UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Fitossanidade

Dissertation

Biochemical, Molecular, Transcriptomic, and Bioinformatics Studies to Identify Genes-Related to Glyphosate-Resistance Mechanisms in *Conyza bonariensis*

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Pelotas, 2019

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Tese apresentada ao Programa de Pós-Graduação em Fitossanidade da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Fitossanidade, área do conhecimento Herbologia.

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À minha mãe, Valéria Piasecki e à
todos meus familiares, Aleixo, Albino,
Claudete, Maria Cecília, Aline, Martina,
Bruno, Silvestre, Lúcia (*in memoriam*),
Gentil, Verônica, Teresinha, Fernanda,
Cálita, Luiz Fernando, Mariano e Ivone

OFEREÇO E DEDICO.

Quantas vezes eu estive
Cara a cara com a pior metade?
A lembrança no espelho
A esperança na outra margem

Quantas vezes a gente sobrevive
À hora da verdade?
Na falta de algo melhor
Nunca me faltou coragem

Se eu soubesse antes o que sei agora
Erraria tudo exatamente igual

Tenho vivido um dia por semana
Acaba a grana, mês ainda tem
Sem passado nem futuro
Eu vivo um dia de cada vez

Quantas vezes eu estive
Cara a cara com a pior metade?
Quantas vezes a gente sobrevive
À hora da verdade?

Se eu soubesse antes o que sei agora
Iria embora antes do final

Surfando karmas e DNA
Eu não quero ter o que eu não tenho
Eu não tenho medo de errar!

Surfando karmas e DNA
Não quero ser o que eu não sou
Eu não sou maior que o mar

Na falta do que fazer, inventei a minha liberdade!

Hengenheiros do Hawaii

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Abstract

PIASECKI, Cristiano. **Biochemical, Molecular, Transcriptomic, and Bioinformatics Studies to Identify Genes-Related to Glyphosate-Resistance Mechanisms in *Conyza bonariensis*.** 2019. 148p. Dissertation (Doctorate) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas.

The hairy fleabane [*Conyza bonariensis* (L.) Cronq.] is among the most widespread economically important glyphosate-resistant weeds in the world. This weed-species has a cosmopolitan distribution, is very competitive and reduce the crop yield substantially. The evolved glyphosate resistance hairy fleabane hinders its management, contribute to increasing the production costs, and consequently reduce agriculture profit. The mechanisms of glyphosate-resistant in hairy fleabane remains unknown and has been attributed to the non-target site (NTSR). Transcriptomic approaches such as RNA sequencing (RNA-Seq) have been applied in molecular studies in non-model plants as weeds, which have a limited molecular database available. The RNA-Seq methodology does not require a reference genome to be assembled and provide high quality and quantity data. Using bioinformatics analysis is possible to identify genes differentially expressed in response to glyphosate treatment. Thus, comparisons between glyphosate-resistant (GR) and -sensitive (GS) biotypes can identify genes related to glyphosate resistance mechanisms. Other RNA-Seq studies were performed in *C. bonariesnsis*; however, its glyphosate-resistance mechanisms remain unclear. Those works were performed up to 48 h after glyphosate treatment. We hypothesized that a short time after treatment was not enough to capture all plant transcriptional responses to glyphosate induced-stress. In this way, we performed experiments to validate our hypothesis. Shikimic acid is the first known metabolite produced after glyphosate inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). In two time-course experiments performed up to 552 h after glyphosate treatment using different doses, we tracked the shikimic acid content in both GR and GS biotypes. The results indicate that the shikimic acid content was transitory, increasing substantially at 24 h after treatment, peaked at 96 h, and at 288 h presented similar content as the untreated plants. In contrast the GS biotype had similar initial shikimic acid levels as GR plants at 24 h and 96 h, but it peaked at 192 h and died after that time-point. Glyphosate treatment leads to reactive oxygen species (ROS) overproduction, which might degrade the RNA integrity and compromise further analysis. Thus, we evaluated the ROS production, oxidative stress, antioxidant enzyme activity, and RNA integrity. The ROS production was higher in GS biotype, whereas in GR increased the antioxidant enzyme activity,

which might be involved in the glyphosate resistance mechanisms. RNA integrity was not negatively affected by glyphosate treatment in both biotypes. Thus, the RNA-Seq and qRT-PCR were performed in a time course, based on shikimic acid results up to 288 h after treatment. The RNA-Seq study revealed 41 candidate NTSR genes related to transport and metabolism. Among them, 19 ABC transporters, 10 CYP450, one glutathione, and five glycosyltransferases. In addition, we report the transcription results of two genes coding for antioxidant enzyme catalase, peroxidase, and superoxide dismutase. These selected target genes might be acting in a complex association to confer glyphosate-resistance in *C. bonariensis.* The gene expression varies among gene groups and within the same group, between biotypes, is responsive to glyphosate and time after treatment. In general, the highest expression responses occurred between 96 h to 192 h after treatment. The present study is the first to associate CYP450, ABC transporters, GT, GST, and antioxidant system to glyphosate-resistance in *C. bonariensis.*

Keywords: *Conyza bonariensis,* Glyphosate-resistance, Antioxidant system, Shikimic-acid, RNA integrity, RNA-Seq, Differential expression, Candidate genes

Resumo

PIASECKI, Cristiano. **Estudos Bioquímicos, Moleculares, Transcriptomicos e de Bioinformática para Identificar Genes Relacionados com os Mecanismos de Resistência ao Glyphosate em *Conyza bonariensis*.** 2019. 148p. Tese (Doutorado) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas.

A buva (*Conyza bonariensis* (L.) Cronq.) está entre as ervas daninhas resistentes ao glyphosate economicamente mais difundidas no mundo. Esta espécie de planta daninha tem uma distribuição cosmopolita, é muito competitiva e reduz substancialmente o rendimento das culturas. A resistência ao glyphosate dificulta o manejo da buva, contribui para o aumento dos custos de produção e, consequentemente, reduz o lucro da atividade agrícola. Os mecanismos de resistência glyphosate na buva permanecem desconhecidos e são atribuídos ao local não alvo. Abordagens transcriptômicas como o sequenciamento de RNA (RNA-Seq) têm sido aplicadas em estudos moleculares em plantas não-modelo como ervas daninhas, que possuem um limitado banco de dados moleculares disponível. A metodologia do RNA-Seq não requer genoma de referência para ser montado, e produz resultados qualitativos e quantitativos. Utilizando análises de bioinformática, é possível identificar genes diferencialmente expressos em resposta ao tratamento com glyphosate. Assim, comparações entre biótipos resistentes (GR) e sensíveis (GS) ao glyphosate podem identificar genes relacionados aos mecanismos de resistência. Outros estudos de RNA-Seq foram realizados em *C. bonariensis*, no entanto, os mecanismos de resistência ao glyphosate permanecem desconhecidos. Esses trabalhos foram realizados até 48 horas após o tratamento com glyphosate. Nossa hipótese foi de que o curto período de tempo após o tratamento não foi suficiente para capturar todas as respostas transcricionais da planta ao estresse induzido pelo glyphosate. Desta forma, realizamos experimentos para validar a hipótese. O ácido chiquímico é conhecido como o primeiro metabólito produzido após o glyphosate inibir a enzima 5-enolpiruvilchiquimato-3-fosfato sintase (EPSPS). Em dois experimentos realizados até 552 h após o tratamento com diferentes doses de glyphosate, nós avaliamos o conteúdo de ácido chiquímico em ambos os biótipos. Os resultados indicam que o conteúdo de ácido chiquímico no biótipo resistente foi transitório, aumentando substancialmente em 24 h após o tratamento, atingiu o pico em 96 h, e em 288 h apresentou conteúdo semelhante ao das plantas não tratadas. Em contraste, o biótipo sensível teve níveis de ácido chiquímico iniciais semelhantes às plantas do resistent às 24 e às 96 h, mas atingiu o pico em 192 h e morreu após esse período. O tratamento com glyphosate leva à superprodução de espécies reativas de oxigênio (EROS), o

que pode degradar a integridade do RNA e comprometer futuras análises. Assim, avaliamos a produção de ROS, o estresse oxidativo, a atividade de enzimas antioxidantes e a integridade do RNA. A produção de EROS foi maior no biótipo sensível, enquanto no resistente apresentou maior atividade das enzimas antioxidantes, que podem estar envolvidas nos mecanismos de resistência ao glyphosate. A integridade do RNA não foi afetada negativamente pelo tratamento com glyphosate em ambos os biótipos. Assim, os estudos de RNA-Seq e qRT-PCR foram realizados em um curso de tempo, com base nos resultados de ácido chiquímico, até 288 h após o tratamento. O estudo RNA-Seq revelou 41 genes candidatos não envolvidos ao local alvo do herbicida e relacionados ao transporte e metabolismo. Entre eles, 19 transportadores ABC, 10 CYP450, uma glutationa e cinco glicosiltransferases. Além disso, relatamos os resultados da transcrição de dois genes que codificam a enzima antioxidante catalase, peroxidase e superóxido dismutase. Esses genes-candidatos podem estar atuando em uma complexa associação para conferir resistência ao glyphosate em *C. bonariensis*. A expressão gênica variou entre os grupos de genes e dentro do mesmo grupo, entre os biótipos, é responsiva ao glyphosate e ao tempo após o tratamento. Em geral, a maior expressão gência ocorreu entre 96 h e 192 h após o tratamento. O presente estudo é o primeiro a associar CYP450, transportadores ABC, GT, GST e sistema antioxidante à resistência ao glyphosate em *C. bonariensis*.

Palavras-chave: *Conyza bonariensis,* Resistência ao glyphosate, Sistema antioxidante, Ácido chiquímico, Integridade do RNA, RNA-Seq, Expressão diferencial, Genes candidatos.

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Summary

General Introduction

In the last few years, genomic approaches have revolutionized plant biology. From the model plant, *Arabidopsis thaliana*, to important crop plants such as rice (*Oryza sativa*), soybean (*Glycine max*) and maize (*Zea mays*); powerful complete genome data sets and tools are yielding the unprecedented ability to explore plant form and function. Furthermore, the genome sequence of additional plant species will be available from in-progress large-scale sequencing projects (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html). One critical group of plants that has been largely overlooked in the genomics revolution is economically significant weeds (STEWART, 2009; STEWART et al., 2009). Weeds are plants that possess particular traits enabling them to persist and be problematic in cultivated land. Of some 250,000 angiosperms, only a couple hundred (i.e., about 0.1%) are weeds of major significance (HOLM et al., 1991; 1997). Thus, relatively few plants possess the suite of biological traits necessary to be noxious weeds.

Weeds are the world's most successful plants: they are among the most persistent, adaptable, stress-tolerant, and competitive. To agriculturalists, weeds are defined as highly competitive plants that persistently adapt to cropping systems and cause crop loss and damage. Weeds cause about $100 billion in annual damage (JESCHKE, 2016; VAN WYCHEN, 2016). The cost is even higher if one includes weeds in pastures, golf courses, aquatic environments, etc. Furthermore, this $100 billion estimate includes only direct costs (i.e., control costs and economic loss) and not indirect costs, such as costs to the environment and human health (JESCHKE, 2016; VAN WYCHEN, 2016). Because of the economic significance of weeds, research has focused on applied aspects, such as how to control a particular weed in a particular crop. With the advent and

accessibility of genomics and transcriptomic tools and resources, we are now poised to identify the genes that underlie weediness traits, thereby answering the fundamental question in weed science, indeed, also in plant biology, “What makes a plant a weed?”

Herbicide application has been a prominent weed control measure in recent decades but, increasingly, herbicide-resistant weeds are challenging this practice. Herbicide resistance refers to the ability of a plant biotype to survive and reproduce under a normally lethal dose of herbicide - either evolving target-or non-target site resistance (YUAN et al., 2007). We have witnessed a dramatic increase in the number of herbicide resistant species and biotypes during the past two decades, especially non-target site resistance of unknown molecular mechanism. More than 500 biotypes of weeds have evolved resistance to one or more of all the major groups of herbicides (HEAP, 2019), among which, resistance to glyphosate is currently of greatest concern.

Glyphosate has become the world’s most widely used herbicide for controlling weeds for several reasons, including its high efficacy and low cost, and because it is environmentally benign (DUKE, 2018). The widespread adoption of transgenic herbicide-resistant crops, such as Roundup-Ready soybean, corn, cotton, and oilseed, has greatly improved the effectiveness of weed management (DUKE, 2018). However, greater glyphosate usage has played a role in the evolution of glyphosate resistance in weedy species (DUKE, 2018; SAMMONS and GAINES, 2014). Horseweed (*Conyza canadensis*), which is in the Asteraceae family, was the first broadleaf weed to evolve glyphosate resistance (GR) in agriculture – United States (VAN GESSEL, 2001). The GR in hairy fleabane (*C. bonariensis*) was first documented in 2005 in Brazil (VARGAS et al., 2007). A third important GR *Conyza* species is sumatran fleabane (*C. sumatrensis*), which was first reported as GR in Spain in 2009 (HEAP, 2019). Together, these three *Conyza* species accounts 63 reports of GR and are the most widely distributed glyphosate-resistant weeds in the world (HEAP, 2019; SHRESTHA et al., 2014).

