# Evolution of target-based resistance mechanism to acetyl-coenzyme A carboxylase herbicides in *Digitaria ciliaris*

by

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## Abstract

*Digitaria ciliaris* (Retz.) Koeler (southern crabgrass) is an annual grass weed of the southern United States, commonly infests all crops, non-crops, and even in turf. This grass is historically controlled in both crop and turf systems for decades by employing postemergence ACCase-targeting herbicides. Resistance to these herbicides is common and continues to develop. *D. ciliaris* has developed resistance to ACCase targeting herbicides. ACCase resistant *D. ciliaris* was recently identified in sod production fields in Georgia. To date, no study has been reported on the possible resistant mechanism for *D. ciliaris* to the ACCase targeting herbicides. The primary goal of this dissertation research is the determination of the ACCase resistance mechanism in *D. ciliaris*. The dissertation, therefore, starts with a short literature review concerning the motivation for this research. In chapter 2 we report on the cross-resistance to pinoxaden herbicide in *D. ciliaris* from the resistant biotypes to sethoxydim and select aryloxyphenoxypropionate (FOPs) herbicides using pinoxaden response evaluation in the greenhouse. Prior selection pressure with other ACCase herbicides could evolve cross-resistance to pinoxaden of *D. ciliaris* in the United States. In addition, the target-based resistance, Ile-1781-Leu amino acid substitution in the ACCase gene is one of the causal mechanisms of resistance in *D. ciliaris* determined by amplification of the plastidic ACCase gene using a standard PCR and Next-Generation Sequencing profile.

Chapter 3 we report on the detection of ACCase enzyme activity in the resistant and susceptible biotypes for the validation of target-based resistance to ACCase herbicides from the malachite green colorimetric functional assay. Greater ACCase enzyme activity in the resistant biotypes to ACCase-targeting herbicides, sethoxydim, clethodim, fluazifop-p-butyl, and pinoxaden conferred the target-based resistance to the resistant biotypes. In addition, the malachite

green functional assay can be used for the measuring of the target-site activity in suspected resistance to ACCase herbicides instead of the $^{14}C$-based radiometric assay. Chapter 4 presents the elucidation of the differential ACCase enzyme inhibition mechanisms in the two resistant biotypes by using the gene expression profile. ACCase gene expression in the R2 biotype was the responsible factor for the inhibition differentiation of the ACCase enzyme from the R1 and S biotypes. Finally, Chapter 5 presents a study on the evaluation of the three different bioassay methods assessing the detection of ACCase-targeting herbicides resistance in *D. ciliaris*. Resistant biotypes were separated from the susceptible with different parameters from the three different bioassays such as agar-based gel box assay, leaf flotation assay, and electrical conductivity assay. Herbicide sensitivity for the suspected resistance population can be primarily screened using these rapid bioassays. The results derived from our research would facilitate increasing our understanding of resistance mechanisms along with genetic variation in the *D. ciliaris* genome.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACCase or ACCs | Acetyl-Coenzyme A carboxylase |
| *D. ciliaris* | *Digitaria ciliaris* |
| POST | Postemergence |
| PRE | Preemergence |
| TSR | Target Site Resistance |
| NTSR | Non-Target Site Resistance |
| ABC | ATP-binding Cassette |
| NGS | Next Generation Sequencing |
| PCR | Polymerase Chain Reaction |
| qRT-PCR | Quantitative Real Time PCR |
| SNV | Single Nucleotide Variant |
| APPs or FOPs | Aryloxyphenoxypropionate |
| CHDs or DIMs | Cyclohexanedione |
| DEN | Phenylpyrazolin |
| CT | Carboxylase Transferase |
| BCC | Biotin Carboxyl Carrier |
| BC | Biotin Carboxylase |
| PMSF | Phenylmethylsulfonyl fluoride |
| PVP | Polyvinylpyrrolidone |
| EDTA | Ethylenediaminetetraacetic Acid |
| BSA | Bovine Serum Albumin |

# Chapter 1

## i. Literature Review

Weeds are a global threat to agriculture. The highly competitive plants that adapt to cropping systems caused the desirable crop yield loss or damage known as weeds (Yuan et al. 2006). On an average, 34% crop yield is reduced for weeds throughout the world. Annually more than 26 billion US$ of crop yield losses occur in USA. For example, due to the weeds, the potential crop yield loss in wheat, rice and maize field is estimated to be 33%, 37%, and 40%, respectively (Oerke 2006; Pimentel et al. 2000). Modern agriculture heavily depends on herbicides application for controlling weeds (Délye et al. 2011). Herbicides play an important role in controlling weeds and preventing crop yield losses. To prevent crop yield loss, growers usually apply either pre-emergence (PRE) or post-emergence (POST) herbicides in their field. Resistance to herbicides in arable weeds is a common phenomenon and widespread exponentially during the last decade in modern cropping systems (Moss 2007; Délye et al. 2013). Herbicide resistance describes the ability of a plant biotype to survive and reproduce under a lethal dose of herbicide to the wild biotype defined by Weed Science Society of America (Yuan et al 2006; Stachler et al 2000).

Resistance to herbicides has been reported to 23 modes of action over 500 weed species in 70 different countries (Gaines et al. 2020). Herbicide resistance is one of the greatest challenges to agriculture worldwide for the future use and new introduction of effective herbicides. According to tactics of resistance managements, the repetitive practice of a single herbicide or group of related herbicides, and the exclusion of other weed control strategies in our agricultural system have resulted in the rapid evolution of resistance to herbicides in weeds (Tranel and Wright 2002). The rapid evolution of resistance creates a severe threat to weed control and crop production.

Management of herbicide-resistant weed, therefore, appears inevitable to control for the resistant alleles before their dissemination and better support for global crop production (Vila-Aiub et al. 2005).

**ACCase Resistance Issue:** With the repeated use of ACCase-targeting herbicides in a variety of grass weeds, many weed populations have evolved resistance (Délye 2005; Llewellyn and Powles 2001; Owen et al. 2007). Only after four years of the introduction of ACCase targeting herbicides into the market, the first case of ACCase resistance was reported in rigid ryegrass (*Lolium rigidum*) to diclofop- methyl from a wheat-growing field in western Australian in 1982 (Heap and Knight 1982). Then, blackgrass (*Alopecurus myosuroides*) evolved resistant to diclofop-methyl from northwestern Europe (Broster and Pratley 2005; Délye et al. 2007). Rigid ryegrass (*L. rigidum*) populations resistant to pinoxaden were reported even before the herbicide was launched in 2006 as shown in studies from 2003 and 2005 in Australia (Boutsalis et al 2012).

Resistance to ACCase targeting herbicides, thus, has been found in 24 distinct genera across six distinct continents (Boutalis 2001; Burgos et al. 2013). To date, 49 grass weeds have been reported as ACCase resistance in the world and fifteen of them are in South America such as sterile oat (*Avena sterilis*), downy brome or cheatgrass (*Bromus tectorum*), smooth crabgrass (*Digitaria ischaemum*), large crabgrass (*Digitaria sanguinalis*), barnyard grass (*Echinochloa crus-galli* var. *crus-galli*), late watergrass (*Echinochloa phyllopogon*), amazon sprangletop (*Leptochloa pancoides*), Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), Persian darnel (*Lolium persicum*), little seed canary grass (*Phalaris minor*), Itch grass (*Rottboellia cochinchinensis*), giant foxtail (*Setaria faberi*), green foxtail (*Setaria viridis*), giant green foxtail (*Setaria viridis* var. *major* (var. *robusta alba*, var. *robustapurpurea*), and johnsongrass (*Sorghum halepense*) (Heap 2021) causing different degrees of failure in weed control.

However, only three cases of ACCase-resistance have been reported in turfgrass systems. For instance, smooth crabgrass (*Digitaria ischaemum* (Schreb.) Schreb. ex Muhl.) biotype was identified on a golf course in southern New Jersey that was resistant to fenoxaprop. The proposed mechanism of resistance was an altered site of action (Derr 2002). This biotype showed cross-resistance to aryloxyphenoxypropionates herbicides but failed to show resistance to cyclohexanediones herbicides (Kuk et al. 1999). Another resistance at turfgrass found in goosegrass (*Eleusine indica* L. Gaertn.) biotype with resistance to sethoxydim on centipedegrass sod field in Georgia. This biotype also showed cross-resistance to clethodim, fenoxaprop, and fluazifop. Target site resistance, the Asp-2078-Gly substitution was responsible for causing the resistance mechanism for this biotype determined (McCullough et al. 2016). McCullough (2016) reported the first case of ACCase resistance in goosegrass from turfgrass system the United States. In 2002, resistant southern crabgrass (*Digitaria ciliaris* (Retz.) Koeler) was first evolved to ACCase-targeting herbicides in Brazil. This grass constitutes serious problems in the soybean and sugarcane production field (Heap 2021). Recently, the resistant population of southern crabgrass, high-level resistance to sethoxydim and selected FOPs herbicides has been identified on the sod production farm in Georgia (Yu et al. 2017).

**Crabgrass.** *Digitaria* spp. (crabgrasses), including large crabgrass (*Digitaria sanguinalis* (L.) Scop.), smooth crabgrass (*Digitaria ischaemum* (Schreb. ex Schweig.) Schreb. ex Muhl.), and southern crabgrass (*Digitaria ciliaris* (Retz.) Koeler) are troublesome weeds in the United States and worldwide (Grichar 1991; Holm et al. 1977; Holm et al. 1979). More than 300 annual and perennial grass species are included in the genus of *Digitaria* distributed over all continents. Of these, some species of *Digitaria* have fodder and food values and others are proven as noxious weeds. The information of the genome size in the *Digitaria* genus has been

documented. For example, *D. ascendens* Rendle and the hexaploid *D. sanguinalis* L contained the nuclear DNA 3.0 and 2.4 pg respectively. In addition, the larger genome size 2.7 and 2.6 pg were identified in *D. lecardii* and *D. ciliaris* species, respectively (Adoukonou-Sagbadja et al. 2007; Bennett et al. 2000). A ploidy series exists within the species of *Digitaria* genus containing the basic chromosome number of x=9. The ploidy level within the genus infers from diploid to hexaploid, for example, *D. iburua* contains chromosome numbers either tetraploid, 2n=4x=36 or hexaploidy, 2n=6x=54. Similarly, a wide range of chromosome numbers has been reported for the species of *D. exilis*, where chromosome counts ranged from diploid to hexaploid levels, 2n =18, 36, and 54 (Adoukonou-Sagbadja et al. 2007). This implies that the *D. ciliaris* could have a different ploidy level and heterozygosity.

Southern crabgrass is a warm-season annual grass that native to Asia but found throughout the Southern United States. This grass belongs to the botanical family Poaceae (Gramineae), the subfamily of Panicoidaea, the Paniceae tribe (Holm et al. 1979; Gleason and Cronquist 1991; Pinto and Fleck 1990). Its principal use to date has been as a pasture and range grass for forage production during the summer. It is also used for conservation plantings because its extensive root system reduced soil erosion. The variety of red rivers considers as high-yielding with improved nutritive-quality forage in the southern regions of the United States (Bouton et al. 2019). Southern crabgrass leads to reduce crop growth and yield both in quantity and quality by sharing water, light, nutrients especially nitrogen, and space.

It also reduces the aesthetic value of lawns and gardens, acts as an alternative host to crop pests and diseases, blocks water flow in ditches, and interferes with harvest (Okumura et al. 1986; ICRISAT 1981; Singh et al. 1996). Nowadays, southern crabgrass is a very problematic, aggressive, and competitive grass weed on sod farms, lawns, pastures, roadsides, old fields, and

waste spaces (Pinto and Fleck 1990; Lepschi and Macfarlane 1997). It retains economic risk to turf units because of its phenotypic variation and the decumbent type of growth habit, stems spread by long stolon or runner that root down at the nodes and form untidy patches. The most common way to control this grass is through the applications of selective herbicides.

**Acetyl-coenzyme A Carboxylase (ACCase) and Its Physiological Function.** Specific graminicide herbicides targeting acetyl-coenzyme A carboxylase (ACCs or ACCase; EC 6.4.1.2) enzyme plays an important role in catalyzing the first committed step for the biosynthesis of *de nevo* fatty acids. The first committed step in long-chain fatty acid biosynthesis is rate-limiting for the pathway and is tightly regulated (Yu et al. 2007; Délye et al. 2011; Petit et al. 2010; Podkowiniki et al. 2003; Konishi et al. 1996; Numa et al. 1965). Inhibition of the ACCase enzyme results in *de novo* fatty acid depletion, leading to rapid cell death due to membrane dysfunction. Unlike in animals, two different isoforms of the ACCase enzyme exist in plants. One is located in the chloroplasts or plastids, which are involved in the biosynthesis of primary fatty acids up to C18 then used as a precursor for lipid biosynthesis in the endoplasmic reticulum. The other is located in the cytosol, which is involved in the synthesis of very long-chain fatty acids up to C32 and secondary metabolites such as flavonoids and suberins (Focke et al. 2003; Harwood, 1988; Sasaki et al. 1995).

More than 80% of total ACCase activity in leaves belongs to the chloroplastic ACCase isoform. The chloroplastic ACCase is a homomeric eukaryotic ACCase enzyme that includes all functional domains in a single polypeptide known as nuclear-encoded eukaryotic ACCase enzyme, or a multi-subunit isoform known as heteromeric plasmid-encoded prokaryotic ACCase enzyme consisting of four subunits, whereas the cytosolic ACCase exist in the multidomain isoform only (Focke et al. 2003; Incledon and Hall 1997; Konishi et al. 1996; Sasaki et al. 1995). Plants

belonging to the Poaceae possess a homomeric or eukaryotic chloroplastic ACCase enzyme (Nikolskaya et al. 1999; Tong 2005; Wakil et al. 1983).

ACCase, a biotin-dependent enzyme catalyzes the formation of malonyl CoA from the acetyl-CoA with the hydrolysis of ATP. ACCase enzyme consists of three catalytic domains: the biotin carboxyl carrier (BCC), biotin carboxylase (BC), and carboxyltransferase (CT, with subunits α and β). The overall reaction is catalyzed by two sequential reactions through the action of three distinct protein components. In the first reaction, the biotin carboxylase domain catalyzes the ATP-dependent carboxylation of a biotin group covalently bound to the BCC domain. In the second reaction, the carboxylated biotin translocate to the CT active site to transfer the carboxyl group from biotin to the acetyl-CoA substrate, producing malonyl-CoA (Délye 2005; Harwood 1988; Howard and Ridley 1990; Nikolau et al. 2003; Yu et al. 2010). The two reactions are presented below:

$$ATP + CO_2 + BCCP = C02\text{-}BCCP + ADP + Pi$$

$$CO_2\text{-}BCCP + \text{acetyl-CoA} = \text{malonyl-CoA} + BCCP$$

**Selectivity:** Dicots have two ACCase enzyme forms, heteromeric or prokaryotic form in the chloroplasts and homomeric or eukaryotic form in the cytoplasm. In contrast, the grasses of the Poaceae family differ from all other plant species with a specific characteristic. They have only the homomeric form of ACCase in the chloroplast. Both chloroplastic and cytosolic ACCase isoforms in grasses of the Poaceae family become active when homodimeric form (Ashton et al. 1994; Egli et al. 1993; De Prado et al. 2004; Zhang et al. 2003). Between the two forms, only the eukaryotic ACCase is the target enzyme to the ACCase targeting herbicides (Collavo et al. 2011). Neither heteromeric chloroplastic nor homomeric cytosolic forms are inhibited by ACCase

targeting herbicides. This phenomenon indicates ACCase-targeting herbicides have selectivity between monocots and dicots (Konishi and Sasaki 1994; Muehlebach et al. 2011; Yu et al. 2010).

Most broadleaf species are naturally resistant to ACCase targeting herbicides due to a less sensitive ACCase enzyme, but the natural tolerance of some grasses appears to be due to either a less sensitive ACCase enzyme or a higher rate of metabolic degradation (Stoltenberg 1989). In some cases, these herbicides caused symptoms on certain broadleaf crops. Some cereal crops can metabolize the herbicide into inactive molecules offered good selectivity between weeds and crops as well (Devine 1997). For example, McCarty et. al (1990) found that the basis of selectivity between centipedegrass and goosegrass was a difference in metabolism. The trace amounts of sethoxydim herbicide <1% only were observed in centipede grass tissue whereas 81-98% was found in goosegrass tissue after 6 hours of treatment with sethoxydim.

**Mechanism of Action (MoA).** Lipids are the water-insoluble, organic, biomolecules found in all cells. The functions of lipid in plants are structural components of membranes and energy storage. The fatty acid biosynthesis pathway is found in any living organism. For this reason, the pathway is known as a primary metabolic pathway. This pathway is essential to plant cell growth. The fatty acids in plant cells generally contain long-chain carbons 16 to 18 lengths, which have between one and three cis double bonds. Fatty acids make up roughly 90% of the acyl chains of structural lipids in most plant membranes (Ohlrogge and Browse 1995).

The synthesis of most lipids starts with the synthesis of fatty acids. Fatty acid plays an important role in respiration. Respiration begins with glycolysis, in which a six-carbon sugar is split into two, three-carbon molecules. These two, three-carbon molecules eventually yield pyruvate, which in turn can yield ethanol in fermentation, enter the citric acid cycle for a complete energy harvest into ATP, and be synthesized into fatty acids. The pyruvate loses one carbon as

carbon dioxide in the Krebs citric acid cycle or fatty acids synthesis. The two-carbon fragment is attached to coenzyme A to yield acetyl coenzyme A. Acetyl CoA is a large molecule of the respirational product that was found after losing pyruvic acid one carbon as a carbon dioxide and serves to move 2-carbon molecules in biochemical pathways. At the fatty acids, synthesis is the conversion of acetyl-CoA into malonyl-CoA. The malonyl-CoA undergoes numerous transformations to become a completed fatty acid (Burton et al. 1989; Stoltenberg et al. 1989; Hopkins and Hunter 2004). In the first step of fatty acids synthesis, acetyl CoA receives carbon dioxide in the combination with ATP to yield malonyl CoA (three carbons molecule). The malonyl CoA reacts with another acetyl-CoA and loses one carbon to yield a 4-carbon molecule. The process continues such that the carbon chain is extended 2 carbons at a time until the chain is approximately 12-18 carbons long. The first step in this process, in which carbon dioxide is added to acetyl-CoA is catalyzed by the enzyme acetyl-CoA carboxylase (Numa et al. 1965; Podkowiniki et al. 2003).

Inhibition of fatty acid biosynthesis with ACCase targeting herbicides is the world's third most important herbicide mode of action (Délye et al. 2011). It presumably blocks phospholipids production, which is used in new membrane construction for cell growth. Fatty acid biosynthesis inhibition with ACCase begins when the herbicide is absorbed by the leaves and started translocation to proliferating meristematic tissues through the phloem. This process damages the cell membrane structure, meristematic activity, and the growth of new leaves in plants (Kukorelli et al. 2013). Plants, therefore, lose the cell membrane integrity, metabolite leakage, and ultimately causes plant cell death (Délye 2005; Kaundun 2014). Inhibition of ACCase activity in grass family are competitively inhibited and interrupted for fatty acid biosynthesis in the target site by the presence of ACCase targeting herbicides or group 1 herbicides (Liu et al. 2007; Senseman 2007).

**Injury Symptoms.** Inhibition of ACCase blocks the biosynthesis of *de novo* fatty acid and prevents the formation of lipid and secondary metabolites in susceptible plants (Cronan and Waldrop 2002). ACCase targeting herbicides are subject to very rapid adsorption into the target weeds. After few days of applying herbicide, the susceptible plant terminates growing due to the inhibition of long-chain fatty acid synthesis that led to the breakdown of the cell membrane, amino acid leakage, and eventually cell dysfunction (McCarty et al. 2010). Injury symptom on a susceptible plant caused by ACCase-targeting herbicides varies depending on the species, exposure rate, and growth stage. In general, symptoms are not apparent until several days after treatment. Safener also has an important role in phytotoxicity development by enhancing the selectivity of herbicide metabolism in grass crops (Délye et al. 2005).

Slow plant death is caused by several days after treatment with characteristic symptoms including chlorosis and necrosis. Chlorosis symptoms can be observed in yellowing near the growth point or leaf primordia of newly formed leaves with possible reddening or purpling of older leaves, whereas necrotic symptoms can be observed in growing tissues after one week of application, with initial chlorosis and subsequent collapse of the leaves (Dayan et al. 2019). Within one to three weeks, the herbicide causes a white to shiny appearance or necrotic symptom resulted from leaf chlorosis or yellowing of older leaves with purpling or reddening. Leaf-sheaths turn brown color and flaccid at the point of attachment to the node and ultimately plant death. In broadleaf plants, symptoms include chlorosis, mottled chlorosis, necrotic spots, leaf crinkling, and leaf distortion (Swisher and Corbin 1982).

**ACCase-targeting Herbicides or Group 1.** ACCase-targeting herbicides or group 1 herbicides are classified into three chemical families, aryloxyphenoxypropionates (AOPP or FOPs), cyclohexanediones (CHD or DIMs), and phenylpyrazolins (DENs). These herbicides

inhibit lipid synthesis. Specifically, they inhibit the enzyme acetyl-coenzyme A carboxylase resulting in reduced production of long-chain fatty acids, which are important for membrane synthesis. All herbicides are primarily used as a postemergence-applied for the control of grass weeds in dicot crops; however, certain selectivity exists allowing for some grass control in turfgrass. These herbicides, therefore, are collectively recognized as the "graminicides" due to their susceptibility to the grass species and the tolerance to all dicots and sedges.

The water solubility of three families of ACCase targeting herbicides is very low, not exceeding 2 ppm for any member of FOPs, approximately 25 ppm for all member DIMs, and approximately 200 ppm for DENs. These herbicides are easily degraded by microbial activity upon entry into soil. The residual activity does not last long more than 14 days in the soil. Due to the low water solubility and rapid microbial degradation, these herbicides have not been reported to have leaching of soil or off-target movement concerns (Kuk et al. 2008). Once these selective herbicides in the soil, they can convert to their acid form. They inhibit meristematic activity by absorbing through either roots and foliage or just the foliage depending on the chemical activity, species, soil characteristic, herbicidal dosage, and rapidly translocating in the phloem to the growing point (Lancaster et al. 2018). FOPs and DIMs were introduced over 45 years ago and widely applied as a post-emergence to control weedy grasses in a variety of field crops since their inception in the late 1970s and 1980s, respectively. A phenylpyrazoline family was introduced in 2006 and consists of a single herbicide, pinoxaden (Hofer 2006; Dayan et al. 2019). All the molecules belonging to these three chemical families consist of a carbon skeleton with polar substituents (Délye 2005). Although these three chemical families target the ACCase enzyme and cause plant death, each family contains distinct structural characteristics from each other (Shaner, 2014).

The chemical family of aryloxyphenoxypropionates (FOPs) included fourteen herbicides such as diclofop-methyl, fluazifop-p-butyl, fenoxaprop-p-ethyl, fenthiaprop, quizalofop-ethyl, quizalofop-p-tefuryl, haloxyfop-etotyl, haloxyfop-methyl, haloxyfop-p-methyl, propaquizafop, isoxapyrifop, metamifop, cyhalofop-butyl, and clodinafop-propargyl (Ruiz-Santaella and Prado 2006). This family has relatively large molecules containing at least two rings with several reactive groups attached to the common structure. Most of these herbicides are found in the methyl, butyl or ester formulated form. The purpose of the formulation is more lipophilicity and increased ability to cross cellular membranes by acid trapping (Takano et al. 2019). Diclofop- methyl is used for controlling the goosegrass within bermudagrass without damaging the bermudagrass. This herbicide is safe to apply to putting greens, for instance, diclofop control goosegrass around 1.3 cm height reported by McCarty 1990. FOPs have low toxicity; the LD50 of all the herbicides is in the 3000 to 5000 milligram per kilogram range. This family of herbicides, however, has other toxicological concerns such as all the FOPs herbicides except quizalofop are categorized as toxic to highly toxic to fish. Moreover, diclofop has a restriction to use as a pesticide due to oncogenicity in laboratory mice.

FOPs-resistant biotypes of several species including red fescue (*Festuca rubra*), Italian ryegrass, and wild oats have emerged i.e., grasses that were previously readily controlled with these herbicides' family, are now resistant (Todd and Stobbe 1977; Heap et al. 1993; Kuk et al 2008). At present, many weeds have evolved resistance to FOPs herbicides such as fluazifop-P, fenoxaprop-P, diclofop, and quizalofop (Cocker et al. 1999; Devine 1997; Leach et al. 1995; Prado et al. 1999; Volenberg and Stoltenberg 2002). Studies have been reported the weeds establishing resistance to different ACCase-targeting herbicides, including those arising from changes or mutations in enzyme sensitivity Ile-2041-Asn and Trp-2027-Cys substitution in *A. myosuroides*, in

addition to homologous substitution in *L. rigidum*, *A. sterilis, Phalaris paradoxa, P. minor* conferred a high degree of resistance to FOPs herbicides (Liu et al. 2007; Petit et al. 2010; Yu et al. 2007b; Hochberg et al. 2009; Zhang and Powles 2006b; Gherekhloo et al. 2012). Generally, FOPs-resistant biotypes frequently are immune to the cyclohexanedione herbicides, for example, Cys- 2008-Arg in *L. rigidum* (Yu et al. 2007; Yu et al. 2013), Trp-1999-Cys in *A. sterilis* (Liu et al. 2007; Kaundun 2013), and Gly-2096-Ala in *A. myosuroides* (Petit et al. 2010) substitutions are conferred a relatively higher rate of resistance to FOPs than the DIMs herbicides.

Nine herbicides such as clethodim, sethoxydim, tralkoxydim, tepraloxydim, alloxydim, butroxydim, cycloxydim, cloproxydim, and profoxydim are included in this **c**yclohexanedione (DIMs) family (O'Donovan et al. 2003; Xiang et al. 2009). These herbicides control grass within broadleaf or dicot crops, many ornamentals (i. e. asparagus and onions), and monocot crops that are not grasses as the crop plants belonging to the orders Liliaceae, Iridaceae, Palmae, Orchidaceae, etc. They provide grass control within a grass crop. The cyclohexanediones have very low toxicity, for instance, the LD50 of these herbicides is in the range of 3000 to 15000 milligram per kilogram. In addition, no negative concerns have been yielded in the long-term feeding studies (Tal et al. 1993).

The herbicide of sethoxydim is registered for the postemergence control of grasses in a very wide variety of agronomic and horticultural crops. This herbicide has activity against some weed grasses in turfgrass. For example, sethoxydim under the trade name Vantage is very active against bentgrass (*Agrostis stolonifera*) and tall fescue (*Festuca arundinacea*) (Hosaka 1984; Hugh et al. 1986). Sethoxydim at the rate of 0.44 kg ha-1 controlled more than 90% of goosegrass (Chernicky et al. 1984). However, some grasses have a tolerance for this herbicide. Sethoxydim at 1.12 kg ha-1 failed to control the red fescue and centipede grass (McCarty et al. 1986; Hugh et al.

1986). Biotypes of several other grass species have emerged that are tolerant to DIMs herbicides. This tolerance is accredited to a change or mutation of the target ACCase enzyme, for example, an Ile-1781-Leu in *A. myosuroides,* along with homologous substitution in *Avena fatua, A. sterilis, L. multiflorum, L. rigidum, Setaria viridis, D. ciliaris* has been identified to considerable tolerance to the sethoxydim herbicide but less tolerance to other FOPs and DIMs herbicides (Liu et al. 2007; Petit et al. 2010; Yu et al. 2007; Zhang and Powles 2006b; White et al. 2005; Christoffoleti et al. 2002; Yu et al. 2013; Basak et al. 2019).

Pinoxaden, an ACCase targeting herbicide is the only molecule containing in the phenylpyrazolin (DENs) chemical family (Locke et al. 2002). This herbicide was launched in 2006 by Syngenta Crop Protection, LLC, Greensboro, NC, USA, under the trade name Axial® XL herbicide. Pinoxaden is first introduced as a selective graminicide for global use as a postemergence control of annual and perennial grassy weeds within cereal crops, wheat, and barley (Hofer et al. 2006; Muehlebach et al. 2011). Based on target grass weed and geographical area, this herbicide is applied at low use rates from 30-60 g ai/ha. Usually, it is used to control a wide variety of grassy weeds in cereals including black grass (*Alopecurus myosuroides*), silky-bent grass (*Apera spica-venti*), wild oats (*Avena* spp.), canary grass (*Phalaris* spp.), ryegrass (*Lolium* spp.) and foxtails (*Setaria* spp.). Pinoxaden herbicide involves a particular adjuvant to optimum spread and translocation of the spray solution into grass weeds, for example, this herbicide is formulated with a built-in proprietary safener such as cloquintocet-mexyl in a ratio of 4:1 herbicide: safener to enhance performance and turf safety (Anonymous 2015). Cloquintocet-mexyl is an easy-to-use liquid formulation, which accelerates the metabolism of pinoxaden into inactive metabolites within wheat and barley, but not in susceptible ryegrass and wild oats (Muehlebach et al. 2011).

Pinoxaden is welcomed at the United Kingdom in turfgrass named manuscript herbicide labeled for annual grass control of ryegrass species (*Lolium* spp.) in maintained fine fescue (*Festuca* spp.) and annual bluegrass (*Poa annua*) turf in 2019 (Hofer et al. 2006; Anonymous 2015). Pinoxaden herbicide under the trade name 'Manuscript' is used for actively treating mature grassy weeds in certain warm-season grasses on golf courses, sod farms, sports turf even in residential or commercial lawns. It is usually applied with Adigor® surfactant (methyl ester of fatty acids, alcohol ethoxylate, and petroleum distillates). Currently, this herbicide is labeled for removal of tropical signal grass (*Urochloa subquadripara*), tropical carpetgrass (*Axonopus compressus*), large and smooth crabgrass, bahiagrass (*Paspalum notatum*), dallisgrass (*Paspalum dilatatum*), and canoe grass (*Paspalum vaginatum*). Pinoxaden is now available for controlling grass in the United States turfgrass market particularly on bermudagrass (*Cynodon sp.*), zoysiagrass (*Zoysia japonica* Steud.) and St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze.). But this herbicide a restricted to use only on bermudagrass and zoysiagrass at fairways, roughs, tee boxes, athletic fields, home lawns, and sod farms. Pinoxaden cannot be applied to putting greens and is restricted to use only sod farm (Anonymous 2018).

Tolerance to pinoxaden has already been reported in Italian ryegrass (*Lolium multiflorum* Lam.), black grass (*Alopecurus myosuroides* Huds.), wild oat (*Avena fatua* L.), and Japanese foxtail (*Alopecurus japonicus*) (Kuk et al. 2008; Kaundun 2010; Petit et al. 2010; Yu et al. 2010; Mohamed et al. 2012; Martins et al. 2014). For example, this tolerance has been attributed to the mutation, Asp-2078-Gly in enzyme sensitivity of the *A. myosuroides*, as well as homologous substitution in *A. sterilis, L. multiflorium, L. rigidum, P. paradoxa* species conferred a high rate of resistance to pinoxaden including all other herbicides in group 1 (Liu et al. 2007; Petit et al. 2010; Yu et al. 2007; Hochberg et al. 2009; Yu et al. 2013). Cross- or multiple-resistance between

members of DIMs or FOPs and pinoxaden herbicide families has also been reported in various grass weed species (Petit et al. 2010; Collavo et al. 2011). For example, Mohamed et al. (2012) found a Japanese foxtail (*A. japonicus*) biotype with cross-resistance to both fenoxaprop and pinoxaden in a wheat field in China. According to the authors' result, the ACCase resistant biotype was 49-fold resistant to fenoxaprop and 16-fold cross-resistant to pinoxaden compared to the susceptible biotype. In most cases, resistance exists in grass weed species before their exposure to pinoxaden (Kaundun 2010; Kuk et al. 2008; Petit et al. 2010; Yu et al. 2010). For instance, the resistant biotypes of black-grass (*A. myosuroides Huds*.) and rye grass (*Lolium* spp.) to pinoxaden herbicide were found in France (Petit et al. 2010) and in Italy (Scarabel et al. 2011), respectively, before the introduction pinoxaden where other DIMs and FOPs herbicide groups had a long history of usage.

**Mechanism of Resistance to ACCase-targeting Herbicides**. Resistance to ACCase targeting herbicides has significant economic consequence because of the limited options for postemergence control of herbicides with an alternative mechanism of action, and their function in managing grass weeds. Resistant biotypes may evolve after continuous using the same herbicides at recommended doses over the six to ten years imposed a strong selection pressure by ACCase targeting herbicides. This kind of event is found in the crop systems where the application of selective herbicides is the only way of grass weed management tool (Devine 2002). Weed biotypes resistant to ACCase-targeting herbicides can evolve from target-site resistance (TSR) and non-target site resistance (NTSR) mechanism.

**Target-site Resistance.** Target-site resistance is directly associated with the ACCase enzyme that can emerge from a mutation (single nucleotide polymorphism) or increased enzyme expression levels. Mutations in the plastidic ACCase gene changing in the enzyme structure at the

herbicide's binding site rendering this enzyme insensitive to the active ingredient. The enzyme primary sequence modifications have been the subject of research for over a decade and are well characterized (Délye, 2005). Grass species, Italian ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), annual ryegrass (*Lolium rigidum*), goosegrass (*Eleusine indica*), sour grass (*Digitaria insularis*), Johnson grass (*Sorghum halepense*), and common wild oat (*Avena fatua*) with target-site resistance have been reported in South America along with other parts of the world. However, ACCase resistance mechanism only has been explained in the *E. indica* in South America by McCullough et al 2016.

With TSR, a single point mutation in the ACCase gene, causing the amino acid alteration that reduces the sensitivity of the ACCase enzyme to ACCase herbicide groups (Preston and Mallory-Smith 2001). The sixteen such modifications have been described so far and most conserved at amino acid positions. The different amino acid alteration, glutamine (Gln)-1756-glutamate (Glu), isoleucine (Ile)-1781-leucine (Leu), tryptophan (Trp)-1999-cysteine (Cys), tryptophan (Trp)-2027-cysteine (Cys), isoleucine (Ile)-2041-aspartate (Asn) or valine (Val), aspartate (Asn)-2078-glycine (Gly), cysteine (Cys)-2088-arginine (Arg), and glycine (Gly)-2096-alanine (Ala) can confer different patterns of resistance among ACCase targeting herbicides (Christoffoleti et al. 2002; Collavo et al. 2011; Délye 2005; Kaundun 2010; Kaundun et al. 2012; Kaundun 2013; Powles and Yu 2010). All the amino acid substitutions associated with resistance are referenced with the corresponding positions following the blackgrass (*A. myosuroides*) plastidic ACCase gene (Genbank accession AJ310767) (Délye 2005).

Each of these single-site mutations makes a difference to the herbicide sensitivity of ACCase. This is the case of Ile-1781-Leu, a mutation in ACCase gene is associated with high-level resistance to clodinafop, haloxyfop, sethoxydim, pinoxaden and low-level resistance to

clethodim in Italian ryegrass (*Lolium multiflorum*) at South America (Kukorelli et al. 2013; Powles and Yu 2010; Scarabel et al. 2011). Similarly, Trp-1999-Cys mutation is associated with only fenoxaprop in perennial ryegrass (*Lolium perenne*) (Kukorelli et al. 2013; Powles and Yu 2010; Xu et al. 2014). Trp-2027-Cys mutation is associated with fenoxaprop, clodinafop, and pinoxaden in goosegrass (*Eleusine indica*) (Kukorelli et al. 2013; Powles and Yu 2010; San Cha et al. 2014), and this mutation also associated with sour grass (*Digitaria insularis* L. Fedde) resistance to haloxyfop, quizalofop, fenoxaprop, and pinoxden herbicides (Takano et al. 2021). Ile-2041-Asn or Val is associated with high-level resistance with clodinafop and haloxyfop, and a low level with pinoxaden herbicide in annual ryegrass (*Lolium rigidum*). In addition, Ile-2041-Asn mutation is associated resistance in Johnsongrass (*Sorghum halepense*) to fluazifop, propaquizafop, quizalofop, and haloxyfop, ACCase-targeting herbicides (Kukorelli et al. 2013; Powles and Yu 2010; Scarabel et al. 2011; Scarabel et al. 2014).

Asp-2078-Gly in ACCase gene is associated with resistance to diclofop, sethoxydim, clethodim, and pinoxaden in Italian ryegrass (*Lolium multiflorum*), this mutation is associated resistance to diclofop, fenoxaprop, cyhalofop, propaquizafop, clethodim, cycloxydim, and pinoxaden in common wild oat (*Aven fatua*), and also associated resistance to fluazifop, haloxyfop, cyhalofop, sethoxydim, clethodim, and tepraloxydim herbicides in goosegrass (*Eleusine indica*) (Cruz-Hipolito et al. 2011; Kaundun 2010; Kukorelli et al. 2013; Osuna et al. 2012; Powles and Yu 2010). Cys-2088-Arg has associated resistance to the clodinafop, haloxyfop, sethoxydim, clethodim, and pinoxaden herbicides in annual ryegrass (*Lolium rigidum*) (Kukorelli et al. 2013; Powles and Yu 2010; Scarabel et al. 2011). Gly-2096-Ala mutation is associated with high-level resistance to clodinafop, fenoxaprop, diclofop, and haloxyfop, and low-level resistance to

sethoxydim in common wild oat (*Avena fatua*) (Beckie et al. 2012; Délye 2005; Kukorelli et al. 2013; Powles and Yu 2010).

