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Hexazinone Resistance in Red Sorrel (*Rumex acetosella*)

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Biannual applications of hexazinone have been applied in many lowbush blueberry fields in Nova Scotia for more than 30 years. Persistent reliance on a single herbicide chemistry may have selected for hexazinone-resistant red sorrel. The recommended rate of hexazinone (1.92 kg ai ha^{-1}) no longer controls red sorrel in many growing regions. Six levels of hexazinone (0, 0.48, 0.96, 1.92, 3.84, and 7.68 kg ai ha^{-1}) were applied to red sorrel plants grown in a greenhouse from seeds collected from three commercial fields and a no blueberry area to determine if they were hexazinone resistant. Red sorrel from two sites where hexazinone had not been applied regularly died at the 0.96 kg ai ha^{-1} rate of hexazinone whereas red sorrel from two commercial fields survived at 7.68 kg ai ha^{-1}. It is concluded that red sorrel is hexazinone-resistant in some wild blueberry fields. A portion of the *psb*A gene was sequenced and it was determined that resistant plants had a Phe to Val substitution at position 255 in the D1 protein. This is the first recorded instance of hexazinone resistance in a perennial broadleaf weed in blueberry fields.

Nomenclature: Hexazinone, Velpar; red sorrel, *Rumex acetosella* L.; lowbush blueberry, *Vaccinium angustifolium* Ait.

Key words: Hexazinone-resistant, mutation, *psb*A.

Weeds compete with blueberries for resources reducing berry yields and quality. Red sorrel, a common weed in blueberry fields, has several other common names, including sheep's sorrel, sour weed and field sorrel. It is a perennial weed that is native to Eurasia and it reproduces by creeping roots and seeds (Love 1983). At present, it is widespread in North America. Red sorrel is considered to be a problematic weed because of its rapid reproduction and spread in blueberry fields. Hexazinone is the predominate herbicide used in commercial wild blueberry fields when it controls localized populations of red sorrel, but suspected resistant populations have been reported throughout Atlantic Canada (Kennedy et al. 2010; McCully et al. 2005).

Group 5 herbicides such as hexazinone inhibit electron flow in photosystem II (Shukla and Devine 2008). This group includes triazines, triazinones, uracils, phenyl-carbamates, ureas, amides, and nitriles (Hess 2000). The site of action of these herbicides is the plastoquinone-binding pocket on the D1 protein in the PS II reaction center of the photosynthetic electron transport chain. When the herbicide binds, transfer of electrons from Q_A to Q_B is blocked and photosynthesis lacks NADPH for CO_2 fixation (Gardner 1981). Subsequent photo-oxidation of membrane lipids then leads to plant death (Shukla and Devine 2008).

Triazine resistance is typically caused by modifications of the herbicide binding site on the target protein (D1 protein) (Devine and Eberlein 1997). The chloroplast *psb*A gene, a maternally inherited gene, encodes the D1 protein in higher plants (Perez-Jones et al. 2009). Triazine-resistance is frequently caused by a Ser_{264} to Gly mutation in the *psb*A gene that alters the conformation of the Q_B and herbicide binding niche (Fuerst and Norman 1991; Trebst 1996). Ser_{264} to Gly mutation decreases the binding of s-triazine and triazinones to this site (Devine et al. 1993). Other mutations at different positions have also been reported to cause resistance to triazine or triazinone herbicides, including Phe_{211}, His_{215}, Val_{219}, Ala_{251}, Phe_{255}, Gly_{256}, Ser_{264}, Phe_{265},Ser_{268}, and Leu_{275} (Devine and Eberlein 1997; Shukla and Devine 2008; Trebst 1991). Amino acid changes in positions from 211 to 275 were conferred in many herbicide-resistant organisms (Trebst 1991). In addition, Ser_{264} to Gly mutation in the *psb*A gene decreases the photosynthetic capacity of resistant plants (Shukla and Devine 2008), and the rate of electron transfer between Q_A and Q_B in resistant plants was lower than in susceptible plants (Bowes et al. 1980).

