OBTAINING SETHOXYDIM RESISTANCE IN SEASHORE PASPALUM

(*PASPALUM VAGINATUM)*

by

DOUGLAS LEE HECKART

(Under the Direction of Paul Raymer and Wayne Parrott)

ABSTRACT

Herbicide resistance has been a sought-after trait for turfgrasses, however attempts to commercialize GM turfgrasses have been unsuccessful. Sethoxydim is a grass-specific herbicide, and resistance results from one of several single base-pair mutations. The most common mutation is an ILE to LEU substitution caused by an A to T mutation at position 1781 of ACCase. Research was initiated to develop a novel source of resistance to sethoxydim in seashore paspalum (*Paspalum vaginatum,* Swartz). Our objectives were to develop *in vitro* selection and regeneration protocols to select for naturally occurring mutations conferring herbicide resistance. A dose response experiment was performed to determine the optimum sethoxydim concentration for selection. Callus was induced from immature inflorescences then plated on callus induction medium containing 10 μM sethoxydim for selection. Green plants were regenerated from resistant callus, the ILE to LEU mutation documented, and expression of herbicide resistance confirmed.

INDEX WORDS: turfgrass, herbicide resistance, mutation breeding, tissue culture

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DEDICATION

I would like to thank God for providing me with many educational opportunities scholastically as well as in life. I would like to thank my family and friends. It was never said that it could not be done and your faith in me was the encouragement I needed. Thank you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Seashore paspalum (*Paspalum vaginatum,* Swartz) is a warm-season turfgrass that is generally adapted to dune environments (Duncan and Carrow, 2000). Specific adaptations of seashore paspalum include tolerance to salt, water logging, and drought (Duncan and Carrow, 2000). These tolerances make paspalum a premium turfgrass candidate for venues with these environmental issues. Seashore paspalum is recommended for new golf courses in tropical or sub-tropical coastal areas where salt or water quality are issues. Additionally, many existing golf courses around the world have replaced bermudagrass (*Cynodon dactylon* (L.) Pers.) with paspalum (Raymer et al., 2008). Compared to bermudagrass, paspalum requires less nitrogen and is more tolerant of irrigation with brackish or poor quality water, reducing management costs and improving irrigation flexibility (Duncan and Carrow, 2000)

The main limitation to replacing bermudagrass with paspalum is bermudagrass re-establishment. Bermudagrass is highly competitive and difficult to eradicate once established (Lowe and Sweet, 2006). Bermudagrass and other weedy grasses can greatly reduce the aesthetic value and quality of a paspalum turf. Currently there are no herbicides available that selectively control bermudagrass in seashore paspalum so development of herbicide-resistant paspalum could be a method to provide an effective means of managing bermudagrass in paspalum. Seashore paspalum that is resistant to a

specific class of herbicides may allow golf course and sporting venues to successfully transition from bermudagrass to seashore paspalum.

Herbicide resistance is a trait that has been actively pursued for many crops. Arguably the most notable of these are Roundup Ready® (RR) products. Glyphosate-resistant crops have a transgene that confers glyphosate resistance. However, the commercial deployment of RR in turfgrasses has yet to occur. The Scotts Company has developed a glyphosate-resistant creeping bentgrass (*Agrostis stolonifera* L.) and in 2003 began field trials in Madras, Oregon. These and subsequent trials have shown that the transgene conferring glyphosate resistance can cross with non-transgenic bentgrass, as its pollen viability is longer than originally expected therefore, pollen travel was also longer than expected (Zapiola et al., 2008). Development of bentgrass with transgenically derived herbicide resistance generated concerns of gene flow and faced regulatory obstacles before the transgenic plants could be marketed. In contrast, environmental release of plants with herbicide resistance derived by non-transgenic means has not been of great concern and is not subject to government regulation. A non-transgenic method of glyphosate resistance has been attempted through gene amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (Dill, 2005). Cell lines of *Daucus carota* L. and *Nicotiniana tabacum* L. were placed in medium containing increasing amounts of glyphosate in an effort to amplify the native EPSPS gene. Cell lines did increase in EPSPS activity, but researchers were unable to obtain plants. (Dill, 2005). Glyphosate resistance requires two single point mutations in the native EPSPS gene (Sidhu et al., 2000). Thus the possibility of obtaining glyphosate resistance through *in vitro* selection is extremely low, due to the fact that both mutations would have to

transpire spontaneously and simultaneously for any level of glyphosate resistance to occur. Thus, for the present study it became important to consider herbicides where resistance can be obtained by single point mutations in a native gene. It also became important to consider instances of herbicide resistance obtained previously through *in vitro* selection. Table 1.1 presents a list of examples where herbicide resistance has been obtained through *in vitro* selection.

Sethoxydim resistance has been achieved in maize (*Zea mays)*, providing an example of a graminaceous crop where weedy grasses can be controlled via herbicide resistance (Parker et al., 1990). Sethoxydim, (2-[1-(ethoxyimino]butyl)-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one) is a graminicide used to control perennial and annual grasses in numerous agricultural and ornamental crops. This herbicide is a member of the cyclohexanedione family. Sethoxydim has been marketed under numerous trade names which include: Vantage®, Poast®, Rezult®. Torpedo®, Ultima®, and Conclude® by manufacturers BASF, Monterey, and TopPro. Currently, sethoxydim is marketed by the BASF Corporation under the trade name Segment®. Sethoxydim-resistance in maize was achieved by selection *in vitro* of cells with, at the time, an unknown mutation in the acetyl-coenzyme A carboxylase (ACCase) gene. Somaclonal variation was the only source for genetic variation, leading to the mutation. The resistance is now understood to originate from a specific mutation occurring in the plastidic ACCase in grasses (Delye et al., 2005).

ACCase is known to exist in two forms: eukaryotic and prokaryotic (Harwood, 1988). The prokaryotic form is made up of four subunits, while the eukaryotic form is a single polypeptide with distinct functional domains (Harwood, 1988). Acetyl-coenzyme

A is carboxylated by ACCase to form malonyl-coenzyme A in the first committed step of lipid biosynthesis. Sasaki et al. (1995) found that ACCase is compartmentalized in two forms in most plants. The chloroplast is known to be the primary site of lipid synthesis; however, there is ACCase present in the cytosol as well. Most plants have the prokaryotic form in the chloroplast and the eukaryotic form in the cytosol. The tetrameric prokaryotic protein is coded for by four distinct genes, one being located in the chloroplast genome. The eukaryotic form is encoded by a nuclear gene approximately 12,000 bp in size (Podkowinski et al., 1996). The Poaceae are unique in that they have the eukaryotic form of ACCase in both the cytosol and chloroplast (Sasaki et al., 1995). The two eukaryotic forms of ACCase in grasses are very similar, as are the genes that code for them (Gornicki et al., 1994). Even though there is homology between the plastidic and cystolic ACCases, the cystolic form is not affected by ACCase-inhibiting herbicides (Delye, 2005).

Sethoxydim is absorbed through leaf surfaces. Transport of the herbicide occurs in the xylem and phloem. Accumulation of sethoxydim is primarily in the meristematic tissue (Ahrens, 1994). The interruption of lipid biosynthesis leads to membrane destruction in these actively growing areas, causing growth to halt and plants to die within 7-21 days. Sethoxydim has a half-life in the soil of 25 days due to breakdown by soil microbial activity (Roslycky, 1986) Sethoxydim does not bind to soil colloids. Despite the lack of adsorption to the soil particles, sethoxydim has shown to have little or no leaching in various soils tested (Koskinen et al., 1993).