Glyphosate resistance mechanisms can be grouped into two general categories: target site (TS) resistance, i.e., caused by changes in the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) coding-gene and shikimic acid metabolism; and non-target site resistance mechanism (NTSR), which

encompasses, essentially, all mechanisms not involving EPSPS-related alterations (DÉLYE et al., 2015; SAMMONS and GAINES, 2014; YUAN et al., 2007). The glyphosate resistance in *Conyza* spp. is due to the NTSR mechanism (AMARO-BLANCO et al., 2018; HEREWARD et al., 2018). To-date, TS resistance has not been discovered in *C. bonariensis*, thus one or more NTSR mechanisms are to blame (AMARO-BLANCO et al., 2018; HEREWARD et al., 2018). The best physiological evidence indicates that NTSR in *C. bonariensis* is caused by subcellular glyphosate sequestration, likely to the vacuole, which prevents it from being translocated to tissues that have not been sprayed (AMARO-BLANCO et al., 2018; HEREWARD et al., 2018; MORETTI et al., 2017).

Genomic efforts on economically important weeds, such as *Conyza* spp., have been extremely limited due mainly to its expensive costs. Weed genomics is critical to understand weed biology and understanding weed biology is critical in weed management (PENG et al., 2014). However, the next generation sequencing (NGS) has offering dramatic increases in cost-effective sequence throughput (MARGULIES et al., 2005; MOROZOVA et al., 2005). The NGS has had a tremendous impact on genomic research for increasing sequencing depth and coverage while reducing time, labor, and cost (MOROZOVA et al., 2005; ROTHBERG and LEAMON, 2008).

Experimental methods, such as transcriptomic, can make collective characterizations of genes is becoming crucial tools to discovery NTSR mechanisms to herbicides (GIACOMINI et al., 2018). A modern technique which has been gradually adopted by scientists in the last years to study non-model plants is the RNA sequencing (RNA-Seq) (WANG et al., 2009). The RNA-Seq approach allows making large scale comparisons of gene expression between herbicide-treated and untreated plants of herbicide-resistant and -sensitive biotypes (GIACOMINI et al., 2018). Thus, the whole-transcriptome comparison performed through the differential expression analysis can reveal NTSR genes.

The transcriptome can be *de novo* assembled, that means that there is no needed reference genome. It is a huge advantage to non-model plants, which have a very limited sequence database. The assembled transcriptome provides sequences of known and unknown genes which can be used in further molecular analysis, such as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (WANG et al., 2009).

In addition to the present work, another two RNA-Seq studies were performed in *C. canadensis* and *C. bonariensis* (PENG et al., 2010; HEREWARD et al., 2018). Despite the power of the RNA-Seq technique, the glyphosate NTSR mechanism remains unknown. Those studies sequenced RNA at 24h and 48h after treatment. In this way, we hypothesized that their unsuccessful is due the short time RNA was sequenced after glyphosate treatment. Thus, most likely, those RNA-Seq studies were not able to capture all gene expression changes in plant defense system in response to herbicide-stress induced.

Glyphosate directly inhibits the enzyme EPSPS (MAROLI et al., 2015), interrupt the shikimic acid pathway (TZIN and GALILI, 2010) and biosynthesis of aromatic amino acids in plants (DUKE et al., 2003). Following occurs the accumulation of shikimic-acid (SCHÖNBRUNN et al., 2001), leading to oxidative stress in plants through the reactive oxygen species (ROS) overproduction (MAROLI et al., 2015). In this way, a long evaluation period after glyphosate treatment is required. However, the ROS generated by the glyphosate' action damage plant cells and can degrade nucleic acids (DNA and RNA). RNA degradation will compromise further molecular analysis (FOYER and NOCTOR, 2005; GILL and TUTEJA, 2010) such as RNA sequencing and qRT-PCR. However, plants use antioxidants systems (non-enzymatic and enzymatic) to cope with oxidative stress induced by ROS (RADWAN and FAYEZ, 2016), and this enzyme group might alleviate the oxidative stress produced by glyphosate action and can be involved with its resistance process (MAROLI et al., 2015).

Thus, considering the agricultural and economic importance of the glyphosate resistance in *C. bonariensis*, the objectives of the present work included: a) evaluate the biochemical alterations, oxidative stress and differential antioxidant enzyme activity in response to glyphosate treatment in glyphosate-resistant and -sensitive biotypes of *C. bonariensis*; b) study the effects of glyphosate treatment on RNA integrity of *C. bonariensis* glyphosate-resistant and -sensitive biotypes; c) perform RNA sequencing, *de novo* assembling of the *C. bonariensis* transcriptome, and identify candidates genes related to glyphosate resistance mechanisms; d) produce a portable tool to identify de glyphosate-resistant *C. bonariensis* plants in the field.

Article 1 – Bragantia [Accepted v. 78(3) (2019)]

Oxidative Stress and Differential Antioxidant Enzyme Activity in Glyphosate-Resistant and -Sensitive Hairy Fleabane as Response to Glyphosate Treatment

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Abstract

Biochemical assays in a time-course were employed to evaluate stresses induced by glyphosate treatment in a resistant (B11R) and sensitive (B17S) biotypes of *Conyza bonariensis.* Two experiments were conducted assessing glyphosate doses and time-course after treatment. The doses of glyphosate ranged from 0 to 11,840 g ae ha^{-1} and assessments performed until 552 h after glyphosate treatment (HAT). The objectives of this study were to evaluate the oxidative stress and differential antioxidant enzyme activity in glyphosate-resistant and -sensitive biotypes of hairy fleabane after glyphosate treatment. After treatment, both studied biotypes accumulated similar levels of shikimic-acid until 96 hours. The sensitive biotype died at 192 HAT. Shikimic-acid and hydrogen

peroxide (H_2O_2) accumulation in glyphosate-resistant biotype were transient and did not differ from those of non-treated plants at 288 and 500 HAT, respectively. In both glyphosate-resistant and sensitive biotypes, a correlation analysis established a cause-and-effect relationship after glyphosate treatment, which leads to shikimic-acid and hydrogen peroxide accumulation, lipid peroxidation (indicates tissue damage) and an enhancement in the activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzyme. However, in the glyphosate-resistant biotype, the oxidative stress and tissue damage were lower, and antioxidant enzyme activities SOD, CAT, and APX were higher than -sensitive biotype. It indicates that antioxidant enzyme in glyphosate-resistant biotype might be related to the glyphosate resistance process. This study is the first report of differential antioxidant enzyme activity in hairy fleabane.

Keywords: *Conyza bonariensis*, lipid peroxidation, shikimic-acid, herbicide resistance.

INTRODUCTION

Hairy fleabane (*Conyza bonariensis* (L.) Cronq.) belonging to the botanical Asteraceae family is native to the Americas and now has a cosmopolitan distribution (Shrestha et al. 2014; Bajwa et al. 2016). Hairy fleabane is highly competitive on crops and is among the most challenging weed species to management around the world (Shrestha et al. 2014; Bajwa et al. 2016; Concenço and Concenço, 2016). According to Trezzi et al. (2015), the yield losses caused by one hairy fleabane plant m^{-2} on soybean can arrive at 36%.

Glyphosate (N-(phosphonomethyl)glycine) is employed in many crop production systems to control a broad spectrum of annual and perennial grasses and broadleaf weeds

(Duke and Powles, 2008; Dill 2005). The widespread use of glyphosate is because of its high efficacy, environmental feature, and low cost (Peng et al. 2010). However, the intensive use of glyphosate has been a factor in weeds evolving resistance, rendering glyphosate treatment ineffective (Baucom and Holt, 2009) in at least 42 weed species (Heap 2018). Of these, glyphosate-resistant from *Conyza* genera. *Conyza canadensis* L. (horseweed) was the first broadleaf weed to evolve glyphosate-resistance (GR), which was observed in the United States in 2000 (Van Gessel 2001). The GR in *C. bonariensis* was first documented in 2005 in Brazil (Vargas et al. 2007).

Glyphosate directly inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Maroli et al. 2015) interrupting the shikimic acid pathway, which is a crucial pathway to carbon and nitrogen metabolism, causing the death in sensitive plants (Tzin and Galili, 2010). In the shikimic acid pathway, EPSPS catalyzes the conversion of the shikimic acid to chorismate, which is the precursor for the biosynthesis of aromatic amino acids tryptophan, phenylalanine, and tyrosine, and secondary metabolites in plants (Duke et al. 2003). Glyphosate acts as a competitive inhibitor concerning phosphoenolpyruvate (PEP), a substrate for EPSPS, to form a complex very stable between enzyme-herbicide and inhibits the product-formation reaction (Sammons et al. 1995).

The interruption in the shikimic acid pathway disrupts the aromatic amino acid biosynthesis and cause alterations in the metabolic stoichiometric of carbon intermediates, which result in system wide-perturbations (Maroli et al. 2015). The EPSPS inhibition by glyphosate and consequently shikimic acid pathway blocking results in the accumulation of shikimic-acid (Schönbrunn et al. 2001), leading to oxidative stress in plants through reactive oxygen species (ROS) production (Maroli et al. 2015). After glyphosate treatment and EPSPS inhibition, there is ROS generation because the

herbicide causes perturbations in the photosynthetic machinery through reducing ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity and 3-phosphoglyceric acid (3-PGA) levels (Servaites et al. 1987; Serviev et al. 2006).

The primary ROS are the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH•), and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010). The ROS are highly reactive toxic molecules, causing lipid peroxidation (LPO), oxidation of DNA, RNA and proteins, damage to cellular structures, and cell death (Foyer and Noctor, 2005; Gill and Tuteja, 2010). When the amount of ROS exceeds the plant's capacity to scavenging it through the action of the antioxidant system, occurs the LPO, the most destructive cellular processes in living organisms, loss of cellular homeostasis, leading to loss of membrane integrity, and cell death (Foyer and Noctor, 2005). Also, the ROS may serve as signaling molecules in response to stresses activating and controlling gene expression (Das and Roychoudhury, 2014).

Plants use antioxidants systems (non-enzymatic and enzymatic) to cope with oxidative stress induced by ROS (Radwan and Fayez, 2016). Among the main enzymatic antioxidants are the superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Gill and Tuteja, 2010; Caverzan et al. 2012; Mittler 2017). SOD provides the first line of defense against the toxic effects of elevated levels of ROS, through removing $O_2^{\bullet -}$, by catalyzing its dismutation, one $O_2^{\bullet -}$ being reduced to H_2O_2 and another oxidized to O_2 (Gill and Tuteja, 2010). CAT directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions (Garg and Manchanda, 2009). APX play the essential role in cope with ROS and protecting cells in higher plants, through scavenging H_2O_2 in water-water and ascorbate-reduced glutathione cycles and utilizes ascorbate as the electron donor (Gill and Tuteja, 2010; Caverzan et al. 2012). APX has a higher affinity for H_2O_2 than CAT and peroxidases (POD), and it may

have a more crucial role in ROS management during stress (Caverzan et al. 2012). The predominant source of ROS production is chloroplasts as a consequence of the high O_2 concentration from photolysis and high energy produced by the photosynthetic electron transport chain. In this way, antioxidant enzyme concentration in the chloroplast is high, but they are also in the cytosol, mitochondria, and peroxisomes (Gill and Tuteja, 2010).

Increase in antioxidant enzyme activity has been related to the protection of glyphosate damages in glyphosate-resistant (GR) *Amaranthus palmeri* biotype, indicating a potential role of antioxidant systems on resistance (Maroli et al. 2015). In hairy fleabane, studies have been reported greater constitutive activity of antioxidants enzyme in paraquat-resistant biotype than in a -sensitive and increased further following paraquat exposure (Shaaltiel and Gressel, 1987; Ye and Gressel, 2000). In these cases, injury symptoms were observed in both biotypes. However, the resistant plants survived to paraquat treatment. To our knowledge, there are no reports of differential antioxidant enzyme activity protecting hairy fleabane of glyphosate perturbations. Investigate the oxidative stress and antioxidant enzyme activity in response to glyphosate treatment could provide useful information about effects os ROS on DNA and RNA degradation. Nucleic-acid degradation evaluations are important, especially previous to molecular studies that investigate herbicide resistance mechanisms in weeds like RNA-Sequencing and quantitative reverse transcription PCR (qRT-PCR), among others. Also, antioxidant enzyme activity could be related to many abiotic stress responses, and in this case of study, the herbicide resistance process in weeds (Maroli et al. 2015).

Thus, it is hypothesized that following glyphosate treatment, the glyphosate-resistant biotype of hairy fleabane suffers lower oxidative stress and tissue damage and has higher antioxidant enzyme activity than -sensitive biotype. Enhance in antioxidant enzyme activity in the resistant biotype could be related to protection against oxidative

stress caused by glyphosate treatment. Therefore, the objectives of this research were to evaluate the oxidative stress and differential antioxidant enzyme activity assessing in glyphosate-resistant and -sensitive biotypes of hairy fleabane followed by glyphosate.

