

## CHAPTER 4

### Resistance to Aryloxyphenoxypropionate Herbicides in Virginia Biotypes of Italian Ryegrass (*Lolium multiflorum*)

I. V. Morozov, E. S. Hagood, H. P. Wilson, P. L. Hipkins, and K. W. Bradley.<sup>1</sup>

**Abstract.** Acetyl-coenzyme A carboxylase (ACCase) assays, and herbicide absorption, translocation, and metabolism studies were conducted to investigate potential mechanism(s) of resistance to the aryloxyphenoxypropionate (APP) herbicides diclofop and quizalofop-P in Italian ryegrass. [<sup>14</sup>C]Quizalofop-P absorption, translocation, and metabolism did not differ between resistant and susceptible Italian ryegrass biotypes. Likewise, Italian ryegrass ACCase activity and percentage inhibition of Italian ryegrass ACCase by diclofop also did not differ between the two biotypes assayed. Results indicate neither herbicide absorption, translocation, and metabolism, nor ACCase sensitivity are responsible for whole-plant resistance.

**Nomenclature:** Acetyl CoA carboxylase [acetyl-CoA-carbon dioxide ligase (ADP forming)], E.C. 6.4.1.2; Diclofop, methyl ester of (±)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid; Quizalofop-P, ethyl ester of (±)-2-[4-[(6-chloro-2-quinoxalinyloxy)phenoxy]propanoic acid; Italian ryegrass, *Lolium multiflorum* Lam. #<sup>2</sup> LOLMU.

**Additional index words:** Absorption, ACCase assay, metabolism, translocation.

---

<sup>1</sup> Graduate Research Assistant (imorozov@vt.edu); Professor; Professor; Senior Research Associate; Research Associate; Dept. Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 – 0331; Assistant Professor, Dept. Agronomy, University of Missouri-Columbia, Columbia, MO 65211.

<sup>2</sup> Letters following this symbol are WSSA-approved computer code from *Composite List of Weeds*, Revised 1989. Available from WSSA, 1508 West University Ave., Champaign, IL 61821 – 3133.

**Abbreviations:** ACCase, acetyl-coenzyme A carboxylase; AC, Amelia County, Virginia; APP, aryloxyphenoxypropionate; DAP, days after planting; DAT, days after treatment; LSD, least significant difference; LSS, liquid scintillation spectrometry; NHC, Northampton County, Virginia; PROC-GLM, general linear models procedure; POST, postemergence; TL, treated leaf; TLC, thin-layer chromatography.

## INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam.), a competitive weed in small grains especially in the northwestern and southeastern United States, affects crop yields, contaminates grain, and interferes with harvest (Appleby and Brewster, 1992; Mersie and Foy, 1985; Stone et al., 1998). Since the early 1980s, the selective control of Italian ryegrass in small grains has been achieved through postemergence applications of diclofop, or diclofop-methyl, a member of the aryloxyphenoxypropionic (APP) family of herbicides (Devine et al., 1993). Several modes of action have been suggested for APP herbicides including inhibition of fatty acid synthetase (Boldt and Barrett, 1991), alteration of cell membrane composition leading to changes in unsaturated fatty acids (Banas et al., 1993; Ruizzo and Gorski, 1988), and inhibition of acetyl CoA carboxylase (ACCcase) (De Prado et al., 2000; Devine and Shimabukuro, 1994; Egli et al., 1993; Gronwald et al., 1992; Somers, 1996).

ACCcase is a multifunctional, biotinylated enzyme that catalyzes the synthesis of malonyl-CoA in the first committed step of the *de novo* fatty acid biosynthesis (Harwood, 1988; Schmid et al., 1997). The enzyme functions in a three-step sequence catalyzing partial reactions involving a kinetic mechanism of carboxybiotin. In an ATP-dependent reaction, ACCcase initiates biotin carboxylation using CO<sub>2</sub> from bicarbonate, followed by the transfer of activated carbon to the

carboxyltransferase site of the biotin, and then from biotin to acetyl-CoA to form malonyl-CoA (Somerville et al., 2000).

Almost all monocot and dicot plants have two isoforms of ACCase: the herbicide-insensitive, heteromeric form in the plastid and the herbicide-sensitive, homodimeric form in cytosol. As an exception, plants from the grass family (Poaceae) were found to have similar homodimeric (herbicide-sensitive) ACCase isoforms in plastids as well as in cytosol. This unique trait had been shown to be the basis for selectivity for APP herbicides in susceptible plant species (Gronwald et al., 1992; Herbert et al., 1997). This herbicide-enzyme relationship is highly specific and any chemical modification of the herbicide or the enzyme can eliminate herbicidal activity.

The selectivity and excellent efficacy of diclofop and other APP herbicides on many grass weeds has led to extensive use of these herbicides. Applied postemergence at 500 to 1500 g/ha, diclofop controlled susceptible Italian ryegrass 80 to 100% and increased wheat yields 20 to 60% (Griffin, 1986, Khodayari et al., 1983, Shaw and Wesley, 1991).

As with a number of other herbicides, the widespread and repeated use of the product alone and in combination with other herbicides targeting the same site of action, has led to the development of herbicide resistance in weed populations. The first occurrence of diclofop-resistant Italian ryegrass was reported in 1987 (Stanger and Appleby, 1989). Currently, diclofop-resistance in Italian ryegrass had been documented in many states throughout the US and worldwide and is rapidly becoming a problem in many agroecosystems (Heap, 1997).

In Virginia, the first diclofop-resistant biotype was reported in 1995 (Hagood, 2004<sup>3</sup>). Subsequently, there has been a significant decline in grower's satisfaction with the diclofop efficacy for Italian ryegrass control. In 1998, greenhouse tests with several ryegrass biotypes

---

<sup>3</sup> Hagood, Edward, S., Jr., 2004. Professor, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA. Personal Communication.

collected from various locations in Virginia were conducted. Diclofop resistance in Italian ryegrass varied among the biotypes and across the range of application rates (Morozov et al., 1999).

Several biochemical mechanisms have been suggested to explain resistance to APP herbicides: (1) increased herbicide metabolism (Hall et al., 1997; Hidayat and Preston, 1997; Menendez and De Prado, 1996); (2) repolarization of the plasma membrane (Devine and Shimabukuro, 1994; DiTomaso, 1993; Wright, 1994); (3) overproduction of the target enzyme (Bradley et al., 2001); and (4) resistance based on reduced ACCase sensitivity (De Prado et al., 2000; Devine and Shimabukuro, 1994; Egli et al., 1993; Gronwald et al., 1992). While some of the mechanisms of resistance mentioned are still subject to scientific debate, in most cases diclofop resistance has been attributed to the presence of insensitive diclofop-resistant ACCase (Devine and Shimabukuro, 1994; Evenson et al., 1994; Evenson et al., 1997; Gronwald et al., 1992).

Although mutations and cross-resistance patterns may vary among the biotypes, the development of resistance appears to be due to changes in the same gene locus, which could be encoded by a single, functionally dominant, nuclear gene (Murray et al., 1996).

