ABSTRACT

BURKE, IAN CRISTOFER. Biology, Physiology, and Pollen Expression of ACCase Resistance in Johnsongrass (*Sorghum halepense*). (Under the direction of Dr. John W. Wilcut)

Greenhouse dose-response experiments were conducted on a biotype of johnsongrass from Washington Co., Mississippi to determine the level of purported resistance to the aryloxyphenoxy propionate herbicide fluazifop-P and cyclohexanedione herbicides clethodim and sethoxydim. Both seedling and rhizome plants were evaluated. Resistant/susceptible ratios (R/S) were 11.0, 5.7, and 5.5 for clethodim, fluazifop-P, and sethoxydim, respectively, for seedling plants. R/S ratios were 15.6, 22.7, and 22.3 for clethodim, fluazifop-P, and sethoxydim, respectively, for rhizome plants. There was no difference between the resistant and susceptible biotypes in the absorption, translocation, or metabolism of ^{14}C-clethodim in the resistant and susceptible biotypes. Specific activity of acetyl Co-A carboxylase (ACCase) from the susceptible and resistant johnsongrass biotypes (means of 0.221 and 0.223 nmol/mg protein/min, respectively) were similar. ACCase from the susceptible biotype was sensitive to clethodim, with an I_{50} value of 0.29 μM clethodim. ACCase from the resistant biotype was less sensitive, with an I_{50} value of 1.32 μM clethodim. The resultant R/S ratio for clethodim was 4.5. These results indicate that resistance to clethodim in this johnsongrass biotype resulted from an altered ACCase enzyme. The relative competitiveness and non-competitive productivity of R and S johnsongrass were assessed in greenhouse and growth chamber experiments. When grown in noncompetitive conditions in growth chamber experiments, photosynthetic rate, net assimilation rate, leaf number, leaf area, specific leaf area, leaf

dry biomass, and shoot dry biomass were similar for R and S biotypes 21, 27, and 35 days after planting. The biotypes were similar in terms of plant height and leaf number. Relative crowding coefficients for above ground dry biomass similar, and a combined t-test indicated that the resistant and susceptible biotype did not differ for above ground dry biomass (t_{lof}=0.54, 1.3; P=0.38, 0.23, respectively). There does not appear to be a fitness penalty associated with the resistance. A seedling bioassay was developed for the determination of resistance to clethodim and fluazifop-P in johnsongrass. The assay was based on differences in the coleoptile length of R and S seedlings exposed to clethodim and fluazifop-P in petri dishes for 5 d. A bioassay concentration of 0.09 mg/L clethodim and 0.18 mg/L fluazifiop-P where chosen as discriminant based on rate responses of each biotype to increasing herbicide dose. At 5 DAT, the R:S ratio for clethodim was 18.7, and the R:S ratio for fluazifop was 35.4. A study was conducted to determine the nuclear state and develop a suitable medium and culture method for *in vitro* germination of johnsongrass pollen. Johnsongrass pollen was trinucleate, and *in vitro* tests for pollen viability using Alexander's stain and a fluorochromatic reaction method indicated johnsongrass pollen was viable (92.6-98.4%). A factorial treatment of four concentrations of sucrose, two concentrations of boric acid, and two concentrations of calcium nitrate was used to determine the optimum pollen germination media. The factorial study was conducted using three different cultural methods: suspension culture, agar culture, and cellophane membrane culture. Germination was highest in a suspension culture with media containing 0.3 M sucrose, 2.43 mM boric acid, and 3 mM calcium nitrate. In a second study, pollen germination using the above media was 78.9% when

harvested from flowers just before anthesis. Three studies were conducted to develop pollen tests for the screening of ACCase target-site resistance in a biotype of johnsongrass using the developed germination media. Pollen from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim, with a GR_{50} of 25.8 (standard error of ±0.6) μM and GR_{50} of 16.4 (standard error of ±1.7) μM clethodim by visual assessment and spectrophotometric assessment, respectively. Minimum R/S values were >3.9 by visual assessment and >6.1 by spectrophotometric assessment. Both assessment methods differentiated the susceptible and resistant biotypes, and ACCase target-site resistance is expressed in johnsongrass pollen.

BIOLOGY, PHYSIOLOGY, AND POLLEN EXPRESSION OF ACCASE RESISTANCE IN JOHNSONGRASS (*Sorghum halepense*)

by

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This dissertation is dedicated to my mother,

the memory of my father,

and my wife

whose dedication to me, my education, and my development

have provided me with the ability and drive necessary

to succeed in any endeavor I choose.

Susan Canal Burke

in memory of Donald Ray Burke

BIOGRAPHY

Ian Cristofer Burke was born on May 12, 1973, on Hill Air Force Base in Ogden, Utah. As his father, Donald R. Burke, held a commission in the United States Air Force, Ian had the opportunity to do a great deal of traveling. After several years in Utah, he endured successive moves to Kansas City, Missouri, Bellville, Illinois, Washington, D. C., Tampa, Florida, Hahn Air Force Base in the then Federal Republic of Germany, and Hampton, Virginia. As both his parents were from Louisiana, Ian spent many days mucking around in Yellow Bayou and the swamps of Lake Ponchetrane. He graduated from Poquoson High School in June 1991.

After three years at the University of South Florida, Ian transferred to Old Dominion University, Virginia, where he met and studied botany under Drs. Lytton Musselman and Rebecca Bray. Dr. Musselman, as an eminent scholar, proved a great inspiration for Ian's education. Ian received a Bachelor's of Science Degree from Old Dominion University in 1996. Ian accepted a position with the North Carolina Employment Security Commission in 1997 as grounds keeper for the Commission's 17 acre campus in Raleigh, North Carolina. His intention was to pursue a higher degree at North Carolina State University. It was in this pursuit that he met Dr. John Wilcut, professor of Weed Science and who eventually became Ian's graduate sponsor and Master's and Doctoral advisory chair.

Ian met his wife in early 2000, Adrienne Dawn Bryan, to which he is happily married. Ian has way too many hobbies and interests for his own good, and they include tinkering on his toy, a 1993 Ford Probe GT, cooking, and especially eating.

At N. C. State, Ian has received numerous honors and awards with respect to his research and academic pursuits, including the Outstanding Graduate Student Award presented by the Weed Science Society of America, the Outstanding Ph.D. Student Award presented by the Southern Weed Science Society, and the Outstanding Master's Student Award presented by the North Carolina Weed Science Society. Ian has also won awards for presentations and posters at the annual meeting of the Southern Weed Science Society and the North Carolina Weed Science Society. Ian has been an author or co-author on 17 refereed journal articles.

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CHAPTER 1

Literature Review

A. Acetyl CoA Carboxylase (ACCase)

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) is an essential enzyme in fatty acid biosynthesis in eukaryotes and prokaryotes (Harwood 1988). In plants, two forms of ACCase have been identified – the first is located in the chloroplast, the primary site of plant fatty acid biosynthesis, and the second is located in the cytosol (Sasaski et al. 1995; Konishi et al. 1996). In most plants, chloroplastic ACCase is a "prokaryotic-type", multi-subunit enzyme. Subunits of the prokaryotic ACCase are encoded in the nuclear DNA, except the β-subunit of carboxyl-transferase (CT), which is encoded by a chloroplastic gene. However, members of the *Poaceae* (*Gramineae*) and also several members of the *Geraniaceae* have only the relatively sensitive eukaryotic ACCase in both the chloroplasts and the cytosol and are killed by ACCase-inhibiting herbicides (Christopher and Holtum 2000).

B. Resistance to ACCase in the *Poaceae*

Widespread and repeated use of ACCase inhibiting herbicides led to the development of different forms of resistance at this site of action. Herbicide resistance can be conferred by several mechanisms, including reduced target site sensitivity (based on a target site mutation), target site amplification, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site (Devine and Shulka 2000; Devine and Eberlein 1997). For example, resistance to ACCase-inhibiting herbicides in *Avena* spp. (Maneechote et al. 1994; Seefeldt et al. 1996; Shukla et al. 1997a), *Eleusine indica* (L.) Gaertn. (Leach et al. 1995), *Lolium* spp. (Gronwald et al. 1992; Tardif

et al. 1993), *Setaria viridis* (L.) Beauv. (Heap and Morrison 1996; Marles et al. 1993), has been attributed to reduced herbicide sensitivity of the ACCase in these resistant biotypes. Metabolism-based resistance to ACCase inhibitors has also been identified in johnsongrass [*Sorghum halepense* (L.) Pers.] (Bradley et al. 2001) and Italian ryegrass [*Lolium multiflorum* (Lam.)] (Preston et al. 1996), and the level of resistance based on enhanced metabolism is usually lower than that observed in plants having target-site based resistance (Shukla et al. 1997b).

Inheritance of graminicide resistance in most species with an altered ACCase is controlled by a single, co-dominant nuclear gene (Barr et al. 1992; Betts et al. 1992; Murray et al. 1995; Parker et al. 1990; Smeda et al. 2000). However, in wild oat (*Avena sativa* L.), resistance is controlled by two genes and appears to be recessive (Warkentin et al. 1988). In *Setaria* spp., sethoxydim resistance in foxtail millet was controlled by a single, completely dominant, nuclear gene (Wang and Darmency 1997). Genetic control of sethoxydim resistance in johnsongrass was determined to be co-dominant (Smeda et al. 2000). Among weed species, the level of resistance and cross-resistance to the aryloxyphenoxypropionates (AOPP) and cylcohexanedione (CHD) herbicides varies, suggesting there are likely multiple sites on ACCase where mutations have occurred. There may be several different mutations at the enzyme level within a species (Devine and Shimabukuro 1994).

In 1992, resistance to the aryloxyphenoxypropionate (AOPP) herbicides and cross-resistance to one of the cyclohexanedione (CHD) herbicides was discovered in two populations of johnsongrass in Mississippi (Smeda et al. 1997). CHD and AOPP herbicides were applied one or more times annually over several years in fields where populations were

detected. Since then, five other ACCase-resistant populations of johnsongrass have been reported (Heap 2005). The population that is the subject of this research also originated in Washington County, Mississippi, and is purportedly resistant to clethodim.

C. Characteristics of Johnsongrass

Johnsongrass occurs in all major agricultural areas of the warm regions of the world and was listed by Holm et al. (1991) as one of the world's ten worst weeds. Johnsongrass reduces crop yields (McWorter and Hartwig 1972; Horowitz 1973) and its pollen contaminates sorghum grown for seed (Rosenow and Clark 1969).

Johnsongrass is described by Vickery (1961) as a perennial, 50-150 cm tall, with culms arising from an extensively creeping and rooting scaly rhizome. Panicles are mostly 10-35 cm long. The spikelets are in pairs, one spikelet being sessile the other pedicellate, except for the terminal group which are in triplets of one sessile and two pedicellate spikelets. Sessile spikelets are distinctly articluate with the subtending joint. Johnsongrass is believed to be a native to the Mediterranean region from the Madeira Islands to Asia Minor, covering southeastern Europe (Piper 1928; Snowden 1936). De Wet and Huckaby (1967) reported that although a tropical ecotype now exist throughout India and Pakistan, the Mediterranean ecotype was introduced to Australia and the Americas.

The pattern of growth and development of johnsongrass seedlings and rhizome sprouts is similar (McWorter 1961; Horowitz 1972), although in the field rhizomes sprouts are thought to emerge earlier and to grow more rapidly than seedlings (McWhorter 1961). Three weeks after emergence of seedlings and sprouts, a rhizome spur is formed and secondary tillers are initiated. Leaf growth is slow during this period. After formation of the rhizome spur, leaf

growth is rapid, equaling or exceeding root growth, while rhizome production is low (McWhorter 1961; Evetts and Burnside 1973). Flowering begins 7 wks after emergence and continues for the rest of the growing season. At flowering rhizome production increases rapidly (McWhorter 1961) although there does not appear to be a causal relationship between the two (Horowitz 1972). The minimum temperature for rhizome formation was between 15 and 20 C (Horowitz 1972). Johnsongrass can produce 8 kg fresh weight and 70 m of rhizomes in one growing season (Horowitz 1973).

Johnsongrass is a closely related species to sorghum [*Sorghum bicolor* (L.) Moench.]. Researchers have shown that hybridization of johnsongrass with other *Sorghum* sp. is possible (Chernicky and Slife 1985; Endrizzi 1957). Arriola and Ellstrand (1996) noted that despite potential differences in ploidy number, a significant rate of interspecific hybridization occurred for adjacent johnsongrass and sorghum plants. Cytochemical studies revealed that the chromosome number for johnsongrass can be 2N=20 or 2N=40, with plants having the latter chromosome number being most common (Monaghan 1979; Warwick and Black 1983).

C. Fitness of Resistant Biotypes

Herbicide resistance in weed biotypes can result in reduced fitness compared to susceptible biotypes (Gressel and Segal 1982). The occurrence of ACCase resistance is associated with reduced fitness in goosegrass (Marshall et al. 1994), but not giant foxtail (*Setaria faberi*) (Wiederholt and Stoltenberg 1996). Cousens et al. (1997) indicated, however, that in order to have confidence that the R biotype is different in terms of fitness, it is necessary to determine the background variation among S populations. That

requirement assumes that evaluations are to be made using traditional fitness studies on an array of dissimilar biotypes. Bergelson and Purrington (1996), in a review of the literature on fitness penalties associated with resistance, observed that if isogenic lines were unavailable then breeding designs utilizing randomized crosses that control the genetic background was more likely to detect costs of resistance. To conduct these studies, resistant biotypes are crossed with one or more susceptible biotypes. Bergelson and Purrington (1996) continued noting that the amount of experimental noise contributed by variation in the genetic background would be dependent on the degree of similarity between resistant and susceptible parents. Variability in johnsongrass is well established – McWhorter (1971) evaluated 55 morphologically distinct biotypes of johnsongrass from across the United States and found them to differ by 2 to 4 fold in height and tiller density.

D. Resistance Testing and Seedling Assays

Resistance testing is very important for the timely implementation of management strategies. When testing for resistance to herbicides, the development of rapid and reliable bioassays is important if growers are to be advised of their herbicide options in a timely manner. The identification of resistance to ACCase-inhibiting herbicides typically involves applying herbicide to plants grown under controlled conditions (Beckie et al. 2000; Letouzé and Gasquez 1999) although it can involve verification in the field (Beckie et al. 2000). Whole plant assays, either in the field or greenhouse, are costly in terms of both labor and time (Beckie et al. 2000; Letouzé and Gasquez 1999). Resistant biotypes can also be identified by measuring the activity of the enzyme in the presence of

increasing herbicide concentrations, a process which is complex, equipment and reagent intensive, and costly (Beckie et al. 2000).

Seedling assays that involve the determination of either coleoptile length or root length as growth parameters to discriminate between resistant and susceptible biotypes have been developed for several AOPP and CHD resistant weed biotypes (Beckie et al. 1990; Murray et al. 1996; Letouzé and Gasquez 1999; Retrum and Forcella 2002), but not for clethodim-resistant johnsongrass

E. Pollen Viability and *In Vitro* Germination

The ability to rapidly assess potentially resistant biotypes is important if a grower is to be informed of treatment options in a timely manner. Since the discovery of target-site ACCase resistance expression in pollen (Richter and Powles 1993), pollen assays have been developed to assess resistance rapidly (Letouze and Gasquez 2000). As several biotypes of acetyl-CoA (ACCase) resistant johnsongrass have been found (Heap 2005), a rapid assay would be useful for quickly determining the presence of target site resistance to ACCase inhibitors. Although much is known about sorghum [*Sorghum bicolor* (L.) Moench] pollen, little is known about johnsongrass pollen other than it contaminates sorghum grown for seed (Rosenow and Clark 1969).

As an important grain crop, sorghum and its reproductive system have been studied throughout the century and are very similar to johnsongrass. The first study on anthesis and pollination in sorghum was reported in the second decade of the 20th century (Graham 1916). Other literature reported in the 1920's and 30's agree with current publications on floral formation, anthesis, and pollination in sorghum (Ayyangar and Rao

1931; Doggett 1988; Patel and Patel 1928; Patil and Gould 1980; Stephens and Quinby 1934). The beginning of the reproductive phase in the genus *Sorghum* is indicated by a characteristic swelling of the apex of the stalk. The swollen area is enclosed by the terminal leaf sheath, whose blade, the flag, is usually small and inconspicuous. Emergence of the flag is followed by the emergence of the boot and the inflorescence. When the head of the inflorescence is completely exerted, the peduncle elongates. Anthesis in the inflorescence is basipetal with the terminal flowers of each panicle opening first, followed by the next flower down shortly thereafter. Johnsongrass floral characteristics differ from that of sorghum in that in addition to a sessile complete spikelet, johnsongrass has a pediceled staminate (or neuter) one (Gleason and Cronquist 1991).

The germination of sorghum pollen has been accomplished in several different media. Lansac et al. (1994) found that sorghum pollen germinated immediately when placed in either distilled water or a growth media. By contrast, Tuinstra and Wedel (2000) were much less successful at germinating sorghum pollen. They were not able to germinate sorghum pollen in distilled water and were only moderately successful at germinating sorghum pollen on an agar media. Neither study noted from what floral stage the pollen was harvested although it was presumably after anthesis. The importance of harvesting fresh sorghum pollen was illustrated by Lansac et al. (1994) – within 15 minutes of the initiation of desiccation *in vitro* sorghum pollen germination reduced by 92%. Others have noted similar trends in other species, for example, in shepard's purse [*Capsella*

bursa-pastoris (L.) Medikus], floral stage was as important for pollen germination as the germination media composition (Leduc et al. 1990).

Germinability of pollen appears to also be related to the nuclear state of pollen. Pollen of angiosperms may be described as either bi- or trinucleate, depending on whether anthesis occurs, respectively, before or after generative cell mitosis (Brewbaker 1957, 1959). Most members of the *Poaceae* are trinucleate (Brewbacker 1967). Brewbaker (1967) noted that while Brewbaker and Kwack (1963) media germinated a number of species, species with trinucleate pollen germinated at a much lower percentage if at all. Trinculeate pollen is also difficult to store – once shed trinucleate pollen germinability *in vitro* decreases rapidly (Fei and Nelson 2003; Lansac et al. 1994; Leduc et al. 1990). The vitality of trinucleate pollen may be related to respiration rate - trinucleate pollen respires at 2 to 3 times the rate as binucleate pollen (Hoekstra and Bruinsma 1975). The difficulty in germinating trinucleate pollen has resulted in a number of different medias and culture methods that are species-specific (Fei and Nelson 2003; Leduc et al. 1990; Muccifora et al. 2003; Letouze and Gasquez 2000).

Several researchers have explored different culture methods, which slow the rate of hydration, to increase the germination of trinucleate pollen on the theory that rapid hydration after extended periods of desiccation destroys important membranes in the pollen (Alexander and Ganeshan 1990; Bar-Shalom and Mattsson 1977). Agar and cellophane-based media are two culture methods that slow the rate of hydration (Alexander and Ganeshan 1990; Bar-Shalom and Mattsson 1977; Shivanna and Rangaswamy 1992).

F. Resistance Testing and Pollen Assays

For plants, gene flow can involve pollen transport or pollen-mediated gene flow (Levin and Kerster 1974; Murray et al. 2002). The reproductive biology of species influences the degree of pollen-mediated gene flow between and within populations (Levin and Kerster 1974). Johnsongrass is a predominantly selfing species with less than a 10% outcrossing rate (Dogget 1988). Even this limited amount of outcrossing could have implications for pollen movement of resistance. Consequently, understanding the biology and physiology of pollen could provide insight into the potential for movement of genes - of special interest are genes imparting herbicide resistance.

Gene expression in the sporophytic generation overlaps with that of the gametophytic generation (Pedersen et al. 1987; Sari Gorla et al. 1986; Tanksley et al. 1981). As a consequence, many single, nuclear encoded genes expressed in the sporophytic generation are also expressed in the gametophytic generation (Richter and Powles 1993). Richter and Powles (1993) showed that ACCase and ALS (acetolactate synthase) genes were expressed in pollen of *Lolium rigidum* Gaud. Furthermore, the enzymes encoded by these genes could be inhibited by herbicides. Richter and Powles (1993) could not detect resistance caused by something other than modification at the target site, which was attributed to a lack of expression of the enzymes conferring resistance. Thus, Letouze and Gasquez (2000) and Richter and Powles (1993) were able to detect target-site resistance in *Alopecurus myosuroides* L. and *Lolium rigidum*, respectively. A bioassay involving pollen could be useful for detecting ACCase target site-resistance in johnsongrass.

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Chapter 2

Cross Resistance of a Johnsongrass (*Sorghum halepense*) Biotype to Aryloxyphenoxypropionate and Cyclohexanedione Herbicides

Abstract: Greenhouse dose-response experiments were conducted on a biotype of johnsongrass from Washington County, Mississippi to determine the level of purported resistance to the aryloxyphenoxy propionate (AOPP) herbicide fluazifop-P and the cyclohexanedione (CHD) herbicides clethodim and sethoxydim. Both seedling and rhizomatous plants were evaluated. Resistant/susceptible ratios (R/S) were calculated based on the GR_{50} values (the rate required to reduce shoot dry biomass, expressed as a percent of the control, 50%). The GR_{50} values for the resistant and susceptible seedling plants were 110 and 10 g ai/ha for clethodim, 193 and 34 g ai/ha for fluazifop-P, and 265 and 48 g ai/ha for sethoxydim, resulting in R/S ratios of 11.0, 5.7, and 5.5 for clethodim, fluazifop-P, and sethoxydim, respectively. The GR_{50} values for the resistant and susceptible rhizomatous plants were 609 and 39 g/ha for clethodim, 657 and 29 g/ha for fluazifop-P, and 668 and 30 g/ha for sethoxydim, resulting in R/S ratios of 15.6, 22.7, and 22.3 for clethodim, fluazifop-P, and sethoxydim, respectively.

Nomenclature: Clethodim; fluazifop-P; sethoxydim; *Sorghum halepense* (L.) Pers. #[1]SORHA, johnsongrass.

[1] Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds*, Revised 1989. Available only on computer disk from WSSA, 810 East 10th Street, Lawrence, KS 66044-8897.

Additional index words: ACCase inhibitors; herbicide resistance; graminicides.

Abbreviations: ACCase, Acetyl coenzyme-A carboxylase; AOPP, aryloxyphenoxypropionate; CHD, cyclohexanedione; R/S, resistant to susceptible ratio; WAT, weeks after treatment.

Introduction

Johnsongrass infests agricultural areas in more than 58 countries throughout the world (Holm et al. 1991). Estimates from the early 1990's of annual losses due to johnsongrass in Arkansas, Louisiana, and Mississippi were approximately $5.8 million in cotton (*Gossypium hirsutum* L.) and $23.7 million in soybean [*Glycine max* (L.) Merr] (McWhorter and Anderson 1993). In North Carolina corn (*Zea maize* L.) production areas, johnsongrass is among the 10 most common weeds, and in grain sorghum [*Sorghum bicolor* (L.) Moench] it is among the most troublesome (Webster 2000). Furthermore, full-season johnsongrass competition may reduce soybean yields by as much as 50% (Williams and Hayes 1984).

In the early 1980s, the selective postemergence control of johnsongrass in soybean first became possible with the registration of several herbicides now called graminicides (Burton 1997). The graminicides are divided into two chemically distinct herbicide classes, the AOPP and the CHD herbicides. Both of these herbicide classes act by inhibiting the enzyme acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2) in susceptible species (Burton 1997). Acetyl coenzyme-A carboxylase catalyzes the first committed step of fatty acid biosynthesis, which is the ATP-dependent carboxylation of

acetyl-CoA to malonyl CoA (Incledon and Hall 1997). Grass species have a eukaryotic type ACCase in the chloroplast, which is sensitive to ACCase inhibitors, while most broadleaf species have a prokaryotic type of ACCase, which is not sensitive to ACCase inhibitors (Incledon and Hall 1997).

Graminicides are currently registered in a wide variety of crops, which often leads to the repeated use of these herbicides (Devine and Shimabukuro 1994). Maxwell and Mortimer (1994) noted that the repeated use of an herbicide or herbicides is one of the most common factors that lead to herbicide resistance. There are now 34 grass weed biotypes resistant to the ACCase inhibitors (Heap 2005). In 1991, resistance to the AOPP herbicides and cross-resistance to the CHD herbicide sethoxydim was discovered in two populations of johnsongrass in Mississippi (Smeda et al. 1997). Cyclohexanedione and AOPP herbicides were applied one or more times annually over several years in fields where populations were detected. However, these populations were not resistant to clethodim (Smeda et al. 1997). Since that time, AOPP-and CHD-resistant johnsongrass has been reported in Kentucky, Louisiana, Mississippi, Tennessee, and Virginia (Heap 2005). Only the biotype from Louisiana, however, is resistant to clethodim (Heap 2005).

In 2000, a sixth purportedly ACCase-resistant population of johnsongrass was found in Washington County, MS. The objectives of this research are to determine the extent of resistance to clethodim of the clethodim-resistant johnsongrass biotype from Mississippi and to quantify the levels of resistance to the AOPP herbicide fluazifop-P and the CHD herbicides clethodim and sethoxydim in both rhizomatous and seedling johnsongrass.

Materials and Methods

Seeds and rhizomes of the Mississippi biotype (designated V99R) were harvested in the fall of 1999. In the spring of 2000, the rhizomes were planted in 12-L pots and grown to 15 to 20 cm in height. The pots were then treated with 280 g/ha clethodim, which corresponds to the maximum registered field use rate for rhizomatous johnsongrass less than 20 cm in height. Treatments were applied with a CO_2-pressurized backpack sprayer that delivered 140 L/ha of spray solution at 146 kPa. Rhizomatous johnsongrass plants that survived these treatments were allowed to grow and produce seed and rhizomes. Rhizomes and seed from these plants were harvested as needed for use in the dose-response studies. Johnsongrass rhizomes and seed were also harvested from a field in Wake County, North Carolina, that had no previous history of graminicide application for use as a susceptible biotype control. All rhizomes were cut into 5- to 7-cm segments and three rhizome segments were planted 1 cm deep in 15-cm diameter plastic pots containing a commercial potting media[2]. Rhizomes that successfully germinated were thinned to one rhizome per pot and watered as needed. Seed populations were established by pregerminating scarified seed in 200-cm round plastic petri dishes placed in an alternating 20/30 C temperature regime. The pregerminated seedlings were then transplanted to 15-cm diameter plastic pots containing the commercial potting media.

[2] Potting media, Metro-Mix 220. Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Road, Marysville, OH 43041.

Two different experiments were conducted, one to evaluate seedling johnsongrass response to increasing graminicide rate, and one to evaluate rhizomatous johnsongrass response to increasing graminicide rate. Herbicide treatments included rates above and below the registered rate for each herbicide. For the seedling experiment, resistant seedling johnsongrass was treated with clethodim at 0, 70, 140 (1X), 280, 560, 1120, 2240, 4500, or 9000 g/ha, fluazifop-P at 0, 140 (0.75X), 280 (1.5X), 560, 1120, 2240, 4500, 9000, or 18000 g/ha, and sethoxydim at 0, 105, 210 (1X), 420, 840, 1680, 3360, 6700, and 13400 g/ha. Susceptible seedlings were treated with clethodim at 0, 70, 140, 280, or 560 g/ha, fluazifop-P at 0 140, 280, 560, or 1120 g/ha, and sethoxydim at 0, 105, 210, 420, or 840 g/ha. To evaluate rhizomatous johnsongrass response to increasing graminicide rate, resistant rhizomatous johnsongrass was treated with clethodim at 0, 18, 35, 70, 140, 280 (1X), 560, 1120, 2240, or 4500 g/ha, fluazifop-P at 0, 13, 26, 53, 105, 210 (1X), 420, 840, 1680, 2520, or 5040 g/ha, and sethoxydim at 13, 26, 53, 105, 210 (1X), 420, 840, 1680, 3360, 6700, or 13400 g/ha. Susceptible rhizomatous johnsongrass was treated with clethodim at 0, 18, 35, 70, 140, or 280 g/ha, fluazifop-P at 0, 13, 26, 53, 105, or 210 g/ha, and sethoxydim at 13, 26, 53, 105, or 210 g/ha.