Hexazinone is repeatedly applied for weed control in wild blueberry, at a recommended rate between 1.44 and 1.92 kg ai ha^{-1}. Preliminary

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experiments conducted by Agriculture and Agri-Food Canada determined that many grassy weeds were already hexazinone-tolerant, including bluegrasses and fescues (McCully et al. 2005). Widespread occurrence of red sorrel combined with variation in its susceptibility to hexazinone suggests that specific populations have developed resistance (N.S. Boyd, personal communication).

The objectives of the experiments were to (1) verify the existence of hexazinone resistant red sorrel in wild blueberry fields in Nova Scotia, and (2) verify whether hexazinone-resistant red sorrel was caused by a by Ser_{264} to Gly mutation in *psb*A gene.

Materials and Methods

Plant Materials. Red sorrel seeds were randomly hand-collected from four different sites in late August 2011 and stored in the dark at 5 C. The sites were a non-blueberry area where hexazinone was never applied (Bible Hill, NS), a relatively new blueberry field near a mature, established field (Debert, NS), and two mature commercial blueberry fields (Collingwood, NS and Dalhousie Mountain, NS). Growers typically applied herbicides every other year. Unfortunately, we do not know how consistently they applied hexazinone in Dahousie Mountain.

Whole-Plant, Dose–Response Assays. Seeds were germinated on two pieces of filter paper in 60 by 15 mm Petri dishes. Filter papers were moistened with 5 ml of a 2% KNO_3 solution. All Petri dishes were placed in a greenhouse at 25/20 C day/night temperatures with a 14-h photoperiod without supplemental lighting in October 2011. After 1 wk, the seeds had germinated and four seedlings at the cotyledon stage were transferred into 12 by 9 cm cell packs with 1 : 2 peat moss : top soil mixture. Plants were grown in the greenhouse for 5 wk under the same conditions. Plants were sprayed with six different hexazinone rates: 0, 0.25×, 0.5×, 1× (1.92 kg ai ha^{-1}), 2×, and 4×, where the 1× rate was the recommended hexazinone rate for commercial blueberry fields. All hexazinone treatments were applied using a hand-held boom with CO_2 pressurized sprayer at 276 kPa pressure with a XR8002VS Teejet nozzle (Wheaton, IL, USA). Hexazinone efficacy on red sorrel was recorded 7, 14, 22, and 27 days after spraying (DAS) using a damage rating scale from 0 to 10, where 0 represents no damage and 10 complete death of all above ground red sorrel shoots. Four red sorrel plants in each pot were compared and the average rates were recorded.

At 27 DAS, above ground tissue of three of the four red sorrel plants was harvested. All dead red sorrel leaves were removed and only living tissue was collected. Shoot biomass was dried at 60 C for 4 d, weighed and plant dry weight recorded. The remaining plants, including control and survived treated plants, were later utilized for *psb*A gene sequencing experiments.

***psbA* Gene Sequencing.** Total DNA was extracted from red sorrel leaf tissue from plants that remained following the hexazinone dose–response experiment. Each sample included two leaf disks from one red sorrel plant. Leaf tissue was disrupted and homogenized in lysis buffer using a MicroSmash bead disruptor MS-100 (Tomy Digital Biology Co., LTD.). followed by extraction using Fujifilm QuickGene DNA tissue kit S and model QuickGene-810 according to the instructions outlined by the manufacturer in the plant tissue protocol (Fujifilm, no date). Each DNA sample was electrophoresed on a 1% agarose gel and a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure DNA concentration.