The objective of this study was to determine the mechanism(s) responsible for resistance to APP herbicides in Virginia biotypes of Italian ryegrass. To achieve this objective, (1) the activity of ACCase was quantified in the presence and absence of diclofop in resistant and susceptible biotypes, and (2) resistant and susceptible biotypes were compared with regard to absorption, translocation and metabolism of quizalofop-P ethyl.

## **MATERIALS AND METHODS**

**Plant Material.** In the summer of 1998 and 1999, Italian ryegrass seed were collected from two different locations, Amelia County (AC) and Northampton County (NHC) in Virginia. The AC

biotype was collected from a field in which diclofop at the label-recommended use-rate did not control Italian ryegrass (Morozov et al., 1999). The NHC biotype was collected from a field with no known prior history of diclofop applications. For the herbicide dose-plant response experiment and the ACCase assay, seeds of each biotype were sown in 10-cm tall plastic pots containing soil substitute<sup>4</sup> with approximately 15 seeds per pot. Plants were grown in the greenhouse and watered as needed until used in the experiments. For herbicide absorption, translocation, and metabolism, Italian ryegrass seedlings of each biotype were grown separately in the greenhouse in 26- by 18-cm greenhouse trays filled with vermiculite soil substitute<sup>5</sup> and watered as needed. For all experiments the average air temperature was maintained at 24°C.

**Herbicide Dose-Plant Response Experiment.** Two weeks after planting, two- to three-leaf leaf seedlings were treated with seven rates of the commercial formulation of diclofop. Herbicide treatments were applied using a stationary track sprayer<sup>6</sup> containing a single 8001 EVS flat fan nozzle tip<sup>7</sup> delivering 234 L/ha of spray solution at 269 kPa. Application rates were 0, 277, 553, 1106, 2212, 4424, and 8848 g a.i./ha, with 553 g a.i. being the label-recommended use rate. The experimental design was a randomized complete block with a factorial arrangement of biotypes and herbicides rates. Each treatment in the experiment was replicated four times and the experiment was repeated once.

**ACCcase Extraction and Assay.** Enzyme extraction and purification operations were performed according to Bradley et al. (2001) with slight modifications. For both biotypes, ACCcase was extracted and assayed in the same buffers, and all procedures were conducted at 4°C.

---

<sup>4</sup> 'General Purpose' Pro Mix BX peat-based growing medium. Premier Horticulture Inc., Red Hill, PA 18076.

<sup>5</sup> Vermiculite sterile growing media (medium grade), The Schundler Company, P.O. Box 513, Metuchen, NJ 08840-0513.

<sup>6</sup> Stationary Track Sprayer. Allen Machine Works. 607 E. Miller Road, Midland, MI 48640..

<sup>7</sup> Spraying Systems Co.®, P. O. Box 7900, Wheaton, IL 60189.

Three g of fresh leaf tissue were harvested from two- to three-leaf seedlings of each Italian ryegrass biotype. Collected tissue was immediately submerged in liquid nitrogen and ground to a powder with a mortar and pestle. Ground powder was extracted with 15 ml buffer (100 mM Tris [pH 8.0]; 1mM ethylenediaminetetraacetic acid [EDTA]; 10 % [v/v] glycerol; 2 mM isoascorbic acid, 0.5% insoluble polyvinylpyrrolidone [PVP], 0.5% [wt/v] PVP-40, 20 mM dithiothreitol [DTT], 0.2 mM phenylmethanesulfonyl fluoride [PMSF]). The extract was filtered through two layers of Miracloth®<sup>8</sup> and centrifuged (27.000 x g, 15 min)<sup>9</sup>. To obtain a partially purified enzyme, the supernatant was gradually brought to 40% ammonium sulfate saturation while being stirred for 30 min at 4°C, and then centrifuged (27.000 x g, 30 min). The pellet was separated from the liquid phase and re-suspended in one ml of elution buffer (50 mM Tricine [pH 8.0]; 2.5 mM MgCl<sub>2</sub>\*6H<sub>2</sub>O; 50mM KCl; and 1 mM DTT). Five ml of the same elution buffer were used to equilibrate Sephadex® G-25 columns<sup>10</sup>. For each biotype, the protein extract was desalted on a Sephadex® G-25 column, eluted, stored on ice, and immediately assayed for total protein content.

The total protein content in the enzyme extract was determined according to Bradford (1976). For each biotype, protein concentration in the enzyme elution was determined in a microassay based on the principle of protein-dye binding using bovine serum albumin as a standard and the Coomassie® Protein Assay Reagent<sup>11</sup> as a dye.

ACCase activity was quantified by measuring the rate of incorporation of <sup>14</sup>C from NaH<sup>14</sup>CO<sub>3</sub><sup>12</sup> into the partially purified enzyme extracts. Aliquots of the partially purified enzyme extracts were incubated at 32 C in an assay buffer (20 mM Tricine-KOH [pH 8.3], 10 mM KCl, 5 mM

---

<sup>8</sup> MiraCloth® filtration material. EMD Biosciences, Inc. 10394 Pacific Center Court. San Diego, CA 92121.

<sup>9</sup> Sorvall RC-58 refrigerated superspeed centrifuge with SS-34 rotor, Sorvall-Kendro Laboratory Products, L.P., 31 Pecks Lane, Newtown, CT 06470-2337.

<sup>10</sup> Sephadex® G-25 column (PD-10, Pharmacia Biotech, Inc. product). Supelco, Inc. Supelco Park, Bellefonte, PA 16823-0048.

<sup>11</sup> Coomassie® Protein Assay Reagent Kit. Pierce Biotechnology, Inc. 3747 N. Meridian Rd., P.O. Box 117, Rockford, IL 61105.

<sup>12</sup> NaH<sup>14</sup>CO<sub>3</sub> radioactive isotope. American Radiolabeled Chemicals, Inc. 11624 Bowling Green, St. Louis, MO 63146-3506.

ATP, 2 mM MgCl<sub>2</sub>, 0.2 [w/v] BSA, 2.5 mM DTT, 3.7 mM NaHCO<sub>3</sub> [including 0.185 MBq of NaH<sup>14</sup>CO<sub>3</sub>]), and herbicide or double-distilled water. For herbicide solutions, diclofop (95% pure, analytical grade)<sup>13</sup> was dissolved in acetone and diluted with double-distilled water to yield final assay concentrations of 0.01, 0.1, 1, 10, and 100 µM. Double-distilled water instead of the herbicide was used in the control samples.

The reaction was initiated with the addition of 5 µl acetyl coenzyme A<sup>14</sup> (acetyl-CoA), followed by a 10 min. incubation period, and stopped with the addition of 20 µl of concentrated HCl. A 110 µl aliquot of assay solution was transferred to a 2.2-cm filter paper disc<sup>15</sup>, which was then placed in liquid scintillation vials and dried in a fume hood. Scintillation cocktail<sup>16</sup> (10 ml) was added, and radioactivity for acid- and heat-stable products was quantified by liquid scintillation spectrometry<sup>17</sup> (LSS). Non-enzymatic <sup>14</sup>C fixation was assessed in vials where acetyl-CoA was substituted with assay buffer. Each biotype was assayed nine times with two subsamples per herbicide concentration.

