CHARACTERIZATION OF THE MECHANISM OF TOLERANCE TO TERBACIL FOR A SELECTED LINE OF ALFALFA

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Bachelor of Science

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Goodwell, Oklahoma

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1993

OKLAHOMA STATE UNIVERSITY

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OF TOLERANCE TO TERBACIL FOR A
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Thesis Approved:

Thesis Adviser

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to his major adviser, Dr. Jim F. Stritzke, for his advice, training, time, and helpful criticism throughout the course of this research. Appreciation is also extended to Dr. Mike P. Anderson for his advice, guidance, assistance, and for the use of his laboratory and equipment in this endeavor. Appreciation is also extended to Dr. Richard Berberet for his suggestions and assistance as a member of the author's graduate committee.

I would like to thank my lovely wife, Tamara, for her love, understanding, and encouragement. I would also like to thank my parents, Mr. and Mrs. Glenford Bensch, for their constant love, support, and assistance. I extend my deepest gratitude to my Lord and Savior, Jesus Christ.

The author would like to thank Ken Nelson, Mark Wayland, Brad Maddux, John Beach, and Rowshan Mallick for their help and ideas in completing this research. Appreciation is also extended to the Department of Agronomy at Oklahoma State University for the research assistantship and for the use of the facilities and equipment that made this research possible.

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CHAPTER I

INTRODUCTION

The development of herbicide tolerant crops is an innovative approach to provide agriculture producers more flexibility in crop production. Some benefits of herbicide tolerant crop include: increased crop safety, expanding currently labeled herbicides to minor crops, and increased weed management flexibility. Herbicide tolerant crops can be developed using either biotechnological or more traditional plant breeding methods.

Alfalfa is a crop for which there are a limited number of labeled herbicides. Terbacil effectively controls most broadleaf and grassy weeds in alfalfa; however terbacil can cause injury to established stands under certain environmental and developmental conditions with rates typically used for weed control. Terbacil application to actively growing plants frequently results in crop injury. Terbacil usage on newly established stands (less than one year old) is not labeled. Development of alfalfa with increased tolerance to terbacil will increase selectivity and allow additional uses of terbacil in alfalfa. The

alfalfa line OK182 has been selected at Oklahoma State University for increased tolerance to terbacil. Terbacil was applied to a seedling stand of 'Cimarron' alfalfa at 0.55 and 0.82 kg ai/ha in 1985. A total of 122 plants survived the terbacil treatments from an original population estimated at 240,000 plants. The survivors were vegetatively propagated and evaluated for terbacil tolerance. Forty-six clones were selected for tolerance based upon chlorosis and regrowth studies following exposure to high rates of terbacil. A half-sib progeny test was conducted to identify the 18 most tolerant original clones. These 18 tolerant clones were interpollinated in the field to yield the line 'OK157'. The concentration of terbacil necessary to kill 50% of the plants (LD_{50}) for OK157 was determined using seven terbacil concentrations incorporated into a soil mix ranging from 0 to 0.5 ppm. The LD_{50} for OK157 was 2.5 times higher than for Cimarron. Approximately 112 plants survived rates of 0.125 ppm or higher and were interpollinated to form syn 1 designated as 'OK182'. Currently OK182 is being developed for further germplasm enhancement.

The mode of action of terbacil is the inhibition of photosynthesis by blocking electron transfer within Photosystem II. Synthesis of ATP and NADPH is prevented, leading to necrosis and death of the plant. Differences in response to herbicides within species have generally been shown to depend on factors that influence levels of

phytotoxic herbicide reaching the site of action within Photosystem II. The most important factors of tolerance are differential uptake, translocation, and metabolism of the herbicides, and biochemical alterations at the site of action. Selection for tolerance to a particular herbicide could result in changes in any of the above mechanisms.

This study examined the physiological differences conferring increased tolerance to OK182. OK182 was developed from Cimarron; therefore, all comparisons will be made to the more susceptible Cimarron line. The objectives of this research were: 1) to determine the degree of terbacil tolerance of the line OK182, and 2) to determine the mechanism of tolerance by examining differences in sensitivity at the the site of action, uptake and translocation, and metabolism of terbacil in OK182 as compared to Cimarron.

CHAPTER II

LITERATURE REVIEW

Development of Herbicide Tolerant Crops:

The development of herbicide tolerant crops will allow more flexibility in developing effective weed control practices. Some reasons for development of herbicide tolerant crops are to permit herbicide usage for weed control in susceptible crops, or to improve the selectivity of a current herbicide. The basis for herbicide selectivity is the ability of crops to survive a specific rate that kills target weeds through an increase in plant tolerance. Fewer herbicides are being labeled for commercial usage due to the high cost of development. The cost of developing a new cultivar has been estimated at 1 to 5% of the cost of a new herbicide (1). Therefore it is more economical to develop a crop with tolerance to a herbicide than to develop a new selective herbicide. In some crop rotations, a residual herbicide applied for weed control in one crop may damage a succeeding crop. This problem could be overcome by planting a cultivar with tolerance to the herbicide as the following crop.

There have been only a few successful attempts to develop crops with tolerance to specific herbicides by conventional breeding techniques (1). A cultivar of bird's-foot trefoil (*Lotus corniculatus* L.), a forage legume, has been selected that recovers from dosages of 2,4-D normally used for weed control (2). A cultivar of perennial ryegrass (*Lolium perenne L.*) has been selected with tolerance to paraquat (3). A cultivar of *L. perenne* has also been bred for increased tolerance to dalapon. This cultivar allows better control of non-desirable grasses (4). Many different crops have potential for selection of varieties with tolerance to specific herbicides that control a broad spectrum of weeds.

Alterations at the site of action of wild *Brassica campestris L.* have been exploited in the breeding of triazine tolerant rapeseed (5). The resistance of the wild *Brassica campestris* was genetically transferred into the rapeseed through conventional breeding techniques. Atrazine tolerance in the wild *B. campestris* line was found to be uniparentally inherited through the female parent, and controlled by cytoplasmic DNA. The triazine tolerant rapeseed will help significantly in controlling the major broadleaf weeds which currently plague this crop in Canada. The transfer of tolerance of wild species to closely related crops through conventional breeding techniques may offer much potential in the development of crops with increased herbicide tolerance.

Plant Tolerance Mechanisms:

The basis for herbicide selectivity is the ability of crops to tolerate the herbicide at a specific rate that kills target weeds. Herbicide selectivity may be altered by formulating and applying the herbicide in a manner whereby the target species receives a greater portion of the herbicide than the non-target species, or by using compounds that are more phytotoxic to the weeds than the crops (6).

Within species there appears to be considerable variation in response among individuals or populations to herbicides. Differences in response to herbicides between different species have generally been shown to depend on factors that influence levels of herbicide reaching the site of action; most important, differential uptake, translocation, and metabolism of the herbicide (7). Biochemical alteration at the site of action is another major factor in differential responses (8).

When considering uptake and translocation, it has been customary to consider two separate routes of translocation in plants. The first is the apoplast, or that "inert continuum comprising the xylem, cell walls and cuticle of a plant that surrounds the protoplast." The second is the symplast, or that "living continuum of interconnected protoplasm within the plasmalemma" (7). Terbacil is primarily translocated in the apoplast due to the inability of the symplast to retain and accumulate the herbicide

within the plasmalemma. Edgington and Peterson (9) claimed that the triazines, similar to terbacil in chemistry and mode of action, can readily shuttle between adjacent xylem and phloem, but as the rate of transpiration exceeds the rate of phloem transport, there is a net movement of the herbicide in the apoplast. Because terbacil movement in the plant is governed by the movement of water in the xylem and cell walls, factors affecting water movement or transpiration would affect the herbicide movement (9).

The symplast is located within the confines of the plasmalemma and forms a continuos system through which substances may move. Long-distance transport in the symplast occurs in the sieve tubes of the phloem. The herbicide must be loaded and retained in the phloem in order to translocated. Several weak acid herbicides are retained in the phloem via an ion trapping mechanism that is pH dependent. The carboxylic acid group on the herbicide aids loading into the phloem where the acid disassociates. The disassociated acid, due to higher internal pH of phloem sieve elements, prevents the herbicide from leaving transport. Retention in the phloem results in long distance transport (9).

Differences in absorption and translocation within species have been reported. A hybrid of corn (PAG-644) absorbed 66% more butylate than a tolerant hybrid (Pioneer 3030) over a 10 hour period (10). A metolachlor tolerant corn hybrid, Cargill 7567, absorbed less metolachlor, than

did a susceptible hybrid, Northrup-King 9283 (11). Differential translocation has been implicated for metribuzin tolerant-susceptible soybean cultivars (12). A diuron tolerant sugar cane cultivar retained twice as much herbicide in the roots as did a susceptible cultivar (13). Differential tolerance in corn hybrids to imazaquin was examined, but the tolerance mechanism was not identified (14). Two lettuce cultivars with differential tolerance to thiobencarb were examined. The susceptible cultivar had more absorption and accumulation of ^{14}C-thiobencarb in the foliage (15). Diclofop tolerance in two biotypes of wild oat was evaluated but the tolerance mechanism could not be determined (16). Bentazon tolerant and susceptible soybean genotypes were studied and differential metabolism was implicated as the tolerance mechanism for the tolerant genotype (17)

Plants metabolize herbicides through a series of intermediates ultimately to insoluble residues. Metabolism refers to the enzymatic or non-enzymatic alteration of the chemical structure of herbicides with plant cells. Metabolism occurs as a three-phase process in plants. Phase I reactions generally detoxify the herbicide or predispose the molecule for conjugation in phase II by means of introducing a reactive group onto the herbicide. In phase II, conjugation of the herbicide usually results in the loss of any phytotoxic activity remaining after phase I reactions. In phase III reactions, conjugates from phase II

reactions are converted to secondary conjugates or insoluble bound residues. It is generally assumed that intermediates involved in phase II and III metabolism are no longer phytotoxic. In many instances, phase I metabolism is considered most important to biological metabolism of herbicides and may be the major factor influencing herbicide selectivity (6).

