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INVESTIGATIONS ON THE MECHANISM AND INHERITANCE OF ATRAZINE TOLERANCE IN CUCUMBER (*CUCUMIS SATIVUS* L.)

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ABSTRACT

INVESTIGATIONS ON THE MECHANISM AND INHERITANCE
OF ATRAZINE TOLERANCE IN CUCUMBER (CUCUMIS SATIVUS L.)

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Tolerance to the triazine herbicides has been reported in a number of economically important weeds that were formerly considered susceptible. We hypothesized that if the capacity to develop tolerance to the triazines existed in susceptible weed species, it might exist in the germplasm of a susceptible crop such as cucumber. The world's cucumber collection was evaluated for atrazine tolerance, and PI 390244 was found to tolerate preemergent applications of up to 0.56 kg/ha. The objectives of this research were to (i) determine if tolerance is due to differences in plant morphology or growth rates, (ii) determine if the differential response of cucumbers was based on differences in uptake, translocation or metabolism of atrazine, (iii) determine if tolerance is manifested at the chloroplast level as it is in the tolerant weed species, (v) determine if PI 390244 is universally tolerant to the triazines and finally (vi) determine the mode of inheritance of atrazine tolerance.

Results of a growth analysis conducted on PI 390244 and 'Marketmore 70' (a susceptible cultivar) revealed that the Plant Introduction had significantly more leaf area but less root biomass during the first 10

days after germination than the susceptible cultivar. The relatively small root biomass of PI 390244 was first hypothesized to be a factor contributing to its selectivity, but subsequent uptake studies with ^{14}C-atrazine refuted this hypothesis.

Isolated chloroplast preparations from both cultivars were equally susceptible to atrazine at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, or 5 x 10^{-5} M.

Although PI 390244 absorbed more total ^{14}C-atrazine, it translocated less radioactivity from the roots. While the concentration of radioactivity found in its roots was 10 to 30% greater than in 'Marketmore 70,' the amount of ^{14}C in the cotyledons, first and second true leaves of 'Marketmore 70' were 53, 34, and 55% higher, respectively, after 48 hours.

A series of autoradiographs were made of the two cultivars. Roots of both cultivars were uniformly labeled with the isotope. However, differences were noted in the foliar distribution of the radioactivity. There was a distinct localization of the radioactivity in the roots and vascular system of the tolerant cultivar, where as the foliage of the susceptible cultivar was uniformly labeled. Compartmentilization of the atrazine away from the major photosynthetic regions appears to be the major mechanism for tolerance in PI 390244.

A metabolism study conducted on both cultivars revealed no differences in the rate of metabolism or nature of metabolites between the two cultivars. A dealkylated metabolite, 2-chloro-4-amino-6-isopropylamino-s-triazine was the only metabolite found and then in only very small quantities (5-10%). The dealkylated metabolite was found only in the shoots and not in the roots of either cultivar.

Intraspecific difference in tolerance to several triazines was observed between the two cultivars. PI 390244 exhibited tolerance to several of the s-triazines and metribuzin. Tolerance in PI 390244 was restricted to the chlorine substituted s-triazines (atrazine, simazine and propazine) and metribuzin. At the Castle Hayne, NC location, atrazine, simazine and propazine reduced the fresh weight of the Plant Introduction an average of 28, 6, and 23%, respectively, while 'Marketmore 70' was reduced 60, 25, and 57%. At the East Lansing, MI location where an additional treatment of metribuzin was added, the Plant Introduction was reduced 44, 26, and 32% by atrazine, simazine and metribuzin as compared to 'Marketmore 70' which was reduced 74, 52, and 77%, respectively. Prometryn, dipropetryn, ametryn and prometon did not significantly reduce the fresh weight of either cultivar at rates of 0.14, 0.28, and 0.56 kg/ha.

The inheritance of tolerance to atrazine is most likely determined by two dominant genes with epistasis.

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INTRODUCTION

The herbicidal activity of the s-triazines was discovered by the J. R. Geigy Co. in Basle, Switzerland in 1952. The principal uses of the substituted s-triazine herbicides are the selective preemergence control of seedling grasses and broadleaf weeds in certain croplands, and with certain of these herbicides, the nonselective control of vegetation in noncroplands.

Extensive testing of the chloro-s-triazines showed the superiority of simazine, propazine and atrazine for weed control in corn. Because of certain special considerations, namely the greater water solubility and post-emergence activity, atrazine was selected as the prime candidate for use in corn. Recent figures show that in 1976, 90.3 million pounds of atrazine were applied to 75.7 million acres of corn in the United States (15). Simazine showed great promise for use in perennial crops and propazine in sorghum.

The s-triazine herbicides are readily absorbed by the roots and translocated upward to the leaves of plants via the transpiration stream. These herbicides are not translocated from the leaves after foliar application but do accumulate (2) and inhibit the Hill reaction in photosynthesis (45, 46).

Recently, there has been a renewed interest in the s-triazine herbicide group. In the early 1970's triazine tolerance was reported in a

number of economically important weeds that were formerly considered susceptible (5, 6, 31, 48, 50, 51, 52, 53, 56, 68, 70, 74, 75).

Because of this, it appeared that tolerance might also occur in crops that were considered susceptible. The objectives of this study were to determine if triazine tolerance existed in the usually sensitive cucumber, (Cucumis sativus L.)

Additional objectives of this study were to (i) determine the mechanism for tolerance to atrazine in cucumber, (ii) determine if tolerance is of a general nature for the triazine group and finally (iii) determine the mechanism for the inheritance of tolerance and the feasibility of developing tolerant cultivars.

CHAPTER 1

LITERATURE REVIEW

I. RESISTANCE TO HERBICIDES IN CROP AND WEEDS

Crop selectivity to the s-triazines is achieved both by physiological tolerance (relatively rapid detoxication of the absorbed chemical) by certain plants and by herbicide placement. Corn and sorghum have been shown to possess a high physiological resistance to atrazine, while cotton and peas exhibit intermediate tolerance. Cucumbers and oats have been found to be very susceptible to injury (66).

Intraspecific differences in response to herbicides exist in both weeds and crop plants. The first reported differential tolerance to a herbicide was with 2,4-D in Agrostis stonolifera L. (1). Since several biotypes of weeds and crops have been found to be tolerant to 2,4-D (13, 28, 59, 76, 77), dalapon (7, 24, 55, 57), and siduron (58).

The inability to control common groundsel (Senecio vulgaris L.) with simazine was first observed in a nursery in Washington State in 1968 (56). Subsequent testing revealed that the resistant biotype of groundsel also exhibited tolerance to atrazine. By 1978, the resistant biotype had spread to over 200,000 ha in the state (6). Soon to follow were reports of resistance in redroot pigweed (Amaranthus retroflexus L.) (48), lambsquarters (Chenopodium album L.) (5), common ragweed (Ambrosia artemsiifolia L.) (69), bird's rape (Brassica campestris L.) (68, 70)

and lateflowering goosefoot (Chenopodium strictum Roth var. glaucophyllum (Aellen) Wahl.) (6). The occurrence of resistance should be of little surprise since Harper in 1954 (24) hypothesized that resistance may develop in weed populations that have been repeatedly exposed to certain persistant herbicides, as has occurred in insect and microbial populations exposed to other pesticides.

Triazine-resistant biotypes first appeared as scattered plants in fields where a triazine herbicide had been used for more than six years and where no inter-row cultivation had been performed thus allowing these species to flower and produce seed (6). Without the selection pressure of the herbicide, the resistant biotypes would be in very low numbers or non-existent due to the lack of competitive ability of these biotypes in comparison to susceptible biotypes. Several studies (27, 51) have shown the susceptible biotypes to have a competitive advantage over the resistant biotypes.

Hensley and Counselman (27) have reported an allelopathic interaction between triazine resistant and susceptible biotypes of redroot pigweed. When seedlings from resistant and susceptible biotypes were grown together in a hydroponic system, only the susceptible biotype survived after 4 weeks. Similar effects were seen in a soil system, but a longer time period was required.

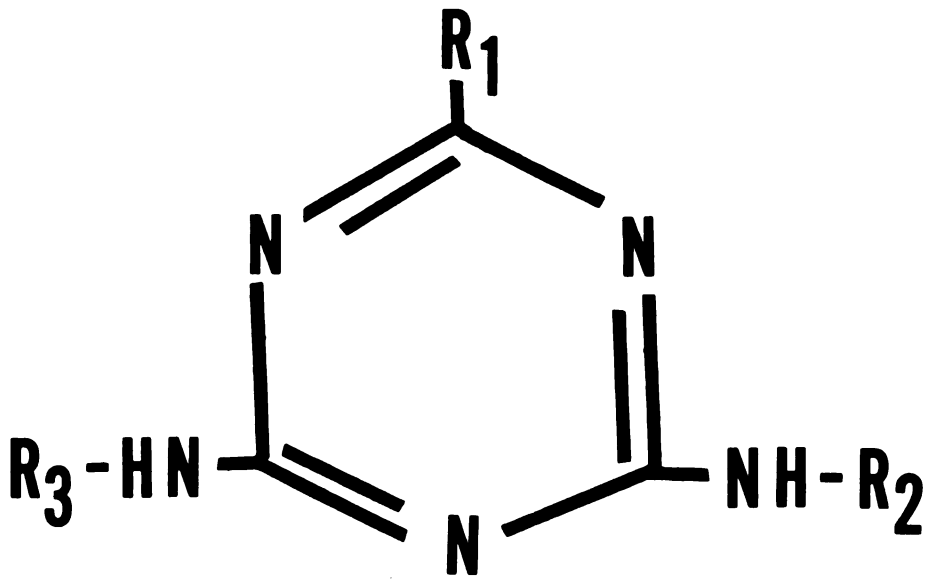
The triazine herbicides are known to be potent photosynthetic inhibitors (16, 17, 29), acting as an inhibitor of electron transport in PS II (44). Photosynthesis was measured in susceptible and resistant biotypes of lambsquarters, pigweed and common groundsel treated with atrazine and simazine. There was no inhibition observed in the resistant biotypes (50, 52, 53). The effect of atrazine on isolated chloroplasts

was also investigated (31, 51, 52, 53, 69, 74, 75). Similar findings were observed at this level also, regardless of species examined. Radosevich (52) hypothesized that structural or confirmational changes associated with the chloroplast membrane may account for the differential inhibition of the Hill reaction.

Studies on the mechanism of s-triazine tolerance in common lambsquarters, common groundsel and redroot pigweed established no differences in absorption or metabolism between biotypes of the three species (32). Jensen et al. (33) has reported that both biotypes of common lambsquarters detoxify ^{14}C-atrazine by hydroxylation, n-dealkylation and conjugation with glutathione. Parent atrazine accounted for greater than 83% of the ^{14}C-activity extracted from the leaves of both biotypes. Jensen concluded that since there was no differential absorption, translocation or metabolism between the two biotypes there must be some other mechanism responsible for resistance.

Pfister et al. (49) have reported that in susceptible biotypes of common groundsel, atrazine and diuron compete for the same binding site on the chloroplast membrane. However, "chloroplasts from triazine-resistant plants showed no atrazine binding over low herbicide concentration ranges, in agreement with a lack of herbicide effects on electron transport." Pfister also suggested that there may be a selective alteration of the chloroplast membrane in the resistant biotypes thus preventing the binding of the triazines. Arntzen et al. (4) has identified the component responsible for the differential binding of the chloroplast membranes. They suggest that a modification in a polypeptide component of the chloroplast membrane could be selectively modified in such a manner to alter a specific herbicide from binding on the membrane.

II. PHYSICAL AND CHEMICAL PROPERTIES OF TRIAZINES



Gysin and Knusli (19) demonstrated that if the two side chains are bisalkylated (equal), substituents at R_1 may be ranked in the following order of increasing inhibitory effectiveness: C_2H_5 > $CH(CH_3)_2$ > CH_3 > Cl > OCH_3 > Br > SCH_3. There is no inhibitory effect on the Hill reaction if H or OH is substituted at R_1. All economically important s-triazines may be placed in one of three subgroups based on whether the substitution at the R_1 position of the ring structure is a chlorine (Cl) atom, a methoxy (O-CH_3) group, or a methylthio (S-CH_3) group.

Two imido hydrogens are required for maximum inhibitory effectiveness, this places strict limitations on the substitutions at R_2 and R_3 (30). Mixed short chain alkyl or symmetrical substitutions favoring lipid solubility increase the inhibitory effectiveness, such that, isopropyl > ethyl > n-propyl > methyl (30).

The following are the common and chemical names of some members of the s-triazine herbicide family.