**Herbicide Absorption and Translocation.** The absorption and translocation experiments were performed according to Bradley et al. (2001) with slight modifications. At the two- to three-leaf stage, seedlings of each biotype were removed from vermiculite, their roots washed in water, and transplanted into glass jars covered with aluminum foil and filled with 100 ml of Hoagland's nutrient solution (1/4 strength, pH 6.3). To reduce the effect of stress from transplanting, a four to five-day acclimation period was allowed prior to herbicide application. At the four-leaf stage, hydroponically-grown Italian ryegrass seedlings were sprayed with 0.38 kg/ha of quizalofop-P, a

---

<sup>13</sup> Diclofop-methyl. Bayer CropScience LP. P.O. Box 12014, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709.

<sup>14</sup> Acetyl Coenzyme A, trilithium salt (C2:0, 95%). Sigma-Aldrich. 3050 Spruce Street., St. Louis, MO 63103.

<sup>15</sup> Whatman® binder-free glass microfiber filter paper (Type GF/A). Fisher Scientific Co. P.O. Box 1768, Pittsburgh, PA 15230.

<sup>16</sup> ScintiVerse ® BD. Fisher Scientific, 1 Reagent Lane, Fair Lawn, NJ 07410.

<sup>17</sup> Liquid scintillation counter, Beckman LS 5000TA Model. Beckman Instruments, 4300 N. Harbor Blvd., Fullerton, CA 92634.

sub-lethal dose that constitutes approximately 50% of the field use rate. The herbicide application was made using a stationary track sprayer<sup>18</sup> containing a single 8001 EVS flat fan nozzle tip<sup>19</sup> delivering 234 L/ha at 269 kPa. Approximately 3 h after spraying, 10 µl of [<sup>14</sup>C]quizalofop-ethyl<sup>20</sup> containing approximate radioactivity of 3,700 Bq/µl (0.01 µmol) was spotted on the second leaf of each of the AC and NHC Italian ryegrass seedlings. Prior to the application, the [<sup>14</sup>C]quizalofop-ethyl was diluted in 5% acetone in an aqueous solution containing 0.5% (v/v) Tween 20<sup>21</sup>. For each biotype, twelve seedlings were spotted, with four of the seedlings randomly selected for harvest at 6, 24, 48 and 72 HAT with three replications per harvest timing. At each harvest, seedlings were divided into treated leaf, the remaining shoot and leaves, and roots. Each of the treated leaves was agitated for 30 s in a 10 ml wash solution (10% ethanol and 0.1% Tween 20) to remove unabsorbed [<sup>14</sup>C]quizalofop-ethyl. A 1 ml aliquot of each leaf rinse was added to 10 ml of scintillation cocktail<sup>22</sup>, and the total unabsorbed radioactivity was quantified through liquid scintillation spectrometry<sup>23</sup> (LSS). The remaining harvested tissues were placed in oxidizer cups, covered with filter paper, dried for 72 h at 60°C and combusted separately in a biological sample oxidizer<sup>24</sup>. The total radioactivity in the samples was determined by the LSS. The absorbed radioactivity was expressed as a percentage of the recovered radioactivity, whereby the count of <sup>14</sup>C recovered from the corresponding plant section was divided by the total LSS counts from <sup>14</sup>C from leaf wash and oxidized plant sections. The translocation of [<sup>14</sup>C]quizalofop-ethyl was expressed as a percent of absorbed radioactivity and

---

<sup>18</sup> Stationary Track Sprayer – Allen Machine Works, 607 E. Miller Road, Midland, MI 48640.

<sup>19</sup> Spraying Systems Co.®, P. O. Box 7900, Wheaton, IL 60189.

<sup>20</sup> Quizalofop-P. DuPont Agricultural Products, 1007 Market St., Wilmington, DE 19898.

<sup>21</sup> Polysorbate 20 [polyoxyethylene (20) sorbitan monolaurate], ICI America, Inc., Wilmington, DE 19899

<sup>22</sup> ScintiVerse ® BD, Fisher Scientific, Fair Lawn, NJ 07410.

<sup>23</sup> Liquid scintillation counter, Beckman LS 5000TA Model, Beckman Instruments, 4300 N. Harbor Blvd., Fullerton, CA 92634.

<sup>24</sup> Biological Oxidizer Model 307, Packard Instrument Co., 2200 Warrenville Road, Downers Grove, IL 60515.

was calculated by dividing the LSS count of  $^{14}\text{C}$  recovered in a corresponding plant section by the total LSS counts of  $^{14}\text{C}$  recovered from all oxidized plant tissues of that sample.

**Herbicide Metabolism.** Treated tissues of each biotype were prepared and harvested as described above in the herbicide absorption and translocation experiment. Based on the data from the absorption and translocation studies, only leaves that were spotted with radiolabeled quizalofop-P were used in the herbicide metabolism analyses. These leaves accounted for approximately 84% of the total radioactivity recovered.

Following leaf rinse, the excised treated leaves (TL) of twelve seedlings of each biotype were stored at  $-20^{\circ}\text{C}$  before used in the experiments. Each TL was homogenized in liquid nitrogen and extracted in 5 ml of acetone and water (4:1, v/v), and centrifuged for 10 min at  $13,000 \times g$ <sup>25</sup>. The supernatant was removed and the extraction procedure was repeated two more times. The supernatant from the three extractions was pooled, and then evaporated to dryness under  $\text{N}_2$  on a sample evaporator<sup>26</sup> and re-dissolved in 200  $\mu\text{l}$  of absolute ethanol. One hundred microliters of each TL extract were then spotted on silica gel thin-layer chromatography (TLC) plates<sup>27</sup> in addition to 5  $\mu\text{l}$  of the [ $^{14}\text{C}$ ]quizalofop-ethyl standard. Prior to spotting, each TLC plate was baked for one hour at  $110^{\circ}\text{C}$ . Each spotted TLC plate was developed in a solvent system comprised of toluene, acetonitrile, methanol, glacial acetic acid and double-distilled water (40:25:15:10:15, v/v) according to Ruizzo and Gorski (1988). Each developed TLC plate was dried for 5 minutes at  $30^{\circ}\text{C}$  and analyzed with a radiochromatogram scanner<sup>28</sup> to determine

---

<sup>25</sup> Sorvall RC-58 refrigerated superspeed centrifuge with SS-34 rotor, Sorvall-Kendro Laboratory Products, L.P., 31 Pecks Lane, Newtown, CT 06470-2337.

<sup>26</sup> Meyer N-EVAP 111 analytical evaporator, Organomation Associates, Inc., P.O. Box 5, Tpk. Sta., Shrewsbury, MA 01545.

<sup>27</sup> Silica gel 60F<sub>254</sub> pre-coated glass plates for thin layer chromatography, EM Science, 480 Democrat Road, Gibbstown, NJ 08027.

<sup>28</sup> BioScan System 200 Imaging Scanner with BioScan Autochanger 1000, BioScan Inc., 4590 MacArthur Blvd. NW, Washington, D.C. 20007.

radioactive positions of the metabolite(s) and parent compound. WinScan® software<sup>29</sup> was used to calculate peak areas with equation parameters set to 13-pt cubic smoothing and rejection of peaks that were below 1.5% of total radioactivity. Parent material was identified as the peak observed in the [<sup>14</sup>C]quizalofop-ethyl standard. Metabolites were separated by their R<sub>f</sub> values and the distribution of radioactivity recovered in each metabolite fraction was expressed as a percentage of the absorbed radioactivity in the TLC analysis. The herbicide metabolism experiment was repeated once with three replications per harvest timing per experiment.