The *psb*A gene sequences from other plants were obtained including: *Poa* (GenBank accession AF131887), *Zea* (AF543684), *Hordeum* (X07 521), *Prunus* (AF410200), *Amaranthus* (AY336 946, K01200) and *Ambrosia* (AB427162 in DDBJ database) (Tian and Darmency 2006; Cseh et al. 2009). Gene sequences for *psb*A were found in NCBI, imported into Sequencer v. 4.7 (Gene Codes Corporation, MI, USA) and aligned. Primers were designed to target regions of *psb*A where nucleotide sequence was conserved in the plants for which sequence data was available (forward: *psb*AF 5′-CCTCCAGTAGATATTGATGGATTCG-3′; reverse: *psb*AR(5′-TGAGCATTACGTTCGTGCAT-3′). The 845 bp amplified sequence spanned nucleotides where mutations associated with resistance to group 5 herbicides were previously reported.

Polymerase Chain Reaction (PCR) amplification reactions contained 50 to 75 ng of template DNA, 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of Taq DNA polymerase (Promega GoTaq, Madison, WI, USA), 1 µM of each primer in a final volume of 20 µl. PCR thermal cycling was carried out in Dyad Disciple (Bio-Rad® Laboratories, Mississauga, ON, Canada) with the following conditions: 1 cycle at 95 C for 5 min, followed by

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35 cycles of 20 s denaturation at 95 C, annealing at 58 C for 15 s and extension for 1 min 30 s at 72 C, followed by a final extension for 10 min at 72 C. The PCR products were separated on a 1.2% agarose gel. PCR products were purified with Qiagen PCR spin columns (Qiagen, Germantown, MD, USA) according to the procedure outline by the manufacturer. Purified PCR product was sequenced in both directions using *psb*AF and *psb*AR and the ABI Big Dye Terminator Kit v. 3.1 (Life Technologies, Burlington, ON, Canada). Sequencing reactions were cleaned-up using a standard sodium acetate/EDTA/ethanol precipitation and separated by capillary electrophoresis on an ABI 3130 nucleic acid analyzer (Life Technologies). Sequencing was performed on 12 hexazinone resistant and 12 susceptible samples which remained from the hexazinone resistant experiment. The samples included five and seven resistant red sorrel leaves from Debert and Collingwood, respectively, and eight and four susceptible red sorrel leaves from Bible Hill and Dalhousie Mountain, respectively. Sequences were imported into Sequencer and aligned to *psb*A from other species. The nucleotide sequences of resistant and susceptible plants were compared. Differences in nucleotide sequence between susceptible and resistant plants were recorded. The nucleotide sequence was imported into BioEdit (Hall 1999) and translated *in silico* to verify the amino acid sequence. Observed nucleic acid substitutions were made and the resulting coding region was translated *in silico* to determine if the nucleic acid substitution resulted in an amino acid change.

Statistical Analysis. The greenhouse study was a randomized complete block design with four blocks and was repeated once. All damage ratings and biomass data were analyzed using Proc Npar1way (Kruskal-Wallis test) in SAS v. 9.3 (SAS Institute Inc., Cary, NC) because of lack of homogeneous variance. All analyses were considered significant at $\alpha = 0.05$. Biomass of control treatments among sites were analyzed using PROC MIXED in SAS v. 9.3. Sites were fixed effects and blocks were random effects. Tukey's test was used to test for site differences at $\alpha = 0.05$. Dose–response curves were obtained with nonlinear regression in SigmaPlot v. 12.1 (Systat Software Inc., Chicago, USA) using the log–logistic Equation1 (Seefeldt et al. 1995):

$$y = C + (D - C)/[1 + (x/I_{50})^b] \qquad [1]$$

where y represents shoot dry weight expressed as a percentage of the untreated control, C is the mean response at high level hexazinone rate, D is the mean response when the herbicide rate is zero, x is the herbicide dose, b is the slope of the line at I_{50}, and I_{50} is the herbicide rate required for 50% growth reduction. Most of the plants from Bible Hill and Dalhousie Mountain died following hexazinone application and had zero biomass and as a result it was not possible to construct dose response curves for those sites. The difference in I_{50} value from Debert and Collingwood biomass was analyzed using a 2 samples t-test in Minitab v. 16 (Minitab Inc., PA, USA).

