# In Vitro Selection of Sethoxydim-Resistant Creeping Bentgrass

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#### ABSTRACT

Creeping bentgrass (Agrostis stolonifera L.) is a fine-textured, cold-hardy turfgrass used by the golf industry in the transition zone and farther north. Grassy weeds are a recurrent problem for seed producers and golf course managers. Herbicide-resistant creeping bentgrass would provide the seed producer and golf course manager with an additional tool to control grassy weeds. In the present study, in vitro selection was used to obtain mutants of creeping bentgrass via somaclonal variation that were resistant to the acetyl coenzyme A carboxylase (ACCase) inhibitor, sethoxydim {2-[1-(ethoxyimino] butyl)-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one}. Selection was imposed on creeping bentgrass calli (total of 13,725) by growing them on induction medium containing 10 µM sethoxydim. Four independent sethoxydimresistant calli were identified and subsequently sequenced toconfirm the presence of an A to C mutation in the first position of the 1781 amino acid codon causing an isoleucine to leucine substitution. Whole plant dose response experiments showed the sethoxydim-resistant event, SR1, was resistant to sethoxydim at rates of >3200 g a.i. ha<sup>-1</sup>. SR1 also showed cross resistance to the ACCase-inhibitor herbicide, fenoxaprop at rates of > 800 g a.i. ha<sup>-1</sup>.

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**Abbreviations:** ACCase, acetyl coenzyme A carboxylase; APP, aryloxyphenoxypropionate; bp, base pairs; CHD, cyclohexandione; CT, carboxyl transferase; DAT, days after treatment; Ile, isoleucine; Leu, leucine; SR, sethoxydim resistant; PT, parental type.

**C**REEPING BENTGRASS is a cool season turfgrass used widely around the United States for golf course greens, tees, and fairways. Weed infestation is a recurrent problem for golf course managers. Broadleaf weeds can be controlled through application of selective herbicides, such as 2,4-D (2,4-dichlorophenoxyacetic acid). In contrast, control of weedy grasses is limited to mechanical control and application of non-selective herbicides. The development of herbicide-resistant creeping bentgrass would allow the end user to maintain a more aestheticly pleasing turf and seed producers to maintain better seed purity.

Acetyl-coenzyme A carboxylase inhibitors are grass-specific herbicides used to control weedy grasses in broadleaf crops. Overreliance on ACCase-inhibitors in cropping systems has resulted in numerous weedy species developing resistance to these herbicides, due to mutations in the ACCase gene (Delye and Michel, 2005). Resistance to ACCase-inhibiting herbicides has been purposely obtained in maize (*Zea mays* L.) (Parker et al., 1990) and in seashore paspalum (*Paspalum vaginatum* Sw.) (Heckart et al., 2010) by in vitro selection.

Currently, there are eight known mutations in ACCase that confer resistance to these herbicides (Powles and Yu, 2010). The

Published in Crop Sci. 56:1-6 (2016).

doi: 10.2135/cropsci2015.04.0208

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most commonly reported mutation is a single isoleucine (Ile) to leucine (Leu) substitution at amino acid position 1781 in the carboxyl transferase (CT) domain of the ACCase protein (Delye and Michel, 2005). Other known mutations in the ACCase CT domain are located at 1999, 2027, 2041, 2078, 2088, and 2096 amino acid residue positions. Whole-plant screening for herbicide-resistant bermudagrass (*Cynodon dactylon* L.) revealed a genotype resistant to ACCase inhibitors that did not possess any of the previously mentioned mutations (Grimshaw et al., 2014). The authors suggested that a metabolic response or a reduced herbicide uptake mechanism may be responsible for expression of herbicide resistance in this genotype.

The current study focuses on two groups of ACCase inhibitors, the cyclohexanediones (CHD) and the aryloxyphenoxypropionates (APP). The CHD sethoxydim is used to control perennial and annual grasses in agricultural and ornamental crops. The goals of the research reported here were to select for naturally occurring sethoxydim-resistant creeping bentgrass mutants using in vitro selection of callus tissue, and to regenerate novel plant suitable for use in breeding efforts to develop herbicide-resistant cultivars.

## MATERIALS AND METHODS Plant Material

Explant tissue used for callus induction were surface sterilized seeds of genotypes Penn A-4, Penn A-1, Penncross, Crenshaw, Pick 96-2, and L93creeping bentgrass. Seeds were surface sterilized using a 10% v/v solution of Clorox with several drops of Tween-20, rinsed three times with sterile water, and placed on modified MS callus induction medium, as described by Wang et al. (2003). All media were brought to pH 5.8, solidified with 2.5 g L<sup>-1</sup> Gelzan (Caisson Laboratories, North Logan, UT), and autoclaved. Induction medium was poured into 100 × 15 mm Petri dishes. Approximately 100 seeds were placed on each plate and sealed with 3 M Micropore tape (St. Paul, MN). Seeds were placed in the dark at 27°C and induced callus tissue was subcultured every 3 wk.

