Cellular effects in perennial ryegrass (*Lolium perenne* L.) associated with the root inhibiting compound alaninyl-alanine

by

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ABSTRACT

Corn gluten meal (CGM), a byproduct of corn (*Zea mays* L.) wet-milling, has been shown to be an effective natural preemergence herbicide and fertilizer for various plant production systems. Alaninyl-alanine (Ala-Ala), along with four other dipeptides, were isolated from CGM and identified as being the inhibitory compounds. The herbicidal effects of these natural compounds are seen as growth-regulating, root inhibitors that have minimal effect on shoot growth at low concentrations. However, little is known about the precise inhibitory action of CGM or Ala-Ala. The overall objective of this research was to determine the cellular effects in perennial ryegrass (*Lolium perenne* L.) associated with the root inhibiting compound, Ala-Ala.

The objective of the first phase of this research was to elucidate morphological and anatomical differences in perennial ryegrass seedlings treated with Ala-Ala using light and transmission electron microscopy, as well autoradiographic studies using [$^{3}H^{+}$]-Ala-Ala. The results from these experiments described the treated root tips as being void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, treated root tips had extreme cell wall abnormalities including uneven thickening and breakage. The autoradiographs suggested that at the high treatment concentrations causing epidermal tissue damage, there was minimal inward movement of the dipeptide. At lower concentrations, root tip epidermal necrosis was not evident, and inward movement of Ala-Ala is not impeded.

The objectives of the second phase of this research were to use time-course studies to

monitor the mitotic activity of perennial ryegrass roots treated with Ala-Ala; to use light and transmission electron microscopy to describe Ala-Ala induced changes in root cell ultrastructure in an effort to elucidate the mode of action of this dipeptide; and to make comparisons with other reported modes of action of commonly used synthetic herbicides. Results showed that Ala-Ala exhibited activity on mitosis within 4 h of exposure, and by 6 h, reduction in the number of mitotic figures was nearly 100%, resulting in only interphase cells. Microscopic analysis revealed profound treatment effects. By 12 h, dense droplets, presumably membrane lipids, were visible in vacuoles and intercellular spaces. After a 48 h exposure, epidermal and cortical cell elongation in treated roots appeared to occur perpendicular to the normal elongation plane, possibly resulting from a loss of cell polarity. Root lateral branching, similar to effects of synthetic preemergence herbicides, was also noted after a 48 h exposure time. Root tips, however, showed no gross external abnormalities until after a 96 h exposure to Ala-Ala.

GENERAL INTRODUCTION

In 1986, researchers at Iowa State University were attempting to introduce *Pythium* into a new putting green located at the Iowa State University Horticulture Research Station (Christians, 1993). The *Pythium* fungus was cultured in the laboratory on food grade corn meal and this inoculated corn meal was then spread over the surface of the putting green, tilled into the upper several inches of soil, and seeded with creeping bentgrass (*Agrostis palustris* L.). Plots consisted of an untreated control, corn meal inoculated with *Pythium*, and a second control plot with an equal amount of fresh, noninoculated corn meal.

The attempt to establish a *Pythium* population was a failure; however, it was noticed that plots treated with the fresh corn meal had very low germination rates when compared to the other two treatments. The reason for this inhibition was uncertain, and the possibility that corn meal contained some type of inhibitory organic compound was hypothesized. After further study, it was concluded that the protein fraction of corn, corn gluten meal, did indeed contain an inhibitory compound.

In 1989, graduate student Dianna Liu, initiated studies to elucidate the inhibitory compound(s). In 1992, five chemical structures were identified as being the inhibitory compounds. These compounds were glutaminyl-glutamine, alaninyl-asparagine, alaninyl-glutamine, glycinyl-alanine, and alaninyl-alanine (Ala-Ala) (Liu *et al.*, 1994). The dipeptides, as well as corn gluten meal, exhibit their activity by stopping root formation in germinating seeds. The mechanism(s) by which corn gluten meal and the biologically active dipeptides exert their inhibitory effects are unknown. The overall objective of this work was to determine

the cellular effects in perennial ryegrass associated with the root inhibiting compound, Ala-Ala.

Dissertation Organization

The following dissertation consists of a review of the literature and two research manuscripts prepared for partial fulfillment of the requirements for the degree, Doctor of Philosophy. The author of the dissertation is J. Bryan Unruh. Dr. Nick E. Christians served as major professor and Dr. Harry T. Horner served as a committee member and provided technical and consulting expertise for the microscopic aspects of the studies. The first section is a review of the literature concerning weed control, herbicide action, and the cellular effects of, and methods of studying herbicides that affect cell division processes. The second section is a manuscript to be submitted to *Crop Science* that reports on a series of experiments that were designed to elucidate morphological and anatomical differences in perennial ryegrass (*Lolium perenne* L.) seedlings treated with the biologically active dipeptide, Ala-Ala. The third section, also a manuscript to be submitted to *Crop Science*, reports on time-course studies to monitor the mitotic activity and the changes in cellular ultrastructure of perennial ryegrass roots treated with Ala-Ala. Following the third section are general conclusions and a list of references cited in the general introduction and conclusions.

LITERATURE REVIEW

Weed Control

A weed is a plant growing where it is not wanted. In production agriculture, weed control is aimed at improving crop production, while minimizing the cost of producing the agriculture products. Specific problems caused by weeds include lower crop and animal yields, less efficient land use, higher costs of insect and plant disease control, poorer quality products, more water management problems, and lower human efficiency (Ashton and Monaco, 1991). These problems constitute a 7.5 billion dollar annual yield loss in the United States alone (Ashton and Monaco, 1991).

Weed control in turfgrass management is markedly different than in production agriculture. In turfgrass systems, the traditional definition of a weed is often expanded to a plant that is undesirable because of its disruptive effect on the aesthetic appearance, stabilizing capacity, or overall utility of a turf (Turgeon, 1985). One of the most important components of turfgrass quality is uniformity. A plant is usually called a weed when it disrupts the uniformity of a turf due to a substantially different leaf width and/or shape, growth habit, or color (Beard, 1973). In a typical city in the United States, 70% of the total turfgrass acreage is residential lawns (Cockerham and Gibeault, 1985). This turfgrass is planted to increase the aesthetic appearance of the residence, and weeds distract from this making them undesirable. Sports turf installations such as ball fields and golf courses depend on uniform turf. Weed infestations greatly disrupt putting greens leading to dissatisfied players. In addition to detracting from the

aesthetic appearance and uniformity, weeds also compete with the desirable turfgrass species for light, soil moisture, soil nutrients, and carbon dioxide (Beard, 1973).

Weed Management Practices

Most conventional weed management practices used in crop production do not lend themselves to turfgrass systems. Agronomic crops, such as wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.), are grown as annuals which provide seasonal opportunities for tillage operations. Conventional row crop cultivation kills weeds by breaking the contact between the roots and soil by cutting the tops from the roots, and by burial of the aerial growing plants and leaves with soil, leading to weed desiccation and depletion of plant reserves (Ashton and Monaco, 1991). Because of the intended perennial use of turfgrasses, the only time that is available for conventional tillage operations is prior to planting. This, however, is not generally done for the purpose of weed control, but rather in preparation of the seed bed.

Weed control in turfgrass systems is generally limited to a number of methods involving a combination of sanitary practices, cultural regulation, as well as mechanical and chemical control methods (Beard, 1973). Sanitary practices that minimize the spread of weed seeds or plant parts should be implemented. These include obtaining weed-free seed, sod, and topdressing sand, as well as minimizing weed transfer from equipment or adjacent unmowed areas.

Weed encroachment into an established turf is minimized when the cultural practices favor a dense, vigorous sward. Cultural practices that ensure such a turf include the proper (a)

turfgrass species; (b) cutting height and frequency; (c) soil fertility and pH level; (d) irrigation frequency and intensity; (e) disease, insect, and nematode controls; and (f) cultivation of compacted areas (Beard, 1973; p. 552).

Mechanical Weed Control

Mechanical weed control methods include the manual removal of the weeds and proper mowing. Hand pulling weeds is a labor intensive control method, but can be effectively used on annual weeds, particularly broadleaf weeds. Spudding, a method in which a metal blade with a sharpened fork (a spud) is used to sever the roots prior to pulling the weed, is also used. These two manual methods are effectively used (a) for controlling annual broadleaf weeds, (b) on relatively small turfgrass areas, (c) where only a few occasional weeds develop in more extensive turfgrass areas, and (d) in turfs adjacent to valuable ornamental plantings that can be seriously damaged if a herbicide is used (Beard, 1973; p. 557).

In addition to manual removal, routine mowing is effective in controlling many weeds. Most large, erect weeds such as common lambsquarters (*Chenopodium album* L.) and smooth pigweed (*Amaranthus hybridus* L.) simply cannot tolerate frequent, close mowings which decapitate the plant. Prostrate weed species such as prostrate spurge (*Euphorbia maculata* L.) are generally not affected by mowing, thus posing a more serious problem.

Chemical Weed Control

Chemical weed control dates back to ancient times. The Roman Army would prevent

the production of crops grown by their enemies by salting the fields. In the early twentieth century, inorganic compounds such as sulfuric acid, were used to selectively control weeds in a limited number of crops (Ashton and Monaco, 1991).

The modern era of herbicide technology began with the discovery of the herbicidal properties of 2-methyl-4-chloro-phenoxyacetic acid (MCPA) and 2,4-dichloro-phenoxyacetic acid (2,4-D) during World War II. In 1943, Templeman and Sexton, both working for Imperial Chemical Industries in England, independently discovered the herbicidal activity of the phenoxyacetic acids (Cremlyn, 1978). After the introduction of 2,4-D, chemical methods for effective weed control evolved rapidly, becoming the dominating force in turfgrass weed control programs.

In 1958, atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), the first of the triazine herbicides, and diquat (1,1'-ethylene-2,2'-bipyridylium ion) and paraquat (1,1'-dimethly-4,4'-bipyridinium ion) the first of the bypyridylium herbicides were introduced by Geigy AG and Imperial Chemical Industries Ltd., respectively (Fedtke, 1982; Ware, 1994; Cremlyn, 1978). The herbicidal properties of the 2,6-dinitroanilines were first reported in 1960 by representatives of Eli Lilly and Company (Ashton and Crafts, 1981).