MATERIAL AND METHODS

Biotype Selection

As recommended by Burgos et al. (2013), we collected seeds of 54 biotypes of *Conyza sp.* (F_0 generation) in the Rio Grande do Sul State (RS), South of Brazil in March 2016. Seeds from glyphosate-resistant (GR) biotypes were collected in agricultural areas with farmer complaints about hairy fleabane control in soybean cultivation that had been treated with glyphosate for a minimum of 5 years. The glyphosate-sensitive biotypes were collected in areas without glyphosate application. In both cases, seeds from each plant were placed in an individual paper bag and stored in a refrigerator (~4 $^{\circ}$C) for two weeks. Subsequently, seeds of each biotype were germinated in trays containing sterilized soil and commercial substrate (Mac plant – Mec Prec, Brazil) 3:1, and watered daily in a greenhouse at 30°C/20°C day/night (± 4 $^{\circ}$C) with 12-hour photoperiod. Thirty days after emergence (30 DAE) seedlings of each biotype were transplanted to pots containing 2.0 L of soil and substrate (according described above). Thirty days after (60 DAE; rosette stage - plants 6 to 8 cm in diameter) plants were treated with glyphosate (1,480 g ae ha^{-1} – Roundup Original DI 370 g ae L^{-1}; Monsanto) using CO_2 sprayer and 150 L ha^{-1} of spray volume. Glyphosate-treated plants were considered resistant if they survived at 28 days after treatment (DAT).

Two biotypes of interest were selected from the municipality of Pelotas 48 km apart from each other: B11R - glyphosate-resistant 32º04’05.91” S, 52º52’59.14” W; B17S -

glyphosate-sensitive 31º49'15.15" S, 52º27'39.55" W. The *Conyza bonariensis* species of two selected biotypes was determined using molecular simple sequence repeats (SSR) markers through genotyping approach (Abercrombie et al. 2009; Marochio et al. 2017).

During two generations (F_0 and F_1) B11R and B17S non-treated plants were self-pollinated and GR segregation assessed by glyphosate treatment. In F_0 generation, segregation was assessed in 94 plants in each biotype (B11R and B17S) and F_1 generation, in 220 plants per biotype. Following glyphosate treatment (1,480 g ae ha^{-1}, 60 DAE; rosette stage) in both generations was evaluated whether plants alive or died at 28 DAT. After that, percentual segregation was calculated for B11R and B17S biotypes in both generations.

Glyphosate Dose-Response

Plants of the F_1 generation of B11R and B17S were cultivated until 60 DAE in a greenhouse. Plants were treated with glyphosate (Roundup Original DI 370 g ae L^{-1}; Monsanto) at the following doses: 0; 92.2; 185; 370; 740; 1,480; 2,960; 5,920; 11,840; and 23,680 g ae ha^{-1}, with a CO_2 sprayer and 150 L ha^{-1} of spray volume. The experiment was performed twice using a completely randomized design with four replicates (three plants per pot formed each replicate). Shoot material was harvested at 28 DAT and dried at 60 °C until constant weight to determine dry weight reduction compared with the non-treated control.

Dose-response curves for the plant bioassays were obtained by a non-linear regression using the log-logistic following equation:

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

Where *C* is the lower limit, *D* is the upper limit, *b* is the slope at the GR_{50}, and GR_{50} indicates the glyphosate dose necessary to reduce dry weight by 50%. The resistance

factor (RF) was calculated by the GR_{50} of the R population divided by that of the S population to estimate the resistance levels. The regression parameters for each biotype were obtained using Sigma Plot® (version 12.5, SPSS Inc, Chicago, IL, USA).

Experimental Design and Evaluated Variables

Two time-course experiments were performed in Pelotas, RS State, Brazil, in a greenhouse at 25 °C/15 °C day/night (± 3 °C) with 12-hour photoperiod with five replicates (three plants per pot formed each replicate) in a completely randomized design. For both experiments, plants of the F_1 generation of B11R and B17S were grown until 60 DAE according previously described. Experiment 1 (E1) was arranged in a three-factorial scheme 2 x 5 x 5. The first factor comprised two *C. bonariensis* biotypes (B11R and B17S); second factor comprised five glyphosate doses: 0; 370; 1,480; 5,920 and 11,840 g ae ha^{-1}; and third factor comprised evaluations of variables in five time-points after glyphosate treatment: 0; 12; 24; 48 and 96 hours after treatment (HAT).

Experiment 2 (E2) was arranged in a three-factorial scheme 2 x 2 x 8. The first factor comprised two *C. bonariensis* biotypes (B11R and B17S); the second factor comprised two glyphosate doses: 0 and 1,480 g ae ha^{-1}; the third factor comprised evaluations of variables in eight times-points after glyphosate treatment: 0; 24; 48; 96; 192; 264; 384 and 552 HAT.

The evaluated variables in both E1 and E2 were: a) shikimic-acid content (SAC); oxidative stress and tissue damage according to b) ROS production - H_2O_2; and c) lipid peroxidation measured using thiobarbituric acid-reactive substances (TBARS); activity of antioxidant enzymes – d) SOD; e) CAT; and f) APX.

For enzyme evaluations, the second and third fully expanded leaves (from the apex) were harvested after glyphosate treatment according to each treatment. Enzyme

evaluations were performed with five technical replicates, which were recorded on a Spectrophotometer Ultrospec 2000 UV-Visible (Pharmacia Biotech) in a 2-mL cuvette.

Whole-Plant Shikimic-Acid Bioassay

Shikimic-acid content (SAC) quantification was performed according to Singh and Shaner (1998) and Perez-Jones (2007) with some modifications. Leaves of B11R and B17S biotypes were harvested after each treatment and stored at -80 ^{o}C. Leaf tissues were chopped, and 0.25 g of fresh weight samples were placed in 15-ml tubes containing 5 ml of 1.25 N HCl. The samples were mixed, placed at -80 ^{o}C until frozen, thawed at room temperature, and incubated at 37 ^{o}C for 45 min. Five technical samples of 125-µL were extracted from the tubes and mixed with reaction buffer [0.25% (w/v) periodic-acid and sodium(meta)periodate solution] and incubated at 37 ^{o}C for 30 min to allow shikimic acid oxidation. After incubation, samples were mixed with 1000-µL 0.6 N NaOH/0.22 M Na_2SO_3 and measured spectrophotometrically at 380 nm. The shikimic-acid content was determined based on a standard curve and expressed in µg g^{-1} fresh weight (µg g^{-1} FW).

Oxidative Stress and Tissue Damage

Oxidative stress was determined according to levels of H_2O_2, as described by Sergiev et al. (1997) and tissue damage according to lipid peroxidation (Heath and Packer, 1968). Lipid peroxidation was measured using species reactive to thiobarbituric acid (TBARS) by the accumulation of malondialdehyde (MDA) a product of lipid peroxidation. The solution of 10 mL of 0.1% trichloroacetic acid (TCA) was added to 1.0 g of processed leaf tissue and vortexed. Samples were centrifuged at 14,000 rpm for 25 min at 4 ^{o}C. For H_2O_2 quantification, the supernatant was collected and five technical samples of 0.2 mL added to 0.8 mL of phosphate buffer 10 mM (pH 7.0), and 1.0 mL of

potassium iodide 1 M. The solution was allowed to stand for 10 min at room temperature. Absorbance was recorded at 390 nm. The concentration of H_2O_2 was determined using a standard curve with known concentrations of H_2O_2 and expressed in millimoles per gram of fresh weight (mM g^{-1} FW). For the determination of TBARS, the supernatant was collected and five technical samples of 0.5 mL added to 1.5 mL of TBARS 0.5%, and TCA 10%. Samples were placed in a 90 ^{o}C water bath for 20 min and after immediately cooled in an ice bath for 10 min. Absorbance was recorded at 532 and 600 nm. Non-specific absorption at 600 nm was subtracted from the reading at 532 nm, and the MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}, and the results were expressed as nM MDA g^{-1} of fresh weight (nM MDA g^{-1} FW).

Enzyme Assays

To determine the activity of antioxidant enzymes SOD, CAT, and APX, extraction was performed, and from this extract, the activities of the enzyme were calculated and expressed in active units (AU) per milligram of fresh weight (AU mg^{-1} FW). To 1.0 g of processed leaf tissue with 0.1 g of polyvinylpyrrolidone (PVP), 4.5 mL of 200 mM phosphate buffer (pH 7.8), 90 µL of 10 mM EDTA, 900 µL of 200 mM ascorbic acid, and 3.51 mL of ultrapure water was added and mixed, and after centrifuged at 14,000 rpm at 4 °C for 25 min. The supernatant was collected and used for all enzyme assays.

Total SOD activity was measured according to Peixoto (1999) in a 2-mL reaction mixture containing 1.0 mL phosphate buffer 100 mM (pH 7.8), 400 µL of methionine 70 mM, 20 µL of ethylenediaminetetraacetic (EDTA) 10 µM, 390 µL of ultrapure water, 150 µL *ρ*-nitro blue tetrazolium chloride (NBT) 1 mM, 20 µL of enzyme extract. After the addition of 20 µL of riboflavin 0.2 mM last, the reaction was initiated. The samples were illuminated with fluorescent lamps at 4,000 lx, 15 Watts for 10 min, and after the

absorbance at 560 nm was recorded. One activity unit (AU) of SOD was equivalent to the amount of enzyme necessary to inhibit 50% of NBT reduction at 560 nm. The results were expressed in AU mg^{-1} of fresh weight min^{-1} (AU mg^{-1} FW min^{-1}).

Catalase and ascorbate peroxidase activities were determined according to Azevedo et al. (1998). Catalase activity was evaluated according to the decline in absorbance for 1.5 min at 240 nm, which indicates the H_2O_2 consumption (extinction coefficient: 39.4 mM cm^{-1}). The 2-mL reaction mixture contained 1.0 mL of potassium phosphate buffer 200 mM (pH 7.0), 850 µL of ultrapure water, 100 µL of H_2O_2 250 mM, and 50 µL of enzyme extract last to initiate the reaction.

Following the decrease in absorbance at 290 nm for 1.5 min, the ascorbate peroxidase activity was measured. The 2.0-mL reaction mixture contained 1.0 mL of potassium phosphate buffer 200 mM (pH 7.0), 750 µL of ultrapure water, 100 µL of ascorbic-acid (ASC) 10 mM, 50 µL of enzyme extract, and 100 µL of H_2O_2 2.0 mM last to initiate the reaction. The APX activity was calculated using an extinction coefficient of 2.9 mM^{-1} cm^{-1}.

For CAT and APX activities calculation purposes, the decrease of one absorbance unit was considered equivalent to one active unit (AU). From the total extract, the activities were calculated by the amount of extract that reduced the absorbance reading by one AU and expressed in AU mg^{-1} of fresh weight min^{-1} (AU mg^{-1} FW min^{-1}).

Statistical Analysis

Statistical analysis was performed using the GLM package statement from SAS (version 9.0, SAS Institute Inc, Cary, NC, USA), and results fitted using Sigma Plot®. To test for normality (Shapiro-Wilk's test) and homogeneity of variance (Hartley's test), SAS Proc Univariate was used. Analysis of variance (ANOVA) was performed at F test

($p \leq 0.05$) using Proc Mixed. Test-t were applied in regressions at $p \leq 0.05$. Interactions among factors were verified at $p \leq 0.05$, and characters that presented significant interaction were split to simple effects referents to qualitative factor (biotypes). Effects of quantitative factors (glyphosate doses and times after treatment) were split using linear regressions at the highest significant polynomial degree, and equations from each level of factors in interaction were split ($p \leq 0.05$). Significant characters were submitted to linear correlations analysis and effects of biotypes and glyphosate doses isolated to verify the tendency of association between interest characters at $p \leq 0.05$. To identify multivariate relation was performed a Step-Wise multiple regression, where it was fixed how dependent variable H_2O_2, while SAC, TBARS, SOD, CAT, and APX were considered explanatory from the model.

For both E1 and E2, the ANOVA demonstrated interactions among factors at $p \leq 0.05$ for all evaluated enzymatic variables (Table 2 and 3). Polynomial models of the second, third, and fourth order were applied to describe enzyme results (Supplementary Tables 1 and 2).

RESULTS AND DISCUSSION

Biotype Selection and Glyphosate Dose-Response

From 54 collected hairy fleabane biotypes, two of them from the same geographical region were selected according to their high and low sensitivity to glyphosate, respectively for glyphosate-resistant B11R and glyphosate-sensitive B17S biotypes based on presenting symptoms after glyphosate treatment. Segregation experiments performed in the first (F_0) and second (F_1) self-pollinating generations evaluated at 28 DAT

presented results of 100% of alive in B11R and 100% of death in B17S. This result indicates that both biotypes are non-segregating.

To determine the level of glyphosate resistance in the selected biotypes of hairy fleabane, we conducted dose-response experiments. The data were fitted to the non-linear log-logistic model, and after that used to estimate resistance factor (RF) (Figure 1; Table 1). Shoot dry weight (SDW) in both biotypes decreased when the glyphosate dose increased. However, there was a different dose-response between the glyphosate-resistant and sensitive biotypes, which could be observed in curve slope and confidence intervals (Figure 1). On average of two experiments, the RF (GR_{50}) of glyphosate-resistant biotype was 18.4 (Table 1). According to HRAC (Herbicide Resistance Action Committee 2012; http://hracglobal.com/ herbicide-resistance/confirming-resistance), an RF >10 is considered a high-level of resistance.