Subgroup			
R_1	R_2	R_3	Common Name
2-Cl	CH_2-CH_3	$CH(CH_3)_2$	atrazine
2-Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	simazine
2-Cl	CH_2-CH_3	CH_2-CH_3	propazine
2-O-CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	prometon
2-S-CH_3	CH_2CH_3	$CH(CH_3)_2$	prometryn
2-S-CH_3-CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	dipropetryn

Atrazine, the most economically important s-triazine, is synthesized by reacting cyanuric chloride, with one equivalent of ethylamine and one equivalent of isopropylamine in the presence of an acid acceptor. Atrazine has low water solubility at 33 ppmw, however, it is much more soluble than simazine (5 ppmw) (3).

III. MECHANISMS OF SELECTIVITY

There are three major metabolic pathways (hydroxylation, dealkylation and conjugation with glutathione) for the detoxication of atrazine and simazine (61, 66).

Corn and sorghum have been shown to be extremely tolerant to atrazine with peas and cotton exhibiting intermediate tolerance and wheat as being susceptible (12, 20, 63, 66). Shimabukuro et al. (66) have demonstrated that in corn, all three detoxication pathways are utilized.

Hydroxy atrazine and hydroxy simazine were the first degradation products reported in higher plants (9, 19, 43). Hydroxylation has been shown to occur only with the chlorine substitution at R_1 (18).

Hydroxylation of the chloro-s-triazines occurs non-enzymatically by the cyclic hydroxamate, benzoxazinone (2,4-dihydroxy-3-deto-7-methoxy-1,4-Benzoxazinone) and/or its 2-glucoside. It is suggested (10) that the catalyst participates in the nucleophilic attack of the chlorine ion. The rate of conversion is proportional to the endogenous levels of benzoxazinone and its 2-glucoside (20, 21, 47, 61). Roots of certain species have been found to contain higher quantities of the compound than do shoots (64).

Dealkylation of atrazine and simazine occurs to some extent in all higher plants (61, 65). Dealkylation involves the loss of either an n-ethyl or n-isopropyl sidechain. It is enzymatically catalyzed (61) and results in only partially detoxified metabolites (60).

Glutathione conjugation is another means by which plants may detoxify atrazine (41, 66). An enzyme, glutathione S-transferase, has been isolated from corn leaves and identified. Glutathione S-transferase has also been found in large quantities in leaf extracts of sorghum and sugar cane (40). No detectable levels of the enzyme were found in peas, oats, wheat or barley. The conjugated metabolites which were isolated and identified were S-(4-ethylamino-6-isopropyl-s-triazinyl-2)-glutathione (GS-atrazine) and S-λ-L-glutamyl-(4-ethylamino-6-isopropylamino-s-triazinyl-2)-L-cysteine (40). Reaction studies have shown that this enzyme has a specific requirement for a chlorine at the 2 position. This enzyme is strongly inhibited if the substitution at the 2 position is a methylthio group.

The rate of atrazine metabolism and detoxication appears to determine the basis for resistance in corn and sorghum (61). The rate of metabolism must be rapid enough to prevent the accumulation of a lethal

level of atrazine in the plant, and the pathway of metabolism must result in the formation of nonphytoxotic metabolites (61). Studies conducted on corn have shown that very little parent atrazine remained after 48 hours (61). Atrazine metabolism was monitored in corn leaf discs by Shimabukuro et al. (66). They reported that at the end of a 5 hr incubation period where the discs were incubated in 55 μM ^{14}C-atrazine, only 23% of the parent atrazine was extracted. The remaining 77% was in the form of glutathione conjugated atrazine.

In sorghum and pea, dealkylation occurred readily (61, 63, 68). Shimabukuro et al. (66) summarized the results obtained on corn, sorghum, peas and wheat that were treated with ^{14}C-atrazine either in the root media or foliarly. When corn and sorghum plants were treated foliarly, the major mechanism of detoxication was the conjugation of atrazine with glutathione. Conversely, with ^{14}C-atrazine nutrient culture treatments on corn and sorghum, corn metabolized the ^{14}C-atrazine non-enzymatically with benzoxazinone to the nonphytotoxic hydroxyatrazine. Sorghum was unable to detoxify the atrazine to any large extent. A small amount of dealkylation occurred in both roots and shoots of corn and sorghum.

The major metabolite of atrazine in both roots and shoots of young peas (which exhibit intermediate tolerance to atrazine) was 2-chloro-4-amino-6-isopropylamino-s-triazine, a dealkylated metabolite (60). No hydroxyatrazine was found, and only a small quantity of glutathione conjugated atrazine was present. The dealkylated metabolite in this form is only partially detoxified and must be further dealkylated to a nonphytotoxic form (60). This may explain the intermediate degree of tolerance exhibited in peas. Very small quantities of hydroxyatrazine, conjugated atrazine and dealkylated metabolites were found in wheat, a

susceptible species (66). These were present in such minute quantities that their presence did not alter the susceptibility of the crop (66).

Thompson (72) has reported that in six Setaria species and three Panicium species treated with atrazine, no differences were found in absorption or translocation between the nine species. Glutathione conjugation was the only major metabolite found. Small quantities of hydroxyatrazine were also found.

The rate of atrazine and propazine metabolism by Setaria and Panicium species correlates highly with their tolerance to these herbicides.

The metabolism of atrazine and simazine by wild cane resulted in the detoxication of atrazine by hydroxy derivatives and glutathione conjugation. Only 30% of the simazine absorbed by the wild cane was detoxified (73).

Jensen (32) has reported that in 53 grass species, dealkylation, hydroxylation and glutathione conjugation occurred in all species although rates of these metabolic pathways varied among the species tested. The recovery of net CO_2 exchange was correlated with formation of glutathione atrazine conjugates. Conjugation was the major detoxication pathway in species exhibiting tolerance to atrazine.

IV. FATE OF S-TRIAZINE HERBICIDES IN SOIL AND PLANTS

The fate of the s-triazines in soil is characterized by enormous complexity in both systems.

Several factors are known to influence the fate and behavior of herbicides in soil systems: 1) photochemical decomposition, 2) chemical

degradation, 3) microbial degradation, 4) volatilization, 5) movement, 6) adsorption, 7) plant or organism uptake (2).

There is evidence for non-biological detoxication of the s-triazine herbicides via photodecomposition, volatilization, hydroxylation and dealkylation (25, 26, 34, 38).

Adsorption appears to be one of the major factors involved in the fate of the s-triazine compounds in soil. Many excellent reviews have been written on this subject (18). Talbert and Fletchall (71) have reported that in the case of a Marshall silty clay loam with an organic matter content of 4.2%, adsorption of five s-triazine herbicides decreased in the order: prometryn > prometone > simazine > atrazine > propazine. Prometryn and prometon were bound significantly more than the others. The effect of chlorosubstitution (atrazine, simazine and propazine) appears to lessen the attractive forces between the herbicide and soil particles (25).

The fact that adsorption has a great influence on the leaching and movement of the s-triazine compounds can be observed. It is felt that adsorption, and not solubility regulates the extent of movement (25).

Detoxication of the chloro-s-triazine compounds may occur chemically in the soil through hydrolysis to form nonphytotoxic hydroxyanalogs (26). Harris (26) has reported that within a few weeks of treatment, hydroxy derivatives of atrazine, simazine and propazine were extracted from soil samples. Recovery at 8 weeks accounted for almost 50% of the radioactivity.

Microbial degradation is also an important component in the detoxication process in soil. Numerous soil microorganisms have been identified as having the capacity to degrade the s-triazines (18). Soil fungi appear to be the major participants in the degradation of the s-triazine compounds (8, 14, 35, 36, 37, 54).

McCormick and Hilbold (42) have reported that the decomposition of triazine herbicides was directly related to the breakdown of soil organic matter. They believe the triazines are passively degraded, incidental to the metabolism of soil-derived substances and are non-stimulatory to the growth or enzymatic capability of the microflora. They also believe that possibly the only reason microorganisms would actively degrade the s-triazines would be as a source of energy.

Many methods have been developed to study the degradation capacity of microorganisms: 1) isolates of microorganisms in nutrient cultures where the s-triazines were used as the sole source of carbon or nitrogen (35, 43), 2) evolution of $^{14}CO_2$ increased oxygen consumption, 3) the use of bioassays to follow oxygen consumption, 4) the use of bioassays to follow the dissipation of the compounds from microbial culture solutions (35).

Various reports can be cited in the literature as to the ability of soil fungi to utilize the s-triazines as a source of nitrogen or carbon, and it can be concluded that soil microorganisms can indeed utilize these compounds as sources of energy (37).

There are three methods that have been documented by which microorganisms in the soil may detoxify the s-triazine compounds: dealkylation, hydroxylation or cleavage of the ring.

Dealkylation is the major mechanism involved in the microbial degradation of the chloro-s-triazines (35, 36, 37, 39). Simazine detoxication has been reported to occur almost immediately after soil microorganisms were exposed to the compound (37). The most effective soil fungi reported by Kearney et al. (37) was Aspergillus fumigatus which could almost completely detoxify simazine in 12 days. The major

metabolite identified was 2-chloro-4-amino-6-ethylamino-s-triazine (39).

Hydroxylation is also a common mechanism by which microorganisms may detoxify herbicides. It is frequently the initial reaction step in the degradation of halogenated pesticides (18).

Ring cleavage occurs only after the chloro-s-triazines are first converted to the hydroxyanalogs (43). During the first 91 hours after exposure, the rate of ^{14}C-ring labeled simazine decomposition by corn, cucumbers and soil microorganisms to $^{14}CO_2$ was high, after which the rate decreased dramatically.

Kearney et al. (39) found $^{14}CO_2$ being evolved from organisms that have been exposed only to chain-labeled simazine but not from ring-labeled simazine. These were the same soil fungi which could dealkylate simazine very readily.

Evolution of low levels of $^{14}CO_2$ from microbial systems treated with ^{14}C-ring labeled s-triazines have been reported (11). Small quantities (less than 5%) of the ^{14}C-labeled prometryn, atrazine and simazine were evolved as $^{14}CO_2$ (42). They hypothesized that the s-triazine ring is somewhat resistant to microbial attack.

The fate of the s-triazine herbicides in plants has been fairly well documented. The detoxication processes associated with higher plants have been previously discussed. In summary, the s-triazine herbicides may remain in plants as unchanged material, partially or totally nonphytotoxic forms as hydroxyanalogs, dealkylated compounds or conjugated with glutathione.

Many factors determine the ultimate fate of the s-triazine compounds. It is the interaction of many components which lead to the

degradation of these herbicides into the nonphytotoxic compounds which are found in plant residues and soil.

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CHAPTER 2

INTRASPECIFIC DIFFERENCES IN TRIAZINE TOLERANCE WITHIN CUCUMBER (*CUCUMIS SATIVUS*) GERMPLASM

ABSTRACT

Tolerance to several *s*-triazine herbicides was determined on two cultivars of cucumber, PI 390244 and 'Marketmore 70' (a susceptible cultivar). PI 390244 has been previously reported tolerant to atrazine at 0.56 kg/ha preemergence. These studies indicated that tolerance in PI 390244 was restricted to the chlorine substituted *s*-triazines (atrazine, simazine, and propazine) and metribuzin. At the Castle Hayne, NC location, weights of the PI were reduced only 28, 6, and 23% by atrazine, simazine and propazine, respectively, as compared to 63, 24, and 57% for 'Marketmore 70'. Weights of the PI at the East Lansing, MI location were reduced 44, 26, and 32% by atrazine, simazine and metribuzin while 'Marketmore 70' was reduced 74, 52, and 77%, respectively. Prometryn, dipropetryn, prometon, and ametryn did not significantly reduce the fresh weight of either cultivar at rates of 0.14, 0.28, and 0.56 kg/ha.

INTRODUCTION

Intraspecific differences in herbicidal response exist in both weed and crop plants. Biotypes tolerant to the s-triazines have been reported in common groundsel (Senecio vulgaris L.) (5), lambsquarters (Chenopodium album L.) (1, 2), common ragweed (Ambrosia artemisiifolia L.) (7), redroot pigweed (Amaranthus retroflexus L.) (3), bird's rape (Brassica campestris L.) (6, 7) and cucumber (Cucumis sativus L.) (9).

The response of two biotypes of common groundsel to six s-triazines has been reported by Radosevich and Appleby (4). The sensitive biotype of common groundsel was found significantly more susceptible to all triazines except terbutryn. Universal s-triazine tolerance has also been demonstrated in biotypes of bird's rape (6). The atrazine tolerant biotype did not show phytotoxic symptoms with the methoxy, chloro and methylthio-s-triazines. Similar results were also observed in atrazine tolerant biotypes of lambsquarters (1).

This study was conducted to determine if a cucumber cultivar relatively tolerant to atrazine was also tolerant to other s-triazines and metribuzin.

