing 46% growth inhibition. Growth stimulation was also observed when the mean standardized growth rates were greater than one. A mean standardized growth rate of 1.10 was obtained when RM-14 calli were grown on 0.1 mM ferulic acid MS plates. This implied that the mean logarithmic growth rate was 10% more than the control mean logarithmic growth rate.

Each of the eighteen callus experiments was repeated once or twice. This generated a total of forty-two growth rate curves. To compare the mean logarithmic growth rates from replications of the same variety of callus, a balanced data set was needed for statistical purposes. This was obtained by using mean logarithmic growth rates within the

phytotoxin concentration range of 0 mM to 5 mM since not all growth rate curves had values at 7 mM and 10 mM concentrations. When the individual concentration levels were compared between replications, significance (DMR: α=0.05) occurred mainly at phytotoxin concentration levels of 1 mM and 5 mM. Since significant differences were observed in the replications, any further analyses had this effect statistically removed.

To compare the varietal response of the calli to specific concentration levels of phytotoxin, the standardized growth rates for all replications were combined to generate one mean (combined growth rate) for each varietal phytotoxin concentration level. The combined growth rates plotted as a function of phytotoxin concentration for each phytotoxin are shown in Figures 9, 10, and 11. Statistical analyses (DMR: α=0.05) performed on these combined growth rates indicated that for a specific phytotoxin there existed significant differences in varietal growth responses (Tables 8, 9, and 10).

In order to compare the effects of different phytotoxins on the combined growth rates for each variety, Figures 9, 10, and 11 were replotted according to the six varieties (Figures 12 to 17). Statistical analyses (DMR: α=0.05) indicated that for a specific variety there existed significant differences in the growth response to the different phytotoxins (Tables 11 to 16). In fact, when considering only 0 mM to 5 mM concentration range of all the varieties and phytotoxins (balanced data set), the average ferulic acid combined growth rate was significantly higher (DMR: α=0.05) than the *p*-coumaric acid and umbelliferone combined growth rates (Table 17). This was clearly evident at phytotoxin concentrations of 5 mM or greater. Additionally, the average varietal combined growth rates were significantly different (Table 17).

Selection for Phytotoxin-Resistant Calli

An attempt was made to obtain alfalfa regenerants resistant to ferulic acid, *p*-coumaric acid, and umbelliferone *via* somaclonal variation. Over a period of nine to thirteen months, healthy callus tissue was sequentially selected at progressively higher

phytotoxin concentration levels incorporated into the MS plates. Calli that grew well on plates containing 5 mM or 10 mM phytotoxin were selected for callus growth analyses (Table 18). The procedures performed for the callus growth analyses were identical to those indicated in the Materials and Methods section. After 25 days of exposure to the phytotoxin, only the RG(S8)-5 of the previously exposed calli exhibited growth (Table 19).

To prove the resistance of this line to 5 mM ferulic acid, a more detailed experiment was constructed. RG(S8)-5 never exposed to a phytotoxin was inoculated onto plates containing no phytotoxin as well as plates containing 5 mM ferulic acid, *p*-coumaric acid, or umbelliferone. RG(S8)-5 previously exposed to ferulic acid was also inoculated onto 5 mM phytotoxin plates. The *p*-coumaric acid and umbelliferone plates were included to determine whether cross-reactivity existed between the phytotoxins. This would be indicated if calli selected for resistance to ferulic acid also displayed resistance to *p*-coumaric acid or umbelliferone. Additionally, to demonstrate whether the resistance to 5 mM ferulic acid was stable, calli exposed to ferulic acid and then subsequently removed from this phytotoxin were also employed in this experiment. The results showed no resistance of calli previously exposed to ferulic acid as well as no resistance of the exposed and subsequently removed calli (Table 20). In fact, the only observable growth of calli on the phytotoxic MS plates was from the calli that was never exposed to phytotoxins. These results conflict with those in Table 19.

Cell Suspension Experiments

Cell Suspension Bioassay

To quantify growth in a cell suspension culture, various techniques can be employed. Three of the most commonly used techniques are cell count, packed cell volume, and dry weight measurements. Difficulties in cell count measurements arise when

cells grow in multicellular clumps. On the other hand, packed cell volume measurements are relatively easy but require special conical centrifuge tubes. Thus, dry weight measurements were chosen based on their ease in quantification and their use of inexpensive equipment.

The fifteen growth curve graphs constructed from dry weight measurements were all quite different. These differences somewhat reflected the variation in dry weight of the inocula. Inoculation weights between 0 mg/ml and 1 mg/ml showed considerable variability with respect to the presence or absence of a lag phase in the control (0 mM) cultures (Figure 18). This lag phase was not as apparent in control cultures with inoculation weights over 1 mg/ml. Although, with this inoculation weight, the stationary phase was often present (Figure 19). However, the inoculation weight was not the only factor contributing to the variability of these graphs. Figure 20 shows the replications performed on Spredor-2 cell suspensions. The inoculation weight for both of these growth curve graphs were within 0.2 mg/ml of each other. Yet, Figure 20b demonstrated a much slower growth than that of Figure 20a. This may be attributed to the growth phase of the cells used in the inoculation or even the overall age of the cell cultures.

What was also apparent from these graphs was that more than one growth phase could be present during a single time interval. This was exhibited by the growth curves in Figure 19c during the fifteenth to twentieth day interval. In fact, one growth curve at a specific phytotoxin concentration could exhibit more than one growth phase during a single time interval since there were not enough points on the graphs to identify clearly when each phase occurred. Therefore, statistical analysis could not correct for these variations in growth phases because the growth rates were not constant within any one time interval. Statistical analyses could, however, be performed on mean dry weight measurements from any specific day. This would indicate whether there existed significant differences between the growth of the cell suspensions in different concentrations of phytotoxin. However, any varietal or phytotoxic comparisons would be invalid because of the variations in the growth

phases. For these reasons, this cell suspension bioassay was disregarded as a method to quantify allelopathy.

pH Measurements

To further monitor the growth of the cell suspensions, pH measurements of the primary filtrates were also recorded. These were obtained from one of the five samples from each phytotoxin concentration. The same sample was used in all the measurements for the duration of the experiment (Figure 21a). Generally, there was no change or a slow decrease in the pH of the filtrate if no growth was observed. If growth was occurring, the pH rapidly decreased and then subsequently increased (Figure 21b). This demonstrated that the cell suspensions were able to utilise the nitrogen source in the phytotoxic MS media. The decrease in pH indicated that NH_4^+ was preferentially utilised. When this nitrogen source was exhausted, the cells utilised NO_3^- which resulted in an increase in pH (Bayley, King, & Gamborg, 1972).

Germination Bioassay

Germination measurements were performed on only three of the six alfalfa varieties due to the unavailability of seeds from three of the varieties. The number of seeds germinated during the twelve day bioassay were expressed as daily cumulative percent germinations. The mean percent germination of the four replicates plotted against the duration of the bioassay is displayed in Figure 22a and Figure 22b. These graphs showed that the variances of the means were unequal, with small variances for very large or very small means and larger variances for intermediate means. Thus, to compare the germination results a natural logarithmic transformation was needed. Since some percent germination values were zero, the transformation in Equation 4 was used.

$$LPG = \ln (PG + 1) \quad (4)$$

where: LPG = logarithmic percent germination
PG = percent germination

Statistical analyses were performed separately on the mean logarithmic percent germinations generated from the second and twelfth day. Both of these measurements were required to define a delay or inhibition in germination similarly defined by Williams and Hoagland (1982). The second day measurement was used to determine whether a phytotoxin caused a delay in germination. This day was chosen because less than 5% of the control Genelle seeds had germinated on the first day, whereas the Spredor-2 control seeds had almost reached a maximum mean percent germination by the third day. Therefore, on the second day the controls of all three alfalfa varieties had an intermediate mean percent germination. Using these second day measurements, a delay in germination was defined as a significant difference (DMR: α=0.05) between the control (0 mM phytotoxin) mean logarithmic percent germination and the mean logarithmic percent germinations obtained from seeds exposed to a phytotoxin. Additionally, these mean logarithmic percent germinations should show a significant difference at the second day, which was not apparent at the twelfth day.

The measurement on the twelfth day was used to determine if a phytotoxin caused an inhibition in the percent germination. By this time, germination had ceased for all varieties. Thus, an inhibition in germination was defined as a significant difference in the aforementioned mean logarithmic percent germinations at the twelfth day. From these definitions, it was also possible for a delay as well as an inhibition in germination to simultaneously occur. However, the delay in germination would be masked by the inhibition effect. The results of these analyses are shown in Table 21 with the corresponding designations of delay and inhibition in germination given in Table 22. According to these definitions, the mean logarithmic percent germinations generated from Figure 22a displayed a delay in germination with 5 mM ferulic acid, whereas those

generated from Figure 22b displayed an inhibition in germination with 3 mM *p*-coumaric acid. Additionally, no significant stimulation (DMR: α=0.05) in germination was noted between the control mean logarithmic percent germinations and the mean logarithmic percent germinations generated from alfalfa seeds exposed to a phytotoxin. It is also important to note that the results of these statistical analyses apply directly to the mean logarithmic percent germinations and only reflect possible significances in the percent germination.

To compare the varietal and phytotoxic effects on the logarithmic percent germinations, only the twelfth day measurements were used. To make these comparisons, the logarithmic percent germinations were adjusted by Equation 5 because significant differences (DMR: α=0.05) existed between the control mean logarithmic percent germinations from each variety. Thus, once again the logarithmic percent germinations were standardized by generating a mean standardized percent germination value of 1.00 for each control.

$$SPG = \frac{LPG}{MLPG_C} \tag{5}$$

where: SPG = standardized percent germination
LPG = logarithmic percent germination
$MLPG_C$ = control mean logarithmic percent germination

Figures 23 to 25 illustrate the effects of the phytotoxins on the varietal mean standardized percent germinations. Statistical analyses of these graphs indicated that for a specific phytotoxin there existed significant differences in the varietal germination response (Tables 23 to 25). Once again these mean standardized percent germinations were replotted according to the variety for comparison of the phytotoxic effects (Figures 26 to 28). Statistical analyses of these graphs indicated that for a specific variety there existed significant differences in the germination response to the phytotoxins (Tables 26 to 28).

Overall comparisons were performed on these standardized percent germinations. When only the varietal effect was considered, Genelle was the least sensitive variety to the phytotoxins (Table 29). Alternatively, when only the phytotoxins were considered, *p*-coumaric acid proved to be the most effective phytotoxin in inhibiting growth.

Extension Bioassay

The extension bioassay used the same three alfalfa varieties as did the germination bioassay. Twenty germinated seeds were used for each phytotoxin concentration level. At three weeks, the shoot extensions and root extensions were measured. To give a visual representation of these three-week-old seedlings, photographs were taken of a typical seedling from each phytotoxin concentration level (Plates 1 to 3).

At three weeks of age, seedlings were of nonuniform total length with a maximum of six outliers in any control (0 mM phytotoxin) group (Figure 29a). Additionally, dead seedlings were prevalent at 5 mM phytotoxin. Since only the extension in the presence of the phytotoxin was of importance to this project, the 5 mM phytotoxin concentration level was disregarded. However, a few seedlings also died at 3 mM phytotoxin. To eliminate these seedlings as well as any other outliers, six seedlings were disregarded from each concentration level. These seedlings displayed the six largest deviations from the mean of the seedling total length. Figures 29 and 30 are scatter plots that illustrate the worst and best control groups, respectively, before and after this elimination procedure.

The extension bioassay was repeated once. Statistical analyses performed on these extension data indicated that significant stimulation (DMR: α=0.05) existed in some root extensions at 0.1 mM or 1.0 mM ferulic acid when compared to the control (0 mM ferulic acid). Figure 31 shows the significant stimulations (DMR: α=0.05) which were not significant in the replications, whereas Figure 32a shows a highly significant (DMR: α=0.01) stimulation that was also present in the replication (Figure 32b). Statistical

analyses (DMR: α=0.05) performed on the varietal control mean extensions indicated that significant differences existed. Hence, once again standardization was performed by adjusting the corresponding control means to a value of 1.00 (Equations 6 and 7).

$$SSE = \frac{SE}{MSE_C} \tag{6}$$

where: SSE = standardized shoot extension
SE = shoot extension
MSE_C = control mean shoot extension

$$SRE = \frac{RE}{MRE_C} \tag{7}$$

where: SRE = standardized root extension
RE = root extension
MRE_C = control mean root extension

In a similar fashion to the callus bioassays, these standardized values for all replications were combined to generate one mean (combined shoot extension and combined root extension) for each varietal phytotoxin concentration level. These combined values plotted as a function of phytotoxin concentration for each phytotoxin are shown in Figures 33 to 35. Statistical analyses (DMR: α=0.05) on these combined values indicated that for a specific phytotoxin there existed significant differences in the varietal extension responses (Tables 30 to 32).

In order to compare the effects of different phytotoxins on the combined values for each variety, Figures 33 to 35 were replotted according to the three varieties (Figures 36 to 38). Statistical analyses (DMR: α=0.05) were again performed (Tables 33 to 35). These results indicated that for a specific variety there existed significant differences in the extension response to the phytotoxins. In the overall comparisons in Table 36, Roamer had a significantly lower (DMR: α=0.05) shoot and root extension in the presence of a

phytotoxin when compared to Genelle and Spredor-2. The last overall comparison involved the phytotoxins. These results showed that all the phytotoxins affected the shoot and root extensions in a similar manner. Ferulic acid was the least effective phytotoxin, whereas umbelliferone was the most effective phytotoxin in inhibiting seedling extension (Table 36).

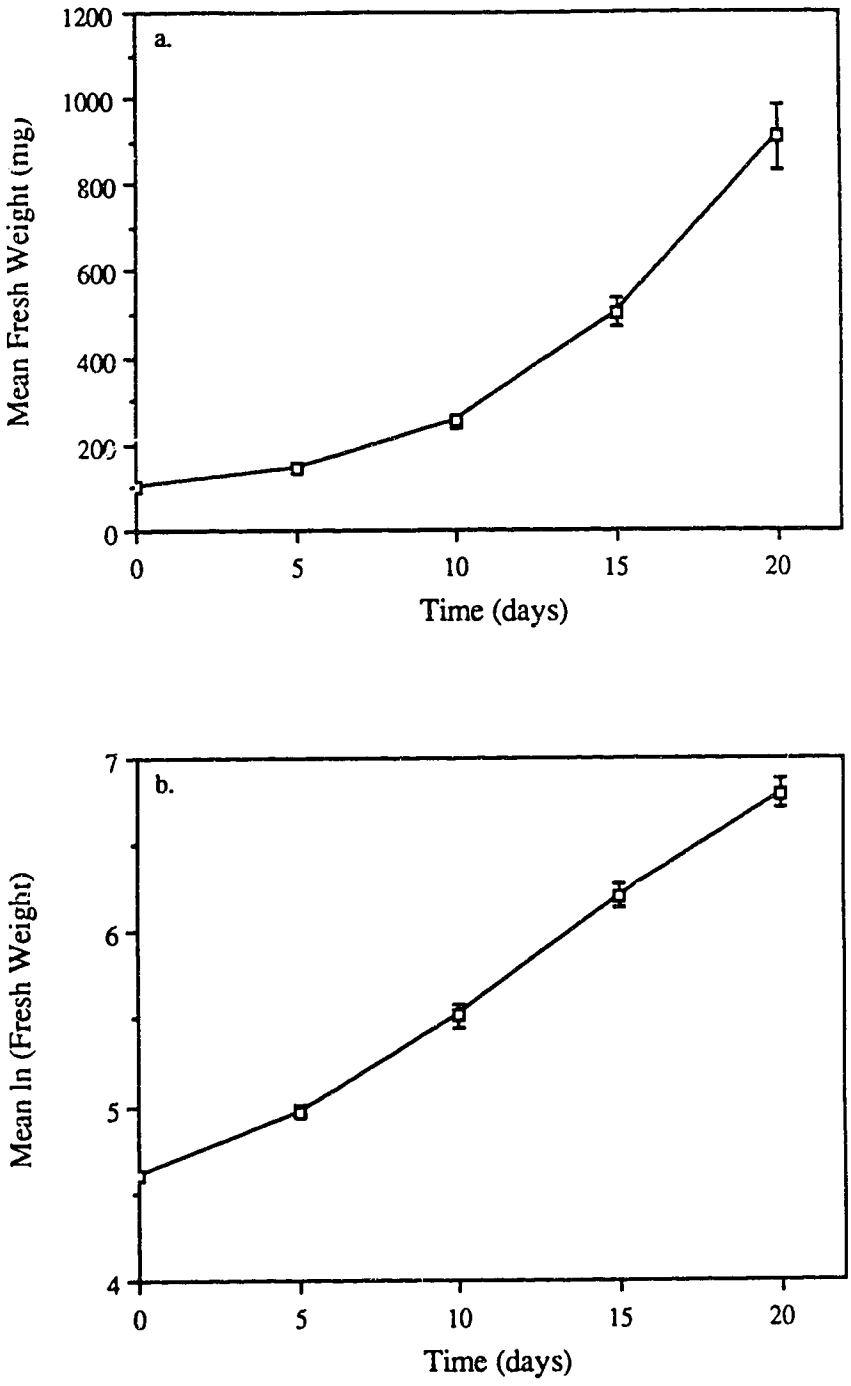
Table 7 Comparison of Fresh and Dry Weight Measurements of MS-15 Calli[a]

Phytotoxin Concentration (mM)	Ferulic Acid		*p*-Coumaric Acid		Umbelliferone	
	FW	DW	FW	DW	FW	DW
0	100	100	100	100	100	100
0.1	81	78	96	91	93	95
1	77	79	74	70	75	78
5	23	26	10	9	10	8
7	20	22	7	4	-	-
10	16	19	-	-	-	-

Note.

[a]Fresh weight (FW) and dry weight (DW) measured at 20 days were expressed as a percentage of control (0 mM) fresh weight or dry weight, respectively.

Figure 3 Mean Growth of SP(S4)-1 Calli



Note.
Error bars indicated were one standard error (Equation 1).

Figure 4 Effect of Ferulic Acid on the Growth of RM-14 Callus Samples

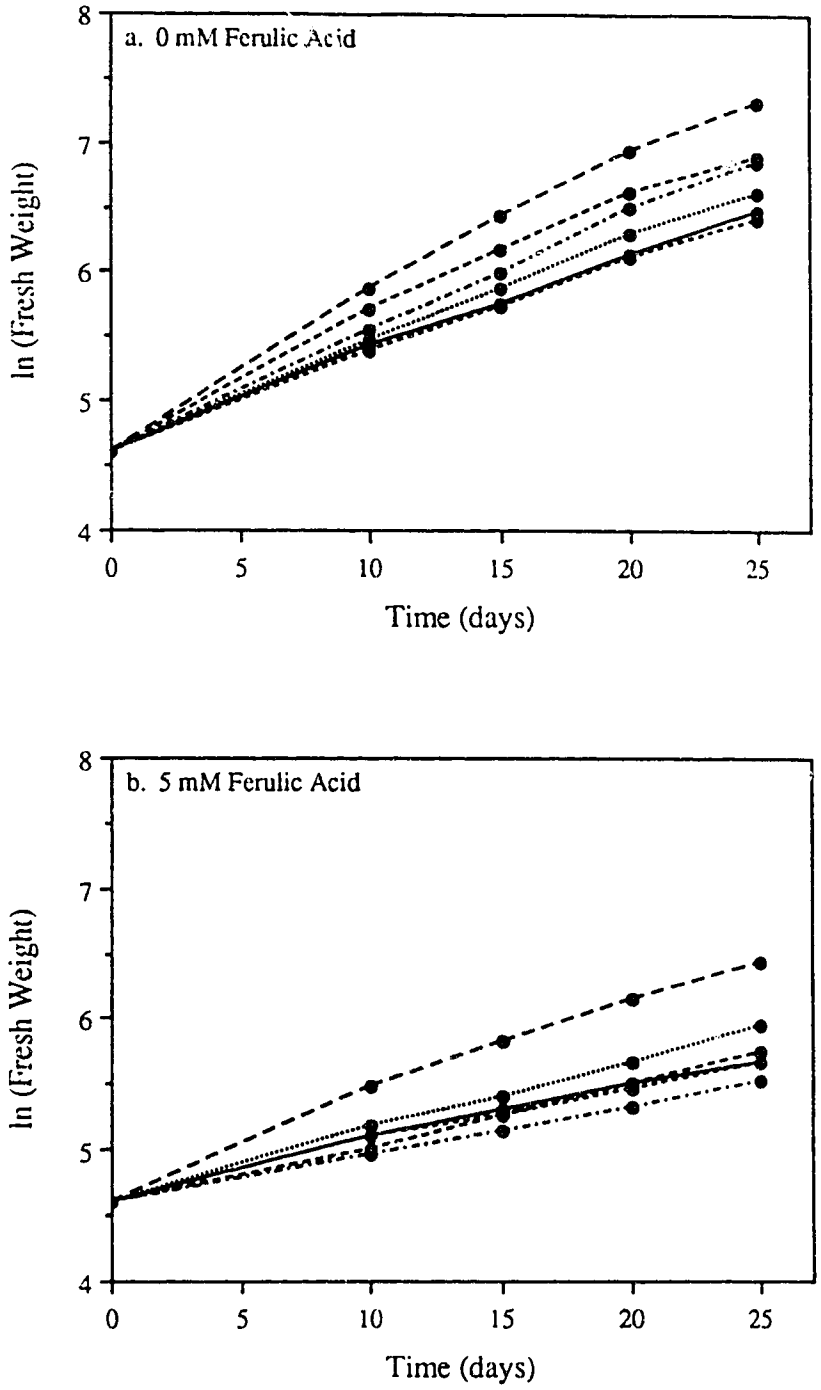
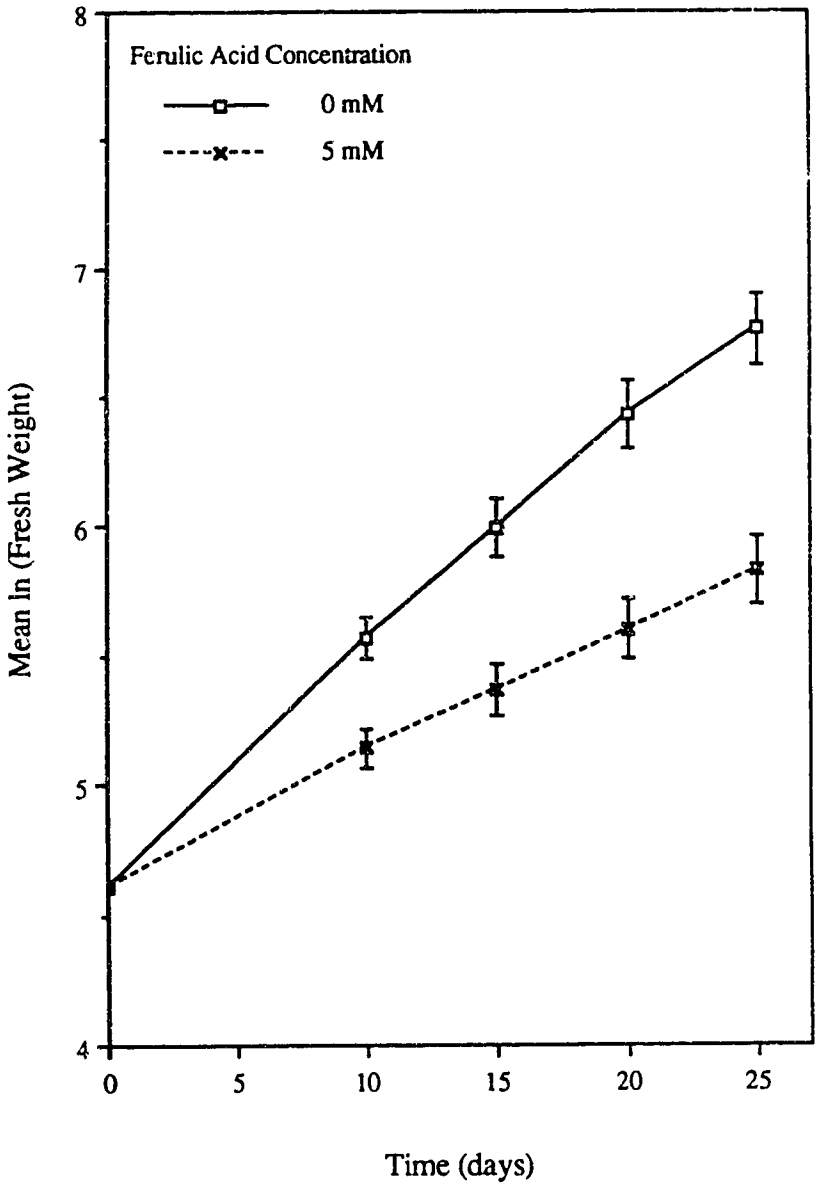
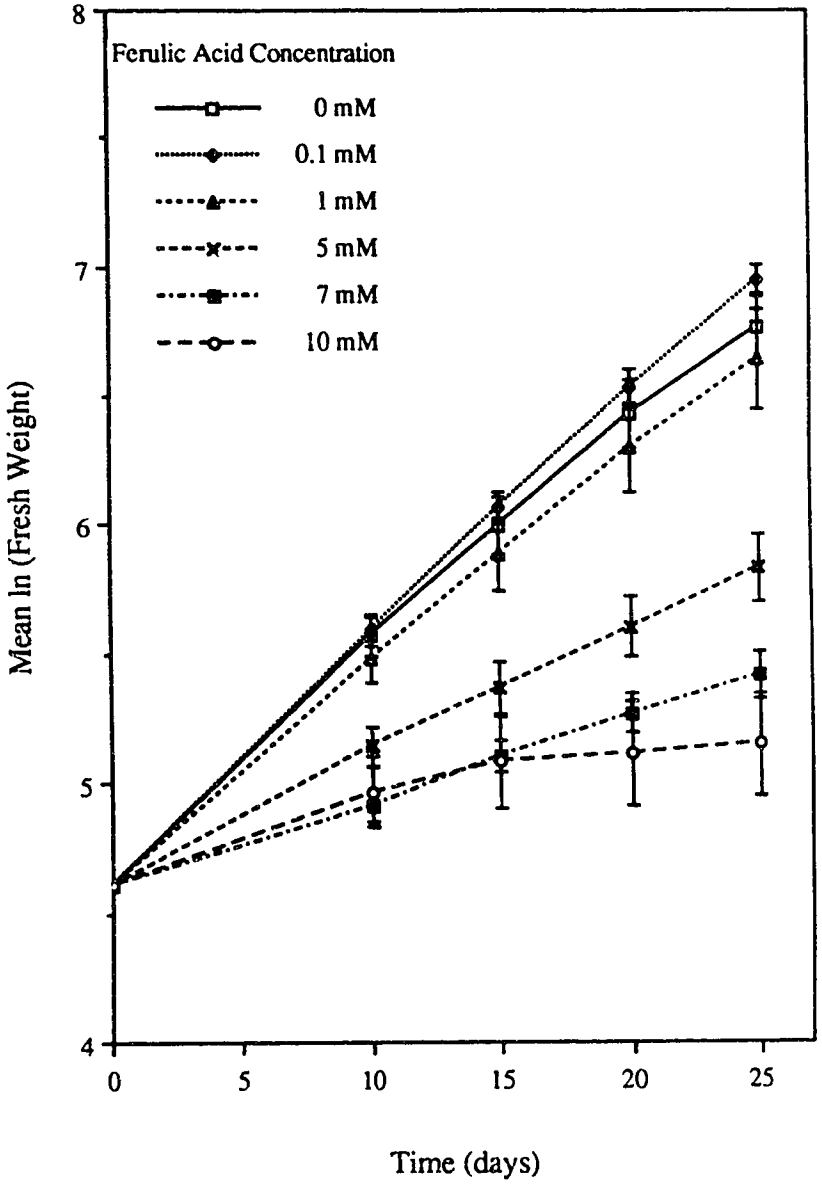


