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Expression of Herbicide Target-Site and Chloroplastic Genes in Response to Herbicide Applications in Italian Ryegrass [*Lolium multiflorum* ssp. *multiflorum* (Lam.)]

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Abstract

Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.)] is a problematic C3 annual weed in winter wheat and other crops in the US, continental Europe. The objective of this research was to assess the expression profiles of herbicide target-site genes upon treatment with field rates of ALS- (*e.g.*, chlorsulfuron), PSII- (*e.g.*, atrazine), EPSPS- (*e.g.*, glyphosate), and HPPD- (*e.g.*, mesotrione) inhibitors, respectively in Italian ryegrass. Furthermore, the expression of the genes in chloroplasts that contribute to photosynthesis and CO_2 assimilation was also measured in response to these herbicide treatments. *LmALS* was upregulated (> 3-fold) whereas *LmpsbA* was downregulated (> 40-fold) at 8 h after chlorsulfuron and atrazine treatments respectively. However, *LmEPSPS* gene expression remained similar to control levels at 8 h after glyphosate treatment. *LmHPPD* gene showed > 1-fold higher expression at 8 h after mesotrione treatment. *LmCAB*, a major component of the photosystem II light-harvesting complex, was downregulated immediately (8 h) after chlorsulfuron, atrazine, glyphosate, and mesotrione treatments respectively. Similarly, *LmRubisco*, was downregulated after glyphosate and mesotrione treatments. However, *LmRubisco* was upregulated 0.4-fold after chlorsulfuron treatment and remained unaffected after atrazine treatment. Overall, the data from this study suggest that the treatment with herbicides showed variation in expression of herbicide target genes and those that are involved in the critical photosynthetic pathway.

Keywords: *Lolium perenne* ssp. *multiflorum* (Lam.), gene expression patterns, herbicides, photosynthesis

1. Introduction

Photosynthetic organisms can be broadly classified into two types based on how carbohydrates are synthesized from CO_2, *i.e.*, C3 and C4. C3 photosynthetic plants fix atmospheric CO_2 through Calvin-Benson cycle, catalyzed by the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) inside the chloroplast of their mesophyll cells (Wang et al., 2012). In general the photosynthesis is partitioned in mesophyll and bundle sheath cells to reduce loss due to photorespiration and increase the efficiency of carbon assimilation (Yin et al., 2011). Rubisco is one of the most abundant enzymes in chloroplasts of mesophyll cells and can constitute as much as 50% of the total soluble protein in some C3 species (Carmo-Silva et al., 2015).

Photosynthesis is the major biochemical process occurring in photoautotrophic organisms and is known to be affected by various anthropogenic factors. Photosynthetic mechanisms such as C3 and C4 have been well studied and characterized in model species and crops; nonetheless, some specific processes related to gene expression in weeds in response to herbicide treatments is not completely known. The effects of herbicide application on the expression of herbicide target-site and photosynthetic genes such as *Rubisco* and chlorophyll a/b binding protein (*CAB*) needs to be investigated in order to better understand the factors leading to plant death. Previously we reported (Varanasi et al., 2017) expression of herbicide target genes as well as those involved in photosynthesis in response to herbicide treatments in a broadleaf weed *Kochia scoparia* (L.) Schrad.

Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.)] is a cool season C3 annual grass, widely distributed throughout the US (USDA-NRCS, 2002). Italian ryegrass (family Poaceae) is native to central and southern Europe, northwest Africa, and southwest Asia (Hubbard, 1968). Ryegrasses are cultivated for turf and forage and are sometimes grown as a cover crop (USDA-NRCS, 2014). Significant yield losses (up to 90%) in winter wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) have been reported due to the presence of Italian ryegrass (Nandula, 2014; Perez-Fernandez & Coble, 1998; Hashem et al., 1998; Appleby et al., 1976). Italian ryegrass also causes substantial losses in vegetable crops and orchards (Bell, 1995; Perez-Jones et al., 2005). Control of Italian ryegrass has traditionally been achieved by chemical means (Bond et al., 2014). Due to its large genetic variability, worldwide distribution, and extensive use of herbicides, Italian ryegrass has recently evolved resistance to herbicides with different sites of action (SOA) (Jasieniuk et al., 2008; Rauch et al., 2010).

Many of the existing herbicides have SOA that target sections of light-dependent photosystem II (PSII) reaction. For example, PSII-inhibitors such as atrazine inhibit photosynthesis by binding to the secondary quinone acceptor Q_B within the D1 protein encoded by the *psbA* gene and blocking the transport of the electrons to the plastoquinone, leading to cellular damage by oxidative stress (Hess, 2000). The broad-spectrum herbicide glyphosate, inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a critical enzyme of the shikimate pathway catalyzing the biosynthesis of aromatic amino acids (Schönbrunn et al., 2001; Steinrucken & Amrhein, 1980). Glyphosate affects the photosynthetic pathway indirectly by inhibiting the biosynthesis of carotenoids, chlorophylls, fatty acids, amino acids, and secondary metabolites such as quinones (Fedtke & Duke, 2005; Dewick, 1998). Acetolactate synthase (ALS) or acetohydroxyacid synthase (AHAS)-inhibitors are one of the most commonly used herbicides for controlling weeds in agronomic crops (Lamego et al., 2009). These herbicides inhibit the ALS enzyme, which catalyzes the biosynthesis of branched-chain amino acids leucine, valine, and isoleucine (Devine & Eberlein, 1997). The HPPD-inhibitors such as mesotrione (a triketone) suppress the 4-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in the biosynthesis of tocopherols and plastoquinone in the carotenoid pathway (Beaudegnies et al., 2009). Plastoquinone is known to be essential for the functioning of the photosynthetic electron transfer process (Moran, 2014).