Whole-Plant Shikimic-Acid Bioassay

Plants of both resistant (B11R) and sensitive (B17S) biotypes treated with glyphosate accumulated higher levels of shikimic-acid content (SAC) than their respective non-treated control (Figures 2a, 3a; Tables 2 and 3; Supplementary Table 1 and 2). In E1, both biotypes (B11R and B17S) had similar patterns of shikimic-acid accumulation after glyphosate treatment. Results presented in Table 2 suggest that there are differences between glyphosate doses, but it isn't a clear pattern. At low glyphosate concentrations (370 g ae ha^{-1}), the SAC in B11R biotype response was slower than B17S at 24 HAT. It also appears that the B11R never reaches the highest level of shikimic acid concentrations seen in B17S biotype at 96 HAT except for highest studied dose. These results could indicate reduction of glyphosate absorption, transport or sequestering as reported in other *Conyza* species studies (Feng et al. 2004; Ferreira et al. 2008; Ge et al.

2014; Cardinali et al. 2015; Tani et al. 2015; Moretti et al. 2017; Kleinman and Rubin, 2017). However, when glyphosate dose increased, the levels of SAC in both B11R and B17S biotypes were similar (Table 2). It indicates that the reduction in glyphosate absorption, transport, or sequestering are not preventing glyphosate reach their target enzyme EPSPS.

On average (E2 - 0 to 192 HAT), SAC levels were 9.3 and 13.3-fold higher than untreated plants for B11R and B17S biotypes, respectively. After 96 HAT, the SAC levels decreased gradually in B11R until 288 HAT when did not differ from non-treated plants (confident interval not shown). In B17S, the SAC abruptly reduced after 192 HAT because the plants died (Figure 3a). The primary consequence of blocking the shikimate pathway, shikimic-acid accumulation, in B11R was transient and at 288 HAT (~12 days) did not differ from non-treated control on E2 (Figure 3a). The EPSPS inhibition could be reduced with time, allowing the enzyme to process the available shikimate-3-phosphate (S_3P), thereby reducing its concentration (Mueller et al. 2003).

Shikimic-acid accumulation in plants tissue was linearly correlated with glyphosate concentration and is a strategy to evaluate the glyphosate-resistance mechanism (Nol et al. 2012). In this way, our results of SAC levels indicate that the glyphosate-resistance mechanism of B11R biotype has non-target site resistance (NTSR). However, further studies are necessary to probe the glyphosate-resistance mechanism such as determining EPSPS gene sequence and expression patterns as well as glyphosate absorption and transport patterns. The glyphosate target-site resistance alleles have low to no known natural variation, which has led to a few cases of target-site resistance (TS). Thus, NTSR might represent the main mechanism of resistance to glyphosate (Yuan et al. 2007). Also, the glyphosate resistance in *C. canadensis* in the United States (Peng et al. 2010; Moretti et al. 2017), Greece (Nol et al. 2012), and *C. bonariensis* in the United States (Moretti et

al. 2017), Australia (Hereward et al. 2018), and Israel (Kleinman and Rubin, 2016) is not the result of EPSPS mutations or overexpression, but due to a NTSR.

Oxidative Stress and Tissue Damage

In E1 higher levels of H_2O_2 (indicate oxidative stress) and TBARS (indicate lipid peroxidation) were observed in B17S than B11R at 96 HAT for all glyphosate-doses (Figure 2b and c; Figure 3b and c; Tables 2 and 3; Supplementary Tables 1 and 2). On E2, the accumulation of H_2O_2 and TBARS in B17S were on average (from 0 to 552 HAT) 1.7 and 1.4-fold higher than in B11R, while at 96 HAT (peak) were 3.3 and 2.5-fold, respectively (Figure 3b and c; Table 3). The H_2O_2 and TBARS levels at 96 HAT in B11R were 3 and 3.2-fold higher than non-treated plants, and on average (from 0 to 552 HAT) the levels were 2.7 and 2.5-fold higher than non-treated, respectively. In B17S, the H_2O_2 and TBARS levels at 96 HAT were 12.3 and 7.7 higher than non-treated plants, and on average (from 0 to 552 HAT) 5.2 and 3.2-fold higher than non-treated, respectively (Figure 3b and c).

In plants, glyphosate action also leads to ROS production and oxidative stress, which may be a secondary effect of blocking the shikimate pathway (Ahsan et al. 2008). Therefore, SAC accumulation (primary effect) led to higher H_2O_2 levels (secondary effect), and consequent lipid peroxidation (LPO), the most damaging ROS process known (Gill and Tuteja, 2010), indicated by TBARS levels (Figure 2b and c; Figure 3b and c). The lower levels of ROS production (H_2O_2) and lipid peroxidation (TBARS) in glyphosate-treated B11R than B17S indicates that the resistant biotype had minor tissue damages after glyphosate treatment. Although B11R had lower levels of H_2O_2 and TBARS regarding B17S, it presented higher levels than non-treated control plants

indicating which oxidative stress also occurred, however, in a lesser scale than B17S which died (Figure 1; Table 1).

In the B17S, H_2O_2 and TBARS levels peaked at 96 HAT and decreasing abruptly after this time (E2) until reaching zero at 264 HAT, which matches with the death of plants. Irreversible damage to cellular tissues may have occurred beginning at this time, such as a loss of cellular homeostasis leading to cell death in sensitive plants (Figure 3b and 3c). In plants, low concentrations of H_2O_2 acts as a signal molecule, and at high concentrations leads to plant cell death (Gill and Tuteja, 2010). In E2 at 500 HAT (~21 days), the levels of H_2O_2 and TBARS in B11R non-differed from the non-treated control. Thus, this result suggests that the detoxification process of the H_2O_2 in glyphosate-resistant biotype took around 500 hours after glyphosate treatment. However, it appears that even after 500 hours, H_2O_2 levels is still 2-fold untreated. It looks like ROS is still being produced, just at a lower rate.

Antioxidant Enzyme Activities

In E1 both glyphosate-treated biotypes when compared with non-treated controls had increased SOD, CAT, and APX activities, independently of dose and time after glyphosate treatment (Figure 2d-f and 3d-f; Tables 2 and 3; Supplementary Table 1 and 2). In E2 at 96 HAT the activities of SOD, CAT, and APX in B11R were 2.7, 6.2, 15.3-fold higher than non-treated control, and in B17S 1.5, 7.5, 13.7-fold higher than non-treated control, respectively (Figure 3d-f; Table 3). On E2, on average (from 0 to 552 HAT) SOD, CAT and APX activities in B11R were 2.5, 4.8, and 11.3-fold higher than non-treated control, and in B17S 1.7, 2.9, and 4.5-fold higher than non-treated control. Comparisons between treated biotypes activities of SOD, CAT, and APX after glyphosate treatment showed higher activities in B11R than in B17S, mainly after 96 HAT (Figure

3d-f; Table 3). On average (from 0 to 552 HAT) in E2, the activities of SOD, CAT, and APX in the B11R were 1.6, 1.5, and 2.4-fold higher than in B17S, respectively. In general, the SOD, CAT, and APX activities decline after 384 HAT, indicating a transient response to oxidative stress. In fact, all oxidative stress markers are still high after 500 hours.

Other studies have been reported the transient antioxidant enzyme activities in *C. bonariensis* and *Ambrosia trifida* in response to paraquat and glyphosate treatment, respectively (Ye and Gressel, 2000; Harre et al. 2018). Glyphosate-mediated changes in antioxidant status have been reported to other species. In maize leaves, glyphosate treatment resulted in increased of H_2O_2 levels, and lipid peroxidation (Sergiev et al. 2006), and similar results were reported in rice (Ahsan et al. 2008), and peanut (Radwan and Fayez, 2016). Also, a potential role of antioxidant systems in glyphosate resistance has been reported in *Amaranthus palmeri* (Maroli et al., 2015).

In the present study, plants of both glyphosate-resistant and sensitive biotypes treated with glyphosate presented significative positive correlation among SAC, H_2O_2, and TBARS contents, and the activities of SOD, CAT, and APX (Supplementary Table 3). It establishes a relation of cause-effect between glyphosate treatment and SAC, H_2O_2 and TBARS production, as well as between treatment and SOD, CAT and APX antioxidant activities in both glyphosate-resistant and sensitive biotypes. Therefore, according to correlation results, the glyphosate treatment causes an accumulation of shikimic-acid in leaf tissue, leading to increasing in oxidative stress, and consequent lipid peroxidation, followed by enhancing in antioxidant enzyme activities (SOD, CAT, and APX) in both studied glyphosate-resistant and -sensitive hairy fleabane biotypes (Supplementary Table 3).

The lower oxidative stress, tissue damage, and higher antioxidant enzyme activities in B11R than in B17S, indicates that antioxidant systems in glyphosate-resistant biotype

could be related to resistance, and is playing an important role in the glyphosate-resistance process. A recent study related the role of the antioxidant enzyme in reducing the herbicide damage in glyphosate resistance process (Délye 2013; Maroli et al. 2015). To our knowledge, there are no reports of resistance to glyphosate in *Conyza* spp. related to antioxidant mechanisms. To better understanding, this is the first report of differential antioxidant enzyme activity which could be related to glyphosate resistance in hairy fleabane. This type of resistance might pose a more significant threat to agriculture because of the multi-herbicide resistance and multi-gene involvement in the mechanisms, is considered the worst type of resistance (Yuan et al. 2007; Duke 2011; Délye 2013; Délye et al. 2013).

CONCLUSION

Glyphosate treatment on glyphosate-resistant and -sensitive biotypes resulted in a similar pattern of shikimic-acid accumulation until 96 hours after treatment for all glyphosate studied doses, but it is not a clear pattern. Shikimic-acid content in resistant biotype was transient and did not differ from non-treated control at 288 hours after treatment (~12 days). The shikimic-acid accumulation leads to oxidative stress and tissue damage occurrence in both biotypes. However, the oxidative stress and tissue damage occurred in glyphosate-sensitive biotype were higher than in -resistant. In response to glyphosate-induced stresses, the activities of antioxidant enzyme superoxide dismutase, catalase, and ascorbate peroxidase increase in glyphosate-resistant and -sensitive biotypes, however, the enzyme activities in glyphosate-resistant biotype were higher than in -sensitive. Thus, the results of the present research indicate that antioxidant enzyme might be related to glyphosate-resistance in hairy fleabane.

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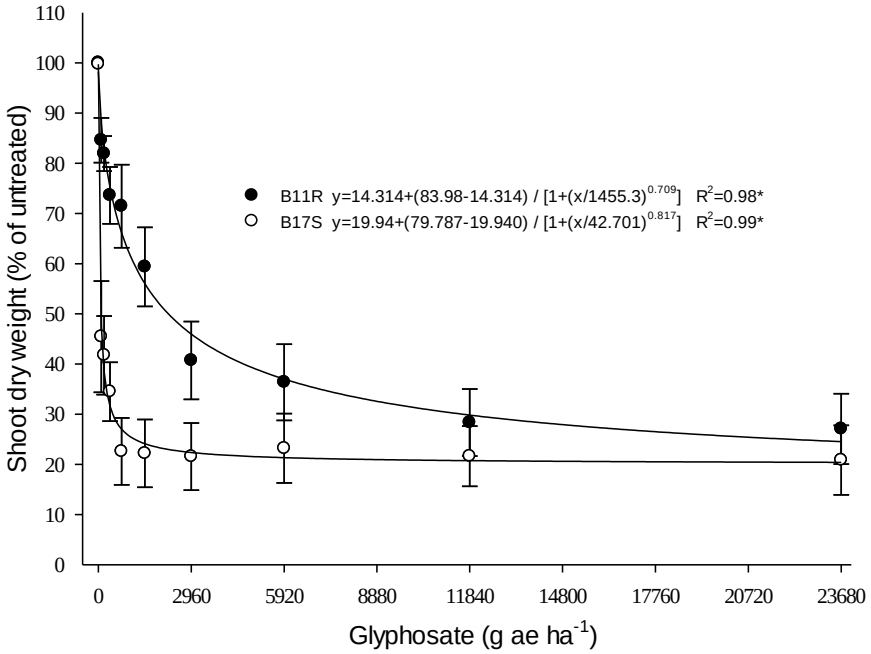

Figure 1. Glyphosate dose-response of glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes at 28 days after treatment (DAT). Lines are the response curves predicted from non-linear regression. Symbols represent mean dry weight (% reduction of untreated) of four replicates and bars the confidence intervals (CI) with 95 %.