Figure 5 Effect of Ferulic Acid on the Mean Logarithmic Growth of RM-14 Calli



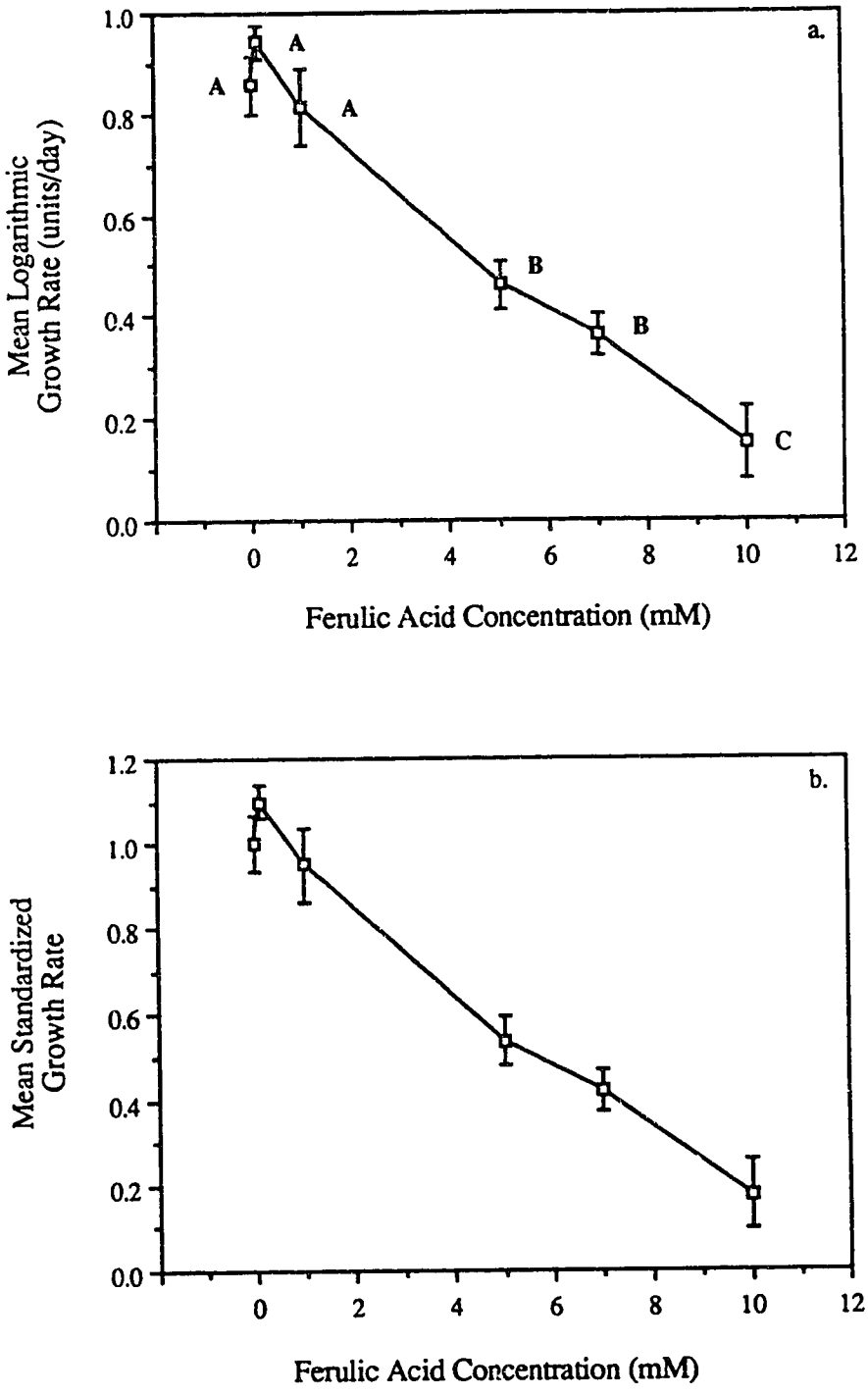
Note.
Error bars indicated were one standard error (Equation 1).

Figure 6 Further Effects of Ferulic Acid on the Mean Logarithmic Growth of RM-14 Calli



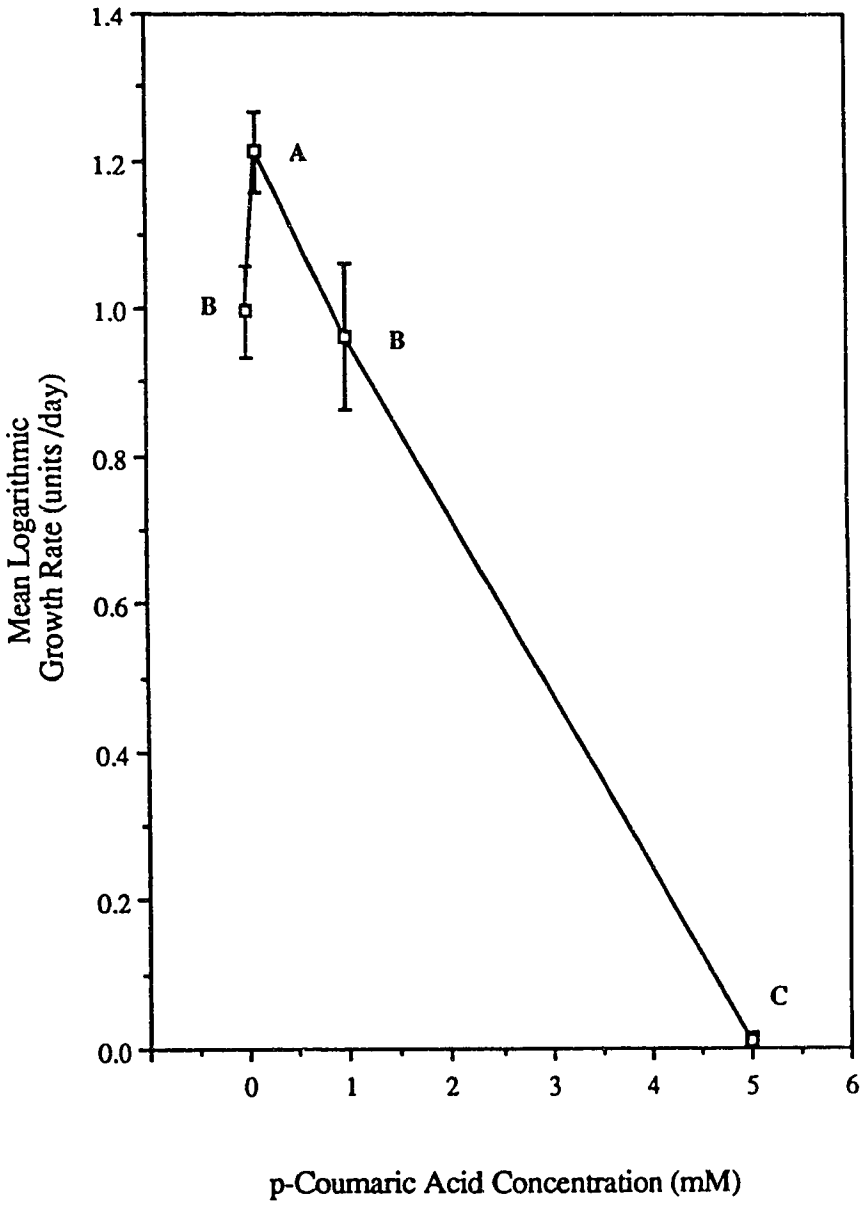
Note.
Error bars indicated were one standard error (Equation 1).

Figure 7 Effects of Ferulic Acid on the Mean Logarithmic and Standardized Growth Rates of RM-14 Calli



Note.
Mean logarithmic growth rates with the same letter were not significantly different (DMR: α=0.05). Error bars indicated were one standard error (Equation 1).

Figure 8 Effect of *p*-Coumaric Acid on the Mean Logarithmic Growth Rate of MS-2 Calli



Note.
Mean logarithmic growth rates with the same letter were not significantly different (DMR: α=0.05). Error bars indicated were one standard error (Equation 1).

Figure 9 Effects of Ferulic Acid on the Varietal Combined (rowth Rates

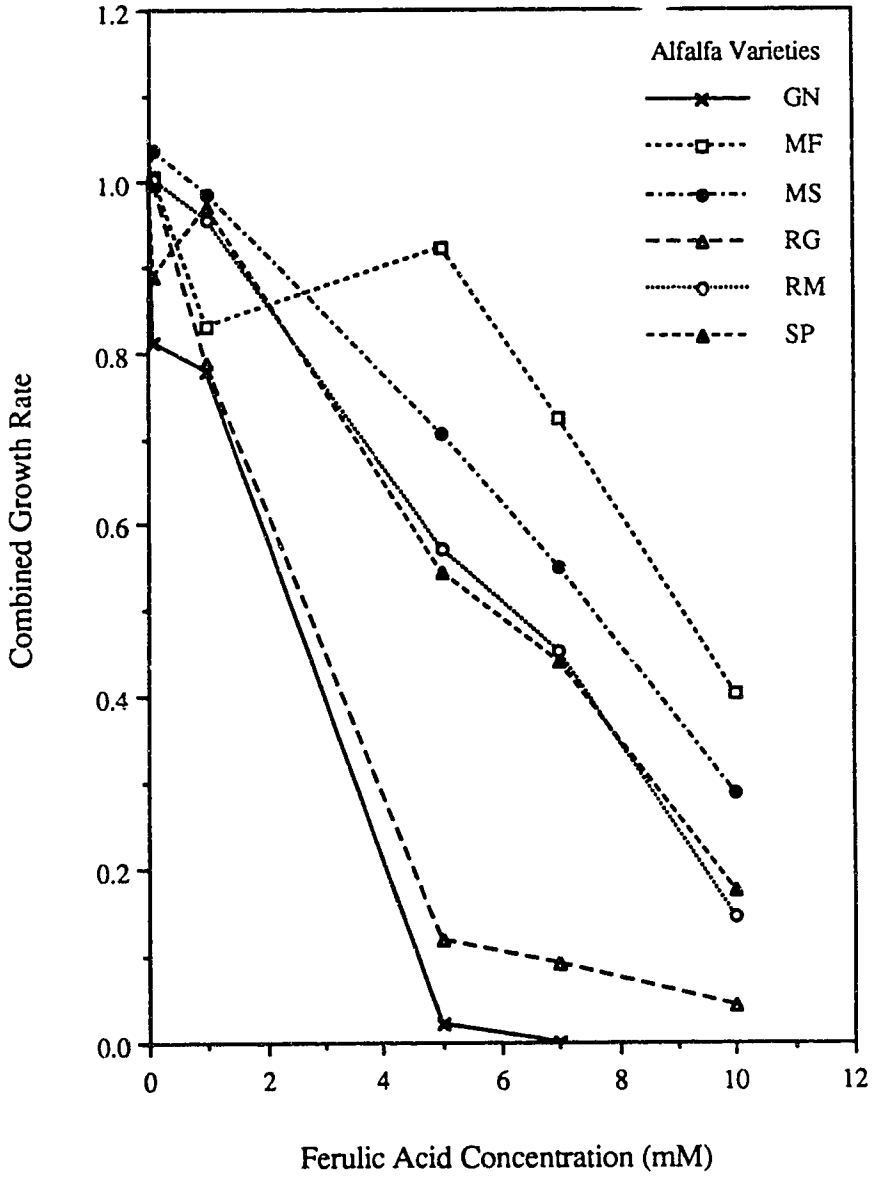


Figure 10 Effects of *p*-Coumaric Acid on the Varietal Combined Growth Rates

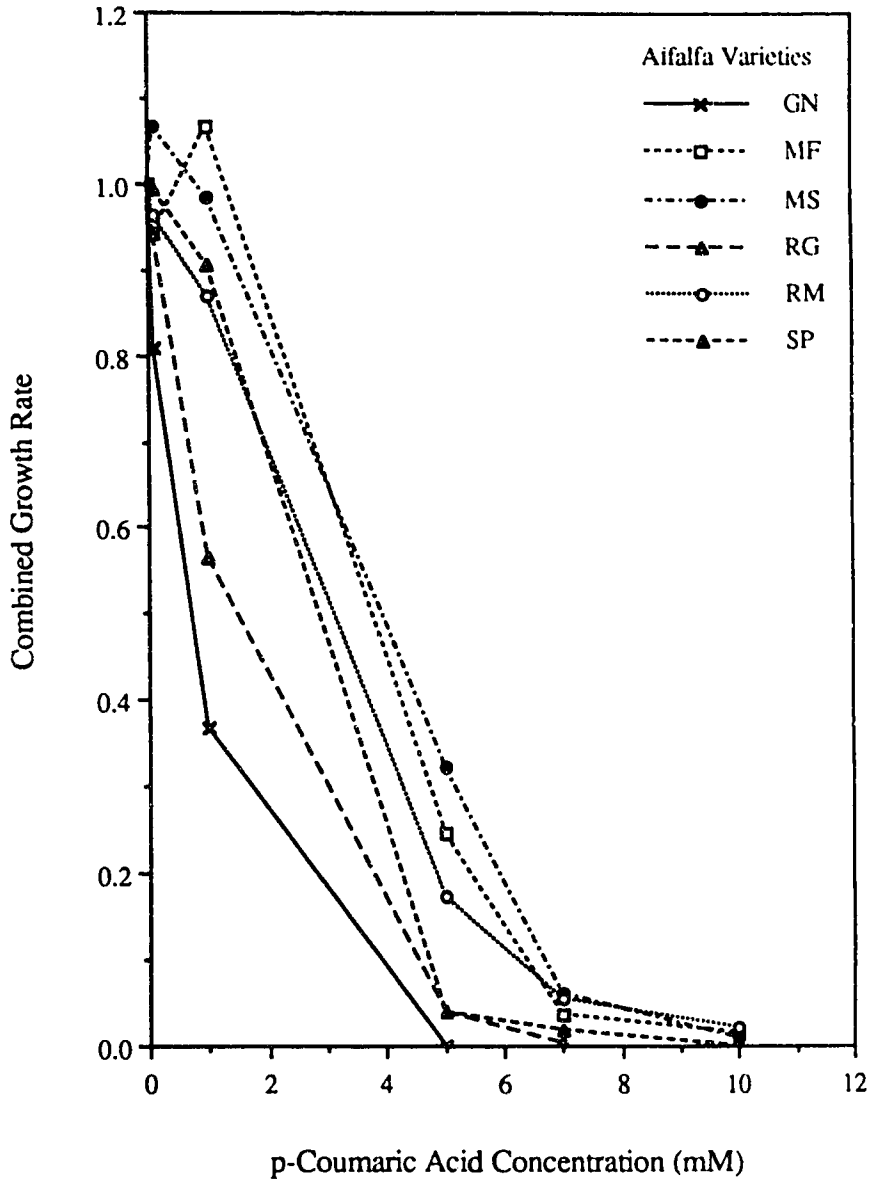


Figure 11 Effects of Umbelliferone on the Varietal Combined Growth Rates

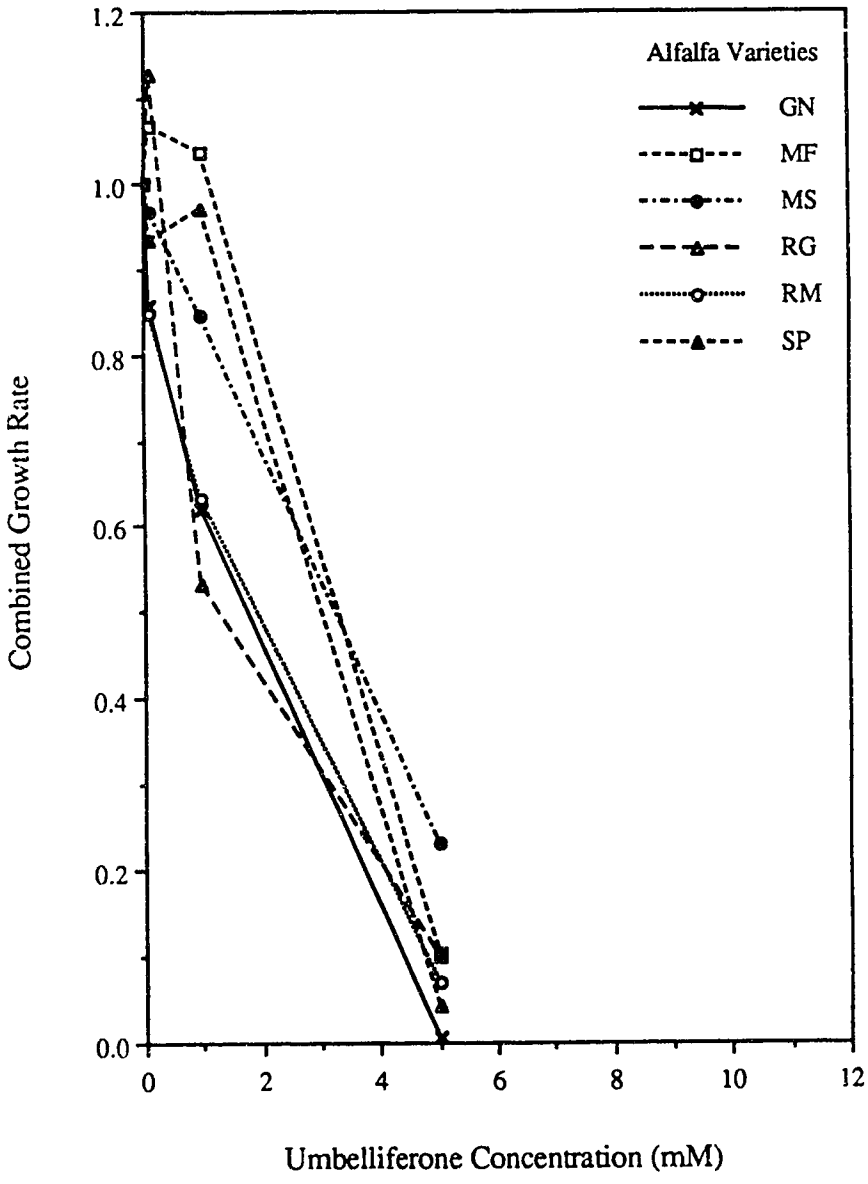


Table 8 Statistical Analyses on the Varietal Combined Growth Rates Based on Alfalfa Calli Grown on Ferulic Acid MS Plates

Ferulic Acid Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Combined Growth Rates[a]
0.1	0.812 0.886 0.995 1.003 1.006 1.037 GN SP RG RM MF MS
1.0	0.779 0.787 0.830 0.954 0.969 0.984 GN RG MF RM SP MS
5	0.022 0.118 0.543 0.571 0.705 0.920 GN RG SP RM MS MF
7.0	0.000 0.091 0.439 0.454 0.550 0.723 GN RG SP RM MS MF
10.0	0.043 0.147 0.176 0.289 0.404 RG RM SP MS MF
0 to 5[b]	0.653 0.737 0.849 0.882 0.931 0.939 GN RG SP RM MS MF

Note.

[a]Varietal combined growth rates depicted above the variety symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined growth rates from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 9 Statistical Analyses on the Varietal Combined Growth Rates Based on Alfalfa Calli Grown on *p*-Coumaric Acid MS Plates

p-Coumaric Acid Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Combined Growth Rates[a]
0.1	0.807 GN, 0.943 RG, 0.943 MF, 0.964 RM, 0.994 SP, 1.066 MS
1.0	0.369 GN, 0.564 RG, 0.869 RM, 0.904 SP, 0.985 MS, 1.066 MF
5.0	0.000 GN, 0.038 SP, 0.040 RG, 0.172 RM, 0.247 MF, 0.321 MS
7.0	0.003 RG, 0.019 SP, 0.037 MF, 0.055 RM, 0.061 MS
10.0	0.001 SP, 0.008 MS, 0.015 MF, 0.021 RM
0 to 5[b]	0.538 GN, 0.636 RG, 0.734 SP, 0.751 RM, 0.814 MF, 0.843 MS

Note.

[a]Varietal combined growth rates depicted above the variety symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined growth rates from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 10 Statistical Analyses on the Varietal Combined Growth Rates Based on Alfalfa Calli Grown on Umbelliferone MS Plates

Umbelliferone Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Combined Growth Rates[a]
0.1	0.849 0.857 0.932 0.965 1.065 1.126 RM GN SP MS MF RG
1.0	0.533 0.619 0.633 0.844 0.970 1.037 RG GN RM MS SP MF
5.0	0.006 0.043 0.071 0.102 0.099 0.231 GN SP RM MF RG MS
7.0	—
10.0	—
0 to 5[b]	0.621 0.638 0.680 0.736 0.760 0.801 GN RM RG SP MS MF

Note.