Each experiment was a split-block design with herbicide rate as the whole plot and johnsongrass biotype as the subplot. The experiment had four replications of treatments, and johnsongrass plants were blocked by visual estimation of size. Both seedling and rhizomatous plants from each biotype were sprayed at the 3 to 4 leaf stage (20 to 40 cm in height). Applications were made in a spray chamber with a single 8001EVS flat-fan

nozzle[3] calibrated to deliver 186 L/ha at 193 kPa. All treatments were applied with 1% (v/v) crop oil concentrate[4]. Visual estimates of johnsongrass control were recorded based on a scale of 0% (no control) to 100% (plant death) 1 and 2 wks after treatment (WAT) (Frans et al. 1986). At 3 WAT, treated shoots were cut at the soil surface, dried for 7 d at 40 C, and weighed. The experiment conducted twice.

Shoot dry weights were expressed as percent of the control and were subjected to an analysis of variance (ANOVA) using the general linear models procedure in SAS (1998), and sums of squares were partitioned to evaluate the effect of trial repetition, herbicide rate, and johnsongrass biotype. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Both non-transformed and arcsin-transformed percent shoot dry weight reduction were examined, and transformation did not improve homogeneity. Analysis of variance was therefore performed on non-transformed percent dry shoot weight reduction. Trial repetition and linear, quadratic, and higher order polynomial effects of percent shoot dry weight reduction over graminicide rate were tested by partitioning sums of squares (Draper and Smith 1981). Regression analysis was performed when indicated by the ANOVA. Nonlinear models were used if the ANOVA indicated that higher order polynomial

[3] Nozzles, TeeJet spray nozzles. Spraying Systems Co., P. O. Box 7900, Wheaton, IL 60189.

[4] Crop oil concentrate, Agri-Dex (83% paraffin-base petroleum oil and 17% surfactant blend). Helena Chemical Co., 5100 Poplar Avenue, Memphis, TN 38137.

effects of percent shoot dry weight reduction were more significant than linear or quadratic estimates. Estimation used the Gauss-Newton algorithm, a nonlinear least squares technique (SAS 1998).

The ANOVA indicated higher order polynomial effects for percent shoot dry weight reduction resulting from increasing herbicide rate. Thus, percent dry shoot weight reduction was modeled using the logistic function:

$$y = A + B / [1 + (x / GR_{50})^{d}] \quad [1]$$

where y is the response at dose x, A is the lower limit for y, B is the upper limit for y, d is the slope, and the GR_{50} is the dose giving 50% injury or inhibition (Seefeldt et al. 1995). The GR_{50} is most commonly referred to because it is the most accurate estimate of plant sensitivity to a herbicide (Seefeldt et al. 1995). When the logistic function was fit to the data, an approximate R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from one (Draper and Smith 1981).

Results and Discussion

There was not a significant treatment by experiment interaction, therefore the data were pooled over experiment runs for both the seedling and rhizomatous study. For each herbicide, ANOVA indicated a significant difference between the susceptible and resistant biotypes. Resistant rhizomatous plants exhibited varying levels of resistance to clethodim, fluazifop-P, and sethoxydim, and resistant seedling plants were found to be resistant to clethodim, fluazifop-P, and sethoxydim (Table 1). For clethodim, fluazifop-P, and sethoxydim, R/S ratios were higher for rhizomatous plants than for seedling plants.

The clethodim GR_{50} values for the resistant and susceptible seedlings were 110 and 10 g/ha, respectively, indicating the resistant biotype was 11 times more tolerant of clethodim than the susceptible biotype (Table 1, Figures 1 and 2). The GR_{50} for clethodim on resistant johnsongrass seedlings was lower than the registered use rate for that herbicide (140 g/ha). At 140 g/ha, the susceptible johnsongrass seedlings were completely controlled. The clethodim GR_{50} values for the resistant and susceptible rhizomatous plants were 609 and 39 g/ha, indicating that the resistant biotype was 15.6 times more tolerant than the susceptible biotype and that johnsongrass plants arising from the rhizomes would require more than twice the registered rate of 280 g/ha of clethodim for a 50% reduction in growth.

The fluazifop-P GR_{50} values for the resistant and susceptible seedlings were 193 and 34 g/ha, respectively, indicating the resistant biotype was 5.7 times more tolerant of fluazifop-P than the susceptible biotype (Table 1, Figures 3 and 4). In contrast to clethodim, the GR_{50} for fluazifop-P on resistant johnsongrass seedlings was higher than the registered use rate for that herbicide, which, at 100 g/ha, would require nearly twice the registered rate of fluazifop-P for a 50% reduction in control. For the resistant and susceptible rhizomatous plants, the fluazifop-P GR_{50} values were 657 and 29 g/ha, indicating that the resistant biotype was 22.7 times more tolerant than the susceptible biotype. As with clethodim and fluazifop-P, johnsongrass plants arising from the rhizomes would require more than twice the registered rate of 280 g/ha of fluazifop-P for a 50% reduction in growth.

The sethoxydim GR_{50} values for the resistant and susceptible seedlings were 265 and 48 g/ha, respectively, indicating the resistant biotype was 5.5 times more tolerant of sethoxydim than the susceptible biotype (Table 1, Figures 5 and 6). The GR_{50} for the resistant johnsongrass seedlings for sethoxydim was higher than the registered use rate for that herbicide (210 g/ha). At 210 g/ha, the susceptible johnsongrass seedlings were completely controlled. The sethoxydim GR_{50} values for the resistant and susceptible rhizomatous plants were 668 and 30 g/ha, indicating that the resistant biotype was 22.3 times more tolerant than the susceptible biotype and that johnsongrass plants arising from the rhizomes would require more than twice the registered rate of 210 g/ha of sethoxydim for a 50% reduction in growth.

The high but variable levels of resistance exhibited by the V99R johnsongrass population to clethodim, fluazifop-P, and sethoxydim is consistent with altered sites of action as the mechanism of resistance. Patterns of ACCase herbicide resistance were first described by Bourgeois et al. (1997) using wild oat (*Avena fatua* L.). Using 85 different ACCase-resistant wild oat biotypes, groupings of resistant biotypes were proposed based on whole plant cross-resistant patterns to AOPP and CHD herbicides. Four distinct groups were described: biotypes highly resistant to a broad spectrum of AOPP and CHD herbicides, biotypes highly resistant to sethoxydim but with low levels of resistance to other AOPP and CHD herbicides, biotypes highly resistant to fluazifop-P but with lower levels of resistance to other AOPP and CHD herbicides, and biotypes resistant to AOPP herbicides but not to CHD herbicides (Bourgeois et al. 1997; Devine and Shulka 2000). The johnsongrass biotype V99R falls into the first category as it has moderate to high

levels of resistance to both AOPP and CHD herbicides. Other weeds with moderate to high levels of resistance to the AOPP and CHD herbicides have been reported. The ACCase enzyme from a biotype of green foxtail [*Setaria viridis* (L.) Beauv.] from Mannitoba, Canada, was highly resistant to a broad spectrum of AOPP and CHD herbicides (Marles et al. 1993). A biotype of large crabgrass [*Digitaria sanguinalis* (L.) Scop.] was found to be 7-, 59-, 25-, and 337-fold resistant to clethodim, fluazifop-P, quizalofop-P, or sethoxydim, respectively (Wiederholt and Stoltenberg 1995).

Each of the five ACCase-resistant populations of johnsongrass that have been reported (Heap 2005) differ considerably in the resistance pattern to ACCase herbicides. For instance, the first reported johnsongrass biotype resistant to ACCase inhibitors occurred in 1991 and was found to be highly resistant to fluazifop-P and quizalofop-P, slightly to moderately resistant to sethoxydim, and susceptible to clethodim (Smeda et al. 1997). Second and third biotypes found in Kentucky 1991 and Tennessee in 1995, respectively, were resistant primarily to fluazifiop-P (Heap 2005). A fourth biotype found in Virginia in 1995 had a low level of resistance to sethoxydim, however, the Virginia biotype's mechanism of resistance was due to overexpression of the target enzyme rather than a modification of the target site (Bradley et al. 2001; Bradley and Hagood 2001). A fifth biotype, from Louisiana, was found to be resistant to clethodim and fluazifop-P (Donnie Miller, personal communication). Based on the cross-resistance patterns for the different biotypes of ACCase-resistant johnsongrass, there are likely different enzyme modifications conferring resistance to ACCase inhibitors for each biotype of johnsongrass with the exception of the Virginia biotype.

Continuous selection pressure through repeated use is allegedly one of the major contributors to increasing populations of weeds resistant to herbicides (Maxwell and Mortimer 1994). Johnsongrass resistant to ACCase inhibitors can be controlled with other selective and non-selective herbicides with different modes of action. Glyphosate, glufosinate, imazapic, imazethapyr, and nicosulfuron are all options for controlling ACCase resistant johnsongrass in the respective crops in which they are registered (Barrentine et al. 1996; Smeda et al. 1997; Wilcut et al. 1999). However, imazapic, imazethapyr, and nicosulfuron inhibit acetolactate synthase (ALS), and resistance to this mode of action has been common (Devine and Shulka 2000). Good stewardship is imperative to protect the ability to control ACCase-resistant johnsongrass with ALS-inhibitors as well as other herbicides with different modes of action.

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Table 1. Regression parameters (and standard errors) and resistant/susceptible (R/S) ratios for seedling and rhizomatous johnsongrass populations resistant and susceptible to graminicides.

			Regression parameters (and standard errors)[a]					
Herbicide	Biotype	Plant Source	*a*	*b*	*d*	GR_{50}	R^2	R/S Ratio
Clethodim	Resistant	Seedling	26 (3.1)	75 (7.1)	1.98 (0.6)	110 (18.7)	0.97	11.0
	Susceptible	Seedling	21 (1.1)	80 (1.9)	1.33 (0.6)	10 (9.3)	0.99	
	Resistant	Rhizomatous	17 (7.2)	80 (8.6)	1.82 (0.53)	609 (11.2)	0.99	15.6
	Susceptible	Rhizomatous	17 (2.9)	83 (3.2)	1.00 (0.75)	39 (3.4)	0.96	
Fluazifop-P	Resistant	Seedling	11 (1.5)	89 (2.4)	0.92 (0.1)	193 (14.8)	0.99	5.7
	Susceptible	Seedling	21 (5.7)	79 (6.9)	1.37 (2.1)	34 (6.52)	0.99	
	Resistant	Rhizomatous	1 (11.9)	99 (13.5)	0.76 (0.14)	657 (23.8)	0.99	22.7
	Susceptible	Rhizomatous	18 (16.1)	80 (19.9)	1.46 (0.84)	29 (12.8)	0.96	
Sethoxydim	Resistant	Seedling	17 (6.8)	84 (8.6)	0.69 (0.2)	265 (8.1)	0.99	5.5
	Susceptible	Seedling	20 (1.0)	81 (1.4)	2.26 (0.7)	48 (12.5)	0.99	
	Resistant	Rhizomatous	4 (13.4)	91 (16.2)	0.64 (0.2)	668 (36.9)	0.97	22.3

Susceptible	Rhizomatous	24 (2.0)	75 (2.6)	1.68 (0.2)	30 (1.7)	0.99

[a] The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass (expressed as a percent of the control) 50%.

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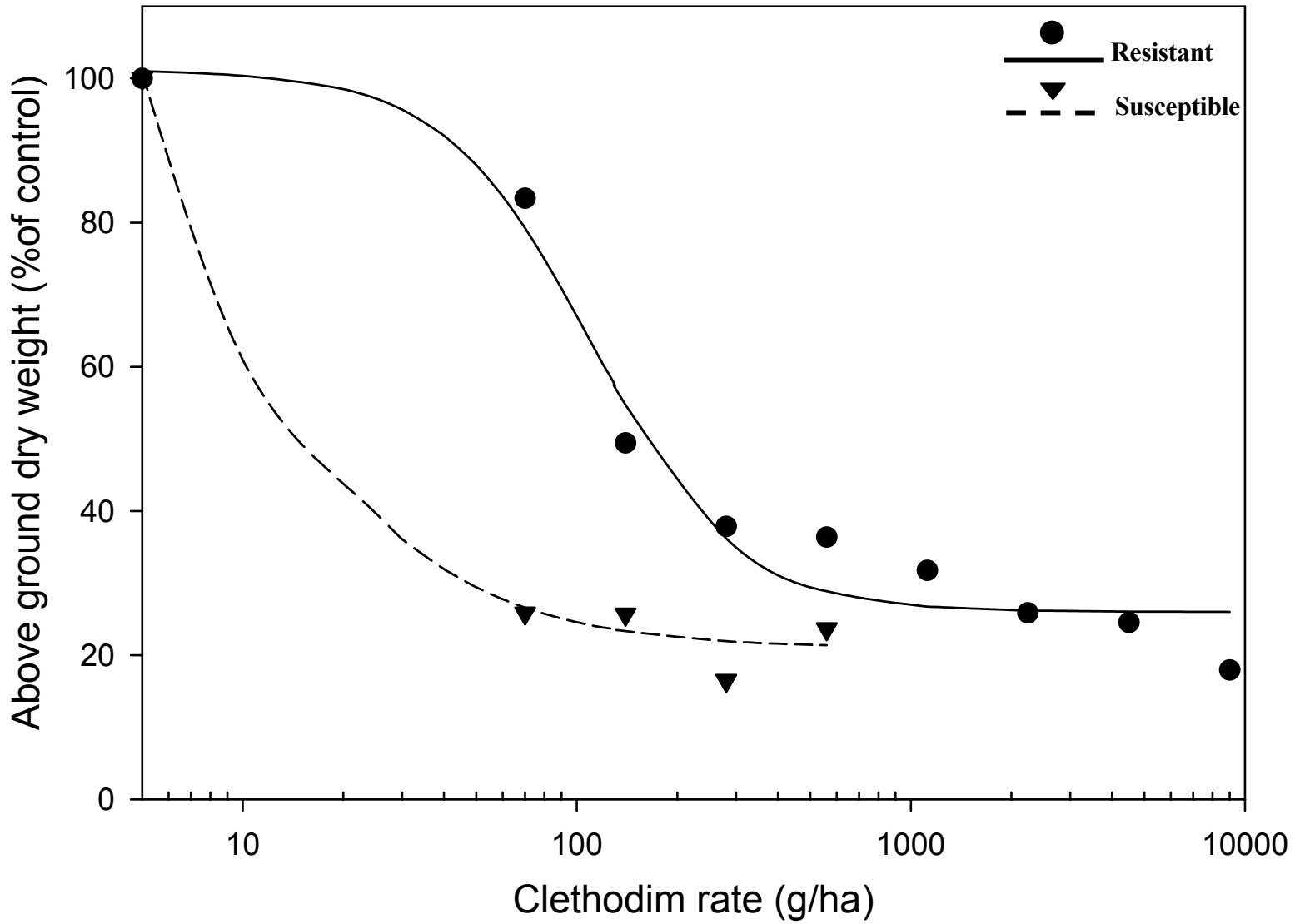

Figure 1. Effect of increasing clethodim rate on susceptible and resistant seedling johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

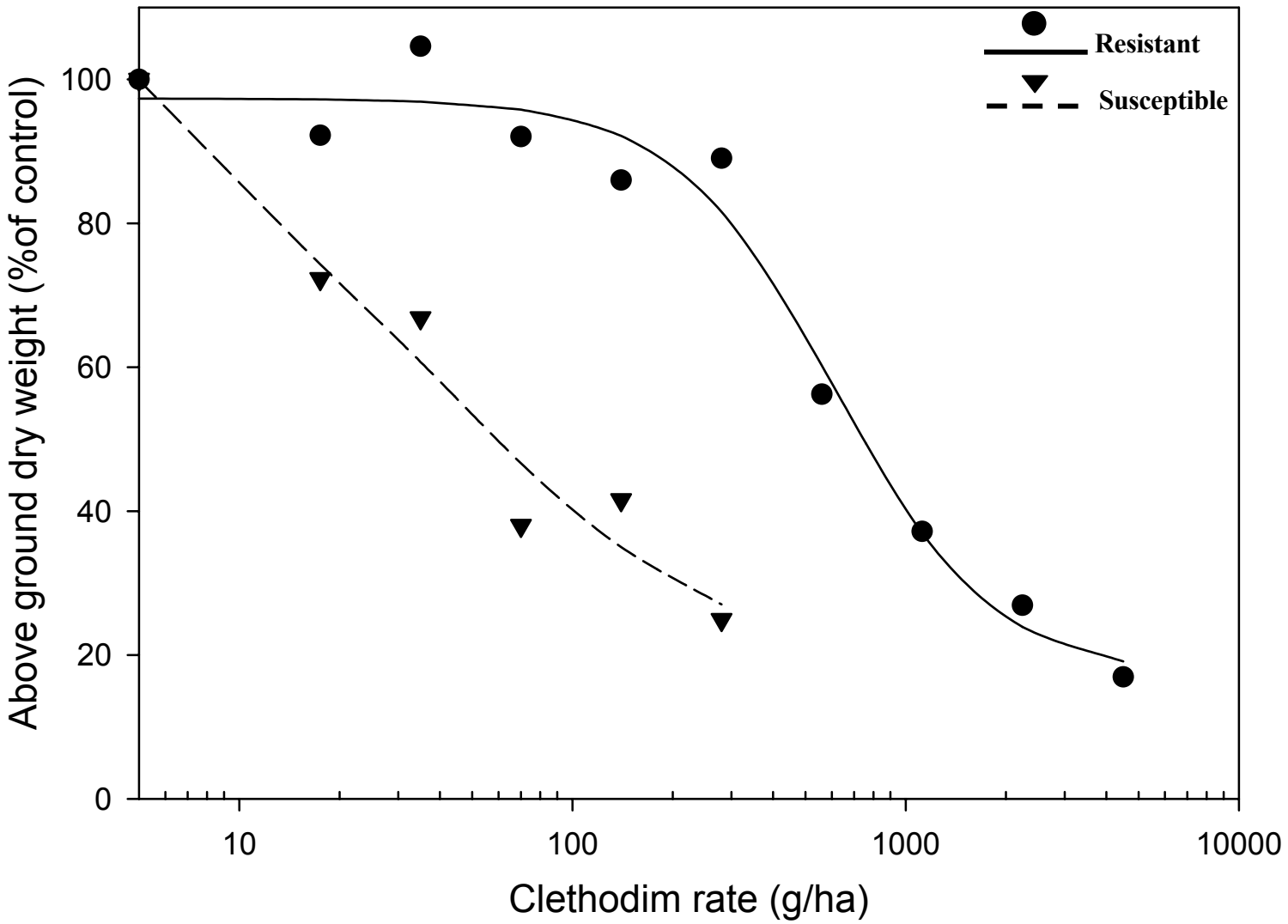

Figure 2. Effect of increasing clethodim rate on susceptible and resistant rhizomatous johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

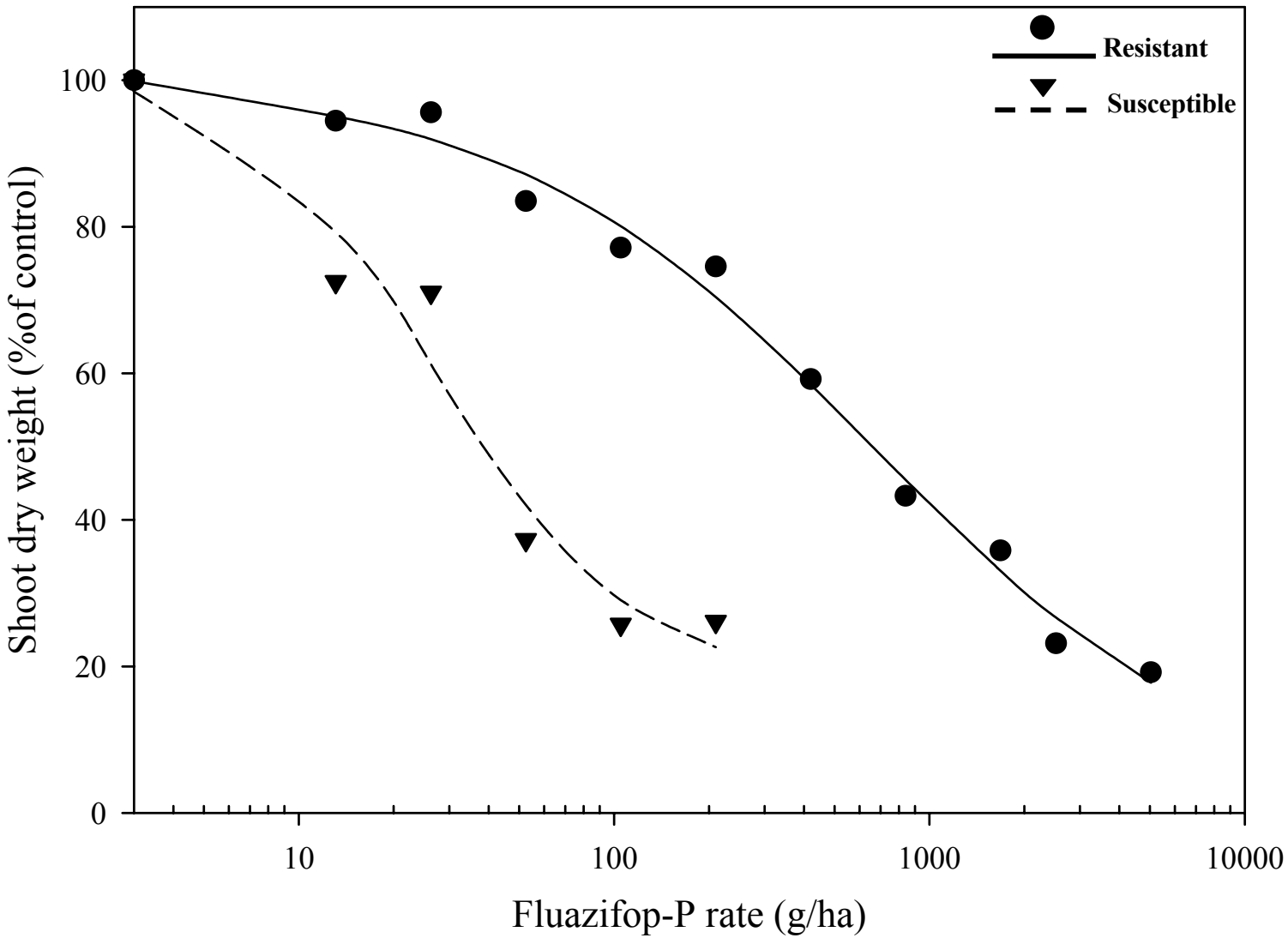

Figure 3. Effect of increasing fluazifop-P rate on susceptible and resistant seedling johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

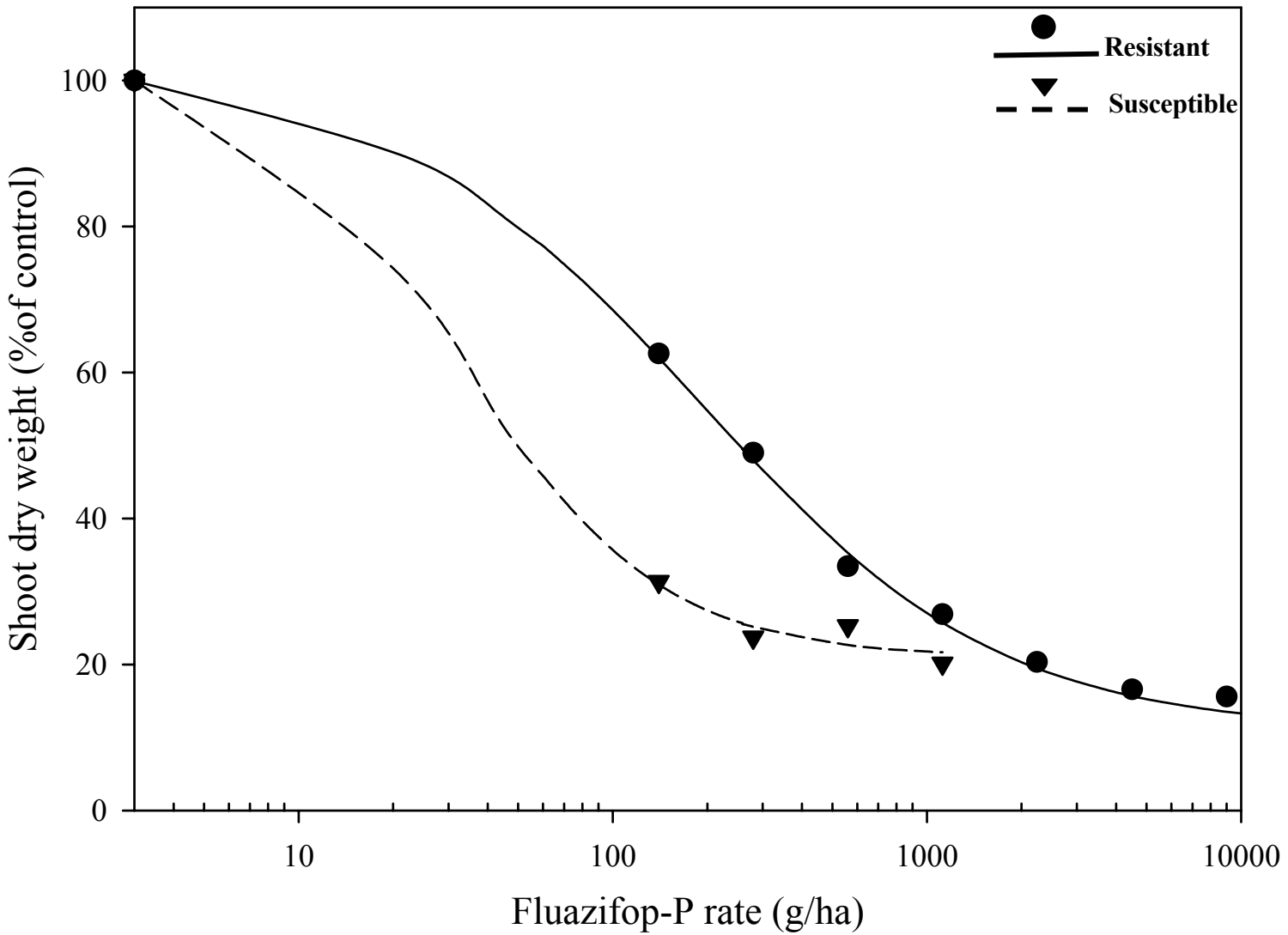

Figure 4. Effect of increasing fluazifop-P rate on susceptible and resistant rhizomatous johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

