Chapter III

Characterization of ALS Resistance in Several Smooth Pigweed Biotypes

Abstract: Experiments were conducted to identify acetolactate synthase (ALS, EC 2.2.1.6 [formerly EC 4.1.3.18]) mutation sites in eight biotypes of smooth pigweed and correlate these mutations with patterns of herbicide cross-resistance. ALS-inhibiting herbicide-resistant smooth pigweed biotypes (R5, R6, R7, R8) collected from fields in Virginia, Delaware, and Maryland and one susceptible smooth pigweed biotype (S) were analyzed for response to postemergence imazethapyr, pyrithiobac, chlorimuron, thifensulfuron, and cloransulam applications. All R biotypes were 261- to 537-fold resistant to imazethapyr and 29- to 88-fold resistant to pyrithiobac. Resistant biotypes also had reduced sensitivity to chlorimuron and thifensulfuron of 2- to 14-fold and 10- to 25-fold, respectively, relative to the S biotype. Biotypes R6, R7, and R8 had reduced sensitivity of 3- to 10-fold to cloransulam relative to the S biotype, whereas R5 had increased sensitivity. All of these biotypes were found to have a serine to asparagine substitution at amino acid position 653, as numbered relative to the protein sequence of *Arabidopsis thaliana*. Four other imidazolinone (IMI)-resistant smooth pigweed biotypes (R1, R2, R3, R4) collected from fields in Somerset County, MD, that had been previously characterized at the whole-plant level with high-level resistance to IMI herbicides, increased sensitivity to PTB and TP herbicides, and low to no cross-resistance to SU herbicides, were found to have an alanine to threonine substitution at position 122.
Nomenclature: Chlorimuron; cloransulam; imazethapyr; pyrithiobac; thifensulfuron; *Amaranthus hybridus* L. AMACH, smooth pigweed.
Key words: Acetolactate synthase, ALS, cross-resistance, herbicide resistance.

Introduction

Acetolactate synthase, the first common enzyme in the biosynthetic pathway of the branched-chain amino acids, valine, leucine, and isoleucine (Durner et al. 1990), is the primary target of more than 50 commercial herbicides spanning five structurally distinct classes of chemicals (Heap 2004). These classes include the imidazolinone (IMI) (Shaner et al. 1984), sulfonylurea (SU) (Chaleff and Muvais 1984),

pyrimidinylthiobenzoate (PTB) (Stidham et al. 1999), triazolopyrimidine sulfonanilide (TP) (Gerwick et al. 1990), and sulfonylaminocarbonyltriazolinone (Santel et al. 1999) herbicides. ALS herbicides are the major mode of action group in use today (Heap 2004), primarily due to their high efficacy in a large number of crops, low application rates, low mammalian toxicity, and environmental safety (Saari et al. 1994). However, the high efficacy specific for a single target enzyme and repeated widespread use of these classes has led to the selection of many ALS-resistant weed species.

There are currently 86 weed species, including monocots and dicots, documented with resistance to one or multiple ALS-inhibiting herbicide classes (Heap 2004). In most cases, ALS-inhibitor resistance has resulted from an altered ALS enzyme with reduced sensitivity to the herbicides (Saari et al. 1994), but enhanced rates of ALS-inhibiting herbicide metabolism have also been reported (Christopher et al. 1991, 1992; Menendez et al. 1997; Veldhuis et al. 2000). Investigations of target site based ALS-inhibitor resistance in field-selected weed biotypes have revealed single amino acid substitutions in one of six positions along the ALS gene (Shaner 1999, Whaley et al. 2004). These six conserved amino acids are Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Trp_{574}, and Ser_{653}, using ALS of *Arabidopsis thaliana* (Sathasivan et al. 1990) as a reference for amino acid numbering.

Variable patterns of cross-resistance between ALS-inhibitor classes have occurred depending on the amino acid position affected and the specific substitution (Shaner 1999). For example, an amino acid change of Ala_{122} or Ser_{653} conferred resistance to IMI herbicides with no or low-level cross-resistance to SU herbicides (Bernasconi et al. 1995; Devine and Eberlein 1997; Patzoldt and Tranel 2001), whereas substitution of Pro_{197} conferred resistance to SU herbicides (Guttieri et al. 1992) with low or no-cross resistance to IMI herbicides. Substitution of Trp_{574} or Ala_{205} has conferred broad cross-resistance (Bernasconi et al. 1995; Woodworth et al 1996); however, substitutions of Ala_{205} have conferred much lower levels of resistance than Trp_{574}.