[a]Varietal combined growth rates depicted above the variety symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined growth rates from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Figure 12 Phytotoxic Effects on Genelle Combined Growth Rates

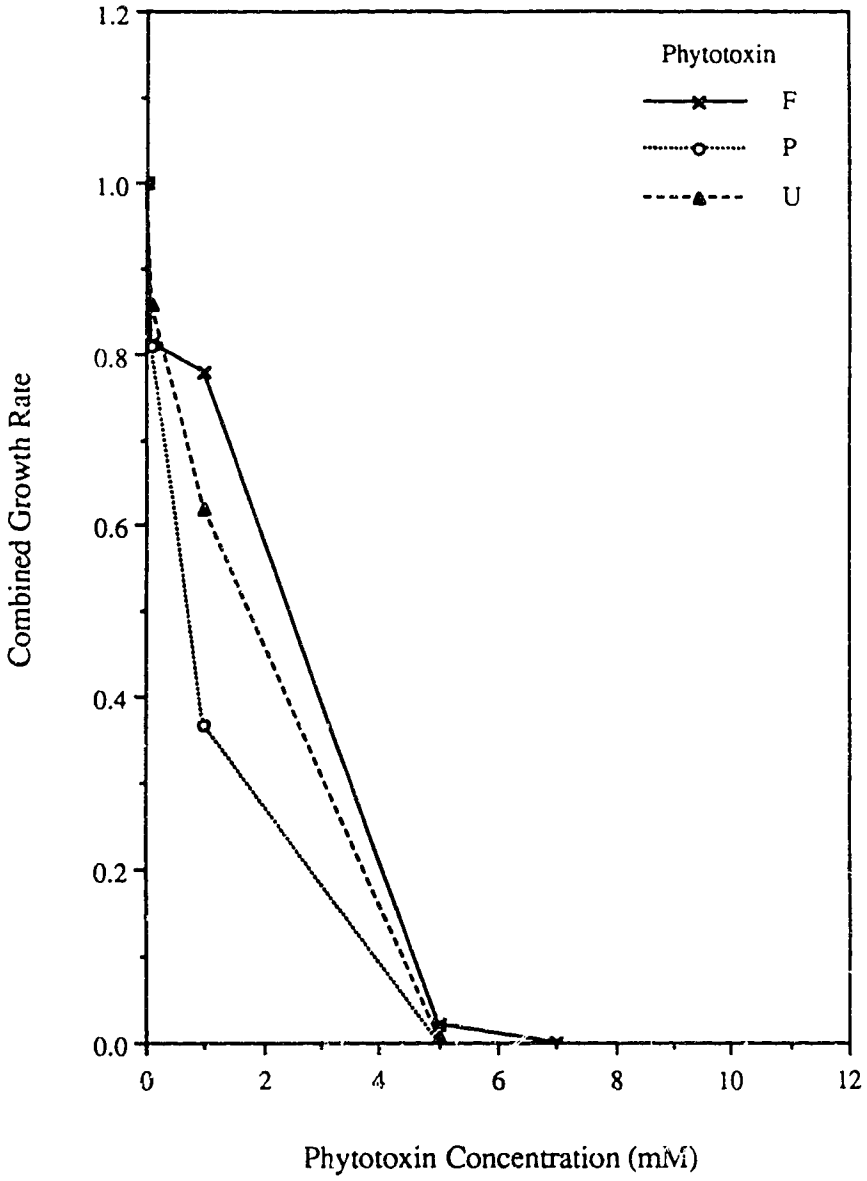


Figure 13 Phytotoxic Effects on *Medicago falcata* L.-127 Combined Growth Rates

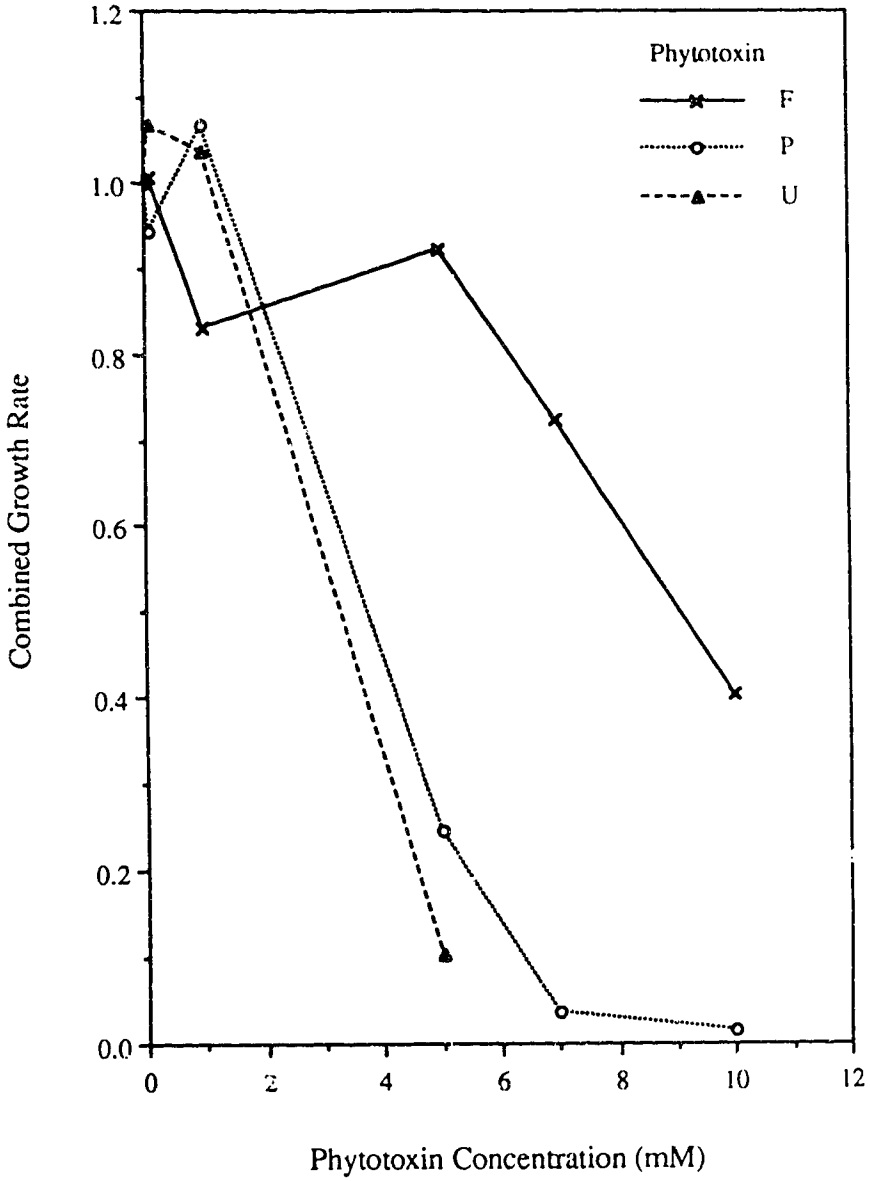


Figure 14 Phytotoxic Effects on *Medicago sativa* L.-503 Combined Growth Rates

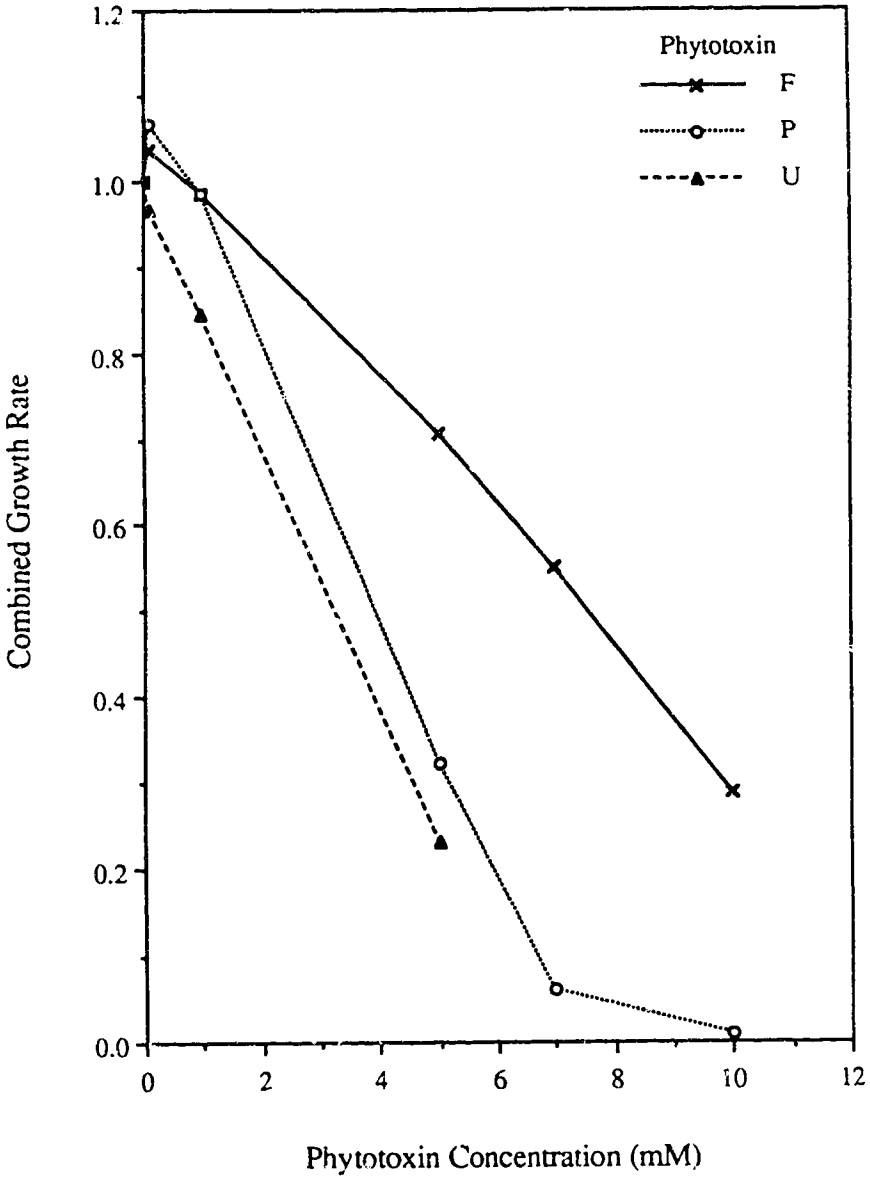


Figure 15 Phytotoxic Effects on Regen-S Combined Growth Rates

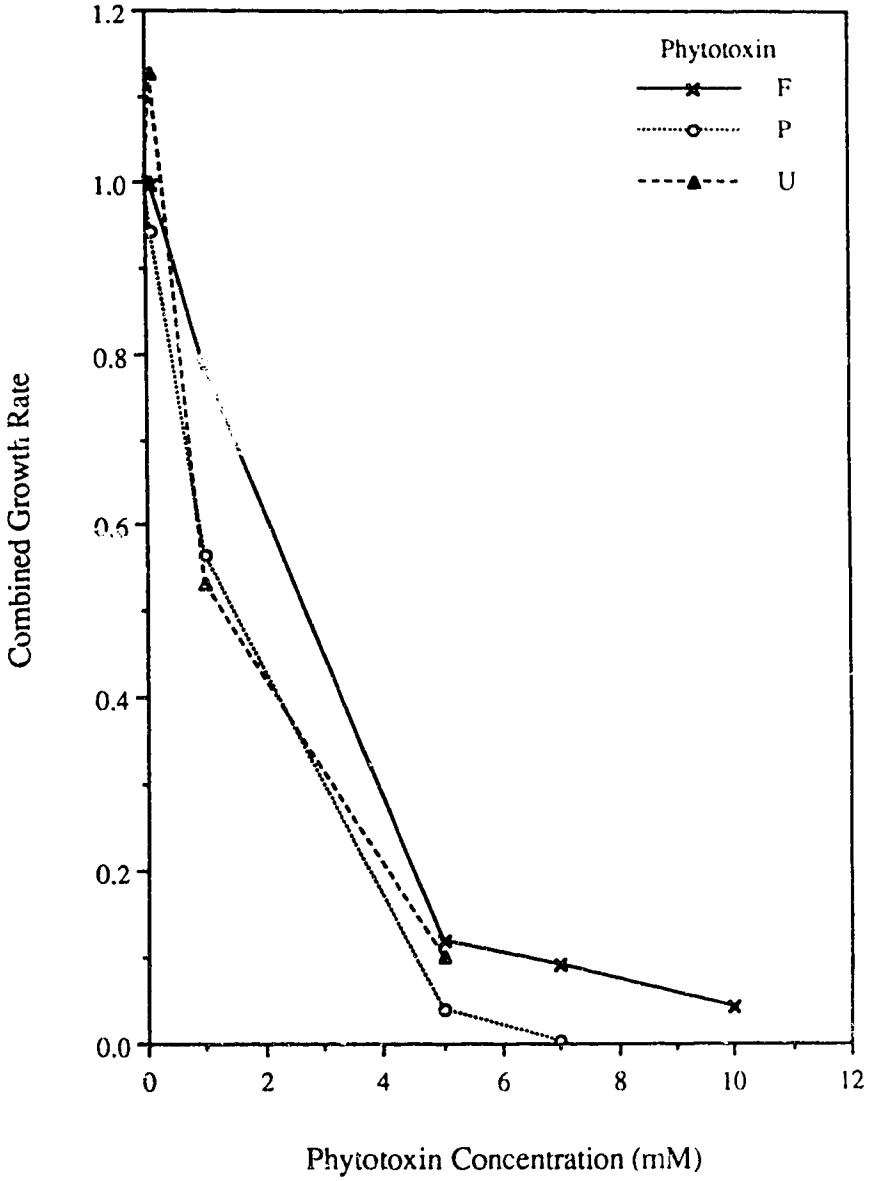


Figure 16 Phytotoxic Effects on Roamer Combined Growth Rates

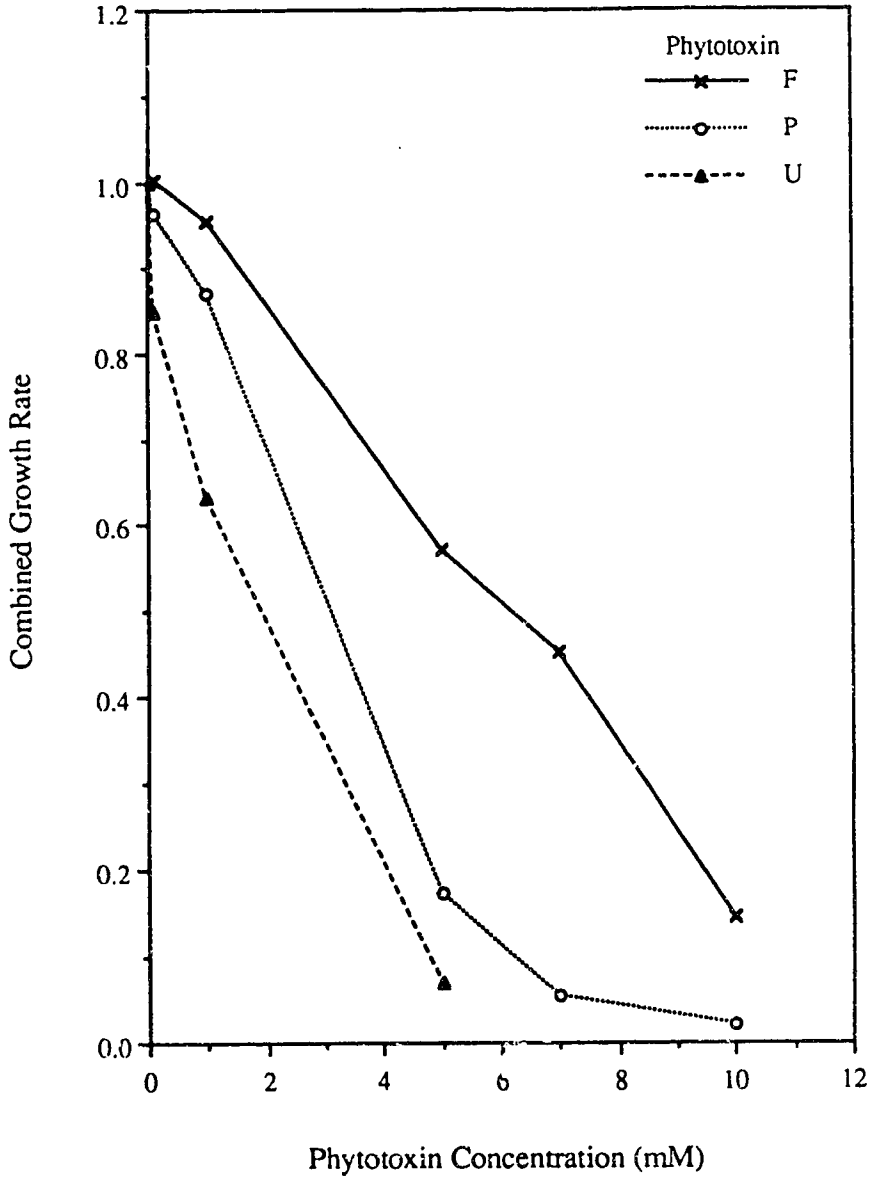


Figure 17 Phytotoxic Effects on Spredor-2 Combined Growth Rates

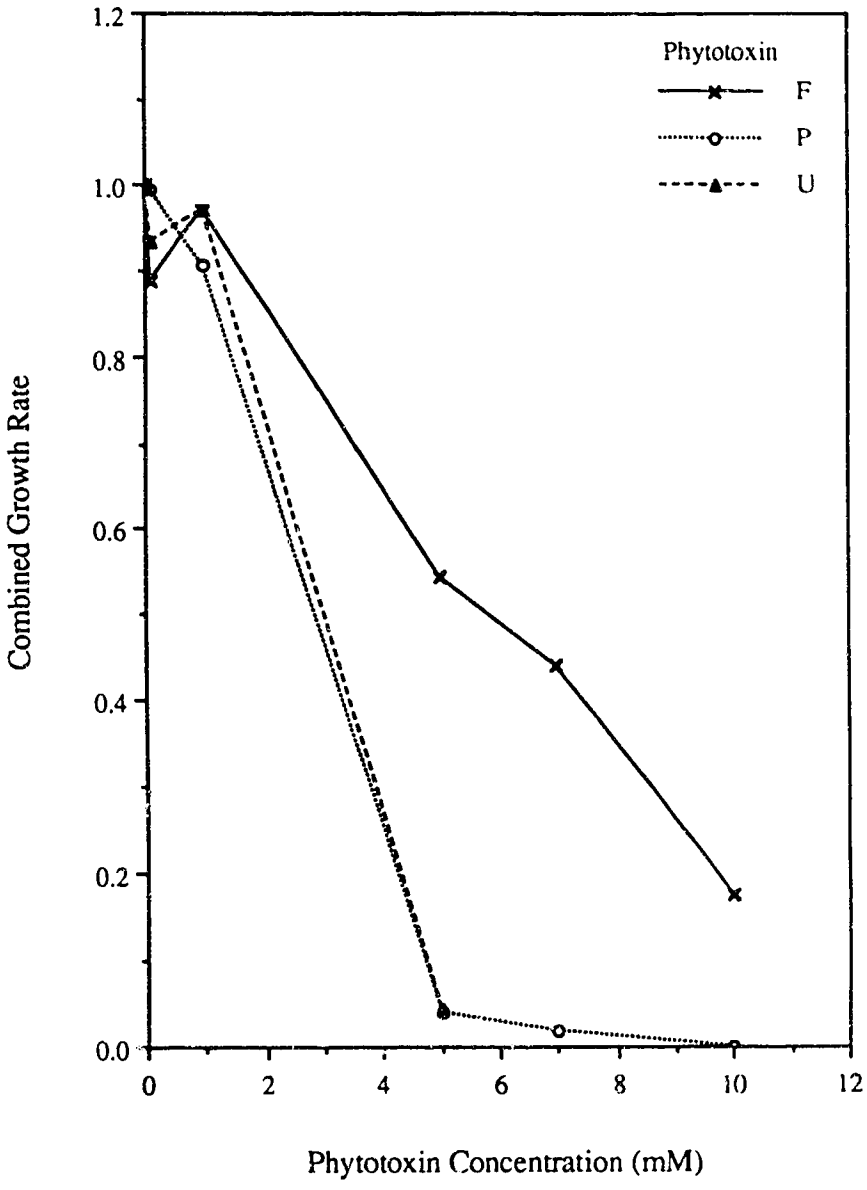


Table 11 Statistical Analyses on Genelle Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.807 0.812 0.857 P F U
1.0	0.369 0.619 0.779 P U F
5.0	0.000 0.006 0.022 P U F
7.0	—
10.0	—
0 to 5[b]	0.538 0.621 0.653 P U F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 12 Statistical Analyses on *Medicago falcata* L.-127 Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.943 1.006 1.065 P F U
1.0	0.830 1.037 1.066 F U P
5.0	0.102 0.247 0.920 U P F
7.0	0.037 0.723 P F
10.0	0.015 0.404 P F
0 to 5[b]	0.801 0.814 0.939 U P F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 13 Statistical Analyses on *Medicago sativa* L.-503 Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.965 1.037 1.066 U F P
1.0	0.844 0.984 0.985 U F P
5.0	0.231 0.321 0.705 U P F
7.0	0.061 0.550 P F
10.0	0.008 0.289 P F
0 to 5[b]	0.760 0.843 0.931 U P F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 14 Statistical Analyses on Regen-S Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.943 0.995 1.126 P F U
1.0	0.533 0.564 0.787 U P F
5.0	0.040 0.099 0.118 P U F
7.0	0.003 0.091 P F
10.0	—
0 to 5[b]	0.636 0.680 0.737 P U F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 15 Statistical Analyses on Roamer Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.849 0.964 1.003 U P F
1.0	0.633 0.869 0.954 U P F
5.0	0.071 0.172 0.571 U P F
7.0	0.055 0.454 P F
10.0	0.021 0.147 P F
0 to 5[b]	0.638 0.751 0.882 U P F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 16 Statistical Analyses on Spredor-2 Phytotoxic Combined Growth Rates

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Combined Growth Rates[a]
0.1	0.886 0.932 0.994 F U P
1.0	0.904 0.969 0.970 P F U
5.0	0.038 0.043 0.543 P U F
7.0	0.019 0.439 P F
10.0	0.001 0.176 P F
0 to 5[b]	0.734 0.736 0.849 P U F

Note.

[a]Phytotoxic combined growth rates depicted above the phytotoxin symbols were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined growth rates from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 17 Statistical Analyses on the Varietal and Phytotoxic Combined Growth Rates

Phytotoxin Concentration[a] (mM)	Duncan's Multiple Range Test Performed on the Varietal and Phytotoxic Combined Growth Rates[b]
0 to 5	0.605 0.684 0.758 0.773 0.845 0.851 GN RG RM SP MS MF
0 to 5	0.712 0.730 0.841 U P F

Note.

[a]Combined growth rates from the 0 mM to 5 mM concentration range were used in the overall varietal and phytotoxic analyses. For the varietal analysis, the replication, phytotoxic, and concentration effects were statistically removed. The replication, varietal, and concentration effects were statistically removed for the phytotoxic analysis.

[b]Varietal and phytotoxic combined growth rates depicted above the variety and phytotoxin symbols respectively, were ranked in increasing value from left to right. Growth rates underscored by the same line were not significantly different, while growth rates not underscored by the same line were significantly different (DMR: α=0.05).

Table 18 History of Calli Tested for Phytotoxin Resistance

Calli[a]	Final Concentration of Phytotoxin in Plates	Time Growing on Final Phytotoxic MS Plates (days)	Total Time Growing on Phytotoxic MS Plates (days)
RG(S8)-5	5 mM ferulic acid	137	378
SP-12	10 mM ferulic acid	137	274
SP-12	5 mM *p*-coumaric acid	137	274
SP-12	5 mM umbelliferone	137	274
RM-10	5 mM *p*-coumaric acid	137	274

Note.

[a]Calli were grown on phytotoxic MS plates. Healthy tissue was transferred to fresh plates with sequential increases in the phytotoxin concentration. When the final phytotoxin concentration was reached, calli were maintained at this concentration for 137 days. Transfer numbers of calli were not increased after the first exposure of the calli to a phytotoxin.

Table 19 Test for Resistance in Calli Growing on Phytotoxic MS Plates[a]

Calli[b]	Phytotoxin Concentration	Fresh Weight of Never Exposed Calli[c] (mg)	Fresh Weight of Previously Exposed Calli[d] (mg)
RG(S8)-5	5 mM ferulic acid	101 ± 2	358 ± 99
SP-12	10 mM ferulic acid	103 ± 5	119 ± 9
SP-12	5 mM *p*-coumaric acid	101 ± 4	112 ± 13
SP-12	5 mM umbelliferone	93 ± 4	114 ± 3
RM-10	5 mM *p*-coumaric acid	148 ± 38	82 ± 2

Note.

[a]Fresh weight was measured at 25 days and displayed with one standard error (Equation 1).

[b]Transfer numbers of the never exposed and previously exposed calli were not increased after the first exposure of the previously exposed calli to a phytotoxin.

[c]Calli were never exposed to a phytotoxin before the start of this experiment.

[d]Calli were previously exposed to a phytotoxin before the start of this experiment (Table 18).