MATERIALS AND METHODS

Initial Screening

A collection of 424 accessions of the world's cucumber germplasm was obtained from the Regional Plant Introduction Station at Ames, Iowa and 41 selected cultivars were obtained from Dr. L. R. Baker, Michigan State University, East Lansing, MI and Asgrow Seed Company, Kalamazoo, MI.

The seeds were sown on July 8, 1977 at East Lansing, MI on a Miami silt loam with an organic matter content of 2.1%. The number of seeds planted per accession varied from 10-200 seeds. Seeds were sown 1.9 cm deep using a V-belt Planter Jr. Atrazine was applied preemergence at 0.56 kg/ha in a spray volume of 333 l/ha over the entire planting.

Evaluation of the Triazines

Cucumber seed of both cultivars, relatively tolerant PI 390244 (The survivors of the initial screening were selfed and maintained individually for three generations - The most tolerant and most homozygous line was selected for this study.) and susceptible 'Marketmore 70' (obtained from the Asgrow Seed Company, Kalamazoo, MI) were sown 1.9 cm deep in individual rows 6.2 m long spaced 30.4 cm apart using a V-belt Planter Jr. Each plot contained 90 seeds of each cultivar.

Seven s-triazines: 2-chloro-4,6-bis(isopropylamino)-s-triazine (propazine), 2,4-bis(isopropylamino)-6-(methylthio)-s-triazine(prometryn), 2-(ethylamino)-4,6-bis(isopropylamino)-s-triazine(dipropetryn), 2,4-bis (isopropylamino)-6-methoxy-s-triazine(prometon), 2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine(ametryn), 2-chloro-4,6-bis (ethylamino)-s-triazine(simazine), and 2-chloro-4-(ethylamino-6-(isopropylamino)-s-triazine(atrazine) were applied preemergence at rates of 0, 0.14, 0.28, and 0.56 kg/ha. The first experiment was conducted in Castle Hayne, NC on April 19, 1979 on a Portsmith fine sandy loam with an organic matter content of 3.2 to 4.4%. The experiment was blocked to minimize variation through the organic matter gradient. The second experiment was initiated in East Lansing, MI on June 16, 1979 on Miami silt loam with an organic matter content of 2.1%. The second

study included 4-amino-6-tert-butyl-3-(methylthio- s -triazin-5(4H)-one (metribuzin) as a chemical treatment. Three replicates were used in each experiment. Plants were harvested at the second leaf stage. Data obtained were number of surviving plants and fresh weight per plot. Analysis of variance were determined on all data and means were compared using the LSD test at the 5% level (8).

RESULTS AND DISCUSSION

The initial study conducted on the world's cucumber collection and selected cultivars for atrazine tolerance resulted in several survivors from the various Plant Introductions. At 17 and 36 days after emergence, plants from 28 accessions had survived the 0.56 kg/ha of atrazine. However, most of these accessions contained either one or two plants and were considered escapes. One accession, PI 390244 exhibited the best tolerance to atrazine (Table 1). Of the 12 seeds initially planted, 9 seeds germinated and 7 plants survived the treatment. The survivors of the Plant Introduction were selfed and the seed obtained was used for all subsequent studies.

Table 1. PI 390244 tolerance to 0.56 kg/ha atrazine.

Number of seed planted	12	
Number of seed emerged	9	
Number of surviving seedlings	7	78% survival

Applications of the s-triazines on both cultivars yielded similar results at both locations (Figures 1 and 2). There was a two-fold difference in soil organic matter content between sites which may explain the slight increase in activity obtained at East Lansing.

It was found that several of the s-triazines did not injure either cultivar at the rates we selected. Prometryn, dipropetryn, prometon and ametryn did not significantly reduce the fresh weight of either cultivar at either location. Higher rates of application may have produced differences. However, the chloro-substituted triazines, atrazine simazine and propazine at the three rates tested greatly reduced the fresh weight of 'Marketmore 70,' the susceptible cultivar, in comparison to the Plant Introduction at both locations (Figure 3). At Castle Hayne, PI 390244 had been reduced 28, 6, and 23%, respectively, while 'Marketmore 70' was reduced by 63, 24, and 57% (Figure 1). The chloro-triazines and metribuzin were also much more toxic to 'Marketmore 70' at the East Lansing location. Atrazine, simazine and propazine decreased the fresh weight of 'Marketmore 70' by 74, 52, and 50%, respectively, as compared to the Plant Introduction which was reduced 44, 26, and 26%. Metribuzin, which is an asymmetrical triazine was extremely toxic to 'Marketmore 70.' Reductions in fresh weight of 'Marketmore 70' were 77% while PI 390244 was reduced only by 32% (Figure 2).

Although both cultivars manifested some injury symptoms, the susceptible cultivar showed extensive chlorosis, necrosis, and high mortality. PI 390244 had a low mortality rate and showed only limited chlorosis.

When summarizing all the data over two locations (excluding metribuzin), the interaction of cultivar x rate x chloro-triazines versus

Figure 1. The interaction of cultivar x chemical at the Castle Hayne, NC location. Data are averages for the three rates of application. * indicates that means are significantly different at the 5% probability level.

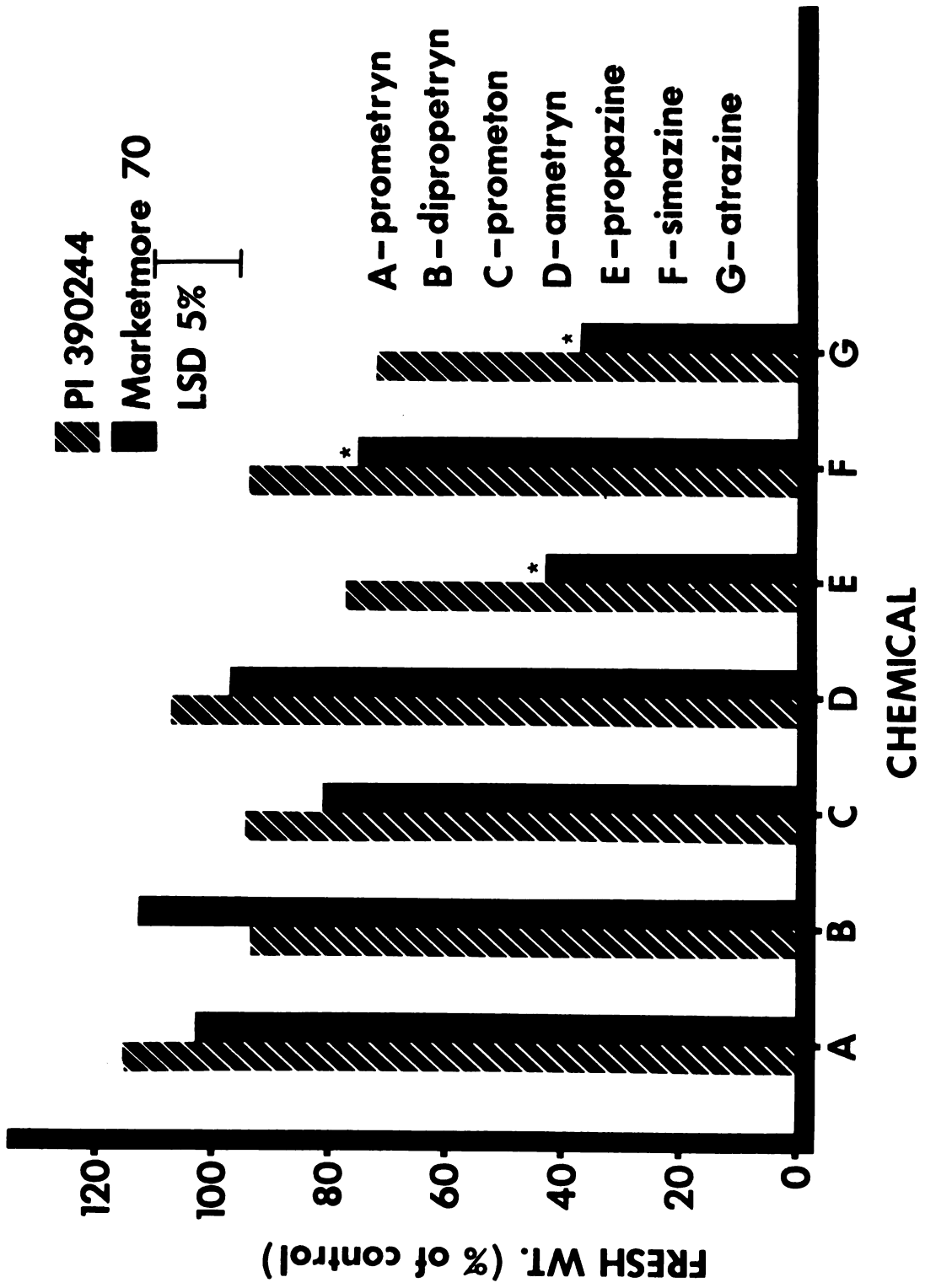

PI 390244
Marketmore 70
LSD 5%
A-prometryn
B-dipropetryn
C-prometon
D-ametryn
E-propazine
F-simazine
G-atrazine
FRESH WT. (% of control)
120
100
80
60
40
20
0
A
B
C
D
E
F
G
CHEMICAL

Figure 2. The interaction of cultivar x chemical at the East Lansing, MI location. Data are averages for the three rates of application. * indicates that means are significantly different at the 5% probability level.