The basic biochemical reactions in higher plants that generally result in herbicide detoxification are oxidation, reduction, hydrolysis, and conjugation. The first three reactions are generally associated with phase I metabolism and conjugation in phase II metabolism. Oxidation reactions of herbicides frequently are primary reactions that result in either detoxification or activation of the herbicide. The major oxidative reactions are N-dealkylation, aromatic hydroxylation, alkyl oxidation, epoxidation, sulfur oxidation, and O-dealkylation. These reactions are catalyzed by monooxygenases collectively referred to as mixed-function oxidases. Reduction is less common than oxidation in the metabolism of herbicides and does not appear to be an important detoxification mechanism. Hydrolysis of ester, amide, and nitrile herbicides is a common phase I reaction in plants that is important as a selective mechanism for some herbicides (18).

Conjugation is the *in vivo* reaction of a pesticide metabolite, usually resulting from a phase I reaction, with an endogenous substrate(s) to form a new compound of higher

molecular weight. Generally, conjugation is a mechanism whereby plants convert lipophilic parent herbicides into more polar, water soluble metabolites (18). Few herbicide conjugates found in plants have been characterized successfully due to the difficulty in isolating and identifying these compounds (6).

Conjugation occurs predominately with glucose, amino acids, and glutathione. Glucosides often account for a major portion of the pesticide metabolites in plants. Glucoside production in plants almost invariably follows transformations of herbicides to a hydroxylated derivative. Because of the inherent instability of glucose esters of acidic herbicides, the formation of such esters does not constitute an effective detoxification mechanism. Glycosidation may contribute to detoxification by virtue of the enhanced water solubility of the products which facilitates their disposal in the vacuole (18).

Herbicides known to form amino acid conjugates through an α-amide bond in plants are predominantly acidic herbicides. Amino acid conjugation of 2,4-D is a well known example of a herbicide undergoing such a reaction. Glutathione (GSH) conjugation is a major herbicide detoxification pathway in plants for several herbicide classes. Conjugates of the chlorotriazines in maize and related species are primarily GSH conjugates (6). GSH conjugation is extremely important in plants because 1) the reaction has a wide range of potential substrates, 2) it is

a detoxification mechanism and a major factor in herbicide selectivity, and 3) it influences the nature of terminal herbicide residues in plants (7).

Tolerance may be due to an physiological alteration of the herbicide binding site. Biochemical alterations at the site of action are the predominant resistance mechanism of weeds. The triazine herbicides are a major herbicide family subject to this tolerance mechanism (19). Triazines are similar to terbacil in that they inhibit the flow of electrons within Photosystem II by binding to a 32 kiolodalton D1 protein inhibiting electron passage from the plastoquinone Q_A and Q_B. Binding sites of uracils and triazines on the D1 protein overlap so that a single mutation on the protein can result in tolerance to both herbicide families (20). The altered D1 protein confers tolerance and tolerant biotypes are often 1000 fold more tolerant than the susceptible biotypes (8).

Herbicide resistance has become well known in scientific and agricultural communities since the discovery and report of triazine resistance in common groundsel (*Senecio vulgaris* L.) in 1970 (21). At least 57 weed species have been reported to have biotypes with tolerance to the triazine herbicides primarily due to changes at the site of action. In addition, at least 47 species have been reported to have biotypes tolerant to one or more of 14 other herbicides or herbicide families. These herbicides include the aryloxyphenoxypropionics, bipyridiliums,

dinitroanilines, phenoxys, substituted ureas, and sulfonylureas (22).

Terbacil Characteristics:

Terbacil (5-chloro-3-(1,1-dimethylethyl)-6-methyl-2,4(1H,3H)-pyrimidinedione) is used for selective control of many annuals and some perennial weeds in apples, blueberries, peaches, citrus, mint, sugarcane, and alfalfa. It is a member of the substituted uracil family of herbicides. It is most readily absorbed through the root system and translocated upward to the leaves via the xylem (24).

Injury symptoms of terbacil on plants develop on lower more mature leaves first. The most prominent symptom on individual leaves first appears at the leaf tips and margins, followed by interveinal chlorosis. The chlorotic tissue becomes necrotic and the leaf dies from the tip and margin inward (25).

The mode of action of terbacil involves inhibition of photosynthesis by the disruption of electron flow (26,27). Approximately 50% of currently labeled herbicides are photosynthesis inhibitors (25). The mechanism of phytotoxicity is to block the synthesis of ATP and NADPH, thus preventing the plant from fixing CO_2. Exposure to light and the herbicide results in carotenoid destruction, chlorophyll bleaching and membrane deterioration accompanied by increased lipid peroxidation. The lipid peroxidation is

not the first step in the process by which the herbicide kills the plant, but is a secondary event related to subsequent tissue deterioration. Lipid peroxidation is probably a consequence of increased singlet O_2 formation due to the blocking of the electron transport. Ultimately it is the light-dependent degeneration of the electron transport system that results in plant death (25).

Terbacil Tolerant Alfalfa:

Terbacil is used as a dormant season herbicide in alfalfa for the control of many weedy grasses and certain broadleaf weeds. Weeds can reduce yield approximately 0.25 to 0.5 kg for every 0.5 kg weeds present. Terbacil will control most cool season weeds such as mustards and annual bromes. If higher rates are used it will give some early summer control of warm-season grasses. Terbacil is normally applied to dormant alfalfa. It can cause some chlorosis when applied to actively growing alfalfa, resulting in yield and even plant losses at rates over 1.1 kg/ha. Increased alfalfa plant tolerance to the herbicide could greatly increase the flexibility and utility of this chemical. It could be applied during a longer period of time and to a greater array of alfalfa growth stages with minimal damage if tolerant cultivars were available.

An alfalfa cultivar with tolerance to terbacil has been selected at Oklahoma State University (23). Terbacil was applied to a seedling stand of 'Cimarron' alfalfa at 0.05

and 0.14 kg ai/ha in 1985. A total of 122 plants survived from the original population of approximately 240,000 plants. These surviving plants were transferred to a greenhouse and vegetatively propagated for further evaluations. Forty-six clones were selected for tolerance and subjected to an additional cycle of selection to obtain the cultivar OK157. The LD_{50} of OK157 was approximately 2.5 times higher than that of Cimarron in a growth chamber bioassay (23). Cycle 3 was initiated by treating OK157 plants in a growth chamber bioassay with seven rates of terbacil. Approximately 112 plants survived the herbicide rate of 0.125 ppm and were interpollinated in the greenhouse by hand to form the cultivar OK182. The selection OK182 will be used as an alfalfa germplasm source to develop cultivars having tolerance to terbacil.

Plants With Terbacil Tolerance:

The basis of tolerance to terbacil has been examined in several crops and weed species, e.g. orange (*Citrus sinensis* L. 'Koethen Sweet orange') (28), peppermint (*Mentha piperita* L.) (29), sugarcane (*Saccharum officinarum* L.) (30), alfalfa (*Medicago sativa* L.) (31), strawberry (*Fragaria* x *ananassa* Duchesne) (32,33), goldenrod (*Solidago fistulosa* Miller) (32,33), purple nutsedge (*Cyperus rotundus* L.) (34), Powell amaranth (*Amaranthus powellii* S. Wats.)(35), watermelon (*Citrullis lanatus* Thunb. 'Charleston gray and

Jubilee')(36), and field violet (*Viola arvensis* Murr.)(37). Tolerance to terbacil may be due to insensitivity at the site of action, reduced uptake and translocation, and/or enhanced metabolism (8).

Rhodes (31) examined the metabolism of terbacil in alfalfa. Alfalfa was treated in the field with 2-C^{14} terbacil in the dormant stage at a rate of 0.18 kg ai/ha. An average total radiochemical residue equivalent to 2.2 ppm (calculated as terbacil) in the alfalfa was harvested 6 months after treatment and 0.4 ppm 8 months after treatment. Three compounds were identified by mass spectral analyses: terbacil (3-tert-butyl-5-chloro-6-methyluracil), metabolite A (3-tert-butyl-5-chloro-6-hydroxymethyl uracil), and metabolite B (6-chloro-2,3-dihydro-7-(hydroxymethyl)-3,3-dimethyl-5H-oxazolo (3,2-a)pyrimidin-5-one). Two additional metabolites, metabolite C (6-chloro-2,3-dihydro-7-methyl-3,3-dimethyl-5H-ozazolo-{3,2-a}pyrimidin-5-one, and metabolite D (3-tert-butyl-6-hydroxymethyluracil) were identified by comparison of their thin layer chromatography (TLC) R_f values with R_f values of reference standards. Terbacil comprised 12.5% of the C^{14} in the whole plant extract, while metabolite A comprised 11.9%, metabolite B 41.2%, metabolite C 18.3%, and metabolite D 5.6%. The remaining 10.5% was material that remained at the origin on the TLC plate (31).