Table 20 Further Analysis into the Growth of RG(S8)-5 Calli[a]

History[b]	Phytotoxin Concentration	Fresh Weight of Calli[c] (mg)
NE	0 mM phytotoxin	1265 ± 69
NE	5 mM ferulic acid	271 ± 37
NE	5 mM *p*-coumaric acid	NG
NE	5 mM umbelliferone	NG
PE	5 mM ferulic acid	NG
PE	5 mM *p*-coumaric acid	NG
PE	5 mM umbelliferone	NG
ER	5 mM ferulic acid	NG

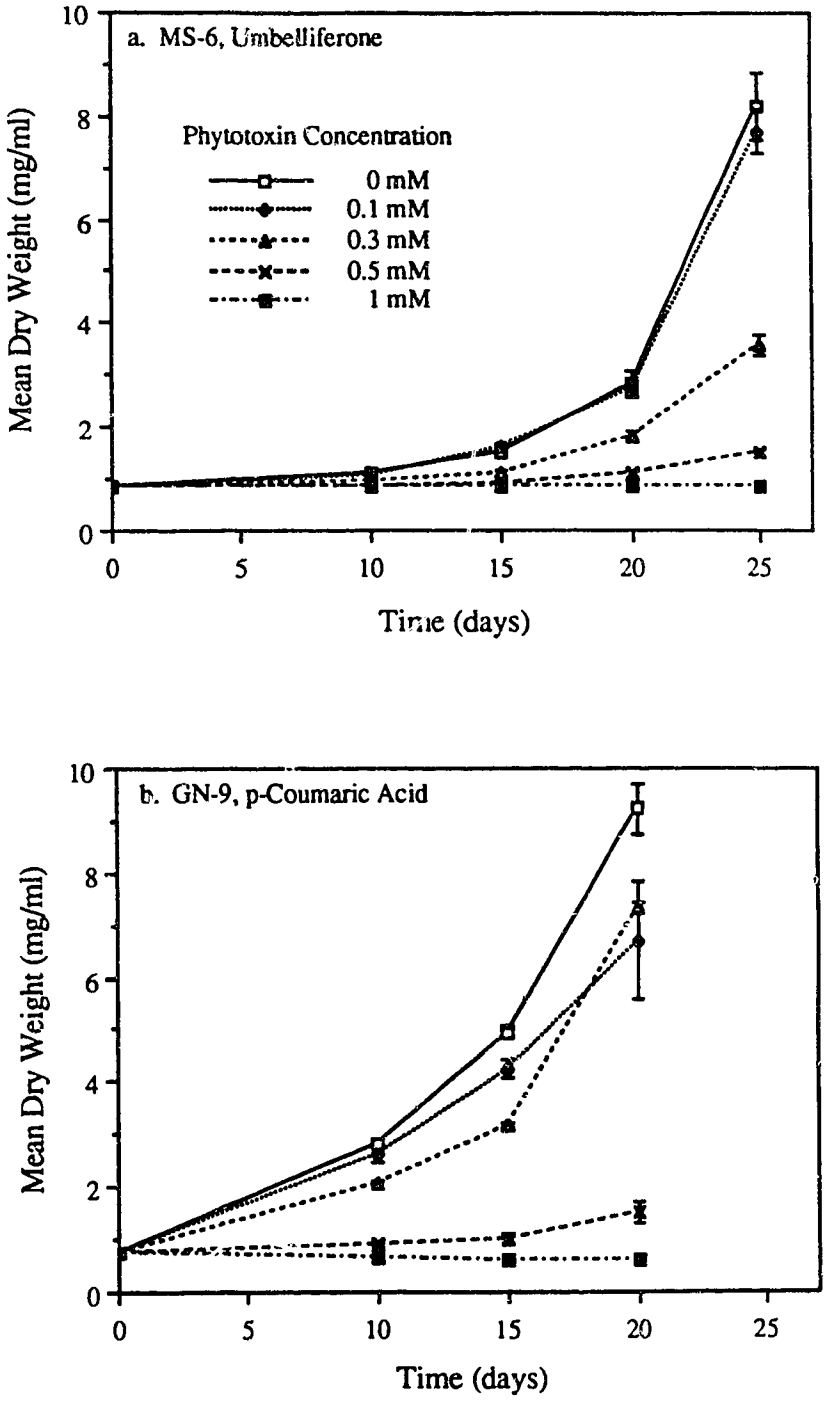
Note.

[a]Transfer number was not increased after the first exposure of the callus to ferulic acid.

[b]Three types of calli were used: (NE) Callus that was never exposed to a phytotoxin before this experiment, (PE) callus that was previously exposed to 5 mM ferulic acid for 184 days with a total exposure to ferulic acid of 425 days, and (ER) callus that was exposed to 5 mM ferulic acid for 95 days with a total exposure to ferulic acid of 336 days and subsequently removed from ferulic acid for 89 days.

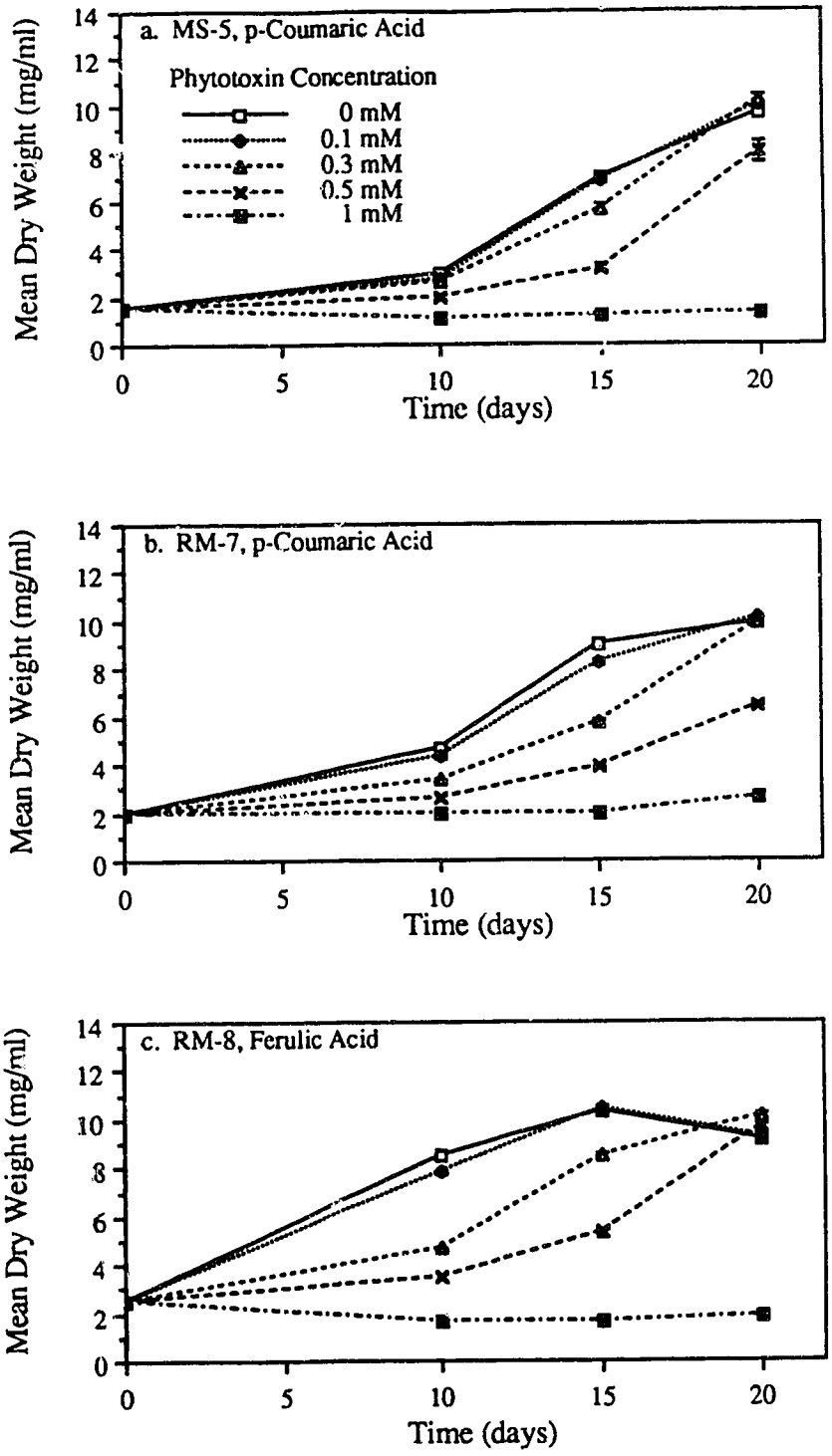
[c]Fresh weight was measured at 20 days and displayed with one standard error (Equation 1). Calli with measurements less than 100 mg (inoculation weight) were designated by no growth (NG).

Figure 18 Variation in Lag Phase Demonstrated by MS-6 and GN-9 Cell Suspensions



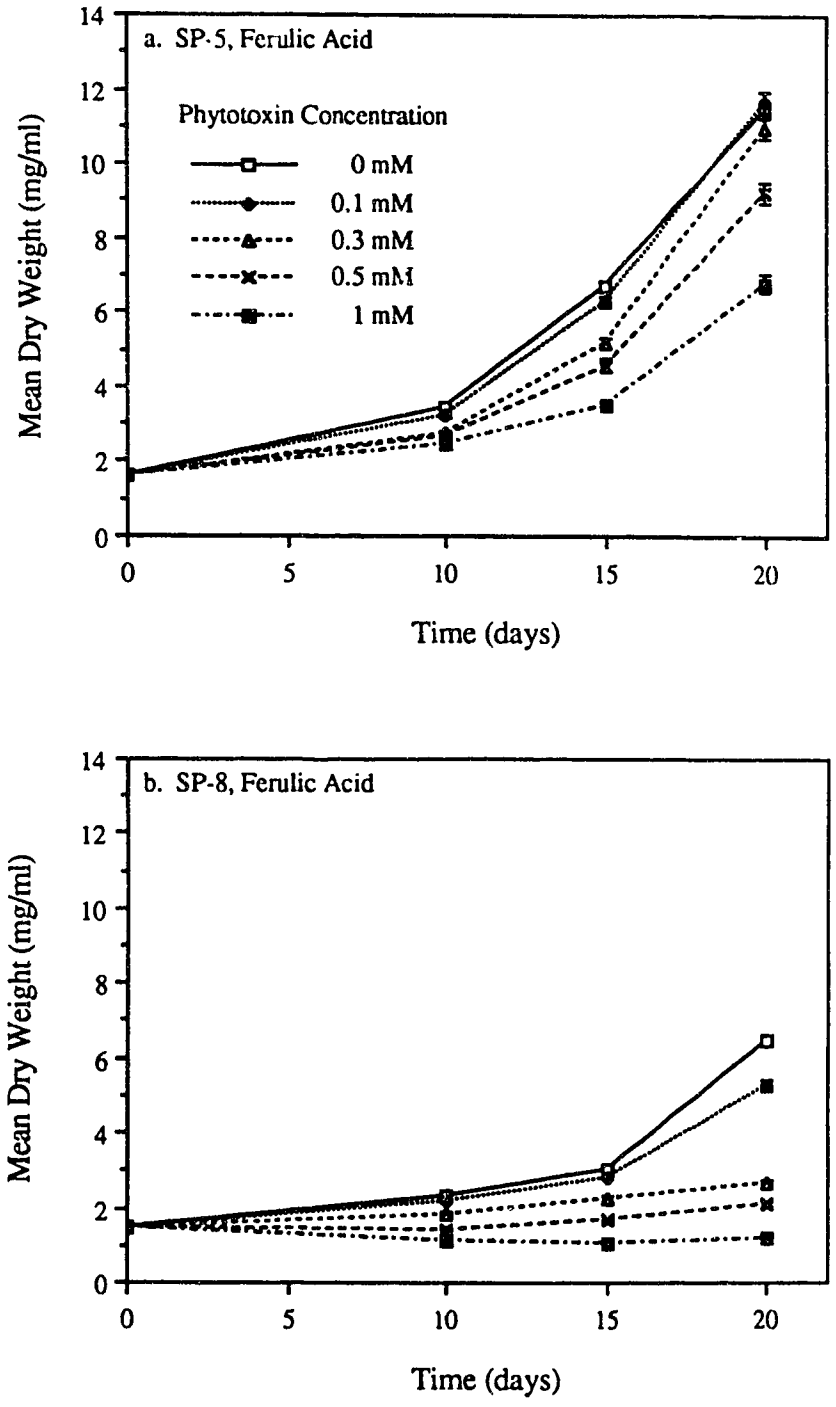
Note.
Error bars indicated were one standard error (Equation 1).

Figure 19 Variation in Stationary Phase Demonstrated by MS-5, RM-7, and RM-8 Cell Suspensions



Note.
Error bars indicated were one standard error (Equation 1).

Figure 20 Variation in the Replications of Spredor-2 Cell Suspensions



Note.
Error bars indicated were one standard error (Equation 1).

Figure 21 Variation in pH with Growth of GN-9 Cell Suspensions

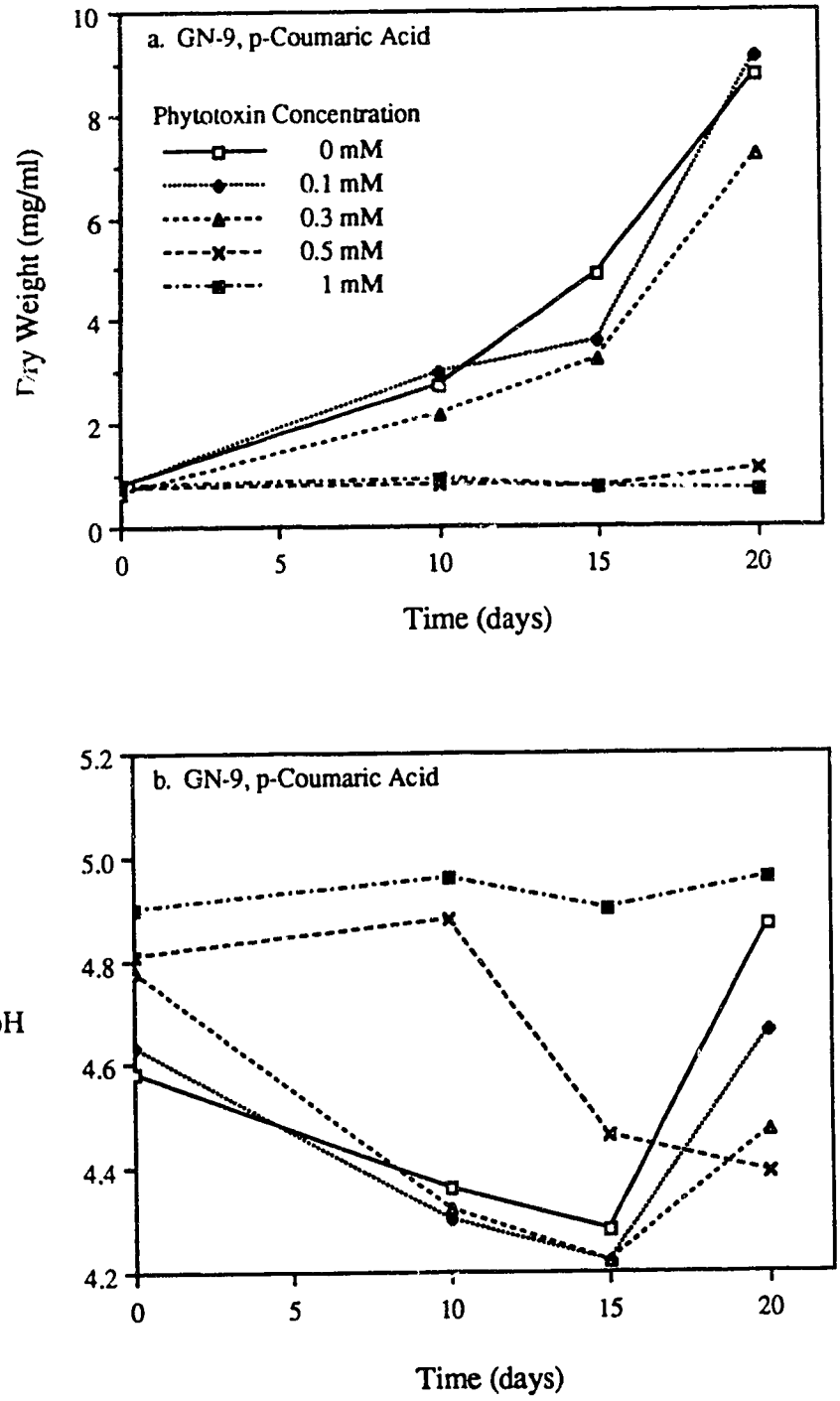
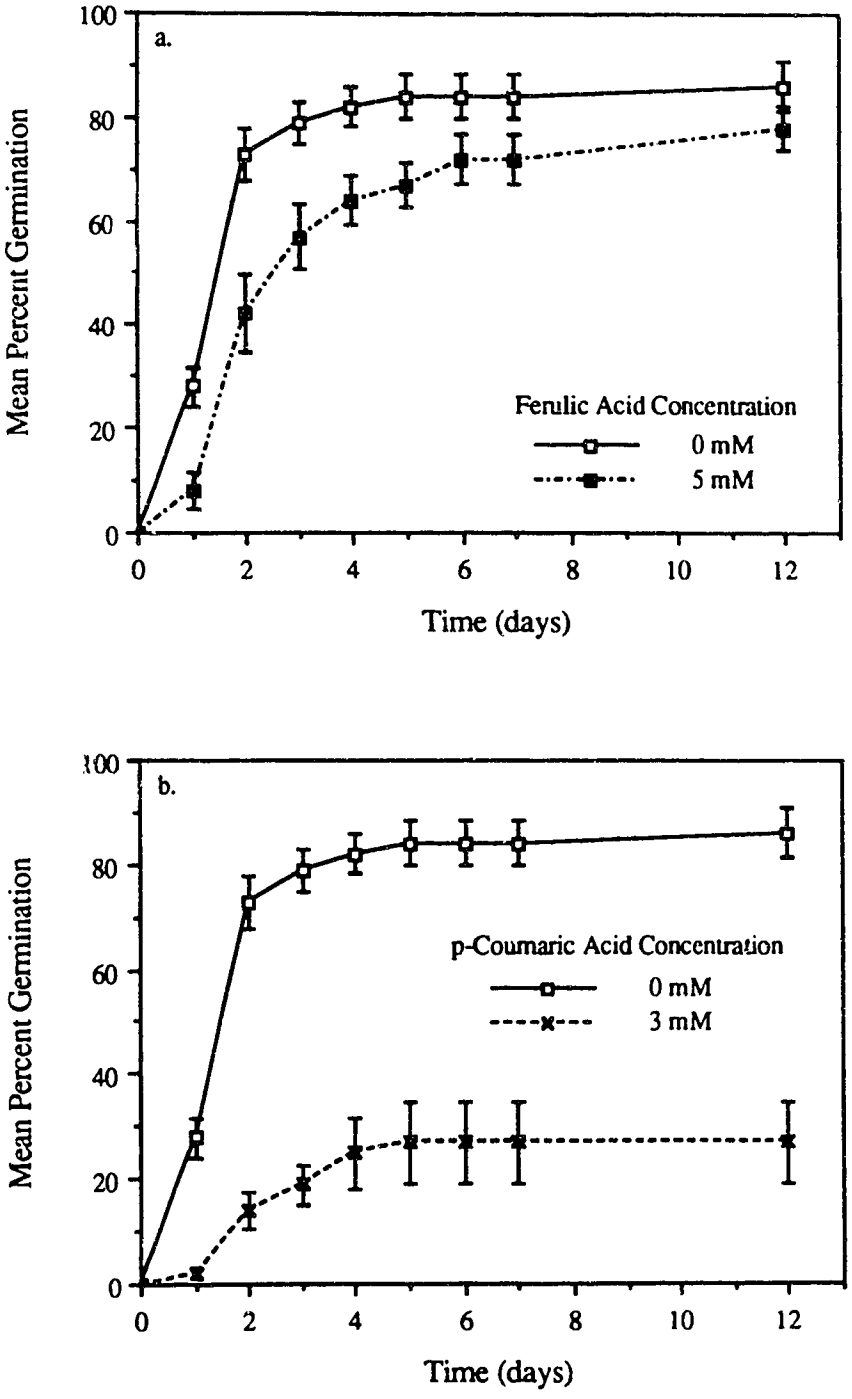


Figure 22 Mean Percent Germination of Roamer Alfalfa



Note.
Error bars indicated were one standard error (Equation 1).

Table 21 Statistical Analyses on the Mean Logarithmic Percent Germination[a]

Variety	Phytotoxin	Phytotoxin Concentration							
		0.1 mM		1 mM		3 mM		5 mM	
		Day 2	Day 12	Day 2	Day 12	Day 2	Day 12	Day 2	Day 12
GN	F	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
GN	P	NSD	NSD	*	NSD	**	NSD	**	*
GN	U	NSD	NSD	*	NSD	**	NSD	**	*
RM	F	NSD	NSD	NSD	NSD	NSD	NSD	**	NSD
RM	P	NSD	NSD	NSD	NSD	**	**	**	**
RM	U	NSD	NSD	NSD	NSD	**	NSD	**	**
SP	F	NSD	NSD	NSD	NSD	NSD	NSD	*	NSD
SP	P	NSD	NSD	NSD	NSD	**	NSD	**	**
SP	U	NSD	NSD	NSD	NSD	**	NSD	**	NSD

Note.

[a]Results of the analyses were designated by NSD (no significant difference (DMR: α=0.05)), * (significant difference (DMR: α=0.05)), and ** (highly significant difference (DMR: α=0.01)), when comparing the mean logarithmic percent germinations from unexposed seeds to those exposed to a phytotoxin.

Table 22 Effects of Phytotoxins on Alfalfa Germination[a]

Variety	Phytotoxin	Phytotoxin Concentration			
		0.1 mM	1 mM	3 mM	5 mM
GN	F	NE	NE	NE	NE
GN	P	NE	D	D	I
GN	U	NE	D	D	I
RM	F	NE	NE	NE	D
RM	P	NE	NE	I	I
RM	U	NE	NE	D	I
SP	F	NE	NE	NE	D
SP	P	NE	NE	D	I
SP	U	NE	NE	D	D

Note.

[a]Results of the analyses were represented by NE (no effect), D (delay in germination), and I (inhibition in germination). A delay in germination was defined as a significant difference (DMR: α=0.05) in the mean logarithmic percent germinations at the second day which was not apparent at the twelfth day, whereas an inhibition in germination was defined as a significant difference (DMR: α=0.05) at the twelfth day.