**Experimental Design and Analysis.** Data were tested for variance homogeneity by plotting residuals and either arcsine square-root or log transformations were used to stabilize variance. To test for interactions of experiment repetition, combined analyses of variance were conducted using the PROC-GLM in SAS<sup>30</sup>. Where appropriate, experiment repetition was considered random and interactions were tested with the mean square that is appropriate for the respective factorial or split plot treatment design (McIntosh 1983). Where possible, quantitative treatment effects were explained with appropriate regressions, otherwise, means were separated with Fisher's Protected LSD test at P = 0.05. For brevity, data from 25 DAT in the herbicide dose-plant response experiment are presented.

## RESULTS AND DISCUSSION

**Herbicide Dose-Plant Response Experiment.** The response to diclofop treatments varied among biotypes and across the range of application rates for the NHC Italian ryegrass biotype

---

<sup>29</sup> LabLogic® WinScan Radio TLC Version 2.2(5), distributed by BioScan Inc., 4590 MacArthur Blvd. NW, Washington, D.C. 20007.

<sup>30</sup> Statistical Analysis Systems (SAS) software, version 6.12. SAS Institute, Inc. P.O. Box 8000, SAS Circle, Cary, NC 27513.

(Figure 4.1). While the control of the NHC Italian ryegrass biotype was low at the label-recommended use rate (0.56 kg a.i./ha), it was significantly higher than that observed in the AC Italian ryegrass biotype. The level of control of the NHC Italian ryegrass biotype increased with increasing diclofop application rate, resulting in 39, 74, 93 and 99% control by 1.12, 2.24, 4.48, and 8.96 kg a.i./ha of diclofop, respectively.

The level of control of AC Italian ryegrass biotype did not statistically vary among the range of application rates of diclofop, and was not significantly greater than that observed in the control treatment. Even at the highest application rate of diclofop (8.96 kg a.i./ha; 16 times the label-recommended use rate), the control of the AC Italian ryegrass biotype observed was not in excess of 7%. The differential response of AC and NHC Italian ryegrass biotypes to diclofop provided basis for considering the AC biotype as diclofop-resistant and the NHC biotype as diclofop-sensitive for the purpose of ACCase assay and absorption, translocation, and metabolism experiments.

**ACCase Extraction and Assay.** The interaction of Italian ryegrass biotype and herbicide dose (Figure 4.2) was not significant ( $P = 0.8495$ ) nor was the main effect of Italian ryegrass biotype ( $P = 0.1638$ ) for percentage enzyme inhibition and total enzyme activity (Figure 4.3). Similar observations were reported for a quizalofop-resistant johnsongrass (*Sorghum halepense*) biotype from New Kent County, Virginia (Bradley et al., 2001). However, in that study, the specific ACCase activity was two- to three-fold greater in the resistant biotype than that observed in the quizalofop-susceptible biotype. Nevertheless, our results do not indicate that ACCase overproduction could be considered to be the mechanism of resistance to diclofop observed in the AC Italian ryegrass biotype in the herbicide dose-plant response experiment. Regardless of Italian ryegrass biotype, both percent enzyme inhibition and total ACCase activity declined with increasing herbicide concentration.

**Herbicide Absorption and Translocation.** Analysis of variance indicated that Italian ryegrass biotypes did not significantly influence absorption and translocation of [<sup>14</sup>C]quizalofop-ethyl over time ( $P > 0.05$ ). However, the interaction of time and plant part was significant ( $P < 0.0124$ ). Irrespective of Italian ryegrass biotype, most of the quizalofop-P was absorbed into treated leaves within 6 HAT and minimal absorption or translocation occurred during the remaining 66 hours (Figure 4.4). Within 6 HAT, 83% of recovered <sup>14</sup>C had absorbed into treated leaves and only 4% additional <sup>14</sup>C accumulated in treated leaves by 72 HAT. Likewise, less than 3 % of recovered <sup>14</sup>C was detected in plant shoots and roots during the 72-hour period.

These results indicate that the differential absorption and translocation of quizalofop-P does not explain observed differences between control of AC and NHC Italian ryegrass biotypes by diclofop. The observed results are consistent with previous reports that the resistance in many grass species, including Italian ryegrass, to APP herbicides is not conferred by altered herbicide absorption and translocation (Bradley et al., 2001; Devine, 1997; Devine and Shimabukuro, 1994; Gronwald et al., 1992; Inledon and Hall, 1997; Kuk et al., 1999).

**Herbicide Metabolism.** [<sup>14</sup>C]quizalofop-P was applied as an ester form of the parent acid. By itself, an ester formulation is not phytotoxic; however, it facilitates the absorption of the compound into the plant. Once inside a plant, the ester form is rapidly hydrolyzed into the phytotoxic acid form (Devine and Shimabukuro, 1994).

Based on previously reported Rf values (Bradley et al., 2001) and labeled and non-labeled quizalofop standards, quizalofop-ethyl (Rf = 0.9) and quizalofop acid (Rf = 0.5) were identified as primary metabolites in both Italian ryegrass biotypes. Peaks obtained with other Rf values were considered metabolites and their values (percent of total radioactivity detected) were pooled for each harvest time within each repetition. This study did not attempt to identify the metabolites, or assess their phytotoxicity. The interaction of harvest timing and herbicide

metabolite was significant ( $P < 0.0001$ ) and data were pooled over Italian ryegrass biotypes and experiment repetition for regression analysis (Figure 4.5).

[ $^{14}\text{C}$ ]Quizalofop-P was rapidly de-esterified by both AC and NHC Italian ryegrass biotypes (Figure 4.5). Based on area peak separations, parent acid decreased 0.6% per hour with a concomitant increase in metabolites between 6 and 72 HAT. Lack of the significant effect of Italian ryegrass biotype ( $P > 0.05$ ) indicates that differential metabolism does not explain the differences in response to diclofop treatments between AC and NHC Italian ryegrass biotypes observed in the herbicide dose-plant response experiment.

It is possible that the AC Italian ryegrass biotype possesses a herbicide-specific mechanism of resistance or produces more ACCase than susceptible biotypes. This biotype could metabolize diclofop, but remain sensitive to quizalofop, irrespective of the fact that both herbicides have similar modes of action. As reported by Shimabukuro et al. (1987), wheat readily metabolizes diclofop to non-toxic conjugates through aryl hydroxylation and subsequent conjugation of parent diclofop acid. Scientific evidence suggests that in wheat diclofop acid hydroxylation is catalyzed by a cytochrome P450 monooxygenase (Cyt P450) (McFadden et al., 1989; Zimmerlin and Durst, 1992). In support of these reports, experiments testing the effects of tetcyclacis, a Cyt P450 inhibitor, on growth of intact plants indicated a significant decrease in diclofop metabolism and an increase in diclofop phytotoxicity in a diclofop-resistant biotype of *Avena sterilis* (Maneechote et al., 1995).