Reduced uptake and translocation were evaluated as possible basis of tolerance in two strawberry cultivars,

'Sunrise' and 'Guardian', and in goldenrod (32). Reported tolerance in 'Sunrise' and susceptibility in 'Guardian' was also evaluated. The tolerance of goldenrod to terbacil was at least five-fold that of strawberry. Radiolabeled terbacil was used to determine the distribution patterns within the plants. The tolerance of strawberry and goldenrod to terbacil was shown to be at least partially attributable to restricted translocation of herbicide to the site of action in mesophyll chloroplasts. Uptake by roots did not appear to be a factor in tolerance to terbacil. The five-fold greater tolerance of goldenrod vs. strawberry to the herbicide was unrelated to the degree of restriction of the compound to roots and leaf veins. Neither phytotoxicity nor uptake and distribution studies provided evidence for greater susceptibility to terbacil in 'Guardian' than 'Sunrise' strawberry.

Further studies on the strawberries and goldenrod indicated that the reduced translocation may have been due to enhanced metabolism in roots, resulting in less terbacil available for translocation throughout plants (33). Terbacil metabolism was evaluated in the two species, strawberry and goldenrod. Reported cultivar variation in strawberry tolerance to terbacil was examined by comparing herbicide metabolism patterns. A terbacil-sensitive plant, cucumber, was used as a basis for comparison with the strawberry and goldenrod species. Using gradient elution high performance liquid chromatography (HPLC), two terbacil

metabolites were separated and quantified from methanol extracts of the three species treated with C^{14} terbacil via roots in solution culture. A minor metabolite was identified as the non-phytotoxic derivative, 3-tert-butyl-5-chloro-6-hydroxymethyluracil, based on its co-migration with authentic 3 -tert-butyl-5-chloro-6-hydroxymethyluracil in two chromatographic systems. The major metabolite was a glycoside which yielded the hydroxylated derivative upon B-glucosidase hydrolysis. In all species, metabolites accumulated more rapidly and extensively in roots than in leaves. Metabolism was greater in the two tolerant species than in cucumber. However, the greater tolerance of goldenrod to terbacil compared to that of strawberry was apparently unrelated to differences in herbicide metabolism.

Metabolism of terbacil was examined in orange seedlings (28). The seedlings were cultured in aqueous solutions treated with terbacil and C^{14} terbacil. Radioactivity was distributed throughout the plant with the largest amount in the roots and the smallest amount in the leaves. Terbacil was metabolized primarily in the roots to form 3-tert-butyl-5-chloro-6-hydroxymethyl uracil, which was conjugated to form a β-glucoside. Identification of the metabolite was made by infrared and mass spectrometry after isolation and purification by column chromatography followed by TLC. An additional, unidentified water-soluble material accumulated in the plant.

A Powell amaranth line has been found to be tolerant to terbacil and bromacil (35). The Photosystem II (PS II) activity of isolated thylakoids from the tolerant line was 55-times more tolerant to terbacil and bromacil than PS II activity from a susceptible line. Isolated thylakoids from the tolerant line also had a lower binding affinity for C^{14} terbacil than thylakoids from the susceptible biotype. These results indicate that the tolerance is probably a result of an altered binding site on the Q_b protein, resulting in reduced terbacil binding.

Differences in tolerance of watermelon cultivars to terbacil have been reported (36). Small seeded 'Crimson sweet' watermelon had 50% growth reduction at 0.16 kg/ha in a logarithmic rate screening trial; whereas, the large seeded cultivars Jubilee and 'Charleston gray' required 0.30 and 0.22 kg/ha, respectively, for 50% growth reduction.

The distribution of C^{14} terbacil in sugarcane has been reported (30). Radiolabeled terbacil was introduced through a small core at the fifth node. Treatments were 0, 100, and 210 ug of 2-C^{14} terbacil per plant. After three weeks, aliquots of the juice, pulp, and leaves from each series were analyzed for total C^{14} by direct combustion followed by liquid scintillation counting. About 90% of the recovered activity in the plants was in the leaves indicating that terbacil was rapidly translocated. The juice and pulp of the plant contained 3 and 7%, respectively.

Terbacil content in treated and untreated pairs of purple nutsedge plants connected by rhizomes indicated translocation (34). Approximately 10% as much terbacil was found in untreated plants as in treated plants.

When terbacil was applied in an isoparaffinic oil to susceptible ivyleaf morningglory (*Ipomoea hederacea* L.), photosynthesis was inhibited; however, photosynthesis in peppermint, a tolerant species, was decreased only temporarily (29). Terbacil was readily absorbed by leaves of both plants; however, there was little or no movement out of the treated peppermint leaves. Terbacil was translocated out of the treated leaves of ivyleaf morningglory to the untreated leaves and shoot apex. Terbacil was metabolized in both plants but at a higher rate in peppermint. Therefore, it appears that foliar-applied terbacil may be bound in peppermint leaves and this, together with the higher rate of metabolism, may contribute to its tolerance. When terbacil was applied to the roots it was metabolized in both plants, but at a higher rate in peppermint. However, the rate of metabolism in roots does not appear to be sufficient to account for the observed tolerance.

Differential uptake, translocation, and metabolism of terbacil was evaluated in field violet at the 3-leaf and 12-leaf growth stage (37). Field violet can be controlled early-postemergent; however, as plants progress in size their susceptibility to typical field application rates decreases. Field violet plants at the 12-leaf growth stage

absorbed less ^{14}C-terbacil/g fresh wt than did plants at the 3-leaf growth stage. However, twice as much radioactivity was translocated to the foliage in the 3-leaf plants as compared with the 12-leaf plants. Plants at both growth stages contained >50% polar metabolites in the foliage, indicating rapid metabolism of the herbicide. This study indicated that tolerance of older plants may be explained by lower total plant uptake of terbacil, increased herbicide metabolism, and restricted translocation to the shoots.

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CHAPTER III of this thesis is a manuscript to be submitted for publication in Pesticide Biochemistry and Physiology.

CHAPTER III

CHARACTERIZATION OF THE MECHANISM OF TOLERANCE TO TERBACIL FOR A SELECTED LINE OF ALFALFA

Abstract. Important factors affecting tolerance of plants to herbicides include differential uptake and translocation, metabolism, and biochemical alterations at the site of action. This study investigated the mechanism of terbacil tolerance in an alfalfa line, 'OK182. Enhancement of tolerance was confirmed by comparison of the growth response of the tolerant line to Cimarron at various terbacil concentrations. Results of the growth response study indicated that OK182 possessed approximately 80% greater tolerance to terbacil than Cimarron. The site-of-action was examined as a possible basis for tolerance, but results indicated no significant difference between lines. Uptake, translocation, and metabolism were then examined using radiolabeled terbacil applied to the nutrient solution of hydroponically grown plants. The roots, stems, and leaves were harvested and extracted and the radioactivity within each plant component determined using liquid scintillation

spectroscopy. Terbacil was taken up rapidly and significant amounts of radioactivity were detected in the leaves of both lines after 1 day. OK182 took up 12,14,13, and 11% less radiolabel 1,2,4, and 6 days respectively following treatment than did Cimarron. Translocation determined as a percentage of uptake was not significantly different between lines. Metabolism was examined in both lines by separating terbacil and its metabolites using thin layer chromatography. The terbacil concentration in leaf tissue of OK182 was 33% less than in Cimarron. Terbacil in leaf tissue was rapidly metabolized with no significant difference in metabolite concentration between lines. One polar metabolite predominated consisting of more than 80% of the total radioactivity. Enhanced tolerance of the alfalfa line OK182 is at least partially due to decreased uptake.

Nomenclature: terbacil, 5-chloro-3-(1,1-dimethylethyl)-6-methyl-2,4(1H,3H)-pyrimidenedione; alfalfa, *Medicago sativa* L.; DCPIP, dichloro-phenol-indophenol.

Additional index words. Herbicide tolerant crop, site-of-action, uptake, translocation, recurrent selection.

INTRODUCTION

Some important factors affecting plant tolerance to herbicides are differential uptake, translocation, and metabolism, and biochemical alterations at the site of action (1,2). Lines with increased tolerance to herbicides have been obtained using biotechnological techniques or traditional plant breeding methods.

Terbacil tolerance has been reported in several crop and weed species. Rhodes examined the metabolism of radiolabeled terbacil in alfalfa (*Medicago sativa L.*) (4). Six radiolabeled areas were identified in plant extracts of alfalfa treated with 1.1 kg/ha radiolabeled terbacil 6 and 8 months following treatment. In strawberry (*Fragaria* x *ananassa* Duchesne) and goldenrod (*Solidago fistulosa* Miller) tolerance was shown to be at least partially attributable to reduced translocation (5). Further studies (6) indicated enhanced metabolism in the roots and two terbacil metabolites, hydroxylated terbacil and a glycoside, were identified. Differential uptake, translocation, and metabolism of terbacil were evaluated in field violet (*Viola arvensis* Murr.) at the 3-leaf and 12-leaf growth stages (7). Field violet plants at the 12-leaf growth stage absorbed and translocated less ^{14}C-terbacil/g fresh wt than did plants at the 3-leaf growth stage. A Powell amaranth (*Amaranthus powellii* S. Wats.) weed biotype has been found with

resistance to terbacil and bromacil (8). The tolerant line was 55 times more tolerant than the susceptible biotype determined by Photosystem II activity of isolated thylakoids. Results indicate that the resistance is probably due to an alteration of the binding site. Terbacil tolerance has also been examined in orange (*Citrus sinensis* L. 'Koethen Sweet orange')(9), peppermint (*Mentha piperita* L.)(10), sugar cane (*Saccharum officinarum* L.) (11), purple nutsedge (*Cyperus rotundus* L.) (12), and watermelon (*Citrullis lanatus* Thunb. 'Charleston gray and Jubilee') (13).