Figure 23 Effects of Ferulic Acid on the Varietal Standardized Percent Germination

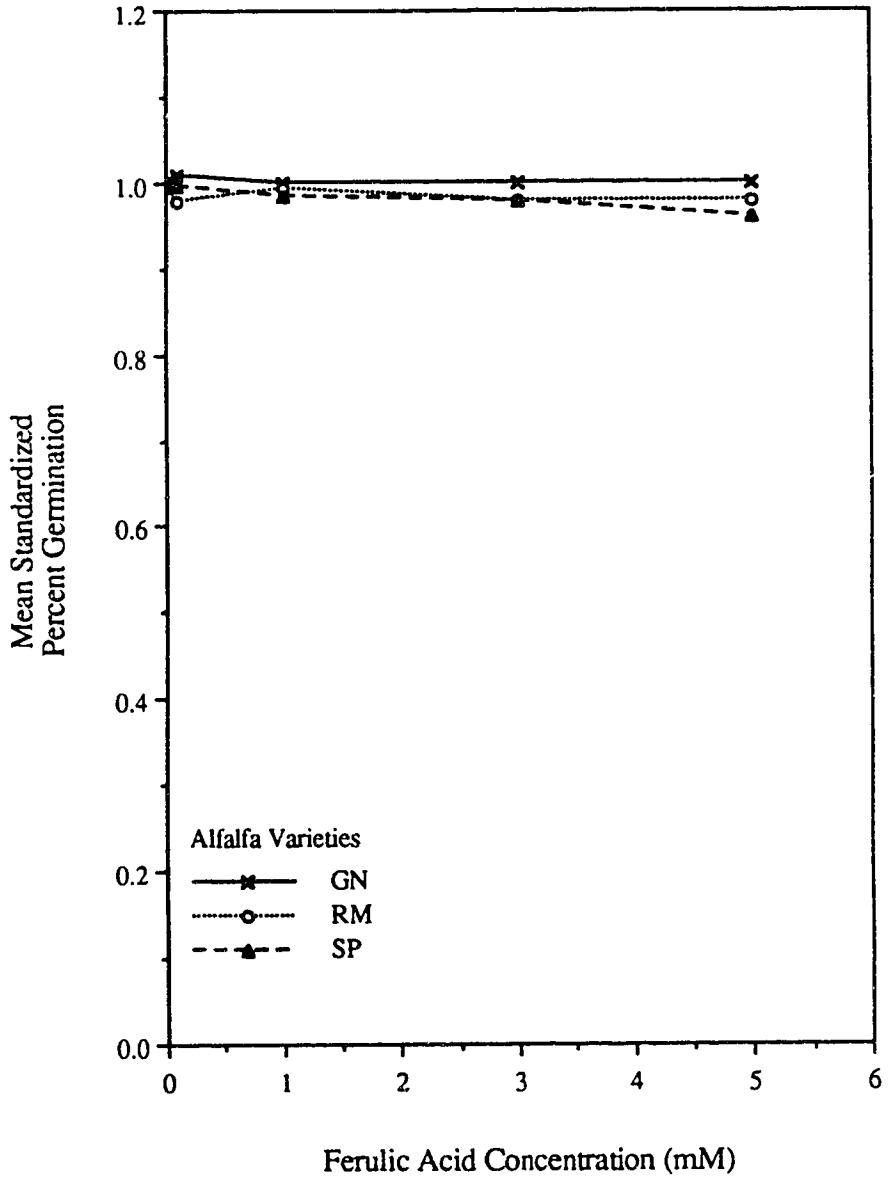


Figure 24 Effects of *p*-Coumaric Acid on the Varietal Standardized Percent Germination

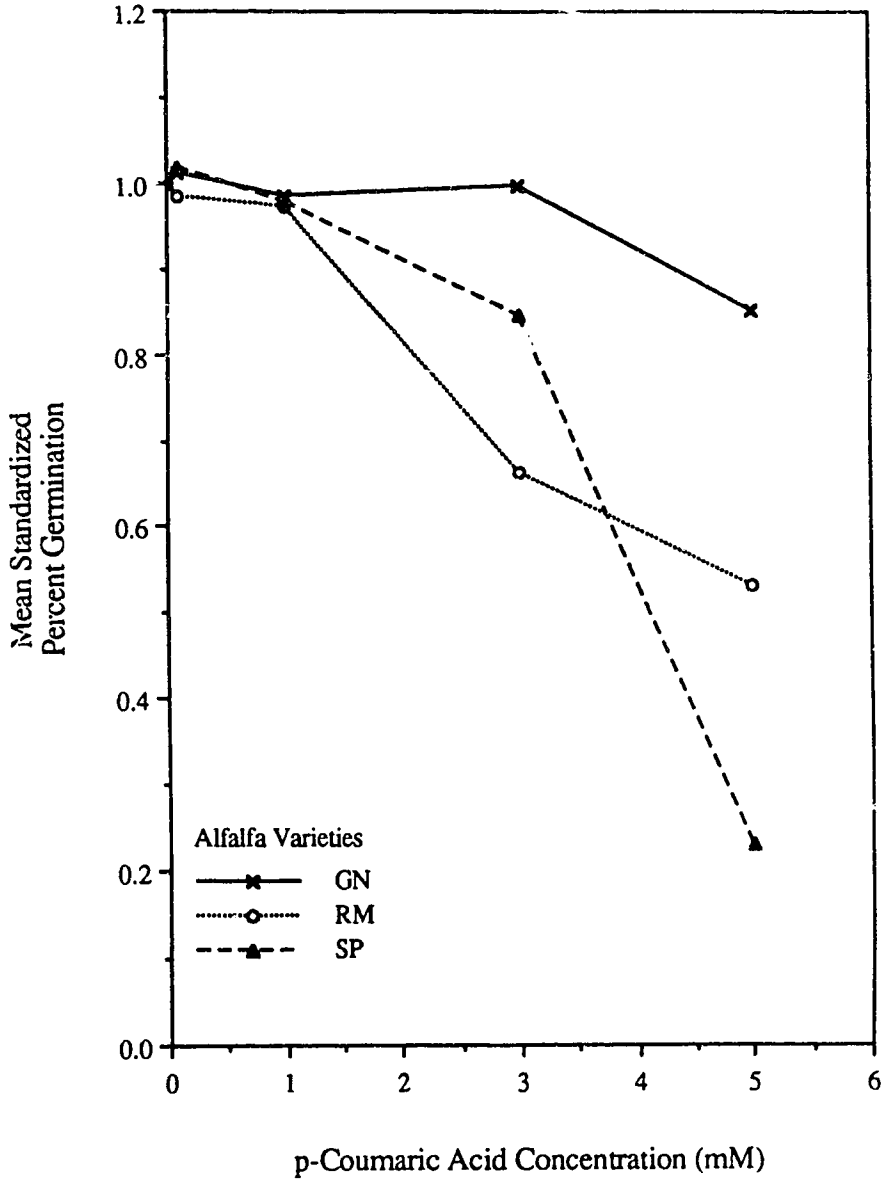


Figure 25 Effects of Umbelliferone on the Varietal Standardized Percent Germination

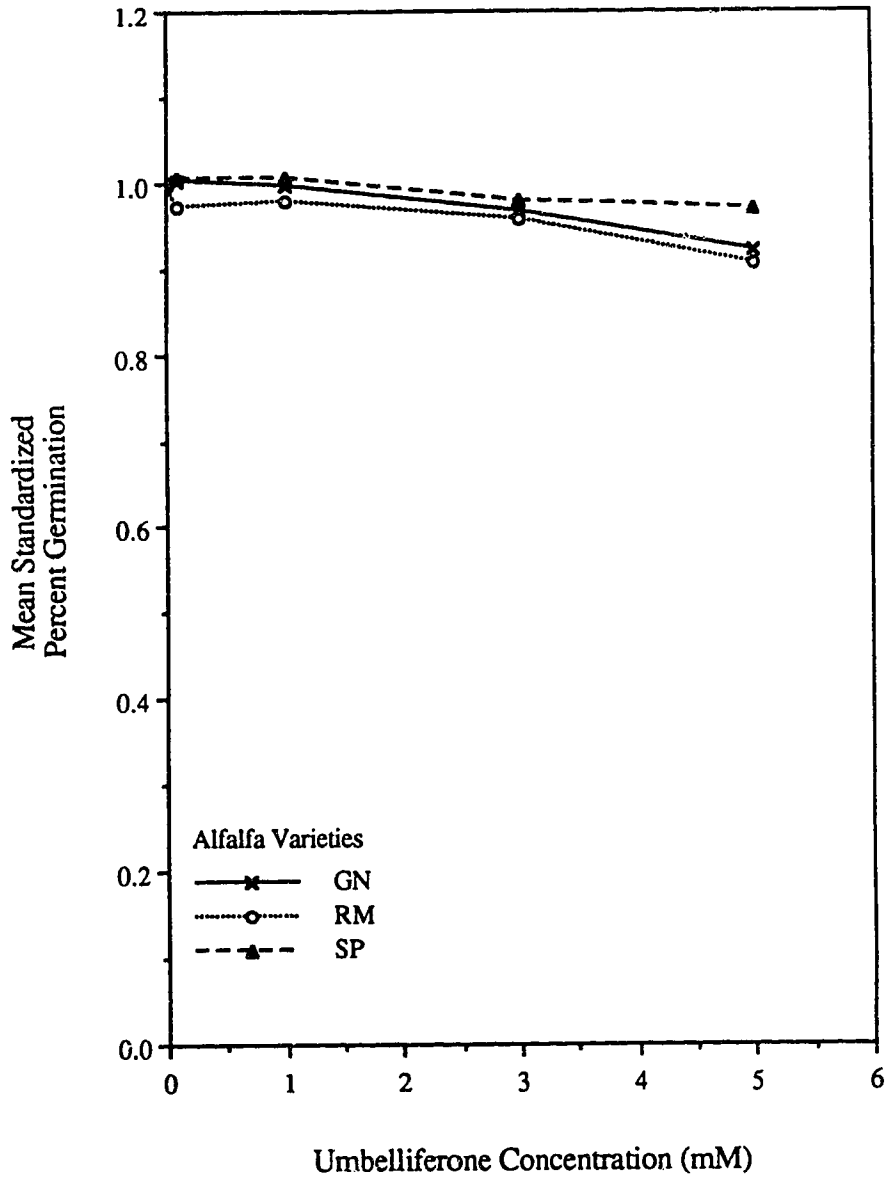


Table 23 Statistical Analyses on the Varietal Standardized Percent Germination From Alfalfa Seeds Germinated in Nutritive Ferulic Acid Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Mean Standardized Percent Germination[a]
0.1	0.979 0.995 1.010 RM SP GN
1.0	0.984 0.993 1.000 SP RM GN
3.0	0.977 0.979 1.000 RM SP GN
5.0	0.960 0.978 1.000 SP RM GN
0 to 5[b]	0.984 0.985 1.002 SP RM GN

Note.

[a]Varietal mean standardized percent germinations depicted above the variety symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the concentration effect was statistically removed.

Table 24 Statistical Analyses on the Varietal Standardized Percent Germination From Alfalfa Seeds Germinated in Nutritive *p*-Coumaric Acid Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Mean Standardized Percent Germination[a]
0.1	0.984 1.012 1.017 RM GN SP
1.0	0.971 0.979 0.984 RM SP GN
3.0	0.661 0.845 0.997 RM SP GN
5.0	0.231 0.531 0.850 SP RM GN
0 to 5[b]	0.814 0.829 0.969 SP RM GN

Note.

[a]Varietal mean standardized percent germinations depicted above the variety symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the concentration effect was statistically removed.

Table 25 Statistical Analyses on the Varietal Standardized Percent Germination From Alfalfa Seeds Germinated in Nutritive Umbelliferone Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Varietal Mean Standardized Percent Germination[a]
0.1	0.973 1.002 1.005 RM GN SP
1.0	0.979 0.995 1.005 RM GN SP
3.0	0.958 0.965 0.978 RM GN SP
5.0	0.905 0.920 0.968 RM GN SP
0 to 5[b]	0.963 0.976 0.991 RM GN SP

Note.

[a]Varietal mean standardized percent germinations depicted above the variety symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall varietal analysis. For this analysis, the concentration effect was statistically removed.

Figure 26 Phytotoxic Effects on Genelle Standardized Percent Germination

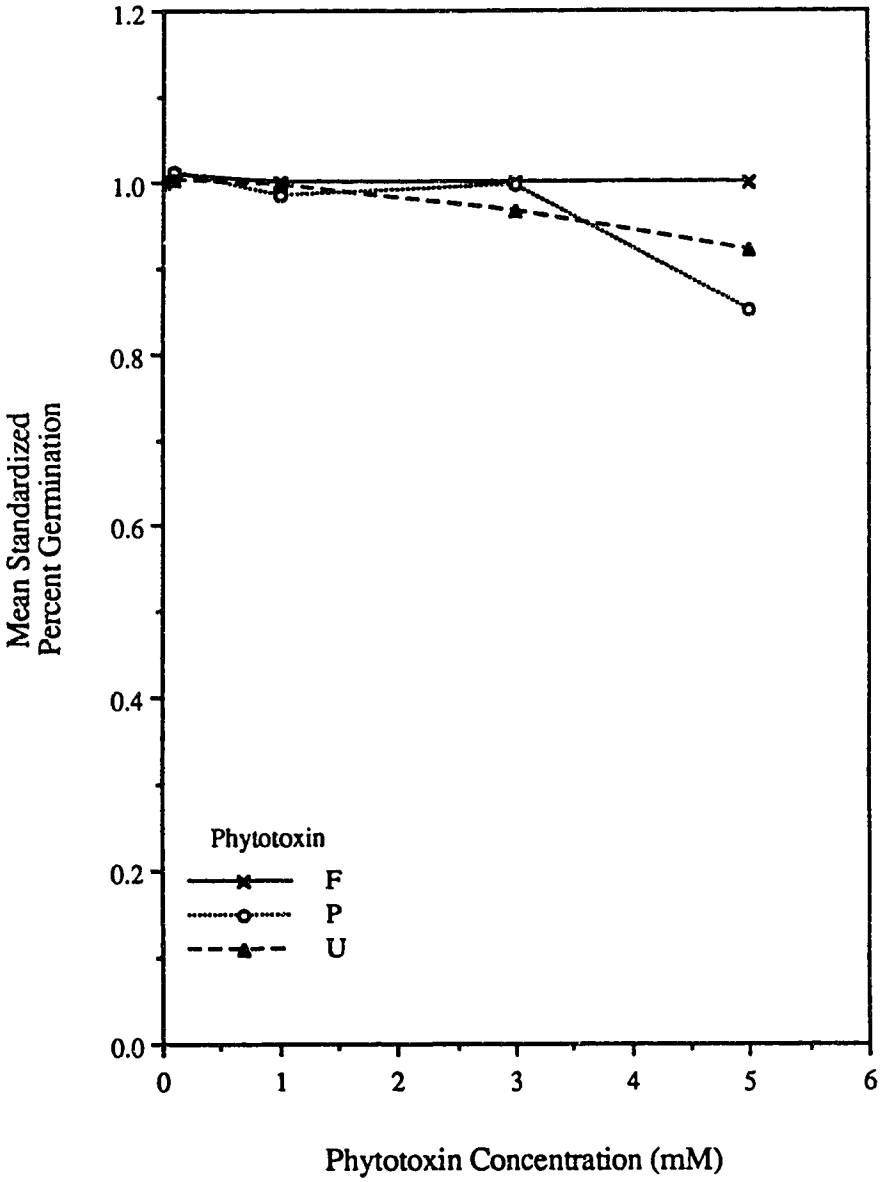


Figure 27 Phytotoxic Effects on Roamer Standardized Percent Germination

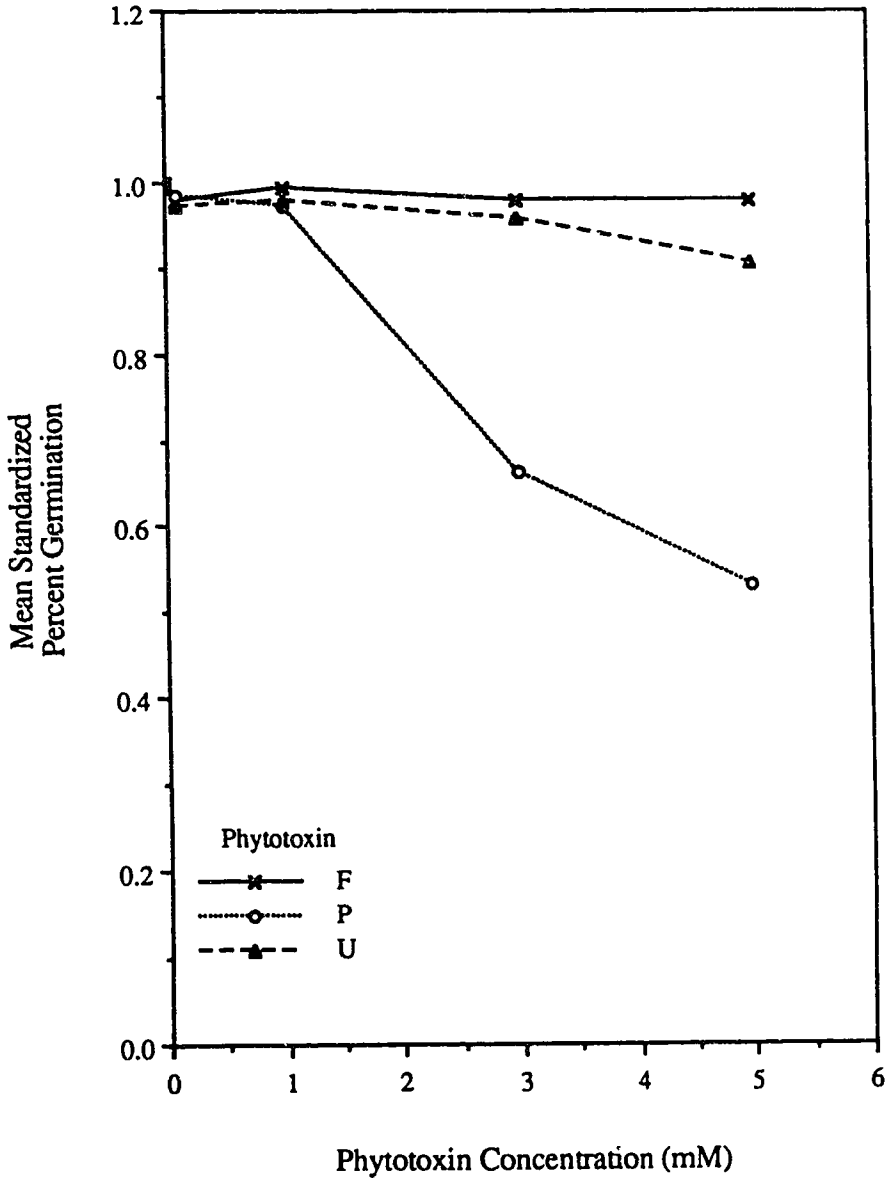


Figure 28 Phytotoxic Effects on Spredor-2 Standardized Percent Germination

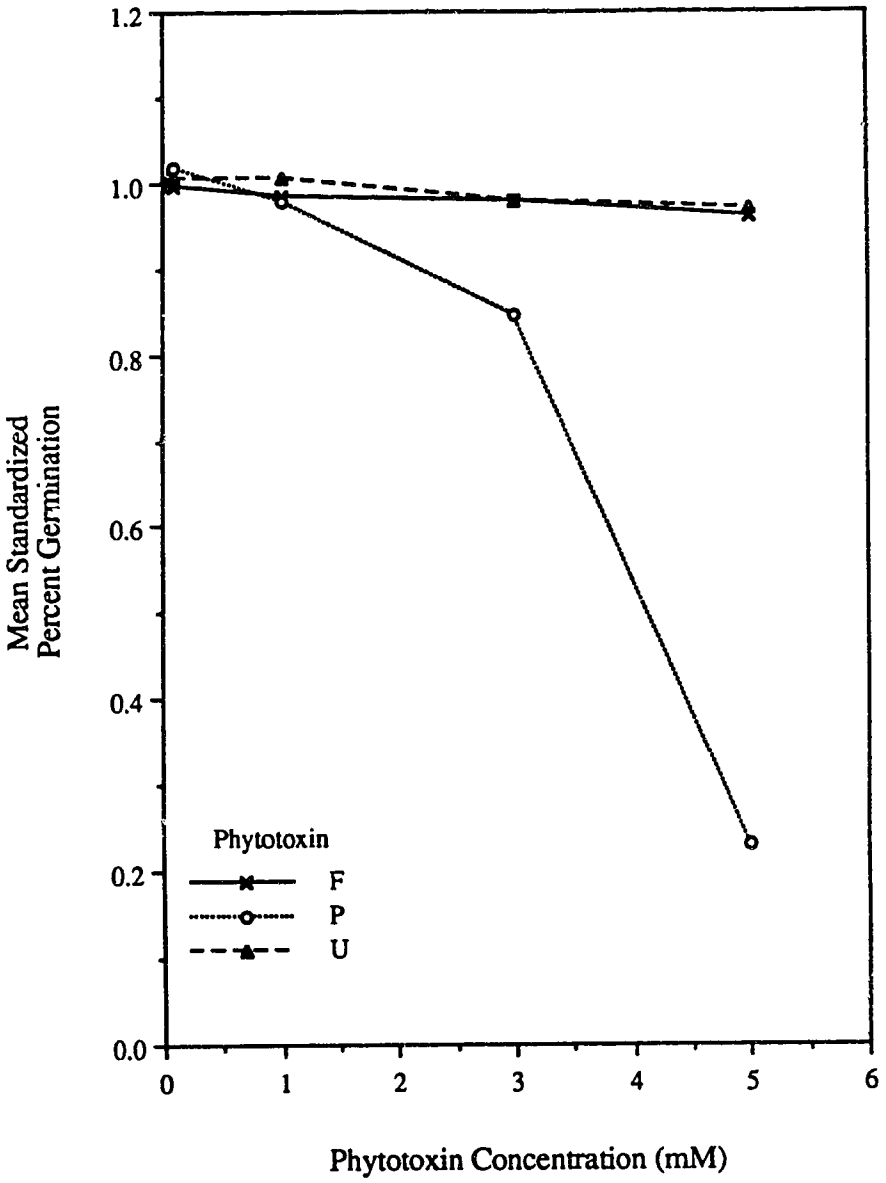


Table 26 Statistical Analyses on Genelle Phytotoxic Standardized Percent Germination

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Mean Standardized Percent Germination[a]
0.1	1.002 1.010 1.012 U F P
1.0	0.984 0.995 1.000 P U F
3.0	0.965 0.997 1.000 U P F
5.0	0.850 0.920 1.000 P U F
0 to 5[b]	0.969 0.976 1.002 P U F

Note.

[a]Phytotoxic mean standardized percent germinations depicted above the phytotoxin symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the concentration effect was statistically removed.

Table 27 Statistical Analyses on Roamer Phytotoxic Standardized Percent Germination

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Mean Standardized Percent Germination[a]
0.1	0.973 0.979 0.984 U F P
1.0	0.971 0.979 0.993 P U F
3.0	0.661 0.958 0.977 P U F
5.0	0.551 0.905 0.978 P U F
0 to 5[b]	0.829 0.963 0.985 P U F

Note.

[a]Phytotoxic mean standardized percent germinations depicted above the phytotoxin symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the concentration effect was statistically removed.

Table 28 Statistical Analyses on Spredor-2 Phytotoxic Standardized Percent Germination

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on Phytotoxic Mean Standardized Percent Germination[a]
0.1	0.995 1.005 1.017 F U P
1.0	0.979 0.984 1.005 P F U
3.0	0.845 0.978 0.979 P U F
5.0	0.231 0.960 0.968 P F U
0 to 5[b]	0.814 0.984 0.991 P F U

Note.

[a]Phytotoxic mean standardized percent germinations depicted above the phytotoxin symbols were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall phytotoxic analysis. For this analysis, the concentration effect was statistically removed.

Table 29 Statistical Analyses on the Varietal and Phytotoxic Standardized Percent Germinations

Phytotoxin Concentration[a] (mM)	Duncan's Multiple Range Test Performed on Varietal and Phytotoxic Standardized Percent Germinations[b]
0 to 5	0.926 0.930 0.982 SP RM GN
0 to 5	0.871 0.977 0.990 P U F

Note.

[a]Standardized percent germinations from the 0 mM to 5 mM concentration range were used in the overall varietal and phytotoxic analyses. For the varietal analysis, the phytotoxic and concentration effects were statistically removed. The varietal and concentration effects were statistically removed for the phytotoxic analysis.

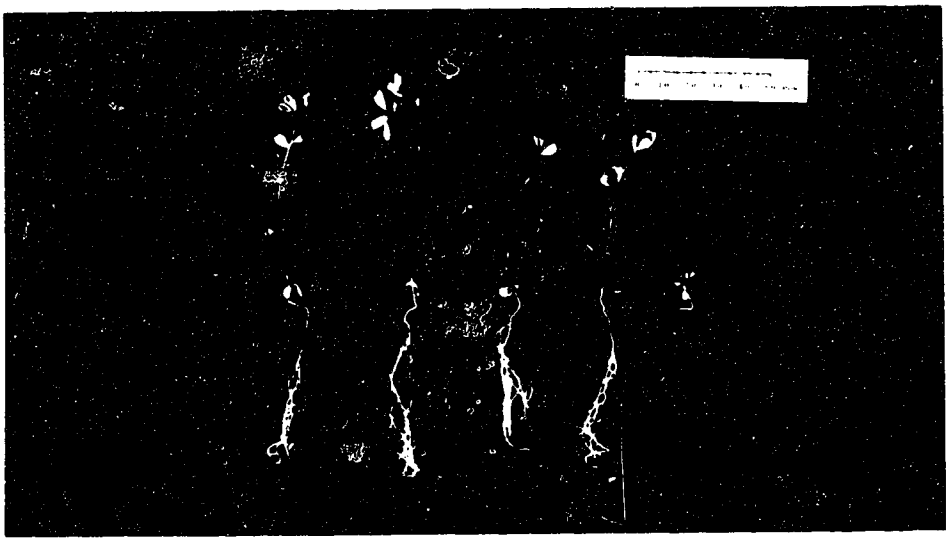
[b]Varietal and phytotoxic mean standardized percent germinations depicted above the variety and phytotoxic symbols respectively, were ranked in increasing value from left to right. Percent germinations underscored by the same line were not significantly different, while percent germinations not underscored by the same line were significantly different (DMR: α=0.05).