Cross-resistance to ACCase herbicides is usually caused by the two categories of TSR (Beckie et al. 2012; Chen et al. 2017) and NTSR mechanisms (Iwakami et al. 2019). Most cross-resistance studies in grass weeds to ACCase targeting herbicides describe TSR and subsequent reduction in enzyme sensitivity to these herbicides as the underlying cause (Yu et al. 2007; Kaundun 2014; Chen et al. 2017). In most instances, the cross-resistance phenomenon has been attributed to the changes or mutations Ile-1781-Leu, Trp-2027-Cys, and Ile-2041-Asn (Chen et al. 2017; Liu et al. 2007; Kaundun 2013). In addition to the mutations, Asp-2078-Gly and Cys-2088-Arg provided a broad spectrum of resistance to all herbicides in group 1 or ACCase targeting herbicid (Délye et al. 2008; Kaundun 2010; Kaundun et al. 2012; Scarabel et al. 2011; Osuna et al. 2012; Yu et al. 2007; Yu et al. 2013).

The resistance levels are not merely reliant on the mutations. There are some other factors responsible for influence herbicide resistance levels such as allele number and initial frequency, recessive, and dominant allele interactions, weed species, plant growth stage, and herbicidal recommended dose (Kaundun 2014). Furthermore, frequencies of ACCase gene mutation naturally vary for different weed species, sites, and the geography of herbicide selection pressure (Délye et al. 2011). The same mutation varies in herbicide sensitivity with weed species. Ile-2041-Asn, mutation causes resistance to cycloxydim herbicide in canary grass (*Phalaris paradoza*) but not in black grass (*Alopecurus myosuroides*) (Délye et al. 2008; Hochberg et al. 2009). Zygosity levels in weed species may result in contrasting herbicide sensitivity levels when plants are compared. This is the case of the annual ryegrass (*L. rigidum*) that results in control the homozygous plants

at the recommended doses of clethodim herbicide in a field in Australia but not in the heterozygous Ile-1781-Leu plants (Yu et al. 2007).

**Non-Target Site Resistance:** Non-target site resistance (NTSR) mechanism is associated with all other resistance mechanisms or no alteration to the target site. This type of resistance is identified as being the predominant resistance mechanism for ACCase herbicide resistance (Délye et al. 2011). NTSR encompasses a range of diverse mechanisms such as enhanced metabolism of toxophores, herbicide detoxification, reduced penetration, and translocation (Powles and Yu 2010; Kukorelli et al. 2013; Kaundun 2014). This resistance is often present in the same population, which has already contained one of TSR alleles such as *L. rigidum* (Han et al. 2016).

Compared with TSR, NTSR levels are low because herbicides may control the early growth stage of plants. Herbicide resistance level depends on the application of dose level. The higher dose application accelerates TSR development, and the lower dose application accelerates metabolic resistance (Gardner et al. 1998). For example, metabolic resistance has been shown to evolve rapidly in *L. rigidum* when herbicides are used at low rates (Neve and Powles 2005). Herbicide application with high temperature also developed resistance in weed species. Due to the biochemical pathways, oxidation and glucose conjugation are considerably increased after the application of pinoxaden herbicide at high temperatures. The temperature-dependent pinoxaden resistance presents in brome (*Brachypodium hybridum*) reported by Matzrafi et al. 2017.

Resistant biotypes are characterized by increased protein expression, protein abundance, post-translational modification of the existing protein, enhanced metabolism of the toxophores, and increased metabolic enzyme expression. One example of this is a population of large crabgrass (*D. sanguinalis*) that evolved ACCase herbicides resistance. ACCase gene expression in resistant populations was higher (5.2) than in susceptible (2.6). The overexpression ratio of 3.9 to 8.9 was

in the resistant population. ACCase resistance had been confirmed in *D. sanguinalis* from target gene overexpression to fluazifop herbicide (Laforest et al. 2017). Enzymatic activities of cytochrome P450 monooxygenase, glutathione-S-transferases, and glycosyl-transferases are involved in herbicide metabolism by increased expression levels (Devine 1997; Preston 2003; Brazier et al. 2002; Kukorelli et al. 2013; Kaundun 2014). Different weed species such as *Avena* spp., *E. phyllopogon*, and *L. rigidum* are confirmed resistant with cytochrome P450-related increased metabolism (Menendez and De Prado 1996; De Prado et al. 2005; Ahmad-Hamdani et al. 2012). Cytochrome P450 monooxygenase, glutathione-S-transferases, and glycosyl-transferase enzymatic activities are also related with multiple resistance in many weed species (Délye 2005; Powles and Yu 2010).

NTSR leads to cross-resistance to multiple herbicides with the activation of P450s that enzymatic reaction can metabolize different classes of herbicides such as late watergrass (*Echinochloa phyllopogon*). Cross and multiple-resistance patterns associated with NTSR are often unpredictable as this type of resistance is conferred by the metabolization rates of specific herbicides by those enzymes without considering their MoA. This is a case of *L. rigidum* population associated with cytochrome P450-mediated with enhanced metabolism for resistance to herbicides with different MoAs, including photosystem II, ALS, ACCase, and microtubule targeting herbicides (Preston and Powles 2002; Powles and Yu 2010; Iwakami et al. 2019). Several reports, however, have highlighted for a better understanding and elucidating the mechanisms of NTSR-based cross-resistance (Yu and Powles 2014; Iwakami et al. 2019).

In most cases of NTSR to ACCase herbicides, the resistance mechanism is involved in herbicide detoxification that the increased ability of the plant to digest ACCase targeting herbicides into nontoxic compounds (Powles and Yu 2010). In this process, herbicide detoxification occurs

where its metabolism rates increase, and the active ingredient of herbicide or toxic component concentration is reduced into a non-toxic molecule with hydrolases, oxidases, and peroxidases (Devine 1997; Preston 2003; Powels and Yu 2010; Kukorelli et al. 2013; Kaundun 2014). Later, metabolites are linked with a glutathione tripeptide, a sugar molecule, or an amino acid that is no longer efficient in inhibiting vital metabolic pathways (Délye 2005). The frequent application of the same herbicide or group of herbicides with the same or different modes of action to a population causes herbicide resistance as well. Preston et al. (1996) reported that rigid ryegrass (*L. rigidum*) has been evolved ACCase herbicides resistance after several years of continued use of the same herbicides. The same population was also found to have resistance to ALS targeting herbicides even though the population never being previously exposed to these herbicides. NTSR mechanism still requires further investigation as the molecular basis remains unknown.

## ii. Dissertation objective

Evolution of target-based resistance mechanism to acetyl-coenzyme A carboxylase herbicides in *Digitaria ciliaris*

Types by Suma Basak

Directed by J. Scott McElroy

Giving priority to the above facts, the present research work is framed and materialized to study the resistance mechanism of *D. ciliaris* followed by establishing a biotype independent mutation detection, enzyme assay, gene profiling, and rapid tests including agar-based gel box, leaf flotation, and electroconductivity assays for the same. So, this dissertation mainly pursues to achieve four goals: 1) To evaluate the two primarily sethoxydim-resistant biotypes of *D. ciliaris* response to pinoxaden and determine if a target-site mutation is present that is commonly associated with ACCase resistance 2) To validate the target-site resistance mechanism of two resistant biotypes containing Ile-1781-Leu amino acid substitution to ACCase-targeting herbicides in a malachite green colorimetric functional assay 3) To explain the different inhibition in the two resistant biotypes of *D. ciliaris* using ACCase gene expression profile 4) To evaluate the three different bioassay methods for assessing the detection of ACCase herbicides resistance in *D. ciliaris*

# Chapter 2

## Evaluation of the sethoxydim-resistant *Digitaria ciliaris* biotypes response to pinoxaden and determination of the resistance mechanism associated with ACCase targeting herbicides

[This chapter has been updated from its published form, Suma basak, J. Scott McElroy, Austin. M. Brown, Clebson G. Gonçalves, Jinesh D. Patel, and Patrick McCullough. 2020. Plastidic ACCase Ile-1781-Leu is present in pinoxaden-resistant southern crabgrass (*Digitaria ciliaris*). Weed Sci 68: 41–50. doi: 10.1017/wsc.2019.56]

### i. Abstract

*D. ciliaris* (Retz.) Koeler (Southern crabgrass) is an annual grass weed that commonly infests turfgrass, roadsides, wastelands and cropping systems throughout the southeastern United States. Two biotypes of *D. ciliaris* (R1 and R2) with known resistance to cyclohexanediones (DIMs) and aryloxyphenoxypropionates (FOPs) previously collected from sod production fields in Georgia were compared to a separate susceptible biotype (S) collected from Alabama for the responses to pinoxaden and to explore the possible mechanisms of resistance. Increasing rates of pinoxaden (0.1 to 23.5 kg $ha^{-1}$) were evaluated for control of R1, R2 and S. The resistant biotypes, R1 and R2 were resistant to pinoxaden relative to S. The S biotype was completely controlled at rates 11.8 and 23.5 kg $ha^{-1}$, resulting in no aboveground biomass at 14 DAT. Pinoxaden rates at which tiller length and above-ground biomass would be reduced 50% ($I_{50}$) and 90% ($I_{90}$) for R1, R2 and S ranged from 7.2 to 13.2 kg $ha^{-1}$, 6.9 to 8.6 kg $ha^{-1}$ and 0.7 to 2.1 kg $ha^{-1}$, respectively for tiller length and 7.7 to 10.2 kg $ha^{-1}$, 7.2 to 7.9 kg $ha^{-1}$ and 1.6 to 2.3 kg $ha^{-1}$, respectively for above-ground biomass. Prior selection pressure from DIMs and FOPs herbicides could result in the

evolution of *D. ciliaris* cross-resistance to pinoxaden herbicide. Amplification of the carboxyl-transferase domain of the plastidic ACCase by standard PCR identified a point mutation resulting in an Ile-1781-Leu amino acid substitution only for the resistant biotype, R1. Further cloning of PCR product surrounding the 1781 region yielded two distinct ACCase gene sequences such as Ile-1781 and Leu-1781. The amino acid substitution, Ile-1781-Leu in both resistant biotypes (R1 and R2), however, was revealed by next-generation sequencing (NGS) of RNA using Illumina platform. A point mutation in Ile-1781 codon leading to herbicide insensitivity in ACCase enzyme has been previously reported in other grass species. Our research confirms that Ile-1781-Leu is present in pinoxaden-resistant *D. ciliaris*.

Nomenclature: Acetyl-coenzyme A carboxylase; Fluazifop-p-butyl; Sethoxydim; Pinoxaden; Axial; Southern crabgrass; *Digitaria ciliaris*

Keywords: Pinoxaden resistance; herbicide resistance; turfgrass; post-emergence; ACCase inhibitor; target site mutation; polymerase chain reaction; cloning; next-generation sequencing

## ii. Introduction

*Digitaria* spp. (crabgrasses) are summer annual grass weeds that commonly infest both turfgrass and landscape environments as well as all crops (Lepschi and Macfarlane 1997). *Digitaria ciliaris* (Retz.) Koeler (southern crabgrass) is a $C_4$ herbaceous, monocotyledonous, annual in the Poaceae family (Bantilan et al.1974; Shetty et al. 1982; Murphy et al. 2014; Watson and Dallwitz 1992). Native to Asia, *D. ciliaris* is distributed in tropic and subtropic of both hemispheres and now found throughout the Midwest and Southern United States (Gleason and Cronquist 1991; Webster 2005). Due to its prodigious seed production and vigorous growth rate, this grass species inhabits and competes with desirable crops. Furthermore, its bunch-type growth and light-green color reduce turfgrass uniformity, and it also has allelopathic properties that can act against crops, other weeds, nitrifying bacteria and Rhizobium (Ito et al. 1987; Ito and Ichikawa 1994).

In modern agriculture, the control of grass weed species, especially the Poaceae family, largely depends on applications of selective herbicides (Délye et al. 2011). Acetyl-coenzyme A carboxylase (ACCs or ACCase; EC 6.4.1.2) inhibitors (often referred to as graminicides) are a unique herbicide mode of action that is primarily active on grasses. These herbicides are predominantly used as a selective graminicide for postemergence (POST) grass weed control in broad-leaved crops, however, certain selectivity exists allowing for some grass control in turfgrass (Délye 2005; Powles and Yu 2010; Kaundun 2010). Based on chemical structure, ACCase inhibitors (the group 1 herbicide according to WSSA classification) are broadly classified into three distinct chemical families, namely, aryloxyphenoxypropionate (APPs or FOPs), cyclohexanedione (CHDs or DIMs), and phenylpyrazolin (DEN) (Hofer et al. 2006, Liu et al.

2007; Hochberg et al. 2009; Powles and Yu 2010; Tang et al. 2014). The herbicidal action of these herbicides mainly depends on the selective binding of the carboxylase transferase (CT) domain of plastidic ACCase isoforms (Nikolskaya et al.1999).

Pinoxaden is a selective grass active compound discovered by Syngenta Crop Protection AG, Basel, Switzerland, a relatively new chemical and the only herbicide in the DEN family (Hofer et al. 2006; Senseman 2007; Petit et al. 2010; Tang et al. 2014). In 2006, pinoxaden was globally introduced as AXIAL® for the control of annual grass weeds in cereal crops. At recommended rates of 30-60 g $ha^{-1}$, AXIAL® is active against a wide range of important grass weed species such as *Alopecurus myosuroides* Huds. (black grass), *Apera spica-venti* (L.) P. Beauv (common wind grass), *Avena* spp. (loose silky-bent), *Lolium* spp. (ryegrass), *Phalaris* spp. (canarygrass), and *Setaria* spp. (foxtail). Due to its effective postemergence activity against a broad spectrum of grass weeds, pinoxaden is originally labeled for annual grass weed control in cereal crops including *Triticum aestivum* L. (wheat) and *Hordeum vulgare* L. (barley) (Locke et al. 2002; Porter et al. 2005; Hofer et al. 2006; Kuk et al. 2008). Pinoxaden interacts with the CT domain of homomeric ACCase enzyme with a similar binding mode in grass chloroplasts similar to the DIMs and FOPs (Hofer et al. 2006; Yu et al. 2010; Muehlebach et al. 2009).

Resistance to ACCase-inhibiting herbicides involves non-target site resistance (NTSR) mechanism by changing the metabolic activity of the protein and/or target-site based resistance (TSR) mechanism by altering to the target-site protein structure at the herbicide's binding site rendering it less sensitive to herbicidal activity (Devine 1997; Délye 2005). Resistance to the grass specific ACCase-inhibiting herbicides depends on NTSR mechanism, which is initiated without structural alteration of a target site protein (Yuan et al.2006; Délye et al. 2011). NTSR can be but is not limited to increased protein expression, increased protein abundance, post-translational

modification of the existing protein, increased herbicide metabolism, reduction of herbicide diffusion rate into the plant, repolarization of plasma membrane electrogenic potential (*Em*), or reduced rate of herbicide translocation (Bradley et al. 2001; Délye et al. 2013). Researchers have reported NTSR, such as enhances degradation of ACCase-inhibiting herbicides in the resistant biotypes of *Alopecurus myosuroides* Huds. (blackgrass), *Lolium* spp. (ryegrass), *Avena fatua* L. (wild oat), *Digitaria sanguinalis* (L.) Scop. (large crabgrass), as compared to susceptible biotypes (Mendez and DePrado 1996; Cummins et al. 1997; Hidayat and Preston 1997; Preston and Powles 1998; Cocker et al. 2000; Letouze´ and Gasquez 2003; DePrado et al. 2005; Petit et al. 2010).

TSR is most commonly a single point mutation in the plastidic ACCase gene, causing the amino acid alteration that reduces sensitivity of the ACCase enzyme to these herbicide groups (Preston and Mallory-Smith 2001; Yuan et al. 2006). To date, eight conserved amino acid substitutions at seven positions in the CT domain of the ACCase gene have been documented to the group 1 herbicide resistance in a variety of grass weed species (Beckie and Tardif 2012). The different known amino acid alteration, Gln-1756-Glu, Ile-1781-Leu, Thr-1805-Ser, Lys-1930-Arg, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala can confer different patterns of resistance among ACCase-inhibitors (Délye et al. 2002a, 2002b, 2000c; Délye 2005; White et al. 2005; Zhang and Powles 2006a, 2006b; Liu et al. 2007; Yu et al. 2007; Hochberg et al. 2009; Kaundun 2010; Petit et al. 2010; Collavo et al. 2011; Délye et al. 2011; Beckie and Tardif 2012; Gherekhloo et al. 2012; Kaundun et al. 2013; Yu et al. 2013). While the amino acid alterations Ile-1781-Leu or Val, Asn-2078-Gly, and Cys-2088-Arg confer resistance to all ACCase-inhibitors, the amino acid alterations, Trp-1999-Cys or Leu, Trp-2027-Cys, Ile-2041-Asn or Val, or Gly- 2096-Ala endow resistance to one or more FOPs but not to DIMs or DEN herbicides families (Powles and Yu 2010; Collavo et al. 2011).

Two biotypes of *D. ciliaris* with suspected resistance to DIMs and FOPs herbicide groups were identified in an *Eremochloa ophiuroides* (Munro) Hack. (centipedegrass) sod production fields, Georgia, United States (Yu et al. 2017). Sethoxydim applied at 315 and 945 g $ha^{-1}$ did not control these *D. ciliaris* biotypes >20% in field experiments. Yu et al. (2017) also reported that the shoot biomass production treated with sethoxydim from dose-response data was >64-times higher in both resistant populations than the susceptible population. Resistance to pinoxaden has already been identified in *Lolium multiflorum* Lam. (Italian ryegrass), *Alopecurus myosuroides* Huds. (black grass), *Avena fatua* L. (wild oat), and *Alopecurus japonicus* (Japanese foxtail) (Kuk et al. 2008; Kaundun 2010; Petit et al. 2010; Yu et al. 2010; Mohamed et al. 2012; Martins et al. 2014). Pinoxaden is now available for grass control in the US turfgrass market particularly in *Cynodon sp.* (bermudagrass), *Zoysia japonica* Steud. (zoysiagrass) and *Stenotaphrum secundatum* (Walt.) Kuntze. (St. Augustinegrass). We hypothesized that the two populations of *D. ciliaris* previously confirmed sethoxydim resistant will be cross-resistant to pinoxaden. Further based on the pattern of resistance to sethoxydim and other ACCase inhibitors, we hypothesized that these populations would possess a common mutation previously associated with ACCase herbicide resistance. The objectives of our research, therefore, was to evaluate the two primarily sethoxydim-resistant biotypes of *D. ciliaris* response to pinoxaden and determine if a target-site mutation is present that is commonly associated with ACCase resistance.

## iii. Materials and Methods

**Plant Materials and Growth Condition.** This research utilized two biotypes, R1 and R2 of *D. ciliaris* with confirmed resistant to sethoxydim and FOPs herbicides in previous publications (Yu et al. 2017) collected from two undisclosed fields of ‘TifBlair’ centipedegrass in Georgia. The fields were around 160 km apart in central Georgia where sethoxydim and FOPs herbicides had

been used annually for over two decades and control failure was evident in these fields. The plants from both biotypes were uninjured from sethoxydim and FOPs herbicides at a standard use rate range from 315 and 945 g $ha^{-1}$ applied approximately 3 weeks prior to collection from these fields. A separate susceptible (S) biotype of *D. ciliaris* was collected in Auburn, Alabama with no known history of exposure to ACCase inhibitors. Per an agreement with the landowners where the resistant types resided, it was agreed that the location of the resistant types would not be reported in any form in the future. The collected plants were propagated separately in a greenhouse environment to increase seed lots for experiments. Seeds from mature plants were collected randomly by hand, then was air-dried and stored in paper bags at 4$^{o}$C until planted.

The research was conducted at the Auburn University Weed Science greenhouse (32.35°N, 85.29°W) in Auburn, Alabama. Three biotypes were seeded in separate plastic flats containing commercial potting soil and peat moss (2:1 v/v). The plastic flats were placed in a greenhouse set for 32/25$^{o}$C (day/night) with no supplementary lighting. Plastic flats were overhead irrigated three times daily (around 0.2 cm per cycle) to prevent moisture deficiencies. Four weeks later, single seedlings at a three to four leaf stage were transplanted individually into plastic pots (10 cm × 10 cm × 8.5 cm) filled with the surface horizon of a marvyn sandy loam (fine-loamy, kaolinitic, thermic Typic Kanhapludults) with a pH of 6.5 and 1.1% organic matter. The potted seedlings were irrigated daily and fertilized weekly to promote growth. Plants reached a one- to two-tiller growth stage before treatments. Some plants were also allowed to produce flower and seed. All seeds from dehiscent inflorescences were cleaned, air-dried, and stored at 4°C until used in subsequent experiments.

**Pinoxaden Response Evaluation.** The responses of the three *D. ciliaris* biotypes were evaluated from a rate titration of pinoxaden (Axial®, Syngenta, Greensboro, NC) herbicide**.** Axial

also contains the safener, 25 g $L^{-1}$ (2.45% w/w) cloquintocet- mexy. Treatments were applied with a $CO_2$ pressurized sprayer calibrated to deliver 280 L $ha^{-1}$ from a handheld four-nozzle boom at 32 psi (TeeJet TP8003VS nozzles with 25 cm spacing; Spraying Systems Company, Wheaton, IL). All treatments included a nonionic surfactant (Induce, Helena® Chemical Company, Collierville, TN) at 0.25% v/v. Pinoxaden at 0.1, 0.2, 0.4, 0.7, 1.5, 2.9, 5.9, 11.8, and 23.5 kg $ha^{-1}$ was applied to both resistant and susceptible plants. Non-treated checks of the three biotypes were included as control treatments and sprayed with water. Plants were returned to the greenhouse after herbicide application, and irrigation was withheld for 24 hours. Control data were collected 14, 28, and 42 days after treatment (DAT) on a 0 to 100 percent scale in which 0% corresponded to no control and 100% corresponded to complete plant death or desiccation. The tiller length from the shoots was collected by measuring the length of the longest tiller from the base to the termination of the tiller at 42 DAT. The foliar weight of each plant was measured to determine the total above-ground biomass. The shoots, therefore, were clipped at the soil surface and were weighted using an analytical balance.

The greenhouse experimental design was conducted twice in time as a completely randomized factorial design. All treatments were replicated on three plants per biotype. Data analyses were performed using the PROC GLM procedure through SAS (SAS version 9.4, SAS Institute Inc., Cary, NC). Since differences between the data of the two experimental runs were not detected in the analysis of variance (ANOVA) at the 0.05 probability level, the data were pooled overruns for subsequent analysis. Pinoxaden rates were log-transformed to produce equal spacing among treatments prior to regression analysis. The non-treated control (0 kg $ha^{-1}$) was transformed to −1.36 to maintain equal spacing among log treatment rates. Tiller length (cm) and above-ground biomass (g) weight were converted to percent relative to the non-treated plants,

respectively. The nontreated mean of each biotype was used for conversion calculations to determine relative measures of each treatment and a model was selected that characterized the relationship of the response curves with pinoxaden herbicide rate after plotting treatment means. All measurements relative to nontreated were used for the regression model. Percent control data were fitted to an exponential growth model (equation 1) with two-parameter and percent data of the tiller length and the above-ground biomass were fitted to a sigmoidal equation with three parameters (equation 2) in SigmaPlot 13 (Systat Software Inc, London, UK):

$$y = a * exp^{bx} \quad (1)$$

$$y = \frac{a}{1+e^{-(\frac{x-x0}{b})}} \quad (2)$$

In equation 1, y is the control (%) of *D. ciliaris* biotype, x is log-transformed pinoxaden herbicide rate (kg $ha^{-1}$), and b is the y-intercept. In equation 2, y is the length/weight (%) of *D. ciliaris* biotype, x is log-transformed pinoxaden herbicide rate (kg $ha^{-1}$), $x_0$ is the asymptote, and b is the y-intercept. 95% confidence intervals ($\alpha$=0.05) for the estimates were calculated for regression model parameters. Regression equations were used to calculate inhibition values at 50% and 90% (referred to as $I_{50}$ and $I_{90}$ values) compared to that of the nontreated for each biotype and pinoxaden $I_{50}$ R/S value and $I_{90}$ R/S value was determined by each resistant biotype versus susceptible biotype.

**Target-Site Based Resistance.** Experiments were conducted to explore the potential target-site mechanism commonly associated with ACCase inhibiting herbicide sensitivity. RNA for the three *D. ciliaris* biotypes (R1, R2, and S) were isolated from leaf samples (approximately 0.1 g) using the TRIzol method (Trizol, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of total RNA were determined with gel

electrophoresis, a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA), and a Qubit 2.0 fluorometer (Invitrogen, Life Technologies). Then, the high-quality RNA was converted to complementary DNA (cDNA) through reverse transcriptase-polymerase chain reaction (RT-PCR) conversion using Proto Script II First Strand cDNA Synthesis Kit (New England Biolabs, Inc., Ipswich, MA, USA). PCR techniques were employed with some variations previously stated by Zhang and Powles (2006a). Sections of the ACCase gene were amplified with forward and reverse primers produced using the NCBI design tool and were sequenced to evaluate potential known single nucleotide polymorphisms (SNPs) for conferring herbicide resistance. The primers (listed in Table 1) were designed to amplify highly conserved regions of *D. ciliaris*, covering known resistance-conferring mutation site using ACCase gene sequences of *Echinochloa crus-galli* (L.) Beauv (NCBI accession number: KU198448).

PCR was conducted using plant cDNA amplification in a 25 µl volume. The total volume of each PCR reaction mixture contained 1X standard reaction buffer, 200 µM dNTP, 0.5 µM forward and reverse primer, 250 ng of cDNA, and 0.125 U Taq DNA polymerase (New England Biolabs, Inc., Ipswich, MA, USA). The cycling program consisted of an activation step of 95°C for 30 sec followed by 35 cycles of 20 sec at 95°C, 1 min annealing at 58-62 °C depending on primers used, and 1 min at 68°C, followed by a final extension step of 5 min at 68°C. The PCR product was visualized on a 1.3% agarose gel in Tris-acetate-EDTA (TAE) buffer and 1% ethidium bromide solution. The specific band size of PCR product from the gel was extracted using E.Z.N.A.® Gel DNA Extraction Kit. Each extracted PCR product was sequenced in forward and reverse directions at Eurofins Genomic. Co., Ltd., Louisville, KY, USA. The sequences data of resistant and susceptible *D. ciliaris* biotypes were compared to determine if there was a nucleotide substitution. Nucleotide sequences predicted from the ACCase gene sequences of *D. ciliaris* were

subjected to the nucleotide (blastn) and amino acid (blastx) searches using NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) to identify nucleotide and transcribed amino acid positions, respectively. The nucleotide sequence for each *D. ciliaris* biotypes then were further aligned and compared using CLC Genomics workbench v. 6.5.2 (CLC Bio, Aarhus, Denmark).

PCR products generated with Taq polymerase were ligated into the pGEMT®Easy Vector (Promega Corp., Fitchburg, WI) and transformed in *Escherichia coli* (JM109 High-Efficiency competent cells, Promega Corp.). According to manufacture instruction, 100 µL aliquot of each bacterial suspension was plated on media containing LB broth, ampicillin, X-gal, and IPTG. The white colonies with putative inserts were selected from the transformed plates and incubated at $37^0$C overnight (Figure 6). The plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen N.V., Venlo, Netherlands). The plasmids containing ACCase putative mutated cDNA fragments were sequenced, analyzed, compared with cDNA fragment of susceptible biotype in a similar method as earlier discussed. As the standard PCR based resistance mechanism determination in the R2 biotype was unable to detect the amino acid substitution in Ile-1781 codon, we carried out further studies to investigate the amino acid substitution using transcriptome analysis.

**Transcriptome Profiling.** *D. ciliaris* is a suspected polyploid species with potentially multiple copies of plastidic ACCase in subgenomes. To fully account for all expressed plastidic ACCase, mRNA was sequenced using the next-generation sequencing. The two resistant biotypes, R1 and R2 were sequenced in the Genomic Service Laboratory using Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA) at HudsonAlpha Institute for Biotechnology (Cummings Research Park, Huntsville, AL, USA). After receiving large quantities of data files, the raw reads qualities were checked by FastQC v.0.11.1 (Andrews 2010). The reads then were

processed by Trimmomatic v.0.33 to remove adaptors contamination, unqualified reads, and sequences (Bolger et al. 2014). Again, the trimmed reads were qualified for high throughput sequence data with FastQC. The sequence data were normalized with Trinity's in silico read normalization (Grabherr et al. 2011), with maximum coverage of 30 and k-mer of 25. Each biotype was assembled using three de novo assembler's transcriptome assemblers Trinity 2014-04-13p1, Velvet 1.2.08_maxkmer101, and CLC Genomic workbench (Zerbino and Birney 2008; Grabherr et al. 2011). Trinity k-mer size was 25, Velvet k-mer size was 2 to 81 with a step size of 10, and CLC k-mer size was 14 to 64 with a step size of 5. N50s and contig length distributions of the assemblies were calculated for estimating the quality of the assembly with the script Count_fasta.pl. Consensus regions (contigs) bigger than 200 bp were considered from all assemblies.

All the assemblies were pooled into one merged assembly for each species individually. The merged assembly was processed by EvidentialGene tr2aacds pipeline. Using the transcript fasta file from any of the transcript assemblers, this pipeline can produce the coding DNA sequence (CDS) and amino acid sequences from each input contig. It then can use fastanrdb for reducing duplicate sequences, cd-hit and cd-hit-est and blastn to search the similar CDS among sequences. The output transcripts were three classes: Main (the best transcripts with the unique CDS, which is close to a biologically real set), Alternate (possible isoforms), and Drop (the transcripts did not pass the internal filter). The Main and Alternate sets were submitted to the NCBI Transcriptome Shotgun Assembly (TSA) database. Sequences flagged by TSA as duplicates or moderate to strong matches with Univac vectors were masked or removed as per TSA requirements. For R1 and R2 assemblies, 629 of 485564 sequences, and 575 of 367801 were modified respectively to meet submission requirements for submission to TSA.

For extracting contigs, each assembly was searched for homologous ACCase gene sequences from *Alopecurus myosuroides* Huds. (AJ310767), *Setaria viridis* (L.) P. Beauv. (AM408428), and *Echinochloa crus-galli* (L.) Beauv. (KU198448) using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/) and SwissProt using ncbi-blast-2.2.29+ with an E-value threshold of $1e^{-5}$. The blast outputs were processed with Trinity downstream analyses. Main and alternate sequence sets were annotated with the NCBI nonredundant (Nr) protein database using ncbi-blast-2.2.29+ at E-value threshold of $1e^{-5}$ where 20 maximum hits found for each query. The Nr blast results were processed using Blast2 Gene Ontology (GO) v. 3.0 (Götz et al. 2008) to analyze the gene functions and compare to reference ACCase genes from the three species downloaded from the NCBI. Open reading frames (ORF) were projected using CLC Genomics Workbench and confirmed by comparing to mRNA of *E. crus-galli* (KU198448). *D. ciliaris* ACCase were aligned to *E. crus-galli* (KU198448) genomic DNA. All reads were mapped to the putative assembled plastidic ACCase contiguous sequence to identify single nucleotide variations associated with herbicide resistance possibly not previously identified using standard PCR sequencing. Reads mapping and Single nucleotide variantions detection or other related mutations were conducted using 'map reads to reference' and 'probabilistic variant detection' tools in CLC Genomics Workbench v. 6.5.2 (Li 2013). The mapping parameters were selected to 'Mismatch cost=3, Insertion cost=3, Deletion cost=3, Length fraction=0.95, Similarity fraction=0.95'. The parameters of variants calling were set to 'Minimum coverage=30, Variant probability=90'.

### iv. Results and Discussion

**Pinoxaden Response Evaluation.** Pinoxaden herbicide treatment by experimental run interactions was not significant ($P > 0.05$) for control, tiller length and aboveground biomass; therefore, data were pooled over experimental run and reported as a combined means. However,

the pinoxaden herbicide treatment by resistant and susceptible biotypes of *D. ciliaris* interactions was highly significant ($P < 0.05$) values observed for control, tiller length and aboveground biomass. Pinoxaden controlled and reduced the measured variables of R1 and R2 less than the S across all rates (Figure 1 and 2). At 14 DAT, no control was observed for the R1 and R2 biotypes whereas, 8% control was observed in S biotype at a rate of 0.1 kg $ha^{-1}$. At the rates of 0.2, 0.4, and 0.7 kg $ha^{-1}$, pinoxaden controlled R1 and R2 biotypes less than 5% but controlled the S biotype 32, 37 and 40%, respectively. Pinoxaden at the rates of 1.5, 2.9, 5.9, 11.8, and 23.5 kg $ha^{-1}$ controlled the R1 biotype 3, 20, 23, 50, and 60%, respectively and the R2 biotype 12, 20, 43, 80, and 88%, respectively while the S biotype was controlled 67, 75, 97, 100, and 100%, respectively. The S biotype was completely controlled at the rates 11.8 and 23.5 kg $ha^{-1}$ resulting in no aboveground biomass at these rates, while no rate completely controlled the R1 and R2 biotypes at 14 DAT.

A similar trend in the biotype's response at 28 and 42 DAT was observed for R1, R2, and S. For example, pinoxaden at 1.5, 2.9, and 5.9 kg $ha^{-1}$ at 28 DAT controlled the R1 biotype 12, 32, and 38%, respectively, the R2 biotype 22, 33, and 63%, respectively, compared to the S biotype 55, 77, and 97%, respectively. Pinoxaden at the rate of 2.9 to 23.5 kg $ha^{-1}$ controlled the S biotype 100% at 42 DAT, but R1 and R2 controlled only ≤40%. Percent control relative to the nontreated response to pinoxaden rate was modeled for all three biotypes through exponential growth functions. The 50% inhibition ($I_{50}$) and 90% percent inhibition ($I_{90}$) values were calculated with the exponential growth function equation (Figure 1) presented in Table 2. The level of resistance was determined by $I_{50}$ and $I_{90}$ values of each resistant biotype versus susceptible biotype presented as a R/S ratio. The S biotype contained less $I_{50}$ and $I_{90}$ values than R1 and R2 biotypes. Obtaining 50% inhibition of S biotype by 14 DAT, 28 DAT, and 42 DAT required 1.1, 0.5, and 0.2 kg $ha^{-1}$,

respectively, which were much lower rates than the R1 biotype 16.2, 7.4, and 8.5 kg $ha^{-1}$, respectively and the R2 biotype 8.3, 4.9, and 6.9 kg $ha^{-1}$, respectively. The resistant biotypes were significantly less sensitive to pinoxaden than S biotype. The level of resistance as expressed by R/S ratios at 14 DAT, 28 DAT, and 42 DAT was 14.6, 14.3 and 56.9 for R1 biotype and 7.4, 9.5, 46.2 for R2 biotype, respectively. Similarly, the required amount of pinoxaden was also lower for 90% inhibition at 14 DAT, 28 DAT, and 42 DAT for the S biotype 9.2, 6.8, and 4.9 kg $ha^{-1}$, respectively compared to the R1 biotype 37.7, 18.2, and 19.7 kg $ha^{-1}$, respectively and R2 biotype 20.8, 15.7, and 16.9 kg $ha^{-1}$, respectively. The resistant biotypes had high resistance level to pinoxaden compared to S biotype. The ratio of R/S at 14 DAT, 28 DAT, and 42 DAT was 4.1, 2.7, and 4.0 for R1 biotype and 2.2, 2.3, and 3.4 for R2 biotype, respectively.

Similar trends compared to control data of R1, R2, and S response were observed in above-ground biomass and tiller length. Pinoxaden at a rate of 1.5 kg $ha^{-1}$ reduced R1 and R2 biotypes above ground biomass 8 and 37 compared to 71% for S biotype. No tillers were produced at rates $\geq$ 2.9 kg $ha^{-1}$ of pinoxaden, which resulted in no above-ground biomass produced in S biotype; however, R1 and R2 biotypes did produce above-ground biomass at the rates of 2.9 and 5.9 kg $ha^{-1}$. Percent maximum tiller length and percent above-ground biomass relative to the non-treated response to the increasing rate of pinoxaden were modeled for all three biotypes using sigmoidal functions. $I_{50}$ and $I_{90}$ values were calculated through the sigmoidal equation (Figure 2) presented in Table 3. The S biotype contained less pinoxden for $I_{50}$ and $I_{90}$ reduction than R1 and R2 biotypes both in tiller length and above-ground biomass. In case of tiller length, pinoxaden $I_{50}$ for S biotype was 0.7 kg $ha^{-1}$ compared to 7.2 and 6.9 kg $ha^{-1}$ for R1 and R2 biotypes, respectively. R1 and R2 biotypes were 10.4 and 9.9 more resistant, respectively than S biotype based on R/S ratios. Pinoxaden tiller length $I_{90}$ for S biotype was 2.1 kg $ha^{-1}$ compared 13.2 and 8.6 kg $ha^{-1}$ for R1 and

R2 biotypes, respectively. R1 and R2 biotypes were 6.2 and 4.1 more resistant, respectively than S biotype based on R/S ratio. In the case of above-ground biomass, pinoxaden $I_{50}$ values for S biotype was 1.6 kg $ha^{-1}$ compared 7.7 and 7.2 kg $ha^{-1}$ for R1 and R2 biotypes, respectively. R1 and R2 biotypes were 4.8 and 4.5 more resistant than S biotype based on R/S ratios, respectively. Pinoxaden above ground biomass $I_{90}$ for S biotype was 2.3 kg $ha^{-1}$ compared to 10.2 and 7.9 kg $ha^{-1}$ for R1 and R2 biotypes, respectively. R1 and R2 biotypes were 4.5 and 3.5 more resistant than S biotype based on R/S ratios, respectively.