Caddel, et al. (3) reported on alfalfa selected for tolerance to terbacil. A tolerant line, 'OK157', was developed by screening field-grown plants from the cultivar 'Cimarron', followed by intercrossing using recurrent selection techniques. The lethal dosage of terbacil necessary to kill 50% of the plants (LD_{50}) was 2.5 times higher for OK157 than for Cimarron. Plants of OK157 that survived terbacil screening tests were then interpollinated to form syn 1 of the line 'OK182'.

This study was initiated to examine the physiological differences between the line OK182 and its parental population, Cimarron. The objectives of this study were to 1) determine the degree of terbacil tolerance in OK182 and Cimarron, and 2) evaluate the mechanism of tolerance by examining for differences in the site of action, uptake and

translocation, and metabolism in OK182 as compared to the cultivar Cimarron.

MATERIALS AND METHODS

Seed was provided by Dr. J.L. Caddel for the line OK182 and Cimarron. The 2-(C^{14})-terbacil and analytical grade terbacil were furnished by E. I. duPont de Nemours & Co., Wilmington, DE. Assay and extraction chemicals for the site of action experiments were obtained from the Sigma Chemical Co. (St. Louis MO).

Growth response. Alfalfa seeds from the two lines were germinated in a soil mixture consisting of three parts peat moss, three parts pearlite, eight parts sterilized sand, 1/2 cup lime, and 3 cups fertilizer formulation of 15-15-15. The plants were grown under continuous fluorescent lighting at a PPFD of 300 $\mu E/m^2 \cdot sec$. and maintained at a temperature ranging from 25-35°C. After 2 weeks, plants were transferred to a modified Wych and Rains (14) nutrient solution.

The nutrient solution volume (420 ml/cup) was kept constant by adding additional nutrient solution every day and was aerated for maximal root growth. Plants were allowed to adjust to the hydroponics for approximately 1 week and then were treated with six terbacil rates between 0.1 and 1 μM. Plants were allowed to grow for approximately

2 weeks before being harvested. The experimental design utilized was a randomized complete block design with three replications. Each cup contained four plants and measurements were based on a per cup basis. The fresh and dry weights of top growth were used to evaluate response of the two lines to terbacil. Data were subjected to analysis of variance and means were separated using an LSD (0.05). The top growths were expressed as a percentage of Cimarron and the concentration of terbacil needed to inhibit growth 50% (GR_{50}) was determined. This experiment was repeated three times with similar results; so, only data from the first experiment are reported.

Site of action. The thylakoid membranes were examined for sensitivity to terbacil using leaf tissue of the two lines. Leaf tissue was grown under the conditions described for the growth response experiments. The thylakoid membranes were extracted from the leaf tissue and a modified DCPIP assay was used to evaluate the sensitivity at the site of action (15). Chlorophyll content of the extract was determined and 2 µg chlorophyll used in each assay. The assay mixture consisted of 170 µl of 80 µM DCPIP assay media, 10 µl of herbicide or water, and 20 µl of thylakoid membrane extract into each well of a microtiter plate. Herbicide concentrations ranged between 0 and 125 µM with eight replicates per treatment. The contents were mixed gently with a slow rotary motion and initial absorbance at 600 nM

was determined using a Bio Rad® 2550 EIA reader. Electron flow was initiated by illuminating the thylakoid membranes for 1 minute using a Sylvania® narrow spot lamp with a PPFD of 600 $\mu E/m^2 \cdot sec$. Final absorbance was determined after the 1 minute illumination period. The net absorbance reflects the degree of photosynthetic electron transport in the thylakoid membranes. The final results were expressed as a percentage of the absorbance obtained from the untreated membranes. Data were subjected to analysis of variance and means were separated using an LSD (0.05). The I_{50} was determined as the concentration of terbacil at which net absorbance was inhibited by 50% using the slope of the linear portion of the assay as determined by regression analysis. This experiment was repeated three times with similar results; but, only data from one experiment is reported.

Uptake and translocation. The two lines were examined for differential uptake and translocation using 2-^{14}C-terbacil. Seed was germinated and allowed to grow for 2 weeks in the soil mixture described in growth response experiments. Plant roots were washed free of soil under tapwater immediately before being placed into hydroponics and allowed to adjust to the hydroponics for approximately two weeks. Plants were then placed into fresh nutrient solution containing approximately 0.42 μCi of 2-^{14}C-terbacil and 560 nM of terbacil, in experiment 1, and 0.63 μCi of 2-^{14}C-

terbacil and 560 nM of terbacil in experiment 2. The specific activity of the terbacil was 3.22 and 4.68 μCi/mg in experiments 1 and 2 respectively.

The hydroponic solution volume was maintained at 420 ml/cup by adding fresh nutrient solution daily to replace transpirational losses. There were three cups per harvest date with four plants/cup and measurements based on a per cup basis. The plants were harvested 1,2,4 and 6 days following treatment. The roots were rinsed twice in fresh distilled water to remove unretained radioactive terbacil. For analysis, the nutrient solution was sampled and the plants were separated into roots, stems, and leaves and individual fresh weights recorded. Plant tissue harvested from each cup was extracted in 15 ml of methanol using a homogenizer blender. The extract was centrifuged for 5 minutes at 5000 RPM and the supernatant filtered through a #1 Whatman filter. The pellot was resuspended in 5 ml of methanol and centrifuged a second time. The supernatant was filtered through the Whatman filter and combined with the 15 ml already collected. A 1 ml aliquot of the total methanol extract from each sample was removed and the remaining extract refrigerated for further use in metabolism experiments. Each 1 ml sample of extract received 50 μl of a 5% sodium hypochlorite solution and was placed under fluorescent light for 1 hour to bleach the pigments (16). Twelve ml of Ecolite® Liquid Scintillation Cocktail was then added to the bleached extract and the total

radioactivity determined by Liquid Scintillation Spectroscopy (LSS) using a Beckman® LS 6000 liquid scintillation counter. Disintegrations per minute (DPM) were corrected for background radiation and quenching. Data were subjected to analysis of variance and means were separated using an LSD (0.05). Results from the two experiments were pooled. Plant extracts were refrigerated at 4°C for further use.

Metabolism. The extracts of each of the two lines saved from the uptake and translocation experiments were utilized to determine metabolism of the radiolabeled terbacil. A 100 µl aliquot of each methanol extract was spotted twice onto the preadsorbent zone of a Whatman thin layer chromatography (TLC) plate (250 µM). The plate was developed in a hexane/ethyl acetate/methanol (10:10:1) solvent. The radioactive zones were located using a Bioscan® System 200 imaging TLC plate scanner. The radioactive zones were scraped and placed into 12 ml of Ecolite® Liquid Scintillation cocktail and counted for 20 minutes on a Beckman® LS 600 liquid scintillation counter. DPM's were corrected for background radiation and quenching. Data were subjected to analysis of variance and means were separated using an LSD (0.05). Results from the two experiments were pooled.

RESULTS

Growth response. Top growth of Cimarron was reduced at lower concentrations of terbacil than top growth of OK182 (Figure 1). The terbacil concentration inhibiting growth 50% (GR_{50}) for Cimarron and OK182 was 353 and 640 nM, respectively. Based on the GR_{50}, OK182's tolerance to terbacil was enhanced by 80% when compared to Cimarron. Growth of Cimarron was completely inhibited at a concentration of 600 nM; whereas, this same concentration had little effect on growth of OK182.

Site of action. The I_{50} for Cimarron and OK182 was 75 nM and 61 nM respectively (Figure 2). There was no significant difference in the I_{50}'s between lines using a 95% confidence interval.

Uptake and translocation. The roots, stems, leaves, and total plant weights for Experiments 1 and 2 are given in Tables 1 and 2, respectively. The DPM's/g fresh weight 6 days following treatment are summarized in Tables 3 and 4. Approximately 95% of the radiolabel in the plants was accounted for in the methanol extracts. The radiolabel was translocated very rapidly to the leaves where it accumulated throughout the 6 day labeling period. Six days following treatment approximately 77% of the radioactivity of the plant was in the leaves, 20% in stems, and 3% in roots for

both lines (Figure 3)(Table 3 and 4). OK182 took up 12,14,13, and 11% less radiolabel than Cimarron on days 1,2,4 and 6 respectively (Figure 4). There was no significant difference between lines in translocation when determined as a percentage of total uptake (Figure 5).

Metabolism. The TLC separation yielded three major bands of radioactivity (Figure 6). The three radioactive components comprised greater than 95% of the total radioactivity on the TLC plate. Terbacil was identified as one of the components based on co-chromatography with an authentic standard (R_f=0.52). Metabolite I was a polar metabolite that did not migrate from the point of origin. This metabolite consisted of more than 80% of the total radioactivity. The second metabolite, metabolite II, migrated to an R_f of 0.24.

Both lines showed a high level of terbacil metabolism, and by day 6 less than 20% of the radiolabel was unmetabolized terbacil (Figure 7). The terbacil concentration in the leaf extract was 33% less in OK182 as compared to Cimarron 6 days following treatment (Figure 8). The DPM's/g fresh weight of terbacil and metabolites 1,2,4, and 6 days following treatment are summarized in Tables 5 and 6.