Plates 1 to 3 Three-Week-Old Alfalfa Seedlings Grown in Nutritive Phytotoxic Solution

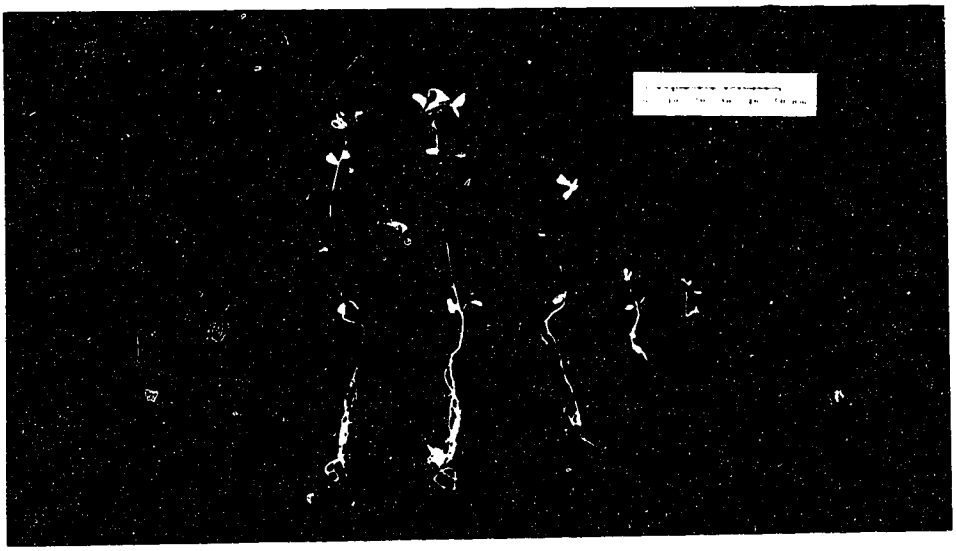
Note.
The three alfalfa varieties, Genelle (Plate1), Roamer (Plate 2), and Spredor-2 (Plate 3), were all grown in nutritive phytotoxic solutions consisting of ferulic acid (Plates 1a, 2a, and 3a), *p*-coumaric acid (Plates 1b, 2b, and 3b), or umbelliferone (Plates 1c, 2c, and 3c). The different phytotoxin concentrations utilised were 0 mM, 0.1 mM, 1 mM, 3 mM, and 5 mM. The resulting three-week-old alfalfa seedlings are displayed from left to right, respectively.

Plate 1

a



b



c

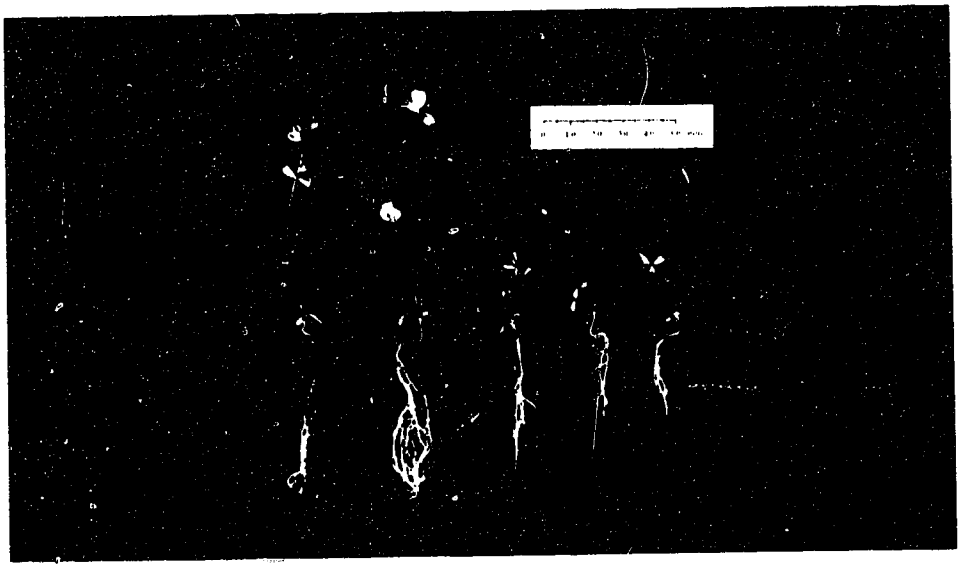
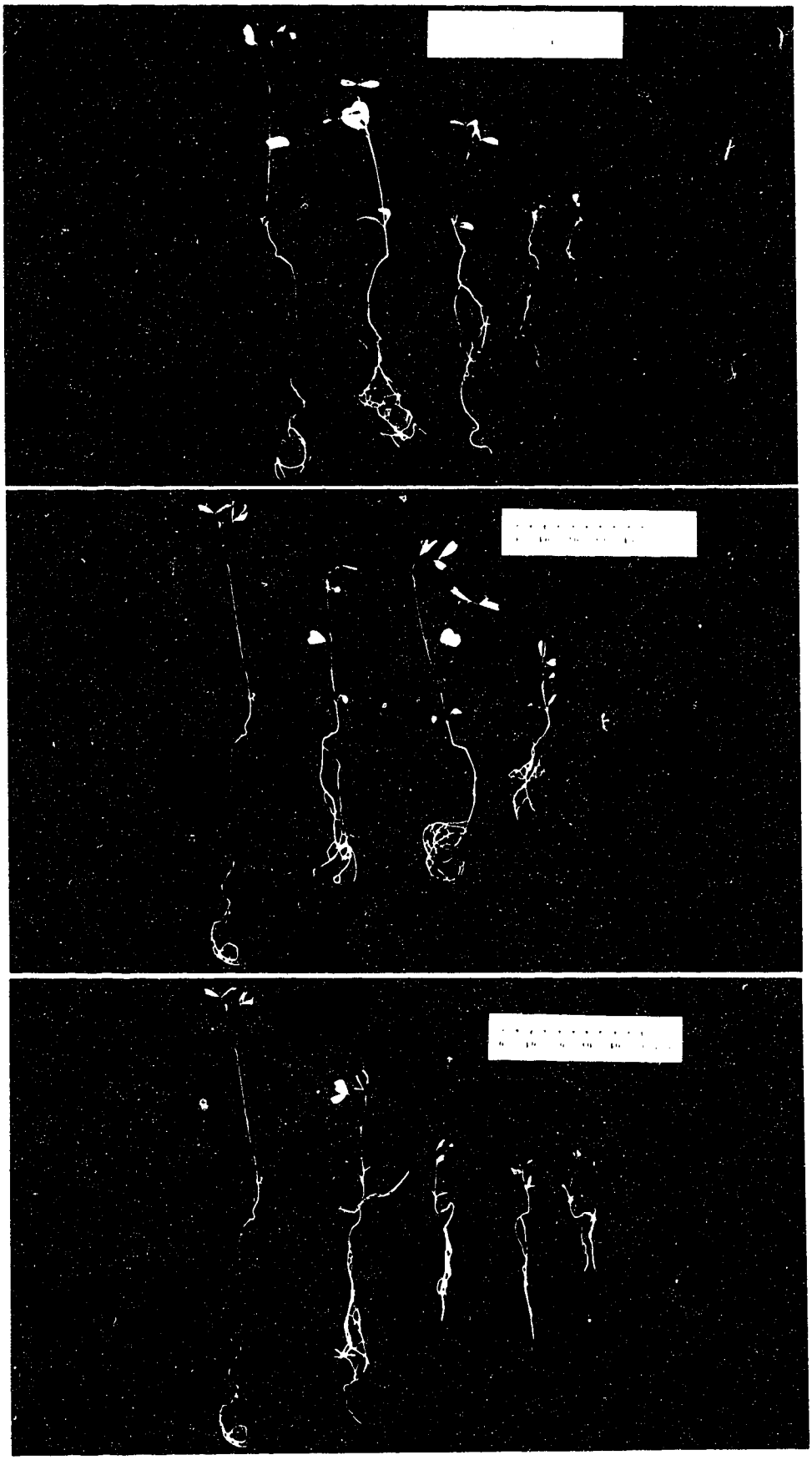


Plate 2



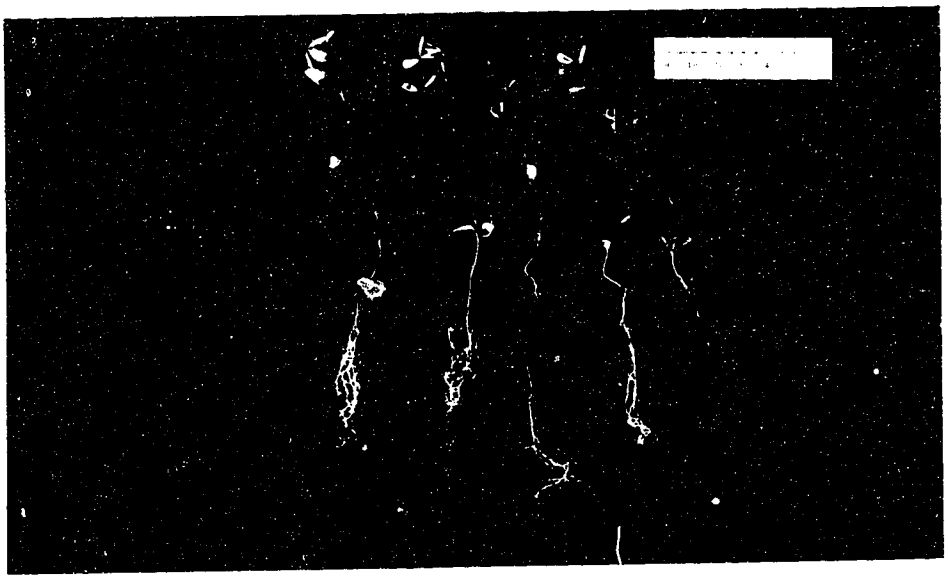
a

b

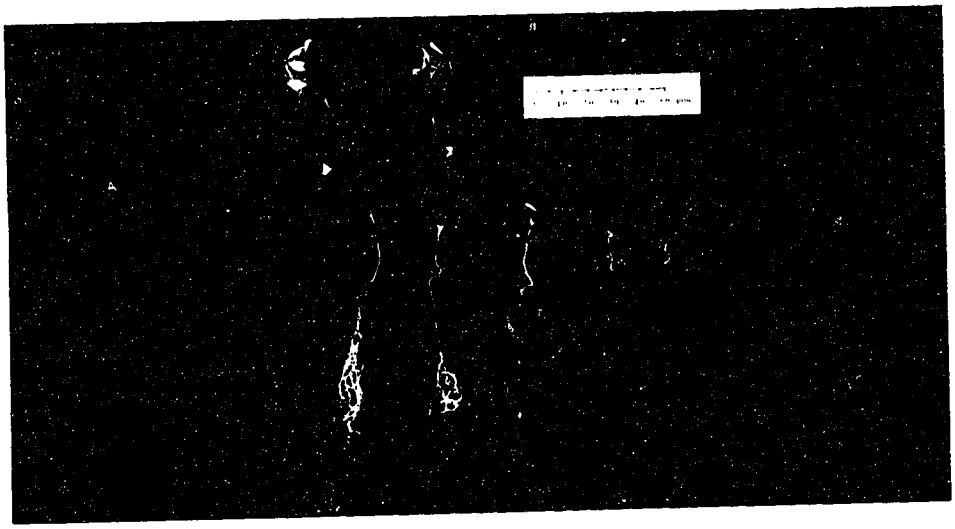
c

Plate 3

a



b



c

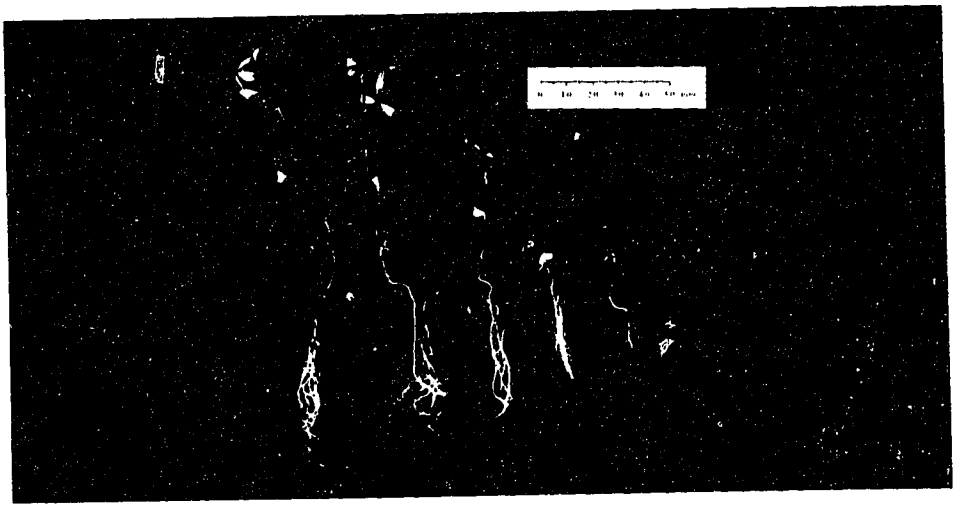


Figure 29 Variation in Three-Week-Old Genelle Seedling Total Length

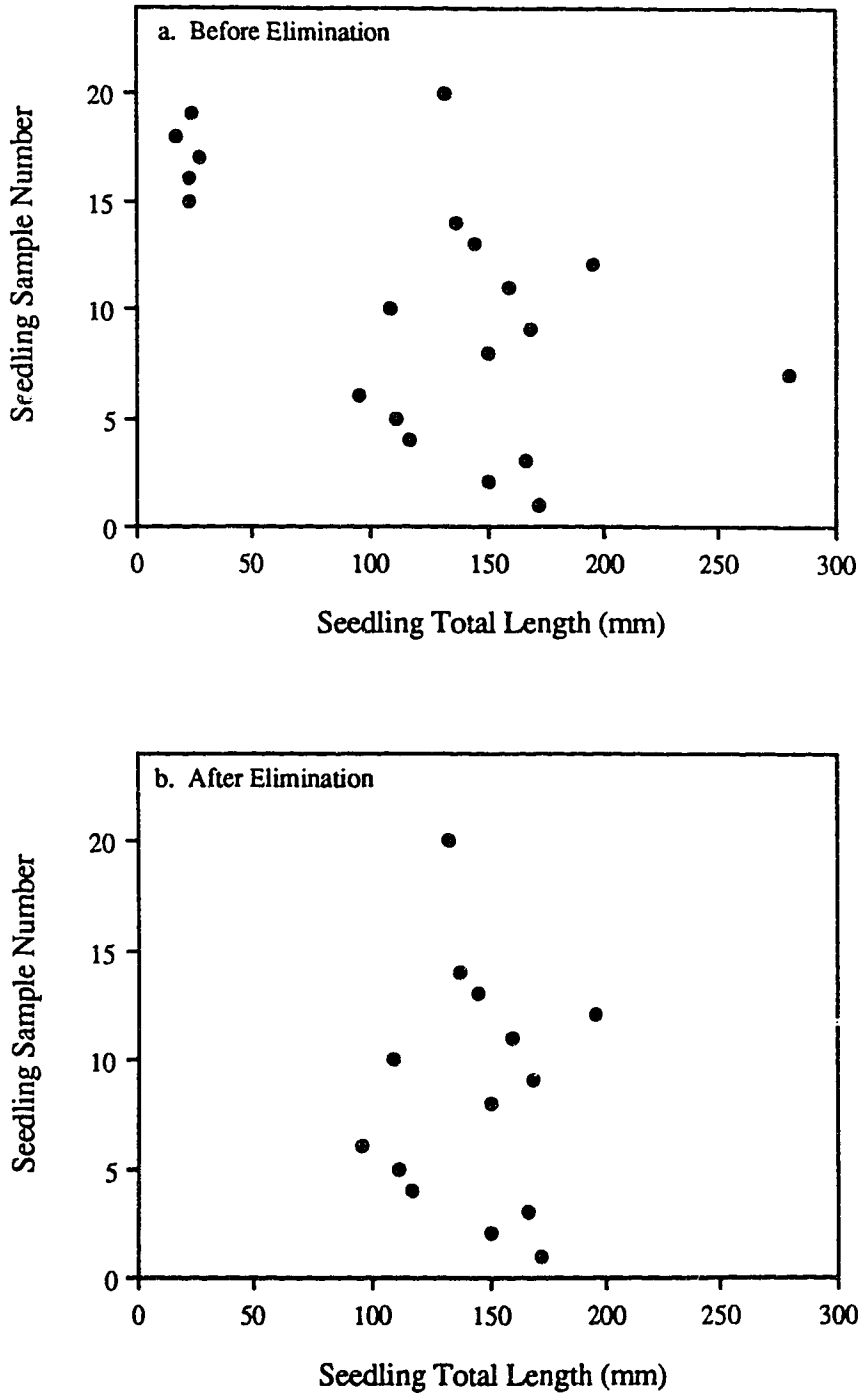


Figure 30 Variation in Three-Week-Old Spredor-2 Seedling Total Length

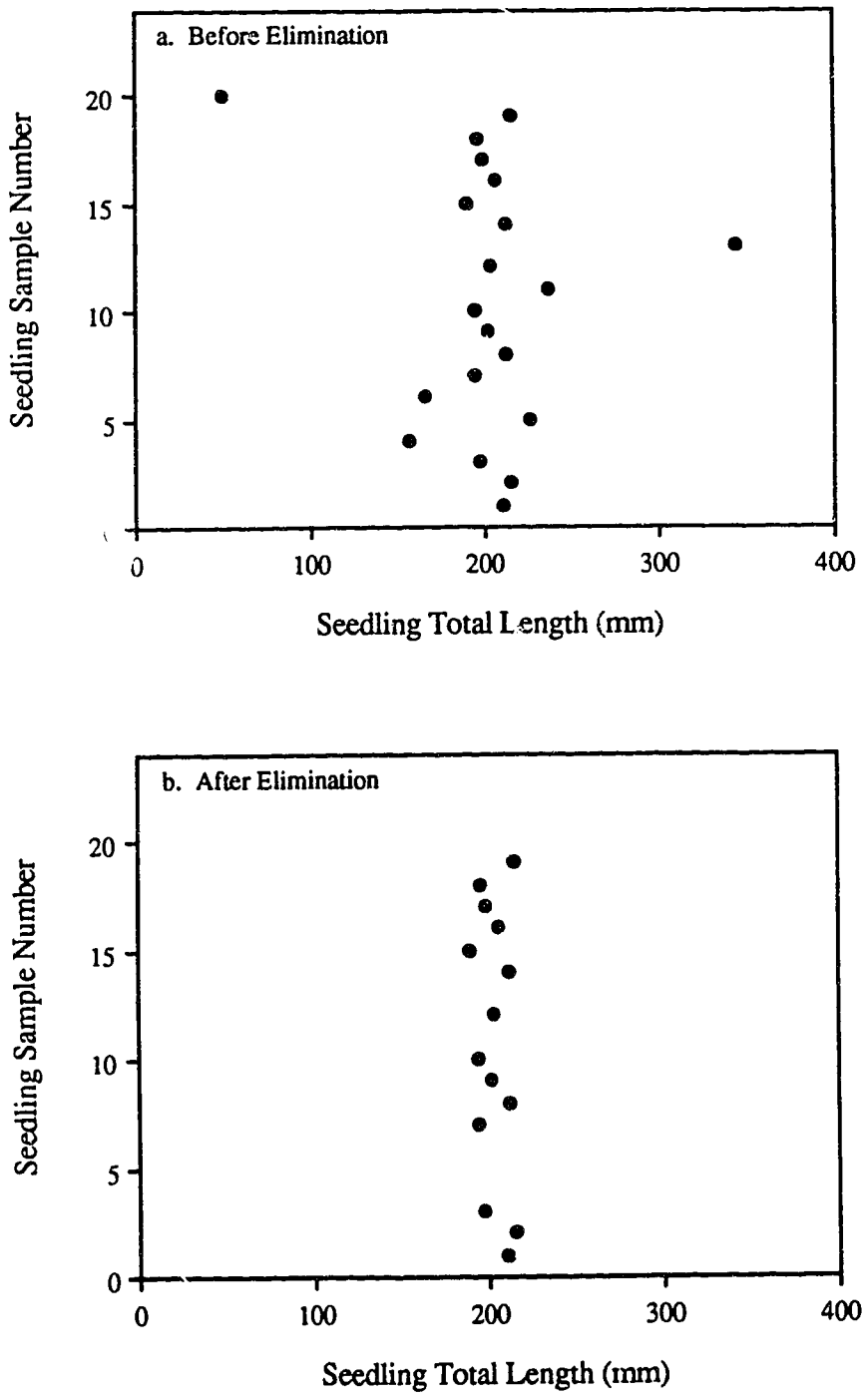
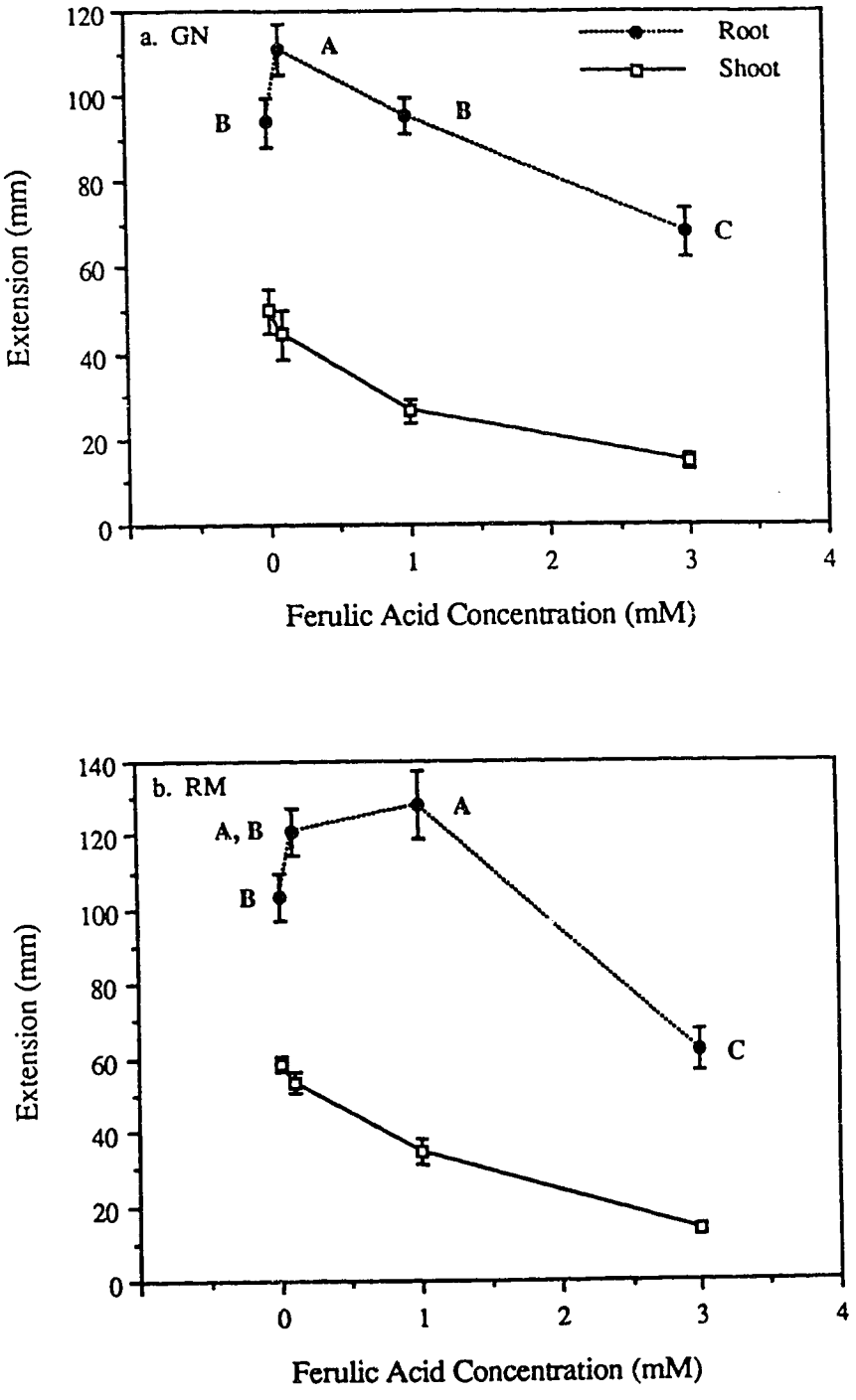
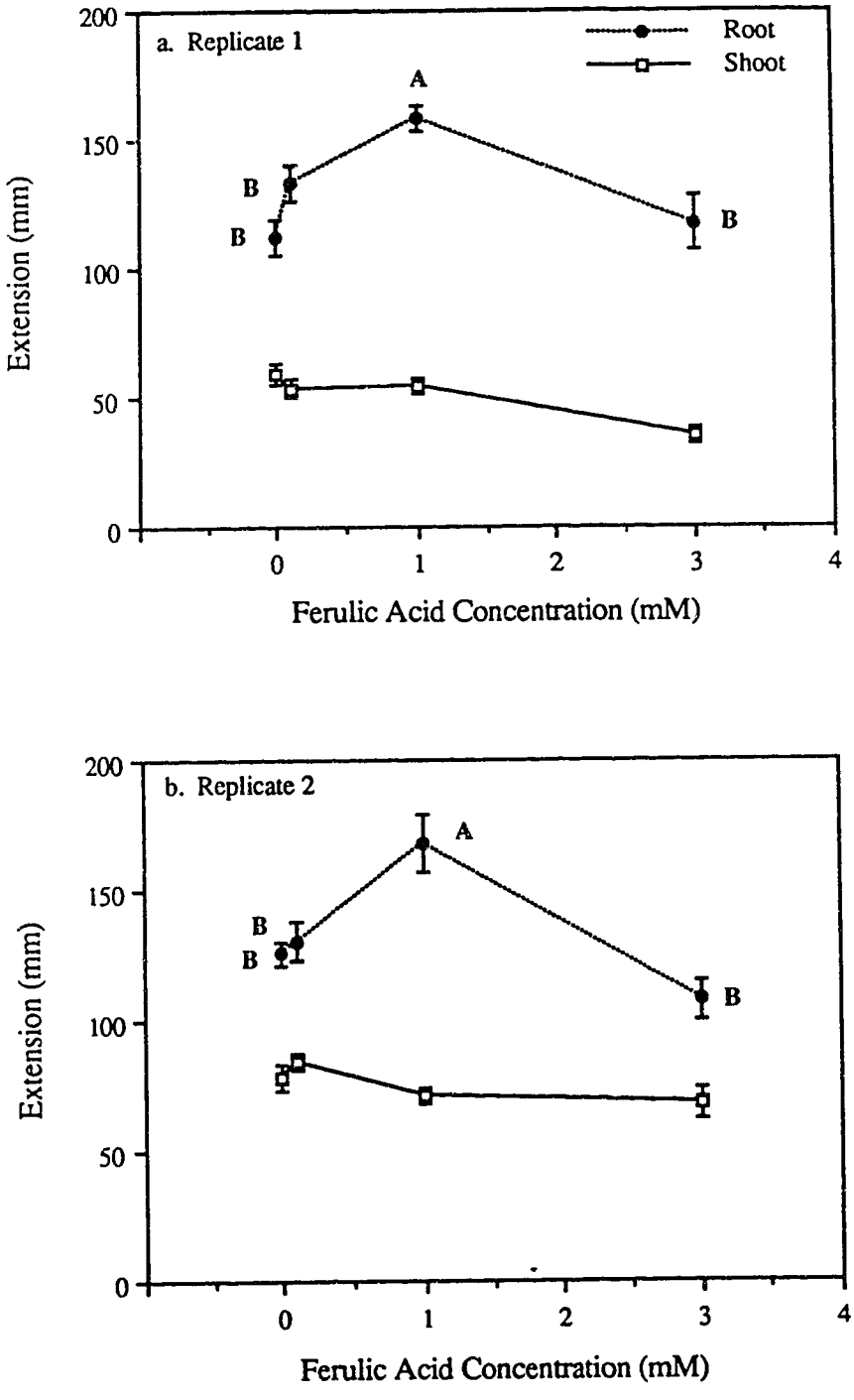