**Target-Based Resistance.** PCR-based sequencing was conducted in the ACCase carboxyltransferase (CT) domain coding region containing known amino acid substitutions conferring resistance to ACCase-inhibiting herbicides (Délye et al. 2011; Liu et al. 2007; Powles and Yu 2010). Sequencing of plastidic ACCase revealed a double peak in the sequencing chromatogram in a single nucleotide for the Ile-1781 codon from cDNA sequencing in the R1 resistant biotype (Figure 3), but not in R2 or S biotypes (Figure 8). Cloning of the PCR product was conducted to confirm the resistant and susceptible alleles in the R1 resistant biotype. A 914bp cDNA fragment containing the Ile-1781 codon was cloned as previously described. Twelve ACCase putative mutated cDNA fragments were sequenced, analyzed, compared with susceptible biotype along with several homologs of ACCase genes, *A. myosuroides* (AJ310767), *E. crus-galli* (KU198448) and *S. viridis* (AM408428). Cloned cDNA fragment from the sequencing resulted in two separate nucleotide sequences surrounding the Ile-1781 codon. Out of the twelve ACCase putative mutated cDNA fragments, eight cloned cDNA fragments contained codon CTA for Leu-1781 amino acid, and four cloned cDNA fragments contained ATA for Ile-1781. Other polymorphisms between cloned amplicons are presented in Figure 4, 5, and 7. Sequences of ACCase resistant *D. ciliaris* were submitted to NCBI database (accession numbers: MK558087

and MK558088). The presence of a double peak on the sequencing chromatogram can indicate heterozygosity between two homologs of a diploid organism or an allelic difference between homoeologs of a polyploid organism. Based on sequencing of PCR products generated by various primers, we found only the Ile-1781-Leu substitution in the resistant biotype R1 representing potential herbicide resistance mutation in *D. ciliaris*. However, no double peaks or amino acid substitutions were found in R2 biotype. Considering both R1 and R2 response to ACCase-inhibiting was similar, we theorized that standard PCR was not able to amplify all homoeologs in R2 resistant biotype. So, next generation sequencing using Illumina platform was performed to sequence all expressed plastidic ACCase in R2 resistant biotype.

**Transcriptome Profiling.** Two de novo assembly transcriptomes were assembled separately for both R1 and R2 biotypes. Number of reads obtained for the resistant biotypes R1 and R2 were 484935 and 367226, respectively. ACCase contigs were extracted via BLAST analysis with plastidic ACCase genes from three species, NCBI: *A. myosuroides* (AJ310767); *E. crus-galli* (KU198448); and *S. viridis* (AM408428). The ACCase transcript of *D. ciliaris* coding for the complete protein was recovered, showing 91.5% similarities and 83.0% identity with *A. myosuroides*. Sequence analysis produced an open reading frame (ORF) containing coding for a 157-amino acid long peptide for the ACCase CT-domain with 93.9% homology to *E. crus-galli*. Mapping of sequence reads from extracted contigs revealed heterozygosity in Ile-1781 codon conferring a nonsynonymous substitution of Leu at this position (Figure 9) along with 26 synonymous substitution (T to C at 1456, A to G at 1516, C to T at 1543, G to A at 1555, T to C at 1705, C to T at 1849 A to G at 1852, C to A at 1900, T to G at 1903, T to A at 1969, C to T at 1981, C to T at 2023, A to T at 2293, A to T at 2311, T to A 2323, C to T at 2374, A to G at 2431, T to C at 2474, C to T at 2549, C to T at 2593, A to G at 2617, T to C at 2650, T to C at 2719, C

to G at 2932, and C to G at 2971) in the carboxyl transferase domain of the plastid ACCase for both R1 and R2 biotypes (Figure 10).

Identification of an Ile-1781-Leu substitution in R2 supported our initial hypothesis of target-based resistance mechanism determination and our suspicions of a lack of adequate amplification to all homeologs of plastidic ACCase mRNA with standard PCR. Sequencing reads of R1 and R2 biotypes have been submitted to NCBI Sequence Read Archive (SRA) database (accession numbers PRJNA524359 and PRJNA524643, respectively). The sequence assemblies to NCBI Transcriptome Shotgun Assembly (TSA) project have been deposited at DDBJ/EMBL/GenBank under the accession GHOH00000000. The version described in this paper is the first version, GHOH01000000. The amino acid substitution, Ile-1781-Leu, therefore, in the resistant biotypes could be a possible causal resistance mechanism to ACCase-inhibiting herbicides in *D. ciliaris*. SNPs in ACCase mapping of both resistant biotypes were also found in a 1:2 ratio where approximately 33% of mapped reads contains SNPs. A 1:2 ratio likely indicates that *D. ciliaris* is a hexaploid as well, but futher research is needed to confirm this hypothesis.

## v. Conclusion

The insensitivity of the ACCase target site has been reported as the most common mechanism of resistance to ACCase inhibiting herbicides (Kuk et al. 2008). Using a combination of standard PCR based sequencing, vector sequencing, and Illumina sequencing, we identified a common amino acid, Ile-1781-Leu substitution in the plastid ACCase gene of *D. ciliaris* resistant biotypes. The double peak in the PCR chromatogram (expected as A or C) in the center of the nucleotide at position 1781 in the resistant biotypes R1, suggesting the resistant biotypes can express at least two different plastidic ACCase gene. Our finding of ACCase resistance Ile-1781-Leu substitution to DIMs and FOPs is consistent with the vast majority of literature on the subject

in *A. myosuroides* Huds. (black grass) (Brown et al. 2002; Délye et al. 2002a, 2002b; Petit et al. 2010), *L. rigidum* Gaud. (rigid ryegrass) (Zagnitko et al. 2001), *L. multiflorum* Lam. (Italian ryegrass) (White et al. 2005; Zhang and Powels 2006a, 2006b; Yu et al. 2007), *S. viridis* (L.) P. Beauv. (foxtail millet) (Délye et al. 2002c), *A. fatua* L. (wild oat) (Christoffers et al. 2002), *A. sterilis* ssp. Ludoviciana (sterile oat) (Liu et al. 2007; Torres-García et al. 2018), and *Zea mays* L. (maize) (Genbank accession numbers. AF359517 and AF359518; Zagnitko et al. 2001). A target-site mutation does not preclude other possible resistance mechanisms contributing to overall resistances response for examples, Maneechote et al. (1999) found that the FOPs resistant *A. sterilis* biotype with target-site ACCase mutations and enhanced metabolisms, but Cyt-P450 inhibitors reduced tolerance to diclofop in the resistant biotype. Yu et al. (2013) tested hexaploid *A. fatua* and concluded that the lower level of target-site ACCase resistance in polyploids than diploids weed species especially Poaceae family due to the herbicide dilution effect. Torres-García et al. (2018) found that the ACCase gene alteration with Ile-1781-Leu showed enhanced metabolism in *A. fatua* for resistance test. All the experiments implied ACCase gene mutations metabolic adaptation, as well as detoxification mechanisms, can be contributed to resistance in the resistant biotype.

Based on our finding, we reached three major conclusions, first the *D. ciliaris* biotypes (Yu et al. 2017) previously reported as resistant to sethoxydim and mildly resistant to some FOP herbicides are also resistant to pinoxaden relative to the susceptible biotype. The *D. ciliaris* biotypes responded similarly to pinoaxden and other ACCase-inhibiting herbicides. Prior selection pressure with DIMs and FOPs herbicides could result in the evolution of *D. ciliaris* cross-resistance to pinoxaden herbicide in United States. Secondly, the amino acid substitution, Ile-1781-Leu in the ACCase gene is the likely causal mechanism of resistance in *D. ciliaris.* Mutation at

Ile-1781 is a common substitution site that yielded resistance to ACCase-inhibiting herbicides in weed species tested. The authors acknowledge that other NTSR mechanism may be simultaneously occurring contributing to resistance. Thirdly, while not an initial part of our original research goals, reliance on PCR to amplify all expressed copies of plastidic ACCase in a weed species could lead to erroneous conclusions, whereas NGS transcriptome profiling was able to identify polymorphism previously missed by the standard PCR. Such a conclusion is especially important with respect to ACCase resistance where both target and non-target resistance mechanisms can occur simultaneously, and a lack of adequate PCR-based amplification could cause one to conclude target-site resistance is absent in a population. This is the first reported case of cross-resistance to pinoxaden from previously identified ACCase resistant biotypes and the target-based resistance to ACCase-inhibiting herbicides in *D. ciliaris* species from managed turfgrass.

**Table 1.** List of five primer pair used in this study to detect single nucleotide polymorphisms in *D. ciliaris*

| Primer name | Forward (5′ to 3′) | Reverse (5′ to 3′) | Target Site Substitutions | Annealing Temperatures (°C) |
|---|---|---|---|---|
| ACCase1781 | CCCTGAACGTGGGTTTCAGT | GCCATGATCTTAGGACCACCC | Ile-1781-Leu | 59 |
| ACCase1 | GGCATAGCGGATGAGGTGAA | GCAGCCATTCTGAGGGAAGT | Ile-1781-Leu, Thr-1805-Ser, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly | 61 |
| ACCase2 | CCCATATGCAGTTGGGTGGT | CATGCAACTCAGCAAACCGT | Lys-1930-Arg, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly | 58 |
| ACCase3 | TGGGTGGTATGTTCGACAAAG | ACCCAGCCTGAAGAATCCCTT | Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val | 60 |
| ACCase14 | CAGTGGTTACTGGCAGAGCA | TCCATTTCCAACCTTTGCACC | Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly Gly-2096-Ala | 58 |

**Table 2.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing pinoxaden rate relative to the nontreated control was measured through the model with equations of exponential growth for percent control. The required rate of pinoxaden was also calculated by 50% ($I_{50}$) and 90% ($I_{90}$) based on control at 14, 28 and 42 days after treatment. Parameter estimates, 95% confidence intervals (CI), as well as values of $I_{50}$ and $I_{90}$ are presented as means of model comparison

| Control[a] | Biotype[b] | Equation[c] | $R^2$ | Parameter estimates and confidence intervals | | | | Inhibition[d] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $y = a * exp^{bx}$ | | a | 95% CI | b | 95% CI | $I_{50}$ (kg) | R/S ratio | $I_{90}$(kg) | R/S ratio |
| 14 DAT | R1 | $y = 7.2 * exp^{1.6x}$ | 0.94 | 7.2 | (3.4, 11.1) | 1.6 | (1.2, 2.0) | 16.2 | 14.6 | 37.7 | 4.1 |
| | R2 | $y = 13.1 * exp^{1.5x}$ | 0.95 | 13.1 | (7.2, 18.9) | 1.5 | (1.1, 1.8) | 8.3 | 7.4 | 20.8 | 2.3 |
| | S | $y = 48.5 * exp^{0.6x}$ | 0.87 | 48.5 | (3.9, 58.9) | 0.6 | (0.4, 0.9) | 1.1 | | 9.2 | |
| 28 DAT | R1 | $y = 14.9 * exp^{1.4x}$ | 0.95 | 14.9 | (9.5, 20.5) | 1.4 | (1.1, 1.7) | 7.4 | 14.3 | 18.2 | 2.7 |
| | R2 | $y = 22.1 * exp^{1.2x}$ | 0.95 | 22.1 | (15.1, 29.0) | 1.2 | (0.9, 1.4) | 4.9 | 9.5 | 15.7 | 2.3 |
| | S | $y = 58.0 * exp^{0.5x}$ | 0.81 | 58.0 | (45.9, 70.2) | 0.5 | (0.3, 0.7) | 0.5 | | 6.8 | |
| 42 DAT | R1 | $y = 9.5 * exp^{1.8x}$ | 0.92 | 9.5 | (2.4, 16.6) | 1.8 | (1.2, 2.4) | 8.5 | 56.9 | 19.6 | 4.0 |
| | R2 | $y = 13.9 * exp^{1.5x}$ | 0.91 | 13.9 | (5.2, 22.7) | 1.5 | (1.0, 2.0) | 6.9 | 46.2 | 16.8 | 3.4 |
| | S | $y = 68.6 * exp^{0.4x}$ | 0.72 | 68.6 | (55.9, 81.2) | 0.4 | (0.2, 0.6) | 0.2 | | 4.9 | |

[a] Abbreviations: Days after treatment (DAT), [b] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype. [c] In the exponential growth equation, x represents pinoxaden rate, y represents the response variable of control at 14, 28, and 42 days after treatment, [d]Inhibition rate: $I_{50}$ and $I_{90}$ values were calculated using exponential growth equation; R/S ratios, resistant/susceptible ratios

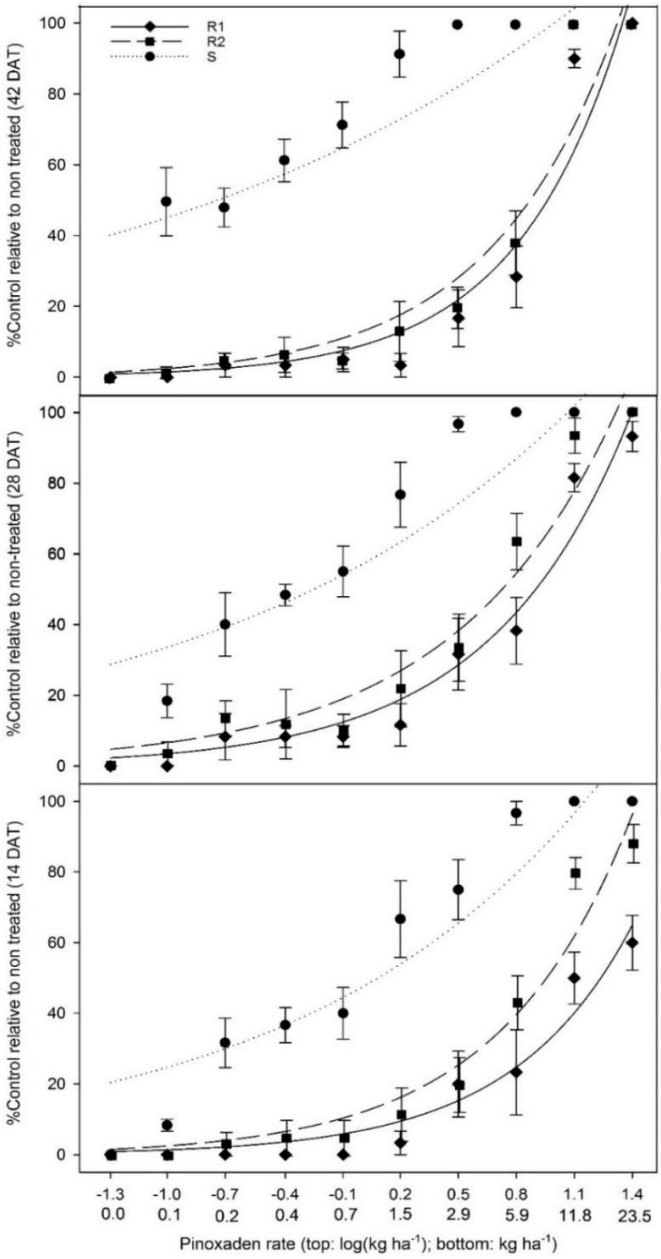



**Figure 1.** Percent visible control response relative to non-treated of resistant and susceptible *D. ciliaris* biotypes with increasing rates of pinoxaden at 14, 28, and 42 days after treatment. The response was modeled based on the log rate of pinoxaden to create equal spacing between rates using exponential growth regression. Results were pooled over experimental runs. Vertical bars represent the standard errors (P=0.05) of the means. Means (n=6) are represented by differing symbols for each biotype and regression equation model are represented by differing line type for each biotype. *D. ciliaris* biotypes: Resistant biotypes, R1 and R2, Georgia, and susceptible biotype, S, Alabama

**Table 3.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing pinoxaden rate relative to the nontreated control measured through the model with equations of sigmoidal for percent length of the tiller and above-ground biomass. The required rate of pinoxaden to reduce the measured variables of *D. ciliaris* biotypes was also calculated by 50% ($I_{50}$) and 90% ($I_{90}$). Parameter estimates and 95% confidence intervals (CI) are presented as means of model comparison

| Rating | Biotype[a] | Equation[b] | $R^2$ | Parameter estimates and confidence intervals | | | | | | Inhibition[c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $y = \frac{a}{1 + e^{-(\frac{x-x0}{b})}}$ | | A | 95% CI | b | 95% CI | $x_0$ | 95% CI | $I_{50}$ (kg) | R/S ratio | $I_{90}$ (kg) | R/S ratio |
| Tiller length | $R_1$ | $y = \frac{102.8}{1 + e^{-(\frac{x-0.9}{-0.1})}}$ | 0.98 | 102.8 | (108.2, 97.4) | -0.1 | (-0.06, -0.18) | 0.9 | (0.9, 0.8) | 7.2 | 10.4 | 13.2 | 6.2 |
| | R2 | $y = \frac{101.6}{1 + e^{-(\frac{x-0.8}{-0.1})}}$ | 0.99 | 101.6 | (105.7, 97.5) | -0.1 | (0.09, -0.18) | 0.8 | (1.0, 0.6) | 6.9 | 9.9 | 8.6 | 4.1 |
| | S | $y = \frac{100.9}{1 + e^{-(\frac{x-(-0.2)}{-0.2})}}$ | 0.99 | 100.9 | (109.1, 92.8) | -0.2 | (-0.15, 0.29) | -0.2 | (-0.1, -0.3) | 0.7 | | 2.1 | |
| Above-ground biomass | $R_1$ | $y = \frac{128.7}{1 + e^{-(\frac{x-0.9}{-0.1})}}$ | 0.91 | 128.7 | (142.9, 114.4) | -0.1 | (0.07, -0.19) | 0.9 | (1.1, 0.7) | 7.7 | 4.8 | 10.2 | 4.5 |
| | R2 | $y = \frac{121.0}{1 + e^{-(\frac{x-0.9}{-0.02})}}$ | 0.93 | 121.0 | (132.4, 109.7) | -0.02 | (32.15, -32.19) | 0.9 | (140.9, -139.2) | 7.2 | 4.5 | 7.9 | 3.5 |
| | S | $y = \frac{145.0}{1 + e^{-(\frac{x-0.2}{-0.1})}}$ | 0.93 | 145.0 | (165.6, 124.4) | -0.1 | (0.16, -0.31) | 0.2 | (0.3, 0.1) | 1.6 | | 2.3 | |

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype. [b] In the sigmoidal equation, x represents pinoxaden rate, y represents the response variable of tiller length and above-ground biomass relative to the non-treated check. [c]Inhibition rate: $I_{50}$ and $I_{90}$ values were calculated using sigmoidal equation; R/S ratios, resistant/susceptible ratios

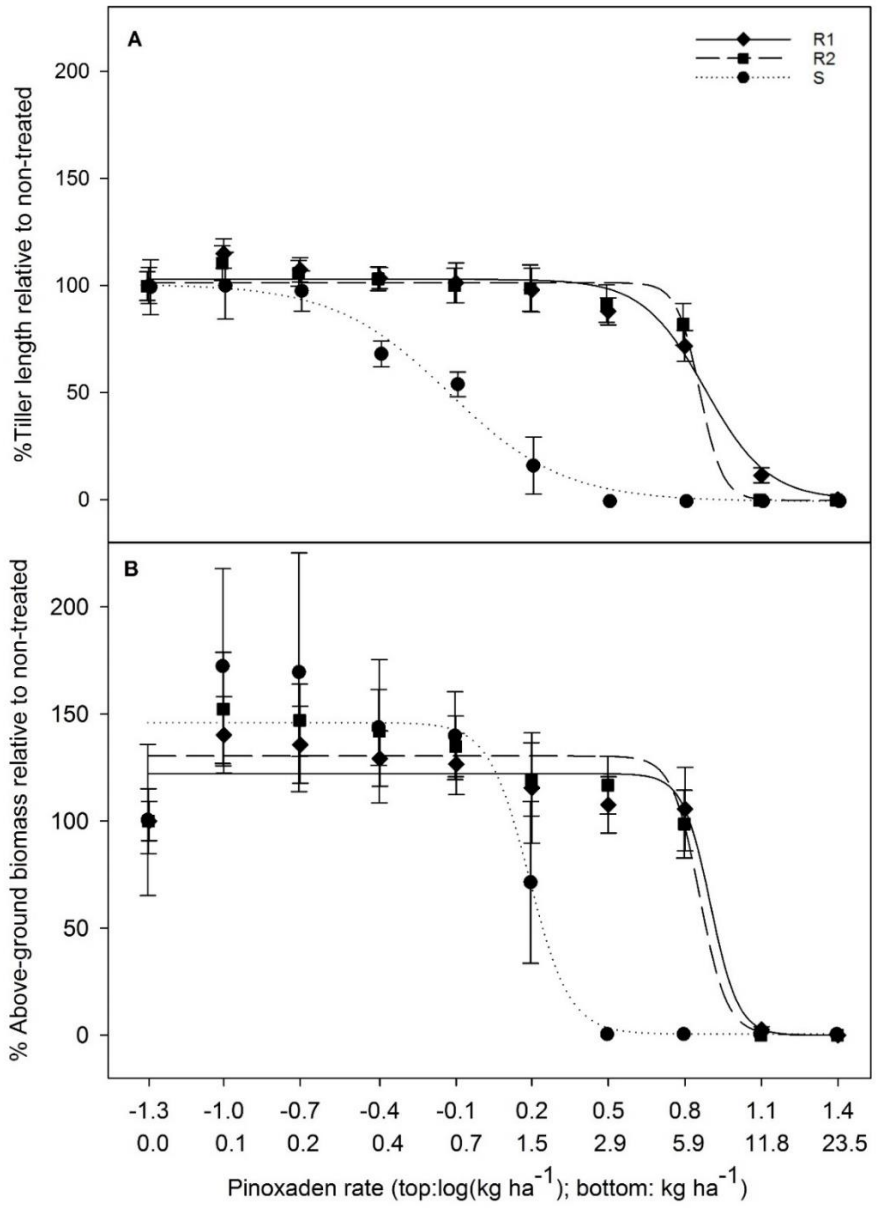



**Figure 2.** Percent tiller length (A) and percent above-ground biomass (B) response in resistant and susceptible *D. ciliaris* biotypes 42 days after treatment with increasing rates of pinoxaden. The tiller length (cm) and above-ground biomass weight (g) were expressed as a percentage (%) of the non-treated control. The response was modeled based on the log rate of pinoxaden to create equal spacing between rates using sigmoidal regression. Results were pooled over experimental runs. Vertical bars represent the standard errors (P=0.05) of the means. Means (n=6) are represented by differing symbols for each biotype and regression equation model are represented by differing line type for each biotype. *D. ciliaris* biotypes: Resistant biotypes, R1 and R2, Georgia, and susceptible biotype, S, Alabama

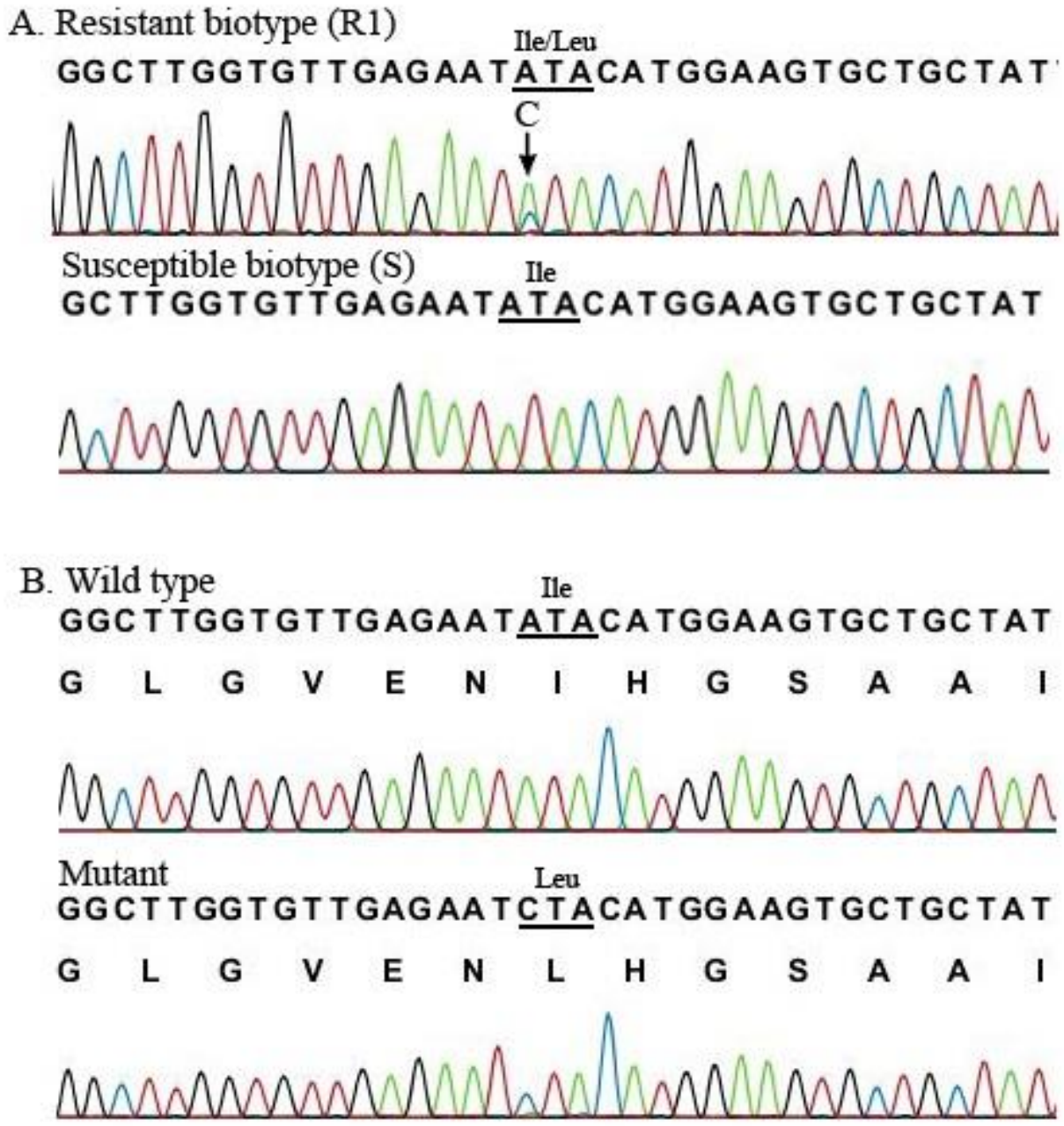



**Figure 3.** Chromatogram of nucleotide sequence surrounding ATA codon encoding Ile-1781. The double peak indicates an amino acid substitution of CTA coding for Leu-1781 in the resistant biotype R1 (A). Nucleotide sequences from cloned cDNA fragments of transgenic and non-transgenic alleles surrounding the Ile-1781 codon. An Ile-1781 to Leu substitution confers to ACCase-inhibiting herbicides, DIMs, FOPs, and pinoxaden resistance in *D. ciliaris*. Cloned cDNA fragments showed the ATA codon for Ile in non-transgenic allele (wild type) and the CTA codon for Leu in transgenic allele (mutant) at position 1781 (B)

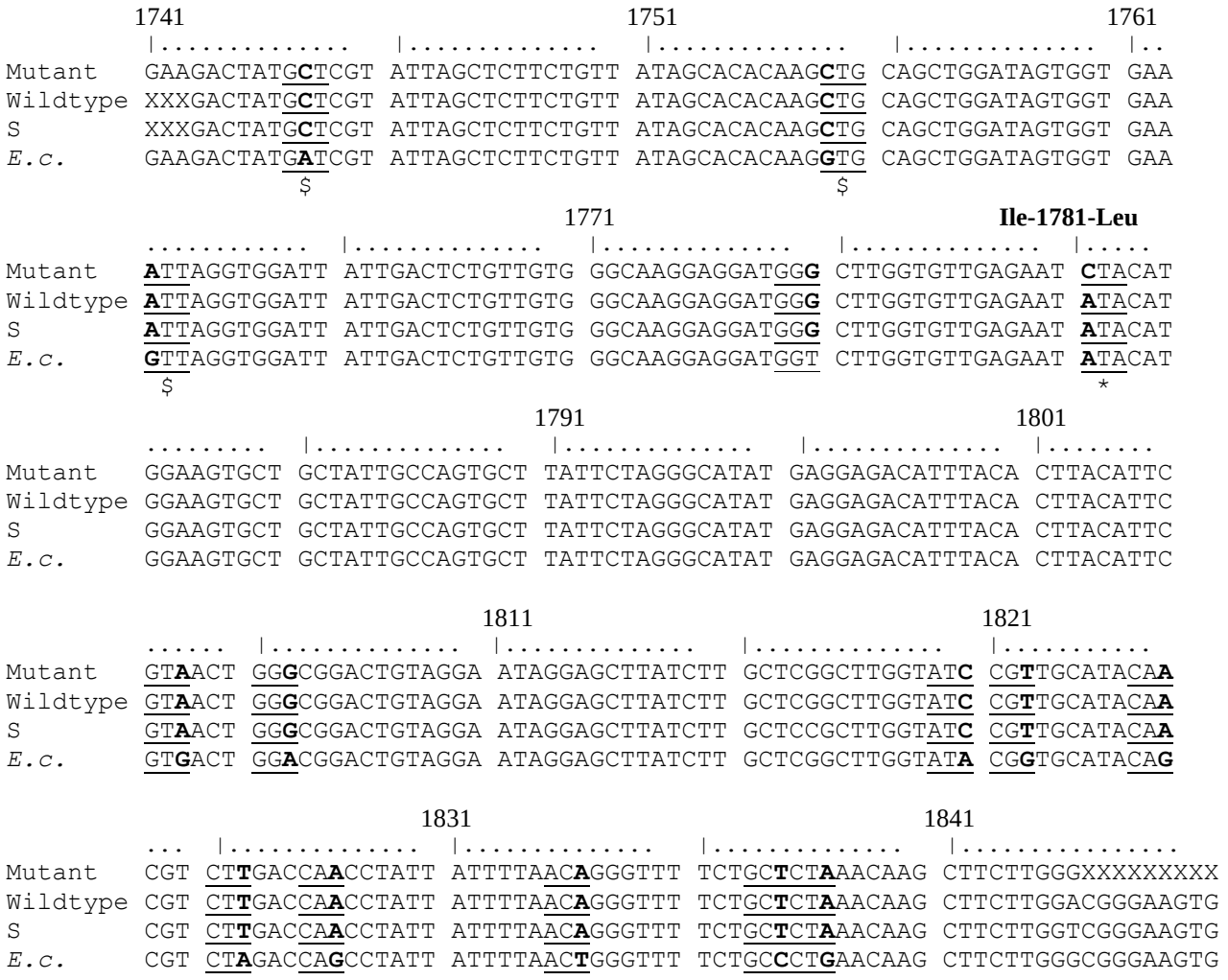



**Figure 4.** Nucleotide sequences of mutated and wild type cDNA fragments surrounding the Ile1781 to Leu substitution in resistant biotype, R1. Sequences were aligned with susceptible biotype (S), and *E. crus-galli* (*E.c.*) (accession number: KU198448). Silent codons were bolded, and underlined, missense codons were bolded, underlined, and marked by using '$', and amino acid substitution (ATA to CTA) were bolded, underlined, and marked with '*'.

```
                1741         1751          1761         1771          Ile-1781-Leu
          .... |.......... |.......... |.......... |.......... |..........
Mutant    ..TE EDYARISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN LHGSAAIASA
Wildtype  ..XX XDYARISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN IHGSAAIASA
S         ..XX XDYARISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN IHGSAAIASA
E.c.      ..TE EDYDRISSSV IAHKVQLDSG EVRWIIDSVV GKEDGLGVEN IHGSAAIASA
                    $            $        $                      *

          1791         1801         1811         1821         1831         1841
          |.......... |.......... |.......... |.......... |.......... |........
Mutant    YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGXXX..
Wildtype  YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREV..
S         YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREV..
E.c.      YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREV..
```

**Figure 5.** Amino acid sequences of mutated and non-transgenic wild type cDNA fragments of resistant biotype, R1. Transcribed amino acid sequences were aligned with susceptible biotype (S) and *E. crus-galli* (*E.c.*) (accession number: KU198448). Silent codons in amino acids were underlined. Missense codons, which were presented in both susceptible and resistant biotypes underlined and marked by using '$'. The amino acid substitution lle-1781 to Leu were bolded, underlined and marked with '*'.