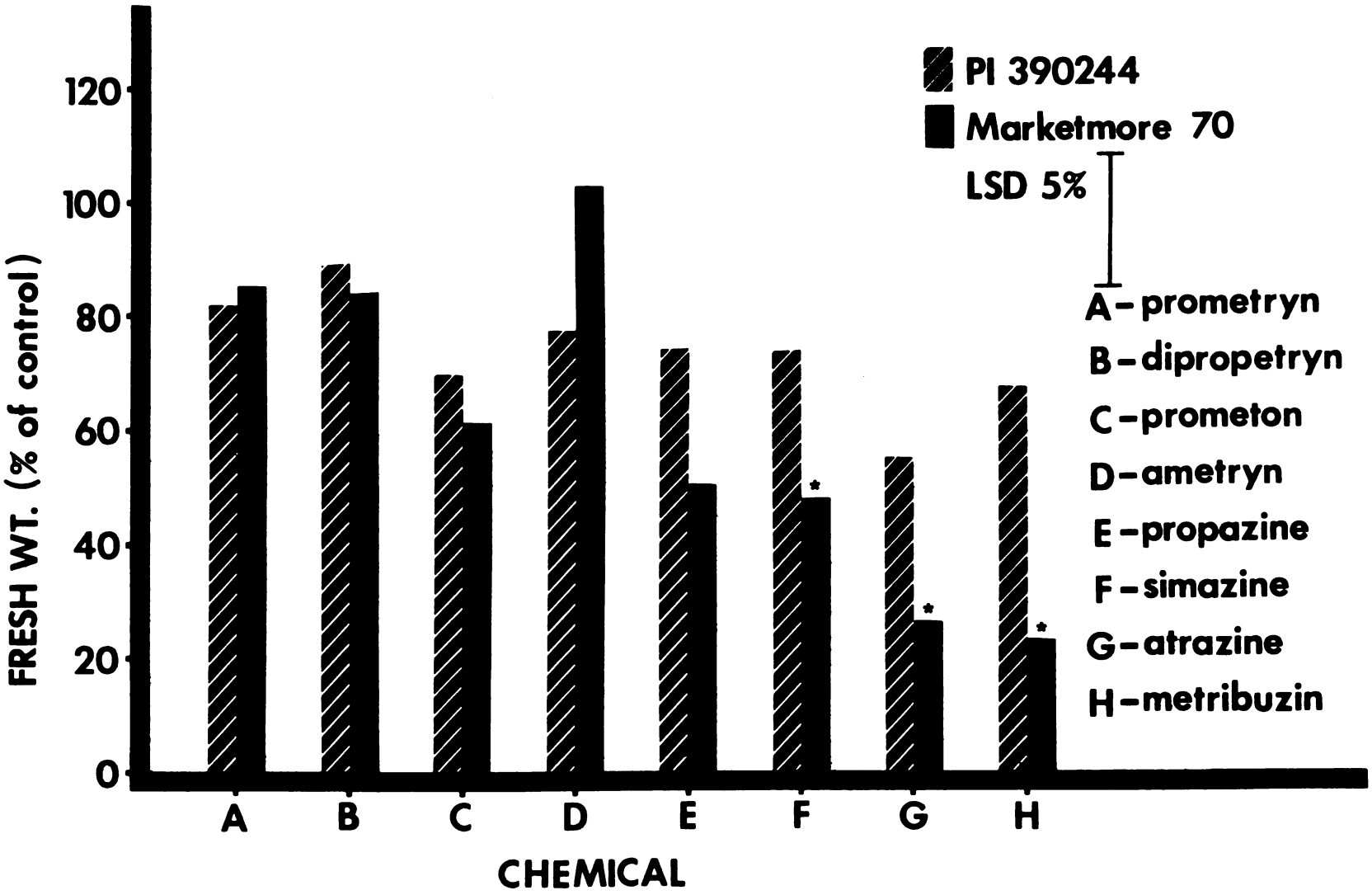
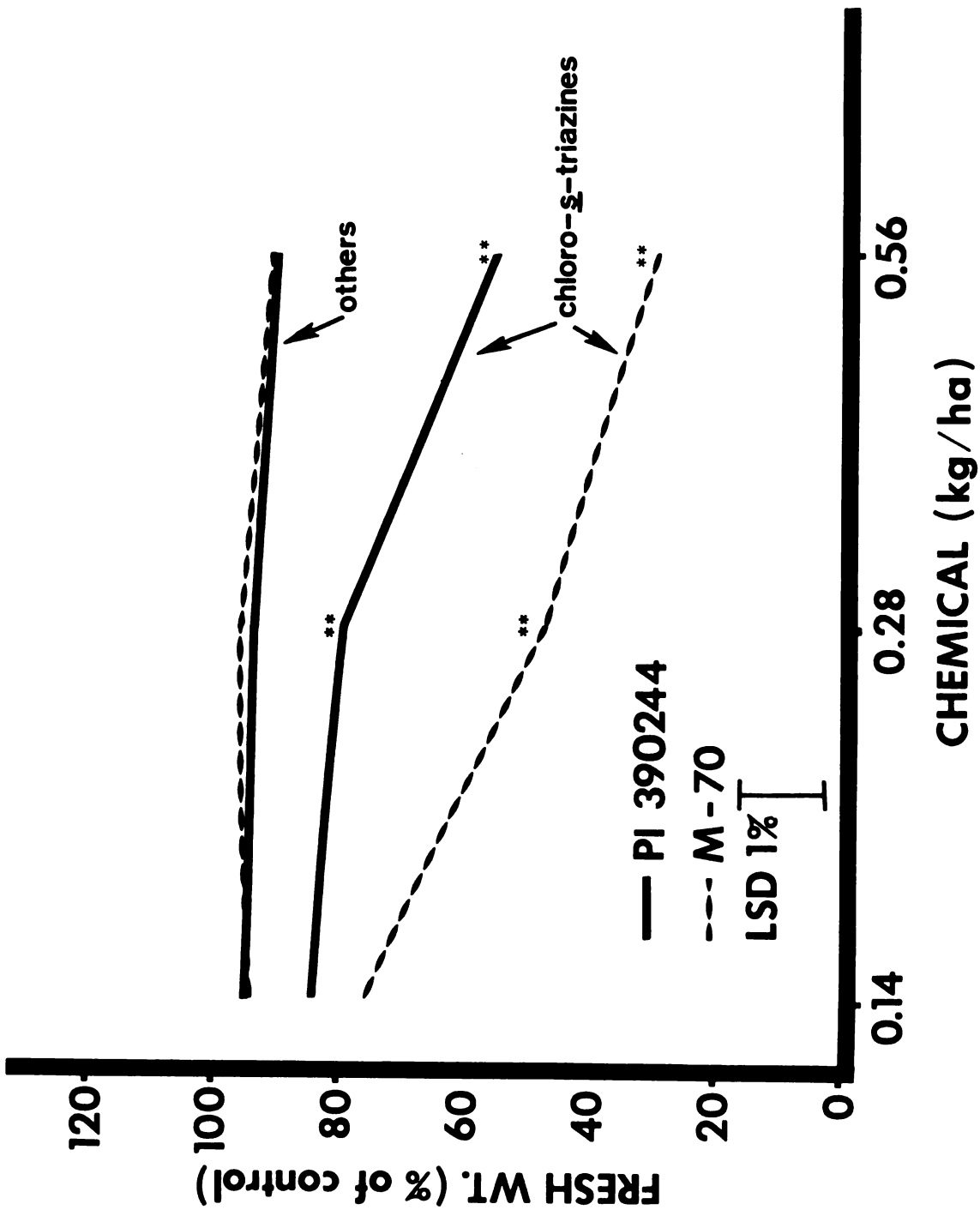

PI 390244
Marketmore 70
LSD 5%
A-prometryn
B-dipropetryn
C-prometon
D-ametryn
E-propazine
F-simazine
G-atrazine
H-metribuzin
FRESH WT. (% of control)
120
100
80
60
40
20
0
A
B
C
D
E
F
G
H
CHEMICAL
*
*
*

Figure 3. The interaction of triazine type x cultivar x rate. Data are averages of both locations. Metribuzin data has been omitted. ** indicates that means are significantly different at the 1% probability level.


FRESH WT. (% of control)
120
100
80
60
40
20
0
0.14
0.28
0.56
CHEMICAL (kg/ha)
others
chloro-s-triazines
**
**
**
**
PI 390244
M-70
LSD 1%

others was significant at the 1% level (Figure 3). Although both cultivars were affected by the chloro-triazines, 'Marketmore 70' was much more susceptible at the higher rates of 0.28 and 0.56 kg/ha.

Since only atrazine, simazine, propazine (all chlorine substituted s-triazines), and metribuzin were the only triazines which affected the growth of both cultivars, it may have been that we erroneously selected poor rates of the others. Differences might have been noted with the other s-triazines had they been applied at higher rates. Due to a shortage of PI 390244 seed, this has not been accomplished.

Resistant biotypes of lambsquarters, bird's rape, and common groundsel have been reported very tolerant to all of the triazines. The resistance in these particular weeds occurs at the chloroplast level. The tolerance to the triazines exhibited among cucumbers is of a different nature. A previous study (10) has shown that the mechanism of tolerance in PI 390244 may be related to its ability to sequester the herbicide in its vascular system thus preventing the movement of the compound into the leaves and the chloroplasts. This is the most likely mechanism responsible for the relatively good tolerance manifested by PI 390244.

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CHAPTER 3

MECHANISM FOR DIFFERENTIAL ATRAZINE TOLERANCE WITHIN CUCUMBER (*CUCUMIS SATIVUS*)

ABSTRACT

The cucumber (*Cucumis sativus* L.) accession PI 390244 tolerated up to 0.56 kg/ha atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] whereas most cultivars and accessions were killed by 0.14 kg/ha. The possible mechanisms for tolerance were investigated by comparing growth rates, effects on photosystem II in isolated chloroplasts, and differences in uptake and translocation of ^{14}C-atrazine between tolerant PI 390244 and the susceptible cultivar 'Marketmore 70.' PI 390244 had significantly more leaf area than 'Marketmore 70,' but less root biomass. Isolated chloroplast preparations from both cultivars were equally susceptible to the herbicide. Although PI 390244 absorbed more total ^{14}C-atrazine, it translocated less radioactivity from the roots. While the concentration of ^{14}C found in the PI 390244 roots was 10 to 30% greater than in 'Marketmore 70,' the amount of radioactivity in the cotyledons, first and second true leaves of 'Marketmore 70' were 53, 34, and 55% higher, respectively, after 48 hours.

INTRODUCTION

Intraspecific differences in response to herbicides exist in both weeds and crop plants. The first reported differential tolerance to an herbicide was with 2,4-D (2,4-dichlorophenoxy)acetic acid in creeping bentgrass (Agrostis stolonifera L.) (1). Since then, several biotypes of weeds and crops have been found to be tolerant to 2,4-D (10, 22, 30, 31), dalapon (2,2-dichloropropionic acid) (4, 7, 18, 20), and siduron [1-(2-methyl-cyclohexyl)-3-phenylurea] (21). Tolerance to the triazines has been reported in a number of important weeds that were formerly considered susceptible. Biotypes tolerant to the 2-chloro-s-triazines were first reported in 1970 by Ryan (19) in common groundsel (Senecio vulgaris L.). Other broadleaf weed species with triazine-tolerant biotypes are common lambsquarters (Chenopodium album L.) (3), common ragweed (Ambrosia artemisiifolia L.) (23), redroot pigweed (Amaranthus retroflexus L.) (14), and bird's rape (Brassica campestris L.) (23, 25).

Studies on the mechanism of s-triazine tolerance in common lambsquarters, common groundsel and redroot pigweed established no differences in absorption or metabolism between biotypes of the three species. Isolated chloroplasts of the resistant biotypes were not affected by the triazines (12, 15, 16, 17, 24, 26, 29).

The development of tolerance to herbicides is not surprising. Harper (8) hypothesized that resistant biotypes may be selected in weed populations that have been exposed repeatedly to certain persistant herbicides as has occurred in insect and microbial populations.

We hypothesized that if the capacity to develop tolerance to triazines existed in susceptible weed species, it might also exist in

germplasm of susceptible crops. Previous research (27) has revealed tolerance to s-triazines in the usually sensitive cucumber. The world's cucumber collection, obtained from the Regional Plant Introduction Station, Ames, Iowa, was evaluated in the summer of 1977 and Plant Introduction (PI) 390244 was found to tolerate 0.56 kg/ha atrazine, whereas most other PIs were killed at one fifth that rate.

Studies described in this paper were conducted to (i) determine whether the differential response of atrazine tolerant cucumbers was based on differences in uptake and translocation of atrazine, (ii) determine if tolerance was due to differences in plant morphology or growth rates, (iii) determine if tolerance is at the chloroplast level as reported by Souza Machado et al. (24) in common lambsquarters.

MATERIALS AND METHODS

Differential Cultivar Tolerance

Initial evaluations in the field and greenhouse demonstrated atrazine tolerance in cucumber PI 390244 (27). A more definitive test was established by planting five cucumber seeds per pot in Dryden sandy loam soil and preemergence application of atrazine at 0, 0.14, 0.28, 0.56, 0.84, and 1.12 kg/ha in 926 l/ha. Five replications of PI 390244 and the susceptible cultivar 'Marketmore 70' were assigned to a randomized complete block design and maintained under a 16 hour photoperiod with metal halide lights (842 $\mu E\ m^{-2}\ sec^{-1}$) supplementing natural sunlight in the greenhouse.

To study cultivar tolerance in nutrient culture, 2 day-old seedlings were rinsed with distilled water and transferred directly into 220 ml

cups wrapped in aluminum foil containing 180 ml of 0, 0.1, 0.2, or 0.3 µM atrazine in half-strength Hoagland's solution (9). Three seedlings were suspended in the solution by a sponge rubber disc. The nutrient solution was replenished at 2-day intervals. Plants were grown in a growth chamber with a 16 hour photoperiod maintained with cool-white fluorescent and 25 W incandescent lamps (225 µE m^{-2} sec^{-1}) and day and night temperatures of 31 and 21 $\pm$ 2°C, respectively. A randomized complete block design with five replications was used. The fresh weights of the shoots were recorded after 16 days. The experiment was repeated twice.

Growth Analysis

A growth analysis was conducted to compare PI 390244 and 'Marketmore 70.' Plant growth was monitored for 10 days after seedling emergence. Plants were grown individually in 10 cm styrofoam pots containing vermiculite. A randomized complete block design (five replications) was used with five harvest dates and two nutrient regimes, distilled water and half-strength Hoagland's containing 12 mM NO_3^- (pH 6.5). The pots were initially watered with 200 ml of either distilled water or half-strength Hoagland's solution. At 2-day intervals, 100 ml of the nutrient treatments were surface applied to the pots. The pots were placed in a growth chamber under the conditions described previously.

Plants were harvested at 2-day intervals after the seedlings had emerged from the growth medium. Leaf area and shoot and root dry weights were recorded. Plant parts were oven-dried for 2 days at 45°C. Leaf area ratio (LAR), net assimilation rate (NAR), and relative growth rate (RGR) were calculated (6). The dry weight data obtained for the shoots

and roots were analyzed using planned comparisons with the LSD test (5% level). Each growth analysis was repeated two or more times.