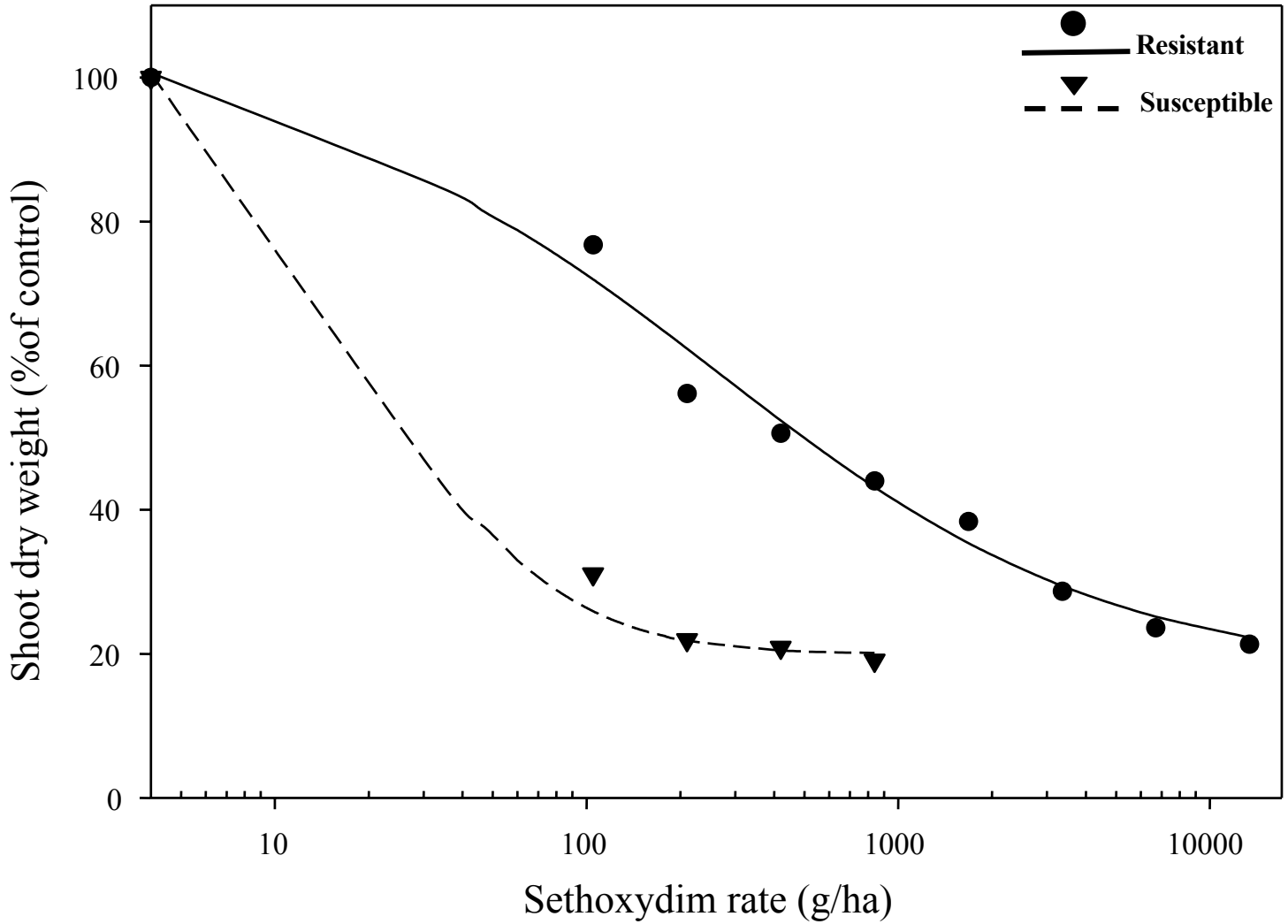

Figure 5. Effect of increasing sethoxydim rate on susceptible and resistant seedling johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

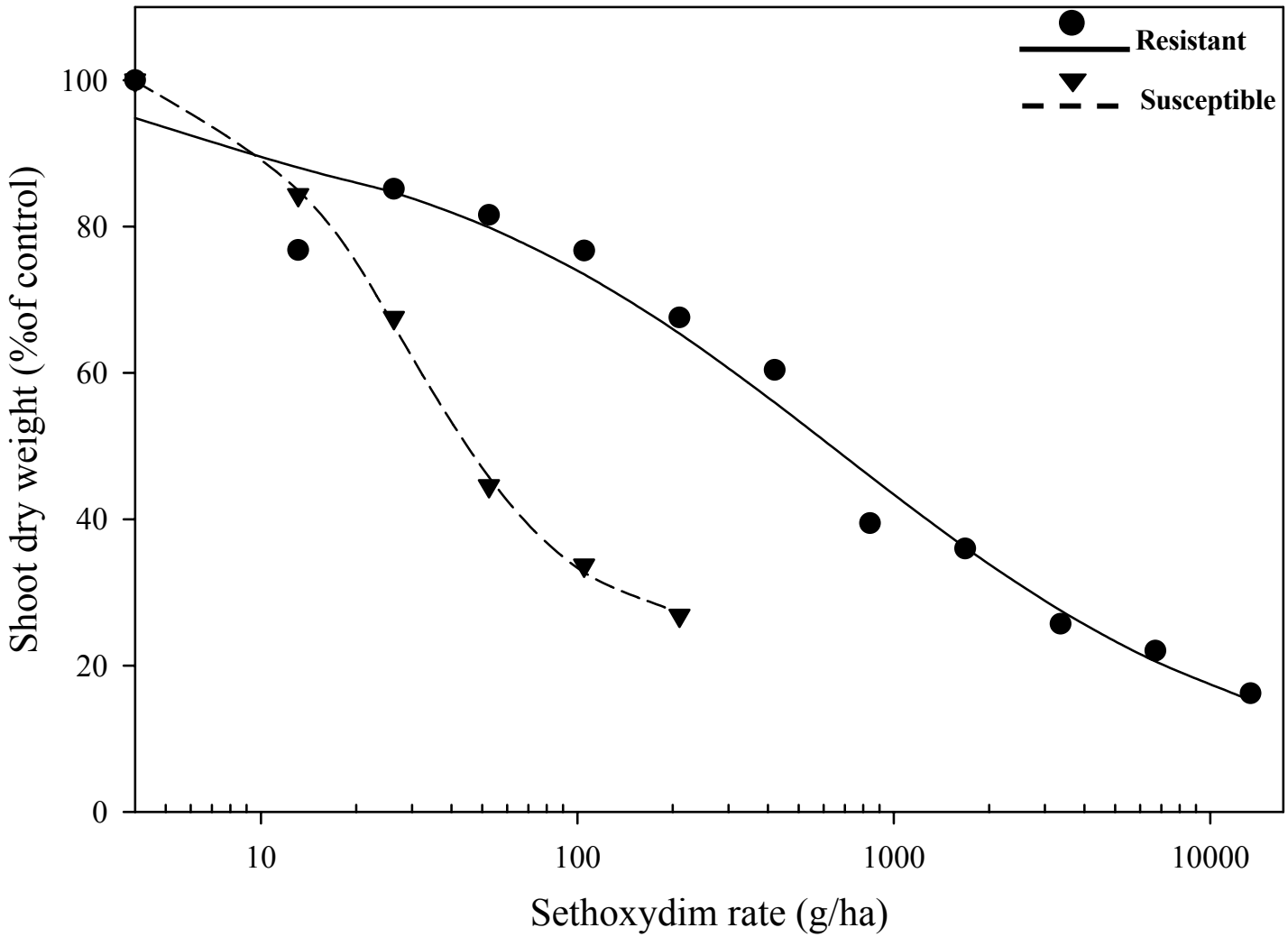

Figure 6. Effect of increasing sethoxydim rate on susceptible and resistant rhizomatous johnsongrass ascension shoot dry weight. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the shoot dry weight in grams, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing biomass, and GR_{50} is the rate required to reduce shoot dry biomass 50%. See Table 1 for regression parameters and resistant/susceptible ratios.

CHAPTER 3

Mechanism of Resistance to Clethodim in a Johnsongrass (Sorghum halepense) Biotype.

Abstract. A biotype of johnsongrass cross-resistant to clethodim, sethoxydim, quizalofop-P, and fluazifop-P was identified in several fields in Washington County, Mississippi. Absorption, translocation, and metabolism studies using ^{14}C-clethodim and acetyl-coenzyme A carboxylase (ACCase) activity assays were conducted to determine the mechanism of resistance. Absorption of ^{14}C-clethodim was higher in the resistant than the susceptible biotype 4 hours after treatment (HAT), but at 24, 48, and 72 HAT, similar levels of radioactivity were detected in both johnsongrass biotypes. Consequently resistant plants had more radioactivity present in the treated leaves at 4 and 24 HAT. However, there was no difference between resistant and susceptible biotypes in the translocation of ^{14}C-label, as a percentage of total absorbed, out of the treated leaf at 4, 8, 24, 48, and 72 HAT. Metabolism of clethodim, both metabolites formed and percentage of clethodim metabolized, was similar in the resistant and susceptible biotypes. There was no difference in the specific activity of ACCase from the susceptible and resistant johnsongrass biotypes (means of 0.221 and 0.223 nmol/mg protein/min, respectively). ACCase from the susceptible biotype was sensitive to clethodim, with an I_{50} value of 0.29 μM clethodim. The ACCase enzyme from the resistant biotype was less sensitive, with an I_{50} value of 1.32 μM clethodim. The resultant R/S ratio for clethodim was 4.5. These results indicate that resistance to clethodim in this johnsongrass biotype resulted from an altered ACCase enzyme that confers resistance to clethodim.

Nomenclature: Clethodim; johnsongrass, *Sorghum halepense* (L.) Pers. SORHA.

Keywords: Absorption, ACCase, acetyl-CoA carboxylase, enzyme activity, herbicide resistance, metabolism, translocation.

Introduction

Cyclochexanedione (CHD) herbicides are typically used to control grass weeds post-emergence in broad-leaved crops (Burke et al. 2003; Burke et al. 2004; Vidrine et al. 1995). In most broad-leaved species, selectivity of CHD herbicides results from an insensitive target enzyme acetyl-coenzyme A carboxylase (ACCase; EC 6.4.1.2). Grass species have a eukaryotic type ACCase in the chloroplast, which is sensitive to ACCase inhibitors, while most broadleaf species have a prokaryotic type of ACCase which is not sensitive to ACCase inhibitors (Incledon and Hall 1997; Konishi and Sasaki 1994).

Herbicides that inhibit ACCase include both the aryloxyphenoxy propionate (AOPP) and CHD herbicides. Widespread and repeated use of ACCase-inhibiting herbicides led to the development of different forms of resistance at this site of action (Devine and Shimabukuro 1994). Herbicide resistance can be conferred by several mechanisms, including reduced target site sensitivity (based on a target site mutation), target site amplification, increased rate of herbicide detoxification, decreased rate of herbicide activation, or sequestration of the herbicide away from the target site (Devine and Eberlein 1997). Several of these mechanisms of resistance have been reported for ACCase inhibitors. For example, resistance to ACCase-inhibiting herbicides in *Lolium* spp. (Gronwald et al. 1992; Tardif et al. 1993), green foxtail [*Setaria viridis* (L.) Beauv.] (Heap and Morrison 1996; Marles et al.

1993a), *Avena* spp. (Maneechote et al. 1994; Seefeldt et al. 1996), and goosegrass [*Eleusine indica* (L.) Gaertn.] (Leach et al. 1995) has been attributed to reduced herbicide sensitivity of the ACCase enzyme in these resistant biotypes. Metabolism-based resistance to ACCase inhibitors has also been identified in johnsongrass (Bradley et al. 2001), large crabgrass [*Digitaria sanguinalis* (L.) Scop.] (Hidayat and Preston 1997), and Italian ryegrass (Preston et al. 1996). Metabolism-based resistance is also the mechanism by which wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) escape injury (MacFadden et al. 1989), although the level of resistance based on enhanced metabolism in johnsongrass is lower than site-based resistance in johnsongrass and other species (Bradley et al. 2001; Smeda et al. 1997).

Johnsongrass resistant to AOPP herbicides was first reported in 1991. Since then, five other johnsongrass biotypes have been found to be resistant to ACCase inhibitors (Heap 2005). Each johnsongrass biotype is resistant to different individuals or groups of AOPP and CHD herbicides. The johnsongrass biotype that is the subject of this research has been found to have high levels of resistance to clethodim as well as fluazifop-P and sethoxydim (Burke et al. 2005). The objective of this study was to determine the mechanism of resistance in this biotype by comparing absorption, translocation, and metabolism of clethodim in the resistant biotype and a known susceptible biotype, and by quantifying the sensitivity of ACCase activity in both biotypes in the presence of increasing concentrations of clethodim.

Methods and Materials

Plant Material

Three-cm lengths of rhizomes from two biotypes (clethodim-resistant and clethodim-susceptible) of johnsongrass were planted in a 7:3 mixture of pure sand and Norfolk loamy sand (fine-loamy, siliceous, thermic, Typic Paleudults) in 10-cm round plastic pots. Upon emergence, plants were thinned to one per pot. Plants were watered daily and maintained in a glasshouse at approximate daily minimum and maximum temperatures of 20 and 32 C, respectively, in a 14-h photoperiod. Natural lighting was supplemented with metal halide lighting with an average midday photosynthetic photon flux density of 700 μmol m^{-2} s^{-1}. All pots received 10 ml of a 25 g L^{-1} commercial fertilizer[1] at emergence and 17 d later. Two studies were conducted as randomized complete blocks with three replications of treatments to evaluate absorption, translocation, and metabolism of clethodim within each biotype of johnsongrass. Each study was conducted twice.

Clethodim Absorption and Translocation

At the four-leaf growth stage, a 2-cm^2 area of the second fully expanded johnsongrass leaf was covered using aluminum foil. Nonlabeled clethodim at 140 g ai ha^{-1} [a 1X rate of clethodim (Anonymous 2005)] was applied using a spray chamber equipped with a single 8001E flat fan nozzle[2] calibrated to deliver 160L ha^{-1} at 200 kPa. Crop oil concentrate[3] at 1.0% (v/v) was included. Immediately after application, the aluminum foil was removed and five 1-μL droplets of ^{14}C-clethodim solution, with each droplet containing approximately 1.0 kBq of radioactivity, was applied to the adaxial side of the

second fully expanded johnsongrass leaf with a microliter syringe. The solution was prepared by diluting clethodim, labeled with ^{14}C in the phenyl ring [Ring-4,6-^{14}C] and a specific activity of 2.1 kBq μmole^{-1}, with HPLC-grade water and commercially formulated clethodim. The total amount of clethodim applied to the treated leaf was equivalent to 140 g ai ha^{-1}. Crop oil concentrate[3] was included in the spotting mixture 1% (v/v). At the beginning of the ^{14}C-label application for each treatment, five 1-μL aliquots of solution were used to calculate the amount of ^{14}C applied to each plant. Radioactivity was determined by liquid scintillation spectrometry (LSS)[4].

Plants were removed from soil 4, 8, 24, 48, and 72 h after treatment (HAT) and were divided into treated leaf blade, roots, and aerial portions above and below the treated leaf blade. The treated leaf sections were rinsed for 15 s with 10 ml methanol:water (1:1, v/v) and 0.25% (v/v) nonionic surfactant[5] to remove nonabsorbed clethodim. A 1 ml aliquot of the rinse was added to 15 ml scintillation fluid[6] and radioactivity was quantified via LSS[4]. All plant parts, including washed roots, were dried for 48 h at 40 C, weighed, and combusted with a biological sample oxidizer[7]. Radioactivity in the oxidized samples was quantified by LSS[4].

Clethodim Metabolism

Clethodim application and plant harvesting methods were similar to the uptake and translocation experiments, except each droplet applied to the treated leaf contained approximately 2.1 kBq of radioactivity. Plants were removed from soil 4, 8, 24, and 72 HAT. All plant parts, including washed roots, were frozen at –20 C. Extraction was performed under reduced lighting without direct fluorescent light or sunlight. Plant parts

were ground in a tissue homogenizer[8] with 10 ml of cold methanol:water (8:2, v/v). The homogenate was then rinsed into a vacuum filtration apparatus containing a single piece of filter paper[9] with an additional 10 to 15 ml of solvent. The residue and filter paper[9] were air dried wrapped in aluminum foil and stored at room temperature. The filtrate was evaporated to less than 10 mL under a stream of nitrogen. Ammonium sulfate (0.07 g/mL fluid) and 20 mL ethyl acetate were added. The ethyl acetate-soluble fraction was separated using a separatory funnel, evaporated under air to dryness and then re-dissolved with 0.5 mL ethyl acetate. To evaluate the potential effects of the extraction process on herbicide degradation, fresh plant leaves were harvested, spotted with 5 uL of the ^{14}C herbicide solution and immediately processed in parallel with the study samples.

A 200-uL aliquot of each sample was spotted on 20 by 20 cm silica gel thin layer chromatography (TLC) plates[10] and developed to a 17-cm solvent front. The solvent consisted of hexane – acetone – acetic acid (49.5:49.5:1) based on Valent USA recommendation (M. A. F. Jalal, personal communication). Plates were partitioned into 9 1-cm wide lanes. A standard that consisted of 1 uL of stock radiolabeled herbicide solution dissolved in 1 ml acetonitrile was spotted on the center lane of each plate. The remaining lanes received a single replicate of treated-leaf samples from each johnsongrass biotype from the two runs of the study. In addition, extracts from freshly-spotted leaves were spotted adjacent to the standard to assess effects of extraction procedures on herbicide degradation. Finally, a non-radiolabeled standard was spotted to allow visual verification of radiolabeled parent compound.

Plates were air dried and radioactive positions, proportions, and corresponding R_f values were determined by scanning TLC plates with a radiochromatogram scanner[11]. Radioactive trace peaks were integrated with radiochromatogram scanner software[12] with smoothing set to 9-point cubic and background excluded from peak area calculation (Askew and Wilcut 2002). Peaks below 1% of total radioactivity were rejected. Parent herbicide was identified by comparing R_f values from the corresponding standard. Parent herbicide R_f values were then verified using a non-radiolabeled standard and an ultraviolet fluorescence cabinet.

ACCase Enzyme Extraction and Assay

Enzyme extractions and assays were performed according to Burton et al. (1989) with modifications. The extraction and assay were repeated twice. ACCase from the susceptible and resistant biotypes was extracted and assayed in the same buffers. All enzyme extraction procedures were conducted at 4 C. Five grams of leaf tissue from fresh rhizome sprouts were ground in liquid nitrogen with a mortar and pestle and extracted with 20 ml of extraction buffer (0.1 M Tricine-KOH adjusted to pH 8.0 with KOH, 0.3 M glycerol, 5 mM dithiothreitol (DTT), 1 mM ethylinediaminetetraacetic acid (EDTA), and 5 mM MgCl). The homogenate was filtered through two layers of miracloth[13] and centrifuged at 27,000 g for 30 min. Ammonium sulfate was added to the supernatant to 40% ammonium sulfate, allowed to gently stir for 30 min, and centrifuged at 27,000 g for 30 min to precipitate proteins. The resulting pellet was resuspended in 1 ml of elution buffer (0.05 M Tricine-KOH [pH 8.3], 0.5 glycerol, 0.1 M KCl, and 1 mM DTT) and desalted on a size exclusion column[14] previously equilibrated with 7 ml of the

same buffer. The partially purified enzyme extracts were immediately assayed for ACCase activity. Protein concentrations in the enzyme extracts were determined by the method described by Smith et al. (1985) method using bovine serum albumin (BSA) as a standard.

ACCase activity was quantified by measuring the rate of incorporation of ^{14}C from $NaH^{14}CO_3$ into an acid- and heat-stable product (Burton et al. 1989). Assays were conducted in 7-ml glass scintillation vials containing 0.2 M tricine-KOH, pH 8.3, 1.0 M glycerol, 0.2 M KCl, 5 mM ATP, 2.5 mM acetyl CoA, and 10 mM HCO_3 (0.5 mCi/mmol), and 50 μg protein extract in a final volume of 0.2 ml. All ingredients except acetyl-CoA were added to the reaction vial and allowed to incubate for 3 m. Reactions were initiated with the addition of acetyl-CoA. Activity was assayed for 4 min at 30 C and the reaction was stopped by adding 50 μl of 6 N HCl. The sample was dried under a stream of air at 30 C. Ethanol:water (1:1, v/v, 0.5 ml) was added to the vial followed by addition of 6 ml scintillation fluid[6]. Radioactivity was determined by LSS[4]. Background dpm (acid stable counts in the absence of acetyl CoA) were subtracted from each treatment. Enzyme activity was expressed in μmol bicarbonate mg^{-1} min^{-1}.

Herbicide solution that contained technical grade clethodim was prepared by dissolving clethodim in acetone and adding 0.5 M tricine-KOH (pH 8.0) to produce an acetone concentration of 33% (v/v). Clethodim dilutions were prepared in acetone, 0.5 M tricine-KOH (pH 8.0) and distilled water to maintain a consistent acetone content in each herbicide dilution. Clethodim concentrations ranged from 0 to 0.1 mM and were replicated 3 times. The final concentration of acetone in all assays was 0.7% (v/v).

Clethodim solutions were stored in the dark at 4 C until use. Clethodim solutions were added to assay mixtures before the 3 min incubation period.

Experimental Design and Analysis

Absorption, translocation, and metabolism data were subjected to analysis of variance (ANOVA) with sums of squares partitioned to reflect a split-plot treatment structure and trial effects. The five harvest timings were considered main plots, the four plant portions quantified subplots, and the two biotypes considered subsubplots. Log transformation did not improve homogeneity of variance based on visual inspection of plotted residuals, therefore, non-transformed data was used in the ANOVA. Trial effects were considered random and mean squares were tested appropriately based on the treatment design (McIntosh 1983).

ACCase assays were arranged in a completely randomized design, and data were subjected to an analysis of variance using the general linear models procedure in SAS (1998), and sums of squares were partitioned to evaluate the effect of trial repetition, herbicide rate, and johnsongrass biotype. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Both non-transformed and log-transformed ACCase specific activity were examined, and transformation did not improve homogeneity. ANOVA was therefore performed on non-transformed ACCase specific activity. Trial repetition and linear, quadratic, and higher order polynomial effects of ACCase specific activity over clethodim concentration were tested by partitioning sums of squares (Draper and Smith 1981). Regression analysis was performed when indicated by the ANOVA. Nonlinear models were used if the ANOVA

indicated that higher order polynomial effects of specific activity were more significant than linear or quadratic estimates. Estimation used the Gauss-Newton algorithm, a nonlinear least squares technique (SAS 1998).

ANOVA indicated higher order polynomial effects for specific activity resulting from increasing herbicide rate. Thus, specific activity was modeled using the logistic function:

$$y = A + B / (1 + (x / I_{50})^{d}) \quad [1]$$

where y is the response at dose x, A is the lower limit for y, B is the upper limit for y, d is the slope, and the I_{50} is the dose giving 50% injury or inhibition (Seefeldt et al. 1995). The parameter I_{50} is most commonly referred to because it is the most accurate estimate of plant sensitivity to a herbicide (Seefeldt et al. 1995). When the logistic function was fit to the data, an approximate R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from one (Draper and Smith 1981).

Results and Discussion

Clethodim Absorption and Translocation

Recovery of ^{14}C ranged from 91.4 to 99.9% of applied (Table 1). Analysis of variance indicated that differences in absorption existed between each biotypes, thus data are presented by biotype averaged over experimental runs. Absorption of ^{14}C-label was significantly greater in the resistant than the susceptible biotype 4 HAT (Figure 1, Table 1). But by 24 HAT, however, similar levels of radioactivity were detected in both johnsongrass biotypes (Figure 1, Table 1). Absorption was biphasic, with more than 67%

of the applied radiolabel absorbed by 24 HAT. There was only a small increase in absorption after 24 HAT. Clethodim and other cyclohexanedione herbicides are rapidly absorbed initially, with little continued absorption after 8 h (Burke and Wilcut 2003a, b; Campbell and Penner 1987; Culpepper et al. 1999, Wanamarta and Penner 1989).

Analysis of variance indicated no difference between the resistant and susceptible biotypes in the translocation of ^{14}C-label out of the treated leaf to other plant portions at any sampling time. The highest amount translocated into the portion of the shoot below the treated leaf at any harvest interval was 1.8 % of applied ^{14}C (Table 1). The shoot below the treated leaf includes the intercalary meristem (Esau 1977), the location where phytotoxicity is expressed as a result of ACCase inhibition. At each sampling time, the majority of the absorbed radioactivity remained in the treated leaf. These results are consistent with the absorption and translocation of clethodim and sethoxydim. Neither differential absorption nor translocation has been reported as conferring resistance to any other ACCase resistant weed biotype identified (Devine 1997; Devine and Shimabukuro 1994), and it does not appear to be the cause of the resistance in this johnsongrass biotype.

Clethodim Metabolism

Based on R_f values provided by Valent USA, radiolabeled, and nonradiolabeled standards, clethodim (R_f = 0.83, 0.56) was identified as a major component of the 4 and 8 HAT harvest (Figure 2). By 24 HAT, less than 5% of the recovered radioactivity was recovered as clethodim, and by 72 HAT no clethodim was recovered from either biotype. Eight other major and minor metabolites were detected with varying R_f values (data not

shown). All compounds identified by thin-layer chromatography that were not clethodim were classified as metabolites and were combined and expressed as percent of recovered radioactivity. As the amount of radioactivity recovered as clethodim decreased, there was an increase in the amount of radioactivity recovered as metabolites. At each harvest interval, similar amounts of clethodim were recovered from the susceptible and resistant biotype. Metabolism of ^{14}C-clethodim into metabolites also occurred similarly in resistant and susceptible biotypes. Others have observed rapid conversion of cyclohexanedione herbicides to metabolites - typically less than 45% recovery of the active parent herbicide 4 HAT (Burke and Wilcut 2003a, b; Campbell and Penner 1985). Differential metabolism does not appear to be the cause of the resistance in this biotype of johnsongrass.

ACCase Extraction and Assay

The specific activity of ACCase from the susceptible and resistant johnsongrass biotypes was similar (means of 0.221 and 0.223 nmol mg^{-1} protein min^{-1}, respectively). ACCase enzyme from the susceptible biotype was highly sensitive to increasing concentrations of clethodim, with an I_{50} value of 0.29 μM clethodim (Figure 3). In contrast, ACCase from the resistant biotype was less sensitive, with an I_{50} value of 1.32 μM clethodim. The resultant R/S ratio was 4.5. The response of ACCase from the resistant biotype of johnsongrass was less than that of the whole-plant level, which was found to be 11.0-fold and 15.9-fold for seedling and rhizome plants, respectively, of the resistant biotype (Burke et al. 2005).

The mechanism of resistance to AOPP and CHD herbicides appears to differ between the first reported resistant johnsongrass biotypes from Mississippi and Virginia (Bradley et al. 2001; Marles et al. 1993b) and this second biotype from Mississippi. The mechanism of the first reported instance of resistance to AOPP and CHD herbicides in a biotype of johnsongrass from Washington County, Mississippi, was also a less sensitive form of the ACCase enzyme (Marles et al. 1993b). That biotype was found to be highly resistant to fluazifop-P, less resistant to quizalofop-P and sethoxydim, and susceptible to clethodim at the whole plant level (Smeda et al. 1997). An alternative mechanism of resistance was suggested for a resistant johnsongrass biotype from Virginia. That biotype was found to be resistant due to an overproduction of ACCase enzyme (Bradley et al. 2001). However, the Virginia biotype had lower overall resistance to AOPP and CHD herbicides, and was also susceptible to clethodim. The johnsongrass biotype that is the subject of this research is resistant to clethodim, and the mechanism of resistance appears to be a less sensitive form of the ACCase enzyme.

Patterns of ACCase herbicide resistance were first described by Bourgeois et al. (1997) using wild oat (*Avena fatua* L.). Using 85 different ACCase resistant wild oat biotypes, groupings of resistant biotypes were proposed based on whole plant cross-resistant patterns to AOPP and CHD herbicides. Four distinct groups were described: biotypes highly resistant to a broad spectrum of AOPP and CHD herbicides; biotypes highly resistant to sethoxydim but with low levels of resistance to other AOPP and CHD herbicides; biotypes highly resistant to fluazifop-P but with lower levels of resistance to other AOPP and CHD herbicides; and biotypes resistant to AOPP herbicides but not to

CHD herbicides (Bourgeois et al. 1997; Devine and Shulka 2000). The johnsongrass biotype that is the subject of this research falls into the first category as it has moderate to high levels of resistance to both AOPP and CHD herbicides and a similar level of resistance at the enzyme level (R/S ratio of 4.5) to other species resistant to clethodim (Tal and Rubin 2004; Volenberg and Stoltenberg 2002).