Herbicide development over the past few decades has seen a trend toward pesticides that are quite specific in their activity allowing for the use of very low concentrations of material. In 1982, E. I. du Pont de Nemours and Company commercialized the sulfonylurea herbicides, chlorsulfuron {2-chloro-N[[(4-methoxy-6-methyl-1,3,5 triazin-2-yl)amino]carbonyl]-benzenesulfonamide} and sulfometuron methyl {methyl 2[[[[(4,6-

dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl] benzoate}. Chlorsulfuron was reported to be 10 to 100 times more active than most herbicides available at that time (Anonymous, 1982; Palm, 1982).

At about the same time the sulfonylureas were being developed, researchers at American Cyanamid were developing the family of imidazolinone herbicides which includes the herbicide imazaquin {2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-quinoline carboxylic acid}. The similarities between the modes of action of the sulfonylureas and imidazolinones are striking, yet structurally they are quite different.

Natural Weed Control

The use of natural products for weed control is seeing increasing popularity (Duke, 1986). Rice (1984) suggested that the use of plant-derived compounds as natural herbicides is an environmentally sound approach. A number of thorough reviews have been written on this subject (Liu, 1993; Duke, 1986). One particular plant-derived natural herbicide which produces promising results is corn gluten meal (CGM) (Christians, 1993).

Corn gluten meal, the protein fraction of corn (*Zea mays* L.), is a by-product of corn wet-milling and is commonly used as a feed material for poultry, livestock, dogs, and fish. The herbicidal efficacy of CGM was tested on 22 weed species, 10 dicotyledonous and 12 monocotyledonous species, with all species exhibiting some susceptibility (Bingaman and Christians, 1995). Additionally, CGM was evaluated as a weed control product in strawberry (*Fragaria X ananassa*) production systems with results indicating that weed control in this

crop was possible (Nonnecke and Christians, 1993). Typical symptoms of seedlings germinated in the presence of CGM were normal shoot development with severely inhibited rooting. This leads to seedling death upon moisture stress. A patent on the use of CGM as a natural preemergent herbicide was issued in July 1991 (Christians, 1991).

Subsequent work has resulted in a water-soluble (hydrolyzed) component of CGM, as well as the isolation and identification of the root inhibiting compounds from the hydrolyzed corn gluten (Liu *et al.*, 1994; Liu and Christians, 1994). These inhibitory compounds were identified as the dipeptides: glutaminyl-glutamine, alaninyl-asparagine, alaninyl-glutamine, glycinyl-alanine, and alaninyl-alanine.

Relatively little is known about the precise inhibitory action of CGM or the root-inhibiting dipeptides. For many years, the inhibitory effects of corn stover on both shoot and root elongation of germinating plants has been recognized (Bonner, 1950; Nielsen *et al.*, 1960). In 1976, Chou and Patrick reported that lettuce (*Latuca sativa* L. cv. Great Lakes) seedlings germinated in aqueous extracts of decomposing corn residues developed abnormal radicles which lacked root hairs and had necrotic root tips. Their findings are similar to earlier work by Patrick and Koch (1958) and Patrick (1971). Work by Muller (1971) suggested that the radicle damage was localized to the meristematic tissue, resulting in suppressed root elongation. Results from these reported experiments coincide with observations by Christians (1993) and Liu and Christians (1994) on the herbicidal effects of CGM. The herbicidal properties of the aforementioned dipeptides had not previously been reported in the literature.

Herbicide Action

The terms mode of action, mechanism of action, and site of action mean different things to those who work within the scientific community. These terms are often used interchangeably and are considered to have the same meaning. The National Academy of Science book, entitled "Weed Control" (Anonymous, 1968), however, stated "The term 'mode of action' refers to the entire sequence of events from introduction of a herbicide into the environment to the death of plants. 'Mechanism of action' refers to the primary biochemical or biophysical lesion leading to death." Furthermore, the term 'site of action' refers to the specific enzyme or enzyme systems being inhibited, metabolic step, or binding site that is impacted by the herbicide (Fedtke, 1982). Ashton and Crafts (1981), in their book "Mode of Action of Herbicides," consider the 'mode of action' to comprise the sum total of anatomical, physiological, and biochemical responses that make up the phytotoxic action of a chemical, as well as the physical (location) and molecular (degradation) fate of the chemical in the plant.

Regardless of the terminology, herbicide action usually refers to the inhibition of a sensitive pathway leading to the death of the weed. Logistically, this is seen as an interdependency problem with three components: herbicide, measurable effects, and visible effects (Fedtke, 1982). The ability of a herbicide to produce desired results is a reflection of its ability to penetrate into plant tissues, resist detoxification (or be transformed into toxic products), and interfere with physiological and biochemical processes in the plant (Devine *et al.*, 1993). These processes must occur in order to produce the measurable and visible effects. Research conducted to generate data from measurable effects such as chlorophyll

concentration, CO_2 fixation, O_2 evolution, or electron transport, should be arranged in a sequence of cause-response relations to explain some descriptive visible effect like necrosis, chlorosis, epinastic movement or wilting. One must be mindful of the possibility, however, that a herbicide may cause lethal damage without any visible effect on the plant.

When conducting research to investigate how a herbicide exhibits its activity, one must assimilate all of the available information and assess whether the effect is primary, secondary, tertiary, etc. The primary molecular site of action is the single metabolic reaction that is affected at a herbicide concentration lower than any other reaction, or the first reaction affected at a given low concentration. Other reactions affected at higher herbicide concentrations, or later in time at a given low concentration, are considered to be secondary in nature (Ashton and Crafts, 1981). The task of establishing an unequivocal cause and effect relationship between the primary and secondary sites of action is indeed difficult. The primary site may also be associated with other secondary and/or tertiary sites of action depending on the dose administered (Fedtke, 1982; Streibig, 1992). Ashton (1967) stated that in biological systems, it is known that there are a number of metabolic bypasses and, therefore, the primary site of action may not be the herbicidal site of action. Furthermore, even though the primary site of action is of considerable scientific interest, the secondary effects undoubtably contribute to the overall action of the herbicide (Ashton and Crafts, 1981). Most often, when assessing the sequence of events of herbicidal activity, time- and concentration-course studies are conducted to describe, in detail, what is taking place.

Fedtke (1982) stated that it is helpful to classify plant metabolism into "basic,"

"intermediate," and "secondary" metabolism when trying to understand data obtained from physiological and biochemical investigations. He contended that basic metabolism includes the production of organic carbon compounds, the generation of high-energy chemical bonds, and the synthesis of the basic cellular polymers such as proteins, nucleic acids, starch, and cellulose. Intermediate metabolism is the connection of all degrading and synthesis pathways which includes all of the small organic molecule interconversion and incorporation into new building blocks. The generation of specific plant compounds like alkaloids, pectins, lignins, and growth hormones, constitutes secondary metabolism. The metabolic pathways that are specific for plant tissues, which are generally affected by herbicides, are most commonly found in basic and secondary plant metabolism.

In elucidating the mechanism of herbicide action, the experimentation on metabolism systems can be defined by the potential amount of information that can be gathered. The information revealed by intact plants, plant tissue, or preparations from disrupted plant tissue, classified as "supercomplex," "complex," or "defined" systems, respectively, are identified in Table 1 (Fedtke, 1982). The level of complexity is greatly minimized when investigations are focused on one or two specific reactions, thus limiting many unknown factors.

Fedtke (1982) also classified six different types of data (Table 2) that can be used when elucidating the mechanism of action of a new herbicide. Information gained from

Table 1. Classification of plant tissues, plant tissue preparations, and methods of investigation used in the search for the mechanism of herbicide action. (From Fedtke, 1982).

Classification	**Data relevance**	**Type of experimentation**
Supercomplex systems	Growth related	Anatomy, dry weight, morphology, etc.
Complex systems	Metabolism related	Tracer studies, gas exchange, estimation of cell components, etc.
Defined systems	Site directed	In vitro studies with enzymes or organelles, binding studies, etc.

the application technique includes herbicide timing and placement. In turfgrass systems, two timing schemes, preemergence and postemergence, are employed. Preemergence herbicides are applied prior to weed emergence from the soil, while postemergence herbicides are applied after emergence of the weed. Herbicide timing provides

Table 2. Information on the mode of action of herbicides as obtained from different types of observations and test systems. (From Fedtke, 1982)

Information source	**Information type**
a. Application technique	Sensitive growth phase or organ
b. Damaged tissue	Sensitive cell type: meristematic, elongating, assimilating, etc.
c. Cytological and microscopical observations	Cellular and subcellular actions
d. Physiological experimentation	*In vivo* inhibited metabolic pathways
e. Biochemical experimentation	*In vitro* inhibited metabolic pathways, effects on biochemical composition
f. Protein/enzyme interactions with herbicides	Sensitive enzymatic reaction and/or binding protein

information on the sensitive growth phase of the treated plants, whereas herbicide placement generally indicates which plant organs are affected (i.e., roots or shoots). The application technique and sensitive tissue, collectively, provide an indication of a herbicide's mechanism, or mode of action, and can allow it to be further classified as a germination inhibitor, contact herbicide, bleaching herbicide, desiccant, or hormone killer (Fedtke, 1982). The dinitroaniline herbicides, for example, have little or no effect when applied to the foliage of mature plants, yet they are active on germinating grass seedling roots that come into contact with them. This leads one to deduce that the herbicide action might be related to root meristematic tissue, which is indeed the case for the dinitroaniline herbicides.

Cytological and microscopic observations can provide data that suggest the

physiological and biochemical events that are modified by the herbicide (Ashton and Crafts, 1981), but caution must be taken in making assertions that manifestation of visual damage is a primary effect of the herbicide. Visual damage in a cellular organelle does not imply that the organelle is spatially close to the site of action (Fedtke, 1982).

Physiological and biochemical information are generally obtained from *in vivo* and *in vitro* experimentation, respectively. The physiological answer to the mechanism of action aims at a particular metabolic pathway such as photosynthesis, respiration, protein synthesis, mitosis, etc. (Fedtke, 1982; Streibig, 1992). Herbicide induced inhibition of a metabolic pathway does not imply a primary herbicidal action on the pathway, but may in fact be the consequence of another metabolic block with which the investigated pathway depends (Fedtke, 1982).