Some of the best characterized ALS-inhibitor resistant species are members of the *Amaranthus* family. *Amaranthus* species are very sensitive to ALS-inhibiting herbicides and possess characteristics that predispose them to have herbicide resistant biotypes such as high genetic variability, prolific seed production, and efficient pollen and seed distribution (Lovell et al. 1996). Smooth pigweed biotypes with resistance to ALS-

inhibiting herbicides have been reported with amino acid substitutions at $Ala_{122,}$ Asp_{376}, and Trp_{574} of the ALS gene (Schmenk et al. 1997; Tranel et al. 2004; Whaley 2005).

ALS-inhibiting herbicides are a major component of weed management programs worldwide; however, development of resistance to this herbicide group has had a negative impact on their use. Characterization of field-selected ALS-inhibitor resistant weed biotypes provides information useful in the development of strategies to manage and prevent resistance to these important herbicides. The objectives of this research were: (1) to characterize the response of suspected ALS-inhibitor resistant smooth pigweed biotypes (R5, R6, R7, R8) from Virginia, Delaware, and Maryland and an ALS-inhibitor susceptible biotype (S) to herbicide representatives of IMI, PTB, SU, and TP herbicide classes and (2) to determine the mechanism and molecular basis for resistance of these four biotypes and four resistant biotypes (R1, R2, R3, R4) previously characterized by Poston et al. (2000).

Materials and Methods

Seed Sources

Seed from smooth pigweed biotypes with suspected resistance to ALS-inhibiting herbicides was collected in Virginia, Delaware, and Maryland from four separate fields with a history of repeated ALS-inhibiting herbicide use. Biotypes were labeled R5, R6, R7, and R8. Farm records indicated that R5, R6, R7, and R8 biotypes were selected with a continuous use of imazethapyr. Seed was collected from approximately 40 different plants at each location that had survived imazethapyr treatment. Seed from the S biotype was collected from a field at the Eastern Shore Agricultural Research and Extension Center near Painter, VA that had no history of ALS-inhibitor herbicide use. Seed was stored at 4 C. Seed from R1, R2, R3, and R4 smooth pigweed biotypes were collected as described by Poston et al. (2000).

Dose-Response Experiments

Greenhouse experiments were conducted in 2000, 2002, and 2003 to evaluate the response of R5, R6, R7, R8, and S to four classes of ALS-inhibiting herbicides. Seed were sown into separate 43- by 53-cm greenhouse flats containing potting medium.[1]

Three evenly sized seedlings with one true leaf were transplanted into 11.4- by 11.4-cm pots containing potting medium. Plants were maintained in the greenhouse under natural sunlight and sprinkler irrigation, and were fertilized weekly with a complete fertilizer.[2]

ALS-inhibiting herbicide rates were based on a logarithmic scale of 0.01, 0.1, 1, 10, and 100 times the registered postemergence commercial use rate. Herbicides and rates were: imazethapyr at 0.7, 7, 70, 700, and 7000 g ai ha^{-1}; pyrithiobac at 0.7, 7, 70, 700, and 7,000 g ai ha^{-1}; chlorimuron at 0.09, 0.9, 9, 90, and 900 g ai ha^{-1}; thifensulfuron at 0.045, 0.45, 4.5, 45, and 450 g ai ha^{-1}; and cloransulam at 0.18, 1.8, 18, 180, and 1,800 g ai ha^{-1}. All herbicide treatments included 0.25% v/v non-ionic surfactant.[3] Herbicides were applied to 4-8 cm smooth pigweed plants with four to six true leaves using a compressed air, moving nozzle, cabinet sprayer equipped with one 8002EVS[4] nozzle and calibrated to deliver 171 L/ha at 289 kPa. Above-ground biomass from the three plants in each pot was harvested 21 d after treatment (DAT) and dried at 65 C for 72 h. Dry biomass data were expressed as a percentage of the nontreated control within each biotype.