There was no significant difference in inhibition of photosynthesis between analytical terbacil and terbacil derived from the TLC separation at equal concentrations. This suggests that if there is an additional metabolite

(such as hydroxylated terbacil) that co-chromatographed with terbacil, its quantity is limited.

DISCUSSION

Selected line OK182 appeared to possess more tolerance to terbacil than Cimarron. It repeatedly produced more biomass throughout the range of terbacil concentrations than did Cimarron in all experiments. A biochemical alteration at the site of action would not explain this increased tolerance, since no significant difference in sensitivity at the site of action was detected between the lines.

There was significantly less uptake of radioactivity in OK182; however, the rate of translocation, expressed as a percentage of total radiolabel taken up, was not significantly different. Results indicate differential uptake as a possible mechanism of tolerance and may partially explain the observed tolerance. Increased tolerance due to uptake has been reported in corn. A susceptible hybrid of corn (PAG-644) absorbed 66% more butylate than a tolerant hybrid (Pioneer 3030) over a 10-hour period (17). A corn hybrid tolerant to metolachlor, Cargill 7567, absorbed less than did a susceptible hybrid, Northrup-King 9283 (18). Terbacil, a non-polar compound, is absorbed into the roots by simple diffusion (19) and then translocated in the xylem to the site of action in the leaves (11). Results indicate rapid translocation to the

leaves with little radioactivity accumulation detected in the roots or stems throughout the 6 day time period. Factors affecting water movement or transpiration would affect the movement of terbacil in the plant (20).

Transpirational differences between the selected and non-selected lines could account for the reduction of terbacil uptake and translocation. Transpiration has been correlated to atrazine and linuron uptake in lettuce, turnip, parsnip, and carrot seedlings where the amount of water transpired was proportional to herbicide uptake (21). Anatomical, morphological, and physiological differences in the leaves and stomata could account for possible transpirational differences. Root area differences could also affect the amount of uptake.

There was no significant difference between lines in the amount of metabolites. Terbacil was rapidly metabolized to a polar metabolite (Metabolite I). Genez and Monaco reported a glycoside conjugate of terbacil which yielded hydroxylated terbacil upon β-glucosidase hydrolysis in strawberry (6). Metabolite II showed significant levels of radioactivity but was not identified.

In conclusion, the observed tolerance observed in the line OK182 is primarily due to the lower concentration of terbacil at the site of action. Leaf extracts contained 33% less terbacil. This lower concentration of terbacil is likely the result of less uptake of the herbicide.

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Table 1. Root, stem, leaf, and total plant weights(g) 1,2,4, and 6 days following treatment. (experiment 1)[a]

	Cimarron				OK182			
day	root	stems	leaves	total plant	root	stems	leaves	total plant
1	1.90 ±0.58	0.92 ±0.19	1.08 ±0.17	3.90 ±0.91	2.44 ±0.22	1.70 ±0.15	1.85 ±0.10	5.99 ±0.34
2	1.67 ±0.47	0.76 ±0.14	0.95 ±0.13	3.38 ±0.74	3.03 ±0.38	2.16 ±0.09	1.70 ±0.13	6.89 ±0.48
4	2.17 ±0.50	1.29 ±0.34	1.43 ±0.28	4.89 ±1.11	3.14 ±0.23	2.30 ±0.23	1.95 ±0.20	7.39 ±0.49
6	2.17 ±0.57	1.09 ±0.19	1.30 ±0.13	4.56 ±0.87	3.74 ±0.30	2.02 ±0.09	1.62 ±0.10	7.38 ±0.29

[a]Means of three replications ± their standard errors.

Table 2. Root, stem, leaf, and total plant weights(g) 1,2,4, and 6 days following treatment. (experiment 2)[a]

	Cimarron				OK182			
day	root	stems	leaves	total plant	root	stems	leaves	total plant
1	2.39 ±0.25	1.50 ±0.07	1.51 ±0.03	5.40 ±0.25	3.07 ±0.68	1.65 ±0.10	1.75 ±0.16	6.47 ±0.92
2	3.31 ±0.97	1.64 ±0.20	1.81 ±0.23	6.76 ±1.40	3.50 ±0.96	1.87 ±0.15	1.48 ±0.22	6.85 ±1.17
4	2.41 ±0.19	1.86 ±0.08	1.45 ±0.20	5.71 ±0.31	4.75 ±1.50	2.07 ±0.20	2.08 ±0.39	8.90 ±2.02
6	3.81 ±0.78	1.81 ±0.21	2.19 ±0.04	7.81 ±0.67	4.14 ±0.55	1.96 ±0.28	2.11 ±0.34	8.21 ±1.05

[a]Means of three replications ± their standard errors.

Table 3. DPM/g fresh weight 6 days following treatment. (experiment 1)[a]

Cimarron					OK182			
	leaf	stem	root	nutrient soln*	leaf	stem	root	nutrient soln*
terbacil	23007 ±3988	8128 ±1395	1810 ±249	------	19267 ±1698	5924 ±659	1288 ±66	------
metabolite I	104298 ±18298	42951 ± 4308	15681 ±2878	4010 ±450	94832 ±13669	26599 ±1989	5125 ±942	2807 ±200
metabolite II	19216 ±2715	4409 ±834	918 ±224	616 ±103	15533 ±2634	2363 ±71	692 ±41	588 ±12

[a]Means of three replications ± their standard errors.
*dpm/g plant fresh wt ± standard error.

Table 4. DPM/g fresh weight 6 days following treatment. (experiment 2)[a]

	Cimarron				OK182			
	leaf	stem	root	nutrient soln*	leaf	stem	root	nutrient soln*
terbacil	35475 ±4500	8945 ±629	1520 ±226	------	14060 ±7145	8699 ±552	1064 ±11	------
metabolite I	126999 ±14907	34546 ±3848	9252 ±1770	2136 ±384	124756 ±18493	38910 ±4536	8654 ±1338	2612 ±331
metabolite II	23055 ±5955	2596 ±457	671 ±148	432 ±79	20797 ±2632	3469 ±220	688 ±53	865 ±152

[a]Means of three replications ± their standard errors.
*dpm/g plant fresh wt ± standard error.

Table 5. Terbacil and total metabolite DPM/g fresh weight for leaves 1,2,4 and 6 days following treatment. (experiment 1)[a]

	Cimarron		OK182	
day	terbacil	total metabolites	terbacil	total metabolites
1	12348 ±1892	14781 ±2049	12749 ±1640	21274 ±6219
2	17857 ±5483	32703 ±4371	16466 ±5055	27508 ±7617
4	21286 ±1909	66858 ±16386	22207 ±2763	73881 ±1168
6	23007 ±3988	123513 ±19879	19267 ±1698	110364 ±15198

[a]Means of three replications ± their standard errors.

Table 6. Terbacil and total metabolite DPM/g fresh weight for leaves 1,2,4 and 6 days following treatment. (experiment 2)[a]

	Cimarron		OK182	
day	terbacil	total metabolites	terbacil	total metabolites
1	28370 ±6021	33139 ±3603	18400 ±1178	24526 ±2156
2	40797 ±6041	54274 ±2541	27045 ±3262	63609 ±5557
4	50883 ±3665	116593 ±13347	23498 ±3230	113399 ±24306
6	35475 ±4500	150055 ±20857	19647 ±1157	145553 ±19344

[a]Means of three replications ± their standard errors.