Only *in vitro* biochemical investigations using an isolated enzyme system will give reliable data on the primary herbicidal influence on a metabolic pathway (Fedtke, 1982). The conclusive explanation of herbicidal action is a description of the molecular site affected by the herbicide. This is usually done by studying the binding of a compound to a relevant enzyme or protein fraction, by demonstrating the chemical structure correlations, by determining the binding site constants, and by correlating binding with the inhibitory or regulatory activity (Fedtke, 1982).

A thorough understanding of the metabolic pathway(s) affected by herbicides, however, is complicated by a lack of knowledge of specific methods to study such system(s), or, unidentified enzyme reactions. Until such time that basic plant metabolic pathways are understood, the gathering of conclusive evidence of herbicide action will be limited.

Interestingly, herbicides and herbicide-resistant mutants have become increasingly important in recent years as tools for studying physiological and biochemical processes in plants (Devine *et al.*, 1993).

Herbicides Affecting Cell Division

Cellular Effects

Plant growth is generally defined as an irreversible increase in size which occurs as a result of cell division and cell enlargement. For sustained growth to occur, plant seedlings require a constant supply of new cells generated by cell division (mitosis) in the meristematic tissue of roots and shoots. Many frequently used herbicides influence growth by affecting these cellular processes. In an effort to provide definitive terminology for these effects, Hess (1983) proposed that herbicidal effects on cell division and cell enlargement be divided into either a disruption or inhibition of these processes. Hess contended that if a herbicide causes an inhibition of cell division, the cell cycle analysis of treated meristems will yield only interphase cells, giving rise to the name "preprophase inhibitors." Plants treated with herbicides that cause disruption of cell division have mitotic stages present, however, one or more stages normally present will be absent or aberrant.

The mechanisms by which inhibition and disruption of cell division occurs are unique from each other. The inhibition of cell division is a secondary effect caused by some disturbance of a plant metabolic process (Hess, 1983; Kim and Bendixen, 1987). Commonly reported metabolic disturbances include DNA, RNA, and protein synthesis, as well as energy

metabolism (ATP synthesis and utilization) (Hess, 1983). As previously stated, herbicide-inhibited cell division results in only interphase cells. Interphase, the phase between successive mitotic divisions, can be divided into three stages, G_1 (Gap 1; pre-DNA synthesis period), S (Synthesis; DNA replication), and G_2 (Gap 2; pre-mitotic period). Protein and RNA are synthesized in the G_1 stage and used in the S stage. If this synthesis is blocked, DNA replication will cease, ensuing proteins will not be made, and subsequent cell division will not occur (Hess, 1983; Rost, 1984; and Kim and Bendixen, 1987). Alachlor [2-chloro-2'-6'-diethyl-N-(methoxymethyl)-acetanilide], metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide], and chlorsulfuron are examples of herbicides reported to inhibit mitosis in root meristem tissue (Hess, 1983).

Disruption of cell division is usually caused by a primary effect on the mitotic apparatus (Hess, 1983). This apparatus is composed of " and $ subunits of tubulin molecules assembled into long tubular structures (microtubules), which are in association with other proteins. Tubulin, a heterodimeric protein with a molecular weight of 110,000, is composed of protein synthesized during the G_2 stage. This apparatus organizes and separates the chromosomes during mitosis (Devine *et al.*, 1993), determines the plane of cell division, and orients cellular microfibril deposition in growing plant cells (Appleby and Valverde, 1989). Herbicides that block protein synthesis during the G_2 stage, produce cells that are disrupted at prophase, metaphase, or anaphase, but with many cells remaining at G_2 (Kim and Bendixen, 1987).

Vaughn and Lehnen (1991) described four arrays of microtubules: cortical (interphase), preprophase, spindle, and phragmoplast. Each array has specific cellular

functions that can be altered by herbicides (Table 3). Lehnen and Vaughn (1992)

Table 3. Microtubule arrays, their roles in cellular processes, and consequences of their loss. (From Vaughn and Lehnen, 1991).

Array	Function(s)	Consequences of loss/ alteration
Cortical	Organizing cellulose microfibril deposition and orientation, setting cell shape.	Uneven thickening of wall, isodiametric cells.
Preprophase	Setting plane for subsequent cell division.	? division plane not set.
Spindle and kinetochore	Movement of chromosomes during mitosis.	No chromosome movement; tetraploid, reformed nucleus (generally lobed). Multipolar mitosis.
Phragmoplast	Organizing the new cell plate formation after mitosis.	Incomplete or no cytokinesis, abnormally oriented cell wall.

described three classes of mitotic disruption based on the type of effect a herbicide elicits. The most common type, characteristic of the dinitroaniline herbicides and other structurally unrelated herbicides such as pronamide [3,5-dichloro-N-(1,1-dimethyl-2-propynyl) benzamide] and dithiopyr [S,S-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-pyridinedicarbothioate], is the inhibition of the polymerization of tubulin subunits into mircotubules. This leads to disrupted microtubule arrays causing cessation of mitosis because the chromosomes cannot divide. Additionally, cell elongation does not take place because of a lack of cortical microtubules, and the cell plate is absent or malformed because of a lack of phragmoplast microtubule arrays. A second class,

common to some carbamate herbicides, is a disruption in the spindle microtubules resulting in either multipolar or starburst arrays. This disruption leads to abnormally oriented phragmoplast arrays because they develop around the portions of multiple nuclei, or in and around the arms of star telophase figures. Cortical and preprophase microtubules are unaffected. Vaughn and Lehnen (1991) described a third class, characteristic only of the herbicide DCPA (dimethyl 2,3,5,6-tetrachloro-1,4-benzenedicarboxylate). The effects of this herbicide are seen on both the stability and organization of phragmoplast microtubule arrays resulting in misoriented, mismatched cell plates. Regardless of which process is affected, both processes result in a reduced supply of new cells in the root meristem leading to an eventual inhibition of growth.

Methods of Studying Herbicides Which Affect Cell Division

Herbicidal effects on cell division in roots can be observed and studied using several techniques. The simplest approach is to determine the herbicidal effect on mitosis in root meristematic tissues, with emphasis on chromosomal patterns. Mitotic indices (number of cells with mitotic figures per 1,000 cells counted) are frequently used to report mitotic activity. Methods for mitotic index analysis are variable. They generally involve paraffin sections or squash preparations of root tips (Setterfield *et al.*, 1954). In large studies where rapid determination is of importance, paraffin sectioning might prove to be cumbersome. Squash preparations, however, provide results within a small amount of time. Generally, squash methods follow this sequence. At selected times after treatment (or various concentrations of

herbicide), root tips are fixed in an absolute ethanol:glacial acetic acid solution (3:1), after which they are placed in 70% ethanol for storage. Prior to analysis, root tips are hydrolyzed in HCl, stained in Schiff's reagent, and placed on a glass slide and squashed. The slide is then scored and data are plotted as mitotic index versus time (or concentration), which provides information on the lethal concentration (LC_{50}) and time-course effects. Additional information such as percentage of mitotic figures arrested in a specific phase, or relative distribution of mitotic figures at metaphase, anaphase, etc., compared to the cumulative total can also be obtained. This type of information allows the researcher to ascertain the effect of the herbicide on cell division as being an inhibitor or a disrupter.

Microscopic analysis is another method for studying the effects of herbicides on cell division. Many authors including Lehnen and Vaughn (1992), Elmore and Bayer (1992), Armbruster *et al.* (1991), and Hillman and Ruthmann (1982) have used microscopy in elucidating the effects of herbicides on mitosis. Microtechnique is as varied as the researcher doing the work. Often times, protocols must be developed for each specific species being studied. Although microscopy is very useful for showing cellular effects, caution must be taken when interpreting the results, so that direct and indirect effects are not confused.

In addition to using general light microscopy in studying herbicidal effects on roots (specifically microtubules), immunofluorescence microscopy has substantially enhanced analysis of mitotic disrupter herbicides (Sherman and Vaughn, 1991). These authors, along with Lehnen and Vaughn (1992) described methods by which monoclonal anti-" -tubulin primary and goat anti-mouse IgG fluorescein-conjugated secondary antibodies are used to stain

tubulin. When viewed with a microscope using a short-wavelength (blue or ultraviolet energy) light source, the fluorescent tracer, bound to the tubulin, illuminates allowing the microtubule arrays to be seen, and aberrations detected.

Another technique by which herbicidal effects can be studied is autoradiography. This technique allows localization of a herbicide which contains a radioisotope, such as ^{14}C, ^{3}H, and ^{35}S, that has been incorporated into the specimen. Localization is obtained by covering a thin section a of radioisotope-incorporated specimen with a thin layer of photographic emulsion. As the radioisotope decays (disintegrates), energy particles (electrons) of radiation are emitted producing latent images in the emulsion. After developing the emulsion, microscopic examination reveals silver grains overlaying the tissue section where the radioisotope is incorporated.

Although this technique can provide much information, there are also a number of problems that can be encountered. One such problem is that conventional histological techniques for fixation and dehydration may result in loss of, or alter the true position of, soluble labelled herbicides (Radwan *et al.*, 1960; O'Donovan and Vanden Born, 1981). A second consideration is that the localization is of the radioactive atom only (O'Donovan and Vanden Born, 1981). Herbicides which are rapidly metabolized might yield two or more metabolites, one of which might contain the radioisotope, the other might contain the active site of the molecule. A third concern is the difficulty in accurately interpreting the autoradiographs. The major obstacle to accurate interpretation is the precise determination of the source of the disintegration (Bozzola and Russell, 1992). As the radioisotope decays, the disintegrations are

randomly directed and may travel some distance before striking a silver halide crystal contained in the emulsion, thus causing inaccurate localization.

Literature Cited

Anonymous. 1968. Principles of plant and animal pest control, Vol. 2, weed control, Publ. 1975. Natl. Acad. Sci., Washington, D.C. p. 471.

Anonymous. 1982. Glean technical bulletin. E.I. du pont de Nemours and Co., Wilmington, DE.

Appleby, A.P. and B.E. Valverde. 1989. Behavior of dinitroaniline herbicides in plants. Weed Technol. 3:198-206.

Armbruster, B.L., W.T. Molin, and M.W. Bugg. 1991. Effects of the herbicide dithiopyr on cell division in wheat root tips. Pestic. Biochem. and Physiol. 39:110-120.

Ashton. F.M. 1967. Localization of primary site of action of herbicides. Proc. 19th Annu. Calif. Weed Conf. pp. 80-84.