#### **Dose Response**

The response of bentgrass callus tissue to sethoxydim concentration was determined using Penn A-4 callus tissue.. Laboratory-grade sethoxydim (Chemservice Inc. West Chester, PA) was diluted with methanol to 1 mg mL<sup>-1</sup>. Sethoxydim selection medium consisted of callus induction medium supplemented with 0, 2.5, 5, 7.5, or 10 µM sethoxydim filter sterilized and added following autoclaving. Each dose level was replicated seven times and each plate contained 0.25 g of callus tissue. Each Petri dish contained 16 equal pieces of callus that were plated in a four by four pattern. Calli were weighed and 3-wk intervals and 0.25 g of the callus was subcultured to fresh medium. The initial weight was subtracted from the weight at 3 wk and then divided by the initial weight to obtain the fold growth change (Parrott and Bouton, 1990). Fold growth measurements were modeled using a negative exponential decay function using nonlinear regression (SAS Institute, 2008).



Figure 1. Bentgrass calli growing of selection media containing 10  $\mu$ M sethoxydim. Arrow indicates sethoxydim-resistant callus.

#### Selection of Sethoxydim-resistant Cells

Four-month-old callus tissue was subcultured onto selection medium containing10  $\mu$ M sethoxydim. Two-hundred and twenty-five calli pieces, each approximately 4 mm in diameter, were placed in 245 by 245 mm square bioassay dishes (Corning, Corning, NY) in a 15 by 15 grid. After 3 wk, the calli were subcultured to fresh plates, and the process was repeated three times. Resistant calli (Fig. 1) were increased by subculturing for an additional 3 wk on 10  $\mu$ M sethoxydim.

Callus of each sethoxydim-resistant (SR) line was divided into 4-mm pieces and placed on shoot initiation medium (Wang et al., 2003). The plates containing the calli were transferred to a growth chamber at 25°C with a 1-h dark, 23-h light photoperiod, and a light intensity of 66 to 95  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>. Shoots were subcultured on MS basal medium without growth regulators to permit root formation. Rooted plants regenerated from each cell line were removed from the medium and placed in pots containing a 1:1 mix of Fafard 3B (Sun Gro Horticulture, Agawam, MS) mix and sand. Potted plants were acclimated in a growth chamber for 1 wk at 25°C with a 8-h dark, 16-h light photoperiod, and a light intensity of 66 to 95  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>, then transferred to a greenhouse at 24°C to 32°C with a 10-h light, 14-h dark photoperiod.

#### **Whole Plant Evaluation**

DNA was extracted from leaves of regenerated plants via the CTAB method as described by Lassner et al. (1989). Previously published primers were used to amplify regions around the known locations of single-nucleotide-polymorphisms (SNPs) that confer resistance to ACCase-inhibiting herbicides (Heckart et al., 2010).

Sethoxydim-resistant plants were regenerated from each of four SR events. Only plants from the first resistant event, SR1, were analyzed for whole-plant resistance, because all events possessed the same resistance mutation in ACCase. All plants used in dose response experiments appeared uniform in growth and development and were regenerated from a single cell line (SR1) tracing back to/a single SR callus. Dose-response experiments were conducted in a greenhouse using four ACCase-inhibiting herbicides. These were two CHD herbicides, sethoxydim and cycloxydim (Focus Ultra, BASF, Research Triangle Park,



Figure 2. Response of creeping bentgrass callus tissue after 9 wk on selection medium containing varying concentrations of sethoxydim. Callus tissue was derived from seeds of Penn A-4, and 0.25 g of callus was placed on each plate. Calli were transferred after 3 wk and underwent three cycles of selection. Data from five replicate plates for each sethoxydim concentration were modeled using and exponential decay function.