Spontaneous sethoxydim resistance has been observed in numerous weedy species (Table 1.2.) and results after the large-scale application of sethoxydim in

cropping systems on an annual or semi-annual basis. The most commonly reported mutation is a single isoleucine to leucine substitution at position 1781 in the carboxyl transferase (CT) domain. An adenine to thymine transversion in the first position of the codon accounts for this substitution. Unfortunately, none of the commonly used mutagens induces transversion mutations. An asparigine to glycine substitution at ACCase amino acid position at 2078 has also been shown to provide resistance. This has only been shown in *Alopecurus myosuroides* Huds (Black grass) (Delye, 2005).

Much of what is known about sethoxydim resistance in grasses has been discovered through investigation of weedy species. Continual use of herbicides in the same area for consecutive years in agricultural situations can lead to herbicide resistant weeds through selection pressure. This raises the concern that the use of sethoxydim in turfgrass will foment the development of sethoxydim-resistant weeds. This is a possibility however; application of sethoxydim on sethoxydim-resistant (SR) paspalum would likely be in small areas to control invading grass weeds. Application in such small areas would minimize the risk of such resistance developing in bermudagrass and other weedy grasses.

Transgenic approaches for sethoxydim resistance are not practical with current technology. First, ACCase is large (~12,000 bp), which makes it difficult to manipulate with current transformation procedures. The cDNA sequences available from GenBank are also exceedingly large, at around 7,000 bp, not including the vector backbone. Secondly, there is a high level of homology that would exist between the inserted gene and the native genes. Since only 20-26 bp of homology is needed to cause gene silencing of both the inserted gene and the native gene, this approach would likely result in

silencing (Brodersen and Voinnet, 2006) and be lethal. Engineering of the herbicide-resistant prokaryotic ACCase genes is also not an easy option, as it requires the coordinated transformation of four genes into the nuclear and chloroplastic genomes.

Two remaining options are to modify the native gene *in vivo* or to select for mutants *in vitro. In vivo* modifications can include chimeraplasty (Dong et al., 2006) or zinc fingers (Durai et al., 2005). As these technologies are in their infancy it did not appear to be appropriate for this project. Given the previous success of *in vitro* selection for sethoxydim resistance, this approach was deemed the most practical to obtain sethoxydim-resistant paspalum. Such an approach would have the added benefit that any naturally occurring mutant plant expressing sethoxydim resistance could be used immediately, without any of the regulatory hurdles associated with transgenic approaches.

The overall goal of this research was to obtain sethoxydim-resistant seashore paspalum, which could be used in a breeding program for the development of herbicide resistant cultivars. The objectives were to 1) develop protocols select and regenerate naturally occurring mutants which confer herbicide resistance and 2) utilize these protocols to obtain sethoxydim-resistant seashore paspalum.

REFERENCES

Ahrens, W. 1994. Herbicide handbook of the weed science society of America. Weed Sci. Soc.America, Champaign, IL, USA.

Brodersen, P., and O. Voinnet. 2006. The diversity of RNA silencing pathways in plants. Trends Genet. 22:268-280.

Delye, C. 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. Weed Sci. 53:728-746.

Delye, C., X.Q. Zhang, S. Michel, A. Matejicek, and S.B. Powles. 2005. Molecular bases for sensitivity to acetyl-coenzyme a carboxylase inhibitors in black-grass. Plant Physiol. 137:794-806.

Dill, G. 2005. Glyphosate-resistant crops: history, status and future. Pest Manag. Sci. 61:219-224.

Dong, C., P. Beetham, K. Vincent, and P. Sharp. 2006. Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. Plant Cell Rep. 25:457-465.

Duncan, R., and R. Carrow. 2000. p. 1-94 Seashore Paspalum: The Environmental TurfgrassWiley, Hoboken NJ.

Durai, S., M. Mani, K. Kandavelou, J. Wu, M. Porteus, and S. Chandrasegaran. 2005. Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. Nucleic Acids Res. 33:5978.

Gornicki, P., J. Podkowinski, L.A. Scappino, J. Dimaio, E. Ward, and R. Haselkorn. 1994. Wheat acetyl-coenzyme-A carboxylase cDNA and protein structure. Proc. Natl. Acad. Sci. U. S. A. 91:6860-6864.

Harwood, J.L. 1988. Fatty Acid Metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:101-138.

Koskinen, W., D. Buhler, and D. Wyse. 1993. Persistence and movement of sethoxydim residues in three Minnesota soils. Weed Sci.:41:634-640.

Lowe, T. and Sweet, K. 2006. Converting Bermudagrass to Seashore Paspalum. Green Section Record Nov/Dec. 44:19-21.

Parker, W.B., D.A. Somers, D.L. Wyse, R.A. Keith, J.D. Burton, J.W. Gronwald, and B.G. Gengenbach. 1990. Selection and characterization of sethoxydim tolerant maize tissu cultures. Plant Physiol. 92:1220-1225.

Podkowinski, J., G. Sroga, R. Haselkorn, and P. Gornicki. 1996. Structure of a gene encoding a cytosolic acetyl-CoA carboxylase of hexaploid wheat, pp. 1870-1874, Vol. 93. National Acad Sciences.

Raymer, P.L., S. K. Braman, L. L. Burpee, R. N. Carrow, Z. Chen, and T. R. Murphy. 2008. Seashore Paspalum: Breeding a Turfgrass for the Future. USGA Green Section Record:22-26.

Roslycky, E.B. 1986. Microbial response to sethoxydim and its degradation in soil. Can. J. Soil Sci. 66:411-419.

Sasaki, Y., T. Konishi, and Y. Nagano. 1995. The compartmentation of acetyl coenzyme-A carboxylase in plants. Plant Physiol. 108:445-449.

Sidhu, R., B. Hammond, R. Fuchs, J. Mutz, L. Holden, B. George, and T. Olson. 2000. Glyphosate-tolerant corn: The composition and feeding value of grain from glyphosate-tolerant corn is equivalent to that of conventional corn (Zea mays L.). J. Agric. Food Chem 48:2305-2312.

Zapiola, M.L., C.K. Campbell, M.D. Butler, and C.A. Mallory-Smith. 2008. Escape and establishment of transgenic glyphosate-resistant creeping bentgrass Agrostis stolonifera in Oregon, USA: a 4-year study. J. Appl. Ecol. 45:486-494.

Table 1.1. List of crops where herbicide resistance has been gained through *in vitro* selection techniques.

Crop	Species	Herbicide	Year	Reference
Tobacco	*Nicotiana tobacum* L.	picloram	1978	(Chaleff and Parsons, 1978)
Tobacco	*Nicotiana tobacum* L.	paraquat	1980	(Miller and Hughes, 1980)
Tobacco	*Nicotiana tobacum* L.	chlorsulfuron	1984	(Chaleff and Ray, 1984)
Rape Seed	*Brassica napus* L.	chlorsulfuron	1988	(Swanson et al., 1988)
Rape Seed	*Brassica napus* L.	imidazolinone	1989	(Swanson et al., 1989)
Maize	*Zea mays* L.	sethoxydim	1990	(Parker et al., 1990)
Maize	*Zea mays* L.	imidazolinone	1991	(Newhouse et al., 1991)
Cotton	*Gossypium hirsutum* L.	primsulfuron	1996	(Rajasekaran et al., 1996)
Sugarbeet	*Beta vulgaris* L.	imidazolinone	1998	(Wright and Penner, 1998)

Table 1.2. Reported mutations conferring resistance to sethoxydim in weedy species.