The results of many ACCase inhibition studies referenced herein suggest that different patterns of resistance may be conferred by separate mutations in the gene for plastidic ACCase (Devine and Shulka 2000). Recent reports indicate that a single modification (a single isoleucine-to-leucine substitution) confers cross-resistance to the AOPP and CHD herbicides in *Avena fatua* L. (Christoffers et al. 2002), *Lolium rigidum* Gaud. (Delye et al. 2002b; Tal and Rubin 2004; Zagnitko et al. 2001), green foxtail (Delye et al. 2002c), and *Alopecurus myosuroides* Huds (Delye et al. 2002a). A second substitution (a leucine-to-isoleucine substitution) was associated with AOPP, but not CHD, resistance (Delye et al. 2003). Further studies are needed to assess the modification and/or modifications responsible for the target site-based resistance in this johnsongrass biotype.

Sources of materials

[1] Peters Professional 20-20-20, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[2] Tee Jet Flat Fan Nozzles, TeeJet spray nozzles. Spraying Systems Co., P. O. Box 7900, Wheaton, IL 60189.

[3] Crop oil concentrate, Agri-Dex (83% paraffin-base petroleum oil and 17% surfactant blend). Helena Chemical Co., 5100 Poplar Avenue, Memphis, TN 38137.

[4] Packard TRI-CARB 2100TR Liquid Scintillation Spectrometer, Packard Instrument Company, 2200 Warrenville Road, Downers Grove, IL 60515.

[5] Induce® nonionic low foam wetter-spreader adjuvant contains 90% nonionic surfactant (alkylarypolyoxyalkane ether and isopropanol), free fatty acids, and 10% water, Helena Chemical Company, Suite 500, 6075 Poplar Avenue, Memphis TN 38137.

[6] ScintiVerse® SX18-4 Universal Liquid Scintillation Cocktail, Fisher Scientific, 1 Regeant Road, Fair Lawn, NJ 07410.

[7] Model OX-500 Biological Material Oxidizer, R. J. Harvey Instrument Corp., 123 Patterson Street, Hillsdale, NJ 07462.

[8] Pyrex® Tissue Homogenizer No. 7727-40, Corning Inc., Corning, NY 14831.

[9] Whatman® Filter Paper 5 m, Whatman International Ltd., Whatman Holst Leonards Road Allington Maidstone, Kent ME160LS, U. K.

[10] Whatman K6F Silica Gel 60A thin layer chromatography plates, Whatman Inc., 9 Bridewell Place, Clifton, NJ 07013.

[11] BioScan System 200 Imaging Scanner, Bioscan, 4590 MacArthur Boulevard NW, Washington, DC 20007.

[12] Win-Scan software, LabLogic® Win-Scan Radio TLC Version 2.2(5) 32-bit, BioScan, 4590 MacArthur Boulevard NW, Washington, DC 20007.

[13] Miracloth, Calbiochem, EMD Bioscineces, Inc. 10394 Pacific Center Court, San Diego, CA 92121.

[14] Sephadex G-25 Chromatography Column, Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865.

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Table 1. Distribution of applied ^{14}C-label by johnsongrass biotypes (clethodim-resistant and clethodim-susceptible) at five timings after application of ^{14}C-clethodim.

	^{14}C as affected by time after treatment (h)[a]									
	Plant portion									
	Leaf wash		Treated leaf		Shoot above		Shoot below		Root	
Time (h)	Suscep.	Resistant	Suscep.	Resistant	Suscep.	Resistant	Suscep.	Resistant	Suscep.	Resistant
	% of applied									
4	60.9*	36.2	37.7*	59.8	0.2	2.1	0.5	0.5	0.6	1.2
8	40.9	30.1	48.8*	62.9	0.9	1.9	0.3	1.0	0.5	1.1
24	28.8	20.9	65.5	74.4	3.1	1.2	1.9	2.0	0.7	1.4
48	25.3	31.2	70.9	65.8	0.9	0.8	0.9	0.4	1.9	1.8
72	24.9	19.7	71.3	76.6	1.4	2.7	0.6	0.7	1.8	0.7
LSD (0.05)	19.5	11.2	23.1	8.3	NS	NS	NS	NS	NS	NS

[a]Clethodim was applied at 140 g ai ha^{-1}. A '*' denotes differences between the susceptible and resistant johnsongrass biotypes for each plant portion at P = 0.05.

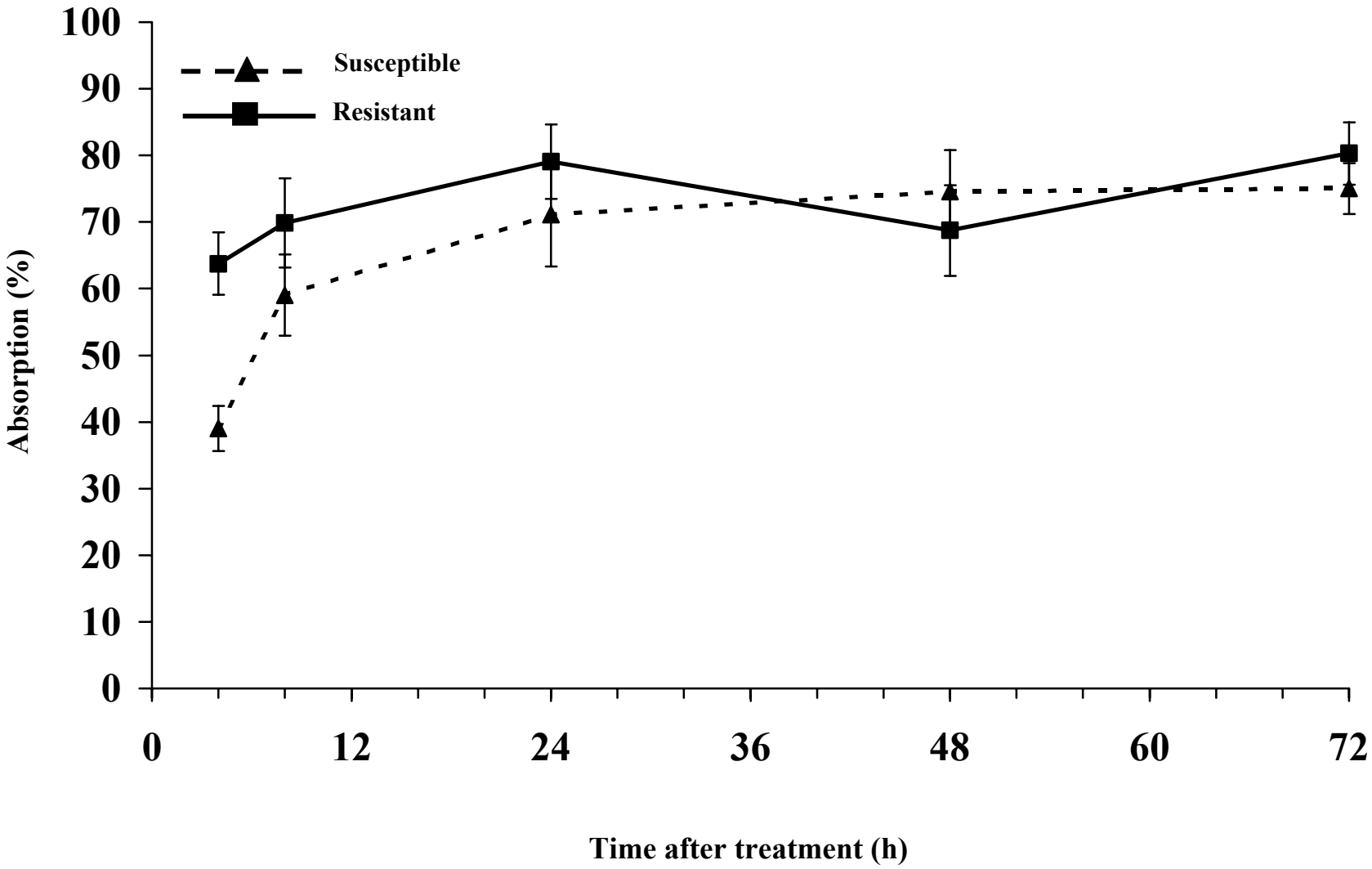

Figure 1. Foliar absorption of ^{14}C-clethodim based on leaf-wash recovery expressed as percent of applied in resistant (R) and susceptible (S) johnsongrass.

.

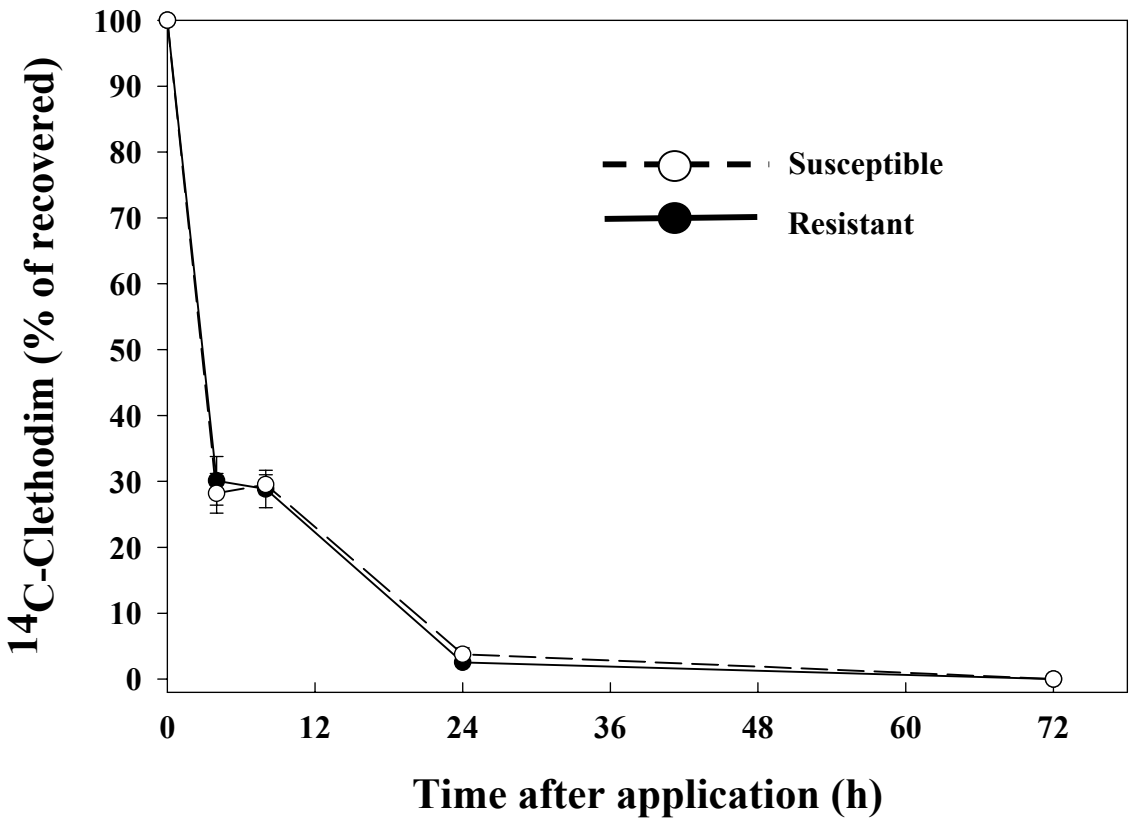

Figure 2. Metabolism of ^{14}C-clethodim in resistant (R) and susceptible (S) johnsongrass (*Sorghum halepense*) biotypes. Percentages of absorbed ^{14}C as clethodim is illustrated. Error bars represent the standard error of the mean (*n*=6).

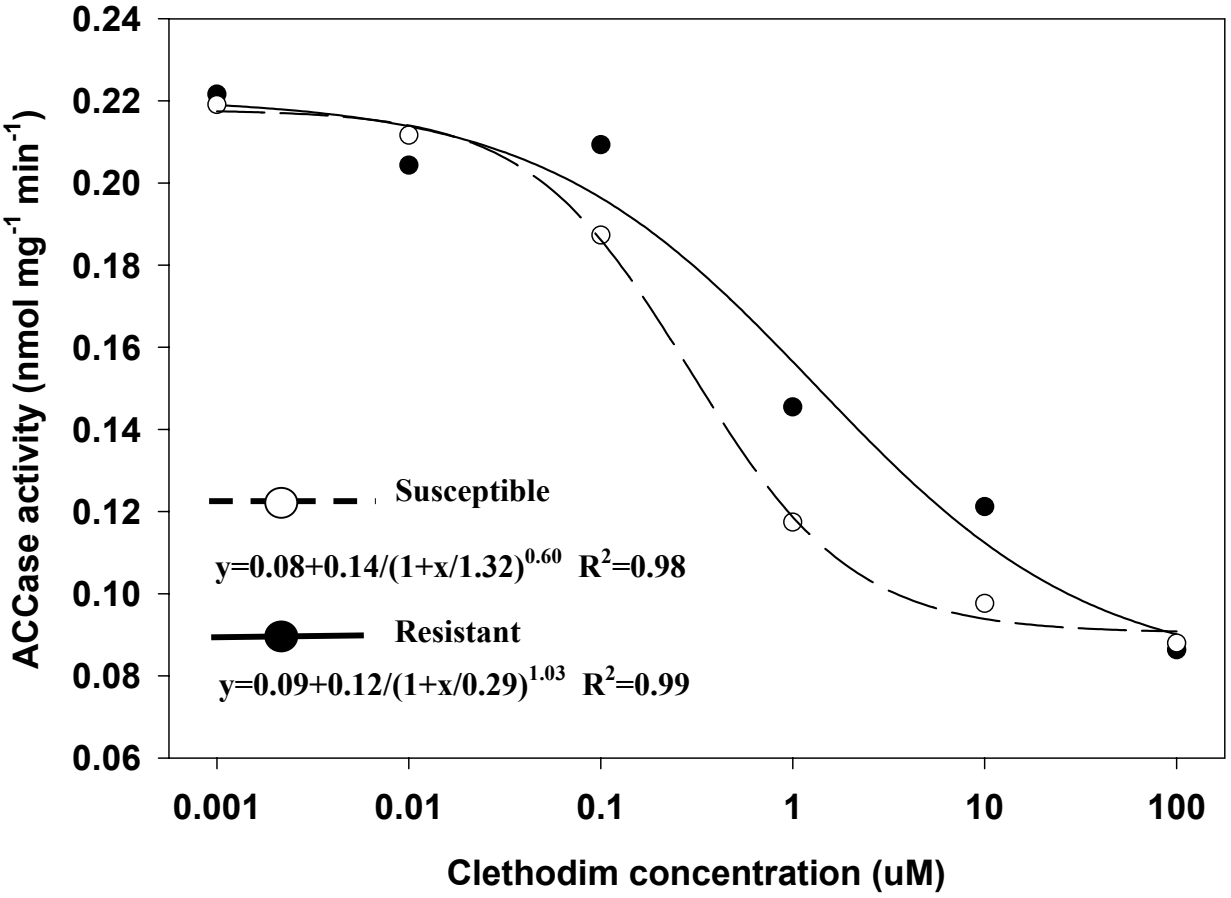

Figure 3. Inhibition of ACCase activity from resistant (R) and susceptible (S) johnsongrass (*Sorghum halepense*) biotypes by clethodim. Error bars represent the standard error of the mean.

CHAPTER 4

Early Vegetative Growth and Competitiveness of ACCase-Resistant and –Susceptible Johnsongrass (*Sorghum halepense*) Biotypes

Abstract. The relative competitiveness and non-competitive productivity of acetyl coenzyme A carboxylase (ACCase)-resistant and ACCase-susceptible johnsongrass were assessed in greenhouse and growth chamber experiments. When grown in noncompetitive conditions in growth chamber experiments, photosynthetic rate, net assimilation rate (NAR), leaf number, leaf area, specific leaf area, leaf dry biomass, and shoot dry biomass were similar for resistant (R) and susceptible (S) biotypes 21, 27, and 35 days after planting (DAP). Competition between biotypes was evaluated using a set of replacement series experiments with seven ratios of R:S plants (6:0, 5:1, 3:1, 1:1, 1:3, 1:5, 0:6) all at a density of 85 plants/m^2. The lack of curvature in the R or S biotype lines of the replacement series diagrams indicates that neither biotype has a competitive advantage in terms of leaf number or plant height. Relative crowding coefficients for above-ground dry biomass were not different from 1, and a combined t-test indicated that the R and S biotype did not differ from the theoretical equal competition line for above-ground dry biomass (t_{lof}=0.54, 1.3; P=0.38, 0.23, respectively). Although it is not possible to conclude from this study that ACCase resistance has or has not reduced the fitness of the R biotype, the two biotypes have very similar growth parameters.

Nomenclature: *Sorghum halepense* (L.) Pers. #[5]SORHA, johnsongrass.

Additional index words: Fitness; relative crowding coefficients; replacement series; deWit diagrams.

Abbreviations: ACCase, acetyl coenzyme A carboxylase; AOPP, aryloxyphenoxy propionate; CHD, cyclohexanedione; DAP, days after planting; LAR, leaf area ratio; NAR, net assimilation rate; RCC, relative crowding coefficient; RGR, relative growth rate; R, resistant; SLA, specific leaf area; S, susceptible.

Introduction

Johnsongrass [*Sorghum halepense* (L.) Pers.] occurs in all major agricultural areas of the warm regions of the world and was listed by Dowler (1998) as one of the world's ten worst weeds. Johnsongrass reduces crop yields (Horowitz 1973; McWorter and Hartwig 1973) and its pollen contaminates sorghum grown for seed (Rosenow and Clark 1969). In 1991, resistance to the aryloxyphenoxy propionate [AOPP] herbicides fluazifop-P and quizalofop was discovered in two populations of johnsongrass in Mississippi (Smeda et al. 1997). Since then, five other ACCase-resistant populations of johnsongrass have been reported (Heap 2005). The population that is the subject of this research originated in

[5] Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds*, Revised 1989. Available only on computer disk from WSSA, 810 East 10th Street, Lawrence, KS 66044-8897.

Washington County, Mississippi, and is resistant to both AOPP and cyclohexanedione (DIM) herbicides, including clethodim.

Herbicide resistance in weed biotypes can result in reduced fitness compared to susceptible biotypes (Gressel and Segal 1982). Cousens et al. (1997) indicated, however, that in order to have confidence that the R biotype is different in terms of fitness, it is necessary to determine the background variation among S populations. That requirement assumes that evaluations are to made using traditional fitness studies on an array of dissimilar biotypes. Bergelson and Purrington (1996) observed that if isogenic lines were unavailable, breeding designs that control the genetic background through randomized crosses were more likely to detect costs of resistance. To conduct these studies, resistant biotypes are crossed with one or more susceptible biotypes. Bergelson and Purrington (1996) continued noting that the amount of experimental noise contributed by variation in the genetic background would be dependent on the degree of similarity between resistant and susceptible parents.

Variability in johnsongrass is well established. McWhorter (1971) evaluated 55 morphologically distinct biotypes of johnsongrass from across the United States and found them to differ by 2 to 4 fold in height and tiller density. Prepatory to conducting fitness experiments as described by Bergelson and Purrington (1996) with this biotype of ACCase-R johnsongrass, it is necessary to find a susceptible biotype that does not differ phenologically from the R biotype. As ACCase resistance is a co-dominant trait and a seedling population that has an intermediate level of resistance is most likely segregating (Smeda et al. 2000), preliminary attempts were made to recover susceptible seedlings

from a seedling collection of the resistant johnsongrass biotype. These attempts were not successful. Therefore, a biotype of johnsongrass from Washington County, Mississippi was obtained with no known previous exposure to ACCase-inhibiting herbicides. Separate experiments were conducted in 2004 and 2005 to evaluate early season non-competitive productivity and competitiveness the R and S johnsongrass biotypes from Washington County for suitability of performing further experiments on the fitness of the ACCase resistance.

Materials and Methods

Growth of S and R Johnsongrass under Noncompetitive Conditions.

The experiment was conducted in the North Carolina State University Southeastern Plant Environmental Laboratory, Raleigh, North Carolina from January to March 2005. Two identical chambers, 3 m^2 with a height of 2.1 m, were used to grow johnsongrass under optimum (high temperature, HT) and suboptimum (low temperature, LT) temperature regimes of 34/24 ± 2 C and 24/18 ± 2 C. Lighting was provided by a combination of fluorescent and incandescent lamps (380 umol/m^2/s photosynthetic photon flux), and both chambers were set for a 14-hr photoperiod. Each chamber contained 24 pots with a diameter of 25.4 cm. Johnsongrass seeds of both biotypes were pregerminated and transplanted one per pot. Each pot contained a substrate of steam-sterilized and washed #16 gravel and a proprietary blend of peat and vermiculite. All pots were watered with a standard nutrient solution twice daily throughout the study.

Treatments consisted of a factorial arrangement of two biotypes (R and S), two temperature regimes (HT and LT), and three destructive sampling dates. Photosynthetic net assimilation rate (NAR), leaf area, leaf number, tiller number, plant height, leaf fresh biomass, stem fresh biomass, total above-ground fresh biomass, leaf dry biomass, stem dry biomass, total above ground dry biomass, and root dry biomass were measured 21, 28, and 36 DAP. Photosynthetic NAR was measured on the highest fully expanded leaf using a LI-COR LI-6200 portable photosynthesis system[6], and total leaf area was measured with a LI-COR 310 leaf area meter[7]. Above-ground and root dry biomass were determined after drying at 65C for 5 d. Relative growth rate (RGR), NAR, and specific leaf area (SLA) were determined as described by Radford (1967).

The experimental design was a split-plot with temperature regimes as whole plots and biotypes and sample dates as sub-plots. Within each chamber (or whole plot), biotype and sampling date were replicated four times and seedlings were blocked according to size at the beginning of the experiment. The experiment was conducted twice. Data were square-root transformed to improve variance homogeneity and then subjected to ANOVA using PROC MIXED in SAS (1998) with partitioning appropriate for a two by two by three factorial treatment arrangement of temperature regime, biotype, and sampling date. Non-transformed means are presented for clarity.

[6] Model LI-6200 portable photosynthesis system, LI-COR, P.O. Box 4425, Lincoln, NE 68504.

[7] Model LI-310 leaf area meter, LI-COR, P.O. Box 4425, Lincoln, NE 68504.

Growth of S and R Johnsongrass Biotypes under Competitive Conditions.

The experiment was conducted in greenhouses at North Carolina State University from September to December 2004. Seeds of each biotype were pregerminated and planted into 30-cm diameter pots filled with 12 L of potting medium. All pots received 2 g of a commercial greenhouse fertilizer[8] 7 d after planting. Plants were grown with approximate day/night temperatures of 32/25 C and were watered over the top daily. Natural light in the greenhouse was supplemented for a 12-h photoperiod by metal halide lamps (giving an additional 250 umol/m^2/s photon flux density at soil level).

Each pot contained six plants, which gave a density of 85 plants/m^2 in the following R:S mixtures: 6:0, 5:1, 4:2, 3:3, 2:4,1:5, and 0:6. Pots were planted with one or both biotypes to give a conventional replacement series experiment arranged in a randomized complete block design (de Wit 1960; Cousens 1991; Radosevich 1987). Plants of each biotype were identified with color-coded plastic markers. Plant height and leaf number were determined 14, 21, 28, 35, and 42 DAP, and leaf and stem matter were harvested 42 DAP and dried for 7 d at 60 C. Treatments were replicated four times, and the experiment was conducted twice.

Replacement series diagrams were constructed for each sampling period for leaf number, and for leaf dry biomass and total above-ground dry biomass 42 DAP.

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[8] Peters Professional 20-20-20, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

Homogeneity of variance between the experimental runs was confirmed using ANOVA. Where visual evaluation of replacement series diagrams has been unsatisfactory, traditional approaches of statistical analysis have included regression to test for non-linearity of the response, or a series of t-tests comparing observed and expected relative yields separately at each plant ratio (Roush et al. 1989; Weiderholt and Stoltenberg 1996a, b). As the separate t-tests can lack power or lead to ambiguous results (Roush et al. 1989), a single t-test comparing observed and expected results combined over all plant proportions, except monocultures, was performed. Data were converted to relative values (with monoculture above ground dry biomass set equal to 1 separately for each run) and relative values were averaged over replication for each proportion within each run. ANOVA was conducted for each biotype on the mean leaf dry biomass or shoot dry biomass for all proportions except monocultures using the PROC MIXED procedure in SAS (Littell et al. 1996) with experimental runs treated as replicates and proportion as a fixed effect. Means were summed over proportions and compared to the sum of the expected relative values using the appropriate standard error and degrees of freedom from PROC MIXED to carry out a t-test.

A third method used to quantify the similarity of one weed biotype with another when grown in mixture is known as the relative crowding coefficient (RCC) (Harper 1977). The RCC is calculated as:

$$\frac{((5)X_S^{5:1}/X_R^{5:1} + (2)X_S^{4:2}/X_R^{4:2} + X_S^{3:3}/X_R^{3:3} + X_S^{2:4}/(2)X_R^{2:4} + X_S^{1:5}/(5)X_R^{1:5})/N}{(X_S^{6:0}/X_R^{0:6})} \quad [1]$$

where $X_R^{r:s}$ is sum of the plant height, leaf number, or above-ground dry biomass for the R biotype of johnsongrass at a ratio of r:s. $X_S^{r:s}$ is the sum of the plant height, leaf number, or above ground dry biomass for the S biotype of johnsongrass at a ratio of r:s (Novak et al. 1993). N is equal to the number of mixed species comparisons; for this study N=5. Values of RCC greater than 1 indicate that the S biotype is more competitive than the R biotype; RCC values less than 1 indicate that the R biotype is more competitive. An RCC of 1 indicates that the biotypes are similar.

Results and Discussion

Growth of S and R Johnsongrass under Noncompetitive Conditions.

Two temperature regimes were used to determine if differences in non-competitive productivity might occur between biotypes in optimum and sub-optimum conditions. The RGR temperature regime by biotype interactions were significant, consequently RGR is presented by biotype and temperature (Table 1). Relative growth rate expresses dry weight accumulation over a specific time interval and considers the initial weight at the start of the time interval (Gardner 1985). Relative growth rate was higher in the optimum temperature regime for the R biotype, but the S biotype RGR was similar in both temperature regimes and also similar to the resistant biotype in the optimum temperature regime. There was also a temperature regime by biotype interaction for NAR. In the optimum temperature regime, NAR was higher for the susceptible biotype, while NAR was similar for each biotype in the sub-optimum temperature regime (Table 1). The main effect of temperature was significant for photosynthetic rate (Table 2). For

clarity, photosynthetic rate is shown by biotype. Specific leaf area was not significantly different among temperature regimes or biotypes (Table 1).

Temperature regime by biotype interactions were not significant for leaf number, tiller number, plant height (Figure 1), leaf fresh biomass, stem fresh biomass, total above ground fresh biomass (Figure 2), leaf dry biomass, stem dry biomass, total above ground dry biomass (Figure 3), leaf area or root dry biomass (Figure 4), thus the main effect of biotype was averaged over temperature regime at each sampling period. For comparison, each biotype is shown separately.

These results suggest that under noncompetitive conditions, early season growth (<35 DAP) was similar for R and S biotypes. The S biotype produced more tillers early (Figure 1), but by 35 DAP each biotype had similar numbers of tillers. The biotypes were not different at each harvest interval for all parameters, although the susceptible biotype had higher numerical parameters at the 35 DAP harvest interval.

Growth of S and R Johnsongrass Biotypes under Competitive Conditions.

The lack of curvature in the R or S biotype lines of the replacement series diagrams indicates that neither biotype has a competitive advantage in terms of leaf number (Figure 5a) or plant height (Figure 5b). The plot of above-ground dry biomass (Figure 5c) shows that the intersection point of the R and S lines is to the left of the 3:3 ratio, however, neither biotype is different from the expected 3:3 ratio. Visual evaluation of the de Wit diagram of relative above-ground dry biomass was therefore inconclusive and necessitated further analysis. The data were compared to theoretical equal competition values using a combined t-test. Results of the combined t-test indicate that the R and S

biotype did not differ from the theoretical equal competition line for above-ground dry biomass (t_{lof}=0.54, 1.3; P=0.38, 0.23, respectively). The third method of evaluating competition, RCC values, agreed with the other methods of evaluation. The RCC values for leaf number, plant height, or above ground dry biomass were not different from 1 (Table 3), indicating lack of a competitive advantage for either biotype. Wiederholt and Stoltenberg (1996a, b) reported that resistant biotypes of large crabgrass and giant foxtail also produced similar biomass as susceptible biotypes.