This naturally occurring ability to rapidly detoxify diclofop, however, does not confer tolerance to other APP herbicides. While tolerant to diclofop, wheat is susceptible to haloxyfop, fluazifop, and other APP herbicides because of its inability to rapidly detoxify these herbicides (Devine and Shimabukuro, 1994). It is possible that the AC Italian ryegrass biotype possesses a similar herbicide-specific mechanism of resistance and further research to elucidate this mechanism is necessary.

## **ACKNOWLEDGEMENTS**

This study was sponsored in part by the Virginia Small Grains Board. Authors thank Bayer CropScience and DuPont Agricultural Products for providing necessary chemicals for the study. Authors also thank the help of Drs. S. Askew, N. Call and S. R. King for their assistance in our work.

## SOURCES OF MATERIALS

Acetyl Coenzyme A, trilithium salt (C2:0, 95%). Sigma-Aldrich. 3050 Spruce Street., St. Louis, MO 63103.

Coomassie® Protein Assay Reagent Kit. Pierce Biotechnology, Inc. 3747 N. Meridian Rd., P.O. Box 117, Rockford, IL 61105

Diclofop-methyl. Bayer CropScience LP. P.O. Box 12014, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709.

NaH<sup>14</sup>CO<sub>3</sub> radioactive isotope. American Radiolabeled Chemicals, Inc. 11624 Bowling Green, St. Louis, MO 63146-3506.

Polysorbate 20 [polyoxyethylene (20) sorbitan monolaurate], ICI America, Inc., Wilmington, DE 19899

Quizalofop-P. DuPont Agricultural Products, 1007 Market St., Wilmington, DE 19898.

## LITERATURE CITED

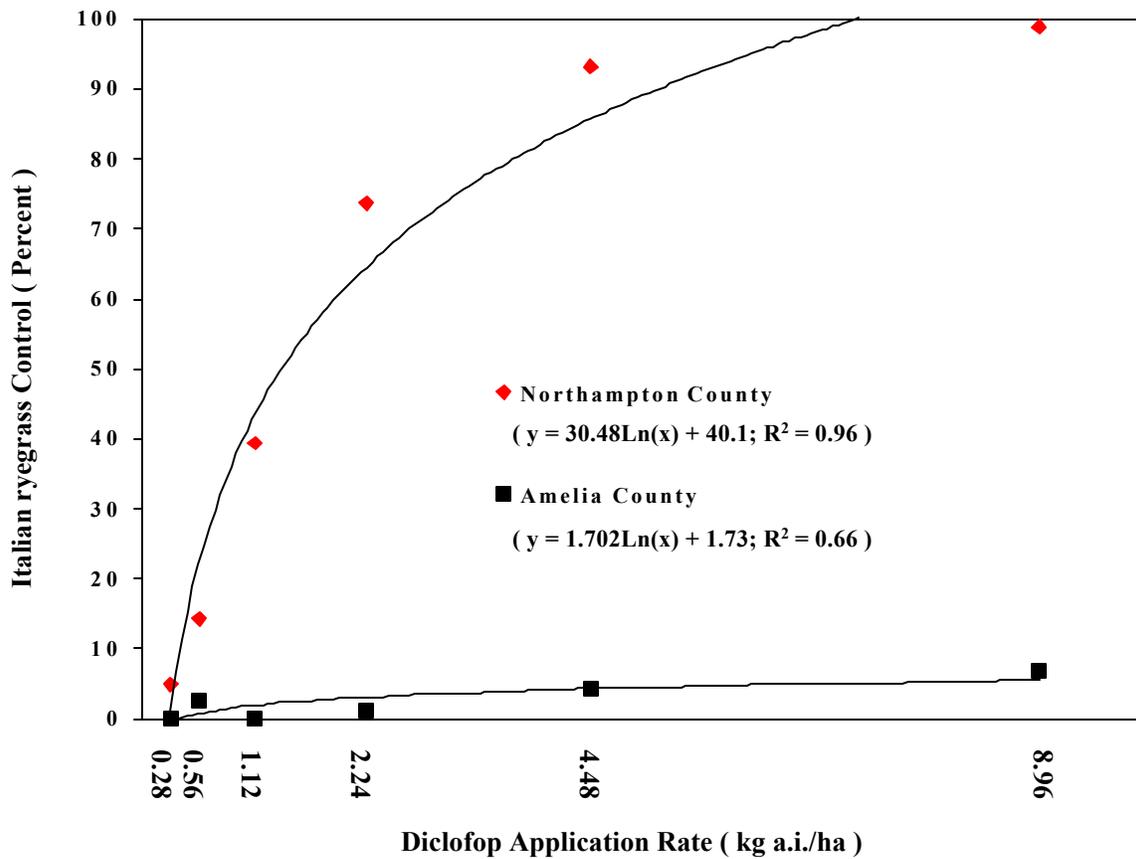
- Appleby, A. P. and B. D. Brewster. 1992. Seeding arrangement of winter wheat (*Triticum aestivum*) grain yield and interaction with Italian ryegrass (*Lolium multiflorum*). *Weed Tech.*, 6:820-823.
- Banas, A., I. Johansson, G. Stenlid, and S. Stymne. 1993. The effect of haloxyfop and alloxydim on growth and fatty acid composition of wheat roots. *Swedish J. Agric. Res.*, 23:55-65.
- Boldt, L. D. and M. Barrett. 1991. Effects of diclofop and haloxyfop on lipid synthesis and in corn (*Zea mays*) and bean (*Phaseolus vulgaris*). *Weed Sci.*, 39:143-148.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, 72:248-254.
- Bradley, K. W., J. Wu, K. K. Hatzios, and E. S. Hagood. 2001. The mechanism of resistance to aryloxyphenoxypropionate and cyclohexanedione herbicides in a johnsongrass biotype. *Weed Sci.*, 49:477-484.
- Browse, J. and C. Somerville. 1991. Glycerolipid synthesis: biochemistry and regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 42:267-506.
- De Prado, R. J. Gonzales-Gutierrez, J. Menendez, J. Gasquez, J. W. Gronwald, and R. Gimenez-Espinosa. 2000. Resistance to acetyl CoA carboxylase-inhibiting herbicides in *Lolium multiflorum*. *Weed Sci.*, 48:311-318.
- Devine, M. D. 1997. Mechanisms of resistance to acetyl-coenzyme A carboxylase inhibitors: a review. *Pestic. Sci.*, 51:259-264.
- Devine, M. D. and R. H. Shimabukuro. 1994. Resistance to acetyl-Coenzyme A carboxylase inhibiting herbicides. In: *Herbicide Resistance In Plants : biology and biochemistry*. S. Powles and J. Holtum (eds.). CRC Press, Boca Raton FL. pp. 141-170.