Species	Common name	Mutation		Herbicide	Reference
		Position	Substitution		
Alopecurus myosuroides Huds.	Black grass	1781	Ile to Leu	Sethoxydim	Delye et al. 2002B
Setaria virdis L.Beav.	Green foxtail	1781	Ile to Leu	Sethoxydim	Delye et al. 2002B
Avena fatua L.	Wild oat	1781	Ile to Leu	Sethoxydim	Christoffers et al. 2002
Lolium spp.	Ryegrass	1781	Ile to Leu	Sethoxydim	Zagnitko et al. 2001
Poa annua L.	Annual bluegrass	1781	Ile to Leu	Sethoxydim	Delye et al. 2005
Festuca rubra L.	Red fescue	1781	Ile to Leu	Sethoxydim	Delye et al. 2005
Alopecurus myosuroides Huds.	Black grass	2078	Asp to Gly	Clethodim/Cyclodim	Delye et al. 2005

CHAPTER 2

OBTAINING SETHOXYDIM RESISTANCE IN SEASHORE PASPALUM (*PASPALUM VAGINATUM*)[1]

[1]Heckart, D.L., W.A. Parrott, and P.L. Raymer. To be submitted to *Crop Science*.

ABSTRACT

Herbicide resistance has been a sought-after trait for turfgrasses, however attempts to commercialize GM turfgrasses have been unsuccessful. Sethoxydim is a grass-specific herbicide, and resistance results from one of two possible single base-pair mutations. The most common mutation is an ILE to LEU substitution caused by an A to T mutation at position 1781 of acetyl coenzyme A carboxylase. Research was initiated to develop a novel source of resistance to sethoxydim in seashore paspalum (*Paspalum vaginatum,* Swartz). The objectives of the present study were to develop *in vitro* selection and regeneration protocols to select for naturally occurring mutations conferring herbicide resistance. A dose response experiment was performed to determine the optimum sethoxydim concentration for selection. Callus was induced from immature inflorescences then plated on callus induction medium containing 10 µM sethoxydim for selection. Green plants were regenerated from resistant callus, the ILE to LEU mutation documented, and expression of herbicide resistance confirmed.

INDEX WORDS: turfgrass, herbicide resistance, mutation breeding, tissue culture

INTRODUCTION

Seashore paspalum (*Paspalum vaginatum,* Swartz) is a warm-season turfgrass that is adapted to dune environments (Duncan and Carrow, 2000) Desirable attributes of seashore paspalum include tolerance to salt, water logging, and drought (Duncan and Carrow, 2000). Such tolerances to abiotic stress make paspalum a premium turfgrass candidate for venues where any or all of these factors otherwise limit turfgrass growth. Compared to bermudagrass (*Cynodon dactylon* (L.) Pers.), paspalum requires less nitrogen and is more tolerant of irrigation with brackish or waste water, reducing management costs and improving irrigation flexibility (Duncan and Carrow, 2000). Golf course architects recommend seashore paspalum for new courses in tropical or sub-tropical coastal areas where salt or water quality are issues (Duncan and Carrow, 2000). Additionally, many existing golf courses around the world have replaced bermudagrass with paspalum (Raymer, 2008).

The main difficulty in replacing bermudagrass with paspalum is bermudagrass re-establishment. Bermudagrass is highly competitive and difficult to eradicate once established (Lowe and Sweet, 2006), and invasion by bermudagrass and other weedy grasses can greatly reduce the aesthetic value and quality of paspalum turf. Currently there are no herbicides available that selectively control bermudagrass in seashore paspalum. Development of herbicide-resistant paspalum could provide an effective means of managing bermudagrass in paspalum, and allow golf course and sporting venues to transition from bermudagrass to seashore paspalum.

Sethoxydim, (2-[1-(ethoxyimino]butyl)-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one) has many of the traits necessary to select against different grasses and

facilitate the replacement of bermudagrass with paspalum. It is a graminicide used to control perennial and annual grasses in numerous agricultural and ornamental crops. This herbicide is a member of the cyclohexanedione (CHD) family. Sethoxydim has been marketed under numerous trade names, including Vantage®, Poast®, Rezult®, Torpedo®, Ultima®, Conclude®, and Segment®. Sethoxydim and other acetyl coenzyme A carboxylase (ACCase) inhibitors cause cell death by competing with ACCase for its binding site in the in the ACCase carboxylase transfer (CT) domain (Delye, 2005). In turn, this first step of fatty acid biosynthesis is halted and the product, malonyl coenzyme A, is not produced. Cell membranes are compromised without fatty acids and cell death ensues.

Spontaneous sethoxydim resistance has been observed in numerous weedy species. Such resistance has arisen after the large-scale application of sethoxydim on an annual or semi-annual basis in various cropping systems (Delye and Michel, 2005). The most commonly reported mutation is a single isoleucine (Ile) to leucine (Leu) substitution at position 1781 in the carboxyl transferase domain (Delye and Michel, 2005) of the ACCase gene. An adenine to thymine transversion in the first position of the codon accounts for this substitution. Additionally, a mutation at the 2078 codon, which normally codes for asparagine (Asp), also confers resistance to sethoxydim. Both of these mutations also confer resistance to aryloxyphenoxypropionate (APP) herbicides.

Transgenic approaches for obtaining sethoxydim resistance are not practical. First, ACCase is large (~12,000 base pairs (bp)), which makes it difficult to manipulate with current transformation procedures. The cDNA sequences available from GenBank are also large, (~7,000 bp), not including the vector backbone. Secondly, there is a high level of homology that exist between the inserted gene and the native genes. Since only

20-26 bp of homology are needed to cause gene silencing of both the inserted gene and the native gene, this approach would likely result in silencing (Brodersen and Voinnet, 2006) and be lethal.

ACCase exists in two forms: eukaryotic and prokaryotic (Harwood, 1988). The prokaryotic form is made up of four subunits and is coded by four genes, one of which is located in the chloroplast genome. The eukaryotic form is a single polypeptide with distinct functional domains (Harwood et al., 1988), and is encoded by a nuclear gene approximately 12,000 bp in size (Podkowinski et al., 1996). Sasaki et al. (1995) found that ACCase is compartmentalized in most plants, such that the prokaryotic form is in the chloroplast and the eukaryotic form in the cytosol. The poaceae are unique in that they have the eukaryotic form of ACCase in both the cytosol and chloroplast (Sasaki et al., 1995). The two eukaryotic forms of ACCase in grasses are very similar, as are the genes that code for them (Gornicki et al., 1994). Even though there is homology between the plastidic and cystolic ACCases, the cystolic form is not affected by ACCase-inhibiting herbicides (Delye, 2005). Thus, engineering of the herbicide-resistant prokaryotic ACCase genes is also not an option, as it requires the coordinated transformation of four genes into the nuclear and chloroplastic genomes.

Sethoxydim resistance was obtained in maize (*Zea mays* L.*)* by *in vitro* selection, providing an example of a graminaceous crop in which weedy grasses can be controlled via herbicide resistance (Parker et al., 1990). The resistance is now understood to originate from the single Ile to Leu substitution at position 1781 mutation occurring in the plastidic ACCase in grasses (Delye et al., 2005).

The goal of this research was to identify naturally occurring mutants conferring sethoxydim-resistant in seashore paspalum via *in-vitro* selection. Once identified, these resistant plants could be used in a breeding program for the development of herbicide-resistant cultivars.

MATERIALS AND METHODS

Callus Induction

Explants were obtained from 10 genotypes including: eight experimental lines from The University of Georgia seashore paspalum breeding program, one collected ecotype, Mauna Kea (PI 647892), and the commercial seeded cultivar, 'Seaspray' (Table 2.1)**.** Immature inflorescences were removed from floral shoots prior to emergence. The two spikes were separated and surface-sterilized with 10% commercial bleach with several drops of Tween 80 for 10 minutes then rinsed with sterile water.

The induction medium consisted of MS basal salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg et al., 1968) and 2 mg L^{-1} 2,4-D (Cardona and Duncan, 1997). The medium was brought to a pH of 5.8 and 2 g of Gelzan™ (Caisson Laboratories Inc. North Logan, UT) gelling agent was added. Following autoclavation, the medium was poured into 100 x 15 mm Petri dishes. Four explants were placed on each plate and the plates were sealed with Nescofilm™ (Karlan Research Products Co; Cottonwood, AZ). The explants were placed in the dark at 27˚C. The callus generated was given a cell line designation based on the genotype and the date the explant was placed on induction medium.