Figure 31 Significant Stimulation in Root Extensions of Two Alfalfa Varieties



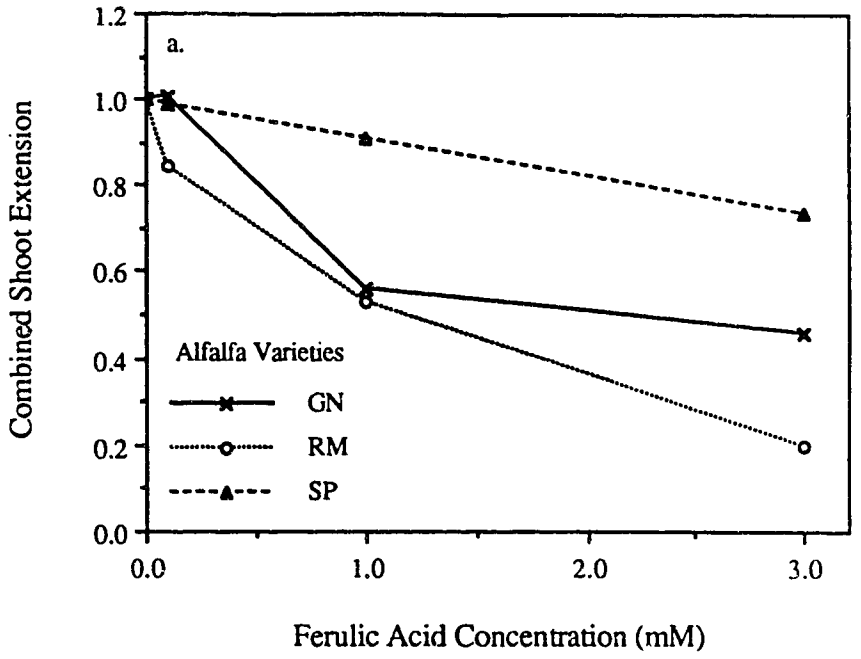
Note.
Mean root extensions with the same letter were not significantly different (DMR: α=0.05).
Error bars indicated were one standard error (Equation 1).

Figure 32 Significant Stimulation in Root Extensions In Spredor-2 Replicates



Note.
Mean root extensions with the same letter were not significantly different (DMR: α=0.05).
Error bars indicated were one standard error (Equation 1).

Figure 33 Effects of Ferulic Acid on the Extensions of Alfalfa Seedlings



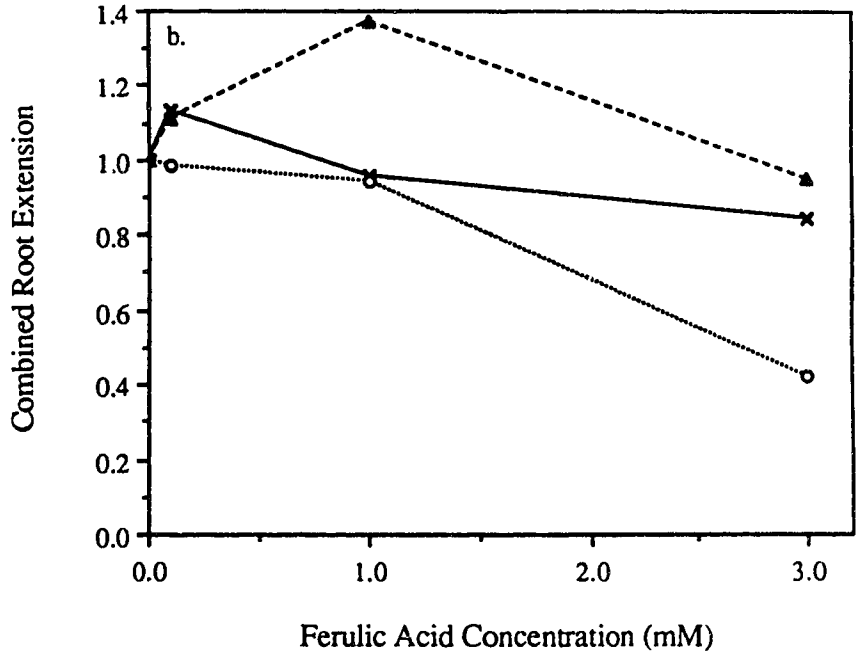
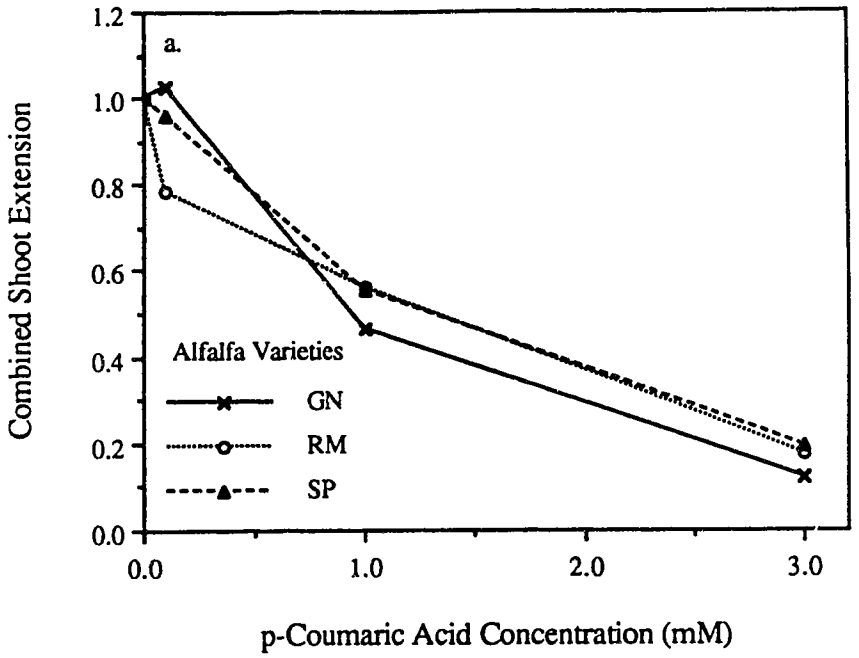


Figure 34 Effects of *p*-Coumaric Acid on the Extensions of Alfalfa Seedlings



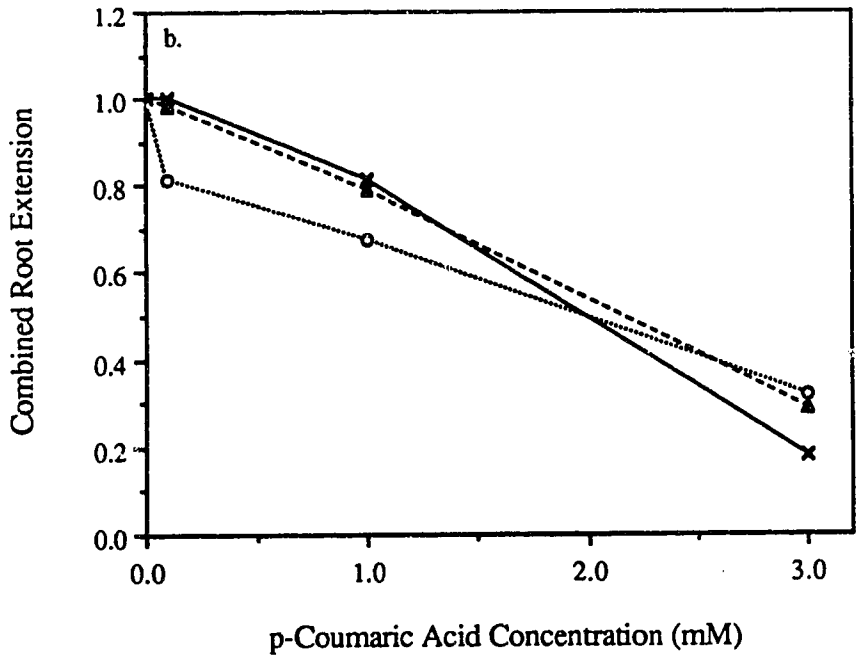
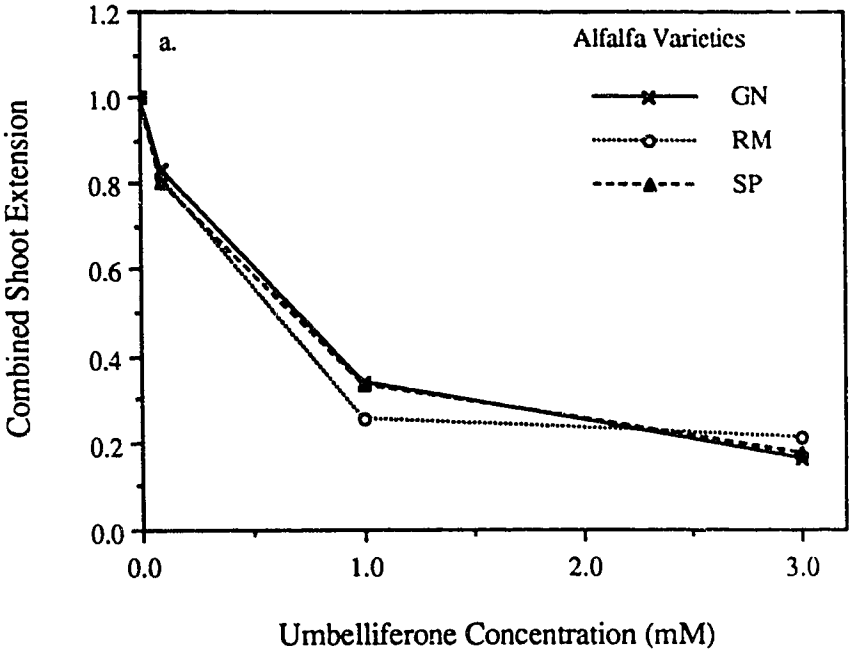


Figure 35 Effects of Umbelliferone on the Extensions of Alfalfa Seedlings



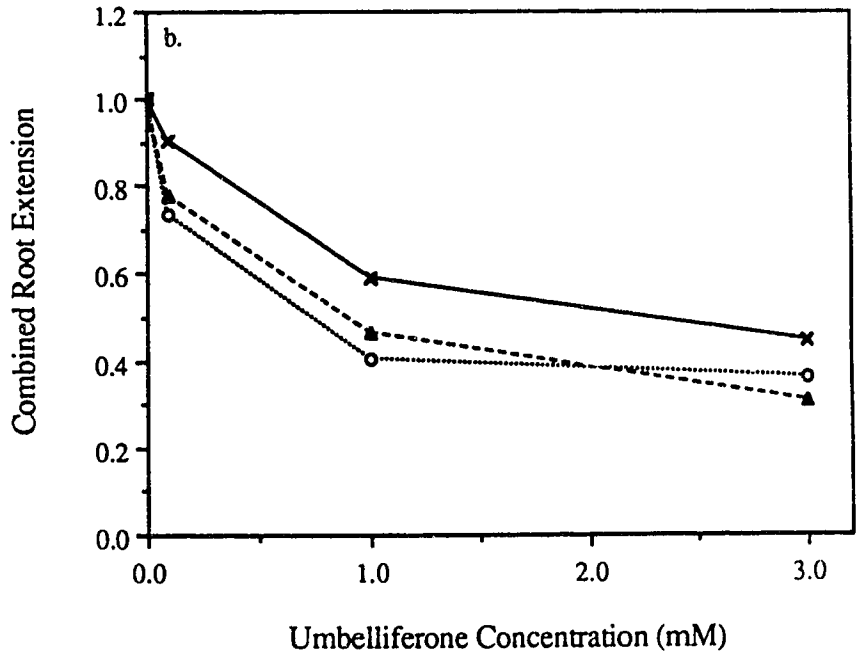


Table 30 Statistical Analyses on the Combined Extensions of Alfalfa Seedlings Grown in Nutritive Ferulic Acid Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Varietal Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.844 0.989 1.006 RM SP GN	0.983 1.110 1.135 RM SP GN
1.0	0.533 0.563 0.913 RM GN SP	0.946 0.958 1.372 RM GN SP
3.0	0.201 0.459 0.734 RM GN SP	0.425 0.842 0.951 RM GN SP
0 to 3[b]	0.644 0.757 0.909 RM GN SP	0.839 0.984 1.108 RM GN SP

Note.

[a]Varietal combined extensions depicted above the variety symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined extensions from the 0 mM to 3 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 31 Statistical Analyses on the Combined Extensions of Alfalfa Seedlings Grown in Nutritive *p*-Coumaric Acid Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Varietal Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.784 0.958 1.027 RM SP GN	0.812 0.983 0.998 RM SP GN
1.0	0.464 0.552 0.558 GN SP RM	0.677 0.791 0.815 RM SP GN
3.0	0.123 0.175 0.195 GN RM SP	0.179 0.292 0.322 GN SP RM
0 to 3[b]	0.629 0.653 0.676 RM GN SP	0.703 0.748 0.767 RM GN SP

Note.

[a]Varietal combined extensions depicted above the variety symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined extensions from the 0 mM to 3 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 32 Statistical Analyses on the Combined Extensions of Alfalfa Seedlings Grown in Nutritive Umbelliferone Solution

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Varietal Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.804 0.812 0.834 SP RM GN	0.736 0.779 0.904 RM SP GN
1.0	0.252 0.333 0.338 RM SP GN	0.402 0.467 0.591 RM SP GN
3.0	0.162 0.174 0.213 GN SP RM	0.307 0.363 0.446 SP RM GN
0 to 3[b]	0.569 0.578 0.583 RM SP GN	0.625 0.638 0.735 RM SP GN

Note.

[a]Varietal combined extensions depicted above the variety symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Varietal combined extensions from the 0 mM to 3 mM concentration range were used in the overall varietal analysis. For this analysis, the replication and concentration effects were statistically removed.

Figure 36 Phytotoxic Effects on Genelle Seedling Extensions

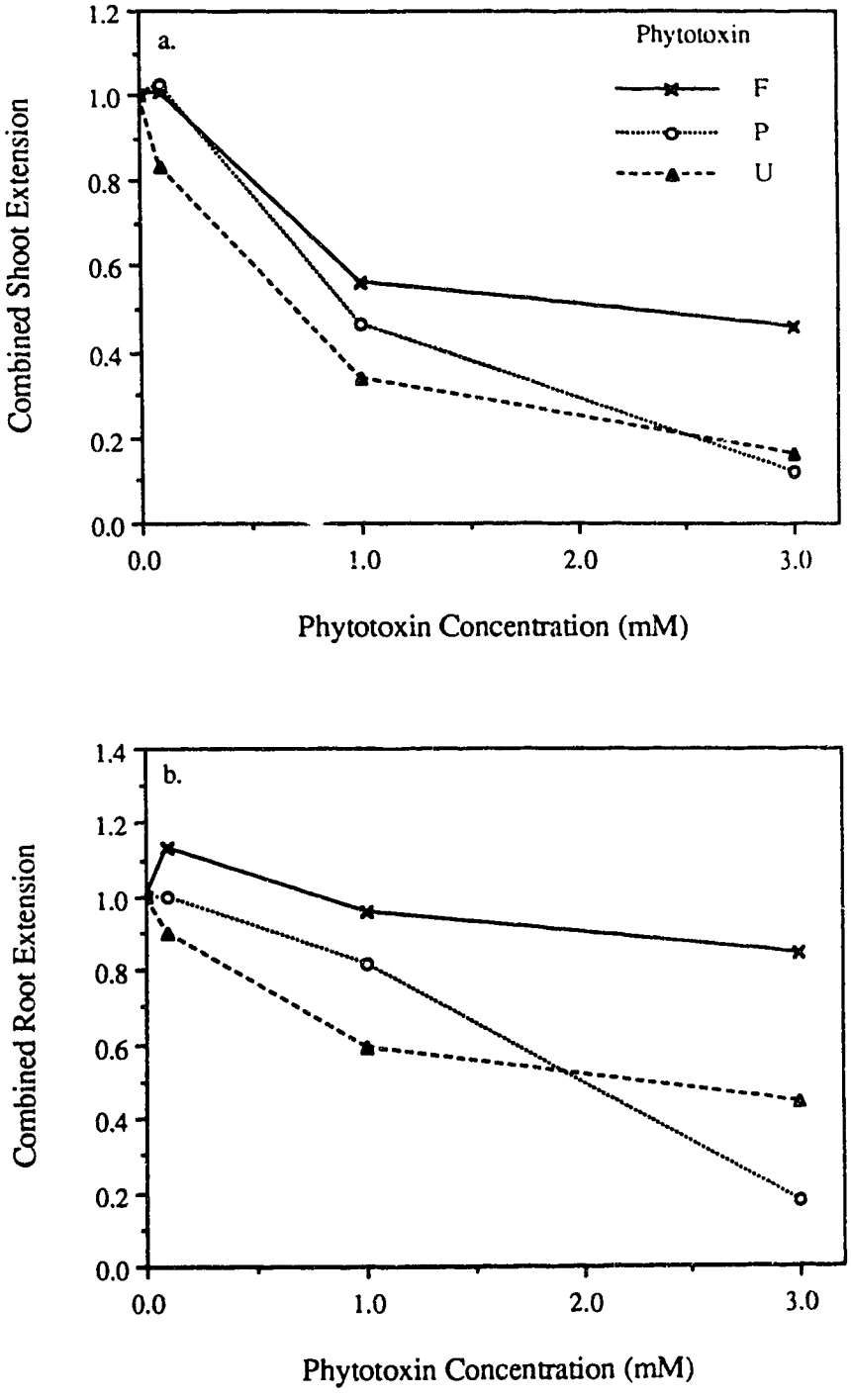
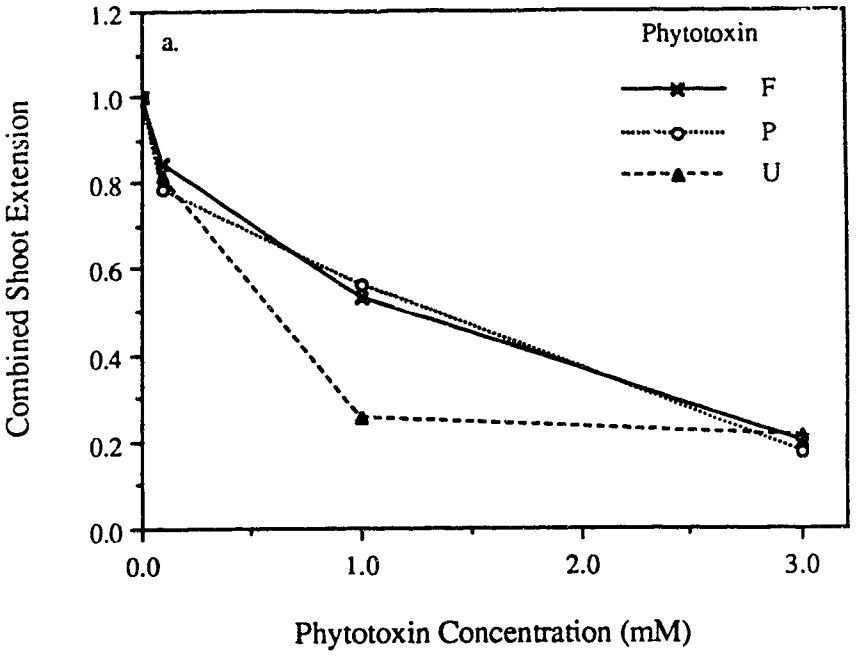


Figure 37 Phytotoxic Effects on Roamer Seedling Extensions



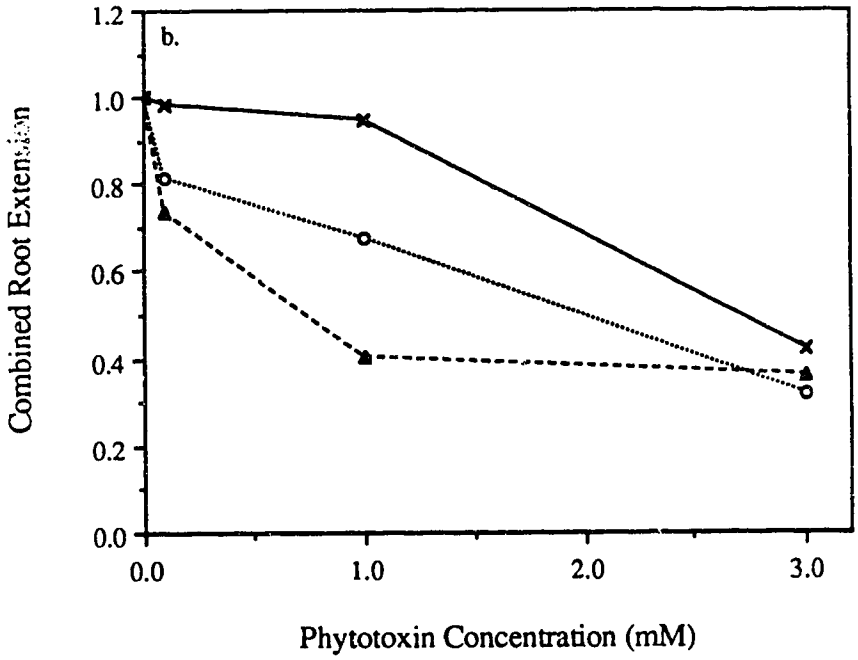


Figure 38 Phytotoxic Effects on Spredor-2 Seedling Extensions

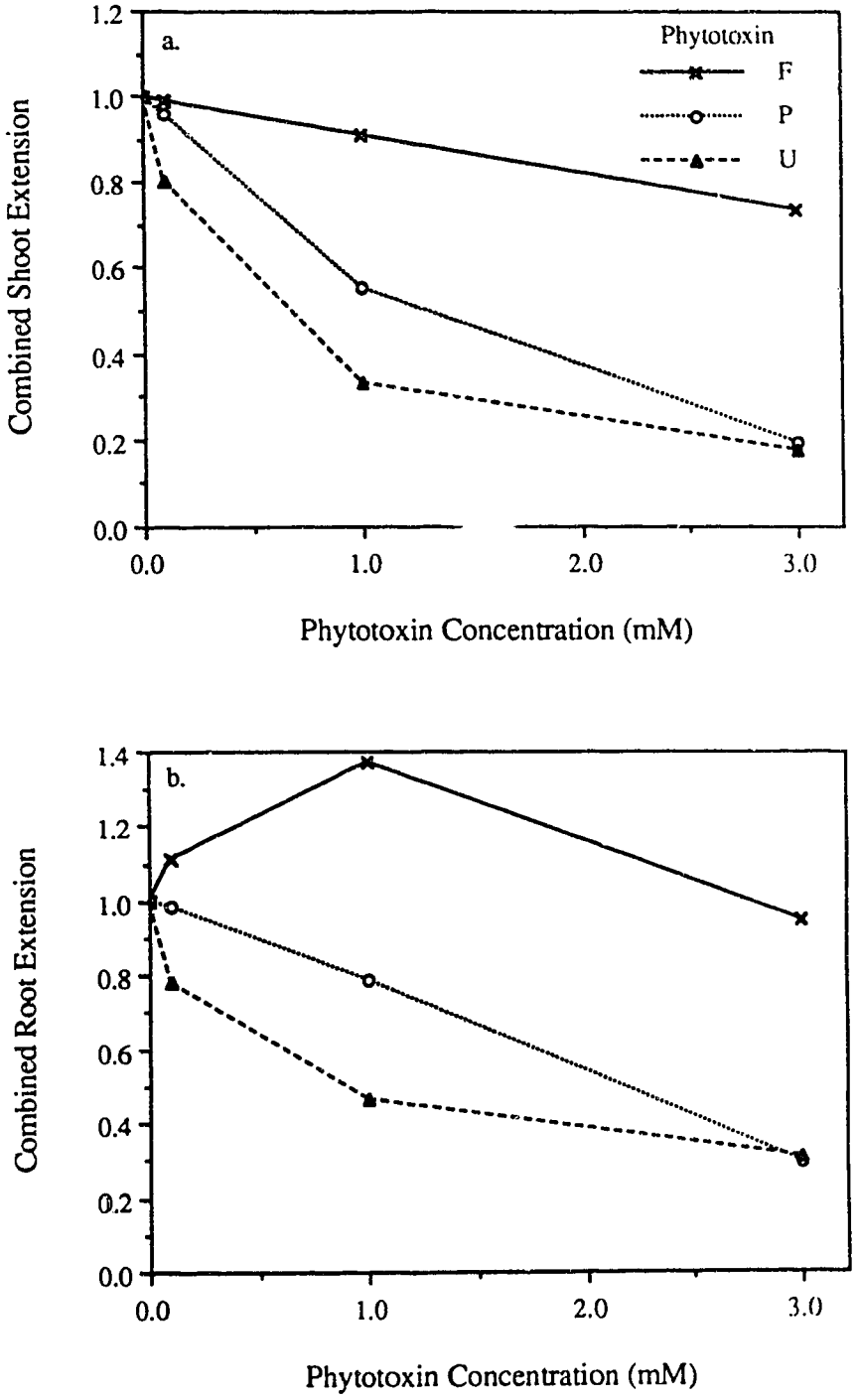


Table 33 Statistical Analyses on Gerelle Combined Seedling Extensions

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Phytotoxic Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.834 1.006 1.027 U F P	0.904 0.998 1.135 U P F
1.0	0.338 0.464 0.563 U P F	0.591 0.815 0.958 U P F
3.0	0.123 0.162 0.459 P U F	0.179 0.446 0.842 P U F
0 to 3[b]	0.583 0.653 0.757 U P F	0.735 0.748 0.984 U P F

Note.