Isolated Chloroplasts

Chloroplasts were isolated from mature leaves of 3 week-old cucumber (PI 390244 and 'Marketmore 70') plants that had been grown in coarse grade vermiculite, and were watered daily with half-strength Hoagland's solution. Plants had been grown for 3 weeks in a growth chamber with a 16 hour photoperiod using cool white fluorescent light (300 E m^{-2} sec^{-1}) with day and night temperatures of 30 and 20 $\pm$ 2°C, respectively. Chloroplasts were isolated according to Izawa and Good (11) with the following modifications: plants were harvested at the end of the dark period, but before exposure to light. Fifty grams of deveined leaf tissue were ground for 5 sec in a Waring blender with 75 ml 0.3 mM NaCl, 0.03 M tricine (N-tris[Hydroxymethyl]methyl glycine), 3 mM $MgCl_2$, 0.5 mM EDTA (pH 7.8). The homogenate was squeezed through 16 layers of cheese-cloth and then centrifuged at 2500 x g for 90 seconds. The sediment was resuspended in 10 ml of a medium consisting of 0.2 M sorbitol, 0.005 M HEPES (N-2-hydroxyethyl piperazine-N^1-2ethanesulfonic acid), 2 mM $MgCl_2$ and bovine serum albumin (BSA) (0.5 g/100 ml) (pH 7.4) and centrifuged at 2000 x g for 45 seconds. The supernatant was then filtered through tissue paper. The filtered supernatant solution was centrifuged at 2500 x g for 3 minutes. The supernatant solution was decanted, the pellet resuspended in a final volume of 1.5 ml of suspension medium, and the chloroplast content was determined (2). All extractions were made in a cold room at 0°C. Treatments consisted of 0, 10^{-8}, 10^{-7}, 10^{-6}, or 5 x 10^{-5} M atrazine.

The Hill reaction assay was carried out in a darkened room. The reaction mixture was made up in a 13 by 100 mm cuvette. The reaction mixture (2 ml final volume) consisted of 15 µg/µl chloroplast suspension, 50 mM HEPES, 2 mM $MgCl_2$, 0.5 mM ferricyanide, and 0.2 M sorbitol, with the various concentrations of atrazine. The reaction was measured on a modified Bausch and Lomb Spectronic 505 Spetrophotometer at 420 nm. The experiment was repeated twice for all concentrations.

Uptake and Translocation

Cucumber seeds (PI 390244 and 'Marketmore 70') were germinated in 77 ml plastic cups containing coarse grade vermiculite and watered with half-strength Hoagland's solution. Two days after emergence, the seedlings were rinsed carefully with distilled water to remove the vermiculite. The seedlings were suspended by sponge rubber discs in aluminum foil-wrapped cups that contained 180 ml nutrient solution containing 12 mM NO_3^- (pH 6.5). The nutrient solution was changed every two days. Seedlings were placed in a growth chamber under conditions described previously for the chloroplast study. At 16 days, the seedlings were placed in 170 ml of nutrient solution to which 0.2 µCi of (ring - ^{14}C) fortified atrazine with a specific activity of 27.2 µCi/4.63 M had been added. Corrections for evaporation were made following each exposure time using a control treatment that was identical to the others except that it contained no plants. All tests were initiated at the beginning of the light period. A randomized complete block design containing four replications (three seedlings/replication) was used. Plants were harvested at 6, 12, 24, and 48 hours after treatment and the roots were rinsed three times with distilled water. Solution volume was

recorded at harvest after the removal of the plants, and absorption calculated by difference, after correcting for evaporation. Harvested plants were divided into roots, stem plus cotyledons, first leaf and second leaf, and the parts individually frozen in vials with dry ice and acetone and lyophilized. Dry weights were recorded and samples were combusted in a Harvey Biological Oxidizer. The $^{14}CO_2$ released by combustion was captured in 15 ml of Carbon 14 Cocktail[1] and quantitated by liquid scintillation. Data from two experiments were averaged and analyzed as a three-way factorial. Planned comparisons were made using the LSD test (5% level).

Plants of PI 390244 and 'Marketmore 70,' grown as previously described, were suspended in ^{14}C-atrazine (0.2 μCi/40 ml) solution for 24 hours. Following exposure, the roots were rinsed three times with distilled water, blotted, pressed, frozen, lyophilized, and mounted. The plants were then exposed for one week to Kodak no-screen x-ray film. The treatments were replicated four times.

RESULTS AND DISCUSSION

Differential Cultivar Tolerance

'Marketmore 70' cucumber was injured more extensively than PI 390244 by an atrazine treatment of 0.14 kg/ha (Table 1). Several PI 390244 plants survived treatment with 0.56 kg/ha, but there was considerable variability among siblings of this cultivar. Plants of the two cultivars grown for 16 days in half-strength Hoagland's solution containing 0.1,

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[1]R. J. Harvey Instrument Company; Hillsdale, NJ 07642.

Table 1. Differential tolerance of cucumber PI 390244 and 'Marketmore 70' to atrazine in Dryden sandy loam and in nutrient culture solution.

Soil Treatment

Atrazine rate (kg/ha)	Plant survival[a]	
	'Marketmore 70'	PI 390244
	----------(%)----------	
0.14	12	75
0.28	0	50
0.56	0	16
0.84	0	0
1.12	0	0

[a]F value for cultivar is significantly different at the 1% level.

Nutrient Culture

Atrazine (μM)	Fresh wt/plant	
	'Marketmore 70'	PI 390244
	----------(g)----------	
0	10.6	14.0
0.1	11.0	13.3
0.2	5.1	11.6
0.3	0.9	6.8
L.S.D. at .01	1.82	1.82

0.2, or 0.3 M atrazine responded quite different (Table 1). None of the susceptible cultivar 'Marketmore 70' survived treatment with 0.3 µM atrazine, but PI 390244 was reduced in fresh weight to only 48% of the control. At the lowest and intermediate concentrations of atrazine, decreases noted on PI 390244 were 95% and 83% of control compared to 104% and 48% exhibited by 'Marketmore 70.'

Growth Analysis

The net assimilation rates of the two cultivars were not significantly different regardless of nutrient treatment. Highly significant differences in the relative growth rates were observed between different nutrient regimes.

PI 390244 had approximately 15% more leaf area than 'Marketmore 70' after 8 to 10 days, when averaged over both nutrient regimes (Figure 1). Both cultivars produced greater leaf areas with nutrient solution than with distilled water.

'Marketmore 70' had significantly more root biomass than PI 390244 at 6 days and thereafter (Figure 2). The nutrient solution increased root biomass of both cultivars.

The relatively small root biomass of PI 390244 at 6 to 10 days after seedling emergence was first hypothesized to be a factor contributing to the selectivity of this cultivar to atrazine by restricting absorption and dilution of the quantity absorbed throughout the larger leaf area. Later uptake studies with ^{14}C-atrazine refuted this hypothesis.

Figure 1. Leaf area ratios (LAR) from atrazine-tolerant PI 390244 and atrazine-susceptible 'Marketmore 70' cucumber in distilled water and half-strength Hoagland's solution two to ten days after emergence. Asterisks indicate values that are significantly different at the 5% level.

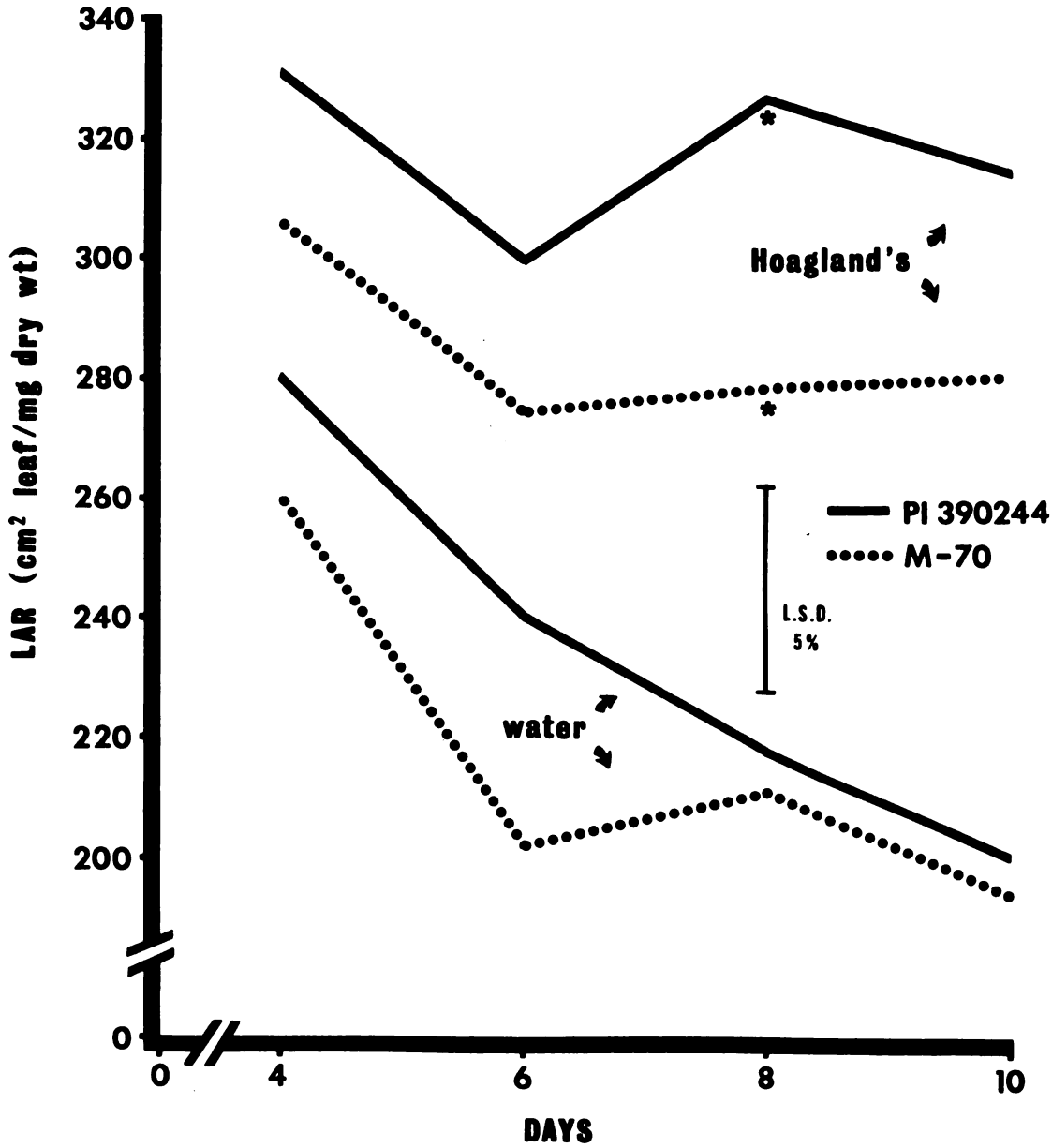
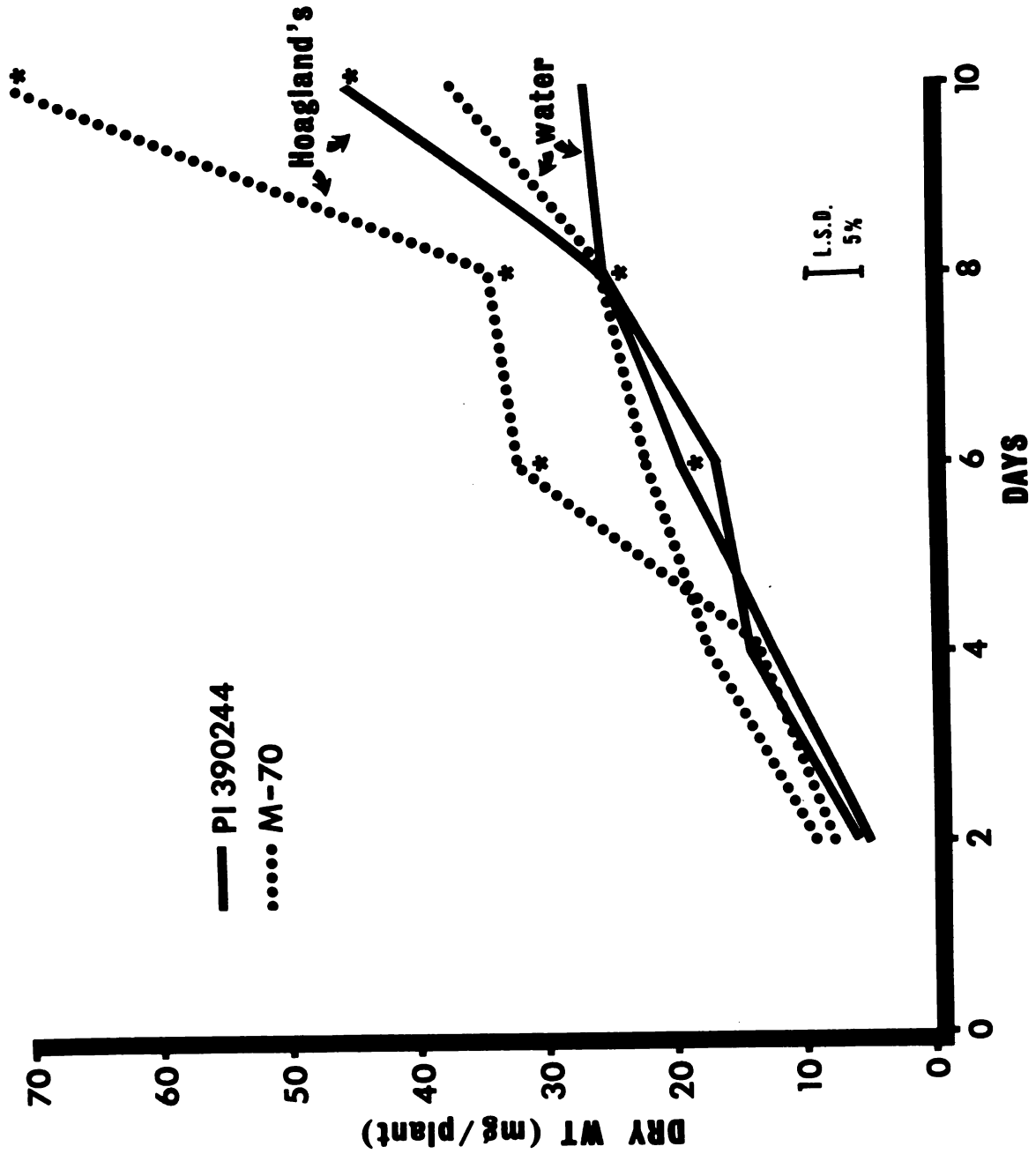

340
320
300
280
260
240
220
200
0
LAR (cm² leaf/mg dry wt)
Hoagland's
water
PI 390244
M-70
L.S.D.
5%
0
4
6
8
10
DAYS

Figure 2. Root biomass from atrazine-tolerant PI 390244 and atrazine-susceptible 'Marketmore 70' in distilled water and nutrient solution two to ten days after emergence. Asterisks indicate values that are significantly different at the 5% level.