Dose Response

The response of paspalum tissue to sethoxydim rate was determined, using callus tissue generated from the variety 'Seaspray'. Laboratory grade sethoxydim was obtained from Chemservice Inc. (West Chester, PA) and diluted with methanol to a concentration of 1 ml methanol to1 mg sethoxydim. The sethoxydim concentrations evaluated were: 0, 2.5, 5, 7.5, 10, 25, 50, and 100 μM. The medium was the same as used for callus induction, with sethoxydim added after the autoclaved medium had cooled to approximately 55˚C, thus preventing loss of activity from heat degradation (Campbell and Penner, 1985).

To measure callus growth, one-half gram of callus tissue was weighed, separated into nine equal pieces, and placed in a 3 x 3 pattern on the medium of each plate. Six replicate plates for each of the eight sethoxydim concentrations were distributed on a rack in a growth room in a completely randomized design. At the three-week subculture period, the tissue from each plate was weighed and recorded. Only 0.5 g was subcultured from each plate and taken to the next growth period. This process was continued for nine weeks, providing three growth measurements for each plate. The weight from each plate at each of the measurement points (3, 6, and 9 wks) was divided by the initial weight to obtain the percent change for a 3-week period (Parrot and Bouton, 1990). Callus growth in response to sethoxydim concentration was fitted to a negative exponential decay function using non-linear regression (SAS Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC).

Selection of Sethoxydim-Resistant cells

Callus tissue, approximately 6 months old, was removed from MS/B5 induction medium and placed on callus induction medium with a sethoxydim concentration of 10 μM, as determined by the dose response study. Calli approximately 4 mm in diameter were placed on callus induction medium on 245 mm x 245 mm square bioassay plates in a 15 x 15 grid for a total of 225 calli per plate. After three weeks, the calli were subcultured to fresh plates. The process was repeated three times for a total selection period of 9 weeks. Resistant calli were subcultured into 100 x 15 mm Petri dishes containing callus induction medium supplemented with 10 μM sethoxydim for one month in order to obtain sufficient callus. This provided a total selection time of 12 wks or more.

Estimation of Resistant Calli Formation Frequency

Individual cells were measured to determine cell size and an estimate of the frequency of resistant cells compared to total number of cells placed on selection medium. To obtain individual cells, two grams of callus tissue were added to 25 ml of liquid induction medium and placed on a shaker at 112 RPM. After growing in the dark at 25 ˚C for five days, clear medium, absent of callus clusters, was extracted. Fifteen microliters of medium were placed on a slide and mixed with 2 μL of modified carbol fuchsin stain (Kao, 1975). Cells were observed in a microscope at 40X and measured using Image Pro Plus© (Media Cybernetics Inc. Bethesda MD). The diameter from a total of 100 cells was measured twice to calculate an average cell diameter. This value was used to estimate the volume of a single cell based on the assumption that cells were spherical. To find the approximate volume of a 4 mm callus piece, 100 representative calli were placed in a

graduated cylinder to find the displacement in water and obtain the callus volume, which was divided by the volume of a single cell. This provided an estimate of the number of cells in a 4 mm callus piece.

Data describing the number of resistant calli observed per plate completing the selection process were modeled as the probability of a resistant callus event per trial (plate of 225 calli) and tested for differences among genotypes using Proc GLIMMIX SAS (Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC). In this analysis each plate of 225 calli was considered a replicate. Replicate numbers varied with genotype due to differences in the amount of tissue available for selection.

Regeneration

The medium originally used for the induction of callus from bahiagrass (*Paspalum notatum,* Flugge) (Altpeter and James, 2005) was modified by removing the auxin, thus allowing it to serve as the regeneration medium. The medium consists of MS/B5 basal medium supplemented with 1.24 mg L^{-1} $CuSO_4$, and 1.125 mg/L^{-1} BAP (6-benzylaminopurine). Calli of each sethoxydim-resistant (SR) line were placed on regeneration medium in a 4 x 4 grid, using five plates per line. Each callus was approximately 4 mm in size. These were placed in a growth chamber at 25 ˚C with a 1-h dark, 23-h light photoperiod, and a light intensity of 66-95 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes. All plates were evaluated for regeneration at the end of a 30-day period. If shoots appeared the cell lines were subcultured for an additional month on regeneration medium. To induce root growth, the shoots were transferred to MS/B5 basal medium without growth regulators. When root growth was adequate (~30 days) the

plants were removed from the medium and placed in pots containing a 1:1 mix of Fafard® 3B (Agawam, MS) mix and sand. The potted plants were maintained in a growth chamber for one week as described, after which the plants were transferred to a greenhouse with a 10-hr light, 14-hr dark photoperiod at 24 ˚C to 32 ˚C.

Molecular Characterization of Resistant Calli

Once SR paspalum lines were selected, the mutation causing the resistance was characterized. DNA was extracted from callus or leaves of regenerated plants via the CTAB method as described by (Lassner et al., 1989).

Primers used for PCR amplification of the region surrounding the 1781 aa position were designed based on the amino acid sequence as described by Delye et al. (2005). Two regions flanking the 1781 position were selected for forward and reverse primers due to their conservation among species. The nucleotide sequence from *Setaria viridis ACCase* (GenBank AF294805) (Delye et al., 2002) was translated using Generunner© V.3.05 (Hastings Software 1994 Hastings, NY) to find the nucleotide sequence corresponding to the conserved amino acid region. Twenty base pairs were selected and in each homologous region and the BLAST function was used on GenBank to check homology between species. Individual bases were changed to match the highest number of grass species possible. The resulting primers amplify a 384-bp fragment of the ACCase gene that spans the A to T transversion which causes the Ile to Leu substitution at the 1781 position. The primers were designated SV384F (5’ CGGGGTTTCAGTACATTTAT 3’) and SV384R (5’ GATCTTAGGACCACCCAACTG 3’). Annealing temperature was 53˚C with an extension time of 30 sec and 35 cycles. The same process was used to sequence

the 2078 region. The primers developed for sequencing the 2078 region were SVAC2F (5'AATTCCTGTTGGTGTCATAGCTGTGGAG 3') and SVAC1R (5' TTCAGATTTATCAACTCTGGGTCAAGCC 3'). These primers amplified a fragment 520 bp in length that spans the coding region of the 2078 position.

Whole Plant Evaluation

SR plants regenerated from a sethoxydim-resistant cell line, SR11, were tested for resistance at the whole plant level in a dose response experiment conducted in a greenhouse. In this experiment, SR11 was compared to two herbicide-susceptible controls; the original parental line, Mauna Kea (PT); and a Mauna Kea line regenerated from tissue culture (TC). Plants were transplanted to Cone-tainers™ measuring 4 x 14 cm and tapering to 1 cm (Stuewe and Sons Inc., Corvallis, Oregon) containing a 1:1 mix of Fafard® 3B mix and sand and placed on benches under sodium lights in a greenhouse with a 16-h photoperiod maintained at 27/32 °C day/night for two weeks prior to treatment applications.

Each of the three genotypes, SR11, PT, TC, were treated with 0, 50, 100, 200, 400, 800, 1600, and 3200 g ai ha^{-1} rates of sethoxydim using Segment ™ herbicide (BASF Corp., Florham Park, NJ). All herbicide rates were applied at a spray volume of 187l ha^{-1} in an experimental spray chamber, and after drying, returned to the greenhouse bench and maintained under the conditions described above. Visual estimates of crop injury were recorded at 7, 14, 21, and 28 d after treatment (DAT) using a scale of 0 to 100, where 0 equals no injury and 100 equals complete death. The experiment was a three by eight factorial with three genotypes and eight herbicide rates. Treatments were arranged in a

randomized complete block design. Only two replications of TC were possible due to limited plant materials; otherwise four replications were used for the other two genotypes.