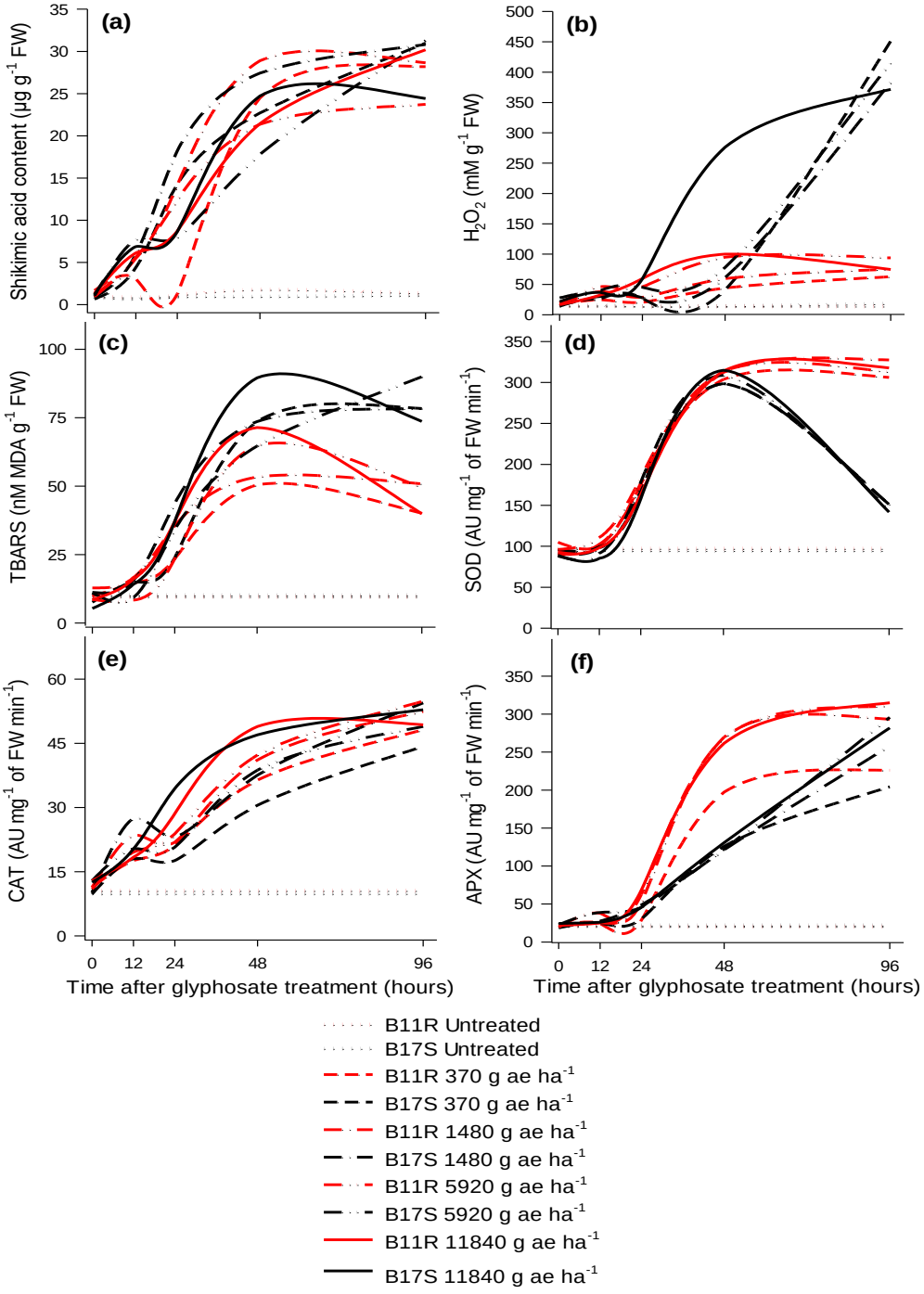

Figure 2. Tissue damage and antioxidant enzyme activities of glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes in a range of time points after glyphosate treatment and doses. Regressions equations and statistics are presented in Supplementary Table 1. Experiment 1. H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase.

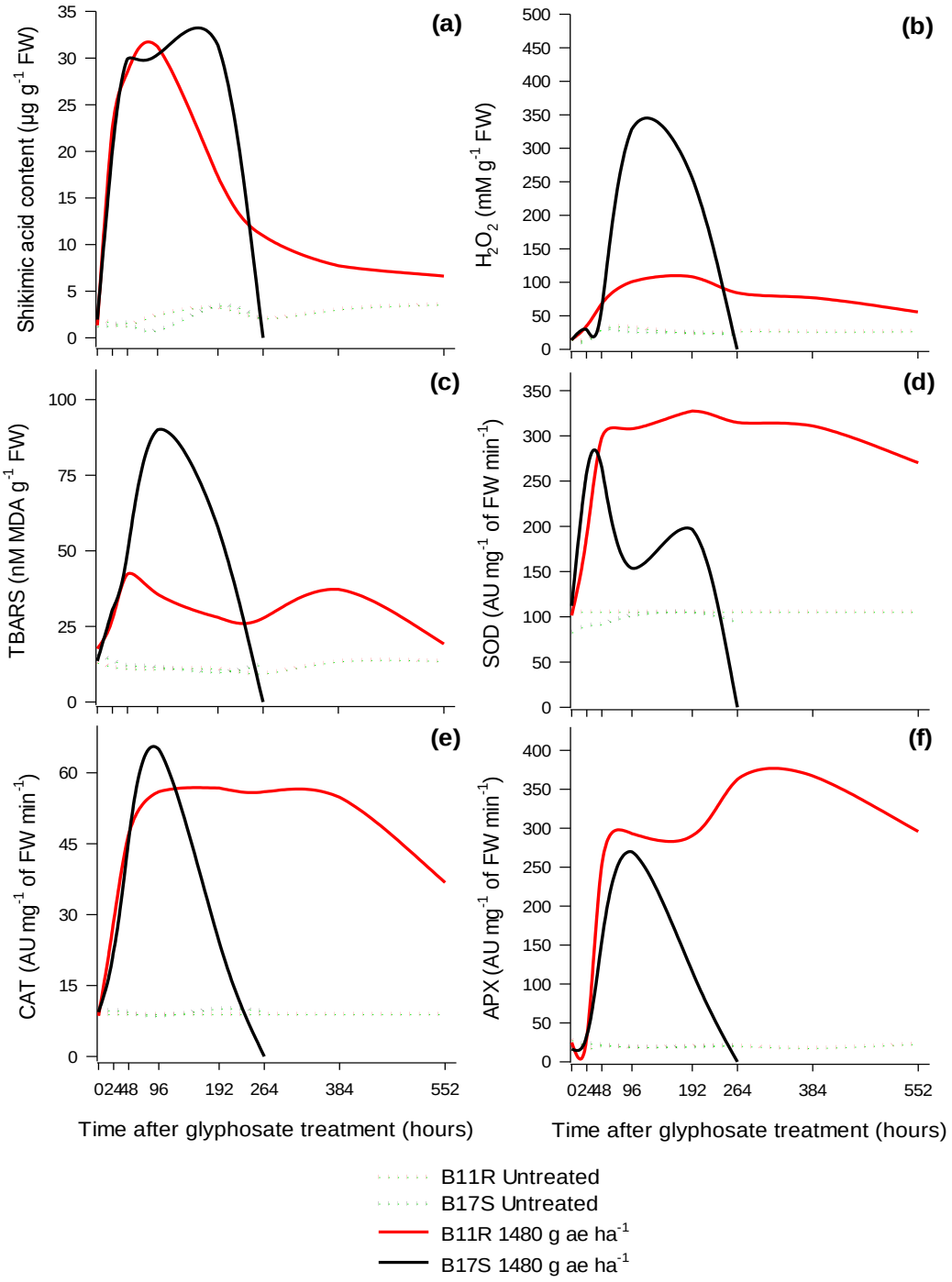

Figure 3. Tissue damage and antioxidant enzyme activities of glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes in a range of time points after glyphosate treatment and doses. Regressions equations and statistics are presented in Supplementary Table 2. Experiment 2. H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase.

Table 1. Parameter estimates of glyphosate dose resulting in 50% reduction of shoot dry weight (GR_{50}) in glyphosate-resistant (B11R) and -sensitive (B17S) *C. bonariensis* biotypes determined at 28 days after treatment and based on a four-parameter log-logistic model shown in Equation 1.

Herbicide	B11R	B17S	RF[b]
	GR_{50}[a]	GR_{50}[a]	
Glyphosate	2,502 (954.5-4,050)	135.8 (65.5-206.2)	18.4

Resistance levels were indicated by the resistance factor (RF). GR_{50} values with different letters are significantly different at P = 0.05 significant level.

[a] GR_{50}, herbicide dose causing 50 % growth reduction of plants;

[b] RF (resistance factor) = GR_{50} (R)/GR_{50} (S).

Table 2. The simple qualitative effect of separating the of tissue damage and activity of antioxidant enzymes as a response of glyphosate treatment in resistant (B11R) and -sensitive (B17S) biotypes of *Conyza bonariensis* as a function of time after treatment and doses. Experiment 1.

Time after treatment (hours)	Biotype									
	B11R					B17S				
	Glyphosate doses (g ae ha^{-1})									
	0	370	1,480	5,920	11,840	0	370	1,480	5,920	11,840
Shikimic acid (µg g^{-1} FW)										
0	0.98 α	1.67 α	1.22 α	0.95 α	1.34 α	0.88 α	0.66 α	0.85 α	1.05 α	1.20 α
12	0.87 α	3.04 α	5.49 α	5.62 β	6.33 α	0.66 α	4.41 α	6.02 α	7.91 α	6.88 α
24	1.76 α	1.26 β	14.13 β	14.13 α	12.40 α	0.91 α	14.26 α	18.65 α	7.96 β	8.64 β
48	1.77 α	21.48 α	29.33 α	21.89 α	21.54 β	0.94 α	22.71 α	28.08 α	18.68 β	24.50 α
96	1.33 α	28.20 β	28.84 β	23.85 β	30.19 α	1.11 α	31.00 α	32.27 α	31.29 α	25.06 β
CV (%)	9.62									
H_2O_2 (mM g^{-1} FW)										
0	13.02 α	17.00 α	15.34 α	19.66 β	16.68 β	15.00 α	19.68 α	14.36 α	28.00 α	21.66 α
12	14.02 α	24.66 α	38.32 α	46.68 α	33.00 β	15.68 α	26.68 α	30.34 β	40.68 β	36.68 α
24	13.68 α	20.66 β	28.98 α	46.32 α	59.68 α	14.02 α	27.00 α	27.98 α	45.34 α	59.34 α
48	14.34 α	44.32 α	59.68 α	95.00 α	100.10 β	14.34 α	40.66 β	61.02 α	78.00 β	276.32 α
96	14.68 β	63.02 β	75.22 β	94.00 β	74.66 β	18.32 α	451.00 α	383.00 α	416.02 α	371.66 α
CV (%)	3.74									
TBARS (nM MDA g^{-1} FW)										
0	8.87 α	9.80 α	12.85 α	8.67 α	8.57 α	9.44 α	7.63 α	11.25 α	10.99 α	5.32 α
12	8.16 α	14.71 α	16.67 α	8.46 α	16.15 α	8.41 α	15.02 α	15.69 α	9.34 α	14.19 α
24	10.22 α	23.48 α	35.53 β	23.53 β	37.12 α	9.81 α	23.84 α	43.71 α	34.17 α	37.01 α
48	10.73 α	50.53 β	53.41 β	64.92 α	71.43 β	11.81 α	73.65 α	65.54 α	64.72 α	89.60 α
96	9.96 α	40.10 β	50.89 β	49.50 β	39.89 β	11.41 α	78.40 α	97.70 α	90.01 α	73.65 α
CV (%)	8.83									
SOD (AU mg^{-1} of FW min^{-1})										
0	95.80 α	104.91 α	93.03 α	96.37 α	90.19 α	96.02 α	91.21 β	96.18 α	88.12 α	88.91 α
12	96.02 α	101.66 α	102.77 α	110.99 α	98.37 α	96.84 α	98.22 α	99.68 α	91.56 β	85.32 β
24	98.73 α	176.49 α	175.13 α	183.41 α	163.08 α	95.54 α	167.51 α	163.91 α	177.61 α	158.02 α
48	98.06 α	303.83 α	312.21 α	313.80 α	313.96 α	90.47 α	298.64 α	301.63 α	308.74 α	314.98 α
96	96.82 α	306.22 α	327.57 α	312.97 α	317.85 α	93.34 α	151.29 β	161.93 β	144.44 β	141.64 β
CV (%)	6.17									
CAT (AU mg^{-1} of FW min^{-1})										
0	9.40 α	11.35 α	11.36 α	10.96 α	12.92 α	10.57 α	9.79 α	10.18 α	12.92 α	12.53 α
12	10.96 α	17.62 α	23.49 α	19.58 α	18.40 α	9.40 α	18.03 α	27.41 β	20.36 α	20.36 α
24	11.35 α	21.14 α	21.93 α	23.89 α	28.56 β	9.79 α	17.62 β	23.10 α	20.75 α	34.46 α
48	10.57 α	36.42 α	41.12 α	42.29 α	48.95 α	9.39 α	30.54 β	37.59 β	38.77 β	46.99 α
96	10.55 α	48.16 α	52.47 α	54.82 α	49.34 β	10.18 α	44.25 β	54.43 α	48.95 β	52.86 α
CV (%)	10.73									
APX (AU mg^{-1} of FW min^{-1})										
0	21.28 α	20.22 α	19.68 α	21.81 α	21.81 α	23.41 α	18.62 α	22.88 α	22.88 α	24.47 α
12	19.68 α	23.94 α	37.77 α	26.60 α	25.00 α	21.81 α	24.47 α	38.83 α	27.66 α	26.07 α
24	20.75 α	28.73 α	52.14 α	62.77 α	68.63 α	21.81 α	30.32 α	45.75 α	48.95 β	45.75 β
48	20.74 α	197.38 α	269.73 α	269.20 α	261.75 α	22.88 α	126.09 β	121.30 β	122.36 β	130.88 β
96	19.15 α	226.11 α	293.14 α	310.70 α	314.95 α	20.22 α	204.29 β	258.03 β	295.80 β	281.97 β
CV (%)	6.85									

H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; *Significant at p≤0.05; ^{NS} non-significant at p≤0.05; CV: coefficient of variation; Different symbols in each glyphosate dose and time indicate the difference between biotypes.