[a]Phytotoxic combined extensions depicted above the phytotoxin symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined extensions from the 0 mM to 3 mM concentration range were used in the overall phytotoxin analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 34 Statistical Analyses on Roamer Combined Seedling Extensions

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Phytotoxic Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.784 0.812 0.844 P U F	0.736 0.812 0.983 U P F
1.0	0.252 0.533 0.558 U F P	0.402 0.677 0.946 U P F
3.0	0.175 0.201 0.213 P F U	0.322 0.402 0.425 P U F
0 to 3[b]	0.569 0.629 0.644 U P F	0.625 0.703 0.839 U P F

Note.

[a]Phytotoxic combined extensions depicted above the phytotoxin symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined extensions from the 0 mM to 3 mM concentration range were used in the overall phytotoxin analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 35 Statistical Analyses on Spredor-2 Combined Seedling Extensions

Phytotoxin Concentration (mM)	Duncan's Multiple Range Test Performed on the Phytotoxic Combined Extensions[a]	
	Combined Shoot Extension	Combined Root Extension
0.1	0.804 0.958 0.989 U P F	0.779 0.983 1.110 U P F
1.0	0.333 0.552 0.913 U P F	0.467 0.791 1.372 U P F
3.0	0.174 0.195 0.734 U P F	0.292 0.307 0.951 P U F
0 to 3[b]	0.578 0.676 0.909 U P F	0.638 0.767 1.108 U P F

Note.

[a]Phytotoxic combined extensions depicted above the phytotoxin symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

[b]Phytotoxic combined extensions from the 0 mM to 3 mM concentration range were used in the overall phytotoxin analysis. For this analysis, the replication and concentration effects were statistically removed.

Table 36 Statistical Analyses on the Varietal and Phytotoxic Combined Seedling Extensions

Phytotoxin Concentration[a] (mM)	Duncan's Multiple Range Test Performed on the Varietal and Phytotoxic Combined Extensions[b]	
	Combined Shoot Extension	Combined Root Extension
0 to 3	0.614 0.665 0.721 RM GN SP	0.722 0.822 0.838 RM GN SP
0 to 3	0.577 0.653 0.770 U P F	0.666 0.739 0.977 U P F

Note.

[a]Varietal and phytotoxic combined extensions from the 0 mM to 3 mM concentration range were used in the overall varietal and phytotoxic analyses. For the varietal analysis, the replication, phytotoxic, and concentration effects were statistically removed. The replication, varietal, and concentration effects were statistically removed from the phytotoxic analysis.

[b]Varietal and phytotoxic combined extensions depicted above the variety and phytotoxin symbols were ranked in increasing value from left to right. Extensions underscored by the same line were not significantly different, while extensions not underscored by the same line were significantly different (DMR: α=0.05).

DISCUSSION

The results from the *in vitro* bioassays of allelopathy indicated that the cell suspension bioassay could not adequately quantify allelopathy in a fashion similar to the callus bioassay. The callus, germination, and extension bioassays all demonstrated significant differences in the varietal responses to the phytotoxins. As well, significant differences existed in the potency of the phytotoxins in inhibiting alfalfa growth or germination. Comparisons between these *in vitro* and *in vivo* bioassays in quantifying allelopathy will be discussed along with the failure to achieve a stably resistant callus line and other interesting features of the bioassays.

Callus Bioassays

The results obtained from the callus bioassays indicate that there exists significant variability in the sensitivity of the alfalfa varieties to the phytotoxins (Table 17). Consequently, a genetic basis is indicated for this variability in sensitivity. This result was surprising because previous findings (Miller, 1983; Goplen & Webster, 1969) from field experiments measuring resistance to autotoxicity, indicated that no major genetic differences existed among alfalfa cultivars. However, what is most interesting in the results of the callus bioassays is the relative order of sensitivity displayed by the varieties. The two wild isolates (*Medicago falcata* L. and *Medicago sativa* L.) were the most tolerant to the phytotoxins, with a lower tolerance expressed by the two commercial cultivars (Roamer and Spredor-2) and an even lower tolerance expressed by the two noncommercial cultivars (Regen-S and Genelle).

Somewhat similar results between wild isolates or accessions and commercial cultivars have also been noted when the allelopathic trait being studied was the production of allelochemicals rather than the resistance to allelochemicals. Fay and Duke (1977) found

four accessions of *Avena* that exuded up to three times as much of the allelochemical scopoletin as did the standard oat cultivar "Garry" (*Avena sativa* L.). Similarly, Lockerman and Putnam (1981a, 1981b) evaluated the allelopathic effects of a wild accession with superior weed-suppressing ability to the standard cucumber cultivar "Pioneer" (*Cucumis sativus* L.). Again, the wild accession exudate was more allelopathic to the indicator species than the standard commercial cultivar exudate.

The results of the callus bioassays support the hypothesis of Putnam and Duke (1974). They hypothesized that during the process of breeding and selecting for desirable characteristics in a weed-free environment, the capacity to produce allelochemicals has been reduced or lost, resulting in cultivars with low competitive ability with weeds. However, not all species demonstrate this loss of allelopathic potential in commercial cultivars. Leather (1983) has shown that sunflower (*Helianthus annuus* L.) cultivars retained and even increased their allelopathic potential when compared to the native sunflower. Therefore, with these observations in mind, it is possible that the breeding of alfalfa cultivars (Table 3) for certain characteristics like high yield and resistance to insects and diseases may inadvertently selected against allelopathic resistance. However, it is also possible that some cultivars not yet characterized may have retained or even increased their allelopathic potential. Additionally, it is conceivable that the two wild isolates characterized are not representative of the native alfalfa isolates and are in fact relatively more resistant. Therefore, more wild isolates and cultivar characterizations should be performed.

Comparisons Between Callus and Cell Suspension Bioassays

Even though the cell suspension bioassays could not be statistically analysed, with improvements, this bioassay may be a feasible method for an *in vitro* quantification of allelopathy. Possible improvements or alterations to the design of this bioassay include a constant inoculation density and packed cell volume measurements of growth. These

factors were included in a cell suspension bioassay by Zilkah and Gressel (1977b). They utilised the Spearman ranking procedure for statistical analyses. When compounds specifically inhibiting *in vitro* cells were ignored, they obtained significant correlations in the rank of phytotoxicity on a species between cell suspension, callus, and seedling bioassays.

Cell suspension bioassays have a few advantages over callus bioassays. Cell suspensions offer easier sampling of the tissue under sterile conditions. As well, such cell suspensions are more homogeneous than calli. This is especially true with fine cell suspensions, which are characteristic of the alfalfa varieties chosen. Calli are more heterogeneous because they often contain nodules of high meristematic activity. Therefore, even though the callus used for the sample inocula was from one source, variability in the samples' growth curves would still occur. This heterogeneity in the callus inoculum is probably the major source of variation expressed in the growth curves of the callus samples. It is depicted by the standard error bars of the growth rates and is clearly larger for the calli (Figure 4a) than the cell suspensions (Figure 18). In fact, the cell suspensions often generated standard errors too small to be indicated as error bars on the graphs.

One other major difference between callus and cell suspension bioassays is the availability of the phytotoxin to the cells. In calli, only a few cells are in direct contact with the medium. Thus, components in the medium must diffuse or translocate through the callus to reach cells further away from the medium. This could be problematic if the phytotoxin being studied is not easily translocated. This potential problem does not exist in fine cell suspensions because all of these cells are in contact with the medium. This may be one reason why cell suspensions are more sensitive to phytotoxins than calli.

The cell suspension bioassays performed all used 1 mM as an upper concentration limit for measuring phytotoxicity, whereas the callus bioassays performed often used 10 mM phytotoxin to achieve the same effect. This may be due to the translocation differences mentioned previously. More evidence supporting this view comes from Zilkah

and Gressel (1977a). They noticed that if a larger callus inoculum was used, a higher concentration of growth regulator was required to achieve the same amount of inhibition.

This difference in the upper concentration limit may also be due to the lower pH of the cell suspensions. The calli were inoculated onto phytotoxic MS media with a pH of 5.65 ± 0.05, whereas the cell suspension pH was 5.05 ± 0.05. It has been shown (Blum, Dalton, & Shann, 1985) that when the pH was lowered from 7.0 to 5.5, ferulic acid and *p*-coumaric acid had more inhibitory activity on the growth of cucumber seedlings. Since the ferulic acid pKa is approximately 4.85, they postulated that decreased solubility and ionization of the phenolic acids at the lower pH was the cause of this increased inhibitory activity. Furthermore, they suggested that the seedling cell membranes were more permeable to the undissociated form of the phenolic acids which were present to a greater degree at the lower pH. This theory was further substantiated by experiments showing that the greatest depletion of the phenolic acids from the culture solution occurred at the lowest pH. As well, subsequent studies (Shann & Blum, 1987) using a pH range of 4.0 to 7.0 proved that the greatest ferulic acid uptake occurred at the lowest pH studied. Therefore, it is reasonable to assume that the lower pH of the cell suspensions might contribute to the lower thresholds of phytotoxic activity exhibited in this *in vitro* bioassay.

With the lower thresholds of phytotoxic activity in the cell suspensions, smaller quantities of phytotoxin need to be used. This could prove to be another potential advantage for cell suspension bioassays, especially if only small amounts of the phytotoxin are available. Thus, if the cell suspension bioassay described in the Materials and Methods section is revised, it may adequately quantify allelopathy. However, this revision would have to involve synchronizing the inocula to the same growth phase (preferably late exponential phase cells) and using a constant inoculation density. As well, daily measurements need to be performed so that the exponential growth phase of the cell suspensions can be easily identified. Unfortunately, this last requirement will probably make this bioassay not very feasible for a rapid and easy technique to quantify allelopathy.

Selection for Phytotoxin-Resistant Calli

Since variation exists in the response of tissue cultures from different varieties to phytotoxins, it is reasonable to assume that genes are involved in producing this response. Therefore, it should be possible to mutate or amplify these genes to obtain resistance to these allelochemicals. Tissue culture naturally supplies a source of variation and regenerated plants often retain these unique somaclonal variations. Hence, it may be possible to obtain resistant alfalfa regenerants *via* somaclonal variation if the resistance expressed *in vitro* is also expressed by the regenerants. Unfortunately, when callus cultures were tested for resistance, none was indicated.

The appearance of apparent resistance in the selected callus cultures could have been caused by the size of the callus transferred and the frequency of transfers to fresh phytotoxic MS plates. When the calli were sequentially selected for resistance to progressively higher phytotoxin concentration levels, roughly 200 mg of callus were transferred to fresh phytotoxic MS plates each month. However, when these same calli were tested for resistance, 100 mg of calli were inoculated and subsequently transferred to fresh phytotoxic MS plates every five days. It is possible that during selection, a one month period between transfers could result in degradation of the phytotoxin in the MS plates, thereby enabling callus to grow. Additionally, this larger callus may have provided enough mass for some fortuitous cells to be displaced far enough away from the phytotoxin source.

The phytotoxin in the MS plates would first have to diffuse or translocate through the cells proximal to the medium in order to reach the distal cells. Furthermore, the phytotoxin would have to retain its activity and hence, not be degraded by the proximal cells. Presumably, the concentration of phytotoxin in the distal cells could be sufficiently lower to enable growth of some sensitive alfalfa cells (escapes). This view was corroborated by the findings of Zilkah and Gressel (1977a), who noted that larger callus

inocula required a higher concentration of growth regulator to achieve the same inhibition produced with smaller callus inocula.

Further experiments involving the kinetics of inhibition of callus growth by growth regulators (Zilkah & Gressel, 1977a) show that after a period of exposure to high concentrations of the growth regulators, recovery can occur. As well, they suggested that "the recovery is probably not due to selection of resistant cells from the whole population, as the whole callus piece recovers simultaneously and not isolated spots on the callus". Since this response was also noted with the calli selected for the resistance test (Table 18), it is very plausible that the apparently resistant calli isolated were not resistant to the phytotoxins, but were in fact fortuitous escapes.

If fortuitous escapes exist, then it is reasonable to assume that the physical dimensions of the calli can affect the growth of these escapes. In other words, 200 mg of callus that was fairly flat would have more cells exposed to the phytotoxic MS medium than 200 mg of callus that was more globular. Under these conditions, the latter callus would more likely exhibit growth of escapes due to the lower concentration of phytotoxin exposed to the distal cells.

When testing for resistance, an overall difference in physical dimensions of the callus inocula in Tables 19 and 20 may somewhat explain the alternating growth behaviours exhibited by the Regen-S calli previously exposed to 5 mM ferulic acid and the Regen-S calli never exposed to ferulic acid. As well, the effect of the physical dimensions of the callus inocula will contribute to the large variability noticed in the callus growth curves. This high variability was noticed in Regen-S that was previously exposed to 5 mM ferulic acid (Table 19). In this experiment the fresh weights of the six samples at 25 days were 65, 750, 219, 255, 347, and 509 mg. Nevertheless, the variabilities expressed in the other Regen-S fresh weights were considerably lower. Hence, another factor is involved in these alternating growth responses.

This other factor may be the growth phase of the callus inocula. Growth of calli

and cell suspensions measured by fresh or dry weights follows a sigmoidal curve (Lindsey & Yeoman, 1985) with characteristic lag, exponential, linear, and stationary phases. Each of these phases differ in their metabolic activity. For instance, lignin biosynthesis and the accumulation of most secondary metabolites preferentially occur in the stationary phase. It has been demonstrated (Zilkah & Gressel, 1977a) that inocula of exponentially growing *Chrysanthemum segetum* L. and *Cirsium avrense* L. calli exhibited growth at 0.1 mM of exogenously applied growth regulator, whereas no growth was exhibited with stationary callus inocula. Furthermore, the phytotoxic activity of ferulic acid, *p*-coumaric acid, and umbelliferone varied with the different growth phases of yeast (Van Sumere, Cottenie, De Greef, & Kint, 1972). Therefore, it is plausible that the growth phase of the callus inocula could affect the inhibitory activity of the phytotoxins resulting in the alternating growth behaviours observed in Tables 19 and 20.

It was unfortunate that selection of a callus line for resistance to phytotoxins was not achieved. The technique used to detect resistance to these allelochemicals was appropriate since other resistances or tolerances in various species have previously been achieved by similar methods using callus tissue (Carlsbach, Green, & Donovan, 1977; Chen, Gavinlertvatana, & Li, 1979; Chaleff & Ray, 1984; Chandler & Vasil, 1984). As well, somaclonal variation in alfalfa regenerants has been well documented (Groose & Bingham, 1984; Nagarajan & Walton, 1987; Johnson, Stuteville, Schlarbaum, & Skinner, 1984). One particular experiment was extremely similar in the protocols for obtaining resistance and even utilised Regen-S alfalfa calli (Hartman, McCoy, & Knous, 1984). This experiment obtained stable disease resistance, previously shown to be controlled by only two genes (Hijano, Barnes, & Frosheiser, 1983), to the toxin(s) or allelochemical(s) produced by *Fusarium oxysporum* f. sp. *medicaginis*. This experiment also utilised a larger amount of calli (150 ± 50 mg) for the selection of resistance than in the evaluation of resistance (105 ± 50 mg). Additionally, this alfalfa callus was maintained on selective media for about the same duration as the alfalfa calli in Table 18. Further similarities also

occur in the method of resistance evaluation; both methods measured fresh weights of approximately three- to four-week-old calli to evaluate resistance. Thus, perhaps the reason why no stably resistant callus line was obtained lies in the genetic basis of this resistance. It is possible that some resistant cells were produced but they had a slow growth rate. This last characteristic would result in a gradual decline in the proportion of resistant cells because the faster growing sensitive cells would soon outnumber these resistant cells. Additionally, other explanations exist as to why no stably resistant callus line was obtained.

Perhaps a better method would involve alfalfa cell suspensions rather than calli because no fortuitous escapes could grow under these conditions. Alfalfa cell suspensions have been used for the selection of stable herbicide resistance (Donn, Tischer, Smith, & Goodman, 1984) as well as for the selection of salt tolerance (Croughan, Stavarek, & Rains, 1978) and freezing tolerance (Orr, Singh, & Brown, 1985). Additionally, disease resistance has been achieved using suspensions of alfalfa protoplasts (Latunde-Dada & Lucas, 1983). Therefore, if alfalfa cell suspensions were sequentially selected at progressively higher phytotoxin concentration levels incorporated into the medium, it may prove to be a better method for selecting phytotoxin resistance than the callus method employed.

Comparisons Between *In Vitro* and *In Vivo* Bioassays

Fairly good correlations have been obtained by Zilkah, Bocion, and Gressel (1977) between the phytotoxic effects on calli and seedlings when utilising the Spearman ranking procedure. In the few cases where significant correlations did not exist in the species rank between these bioassays they ascertained reasons for this lack of correlation as being due to photosynthesis inhibitors affecting seedlings more than the nonphotosynthetic calli and also due to inhibitors which were not penetrated or translocated in seedlings to the extent they

were in calli. Despite these reported correlations, no correlation was obtained in this research project between the *in vitro* and *in vivo* responses of the alfalfa varieties to the phytotoxins. For instance, when considering only the three alfalfa varieties consistently used in all the bioassays, Tables 17, 29, and 36 show that Genelle was the most sensitive variety to the phytotoxins in the callus bioassays. However, this variety was the least sensitive in the germination bioassays and it was of intermediate sensitivity in the extension bioassays. In fact, the only consistency between the bioassays existed in the relative potency of ferulic acid. This phytotoxin was the least potent in the overall statistical analyses performed; either umbelliferone or *p*-coumaric acid were the most potent phytotoxin. This relative order of potency has been observed elsewhere (Zenk & Müller, 1963; Demos, Woolwine, Wilson, & McMillan, 1975; Moreland & Novitzky, 1987). However, it is species and concentration dependent (Van Sumere, Cottenie, De Greef, & Kint, 1972).

There are potentially many factors that may contribute to the inconsistencies observed in the varietal responses to the phytotoxins between different bioassays. Probably the most important factor involves the phytotoxic effects on photosynthesis. The callus cultures assayed were all heterotrophic (nonphotosynthetic). As well, no photosynthesis occurred in the germination bioassays since they were conducted in the dark. In contrast, the extension bioassays were conducted in alternating light and dark photoperiods resulting in the accumulation of seedling dry weight at 7 to 8 days. Since it has been shown (Einhellig & Rasmussen, 1979; Toro, Leather, & Einhellig, 1988) that the phytotoxins employed can affect photosynthesis, this lack of conformity between bioassays will contribute to the absence of correlation in the varietal responses to the phytotoxins. Moreover, if the allelochemicals are photolabile, this will further contribute to this absence of correlation.

Another major contributing factor to the inconsistencies observed in the varietal responses to the phytotoxins between different bioassays involves the tissue differences in

the bioassays. Callus tissue has no cuticle which results in less permeation and translocation problems than that which occurs in seeds or seedlings. Differences in seed coats may also give differences in varietal responses, that are not apparent in seedlings. As well, translocation problems will occur to a greater extent in seedlings where the phytotoxin or some secondary chemical must be translocated in order for the response to be visible in the shoot extension.

Additionally, these tissues all exhibit different metabolisms. Calli somewhat resemble meristematic tissue, whereas seedlings only have isolated regions of meristematic tissue. Moreover, protrusion of the radicle through the seed coat involves elongation and not cell division. These differences in growth kinetics may well contribute to the inconsistencies because it has previously been mentioned (Van Sumere, Cottenie, De Greef, & Kint, 1972; Zilkah & Gressel, 1977a) that the activity of the phytotoxins is affected by the growth phases of the tissues being assayed.

A further effect resulting from the utilisation of different alfalfa tissue in each bioassay is the variation in threshold levels of phytotoxic activity. This variation is reflected in the range of phytotoxin concentrations employed in the bioassays since these ranges were experimentally determined to generate similar responses. For example, to achieve a 50% reduction in the growth or germination rate, generally a lower concentration of phytotoxin was required to produce this effect in the shoot extension, with a slightly higher concentration required by the root extension and callus bioassays, and an even higher concentration required by the germination bioassays. Thus, the shoot extension bioassay was the most sensitive, whereas the germination bioassay was the least sensitive to the phytotoxins. These different levels of sensitivity expressed by the extension and germination bioassays corroborates other findings (Leather & Einhellig, 1985).

Conclusions

Considering the aforementioned factors involved in the discrepancies between bioassay results, my opinion is that the alfalfa callus bioassay described in the Materials and Methods section is not a good method for the quantification of allelopathy, as it does not reflect the *in vivo* growth responses. However, I also feel that no one bioassay can adequately define the allelopathic potential of a chemical. As well, since there exist differences in the response to allelochemicals between species, more than one organism should be used to evaluate an allelochemical. Alternatively, only one organism could be utilised if this organism was shown to be sufficiently sensitive and thus respond to all allelochemicals.

The callus bioassay can be improved by using photosynthetic calli (if available) and callus inocula from one growth phase (preferentially late exponential phase). With these improvements, the callus bioassay may adequately reflect the *in vivo* growth responses. Additionally, the cell suspension bioassay may be improved to adequately quantify allelopathy. These improvements should involve synchronous inocula at a constant inoculation density as well as additional measurements to those described in the Materials and Methods section. Furthermore, these improved *in vitro* bioassays could be analysed by a different statistical test. One such test could be the Spearman's ranking procedure used by Zilkah, Bocion, and Gressel (1977). Therefore, if the improved *in vitro* bioassays are used in concert with other bioassays, the allelopathic potential of an allelochemical may be adequately measured.

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