DRY WT (mg/plant)
70
60
50
40
30
20
10
0
0
2
4
6
8
10
DAYS
PI 390244
M-70
Hoagland's
water
L.S.D. 5%

Isolated Chloroplasts

The s-triazines are known to be potent inhibitors of the Hill reaction (12). There were no differences in response between the two cultivars (Figure 3). Therefore, unlike the results reported with common lambsquarters (16, 23), the differential response is not manifested at the chloroplast level.

Uptake and Translocation

Atrazine was readily absorbed from the nutrient solution by the roots and translocated aceopetally to the leaves of both cultivars. PI 390244 consistently absorbed more water and more ^{14}C-atrazine than 'Marketmore 70' (Figure 4), even though PI 390244 had less root biomass at the time of treatment. Perhaps, greater transpiration losses from larger leaf areas caused an increase in root uptake. PI 390244 contained 10 to 13% more ^{14}C/plant than 'Marketmore 70' through the duration of the experiment (Figure 4).

There were no significant differences between cultivars in distribution of radioactivity (dpm/mg) within a plant part 6 or 12 hours after treatment. However, at 24 and 48 hours, PI 390244 accumulated more ^{14}C than 'Marketmore 70' in the roots (Figure 5a). Although no ^{14}C-activity accumulated in the roots after 24 hours in either cultivar, ^{14}C-activity continued to accumulate in both the cotyledons and leaves. 'Marketmore 70' had 32% more dpm/mg in the cotyledons after 48 hours than PI 390244 (Figure 5b). The ^{14}C concentration in the first and second leaves was not significantly different between cultivars at 6 and 12 hours after treatment, but after 24 and 48 hours, 'Marketmore 70' had consistently more dpm/mg in these two areas (Figures 5c and 5d).

Figure 3. Photochemical activity of isolated chloroplasts from atrazine-tolerant PI 390244 and atrazine-susceptible 'Marketmore 70' cucumber leaves exposed to several atrazine concentrations.

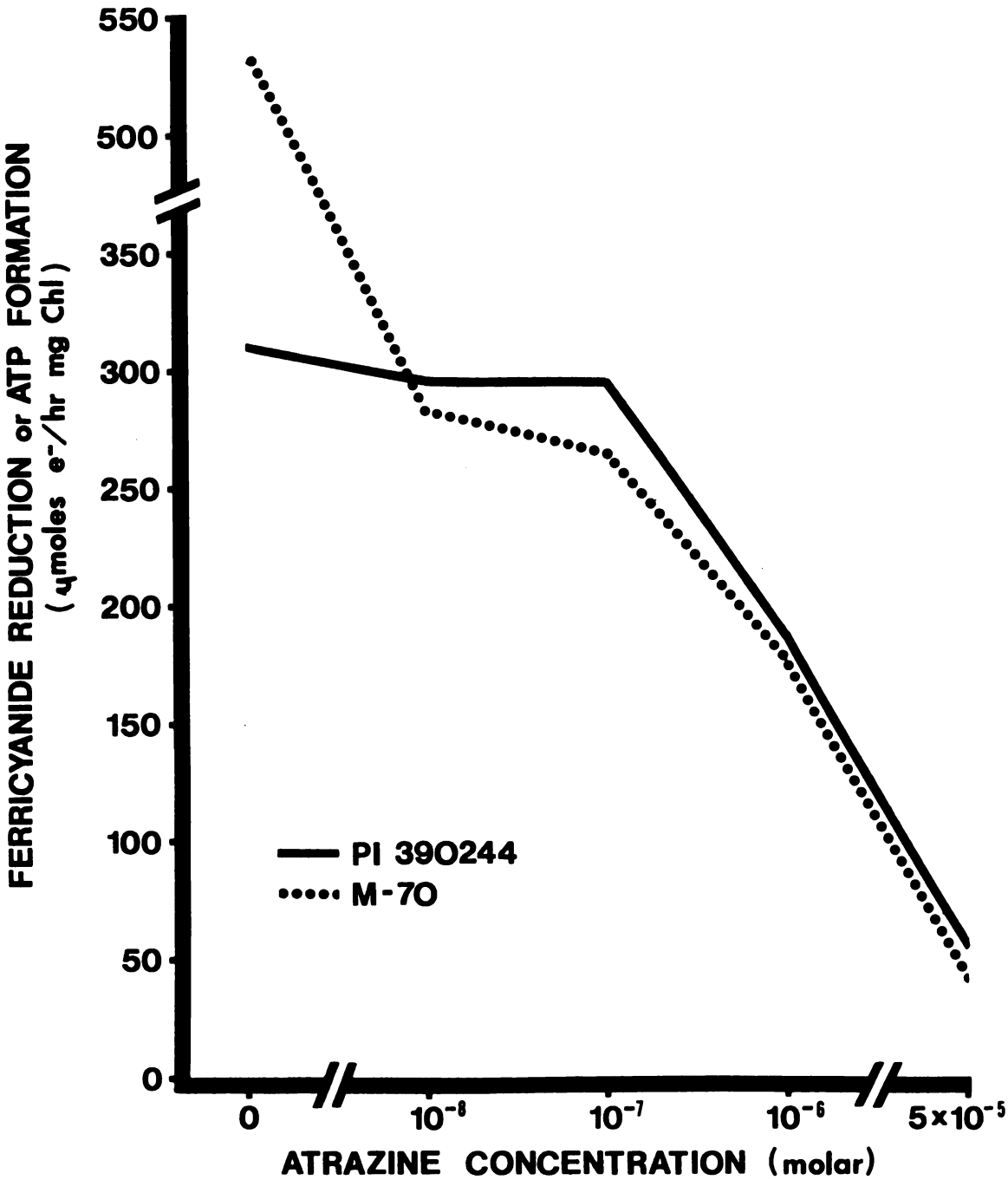

FERRICYANIDE REDUCTION or ATP FORMATION
(μmoles e⁻/hr mg Chl)
550
500
350
300
250
200
150
100
50
0
PI 390244
M-70
0
10⁻⁸
10⁻⁷
10⁻⁶
5×10⁻⁵
ATRAZINE CONCENTRATION (molar)

Figure 4. Uptake of water and ^{14}C-atrazine by atrazine-tolerant PI 390244 and atrazine-susceptible 'Marketmore 70' cucumber from 6 to 48 hours.

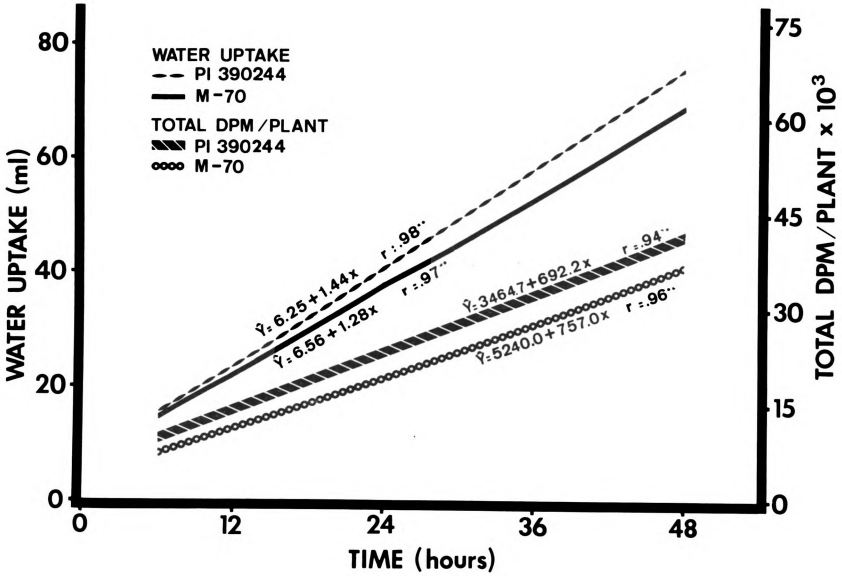
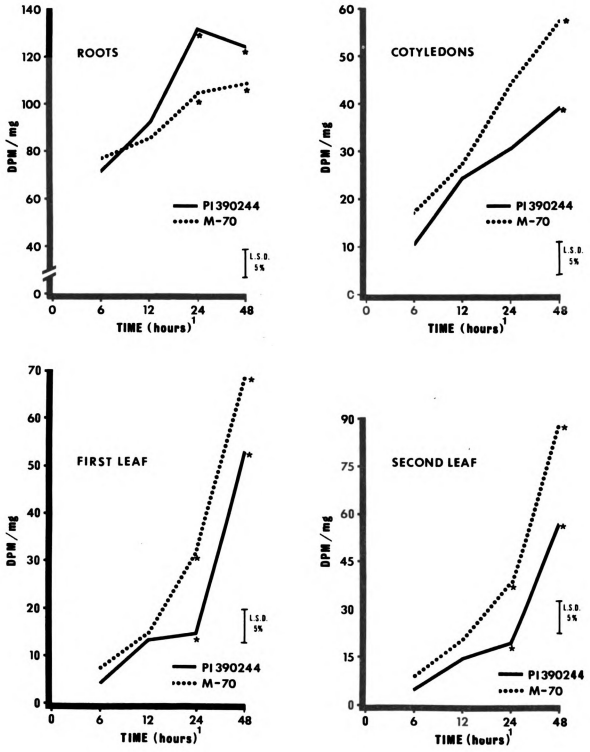

WATER UPTAKE
PI 390244
M-70
TOTAL DPM/PLANT
PI 390244
M-70
Ŷ=6.25+1.44x r=.98**
Ŷ=6.56+1.28x r=.97**
Ŷ=34647+692.2x r=.94**
Ŷ=5240.0+757.0x r=.96**
WATER UPTAKE (ml)
TOTAL DPM/PLANT x 10³
TIME (hours)
0
12
24
36
48
0
20
40
60
80
0
15
30
45
60
75

Figure 5. Accumulation of ^{14}C in a) roots; b) cotyledons; c) first leaf; and d) second leaf of atrazine-tolerant PI 390244 and atrazine-susceptible 'Marketmore 70' cucumbers.



[1] TIME DISPLAYED ON LOG SCALE

Although PI 390244 contained more total radioactivity than the 'Marketmore 70,' there were pronounced differences in the distribution of the isotope. Differences in atrazine tolerance by the two cultivars does not seem to be explained by differential uptake, but may be related to differential translocation.

The differential tolerance of plants to the s-triazines has not previously been associated with differences in uptake and translocation. Studies conducted by Thompson et al. (26) with ^{14}C-atrazine and ^{14}C-simazine on resistant and susceptible strains of redroot pigweed did not reveal differences in uptake or subsequent translocation. Jensen et al. (12) found no difference between two selections of common lambsquarters in foliar or root uptake of ^{14}C-atrazine or in translocation and accumulation of ^{14}C-atrazine within the plants following root uptake. Radosevich and Appleby (15) found no difference in uptake of ^{14}C-simazine in two biotypes of common groundsel.