In this study, we investigated the expression profiles of herbicide target genes (*LmpsbA*, *LmALS*, *LmEPSPS*, and *LmHPPD*) and photosynthetic genes (*LmRubisco* and *LmCAB*) in *L. multiflorum* after treatment with four SOA herbicides (PSII-, ALS-, EPSPS-, and HPPD-inhibitors). We also assessed the changes to the photosynthetic apparatus, *i.e.*, leaf chlorophyll index and chlorophyll fluorescence (F_v/F_m) in response to above herbicide applications. The objective of the current study was therefore to investigate how changes in the target-site gene expression after herbicide application would affect critical pathways (such as photosynthesis) in *L. multiflorum*. An understanding of the gene expression in response to various herbicides could better elucidate the cross-talk between different pathways and the underlying mechanism(s) contributing to plant death.

2. Materials and Methods

2.1 Plant Material

Seeds of *L. multiflorum* (resistant to atrazine, mesotrione, and chlorsulfuron but sensitive to glyphosate) were germinated in trays (20 cm × 10 cm) in the greenhouse conditions (29/25 ^{o}C temperature maximum/minimum; 15/9 h light day/night, supplemented with 1200 µmol m^{-2} s^{-1} illumination using sodium vapor lamps). After emergence, the seedlings were transplanted individually to 6.5 cm × 6.5 cm × 6.5 cm pots for herbicide treatments.

2.2 Herbicide Treatments and Tissue Collection

L. multiflorum seedlings were raised as described above and 10-12 cm tall seedlings were used for herbicide treatments. Three seedlings (replications) each of *L. multiflorum* were separately treated with recommended field rates (1×) of four herbicides: a) chlorsulfuron (Glean XP at 18 g ai ha^{-1}; 0.25% v/v nonionic surfactant [NIS])—an ALS-inhibitor, b) glyphosate (Roundup Weathermax at 840 g ae ha^{-1}; 2% w/v ammonium sulfate [AMS])—an EPSPS inhibitor, c) atrazine (Aatrex 4 L at 2240 g ai ha^{-1}; 1% v/v crop oil concentrate [COC])—a PSII-inhibitor, and d) mesotrione (Callisto at 105 g ai/ha; 1% v/v COC + 0.85% w/v AMS)—an HPPD-inhibitor. Herbicides were applied using a bench-type sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing, Hollandale, MN, USA) equipped with a single flat-fan nozzle (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL) delivering 187 L ha^{-1} at 240 kPa in a single pass at 4.75 km h^{-1}. Additionally, three seedlings were used as non-treated control and three other seedlings were treated with only adjuvant (control). The leaf tissue (new and fully expanded) was collected from non-treated, adjuvant only treated as well as herbicide treated seedlings (a total of three biological replicates) at 8 h and 7 d after treatment (DAT) and

immediately frozen in liquid nitrogen (-196 ^{o}C). All the harvested tissue was stored at -80 ^{o}C until total RNA isolation.

2.3 Total RNA Isolation

To isolate RNA the leaf tissue that was maintained at -80 ^{o}C was homogenized in liquid nitrogen using a pre-chilled mortar and pestle. To isolate total RNA using a TRIazol® reagent (Thermo Fisher Scientific, Waltham, MA, USA) the powdered tissue was collected into 1.5 mL microcentrifuge tube and the manufacturer's instructions were followed in this procedure. To remove any DDNA contamination, the extracted RNA was treated with DNase 1 enzyme (Thermo Fisher Scientific). The quality of RNA was determined using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific) and agarose gel (1%) (Agarose Products, MS, USA) electrophoresis. The isolated RNA was stored at -80 ^{o}C.

2.4 cDNA Synthesis

Complementary DNA (cDNA) was synthesized from 1 µg of the total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA was then diluted with molecular grade water in a 1:5 ratio and used in a quantitative PCR (qPCR) reaction to study the expression of herbicide target-site and photosynthetic genes in *L. multiflorum*.

2.5 Gene-Specific qPCR Primer Designing

The primers for quantitative real-time PCR (qPCR) (Table 1) for *LmALS* and *LmpsbA* genes were designed based on the sequence information obtained from GenBank (accession numbers AF310684.1, EU360732.1). Primers for *LmHPPD* gene were designed by alignment of nucleotide sequences from *L. multiflorum* (Italian ryegrass; accession number AX458019.1), *Brachypodium distachyon* (still brome) (accession number XM_003569953.2), *Triticum aestivum* (wheat; accession number AM084898.1), and *Zea mays* (maize; accession number AJ634707.1) respectively. Nucleotide sequences from *B. distachyon* (accession number XM_003578544.2) and *T. durum* (durum wheat; accession number AJ635207.1) were utilized for designing conserved primers for *LmCAB*. Primers for the large subunit (LSU) of the *LmRubisco* gene were designed on the basis of nucleotide sequence obtained from *L. perenne* (perennial ryegrass; accession number HM850132.1). The *EPSPS* gene in *L. multiflorum* was amplified by using previously reported primers (Salas et al., 2012). Primers for *LmActin*, the reference gene used for qPCR, were designed from the nucleotide sequence of *L. perenne* (accession number EF460315.1) available at GenBank.