Experiments were arranged in a completely randomized design with four replications and were repeated three times. Dry biomass data expressed as a percentage of the nontreated control were subjected to ANOVA to test for treatment by run interactions. No significant interactions were present so data were pooled over repeated experiments. Non-linear regression was used to generate herbicide dose-response curves of the resistant and susceptible biotype for each herbicide. The herbicide-dose required for 50% growth reduction (GR_{50}) was calculated from regression equations. Resistance ratios, GR_{50} resistant / GR_{50} susceptible, were calculated to indicate the level of resistance. Differences in dry biomass reductions between biotypes were subjected to ANOVA and treatment means within a given herbicide rate were separated using Fisher's LSD test at P = 0.05 significance level.

DNA Analysis

Plant Materials

ALS genes were sequenced to determine the molecular basis for resistance. Eight resistant (R1, R2, R3, R4, R5, R6, R7, R8) smooth pigweed biotypes and one S biotype

were grown as described above. All plants were treated at the four- to five-leaf stage with imazethapyr at 70 g ha^{-1} plus a non-ionic surfactant at 0.25% v/v to verify resistance, and all responded similarly, indicating homogenous populations. Young leaf tissue (120 mg) was harvested from treated R plants 14 DAT and from nontreated S plants for genomic DNA isolation.

Isolation and Sequencing

Genomic DNA was isolated using a Qiagen DNeasy Plant Mini Kit.[5] Two polymerase chain reaction (PCR) primers were designed from *Amaranthus spp*. nucleotide sequence (GeneBank Accession number; U55852) to amplify an approximately 2 kb section of DNA from each smooth pigweed biotype. Primers used for amplification were Forward 1 and Reverse 1 listed in Table 3.1. Each PCR reaction contained 25 ng genomic DNA, 12 µM of each forward and reverse primer, 20 mM dNTPs, 0.5 µl *PfuTurbo*® DNA polymerase[6] with 1x concentration of supplied buffer in a final volume of 25 µl. PCR reactions were subjected to a 5-min denaturation at 94 C; 30 cycles of 1 min at 94 C, 1.5 min at 67 C, and 2 min at 72 C; then a final 5 min at 72 C.

PCR products were purified by gel electrophoresis followed by a Qiagen Gel Purification Kit[5] and sequenced directly. Sequencing reactions included 50 ng of DNA from the gel-purified band, 2 µM of primer, and 4 µl ABI Prism® Big Dye™ Terminator[7] (v. 3.0) reagent in a final volume of 15 µl. Eight separate reactions were conducted for each biotype, including four forward and four reverse primers to ensure complete coverage of both DNA strands (Table 3.1). Sequencing gels were run by the Core Laboratory Facility at the Virginia Bioinformatics Institute[8] using an ABI 377 automated sequencer. Sequences were aligned and compared using Sequencher™ 3.1 software.[9]

Results and Discussion

Whole Plant Response

Dose-response experiments were conducted in the greenhouse with representative herbicides from four ALS-inhibitor classes to confirm resistance and to determine cross-resistance patterns for R5, R6, R7, and R8 smooth pigweed biotypes. All R biotypes were between 261- to 537-fold resistant to imazethapyr relative to the S biotype (Table

3.2). Imazethapyr applied at the commercial POST rate of 70 g ha^{-1} reduced dry biomass in the S biotype 97% while R6, R7, R8, and R9 biotypes all responded similarly with a biomass reduction of 20 to 34% (Table 3.3). Approximately 0.9 g ha^{-1} imazethapyr was required to reduce dry biomass 50% in the S biotype compared to more than 234 g ha^{-1} in all R biotypes (Table 3.4). Poston et al. (2000) reported high-level resistance to imazethapyr with R1, R2, R3, and R4 smooth pigweed biotypes from 730- to 1,350-fold relative to an S biotype.

All R biotypes were 29- to 88-fold resistant to pyrithiobac relative to the S biotype (Table 3.2). Pyrithiobac applied at the commercial use rate of 70 g ha^{-1} reduced dry biomass 98% in the S biotype while dry biomass was reduced 75, 69, 59, and 56% in R5, R6, R7, and R8 biotypes, respectively (Table 3.3). Approximately 0.9 g ha^{-1} pyrithiobac was required to reduce dry biomass in the S biotype 50%, whereas approximately 30, 26, 72, and 79 g ha^{-1} was required in R5, R6, R7, and R8 biotypes, respectively (Table 3.4). Poston et al. (2000) reported that R1, R2, R3, and R4 smooth pigweed biotypes exhibited increased sensitivity to pyrithiobac relative to an S biotype, particularly at low herbicide concentrations.