NC) and two APP herbicides fluazifop-p-butyl (Fusilade II, Syngenta Crop Protection, Inc., Greensboro, NC) and fenoxaprop-p-ethyl (Acclaim Extra, Bayer Environmental Science, Montvale, NJ). The SR1 plants were compared to plants from the susceptible, original parental line, Penn A-4. Rooted plants were transferred to 4 by 14 cm Cone-Tainers (Stuewe and Sons Inc., Corvallis, Oregon) with a 1:1 mix of Fafard 3B mix (Sungro Horticulture, Agawam, MA) and sand. Cone-Tainers were placed under high pressure sodium lights in a greenhouse with a 16-h photoperiod maintained at 27/32°C day/night. Herbicides were applied at a spray volume of 187 L ha<sup>-1</sup> in a spray chamber. Each dose response experiment was a  $2 \times 8$  factorial with two genotypes and eight herbicide rates including: 0, 50, 100, 200, 400, 800, 1600, and 3200 g a.i. ha<sup>-1</sup>. Treatments were arranged in a randomized complete block design with four replications. Following treatment with herbicides, plants were allowed to dry and returned to the greenhouse. Visual estimates of crop injury were recorded at 21 and 28 d after treatment (DAT) and dose-response experiments were repeated twice. Injury was rated using a scale of 0 to 100%, where 0 equals no injury and 100 equals complete death. Data for all whole plant response experiments were analyzed using a three-way analysis of variance (SAS Institute, 2008) Factors were DAT, experiment, and genotype in response to each herbicide and were considered significant at  $p \leq 0.05$ . Means were separated with a protected Fisher's LSD if a factor was found to be significant. The dose response data for Penn A-4 were fitted to a Mitscherlich model (Grey et al., 2006), and SR1 injury data were fitted to a linear model for response to each herbicide.

## RESULTS

#### Callus Dose Response

Figure 2 shows callus response to varying concentrations of sethoxydim. A sethoxydim concentration of 2.5 to 5  $\mu$ M greatly reduced callus growth. There was no callus growth at 7.5  $\mu$ M, and this level may have been adequate for selection; however, previous experience with in vitro

 Table 1. Summary of creeping bentgrass cell lines completing in vitro selection for sethoxydim resistance.

Genotype	Calli through selection <sup>†</sup>	SR Calli <sup>‡</sup>	SR calli regenerated§	Calli positive for leucine 1781
Penn A-4	12,825	4	4	4
Penn A-1	675	0	0	0
Penncross	225	0	0	0
Pick 96-2	0	0	0	0
L93	0	0	0	0
Crenshaw	0	0	0	0

<sup>+</sup>Number of calli pieces completing three 3-wk cycles of selection for sethoxydim resistance.

<sup>‡</sup> Number of calli demonstrating exponential growth after three cycles of selection, meaning they are sethoxydim resistant.

<sup>§</sup>Number of sethoxydim-resistant calli producing viable plants after more than 4 wk on regeneration medium.

selection of seashore paspalum (Heckart et al., 2010) has shown that increasing the concentration to 10  $\mu$ M will prevent escapes. Data from the dose-response experiment were fitted to a negative exponential decay function. The regression equation was  $\gamma = (\beta_0 e^{-\beta_1 X}) = (12.5 \exp^{-1.25 X})$ .

## Selection of Sethoxydim-Resistant Cells

Three out of six cultivars formed calli on callus induction medium (Table 1). Enough callus was obtained from Penn A-4 to obtain 57 plates, each containing 225 4-mm-diam. calli (12,825 total). In contrast only 675 and 225 calli completed the selection process for Penn A-1 and Penncross, respectively. During the third cycle of selection, four resistant calli were selected from Penn A-4. These were subcultured on callus induction medium supplemented with 10  $\mu$ M sethoxydim for an additional 3 wk to confirm resistance and to increase callus for regeneration. Sequencing confirmed the transversion of A (adenine) to C (cytosine) at position 1781 causing an Ile to Leu substitution in all four lines (Fig. 3).



Figure 3. Alignment of polymerase chain reaction (PCR)-amplified sequences from wild-type (WT) Penn A-4 and sethoxydim-resistant events SR1–4. Creeping bentgrass has multiple alleles; therefore, a mutation is indicated by a double peak at the first position of the 1781 codon. The A to C mutation causes an isoleucine to leucine substitution, known to confer resistance to ACCase-inhibiting herbicides. All four cell lines appear to be heterozygous for the mutation.

### Whole Plant Response to Acetyl Coenzyme A-Inhibiting Herbicides

All four SR lines possessed the same nucleotide mutation; therefore, only SR1 was compared to the parental Penn A-4. Comparisons among visual injury estimates taken at 21 and 28 DAT were not significantly different. Likewise, there were no significant differences between repetitions of the dose response. Since DAT and replication were not significant, data were combined for each genotype at each rate. Conversely, comparisons between genotype and visual injury ratings showed significant differences between means of each genotype for all herbicides.