Table 1. Quantitative Real-Time PCR primer sequences used for studying the expression of herbicide target-site and photosynthetic genes in *L. multiflorum.* LSU refers to the large subunit of *LmRubisco*

Gene	Primer Sequences: 5′→3′	Amplicon Size (bp)	T_m (^{o}C)
LmEPSPS	F: CTG ATG GCT CGT CCT TTA GCT C R: CCC AGC TAT CAG AAT GCT CTG C	136	60.0
LmpsbA	F: TTG GCT GCT TGG CCT GTA GTA G R: GCT CGG TTG ATG ATA TCA GCC CAA G	149	59.0
LmALS	F: AGG AGT GAA GTC CGT GCA GC R: GCA ATC CTG CCA TCA CCT TCC AT	155	59.0
LmHPPD	F: TCA GGA CGC TCA GGG AGA TG R: AGC TCC TGG CAT TCC TTG ATC TG	145	59.0
LmCAB	F: AGA CCT TCG CCA AGA ACC GC R: TTG CCG AGG TAG TCA AGG CC	178	59.0
LmRubisco (LSU)	F: ACC CCG CAG TAT GAA ACC AAG GAT A R: TTG TCT TCC CCA GCA ACA GGC	209	59.0
LmActin	F: CTG ACT GAG GCA CCC CTG AA R: GCT GAC ACC ATC ACC AGA ATC CAA C	168	60.0

Note. T_m (^{o}C): melting temperature.

2.6 Quantitative Real-Time PCR

To determine the expression of herbicide target-site and photosynthetic genes in *L. multiflorum.* a StepOnePlus™ real-time detection system (Thermo Fisher Scientific), was used. The qPCR reaction mix (14 µL) consisted of 2

µL of cDNA, 2 µL each of forward and reverse primers (5 µM), and 8 µL of PowerUp SYBR Green master mix (Applied Biosystems, Waltham, MA, USA), The primers for herbicide target-site, photosynthetic, and reference genes (as discussed above) were used in the qPCR reactions (Table 1). A standard curve with different cDNA concentrations was developed for all genes tested, to verify reaction efficiency of different primers used in this study. QPCR conditions were maintained as follows: 95 ^{o}C for 10 min and 40 cycles of 95 ^{o}C for 15 s followed by an annealing at 59/60 ^{o}C for 1 min (Table 1). To determine the specificity of the qPCR reaction, a melt curve profile was generated following the thermal cycling protocol.

2.7 Leaf Chlorophyll Index

The chlorophyll index in the leaves of *L. multiflorum* was measured at 1, 2, 3, 4, 5, 7, 9, 12, and 14 days after treatment (DAT) with glyphosate, chlorsulfuron, atrazine, and mesotrione. We used a chlorophyll meter SPAD-502 plus (Konica Minolta Optics Inc., Japan) (Ling et al., 2011) to record the chlorophyll index from the intact leaves on the plant.

2.8 Measurement of Chlorophyll Fluorescence (F_v/F_m)

The efficiency of photosynthetic light reaction (Photosystem II), which is sensitive to oxidative stress (Krause & Weis, 1991) was measured in *L. multiflorum* using a chlorophyll fluorometer OS30p$_+$ (Opti-Sciences Inc., Hudson, NH, USA) from 1 to 14 DAT with glyphosate, chlorsulfuron, atrazine, and mesotrione.

2.9 Statistical Analysis

As an endogenous reference gene (housekeeping gene), *LmActin* was used in order to normalize the gene expression data. Relative gene expression was evaluated using the formula for fold induction ($2^{\Delta Ct}$) (Livak and Schmittgen 2001). The transcript levels were measured relative to the adjuvant treated controls. A calibrator sample (untreated-no adjuvant) was also included to represent 1x expression of gene of interest. One-way ANOVA in SigmaPlot (version 12.3) was used for gene expression analysis. Fisher's LSD ($p < 0.05$) was used for mean separation. Three biological ($n = 3$) and nine technical replicates (each biological sample had three technical replicates) were included to calculate standard deviations (SDs). To calculate mean and SD values for chlorophyll index and fluorescence, four biological replicates ($n = 4$) were used at each time point for each herbicide treatment.

3. Results

The expression profiles of herbicide target (*ALS*, *psbA*, *EPSPS*, and *HPPD*) and the photosynthetic genes (*CAB* and *Rubisco*) was measured at 8 h and 7 DAT in Italian ryegrass after treatment with 1× rates of herbicides such as chlorsulfuron, atrazine, glyphosate, and mesotrione, representing different SOA. The data on *Rubisco* expression at 7 d after all herbicide treatments was not included because the transcripts were undetectable at that time point.

3.1 Gene Expression After Chlorsulfuron Ttreatment

LmALS was upregulated (> 3-fold, $p \leq 0.001$) relative to NIS treated control 8 h after treatment with chlorsulfuron (Figure 1). The two photosynthetic genes, *LmCAB* and *LmRubisco* showed a contrasting response after chlorsulfuron treatment. *LmCAB* was downregulated (> 1-fold, $p \leq 0.001$) at 8 h after chlorsulfuron treatment, whereas the expression of the *LmRubisco* was slightly increased (1.4-fold, $p \leq 0.001$) (Figure 1).