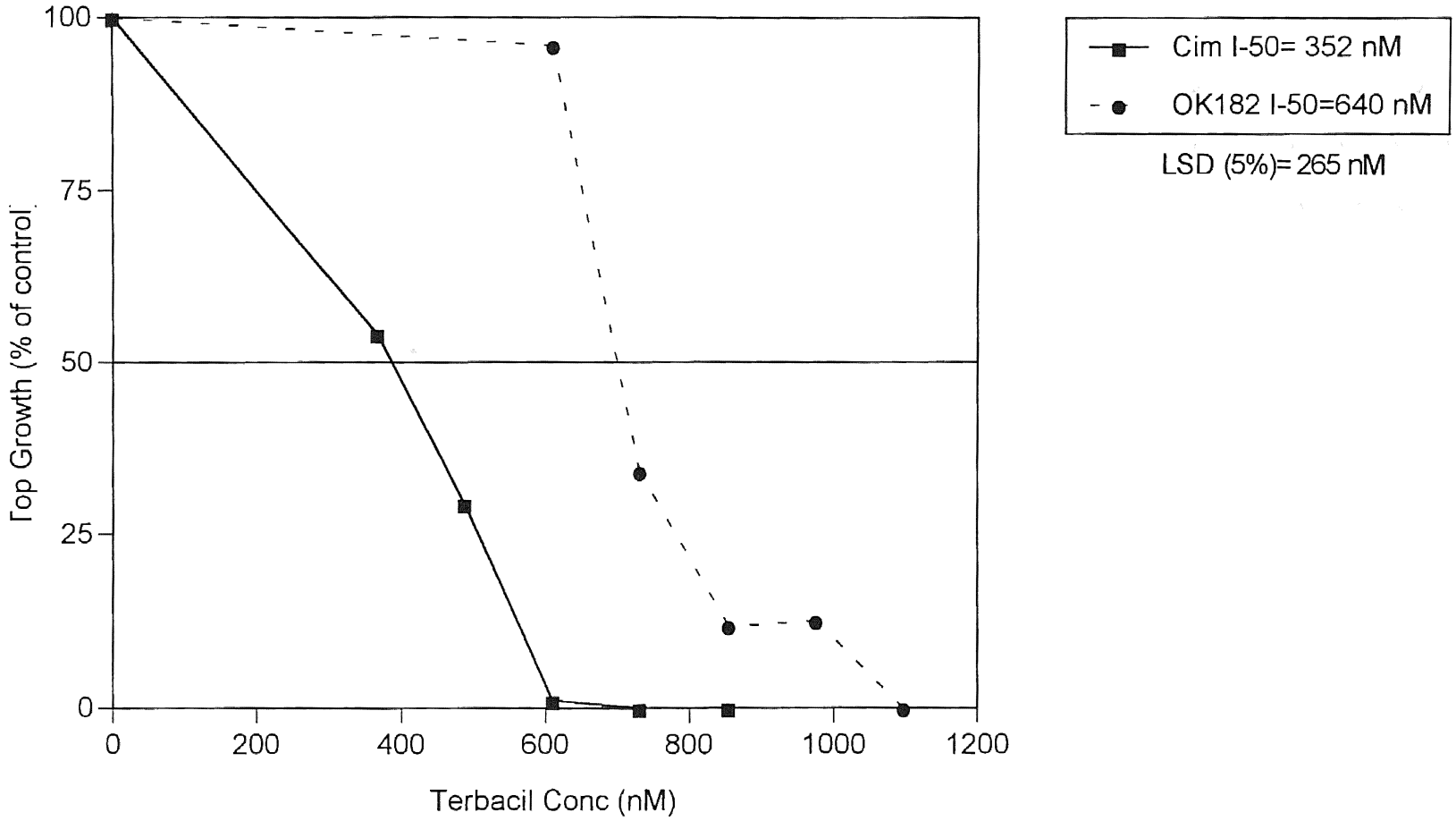

Figure 1. Growth response differences of Cimarron and OK182 to a range of terbacil concentrations from 0 to 1200 nM.

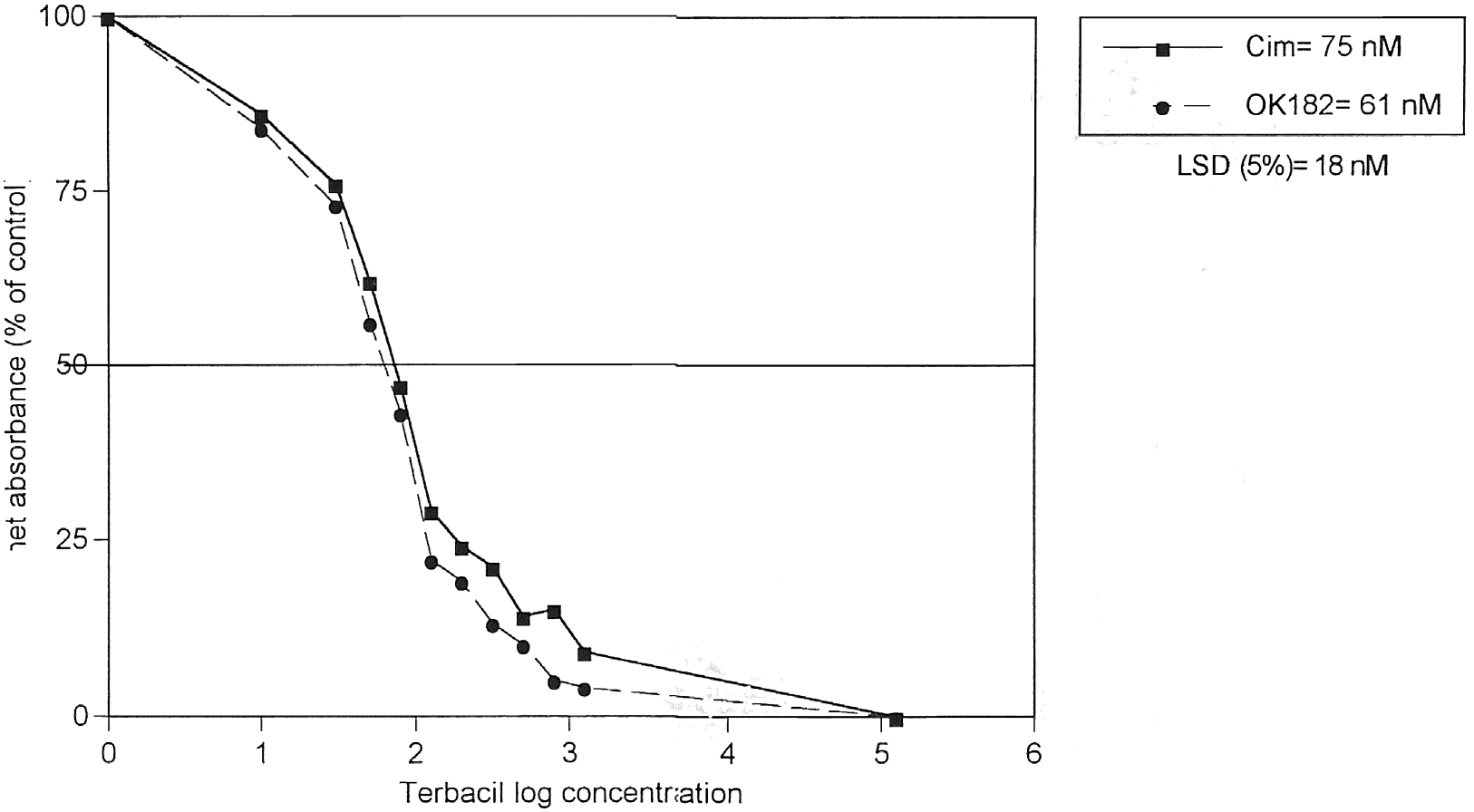

Figure 2. Active site titration with terbacil. DCIPIP net absorbance spectrophotometrically measured indicating Photosystem II activity for Cimarron and OK182.

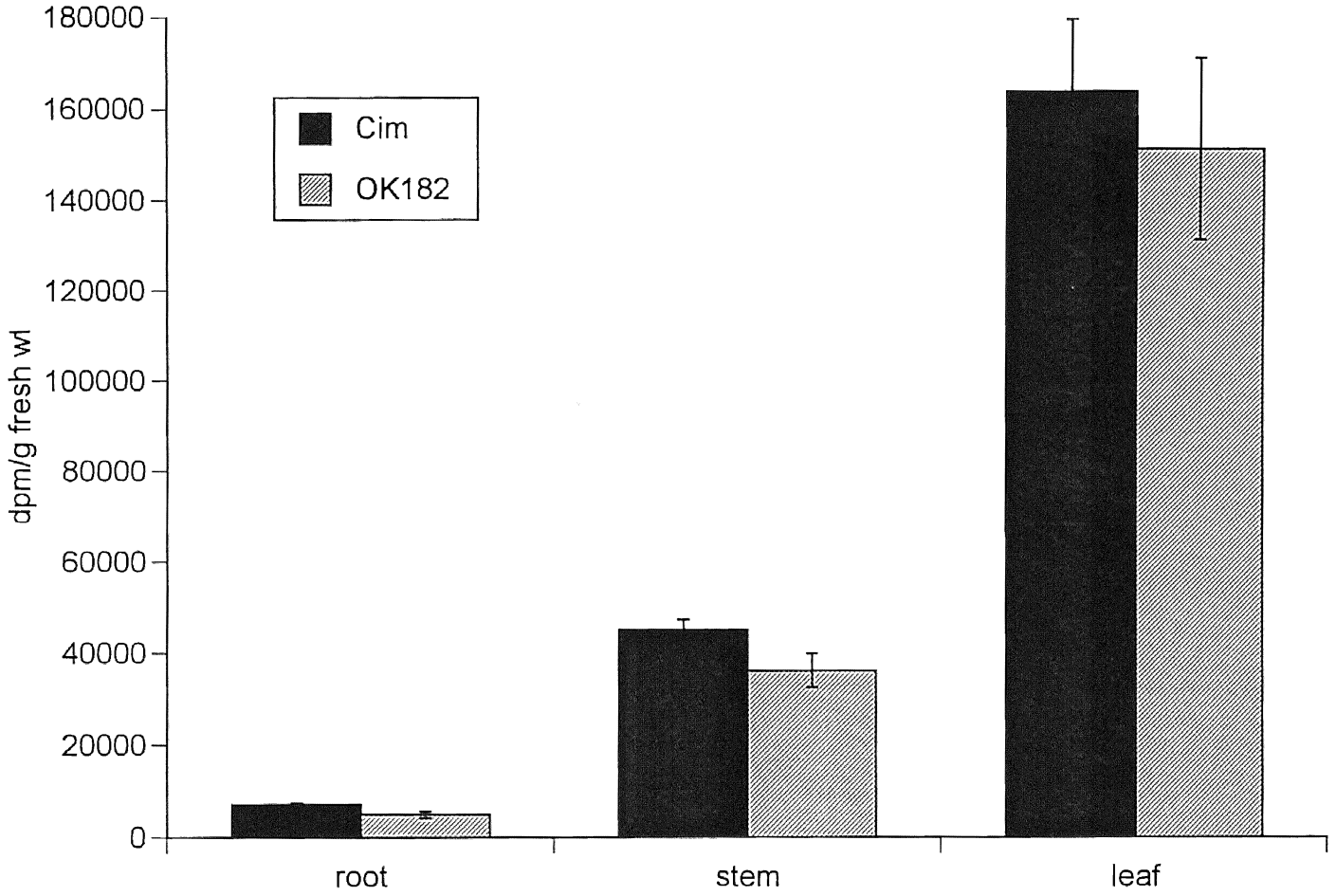

Figure 3. Radioactivity in the root, stem, and leaf extracts 6 days following treatment. (exp 1+2)