A series of autoradiographs were made of the two cultivars to further elucidate translocation differences. Roots of both cultivars were uniformly labeled with the isotope (Figure 6). However, differences were noted in the foliar distribution of the ^{14}C. There appeared to be a high accumulation of radioactivity distributed uniformly throughout the leaves of the susceptible cultivar while the tolerant cultivar exhibited localization of radioactivity only in the vascular regions of the plant. Similar symptomology was seen in a greenhouse experiment using formulated atrazine (Figure 7).

Differential atrazine tolerance in cucumber may be based on several factors: (i) differences in plant morphology between the two cultivars with PI 390244 possessing less root biomass and more leaf area than the

Figure 6. Treated plants (above) and autoradiographs (below) of atrazine-susceptible 'Marketmore 70' (left) and atrazine-tolerant PI 390244 (right) 24 hours after root treatment with ^{14}C-atrazine.

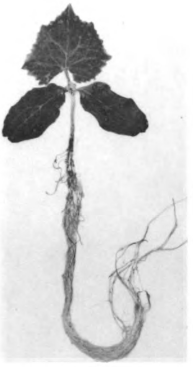


Figure 7. A plant of PI 390244 that survived 0.56 kg/ha preemergent atrazine treatment. Chlorosis is localized in the vascular system of the leaf.

more susceptible cultivar; (ii) differences in translocation of ^{14}C-atrazine; and lastly (iii) evidence from autoradiographs suggests that ^{14}C-activity in PI 390244 was more localized in the roots and xylem of the plant. Possibly, there is some type of binding of the herbicide in the xylem. Further research is needed to determine if cucumber tolerance to the triazine group is of a general nature and to determine if metabolism may also contribute to the tolerance in PI 390244. A genetic study has ascertained the inheritance of tolerance to atrazine is most likely determined by two dominant genes with epistasis (28).

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CHAPTER 4

ATRAZINE METABOLISM IN A SUSCEPTIBLE AND RELATIVELY TOLERANT CUCUMBER CULTIVAR

ABSTRACT

Metabolism of the herbicide 2-chloro-4-ethylamino-6-isopropylamino-s-triazine (atrazine) was investigated in two cultivars of cucumber (Cucumis sativus L.), PI 390244 which has been identified previously as exhibiting tolerance to the herbicide and 'Marketmore 70,' a susceptible cultivar. This study revealed no differences in the rate of metabolism or nature of metabolites between the two cultivars. The only metabolite found was 2-chloro-4-amino-6-isopropylamino-s-triazine and then in only very small quantities (5-10%). No hydroxy-atrazine or glutathione-conjugated atrazine was found. The dealkylated metabolite was found only in the shoots and not in the roots of either cultivar during the length of the experiment.

The slight metabolism and minimal differences found between the two cultivars cannot explain the differences in tolerance between the two cultivars.

INTRODUCTION

Dealkylation of atrazine occurs to some extent in all higher plants investigated (2, 4). The rate of atrazine metabolism and detoxication appears to determine the basis for resistance in corn, sorghum and peas (2). The rate of metabolism must be rapid enough to prevent the accumulation of a lethal level of atrazine in the plant, and the pathway of metabolism must result in the formation of non-phytotoxic metabolites (2, 5).

The major metabolite of atrazine in both roots and shoots of young peas, which exhibit intermediate tolerance to atrazine, was 2-chloro-4-amino-6-isopropylamino-s-triazine, a dealkylated metabolite (4). No hydroxyatrazine or glutathione conjugated atrazine was present. The dealkylated metabolite in this form is only partially detoxified and must be further dealkylated to a non-phytotoxic form (4). Shimabukuro (3) suggests this may be the basis for the intermediate tolerance exhibited by peas.

Atrazine tolerance in cucumber has recently been reported in PI 390244 obtained from the Regional Plant Introduction Station at Ames, Iowa (7). It was hypothesized that the rate of metabolism and detoxication of atrazine might be responsible for the differential response observed in cucumber.

MATERIALS AND METHODS

Cucumber seeds (PI 390244 and 'Marketmore 70,' a susceptible cultivar) were germinated in 77 ml plastic cups containing coarse grade

vermiculite and watered with half-strength Hoagland's solution (1). Two days after emergence, the seedlings were rinsed carefully with distilled water to remove the vermiculite. The seedlings were transplanted to 220 ml cups wrapped in aluminum foil which contained 180 ml nutrient solution containing 12 mM nitrate nitrogen (pH 6.5). A sponge rubber disc was used to suspend seedlings in the nutrient solution, which was changed every two days. Seedlings were placed in a growth chamber with a 16 hour photoperiod from cool white fluorescent light (300 $\mu E\ m^{-2}\ sec^{-1}$) with day and night temperatures of 31° and 21°C $\pm$ 1°C, respectively. After 11 days, the cucumber seedlings were placed in 150 ml of nutrient solution that had 0.2 μCi of uniformally ring-labeled ^{14}C-atrazine with a specific activity of 27.2 μCi/4.63 M added. All tests were initiated at the beginning of the light period. The experimental design was a split-split plot containing four replications (two seedlings/replication). Plants were harvested at 1, 3 and 5 days after treatment. At harvest, the roots were rinsed three times with distilled water. Water uptake was recorded at the time of harvest after the removal of the plants by measuring the solution remaining and subtracting the water lost by evaporation. Harvested plants were then divided into four parts: roots, stem plus cotyledons, first leaf and second leaf. These plant parts were then frozen in a dry ice and acetone bath and lyophilized. Samples were kept frozen at -25°C until analyzed. The two plant replication were combined prior to storage.

The extraction procedure followed was one furnished by the CIBA-Geigy Corporation of Greensboro, NC and is as follows.

Individual plant parts were homogenized with 15 ml chloroform for 5 minutes in a Sorvall Omni-mixer. The homogenate was filtered through

glass wool and 20 g anhydrous sodium sulfate. The filtrate was evaporated to dryness in a 50 ml round bottom flask on a rotary evaporator at 40°C. The flask was rinsed twice with 50 ml of hexane. The hexane was then decanted into a 125 ml separatory funnel. The flask was also rinsed with 25 ml of acetonitrile and was thus combined with the hexane and swirled for 1 minute. The acetonitrile was decanted into a second 125 ml separatory funnel. The hexane fraction was rinsed with 25 ml acetonitrile for 1 minute and the acetonitrile fractions then were combined. To the separatory funnel containing the acetonitrile, 50 ml hexane was added. The acetonitrile fraction was decanted into a 250 ml round bottom flask. The filtrate was evaporated to dryness. To flasks containing root extractions, 2 aliquots of 10 ml methanol were added. Two aliquots of 10 ml carbon tetrachloride were added to flasks containing chlorophyll. The methanol and carbon tetrachloride extracts were then decanted into vials for storage purposes until analyzed. All samples were stored at -15°C.

Before assaying, all samples were evaporated to dryness under a stream of nitrogen and brought up to volume of 1 ml with methanol. A 50 µl sample was streaked on a 0.250 mm Silica Gel G thin-layer chromatography plate. The plates were developed 15 cm in benzene:acetic acid (50:4 v/v) (Solvent A). The silica gel was scraped at 1 cm intervals and assayed for radioactivity using 15 ml of a 4 g PPO (2,5-diphenyloxazole) plus 50 mg POPOP (1,4-bis[2-(4-methyl-5-phenyloxazoly)]-benzene in 1 liter toluene. A second 50 µl was streaked on a similar plate and developed in benzene:acetic acid:water (50:50:3 v/v) (Solvent B). Standards of ^{14}C-atrazine and the dealkylated metabolite 2-chloro-4-amino-6-isopropylamino-*s*-triazine were also streaked on each plate. The Rf

values of atrazine and the dealkylated metabolite were 0.40 and 0.26, respectively for Solvent A and 0.98 and 0.96 for Solvent B. Rf values of the unknown spots were compared with the standards. The atrazine and the nonlabeled metabolite were detected by viewing the chromatogram under ultraviolet light. Rf values of the unknown spots were compared with the standards. Data were converted to percent (%) unchanged atrazine for direct comparisons. The experiment was conducted twice and significance of the means was determined using the LSD test at the 1% level (6).

RESULTS AND DISCUSSION

Thin-layer chromatographic analysis of chloroform-soluble compounds in the plant extracts showed that only dealkylation occurred in cucumbers. There were no differences in the rate of metabolism or dealkylated metabolites between the two cultivars. The only metabolite found was 2-chloro-4-amino-6-isopropylamino-s-triazine, and then in only very small quantities (5-10%). No hydroxyatrazine or glutathione conjugated atrazine were found.

There was a significant difference (1% level) in the amount of unchanged atrazine only in the first leaf between the two cultivars. In general, 'Marketmore 70' appeared to metabolize more ^{14}C-atrazine although the differences were not significant (Figure 1).

The dealkylated metabolite was not found in the roots of either cultivar during the length of the experiment. The metabolite was found in the other plant parts of both cultivars (Figure 2).

Figure 1. Interaction of cultivar and plant part on the amount of unchanged ^{14}C-atrazine.

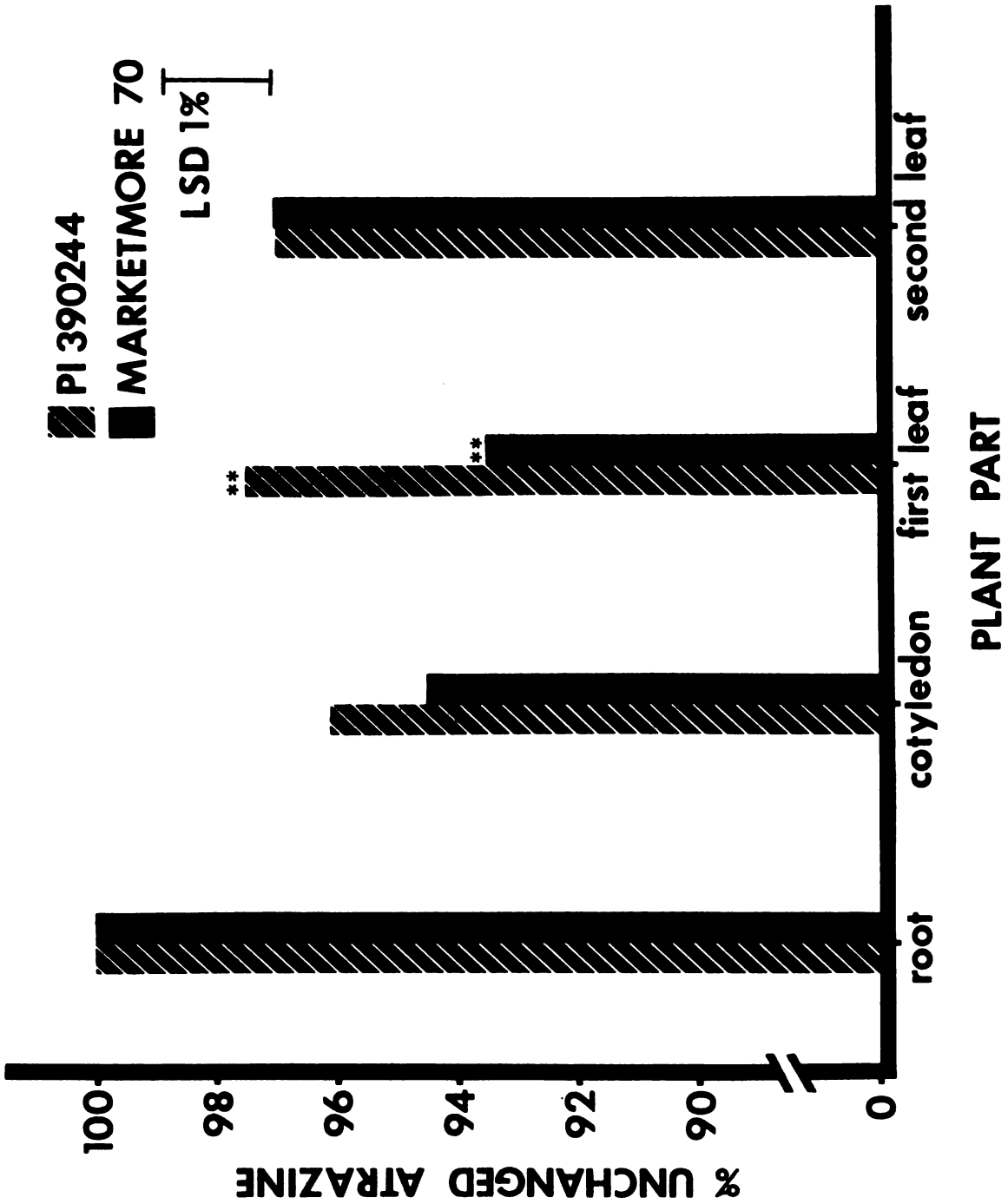
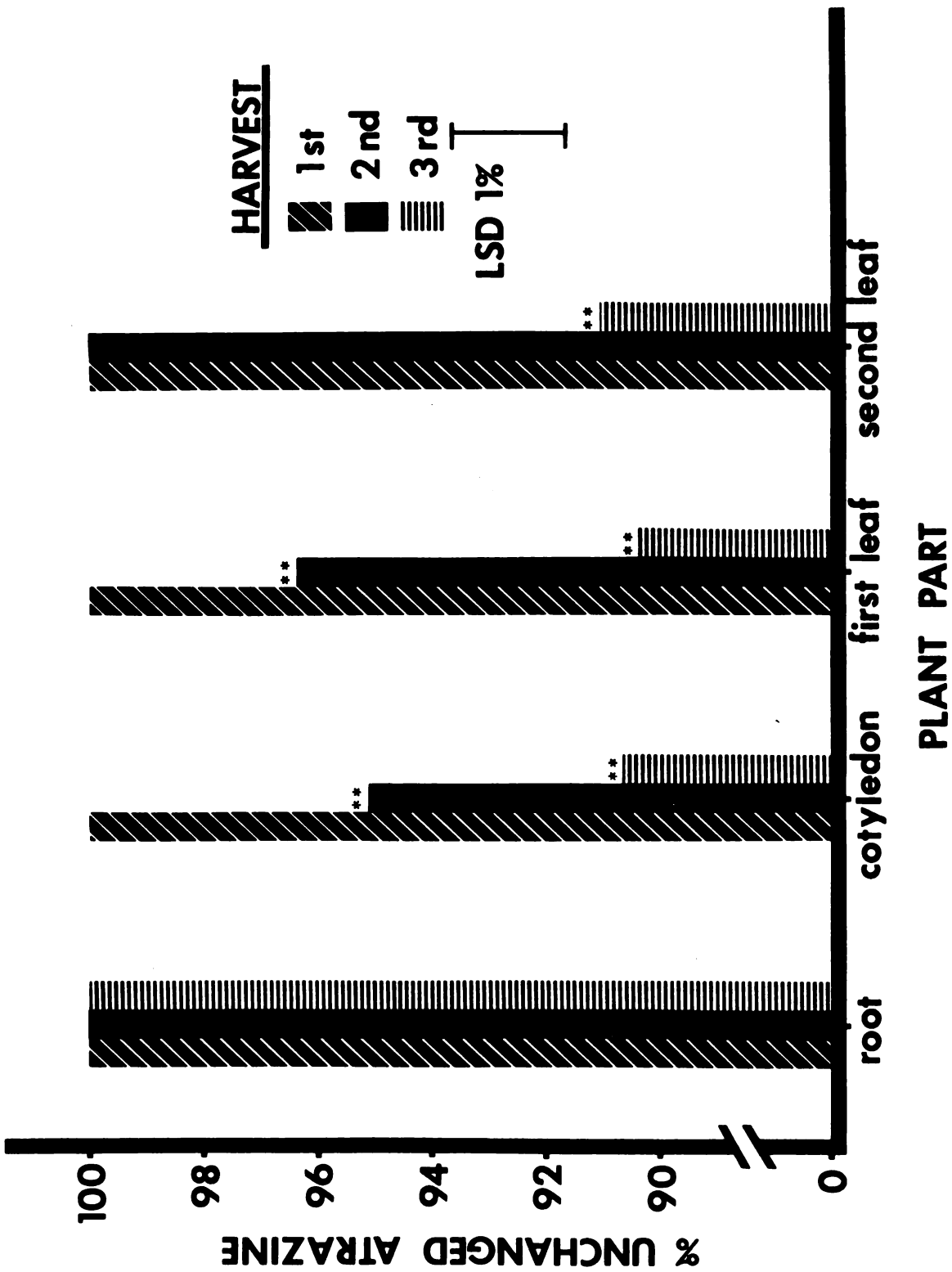