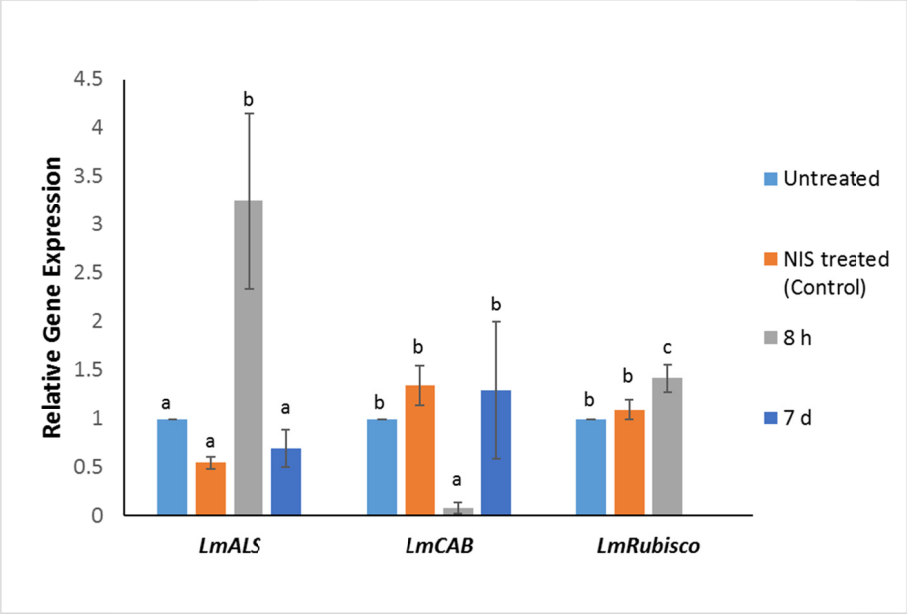

Figure 1. Relative fold expression of the target-site (*LmALS*) and photosynthetic genes (*LmCAB* and *LmRubisco*) in *L. multiflorum* after chlorsulfuron treatment. Letters indicate significant differences ($p < 0.05$) among the means of each gene at different time points (8 h and 7 d after treatment). The expression of the target-site and photosynthetic genes was measured relative to a NIS treated control. Untreated sample (no adjuvant, no herbicide treatment) was used as a calibrator. Error bars represent±standard deviation from the mean (n = 3). Mean values were based upon three biological and nine technical replicates. The qPCR data was normalized using *LmActin* as the reference gene. NIS refers to treatment with non-ionic surfactant. Transcripts of *LmRubisco* were undetectable at 7 DAT and therefore was not shown in this figure

3.2 Gene Expression After Atrazine Treatment

There was a strong induction of *LmpsbA* gene expression by COC treatment (Figure 2). The expression of *LmpsbA* gene was initially downregulated (> 40-fold, $p \leq 0.001$) relative to the COC treated control at 8 h, followed by increased transcript levels at 7 d after atrazine treatment respectively (Figure 2). *LmCAB* was downregulated, whereas the expression of *LmRubisco* remained unaffected relative to COC treated control at 8 h after atrazine treatment ($p \leq 0.002$; $p \leq 0.026$) (Figure 2).

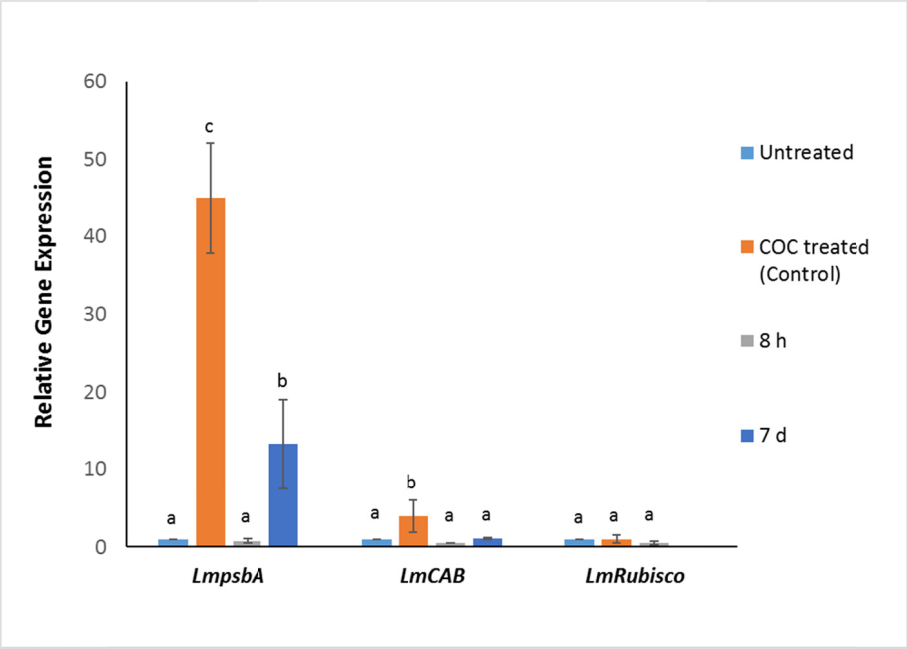

Figure 2. Relative fold expression of the target-site (*LmpsbA*) and photosynthetic genes (*LmCAB* and *LmRubisco*) in *L. multiflorum* after atrazine treatment. Letters indicate significant differences ($p < 0.05$) among the means of each gene at different time points (8 h and 7 d after treatment). The expression of the target-site and photosynthetic genes was measured relative to a COC treated control. Untreated sample (no adjuvant, no herbicide treatment) was used as a calibrator. Error bars represent±standard deviation from the mean (n = 3). Mean values were based upon three biological and nine technical replicates. The qPCR data was normalized using *LmActin* as the reference gene. COC refers to treatment with crop oil concentrate. Transcripts of *LmRubisco* were undetectable at 7 d DAT and therefore was not shown in this figure

3.3 Gene Expression After Glyphosate Treatment

The expression of *LmEPSPS* gene remained similar to control levels at 8 h and then peaked (16.7-fold, $p \leq 0.001$) at 7 d in response to treatment with glyphosate (Figure 3). Both the photosynthetic genes, *LmCAB* and *LmRubisco* were downregulated at 8 h after glyphosate treatment ($p \leq 0.001$) (Figure 3). However, the expression of *LmCAB* was upregulated at 7 d after glyphosate treatment and was comparable to the control levels.