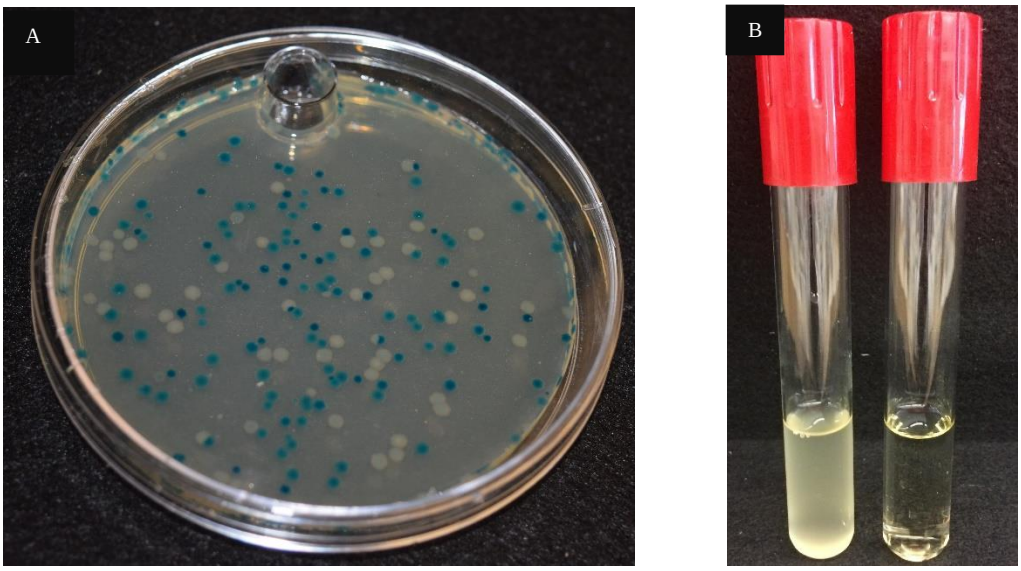



**Figure 6.** Separation of double peaks in resistant biotype with JM109 high efficiency competent cells of *E. coli*. Amplified plasmid vector with ACCase gene produced two different types, white and blue of colonies on media containing LB broth, ampicillin, X-gal, and IPTG (A). Incubation of transformed cell into LB-Amp solution (B)

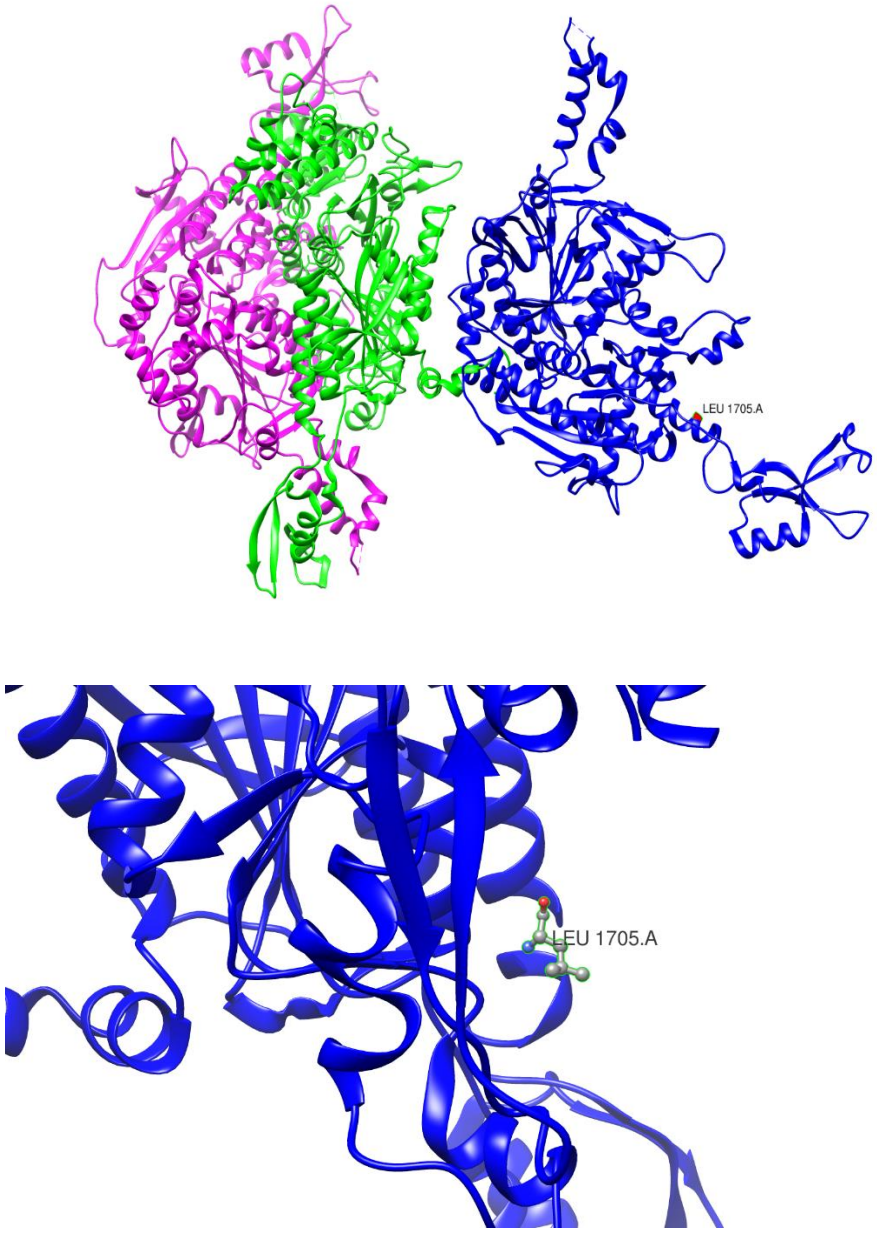
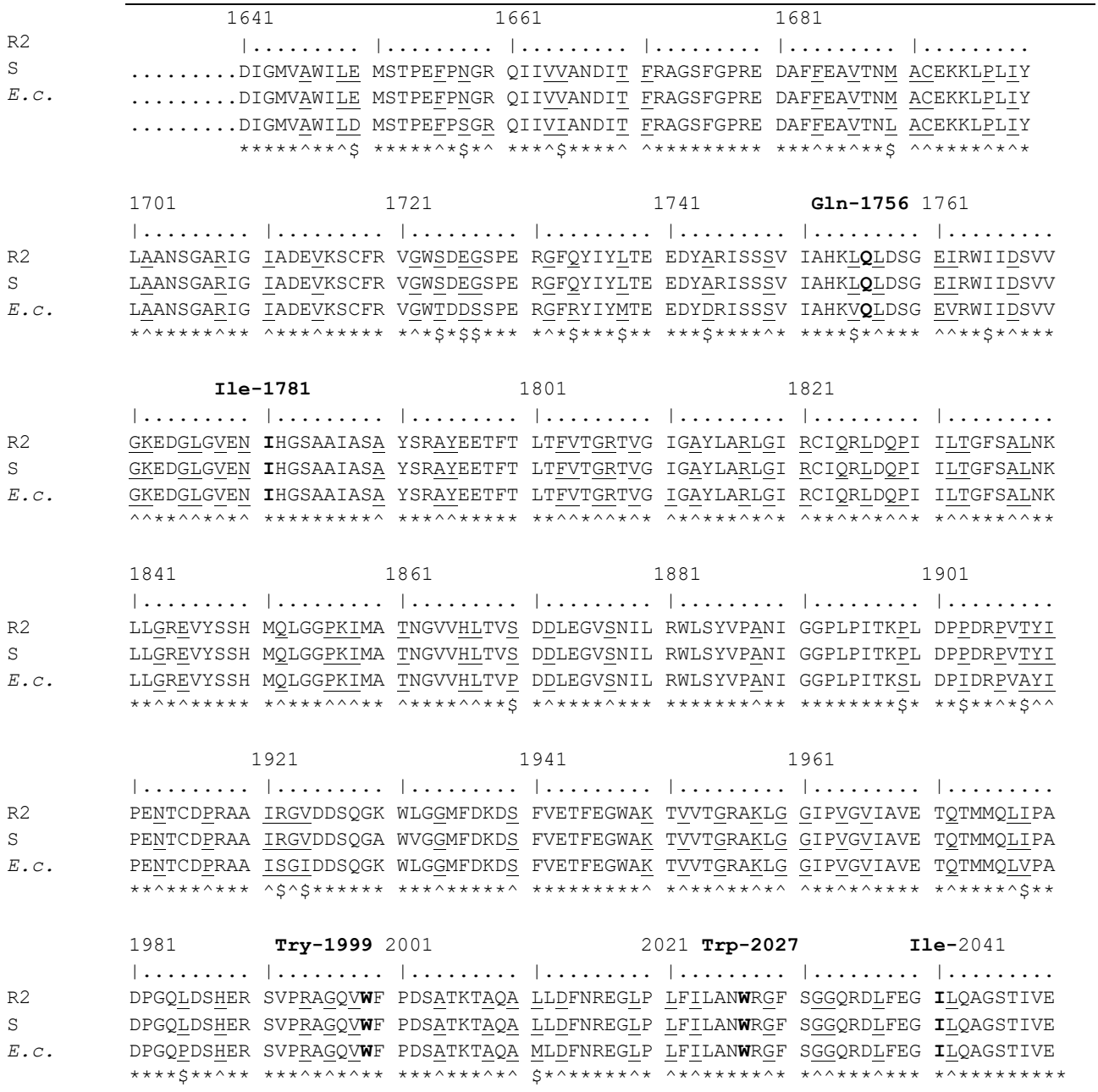



**Figure 7.** An amino acid substitution, Ile1781 to Leu of the 3D structure of CT domain chain A location showing in yeast ACCase (A). Leu-1705 is equivalent to a site of resistance mutations Leu-1781 in *D. ciliaris* and labeled by red color at the position Leu-1705 (B). This diagram was performed with protein data bank ID code 1UYS and Chimera v. 1.12


1641 1661 1681
R2
S
E.c.
.....DIGMVAWILE MSTPEFPNGR QIIVVANDIT FRAGSFGPRE DAFFEAVTNM ACEKKLPLIY
.....DIGMVAWILE MSTPEFPNGR QIIVVANDIT FRAGSFGPRE DAFFEAVTNM ACEKKLPLIY
.....DIGMVAWILD MSTPEFPSGR QIIVIANDIT FRAGSFGPRE DAFFEAVTNL ACEKKLPLIY
1701 1721 1741 Gln-1756 1761
LAANSGARIG IADEVKSCFR VGWSDEGSPE RGFQYIYLTE EDYARISSSV IAHKLQLDSG EIRWIIDSVV
LAANSGARIG IADEVKSCFR VGWSDEGSPE RGFQYIYLTE EDYARISSSV IAHKLQLDSG EIRWIIDSVV
LAANSGARIG IADEVKSCFR VGWTDDSSPE RGFRYIYMTE EDYDRISSSV IAHKVQLDSG EVRWIIDSVV
Ile-1781 1801 1821
GKEDGLGVEN IHGSAAIASA YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK
GKEDGLGVEN IHGSAAIASA YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK
GKEDGLGVEN IHGSAAIASA YSRAYEETFT LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK
1841 1861 1881 1901
LLGREVYSSH MQLGGPKIMA TNGVVHLTVS DDLEGVSNIL RWLSYVPANI GGPLPITKPL DPPDRPVTYI
LLGREVYSSH MQLGGPKIMA TNGVVHLTVS DDLEGVSNIL RWLSYVPANI GGPLPITKPL DPPDRPVTYI
LLGREVYSSH MQLGGPKIMA TNGVVHLTVP DDLEGVSNIL RWLSYVPANI GGPLPITKSL DPIDRPVAYI
1921 1941 1961
PENTCDPRAA IRGVDDSQGK WLGGMFDKDS FVETFEGWAK TVVTGRAKLG GIPVGVIAVE TQTMMQLIPA
PENTCDPRAA IRGVDDSQGA WVGGMFDKDS FVETFEGWAK TVVTGRAKLG GIPVGVIAVE TQTMMQLIPA
PENTCDPRAA ISGIDDSQGK WLGGMFDKDS FVETFEGWAK TVVTGRAKLG GIPVGVIAVE TQTMMQLVPA
1981 Try-1999 2001 2021 Trp-2027 Ile-2041
DPGQLDSHER SVPRAGQVWF PDSATKTAQA LLDFNREGLP LFILANWRGF SGGQRDLFEG ILQAGSTIVE
DPGQLDSHER SVPRAGQVWF PDSATKTAQA LLDFNREGLP LFILANWRGF SGGQRDLFEG ILQAGSTIVE
DPGQPDSHER SVPRAGQVWF PDSATKTAQA MLDFNREGLP LFILANWRGF SGGQRDLFEG ILQAGSTIVE

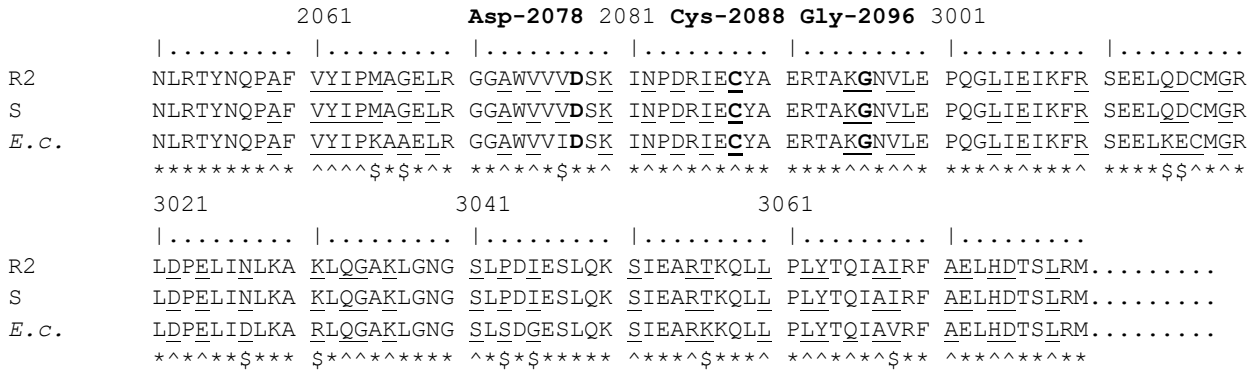



**Figure 8.** Sequence alignment of plastid ACCase gene in the carboxyltransferase domain from the R2, S, *E.c.*. The amino acids, which were identical marked with asterick (*) identity. Silent codons in amino acid were underlined and marked by using '^', and missense codons in amino acid were underlined and marked with '$'. Abbreviations: R2, Resistant biotype2; S, Susceptible biotype; *E.c.*, *E. crus-galli* (accession number: KU198448)


**Figure 9.** Illumina sequencing with single nucleotide variation of adenine (A) to cytosine (C) at 1781 region in both resistant biotypes, R1 and R2. ACCase gene of *D. ciliaris* in both resistant biotypes, R1 and R2 contained CTA codon for the amino acid Leu-1781 instead of ATA codon for Ile-1781. Comparison of Illumina sequencing of *D. ciliaris* transcriptome producing 93.9% homology to the reference gene of *E. crus-galli* (KU198448). Illumina sequence analysis at carboxyl transferase domain revealed the amino acid substitution, Ile-1781 Leu conferring resistance to DIMs, FOPs and pinoxaden in *D. ciliaris*

| Amino acid position | Frequency (Same reference) | Frequency (Different reference) | Codon | Amino acid substitution |
|---|---|---|---|---|
| 1456 | 68.48 | 31.52 | AAT/AAC | N(Asn) |
| 1516 | 66.32 | 33.33 | AGA/AGG | R (Arg) |
| 1543 | 75.65 | 24.36 | TGC/TGT | C (Cys) |
| 1555 | 75.06 | 24.94 | TTG/TTA | L (Leu) |
| 1705 | 75.18 | 24.82 | TCT/TCC | S(Ser) |
| **1781** | **60.21** | **39.58** | **ATA/CTA** | **I/L(lle/Leu)** |
| 1849 | 60.17 | 39.62 | TTC/TTT | F(Phe) |
| 1852 | 68.38 | 31.62 | GTA/GTG | V(Val) |
| 1900 | 58.67 | 41.09 | ATC/ATA | I (Ile) |
| 1903 | 70.07 | 29.93 | CGT/CGG | R(Arg) |
| 1969 | 76.66 | 22.71 | GGT/GGA | G(Gly) |
| 1981 | 69.95 | 30.05 | TAC/TAT | Y(Tyr) |
| 2023 | 70.87 | 29.13 | ACC/ACT | T(Thr) |
| 2293 | 72.8 | 26.99 | ACA/ACT | T(Thr) |
| 2311 | 74.25 | 25.75 | GCA/GCT | A(Ala) |
| 2323 | 55.75 | 43.65 | GGT/GGA | G(Gly) |
| 2374 | 73.56 | 26.27 | ATC/ATT | I(Ile) |
| 2431 | 67.52 | 32.48 | CAA/CAG | Q(Gln) |
| 2474 | 67.54 | 32.46 | TTG/CTG | L(Leu) |
| 2549 | 60.12 | 39.56 | CTG/TTG | L(Leu) |
| 2593 | 76.83 | 23.17 | AAC/AAT | N(Asn) |
| 2617 | 73.08 | 26.37 | GCA/GCG | A(Ala) |
| 2650 | 70.96 | 28.77 | CGT/CGC | R(Arg) |
| 2719 | 75.17 | 24.36 | ACT/ACC | T(Thr) |
| 2932 | 76.23 | 23.77 | ACC/ACG | T(Thr) |
| 2971 | 73.91 | 26.09 | TCC/TCT | S(Ser) |
| Average | 69.75% | 30.09% | | |

**Figure 10.** Single nucleotide variations of *D. ciliaris* ACCase gene in both resistant biotypes, R1 and R2 were shown on RNA-seq. Illumina sequence analysis of the resistant biotypes at CT domain produced 27 single nucleotide variations (SNVs). Out of 27 SNVs, only one non- synonymous mutation, Ile to Leu was found at 1781 position. The amino acid substitution was bolded, and underlined, and silent codons were underlined.

# Chapter 3

## Validation of target-site resistance mechanism to ACCase-targeting herbicides in *Digitaria ciliaris* utilizing a functional malachite green colorimetric assay

[This chapter is accepted by the journal of weed science for publication as Suma Basak, Douglas Goodwin, Jahangir Alam, James Harris, Jinesh D. Patel, Patrick McCullough, and J. Scott McElroy. 2021. Detection of ACCase-targeting herbicides effect on ACCase activity resistance utilizing a malachite green colorimetric functional assay]

### i. Abstract

Research was conducted to evaluate acetyl-Coenzyme A carboxylase (ACCase) enzyme activity using a functional malachite green colorimetric assay previously identified as resistant to sethoxydim, and select aryloxyphenoxypropionate (FOPs) herbicides, fenoxaprop, and fluazifop. Two resistant southern crabgrass (*Digitaria ciliaris*) biotypes, R1 and R2, containing an Ile-1781-Leu amino acid substitution and previously identified as resistant to sethoxydim, pinoxaden, and fluazifop but not clethodim was utilized as the resistant chloroplastic ACCase source compared to known susceptible (S) ACCase. Dose-response studies with sethoxydim, clethodim, fluazifop-p-butyl, and pinoxaden (0.63 to 40 µM) were conducted to compare the ACCase enzyme-herbicides interaction of R1, R2, and S using the malachite green functional assay. Assay results indicated that R biotypes required more ACCase-targeting herbicides to inhibit ACCase activity compared to S. $IC_{50}$ values of all four herbicides for R biotypes were consistently an order of magnitude greater than S. No sequencing differences in the carboxyltransferase domain was observed for R1 and R2, however, R2 $IC_{50}$ values were greater across all biotypes. While past research reported R, biotypes were not resistant to postemergence applied clethodim, $IC_{50}$ values for clethodim were

greater for R biotypes compared to S. These results indicate the malachite green functional assay is effective in evaluating ACCase enzyme activity of R and S biotypes in the presence of ACCase-targeting herbicides, which can be used as a replacement for the $^{14}C$-based radiometric functional assay.

Nomenclature: Acetyl-coenzyme A carboxylase (ACCase); Sethoxydim; Fluazifop-P-butyl; Pinoxaden; Clethodim; Southern crabgrass (*Digitaria ciliaris*)

Keywords: ACCase resistance, ACCase enzyme activity, ACCase-enzyme herbicide interaction**,** Malachite green functional assay

## ii. Introduction

Acetyl-coenzyme A carboxylase (ACCase or ACCs; EC.6.4.1.2) is an essential enzyme that catalyzes the formation of malonyl-CoA. The reaction product, malonyl-CoA is involved in the biosynthesis of *de novo* fatty acids in plastids and the elongation of long-chain fatty and secondary metabolites which are crucial for energy storage, cell, or organelles biomembrane structure composition, and hormonal regulation (Délye et al. 2011; Harwood, 1988; Keereetaweep et al. 2018; Konishi et al. 1996; Ohlrogge and Browse 1995; Podkowinski et al. 2003; Petit et al. 2010; Yang et al. 2018; Ye et al. 2018; Yu et al. 2007). Unlike in animals, two different isoforms of the ACCase enzyme exist in plants. One is located in the chloroplasts or plastids, which are involved in the biosynthesis of primary fatty acids up to C18 then used as a precursor for lipid biosynthesis in the endoplasmic reticulum. The other is located in the cytosol, which is involved in the synthesis of very-long-chain fatty acids up to C32 and secondary metabolites such as flavonoids and suberins (Focke et al. 2003; Harwood, 1988; Sasaki et al. 1995). The chloroplastic ACCase is a homomeric eukaryotic ACCase enzyme that includes all functional domains in a single polypeptide, or a multi-subunit isoform known as heteromeric prokaryotic ACCase enzyme consisting of four subunits whereas, the cytosolic ACCase exist in the multidomain isoform (Incledon and Hall, 1997; Konishi et al. 1996). Plants belonging to the Poaceae possess a homomeric or eukaryotic chloroplastic ACCase which contains three catalytic domains: the biotin carboxyl carrier (BCC), biotin carboxylase (BC), and carboxyltransferase (CT, with subunits α and β). (Nikolskaya et al. 1999; Tong, 2005; Wakil et al. 1983). The biotin-dependent ACCase enzyme catalyzes the production of malonyl-CoA from acetyl-CoA with the hydrolysis of ATP. The overall reaction is catalyzed by two sequential reactions through the action of three distinct protein

components. In the first reaction, the biotin carboxylase domain catalyzes the ATP-dependent carboxylation of a biotin group covalently bound to the BCC domain. In the second reaction, the carboxylated biotin translocate to the CT active site to transfer the carboxyl group from biotin to the acetyl-CoA substrate, producing malonyl-CoA (Délye 2005; Harwood,1988; Howard and Ridley,1990; Nikolau et al. 2003; Yu et al. 2010).

ACCase-targeting herbicides inhibit chloroplastic ACCase activity in grasses of the Poaceae family resulting in a decrease in fatty acid production (Lancaster et al. 2018; Powles, 2005). ACCase-targeting herbicides are commonly applied postemergence to control grass weeds in both crop and turf systems. Herbicide resistance has developed to ACCase-targeting herbicides via target-site (TSR) and non-target site resistance (NTSR) mechanisms. The TSR amino acid substitutions associated with ACCase-targeting herbicides occur in the carboxyl transferase domain with the following have been reported: Gln-1756-Glu, Ile-1781-Leu, Thr-1805-Ser, Lys-1930-Arg, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn or Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala (Beckie and Tardif, 2012; Collavo et al. 2011; Délye 2005; Powles and Yu, 2010, Kaundun 2010; Kaundun et al. 2012; Kaundun et al. 2013). NTSR encompasses a range of processes, including increased enzyme expression, increased enzyme abundance, enhanced metabolism of the herbicides, herbicide detoxification, reduced herbicide uptake, penetration, and impaired translocation. Different enzymatic activities are involved in the development of NTSR including, but not limited to, cytochrome P450 monoxygenase, glutathione-S-transferases, glycosyl-transferases, hydrolases, oxidases, and peroxidases (Cocker et al. 1999; Kaundun 2014; Kukorelli et al. 2013; Powels and Yu 2010; Preston 2003; Yuan et al. 2007).

A research component in herbicide resistance discovery a functional assay evaluating the interaction of the target site with herbicide. Functional assays measure the target-site activity of

suspected resistance biotypes compared to the known susceptible biotypes. Functional assays assess enzymatic activity in the presence of herbicides to determine if amino acid changes affect the enzyme-herbicide interaction. The most used functional assay to assess ACCase enzyme activity is a $^{14}C$ based radiometric assay (Cocker et al. 1999; Cruz-Hipolito et al. 2011; De Prado et al. 2004; Secor and Cséke 1988; Seefeldt et al. 1996; Yang et al. 2007;). The $^{14}C$ based radiometric assay, however, is expensive and requires special $^{14}C$ detection equipment, handling of radioactivity, and the enzyme can be insoluble in scintillation mixtures. Howard and Ridley (1990) found a similar inhibition concentration value comparing $^{14}C$ based radiometric assay and malachite green colorimetric assay in the maize ACCase enzyme-fluazifop-P-butyl interaction test. Comparing the $^{14}C$ based assay, the malachite green assay offers several advantages including simplicity to perform, specificity for inorganic orthophosphate, accuracy, high sensitivity, stability of the reagents, and less expensive because nonradioactive materials are used for the labeling of the enzyme-substrate (Baykov et al. 1988; Carter and Karl 1982; Geladopoulos et al. 1990; Van Veldhoven and Mannaerts, 1987).

Our research objective was to develop the methodology and evaluate the malachite green colorimetric assay as a functional assay for evaluating ACCase-herbicide interaction. To our knowledge, the malachite green functional assay has not been utilized to assess ACCase herbicides resistance as the majority have utilized the $^{14}C$ functional assay. Furthermore, our research will be the first to report ACCase activity for all three families of ACCase-targeting herbicides. Two resistant biotypes with Ile-1781-Leu amino acid substitution and a known susceptible biotype of southern crabgrass (*Digitaria ciliaris*) were utilized as a model for evaluating the effectiveness of the malachite green assay in ACCase resistance detection.

### iii. Materials and Methods

**Seed Sample Collection and Growth Condition.** Previously collected seeds of two resistant biotypes (R1 and R2) of *D. ciliaris* with confirmed resistance to select ACCase-targeting herbicides and one susceptible biotype were included in this study (Yu et al. 2017; Basak et al. 2019). To generate green plant material for enzyme extraction, seeds of resistant and susceptible *D. ciliaris* biotypes were sown in separate plastic flats containing commercial potting soil and peat moss (2:1 v/v). The plastic flats were placed for 2 weeks in a greenhouse set for 32/25$^{o}$C (day/night). Ambient lighting was used throughout the experiment with no supplemental light added. Relative humidity levels alternated between 65% during the day and 75% during the night. No supplementary fertility was provided because of the quick turnaround and no signs of nutrient stress were observed. Plastic flats were irrigated three times daily (around 0.2 cm per cycle) to provide adequate moisture.

**Malachite Green Colorimetric Assay.** Research was conducted in the Department of Chemistry and Biochemistry, Auburn University, Auburn, AL, USA. ACCase enzyme extraction and activity bioassay were performed as described by Howard and Ridley (1990) with some modifications. Enzymes were extracted in the cold chamber at 4$^{o}$C from healthy plants of three *D. ciliaris* biotypes S, R1, and R2. Approximately 10 grams of fresh leaf tissues were harvested and grounded in liquid nitrogen with a mortar and pestle and suspended in ice-cold 40 mL of enzyme extraction buffer [100 mM Tricine, pH 8.0, 5mM Dithiothreitol, 10 mM $MgCl_2.6H_2O$, 1mM $Na_2EDTA$, 0.5% (w/w) polyvinylpyrrolidone (PVP), 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was stirred for 30 min on ice and then filtered through four layers of cheesecloth. The solution was kept on ice until centrifuged at 22000*g* (Optima XE-90 Ultracentrifuge, Beckman Coulter, Inc. USA) for 30 min to remove cell debris. The pellet was

discarded, and the supernatant was collected and adjusted to 30% ammonium sulfate saturation with solid ammonium sulfate. After being stirred for 20 min, the solution was centrifuged at 22000*g* for 30 min. The supernatant was decanted, adjusted to 60% ammonium sulfate saturation, and centrifuged to allow the protein precipitation as previously described. The final pellet after the 60% precipitation was resuspended in 2 ml elution buffer [10 mM Tris, 20 mM mercaptoethanol, 1 mM $Na_2EDTA$, 1 mM Benzamidine, 10 mM $MgCl_2.\ 6H_2O$ and 20 % glycerol]. The enzyme extract was desalted on a Sephadex G-25 column equilibrated with elution buffer solution [10 mM Tris, 20 mM mercaptoethanol, 1 mM EDTA, 1 mM Benzamidine, 10 mM $MgCl_2.\ 6H_2O$ and 10% glycerol]. The enzyme extracts were frozen at -80$^oC$ and assayed within a week of extraction.

The enzyme concentration in the enzyme extracts was measured as described by Bradford assay (Bradford 1976) using bovine serum albumin as a standard. Using SDS-PAGE (Superior Protein Separation-Polyacrylamide gel electrophoresis) analysis, the enzyme extracts from S, R1, and R2 were separated and compared with maize (*Zea mays*). The assay was performed three independent extractions and each treatment was replicated three times per ACCase-targeting herbicides doses such as sethoxydim (Segment®, BASF, Research Triangle Park, NC), clethodim (Envoy ®, Valent, Walnut Creek, CA), fluazifop -p-butyl (Fusillade®, Syngenta, Greensboro, NC), and pinoxaden (Axial®, Syngenta, Greensboro, NC). The assay was carried out using 96 well plates (TECAN®, Morrisville, NC) where each well contained a total of 250 µL of the reaction mixture. Each reaction mixture contained 25 µL of enzyme extracts, 25 µL ACCase-targeting herbicides at a series of concentrations (0, 0.63, 1.25, 2.5, 5.0, 10, 20, and 40 µM) and 150 µL of the enzyme assay buffer [0.1 M Tricine, pH 8.0, 15 mM KCl, 3 mM $MgCl_2.\ 6H_2O$ 1 mM Dithiothreitol, 0.01 BSA 120 mM $NaHCO_3$, 25 mM ATP]. 25 µL of Acetyl CoA (lithium salt, final concentration 4.5 mM) was then added to start the reaction. All the reaction mixtures were incubated at 30$^oC$ for 20

min. The reaction was terminated by the addition of 25 µL malachite green solution [72.9 mg malachite green dissolved in 200 mL molecular grade deionized water, filtered through 0.45 µm PTFE filter, and then mixed with ammonium molybdate in 12.1 M HCl and 10% Triton-X]. Standard curves were generated with inorganic phosphate containing nontreated control, 1.25, 2.5, 0.5, 1.0, 2.0, and 4.0 µM concentration dissolved in water and added to the wells prior to the addition of 25 µL malachite green solution. The absorbance of ACCase-enzyme activity was monitored at 630 nm colorimetrically on a microplate photometer (TECAN®, microplate photometer, Morrisville, NC) and was expressed as a percentage of the nonherbicidal control (Figure 1).

The design of the experiment was replicated twice in time as a completely randomized design with three replications. An analysis of variance (ANOVA) using the GLM procedure in Statistical Analysis System (SAS version 9.4, RTP, NC) was performed on all data to detect the significant differences among the herbicide concentrations and biotypes. Linear model was developed with herbicide treatment, herbicide rates, biotypes, replication, and experiments repeated in time as main effects. Experimental run by herbicide treatment by biotype was evaluated as an indicator of differences in experimental runs. The data were pooled over runs for subsequent analysis since differences between the data of the two experimental runs were not detected in the analysis of variance at the 0.05 probability level. Regression models developed using Prism (GraphPad Software, version 5.0, Inc., La Jolla, CA). ACCase-targeting herbicide concentrations causing 50% inhibition of the ACCase enzyme activity ($IC_{50}$) values were estimated using nonlinear regression models. The following non-linear regression analysis used to calculate the $IC_{50}$ value in the enzymatic experiment:

$$Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC_{50})))$$

Y is the enzyme response (%), x is log-transformed ACCase-targeting herbicides concentration (µM), Top and bottom are the plateaus in the units of the Y-axis, and LogIC50 is the log-transformed ACCase- targeting herbicides concentration (µM). 95% confidence intervals ($\alpha$=0.05) for the estimates calculated for nonlinear-regression model parameters. Regression equations were used to calculate inhibition concentration values at 50% (referred to as $IC_{50}$ values) compared to that of the nontreated for each biotype and each ACCase-targeting herbicide. The $IC_{50}$ and R/S values (ratio of R to S $IC_{50}$ values) were determined for each resistant biotype versus susceptible biotype. Percent ACCase enzyme activity relative to the nontreated response to ACCase-targeting herbicides was modeled for all three biotypes using the least-squares fit model to allow for calculation of $IC_{50'S}$ (Figure 2) presented in Table 1.

### iv. Results and Discussion

Herbicide treatment by biotype by experimental run interactions was non-significant ($P > 0.05$) for ACCase-enzyme activity; therefore, data were pooled over the experimental run. Herbicide treatment by biotype was significant ($P < 0.05$) for all four herbicides tested. Data presented will focus on biotype response to increasing concentrations of the four herbicides tested. ACCase activity in the presence of a given herbicide concentration was expressed as a percentage of enzyme activity reduction relative to no herbicide. In general, 40 µM of all four herbicides resulted in complete diminished ACCase activity for all three biotypes. $IC_{50}$ values were, however, consistently higher for ACCase enzyme activity from R1 and R2 biotypes relative to the S biotype (Figure 2). Depending on the herbicide under evaluation, $IC_{50}$ for R1 ACCase was 7.6–21.9 fold higher than for S ACCase, and $IC_{50}$ for R2 ACCase was from 16.3–58.7 fold higher than for S ACCase (Table 1).

R1 and R2 had higher ACCase enzyme activity to sethoxydim compared to S (Figure 2). For example, sethoxydim at 0.63 µM inhibited S ACCase enzyme activity 41%, while R1 and R2 ACCase activity were inhibited 7.5 and 3.7%, respectively. Sethoxydim at 1.3, 2.5, and 5 µM inhibited S ACCase-enzyme activity 59.5, 73.2, and 81.1%, respectively, while R1 activity was inhibited 11.2, 19.4, and 34.9%, respectively, and the R2 biotype activity was inhibited 6.9, 12.1, 23.4%, respectively. Sethoxydim at 10 and 20 µM inhibited S biotype ACCase enzyme activity 88.3 and 91.2%, respectively, whereas R1 was inhibited 68 and 78.8%, respectively, and R2 was inhibited 38.1 and 54.3%, respectively. R1 and R2 $IC_{50}$ values were 15.3 and 41.1 µM respectively, compared to 0.7 µM for S, which was 21.9-fold higher than S for R1 and 58.7-fold higher than S for R2.

Previous research reported R1 and R2 were not resistant to clethodim when foliar applied (Yu et al. 2017). However, S was relatively more sensitive to clethodim than R1 and R2 (Figure 2). For example, clethodim at 0.63, 1.3, and 2.5 µM inhibited S ACCase enzyme activity 48.3, 64.8, and 75.9%, respectively, while R1 was inhibited 14.9, 27.4, and 44.5%, respectively and R2 10.7, 19.7, and 30.6% respectively. R1 and R2 $IC_{50}$ values for clethodim were 3.5 and 7.5 µM, respectively, compared to 0.46 µM for S, which was 7.6 and 16.3-fold higher for R1 and R2, respectively, than S. The assay was sensitive enough to detect a difference in the inhibition of R1 and R2 by clethodim, which was unexpected based on previous postemergence applications. R1 and R2 ACCase enzyme activity were relatively lower in the presence of clethodim compared to sethoxydim, fluazifop-p-butyl, and pinoxaden which may explain the difference in whole plant response observed previously.

Like sethoxydim, fluazifop-p-butyl and pinoxaden inhibited ACCase activity of S more than that of R1 and R2. Fluazifop-p-butyl at 0.63 to 10 µM inhibited S biotype ACCase enzyme

activity 47.1 to 94.2% while the R1 and R2 biotypes enzyme activity were inhibited 10.8 to 64.4% and 7.1 to 52%, respectively. R1 and R2 $IC_{50}$ values for fluazifop-p-butyl were 8.9 and 17.1 µM compared to 0.5 µM for S, which was 17.8 and 34.2-fold higher for R1 and R2, respectively than S. Pinoxaden at 0.63 to 10 µM inhibited S ACCase enzyme activity 26.4 to 87%, respectively, while R1 activity was inhibited 8.6 to 55.4%, respectively, and R2 activity was inhibited 3.2 to 45.9 %, respectively. R1 and R2 $IC_{50}$ values for pinoxaden were 12.7 and 28.4 µM, respectively, compared to 1.5 µM for S, which was 8.5 and 18.9-fold higher for R1 and R2, than S.

## v. Conclusion

The malachite green assay was effective in differentiating ACCase enzyme activity against ACCase-targeting herbicides for *D. ciliaris* susceptible and resistant biotypes with Ile-1781-Leu amino acid substitution (Yu et al. 2017, Basak et al. 2019). However, two unanticipated results were observed for this assay. First, ACCase enzyme activity of R1 and R2 biotypes were inhibited at different extents for each herbicide. This result can only be because of differential interaction with the ACCase substrate and the tested ACCase-targeting herbicide as no absorption, translocation, or metabolism is at play as would be when screening whole plants. The ACCase carboxyl transferase domains were sequenced and reported for S, R1, and R2 previously (Basak et al. 2019). No other amino acid substitutions except Ile-1781-Leu were observed between R1 or R2 that would explain the difference between these biotypes (Figure 3). Therefore, the specific mechanisms behind a lower ACCase enzyme herbicide inhibition of the R2 biotype compared to R1 and S remain unknown. We theorize that R2 expresses more resistant chloroplastic ACCase homoeologs containing Ile-1781-Leu compared to non-resistant homoeologs. *D. ciliaris* is a polyploid species with ACCase encoded on separate progenitor genomes (Adoukonou-Sagbadja et al. 2007; Bennett et al. 2000). Enzyme extraction for this procedure provides a bulk sample of

all translated ACCase resulting in a mixture of resistant and susceptible chloroplastic ACCase isoforms in the extract. While the total amount of enzyme is the same in each sample it is unknown what the ratio of resistant to susceptible isoforms is in an extracted enzyme sample.

Second, the previous research indicated that R1 and R2 biotypes were not resistant to clethodim, however, our results showed that R1 and R2 ACCase enzyme was inhibited less than S. Based on these results, the Ile-1781-Leu amino acid substitution does reduce the ability of clethodim to inhibit ACCase activity, but not to a degree that would result in less control in the field for the tested biotypes based on past results by Yu et al. (2017). Both unexpected results are seen as positive results as they indicate the sensitivity in detecting subtle ACCase to ACCase-inhibiting herbicide interactions that will be beneficial for determining mechanisms of resistance in the future. Based on these results we conclude that the malachite green assay is a highly sensitive assay for measuring ACCase activity as a functional assay for ACCase-targeting herbicides resistance. While only the Ile-1781-Leu amino acid substitution was evaluated, we see no reason that other known mutations could not be evaluated in the same system. Utilization of the malachite green assay in the future will eliminate the need for a $^{14}C$ based radiometric assay and may uncover other unknown subtle differences in ACCase to herbicide interactions that are still unknown.