Figure 4 summarizes the results of four dose-response experiments using sethoxydim as well as three other ACCase inhibiting herbicides. The response of SR1 was compared to that of the naturally SR centipedegrass (Ermochloa ophiuroides Munro.). The 1X rate of application of sethoxydim on seedling centipedegrass is 210 g a.i. ha<sup>-1</sup>. The SR1 demonstrated a high level of SR with only minimal injury (8.4%) at a 15X rate (Fig. 4 and 5). The  $LD_{50}$  for SR1 was >15X the field rate, compared to that of the parental line, which had an LD<sub>50</sub> between 2X and 4X. The cycloxydim 1X rate was determined by the highest rate labeled (200 g a.i. ha<sup>-1</sup>) to control weedy grasses. Cycloxydim injury to SR1 plants was lower than that of Penn A-4 at all rates from 0.25X to 16X; however, injury at 16X was substantial, with 51.25% injury to the plant. Fluazifop is labeled to control weedy grasses at 280 g a.i. ha<sup>-1</sup> (1X). The SR1 injury was lower when compared to that of Penn A-4 at all fluazifop concentrations, but injury level was above 50% at the 11X rate. Fenoxaprop

experiments showed that injury to SR1plants did not exceed 20% when treated with a 16X rate.

### DISCUSSION

In all, 13,725 calli were screened, resulting in four SR cell lines that regenerated into whole plants. Sequencing revealed a previously unreported nucleotide mutation of A to C at the first position of the 1781 codon, resulting in an amino acid residue substitution of Ile to Leu. While this nucleotide mutation is unique in creeping bentgrass, it results in the same Ile to Leu substitution caused by the A to T mutation known in seashore paspalum and in several weedy species.

Genetic studies of inheritance of this mutation were not conducted on this plant material, however, our work on seashore paspalum (Raymer, unpublished data, 2014) and published work by Wang and Darmency (1997) on foxtail millet (*Setaria viridis* L. P. Beauv.) indicate this mutation is heritable as a single, completely dominate, nuclear gene.

This research demonstrates an efficient method for obtaining a trait conferring resistance to ACCase herbicides in a species not known to possess such a trait. A number of mutations that confer resistance to ACCase herbicides have been reported to arise naturally in several weedy grasses (Powles and Yu, 2010; Delye and Michel, 2005). This implies that selection for resistance to ACCase herbicides in planta (whole plant level) may be possible. However, a mutation arising in planta can only be passed on to the next generation if that mutation arises in a germ line cell (pollen or egg), whereas in tissue culture, any callus cell that mutates can give rise to a whole plant. Heckart et al. (2010) when using in vitro selection to



Figure 4. Whole-plant response of first sethoxydim-resistant event (SR1) and parent type Penn A-4 to four acetyl coenzyme A carboxylase (ACCase)-inhibiting herbicides. Measurements were taken at 21 and 28 d after treatment and herbicide dose experiments were repeated twice, excluding cycloxydim, for which only one experiment was performed. Dose response for Penn A-4 was modeled using a Mitscherlich model and response of SR1 was fitted to a linear model.



Figure 5. Whole-plant response of the first sethoxydim-resistant event (SR1) compared to the parental type Penn A-4 to different rates of sethoxydim ranging from 0 to 3200 g a.i. ha<sup>-1</sup>. Plants were evaluated 28 d after treatment following foliar application of sethoxydim.

obtain SR in seashore paspalum, reported the frequency of an A to T mutation at the first position of the 1781 codon at 1 in 1.3 billion. If we assume the A to C mutation reported here has a similarly low frequency, selection using tissue culture seems a far more efficient approach than in planta. Although this novel mutation could allow control of many weedy grasses in creeping bentgrass, two of the major weeds are annual bluegrass (Poa annua L.) and red fescue (Festuca rubra L.), which also possess the 1781 Leu mutation (Delye and Michel, 2005). The herbicide ethofumesate (Prograss, Bayer CropScience, LP, Research Triangle Park, NC) has been labeled for control of annual bluegrass in creeping bentgrass. Testing of the experimental herbicide, methiozoline (PoaCure, Moghu Research, Daejeon, Korea), on golf course greens in Georgia has shown methiozolin to be highly effective for control of annual bluegrass with minimal turf injury (McCullough et al., 2013). Conversely, creeping bentgrass can also be considered a weed if found in a seed-production field or putting green of another species. Treatment with herbicides with other mechanisms of action, including glyphosate, will still control SR bentgrass.

In summary, creeping bentgrass with high levels of SR was selected in vitro. This creeping bentgrass has cross resistance to other ACCase-inhibiting herbicides, thus permitting flexibility in weed control systems.

#### Acknowledgments

This research was supported by funding from the Georgia Agricultural Experiment Stations and grants from The Univ. of Georgia Cultivar Development Research Program and BASF Plant Sciences L.L.C. We thank Jerry Davis (University of Georgia) for assistance with statistical analysis of dose response data and the staff of the Parrott and Raymer Labs for their technical assistance.

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