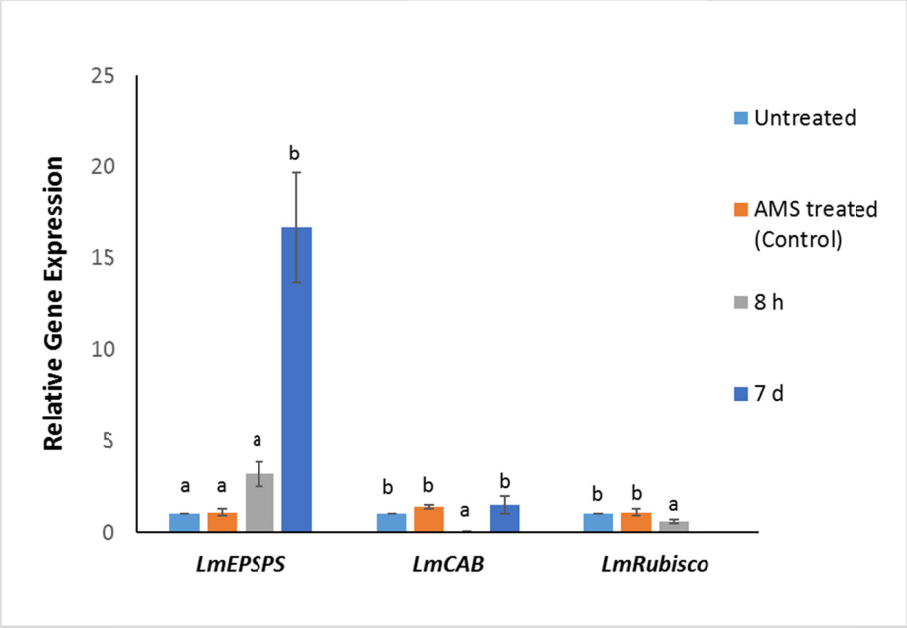

Figure 3. Relative fold expression of the target-site (*LmEPSPS*) and photosynthetic genes (*LmCAB* and *LmRubisco*) in *L. multiflorum* after glyphosate treatment. Letters indicate significant differences ($p < 0.05$) among the means of each gene at different time points (8 h and 7 d after treatment). The expression of the target-site and photosynthetic genes was measured relative to an AMS treated control. Untreated sample (no adjuvant, no herbicide treatment) was used as a calibrator. Error bars represent±standard deviation from the mean (n = 3). Mean values were based upon three biological and nine technical replicates. The qPCR data was normalized using *LmActin* as the reference gene. AMS refers to treatment with ammonium sulphate. Transcripts of *LmRubisco* were undetectable at 7 d DAT and therefore was not shown in this figure

3.4 Gene Expression After Mesotrione Treatment

LmHPPD gene was upregulated at 8 h (> 1-fold compared to adjuvant-only treated control plants) after mesotrione treatment ($p \leq 0.001$) (Figure 4). *LmCAB* was downregulated at 8 HAT with mesotrione ($p \leq 0.001$) (Figure 4). However, the expression of *LmCAB* reached a peak (> 2.5-fold, $p \leq 0.001$) at 7 d after mesotrione application. *LmRubisco* showed a downward trend at 8 h after mesotrione treatment ($p \leq 0.001$) (Figure 4).

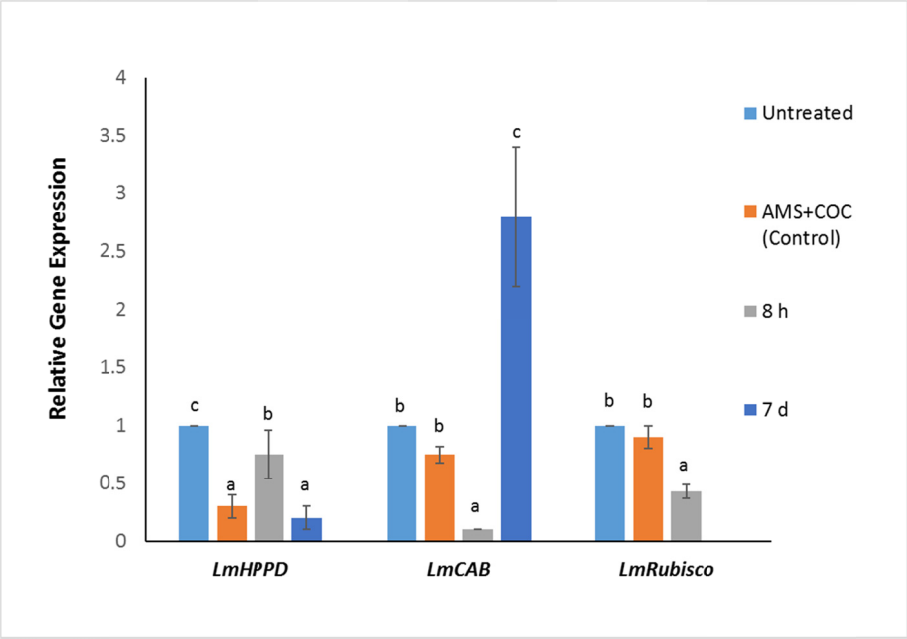

Figure 4. Relative fold expression of the target-site (*LmHPPD*) and photosynthetic genes (*LmCAB* and *LmRubisco*) in *L. multiflorum* after mesotrione treatment. Letters indicate significant differences ($p < 0.05$) among the means of each gene at different time points (8 h and 7 d after treatment). The expression of the target-site and photosynthetic genes was measured relative to an AMS + COC treated control. Error bars represent±standard deviation from the mean (n = 3). Mean values were based upon three biological and nine technical replicates. The qPCR data was normalized using *LmActin* as the reference gene. AMS + COC refers to treatment with both ammonium sulphate and crop oil concentrate. Transcripts of *LmRubisco* were undetectable at 7 d DAT and therefore was not shown in this figure

3.5 Chlorophyll Index and Fluorescence (F_v/F_m)

The leaf chlorophyll index did not significantly change during the two-week period after the application of four herbicides (Figure 5A). However, chlorophyll fluorescence (F_v/F_m) decreased 2 DAT with atrazine (Figure 5B), and then showed an upward trend for the next 12 days, until it reached close to untreated control. A decrease in fluorescence was detected five days after mesotrione treatment, which eventually reached back to control levels by day 12 (Figure 5B). Chlorophyll fluorescence remained unchanged during the 7 and 14 d duration after glyphosate and chlorsulfuron treatments (Figure 5B).