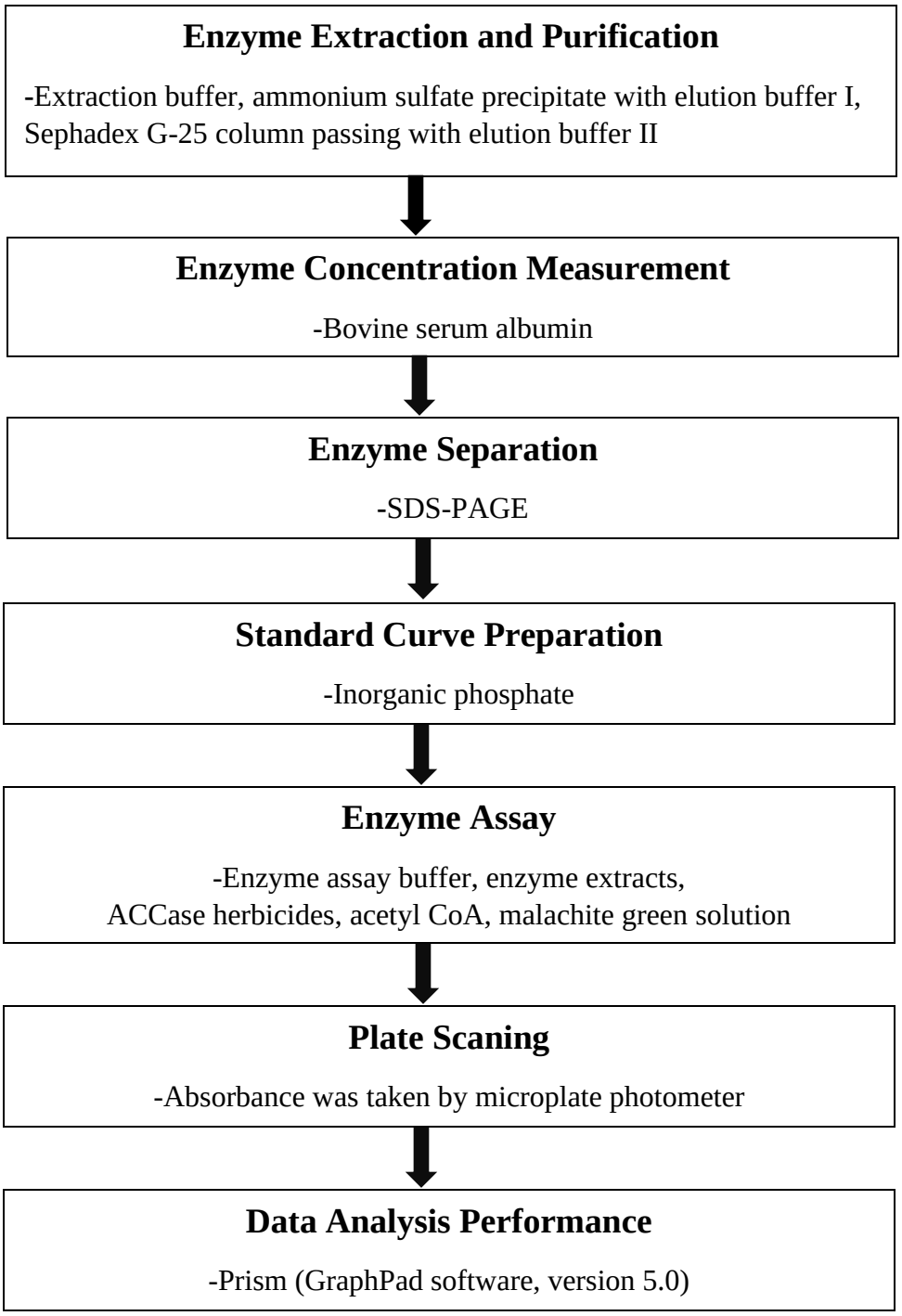



**Figure 1.** Workflow for the measurement of ACCase enzyme activity with malachite green colorimetric assay

**Table 1.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing concentrations relative to the nontreated control measured with the least-squares fit model for percent of ACCase enzyme activity. The concentration of ACCase-targeting herbicides, sethoxydim, pinoxaden, fluazifop, and clethodim required to cause 50% in-vitro inhibition of ACCase enzyme activity ($IC_{50}$) was calculated from concentration-response curves. Parameter estimates, 95% confidence intervals (CI), values of $IC_{50's}$ as well as R/S are presented as means of model comparison

| Biotype[a] | Equation[b] | Parameter estimates and confidence intervals | | | | | | Inhibition[c] | |
|---|---|---|---|---|---|---|---|---|---|
| | Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) | Bottom | 95% CI | Top | 95% CI | Log IC50 | 95% CI | $IC_{50}$ (µM) | R/S ratio |
| | | | | Sethoxydim | | | | | |
| R1 | Y=-38.3+(99.5+38.3)/(1+10^((X-1.2))) | -38.3 | (-42.4, -34.2) | 99.5 | (98.6, 100.5) | 1.2 | (1.1, 1.2) | 15.3 | 21.9 |
| R2 | Y=-103.5+(99.7+103.5)/(1+10^((X-1.6))) | -103.5 | (-117.7, -89.3) | 99.7 | (98.8, 100.6) | 1.6 | (1.6, 1.7) | 41.1 | 58.7 |
| S | Y=-3.3 + (114.2+3.3)/(1+10^((X+0.2))) | 3.3 | (1.4, 5.2) | 114.2 | (109.4,118.9) | -0.2 | (-0.2, -0.1) | 0.7 | |
| | | | | Clethodim | | | | | |
| R1 | Y=-6.9+(102.1+6.9)/(1+10^((X-0.5))) | -6.9 | (-9, -4.8) | 102.1 | (100.3, 103.9) | 0.5 | (0.5, 0.6) | 3.5 | 7.6 |
| R2 | Y=-17.1+(99.1+17.1)/(1+10^((X-0.9))) | -17.1 | (-21.2, -13) | 99.1 | (97.2, 101.1) | 0.9 | (0.8, 0.9) | 7.5 | 16.3 |
| S | Y=4.2+(120.1-4.2)/(1+10^((X+0.3))) | 4.2 | (2.3, 6.1) | 120.1 | (114.2, 125.9) | -0.3 | (-0.4, -0.3) | 0.4 | |
| | | | | Fluazifop-p-butyl | | | | | |
| R1 | Y=-20.4+(99.4+20.4)/(1+10^((X-0.9))) | -20.4 | (-24.5, -16.4) | 99.4 | (97.7, 101) | 0.9 | (0.9, 1.0) | 8.9 | 17.8 |
| R2 | Y=-39.8+(98.2+39.8)/(1+10^((X-1.2))) | -39.8 | (-47.4, -32.3) | 98.2 | (96.7, 99.8) | 1.2 | (1.2, 1.3) | 17.1 | 34.2 |
| S | Y=-0.4+(119.5+0.4)/(1+10^((X+0.3))) | -0.4 | (-1.2, 0.4) | 119.5 | (117.3, 121.8) | -0.3 | (-0.3, -0.3) | 0.5 | |

| | | Pinoxaden | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| R1 | Y=-30.2+ (97.8+30.2)/(1+10^((X-1.1))) | -30.2 | (-36.8, -23.6) | 97.8 | (95.9, 99.7) | 1.1 | (1.0, 1.2) | 12.7 | 8.5 |
| R2 | Y=-67.7 + (99.1+67.7)/(1+10^((X-1.5))) | -67.7 | (-84.5, -50.8) | 99.1 | (97.3, 100.9) | 1.5 | (1.4, 1.5) | 28.4 | 18.9 |
| S | Y=-2.1+ (106.1+2.1)/(1+10^((X-0.2))) | -2.1 | (-3.4, -0.8) | 106.1 | (104.1, 108) | 0.2 | (0.1, 0.2) | 1.5 | |

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype. [b] In the least-squares fit equation, x represents the concentration of ACCase-targeting herbicides, y represents the response variable of ACCase enzyme activity, [c]Inhibition: The required concentration of ACCase-targeting herbicides was calculated by 50% ($IC_{50}$) based on regression curve to fit in the concentration-response inhibition equation; R/S ratios: Resistant/Susceptible ratios

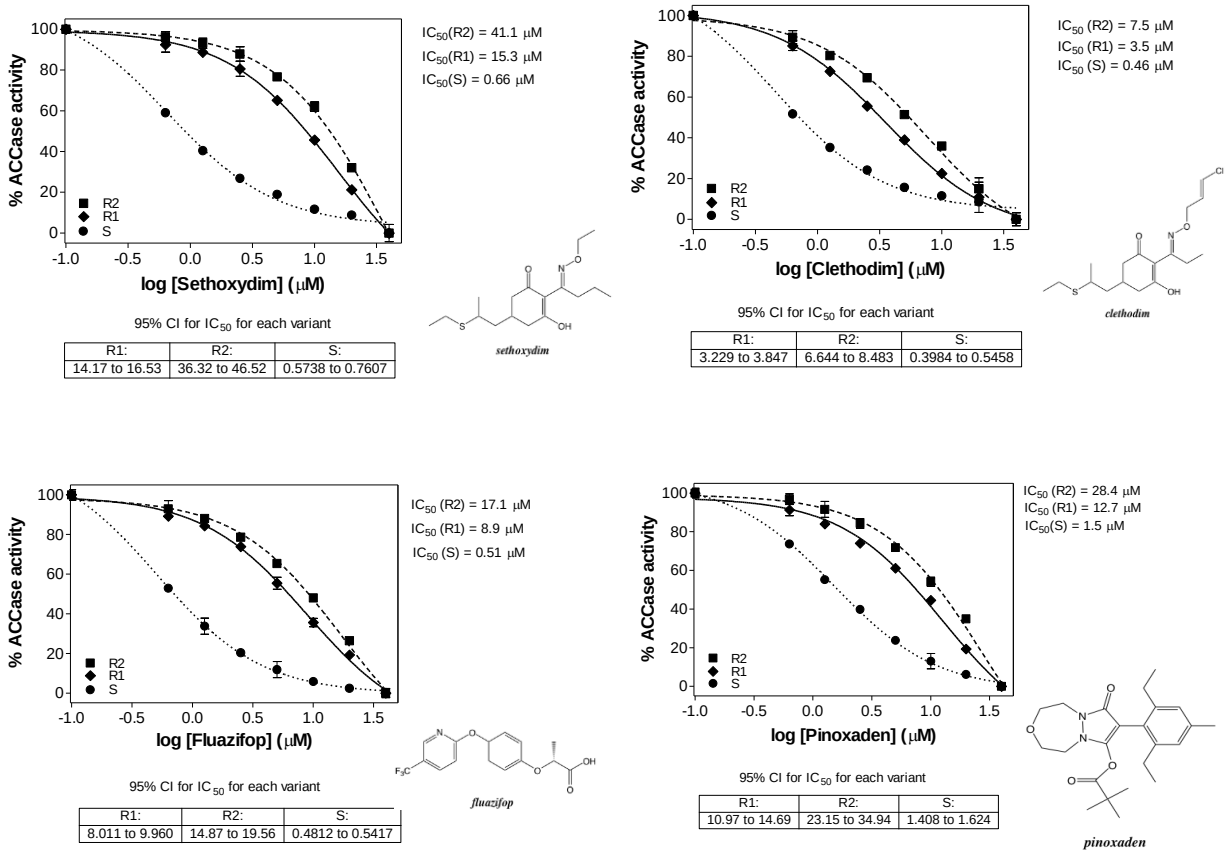



**Figure 2.** Response curves for percent ACCase enzyme activities of resistant and susceptible *D. ciliaris* biotypes to the increasing concentrations of ACCase-targeting herbicides, sethoxydim, pinoxaden, fluazifop-p-butyl, and clethodim. Vertical bars represent the standard errors of the means (n=6). The response was modeled based on the log rate of ACCase herbicides to create equal spacing between rates using least-squares fit. *D. ciliaris* biotypes: Resistant biotypes, R1 and R2, and Susceptible biotype, S

```
                                        1                    21
                          ..... .......... |......... .......... |......... ..........
R2      .................MSQLG LAAAASKALP LLPNLQRSST GTTFSSSALS RPSNRRKSRT RSLRDGGDGV
R1      .................MSQLG LAAAASKALP LLPNLQRSST GTTFSSSALS RPSNRRKSRT RSLRDGGDGV
D.e.    .................MSQLG LAAAASKALP LLPNLQRSSG GATFSSSALS RPSNRRKSRT RSLRDGGDGV
                          ***** ********** *********^ *^********* ********** **********
        41                    61                   81                   101
        |......... .......... |......... .......... |......... .......... |.........
R2      SDAKKHNQSV RQGLAGIIDL PNEATSEVDI SHGSEDPRGP PEPYQMNGII NEAHNGRHAS VSKVVEFCAA
R1      SDAKKHNQSV RQGLAGIIDL PNEATSEVDI SHGSEDPRGP PEPYQMNGII NEAHNGRHAS VSKVVEFCAA
D.e.    SDAKKHNQSV RQGLAGIIDL PNEATSAVDI SHGSEDPRGP SEPYQMNGII NEAHNGRHAS VSKVVEFCAA
        ********** ********** ******^*** ********** ^********** ********** **********
                 121                  141                  161
        .......... |......... .......... |......... .......... |......... ..........
R2      LGGKTPIHSI LVANNGMAAA KFMRSVRTWA NDTFGSEKAI QLIAMATPED MRINAEHIRI ADQFVEVPGG
R1      LGGKTPIHSI LVANNGMAAA KFMRSVRTWA NDTFGSEKAI QLIAMATPED MRINAEHIRI ADQFVEVPGG
D.e.    LGGKTPIHSI LVANNGMAAA KFMRSVRTWA NDTFGSEKAI QLIAMATPED MRINAEHIRI ADQFVEVPGG
        ********** ********** ********** ********** ********** ********** **********
        181                  201                  221                  241
        |......... .......... |......... .......... |......... .......... |.........
R2      TNNNNYANVQ LIVEIAERVG VSAVWPGWGH ASENPELPDA LTAKGIIFLG PPASSMNALG DKVGSALIAQ
R1      TNNNNYANVQ LIVEIAERVG VSAVWPGWGH ASENPELPDA LTAKGIIFLG PPASSMNALG DKVGSALIAQ
D.e.    TNNNNYANVQ LIVEIAERVG VSAVWPGWGH ASENPELPDA LTAKGIIFLG PPASSMNALG DKVGSALIAQ
        ********** ********** ********** ********** ********** ********** **********
                  261                  281                  301
        .......... |......... .......... |......... .......... |......... ..........
R2      AAGVPTLSWS GSHVEVPLEC CLDAIPEEMY RKACVTTTEE AVASCQVVGY PAMIKASWGG GGKGIRKVHN
R1      AAGVPTLSWS GSHVEVPLEC CLDAIPEEMY RKACVTTTEE AVASCQVVGY PAMIKASWGG GGKGIRKVHN
D.e.    AAGVPTLSWS GSHVEVPLEC CLDAIPEEMY RKACVTTTEE AVASCQVVGY PAMIKASWGG GGKGIRKVHN
        ********** ********** ********** ********** ********** ********** **********
        321                  341                  361                   381
        |......... .......... |......... .......... |......... .......... |.........
R2      DDEVRALFKQ VQGEVPGSPI FIMRLASQSR HLEVQLLCDQ YGNVAALHSR DCSVQRRHQK IIEEGPVTVA
R1      DDEVRALFKQ VQGEVPGSPI FIMRLASQSR HLEVQLLCDQ YGNVAALHSR DCSVQRRHQK IIEEGPVTVA
D.e.    DDEVRALFKQ VQGEVPGSPI FIMRLASQSR HLEVQLLCDQ YGNVAALHSR DCSVQRRHQK IIEEGPVTVA
        ********** ********** ********** ********** ********** ********** **********
                 401                  Glu-424              441
        .......... |......... .......... |......... .......... |......... ..........
R2      PRETVKALEQ AARRLAKAVG YVGAATVEYL YSMDTGEYYF LELNPRLQVE HPVTEWIAEV NLPAAQVAVG
R1      PRETVKALEQ AARRLAKAVG YVGAATVEYL YSMDTGEYYF LELNPRLQVE HPVTEWIAEV NLPAAQVAVG
D.e.    PRETVKALEQ AARRLAKAVG YVGAATVEYL YSMETGEYYF LELNPRLQVE HPVTEWIAEV NLPAAQVAVG
        ********** ********** ********** ***$****** ********** ********** **********
        461                  Asp-484              501                  521
        |......... .......... |......... .......... |......... .......... |.........
R2      MGIPLWQIPE IRRFYGMDYG GGYGIWRKTA ALATPFNFDE VDSQWPKGHC VAVRITSENP DDGFKPTGGK
R1      MGIPLWQIPE IRRFYGMDYG GGYGIWRKTA ALATPFNFDE VDSQWPKGHC VAVRITSENP DDGFKPTGGK
D.e.    MGIPLWQIPE IRRFYGMDYG GGYDIWRKTA ASATPFNFDE VDSQWPKGHC VAVRITSENP DDGFKPTGGK
        ********** ********** ***$****** *^* ****** ********** ********** **********
                 541                  561                  Ile-586
        .......... |......... .......... |......... .......... |......... ..........
R2      VKEISFKSKP NVWAYFSVKS GGGIHEFADS QFGHVFAYGL SRSAAITNMA LALKEVQIRG EIHSNVDYTV
R1      VKEISFKSKP NVWAYFSVKS GGGIHEFADS QFGHVFAYGL SRSAAITNMA LALKEVQIRG EIHSNVDYTV
D.e.    VKEISFKSKP NVWGYFSVKS GGGIHEFADS QFGHVFAYGL SRSAAITNMA LALKEIQIRG EIHSNVDYTV
        ********** ***^****** ********** ********** ********** *****$**** **********
        601                  621                  641                  661
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        |......... .......... |......... .......... |......... .......... |.........
R2      DLLNASDFRE NKIHTGWLDT RIAMRVQAER PPWYISVVGG ALYKTVTANA TTVSDYVSYL TKGQIPPKHI
R1      DLLNASDFRE NKIHTGWLDT RIAMRVQAER PPWYISVVGG ALYKTVTANA TTVSDYVSYL TKGQIPPKHI
D.e.    DLLNASDFRE NKIHTGWLDT RIAMRVQAER PPWYISVVGG ALYKTVTANA ATVSDYVSYL TKGQIPPKHI
        ********** ********** ********** ********** ********** ^********** **********
                 681                  701               Met-721
        .......... |......... .......... |......... .......... |......... ..........
R2      SLVNSTVNLN IEGSKYTIET VRTGHGSYRL RMNDSAIEAN VQSLCDGGLL VQLDGNSHVI YAEEEAGGTR
R1      SLVNSTVNLN IEGSKYTIET VRTGHGSYRL RMNDSAIEAN VQSLCDGGLL VQLDGNSHVI YAEEEAGGTR
D.e.    SLVNSTVNLN IEGSKYTIET VRTGHGSYRL RMNDSAIEAN VQSLCDGGLL MQLDGNSHVI YVEEEASGTR
        ********** ********** ********** ********** ********** $********* *^*****^***
        741                761                  781                  801
        |......... .......... |......... .......... |......... .......... |.........
R2      LQIDGKTCLL QNDYDPSKLL AETPCKLLRF LVADGAHVDA DVPYAEVEVM KMCMPLLSPA SGVIHVMMSE
R1      LQIDGKTCLL QNDYDPSKLL AETPCKLLRF LVADGAHVDA DVPYAEVEVM KMCMPLLSPA SGVIHVMMSE
D.e.    LQIDGKTCLL QNDYDPSKLL AETPCKLLRF LVADGAHVDA DIPYAEVEVM KMCMPLLSPA SGVIHVMMSE
        ********** ********** ********** ********** *^********* ********** **********
                 821                  841                  861
        .......... |......... .......... |......... .......... |......... ..........
R2      GQALQAGDLI ARLDLDDPSA VKRAEPFDGI FPQMGLPVAA SSQVHKRYAS SLNAARMVLA GYEHNINEVV
R1      GQALQAGDLI ARLDLDDPSA VKRAEPFDGI FPQMGLPVAA SSQVHKRYAS SLNAARMVLA GYEHNINEVV
D.e.    GQALQAGDLI ARLDLDDPSA VKKAEPFDGI FPQMSLPVAA SSQVHKRYAS SLNAARMVLA GYEHNINEVV
        ********** ********** **^******** ****^****** ********** ********** **********
        881                 901              Lys-921                  941
        |......... .......... |......... .......... |......... .......... |.........
R2      QDLICCLDDP ELPFLQWDEL MSVLATRLPR NLKSELEDKY QEYKLNFYHG KNKDFPSKLL RDIIEANLAY
R1      QDLICCLDDP ELPFLQWDEL MSVLATRLPR NLKSELEDKY QEYKLNFYHG KNKDFPSKLL RDIIEANLAY
D.e.    LDLICCLDDP ELPFLQWDEL MSVLATRLPR NLKSELEDKY KEFKLNFYHG KNKDFPSKLL KDIIEANLAH
        ^********** ********** ********** ********** $*^******** ********** ^*********^
        Ala-957   961                  981                 1001          Asp-1018
        .......... |......... .......... |......... .......... |......... ..........
R2      GSEKEKTTNE RLVEPLMSLL KSYEGGRESH AHFVVKSLFE EYLAVEELFS DGIQSDVIET LRHQHSKNLQ
R1      GSEKEKTTNE RLVEPLMSLL KSYEGGRESH AHFVVKSLFE EYLAVEELFS DGIQSDVIET LRHQHSKNLQ
D.e.    GSEKEKATNE RLVEPLMSLL KSYEGGRESH AHFVVKSLFE EYLSVEELFS DGIQSDVIET LRHQYSKDLQ
        ******$*** ********** ********** ********** ***^******* ********** ****^**$**
        1021                1041   Asn-1050        1061                 1081
        |......... .......... |......... .......... |......... .......... |.........
R2      KVVDIVLSHQ GVRNKAKLVT ALMEKLVYPH PTAYRDLLVR FSSLNHKRYY KLALKASELL EQTKLSELRA
R1      KVVDIVLSHQ GVRNKAKLVT ALMEKLVYPH PTAYRDLLVR FSSLNHKRYY KLALKASELL EQTKLSELRA
D.e.    KVVDIVLSHQ GVRNKAKLVT ALMEKLVYPN PAAYRDLLVR NSSLNHKRYY KLALKASELL EQTKLSELRA
        ********** ********** *********$ *^********* ********** ********** **********
                 1101                 1121                 1141
        .......... |......... .......... |......... .......... |......... ..........
R2      SIARSLSDLG MHKGEMTIKD SMEELVSAPL PVEDALISLF DYSDPTVQQK VIETYISRLY QPLLVKDSIQ
R1      SIARSLSDLG MHKGEMTIKD SMEELVSAPL PVEDALISLF DYSDPTVQQK VIETYISRLY QPLLVKDSIQ
D.e.    SIARSLSDLG MHKGDMTIKD SMEELVSAPL PVEDALISLF DYSDPTVQRK VVETYISRLY QPLLVKDSIQ
        ********** ****^****** ********** ********** *********^* *^********* **********
        Ala-1168            1181                 1201                 1221
        |......... .......... |......... .......... |......... .......... |.........
R2      MKFKESGTFA FWEFYEGHVD TRNGHGAIIG GKRWGAMVVL KSLESASTAI VAALKDSAQF NSSEGNMMHI
R1      MKFKESGTFA FWEFYEGHVD TRNGHGAIIG GKRWGAMVVL KSLESASTAI VAALKDSAQF NSSEGNMMHI
D.e.    MKFKESGAFA FWEFSDEHAD TKNGQEAVLG QKRWGAMVVI KSLESARTAI VDALKDSARH ASSEGNMMHI
        *******$** ****^^^^^* *^***^*^^* ^**********^* ********^*** *^*******^^ ^**********

                1241                 1261                    Glu-1286
```

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        .......... |......... .......... |......... .......... |......... ..........
R2      ALLSAENESN ISGISSDDQA QHRMEKVTKI LKDTSVVNDL RAAGLKVISC IVQRDKVRMP MRHTFLWSDE
R1      ALLSAENESN ISGISSDDQA QHRMEKVTKI LKDTSVVNDL RAAGLKVISC IVQRDKVRMP MRHTFLWSDE
D.e.    ALLSSENENN IRSIASDDQA QHRMEKITKI FKDSGVVNDL RSAGLKVISC IVQRDEVRMP MRHTFLWSDE
        ****^*****^ *^^^^^***** *******^*** ^**^^***** *^********* *****$**** **********
        Glu-1307          1321                1341                1361
        |......... .......... |......... .......... |......... .......... |.........
R2      KGCYEDDQIL RHVEPPLSAL LELDKLKVKG YNEMKYTPSR DRQWHIYTLR NTENPKMLHR VFFRTVVRQP
R1      KGCYEDDQIL RHVEPPLSAL LELDKLKVKG YNEMKYTPSR DRQWHIYTLR NTENPKMLHR VFFRTVVRQP
D.e.    KSCYEEEQIL RHVEPPLSAL LELDKLKVKG YNEMKYTPSR DRQWHIYTLR NTENPKMLHR VFFRTIVRQL
        *^*****$*** ********** ********** ********** ********** ********** *****^****^
        Ile-1377   1381                 Met-1405          1421
        .......... |......... .......... |......... .......... |......... ..........
R2      NADNKFTSAQ ISDAEVGCPE ESLSFTSNSI LRSLITAIEE LELHAIRTGH SHMYLCILKE QKLLDLIPFS
R1      NADNKFTSAQ ISDAEVGCPE ESLSFTSNSI LRSLITAIEE LELHAIRTGH SHMYLCILKE QKLLDLIPFS
D.e.    NADNKFASAQ VSNTEVGGLE ESLSSTSNSI LRSLMTAIEE LELHAIRTGH SHMYLCILKE QKLLDLIPFS
        ******$*** ^*^^^^^^^^* ****^***** ****$***** ********** ********** **********
        1441              1461                  Asp-1488           1501
        |......... .......... |......... .........  |......... .......... |.........
R2      GSAIVDVGQD EATACSLLKS MALKIHKLVG AQMHHLSVCQ WEVKLKLECD GPASGTWRVV TTNVTSHTCT
R1      GSAIVDVGQD EATACSLLKS MALKIHKLVG AQMHHLSVCQ WEVKLKLECD GPASGTWRVV TTNVTSHTCT
D.e.    GSTIVDVGQD EATACSLLKS MALKIHELVG AQMHHLSVCQ WEVKLKLDCD GPASGTWRVV TTNVTSHTCT
        **^******** ********** ******^*** ********** ********$** ********** **********
                 1521                1541                1561
        .......... |......... .......... |......... .......... |......... ..........
R2      VDIYREVEEI ESQKLVYHSA TSSASP——LH GVALNNPYQP LSVIDLKRCS ARNNRTTYCY DFPLAFETAL
R1      VDIYREVEEI ESQKLVYHSA TSSASP——LH GVALNNPYQP LSVIDLKRCS ARNNRTTYCY DFPLAFETAL
D.e.    VDIYREVEDT ESKKLVYHSA SSSASP——LP GVALNNPYQP LSVIDLKRCS ARNNRTTYCY DFPLAFETAL
        *********^ **^******** ^*********^ ********** ********** ********** **********
        Gly-1588              Ala-1610          1621                1641
        |......... .........  |......... .......... |......... .......... |.........
R2      QKSWESNDSS VSKGSESSKS YVKATELVFS EKHGSWGTPI VPMERPAGLN DIGMVAWILE MSTPEFPNGR
R1      QKSWESNDSS VSKGSESSKS YVKATELVFS EKHGSWGTPI VPMERPAGLN DIGMVAWILE MSTPEFPNGR
D.e.    RKSWELSGCG VSKGSESSKP YVKATELVFA EKHGSWGTPI VPMERPAGLN DIGMVAWILE MSTPEFPNGR
        ^*****^$^^ *********^ *********$ ********** ********** ********** **********
        1661               1681       Arg-1694    1701                1721
        |......... .......... |......... .........  |......... .......... |.........
R2      QIIVVANDIT FRAGSFGPRE DAFFEAVTNM ACEKKLPLIY LAANSGARIG IADEVKSCFR VGWSDEGSPE
R1      QIIVVANDIT FRAGSFGPRE DAFFEAVTNM ACEKKLPLIY LAANSGARIG IADEVKSCFR VGWSDEGSPE
D.e.    QIIVVANDIT FRAGSFGPRE DAFFEAVTNM ACERKLPLIY LAANSGARIG IADEVKSCFR VGWSDEGSPE
        ********** ********** ********** ***$****** ********** ********** **********
                1741                1761                  Ile-1781
        .......... |......... .......... |......... .......... |......... ..........
R2      RGFQYIYLTE EDYARISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN LHGSAAIASA YSRAYEETFT
R1      RGFQYIYLTE EDYARISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN LHGSAAIASA YSRAYEETFT
D.e.    RGFQYIYLTE EDYSRISSSV IAHKLQLDSG EIRWIIDSVV GKEDGLGVEN IHGSAAIASA YSRAYEETFT
        ********** ***^******* ********** ********** ********** @********* **********
        1801               1821                1841                1861
        |......... .......... |......... .........  |......... .......... |.........
R2      LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREVYSSH MQLGGPKIMA TNGVVHLTVS
R1      LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREVYSSH MQLGGPKIMA TNGVVHLTVS
D.e.    LTFVTGRTVG IGAYLARLGI RCIQRLDQPI ILTGFSALNK LLGREVYSSH MQLGGPKIMA TNGVVHLTVS
        ********** ********** ********** ********** ********** ********** **********