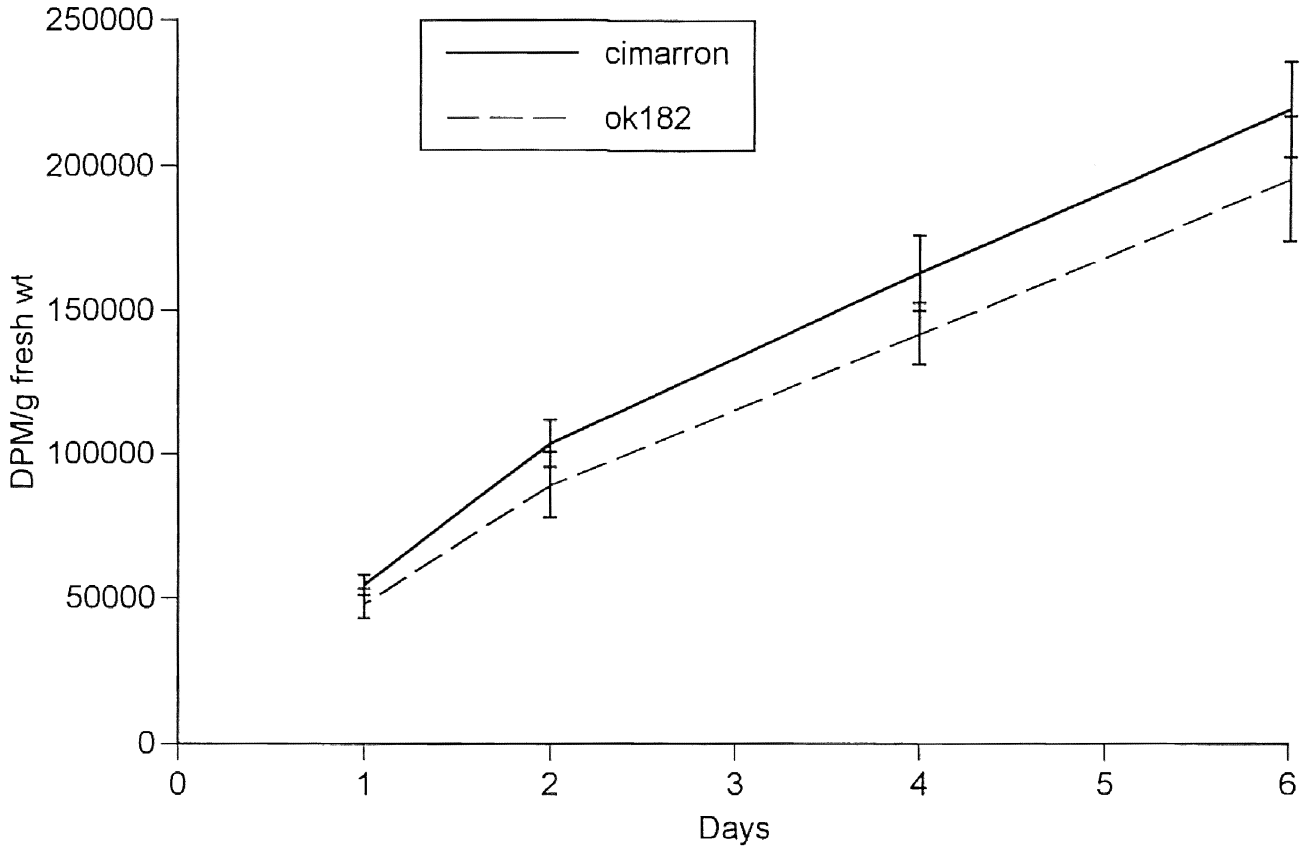

Figure 4. Total radiolabel uptake 1,2,4, and 6 days following treatment for Cimarron and OK182. OK182 took up 12,14,13, and 11% less radiolabel than Cimarron 1,2,4, and 6 days respectively (exp 1+2).

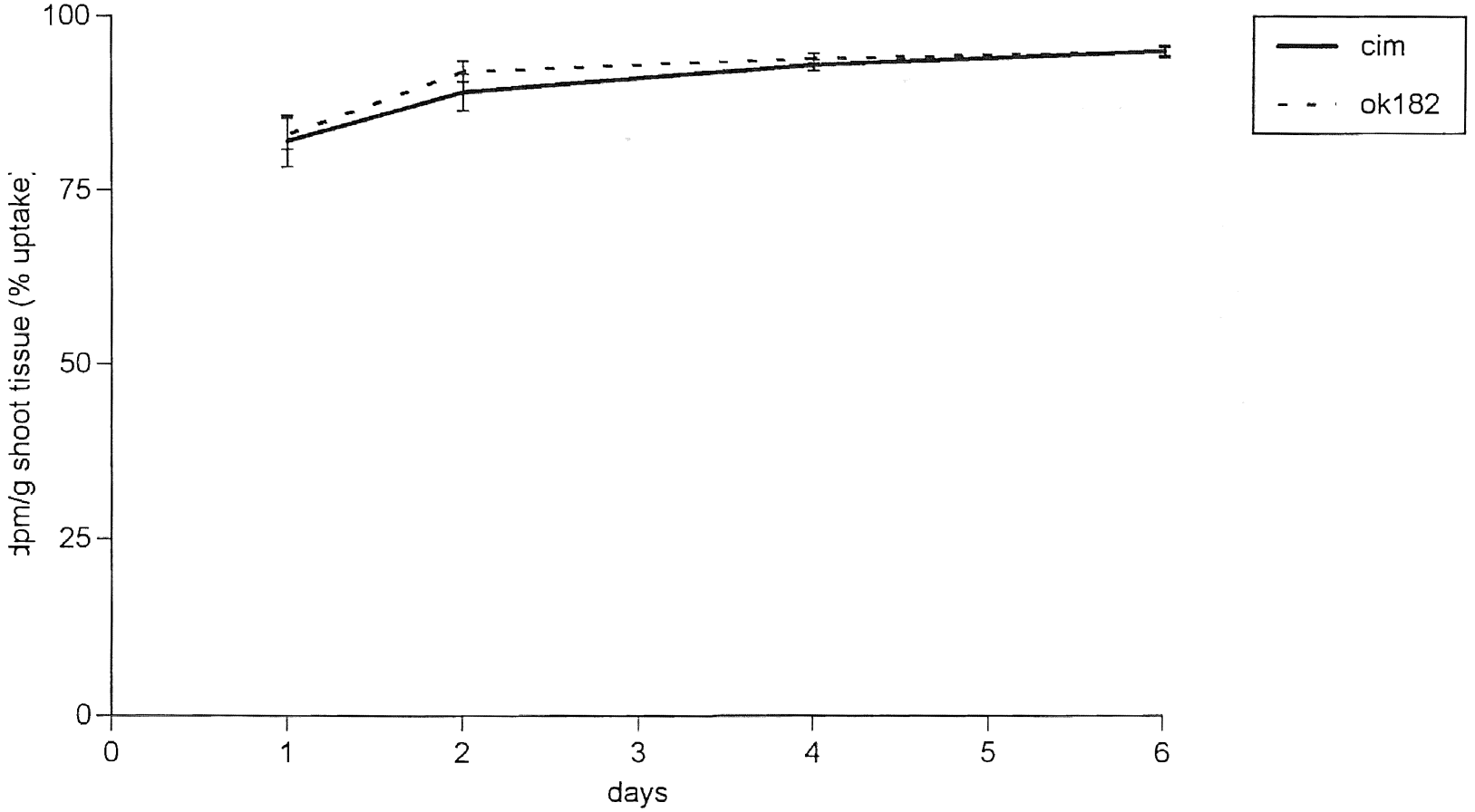

Figure 5. Actual translocation adjusted as a percentage of uptake.