- Devine, M. D., S. O. Duke, and C. Fedtke. 1993. Physiology of the Herbicide Action. PTR Prentice Hall, Englewood Cliffs, New Jersey. pp 441.
- DiTomaso, J. M. 1993. Membrane response to diclofop acid is pH dependent and is regulated by the protonated form of the herbicide in roots of pea and resistant and susceptible rigid ryegrass. *Plant Physiol.*, 102:1331-1336.
- Egli, M. A., B. G. Gengenbach, J. W. Gronwald, D. A. Somers, and D. L. Wyse. 1993. Characterization of maize acetyl-Coenzyme A carboxylase. *Plant Physiol.*, 101:499-506.
- Evenson, K. J., J. W. Gronwald, and D. L. Wyse. 1994. Purification and characterization of acetyl-coenzyme A carboxylase from diclofop-resistant and -susceptible *Lolium multiflorum*. *Plant Physiol.*, 105:671-680.
- Evenson, K. J., J. W. Gronwald, and D. L. Wyse. 1997. Isoforms of acetyl-coenzyme A carboxylase in *Lolium multiflorum*. *Plant Physiol. Biochem.*, 35:265-272.
- Griffin, J. L. 1986. Ryegrass (*Lolium multiflorum*) control in winter wheat (*Triticum aestivum*). *Weed Sci.*, 34:98-100.
- Gronwald, J. W., C. V. Eberlein, K. J. Betts, R. J. Baerg, N. J. Ehlke, and D. L. Wyse. 1992. Mechanism of diclofop resistance in an Italian ryegrass (*Lolium multiflorum* Lam.) biotype. *Pest. Biochem. Physiol.*, 44:126-139.
- Hall, L. M., S. R. Moss, and S. B. Powles. 1997. Mechanisms of resistance to aryloxyphenoxypropionate herbicides in two resistant biotypes of *Alopecurus myosuroides* (blackgrass): herbicide metabolism as a cross-resistance mechanism. *Pest. Biochem. Physiol.*, 57:87-98.
- Harwood, J. L. 1988. Fatty acid metabolism. *An. Rev. Plant Physiol. Plant Mol. Biol.*, 39:101-138.
- Heap, I. A. 1997. The occurrence of herbicide-resistant weeds worldwide. *Pestic. Sci.*, 51:235-243.

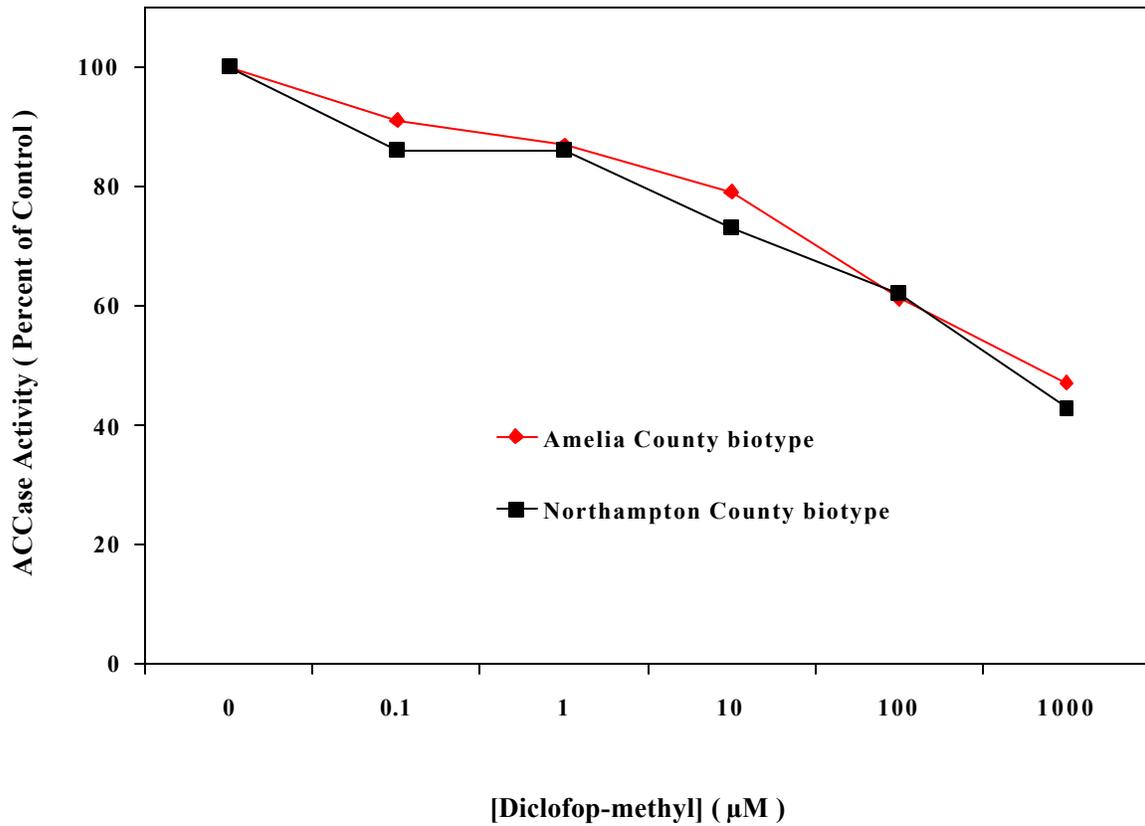
- Herbert, D. K. A. Walker, L. J. Price, D. J. Cole, K. E. Pallett, S. M. Ridley, and J. L. Harwood. 1997. Acetyl Co-A carboxylase – a graminicide target site. *Pestic. Sci.*, 51:67-71.
- Hidayat, I. and C. Preston. 1997. Enhanced metabolism of fluazifop acid in a biotype of *Digitaria sanguinalis* resistant to the herbicide fluazifop-P-butyl. *Pestic. Biochem. Physiol.*, 57:137-146.
- Incedon, B. J. and J. C. Hall. 1997. Acetyl-Coenzyme A carboxylase: quaternary structure and inhibition by graminicidal herbicides. *Pestic. Biochem. Physiol.*, 57:255-271.
- Khodayari, K., R. E. Frans, and F. C. Collins. 1983. Diclofop – a selective herbicide for Italian ryegrass (*Lolium multiflorum*) control in winter wheat (*Triticum aestivum*). *Weed Sci.*, 31:436-438.
- Kuk, Y.I, J. Wu, J. F. Derr, K. K. Hatzios. 1999. Mechanism of fenoxaprop resistance in an accession of smooth crabgrass (*Digitaria ischaemum*). *Pestic. Biochem. Physiol.*, 64:112-123.
- Maneechote, C., C. Preston, and S. B. Powles. 1997. A diclofop-methyle-resistant *Avena sterilis* biotype with a herbicide-resistant acetylene-coenzyme A carboxylase and enhanced metabolism of diclofop-methyl. *Pestic. Sci.*, 49:105-114.
- McFadden, J. J., D. S. Frear, and E. R. Mansager. 1989. Aryl hydroxylation of diclofop by cytochrome P-450 dependent monooxygenase from wheat. *Pestic. Biochem. Physiol.*, 34:92-100.
- Menendez, J. and R. De Prado. 1996. Diclofop methyl cross resistance in a chlortoluron resistant biotype of *Alopecurus myosuroides*. *Pestic. Biochem. Physiol.*, 56:123-133
- Mersie, W. and C. L. Foy. 1985. Interaction of chlorsulfuron and diclofop on Italian ryegrass. *Proc. South. Weed Sci. Soc.*, 38:426-431.
- Morozov I. V., E. S. Hagood, and P. L. Hipkins. 1999. Response of diclofop-resistant Italian ryegrass (*Lolium multiflorum*) to preemergence and postemergence herbicides. *Abstr. Proc., Southern Weed Sci. Soc.*, 52:40.