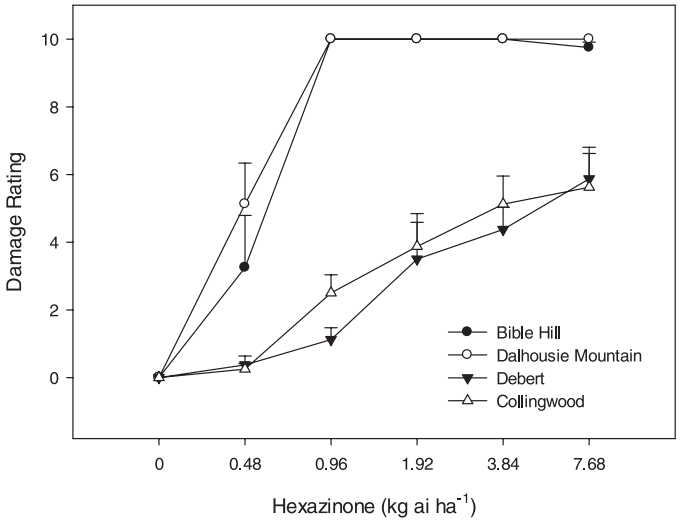


Figure 1. Damage ratings of red sorrel plants 21 days after spraying with different rates of hexazinone. Plants were grown from seed collected at four sites: Bible Hill, Dalhousie Mountain, Debert, and Collingwood, Nova Scotia. Data points are means and vertical bars represent the standard error of the mean.

Results

Whole-Plant, Dose–Response Assays. There was an impact of hexazinone dose on damage ratings at all sites ($P < 0.0001$). Two obvious patterns emerge with very similar damage observed on plants originating from Bible Hill and Dalhousie Mountain and very similar damage ratings from plants originating from Debert and Collingwood (Figure 1). All damage ratings were close to 10 at rates of 0.96, 1.92, 3.84, and 7.68 kg ai ha^{-1} for plants from Bible Hill and Dalhousie Mountain 21 DAS. Plants from both locations were highly susceptible with near complete control observed at one half of the recommended rate and damage at rates as low as one quarter of the recommended rate (Figure 1). We concluded that plants from Bible Hill and Dalhousie Mountain were not resistant to hexazinone. Damage ratings of 4 were observed for plants from Debert and

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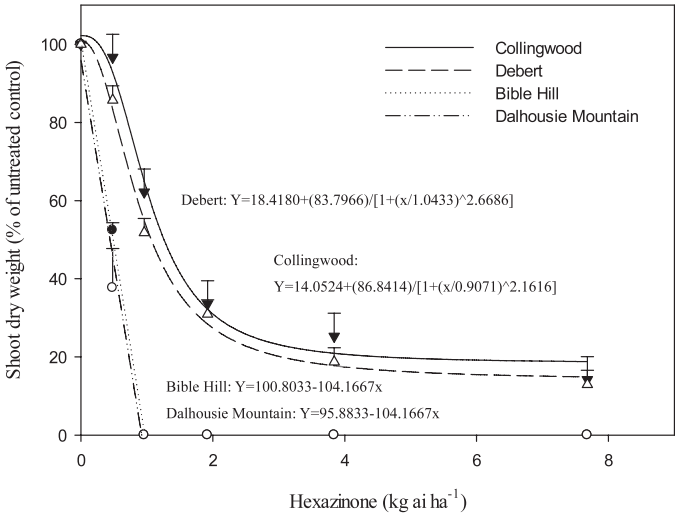


Figure 2. Hexazinone dose–response in red sorrel under greenhouse conditions grown from seeds collected at four sites in Nova Scotia. Shoot dry weights were recorded 31 d after treatment and expressed as a percentage of the untreated control; each point is the mean of four replications. Symbols and lines represent actual and predicted growth responses, respectively.