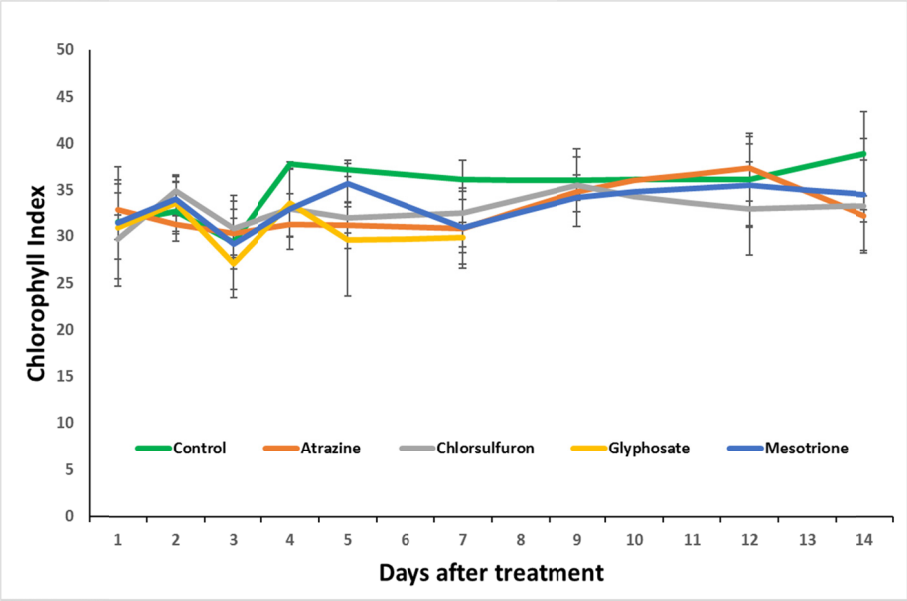

Figure 5A. Chlorophyll index in *L. multiflorum* during the 14 d period after treatment with four modes of action herbicides. Error bars represent±SD from the mean (n = 4)

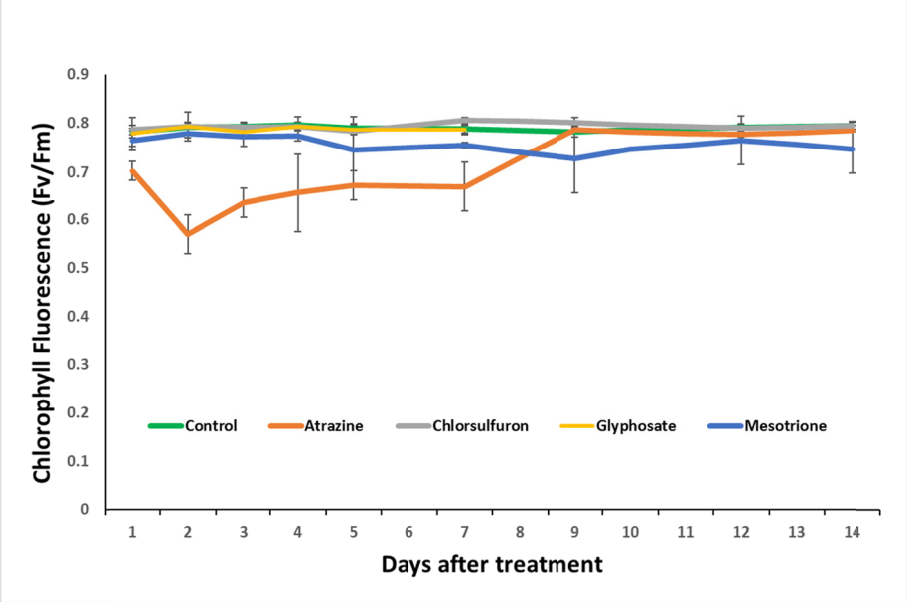

Figure 5B. Chlorophyll fluorescence in *L. multiflorum* during the 14 d period after treatment with four modes of action herbicides. Error bars represent±SD from the mean (n = 4)

4. Discussion

Our understanding of the SOA of the commonly used herbicides for weed control is limited to their effect on their respective target sites. An important question that remains unanswered is the effects of herbicides on critical non-target site pathway genes (such as photosynthesis) important for plant survival. It is generally believed that rapid chloroplast destruction or aromatic amino acid starvation leads to plant death after herbicide application. Does the inhibition of amino acid pathways by herbicide application (such as EPSPS- and ALS-inhibitors) affect the chloroplast and related pathway genes in PSII and Calvin cycle?

Expression profiles of genes in any biosynthetic pathway will be altered if there is inhibition of that specific pathway in plants. Importantly, such inhibition also affects the expression of genes in other related pathways as well (Guyer et al., 1995). Through the use of different molecular and genetic approaches, it has become increasingly apparent that plant signal transduction involves extensive cross-talk between different pathways leading to various physiological and developmental responses to environmental stimuli (Møller & Chua, 1999). Several herbicides have targets in pathways that share some common products produced in those pathways (Jiang et al., 2013). For example, in PSII and carotenoid biosynthesis pathways (Siggel, 1976; Norris et al., 1995), plastoquinone plays an important role, and in these pathways the *psbA* and *HPPD* genes code for the herbicide target proteins. Likewise, chorismate, is an important precursor for the production of various amino acids, which is one of the products produced in the shikimate pathway involving EPSPS, the target site of glyphosate (Tzina & Galilia, 2010). In order to understand the cross-talk between the pathways that involve herbicide targets, it is important to assess the effect of herbicides on the expression of the herbicide targets (Duke et al., 2013; Dayan & Duke, 2014).