- Murray, B. G., A. L. Brule-Babel, and I. N. Morrison. 1996. Two distinct alleles encode for acetyl-CoA carboxylase Inhibitor resistance in wild oat (*Avena fatua*). *Weed Sci.*, 44:476-481.
- Ruizzo, M. A. and S. F. Gorski. 1988. Inhibition of chloroplast-mediated reactions by quizalofop herbicide. *Weed Sci.*, 36:713-718.
- Schmid, K., J. Andrews, and J. Ohlrogge. 1997. Fatty acid and lipid biosynthesis and degradation. *In: Plant Metabolism*. 2<sup>nd</sup> ed. D. T. Dennis, D. B. Layzell, D. D. Lefebvre, and D. H. Turpin (eds.). Addison Wesley Longman Ltd., London, England. pp. 414-429.
- Shaw, D. R. and M. T. Wesley. 1991. Wheat (*Triticum aestivum*) cultivar tolerance and Italian ryegrass (*Lolium multiflorum*) control with diclofop, BAY SMY 1500, and metribuzin. *Weed Tech.*, 5:776-781.
- Shimabukuro, R. H., W. C. Walsh, and A. Jacobson. 1987. Aryl-*O*-glucoside of diclofop: a detoxification product in wheat shoots and wild oat cell suspension culture. *J. Agric. Food Chem.*, 35:393-397.
- Shukla, A., S. Dupont, and M. D. Devine. 1997. Resistance to ACCase-inhibitor herbicides in wild oat: evidence for target site-based resistance in two biotypes from Canada. *Pestic. Biochem. Physiol.*, 57:147-155.
- Somers, D. A. 1996. Aryloxyphenoxypropionate- and Cyclohexanedione-Resistant Crops. *In: Herbicide-resistant Crops*. Duke S. O. (ed.). CRC Press. Boca Raton, FL. pp. 175-188.
- Somerville, C., J. Browse, J. G. Jaworski, and J. B. Ohlrogge. 2000. Lipids. *In: Biochemistry and Molecular Biology of Plants*, B. Buchanan, W. Gruissem, and R. Jones (eds.). American Society Of Plant Physiologists, Rockville, MD. pp. 456 – 527.
- Stanger, C. E. and A. P. Appleby. 1989. Italian ryegrass (*Lolium multiflorum*) accessions to diclofop. *Weed Sci.*, 37:350-352.
- Steel, R. G. D., J. H. Torrie, and D. A. Dickey. 1997. *Principles and Procedures of Statistics, A Biometrical Approach*. (3<sup>rd</sup> Ed.). WCB/McGraw-Hill. New York. pp. 352-399.

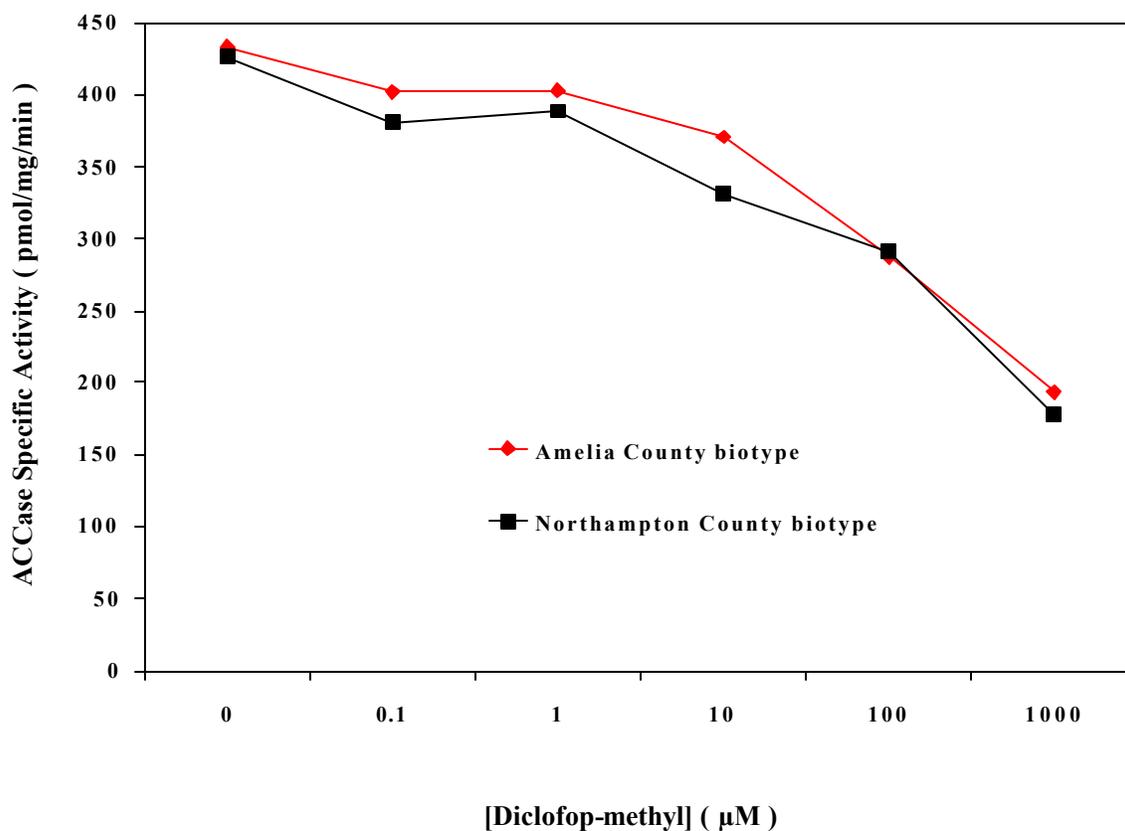
- Stone, M. J., H. T. Cralle, J. M. Chandler, T. D. Miller, R. W. Bowey, K. H. Carson. 1998. Above- and belowground interference of wheat (*Triticum aestivum*) by Italian ryegrass (*Lolium multiflorum*). *Weed Sci.*, 46:438-441.
- Wright, J. P. 1994. Use of membrane potential measurements to study mode of action of diclofop-methyl. *Weed Sci.*, 42:285-292.
- Zimmerlin, A. and F. Durst. 1992. Aryl hydroxylation of the herbicide diclofop by a wheat cytochrome P-450 monooxygenase. *Plant Physiol.*, 100:874-881.