Ashton, F.M. and A.S. Crafts. 1981. Mode of action of herbicides. 2nd ed. John Wiley and Sons, Inc., New York, NY.

Ashton, F.M. and T.J. Monaco. 1991. Weed Science: Principles and Practices. 3rd ed. John Wiley and Sons, Inc., New York, NY.

Bozzola, J.J. and L.D. Russell. 1992. Electron Microscopy: Principles and techniques for biologists. Jones and Bartlett Publishers, Boston, MA.

Beard, J.B. 1973. Turfgrass: Science and Culture. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Bingaman, B.R. and N.E. Christians. 1995. Greenhouse screening of corn gluten meal as a natural weed control product for broadleaf and grass weeds. HortScience 30:1256-1259.

Bonner, J. 1950. The role of toxic substances in the interactions of higher plants. Bot. Rev. 16:51-65.

Chou, C.H. and Z.A. Patrick. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. J. Chem. Ecol. 2: 369-387.

Christians, N.E. 1991. Preemergence weed control using corn gluten meal. U.S. Patent No. 5,030,268 p. 63-65.

Christians, N.E. 1993. The use of corn gluten meal as a natural preemergence weed control in turf. *In* R.C. Carrow, N.E. Christians, and R.C. Shearman (eds.) International Turfgrass Society Research Journal. 7:284-290.

Cockerham, S.T. and V.A. Gibeault. 1985. The size, scope, and importance of the turfgrass industry. p. 7-12. *In* V.A. Gibeault and S.T. Cockerham (eds.) Turfgrass Water Conservation. University of California, Riverside, Division of Agriculture and Natural Resources.

Cremlyn, R. 1978. Pesticides: Preparation and Mode of Action. John Wiley and Sons, Inc., New York, NY.

Devine, M., S.O. Duke, and C. Fedtke. 1993. Physiology of herbicide action. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Duke, S.O. 1986. Naturally occurring chemical compounds as herbicides. p. 17-44. *In* Reviews of Weed Science. Vol. 2. WSSA, Champaign, IL.

Elmore, C.L. and D.E. Bayer. 1992. Mitotic and structural effects of nitralin and butralin on ryegrass (*Lolium perenne* L.). Weed Res. 32:77-86.

Fedtke, C. 1982. Biochemistry and physiology of herbicide action. Springer-Varlag Berlin Heidelberg, Germany.

Hess, F.D. 1983. Mode of action of herbicides that affect cell division. p. 79-84 *in* J. Miyamoto and P.C. Kearney, eds. Pesticide Chemistry. Human Welfare and the Environment. Vol. 3. Mode of action, metabolism and toxicology. Pergamon, Oxford.

Hillman, G. and A. Ruthmann. 1982. Effect of mitotic inhibitors on the ultrastructure of root meristem cells. Planta. 155:124-132.

Kim, J.C. and L.E. Bendixen. 1987. Effects of haloxyfop and CGA-82725 on cell cycle and cell division of oat (*Avena sativa*) root tips. Weed Sci. 35:769-774.

Lehnen, Jr., L.P and K.C. Vaughn. 1992. The herbicide sindone B disrupts spindle microtubule organizing centers. Pestic. Biochem. and Physiol. 44:50-59.

Liu, D.L. 1993. Isolation and identification of root-inhibiting compounds from corn gluten meal. Ph.D. diss. Iowa State Univ., Ames.

Liu, D.L. and N.E. Christians. 1994. Isolation and Identification of root-inhibiting compounds from corn gluten hydrolysate. J. Plant Growth Regul. 13:227-230.

Liu, D.L., N.E. Christians, and J.T. Garbutt. 1994. Herbicidal activity of hydrolyzed corn gluten meal on three grass species under controlled environments. J. Plant Growth Regul. 13:221-226.

Muller, W.H. 1971. Effect of allelopathic substances on seedling development, pp. 73 and 74, *in* Biochemical Interactions Among Plants. Natl. Acad. Sci., Washington, D.C.

Nielsen, K.F., T.F. Cuddy, and W.B. Woods. 1960. The influence of the extract of some crops and soil residues on germination and growth. Can. J. Plant Sci. 40:188-197.

Nonnecke, G.R. and N.E. Christians. 1993. Evaluation of corn gluten meal as a natural, weed control product in strawberry. Acta Hortic. 348:315-320.

O'Donovan, J.T. and W.H. Vanden Born. 1981. A microautoradiographic study of ^{14}C-labelled picloram distribution in soybean following root uptake. Can. J. Bot. 59:1928-1931.

Palm, H.L. 1982. Today's Herbicide: Glean. Weeds Today. Winter 5-6.

Patrick, Z.A. 1971. Phytotoxic substances associated with the decomposition in soil of plant residues. Soil Sci. 111:13-18.

Patrick, Z.A. and L.W. Koch. 1958. Inhibition of respiration, germination and growth by substances arising during the decomposition of certain plant residues in the soil. Can. J. Bot. 36:621-647.

Radwan, M.A., C.R. Stocking, and H.B. Currier. 1960. Histoautoradiographic studies of herbicidal translocation. Weeds 8:657-665.

Rice, E.L. 1984. Allelopathy. 2nd ed. Academic Press, New York, NY.

Rost, T.L. 1984. The comparative cell cycle and metabolic effects of chemical treatments on root tip meristems. III. Chlorsulfuron. J. Plant Growth Regul. 3:51-63.

Setterfield, G., R. Schreiber, and J. Woodard. 1954. Mitotic frequency determinations and microphotometric feulgen dye measurements in root tips. Stain Technol. 29:113-120.

Sherman, T.D. and K.C. Vaughn. 1991. Immunology in weed science. Weed Sci. 39:514-520.

Streibig, J.C. 1992. Quantitative assessment of herbicide phytotoxicity with dilution assay. Dept. of Agric. Sciences, Royal Veterinary and Agricultural University, Copenhagen.

Turgeon, A.J. 1985. Turfgrass Management. Reston Publishing Co., Reston, NJ.

Vaughn, K.C. and L.P. Lehnen. 1991. Mitotic disrupter herbicides. Weed Sci. 39:450-457.

Ware, G.O. 1994. The pesticide book. 4th ed. Thomson Publications. Fresno, CA.

HERBICIDAL EFFECTS OF THE DIPEPTIDE, ALANINYL-ALANINE, ON PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) SEEDLINGS

A paper to be submitted to *Crop Science*

J. B. Unruh, N. E. Christians*, and H. T. Horner[1]

Abstract

Corn gluten meal (CGM), a byproduct of corn (*Zea mays* L.) wet-milling, has been shown to be an effective natural preemergence herbicide and fertilizer for various plant production systems. Alaninyl-alanine (Ala-Ala), along with four other dipeptides were isolated from CGM and identified as being the inhibitory compounds. The herbicidal effects of these natural compounds are seen as growth-regulating, root inhibitors that have minimal effect on shoot growth at low concentrations. However, little is known about the precise inhibitory action of CGM or Ala-Ala. The objective of this research was to elucidate morphological and anatomical differences in perennial ryegrass (*Lolium perenne* L.) seedlings treated with Ala-

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Abbreviations: CGM, Corn Gluten Meal; Ala-Ala, Alaninyl-alanine, LM, Light Microscopy; TEM, Transmission Electron Microscopy.

Ala using light and transmission electron microscopy, as well as autoradiographic studies using [$^{3}H^{+}$]-Ala-Ala. Root length was reduced by at least 42% at all treatment concentrations when compared to the untreated control. The treated root tips were observed to be void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, treated root tips had extreme cell wall abnormalities including uneven thickening and breakage, especially in the epidermal and subepidermal cells. The autoradiographs suggested that at the treatment concentrations causing epidermal tissue damage, there was minimal inward movement of the dipeptide. At low treatment concentrations, root tip epidermal necrosis was not evident, and inward movement of the dipeptide was not impeded.

Introduction

Corn gluten meal, a byproduct of corn wet-milling, has been shown to be an effective natural preemergence herbicide and fertilizer for various plant production systems (Christians, 1993). The CGM is the protein fraction of corn and contains approximately 10% nitrogen by weight. Plots treated with CGM as a natural organic fertilizer exhibited quality better than, or equal to, other commercially available fertilizers (Agnew and Christians, 1993). The herbicidal efficacy of CGM was tested on 22 weed species, 10 dicotyledonous and 12 monocotyledonous species, with all species exhibiting some susceptibility (Bingaman and Christians, 1995). Additionally, CGM was evaluated as a weed control product in strawberry (*Fragaria X ananassa*) production systems with results indicating that weed control in this

crop is possible (Nonnecke and Christians, 1993).

Subsequent work resulted in a water-soluble (hydrolyzed) component of CGM as well as the isolation and identification of the root inhibiting compounds from the hydrolyzed corn gluten (Liu *et al.*, 1994; Liu and Christians, 1994). These inhibitory compounds were identified as the dipeptides: glutaminyl-glutamine, alaninyl-asparagine, alaninyl-glutamine, glycinyl-alanine, and alaninyl-alanine. The herbicidal effects of CGM, both water-insoluble and water-soluble, and the inhibitory dipeptides are seen as growth-regulating, root inhibitors that have minimal effect on shoot growth at low concentrations (Christians, 1993; Liu *et al.*, 1994; Liu and Christians, 1994).

Relatively little is known about the precise inhibitory action of CGM or the root-inhibiting dipeptides. For many years, however, the inhibitory effects of corn stover on both shoot and root elongation of germinating plants have been recognized (Bonner, 1950; Nielsen *et al.*, 1960). In 1976, Chou and Patrick reported that lettuce (*Latuca sativa* L. cv. Great Lakes) seedlings germinated in aqueous extracts of decomposing corn residues developed abnormal radicles which lacked root hairs and had necrotic root tips. Their findings are similar to earlier work by Patrick and Koch (1958) and Patrick (1971). Work by Muller (1971) suggested that the radicle damage was localized to the meristematic tissue, resulting in suppressed root elongation. These results coincide with those reported by Christians (1993) and Liu and Christians (1994) dealing with the herbicidal effects of CGM. The herbicidal properties of the aforementioned dipeptides had not previously been reported in the literature.

In the research reported here, a series of experiments are presented that were designed

to elucidate morphological and anatomical differences in perennial ryegrass seedlings treated with the biologically active dipeptide, Ala-Ala.