Smooth pigweed biotypes R5, R6, R7, and R8 were less than 15-fold and 26-fold resistant to the SU herbicides, chlorimuron and thifensulfuron, respectively, and differential responses compared to the S biotype were more evident at herbicide rates lower than the commercial use rate (Table 3.2). At 0.9 g ha^{-1} chlorimuron, dry biomass of R6, R7, and R8 was reduced 40 to 45% compared to 77% dry biomass reduction in the S biotype (Table 3.3). Biomass reduction of R5 was 63% at 0.9 g ha^{-1} chlorimuron, but was not significantly different than biomass reduction of the S biotype. Biotype S, R5, R6, and R7 dry biomass was reduced 84 to 97% at 9 g ha^{-1} chlorimuron, whereas R8 biomass was reduced 65%. Based on resistance ratios, R5, R6, R7, and R8 biotypes were approximately 2-, 6-, 7-, and 14-fold more resistant to chlorimuron, respectively, relative to the S biotype (Table 3.2). Poston et al. (2000) reported smooth pigweed biotypes R1, R2, R3, and R4 were 4.7- to 7-fold more resistant to chlorimuron than an S biotype, however these biotypes were approximately 1.4- to 3.3-fold more sensitive to thifensulfuron than an S biotype. Manley et al. (1998) also characterized an IMI-resistant smooth pigweed biotype with variable cross-resistance to SU herbicides. This biotype

displayed low-level cross-resistance to chlorimuron, and resistance to rimsulfuron, but was effectively controlled by several other SU herbicides.

Biotypes R5, R6, R7, and R8 were 10-, 25-, 15-, and 20-fold resistant to thifensulfuron, respectively, compared to the S biotype (Table 3.2). At 0.45 g ha^{-1} thifensulfuron, biomass of biotypes R6, R7, and R8 was reduced 50 to 58% compared to a biomass reduction of 82% in the S biotype (Table 3.3). Although not significantly different than the S biotype, R5 biomass was reduced 66%. At the commercial use rate of 4.5 g ha^{-1} thifensulfuron, biomass of all R biotypes and the S biotype was reduced 91 to 98%.

Biomass reduction of all biotypes was 78% or less from the commercial use rate of cloransulam (18 g ha^{-1}) (Table 3.3). Other research has reported similar levels of smooth pigweed control in the greenhouse with cloransulam (Nelson and Renner 1998; Poston et al. 2000). With cloransulam at 1.8 g ha^{-1}, biomass of R6, R7, and R8 biotypes was reduced similarly to the S biotype at 33 to 46%, whereas R5 biomass was reduced 63%. At 18 g ha^{-1} cloransulam, biomass of R6, R7, and R8 was reduced 36 to 53%, whereas biomass of R5 and S was reduced 77 and 64%, respectively. Resistance ratios indicated that R6, R7, and R8 were 3-, 6-, and 10-fold resistant to cloransulam relative to the S biotype (Table 3.2). In contrast, R5 was more sensitive to cloransulam than the S biotype. Poston et al. (2000) also reported increased sensitivity to cloransulam in R1, R2, R3, and R4 smooth pigweed biotypes relative to an S biotype. Further investigations concluded that the increased sensitivity of R2 to cloransulam was due to a more sensitive ALS enzyme compared to an S biotype rather than a difference in cloransulam absorption, translocation, or metabolism rates (Poston et al. 2001).