Data were first analyzed using a two-way analysis of variance (SAS Institute, Inc. 2008. SAS OnlineDoc® 9.2. Cary, NC) and subsequently analyzed within herbicide rate. Differences among genotype means at each herbicide rate were determined using Fisher's Least Significant Difference (LSD).

RESULTS AND DISCUSSION

Herbicide resistance is a trait that has been actively pursued for many crops. Arguably the most notable of these are the Roundup Ready® (RR) products. The commercial deployment of RR in turfgrasses has yet to occur; although, a glyphosate-resistant creeping bentgrass (*Agrostis stolonifera* L.) has been developed. However, development of turfgrass with transgenically derived herbicide resistance traits has generated concerns over gene flow (Zapiola et al., 2008), and such transgenic turfgrasses face steep regulatory concerns before they can be released. In contrast, the environmental release of plants with herbicide resistance obtained by non-transgenic means has not been of great concern and is not subject to government regulation. Thus, *in vitro* selection for herbicide tolerance is an attractive alternative to transformation whenever possible. In addition, mutagenesis is not an option as none of the know mutagens induce the adenine to thymine transversion needed to obtain resistance.

In vitro selection for herbicide resistance has been attempted in numerous crops, including *in vitro* selection for glyphosate resistance has been attempted Glyphosate resistance requires two single point mutations in the native EPSPS gene (Sidhu et al.,

2000). Thus the possibility of obtaining glyphosate resistance through *in vitro* selection is extremely low due to the fact that both mutations would have to transpire spontaneously and simultaneously for any level of glyphosate resistance to occur. In contrast resistance to herbicides has been obtained in several crops (Table 2.2) where single point mutations confer resistance.

While *in vitro* selection has not been overly successful for other herbicides, it appeared to be a good approach for developing sethoxydim resistance. Spontaneous mutations conferring resistance are well-documented and prior results with corn made it clear *in vitro* selection could be a useful strategy. In contrast *in vitro* selection proved unsuccessful in Kentucky bluegrass (*Poa pratensis* L.*)* (Somers, 1996). The failure in the latter was due to improper tissue culture protocols, illustrating the importance of having established callus induction and plant regeneration protocols.

An existing medium formulation for callus induction in bahiagrass proved to be highly suited for paspalum regeneration upon removal of the auxin. Thus, little work was required to optimize paspalum regeneration from cell culture, and work centered on selection for resistance.

Results of the dose response experiment demonstrated that 7.5 μM sethoxydim in the medium prevented all growth of callus tissue (Figure 2.1). The resulting regression equation is: $(B_o e^{-B_1 X}) = y = (1378.0e^{-0.481X})$. A concentration of 10 μM sethoxydim was chosen to ensure the efficacy of selection.

The selection process resulted in 65 SR lines, representing a mutation rate of one resistance event per 312 calli. The six cell lines that produced SR calli were: Mauna Kea, GA 05-025-164, UGA03.539.13, UGA05.025.181, UGA03.525.22, and UGA03.09E-3.

The frequency of SR calli was low in all genotypes and ranged from 0 to 0.0051. Even though the probability of recovering a SR lines was low for all genotypes, the number of SR lines recovered varied and ranged from zero to as high as nine per plate of 225 calli. Statistical analysis for differences in the probability of obtaining a resistant calli event indicated no significant differences ($p=0.35$) among genotypes.

Two of the 65 SR cell lines were lost prior to regeneration and of the 63 SR lines remaining, only three regenerated: SR11, SR31, and SR63. Two lines originated from the same cell line derived from Mauna Kea initiated on 12 January 2008, while line SR63 originated from experimental line UGA 03-098E-3 initiated on 4 March 2008. The callus tissue of the three lines that regenerated was dense and yellow compared to a majority of the lines, which were white and soft. Thus, the two primary qualifiers for *in vitro* selection for a genotype are the capability for regeneration as well as its ability to mutate for resistance.

The average volume of a single callus cell was measured to be 1.3582×10^{-5} μL. This provides an approximation of 258,000 cells per 4 mm callus piece. Thus, the 20,250 calli put through selection contained approximately 5.2 billion cells. Assuming that only a single mutant cell was responsible for each SR cell line, the frequency of resistant cells in this experiment was one per 8×10^7 cells. The frequency of obtaining the A to T mutation at the 1781 aa position was one in 1.74×10^9. Reports on frequency of spontaneous mutations have been highly variable. Ruiter et al. (2003) found the mutation frequency in the native *als* gene of *Brassica napus* L. to be 2×10^7. They also reported spontaneous mutations in the transgenic *bar* gene in *Brassica napus* L. and *Nicotiana tabacum* L. occur at the higher rate of 4×10^4. In contrast, the A to T mutation in paspalum

was 87 and 43,537 fold lower than reported rates for *Brassica and tobacco*, respectively. However, when *in vitro* selection is initiated with an expectation to obtain a single specific base change, the frequency obtained in paspalum is most likely the more accurate estimate. Although the low frequency at which the single specific base change occurs is low, the use of large plates made it possible to efficiently screen large numbers of callus pieces in five months.

ACCase amplicons were obtained from 63 of the 65 SR lines, and only three exhibited the A to T transversion at position 1781 (Fig 2.2), The possibility cannot be excluded that mutations at positions other than 1781 or 2078 also occurred in these SR cell lines. Resistant lines are heterozygous for the mutation, so the chromatogram in Figure 2.2 shows a double peak at the point of mutation, with one peak representing the wild-type allele, and the other the mutated allele. Of the three lines that regenerated, only SR11 possesses the expected Ile to Leu mutation. Lines SR31 and SR63 both have the wild-type sequence at this position. Since sethoxydim resistance can also be conferred by an Asp to Gly mutation at the 2078 position; DNA from SR31 and SR63 was analyzed for presence of this mutation, but neither of them possessed it. The nature of sethoxydim resistance remains undetermined for SR31 and SR 63.

Figure 2.3 illustrates the effect of sethoxydim rate on injury ratings of each of the three tested genotypes at 14 DAT. The two-way analysis of variance indicated significant genotype, herbicide rate, and genotype by herbicide rate effects for injury ratings at 7, 14, 21, and 28 DAT (Table 2.3)(Table 2.4). Line SR11 showed excellent herbicide resistance, even at the highest rate of 3200 g ai ha^{-1} (Fig. 2.4). In contrast, both PT and TC had injury scores of 30 or greater at rates of 200 g ai ha^{-1}, and injury scores of 80% or greater at rates

≥800 g ai ha^{-1}. When mean injury scores were compared for each of the three genotypes at each herbicide rate, SR11 had significantly less injury than PT or TC at all rates of above 100 g ai ha^{-1} at all rating dates. The maximum injury score observed on SR11 was 7.5 at 3200 g ai ha^{-1}, or 15 times greater than the lowest labeled rate for centipedegrass, (*Eremochloa ophiuroides* (Munro) Hack), a turfgrass species naturally tolerant to sethoxydim.

Estimates of LD_{50} for the three genotypes were 189, 276, and >3200 g ai ha^{-1} for PT, TC, and SR11, respectively. These data provide evidence that the level of herbicide resistance present in SR11 is more than adequate to provide effective control of susceptible weedy grasses without concerns over herbicide injury.

Cross resistance to APPs has been reported in several weedy species of plants possessing the 1781 ILE to LEU (Delye, 2005). Resistance to CHDs and APPs may occur differently depending on structure of the herbicide molecule. However, since conformational changes and differences in side chains determine which herbicide molecules are able to interact with ACCase (Delye, 2005), and although the mutation at aa position 1781 has been achieved, resistance to other CHDs and APPs is not guaranteed. Resistance to these other ACCase inhibitors will need to be determined on a case by case basis.