                1881                1901                1921
```

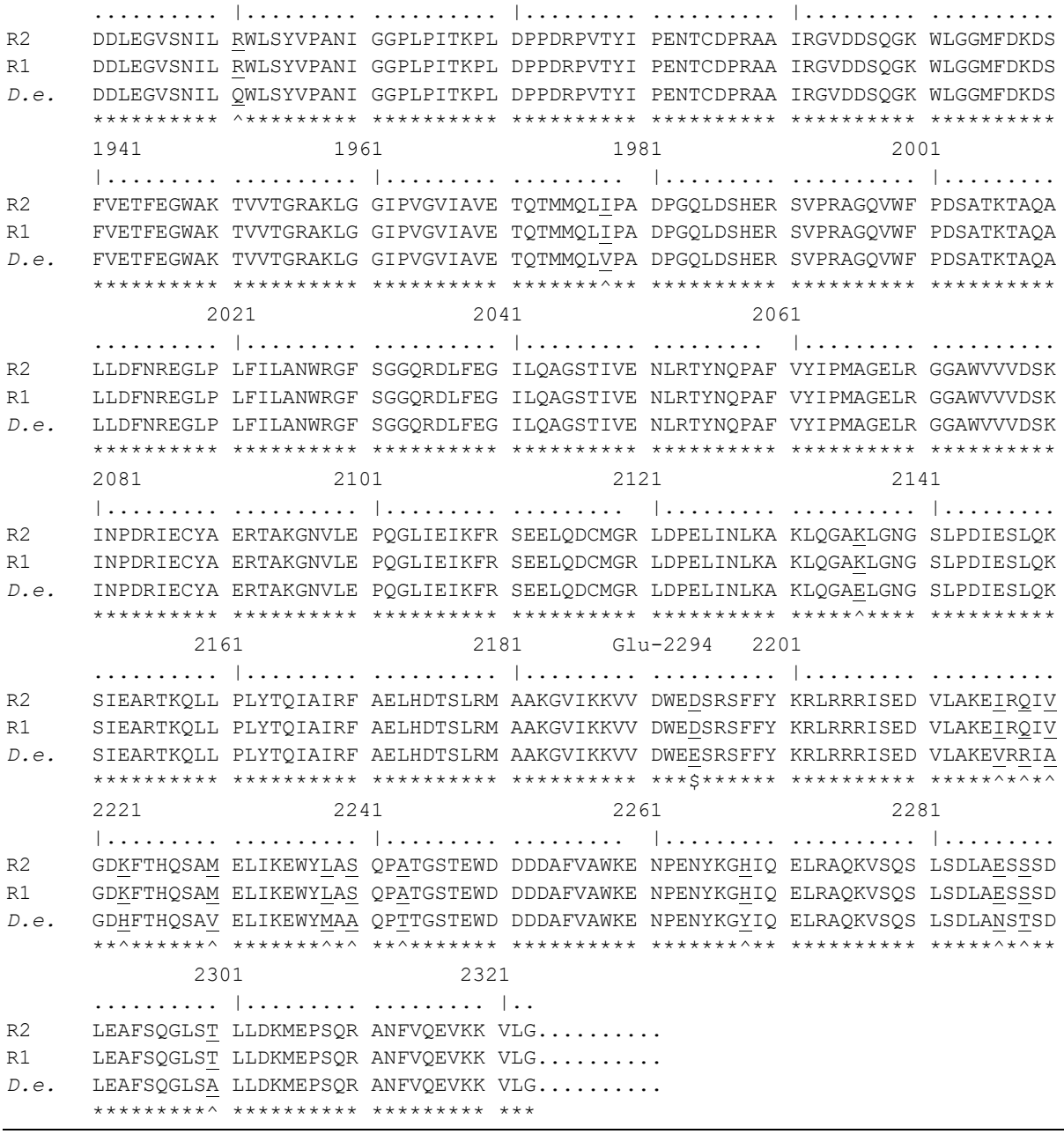


**Figure 3.** Amino acid sequence comparisons of plastid multidomain ACCase gene of *Digitaria ciliaris*, R2 and R1 resistant biotypes. Transcribed amino acid sequences were aligned with the *Digitaria. exilis* (*D.e.*) (GenBank accession number: KAF8662016) and were shown with residue numbering following the *Alopecurus myosuroides* plastid ACCase (GenBank accession number: AJ310767). The amino acids, which were identical marked with asterick (*) identity. Silent codons in amino acid were underlined and marked by using '^' , missense codons in amino acid were underlined and marked with '$', and the amino acid substitution, Ile-1781 to Leu was bolded, underlined, and marked with '@'.

# Chapter 4

## Determination of differential inhibition mechanism in the two resistant *Digitaria ciliaris* biotypes using gene expression profile

**[**This chapter has been prepared for publication. Suma Basak, J. Scott McElroy, Jinesh D. Patel, and Patrick E. McCullough. 2021. Overexpression of acetyl-coenzyme A carboxylase gene confers the differential inhibition mechanism to ACCase targeting herbicides in resistant southern crabgrass (*Digitaria ciliaris*)]

### i. Abstract

The main intent of our study was to explain the inhibition mechanism difference for ACCase resistance between the resistant biotypes, R1 and R2 of southern crabgrass (*D. ciliaris*). Our previous research found that *D. ciliaris* had resistance to ACCase (Acetyl-coenzyme A carboxylase; EC.6.4.1.2)-targeting herbicides due to presence of Ile-1781-Leu mutant allele and greater ACCase enzyme activity. However, the resistant biotypes of *D. ciliaris* were inhibited by ACCase-targeting herbicides at different extents for each herbicide and had less sensitive to clethodim compared to the susceptible biotype. To elucidate the differential inhibition mechanisms in the two resistant biotypes, our research was conducted by measuring the level of ACCase gene expression using quantitative reverse transcriptase PCR (qRT-PCR). The resistant biotypes, R1 and R2 had greater ACCase enzyme expression compared to S biotype. The expression of ACCase gene in resistant biotypes, R1 and R2, were 5 and 9-fold, respectively, greater compared to the susceptible biotype, S. Moreover, the R2 biotype had 1.7-fold greater ACCase gene expression compared to the R1 biotype. No significant differences in ACCase gene copy number were found among S, R1, and R2 biotypes for the ACCase gene. ACCase gene expression assay revealed that

the differential inhibition levels of ACCase enzyme activity for ACCase herbicides in the two resistant biotypes, R1 and R2 were associated due to the enhanced expression of the ACCase gene in the R2 biotype.

Nomenclature: Acetyl-coenzyme A carboxylase (ACCase); Southern crabgrass (*Digitaria ciliaris*)

Keywords: Acetyl-coenzyme A carboxylase, Overexpression, gene profile, ACCase-herbicides resistance

## ii. Introduction

ACCase herbicides resistance mechanism involves the target site-based resistance (TSR) and non-target site-based resistance (NTSR). The mutations at the herbicide-binding site of the target enzymes or the regulation of target genes are considered as TSR (Preston and Mallory-Smith 2001; Beckie 2006). For example, eight conserved amino acid substitutions at seven positions in the carboxyl transferase domain of the target plastidic ACCase gene in about 49 weed species are associated with ACCase targeting herbicides resistance (Heap 2021; Powles and Yu 2010; Collavo et al. 2011). NTSR results in the decrease of the active herbicide that achieves the target enzyme or binding domain and usually associates with the reduction of herbicide absorption and translocation (Kaundun 2014; Kukorelli et al. 2013; Powles and Yu 2010; Ruiz-Santaella et al. 2006), herbicide detoxification (Délye, 2005), and the enhancement of herbicide metabolism such as cytochrome P450 monooxygenase, glutathione S-transferase, and glucosyltransferase enzymatic activities (Ahmad-Hamdani et al. 2012; Brazier et al. 2002; Cummins et al. 1999; Cummins et al. 2009; Iwakami et al. 2019; Yuan et al. 2007; Yu and Powles 2014). Researchers have reported NTSR, such as enhance degradation of ACCase targeting herbicides in resistant biotypes of blackgrass (*Alopecurus myosuroides* Huds.) (Cummins et al. 1997; Letouze´ and Gasquez 2003; Petit et al. 2010), wild oat (*Avena fatua* L.) (Cocker et al. 2000), Asia minor bluegrass (*Polypogon fugax*) (Zhao et al. 2019b), large crabgrass [*Digitaria sanguinalis* (L.) Scop.] (Hidayat and Preston 1997), ryegrass (*Lolium* spp.) (Preston and Powles 2002), and rice barnyard grass [*Echinochloa phyllopogon* (Stapf) Koss] (De Prado et al. 2005; Mendez and De Prado 1996; Iwakami et al. 2019) as compared to the susceptible biotype.

The first step of fatty acid biosynthesis is catalyzed by a key enzyme, acetyl-CoA carboxylase (ACCase; EC.6.4.1.2). ACCase enzyme consists of three functional domains: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase (CT, with subunits α and β). The two reactions, acetyl CoA carboxylation and malonyl CoA formation catalyzed by the BC and CT subunits. First, the biotin carboxylase domain catalyzes the ATP-dependent carboxylation of a biotin group covalently bound to the BCCP domain. Secondly, the carboxyl transferase domain transfers the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA (Harwood 1988; Nikolau et al. 2003; Nikolskaya et al. 1999). ACCase-targeting herbicides with three structural distinct chemical classes, aryloxyphenoxypropionates (FOPs), cyclohexanediones (DIMs), and phenylpyrazolins (DEN) control the grass weed inhibiting ACCase activity by blocking fatty acids biosynthesis, preventing the formation of lipid and secondary metabolites such as flavonoids and suberin.

Plants express chloroplastic and cytoplasmic ACCase isoforms. However, dicots have two forms of the ACCase, heteromeric or prokaryotic form in the chloroplastids, and homomeric or eukaryotic form in the cytoplasm, where each domain of ACCase is encoded by different nuclear or plasmid genes expressed in a coordinated fashion. On the other hand, plants belonging to the Poaceae family, grasses species have only the homomeric chloroplastic ACCase in which the BCCP, BC, and CT domains are localized within a single polypeptide chain and are encoded by a nuclear gene. Both chloroplastic and cytoplasmic ACCase in Poaceae become active when homodimerized. This eukaryotic, homomeric form of ACCase is inhibited by ACCase targeting herbicides (Konishi et al.1996; Incledon and Hall, 1997; Sasaki and Nagano 2004; Zhang et al. 2003). Sometimes, selection pressure imposed by the application of the same ACCase-targeting herbicides or same herbicide concentrations can lead to increased gene-specific activity due to a

higher ACCase gene expression rate. Overexpression of ACCase gene, therefore, allows for sustained fatty acid biosynthesis and the active ingredient is unable to block the ACCase gene physiological function at rates incompatible with cellular metabolism.

*Digitaria ciliaris*, also known as southern crabgrass, is a common summer weed distributed across the Southern part of the United States. This grass, recently, emergences as a resistant to ACCase herbicides and has become an increasing problem in the sod production field, Georgia (Yu et al 2017). Previous research reported that the resistant biotypes, R1 and R2 had cross-resistance to pinoxaden and differential levels of susceptibility to targeting herbicides of ACCase. Also, ACCase resistance in R biotypes was associated with the Ile-1781-Leu, target-site mutation (Basak et al. 2019). Yu et al. 2017, however, reported that the R biotypes were not resistant to clethodim and found acceptable control about 83% when foliar applied. R biotypes had relatively less sensitive to clethodim than S biotype in the malachite green colorimetric assay. Moreover, ACCase enzyme activity of R1 and R2 biotypes were inhibited at different extents for each herbicide, and one of the resistant biotypes, R2 had greater ACCase enzyme activity compared to R1 even though there is no significant difference observed between two resistant biotypes R1 and R2 for the resistance to ACCase-targeting herbicides at green experiment (Yu et al. 2017; Basak et al. 2019). The specific mechanisms behind a greater ACCase enzyme activity of the R2 biotype compared to R1 and S remain unknown. Based on our experimental results, we hypothesize that TSR and NTSR, multiple mechanisms might have probably stacked in this R2 biotype.

Increased gene expression rates may be associated with TSR (San Cha et al. 2014). TSR with ACCase overexpression in grass species has been reported in South America along with other parts of the world. Reported cases of ACCase overexpression include Johnson grass (*Sorghum halepense*) in South America (Bradley et al. 2001), chinese sprangletop (*Leptochloa chinensis*) in

Thailand (Pornprom et al. 2006), and goose grass (*Elusine indica*) in Malaysia (San Cha et al. 2014). Gene expression profiling is the most newly documented that confers resistance by increasing the number of target sites. Increased gene expression rates are a well-documented form of NTSR. Both target-based and non-target-based resistance can be determined through gene expression profiling (Délye et al. 2013; Powles and Yu 2010; Yuan et al. 2007; Zhao et al. 2019 a, b). Laforest et al. (2017), however, reported that ACCase gene expression was greater (5.2) in the resistant population of large crabgrass (*D. sanguinalis*) than in susceptible (2.6). The expression level ratio of 3.9 to 8.9-fold was higher in the resistant population compared to the susceptible population. ACCase resistance had been confirmed in *D. sanguinalis* from target gene overexpression to fluazifop herbicide. The resistance mechanism of glyphosate in the resistant biotype of palmer amaranth (*Amaranthus palmeri*) was the overexpression of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (Gaines et al. 2010).

Yu et al. (2007) reported that clethodim at recommended doses efficiently controlled the homozygous annual ryegrass (*L. rigidum*) in a field in Australia but not heterozygous Ile1781Leu plants. As *D. ciliaris* is under suspicion of different ploidy level with potentially multiple copies of plastidic ACCase in subgenomes (Adoukonou-Sagbadja et al. 2007; Bennett et al. 2000), we hypothesized that the higher gene expression with target-site mutation, Ile to Leu at 1781 position in the resistant biotype, R2 could be differentiation test between the two resistant biotypes, R1 and R2 that in addition to conferring resistance to cyclohexanediones (DIMs) and aryloxyphenoxypropionates (FOPs) herbicides, Ile-1781-Leu also caused resistance to pinoxaden in *D. ciliaris*. To our knowledge, this is the first report of ACCase overexpression from *D. ciliaris* for differentiating the resistance mechanism between the two resistant biotypes R1 and R2. Our

clear objective of this research, therefore, is to investigate the differential level of resistance between the resistant biotypes, R1 and R2.

### iii. Materials and Methods

**Plant Material.** *D. ciliaris* biotypes with resistance to sethoxydim and FOPs herbicides were collected from 'TifBlair' centipedegrass [*Eremochloa ophiuroids* (Munro) Hack.] sod production field in Georgia (Yu et al. 2017). An herbicide susceptible biotype from the Auburn University campus was included in all experiments as well. All plants were propagated into the Auburn University Weed Science greenhouse (32.35°N, 85.29°W) in Auburn, Alabama to increase seed lots for experiments. Seeds were collected by hand and bulked. Seeds were then dispersed over plastic flats filled with commercial potting soil and peat moss (80:20 by volume). The plastic flats were placed in a greenhouse set for 32/25$^{o}$C (day/night) with no supplementary lighting. Four weeks later, the plant materials were transplanted to square plastic pots (10 cm × 10 cm × 8.5 cm) filled with the surface horizon of a marvyn sandy loam (fine-loamy, kaolinitic, thermic Typic Kanhapludults) with a pH of 6.5 and 1.1% organic matter. Plastic pots containing each *D. ciliaris* biotype were watered daily (around 0.2 cm per cycle) to prevent moisture deficiencies until plants reached a one- to two-tiller growth stage before the initiation of the following experiments.

**ACCase Herbicide Dose Response Evaluation.** The responses of the S, R1, and R2 *D. ciliaris* biotypes were evaluated from a rate titration of sethoxydim (Segment®, BASF Corp, Research Triangle Park, NC) herbicide**.** Treatments were applied with a $CO_2$ pressurized sprayer calibrated to deliver 280 L ha$^{-1}$ from a handheld four-nozzle boom at 32 psi (TeeJet TP8003VS nozzles with 25 cm spacing; Spraying Systems Company, Wheaton, IL). All treatments included a nonionic surfactant (Induce, Helena® Chemical Company, Collierville, TN) at 0.25% v/v. Sethoxydim at 0.3, 0.7, 1.3, 2.6, and 5.2 kg ha$^{-1}$ was applied to both resistant and susceptible plants.

Non-treated checks of all biotypes were included as control treatments and sprayed with water. Plants were returned to the greenhouse after herbicide application and irrigation were withheld for 24 h. The tiller number from the shoots was collected by counting the tiller at 27 days after treatment (DAT).

The design in the greenhouse experiments was a completely randomized factorial design with three replications and was conducted twice in time. Data analyses were performed using the PROC GLM procedure through SAS (SAS version 9.4, SAS Institute Inc., Cary, NC). Mean and standard errors were determined. Since the differences between the data of the two runs of the sethoxydim herbicide were not detected in the analysis of variance (ANOVA) at the 0.05 probability level, and thus the data were pooled overruns across the experimental repetitions for subsequent analysis. Sethoxydim rates were log-transformed to create equal spacing between treatments that can also fit into the regression analysis. Tiller number was converted to percent relative to the non-treated plant, respectively. A model was characterized that the relationship of the response curves with sethoxydim rate. All measurements relative to nontreated were used for the regression model. Percent of tiller number data were fitted to a least-squares in the Prism (GraphPad Software, version 5.0, Inc., La Jolla, CA):

$$Y=Bottom + (Top-Bottom)/(1+10^{((X-LogIC_{50}))})$$

where Y represents the response (%) of *D. ciliaris*, x is log-transformed ACCase-targeting herbicides concentration (µM), Top and bottom are the plateaus in the units of the Y-axis, and LogIC50 is the log-transformed sethoxydim (kg $ha^{-1}$). 95% confidence intervals ($\alpha$=0.05) for the estimates were calculated for regression model parameters. Regression equations were used to calculate sethoxydim inhibition values at 50% (referred to as $I_{50}$ values) compared to that of the

nontreated for each biotype. $I_{50}$ and R/S values were determined for resistant biotype versus susceptible biotype presented in Table1.

**ACCase Gene Expression Assay.** Quantitative Reverse Transcriptase-PCR (qRT-PCR) was conducted to measure the level of ACCase gene using endogenous control β-actin gene as a reference. Approximately 0.1 g fresh 14 days young leaf tissues of both susceptible (S) and resistant biotypes (R1 and R2) were homogenized with a mortar and pestle in liquid nitrogen, and RNAs were extracted using the TRIzol method (Trizol, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of total RNAs were evaluated with gel electrophoresis, a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA), and a Qubit 2.0 fluorometer (Invitrogen, Life Technologies). Then, the RNA sample was converted to complementary DNA (cDNA) through reverse transcriptase-polymerase chain reaction (RT-PCR) conversion using qScript™ cDNA Synthesis Kit (New England Biolabs, Inc., Ipswich, MA, USA). The primer pairs ACCase-F1/R1 (ACCase-F1: 5'-ATCATTTGGCCCGAGGGAAG-3' and ACCase-R1: 5'-CATCGGCTATGCCAATCCT-3') and Actin-F1/R1 (Actin-F1: 5'-CGGAGAATAGCATGAGGAAGTG-3' and Actin-R1: 5'-AGTGGTCGAACAACTGGTATTG - 3') were designed to target specifically the cDNA based on the sequences available in the NCBI (National Center for Biotechnology Information) database (GenBank accession KU198448 and KY967696). The qRT-PCR was performed using PerfeCTa SYBR green FastMix, ROX (Quantabio) according to the manufacturer's instructions. A total of three biological replicates were used in this ACCase gene expression experiment. The amplification was performed in three technical replicates per sample on a 96-well plate. qRT-PCR was performed under the following conditions: 2 min at 95$^{o}$C; followed by 40 cycles of 5 sec at 95$^{o}$C and 10 sec at 58$^{o}$C in Applied Biosystems Step One Plus™ Real-Time PCR System. The

ACCase gene expression data were normalized using the comparative $C_T$ ($\Delta\Delta C_T$) method with the endogenous control β-actin gene (Schmittgen and Livak 2008). All data analyses performed using the PROC t-test with the Statistical Analysis System (SAS version 9.4, SAS Institute Inc., Cary, NC). A Duncan's multiple-range test was used for means separation where there were significant differences.

**ACCase Gene Duplication or Copy Number.** Copy number analysis of ACCase gene in resistant and susceptible biotypes was performed using quantitative PCR (q-PCR). Plant genomic DNA was extracted using a Qiagen DNA extraction kit (USA) according to the manufacturer's instructions and quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA), and Gel electrophoresis. The qPCR was performed using PerfeCTa SYBR Green FastMix, ROX (Quantabio) according to the manufacturer's instructions. The DNA concentration of each sample was fixed at 5 ng/µl and the amplification was performed in triplicate on a 96-well plate. The qPCR reaction (20µl) contained 10µl of 2X SYBR Green PCR Master Mix, 0.75µl of each primer at 10µM, 6.5µl of sterilized water, and 2µl of genomic DNA (5ng/µl). A total of three biological replicates with three technical replicates per sample were used. The relative quantification was performed under the following conditions: 8 min at 95$^o$C; followed by 40 cycles of 3 sec at 95$^o$C and 30 sec at 60$^o$C in Applied Biosystems Step One Plus™ Real-Time PCR System. The melt curve analysis was performed using the comparative $C_T$ ($\Delta\Delta C_T$) method with the endogenous control β-actin gene (Laforest et al. 2017; Gaines et al. 2016).

### iv. Results and Discussion

**ACCase Herbicides Response Evaluation.** ACCase herbicide significantly reduced the fresh shoot biomass of susceptible biotype compared to the resistant biotypes R1 and R2 in greenhouse ACCase herbicides response trial (Figure 1). The resistant R1 biotype was the most tolerant to herbicide damage. The dry shoot biomass of the treated susceptible biotype at the recommended doses for sethoxydim was higher than the resistant biotype while the treated resistant R1 and R2 biotypes had very few to no necrotic spots with ACCase herbicides. Non-treated of the resistant biotype showed similar results to non-treated of the susceptible biotype. The treated resistant biotype, therefore, showed a greater level of resistance relative to the susceptible, S biotype in the greenhouse trial at Auburn University. Sethoxydim at the rates of 0.3, 0.7, and 1.3 kg $ha^{-1}$ reduced the tiller number in S biotype 55.6, 72.2, 88.9%, respectively, while the tiller number reduction of 6.3, 18.8, and 31.3%, respectively for R1 biotype and 11.7, 29.4, and 41.1%, respectively for R2 biotype. The S biotype was completely reduced tiller production at the rates of 2.6 and 5.2 kg $ha^{-1}$, while the reduction in the R biotypes ≤50%, resulting in no rate completely reduced the tiller production in the R biotypes at 27 DAT. $I_{50}$ for S biotype was 0.1 kg $ha^{-1}$ compared to 8.1 and 3.4 kg $ha^{-1}$ for the R1 and R2 biotypes, respectively. R1 and R2 biotype were 81 and 34 times more resistant than the S biotype based on R/S ratios.

**ACCase Gene Expression.** ACCase gene overexpression was observed in the resistant biotypes than the susceptible biotype determined by qRT-PCR. Compared with the S, R1 and R2 biotypes had a significant difference ($P < 0.01$) in ACCase gene expression. Our triplicated experiments consistently indicated a higher ΔCt value in the resistant biotypes compared to the susceptible for the ACCase gene, using the endogenous control β-actin gene as a reference. Comparison of ACCase gene with β-actin, the average ΔCt value for resistant biotypes, R1 and

R2, were 4.2 and 5.1, respectively, whereas this value for susceptible biotype, S was 1.9. Also, the average ΔΔCt value plus or minus the standard error specifies the overexpression range that the mean is likely to fall within 14.9 to 24.5, 26.5 to 41.9, and 2.9 to 4.5 for R1, R2, and S, respectively (Figure 3a). Compared with the S biotype, R1 and R2 biotypes had significantly higher ACCase gene expression, being 5.4 and 9.3-fold greater, respectively.

**ACCase Gene Duplication or Copy Number.** ACCase gene copy number can be another factor for influencing the accumulation of ACCase enzymes targeted by ACCase-herbicides. For example, resistant biotypes of *D. sangunalis* contained 5 to 7 folds higher ACCase gene copy number compared to the susceptible biotypes reported by Laforest et al. 2017. Similarly, Gaines et al. (2010) reported that the resistant biotypes of *Amaranthus palmeri*, up to a 160-fold increase in 5-enolpyruvylshikimate3-phosphate synthase (EPSPS) copy number was responsible for glyphosate herbicide resistance compared to the susceptible biotype. Increase EPSPS gene copy number to glyphosate herbicide was found in the resistant biotype of kochia [*Kochia scoparia* (L.)] dicotyledonous species reported by Gain et al. 2016. So, it was imperative to find out either ACCase gene regulation or ACCase gene duplication/copy number was the responsible factor for the ACCase gene overexpression. The qPCR, therefore, was performed to determine the number of copies of the ACCase gene in *D. ciliaris*. However, no relative differences ($P > 0.01$) in ACCase copy number among the resistant biotypes, R1 and R2, and susceptible biotype, S was found in the qPCR study (Figure 3b).

## v. Conclusion

To clarify the differential level of resistance between two resistant biotypes, R1 and R2, qRT- PCR was performed to ACCase and β-actin levels of expression from both R biotypes and susceptible biotypes. ACCase gene overexpression was able to differentiate the level of resistance

between the two resistant biotypes, R1 and R2. Generally, ACCase gene overexpression was observed in both resistant biotypes than the susceptible biotype. Compared with the S biotype, R1 and R2 had significantly higher ACCase gene expression, being 5.4 and 9.3-fold greater, respectively. ACCase gene overexpression in resistant biotypes is one of the possible factors enhancing greater ACCase enzyme activity. In addition, the R2 biotype had 1.7-fold greater ACCase gene expression compared to the R1 biotype. Due to greater levels of ACCase gene expression or transcript stability, the resistant biotype R2 was less inhibited ACCase enzyme activity by ACCase-targeting herbicides compared with those of R1 and S and had the differential level of resistance between the two resistant biotypes, R1 and R2 of *D. ciliaris*. The overexpression of herbicide target gene influenced to the resistance had been reported in the ACCase-targeting herbicide resistant weed, large crabgrass (*D. sanguinalis*) (Laforest et al. 2017), in the glyphosate herbicide resistant weeds such as annual bluegrass (*Poa annua* L.) (Brunharo et al. 2019); common waterhemp (*Amaranthus tuberculatus* (Moq.) J.D. Sauer) (Chatham et al. 2015; Dilon et al. 2017; Lorentz et al. 2014); goosegrass (*Eleusine indica* L. Gaertn.) (Chen et al. 2015); palmer amaranth (*Amaranthus palmeri*) (Gaines et al 2010); ragweed (*Kochia scoparia* L. Schrad.) (Gaines et al. 2016); great brome (*Bromus diandrus* Roth) (Malone et al. 2016); prickly amaranth (*Amaranthus spinosus* L.) (Nandula et al. 2014); italian ryegrass (*Lolium perenne ssp. multiflorum (Lam.) Husnot*) (Salas et al. 2012), and in the ALS (Acetolactate synthase or acetohydroxy acid synthase)-targeting herbicide resistant weed, shortawn foxtail (*Alopecurus aequalis*) (Sprague et al 1997; Iwakami et al. 2017).

On the other hand, the gene duplication or gene copy number of herbicide target gene influenced to the resistance had been reported in the glyphosate herbicide-resistant weeds such as Kochia (*Kochia scoparia* (L.) Schrad.) (Gaines et al. 2016), waterhemp (*Amaranthus tuberculatus*

(Moq.) J.D. Sauer) (Chatham et al. 2017; Dillon et al. 2017; Lorentz et al. 2014), spiny amaranth (*Amaranthus spinosus* L.) (Nandula et al. 2014), Italian ryegrass (*Lolium perenne* ssp. multiflorum (Lam.) Husnot) (Salas et al. 2012), goosegrass (*Eleusine indica* (L.) Gaertn.) (Chen et al. 2015) and great brome (*Bromus diandrus* Roth) (Malone et al. 2016) and the ACCase-targeting herbicide-resistant weed, large crabgrass (*D. sanguinalis*) (Laforest et al. 2017). In this study, no relative difference was observed in ACCase gene copy number among the biotypes S, R1, and R2. Assuming that ACCase gene regulation is one of the possible responsible factors for the ACCase gene overexpression rather than ACCase gene duplication or copy number. The overexpression of the ACCase gene or transcript stability may have contributed to increased accumulation of ACCase enzyme, the contribution of these factors for ACCase resistance level, however, remains unknown. Although greater ACCase gene expression in R biotypes is a possible factor for enhancing ACCase enzyme activity, further studies are needed to support this statement because Kuhlemeier (1992) reported that all transcription does not have always equal translation, and posttranslational regulation also has an important role for actual enzyme level. Further studies are also needed to measure the expression of different homologs within each plant, as we just measured bulk expression, which is no expression of the different homologs within each plant.

Based on our findings, we reached two major conclusions, first greater ACCase-enzyme activity against ACCase targeting herbicides in the R biotypes is associated ACCase gene overexpression along with Ile-1781-Leu, target-site mutation. Secondly, the differential level of resistance with greater ACCase enzyme activity was found in the R2 biotype compared to R1 and S, which was caused by the greater expression of the ACCase gene in this biotype. Other NTSR mechanism may be simultaneously occurring contributing to ACCase resistance in R2 biotype. Thirdly, R1 and R2 biotypes were not resistant to clethodim at foliar application and ACCase

enzyme of R biotypes was inhibited less than S. *D. ciliaris* has the different ploidy level. This implies that the greater ACCase enzyme from the heterozygous Ile-1781-Leu plants of *D. ciliaris* is the main cause of reduced the susceptibility to clethodim for inhibiting the ACCase enzyme activity.

**Table 1.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing sethoxydim rate relative to the nontreated control was measured with the least-squares fit model for percent tiller number. The required rate of sethoxydim to cause 50% reduction of tiller number ($I_{50}$) was calculated from the dose response curves at 27 days after treatment. Parameter estimates, 95% confidence intervals (CI), values of $I_{50,}$ and R/S ratios of $I_{50}$ are presented as means of model comparison

| Biotype[a] | Equation[b] | $R^2$ | Parameter estimates and confidence intervals | | | | | | Inhibition[c] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) | | Bottom | 95% CI | Top | 95% CI | Log IC50 | 95% CI | $I_{50}$ (kg) | R/S ratio |
| R1 | Y=-161.5 + (103.3+161.5)/(1+10^((X-0.9))) | 0.9 | -161.5 | (-439.5, 116.5) | 103.3 | (90.3, 116.3) | 0.9 | (0.2, 1.6) | 8.1 | 81 |
| R2 | Y=-66.3 + (103.1+66.3)/(1+10^((X-5.2))) | 0.8 | -66.3 | (-135.3, 2.6) | 103.1 | (90.9, 115.2) | 0.5 | (0.1, 0.9) | 3.4 | 34 |
| S | Y=-4.3+ (198.7+4.3)/(1+10^((X+0.9))) | 0.7 | -4.3 | (-14.1,5.3) | 198.7 | (89.8, 307.5) | -0.9 | (-1.4, -0.5) | 0.1 | |

[a] *D. ciliaris* biotypes: R2, resistant biotype, S, susceptible biotype. [b] In the least-squares fit equation, x represents the sethoxydim rate, y represents the response variable of tiller number, [c]Inhibition: The required rate of sethoxydim was calculated by 50% ($I_{50}$) based on regression curve to fit in the dose-response inhibition equation; R/S ratios: Resistant/Susceptible ratios