Table 3. The simple qualitative effect of separating the cellular damage and activity of antioxidant enzymes as a response of glyphosate treatment in resistant (B11R) and -sensitive (B17S) biotypes of *Conyza bonariensis* as a function of time after treatment and doses. Experiment 2.

Time after treatment (hours)	Biotype			
	B11R		B17S	
	Glyphosate doses (g ae ha^{-1})			
	0	1,480	0	1,480
Shikimic acid (µg g^{-1} FW)				
0	2.28 α	1.33 α	1.27 α	1.95 α
24	1.47 α	22.55 α	1.37 α	20.12 β
48	1.33 α	28.51 α	1.59 α	29.92 α
96	2.49 α	31.22 α	0.84 α	30.37 α
192	3.27 α	17.31 β	3.49 α	31.37 α
264	2.12 α	10.95 α	2.11 α	0.00 β
384	3.08 α	7.73 α	0.00 β	0.00 β
552	3.61 α	6.91 α	0.00 β	0.00 β
CV (%)	16.23			
H_2O_2 (mM g^{-1} FW)				
0	15.33 α	14.33 α	14.66 α	13.66 α
24	15.00 α	34.66 α	12.00 α	29.66 α
48	30.66 α	67.66 α	29.99 α	59.00 α
96	33.33 α	100.66 β	26.66 α	328.00 α
192	24.33 α	108.33 β	25.99 α	256.66 α
264	27.33 α	84.33 α	20.91 α	0.00 β
384	26.66 α	77.00 α	0.00 β	0.00 β
552	27.33 α	58.66 α	0.00 β	0.00 β
CV (%)	15.55			
TBARS (nM MDA g^{-1} FW)				
0	13.05 α	17.65 α	15.74 α	13.57 α
24	11.71 α	27.45 α	13.88 α	30.55 α
48	11.09 α	42.32 β	12.18 α	49.34 α
96	10.99 α	35.66 β	11.71 α	90.06 α
192	11.09 α	28.02 β	10.01 α	57.75 α
264	9.54 α	27.71 α	11.37 α	0.00 β
384	13.52 α	37.26 α	0.00 β	0.00 β
552	13.67 α	19.61 α	0.00 β	0.00 β
CV (%)	16.57			
SOD (AU mg^{-1} of FW min^{-1})				
0	95.79 α	101.52 α	83.27 α	112.17 α
24	112.90 α	190.13 β	90.86 α	260.09 α
48	98.73 α	298.25 α	92.38 α	265.12 β
96	114.05 α	307.88 α	101.94 α	153.64 β
192	96.81 α	327.47 α	105.48 α	196.63 β
264	114.56 α	314.82 α	103.58 α	0.00 β
384	104.11 α	311.03 α	0.00 β	0.00 β
552	108.44 α	274.26 α	0.00 β	0.00 β
CV (%)	18.53			
CAT (AU mg^{-1} of FW min^{-1})				
0	8.61 α	8.61 α	9.79 α	9.39 α
24	7.83 α	28.58 α	9.79 α	22.32 β
48	9.39 α	46.99 α	9.39 α	45.81 α
96	9.00 α	55.99 β	8.61 α	65.00 α
192	9.39 α	56.78 α	10.18 α	24.28 β
264	9.39 α	55.99 α	8.68 α	0.00 β
384	9.00 α	54.82 α	0.00 β	0.00 β
552	9.39 α	37.69 α	0.00 β	0.00 β
CV (%)	16.41			
APX (AU mg^{-1} of FW min^{-1})				
0	28.19 α	25.00 α	22.87 α	16.49 α
24	17.55 α	31.38 α	23.94 α	34.04 α
48	21.28 α	253.24 α	22.34 α	155.35 β
96	19.15 α	293.14 α	19.68 α	269.73 α
192	20.74 α	290.48 α	19.68 α	115.97 β
264	30.32 α	362.83 α	21.94 α	0.00 β
384	18.08 α	367.09 α	0.00 β	0.00 β
552	23.40 α	301.25 α	0.00 β	0.00 β
CV (%)	25.91			

H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; *Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$; CV: coefficient of variation; Different symbols in each glyphosate dose and time indicate the difference between biotypes.

Supplementary Table 1. Regression[1], mean square error (MSE) and determination coefficient (R^2) for the polynomial model of evaluated variables extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate dose (g ae ha^{-1}) and time after glyphosate treatment. Exp 1.

Variable	Biotype	Rate (g ae ha^{-1})	Regression[1]	MSE	R^2
Shikimic acid	B11R	0	$y=0.81858+0.03063x-0.00026173x^2$	0.388	0.38*
		370	$y=1.6722+0.6551x-0.0659x^2+0.0018x^3-0.000012x^4$	1.085	0.99*
		1,480	$y=0.9968+0.3035x+0.0122x^2-0.0001287x^3$	1.302	0.99*
		5,920	$y=0.9536+0.0013x+0.0435x^2-0.0010x^3+0.0000060x^4$	1.268	0.98*
		11,840	$y=0.87758+0.540913x-0.0024459x^2$	1.160	0.99*
	B17S	0	$y=0.8868-0.0580x+0.0042x^2-0.000089x^3-0.000000532x^4$	0.137	0.58*
		370	$y=0.6646-0.2687x+0.0644x^2-0.0014x^3+0.0000087x^4$	1.373	0.98*
		1,480	$y=0.8522-0.3006x+0.0818x^2-0.0018x^3-0.000011x^4$	0.867	0.99*
		5,920	$y=1.0552+1.2031x-0.0699x^2+0.0015x^3-0.0000096x^4$	1.392	0.99*
		11,840	$y=1.1974+0.9277x-0.0525x^2+0.0013x^3-0.0000089x^4$	1.383	0.98*
H_2O_2	B11R	0	$y=13.4260$	2.799	0.032^{NS}
		370	$y=17+1.8953x-0.1433x^2+0.0034x^3-0.00002231x^4$	2.980	0.98*
		1,480	$y=15.34+4.9067x-0.3309x^2+0.0073x^3-0.00004549x^4$	3.048	0.98*
		5,920	$y=19.66+4.9095x-0.2972x^2+0.0068x^3-0.00004366x^4$	2.137	0.99*
		11,840	$y=16.68+0.4954x+0.0927x^2-0.0018x^3+0.00000915x^4$	2.804	0.99*
	B17S	0	$y=15.4661-0.070588x+0.00104046x^2$	2.144	0.34*
		370	$y=19.4144+1.37006x-0.07104x^2+0.00107921x^3$	2.574	0.99*
		1,480	$y=14.36+3.0102x-0.1836x^2+0.0038x^3-0.0000195x^4$	2.556	0.99*
		5,920	$y=28.4068+1.2928x-0.0396x^2+0.00071086x^3$	2.672	0.99*
		11,840	$y=21.660+2.9912x-0.2520x^2+0.0098x^3-0.0000741x^4$	2.703	0.99*
TBARS	B11R	0	$y=9.037925$	2.560	0.043^{NS}
		370	$y=10.0404-0.01305x+0.03215x^2-0.0002995x^3$	2.277	0.98*
		1,480	$y=12.8516-1.0087x+0.1448x^2-0.00306x^3+0.00001785x^4$	2.789	0.98*
		5,920	$y=8.4072-0.6170x+0.06404x^2-0.0005537x^3$	2.281	0.99*
		11,840	$y=8.5680-0.3868x+0.1069x^2-0.0019x^3+0.000009473x^4$	3.174	0.98*
	B17S	0	$y=9.13664$	2.597	0.134^{NS}
		370	$y=7.6388+0.9554x-0.0493x^2+0.0019x^3-0.000015006x^4$	2.944	0.99*
		1,480	$y=11.2516-1.7686x+0.2352x^2-0.0051x^3+0.00003087x^4$	3.013	0.99*
		5,920	$y=10.9938-2.2873x+0.2302x^2-0.0045x^3+0.00002620x^4$	2.875	0.99*
		11,840	$y=5.0474+0.1905x+0.0601x^2-0.0005697x^3$	3.374	0.99*
SOD	B11R	0	$y=96.78659$	9.834	0.0008^{NS}
		370	$y=104.9096-6.1163x+0.6120x^2-0.0111x^3+0.0000586x^4$	10.285	0.99*
		1,480	$y=93.0266-3.7183x+0.4694x^2-0.0081x^3+0.00004089x^4$	10.316	0.99*
		5,920	$y=96.3716-3.0452x+0.4441x^2-0.0078x^3+0.00003987x^4$	10.881	0.99*
		11,840	$y=88.4491-0.87204x+0.1991x^2-0.001720x^3$	10.986	0.99*
	B17S	0	$y=95.95514$	7.883	0.034^{NS}
		370	$y=91.2108-3.8467x+0.4558x^2-0.00764x^3+0.00003518x^4$	10.690	0.98*
		1,480	$y=94.1578-1.3150x+0.21502x^2-0.0020206x^3$	11.024	0.98*
		5,920	$y=88.1202-6.0333x+0.6647x^2-0.0122x^3+0.00006311x^4$	7.444	0.99*
		11,840	$y=88.9168-5.4785x+0.5250x^2-0.00821x^3+0.00003545x^4$	11.152	0.99*
CAT	B11R	0	$y=10.426350$	2.155	0.0044^{NS}
		370	$y=11.8015+0.2831x+0.0083x^2-0.00007621x^3$	2.789	0.96*
		1480	$y=11.3564+2.3010x-0.1431x^2+0.0032x^3-0.00002011x^4$	3.169	0.96*
		5920	$y=11.5583+0.4319x+0.0081x^2-0.00008273x^3$	2.941	0.97*
		11840	$y=12.7670+0.3265x+0.0173x^2-0.000174x^3$	2.299	0.98*
	B17S	0	$y=9.868320$	1.777	0.00^{NS}
		370	$y=9.79+1.4893x-0.0891x^2+0.0019x^3-0.000012336x^4$	2.670	0.96*
		1,480	$y=10.1816+3.2987x-0.2029x^2+0.0042x^3-0.00002578x^4$	3.007	0.97*
		5,920	$y=13.8922+0.16205x+0.01215x^2-0.00010456x^3$	3.478	0.94*
		11,840	$y=11.3559+1.0519x-0.006447x^2$	3.687	0.95*
APX	B11R	0	$y=20.8820$	2.528	0.045^{NS}
		370	$y=20.2170+2.3584x-0.2789x^2+0.0098x^3-0.00007306x^4$	7.255	0.99*
		1,480	$y=19.6850+4.3251x-0.3722x^2+0.0125x^3-0.00009213x^4$	7.457	0.99*
		5,920	$y=21.8130-0.0430x-0.0115x^2+0.0045x^3-0.00004259x^4$	6.823	0.99*
		11,840	$y=21.8130-1.2041x+0.1068x^2+0.0015x^3-0.00002327x^4$	6.864	0.99*
	B17S	0	$y=22.90360$	3.946	0.045^{NS}
		370	$y=18.6210+1.5862x-0.1480x^2+0.0051x^3-0.00003724x^4$	7.381	0.99*
		1,480	$y=22.8770+2.7618x-0.1724x^2+0.0048x^3-0.00003169x^4$	5.464	0.99*
		5,920	$y=22.7875-0.3235x+0.0669x^2-0.000354x^3$	5.522	0.99*
		11,840	$y=24.8534-1.0792x+0.0976x^2-0.000609x^3$	8.189	0.99*

H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; *Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$.

Supplementary Table 2. Regression[1], mean square error (MSE) and determination coefficient (R^2) for the polynomial model of evaluated variables extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate dose (g ae ha^{-1}) and time after glyphosate treatment. Experiment 2.