Collingwood at the recommended rate of 1.92 kg ai ha^{-1} hexazinone (Figure 1). Four times the recommended rate did not kill red sorrel from either site 21 DAS (Figure 1) although growth was suppressed 42%. Thus, we concluded that red sorrel populations from Debert and Collingwood were resistant to hexazinone but plant growth was suppressed at higher rates.

Hexazinone rate impacted red sorrel above ground biomass at all sites ($P < 0.001$). At 0.48 kg ai ha^{-1} (0.25X) of hexazinone, the red sorrel shoots from Debert and Collingwood decreased by 3 and 14%, respectively (Figure 2). Conversely, biomass decreased by 48% and 62% for red sorrel shoots from Bible Hill and Dalhousie Mountain, respectively (Figure 2). All red sorrel plants from Bible Hill and Dalhousie Mountain died when exposed to 0.96 kg ai ha^{-1} and higher rates of hexazinone and the living biomass was zero. The biomass of plants from Debert and Collingwood sites declined as the rate of hexazinone increased and the biomass from Debert and Collingwood was 14% and 13% of the untreated control at the 7.68 kg ai ha^{-1} rate of hexazinone (Figure 2). However, Biomass was not different between the Debert and Collingwood sites ($P = 0.4429$). Also, the I_{50} value for hexazinone dose was not different ($P = 0.8070$) between Debert and Collingwood at 1.04($\pm$ 0.13) kg ai ha^{-1} and 0.91($\pm$ 0.08) kg ai ha^{-1}, respectively. Data from Bible Hill and Dalhousie Mountain could not be analyzed due to death of plants at the lowest rate. However, Debert and Collingwood biomass was higher than Bible Hill and Dalhousie Mountain ($P < 0.0001$). The required dose to kill 100% of plants from Bible Hill and Dalhousie Mountain red sorrel was only 0.96 kg ai ha^{-1} hexazinone.

***psb*A Gene Sequencing.** Studies of DNA sequencing of *psb*A gene region were conducted on 12 resistant and 12 susceptible plants, which remained from the hexazinone resistance greenhouse experiments. Previous work determined that most triazine resistant plants have a single nucleic acid substituion, which results in a Ser_{264} to Gly mutation (Jia et al. 2007). The primer pair *psb*AF/*psb*AR was designed to amplify an 845 bp fragment of the *psb*A gene which included the nucleotide sequence which is translated into the Q_B binding niche of the D1 protein which is also the site of triazine binding and the region where previous mutations associated with resistance have been reported.

The consensus sequence for the portion of the red sorrel *psb*A gene sequenced in this study has been deposited in the NCBI GenBank database (GenBank Accession KJ566595). The portion of the gene which flanks the target site has been determined to be mutated in other plants as shown in Figure 3. Amino acid residues between 211 and 275 in the Q_B binding niche are shown. Previous reports concurred that a common mutation associated with triazine resistance was Ser_{264} (AGT) to Gly (GGT) (Jia et al. 2007). However, Ser_{264} in resistant red sorrel was not changed (Figure 3) indicating hexazinone resistance in red sorrel was caused by another mutation. The mutation, Phe_{255} (TTT) to Ile (ATT) was reported to cause hexazinone resistance in shepherd's-purse (Perez-Jones et al. 2009). The authors reasoned that the binding of hexazinone to the D1 protein was significantly reduced by Phe_{255} to Ile mutation (Perez-Jones et al. 2009). Amino acid residue 255 was found to differ in hexazinone resistant and susceptible plants in this study. However, the amino acid substitution observed was different than reported in shepherd's purse. Phe (TTT) was shown in all susceptible red sorrel protein sequences at position 255. However, all the resistant plants had the codon GTT at this position, which codes for the amino acid Val. This mutation may decrease the binding of hexazinone, since the mutation in this position affected the binding of triazine herbicides in the Q_B binding site on D1 protein (Gronwald 1997). Hexazinone efficacy was deceased by Phe_{255}