A. Treated plants B. Non-treated plants

**Figure 1.** Injury observed on the susceptible and resistant plants of *D. ciliaris* 9DAT with sethoxydim herbicide (A: treated plants and B: non-treated plants) in greenhouse experiment

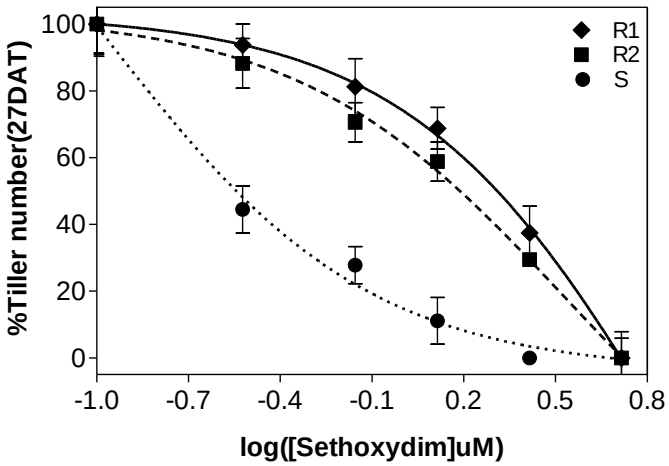


**Figure 2.** Percent tiller number response relative to non-treated of resistant and susceptible *D. ciliaris* biotypes with increasing rates of sethoxydim 27 days after treatment. The response was modeled based on the log rate of sethoxydim to create equal spacing between rates using regression model. Results were pooled over experimental runs. Vertical bars represent the standard errors (P=0.05) of the means. Means (n=6) are represented by differing symbols for each biotype and regression equation model are represented by differing line type for each biotype. *D. ciliaris* biotypes: Resistant biotypes, R1 and R2, Georgia, and susceptible biotype, S, Alabama

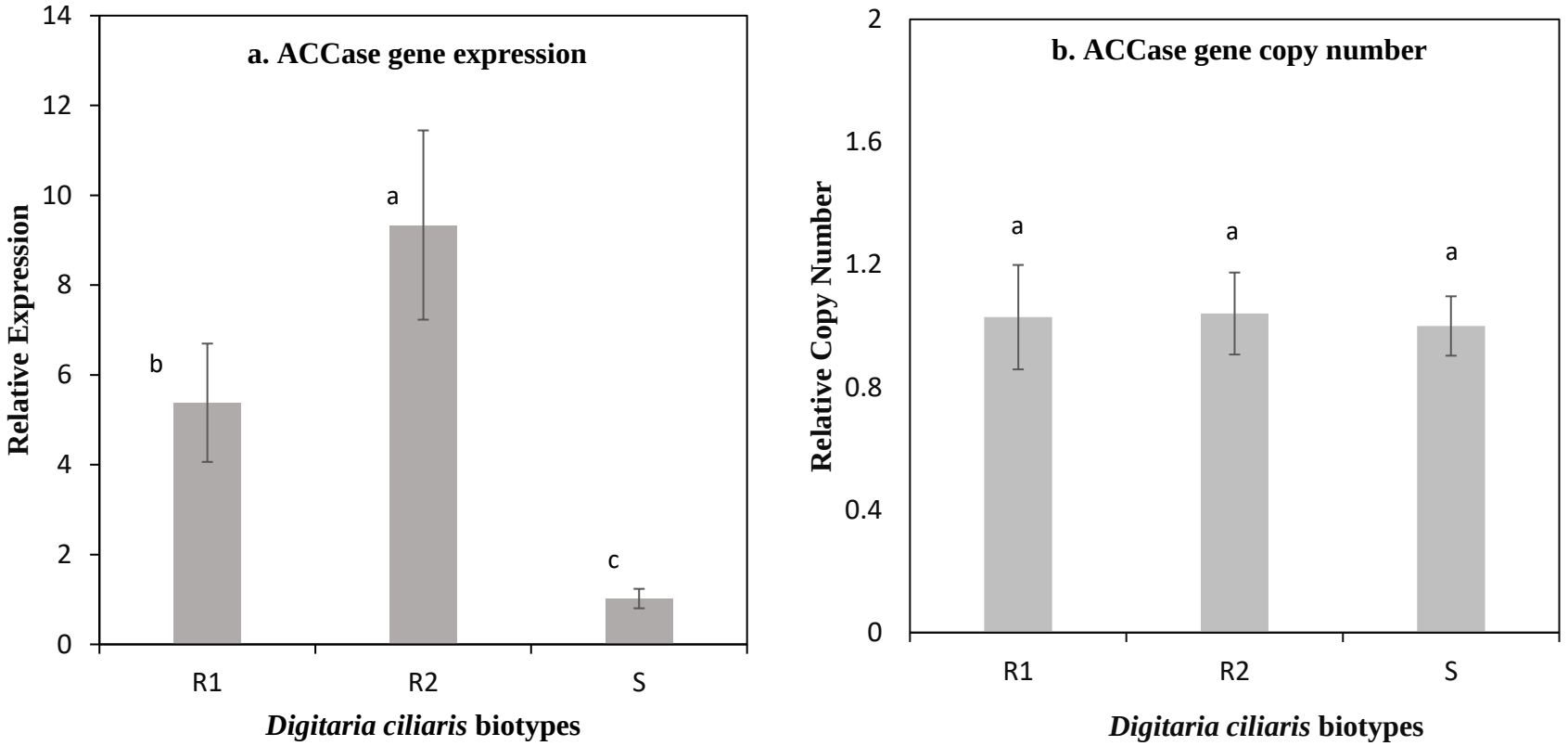


**Figure 3.** Comparison of the relative ACCase gene expression and ACCase gene copy number in the resistant and susceptible biotypes using qRT-PCR to the endogenous control β-actin gene. Vertical bars represent the standard errors of the means (n=3). Bars with the different letter in a column represent the significantly different between resistant and susceptible biotypes according to statistical grouping using single-factor Duncan analysis (P<0.01) for ACCase overexpression and (P>0.01) for ACCase copy number. *D. ciliaris* biotypes: Resistant biotypes, R1 and R2, Georgia, and Susceptible biotype, S, Alabama

# Chapter 5

## Evaluation the three different bioassays for the early-stage detection of ACCase-targeting herbicides resistance in *D. ciliaris*

[This chapter has been prepared for the submission in the journal of weed science, Suma Basak, J. Scott McElroy, Bo Bi, Clebson G. Gonçalves, John Peppers, Jinesh D. Patel, and Patrick E. McCullough. 2021. Evaluation of three diagnostic bioassays for first phase detection of southern crabgrass (*Digitaria ciliaris*) resistance to ACCase-targeting herbicides]

### i. Abstract

Diagnostic bioassays are used to provide an initial screen of the suspected resistance population. These assays are normally conducted at a single dose and are evaluated at a specific time after treatment and must provide a clear and obvious differential response between resistant and susceptible. Three different bioassays were evaluated to assess the detection of acetyl CoA carboxylase (ACCase)-targeting herbicides resistance in southern crabgrass (*D. ciliaris*). Biotypes (R1 and R2) containing Ile-1781-Leu mutation with known resistance to sethoxydim and select aryloxyphenoxypropionate (FOPs) herbicides along with a susceptible biotype (S) were evaluated. R1 and R2 differed from S in all employed bioassays including agar-based gel box assay, leaf flotation assay, and electrical conductivity assay. In the agar-based gel box assay, the susceptible biotype had greater phytotoxicity at the lower concentration relative to the resistant biotypes at 3 days after treatment but differences between resistant and susceptible decreased over time. In the leaf flotation assay, leaves of southern crabgrass from S, R2, and R1 biotypes were placed in separate polypropylene centrifuge tubes containing the discriminating dose of herbicides (0.6 to 9.6 μM). Resistant biotypes floated at the lower concentration on the surface, whereas the leaves

of susceptible biotypes failed to float. For the electrical conductivity assay, the susceptible biotype contained high electrical conductivity due to the high leaching of electrolyte into the water across all four herbicides tested than the resistant biotypes. While these assays were able to separate R and S biotypes, a clear and obvious response was not observed.

Nomenclature: Acetyl-coenzyme A carboxylase (ACCase); Sethoxydim; Fluazifop-p-butyl; Clethodim; Pinoxaden; Southern crabgrass (*Digitaria ciliaris*)

Key words: Rapid test, Agar based gel box, leaf flotation, electrical conductivity, Herbicide resistance test; turf, ACCase-targeting herbicides

## ii. Introduction

Herbicides targeting acetyl-CoA carboxylase (ACCase) are unique selective herbicides utilized as a post-emergence (POST) to control a variety of field crops (Délye 2005; Powles and Yu 2010; Kaundun 2011). More than 50% of grass crops are treated with these herbicides throughout growing seasons (Kaundun 2011). ACCase-targeting herbicides control the grass weed by inhibiting *de novo* fatty acid syntheses. Three distinct chemical classes herbicides, cyclohexanediones (CHD or DIMs), aryloxyphenoxypropionates (AOPP or FOPs), and pyrazolines (DEN) are included in ACCase-targeting herbicides (Hofer et al. 2006, Hochberg et al. 2009; Powles and Yu 2010). Typically, these herbicides control grass weeds by inhibiting the enzyme ACCase, which catalyzes the carboxylation of acetyl-CoA to malonyl Co-A in de novo fatty acid biosynthesis. When susceptible plants are treated with ACCase targeting herbicides, ACCase blocks the biosynthesis of *de novo* fatty acid (Powles 2005; Cronan and Waldrop 2002). Resistance to ACCase-targeting herbicides can be caused to target-site resistance (TSR) or non-target site resistance (NTSR). TSR is generally initiated by mutation (s) in the gene, where a mutation alters the protein structure at the herbicide's binding site rendering this protein insensitive to the active ingredient. NTSR is initiated without alteration to the target site (Delye et al. 2011). This resistance encompasses a range of diverse mechanisms including reduced herbicide uptake, penetration, impaired translocation, and enhanced metabolism of herbicides (Powles and Yu 2010). Both target and nontarget resistance mechanisms can exist in a single population (Powles and Yu 2010; Burnet et al.1994).

Researchers have established resistance testing procedures for various weed and herbicide combinations (Rüegg et al. 2007). Many researchers reported that the ager-based testing assay was

simple, robust, cost-effective, quick, and successful for detecting both target and nontarget site resistance in an array of broadleaf and grass weeds of the cropping system. For example, Kaundun et al. (2011) developed a rapid in-season quick test (RISQ) for resistance screening of *Lolium* spp. (ryegrass) and *Alopecurus myosuroides* Huds. (blackgrass) to acetolactate synthase (ALS) and ACCase targeting herbicides in the growing season. Brosnan et al. (2017) used the agar-based quick test for determining the resistance of EPSPS (5 enolpyruvylshikimate-3 phosphates) and ALS-targeting herbicides in *Poa annua* (annual bluegrass) less than two weeks. Similarly, Hensley (1981) tested the leaf disc flotation assay for identifying the triazine resistant or susceptible biotypes of common groundsel (*Senecio vulgaris* L.), common lambs- quarters (*Chenopodium album* L.), and smooth pigweed (*Amarantbus bybridus* L.). Thus, various alternative procedures have been developed to determine herbicide resistance in whole plants (Boutsalis 2001; Kaunduan and windass 2006; Kaundun et al. 2014), seedling (Letouzé and Gasquez 1999), seed (Bourgeois et al. 1997; Kim et al. 2000; Tal et al. 2000), and pollen bioassays (Letouzé and Gsquez 2000) conducted in a greenhouse environment. For laboratory experiments, tests include plantlet evaluations (Letouzé et al. 1997), seed germination percentage with hypocotyl, and radicle length (Abdurruhman et al. 2018), pollen germination (Richter and Powles 1993), and chlorophyll fluorescence (Clay and Underwood, 1990; Norsworthy et al. 1998; Van Oorschot and Van Leeuwen 1992).

The petri dish bioassay included for resistance test that was related to seed germination on herbicide-saturated media (Murray et al. 1996; Abdurruhman et al. 2018). A major limitation associated with the seed-petri dish bioassay is the time required for seed production, development of seed dormancy, and seed ripening, followed by uniform germination of seed for evaluation (Cutulle et al. 2009). The whole plant assay, however, is the most common technique for detecting

resistance among others (Moss et al. 1998; Beckie et al. 2000). Conventionally, the characterization of weed resistance has mainly relied on greenhouse screening. The herbicide resistance confirmation and evaluation in weeds include the seeds of surviving weeds collection from the treated field after treating with specific herbicides. The collected seeds then are grown under a controlled environment in a glasshouse or a growth chamber. New individuals are treated with either PRE or POST control herbicides and compared their response to herbicide treatment with that of confirmed herbicide susceptible individuals in the same manner (Moss 1995; Burgos et al. 2013). The conventional techniques to diagnose herbicide-resistance weeds are tedious, laborious, time-consuming, require more space, and not very accurate.

Our research objective was to evaluate the three rapid bioassays with ACCase resistance in two biotypes of *D. ciliaris* previously confirmed resistant to sethoxydim and select aryloxyphenoxypropionate (FOPs) herbicide with a mutant allele, Ile-1781-Leu amino acid substitution, and greater ACCase enzyme activity. Further, no reports, to our knowledge have been published for detecting ACCase resistance in *D. ciliaris* with the rapid bioassay including three different assays, an agar-based gel box assay, leaf flotation assay, and electrical conductivity assay. The primary goal of our research, therefore, was to determine the resistance level of *D. ciliaris* to ACCase-targeting herbicides through the rapid bioassay for the field plant population.

## iii. Materials and Methods

Experiments were conducted using the growth chamber at plant genomics laboratory in the Department of Crop, Soil and Environmental Sciences, Auburn, AL, USA. The level of resistance in *D. ciliaris* was diagnosed through rapid bioassays. Two resistant biotypes (R1 and R2) containing Ile-1781-Leu mutant allele and greater ACCase enzyme activity of *D. ciliaris* to sethoxydim and select aryloxyphenoxypropionates (FOPs) herbicides, fenoxaprop and fluazifop

(Yu et al. 2017, Basak et al. 2019), and one susceptible biotype was involved in our study. The resistant biotypes were collected from undisclosed sod production fields of 'TifBlair' centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) in Georgia. A separate susceptible biotype (S) was harvested from the Auburn University campus, Auburn, AL. The collected plants were propagated separately in the greenhouse for increasing seed and stored at 4$^{o}$C for future use.

All greenhouse experiments were conducted at the Auburn University Weed Science Greenhouse located on the main campus of Auburn University, Auburn, Alabama (32.35$^{o}$N, 85.29$^{o}$W). The test plant of *D. ciliaris* was grown in separate plastic flats containing commercial potting soil and peat moss (2:1 v/v) under greenhouse conditions. The plastic flats were placed for 2 weeks in a greenhouse. Daily temperatures in the greenhouse were maintained between a low of 28$^{o}$C and a high of 32$^{o}$C (night/day) (+/- 3C) throughout the study. Relative humidity levels were alternated between 65% during the day and 75% during the night. Ambient lighting was used throughout the experiment with no supplemental light added. Irrigation for plastic flats three times daily (around 0.2 cm per cycle) was provided as needed to maintain a moist soil condition.

The rapid bioassay with three different methods such as an agar-based gel box assay, leaf flotation assay, and electrical conductivity assay was used to evaluate the responses of *D. ciliaris* resistant and susceptible biotypes from a rate titration of four different ACCase-targeting herbicides such as sethoxydim (Segment®, BASF Corp, Research Triangle Park, NC), fluazifop-P-butyl (Fusillade®, Syngenta Crop Protection, Greensboro, NC ), pinoxaden (Axial®, Syngenta Crop Protection, Greensboro, NC) and clethodim (Envoy®, Valent U.S.A. Corp., Walnut Creek, CA). All glassware, conical flasks, beakers, tips of pipettes, metallic instruments like forceps, scalpels were wrapped with aluminum foils and then were sterilized. Each plant culture box was capped with a plastic cap and was autoclaved at a temperature of 121$^{0}$C for 15 minutes at 1.5 kg

$cm^{-2}$ pressure. All aseptic manipulations were carried out in a laminar airflow cabinet which was cleaned with absolute ethyl alcohol to kill the surface contaminants. Instruments were sterilized by the flaming method inside the cabinet and sterilized between each sample. All measures were taken to obtain maximum contamination-free condition during setting up the plantlet into the agar culture or setting up the leaves into the polypropylene centrifuge tubes. All the waste contaminate was sterilized using a liquid cycle in an autoclave before disposing of it.

**Agar-Based Gel Box Assay.** In this study, ACCase resistance for *D. ciliaris* was evaluated using an agar-based gel box assay. Seedlings of each biotype at the 2-3 leaf stage were carefully uprooted from >5 tiller plants with each tiller. Roots were washed under tap water to remove any growing media. The seedlings then were dissected into a single tiller with approximately 7cm of the shoot and 5cm of root and washed with distilled water. Polycarbonate plant culture boxes (Magenta GA-7, Bioworld, Dublin, OH) were used to determine if the agar-based gel box bioassay was useful in detecting the level of ACCase-targeting herbicides resistance in *D. ciliaris*. The agar-based resistance test assay was previously reported by Kaundun et al. 2011 and Brosnan et al. 2017.

MS (Murashige and Skoog 1962) basal medium was prepared as described Brosnan et al. 2017 and added to the solution at a rate of 4.43g $L^{-1}$, and the pH of the medium was adjusted to 6.5 with a pH meter with the help of 0.1 or 10 N sodium hydroxide/ hydrochloric acid solution, whichever was necessary. After adjusting the pH, 5.5 g agar powder (Plant Agar, Duchefa Biochemie, Haarlem, Netherlands) was dissolved in 1 L MS medium to solidify the medium. This amended solution was sterilized properly at $121^0$C for 20 minutes at 1.5 kg $cm^{-2}$ pressure using an autoclave machine (Vaccum Steam Sterilizer, Getinge, Inc, Wayne, NJ). 3 mL of a rifampicin antibiotic and 15 μL azoxystrobin (Heritage TL, Syngenta Professional Products, Greensboro, NC)

were used in the autoclaved agar medium to avoid fungal and bacterial contamination. A total of 70 mL MS media was poured into the polycarbonate plant tissue culture boxes of 10.2 cm x 7.6 cm x 7.6 cm. The commercial herbicide solution was added to the polycarbonate plant tissue culture boxes. Each biotype was treated with each herbicide at a large range of concentrations, i.e., Control, 0.2, 0.4, 0.8, 1.6, 3.3, 6.5, and 12μM. Five single tiller plantlets then were embedded horizontally on top of the agar culture in each plant tissue culture boxes using sterilized scalpel and forceps represented in Figure 1a. The roots below the growing point were then gently pushed into the agar, ensuring that the remaining roots were in contact with the agar. The plant tissue culture boxes then were covered with lids and incubated in the growth chamber configured to provide a constant 16$^{o}$C air temperature, 60% relative humidity, and 16h photoperiod. Phytotoxicity data were recorded at 3, 6, and 9 days after treatment (DAT) on a 0 to 100 percent scale in which 0% corresponded to no damage and 100% corresponded to complete plant death or desiccation.

**Leaf Flotation Assay.** Leaf flotation test was conducted using polypropylene centrifuge 50 mL tubes containing herbicide solution to determine if the time of leaf flotation could be correlated with resistance. The solution was prepared by adding commercial herbicides and surfactants and adjusted to pH 6.5 using 0.1 or 10N sodium hydroxide/ hydrochloric acid solution as necessary. Surfactant (2 μL; Induce, Helena® Chemical Company, Collierville, TN) was added to the solution to reduce the surface tension. A total of 40mL solution was poured into the sterile polypropylene centrifuge tube. Each biotype was treated with each herbicide at six ranges of concentrations, i.e., Control, 0.6, 1.2, 2.4, 4.8, and 9.6μM. The leaf was cut into 2.5-cm long and immediately transferred to deionized water. Three leaves then were embedded horizontally on top of the solution in each tubes using sterilized forceps represented in Figure 2a. The tubes then were

covered with lids and incubated at 25$^{o}$C temperature. At 8, 16, and 32 hours after treatment (HAT), leaf flotation or sinking data was recorded on a 0 or 100 scale in which 0 corresponded to sink and 100 corresponded to float.

**Electrical Conductivity Assay.** The electrical conductivity test was performed on the Extech EC150 conductivity meter (FLIR Systems, Wilsonville, Oregon, USA) according to the manufacturer's instructions. Treated leaves were first placed in a solution containing 0, 0.5, 0.9, 1.9, 3.8, and 7.5µM of each herbicide and incubated for 24h at room temperature. The treated leaves were removed from the herbicide solution, washed with distilled water, and were placed into another polypropylene centrifuge tube containing 40ml distilled water. All the seven tubes containing treated leaves were placed into a glass beaker containing tap water and warmed at 100$^{o}$C for 3-5 minutes with intermittent shaking of tubes. Then, the leaves were discarded from tubes and the solution was cooled down until reaching room temperature. The conductivity meter was placed horizontally into the tubes measuring the values of electrical conductivity for each biotype. The electrical conductivity was monitored with a conductivity meter with sensor cap represented in Figure 3a and the conductivity (µS/cm) was expressed as a percentage of the nonherbicidal control.

**Experimental Design and Data Analysis.** All experiments were established as a complete randomized factorial design. All treatments were replicated three times on five individual plantlets per biotype for the gel box assay, three leaves per biotype for the leaf flotation assay, and three leaves per biotype electrical conductivity test assays that were conducted twice in time. All statistical test was carried out with the PROC GLM procedure through SAS (SAS version 9.4, SAS Institute Inc., Cary, NC). Fisher's protected LSD (P= 0.05) was used to compare the difference among S, R2, and R1. The experiment was repeated two times under controlled

conditions. Since differences between the data of the two experimental runs were not detected in the analysis of variance at the 0.05 probability level, the data were pooled overruns for subsequent analysis. Each herbicide rates were log-transformed to produce equal spacing among treatments prior to regression analysis. The nontreated mean of each biotype was used for conversion calculations to determine relative measures of each treatment. A model was characterized that the relationship of the response curves with herbicide rate after plotting treatment means. All measurements relative to nontreated were used for the regression model.

Regression models were developed using Prism (GraphPad Software, version 5.0, Inc., La Jolla, CA). ACCase-targeting herbicide concentrations causing 50% phytotoxicity, leaf flotation, and electrical conductivity ($IC_{50}$) values were estimated using nonlinear regression models. The following non-linear regression analysis was used to calculate the $IC_{50}$ value in the experiments:

$$Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC_{50})))$$

Y represents the response (%) of *D. ciliaris*, x is log-transformed ACCase-targeting herbicides concentration (µM), Top and bottom are the plateaus in the units of the Y-axis, and LogIC50 is the log-transformed ACCase-targeting herbicides concentration (µM). 95% confidence intervals ($\alpha$=0.05) for the estimates were calculated for nonlinear-regression model parameters. Regression equations were used to calculate inhibition concentration values at 50% (referred to as $IC_{50}$ values) compared to that of the nontreated for each biotype and each ACCase-targeting herbicide. The $IC_{50}$ and R/S values were determined for each resistant biotype versus susceptible biotype. Percent of phytotoxicity from agar-based gel box assay, percent of leaf flotation from leaf flotation assay, and percent data of electrical conductivity from electroconductivity assay relative to the nontreated response to ACCase-targeting herbicides were modeled for all three biotypes using the least-squares fit model, Figure 1b, 2b, and 3b, respectively. The response curves from each biotype were

allowed for calculation of $IC_{50}$'s presented in Tables 1, 2, and 3. The comparison of the three different assays for tested ACCase herbicides resistance is presented in Table 4.

### iv. Results and Discussion

**Agar-Based Gel Box Assay.** Greater phytotoxicity was observed for the S biotype compared to the R biotypes for all the herbicides tested in this study (Figure 1b). Sethoxydim at 0.8, 1.6, and 3.3µM induced 43.3, 56, and 64.7%, phytotoxicity in S biotype, respectively, 12.7, 16, and 25.3% phytotoxicity, respectively, for the R2 biotype and 16.6, 24.6, and 36% phytotoxicity, respectively, for the R1 biotype at 3DAT. Differences in phytotoxicity between S, R2, and R1 were observed at 6 and 9DAT. Sethoxydim at 1.6µM induced 63.3 and 74.6% phytotoxicity at 6 and 9DAT in the S biotype, 20.6 and 24.6% phytotoxicity for the R2 biotype, respectively, and 24 and 36.6%, for the R1 biotype, respectively. $IC_{50}$ values of the S biotype at 3, 6, and 9DAT were 0.7, 0.6, and 0.4µM, respectively, compared to 13.9, 8.0, and 10.7µM, respectively, for the R2 biotype and 4.2, 2.7, and 5.8µM, respectively, for the R1 biotype. Sethoxydim was 19.9, 13.3, and 26.8 times more phytotoxic to S than R2 at 3, 6, and 9DAT, respectively, 6.0, 4.5, and 14.5 times more phytotoxic to S than R1 at 3, 6, and 9DAT, respectively.

In the fluazifop-p-butyl response evaluation, fluazifop-p-butyl at 0.8, 1.6, and 3.3µM induced 47.3, 61.3, and 72.7%, phytotoxicity in the S biotype, respectively, 16, 22, and 33.3% phytotoxicity, respectively, for the R2 biotype and 22.7, 34, and 47.3% phytotoxicity, respectively, for the R1 biotype at 3DAT. Differences in phytotoxicity between S, R2, and R1 were observed at 6 and 9DAT. Fluazifop-p-butyl at 0.8µM induced 50.7 and 64% phytotoxicity at 6 and 9DAT in the S biotype, and 17.3 and 20% phytotoxicity for the R2 biotype, respectively, and 24.6 and 28% for the R1 biotype, respectively. $IC_{50}$ values of the S biotype at 3, 6, and 9DAT were 0.6, 0.5, and 0.3µM, respectively, compared to 4.6, 5.9, and 9.8µM, respectively, for the R2 biotype and 1.8,

2.8, and 3.4µM, respectively, for the R1 biotype. Fluazifop-p-butyl was 7.7, 11.8, and 32.7 times more phytotoxic to S than R2 at 3, 6, and 9DAT, respectively, 3.0, 5.6, and 11.3 times more phytotoxic to S than R1 at 3, 6, and 9DAT, respectively.

Pinoxaden induced phytotoxicity in the S more than that of R biotypes. For example, pinoxaden at 0.8, 1.6, and 3.3µM induced 49.3, 64.7, and 79.3%, phytotoxicity in S biotype, respectively, 14.6, 20.6, and 32.6% phytotoxicity, respectively, for the R2 biotype and 20.6, 31.3, and 45.3% phytotoxicity, respectively, for the R1 biotype at 3DAT. Differences in phytotoxicity between S, R2, and R1 were observed at 6 and 9DAT. Pinoxaden at 3.3µM induced 84.6-94.7% phytotoxicity at 6 and 9DAT, respectively, in the S biotype, only ≤42.6-71.3% phytotoxicity for the R biotypes. $IC_{50}$ values of the S biotype at 3, 6, and 9DAT were 0.6, 0.4, and 0.2µM, respectively, compared to 7.1, 4.7, and 6.1µM, respectively, for the R2 biotype and 3.0, 1.9, and 1.5µM, respectively, for the R1 biotype. Pinoxaden was 14.8, 11.7, and 30.5 times more phytotoxic to S than R2 at 3, 6, and 9DAT, respectively, 5.0, 4.8, and 7.5 times more phytotoxic to S than R1 at 3, 6, and 9DAT, respectively.

Clethodim induced greater phytotoxicity in S biotype than both resistant biotypes. At 3DAT, clethodim at 0.8, 1.6, and 3.3µM induced 56.7, 72, and 83.3% phytotoxicity in S biotype, respectively, 19.3, 30.6, 44% phytotoxicity, respectively, for the R2 biotype and 30.6, 48, 60.6%, phytotoxicity, respectively, for the R1 biotype. Differences in phytotoxicity between S, R2, and R1 were observed at 6 and 9DAT. Clethodim at 1.6µM induced 78 and 90% phytotoxicity at 6 and 9DAT in the S biotype, 36 and 48.6% phytotoxicity for the R2 biotype, respectively, and 50.6 and 72.6%, for the R1 biotype, respectively, indicating R biotypes had a degree of resistance to clethodim. Clethodim induced greater phytotoxicity in both resistant biotypes than other ACCase-targeting herbicides. For instance, clethodim at 0.8µM produced 22.7-35.3% phytotoxicity of

resistant biotypes at 6DAT, while other ACCase-targeting herbicides, sethoxydim, fluazifop, and pinoxaden induced 16-19.3%, 17.3-22.7%, and 18.6-24.7%, respectively in R biotypes. $IC_{50}$ values of the S biotype at 3, 6, and 9DAT were 0.4, 0.3, and 0.1µM, respectively, compared to 3.8, 5.5, and 2.0µM, respectively, for the R2 biotype and 1.5, 1.2, and 0.5µM, respectively, for the R1 biotype. Clethodim was 9.5, 18.3, and 20 times more phytotoxic to S than R2 at 3, 6, and 9DAT, respectively, 3.8, 4.0, and 5.0 times more phytotoxic to S than R1 at 3, 6, and 9DAT, respectively.

**Leaf Flotation Assay.** R biotypes leaves had a greater floatation tendency at lower concentrations, than S (Figure 2b). At 8HAT, sethoxydim at 1.2, 2.4, and 4.8µM inhibited S biotype leaf flotation 55, 68.4, and 75%, while R2 biotype inhibited flotation 5, 8.4, and 15%, respectively, and R1 inhibited flotation 10, 16.7, and 25%, respectively. Inhibition of leaf flotation was observed at 16 and 32HAT for all biotypes. Sethoxydim at 2.4µM inhibited S biotype leaf flotation 78.4 and 85%, while R2 biotype inhibited flotation 16.7 and 35%, respectively, and R1 inhibited flotation 25 and 50%, respectively at 16 and 32HAT. $IC_{50}$ values of the S biotype at 8, 16, and 32HAT were 0.5, 0.4, and 0.3µM, respectively, compared to 16.1, 6.7, and 22.4µM, respectively, for the R2 biotype and 5.1, 2.9, and 5.4µM, respectively, for the R1 biotype. Sethoxydim was 32.2, 16.8, and 74.6 times more inhibited in flotation to S than R2 at 8, 16, and 32HAT, respectively, 10.2, 7.3, and 18 times more phytotoxic to S than R1 at 8, 16, and 32HAT, respectively.

Fluazifop-p-butyl at 1.2, 2.4, and 4.8µM inhibited leaf flotation 50, 60, and 70%, respectively, while R2 biotype inhibited flotation 5, 10, and 20%, respectively, and R1 inhibited flotation 11.7, 20, and 30% at 8HAT. Inhibition of leaf flotation was observed at 16 and 32HAT for all biotypes. Fluazifop-p-butyl at 2.4µM inhibited S biotype leaf flotation 76.7 and 81.7%, while R2 biotype inhibited flotation 20 and 35%, respectively, and R1 inhibited flotation 28.3 and

50%, respectively, at 16 and 32HAT. $IC_{50}$ values of the tS biotype at 8, 16, and 32HAT were 0.6, 0.5, and 0.4µM, respectively, compared to 12.7, 7.5, and 9.7µM, respectively, for the R2 biotype and 4.4, 2.8, and 3.1µM, respectively, for the R1 biotype. Fluazifop-p-butyl was 21.2, 15, 24.3times more inhibited in flotation to S than R2 at 8, 16, and 32HAT, respectively, 7.3, 5.6, and 7.8times more phytotoxic to S than R1 at 8, 16, and 32HAT, respectively.

Pinoxaden inhibited S biotype leaf flotation greater across all concentrations than R biotypes. Pinoxaden at 0.6, 1.2, and 2.4µM inhibited S biotype leaf flotation 35-60% at 8HAT, 45-73.3% at 16HAT, 50-70% at 32HAT, while R2 inhibited flotation 5-16.3% at 8HAT, 10-25% at 16HAT, 15-45% at 32HAT, and R1 biotype inhibited flotation 8.4-25% at 8HAT, 15-36.7% at 16HAT, 20-55% at 32HAT. $IC_{50}$ values of the S biotype at 8, 16, and 32HAT were 0.5, 0.4, and 0.3µM, respectively, compared to 5.1, 4.7, and 5.4µM, respectively, for the R2 biotype and 2.5, 1.3, and 2.3µM, respectively, for the R1 biotype. Pinoxaden was 10.2, 11.8, and 18 times more inhibited in flotation to S than R2 at 8, 16, and 32HAT, respectively, 5.0, 5.3, and 7.7 times more phytotoxic to S than R1 at 8, 16, and 32HAT, respectively.

The S biotype was relatively more sensitive to clethodim herbicide than the R biotypes. Clethodim at 0.6, 1.2, and 2.4µM inhibited S biotype leaf flotation 40, 61.7, and 75%, respectively, at 8HAT; 50, 70, and 81.7%, respectively, at 16HAT; 65, 80, and 86.7%, respectively, at 32HAT, while R2 biotype inhibited 8.3, 15, and 25% flotation, respectively, at 8HAT; 15, 23.7, and 38.3% flotation, respectively, at 16HAT; 20, 35, and 55% flotation, respectively, at 32HAT and R1 biotype inhibited 15, 25, and 35% floatation, respectively, at 8HAT; 23.4, 36.7, and 50% floatation, respectively at 16HAT; 35, 53.3, and 70%, floatation, respectively, at 32HAT. $IC_{50}$ values of the S biotype at 8, 16, and 32HAT were 0.9, 0.2, and 0.1µM, respectively, compared to 3.2, 2.5, and 2.3µM, respectively, for the R2 biotype and 1.4, 1.1, and 0.8µM, respectively, for the