These studies suggest that by 42 DAP these biotypes accumulate biomass similarly in both competitive and noncompetitive conditions. Gressel (2002) argues strongly that there should be some penalty for resistance as the resistance is not the predominate biotype and exists at a much lower frequency. Although the binding site for ACCase herbicides is different than that of the substrate binding site, Gressel (2002) argues that there should still be changes in the Michaelis constant (K_m). Those changes are simply too small to measure *in vitro* or the mutational differences may influence allosteric feedback mechanisms that are not measured as part of *in vitro* K_m (Gressel 2002). Other herbicide target sites demonstrate clear fitness penalties as the site of herbicide activity is also the substrate binding site, such as resistance to photosystem II herbicides (Conrad and Radosevich 1978; Gressel 2002). Although it is not possible to conclude that ACCase resistance has or has not reduced the fitness of the resistant biotype in this study, the two biotypes have very similar growth parameters.

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Table 1. Relative growth rate, specific leaf area, net assimilation rate, and photosynthetic rate of ACCase-resistant and –susceptible johnsongrass 35 d after planting under noncompetitive conditions as affected by temperature[a].

Biotype	Temperature regime	Relative growth rate	Specific leaf area	Net assimilation rate	Photosynthetic rate
	C	g/g/d	cm^2/g	$g/dm^2/d$	umol $CO_2/m^2/s$
R	34/24	0.22 A	408.1 A	13.12 B	15.62 A
	24/18	0.15 B	361.4 A	5.38 C	11.53 B
S	34/24	0.19 AB	392.2 A	18.28 A	15.78 A
	24/18	0.18 AB	335.7 A	4.47 C	11.45 B

[a] Means within a column followed by the same letter are not different according to Fisher's Protected LSD test at $P \leq 0.05$.

Table 2. Relative crowding coefficients for height, leaf number, and above ground dry biomass of ACCase resistant (R) and susceptible (S) johnsongrass biotypes 35 days after planting. Values greater than 1 indicate the S exceeded the R biotype and values less than 1 indicate the S exceeded the R biotype.

S/R Relative Crowding Coefficients[a]		
Height	Leaf number	Aboveground dry biomass
1.01 ±0.21	0.94 ± 0.05	0.99 ±0.13

[a] Means listed with standard error of the mean.

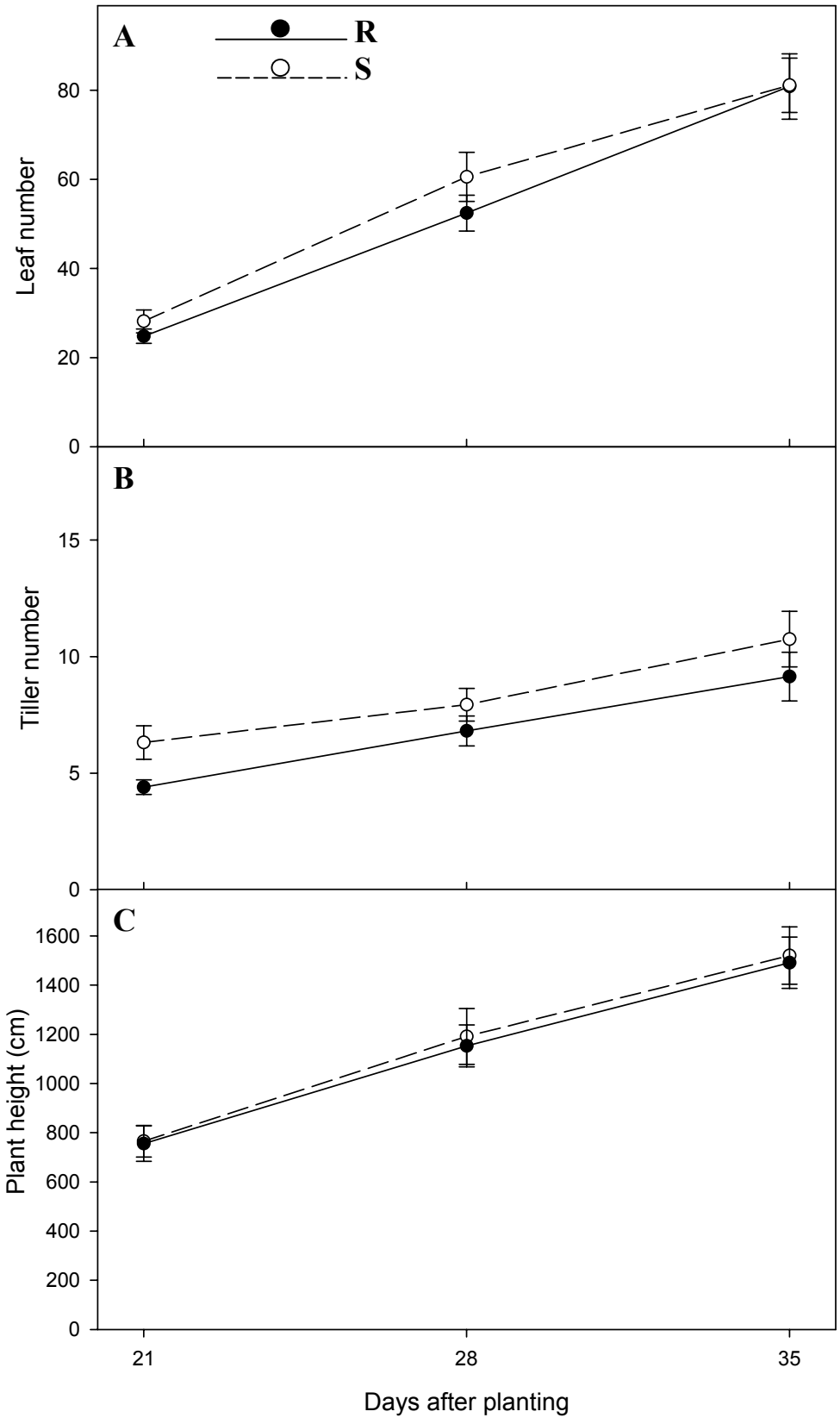

Figure 1. Average leaf number (A), tiller number (B), and plant height (C) of ACCase-resistant and –susceptible johnsongrass grown under noncompetitive conditions. Error bars represent standard error of the mean.

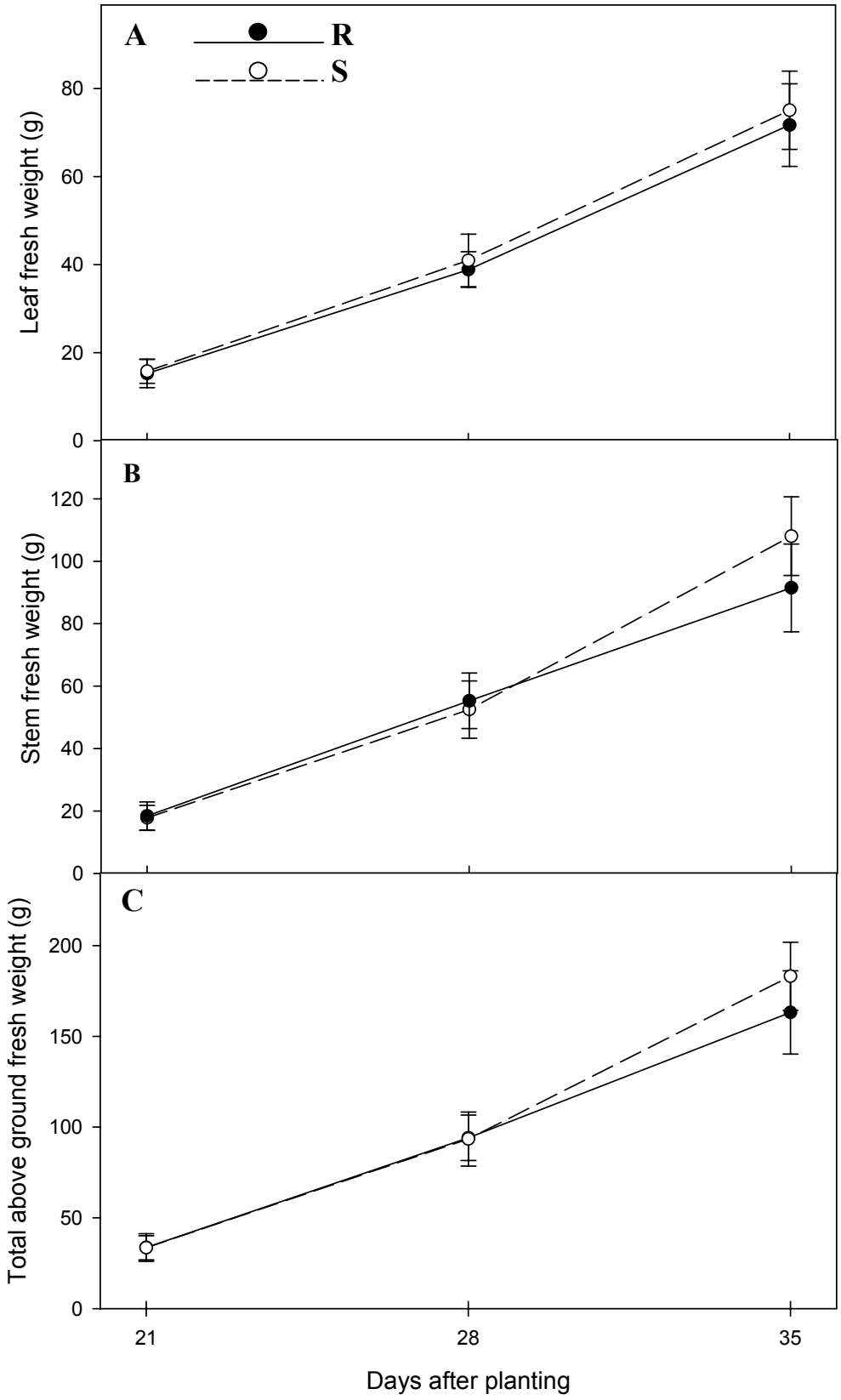

Figure 2. Average leaf fresh weight (A), stem fresh weight (B), and total above ground fresh weight (C) of ACCase-resistant and –susceptible johnsongrass grown under noncompetitive conditions. Error bars represent the standard error of the mean.

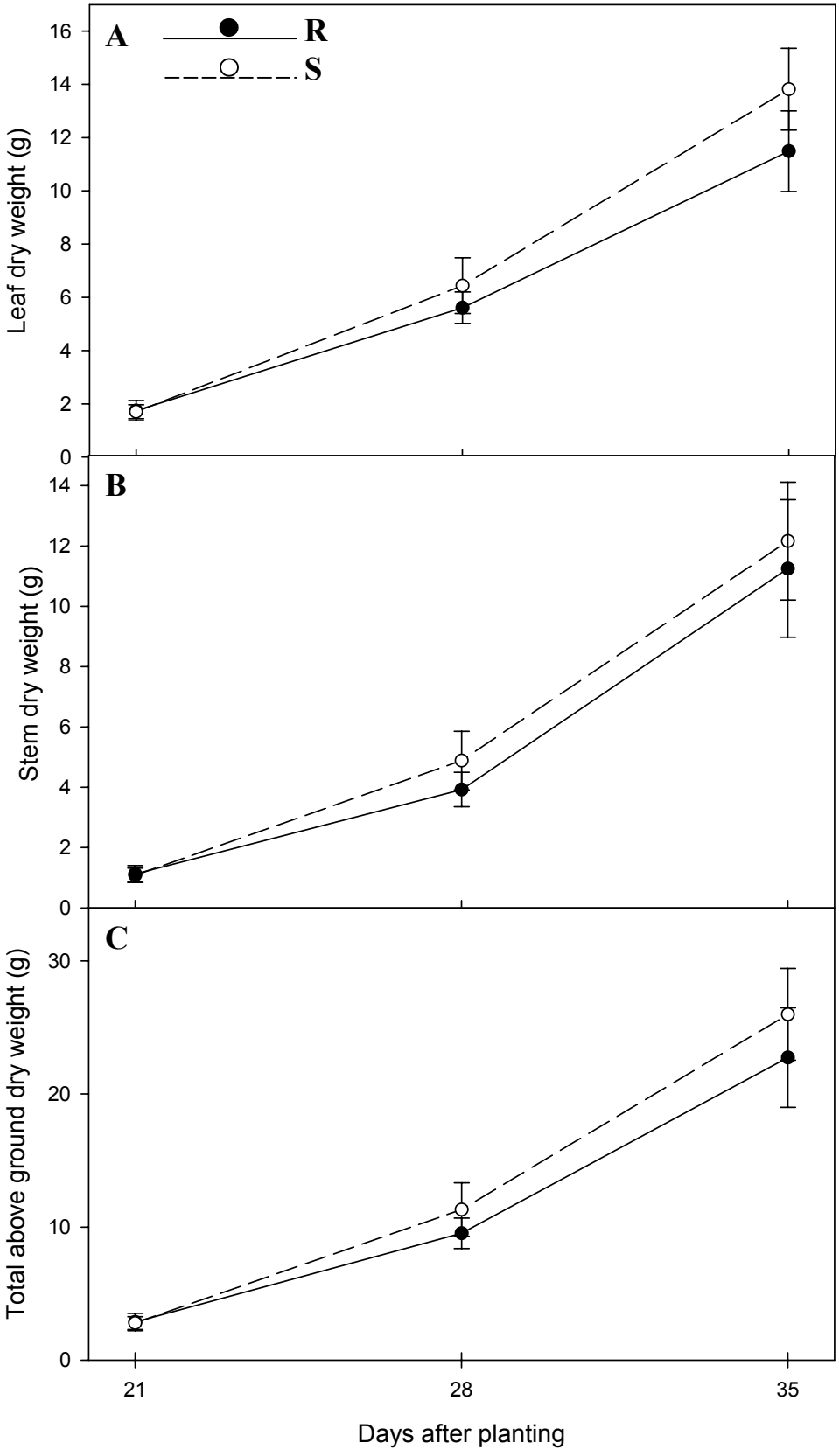

Figure 3. Average leaf dry weight (A), stem dry weight (B), and total above ground dry weight (C) of ACCase-resistant and –susceptible johnsongrass grown under noncompetitive conditions. Error bars represent standard error of the mean.

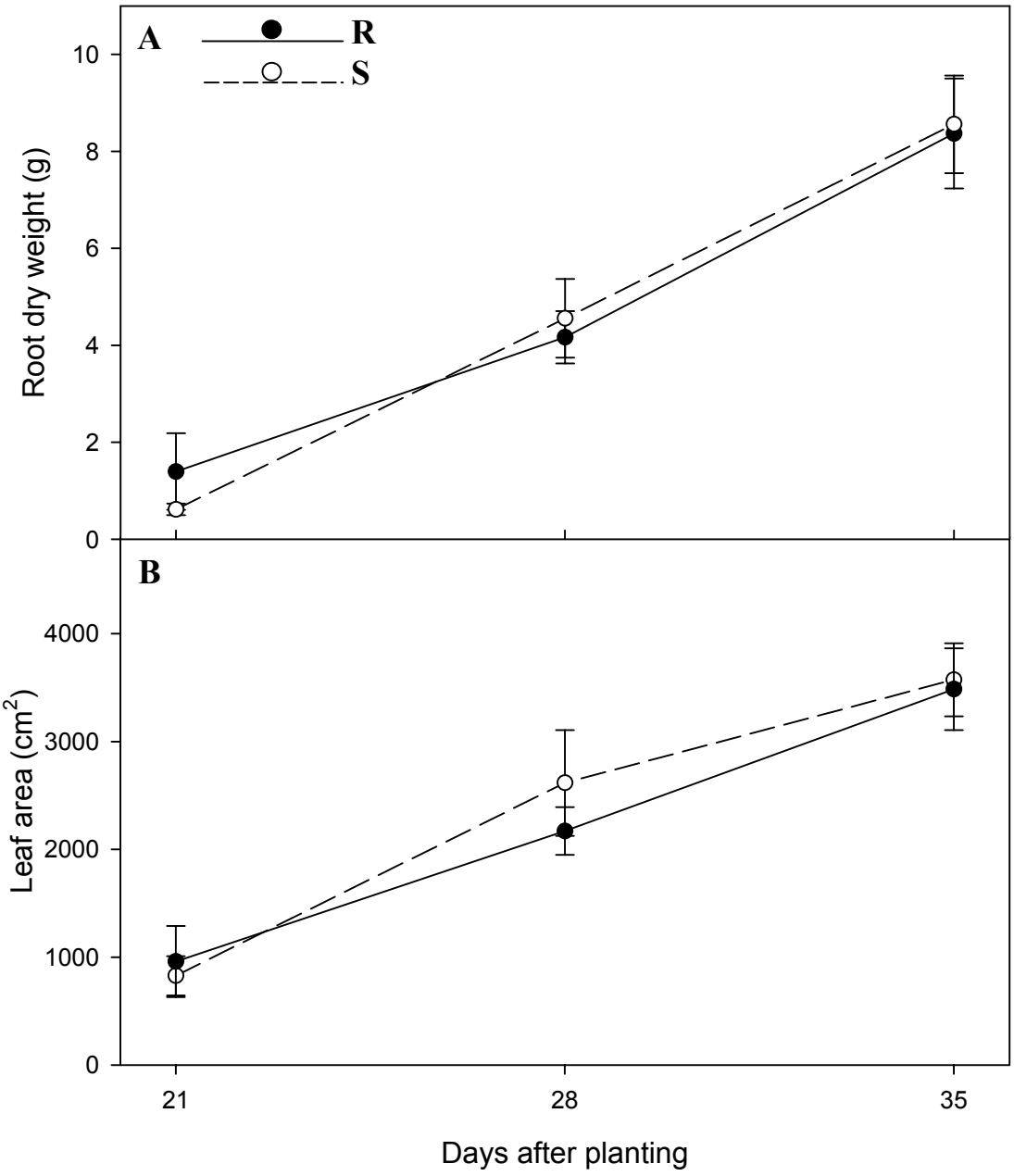

Figure 4. Average root dry weight (A) and leaf area (B) of ACCase-resistant and –susceptible johnsongrass grown under noncompetitive conditions. Error bars represent standard error of the mean.

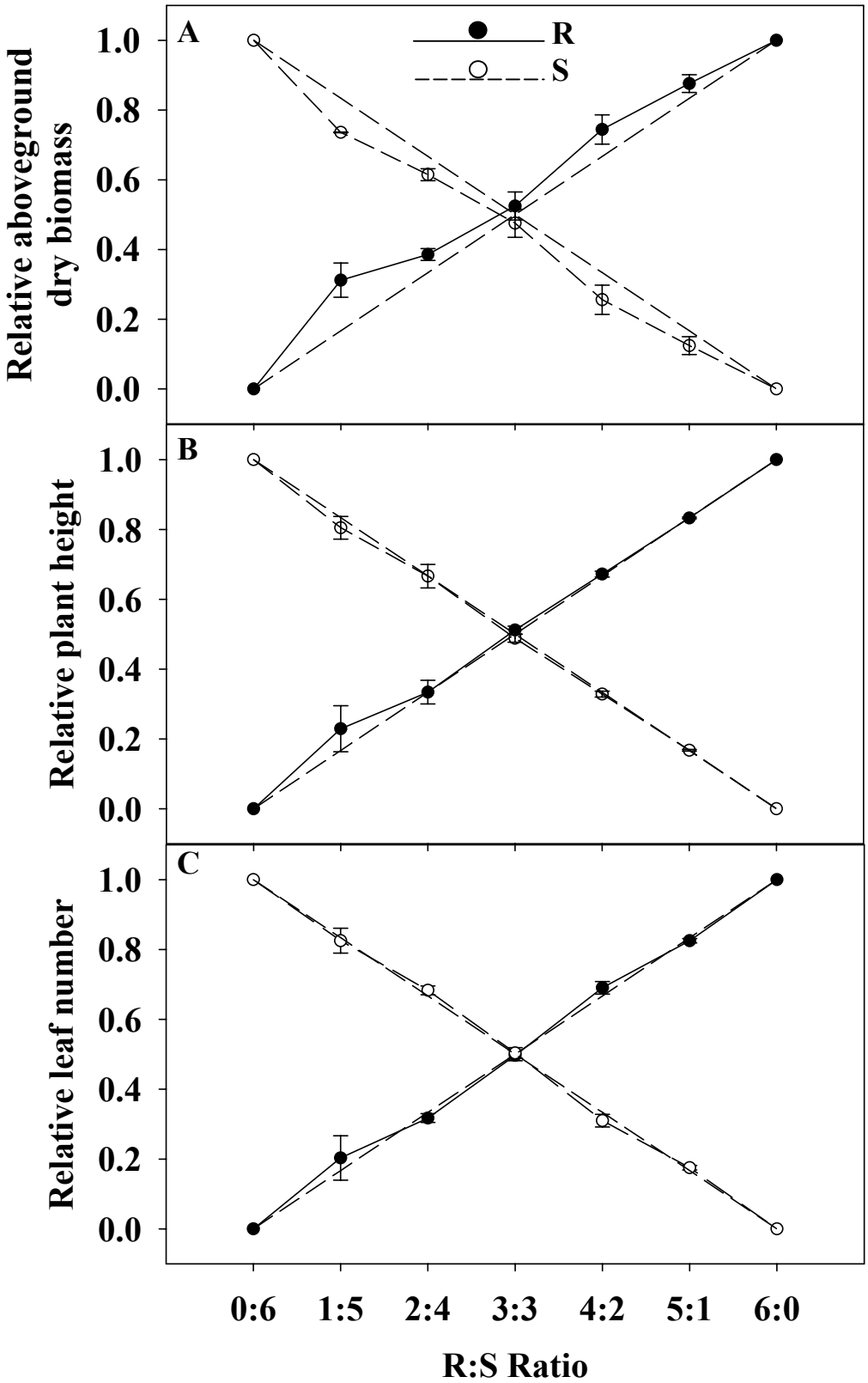

Figure 5. Replacement series diagrams for above ground dry biomass (A), leaf number (B), and plant height (C) of competing ACCase-resistant (R) and –susceptible (S) johnsongrass. Error bars represent the standard error of the mean.

CHAPTER 5

A Seedling Assay to Screen Aryloxyphenoxypropionic Acid and Cyclohexanedione Resistance in Johnsongrass (*Sorghum halepense*)

Abstract. A seedling bioassay was developed for the rapid diagnosis of resistance to clethodim and fluazifop-P in johnsongrass. The assay was based on differences in the coleoptile length of susceptible (S) and resistant (R) seedlings exposed to clethodim and fluazifop-P in petri dishes for 5 d. A bioassay concentration of 0.09 mg/L clethodim and 0.18 mg/L fluazifop-P were chosen as discriminant based on rate responses of each biotype to increasing herbicide dose. At 5 days after treatment (DAT), the clethodim GR_{50} values for the R and S seedlings were 462.5 and 24.8 mg/L, respectively, resulting in an R:S ratio of 18.7. The fluazifop GR_{50} values for the R and S seedlings were 618.7 and 17.5 mg/L, resulting in a R:S ratio of 35.4.

Nomenclature: Clethodim, fluazifop-P, *Sorghum halepense* (L.) Pers. #[9]SORHA, johnsongrass.

Additional index words: ACCase inhibitors, herbicide resistance.

Abbreviations: ACCase, acetyl coenzyme-A carboxylase; AOPP, aryloxyphenoxypropionate; CHD, cyclohexanedione; DAT, d after treatment; R, resistant; S, susceptible.

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[9] Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds*, Revised 1989. Available only on computer disk from WSSA, 810 East 10th Street, Lawrence, KS 66044-8897.

Introduction

The selective postemergence control of johnsongrass in soybean [*Glycine max* (L.) Merr.] and other broadleaf crops first became possible In the late 1970's with the registration of several herbicides that provide postemergence control of grass weeds (Burton 1997). Also called graminicides, these herbicides have since been divided into two chemically distinct herbicide classes, the aryloxyphenoxypropionate (AOPP), which include fluazifop-P and the cyclohexanedione (CHD) herbicides, which include clethodim (Burton 1997). Both of these herbicide classes act by inhibiting the enzyme acetyl coenzyme-A carboxylase (ACCase, EC 6.4.1.2) in susceptible grass species. The enzyme ACCase catalyzes the first committed step of fatty acid biosynthesis, which is the ATP-dependent carboxylation of acetyl-CoA to malonyl CoA (Incledon and Hall 1997). Grass species have a eukaryotic type ACCase in the chloroplast, which is sensitive to ACCase inhibitors, while most broadleaf species have a prokaryotic type of ACCase, which is not sensitive to ACCase inhibitors (Incledon and Hall 1997).

Graminicides are currently registered in a wide variety of crops, which often leads to the repeated use of these herbicides (Devine and Shimabukuro 1994). Maxwell and Mortimer (1994) noted that repeated use of an herbicide or herbicides is one of the most common factors that lead to herbicide resistance. There are now 34 grass weed biotypes resistant to the ACCase inhibitors (Heap 2005). In 1992, resistance to the AOPP herbicides and cross-resistance to the CHD herbicide sethoxydim was discovered in two populations of johnsongrass in Mississippi (Smeda et al. 1997). Several CHD and AOPP herbicides had been applied one or more times annually over several years in fields where

populations were detected. However, these populations were not resistant to clethodim (Smeda et al. 1997). Since that time, AOPP- and CHD-resistant johnsongrass has been reported in Kentucky, Louisiana, Mississippi, Tennessee, and Virginia (Heap 2005). Only the biotype from Louisiana, however, is resistant to clethodim (Heap 2005). In 1999, a single population of johnsongrass survived repeated treatments of clethodim in Washington County, Mississippi. The population was found to be resistant not only to clethodim but also to sethoxydim and fluazifop-P (Burke et al. 2005a). Resistance was due to a less susceptible ACCase enzyme (Burke et al. 2005b).

Resistance testing is very important for the timely implementation of management strategies. When testing for resistance to herbicides, the development of rapid and reliable bioassays is important if growers are to be advised of their herbicide options in a timely manner. The identification of resistance to ACCase-inhibiting herbicides typically involves applying herbicide to plants grown under controlled conditions (Beckie et al. 2000; Letouzé and Gasquez 1999) although it can involve verification in the field (Beckie et al. 2000). Whole plant assays, either in the field or greenhouse, are costly in terms of both labor and time (Beckie et al. 2000; Letouzé and Gasquez 1999). Resistant biotypes can also be identified by measuring the activity of the enzyme in the presence of increasing herbicide concentrations, a process which is complex, equipment and reagent intensive, and costly (Beckie et al. 2000).

Seedling assays that involve determination of either coleoptile length or root length as growth parameters to discriminate between resistant and susceptible biotypes have been developed for several AOPP- and CHD-resistant weed biotypes (Beckie et al. 1990;

Murray et al. 1996; Letouzé and Gasquez 1999; Retrum and Forcella 2002), but not for clethodim-resistant johnsongrass. Therefore, the objective of this research was to identify a suitable clethodim and fluazifop-P concentration for johnsongrass seedling assays for resistance.