CONCLUSIONS

Resistance to sethoxydim would be difficult to obtain by current transgenic technology due to the size and number of the ACCase genes involved. In contrast, past success with *in-vitro* selection, and various documented instances of spontaneous resistance suggested that sethoxydim resistance is a trait that is particularly amenable to *in*

vitro selection. As expected, it was possible to obtain numerous resistant cell lines in a relatively short period of time, and with a reasonable effort. Seashore paspalum now has the benefit of herbicide resistance, which should facilitate its greater adoption in golf courses and sports fields currently planted with the less-desirable bermudagrass. Such *in-vitro* selection should also be practical for other turf grass species with established tissue culture and regeneration protocols. This research is the first instance of herbicide resistance obtained in an otherwise susceptible turfgrass species. Inasmuch as it was obtained without the use of genetic transformation, it may become the first commercial herbicide-resistant turfgrass variety.

REFERENCES

Altpeter, F., and V. James. 2005. Genetic transformation of turf-type bahiagrass (Paspalum notatum Flugge) by biolistic gene transfer. Int. Turfgrass Soc. Res. J 10:1–5.

Brodersen, P., and O. Voinnet. 2006. The diversity of RNA silencing pathways in plants. Trends Genet. 22:268-280.

Campbell, J.R., and D. Penner. 1985. Abiotic transformations of sethoxydim. Weed Sci. 33:435-439.

Cardona, C.A., and R.R. Duncan. 1997. Callus induction and high efficiency plant regeneration via somatic embryogenesis in paspalum. Crop Sci. 37:1297-1302.

Delye, C., and S. Michel. 2005. 'Universal' primers for PCR-sequencing of grass chloroplastic acetyl-CoA carboxylase domains involved in resistance to herbicides. Weed Res. 45:323-330.

Delye, C., T.Y. Wang, and H. Darmency. 2002. An isoleucine-leucine substitution in chloroplastic acetyl-CoA carboxylase from green foxtail (Setaria viridis L. Beauv.) is responsible for resistance to the cyclohexanedione herbicide sethoxydim. Planta 214:421-427.

Delye, C., X.Q. Zhang, S. Michel, A. Matejicek, and S.B. Powles. 2005. Molecular bases for sensitivity to acetyl-coenzyme a carboxylase inhibitors in black-grass. Plant Physiol. 137:794-806.

Délye, C. 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. Weed Sci. 53:728-746.

Duncan, R., and R. Carrow. 2000. p. 1-94 Seashore Paspalum: The Environmental Turfgrass Wiley, Hoboken NJ.

Gornicki, P., J. Podkowinski, L.A. Scappino, J. Dimaio, E. Ward, and R. Haselkorn. 1994. Wheat acetyl-coenzyme-A carboxylase cDNA and protein structure. Proc. Natl. Acad. Sci. U. S. A. 91:6860-6864.

Harwood, J.L. 1988. Fatty Acid Metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:101-138.

Kao, K. 1975. A nuclear Staining Method for Plant Protoplasts, p. 60 -62, *In* O. L. Gamborg, Wetter, L.R. , ed. Plant Tissue Culture Methods. National Research Council of Canada, Saskatoon Canada.

Lassner, M., P. Peterson, and J. Yoder. 1989. Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol. Biol. Report. 7:116-128.

Lowe, T. and Sweet, K. 2006. Converting Bermudagrass to Seashore Paspalum. Green Section Record Nov/Dec. 44:19-21.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15:473-&.

Parker, W.B., D.A. Somers, D.L. Wyse, R.A. Keith, J.D. Burton, J.W. Gronwald, and B.G. Gengenbach. 1990. Selection and characterization of sethoxydim tolerant maize tissu cultures. Plant Physiol. 92:1220-1225.

Parrot, W.A., and J.H. Bouton. 1990. Aluminum tolerance in alfalfa as expressed in tissure culture. Crop Sci. 30:387-389.

Podkowinski, J., G. Sroga, R. Haselkorn, and P. Gornicki. 1996. Structure of a gene encoding a cytosolic acetyl-CoA carboxylase of hexaploid wheat, pp. 1870-1874, Vol. 93. National Acad Sciences.

Raymer, P.L., S. K. Braman, L. L. Burpee, R. N. Carrow, Z. Chen, and T. R. Murphy. 2008. Seashore Paspalum: Breeding a Turfgrass for the Future. USGA Green Section Record:22-26.

Ruiter, R., I. Van Den Brande, E. Stals, S. Delaure, M. Cornelissen, and K. D'Halluin. 2003. Spontaneous mutation frequency in plants obscures the effect of chimeraplasty. Plant Mol. Biol. 53:715-729.

Sasaki, Y., T. Konishi, and Y. Nagano. 1995. The compartmentation of acetyl coenzyme-A carboxylase in plants. Plant Physiol. 108:445-449.

Sidhu, R., B. Hammond, R. Fuchs, J. Mutz, L. Holden, B. George, and T. Olson. 2000. Glyphosate-tolerant corn: The composition and feeding value of grain from glyphosate-tolerant corn is equivalent to that of conventional corn (Zea mays L.). J. Agric. Food Chem 48:2305-2312.

Somers, D.A. 1996. Aryloxyphenoxypropianate and cyclohexandione resistant crops, p. 175 - 188, *In* S. Duke, ed. Herbicide Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical aspects CRC Lewis publishers, . Boca Raton. US.

Zapiola, M.L., C.K. Campbell, M.D. Butler, and C.A. Mallory-Smith. 2008. Escape and establishment of transgenic glyphosate-resistant creeping bentgrass Agrostis stolonifera in Oregon, USA: a 4-year study. J. Appl. Ecol. 45:486-494.

TABLES AND FIGURES

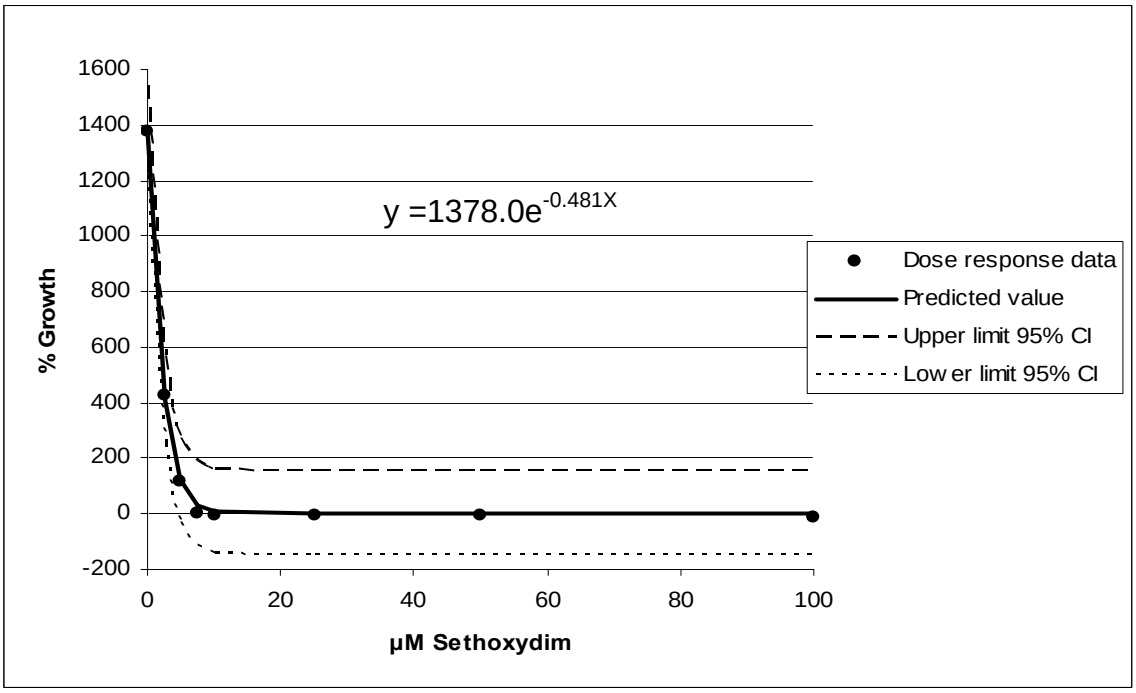

Figure 2.1. Response of seashore paspalum callus tissue to varying concentrations of sethoxydim in the medium.