Variable	Biotype	Rate (g ae ha^{-1})	Regression[1]	MSE	R^2
Shikimic acid	B11R	0	$y=2.2964-0.0615x+0.001208x^2-0.00000724x^3+0.000000017x^4$	0.627	0.64*
		1,480	$y=2.1458+1.0016x-0.0109x^2+0.00004614x^3-0.000000086x^4$	2.430	0.95*
	B17S	0	$y=1.1998+0.0547x-0.0019x^2+0.0000227x^3-0.000000106x^4$	0.409	0.89*
		1,480	$y=1.5440+1.2984x-0.0238x^2+0.000218x^3-0.000000980x^4$	1.647	0.99*
H_2O_2	B11R	0	$y=14.3015+0.0263x+0.0095x^2-0.000125x^3+0.00000060x^4$	6.159	0.52*
		1,480	$y=12.6273+1.1204x+0.000767x^2-0.0000416x^3+0.00000013x^4$	9.045	0.93*
	B17S	0	$y=12.3267+0.30203x-0.001486x^2+0.00000163x^3$	5.892	0.77*
		1,480	$y=21.5298-4.7439x+0.1986x^2-0.001716x^3+0.00000599x^4$	20.172	0.98*
TBARS	B11R	0	$y=12.1651-0.01238x+0.00002926x^2$	2.343	0.16*
		1,480	$y=16.8011+0.7957x-0.00923x^2+0.00004050x^3-0.00000007x^4$	3.931	0.81*
	B17S	0	$y=15.8364-0.1427x+0.00245x^2-0.00002345x^3+0.00000011x^4$	1.241	0.96*
		1,480	$y=14.1699+0.19604x+0.2146x^2-0.000229x^3+0.00000084x^4$	5.347	0.97*
SOD	B11R	0	$y=104.05809$	15.103	0.01^{NS}
		1,480	$y=98.7349+5.6050x-0.0501x^2+0.000203x^3-0.00000038x^4$	23.960	0.92*
	B17S	0	$y=82.4703+0.5084x-0.007305x^2+0.00005262x^3-0.00000016x^4$	12.632	0.93*
		1,480	$y=110.2537+12.4663x-0.3103x^2+0.00294x^3-0.0000127x^4$	43.604	0.86*
CAT	B11R	0	$y=8.723882$	1.520	0.03^{NS}
		1,480	$y=8.0414+1.1566x-0.009980x^2+0.0000377x^3-0.000000067x^4$	2.523	0.98*
	B17S	0	$y=10.2686-0.0573x+0.000734x^2-0.00000284x^3$	1.581	0.88*
		1,480	$y=8.9403+03284x+0.0178x^2-0.000246x^3+0.000001123x^4$	5.673	0.95*
APX	B11R	0	$y=27.5582-0.5382x+0.0115x^2-0.000107x^3+0.000000048x^4$	4.162	0.55*
		1,480	$y=9.6795+1.8663x+0.0848x^2-0.00119x^3-0.000005834x^4$	36.661	0.93*
	B17S	0	$y=22.9555+0.0808x-0.00259x^2+0.00001849x^3-0.000000004x^4$	4.136	0.86*
		1,480	$y=12.7873-1.0183x+0.1368x^2-0.00154x^3+0.00000664x^4$	44.045	0.83*

H_2O_2: hydrogen peroxide; TBARS: thiobarbituric acid-reactive substances; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; *Significant at $p\leq0.05$; *Significant at $p\leq0.05$; ^{NS} non-significant at $p\leq0.05$.

Supplementary Table 3. Pearson correlation coefficients estimates of variables shikimic-acid content (SAC), hydrogen peroxide (H_2O_2), thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and RNA integrity number (RIN) from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate (g ae ha^{-1}) treatment.

Variable[1]	Biotypes	Variable	SAC				H_2O_2				TBARS			
		Biotypes	B11R		B17S		B11R		B17S		B11R		B17S	
		Rates	0	1,480	0	1,480	0	1,480	0	1,480	0	1,480	0	1,480
SAC	B11R	0	-				0.034^{NS}				0.217^{NS}			
		1,480		-				0.401^{*}				0.627^{**}		
	B17S	0			-				0.630^{**}				0.539^{*}	
		1,480				-				0.769^{**}				0.913^{**}
H_2O_2	B11R	0	0.034^{NS}				-				-0.076^{NS}			
		1,480		0.401^{*}				-				0.437^{*}		
	B17S	0			0.630^{**}				-				0.637^{**}	
		1,480				0.769^{**}				-				0.902^{**}
TBARS	B11R	0	0.217^{NS}				-0.076^{NS}				-			
		1,480		0.627^{**}				0.437^{*}				-		
	B17S	0			0.539^{*}				0.637^{**}				-	
		1,480				0.913^{**}				0.902^{**}				-
SOD	B11R	0	-0.132^{NS}				0.039^{NS}				-0.140^{NS}			
		1,480		0.396^{*}				0.849^{**}				0.557^{*}		
	B17S	0			0.687^{**}				0.797^{**}				0.864^{**}	
		1,480				0.797^{**}				0.374^{*}				0.640^{**}
CAT	B11R	0	0.152^{NS}				0.092^{NS}				0.003^{NS}			
		1,480		0.415^{*}				0.899^{**}				0.598^{**}		
	B17S	0			0.678^{**}				0.731^{**}				0.886^{**}	
		1,480				0.849^{**}				0.750^{**}				0.931^{**}
APX	B11R	0	0.017^{NS}				-0.143^{NS}				-0.151^{NS}			
		1,480		0.071^{NS}				0.791^{**}				0.436^{*}		
	B17S	0			0.632^{**}				0.697^{**}				0.886^{**}	
		1,480				0.762^{**}				0.798^{**}				0.883^{**}

Continue

Continued

Variable[1]	Biotypes	Variable	SOD				CAT				APX			
		Biotypes	B11R		B17S		B11R		B17S		B11R		B17S	
		Rates	0	1,480	0	1,480	0	1,480	0	1,480	0	1,480	0	1,480
SAC	B11R	0	-0.132^{NS}				0.152^{NS}				0.017^{NS}			
		1,480		0.396*				0.415*				0.071^{NS}		
	B17S	0			0.687**				0.678**				0.632**	
		1,480				0.797**				0.849**				0.762**
H_2O_2	B11R	0	0.039^{NS}				0.092^{NS}				-0.143^{NS}			
		1,480		0.849**				0.899**				0.791**		
	B17S	0			0.797**				0.731**				0.697**	
		1,480				0.374*				0.750**				0.798**
TBARS	B11R	0	-0.140^{NS}				0.003^{NS}				-0.151^{NS}			
		1,480		0.557*				0.598**				0.436*		
	B17S	0			0.864**				0.886**				0.886**	
		1,480				0.640**				0.931**				0.883**
SOD	B11R	0	-				-0.181^{NS}				-0.118^{NS}			
		1,480		-				0.958**				0.891**		
	B17S	0			-				0.896**				0.831**	
		1,480				-				0.676**				0.563**
CAT	B11R	0	-0.181^{NS}				-				0.021^{NS}			
		1,480		0.958**				-				0.882**		
	B17S	0			0.896**				-				0.851**	
		1,480				0.676**				-				0.938**
APX	B11R	0	-0.118^{NS}				0.021^{NS}				-			
		1,480		0.891**				0.882**				-		
	B17S	0			0.831**				0.851**				-	
		1,480				0.563**				0.938**				-

* correlation coefficients of linear correlation (n=40) significative at 5.00% of probability to t-test.

** correlation coefficients of linear correlation (n=40) significative at 1.00% of probability to t-test.

^{NS} linear coefficients of correlation (n=40) non-significative to the t-test.

Article 2 – Planta Daninha [Accepted (2019)]

Does the Glyphosate Treatment Interfere Negatively on RNA Integrity in Glyphosate-Resistant and -Sensitive *Conyza bonariensis*?

A aplicação de glyphosate interfere negativamente na integridade do RNA de *Conyza bonariensis* resistente e sensível ao glyphosate?

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ABSTRACT - The hairy fleabane (*Conyza bonariensis* (L.) Cronq.) is among the most problematic glyphosate-resistant weeds to manage around the world. In weed science, molecular approaches such as RNA sequencing (RNA-Seq) and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) have been employed to study molecular responses to glyphosate treatment in *Conyza* species. Glyphosate treatment leads to reactive oxygen species (ROS) production in plants which could damage the RNA. Degraded RNA is an issue and can compromise further molecular analysis. Thus, the objective of this study was to evaluate whether glyphosate treatment interferes negatively on RNA integrity of glyphosate-resistant and -sensitive hairy fleabane biotypes. Two experiments were performed using glyphosate doses from 0 to 11,840 g ae

ha^{-1} and evaluated in a time-course until 288 h after treatment. The total of 86 RNA samples were evaluated. The RNA integrity was evaluated in a Bioanalyzer 2100 equipment according to RNA integrity number (RIN) scores and electrophoresis gel. The RIN scores ranged from 5.1 to 9.0. Glyphosate doses do not reduce the RIN scores in both glyphosate-resistant and -sensitive biotypes of hairy fleabane. Visual and automatic analysis of electrophoresis gel show suitable results for all RNA samples, with well-defined bands at 28S and 18S positions and no degradation. The results of the analysis indicate that glyphosate treatment does not affect the RNA integrity of glyphosate-resistant and -sensitive biotypes of hairy fleabane until 288 h and 192 h after glyphosate treatment, respectively. The RNA integrity analysis provides useful results to evaluate the RNA condition for further molecular analysis. The costs for RNA integrity analysis were around US$ 14.25 per sample, considering just reagents. These results are useful for planning future time-course experiments in *Conyza* spp. after glyphosate treatment.

Keywords: hairy fleabane, molecular analysis, RNA integrity number (RIN).

RESUMO – A buva (*Conyza bonariensis* (L.) Cronq.) está entre as plantas daninhas resistentes ao glyphosate mais difíceis de serem manejadas em todo o mundo. Na ciência das plantas daninhas, abordagens moleculares como o sequenciamento de RNA (RNA-Seq) e a reação da transcriptase reversa da polimerase em tempo real (qRT-PCR) têm sido empregadas para estudar as respostas moleculares ao tratamento com glyphosate em espécies de *Conyza*. No entanto, o tratamento com glyphosate leva à produção de espécies reativas de oxigênio (ROS) em plantas que podem danificar o RNA. A degradação do RNA é um problema e pode comprometer futuras análises moleculares. Assim, o objetivo deste estudo foi avaliar se o tratamento com glyphosate interfere negativamente na integridade do RNA de biótipos de buva resistente e sensível ao glyphosate. Dois experimentos foram realizados utilizando doses de glyphosate de 0 a 11.840 g e.a. ha^{-1} e avaliados em um tempo de até 288 horas após o tratamento. O total de 86 amostras de RNA foram avaliadas. A integridade do RNA foi avaliada em um equipamento Bioanalyzer 2100 de acordo com escores de número de integridade de RNA (RIN) e em gel de eletroforese. Os escores do RIN variaram de 5,1 a 9,0. Doses de glyphosate não reduziu os escores do RIN em biótipos de buva resistentes e sensíveis ao glyphosate. A análise visual e automática do gel de eletroforese mostrou resultados adequados para todas as amostras de RNA, com bandas bem definidas nas posições 28S e 18S e sem

degradação. Os resultados da análise indicam que o tratamento com glyphosate não afeta negativamente a integridade do RNA dos biótipos resistentes e sensíveis ao glyphosate da buva até 288 e 192 horas após o tratamento com glyphosate, respectivamente. A análise da integridade do RNA fornece resultados úteis. No entanto, os custos são altos e ficaram em torno de US$ 14,25 por amostra, considerando apenas os reagentes. Estes resultados são úteis para o planejamento de experimentos futuros em *Conyza* spp. após o tratamento com glyphosate.

Palavras-chaves: buva, análises moleculares, número de integridade do RNA (RIN).

INTRODUCTION

Hairy fleabane (*Conyza bonariensis* (L.) Cronq.) belonging to the botanical Asteraceae family is native to the Americas and now has a cosmopolitan distribution (Shrestha et al. 2014; Bajwa et al. 2016). Hairy fleabane is highly competitive on crops and is among the most problematic weed species to management around the world (Shrestha et al. 2014; Bajwa et al. 2016; Concenço and Concenço, 2016). The interferences caused by one hairy fleabane plant m^{-2} can reduce the soybean yield up to 36% (Trezzi et al., 2015).

In agricultural cropping systems, the management of hairy fleabane has been made using herbicides, mainly glyphosate. However, the intensive and widespread use of glyphosate has been a factor in weeds evolving resistance, making glyphosate treatment ineffective (Baucom and Holt, 2009). *C. canadensis* (horseweed) was the first broadleaf weed to evolve glyphosate-resistance (GR) and was observed in the United States in 2000 (Van Gessel, 2001). The GR in hairy fleabane was first documented in 2005 in Brazil (Vargas et al. 2007). Another important GR agricultural weed is sumatran fleabane (*C. sumatrensis*), which was first reported as GR in Spain in 2009 (Heap, 2018). Together, these three species accounts 63 reports of GR around the world (Heap, 2018).

Several studies have been conducted in *Conyza* spp. with the objectives to understand the GR mechanisms (Feng et al. 2004; Ferreira et al. 2008; Peng et al., 2010; Ge et al. 2014; Cardinali et al. 2015; Tani et al. 2015; Moretti et al. 2017; Kleinman and Rubin, 2017; González-Torralva et al., 2017; Hereward et al., 2018; Amaro-Blanco et al., 2018). These studies have found that the GR mechanisms are related to herbicide transport, translocation, uptake, metabolism, and alterations at the 5-

enolpyruvylshikimate 3-phosphate synthase (EPSPS) enzyme, which is the glyphosate target site. However, the specific GR mechanisms remain unclear in *Conyza* spp., even after the adoption of the powerful approaches like RNA-Sequencing (RNA-Seq) and quantitative real-time reverse transcriptase (qRT-PCR) (Peng et al., 2010; Hereward et al., 2018). RNA-Seq and qRT-PCR are molecular approaches used to evaluate changes in gene expression in response to stress, in this case, herbicide treatment. These methods produce quantitative e qualitative data with high accuracy (Nolan et al., 2006; Schmittgen and Livak, 2008; Wang et al., 2009). In general, RNA-Seq is used to provide sequences and identify candidate differentially expressed genes, and further evaluations are performed using qRT-PCR.