Methods and Materials

Experiments were conducted on johnsongrass seedling populations previously identified as R and S (Burke et al. 2005a). The glumes of johnsongrass seed were removed, and the seed pre-germinated in 15x300 mm round plastic petri dishes lined with germination paper in an alternating temperature growth chamber set at 20/30 C. Johnsongrass seedlings with a root length of at least 3 mm were then placed in assay dishes. Seedling assays were prepared by heating distilled water to 70 C and adding agar at 8 g/L. The agar media was boiled and allowed to cool to 45 C. To prepare each assay, a solution of either clethodim (Anonymous 2005b) or fluazifop-P (Anonymous 2005a) in the commercial form were prepared each at 0.5 g ai/L. Using these solutions, treatments were prepared by first adding the appropriate amount of herbicide solution to a 200 mL volumetric flask and then bringing the volume to 200 mL using the agar solution. Twenty mL of agar-herbicide solution were poured into 15x300 plastic petri dishes and allowed to cool. Clethodim assay concentrations were 0, 11, 22, 45, 90, 180, 360, 720, or 1,440 µg ai/L. Fluazifop-P assay concentrations were 0, 23, 47, 94, 180, 380, 750, and 1,500 µg ai/L. The highest rate used in these assays corresponds to 0.15 times the 1X rate for clethodim (140 g ai/ha) (Anonymous 2005a) and fluazifop (280 g ai/ha)

(Anonymous 2005b) on an area basis. Ten pre-germinated johnsongrass seedlings with coleoptiles 3 mm in length were placed in each agar plate, and the plates were then placed in an alternating temperature growth chamber set at 20/30 C. There were four repetitions of each herbicide concentration, and the experiment was conducted twice. At 5 d after treatment (DAT), the dishes were removed and coleoptile length was measured in mm.

Statistical Analysis

Coleoptile length was subjected to an analysis of variance using the general linear models procedure in SAS (1998), and sums of squares were partitioned to evaluate the effect of trial repetition, herbicide rate, and johnsongrass biotype. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Both non-transformed and log-transformed coleoptile were examined, and transformation did not improve homogeneity. ANOVA was therefore performed on non-transformed coleoptile length. Trial repetition and linear, quadratic, and higher order polynomial effects of coleoptile length over graminicide rates were tested by partitioning sums of squares (Draper and Smith 1981). Regression analysis was performed when indicated by the ANOVA. Nonlinear models were used if the ANOVA indicated that higher order polynomial effects on coleoptile length were more significant than linear or quadratic estimates. Estimation used the Gauss-Newton algorithm, a nonlinear least squares technique (SAS 1998).

Analysis of variance indicated higher order polynomial effects for coleoptile length resulting from increasing herbicide rate. Thus, coleoptile length was modeled using the logistic function:

$$y = A + B / (1 + (x / GR_{50})^{d}) \quad [1]$$

where y is the response at dose x, A is the lower limit for y, B is the upper limit for y, d is the slope, and the GR_{50} is the dose giving 50% coleoptile length reduction (Seefeldt et al. 1995). The parameter GR_{50} is most commonly referred to because it is the most accurate estimate of plant sensitivity to a herbicide (Seefeldt et al. 1995). When the logistic function was fit to the data, an approximate R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from one (Draper and Smith 1981). Growth increments for all seedlings were grouped into 5-mm intervals, and frequency histograms were constructed to compare the distributions of growth increments of both biotypes at distinguishing herbicide concentrations.

Results and Discussion

There was not a significant treatment by experiment interaction, therefore data were pooled over experiment runs for both clethodim and fluazifop-P studies. For each herbicide, ANOVA indicated a significant difference between S and R biotypes. At 5 DAT, the clethodim GR_{50} values for the R and S seedlings were 462.5 and 24.8 μg/L, respectively, indicating the resistant biotype was 18.7 times more tolerant of clethodim than the susceptible biotype (Figure 1). The fluazifop-P GR_{50} values for the R and S seedlings were 618.7 and 17.5 μg/L, resulting in a R:S ratio of 35.4 (Figure 2). The R:S

ratios for clethodim and fluazifop-P were higher than the whole plant dose response R:S ratios (11.0 and 5.7, respectively) (Burke et al. 2005a). The differences in R:S ratios could be related to dosage and plant biomass. For each herbicide, a single rate was chosen that allowed differentiation of the two biotypes. The rate selected to differentiate R and S biotypes for each herbicide used in this study is equivalent to 1.9% of the field use rate. Coleoptile growth of the susceptible biotype was inhibited at these concentrations while the resistant biotype was not affected.

Frequency distributions of coleoptile length for R and S biotypes at 0.09 mg/L clethodim and 0.18 mg/L fluazifop-P at 5 DAT, the rates chosen as assay rates, are shown in Figures 3 and 4. Both biotypes have distinct distributions of growth in response to the respective herbicide concentrations. For each herbicide, the resistant and susceptible populations overlapped in their distribution. As the seedling population was collected from plants that had survived several applications of clethodim, the seedling population may be segregating. In johnsongrass, graminicide resistance appears to be a dominant trait (Smeda et al. 2000), and heterozygotes exhibit an intermediate level of resistance. These responses illustrate the need to test populations extensively rather than a limited number of individuals. Retrum and Forcella (2002) also noted overlapping distributions of susceptible and resistant green foxtail [*Setaria viridis*(l.) Beauv.] biotypes in response to a sethoxydim assay. While distribution differences allow detection of resistance at a single rate of herbicide, it is important to develop a rate response for each biotype to determine the level of resistance using regression analysis (Beckie et al. 2000).

The agar growth media provided a suitable substrate for growth of johnsongrass, although root lengths could not be measured. Root growth of S seedlings was strongly inhibited at even the lowest AOPP and CHD rates. The consistency of the method appears to be very high as the coleoptile response of the S biotypes was similar to both clethodim and fluazifop-P herbicides. The identification of concentrations effective at separating resistant and susceptible biotypes is important not only for rapid diagnosis of potential resistance, but also for screening of seed for use in experiments. Coleoptile length was an effective growth parameter to detect clethodim and fluazifop-P resistance in johnsongrass biotypes

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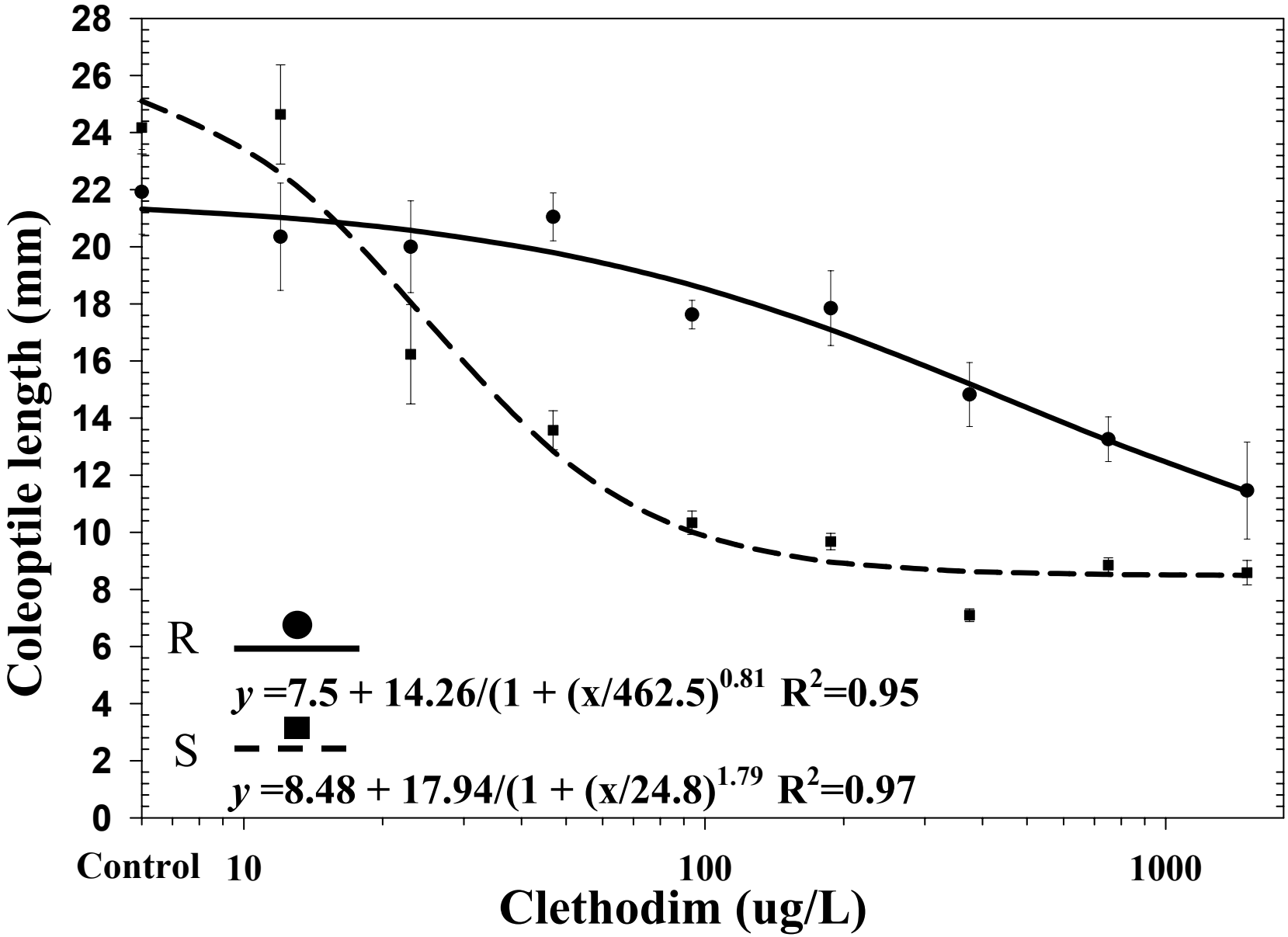

Figure 1. Effect of increasing clethodim rate on susceptible and resistant seedling johnsongrass biotype seedling coleoptile length. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the coleoptile length in mm, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing coleoptile length, and GR_{50} is the rate required to reduce coleoptile length 50%. Error bars represent standard error of the mean (n=8).

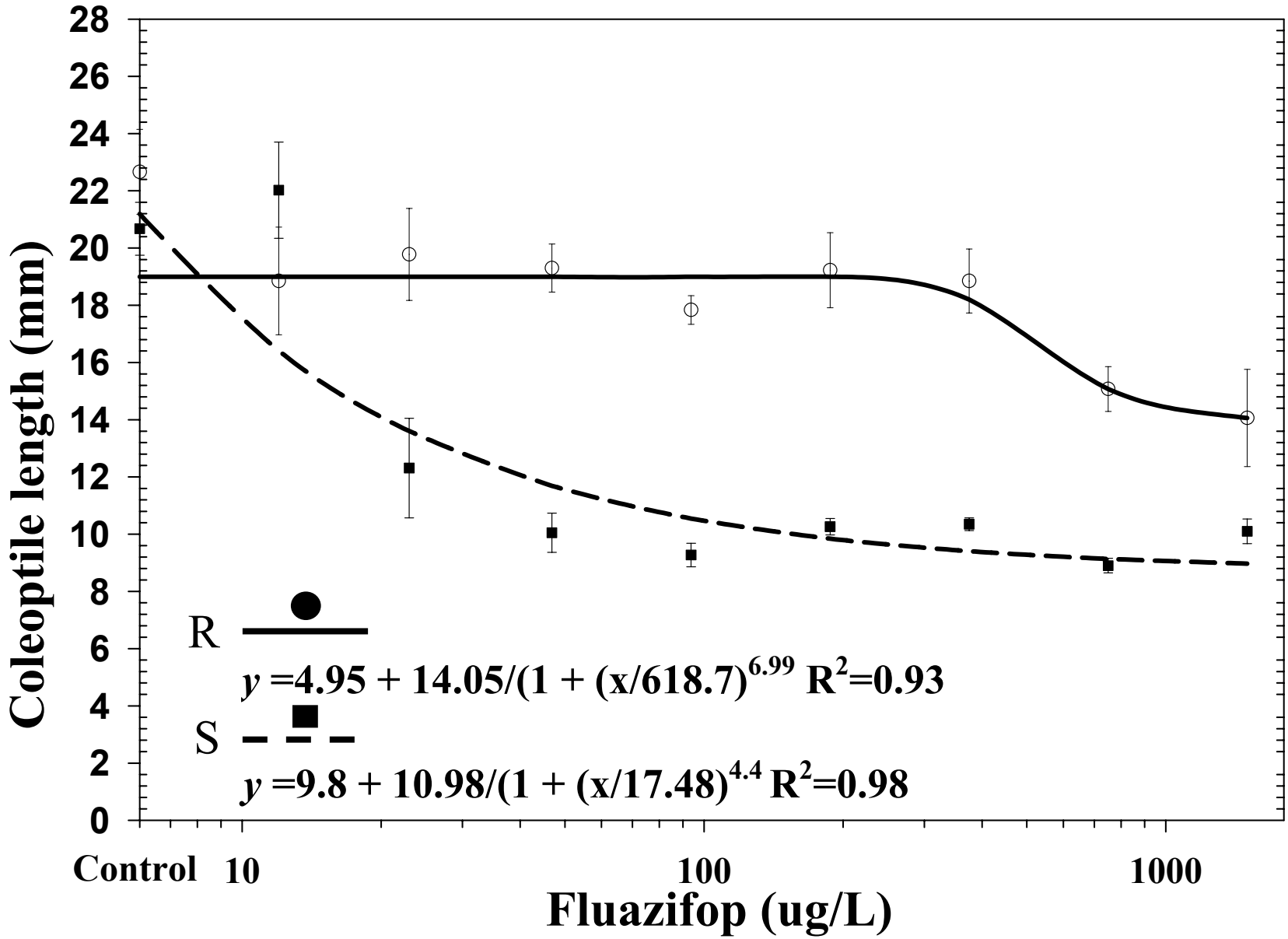

Figure 2. Effect of increasing fluazifop-P rate on susceptible and resistant seedling johnsongrass biotype seedling coleoptile length. The plant response to dose was modeled using the logistic dose-response curve $y = a + b/(1+x/GR_{50})^d$ where y is the coleoptile length in mm, a is the lower asymptote, b is the upper asymptote, d is the rate of decreasing coleoptile length, and GR_{50} is the rate required to reduce coleoptile length 50%. Error bars represent standard error of the mean (n=8).

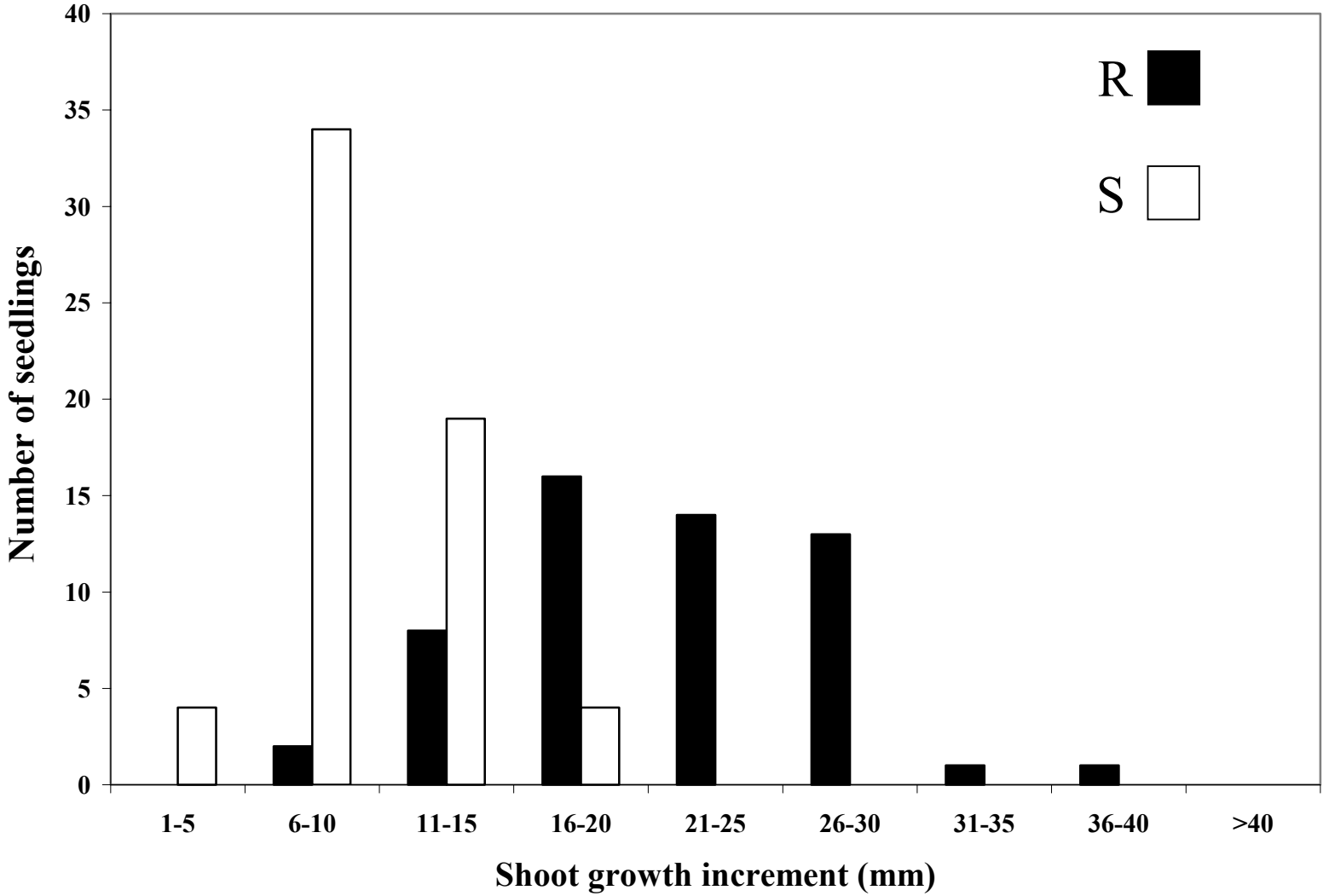

Figure 3. Distributions of coleoptile lengths of resistant (R) and susceptible (S) biotypes of johnsongrass seedlings 5 d after exposure to 0.09 mg ai/L clethodim.

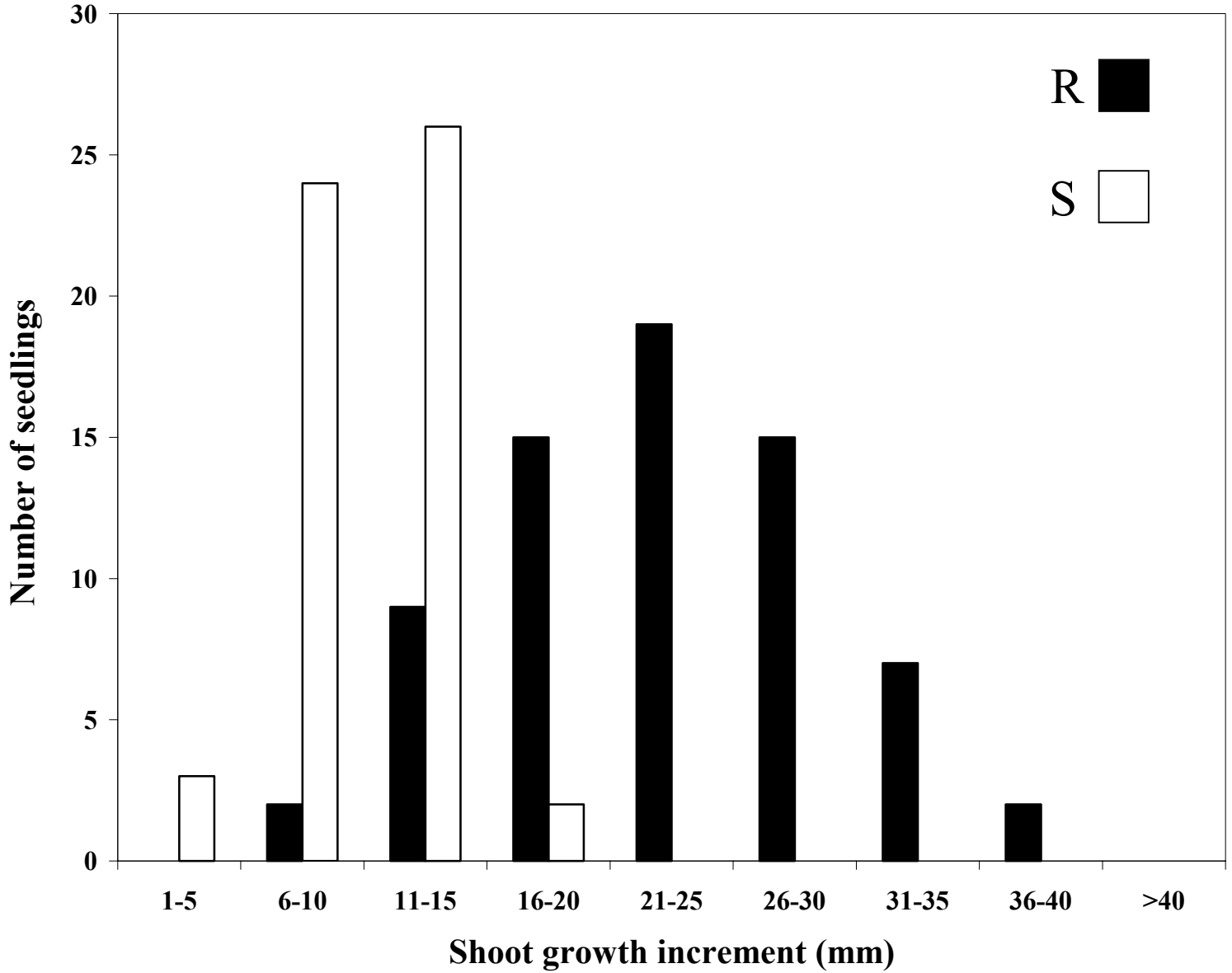

Figure 4. . Distributions of coleoptile lengths of resistant (R) and susceptible (S) biotypes of johnsongrass seedlings 5 d after exposure to 0.18 mg ai/L fluazifop-P.

CHAPTER 6

Viability and *InVitro* Germination of Johnsongrass (*Sorghum halepense*) Pollen

Abstract. To study the physiology of pollen growth, a large proportion of viable pollen grains must germinate. The objectives of this study were to evaluate the nuclear state and develop a suitable medium and culture method for *in vitro* germination of johnsongrass pollen. Johnsongrass pollen was trinucleate, and i*n vitro* tests for pollen viability using Alexander's stain and a fluorochromatic reaction method (FCR) indicated johnsongrass pollen was viable (92.6-98.4%). A factorial treatment arrangement of four concentrations of sucrose, two concentrations of boric acid, and two concentrations of calcium nitrate were used to determine the optimum pollen germination media composition in suspension culture, agar culture, and cellophane membrane culture. Germination was highest in a suspension culture with media containing 0.3 M sucrose, 2.4 mM boric acid, and 3 mM calcium nitrate. Pollen germination using this media was 78.9% when anthers were harvested just before anthesis.

Nomenclature: *Sorghum halepense* (L.) Pers. #SORHA, johnsongrass.

Key Words: pollen tests, agar culture, suspension culture, cellophane membrane culture.

Introduction

Johnsongrass [*Sorghum halepense* (L.) Pers.] occurs in all major agricultural areas of the warm regions of the world (Holm et al. 1991). The registration of acetyl coenzyme-A (ACCase) inhibitors, commonly called graminicides, has allowed growers to selectively remove johnsongrass infestations from broadleaf agronomic crops such as soybean [*Glycine max* (L.) Merr.] and cotton (*Gossypium hirsutum* L.) (Burton 1997). The first reported

incidence of an ACCase-resistant species occurred in the early 1980's (Heap 2005). Over 80 species have since developed resistance to ACCase inhibitors, including six biotypes of johnsongrass. Since the discovery of target-site ACCase resistance expression in pollen (Richter and Powles 1993), pollen assays have been developed to assess resistance (Letouze and Gasquez 2000). Such assays could be important for evaluating gene flow by pollen. As several biotypes of ACCase-resistant johnsongrass have been found (Heap 2005), an assay would be useful for determining the presence of target site resistance to ACCase inhibitors in johnsongrass. Although much is known about sorghum [*Sorghum bicolor* (L.) Moench] pollen, little is known about johnsongrass pollen other than it contaminates sorghum grown for seed (Rosenow and Clark 1969).

As an important grain crop, sorghum and its reproductive system have been studied throughout the century and are very similar to johnsongrass. The first study on anthesis and pollination in sorghum was reported in the second decade of the 20th century (Graham 1916). Other literature reported in the 1920's and 1930's agree with current publications on floral formation, anthesis, and pollination in sorghum (Ayyangar and Rao 1931; Doggett 1988; Patil and Gould 1980; Patel and Patel 1928; Stephens and Quinby 1934). The beginning of the reproductive phase in the genus *Sorghum* is indicated by a characteristic swelling of the apex of the stalk. The swollen area is enclosed by the terminal leaf sheath whose blade, the flag leaf, is usually small and inconspicuous. Emergence of the flag leaf is followed by emergence of the boot and the inflorescence. When the head of the inflorescence is completely excerted, the peduncle elongates. Anthesis in the inflorescence is basipetal with the terminal flowers of each panicle opening first, followed by the next flower down shortly thereafter. Johnsongrass floral

characteristics differ from that of sorghum in that in addition to a sessile complete spikelet, johnsongrass has a pediceled staminate (or neuter) one (Gleason and Cronquist 1991).

The germination of sorghum pollen has been accomplished in several different media. Lansac et al. (1994) found that sorghum pollen germinated immediately when placed in either distilled water or a growth media. By contrast, Tuinstra and Wedel (2000) were much less successful at germinating sorghum pollen. They were not able to germinate sorghum pollen in distilled water and were only moderately successful at germinating sorghum pollen on an agar media. Neither study noted from what floral stage the pollen was harvested although it was presumably after anthesis. The importance of harvesting fresh sorghum pollen was illustrated by Lansac et al. (1994). *In vitro* sorghum pollen germination was reduced by 92% within 15 minutes of the initiation of desiccation. Others have noted similar trends in other species. Floral stage was as important for pollen germination as the germination media composition for Sheppard's purse [*Capsella bursa-pastoris* (L.) Medikus] (Leduc et al. 1990).

Germinability of pollen appears to also be related to the nuclear state of pollen. Pollen of angiosperms may be described as either bi- or trinucleate, depending on whether anthesis occurs, before or after generative cell mitosis, respectively (Brewbaker 1957, 1959). Most members of the *Poaceae* are trinucleate (Brewbacker 1967). Brewbaker and Kwack (1963) media germinated a number of species, however, species with trinucleate pollen germinated at a much lower percentage or failed to germinate (Brewbaker 1967). Trinculeate pollen is also difficult to store. Once shed, trinucleate pollen germinability *in vitro* decreases rapidly (Fei and Nelson 2003; Lansac et al. 1994;

Leduc et al. 1990). The vitality of trinucleate pollen may be related to respiration rate; trinucleate pollen respires at 2 to 3 times the rate of binucleate pollen (Hoekstra and Bruinsma 1975). The difficulty in germinating trinucleate pollen has resulted in a number of different medias and culture methods that are species-specific (Fei and Nelson 2003; Leduc et al. 1990; Muccifora et al. 2003; Letouze and Gasquez 2000).

Several researchers have explored different culture methods, which slow the rate of hydration, to increase the germination of trinucleate pollen on the theory that rapid hydration after extended periods of desiccation destroys important membranes in the pollen (Alexander and Ganeshan 1990; Bar-Shalom and Mattsson 1977). Agar and cellophane-based media are two culture methods that slow the rate of hydration (Alexander and Ganeshan 1990; Bar-Shalom and Mattsson 1977; Shivanna and Rangaswamy 1992). In order for resistance assays using pollen to be useful, a large proportion of viable pollen grains must germinate. Therefore, the objectives of this study were to evaluate the nuclear state of johnsongrass pollen and to develop a suitable medium and culture method for *in vitro* germination of johnsongrass pollen.