Molecular Basis of Resistance

Results from the dose-response experiments confirmed that R5, R6, R7, and R8 were resistant to ALS-inhibiting herbicides and exhibited levels of resistance to imazethapyr and pyrithiobac indicative of an altered ALS enzyme. In other research, ALS resistant smooth pigweed biotypes R1, R2, R3, and R4 were also characterized as having high-levels of resistance to imazethapyr, but without cross-resistance to pyrithiobac (Poston et al. 2000). To identify the molecular basis of resistance in the eight ALS-inhibitor

resistant smooth pigweed biotypes (R1, R2, R3, R4, R5, R6, R7, R8), an 1881-bp coding region that encoded 627 amino acids from the ALS gene of R and S biotypes was sequenced and compared. This region included the six positions where mutations have been reported to confer ALS-inhibitor resistance in other plant species. Only one nucleotide difference existed between all R biotypes and the S biotype, which resulted in single amino acid changes at positions previously reported to confer ALS-inhibiting herbicide resistance. Biotypes R1, R2, R3, and R4 all had an amino acid substitution of alanine (GCA codon) to threonine (ACA codon) at position 122, whereas R5, R6, R7, and R8 all had an amino acid substitution of serine (AGC codon) to asparagine (AAC codon) at positon 653. These results are consistent with the hypothesis that ALS-inhibitor resistance in the eight smooth pigweed biotypes is due to an altered ALS enzyme.

The four smooth pigweed biotypes (R1, R2, R3, R4) with an Ala_{122} to Thr substitution displayed high-level resistance to IMI herbicides, increased sensitivity to PTB and TP herbicides, and low to no cross-resistance to SU herbicides (Poston et al. 2000). An Ala_{122} to Thr substitution in a partially purified ALS enzyme from sugarbeet (*Beta vulgaris*) conferred high-level resistance to imazethapyr, but no cross-resistance to the SU herbicide chlorsulfuron or the TP herbicide flumetsulam (Wright et al. 1998). Other reported cases of an Ala_{122} to Thr substitution were a common cocklebur (*Xanthium strumarium* L.) biotype that was resistant to only IMI herbicides (Bernasconi et al. 1995) and an eastern black nightshade (*Solanum ptycanthum* L.) biotype that was resistant to IMI herbicides with low cross-resistance (<11-fold) to the SU herbicide primisulfuron (Milliman et al. 2000).

The four smooth pigweed biotypes (R5, R6, R7, R8) with a Ser_{653} to Asn substitution exhibited high-level resistance to the IMI herbicide and 29- to 88-fold resistance to the PTB herbicide. These biotypes were also 2- to 25-fold resistant to the SU herbicides, but differences between the S biotype were generally only evident at herbicide rates below the commercial POST use rate. Biotypes R6, R7, and R8 were 3- to 10-fold resistant to the TP herbicide, whereas R5 had increased sensitivity, even though all ALS genes were molecularly identical. Differential sensitivity to cloransulam might be explained by differential absorption, translocation, or metabolism rates. Mutant *Arabidopsis thaliana*

plants with a Ser_{653} to Asn substitution were reported to be 100- to 1,000-fold resistant to IMI and PTB herbicides, but lacked cross-resistance to SU or TP herbicides (Sathasivan et al. 1991; Mourad and King 1992). In other research, a mutant *Arabidopsis thaliana* ALS enzyme with a Ser_{653} to Asn substitution remained sensitive to SU herbicides, although sensitivity to some SU herbicides was reduced 1.6- to 13.1-fold (Chang and Duggleby 1998).

We have concluded that eight smooth pigweed biotypes, collected from separate locations in Virginia, Delaware, and Maryland and selected by repeated use of IMI herbicides, exhibited resistance to ALS-inhibiting herbicides due to an altered ALS enzyme. Two separate ALS mutations conferred IMI resistance in these biotypes, and cross-resistance patterns on the whole-plant level differed based on the specific mutation.

Sources of Materials

[1] Pro-Mix BX. Premier Horticulture, Inc., Red Hill, PA 18076.

[2] Peters 20-20-20 professional soluble plant food. Wetzel, Inc., 1345 Diamond Springs Road, Virginia Beach, VA 23455.

[3] Induce nonionic low-foam wetter/spreader adjuvant with 90% principal functioning agents as a blend of alkyl aryl polyoxylkane ether free fatty acids. Setre Chemical Company, Memphis, TN 38137.

[4] Teejet 8002EVS flat fan spray tip. Spraying Systems Co., North Avenue, Wheaton, IL 60188.

[5] QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355-1106.

[6] Roche Diagnostics GmbH, Roche Molecular Biochemicals, Sandhofer Strasse 116, D-68305 Mannheim, Germany.

[7] PE Corporation, PE Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404.