R1 biotype. Clethodim was 3.6, 12.5, and 23 times more inhibited in flotation to S than R2 at 8, 16, and 32HAT, respectively, 1.6, 5.5, and 8 times more phytotoxic to S than R1 at 8, 16, and 32HAT, respectively.

**Electrical Conductivity Assay.** The S biotype produced a greater electrical conductivity for leaching electrolytes into the water to the ACCase-targeting herbicides relative to the R biotypes (Figure 3b). Sethoxydim at 0.9, 2.0, and 3.8µM produced 59.7, 80.5, and 90.7% electrical conductivity in S, respectively, 11.7, 21.6, and 53.3% electrical conductivity in R2, respectively, and 17.2, 29.8, and 65.2% electrical conductivity in R1, respectively. The $IC_{50}$ value of the S biotype was 0.4µM compared to 31.5 and 11.2µM for the R2 and R1 biotypes, respectively. Sethoxydim was 79 and 28 times more leaching electrolyte to S than R2 and R1 respectively, implying that sethoxydim efficiently caused greater leaching electrolyte into the water from the S.

Fluazifop-P-butyl produced electrical conductivity in the S more than that of R biotypes. Fluazifop-p-butyl at 0.9, 2.0, and 3.8µM produced 47.8, 72, and 88.4% electrical conductivity in S, respectively, 12.3, 23.8, and 50.7% electrical conductivity in R2, respectively, and 17.1, 32.3, and 52% electrical conductivity in R1, respectively. The $IC_{50}$ value of the S biotype was 0.6µM compared to 46.7 and 8.7µM for the R2 and R1 biotypes, respectively. Fluazifop-p-butyl was 77 and 15 times more leaching electrolyte to S than R2 and R1 respectively, indicating that less leaching electrolyte into the water from the R biotypes confers fluazifop-p-butyl resistance.

Pinoxaden caused significantly and increased the measured variables of S in electrical conductivity across all concentrations relative to R biotypes. Pinoxaden at 0.9, 2.0, and 3.8 µM produced 42.4-84.5% electrical conductivity in S, respectively, 11.2-53.2% and 19.4-64.2% electrical conductivity in R2 and R1, respectively. The $IC_{50}$ value of the S biotype was 0.7µM compared to 43.4 and 7.6µM for the R2 and R1 biotypes, respectively. Pinoxaden was 62 and 11

times more leaching electrolyte to S than R2 and R1 respectively, which suggested that a less electrical conductivity by pinoxaden herbicide caused the pinoxaden resistance to the R biotypes.

Clethodim resulted in a significant increase in electrical conductivity for all biotypes comparing all four herbicides used. However, the S biotype had relatively greater clethodim electrical conductivity than the R biotypes. Clethodim at 0.9, 2.0, and 3.8µM produced 50.7, 67.1, and 88% electrical conductivity in S biotype, respectively, 15.6, 31.4, and 57.1% electrical conductivity in R2, respectively, and 26.1, 46.3, and 68.3% electrical conductivity in R1, respectively. The $IC_{50}$ value of the S biotype was 0.97µM compared to 15.7 and 3.5µM for the R2 and R1 biotypes, respectively. Clethodim was 17 and 4 times more leaching electrolyte to S than R2 and R1, respectively. Previous research also reported the R1 and R2 biotypes had differential resistance to clethodim when foliar applied (Yu et al. 2017) and comparatively lower ACCase enzyme activity to clethodim than the other inhibitors sethoxydim, pinoxaden, and fluazifop (Basak et al. 2019).

## v. Conclusion

Rapid diagnosis for grass weed resistance is very critical. Without exposure to herbicides, the separation of the resistant population is difficult, and farmers need to identify herbicide resistance quickly after weeds have been found to escaping an herbicide treatment. Timely detection of herbicide resistance at the first stage of crop production, therefore, is essential to help farmers finding alternative solutions to manage herbicide resistance in their fields. Traditionally, the characterization of grass weed resistance has mainly relied on greenhouse screening, which required time and labor. The current study investigated the feasibility of the rapid bioassay to quickly determine ACCase resistance against the selected ACCase-targeting herbicides in *D. ciliaris*. There are no apparent differences in physical appearance between the two resistant and

susceptible biotypes. Recently, the separation of a susceptible and resistant biotype of the various species is also challenging. To make a visual separation between susceptible and resistant biotypes, we have focused on the two rapid bioassays such as phytotoxicity development in agar-based gel box assay and leaf flotation in leaf flotation-based polypropylene centrifuge tube assay.

Attempts to test the single tiller plantlets of *D. ciliaris* in agar-based gel box assay by embedded them horizontally on top of the agar culture integrating ACCase herbicides, the susceptible biotypes had greater phytotoxicity to ACCase-targeting herbicides than the R biotypes within 9 days after treatment at less expense. But the noticeable separation was not found from this assay. Although phytotoxicity induction was significantly greater in the susceptible biotype compared to R biotypes, phytotoxicity development also was found in R biotypes in the gel box assay. We realized that plantlets not only were inhibited by ACCase herbicide treatment but also experienced wilting because of excessive transpiration from leaves. Also, this visual assessment test assay has required the researcher to create a lot of clones, sacrifice the clones, and need to wait at least 9 days to investigate the ACCase herbicides resistance of the suspected *D. ciliaris* plants.

Likewise, we used a non-destructive leaf flotation assay using a polypropylene centrifuge tube for the discrimination between susceptible and R biotypes, which was required less time than the agar-based gel box assay. This assay allowed to separate between R and S biotypes in visual evaluations to ACCase-targeting herbicides within 32h by avoiding transpiration. The leaf flotation assay also can be used to determine the resistance to ACCase-targeting herbicides in large numbers of plants without destroying the whole plant or preventing reproduction. The rate of leaves sinking was always significantly higher in the susceptible biotype compared to the R biotypes. However, the tendency of leaves sinking was found for both susceptible and resistant biotypes. This unpredicted result indicated that the leaf flotation assay is more suitable for

photosynthesis-targeting herbicides rather than ALS and ACCase-targeting herbicides. With our experiment using the electrical conductivity assay, however, the diagnostic of herbicide resistance is feasible to diagnose herbicide resistance at the first stage of crop production. Because the rate of leaching electrolyte to the selected ACCase-targeting herbicides for the S biotype was constantly greater than the R biotypes and provided results within 24h after treatment. Moreover, farmers can separate the resistant and susceptible field populations easily.

The $IC_{50}$ values for resistant biotypes across all four herbicides were consistently greater than the susceptible biotypes in the three different assays, indicating that the resistant biotypes are resistant to ACCase-targeting herbicides. All the rapid bioassays showed similar trends resistance and the comparisons among the three different assays have been presented in Table 4. The electrical conductivity test assay, however, had a maximum difference between S and R biotypes. Thus, in the future, our findings from the rapid bioassay can be used as tools for routine detection of resistance to post-emergence herbicides in grass weed field populations and this technique of resistance level determination can be exploited successfully. To our knowledge. this is the first report of rapid bioassay with three different assays from *D. ciliaris* to the ACCase-targeting herbicides resistance. Further studies are needed to expand these assays for detecting herbicide resistance in other grass weeds with other modes of action as well as herbicide mixtures. We used only four different ACCase herbicides to test the resistance level in *D. cilliaris* and we have yet to explore the efficiency of this dynamic assay for use with other populations of the same species that can be yielded cross or multiple resistance mechanisms. Further, more research is needed for exploring other modes of action to control the *D. ciliaris*.

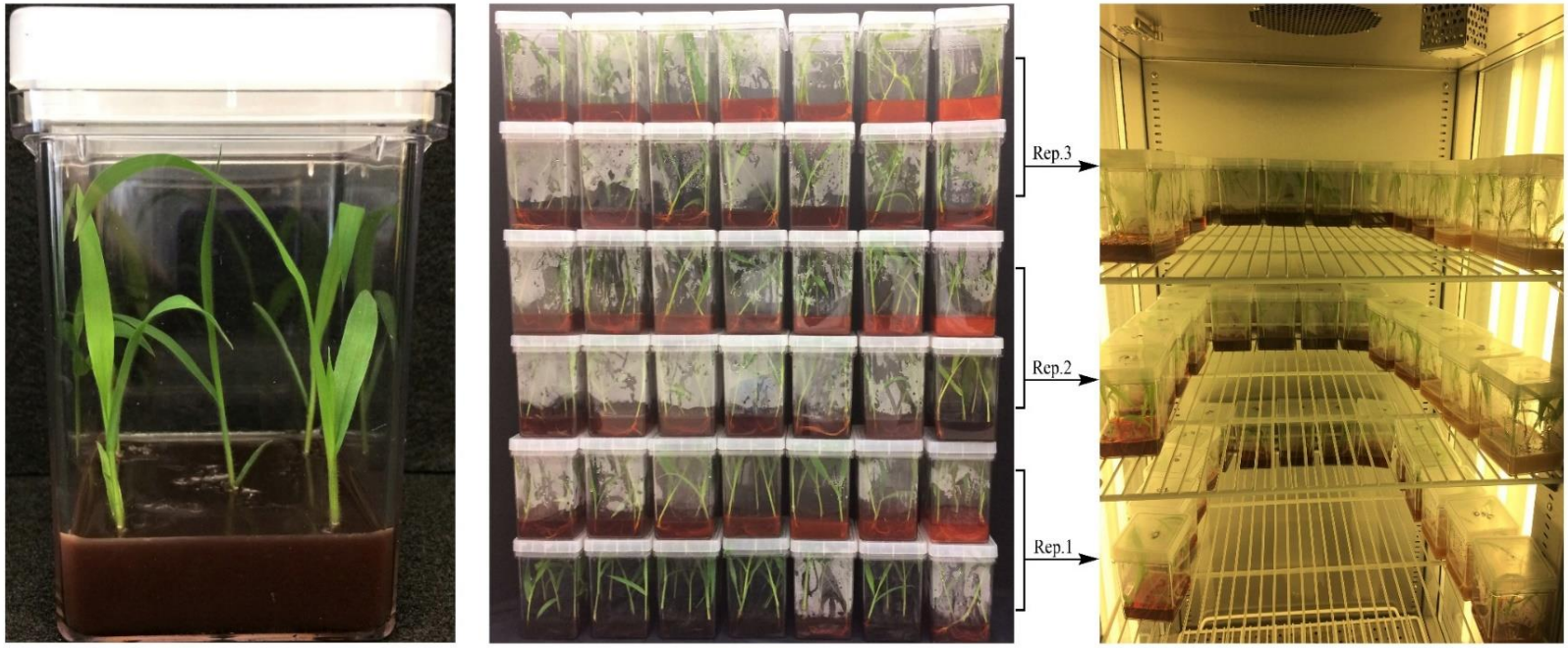


**Figure 1a.** Single tiller plantlet of *D. ciliaris* was inserted into agar medium containing different concentrations of ACCase targeting herbicides inside polycarbonate plant tissue culture box and the boxes were incubated into the growth chamber

**Table 1.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing concentrations of ACCase-targeting herbicides, sethoxydim, pinoxaden, fluazifop-p-butyl, and clethodim relative to the nontreated control measured with least squares fit model for percent of phytotoxicity from agar-based gel box assay. The required concentration of ACCase-targeting herbicides ($IC_{50}$) to cause 50% in vitro inhibition of phytotoxicity at 3, 6, and 9 days after transplanting (DAT) was calculated from concentration response curves. Parameter estimates, 95% confidence intervals (CI), values of $IC_{50}$, and R/S ratio are presented as means of model comparison

| Biotype[a] | Time[b] | Equation[c] | Parameter estimates and confidence intervals | | | | | | Inhibition[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) | Bottom | 95% CI | Top | 95% CI | LogIC50 | 95% CI | $IC_{50}$ (µM) | R/S ratio |
| | | | | | Sethoxydim | | | | | |
| R1 | 3DAT | Y=128.4+(6.3-128.4)/(1+10^((X-0.6))) | 128.4 | (114.1, 142.6) | 6.3 | (2.3, 10.4) | 0.6 | (0.5, 0.8) | 4.2 | 6 |
| R2 | | Y=195.2+(9.8-195.2)/(1+10^((X-1.1))) | 195.2 | (146.3, 244) | 9.8 | (6.9, 12.8) | 1.1 | (0.9, 1.3) | 13.9 | 19.9 |
| S | | Y=104.2+(-11.5-104.2)/(1+10^((X-0.7))) | 104.2 | (99.7, 109.6) | -11.5 | (-19.6, -3.4) | -0.2 | (-0.3, 0.1) | 0.7 | |
| R1 | 6DAT | Y=117.6+(3.3-117.5)/(1+10^((X-0.4))) | 117.6 | (107.6, 127.6) | 3.3 | (-1.2, 7.7) | 0.4 | (0.3, 0.6) | 2.7 | 4.5 |
| R2 | | Y=156.8+(7.4-156.8)/(1+10^((X-0.9))) | 156.8 | (126.6, 186.9) | 7.4 | (3.5, 11.4) | 0.9 | (0.7, 1.1) | 8 | 13.3 |
| S | | Y=102.7+(-11.7-102.7)/(1+10^((X+0.2))) | 102.7 | (99.4, 106.1) | -11.7 | (-17.5, -5.9) | -0.2 | (-0.3, -0.1) | 0.6 | |
| R1 | 9DAT | Y=131+(4-131)/(1+10^((X-0.6))) | 131 | (121, 141) | 4 | (1.3, 6.8) | 0.6 | (0.5, 0.7) | 5.8 | 14.5 |
| R2 | | Y=178.5+(5.4-178.5)/(1+10^((X-1))) | 178.5 | (130.1, 227) | 5.4 | (1.1, 9.7) | 1 | (0.8, 1.3) | 10.7 | 26.8 |
| S | | Y=101.3+(-23-101.3)/(1+10^((X+0.4))) | 101.3 | (99.2, 103.4) | -23 | (-28.8, -17.2) | -0.4 | (-0.5, -0.4) | 0.4 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Fluazifop-p-butyl | | | | | | |
| R1 | 3DAT | Y=112.3+(0.1-112.3)/(1+10^((X-0.3))) | 112.3 | (105.9, 118.6) | 0.1 | (-4.1, 4.3) | 0.3 | (0.2, 0.4) | 1.8 | 3 |
| R2 | | Y=132.2+(6.4-132.2)/(1+10^((X-0.7))) | 132.2 | (119.3, 145) | 6.4 | (3.1, 9.7) | 0.7 | (0.5, 0.8) | 4.6 | 7.7 |
| S | | Y=103.5+(-20.7-103.5)/(1+10^((X+0.3))) | 103.5 | (99.8, 107.1) | -20.7 | (-29.1, -12.1) | -0.3 | (-0.4, -0.2) | 0.6 | |
| R1 | 6DAT | Y=118.3+(3.8-118.3)/(1+10^((X-0.4))) | 118.3 | (110.8, 125.9) | 3.8 | (0.5, 7.1) | 0.4 | (0.3, 0.5) | 2.8 | 5.6 |
| R2 | | Y=142.9+(5.5-142.9)/(1+10^((X-0.8))) | 142.9 | (130, 155.9) | 5.5 | (2.9, 7.9) | 0.8 | (0.7, 0.9) | 5.9 | 11.8 |
| S | | Y=102.5+(-13.9-102.5)/(1+10^((X+0.3))) | 102.5 | (100.1, 104.9) | -13.9 | (-18.4, -9.4) | -0.3 | (-0.3, -0.2) | 0.5 | |
| R1 | 9DAT | Y=123.5+(3.6-12.35)/(1+10^((X-0.5))) | 123.5 | (115.2, 131.8) | 3.6 | (0.7, 6.5) | 0.5 | (0.4, 0.6) | 3.4 | 11.3 |
| R2 | | Y=172+(5.2-172)/(1+10^((X-1))) | 172 | (155.7, 188.3) | 5.2 | (3.5, 6.8) | 1 | (0.9, 1.1) | 9.8 | 32.7 |
| S | | Y=99.5+(-36.7-99.5)/(1+10^((X+0.6))) | 99.5 | (97.2, 101.8) | -36.7 | (-46.7, -26.7) | -0.6 | (-0.7, -0.5) | 0.3 | |
| | | | | | Pinoxaden | | | | | |
| R1 | 3DAT | Y=120.4+(4.4-120.4)/(1+10^((X-0.5))) | 120.4 | (111.1, 129.7) | 4.4 | (0.6, 8.2) | 0.5 | (0.4, 0.6) | 3 | 5 |
| R2 | | Y=149.8+(7.3-1949.8)/(1+10^((X-0.9))) | 149.8 | (132.7, 167) | 7.3 | (4.7, 9.9) | 0.9 | (0.7, 0.9) | 7.1 | 14.8 |
| S | | Y=106.3+(-15.7-106.3)/(1+10^((X+0.3))) | 106.3 | (102.5, 110.1) | -15.7 | (-23, -8.4) | -0.3 | (-0.4, -0.2) | 0.6 | |
| R1 | 6DAT | Y=113.2+(0.5-113.2)/(1+10^((X-0.3))) | 113.2 | (108.3, 118.1) | 0.5 | (-2.5, 3.6) | 0.3 | (0.2, 0.3) | 1.9 | 4.8 |
| R2 | | Y=132.9+(5-132.9)/(1+10^((X-0.7))) | 132.9 | (119.8, 145.9) | 5 | (1.8, 8.3) | 0.7 | (0.6, 0.8) | 4.7 | 11.7 |
| S | | Y=103.3+(-21.1-103.3)/(1+10^((X+0.4))) | 103.3 | (100.6, 106) | -21.1 | (-27.7, -14.5) | -0.4 | (-0.4, -0.3) | 0.4 | |
| R1 | 9DAT | Y=108.5+(-1.5-108.6)/(1+10^((X-0.2))) | 108.6 | (104.4, 112.7) | -1.5 | (-4.8, 1.7) | 0.2 | (0.1, 0.2) | 1.5 | 7.5 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| R2 | | Y=144.4+(4.5-144.4)/(1+10^((X-0.8))) | 144.4 | (132.2, 156.5) | 4.5 | (2.3, 6.8) | 0.8 | (0.7, 0.9) | 6.1 | 30.5 |
| S | | Y=101.8+(-56.4-101.8)/(1+10^((X+0.8))) | 101.8 | (99.6, 103.9) | -56.4 | (-71.1, -41.7) | -0.8 | (-0.8, -0.7) | 0.2 | |
| | | | | Clethodim | | | | | | |
| R1 | 3DAT | Y=108.8+(-2.5-108.8)/(1+10^((X-0.2))) | 108.8 | (104.2, 113.3) | -2.5 | (-6.0, 1.0) | 0.2 | (0.1, 0.3) | 1.5 | 3.8 |
| R2 | | Y=126+(4.1-126)/(1+10^((X-0.6))) | 126 | (115.9, 136.2) | 4.1 | (0.9, 7.4) | 0.6 | (0.5, 0.7) | 3.8 | 9.5 |
| S | | Y=100.9+(-23.3-100.9)/(1+10^((X+0.4))) | 100.9 | (98.5, 103.3) | -23.3 | (-29.6, -17) | -0.4 | (-0.5, 0.3) | 0.4 | |
| R1 | 6DAT | Y=106.7+(-4.0-106.7)/(1+10^((X-0.1))) | 106.7 | (102.3, 111.1) | -4.0 | (-8.1, 0.01) | 0.1 | (0.01, 0.2) | 1.2 | 4 |
| R2 | | Y=139+(4.3-139)/(1+10^((X-0.7))) | 139 | (126, 151.9) | 4.3 | (1.6, 6.9) | 0.7 | (0.6, 0.8) | 5.5 | 18.3 |
| S | | Y=100.9+(-31.2-100.9)/(1+10^((X+0.5))) | 100.9 | (98.8, 103) | -31.2 | (-38.5, -23.8) | -0.5 | (-0.6, -0.4) | 0.3 | |
| R1 | 9DAT | Y=101.2+(-17.7-101.2)/(1+10^((X+0.3))) | 101.2 | (98.8, 103.5) | -17.7 | (-22.6, -12.8) | -0.3 | (-0.4, 0.2) | 0.5 | 5 |
| R2 | | Y=114.5+(-1.7-114.5)/(1+10^((X-0.3))) | 114.5 | (110.4, 118.7) | -1.7 | (-4.1, 0.8) | 0.3 | (0.2, 0.4) | 2.0 | 20 |
| S | | Y=100.8+(-169.5-100.8)/(1+10^((X+1.2))) | 100.8 | (99.3, 102.4) | -169.5 | (-219.1, -119.9) | -1.2 | (-1.3, -1.1) | 0.1 | |

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype; [b] Abbreviations: Days after transplanting (DAT); [c]In the least squares fit equation, x represents the concentration of ACCase-targeting herbicides, y represents the response variable of phytotoxicity; [d]Inhibition: The required concentration of ACCase-targeting herbicides was calculated by 50% ($IC_{50}$) based on regression curve to fit in the concentration response inhibition equation and R/S ratios, resistant/susceptible ratios

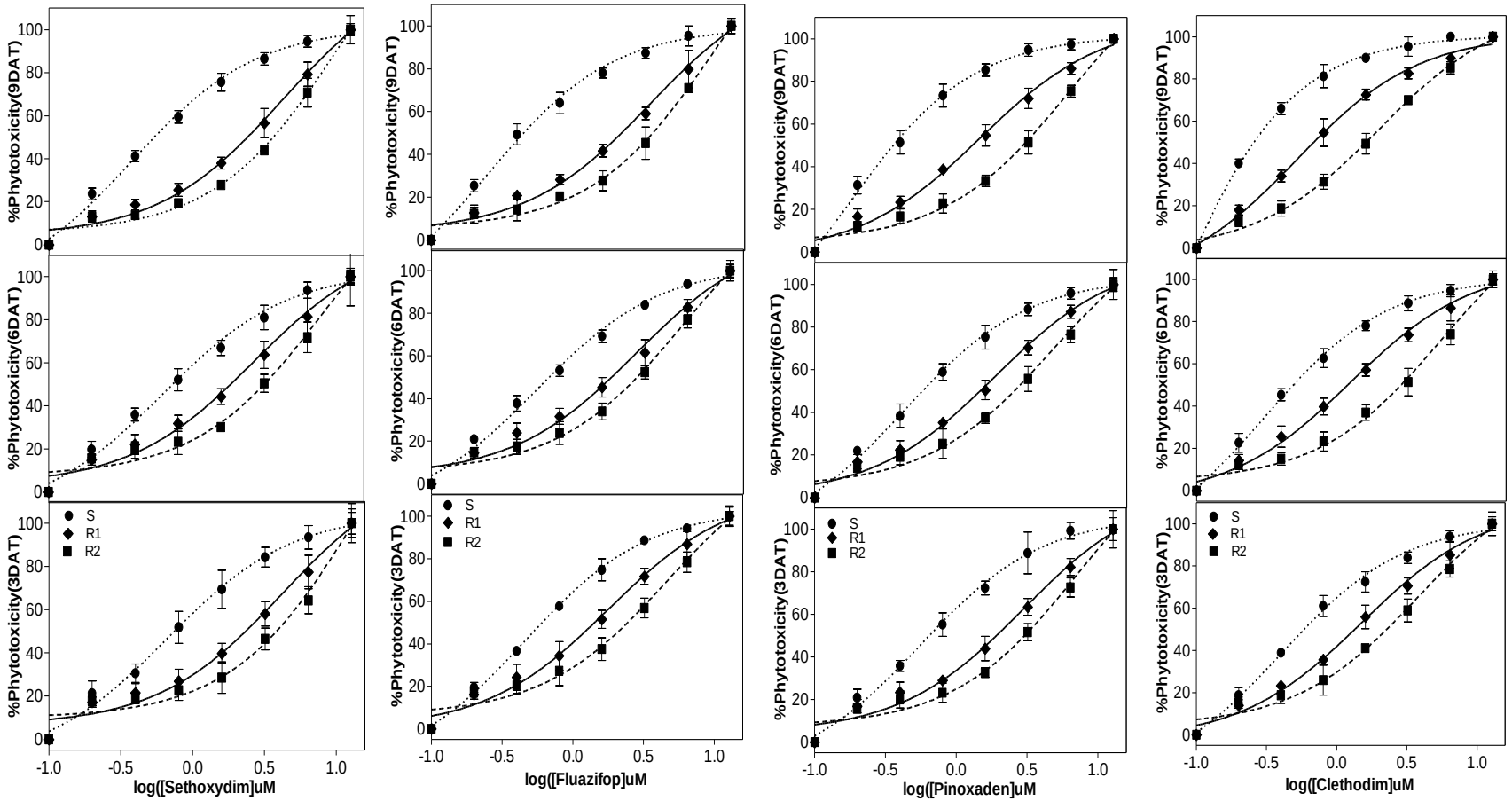


**Figure 1b.** Percent phytotoxicity response relative to non-treated of resistant and susceptible *D. ciliaris* biotypes with increasing concentrations of ACCase-targeting herbicides, sethoxydim, fluazifop, pinoxaden, and clethodim at 3, 6, and 9 days after treatment

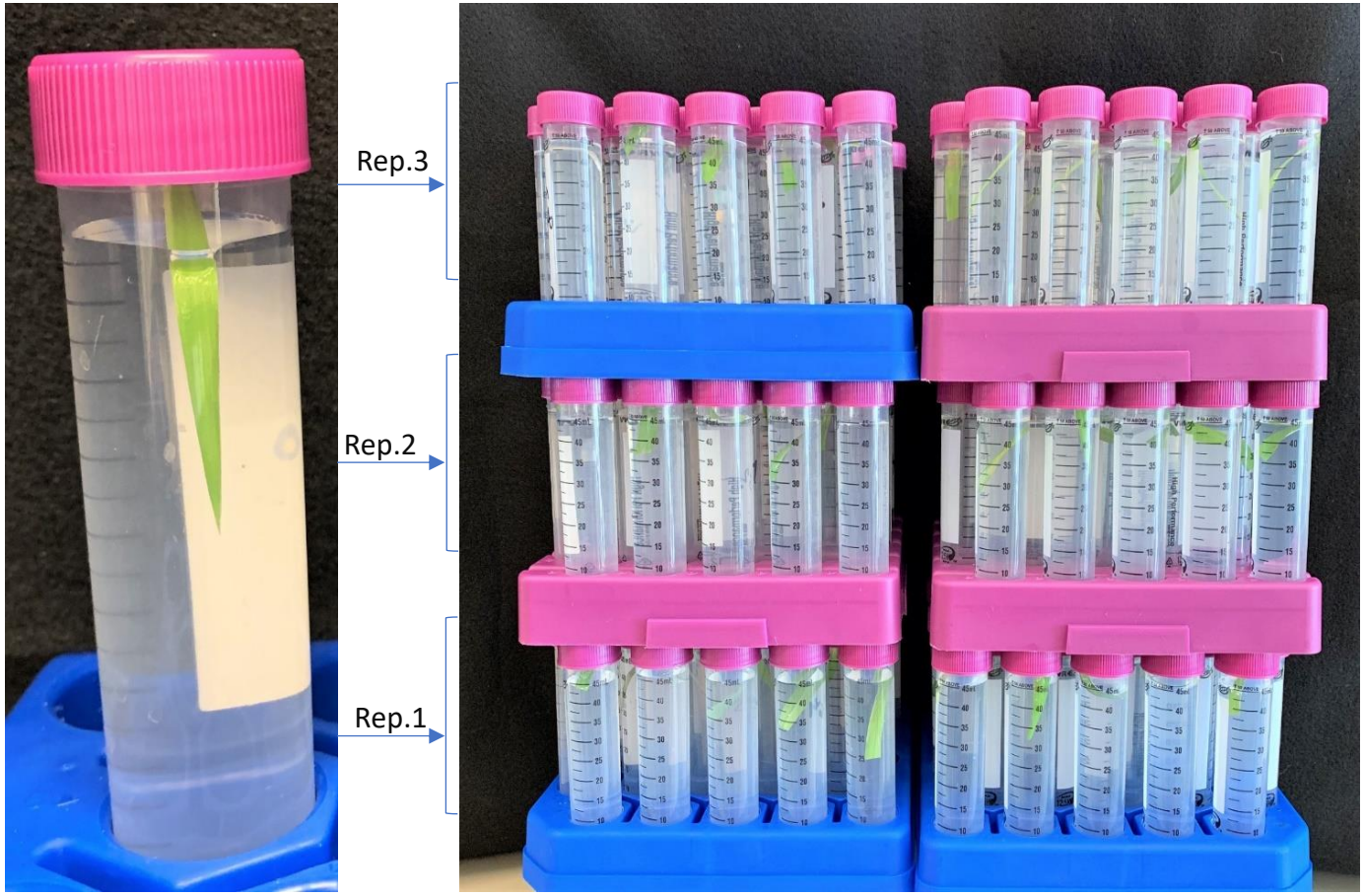


**Figure 2a.** Response of leaves flotation of susceptible and resistant *D. ciliaris* biotypes tested into polypropylene centrifuge tubes containing different concentrations of ACCase-targeting herbicides from leaf flotation assay

**Table 2.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing concentrations of ACCase-targeting herbicides, sethoxydim, pinoxaden, fluazifop, and clethodim relative to the nontreated control measured with least squares fit model for percent of leaf flotation from leaf flotation assay. The required concentration of ACCase-targeting herbicides ($IC_{50}$) to cause 50% in vitro inhibition of phytotoxicity at leaf flotation at 8, 16, and 32 hours after treatment (HAT) was calculated from concentration response curves. Parameter estimates, 95% confidence intervals (CI), values of $IC_{50}$, and R/S ratio are presented as means of model comparison

| Biotype[a] | Time[b] | Equation[c] | Parameter estimates and confidence intervals | | | | | | Inhibition[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) | Bottom | 95% CI | Top | 95% CI | LogIC50 | 95% CI | $IC_{50}$ (µM) | R/S ratio |
| | | | | Sethoxydim | | | | | | |
| R1 | 8HAT | Y=-53.4+(102.2+53.4)/(1+10^((X-0.7))) | -53.4 | (-70.4, -36.4) | 102.2 | (98.5, 106) | 0.7 | (0.6, 0.8) | 5.1 | 10.2 |
| R2 | | Y=-169.4+(101.5+169.4)/(1+10^((X-1.2))) | -169.4 | (-322.2, -16.7) | 101.5 | (95.3, 107.7) | 1.2 | (0.8, 1.6) | 16.1 | 32.2 |
| S | | Y=-5.7+(122.5+5.7)/(1+10^((X+0.3))) | -5.7 | (-7.3, -3.9) | 122.5 | (118.9, 126) | -0.3 | (-0.4, -0.3) | 0.5 | |
| R1 | 16HAT | Y=-29.4+(102.7+29.4)/(1+10^((X-0.5))) | -29.4 | (-38.5, 20.3) | 102.7 | (98.9, 106.6) | 0.5 | (0.4, 0.6) | 2.9 | 7.3 |
| R2 | | Y=-71.5+(101.8+71.5)/(1+10^((X-0.8))) | -71.5 | (-94.9, -48.1) | 101.8 | (98.2, 105.4) | 0.8 | (0.7, 0.9) | 6.7 | 16.8 |
| S | | Y=-5.9+(124.6+5.9)/(1+10^((X+0.4))) | -5.9 | (-7.7, -4.3) | 124.6 | (120.9, 128.4) | -0.4 | (-0.4, -0.3) | 0.4 | |
| R1 | 32HAT | Y=-56.5+(101.3+56.5)/(1+10^((X-0.7))) | -56.5 | (-66.5, -46.4) | 101.3 | (99.2, 103.3) | 0.7 | (0.7, 0.8) | 5.4 | 18 |
| R2 | | Y=-230+(99+230)/(1+10^((X-1.3))) | -230 | (-337.5, -122.5) | 99 | (96.5, 101.5) | 1.3 | (1.2, 1.5) | 22.4 | 74.6 |
| S | | Y=-1.9+(126.7+1.9)/(1+10^((X+0.4))) | -1.9 | (-4.9, 1.0) | 126.7 | (119.1, 134.3) | -0.4 | (-0.5, 0.3) | 0.3 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Fluazifop-p-butyl | | | | | | | | |
| R1 | 8HAT | Y=-46.5+(102.6+46.5)/(1+10^((X-0.6))) | -46.5 | (-63.6, -29.4) | 102.6 | (98, 107.2) | 0.6 | (0.5, 0.8) | 4.4 | 7.3 |
| R2 | | Y=-137+(102+137)/(1+10^((X-1.1))) | -137 | (-209.9, 63.9) | 102 | (97.6, 106.3) | 1.1 | (0.9, 1.3) | 12.7 | 21.2 |
| S | | Y=-5.1+(119.6+5.1)/(1+10^((X+0.3))) | -5.1 | (-7.7, -2.5) | 119.6 | (114.7, 124.5) | -0.3 | (-0.3, 0.2) | 0.6 | |
| R1 | 16HAT | Y=-30.2+(104+30.2)/(1+10^((X-0.5))) | -30.2 | (-39.3, -21) | 104 | (100, 108.1) | 0.5 | (0.4, 0.5) | 2.8 | 5.6 |
| R2 | | Y=-77.2+(100.9+77.2)/(1+10^((X-0.9))) | -77.2 | (-102.8, -51.7) | 100.9 | (97.5, 104.3) | 0.9 | (0.7, 1.0) | 7.5 | 15 |
| S | | Y=119+(-7.4-119)/(1+10^((X+0.2))) | 119 | (-9.9, -4.8) | 119 | (114.5, 123.5) | -0.2 | (-0.3, -0.2) | 0.5 | |
| R1 | 32HAT | Y=-31.2+(102.6+31.2)/(1+10^((X-0.5))) | -31.2 | (-37.8, -24.6) | 102.6 | (99.9, 105.2) | 0.5 | (0.4, 0.5) | 3.1 | 7.8 |
| R2 | | Y=-100.1+(99.3+100.1)/(1+10^((X-0.9))) | -100.1 | (-128.2, -71.9) | 99.3 | (96.7, 101.8) | 1.0 | (0.9, 1.1) | 9.7 | 24.3 |
| S | | Y=-3.6+(120.2+3.6)/(1+10^((X+0.3))) | 0.2 | (-6.4, -0.7) | 120.2 | (114.7, 125.7) | -0.3 | (-0.4, -0.2) | 0.4 | |
| | | Pinoxaden | | | | | | | | |
| R1 | 8HAT | Y=-26.5+(105.9+26.5)/(1+10^((X-0.4))) | -26.5 | (-36.4, -16.6) | 105.9 | (100.6, 111.2) | 0.4 | (0.3, 0.5) | 2.5 | 5.0 |
| R2 | | Y=-53.4+(102.2+53.4)/(1+10^((X-0.7))) | -53.4 | (-70.4, -36.4) | 102.2 | (98.5, 106) | 0.7 | (0.6, 0.8) | 5.1 | 10.2 |
| S | | Y=-5.6+(121.5+5.6)/(1+10^((X+0.3))) | -5.6 | (-8, -3.1) | 121.5 | (116.6, 126.4) | -0.3 | (-0.4, 0.3) | 0.5 | |
| R1 | 16HAT | Y=-22.4+(104.8+22.4)/(1+10^((X-0.3))) | -22.4 | (-27.5, -17.3) | 104.8 | (101.8, 107.8) | 0.3 | (0.3, 0.4) | 1.3 | 5.3 |
| R2 | | Y=-46.7+(100.2+46.7)/(1+10^((X-0.7))) | -46.7 | (-60.4, -32.9) | 100.2 | (96.8, 103.5) | 0.7 | (0.6, 0.8) | 4.7 | 11.8 |
| S | | Y=-2.7+(121.3+2.7)/(1+10^((X+0.3))) | -2.7 | (-5.5, 0.1) | 121.3 | (115.5, 127.2) | -0.3 | (-0.4, -0.3) | 0.4 | |
| R1 | 32HAT | Y=-24.9+ (105+24.9)/(1+10^((X-0.4))) | -24.9 | (-28.3, -21.5) | 105 | (103.2, 106.8) | 0.4 | (0.3, 0.4) | 2.3 | 7.7 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| R2 | | Y=-49.2+(102.1+49.2)/(1+10^((X-0.7))) | -49.2 | (-56.1, -42.2) | 102.1 | (100.4, 103.7) | 0.7 | (0.6, 0.7) | 5.4 | 18 |
| S | | Y=-3.2+(119.8+3.2)/(1+10^((X+0.3))) | -3.2 | (-5.9, -0.5) | 119.8 | (114.5, 125.2) | -0.3 | (-0.4, -0.2) | 0.3 | |
| | | | | Clethodim | | | | | | |
| R1 | 8HAT | Y=-16+(108.5+16)/(1+10^((X-0.1))) | -16 | (-17.5, 14.5) | 108.5 | (107.1, 109.8) | 0.1 | (0.1, 0.2) | 1.4 | 1.6 |
| R2 | | Y=-34.4+(103.7+34.4)/(1+10^((X-0.5))) | -34.4 | (-42.5, -26.2) | 103.7 | (100.6, 106.9) | 0.5 | (0.4, 0.6) | 3.2 | 3.6 |
| S | | Y=-4.9+(119.9+4.9)/(1+10^((X+0.3))) | -4.9 | (-7.9, -1.9) | 119.9 | (114.4, 125.4) | -0.3 | (-0.3, 0.2) | 0.9 | |
| R1 | 16HAT | Y=-7.2+(108.9+7.2)/(1+10^((X-0.04))) | -7.2 | (-14.3, -0.1) | 108.9 | (101.6, 116.3) | 0.04 | (-0.1, 0.2) | 1.1 | 5.5 |
| R2 | | Y=-24.6+(103.4+24.6)/(1+10^((X-0.4))) | -24.6 | (-31.9, -17.3) | 103.4 | (99.8, 107) | 0.4 | (0.3, 0.5) | 2.5 | 12.5 |
| S | | Y=-0.4+(124.3+0.4)/(1+10^((X+0.4))) | -0.4 | (-4.4, 3.5) | 124.3 | (115.1, 133.5) | -0.4 | (-0.5, -0.3) | 0.2 | |
| R1 | 32HAT | Y=-8.1+(113.2+8.1)/(1+10^((X+0.1))) | -8.1 | (-10.3, -5.9) | 113.2 | (110.4, 116) | -0.1 | (-0.1, -0.1) | 0.8 | 8 |
| R2 | | Y=-24.9+(105+24.9)/(1+10^((X-0.4))) | -24.9 | (-28.3, -21.5) | 105 | (103.2, 106.8) | 0.4 | (0.3, 0.4) | 2.3 | 23 |
| S | | Y=1.3+(159.1-1.3)/(1+10^((X+0.8))) | 1.3 | (-1.3, 3.9) | 159.1 | (140.7, 177.6) | -0.8 | (-0.9, -0.7) | 0.1 | |

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype; [b] Abbreviations: Hours after treatment (HAT); [c]In the least squares fit equation, x represents the concentration of ACCase-targeting herbicides, y represents the response variable of leaf flotation; [d]Inhibition: The required concentration of ACCase-targeting herbicides was calculated by 50% ($IC_{50}$) based on regression curve to fit in the concentration response inhibition equation and R/S ratios, resistant/susceptible ratios

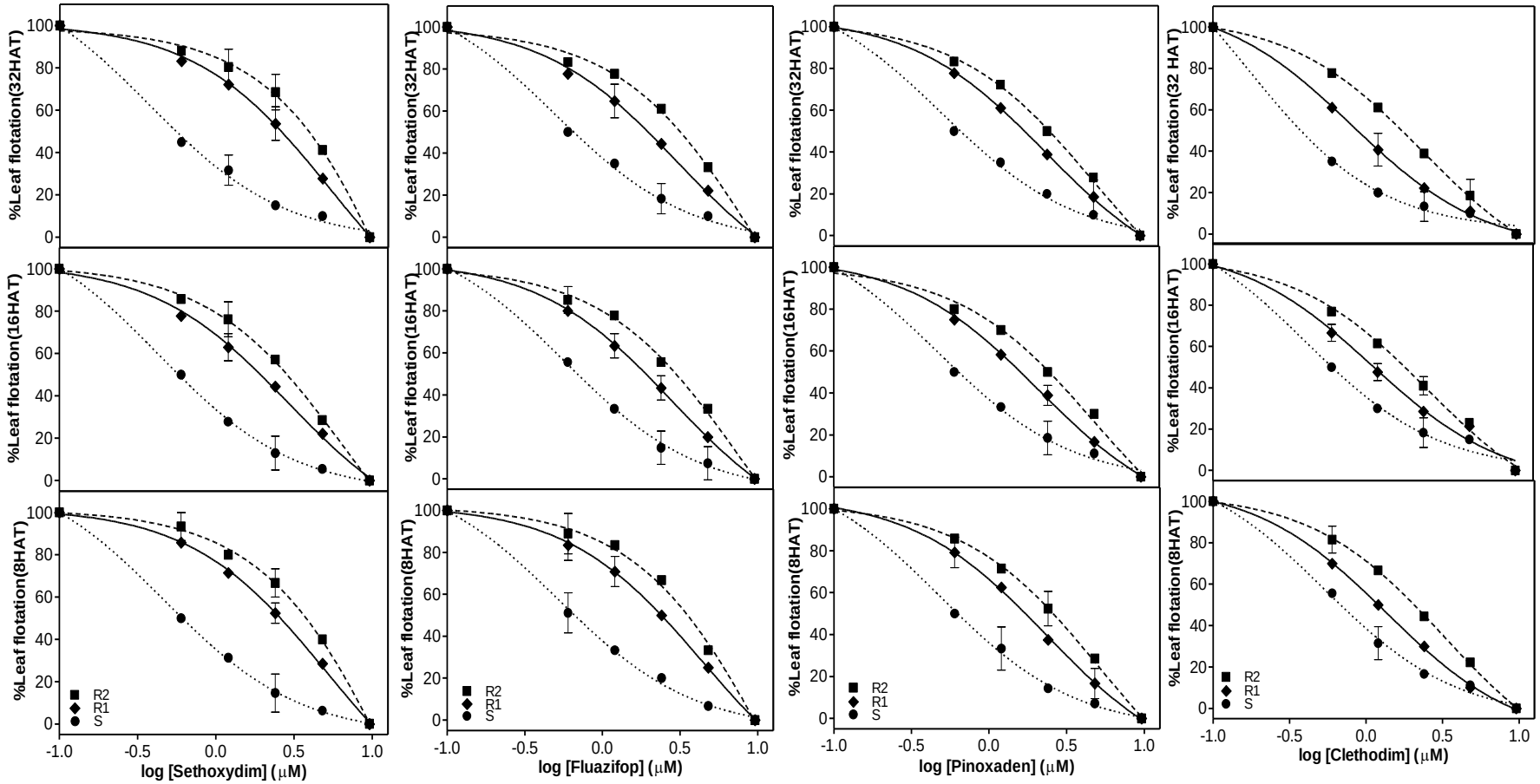


**Figure 2b.** Percent leaf flotation response relative to non-treated of *D. ciliaris* biotypes with increasing concentrations of ACCase-targeting herbicides at 8, 16, and 32 hours after treatment

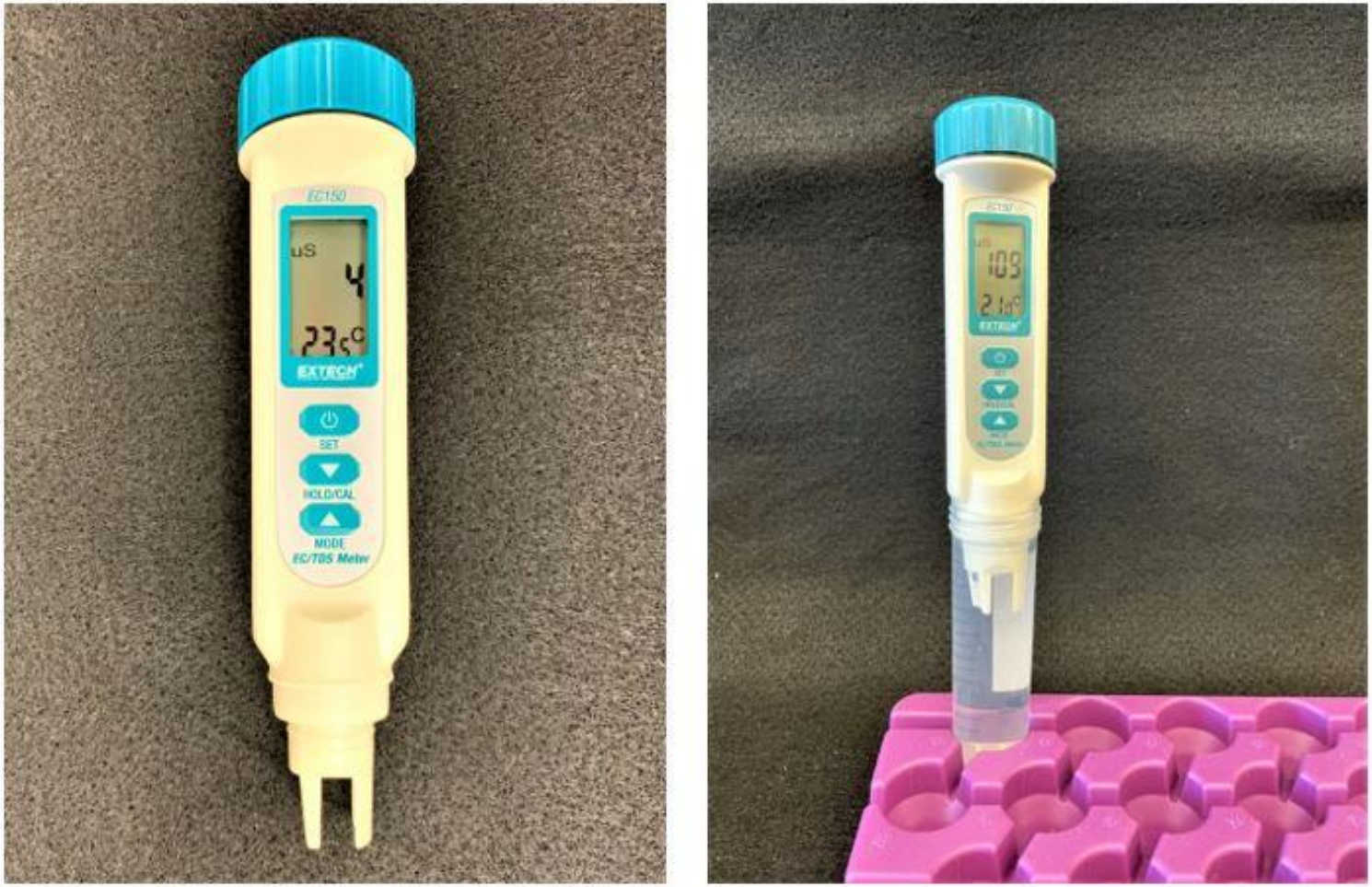


**Figure 3a.** Response of susceptible and resistant *D. ciliaris* biotypes for the leached electrolytes from leaves samples measured with an electroconductivity meter after 24 hours ACCase herbicides treatment in electrical conductivity assay

**Table 3.** Comparison of resistant and susceptible *D. ciliaris* biotypes to increasing concentrations of ACCase-targeting herbicides, sethoxydim, pinoxaden, fluazifop-p-butyl, and clethodim relative to the nontreated control measured with least squares fit model for percent of electrical conductivity from electrical conductivity assay. The required concentration of ACCase-targeting herbicides ($IC_{50}$) to cause 50% in vitro inhibition of electrical conductivity at 24hours after treatment (HAT) was calculated from concentration response curves. Parameter estimates, 95% confidence intervals (CI), values of $IC_{50}$, as well as R/S ratio are presented as means of model comparison

| Biotype[a] | Equation[b] | Parameter estimates and confidence intervals | | | | | | Inhibition[c] | |
|---|---|---|---|---|---|---|---|---|---|
| | Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) | Bottom | 95% CI | Top | 95% CI | LogIC50 | 95% CI | $IC_{50}$ (µM) | R/S ratio |
| | | | | Sethoxydim | | | | | |
| R1 | Y=221.6+(-4.5+221.6)/(1+10^((X-0.9))) | 221.6 | (203.1, 240) | -4.5 | (-5.9, -3.1) | 0.8 | (0.8, 0.9) | 11.2 | 28 |
| R2 | Y=534.4+(-3.6+534.4)/(1+10^((X-1.5))) | 534.4 | (408.4, 660.3) | -3.6 | (-4.7, -2.6) | 1.5 | (1.4, 1.6) | 31.5 | 78.8 |
| S | Y=106.4+(-70.3+106.4)/(1+10^((X+0.5))) | 106.4 | (104.6, 108.2) | -70.3 | (-80.3, -60.2) | -0.5 | (-0.5, -0.4) | 0.4 | |
| | | | | Fluazifop-p-butyl | | | | | |
| R1 | Y=221.6+ (-4.5+221.6)/(1+10^((X-0.9))) | 221.6 | (203.1, 240) | -4.5 | (-5.9, -3.1) | 0.9 | (0.9, 1.0) | 8.7 | 14.5 |
| R2 | Y=557.9+ (-2.5+577.9)/(1+10^((X-1.5))) | 577.6 | (402.5, 753.3) | -2.5 | (-3.6, -1.3) | 1.5 | (1.3, 1.6) | 46.7 | 77.8 |
| S | Y=110.3+(-41.3+110.3)/(1+10^((X-0.2))) | 110.3 | (108.6, 112) | -41.3 | (-45.8, -6.9) | -0.2 | (-0.3, -0.2) | 0.6 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Pinoxaden | | | | | | |
| R1 | Y=204.3+(-4+204.3)/(1+10^((X-0.8))) | 204.3 | (18.32, 225.5) | -4 | (-6, -1.9) | 0.8 | (0.8, 0.9) | 7.6 | 10.9 |
| R2 | Y=704.2+(-4.1+704.2)/(1+10^((X-1.6))) | 704.2 | (431, 977.5) | -4.1 | (-5.3, -2.9) | 1.6 | (1.4, 1.8) | 43.4 | 62 |
| S | Y=112.2+ (-36.1+113.2)/(1+10^((X+0.2))) | 112.2 | (110.2, 114.2) | -36.1 | (-40.5, -31.8) | -0.2 | (-0.2, -0.1) | 0.7 | |
| | | | Clethodim | | | | | | |
| R1 | Y=150+(-7.7+150)/(1+10^((X-0.5))) | 150 | (141.3, 158.6) | -7.7 | (-10.1, -5.2) | 0.5 | (0.5, 0.6) | 3.5 | 3.9 |
| R2 | Y=315.1+(-3+315.1)/(1+10^((X-1.2))) | 315.1 | (269.9, 360.4) | -3 | (-4.3, -1.8) | 1.2 | (1.1, 1.3) | 15.7 | 17.4 |
| S | Y=116.2+(-27.2+116.2)/(1+10^((X+0.01))) | 116.2 | (114.6, 117.9) | -27.2 | (-29.6, -24.8) | -0.01 | (-0.04, 0.02) | 0.9 | |

[a] *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype; [b]In the least squares fit equation, x represents the concentration of ACCase-targeting herbicides, y represents the response variable of electrical conductivity; [c]Inhibition: The required concentration of ACCase-targeting herbicides was calculated by 50% ($IC_{50}$) based on regression curve to fit in the concentration response inhibition equation, and R/S ratios, resistant/susceptible ratios

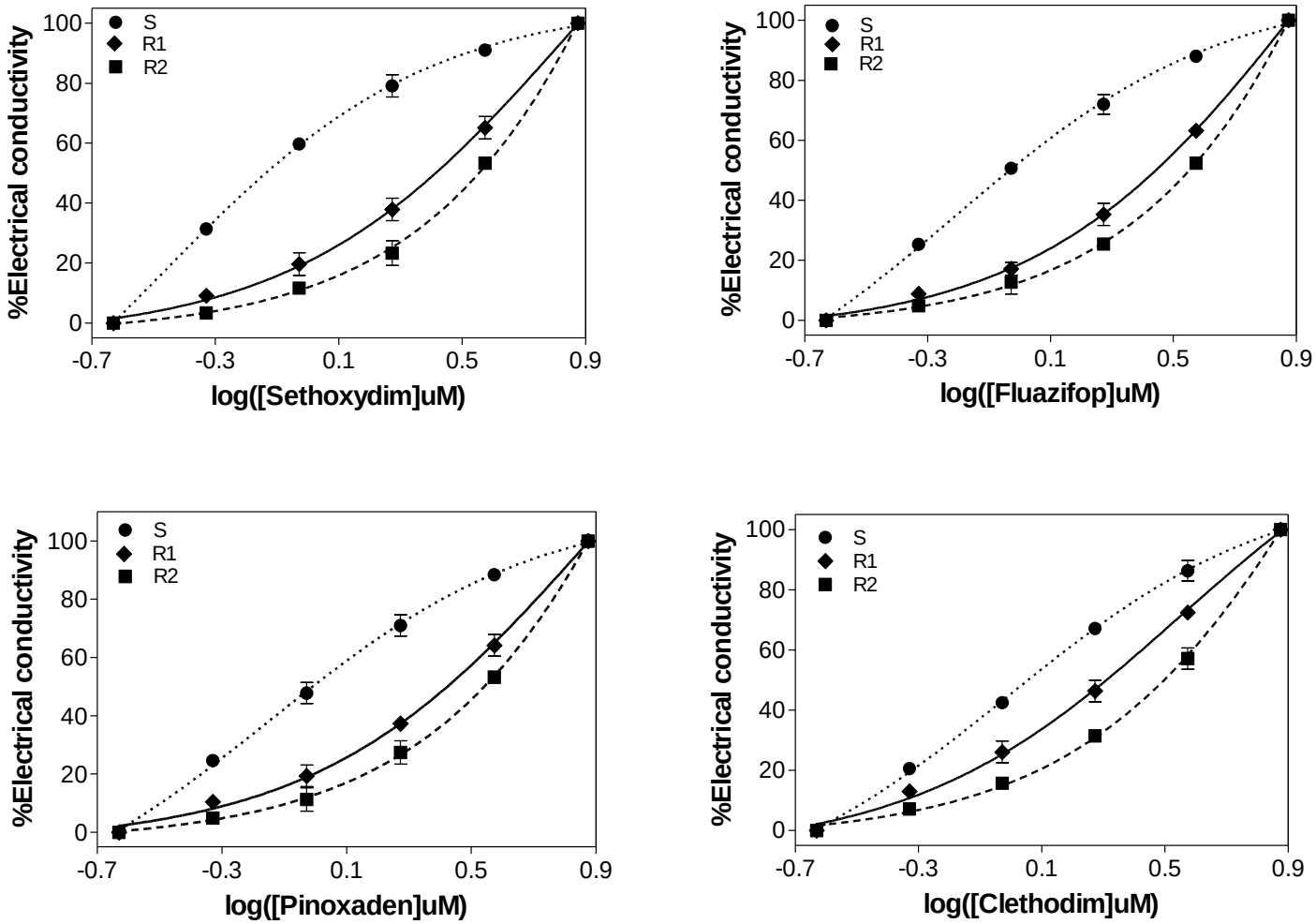


**Figure 3b.** Percent electrical conductivity response relative to non-treated of *D. ciliaris* biotypes with increasing concentrations of ACCase-targeting herbicides at 24 hours after treatment

**Table 4.** Comparison of three different rapid bioassays for testing ACCase herbicides resistance in *D. ciliaris*

| Herbicide[a] | Informative Rate[b] | Resistance for each biotype[c] | | | | | | | | | | | | Pros and cons |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Agar-based gel box assay | | | | | | | | | | | | |
| | | % Phytotoxicity | | | | | | | | | | | | |
| | | 3DAT | | | | 6DAT | | | | 9DAT | | | | |
| | | R1 | R2 | S | LSD (0.05) | R1 | R2 | S | LSD (0.05) | R1 | R2 | S | LSD (0.05) | |
| Sethoxydim | 1.6 | 24.6 | 16 | 50 | 6.2 | 33.3 | 20.6 | 63.3 | 3.5 | 36.6 | 24 | 74.6 | 4.4 | **Advantages:** Quicker response than whole plant bioassay, Carried out by unskilled labor. **Disadvantages:** Required a lot of plantlets, required control environment like growth chamber, and researcher need to wait at least 9 days to get results, tedious, labor intensive, not very accurate |
| Fluazifop-p butyl | 1.6 | 34 | 22 | 61.3 | 7.6 | 35.3 | 24.6 | 66 | 4.1 | 41.3 | 27.3 | 78 | 3.4 | |
| Pinoxaden | 1.6 | 31.3 | 20.6 | 64.7 | 4.0 | 42 | 28.6 | 73.3 | 5.3 | 51.6 | 33.3 | 85.3 | 4.9 | |
| Clethodim | 1.6 | 48 | 30.6 | 72 | 5.2 | 50.6 | 36 | 78 | 3.7 | 72.6 | 49.3 | 90 | 4.2 | |
| | | Leaf flotation assay | | | | | | | | | | | | |
| | | % Flotation inhibition | | | | | | | | | | | | |
| | | 8HAT | | | | 16HAT | | | | 32HAT | | | | |
| | | R1 | R2 | S | LSD (0.05) | R1 | R2 | S | LSD (0.05) | R1 | R2 | S | LSD (0.05) | |
| Sethoxydim | 1.2 | 10 | 5.2 | 55 | 1.6 | 16.3 | 8.3 | 65 | 4.7 | 25 | 16.7 | 68.3 | 6.5 | **Advantages:** Cost-effective, carried out by unskilled labor, utilized for detection of herbicides resistance for large numbers of plants without destroying the whole plants or preventing reproduction. **Disadvantages:** Less accuracy, required polypropylene centrifuge tube for each herbicidal concentration, researcher need to wait at least 32h |
| Fluazifop-p-butyl | 1.2 | 11.7 | 5.8 | 50 | 2.7 | 18.4 | 10 | 60 | 3.3 | 31.7 | 20 | 65 | 4.7 | |
| Pinoxaden | 1.2 | 15 | 10 | 46.7 | 2.6 | 21.6 | 15 | 58.3 | 3.3 | 35 | 25 | 66.7 | 4.6 | |
| Clethodim | 1.2 | 25 | 16.6 | 61.7 | 0.5 | 36.7 | 21.6 | 70 | 1.3 | 53.3 | 35 | 80 | 3.3 | |
| | | Electrical conductivity assay | | | | | | | | | | | | |
| | | % Electrical conductivity | | | | | | | | | | | | |
| | | 24HAT | | | | | | | | | | | | |
| | | R1 | | | R2 | | | S | | LSD (0.05) | | | | **Advantages:** Simple, Quick, Robust, Versatile, highly accurate, required only 24h to get results. **Disadvantages:** Required skilled labor and Electrical conductivity meter |
| Sethoxydim | 2.0 | 29.8 | | | 21.6 | | | 80.5 | | 2.0 | | | | |
| Fluazifop-p-butyl | 2.0 | 32.3 | | | 23.8 | | | 72 | | 1.6 | | | | |
| Pinoxaden | 2.0 | 35.8 | | | 26.3 | | | 69.5 | | 1.3 | | | | |
| Clethodim | 2.0 | 46.3 | | | 31.4 | | | 67.1 | | 1.1 | | | | |

[a]ACCase-targeting herbicides: sethoxydim, pinoxaden, fluazifop, and clethodim; [b]Informative Rate: The concentration of ACCase targeting herbicides [c]Resistance for *D. ciliaris* biotypes: R1 and R2, resistant biotypes, S, susceptible biotype; LSD ($P < 0.05$) value for comparison of means in the biotypes

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