Materials and Methods

Perennial ryegrass seeds were germinated in 100 X 15 mm round plastic petri dishes containing a 70 mm Whatman® No. 1 filter paper (Whatman International Ltd., England)[2] treated with 0.0, 0.5, 1.0, 1.5 and 2.0 mg Ala-Ala (Sigma Chemical Company, St. Louis, MO) applied in 1 ml deionized distilled water (D.D. H_2O) solutions. The dishes were covered with lids, sealed with Parafilm®, and placed in a controlled environment chamber. Photosynthetically active radiation in the growth chamber was 70 µmol m^{-2} s^{-1} provided by high output fluorescent tubes, with 25/15°C day/night temperature and a 8 h photoperiod.

Treated and untreated seedlings were macroscopically examined with an Olympus SZH zoom stereo microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and photographed with Kodak Ektachrome 160T film (Kodak Co., Rochester, NY). Root lengths of five seedlings grown at each treatment concentration were measured to the nearest millimeter after 3, 4, 5, 6, and 7 d of exposure to Ala-Ala. There were three replicates with three dishes for each treatment.

Root tips for microscopic analysis were fixed with 2%:2% paraformaldehyde: glutaraldehyde in 0.1 *M* Na^+ cacodylate buffer, pH 7.2, at 4°C for 20 h. Tissue was

[2]Mention of product and equipment names is for identification purposes only and does not imply a warranty or endorsement to the exclusion of other products that may be similar.

dehydrated in a graded ethanol series, then passed through three changes of acetone, and embedded in Spurr's "hard" resin (Electron Microscopy Sciences, Fort Washington, PA). Median longitudinal sections, 1 µm and 80 nm thick, were cut on a Reichert Ultracut S Microtome (Reichert Division, Leica, Austria), for light microscopy (LM) and transmission electron microscopy (TEM), respectively. The LM sections were mounted on glass slides and stained with 1% toluidine blue in 1% Na^+ borate for 8 s, and then were observed with a Leitz Orthoplan microscope (Leica Canada, Midland, ON), with images recorded on Kodak Technical Pan film (Kodak Co., Rochester, NY). TEM sections were placed on 200-mesh copper grids, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) for 45 min and 15 min, respectively, and observed and photographed using Kodak SO-163 Electron Image film (Kodak Co., Rochester, NY) on a JEOL 1200EX scanning transmission electron microscope operating at 80 kV (JEOL, Peabody, MA).

For autoradiographic studies, perennial ryegrass seeds were cultured in 100 X 15 mm round plastic petri dishes containing a 70 mm Whatman® No. 1 filter paper treated with 0.5, 1.0, 1.5 and 2.0 mg Ala-Ala applied in 1 ml D.D. H_2O solutions. Each treatment contained 259 kBq [$^3H^+$]-Ala-Ala (specific activity = 3.7 X 10^5 kBq $mmol^{-1}$) (Moravek Biochemicals, Brea, CA). The dishes were covered with lids, sealed with Parafilm®, and placed in a controlled environment chamber with conditions being the same as those for previous experiments. After 6 d, root tips were rinsed with 0.1 *M* Na^+ cacodylate buffer, pH 7.2, to remove any exogenous isotope and fixed with 2%:2% paraformaldehyde: glutaraldehyde in the same buffer at 4°C for 20 h. Tissue was dehydrated and embedded as previously described.

All solutions were retained and subjected to liquid scintillation counting to detect any loss of the isotope caused by the fixation/dehydration chemicals.

Median longitudinal serial sections of the roots, 1 µm thick, were cut on a Reichert Ultracut S Microtome, mounted on glass slides and coated with Kodak NTB-2 liquid emulsion (International Biotech, New Haven, Conn.) in the dark. The slides were dried, then packed in light-tight boxes containing a desiccant, and stored in a refrigerator for 6 d. At the end of the exposure period, slides were developed in Kodak Dektol (1:1 with D.D. H_2O) developer (Kodak Co., Rochester, NY) for two min, rinsed in water, fixed in Kodak fixer for four min., and washed in running water. Slides were then dehydrated in a graded ethanol series to xylene, Permount was added, followed by a cover slip, and observed and photographed with a Leitz Orthoplan microscope. Images were recorded on Kodak Technical Pan film.

Results and Discussion

Comparison between treated and untreated roots showed that treated roots were severely inhibited and necrotic (Fig. 1B), compared to the untreated roots (Fig. 1A). Root length was reduced by at least 42% with all treatment concentrations when compared to the untreated control (Table 1). As the duration of exposure increased, the difference between treatments and the control became greater, because the untreated roots continued to grow, whereas growth in the treated roots subsided. Differences in root lengths at the varying concentrations of Ala-Ala were noted at all measurement times except for Day 3. Other than this exception, the 0.5 and 1.0 mg treatments were not different from each other, as was also

the case between the 1.5 and 2.0 mg treatment concentrations. Differences between the lower two and upper two treatment concentrations at 5, 6, and 7 d of exposure consistently resulted in an average of 13 to 14% greater reduction in root length with the higher doses. Higher treatment concentrations at Day 4 resulted in an average of 26% greater reduction in root length.

Elmore and Bayer (1992) described the root apical meristem in perennial ryegrass as having three groups of initials: one giving rise to the root cap; one producing the cells of the epidermis and cortex; and the third, innermost set, forming the vascular cylinder. The observations of Ala-Ala treated perennial ryegrass roots will be presented and discussed in this order.

Root-cap cells in Ala-Ala treated seedlings exhibited disruption similar to the rest of the root (Figs. 1D and E). Cells appeared to be void of cellular components, specifically discernible nuclei, mitotic structures, and amyloplasts. This differed from the untreated control seedling root-cap (Fig. 1C) which was composed of parenchyma cells that had small, uniform nuclei, visible mitotic structures (arrows), and amyloplasts. The absence of amyloplasts was similar to the effects of nitralin (4-methylsulphonyl-2,6-dinitro-N,N-dipropy-laniline) and butralin [4-(1,1-dimethylethyl)-N-(1-methylpropyl)-2,6-dinitrobenzenamine] treated perennial ryegrass seedlings that were observed by Elmore and Bayer (1992). Esau (1965) mentioned that the starch grains in the amyloplasts are rather persistent, in that they are not readily utilized except under conditions of extreme starvation. The lack of amyloplasts in the Ala-Ala treated roots was possibly a result of starch utilization in response to starvation caused by a disruption

in cellular processes, or, they were not produced because of the Ala-Ala induced cellular disruption.

As previously stated, the second group of initials produce the cells of the epidermis and cortex. For discussion purposes, these cell layers have been identified with the numerals 1, 2, and 3 (Fig. 1C). The outermost layer, the epidermis, consisted of large, closely aligned, laterally elongated cells with thin walls. Directly beneath the epidermis were layers of small, less rectangular cortical cells with dense cytoplasm and large nuclei.

Light micrographs of the whole root tip (Figs. 1C - E) show the effects of Ala-Ala on root tips. Cellular necrosis was evident in both 1.0 mg and 2.0 mg Ala-Ala treated roots. This necrosis, however, was much more severe with the 2.0 mg treatment. Abnormal cell sizes and shapes were noted at both treatment concentrations when compared to the untreated control. Additionally, cells in the 1.0 mg Ala-Ala treated roots possessed only interphase nuclei, whereas no nuclei were observed in roots treated with the higher concentration.

Electron micrographs (Figs. 2A and B) from the second layer of cells, approximately 250 ：m from the root tip, showed that differences between the untreated control and 1.0 mg Ala-Ala treatment were minimal. Cell size and shape of treated and untreated roots were comparable and possessed no gross abnormalities. Roots treated with 2.0 mg Ala-Ala, however, possessed cells with extreme cell wall abnormalities including uneven thickening and breakage, along with a wide variation in cell size and shape (Fig. 2C). Although some nuclei were visible, these cells were mostly vacuolate with contents that appeared to be remnants of cellular organelles.

The vascular cylinder, formed from the innermost set of initials, was not evident within 300 ：m of the root initials in the untreated control. In the 1.0 mg treated roots, vascular elements were abundant and could be seen within 130 ：m from the root tip. In the 2.0 mg treated roots, vascular tissue was minimal, and only observed after 438 ：m from the root tip. The 1.0 mg Ala-Ala treatment produced similar effects as nitralin and butralin in that the developmental stages, specifically xylem development, appeared closer to the apex (Elmore and Bayer, 1992). Ashton and Crafts (1981) and Hess (1983) stated that increased differentiation is a characteristic of roots in which mitosis has been affected by herbicide treatment.

Liquid scintillation counting detected appreciable isotope activity only in the pre-fixation buffer wash solutions, indicating that the fixation/dehydration solutions did not dislodge the [$^{3}H^{+}$]-Ala-Ala. The autoradiograph of the 0.5 mg [$^{3}H^{+}$]-Ala-Ala treated root showed uniform diffuse labelling throughout the entire root tip (Fig. 3A), and the radioactivity did not appear to be associated with any specific tissue type. Localization of radioactivity in the 1.0 mg treated roots was less uniform than the 0.5 mg treatment (Fig. 3B), with intense localization along one edge. Fig. 3C shows profuse localization along one side of the 1.5 mg treated root. In the 2.0 mg treatment, a considerable amount of radioactivity was present in the epidermal cells extending back 205 ：m from the tip of the root cap (Fig. 3D). This region revealed very little localization interior to the epidermis. There was, however, increased localization throughout the epidermis, cortex, and vascular region beyond this area of intense localization. Although some variability was observed, these autoradiographs indicate that at

the high treatment concentrations, the epidermal cells were adversely affected, thus decreasing the inward movement of the dipeptide. This effect possibly could be a result of abnormal cell wall thickening common to the epidermal cells in the treated roots, or disrupted cellular transport mechanisms. At the lower treatment concentrations, root tip necrosis was not as evident, and movement of the dipeptide did not seem to be impeded.

The herbicidal effects of the dipeptide, Ala-Ala, on treated perennial ryegrass roots were numerous. Of the observed abnormalities, it seemed that Ala-Ala, like most herbicides, responds differently to different concentrations. Higher concentration levels seemed to cause more of an epidermal necrosis, whereas lower concentrations produced more subtle effects to the cellular processes. Further research will need to be carried out to identify the specific mode of action of this biologically active dipeptide.