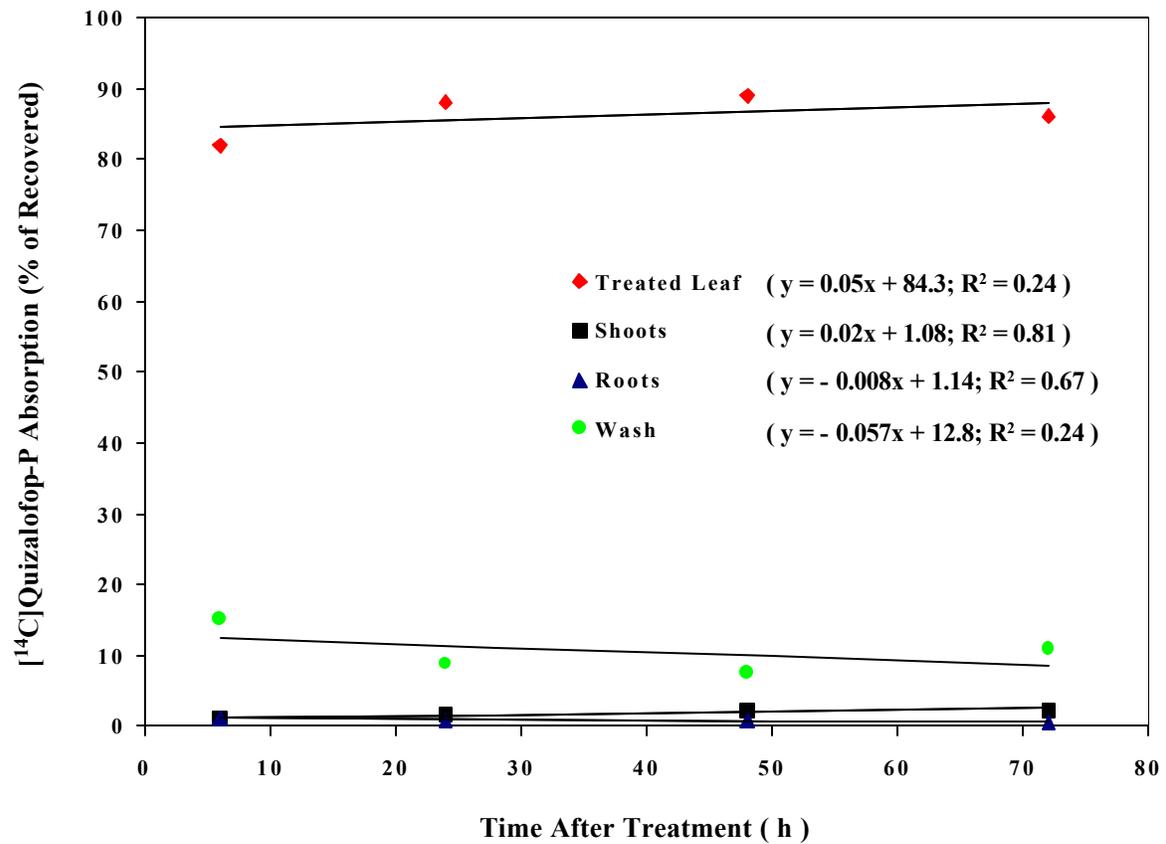
**Figure 4.1.** Estimated control of Amelia County and Northampton County Italian ryegrass (*Lolium multiflorum* Lam.) biotypes at 25 DAT after treatment with six application rates of diclofop. Data represents means averaged over two repetitions of the experiment with four replications of each data point within each repetition.



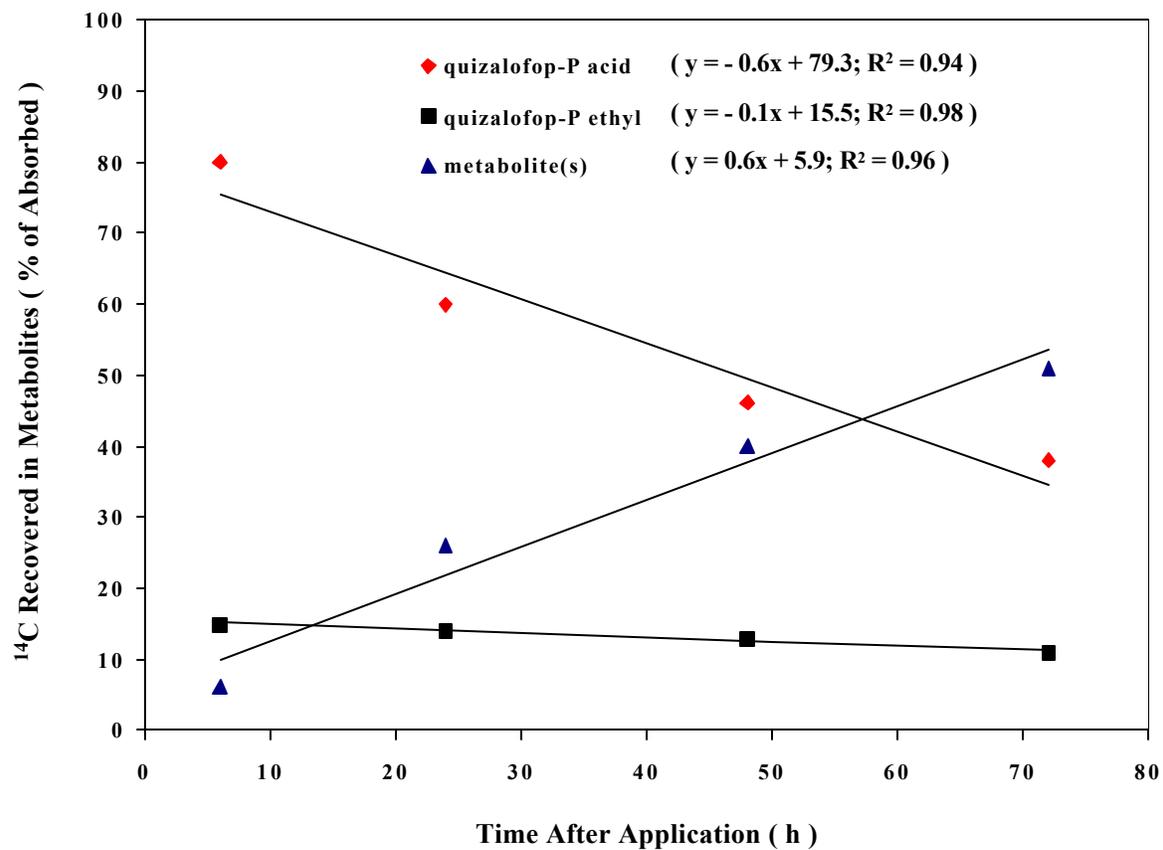
**Figure 4.2.** Inhibition of ACCase activity in Amelia County and Northampton County Italian ryegrass (*Lolium multiflorum* Lam.) biotypes by diclofop. Data represents means averaged over nine repetitions of the experiment with two subsamples per herbicide concentration per repetition for each data point (LSD = 6).



**Figure 4.3.** Inhibition of ACCase specific activity (pmol/mg/min) from Amelia County and Northampton County Italian ryegrass (*Lolium multiflorum* Lam.) biotypes by diclofop. Data represents means averaged over nine repetitions of the experiment with two subsamples per herbicide concentration per repetition for each data point (LSD = 11).



**Figure 4.4.** Absorption and translocation of [<sup>14</sup>C]quizalofop-P pooled over Italian ryegrass (*Lolium multiflorum* Lam.) biotypes at 6, 24, 48, and 72 hours after treatment. Data represents means for two biotypes and two repetitions of the experiment with three replications per harvest timing per experiment.



**Figure 4.5.** Metabolism of [ $^{14}\text{C}$ ]quizalofop-P pooled over Italian ryegrass (*Lolium multiflorum* Lam.) biotypes at 6, 24, 48, and 72 hours after treatment. Data represents means for two biotypes and two repetitions of the experiment with three replications per harvest timing per experiment.