4.1 Expression of Target-Site Genes upon Herbicide Treatments

In this study, we observed a > 3-fold increase in the expression of the *LmALS* gene at 8 h after treatment with chlorsulfuron, ALS-inhibitor (Figure 1). These herbicides inhibit the enzyme ALS thereby the formation of branched-chain amino acids (valine, leucine, and isoleucine). It has been shown that variation in the expression of *ALS* gene in various plant parts of tobaaco, *i.e.*, leaves, young seedlings, and flowers (Keeler et al., 1993). Also, in response to treatment with ALS-inhibitors, an increased protein turnover resulting increased degradation and decreased protein synthesis (Orcaray et al., 2010; Shaner & Reider, 1986; Anderson & Hibberd, 1985; Hofgen et al., 1995; Rhodes et al., 1987). The increased expression of *LmALS* gene found in this study may result in high protein turnover and amino acid accumulation in response to treatment with chlorsulfuron; this may result in lower soluble protein content leading to plant death.

In response to treatment with atrazine, the expression of *LmpsbA* gene was initially downregulated (> 40-fold) at 8 h, although there was an increase in its transcript levels at 7 DAT (Figure 2). The PS-II inhibitors, including atrazine binds to D1 protein, encoded by the chloroplastic gene, *psbA* (Zurawski et al., 1982; Gronwald, 1994), and as a result, the electron transport system is inhibited (Rutherford & Krieger-Liszkay, 2001). Eventually, plant death occurs due to a lack of production of ATP and reducing power, required for carbon fixation. Ultimately the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) will result in lipid peroxidation and plant death (Boulahia et al., 2016; Rutherford & Krieger-Liszkay, 2001). This will also inhibit the degradation of Dl protein and turnover rates (Sundby et al., 1993). This high turnover rate of Dl protein is important for restoring the photoinhibitory damage bestowed by these herbicides. An increased D1 protein turnover is also shown to have increased transcription of the *psbA* gene (Mulo et al., 2012). In this research at 7 d after PS-II inhibitor, atrazine treatment, there was an increased *LmpsbA* gene transcript level which might be because of high D1 protein turnover rates.

In this study, a significant increase in the *LmpsbA* gene expression (45-fold compared to the non-treated sample) was observed after treatment with COC (Figure 2). Crop oil concentrate is a commonly used adjuvant with PSII inhibitors for increasing the herbicide penetration. Previous studies with safeners, used for protecting crops from herbicide injury, indicated the expression of genes related to plant defense and detoxification after herbicide treatments (Riechers et al., 2010). The use of adjuvants may also trigger similar gene expression in the photosynthetic and other pathways. Future studies will help in elucidating the molecular action of adjuvants commonly used along with herbicides.

Glyphosate is also an amino acid biosynthesis inhibitor, similar to ALS-inhibitors, thus treatment with glyphosate can increase the free amino acid content (Shaner & Reider, 1986; Wang, 2001). In response to glyphosate treatment (after 8 h), Gaines et al. (2010) reported no difference in the expression of *EPSPS* gene in Palmer amaranth (*Amaranthus palmeri* S. Wats.). Also, there were no *EPSPS* transcripts induced after glyphosate treatment (21 DAT) in horseweed [*Conyza canadensis* (L.) Cronq.] (Dinelli et al., 2006). Conversely, there was a

2 to 3-fold increase in *EPSPS* gene expression in wild soybean (*Glycine soja*) at 6 h after glyphosate treatment (Gao et al., 2014). Our data suggest that although there was no significant differences in *LmEPSPS* expression at 8 h after glyphosate treatment (Figure 3), nonetheless, there was a 16.7-fold increase in this gene expression at 7 d after treatment with glyphosate (Figure 3), possibly resulting in higher protein turnover and degradation.

HPPD gene expression was previously studied at varying growth temperatures in Palmer amaranth (Godar et al., 2015). Increased *HPPD* transcript levels (3.5 to 15-fold) were detected at 24 h after mesotrione treatment in plants grown under both optimum (32.5/22.5 ^{o}C) and high temperatures (40/30 ^{o}C) (Godar et al., 2015). Our data suggest an upregulation of the *LmHPPD* gene at 8 h after mesotrione treatment (Figure 4).

4.2 Expression of Photosynthetic Genes Upon Herbicide Treatments

Rubisco is the principal enzyme for CO_2 fixation and its expression, in general, remains the same, in both C3 and C4 plants (Patel & Berry, 2008). There are two, large and small subunits of Rubisco protein which are encoded by two separate genes (LSU-large subunit, and SSU-nuclear small subunit) (Patel & Berry, 2008). The factors such as a) expression of *Rubisco* gene, b) mRNA stability, c) polypeptide synthesis, d) post-translational modification, e) active holoenzyme assembly, etc. regulate the rate of biosynthesis and degradation of Rubisco activity (Sheen, 1990; Krapp et al., 1993; Mehta et al., 1992; Eckardt & Pell, 1995; Desimone et al., 1996). *Rubisco* transcript levels also depend on various other factors such as light (Sheen, 1999; Wang et al., 1993), cell and tissue type (Patel et al., 2004; Berry et al., 1997), the efficiency of photosynthetic machinery (Krapp et al., 1993; McCormac et al., 1997), phytohormone and nutrient levels (Sheen, 1999; Imai et al., 2005; Ookawa et al., 2004), and plant developmental stage (Hansel et al., 1993).