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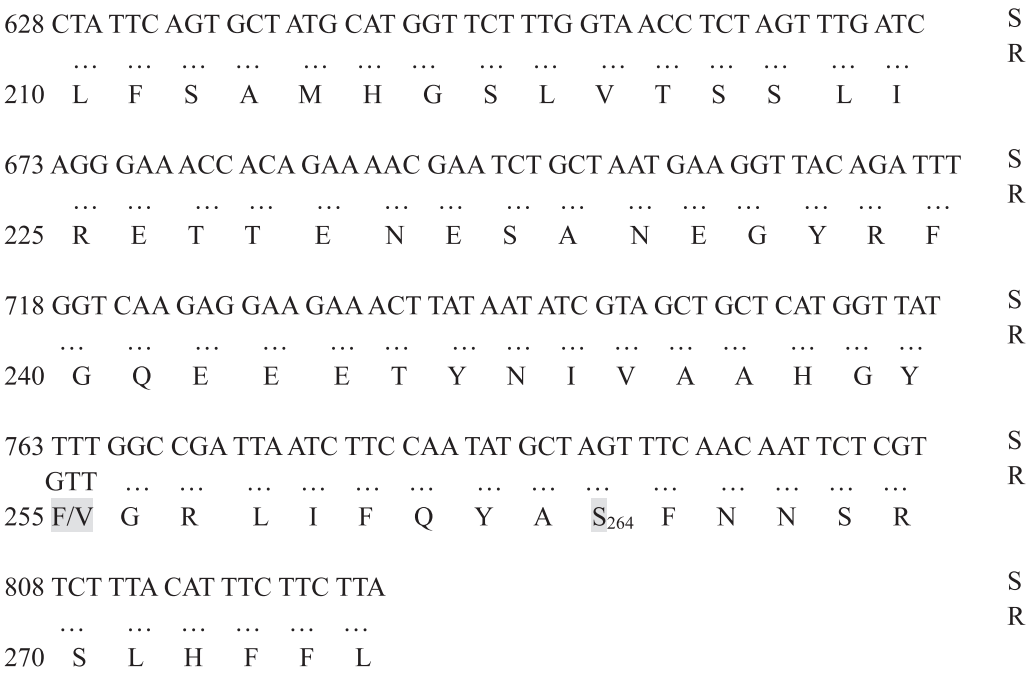


Figure 3. Partial red sorrel *psb*A nucleotide sequence (1st and 2nd row) and deduced amino acid sequence (3rd row) alignment of the *psb*A gene of hexazinone-susceptible (S) and hexazinone-resistant (R) red sorrel populations. The region shown includes the amino acid sequence of the Q_B binding niche of the D1 protein of PS II (210–275). Shaded amino acids indicate a Phe (TTT) to Val (GTT) substitution at position 255 in the R population. There is no difference between R and S at positon 264 Ser (AGT).

to Ile mutation and it may be caused by removing the phenyl ring of Phe (Perez-Jones et al. 2009). Val and Ile have similar structures in that both of them are without the phenyl ring of Phe. Moreover, both resistant and susceptible red sorrel plants had similar size, with an average of 12 leaves per plant.

No other differences in the amplified sequence of the partial *psb*A gene were observed between resistant and susceptible red sorrel except the red sorrel from Bible Hill contained Asn_{230} (AAT) and red sorrel from the other sites had Asn_{230} (AAC). However, this change did not change the amino acid.

In conclusion, red sorrel from some blueberry fields were resistant to hexazinone. No experiments were conducted to measure differences in absorption, translocation and metabolism of hexazinone in red sorrel from the different sites. However, we felt our data provided compelling evidences of resistance caused by a Phe_{255} to Val mutation in the *psb*A gene. Susceptible red sorrel died at 0.96 kg ai ha^{-1} hexazinone. Resistant red sorrel survived at 7.68 kg ai ha^{-1} hexazinone, but growth was suppressed. Alternative red sorrel management options are needed to adequately manage this weed in commercial wild blueberry fields and the industry as a whole needs alternative weed management strategies to delay the onset of resistance in other problematic species.

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