PI 390244
MARKETMORE 70
LSD 1%
**
**
% UNCHANGED ATRAZINE
100
98
96
94
92
90
0
root
cotyledon
first leaf
second leaf
PLANT PART

Figure 2. Interaction of harvest date and plant part on the amount of unchanged ^{14}C-atrazine.


% UNCHANGED ATRAZINE
100
98
96
94
92
90
0
HARVEST
1st
2nd
3rd
LSD 1%
**
root
cotyledon
first leaf
second leaf
PLANT PART

There was very little metabolism of the atrazine by either cultivar during the 5 day period. At five days after treatment the amount of unchanged atrazine was 90%. The slight metabolism and minimal differences found between the two cultivars cannot explain differences in tolerance between the two cultivars. Shimabukuro et al. (2, 4) have stated that slight metabolism occurs in all higher plants investigated, even plants that are considered susceptible to the s-triazines. Another mechanism is more likely responsible for the differential tolerance exhibited by PI 390244. Autoradiograms of both cultivars (treated with ^{14}C-atrazine) revealed that PI 390244 appeared to restrict the movement of atrazine to the vascular system of the plant, thus preventing the movement of the herbicide into the leaves of the plant while 'Marketmore 70,' the susceptible cultivar, had a uniform distribution of the radioactive material throughout the leaves and root system of the plant (8).

The basis for differential tolerance in cucumber is not due to differences in the rate of metabolism or differences in metabolites formed. Slight metabolism did occur at 3 days (95% unchanged atrazine) and continued at 5 days with only 10% of the total as the dealkylated metabolite.

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CHAPTER 5

INHERITANCE OF 2-CHLORO-4-ETHYLAMINO-6-ISOPROPYLAMINO-*S*-TRIAZINE (ATRAZINE) TOLERANCE IN CUCUMBER (*CUCUMIS SATIVUS*)

ABSTRACT

Genetic control of atrazine tolerance appears to be determined by two genes. Analysis of the F_2 population from the cross of PI 390244 (tolerant) and 'Gynoecious 14' (susceptible) suggested that two dominant epistatic genes conditioned atrazine tolerance in cucumber. Variability existed in some of the F_2, BC_1, and BC_2 data. A possible explanation could be that the Plant Introduction was not completely homozygous for the trait or that there may be different levels of gene penetrance or a combination of the two. Penetrance was incomplete in some cases and variable from cross to cross.

INTRODUCTION

Many crop species normally regarded as susceptible to the *s*-triazines may contain significant intraspecific variability for tolerance to these compounds. Genetic variability for *s*-triazine tolerance has been observed recently in soybeans (*Glycine max* Merr. L.) (1), flax (*Linum usitatissimum* L.) (2), wheat (*Triticum aestivum* L.) (3), rape (*Brassica napus* L.) (5), mustard (*Sinapis alba* L.) (3) and cucumber

(Cucumis sativus L.) (6, 7) which are normally considered extremely sensitive.

The genetic investigation of triazine resistance in bird's rape conducted by Souza Machado et al. (5) indicates that resistance is inherited uniparentally, through the female parent. Triazine tolerance in flax was reported to be quantitatively inherited (2).

The purpose of this investigation was to determine the genetic inheritance of atrazine tolerance in cucumber.

MATERIALS AND METHODS

The mode of inheritance of the triazine tolerant character was examined by making crosses with cucumbers that were previously selfed for three generations and screened for homozygosity for atrazine tolerance. PI 390244, the tolerant parent, was crossed with the susceptible 'Gynoecious 14.' Throughout the course of this study the Plant Introduction was maintained by vegetative propagation. Appropriate F_1 hybrids were selfed to obtain F_2 progeny, and also backcrossed as pollen parents to 'Gynoecious 14.' Additional F_1's were used as maternal parents with PI 390244 as the pollen parent. All seeds were sown May 21, 1979 at Castle Hayne, NC on a Portsmith sandy loam with an organic matter content of 3.2 to 4.4%. A randomized complete block design with six replicates was used. Atrazine was applied preemergence at 0.56 kg/ha in a spray volume of 333 l/ha over the entire planting. Parental F_1, F_2 and backcross seedlings were evaluated at three weeks after germination. The seedlings were classified in two phenotypic classes: alive or dead. Family data have been derived from individual F_1 plants which have been selfed. Individual crosses were maintained separately.

RESULTS AND DISCUSSION

Segregation ratios obtained for the individual F_2 populations are found in Table 1. F_2 families of the reciprocal crosses which manifested 9:7 ratios did not deviate significantly from the ratio tested thus suggesting that two dominant genes with epistasis conditioned this factor.

Segregation occurred in both F_1 and F_2 seedlings for atrazine tolerance. The results are shown in Table 1. Segregation ratios obtained for atrazine tolerance in the F_1 population did not deviate significantly from a 1:1 ratio suggesting that possibly one parent was not completely homozygous. When the Plant Introduction was first identified as being atrazine tolerant, the percent survival to 0.56 kg/ha atrazine was 78% (8). The survivors were selfed and maintained individually for three generations and repeatedly tested for atrazine tolerance. The most tolerant and most homozygous line was selected for this study. After the third generation some variability still existed.

Some incongruent data was obtained from the F_2 populations which is presented in Table 2. The cross of PI 390244 x 'Gynoecious 14' for three of the F_2 families tested had a higher total number of survivors at three weeks. They did not fit a 9:7 ratio as did the other crosses but more closely fit a 3:1 ratio. Reciprocal differences were also observed. These F_2 families most closely fit 7:9 ratios, since increased mortality reversed the ratio of the phenotypic classes.

The backcross data is presented in Table 3. The data obtained was also variable but for the most part fit a 1:1 ratio with the exception of one cross of ('Gynoecious 14' x PI 390244) x PI 390244 which fir a 3:1 ratio. A possible explanation for the variability of the data for this

Table 1. Segregation and goodness of fit for tolerance and susceptibility to atrazine of F_1 and F_2 seedlings.

Cross	Ratio	X^2	P	Ratio Tested
'Gy 14' x PI 390244	35:25	0.83	0.40-0.30	1:1
PI 390244 x 'Gy 14'	36:24	1.20	0.40-0.30	1:1
'Gy 14' x PI 390244 family	F_2 data			
1.	88:62	0.17	0.70-0.60	9:7
2.	82:68	0.07	0.80-0.90	
3.	83:67	0.02	0.90-0.80	
PI 390244 x 'Gy 14' family	F_2 data			
1.	86:64	0.05	0.80-0.70	9:7
2.	81:69	0.21	0.70-0.60	
3.	92:58	0.78	0.40-0.30	

Table 2. Incongruent data obtained from the F_2 populations of the cross PI 390244 x 'Gy 14' and 'Gy 14' x PI 390244.

Cross	Ratio	X^2	P	Ratio Tested
PI 390244 x 'Gy 14' family				
1.	100:50	2.08	0.30-0.20	3:1
2.	114:36	0.03	0.80-0.90	
3.	106:44	0.56	0.50-0.40	
'Gy 14' x PI 390244 family				
1.	69:81	0.14	0.70-0.60	7:9
2.	59:91	0.58	0.50-0.40	

Table 3. Segregation and goodness of fit for tolerance and susceptibility to atrazine of backcross seedlings.

Cross	Ratio	X^2	P	Ratio Tested
'Gy 14' x ('Gy 14' x PI 390244)				
1.	52:68	1.06	0.40-0.30	1:1
2.	33:27	0.30	0.60-0.50	
('Gy 14' x PI 390244) x PI 390244				
1.	93:27	0.15	0.70-0.60	3:1
2.	67:53	0.81	0.40-0.30	1:1
'Gy 14' x (PI 390244 x 'Gy 14')				
1.	66:54	0.60	0.50-0.40	1:1
2.	52:68	1.06	0.40-0.30	
3.	62:58	0.06	0.80-0.70	

experiment may be due to different levels of gene penetrance. Penetrance, in this instance was incomplete and variable from cross to cross. Another factor that may have confounded the experiment and the results might have been the variation in organic matter content of the soil used for this study. In an evaluation of PI 390244 versus 'Marketmore 70,' to determine the level of atrazine tolerance in nutrient culture, the Plant Introduction's response was very uniform and no differences were noted among the plants utilized for the study (7). The presence of varying organic matter content, even though no replication differences were noted, may have contributed to the variability expressed. By selection for the proper background genotypes, completely penetrant lines could be developed.

Since the atrazine tolerant trait appears to be dominant, it should not be difficult to incorporate this trait into existing cucumber cultivars. The development of cultivars that possess limited tolerance to the s-triazines would be advantageous in allowing use of low rates for chemical weed control, or the planting of cucumbers in crop rotation systems where a carry-over problem might exist due to the persistance of these herbicides.

Cucumbers have normally been considered so sensitive to the s-triazines that they are used as indicators in bioassays to determine soil persistance. This study demonstrates the importance of selecting cultivars that have been evaluated for triazine susceptibility prior to their use in bioassays.

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