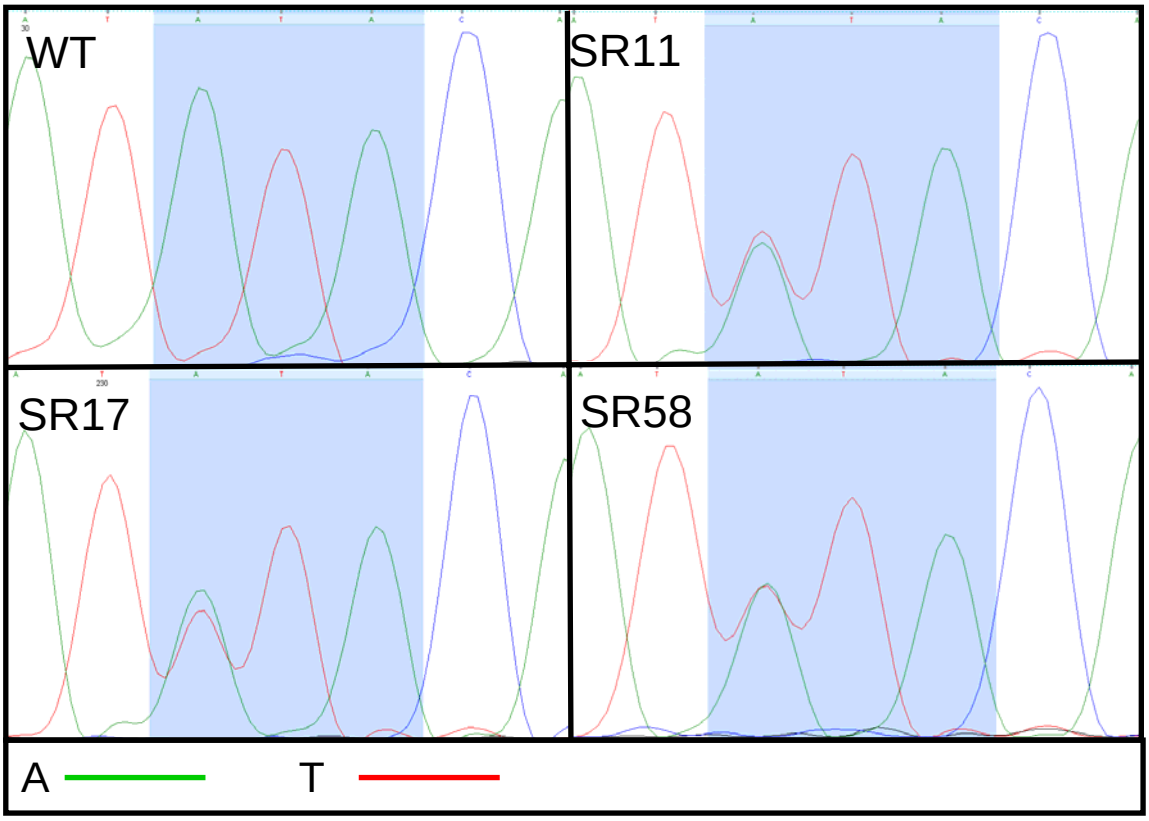

Figure 2.2. Chromatographs of WT and SR lines. The codon for ACCase 1781 aa is highlighted in blue. Since seashore paspalum is a diploid and the mutation occurred in one of the two alleles, the chromatograph shows an equal mix of mutated to non-mutated ACCase genes.

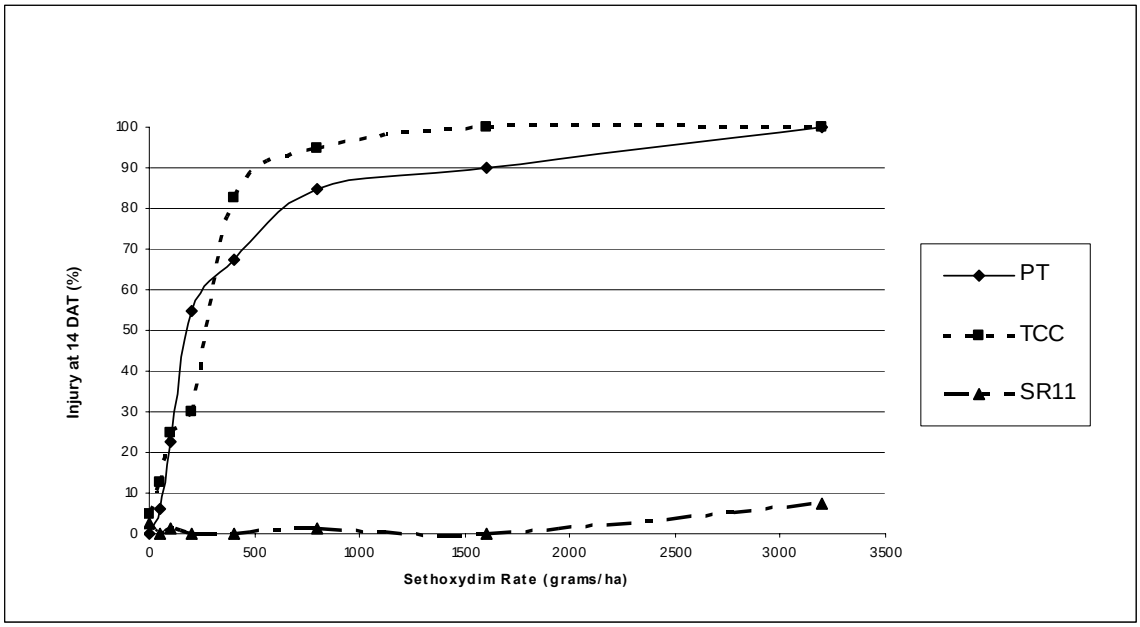

Figure 2.3. Whole-plant response of seashore paspalum to sethoxydim.

Figure 2.4. Whole-plant response to sethoxydim 14 DAT. MK – Mauna Kea parental type, TCC- Mauna Kea tissue-culture-derived control.

Table 2.1. Summary of cell lines which completed selection.

Cell Line	Genotype	Cell Line Initiation	Calli Through Selection	SR Calli	SR Regenerating	Positive for 1781 Mutation
1	Mauna Kea	28 Nov 07	225	0	0	0
2	Mauna Kea	5 Dec 07	1350	3	0	0
3	Mauna Kea	12 Dec 07	225	0	0	0
4	Mauna Kea	9 Jan 08	1125	0	0	0
5	Mauna Kea	12 Jan 08	2025	29	2	2
6	Mauna Kea	21 Jan 08	450	7	0	0
7	Mauna Kea	6 Mar 08	1350	2	0	0
8	Mauna Kea	20 Mar 08	675	0	0	0
9	Seaspray	12 Jan 08	225	0	0	0
10	03-527.8	8 Jan 08	1575	0	0	0
11	03-527.8	21 Jan 08	900	0	0	0
12	03-527.8	16 May 08	225	0	0	0
13	03.539.13	6 Mar 08	3825	11	0	0
14	03.539.13	13 Mar 08	1800	7	0	0
15	05-025-164	20 Mar 08	675	0	0	0
16	05-025-164	9 Apr 08	450	2	0	0
17	05-025-181	4 Mar 08	450	1	0	1
18	03-107C-1	4 Mar 08	450	0	0	0
19	03-098E-3	4 Mar 08	900	2	1	0
20	03-134F.17	4 Mar 08	225	0	0	0
21	03.525.22	20 Apr 08	1125	1	0	0
Total			20250	65	3	3

Table. 2.2. List of crops where herbicide resistance has been gained through *in vitro* selection techniques.