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Literature Cited

Agnew, M.L. and N.E. Christians. 1993. Influence of fertilizer source and core cultivation on the growth of Kentucky bluegrass. *In* R.C. Carrow, N.E. Christians, and R.C. Shearman (eds.) International Turfgrass Society Research Journal. 7:458-463.

Ashton, F.M. and A.S. Crafts. 1981. Mode of action of herbicides. 2nd ed. John Wiley and Sons, Inc., New York, NY.

Bingaman, B.R. and N.E. Christians. 1995. Greenhouse screening of corn gluten meal as a natural weed control product for broadleaf and grass weeds. HortScience 30: 1256-1259.

Bonner, J. 1950. The role of toxic substances in the interactions of higher plants. Bot. Rev. 16:51-65.

Chou, C.H. and Z.A. Patrick. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. J. Chem. Ecol. 2: 369-387.

Christians, N.E. 1993. The use of corn gluten meal as a natural preemergence weed control in turf. *In* R.C. Carrow, N.E. Christians, and R.C. Shearman (eds.) International Turfgrass Society Research Journal. 7:284-290.

Elmore, C.L. and D.E. Bayer. 1992. Mitotic and structural effects of nitralin and butralin on ryegrass (*Lolium perenne* L.) root meristems. Weed Res. 32: 77-86.

Esau, K. 1965. Plant anatomy. 2nd ed. Wiley, New York, NY.

Hess, F.D. 1983. Mode of action of herbicides that affect cell division. p. 79-84 *in* J. Miyamoto and P.C. Kearney, eds. Pesticide Chemistry. Human Welfare and the Environment. Vol. 3. Mode of action, metabolism and toxicology. Pergamon, Oxford.

Liu, D.L. and N.E. Christians. 1994. Isolation and Identification of root-inhibiting compounds from corn gluten hydrolysate. J. Plant Growth Regul. 13:227-230.

Liu, D.L., N.E. Christians, and J.T. Garbutt. 1994. Herbicidal activity of hydrolyzed corn gluten meal on three grass species under controlled environments. J. Plant Growth Regul. 13:221-226.

Muller, W.H. 1971. Effect of allelopathic substances on seedling development, pp. 73 and 74, *in* Biochemical Interactions Among Plants. Natl. Acad. Sci., Washington, D.C.

Nielsen, K.F., T.F. Cuddy, and W.B. Woods. 1960. The influence of the extract of some crops and soil residues on germination and growth. Can. J. Plant Sci. 40:188-197.

Nonnecke, G.R. and N.E. Christians. 1993. Evaluation of corn gluten meal as a natural, weed control product in strawberry. Acta Hortic. 348:315-320.

Patrick, Z.A. 1971. Phytotoxic substances associated with the decomposition in soil of plant residues. Soil Sci. 111:13-18.

Patrick, Z.A. and L.W. Koch. 1958. Inhibition of respiration, germination and growth by substances arising during the decomposition of certain plant residues in the soil. Can. J. Bot. 36:621-647.

Reynolds, E.S. 1963. The use of lead citrate and high pH as an electron opaque stain for electron microscopy. J. Cell Biol. 17:208-212.

Watson, M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4:475-478.

Table 1. Length of perennial ryegrass roots in response to duration of exposure to specific concentrations of alaninyl-alanine (Ala-Ala), expressed as percent of control.

Ala-Ala Concentration	Duration of exposure, d				
	3	4	5	6	7
(mg)	---------- (mm)[†] ----------				
Control	0.5 a	4.1 a	8.5 a	11.9 a	14.6 a
	---------- % of Control ----------				
0.5	46 b	47 bc	33 b	29 b	24 b
1.0	42 b	58 b	35 b	32 b	29 b
1.5	17 b	24 d	19 c	16 c	14 c
2.0	21 b	29 cd	21 c	17 c	14 c

[†]Values within each column sharing the same letter do not differ significantly at $P = 0.05$.

Figure 1. Comparison of alaninyl-alanine (Ala-Ala) treated and untreated perennial ryegrass roots. A. Photomacrograph of untreated seedling. Bar = 231 ：m. B. Photomacrograph of Ala-Ala treated seedling. Bar = 21 ：m. C. Photomicrograph showing median longitudinal section of untreated 5-d old root tip. Epidermal and sub-epidermal cell layers numbered 1, 2, and 3. Arrows = mitotic structures. Bar = 44 ：m. D. Photomicrograph showing median longitudinal section of 1.0 mg Ala-Ala treated 5-d old root tip. Necrosis prevalent in epidermal layer 1. Bar = 40 ：m. E. Photo-micrograph showing median longitudinal section of a 2.0 mg Ala-Ala treated 5-d old root tip. Note extensive epidermal and cortical necrosis. Bar = 40 ：m.

Figure 2. Transmission electron micrographs of layer 2 cortical cells approximately 300 ∶m from the tip of the root cap. Bars = 5 ∶m. A. Untreated. B. 1.0 mg Ala-Ala. C. 2.0 mg Ala-Ala. Little difference noted between untreated control and 1.0 mg Ala-Ala. Extreme cell wall abnormalities in the 2.0 mg treatment include uneven thickening and breakage.

Figure 3. Autoradiographs of [$^{3}H^{+}$]-Ala-Ala treated and untreated perennial ryegrass root tips. RT = Root Tip; Arrows = Epidermis. Bars = 59 ：m. A. 0.5 mg [$^{3}H^{+}$]-Ala-Ala. B. 1.0 mg [$^{3}H^{+}$]-Ala-Ala. C. 1.5 mg [$^{3}H^{+}$]-Ala-Ala. D. 2.0 mg [$^{3}H^{+}$]-Ala-Ala. Diffuse labelling noted throughout entire root tip in 0.5 mg treatment. Intense localization along one side of root tip in the 1.0 and 1.5 mg treatments. In the 2.0 mg treatment, considerable amount of radioactivity present in the epidermal cells, with little localization interior to this layer.

MITOTIC AND ULTRASTRUCTURE CHANGES IN ROOT MERISTEMS OF GRASS SEEDLINGS TREATED WITH ALANINYL-ALANINE

A paper to be submitted to *Crop Science*

J. B. Unruh, N. E. Christians*, and H. T. Horner[1]

Abstract

Alaninyl-alanine (Ala-Ala), is a biologically active dipeptide that inhibits the rooting of germinating seeds. Previous research revealed that treated root tips were void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, treated root tips had extreme cell wall abnormalities including uneven thickening and breakage. Many commonly used synthetic herbicides, such as the dinitroanilines, carbamates, and dithiopyr [S,S-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-pyridinedicarbothioate], produce similar effects on root meristems. The objectives of this investigation were to use time-course studies to monitor the

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Abbreviations: Ala-Ala, Alaninyl-Alanine; LM, Light Microscopy; TEM, Transmission Electron Microscopy.

mitotic activity of perennial ryegrass *(Lolium perenne* L.) roots treated with Ala-Ala; to use light and transmission electron microscopy to describe Ala-Ala induced changes in cellular ultrastructure in an effort to elucidate the mode of action of this biologically active dipeptide; and to make comparisons with other reported modes of action of commonly used synthetic herbicides. Results showed that Ala-Ala exhibited activity on mitosis within 4 h of exposure, and by 6 h, reduction in the number of mitotic figures was nearly 100%, resulting in only interphase cells. Microscopic analysis revealed profound treatment effects. By 12 h, dense droplets, presumably membrane lipids, were visible in vacuoles and intercellular spaces. After 48 h exposure, epidermal and cortical cell elongation in treated roots appeared to occur perpendicular to the normal elongation plane, possibly resulting from a loss of cell polarity. Root lateral branching, similar to effects of synthetic preemergence herbicides, was also noted after a 48 h exposure time. Root tips, however, showed no gross external abnormalities until after a 96 h exposure to Ala-Ala.

Introduction

Alaninyl-alanine (Ala-Ala), a biologically active dipeptide in corn (*Zea mays* L.) gluten meal, was reported to greatly inhibit root growth in germinating seeds (Liu *et al.*, 1994). The morphological and anatomical differences in 6-d old perennial ryegrass seedlings treated with this dipeptide have been described by Unruh (1995). He described the treated root tips as being void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, treated root tips had extreme cell wall

abnormalities including uneven thickening and breakage. These results serve as a basis for further work on elucidating the mechanism(s) by which this biologically active dipeptide exerts herbicidal activity.

Many commonly used synthetic herbicides, such as the dinitroanilines, carbamates, and dithiopyr, produce similar effects on root meristems. Elmore and Bayer (1992) described the mitotic and structural effects of the dinitroaniline herbicides, nitralin (4-methylsulphonyl-2,6-dinitro-N,N-dipropy-laniline) and butralin [4-(1,1-dimethylethyl)-N-(1-methylpropyl)-2,6-dinitrobenzenamine], on perennial ryegrass root meristems. They reported that both herbicides suppressed root elongation, had root radial enlargement, and produced abnormal mitotic figures, resulting in disrupted cell division. In a review of mitotic disrupter herbicides, Vaughn and Lehnen (1991) described carbamate herbicides as disrupting mitosis by producing multipolar mitotic figures which direct the chromosomes toward three or more foci, rather than the two poles at normal anaphase, yielding irregularly shaped and abnormal cell walls. Armbruster *et al.* (1991) described the effects of dithiopyr as disrupting spindle microtubule formation which yields abnormal mitotic profiles, aberrant cell wall deposition patterns, and the cessation of cell elongation.

In an effort to provide definitive terminology for these effects, Hess (1983) proposed that herbicide effects on cell division be divided into either disruption or inhibition. Hess contended that if a herbicide causes an inhibition of cell division, the cell cycle analysis of treated meristems will yield only interphase cells, giving rise to the name "preprophase inhibitors." Plants treated with herbicides that cause disruption of cell division have mitotic

stages present, however, one or more stages normally present will be absent or aberrant.

The mechanisms by which inhibition and disruption of cell division occur are unique from each other. Inhibition of cell division is a secondary effect caused by some disturbance of a plant's metabolic process (Hess, 1983; Kim and Bendixen, 1987). Commonly reported metabolic disturbances include DNA, RNA, or protein synthesis, as well as energy metabolism (Hess, 1983). Disruption of cell division is usually caused by a primary effect on the mitotic apparatus (Hess, 1983). This apparatus organizes and separates the chromosomes during mitosis (Devine *et al.*, 1993), determines the plane of cell division, and orients cellular microfibril deposition in growing plant cells (Appleby and Valverde, 1989). Regardless of which process is affected, both processes result in a reduced supply of new cells in the root meristem leading to an eventual inhibition of growth.