Materials and Methods

Plant material

Rhizomes of johnsongrass from Washington County, MS, were planted in 3.8-L round pots. The plants grown from these rhizomes were allowed to produce flowers. Plants were grown in a glasshouse in Raleigh, NC, with 12 h light/12 h dark regime and fertilized once weekly with a 1 g/L solution of commercial plant fertilizer[1]. Average day and night temperatures were 32 and 18 C, respectively. Flowering commenced 6 to 8

wks after planting and continued indefinitely as long as old shoots and panicles and their roots were trimmed after flowering.

Verification of Nuclear State

Pollen grains at anthesis were stained with a 2.5 ug mL^{-1} solution of 4', 6-diamino-2-phenyl-indole (DAPI) to stain vegetative and generative cells. DAPI stains nuclear material, and, when viewed through a fluorescent light microscope[2] and an ultraviolet light source, allows determination of the nuclear state of the pollen (Coleman and Goff 1985).

Pollen viability

Pollen viability was estimated for each treatment in a factorial combination of sucrose (0, 0.2, 0.3, 0.4 M), $CaNO_3$ (0, 3 mM), and H_3BO_3 (1.62, 2.43 mM) using Alexander's stain (Alexander 1969) and the fluorochromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison 1970). Alexander's stain contains malachite green, which stains cellulose in pollen walls and acid fuchsin, which stains the pollen protoplasm (Alexander 1969). To assess viability using Alexander's stain, newly excerted anthers were harvested and placed in 1 mL of the water-based germination media. Twenty μL of the media containing pollen was placed on a microscope slide and stained with either Alexander's stain or fluorescein diacetate (FDA). For Alexander's stain, six randomly selected fields of view were selected from each slide and pollen visually counted and scored as viable or nonviable. To assess viability using the FCR method, the total number of pollen grains were visually counted in randomly selected fields of view using brightfield microscopy, and then the total number of fluorescent pollen were counted using a fluorescent light source and a FITC filter set (450 nm excitation filter and a 535

nm emission filter). For each treatment, pollen was mounted on three different slides. The study was repeated twice

Pollen germination

Two pollen culture methods, the hanging drop and sitting drop culture methods, do not allow for the germination of large quantities of pollen necessary for physiological or biochemical studies (Shivanna and Rangaswamy 1992). Consequently, to determine the appropriate germination medium and culture technique, the effects of a factorial combination of sucrose (0, 0.2, 0.3, 0.4 M), $CaNO_3$ (0, 3 mM), and H_3BO_3 (1.62, 2.43 mM) were tested in three separate studies using three different cultural methods: a water-based media in culture vials, an agar-based media, and a cellophane membrane impregnated with water-based media. These methods allow for the germination of large quantities of pollen. All media were prepared using distilled water. Each treatment culture of the factorial was prepared with three replicates.

Pollen of sorghum was found to be very sensitive to desiccation (Lansac et al. 1994), consequently, typical methods to harvest pollen were discarded (bulking pollen shed onto a collection plate and then using a fine-haired paint brush to move pollen to different germination media) in favor of harvesting whole anthers and allowing pollen to shed directly into the germination media. To reduce variation of pollen, three whole anthers from three different plants were harvested, bulked, and placed in the appropriate pollen culture treatment. Newly excerted anthers were collected early in the morning between 0730 and 1030 (represented by the floral stage in Figure 1d) as heat of the day reportedly reduced viability in sorghum (Tuistra and Wedel 2000; Sanchez and Smeltzer 1965). Suspension culture methods followed Shivanna and Rangaswamy (1992) with

modifications. Germination of pollen in water-based media was performed in 1.5 mL microcentrifuge tubes containing 0.5 mL of the appropriate germination solution. After 3 h, the anthers were removed from the germination solution and the microcentrifuge tube was placed in a microcentrifuge for 1 min at 3,000 *g*. Twenty μL of the germination solution and pellet, containing both germinated and non-germinated pollen grains, were removed from the microcentrifuge tube using a micropipette. Twenty uL of each treatment replicate media, containing pollen, was placed on three separate microscope slides 3 h after treatment initiation. Six fields each containing 70-100 pollen grains were selected from each slide. Pollen was scored as germinated if the pollen tube was at least half the size of the pollen grain (Cheng and McComb 1992; Richter and Powles 1993).

Surface culture also followed Shivanna and Rangaswamy (1992) with modifications. Two different types of surface cultures were used – an agar-based culture and a cellophane membrane culture. For the agar-based culture, studies were conducted in round plastic 110 mm diameter by 20 mm petri dishes containing a 1% (w v^{-1}) agar medium[3] supplemented with different concentrations of the appropriate germination solution. To prepare the cellophane membrane[4] for use in germination experiments, the membrane was cut into 1 cm square pieces and stapled between two pieces of filter paper[5]. The membrane was then soaked in the appropriate germination solution for 1 h. The soaked membrane assembly was placed on a microscope slide and the upper piece of the filter paper was removed. To reduce variation of pollen, two whole anthers from different flowers on three different plants were harvested, bulked, and placed on the agar or cellophane pollen culture treatment. For each surface culture method, six fields were selected and evaluated from each slide, each field containing 70-100 pollen grains, 3 h

after treatment initiation. Pollen grains in each field were visually counted and scored as germinated or nongerminated. Pollen was scored as germinated if the pollen tube was at least half the size of the pollen grain (Shivanna and Rangaswamy 1992). Each study had a completely randomized design and was conducted twice.

Floral development and pollen viability

In a separate study, pollen viability was assessed at five different floral stages using an optimized germination media containing sucrose (0.3 M), $CaNO_3$ (3 mM), and H_3BO_3 (2.43 mM). The suspension culture method outlined above was used to evaluate pollen germination from flowers well before anthesis (Figure 1a), before anthesis (Figure 1b), just before anthesis (Figure 1c), just after anthesis (Figure 1d), and well after anthesis (Figure 1e). These floral stages represent a continuum of floral development present as the johnsongrass inflorescence (and the sorghums in general) develops (Stephens and Quinby 1934). The study had a randomized complete block design with six replications and was conducted three times.

Statistical analysis

Block effects were considered random, and treatment main effects and interactions were evaluated as fixed effects. The statistical analysis was conducted using PROC GLM procedure (SAS 1998). The appropriate mean squared error term was used to test each of the treatment sources of variation based on expected mean squares for fixed treatment effects (McIntosh 1983). Means were separated using Fisher's Protected Least Significant Difference (LSD) at the 0.05% level of probability.

Results and Discussion

At anthesis, pollen grains of johnsongrass are 50 μm in diameter with a smooth exine. Using an ultraviolet light source and filter on a fluorescent microscope, johnsongrass pollen was determined to be trinucleate (Figure 2). Brewbaker (1967) hypothesized that the second mitotic division trinucleate pollen undergoes (producing the two sperm cells indicated by *Sp* in Figure 2) before anthesis deprives the pollen grain of energy reserves essential for germination and for storage. It is also possible that rapid hydration damages critical membranes (Alexander and Ganeshan 1990). Johnsongrass pollen tubes were generally 50 microns in length or longer (Figure 3). The length of pollen tubes can vary according to the molar concentration of sucrose. For instance, Fei and Nelson (2003) noted that increasing the sucrose concentration from 0.5 M to 1.0 M caused the pollen tubes to be shorter and wider. Media can also affect pollen tube length, and it is thought that agar media gives greater structural support to germinating pollen grains (Shivanna and Rangaswamy 1992).

The FCR method utilizes the stain. When pollen grains are mounted in FDA solution, the nonpolar, nonfluorescent FDA enters the pollen cytoplasm. Cytoplasmic esterases hydrolyze FDA and release fluorescein, which is polar and fluorescent. Fluorescein is relatively more polar, passing only sparingly through an intact membrane. Since fluorescein is more polar and passes only through intact membranes, it accumulates in the cytoplasm of viable pollen grains. When observed using a fluorescent microscope and fluorescein isothiocyanate (FITC) filter, the viable pollen grains give a bright green fluorescence. If the plasma membrane is not intact, fluorescein readily comes out into the mounting medium in the preparation and the pollen grain does not fluoresce. The pollen

grains do not fluoresce if they lack esterases that hydrolyze FDA. Therefore, the FCR test assesses two properties of a pollen grain: the integrity of the plasma membrane of the pollen, and the activity of esterases capable of hydrolyzing the fluorescein ester (Pline et al. 2002; Shivanna and Rangaswamy 1992). Alexander's stain and the FCR method each predicted similar levels of johnsongrass pollen viability (93.1-98.4% and 92.6-97.9%, respectively). As Alexander's stain assumes that the presence of a protoplasm indicates that a pollen grain is viable, this method could overestimate pollen viability (Kearns and Inouye 1993). Immature or incompletely formed pollen grains, which were incompletely stained, were evident (Figure 4). The FCR method was also effective at distinguishing viable and nonviable pollen grains (Figure 5). Others have used the FCR to evaluate differences in pollen morphology. Grains with variable or irregular fluorescence patterns may or may not be viable (Pline et al. 2002). Pline et al. (2002) reported 10 to 20% more pollen germination in cotton than the FCR indicated were viable, indicating that some of the pollen grains that did not stain were capable of germination. No irregular staining was observed in this study, and the FCR method indicated a similar level of germinability as Alexander's stain. However, both Alexander's stain and the FCR method indicated much higher viability than the pollen germination assays (Table 1).

The main effect of sucrose, boric acid, and calcium nitrate were significant while interactions were not significant for germination of pollen in suspension culture or surface culture. Germination increased with increasing sucrose concentration regardless of culture method (Table 1). The highest cumulative germination occurred in suspension culture with a sucrose concentration of 0.3 or 0.4 *M* (14.5 and 13.8%, respectively). Boron and calcium nitrate were also essential for the germination of johnsongrass pollen

in each culture method. Many pollen grains were observed to burst before or during germination without sucrose to act as an osmoticant, and these pollen grains were counted as non-germinated. Johnsongrass pollen germination using surface culture methods was lower than suspension culture methods. The conditions favorable for germination of johnsongrass pollen are very similar to those of sorghum as reported by Tuinstra and Wedel (2000). Germination in johnsongrass also appears to occur much less rapidly than in other plant species, particularly those with binucleate pollen. For instance, cotton pollen germinates so rapidly that the force of the pollen tube growing from the grain can detach the pollen tube from the grain (Pline et al. 2002). The rapid germination of such pollen requires a further test (an aniline blue stain) to verify the presence of callose plugs in the pollen tubes and the addition of that stain can reduce pollen germination (Pline et al. 2002). Johnsongrass pollen that had burst was clearly evident; consequently, the use of aniline blue to verify pollen tube formation was limited to preliminary studies verifying that pollen tube formation occurred (data not shown).

The low level of germination of pollen harvested after anthesis is consistent with reports of other grass species, including sorghum (Tuinstra and Wedel 2000) and creeping bentgrass (*Agrostis stolonifera* L.) (Fei and Nelson 2003). However, viability as assessed by pollen germination was higher in sorghum than johnsongrass. The lack of germination may be due to the short-lived nature of trinucleate pollen found in the genus *Sorghum* and the floral stage at which pollen was harvested. Lansac et al. (1994) reported sorghum pollen percent germination desiccated in an atmosphere of 50 to 55% relative humidity quickly reduced *in vitro* germination and seed set. Pollen with an initial percent germination of 52% dropped significantly to 39, 4, and 0% germination after 5,

15, and 30 min of desiccation (Lansac et al. 1994). As Lansac et al. (1994) reported a large effect of dehydration on sorghum pollen, a further study was initiated to improve johnsongrass pollen germination by examining the effects of floral development on *in vitro* pollen germination.

Using the suspension culture method and the optimum media as determined by the previous experiment, johnsongrass pollen harvested just before anthesis was more germinable than pollen harvested at any other interval (Table 2, Figure 1c). Pollen harvested well before anthesis (Figure 1a) had 4% germination, although Alexander's stain indicated much higher viability. As the flowers matured (Figures 1b and 1c), pollen germination increased. Pollen harvested just after anthesis (Figure 1d) germinated in similar quantity to that of previous experiments using suspension culture. The relative humidity in the greenhouse where the johnsongrass was grown was between 60 and 80%. The level of humidity present in the greenhouse may not have been high enough to maintain johnsongrass pollen viability after anthesis. Pollen of the family *Poaceae* is very sensitive to changes in relative humidity and even short exposures to low relative humidity (50 to 60%) decreased both the vitality and the respiration rate of grass pollen (Hoekstra and Bruinsma 1975). Crowe et al. (1989) found that the most probable cause of imbibitional damage to dry or dehydrated pollen was a phase transition from gel to liquid crystalline upon rehydration of membrane phospholipids, which caused the membranes to leak cellular contents. The sensitivity of johnsongrass pollen to desiccation may be why surface culture experiments using pollen harvested after anthesis failed – damage due to dehydration may have already occurred.

In conclusion, johnsongrass pollen was found to be trinucleate, a state that Brewbaker and Kwack (1963) noted as more difficult to germinate. Johnsongrass pollen is strongly dependent on the addition of sucrose, boric acid, and calcium nitrate for germination. Slowing the rate of hydration by using surface culture methods involving agar or cellophane membranes did not improve johnsongrass pollen germination. Only when the pollen was harvested just before anthesis was sufficient germination achieved for use in physiology studies. More research is needed on pollen biology and physiology, particularly in terms of viability and longevity, to more fully understand potential limitations to gene flow, particularly herbicide resistance, between biotypes and populations, and what environmental factors influence potential gene flow movement by pollen.

Sources of Materials

[1] Peters Professional 20-20-20, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[2] Leica DMIRB, Leica Microsystems, Inc., 2345 Waukegan Rd., Bannockburn, IL 60015.

[3] Granulated agar, Fisher Scientific, P. O. Box 4829, Norcross, GA 30091.

[4] Cellophane membrane CM-20, Fisher Scientific, P. O. Box 4829, Norcross, GA 30091.

[5] Watman #3 filter paper, Fisher Scientific, P. O. Box 4829, Norcross, GA 30091.

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Table 1. The main effects of sucrose, boric acid, and calcium nitrate on pollen germination using three different culture methods.

	Pollen germination – Culture method		
Treatment	Suspension culture	Agar culture	Cellophane culture
	% Pollen viability		
Sucrose (*M*)			
0.0	0.8 b	0.2 c	0.0 c
0.2	2.3 b	0.0 c	1.1 b
0.3	14.2 a	6.7 a	6.1 a
0.4	13.5 a	2.2 b	1.9 b
Boric acid (m*M*)			
1.62	3.0 b	2.1 b	1.6 b
2.43	14.8 a	4.9 ab	3.4 b
Calcium nitrate (m*M*)			
0	2.1 b	0.7 c	0.0 c
3	13.8 a	5.3 a	2.5 b

[a] Means within a column followed by the same letter are not different according to Fisher's protected LSD test at P = 0.05.

Table 2. Pollen viability and germination at different floral stages.

Floral stage[a]	Pollen germination[c]	Alexander's Stain
	———— % Pollen viability[b] ————	
Well before anthesis	4.0 d	73.6 c
Before anthesis	23.9 b	96.5 a
Just before anthesis	78.9 a	93.8 a
Just after anthesis	14.5 c	97.5 a
Well after anthesis	1.7 d	84.0 b

[a] See Figure 1 for a description of the floral stages from which pollen was harvested.

[b]Pollen viability was assessed at five different floral stages using an optimized germination media containing sucrose (0.3 *M*), $CaNO_3$ (3 m*M*), and H_3BO_3 (2.43 m*M*) in a suspension culture.

[c] Means within a column followed by the same letter are not different according to Fisher's protected LSD test at P = 0.05.

Figure 1. Five developmental stages of morphologically complete johnsongrass flowers: a) well before anthesis, b) before anthesis, c) just before anthesis, d) just after anthesis, and e) well after anthesis. Anthesis occurs between stages c and d. Pollen harvested from flowers in stage c was most germinable. A pediceled staminate spikelet is present with the complete spikelet in stage d.

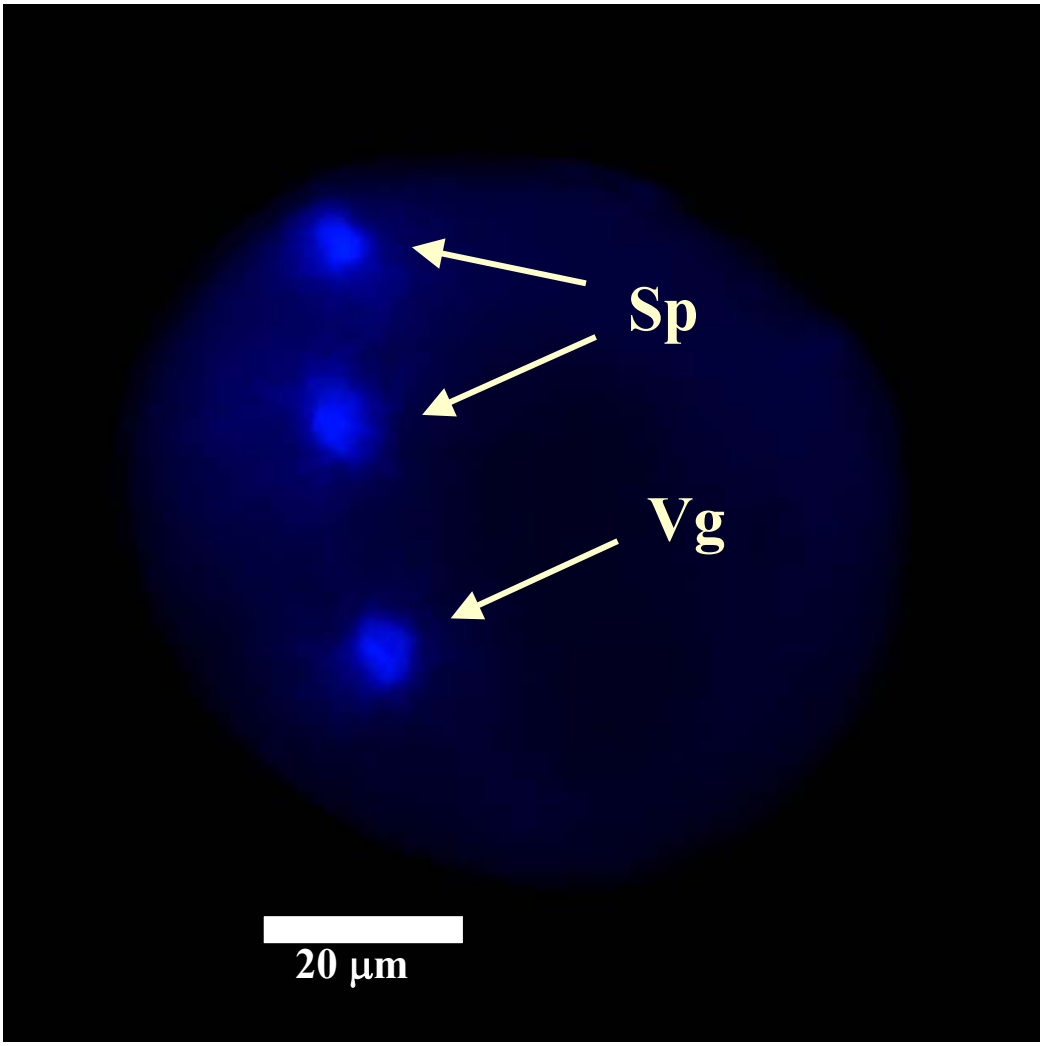

Figure 2. A trinucleate pollen grain of johnsongrass at anthesis. Ultraviolet light revealed the vegetative nuclei (Vg) and two sperm cells (Sp) stained by 4',6-diamino-2-phenyl-indole (DAPI).

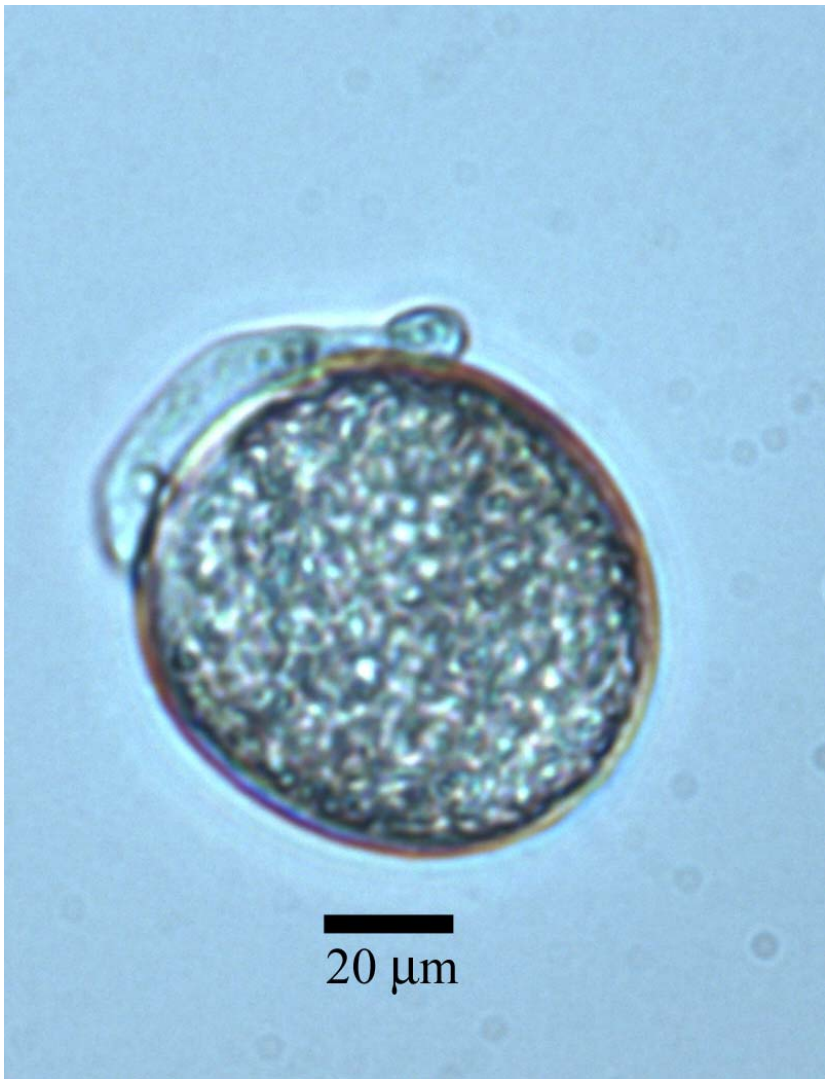

Figure 3. A viable, germinated johnsongrass pollen grain.

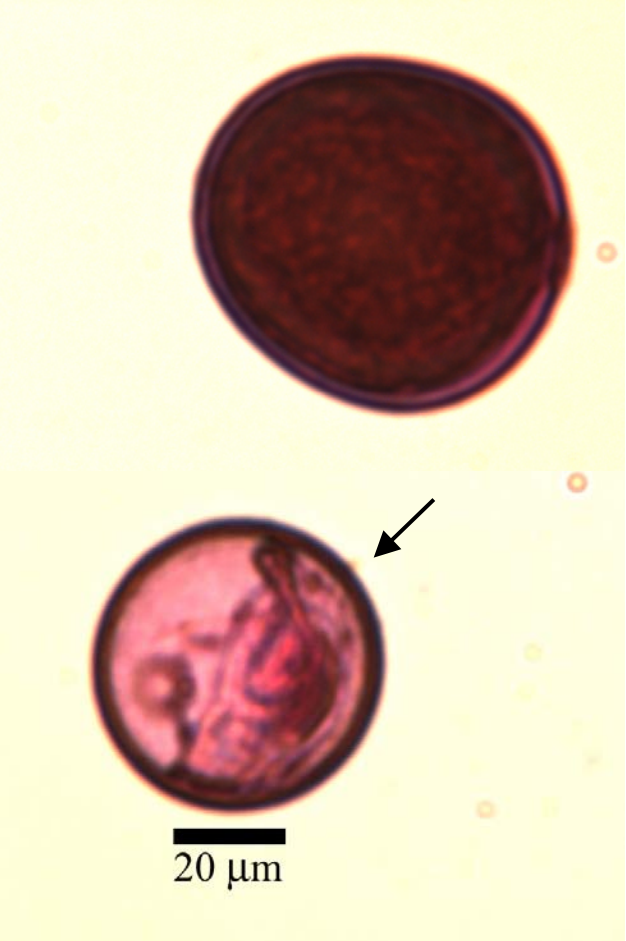

Figure 4. Viable and non-viable (indicated by arrow) johnsongrass pollen assessed by Alexander's stain method.

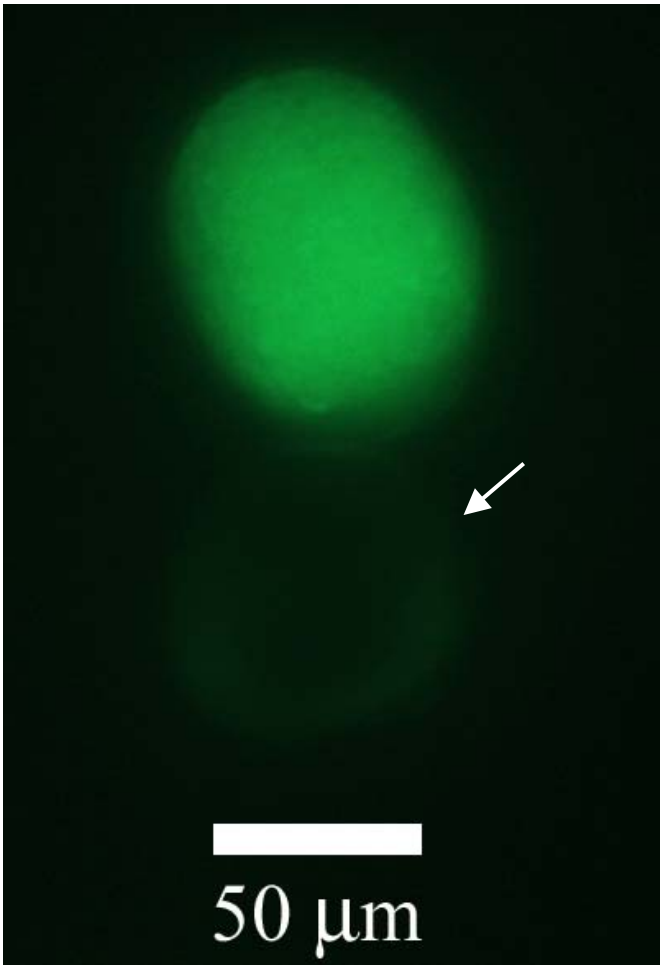

Figure 5. Viable, fluorescing pollen and nonviable, non-fluorescing pollen (indicated by arrow) assessed by the FCR method.

CHAPTER 7

Johnsongrass (*Sorghum halepense*) pollen expresses ACCase target-site resistance

Abstract. Three studies were conducted to develop pollen tests for the screening of acetyl coenzyme A carboxylase (ACCase) target-site resistance in a biotype of johnsongrass. The assays were based on germination of johnsongrass pollen in media supplemented with clethodim. Two different methods were used to evaluate pollen germination – a visual assessment and a spectrophotometric assay. The response of pollen to the germination media was linear for 16 h. At 6 h after treatment, absorbance at 500 nm was nearly 0.5, consequently 6 h was chosen to conduct the pollen assays using the spectrophotometer. Both assessment methods differentiated the susceptible and resistant biotypes. Pollen from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim, with a GR_{50} of 25.8 (standard error of ±0.6) μM and GR_{50} of 16.4 (standard error of ±1.7) μM clethodim by visual assessment and spectrophotometric assessment, respectively. Minimum R/S values were >3.9 by visual assessment and >6.1 by spectrophotometric assessment. ACCase target-site resistance is expressed in johnsongrass pollen.

Nomenclature: *Sorghum halepense* (L.) Pers. #SORHA, johnsongrass.

Key Words: pollen tests, herbicide resistance, ACCase inhibitors.