Crop	Species	Herbicide	Year	Reference
Tobacco	*Nicotiana tobacum* L.	Picloram	1978	(Chaleff and Parsons, 1978)
Tobacco	*Nicotiana tobacum* L.	Paraquat	1980	(Miller and Hughes, 1980)
Tobacco	*Nicotiana tobacum* L.	Chlorsulfuron	1984	(Chaleff and Ray, 1984)
Rape Seed	*Brassica napus* L.	Chlorsulfuron	1988	(Swanson et al., 1988)
Rape Seed	*Brassica napus* L.	Imidazolinone	1989	(Swanson et al., 1989)
Maize	*Zea mays* L.	Sethoxydim	1990	(Parker et al., 1990)
Maize	*Zea mays* L.	Imidazolinone	1991	(Newhouse et al., 1991)
Cotton	*Gossypium hirsutum* L.	Primsulfuron	1996	(Rajasekaran et al., 1996)
Sugarbeet	*Beta vulgaris* L.	Imidazolinone	1998	(Wright and Penner, 1998)

Table 2.3. Response of three genotypes of seashore paspalum to sethoxydim rate.

Herbicide Rate[1]	Plant Injury												Dry Weight		
	7 DAT[2]			14 DAT			21 DAT			28 DAT			42 DAT		
	PT	TC	SR11	PT	TC	SR11	PT	TC	SR11	PT	TC	SR11	PT	TC	SR11
grams	%												----- grams -----		
0	0.0a[3]	0.0a	1.7a	0.0a	5.0a	2.5a	0.0a	2.5a	2.5a	0.0a	0.0a	0.0a	2.1a	2.5a	1.9a
50	5.2a	4.5a	2.9a	6.2b	12.5ab	0.0b	5.0a	2.5a	1.2a	1.2a	0.0a	0.0a	1.6a	2.2a	1.7a
100	7.9b	18.3b	0.8a	22.5b	25.0b	1.2a	13.8a	20.0a	3.8a	6.2a	7.5a	2.5a	1.3b	1.6ab	2.0a
200	20.8b	16.7b	1.7a	55.0b	30.0b	0.0a	52.5b	40.0b	0.0a	43.8b	32.5b	0.0a	0.8b	0.4b	1.9a
400	30.8b	30.8b	3.8a	67.5b	82.5b	0.0a	67.5b	80.0b	2.5a	72.5b	82.5b	0.0a	0.2b	0.1b	2.0a
800	35.0b	60.0c	1.2a	85.0b	87.5b	1.2a	85.0b	95.0b	3.8a	88.8b	100.0c	1.2a	0.3b	0.1b	2.0a
1600	40.8b	43.3b	4.3a	90.0b	100.0c	0.0a	92.5b	100.0c	3.8a	92.5b	100.0b	1.2a	0.2b	0.1b	1.5a
3200	37.9b	46.7b	8.3a	100.0b	100.0b	7.5a	100.0b	100.0b	5.5a	100.0b	100.0b	4.2b	0.1b	0.1b	1.6a

1. Grams a.i. ha^{-1}
2. DAT = days after treatment.
3. Means on the same row (herbicide rate) and within a measured variable group (i.e. 7 DAT) followed by the same letter are not considered to be significantly different at 0.05 according to a protected LSD.

Table 2.4. Summary of ANOVA of seashore paspalum whole plant dose response to sethoxydim.

		Injury				Dry Weight
Source	Df	7 DAT[1]	14 DAT	21 DAT	28 DAT	42 DAT
Rep	3	7	65	60	140	0.03
Genotype	2	4214***	25980***	23961***	24118***	9.00***
Herb. Rate	7	1414***	6345***	7143***	8170***	3.32***
Genotype x Herb. Rate	14	372***	1877***	2014***	2325***	0.88***
ERROR	53	2003	4099	4346	3761	4.10

1. DAT = days after treatment.
*** Significant at 0.001 level of probability.

CHAPTER 3

CONCLUSIONS

The overall goal of this research was to obtain herbicide resistance in seashore paspalum. Transgenic methods were not used because of regulatory issues and subsequent international market limitations of genetically modified cultivars. The documented and well-characterized occurrence of the same single base pair mutation conferring sethoxydim resistance in several weedy grasses and the previous success in obtaining sethoxydim resistance in maize (Parker et al, 1990) provided encouragement that the desired mutation conferring sethoxydim resistance could occur in seashore paspalum. Published estimates of the mutation rate for such a single base pair change were 1 in 20 million (Ruiter et al., 2003). *In vitro* selection was chosen as a plausible approach to identify natural mutations occurring at very low frequencies because of its potential to efficiently screen extremely large numbers of cells.

After numerous setbacks and considerable efforts to develop and refine screening protocols and tissue culture methods, resistant cell lines were obtained in a relatively short period of time with reasonable effort. The low mutation rate previously reported (Ruiter et al., 2003) created a need for large amounts of tissue to be moved through the selection process. The use of 245 mm square bioassay plates during the selection process made the procedure relatively efficient. Sixty-five sethoxydim resistant (SR) cell lines were obtained out of 20,250 calli pieces screened for naturally occurring mutations. As previously described, approximately 5.2 billion cells were screened in this project. The frequency of obtaining a resistant callus was 1 SR line per 312 calli screened or one per 80 million cells. . The approximated frequency of obtaining an A to T mutation at the 1781 amino acid position of the chloroplastic ACCase gene was one per

1.74 billion cells. This is in sharp contrast to one mutation per 20million cells as reported in *Brassica napus* L. (Ruiter et al., 2003)

This research was successful in developing herbicide resistance in seashore paspalum. Herbicide resistance to sethoxydim and possibly other ACCase herbicides should greatly improve weed control of annual weedy grasses and provide a much needed tool for managing even troublesome perennial grasses such as bermudagrass. Herbicide resistant seashore paspalum could lead to greater adoption by golf courses and sports fields with interest in renovating existing bermudagrass courses and fields.

Further research is needed to characterize the nature of resistance in SR 31 and SR63 as well as to determine levels of cross resistance to other ACCase inhibiting herbicides for each of the three resistant lines developed through this research. Extensive field studies will be necessary to develop weed management strategies that will maximize the potential and longevity of this new weed control technology.

In vitro selection should also be practical as a means to develop herbicide resistance in other turfgrass species with established tissue culture and regeneration protocols. Finally, sethoxydim resistant seashore paspalum becomes the first herbicide-resistant turfgrass obtained without the use of genetic transformation. It may become the first commercially available herbicide-resistant turfgrass.

REFERENCES

Parker, W.B., D.A. Somers, D.L. Wyse, R.A. Keith, J.D. Burton, J.W. Gronwald, and B.G. Gengenbach. 1990. Selection and characterization of sethoxydim tolerant maize tissu cultures. Plant Physiol. 92:1220-1225.

Ruiter, R., I. Van Den Brande, E. Stals, S. Delaure, M. Cornelissen, and K. D'Halluin. 2003. Spontaneous mutation frequency in plants obscures the effect of chimeraplasty. Plant Mol. Biol. 53:715-729.