The herbicidal effects of Ala-Ala need to be examined over time so that definitive cause-response relations can be explained and compared to other root inhibiting herbicides. The objectives of this investigation were: to use time-course studies to monitor the mitotic activity of perennial ryegrass roots treated with Ala-Ala; to use light and transmission electron microscopy to describe Ala-Ala induced changes in perennial ryegrass root cell ultrastructure in an effort to elucidate the mode of action of this dipeptide; and to make comparisons to reported modes of action of commonly used synthetic herbicides.

Materials and Methods

Ten perennial ryegrass seeds were germinated in 100 X 15 mm round plastic petri

dishes containing a 70mm Whatman® No. 1 filter paper (Whatman International Ltd., England)[2], treated with 1 ml deionized, distilled water (D.D. H_2O). The dishes were covered with lids, sealed with Parafilm®, and placed in a controlled environment chamber. Photosynthetically active radiation in the growth chamber was 70 µmol m^{-2} s^{-1} provided by high-output fluorescent tubes, with 25/15°C day/night temperature and a 8 h photoperiod. After 72 h, seedlings were transferred to petri dishes containing a filter paper treated with 0.0, 0.5, 1.0, 1.5 and 2.0 mg Ala-Ala (Sigma Chemical Company, St. Louis, MO) applied in 2 ml D.D. H_2O solutions and returned the controlled environment chamber.

For mitotic index analysis, 3-mm long root tips were fixed for 24 h in an absolute ethanol:glacial acetic acid solution (3:1, v/v) at 0, 0.5, 1, 2, 4, and 6 h following the transfer to treatment solutions. Root tips were then stored in 70% ethanol until mitotic index determinations could be made. Prior to analysis, the root tips were placed in distilled water for 20 min to remove the ethanol. Root tips were then hydrolyzed in 5 *M* HCl for 30 min before being stained in Schiff's reagent for 15 min. After staining, a 1-mm root tip was placed in a drop of 45% acetic acid, cover slipped, and squashed into single cells using the eraser end of a pencil to apply pressure. Mitotic index analysis was then performed, and the results were expressed as percentage of mitotic figures per 1000 cells evaluated per slide. Data are the means obtained from counts of three slides per time period.

Root tips for microscopic analysis, cultured as described previously, were fixed at 12,

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[2]Mention of product and equipment names is for identification purposes only and does not imply a warranty or endorsement to the exclusion of other products that may be similar.

24, 48, and 96 h after transfer to either D.D. H_2O (control) or 1.0 mg Ala-Ala treatment solutions, with 2%:2% paraformaldehyde:glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, at 4°C for 20 h, and post-fixed in 0.33% osmium tetroxide in the same buffer at room temperature for 15 min. Root tips were dehydrated in a graded ethanol series and an acetone transition fluid, and embedded in Spurr's "hard" resin (Electron Microscopy Sciences, Fort Washington, PA). Median longitudinal sections, 1 µm and 80 nm thick, were cut on a Reichert Ultracut S Microtome (Reichert Division, Leica, Austria), for LM and TEM, respectively. The LM sections were mounted on glass slides, stained with 1% toluidine blue in 1% Na^+ borate for 8 s and were observed with a Leitz Orthoplan microscope (Leica Canada, Midland, ON), with images recorded on Kodak Technical Pan film (Kodak Co., Rochester, NY). The TEM sections were placed on formvar coated, 50-mesh copper grids, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) for 45 min and 15 min, respectively, and observed and photographed using Kodak SO-163 Electron Image film (Kodak Co., Rochester, NY) on a JEOL 1200EX scanning transmission electron microscope (JEOL, Peabody, MA) operating at 80 kV.

Results and Discussion

The effect of Ala-Ala on cell division in roots of pregerminated perennial ryegrass seedlings is presented in Fig. 1. The Ala-Ala exhibited activity within 4 h of exposure. By 6 h of exposure, reduction in the number of mitotic figures was nearly 100%, resulting in only interphase cells. At treatment concentrations where mitotic figures were present, none were

aberrant, and all four common mitotic phases were seen.

These results suggested that Ala-Ala caused an inhibition of cell division, rather than disruption, since only interphase cells were present. As stated previously, the inhibition of cell division was a secondary effect caused by some disturbance of a plant metabolic process. Hess (1983) asserted that if the effect on plant metabolism causing an inhibition of cell division was expressed immediately after herbicide treatment, the number of cells in mitosis would decrease to zero within 7 to 10 h, indicating that the compound was inhibiting some metabolic process in interphase.

Light micrographs of median longitudinal sections showing time-course effects of Ala-Ala treated and untreated perennial ryegrass roots are shown in Figs. 2 and 3A. Untreated root tips (Fig. 2A), regardless of their age, were quite similar and showed no distinct differences. Profound treatment effects were observed, however, as the duration of exposure to Ala-Ala increased. As early as 12 h of exposure, cells in the region of elongation were greatly disrupted (Fig. 2B). Root cap and meristematic region cells showed no gross abnormalities. Electron micrographs (Figs. 3D and E) from the region indicated by the arrow in Fig. 2B, showed highly vacuolate cells containing dense droplets, possibly derived from membrane lipids. Droplets were also seen in the intercellular spaces. Similar results have been observed by Hillman and Ruthmann (1982) in *Vicia faba* seedlings treated with the plant alkaloid, vinblastine. They speculated that this material may be present due either to a direct effect of vinblastine on lipid metabolism or to vinblastine binding to the membrane which leads to an increased production of membrane lipids.

Roots exposed to Ala-Ala for 24 h (Fig. 2C; Figs. 4A and B) produced results similar to the roots exposed for 12 h. The effects, however, were not as severe as those observed in roots treated for 12 h. The effects of Ala-Ala were much more pronounced after a 48 h exposure. Epidermal and cortical cell elongation in the treated roots appeared to occur perpendicular to the normal elongation plane (Fig. 2D; Figs. 4C and D). This type of effect was noted in other root inhibiting herbicides such as trifluralin (",","-trifluoro-2,6-dinitro-*p*-toluidine) (Lignowski and Scott, 1971), dithiopyr (Armbruster *et al.*, 1991), and nitralin and butralin (Elmore and Bayer, 1992). It is generally agreed that herbicide induced abnormal cell growth patterns are a result of a loss in cell polarity.

Light micrographs of roots exposed to Ala-Ala for 96 h showed the greatest degree of cellular disruption (Fig. 3A). Electron micrographs of the normal orderly cell files as observed in the untreated controls (Fig. 5A), were altered, yielding a disorganized mass of irregularly shaped cells of different sizes (Figs. 5B and C).

Roots exposed to Ala-Ala for 48 h or more developed lateral roots (Figs. 3B and C). This effect was not observed in the untreated controls, regardless of their age, or in roots exposed to Ala-Ala for less than 48 h. Similar results have been reported for a number of preemergence herbicide families including the benzofurans and diamino-s-triazines (Harper, 1982), and the dinitroanilines (Seagle, 1978; Finney, 1991). Wareing and Phillips (1981) and Esau (1965) agreed that the root apical meristem exerts an inhibitory effect on lateral root initiation. We conclude that the cellular disruption, characteristic of the Ala-Ala treated root meristems, disabled this inhibitory effect, resulting in the observed lateral branching.

Figure 6 shows the effects of Ala-Ala on the root apical meristem. Untreated controls at 24 h and 96 h (Figs. 6A and C, respectively) showed little difference from each other, or from a 24 h exposure to Ala-Ala (Fig. 6B). After a 96 h exposure to Ala-Ala, however, the root no longer resembled the untreated control. Cells were much larger, presumably because they had not divided, and also lacked cellular organelles. Both treated and untreated cells possessed many small vacuoles. The vacuoles in the treated roots, both 24 h and to a greater extent 96 h, contain electron dense material, possibly lipid material, as well as degraded cellular components.

The combined results of this study lead to the conclusion that the detrimental effects of Ala-Ala on root meristem growth is the result of an inhibition of cell division rather than a disruption of cell division processes. Because only interphase cells were present and no absent or aberrant mitotic stages were noted, we conclude that Ala-Ala is acting on some metabolic process rather than directly on the mitotic apparatus. The observed effect on mitosis caused by Ala-Ala was different than the dinitroanilines, carbamates, and dithiopyr, all of which affect the mitotic apparatus (Vaughn and Lehnen, 1991), and was similar to the chloracetamides and sulfonylurea herbicides which affect plant metabolic processes (Deal and Hess, 1980; Rost, 1984).

The observed effects on cell walls and membranes, as well as the presence of lipid materials in vacuoles and intercellular spaces, implicates Ala-Ala as having membrane disrupting characteristics. Several classes of herbicides including the aryl-propanoic acids, cyclohexanediones, thiocarbamates, and chloracetamides have been reported to damage

cellular membranes by affecting lipid synthesis (Devine *et al.*, 1993). Whether or not Ala-Ala causes a primary effect on lipid synthesis remains unknown and could be the focus of additional work.

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Literature Cited

Appleby, A.P. and B.E. Valverde. 1989. Behavior of dinitroaniline herbicides in plants. Weed Technol. 3:198-206.

Armbruster, B.L., W.T. Molin, and M.W. Bugg. 1991. Effects of the herbicide dithiopyr on cell division in wheat root tips. Pestic. Biochem. Physiol. 39:110-120.

Deal, L.M. and F.D. Hess. 1980. An analysis of the growth inhibitory characteristics of alachlor and metolachlor on pea and oat seedlings. Weed Sci. 28:168-175.

Devine, M., S.O. Duke, and C. Fedtke. 1993. Physiology of herbicide action. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Elmore, C.L. and D.E. Bayer. 1992. Mitotic and structural effects of nitralin and butralin on ryegrass (*Lolium perenne* L.). Weed Res. 32:77-86.

Esau, K. 1965. Plant anatomy. 2nd ed. Wiley, New York, NY.

Finney, S.B. 1991. Cellular responses of bentgrass and bermudagrass to selected preemergence herbicides. M.S. thesis. Univ. of Tennessee, Knoxville.

Harper, C.A. 1982. Root histology of two turfgrass cultivars in response to selected herbicides. M.S. thesis. Univ. of Tennessee, Knoxville.