Introduction

Johnsongrass [*Sorghum halepense* (L.) Pers.] occurs in all major agricultural areas of the warm regions of the world and was listed by Holm et al. (1991) as one of the world's

10 worst weeds. Johnsongrass reduces crop yields (Horowitz 1973; McWorter and Hartwig 1972) and its pollen contaminates sorghum [*Sorghum bicolor* (L.) Moench] grown for seed (Rosenow and Clark 1969). In the early 1980s, the selective postemergence control of johnsongrass in soybean first became possible with the registration of several herbicides now called graminicides (Burton 1997). The graminicides are divided into two chemically distinct herbicide classes, the aryloxyphenoxypropionate (AOPP) and the cyclohexanedione (CHD) herbicides. Both of these herbicide classes act by inhibiting the enzyme acetyl coenzyme-A carboxylase (ACCase; EC 6.4.1.2) in susceptible species (Burton 1997). Acetyl coenzyme-A carboxylase catalyzes the first committed step of fatty acid biosynthesis, which is the ATP-dependent carboxylation of acetyl-CoA to malonyl CoA (Incledon and Hall 1997). Grass species have a eukaryotic type ACCase in the chloroplast, which is sensitive to ACCase inhibitors, while most broadleaf species have a prokaryotic type of ACCase, which is not sensitive to ACCase inhibitors (Incledon and Hall 1997).

Resistance to the AOPP herbicides fluazifop-P and quizalofop and cross-resistance to the CHD herbicide sethoxydim was discovered in two populations of johnsongrass in Mississippi in 1991 (Smeda et al. 1997). Several CHD and AOPP herbicides were applied one or more times annually over several years in fields where resistant populations were detected. A second biotype was identified in Mississippi in 2000 that was resistant not only to the AOPP herbicides fluazifop-P and quizalofop and the CHD herbicide sethoxydim but also to the CHD herbicide clethodim (Burke et al. 2006a).

For plants, gene flow can involve pollen transport, also called pollen-mediated gene flow (Levin and Kerster 1974; Murray et al. 2002). The reproductive biology of species influence the degree of pollen-mediated gene flow between and within populations (Levin and Kerster 1974). Johnsongrass is a predominantly self-pollinating species with less than a 10% outcrossing rate (Dogget 1988). Even this limited amount of outcrossing could have implications for pollen movement of resistance. Consequently, understanding the biology and physiology of pollen could provide insight into the potential for movement of genes. Of special interest are genes imparting herbicide resistance.

Gene expression in the sporophytic generation overlaps with that of the gametophytic generation (Pedersen et al. 1987; Sari Gorla et al. 1986; Tanksley et al. 1981). As a consequence, many single, nuclear-encoded genes expressed in the sporophytic generation are also expressed in the gametophytic generation (Richter and Powles 1993), through which they are inherited. Richter and Powles (1993) showed that ACCase and acetolactate synthase (ALS) genes were expressed in pollen of rigid ryegrass (*Lolium rigidum* Gaud.). Furthermore, the enzymes encoded by these genes could be inhibited by herbicides. Richter and Powles (1993) could not detect resistance caused by something other than modification at the target site, which was attributed to a lack of expression of the enzymes conferring resistance. Thus, Letouze and Gasquez (2000) and Richter and Powles (1993) were able to detect target-site resistance in blackgrass (*Alopecurus myosuroides* Huds.) and rigid ryegrass, respectively. A bioassay involving pollen could be useful for detecting ACCase target site-resistance in johnsongrass, the impacts of which would be a better understanding of the potential for resistance movement through

johnsongrass pollen. The objectives of these studies were to develop assays for detection of ACCase resistance in johnsongrass pollen to determine if target-site-based resistance is expressed in the gametophytic generation of johnsongrass.

Methods and Materials

Plant material

Two populations of johnsongrass were used in this study: a susceptible population and a resistant population, which is highly resistant to clethodim (Burke et al. 2006a). Both are from Washington County, MS. The ACCase activity of the resistant biotype in the presence of inhibitors was much higher than the susceptible biotype indicating resistance due to an altered target site of action (Burke et al. 2006c).

Rhizomes of ACCase-resistant and susceptible biotypes of johnsongrass were planted in 3.8-L round pots. The plants grown from these rhizomes were allowed to produce flowers. Biotypes were grown in separate glasshouses in Raleigh, NC, with 12 h light/12 h dark regime and fertilized once weekly with a 1 g/L solution of commercial plant fertilizer[1]. Average day and night temperatures were 32 and 18 C, respectively. Flowering commenced 6 to 8 wks after planting and continued indefinitely as long as old shoots and panicles and their roots were trimmed after flowering.

Pollen germination and clethodim assays

Anthers were collected just prior to anthesis from plants that had been verified resistant or susceptible. To ensure consistent start times for each biotype and to prohibit cross contamination, resistant pollen was harvested first, then susceptible pollen. To

reduce variation of pollen, three whole anthers from different flowers on three different plants were harvested, bulked, and placed in the appropriate pollen culture treatment immediately. Germination of pollen was performed in 1.5-mL microcentrifuge tubes containing a water-based media of 0.5 mL of a germination solution containing 0.3 M sucrose, 2.43 mM boric acid, and 3 mM calcium nitrate (Burke et al. 2006b).

Two methods were used to determine pollen germination in response to increasing rates of clethodim. The first method used a visual evaluation of pollen germination, while the second used a spectrophotometer[2] to evaluate germination based on absorbance at 500 nm (Kappler and Kristen 1987). A technical grade clethodim stock solution (100 μM) was freshly prepared and diluted to give final concentrations of 10, 20, 40, 60, 80, and 100 μM when added to the germination solution. The solution that contained technical grade clethodim was prepared by dissolving clethodim in acetone and adding deionized water to produce an acetone concentration of 33% (v/v). Clethodim dilutions were prepared in acetone, pollen germination media, and deionized water to maintain a consistent acetone content in each clethodim dilution. Acetone was used to equalize the concentration of acetone in each clethodim dilution at 0.7%. In preliminary studies, 0.7% concentration of acetone did not affect johnsongrass pollen germination. Clethodim solutions were stored in the dark at 4 C until use.

The first study using visual evaluation of pollen germination in response to increasing clethodim concentrations were arranged in a completely randomized design with three or four replications. Pollen was also germinated in a solution without clethodim and served as a nontreated check. After pollen had incubated 3 h in the germination solution

containing the different clethodim concentrations, the anthers were removed and the microcentrifuge tube was placed in a microcentrifuge for 1 min at 3,000 *g*. Forty μL of the germination solution and pellet, containing both germinated and non-germinated pollen grains, were removed from the microcentrifuge tube using a micropipette and placed on a glass microscope slide. Pollen was scored as germinated if the pollen tube was at least half the size of the pollen grain (Shivanna and Rangaswamy 1992; Richter and Powles 1993). Six fields were selected from each slide, each field containing 70-100 pollen grains. The counts of six fields of each treatment replicate were averaged. Pollen grains in each field were visually counted and scored as germinated or nongerminated. Pollen viability was estimated using Alexander's stain (Alexander 1969). Alexander's stain contains malachite green, which stains cellulose in pollen walls, and acid fuchsin, which stains the pollen protoplasm (Alexander 1969). Only experiments where the control germination was greater than 60% of viable pollen as indicated by Alexander's stain were scored (Richter and Powles 1993). The study was repeated in time.

To develop a spectrophotometric assay, it was necessary to first determine the incubation time necessary to detect pollen tube growth. In a second study, pollen was incubated in the germination solution as described above for 0.5, 1, 2, 4, 6, 8, or 16 h. After each treatment interval, the anthers were removed and the microcentrifuge tube was placed in a microcentrifuge for 1 min at 3,000 *g*. The germination solution was decanted, replaced with 0.25 mL of deionized water, and the pollen and pollen tubes homogenized with a pestle. The volume was brought to 1 mL and viewed in a spectrophotometer at 500 nm. At this wavelength, absorbance is a measurement of turbidity, or the degree of

opacity (Kappler and Kristen 1987). Pollen grains fixed in the culture medium by formaldehyde (final concentration 10 mM) were used as a zero-time control (Kappler and Kristen 1987). The spectrophotometric values of the zero control were subtracted from those obtained in the experiments (Kappler and Kristen 1987). The study was conducted as a completely randomized design with 3 replications and was conducted twice.

In a third study, pollen germination in response to increasing clethodim concentration was evaluated spectrophotometerically (Kappler and Kristen 1987). Pollen was incubated in the germination solution with increasing concentrations of clethodim as described above for 6 h. Pollen was also germinated in a solution without clethodim. Only experiments where the control germination was greater than 60% of viable pollen as indicated by Alexander's stain were assayed (Richter and Powles 1993). The study was conducted as a completely randomized design with three replications and was repeated in time.

Statistical Analysis

For the visual assessment and the spectrophotometric assay, pollen germination was expressed as percent of the control. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Data were ubjected to an analysis of variance using the general linear models procedure in SAS (1998), and sums of squares were partitioned to evaluate the effect of trial repetition, herbicide rate, and johnsongrass biotype. The ANOVA indicated a significant biotype effect for the visual assessment and the spectrophotometric assay. Trial repetition and linear, quadratic, and higher order polynomial effects of percent pollen germination over

graminicide rates were tested by partitioning sums of squares (Draper and Smith 1981). Regression analysis was performed when indicated by the ANOVA. Nonlinear models were used if the ANOVA indicated that higher order polynomial effects of percent pollen germination were more significant than linear or quadratic estimates. Estimation used the Gauss-Newton algorithm, a nonlinear least squares technique (SAS 1998).

ANOVA indicated higher order polynomial effects for percent pollen germination resulting from increasing herbicide rate for susceptible johnsongrass. Thus, percent pollen germination was modeled using the logistic function:

$$y = A + B / (1 + (x / GR_{50})^{d}) \quad [1]$$

where y is the response at dose x, A is the lower limit for y, B is the upper limit for y, d is the slope, and the GR_{50} is the dose giving 50% injury or inhibition (Seefeldt et al. 1995). GR_{50} is most commonly referred to because it is the most accurate estimate of plant sensitivity to a herbicide (Seefeldt et al. 1995). When the logistic function was fit to the data, an approximate R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from one (Draper and Smith 1981). The logistic dose-response model could not be used on the data for the resistant biotype as the pollen from the resistant biotype did not respond to the concentrations of clethodim used in this study.

Results and Discussion

Analysis of variance indicated differences between the resistant and susceptible biotypes for each assessment method, and there was not a trial main effect; consequently,

the response of johnsongrass pollen to increasing clethodim concentrations is presented by biotype averaged over trials. Pollen germination from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim (Figure 1), with a GR_{50} of 25.8 (standard error of ±0.6) μM clethodim. At 3 h after pollen immersion in germination fluid, pollen from the resistant biotype evaluated by visual assessment germinated ≥80% at concentrations of 0 to 100 μM. Consequently, a logistic dose-response model did not fit the data.

The response of pollen to the germination media was linear for 16 h (Figure 2). At 6 h after treatment, absorbance at 500 nm was nearly 0.5, consequently 6 h was chosen to conduct the pollen assays using the spectrophotometer. An absorbance of 0.5 was selected as a balance between the level of absorbance and the time it takes to run the assay. When pollen germination was evaluated by spectrophotometric evaluation 6 h after treatment, percent pollen tube growth inhibition for pollen from resistant johnsongrass was ≤13% at all clethodim concentrations (Figure 3). As in the visual assessment, pollen from the susceptible biotype of johnsongrass was strongly inhibited by increasing concentrations of clethodim (Figure 3), with a GR_{50} of 16.4 (standard error of ±1.7) μM clethodim. The spectrophotometric assay appears to be slightly more sensitive as it indicated pollen tube growth inhibition at a lower concentration than that of the visual assessment for the susceptible biotype. A specific resistant/susceptible (R/S) ratio could not be computed, as the highest clethodim rate did not reduce pollen germination of the resistant biotype 50%. Minimum R/S values (calculated by taking the maximum clethodim concentration used and dividing by the GR_{50} for the susceptible biotype) were

>3.9 by visual assessment and >6.1 by spectrophotometric assessment. By comparison, R/S ratios using seedling assays, seedling whole plant dose response, and rhizome whole plant dose response were 18.7, 11.0, and 15.6 (Burke et al. 2006a, d). The R/S ratio at the enzyme level was found to be 4.5 (Burke et al. 2006c). Both pollen assays were useful for distinguishing the resistant population from the susceptible population.

The resistant biotype of johnsongrass used in this study has a less sensitive form of ACCase (Burke et al. 2006c). The resistant ACCase enzyme appears to be expressed in the pollen of this johnsongrass biotype. Others have noted that biotypes of other species that are resistant due to mechanisms other than target-site resistance are not identifiable using pollen assays (Letouze and Gasquez 2000; Richter and Powles 1993). It may be that the enzyme or enzymes that are responsible for metabolism-based resistance are not expressed in the pollen (Richter and Powles 1993). Therefore, these data lend support to the conclusion that this biotype of johnsongrass is resistant due to an insensitive target site.

Richter and Powles (1993) note that pollen screening will be useful in inheritance studies of target-site resistant traits. As pollen is haploid, heterozygous individuals will have intermediate levels of germination (Richter and Powles 1993). Letouze and Gasquez (2000) were able to detect heterozygous individuals in populations of blackgrass as pollen from the heterozygous individuals germinated at intermediate levels to that of homozygous resistant and susceptible individuals. The population of resistant johnsongrass used in this study was treated with multiple applications of clethodim at 280 g ha^{-1} (two times the registered rate), and any injured plants were removed from the

population prior to the development of the pollen assays. Reports indicate that the ACCase resistance trait is partially dominant, and heterozygous individuals have intermediate levels of resistance at the whole plant level (Smeda et al. 2000; Tal and Rubin 2004). The selection pressure may have removed heterozygous, plants and the plants used in this experiment may be homozygous resistant.

The pollen of johnsongrass and the genus *Sorghum* appears to be short-lived. Burke et al. (2006b) found that pollen harvested just prior to anthesis was most germinable *in vitro*. Lansac et al. (1994) also found a complete loss of the ability of sorghum [*Sorghum bicolor* (L.) Moench] pollen to germinate after desiccation. Furthermore, Lansac et al. (1994) found greatly reduced seed set after 30 min of pollen desiccation. The short-lived nature of pollen of the genus *Sorghum* could have implications for the spread of the ACCase resistance found in the biotype that is the subject of this research. ACCase resistance provides an excellent marker to evaluate the mobility of johnsongrass pollen and the potential for movement of herbicide resistance in a mainly self-pollinating species. The pollen assays provide a good tool to determine resistance, an important part of studying the mobility of pollen (Levin and Kerster 1974). It may be that the short-lived nature of johnsongrass pollen limits the movement of resistance by pollen. Others have found that the contribution of pollen movement to resistance evolution and the spread of resistance in wild oat (*Avena fatua* L.) populations to be relatively small when compared with resistant seed production and dispersal from a resistant plant (Murray et al. 2002). Pollen assays coupled with a study on outcrossing using the ACCase resistance

as a marker would greatly increase the understanding of pollen mediated gene flow in johnsongrass.

As pollen screening provides an indication of target site-based resistance, pollen assays appear to be useful tools for identifying the basis of resistance. These assays, though, are limited to species with germinable pollen. It is also necessary to determine the optimum pollen germination media prior to developing assays. Although pollen techniques are useful, a seedling assay is much more simple for extension personnel and others to perform. In conclusion, target-site based resistance to clethodim, an ACCase inhibitor, is expressed in the pollen of johnsongrass. Two assays were developed to detect that resistance, one a visual assessment using a microscope and a second an absorbance assessment using a spectrophotometer. These assays will be useful in examining the pollen-mediated gene flow of ACCase resistance in populations of johnsongrass in order to gain a better understanding of resistance movement by pollen.

Sources of materials

[1] Peters Professional 20-20-20, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[2] Perkin-Elmer UV/Vis Lambda 10 Spectrometer, 45 William Street, Wellesley, MA 02481-4078.

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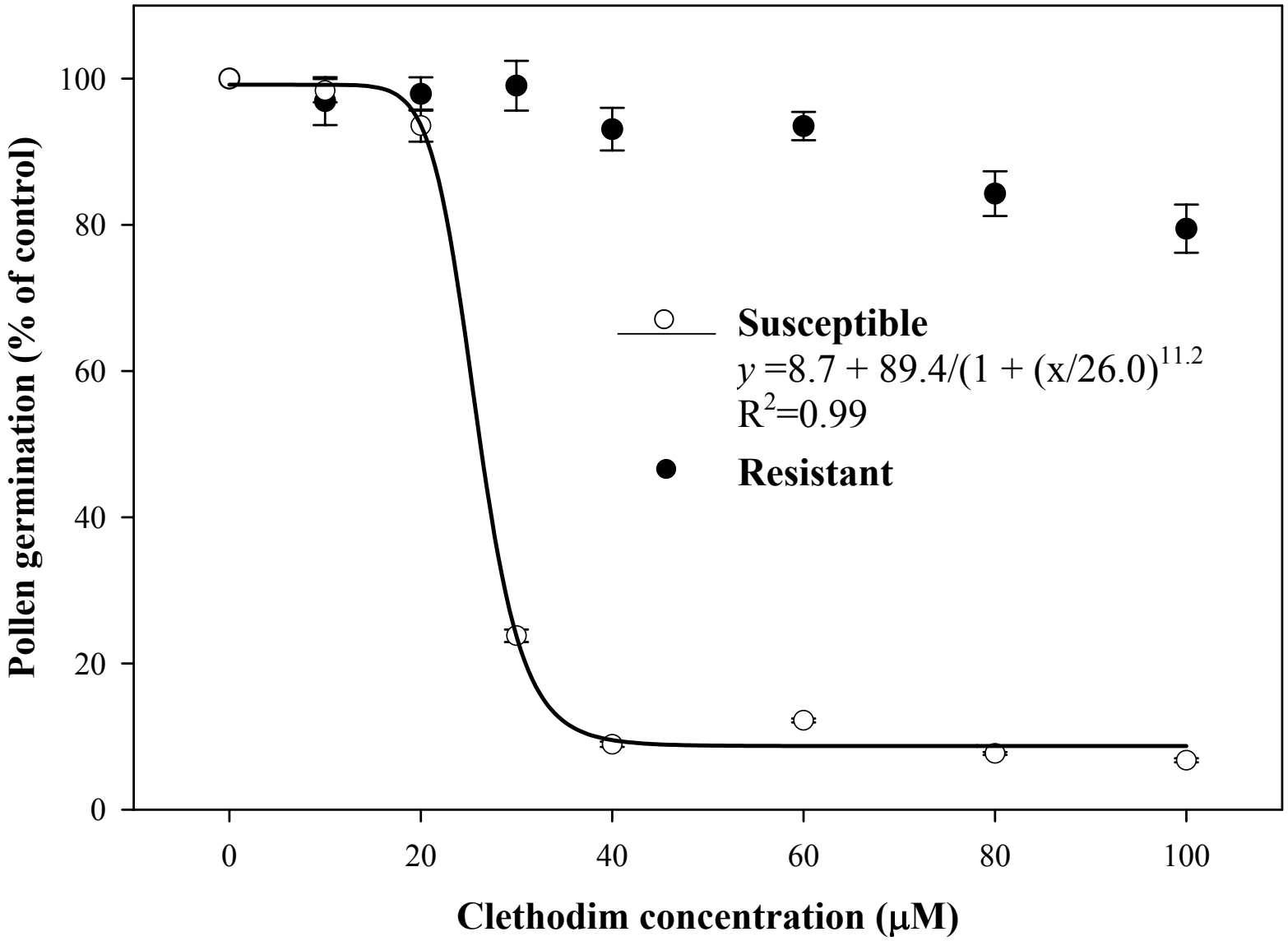

Figure 1. Effect of increasing concentrations of ACCase-inhibiting herbicide clethodim on the germination of pollen from a susceptible and a resistant johnsongrass biotype as evaluated by visual assessment. Vertical bars represent standard error (n=7).

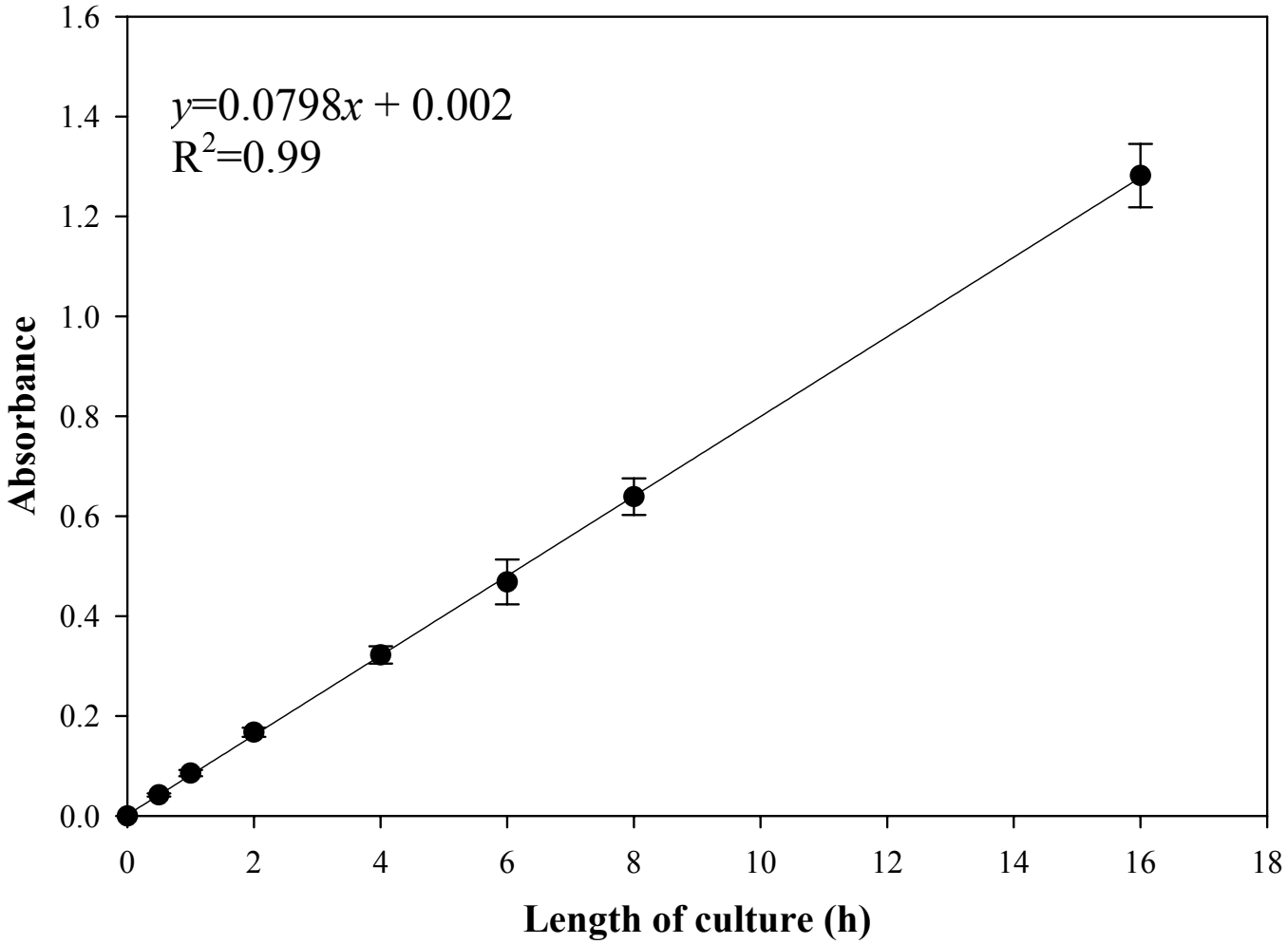

Figure 2. The effect of increasing duration of pollen culture on the optical density at 500 nm of homogenized pollen grains and tubes. Vertical bars represent standard error (n=6).

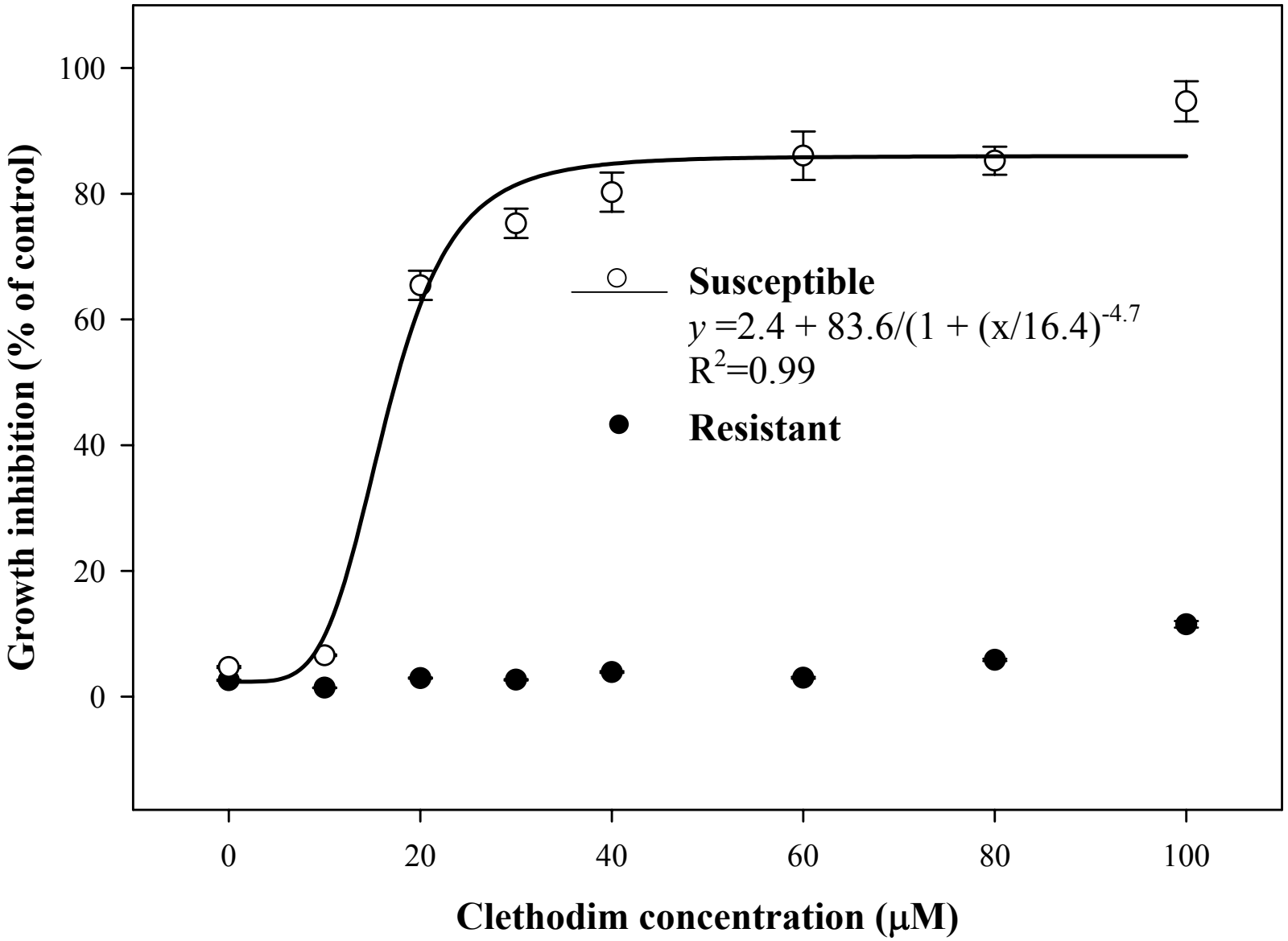

Figure 3. Effect of increasing concentrations of ACCase-inhibiting herbicide clethodim on the germination of pollen from a susceptible and a resistance johnsongrass biotype expressed as growth inhibition as evaluated using a spectrophotometer at 500 nm. Vertical bars represent standard error (n=6).