In response to chlorsulfuron treatment (after 8 h), we found a 1.4-fold increase in *LmRubisco* expression (Figure 1). The small increase in *LmRubisco* expression after chlorsulfuron treatment was most likely caused as a result of the effect of ALS-inhibitors on carbon metabolism. Suzuki et al. (2001) reported an increase in Rubisco content during leaf expansion, reaching a maximum around leaf maturation, then declining gradually during senescence. Inhibition of the CO_2 fixation levels and a decrease in efficiency of PSI and PSII electron transport, and the leaf chlorophyll content were reported two days after treatment with ALS-inhibitor metsulfuron-methyl (Riethmuller-Haage et al., 2006). In this study, the initial *Rubisco* upregulation after ALS-inhibitor treatment was most likely due to the rapid growth of the leaves. No change in chlorophyll index and chlorophyll fluorescence was observed two days after chlorsulfuron treatment (Figures 5A and 5B).

Zhu et al. (2009) reported that the expression of genes associated with the Calvin cycle in soybean significantly varied after atrazine treatment. Rubisco activase and Rubisco-associated proteins displayed mixed expression patterns (either upregulated or downregulated) (Zhu et al., 2009). The current study indicated no significant change in *LmRubisco* gene expression at 8 h after atrazine treatment (Figure 2).

A modest but significant downregulation was observed for *LmRubisco* at 8 h after glyphosate treatment (Figure 3). Ahsan et al. (2008) reported a downregulation (3-4 fold) of *Rubisco* at 12 h after glyphosate treatment in rice (*Oryza sativa* L.). Vivancos et al. (2011), while working with soybean have shown that glyphosate causes the depletion of proteins associated with PSII and an eventual shutdown of photosynthesis. Both the Calvin cycle as well as chlorophyll biosynthesis are affected as a result of decreased CO_2 assimilation and metabolism after glyphosate treatment (Kitchen et al., 1981; Ireland et al., 1986). Inhibitory effects of glyphosate on chlorophyll fluorescence were also reported (Ireland et al., 1986). However, in this study, there was no change in chlorophyll fluorescence and chlorophyll index during the 7 d period after glyphosate treatment (Figures 5A and 5B). Stomatal conductance and CO_2 assimilation were found to be reduced earlier than chlorophyll fluorescence and index in biotypes of *Lolium perenne* L. (Yanniccari et al., 2012).

LmRubisco was downregulated at 8 h after mesotrione treatment (Figure 4). The photooxidative damage caused to chlorophyll pigments and thylakoid membranes due to decreased carotenoid biosynthesis after mesotrione application would possibly result in the downstream inhibition of PSII electron transport pathway leading to a reduction in the ATP and NADPH synthesis and CO_2 fixation by Rubisco.

Chlorophyll a/b binding proteins are the major components of light-harvesting antennae complex of PSII in plants, required for light harvesting and photoprotection via dissipation of excess light energy (Dittami, 2010). Several abiotic stresses such as salinity, metal ions, and ultraviolet radiation can determine the expression of *CAB* gene (Capel et al., 1998; Nott et al., 2006; Staneloni et al., 2008), and is known to be active during the production ROS in chloroplasts (Nott et al., 2006; Kwak et al., 2006; Galvez-Valdivieso & Mullineaux, 2010; Xu et al., 2012). In this study, the transcript levels of *LmCAB* decreased at 8 h after treatment with ALS-, PSII-, EPSPS-, and HPPD-inhibitors (Figures 1, 2, 3, and 4, respectively). In soybean, it was found that the *GmCAB* gene was downregulated at 1, 2, 4, and 8 HAT with atrazine (Zhu et al., 2009). A similar inhibitory effect on

CAB gene expression can be expected with other SOA herbicides such as chlorsulfuron, glyphosate, and mesotrione.

4.3 Chlorophyll Index and Fluorescence (F_v/F_m)

One of the parameters used to evaluate plant health includes the measurement of leaf chlorophyll index (Uddling et al., 2007). Our data suggest that in response to herbicide treatments, no difference in the leaf chlorophyll index of *L. multiflorum* during the 14 d period compared to non-treated plants (Figure 5A). Similarly, in other weed species such as common lambsquarters (*Chenopodium album*) no change in the leaf chlorophyll index after herbicide treatment was reported (Ketel, 1996; Sea et al., 2012).

The photosynthetic capability and the efficiency of PSII in plants can be measured by chlorophyll fluorescence (F_v/F_m) which is highly sensitive to stress (Krause & Weis, 1991; Lu & Zhang, 2000). If there is an inhibition of photosynthetic machinery and an increase in energy dissipation then there will be a decrease in F_v/F_m values (Song et al., 2014). Among the herbicides tested in this research, atrazine is a PSII-inhibitor and can have a more significant effect on the functioning of PSII compared to others (Figure 5B). Upon treatment with atrazine the inhibition of the electron transport chain will affect the functioning of PSII resulting in a decrease in chlorophyll fluorescence values.

The *L. multiflorum* biotype was killed by 1x rate (field rate) of glyphosate, whereas it survived 1x rates of atrazine, chlorsulfuron, and mesotrione (data not shown). In the plants treated with atrazine, chlorophyll fluorescence decreased markedly during the first 24 h, and completely recovered after nine days (Figure 5B). This initial decrease in chlorophyll fluorescence followed by a later recovery of PSII efficiency to control levels in *L. multiflorum* indicates the presence of an inherent herbicide detoxification mechanism. *L. multiflorum* is known to be naturally tolerant to several herbicides (Merini et al., 2009).

5. Conclusions

The data from this study demonstrates differential transcript levels of herbicide target genes in response to herbicide treatment in *L. multiflorum*. Herbicide target genes were found to be upregulated (*LmALS* and *LmHPPD*), downregulated (*LmpsbA*), or remained similar to control levels (*LmEPSPS*) after herbicide treatments. Herbicide treatment can not only affect the expression of herbicide target genes, but can also influence transcript levels of other genes involved in photosynthesis, including the Calvin cycle in plants.

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