[8] Virginia Bioinformatics Institute, Core Laboratory Facility, Washington Street Blacksburg, VA 24061-0477.

[9] Gene Codes Corp, 640 Avis Drive, Ann Arbor, MI 48108.

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Table 3.1. Oligonucleotide primer sequences used in sequencing reactions. Primers were located at approximately 500 bp intervals in forward and reverse directions to sequence both ALS DNA strands of susceptible and resistant smooth pigweed biotypes.

Primer	Sequence
Forward 1	5'-TCCTCGCCGCCCTCTTCAAATC
Forward 2	5'-GTCCGGGTGCTACTAATCTTGTTT
Forward 3	5'-TTGCTAGTACTTTAATGGGGTTGG
Forward 4	5'-CGAAGGGCGATGCGGTTGTAAG
Reverse 1	5'-CAGCTAAACGAGAGAACGGCCAG
Reverse 2	5'-GCATCTGGTCGAGCAACAGCAG
Reverse 3	5'-GTCACTCGATCATCAAACCTAACC
Reverse 4	5'-CTTGGTAATGGATCGAGTTACCTC

Table 3.2. Resistance ratios (R/S)[a] of four resistant (R5, R6, R7, R8) smooth pigweed biotypes to five ALS-inhibiting herbicides based on GR_{50}[b] values.

Herbicide	Class[c]	R5	R6	R7	R8
Imazethapyr	IMI	261	467	537	508
Pyrithiobac	PTB	33	29	80	88
Chlorimuron	SU	2	6	7	14
Thifensulfuron	SU	10	25	15	20
Cloransulam	TP	0.08	3	6	10

[a] Resistance to susceptibility ratios (R/S) were calculated by dividing the GR_{50} of the resistant biotype by the GR_{50} of the susceptible biotype.

[b] GR_{50} refers to the herbicide dose (in g ai ha^{-1}) required for 50% biomass reduction compared to the nontreated control.

[c] Abbreviations: IMI, imidazolinone; PTB, pyrimidinylthiobenzoate; SU, sulfonylurea; TP, triazolopyrimidine.

Table 3.3. Biomass reduction of one susceptible (S) and four resistant (R5, R6, R7, R8) smooth pigweed biotypes by selected rates of five ALS-inhibiting herbicides.[a]

	Biomass reduction[b]									
	Imazethapyr ($g\ ha^{-1}$)		Pyrithiobac ($g\ ha^{-1}$)		Chlorimuron ($g\ ha^{-1}$)		Thifensulfuron ($g\ ha^{-1}$)		Cloransulam ($g\ ha^{-1}$)	
Biotype	7	70	7	70	0.9	9	0.45	4.5	1.8	18
	%									
S	90	97	88	98	77	97	82	98	46	64
R5	16	31	34	75	63	90	66	91	63	77
R6	22	29	43	69	45	84	50	95	35	53
R7	25	34	44	59	40	88	58	96	40	44
R8	22	20	43	56	43	65	53	93	33	36
LSD (0.05)	19	38	19	19	23	14	18	NS	17	19

[a] All herbicide treatments were applied postemergence to four- to six-leaf smooth pigweed plants and included a nonionic surfactant (0.25% v/v).

[b] Values represent above-ground biomass reduction compared to the nontreated control 21 d after treatment.

Table 3.4. Whole-plant response summary for susceptible (S) and resistant (R5, R6, R7, R8) smooth pigweed biotypes to five ALS-inhibiting herbicides. Values were obtained by non-linear regression of dry biomass expressed as a percentage of the nontreated control.

		GR_{50}[b]				
Herbicide	Class[a]	S	R5	R6	R7	R8
		g ai ha^{-1}				
Imazethapyr	IMI	0.9	235	420	483	457
Pyrithiobac	PTB	0.9	30	26	72	79
Chlorimuron	SU	0.2	0.4	1.2	1.3	2.7
Thifensulfuron	SU	0.02	0.2	0.5	0.3	0.4
Cloransulam	TP	4	0.3	12	24	41

[a] Abbreviations: IMI, imidazolinone; PTB, pyrimidinylthiobenzoate; SU, sulfonylurea; TP, triazolopyrimidine.

[b] GR_{50} refers to the herbicide dose (in g ai ha^{-1}) required for 50% biomass reduction compared to the nontreated control.