Hess, F.D. 1983. Mode of action of herbicides that affect cell division. p. 79-84 *in* J. Miyamoto and P.C. Kearney, eds. Pesticide Chemistry. Human Welfare and the Environment. Vol. 3. Mode of action, metabolism and toxicology. Pergamon, Oxford.

Hillman, G. and A. Ruthmann. 1982. Effect of mitotic inhibitors on the ultrastructure of root meristem cells. Planta. 155:124-132.

Kim, J.C. and L.E. Bendixen. 1987. Effects of haloxyfop and CGA-82725 on cell cycle and cell division of oat (*Avena sativa*) root tips. Weed Sci. 35:769-774.

Lignowski, E.M. and E.G. Scott. 1971. Trifluralin and root growth. Plant Cell Physiol. 12:701-708.

Liu, D.L., N.E. Christians, and J.T. Garbutt. 1994. Herbicidal activity of hydrolyzed corn gluten meal on three grass species under controlled environments. J. Plant Growth Regul. 13:221-226.

Reynolds, E.S. 1963. The use of lead citrate and high pH as an electron opaque stain for electron microscopy. J. Cell Biol. 17:208-212.

Rost, T.L. 1984. The comparative cell cycle and metabolic effects of chemical treatments on root tip meristems. III. Chlorsulfuron. J. Plant Growth Regul. 3:51-63.

Seagle, E.D. 1978. Cellular responses in root of bentgrass and bermudagrass to selected herbicides. M.S. thesis. Univ. of Tennessee, Knoxville.

Unruh, J.B. 1995. Cellular effects in perennial ryegrass (*Lolium perenne* L.) associated with the root inhibiting compound alaninyl-alanine. Ph.D. diss. Iowa State Univ., Ames, IA.

Vaughn, K.C. and L.P. Lehnen, Jr. 1991. Mitotic disrupter herbicides. Weed Sci. 39:450-457.

Wareing, P.F. and I.D.J. Phillips. 1981. Growth and differentiation in plants. 3rd ed. Pergamon Press, Elmsford, NY.

Watson, M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. 4:475-478.

Figure 1. Effect of biologically active dipeptide, alaninyl-alanine (Ala-Ala), on mitotic index of perennial ryegrass roots at different durations of exposure.

Figure 2. Photomicrographs of median longitudinal sections showing time-course effects of Ala-Ala treated and untreated perennial ryegrass roots. Arrows indicate regions observed with transmission electron microscope as shown in Figs. 3 - 5. A. Untreated root tip. Bar = 63 ：m. B. 1.0 mg Ala-Ala, 12 h exposure. Bar = 63 ：m. C. 1.0 mg Ala-Ala, 48 h exposure. Bar = 64 ：m. D. 1.5 mg Ala-Ala, 96 h exposure. Bar = 59 ：m.

Figure 3. A-C. Photomicrographs of median longitudinal sections showing time-course effects of Ala-Ala treated and untreated perennial ryegrass roots. A. 1.0 mg Ala-Ala, 96 h exposure. Arrow indicates region observed with transmission electron microscope as shown in Fig. 5. Bar = 65 ：m. B. Lateral root formation (arrow) seen at 48 h exposure. Bar = 65 ：m. C. Lateral root formation after 96 h exposure. Bar = 59 ：m.

D-E. Transmission electron micrographs of Ala-Ala treated perennial ryegrass roots after 12 h exposure. D. Dense droplets, possibly from membrane lipids, denoted by arrow. Bar = 3 ：m. E. Dense droplet shown in D. Bar = 0.5 ：m.

Figure 4. Transmission electron micrographs of perennial ryegrass root tip epidermal cells. A. Untreated control - 24 h exposure. Bar = 4 ：m. B. 1.0 mg Ala-Ala - 24 h exposure. Bar = 6 ：m. C. 1.0 mg Ala-Ala - 48 h exposure. Bar = 6 ：m. D. 1.0 mg Ala-Ala - 48 h exposure. Bar = 5 ：m.

Figure 5. Transmission electron micrographs of perennial ryegrass root tip epidermal cells. A. Untreated control - 96 h exposure. Bar = 5 ：m. B. 1.0 mg Ala-Ala - 96 h exposure. Bar = 5 ：m. C. 1.0 mg Ala-Ala - 96 h exposure. Bar = 4 ：m.

Figure 6. Transmission electron micrographs of perennial ryegrass root tip apical meristem cells. Bars = 5 ：m. A. Untreated control - 24 h exposure. B. 1.0 mg Ala-Ala - 24 h exposure. C. Untreated control - 96 h exposure. D. 1.0 mg Ala-Ala - 96 h exposure.

GENERAL CONCLUSIONS

Results from the experiments reported in the preceding two manuscripts suggest that alaninyl-alanine (Ala-Ala), a biologically active dipeptide derived from corn gluten meal, is a potent inhibitor of rooting. The mechanism(s) by which this dipeptide exerts its activity is/are still unknown, however, the results from these experiments identify some possibilities.

The results from the first set of experiments described the treated root tips as being void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, treated root tips had extreme cell wall abnormalities including uneven thickening and breakage. Autoradiographs suggested that at the higher treatment concentrations, the epidermal tissue was adversely affected, thus decreasing the inward movement of the dipeptide. This could possibly be a result of abnormal cell wall thickening common to the epidermal cells in the treated roots. At the lower treatment concentrations, root tip necrosis was not evident, and inward movement of the dipeptide was not impeded.

In comparing the effects of this dipeptide with many of the commonly used synthetic herbicides, such as the dinitroanilines, carbamates, and dithiopyr, it was noted that many similarities between these root inhibiting compounds and the dipeptide exist. Review of the literature on mode of action studies for these synthetic herbicides provided a basis for further experimental approaches to elucidating the mode of action of this dipeptide. Common to much of this literature were studies to determine the herbicidal effects on mitosis and cell ultrastructure of treated roots, thus leading to the objectives of the second part of this research.

Results from time-course experiments showed that Ala-Ala exhibited herbicidal activity within 4 h of exposure. By 6 h of exposure, reduction in the number of mitotic figures was nearly 100%, resulting in only interphase cells. At treatment concentrations where mitotic figures were present, none were aberrant and all four common mitotic stages were seen.

The combined results of this study lead to the conclusion that the detrimental effects of Ala-Ala on root meristem growth is the result of an inhibition of cell division rather than a disruption of cell division processes. Because only interphase cells were present and no absent or aberrant mitotic stages were noted, we conclude that Ala-Ala is acting on some metabolic process rather than directly on the mitotic apparatus. The observed effect on mitosis caused by Ala-Ala was different than the dinitroanilines, carbamates, and dithiopyr, all of which affect the mitotic apparatus (Vaughn and Lehnen, 1991), and was similar to the chloracetamides and sulfonylurea herbicides which affect plant metabolic processes (Deal and Hess, 1980; Rost, 1984).

The observed effects on cell walls and membranes, as well as the presence of lipid materials in vacuoles and intercellular spaces, implicates Ala-Ala as having membrane disrupting characteristics. Several classes of herbicides including the aryl-propanoic acids, cyclohexanediones, thiocarbamates, and chloracetamides have been reported to damage cellular membranes by affecting lipid synthesis (Devine *et al.*, 1993). Whether or not Ala-Ala causes a primary effect on lipid synthesis remains unknown and could be the focus of additional work.

Literature Cited

Christians, N.E. 1993. The use of corn gluten meal as a natural preemergence weed control in turf. *In* R.C. Carrow, N.E. Christians, and R.C. Shearman (eds.) International Turfgrass Society Research Journal. 7:284-290.

Deal, L.M. and F.D. Hess. 1980. An analysis of the growth inhibitory characteristics of alachlor and metolachlor on pea and oat seedlings. Weed Sci. 28:168-175.

Devine, M., S.O. Duke, and C. Fedtke. 1993. Physiology of herbicide action. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Liu, D.L., N.E. Christians, and J.T. Garbutt. 1994. Herbicidal activity of hydrolyzed corn gluten meal on three grass species under controlled environments. J. Plant Growth Regul. 13:221-226.

Rost, T.L. 1984. The comparative cell cycle and metabolic effects of chemical treatments on root tip meristems. III. Chlorsulfuron. J. Plant Growth Regul. 3:51-63.

Vaughn, K.C. and L.P. Lehnen, Jr. 1991. Mitotic disrupter herbicides. Weed Sci. 39:450-457.

Cellular effects in perennial ryegrass (*Lolium perenne* L.) associated with the root inhibiting compound alaninyl-alanine

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Corn gluten meal (CGM) has been shown to be an effective natural preemergence herbicide and fertilizer for various plant production systems. Alaninyl-alanine (Ala-Ala), along with four other dipeptides, were isolated from CGM and identified as being the inhibitory compounds. The herbicidal effects are seen as growth-regulating, root inhibitors that have minimal effect on shoots at low concentrations. Little is known about the inhibitory action of CGM or Ala-Ala.

The objective of the first phase of this research was to elucidate morphological and anatomical differences in perennial ryegrass seedlings treated with Ala-Ala using light and transmission electron microscopy, as well autoradiographic studies using [$^{3}H^{+}$]-Ala-Ala. Results from these experiments described the treated root tips as being void of cellular components, specifically discernible nuclei and mitotic structures, with an overall loss of cytoplasmic integrity. Furthermore, root tips had extreme cell wall abnormalities including uneven thickening and breakage. Autoradiographs suggested that at high treatment concentrations causing epidermal tissue damage, there was minimal inward movement of

dipeptide. At lower concentrations, root tip epidermal necrosis was not evident, and inward movement of Ala-Ala was not impeded.

The objectives of the second phase of research were to use time-course studies to monitor the mitotic activity of roots treated with Ala-Ala; to use light and transmission electron microscopy to describe Ala-Ala induced changes in root cell ultrastructure; and to make comparisons with other reported modes of action of synthetic herbicides. Results showed that Ala-Ala exhibited activity on mitosis within 4 h of exposure, and by 6 h, reduction in the number of mitotic figures was nearly 100%, resulting in only interphase cells. Microscopic analysis revealed profound treatment effects. By 12 h, dense droplets, presumably membrane lipids, were visible in vacuoles and intercellular spaces. After a 48 h exposure, epidermal and cortical cell elongation in treated roots appeared to occur perpendicular to the normal elongation plane, possibly resulting from a loss of cell polarity. Root lateral branching was also noted after a 48 h exposure time.