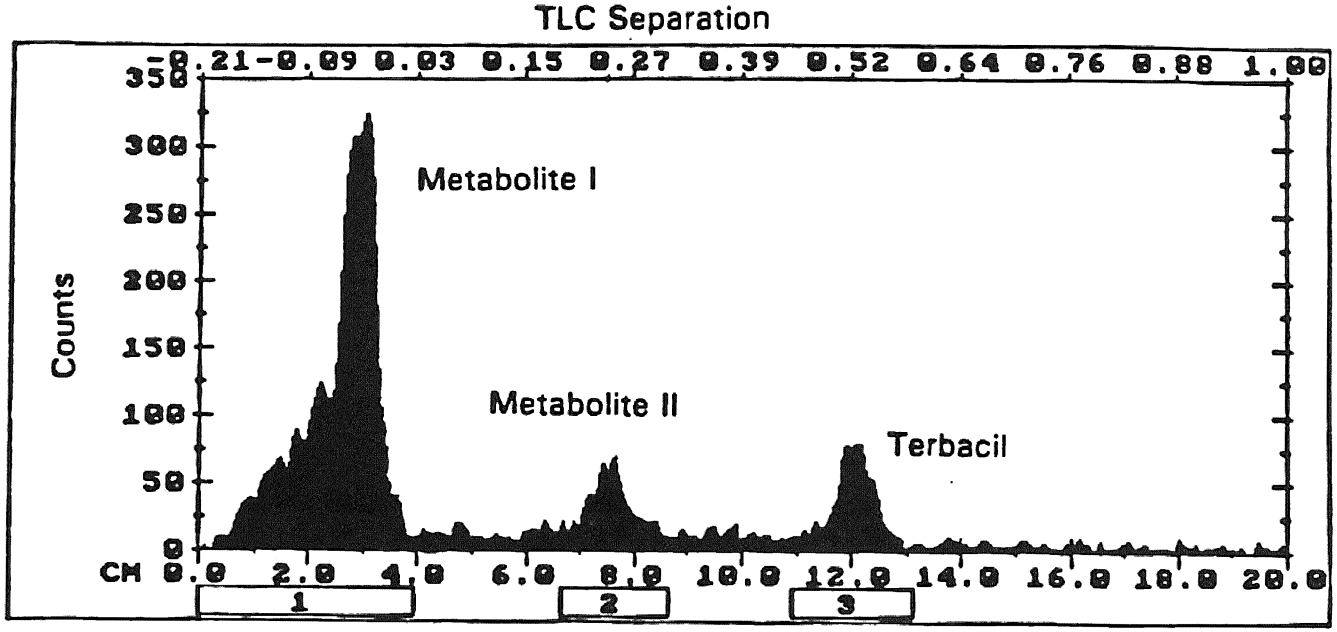

Figure 6. TLC separaton of radioactive components in leaf tissue 6 days following treatment.

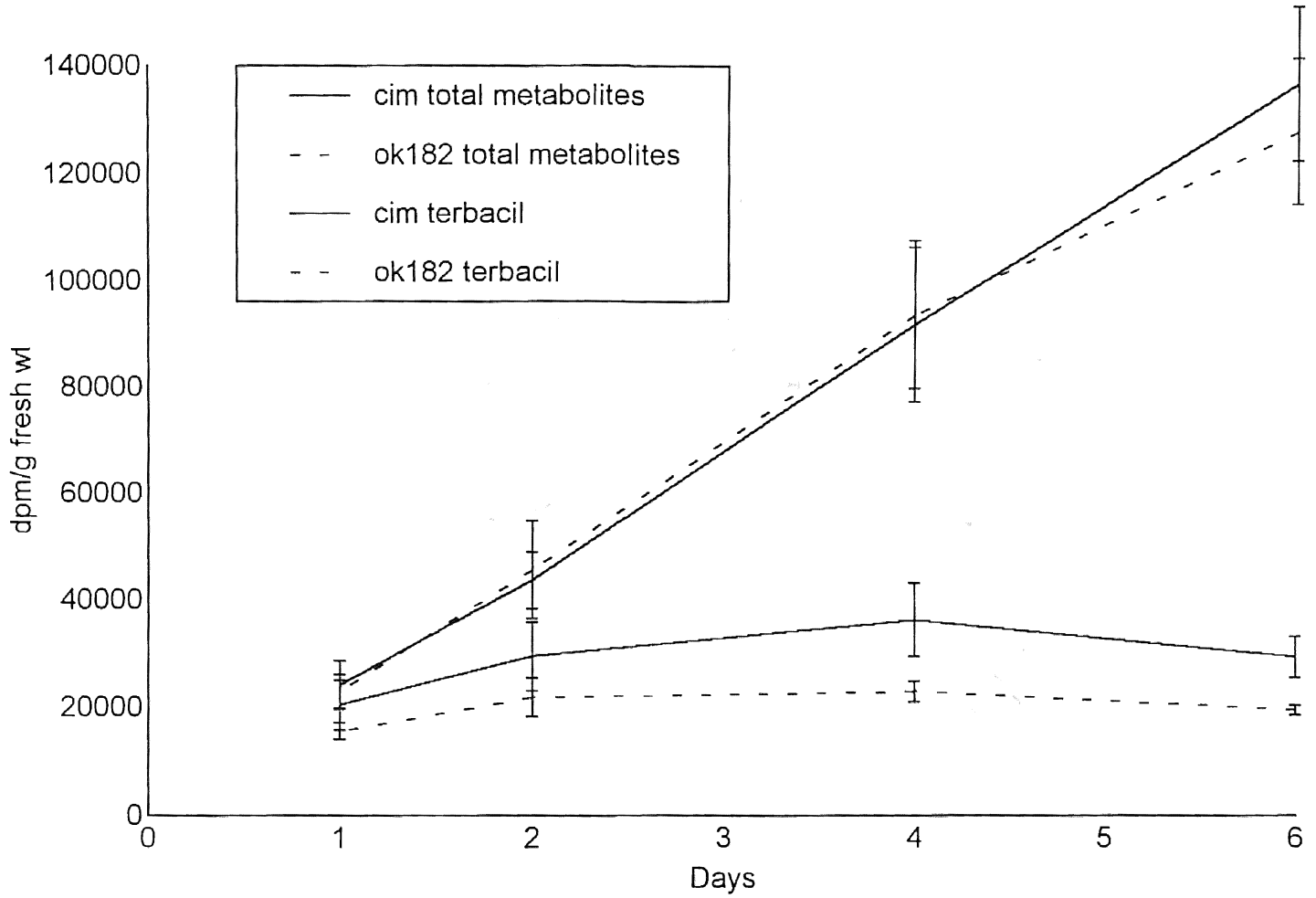

Figure 7. Terbacil and total metabolites in leaf extracts 1,2,4, and 6 days following treatment (exp 1+2).

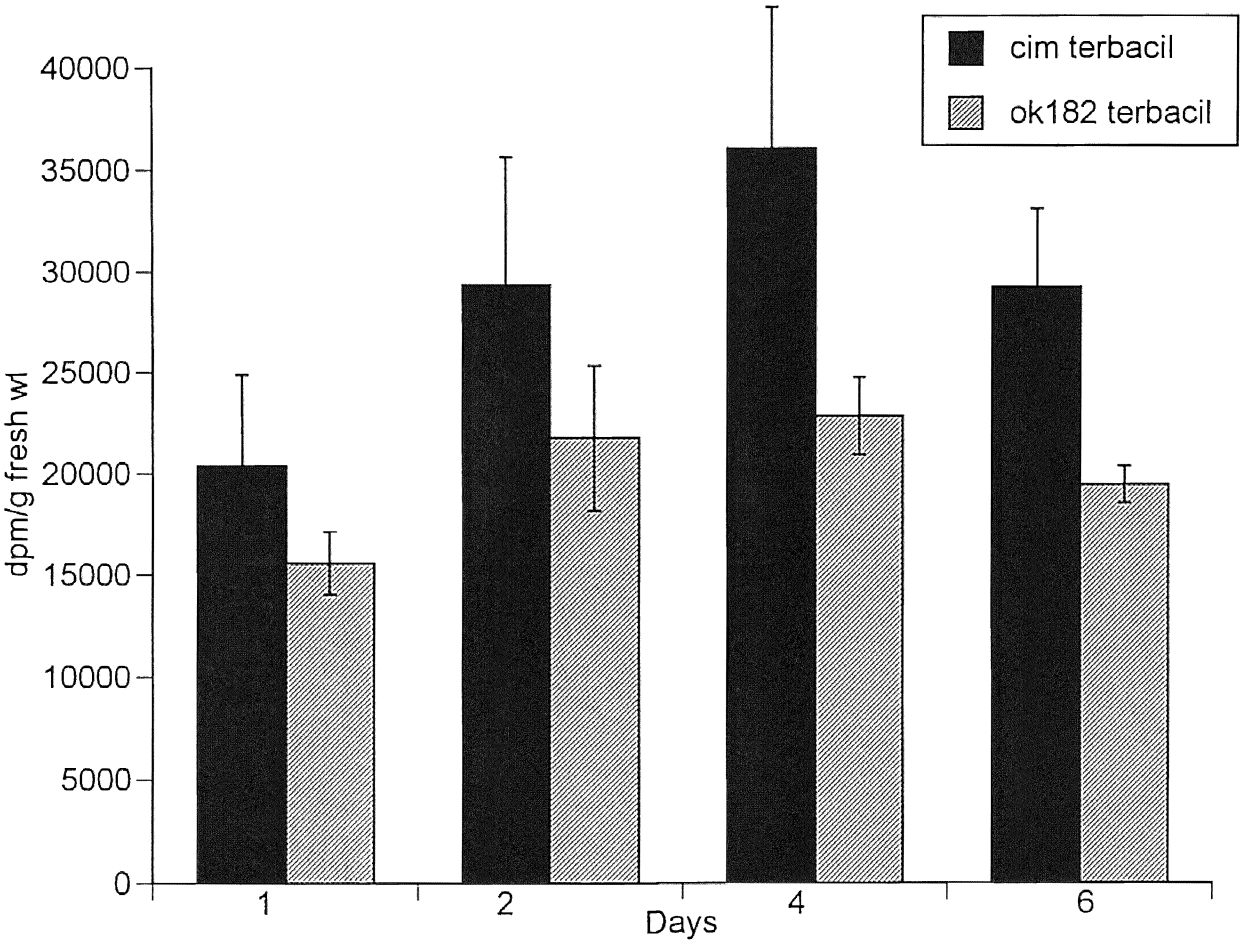

Figure 8. Radiolabeled terbacil in leaf extracts 1,2,4, and 6 days following treatment. (exp 1+2)

VITA

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Master of Science

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