Some articles report the results of RNA-Seq studies to investigate the GR mechanisms in weeds. In those studies, the RNA sequencing was performed from 8 h to 1,008 h after glyphosate treatment (Table 1). However, those works do not reveal the specific(s) mechanism(s) of GR in the studied weed species, including horseweed and hairy fleabane (Table 1). A plausible hypothesis is that RNA-Seq analysis has been performed in a short time after glyphosate treatment, and do not capture all process involved in plant's herbicide detoxification. Studies have shown that shikimic-acid accumulation is transient in GR hairy fleabane, reaching the peak at 96 h after glyphosate treatment and taking around 300 h until their complete detoxification (Piasecki et al., 2019). Thus, whether this process is transient, it could be the first indicator of how long time the plant metabolism takes to deal with the stress generated after glyphosate treatment. In this way, to capture as much as possible the responses of GR hairy fleabane to glyphosate, molecular studies should be performed in more time than 24 h and 48 h after herbicide treatment according to previous studies in horseweed and hairy fleabane, respectively (Table 1).

In another hand, the glyphosate treatment leads to the reactive oxygen species (ROS) production (Cobb and Reade, 2010; Piasecki et al., 2019). The ROS are highly reactive toxic molecules which cause several damages to a plant cell, including the degradation of nucleic acids (RNA and DNA) (Foyer and Noctor 2005; Gill and Tuteja 2010). The RNA degradation by ROS is an issue and could compromise further molecular analysis in a long time after glyphosate treatment.

In that case, the integrity of RNA can be measured using gel electrophoresis and RNA integrity number (RIN) (Schroeder et al., 2006). The first method is well known and is based on the band fragment separation as a function of their molecular weight in

agarose gel through an electrical current. The RIN has evaluated in Agilent 2100 bioanalyzer. The bioanalyzer provides electrophoretic separations of RNA samples in the microchannels, according to their molecular weight and fluorescence laser-induced detection (Schroeder et al., 2006). After, it is used for assessment of the RIN, which is calculated based on an algorithm developed from a Bayesian approach (Schroeder et al., 2006). The use of the ratio of the large (26S) to small (18S) ribosomal RNA subunits (26S/18S) is not considered because has been criticized as it might not reflect the degradation of other types of RNA such as mRNA (Schroeder et al. 2006), which is the target nucleic-acid of transcriptome studies.

The evaluation of RNA integrity is pre-requisite before molecular analysis as RNA-Seq and qRT-PCR. However, there is no information about the RNA integrity in GR and glyphosate-sensitive (GS) hairy fleabane after glyphosate treatment. The action of glyphosate causes great tissue damage in a determined time-point after treatment, especially in GS biotype, could degrade RNA and compromise further comparative analysis against GR and GS biotypes (Figure 1). In this way, the evaluation of RNA integrity in a time-course after glyphosate treatment in GR and GS biotypes of *Conyza* spp. will provide useful information for further molecular studies. Thus, the present study hypothesizes that the glyphosate action leads to the degradation of RNA extracted from GR and GS hairy fleabane. Therefore, the objective of this study was to evaluate whether glyphosate treatment interferes negatively on RNA integrity of glyphosate-resistant and -sensitive hairy fleabane biotypes.

MATERIALS AND METHODS

Two time-course experiments after glyphosate treatment were performed at Universidade Federal de Pelotas (UFPel) in Pelotas, Rio Grande do Sul State, Brazil. The experiments were carried out using plants of two biotypes of hairy fleabane previously characterized as glyphosate-resistant (B11R) and -sensitive (B17S), with a resistance factor of 18.2 (Piasecki et al., 2019). The plants were cultivated in a greenhouse until 60 days after emergence (DAE) at 25 $^{\circ}$C/15 $^{\circ}$C day/night (± 3 $^{\circ}$C) and 12-hours photoperiod.

Experiment 1 (E1) was arranged in a three-factorial scheme 2 x 5 x 5. The first factor comprised two hairy fleabane biotypes (B11R and B17S); second factor comprised five glyphosate doses: 0 (untreated); 370 (1 L pc ha^{-1}); 1,480 (4 L pc ha^{-1}); 5,920 (16 L

pc ha^{-1}), and 11,840 g ae ha^{-1} (32 L pc ha^{-1}); and third factor comprised evaluations of RNA integrity in five time-points: 0; 12; 24; 48; and 96 hours after treatment (HAT).

Experiment 2 (E2) was also arranged in a three-factorial scheme 2 x 2 x 6 — the first factor comprised two hairy fleabane biotypes (B11R and B17S); the second factor comprised two glyphosate doses: 0 (untreated) and 1,480 g ae ha^{-1} (4 L pc ha^{-1}); the third factor comprised evaluations of RNA integrity in six time-points after glyphosate treatment: 0; 24; 48; 96; 192; and 288 HAT. In both E1 and E2 experiments, the herbicide used was Roundup Original DI (Monsanto Company; isopropylamine salt 370 g ae L^{-1}). The glyphosate treatment was performed with a CO_2 sprayer and 150 L ha^{-1} of spray volume.

The RNA was evaluated in both experiments (E1 and E2) using the RNA integrity number (RIN) and gel electrophoresis. In E1 the RIN was assessed until 96 HAT, while in E2 until 192 HAT for B17S and 288 HAT for B11R. In the E1, the evaluation times and glyphosate rates were defined empirically to evaluate the dose-response on RNA integrity. In E2, the time-course experiment was determined based on E1 to determine glyphosate rates. The evaluation time was determined based on results of another study which indicated that shikimic acid content in B11R treated plants did not differ from untreated at 288 HAT and that B17S died after 192 HAT (Piasecki et al., 2019).

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and treated with DNAse I^{TM} (Invitrogen, USA) according to the manufacturer's protocol. RNA concentration (ng μL^{-1}) were checked and standardized to 80 ng μL^{-1} using Nanodrop 2000 spectrophotometer (Nanodrop, USA). The RNA integrity number (RIN) was measured in a commercial laboratory in Bioanalyzer (Agilent Bioanalyzer 2100 system, Agilent Technologies, USA). The electrophoresis evaluations were performed in our laboratory using 1% agarose gel.

In E1 and E2, RNA was obtained from three biological replicates per treatment. In E1, RNA obtained from three plants per treatment was pooled after standardization of concentration to 80 ng μL^{-1}. It resulted in a technical replicate per treatment, producing an amount total of 50 RIN analysis of RIN and 50 for electrophoresis gel. In E2, RIN analysis was performed without pooling RNA, i.e., each treatment had three biological replicates, totaling 36 samples (12 treatments x 3 replicates). However, for electrophoresis gel, the RNA extracted from treated plants according to each biotype were pooled and used three technical replicates. Untreated plants results were provided from

three biological replicates. It was performed because those 12 RNA samples were forwarded to RNA-Sequencing using that experimental design.

The electrophoresis gel was visually and automatically analyzed using the Gel Analyzer software (http://www.gelanalyzer.com). This software was used because they provide high-quality results and is free access. The process of gel image analysis is straightforward and covers all the main aspects of evaluation from automatic lane detection to precise molecular weight calculations. The measurements consisted basically on indicate the peaks observed after scan electrophoresis gel. The intensity (Y-axis) indicates the RNA concentration in each sample, while pixels (X-axis) indicate the position or molecular weight. It is expected to visualize two well-defined RNA lanes (28S and 18S) and a third one at the end of the graph, which indicates typically genomic DNA or extraction residues. The presence of multiple peaks through the graph represent RNA degradation.

Statistical analysis was performed using the GLM package statement from SAS (version 9.0, SAS Institute Inc, Cary, NC, USA), and results fitted using Sigma Plot®. SAS Proc Univariate was used to test for normality (Shapiro-Wilk's test), and homogeneity of variance (Hartley's test). Proc Mixed was used to perform analysis of variance (ANOVA) at F test ($p \leq 0.05$). Test-t were applied in regressions at $p \leq 0.05$. Significant characters were submitted to linear correlations analysis and effects of biotypes and glyphosate doses isolated to verify the tendency of association between interest characters at $p \leq 0.05$. Descriptive statistic was used to describe the electrophoresis gel results.

RESULTS AND DISCUSSION

In E1, the RIN score ranged from 5.1 to 7.2, and the general average was 6.7 (Figure 2). In that experiment were no observed differences on RNA integrity number (RIN) between glyphosate-resistant (GR – B11R) and -sensitive (GS – B17S) biotypes of hairy fleabane until 96 h after treatment (HAT) for untreated samples, and after treatment with 370, 1,480, and 11,840 g ae ha^{-1} of glyphosate (Figure 2 a, b, c, and e). These results indicate that the glyphosate action does not have a relation with RNA integrity. However, after the treatment of 5,920 g ae ha^{-1} of glyphosate, the RIN increased until 96 HAT for both biotypes with no statistical differences between them (Figure 2 d). The increase in RIN on that treatment was an unexpected result, indicating that the glyphosate action

favored to a better score. In this way, that increase can be the result of differences in plant status during the leaves collection, and due to a manual extraction procedure variation.

The RIN score in E2 ranged from 6.1 to 9.0, and the average was 7.9 (Figure 3). In that experiment, plants of hairy fleabane treated with glyphosate do not show significative variations on RIN score until 288 HAT for GR and 192 HAT for GS biotypes (Figure 3). Untreated plants from both GR and GS biotypes presented significative reduction on RIN score at 12 and 24 HAT, respectively. However, there was no difference between biotypes. This result indicates that observed reduction in RIN is not related to glyphosate treatment and could be due to the extraction procedure. Also, treated plants presented higher results for RIN than untreated, demonstrating the non-negative influence of glyphosate action on those results (Figure 3).

In E1 and E2, visual electrophoresis gel analyzes did not show degradation results for all RNA samples in response to different glyphosate doses and evaluated in a time-course after treatment (Figures 4-6). For all results, a clear band can be visualized at the 28S and 18S position (Figure 4 a and b). These results can be compared with an example of degraded and intact RNA (Figure 4 c). Degraded RNA presents an aspect of RNA traces dragged on an electrophoresis gel, and do not show specific bands at expected positions. In another hand, intact RNA presents well defined two bands at 28S and 18S positions, with no traces through the gel (Figure 4 c). In addition, the Gel Analyzer graph results also showed well-defined peaks for 28S and 18S positions and indicated no degradation through all RNA samples (Figures 5 and 6). These results show some variations on peaks high, which means differences in RNA concentration at that point. It could occur because we standardized the RNA concentration using Nanodrop, and this equipment is subject to variations on results in addition to manual manipulation. A third peak appears in the gel and graphs results indicating extraction residues, which is common in Trizol RNA extraction.

RIN scores range from 0 to 10, where 0 is poorly RNA integrity, and 10 is considered excellent. Thus, higher results as possible are desired for good further molecular analysis with low RNA degradation. The degradation process of RNA is only partly known because it depends on the type of RNase enzyme present. RNA degradation is a continuous process and implies which there are no natural integrity categories (Schroeder et al. 2006). Also, the quality of RNA varies extensively from one extraction to another because of manual operations. Also, RNA quality and integrity depend on

employed methods during extraction, reagents, and environment conditions (Johnson et al., 2012).

Isolate RNA to transcriptomic experiments with quality (i.e., non-degraded, free of impurities) and yield is a challenge. It is because of RNases, which degrade the RNA rapidly, are present in large scale in nature and laboratories (Johnson et al., 2012). Plant secondary metabolites such as phenols and polysaccharides are also an issue, and these metabolites vary dramatically within and among species (Agrawal, 2011). In the case of the present study, stress treatment such as glyphosate action on GR and GS plants of hairy fleabane was supposed to cause negative effects on RNA integrity and yield, especially because of ROS production.

The ROS production occurs after glyphosate inhibits the shikimic acid pathway through the direct inhibition of the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Cobb and Reade, 2010). As a consequence of the shikimic acid pathway inhibition, occurs the accumulation of shikimic-acid and reducing power (NADPH+H). The biosynthesis of aromatic amino acids phenylalanine, tyrosine, and tryptophan is interrupted. The lack of tyrosine inhibits the synthesis of plastoquinone, which is an electron acceptor in the photosynthetic electron transport chain in the photosystem II (PSII). The non-regeneration of plastoquinone in the PSII interrupts the electron transport, leading to energy accumulation. Therefore, both processes, reducing power accumulation and PSII blockage, leads to the production of ROS, oxidative stress, cell damage, and plant death (Cobb and Reade, 2010).

The ROS production after glyphosate treatment has been demonstrated in GR and GS biotypes of hairy fleabane (Piasecki et al., 2019) and the literature indicates that the ROS leads to RNA degradation in plants (Foyer and Noctor 2005; Gill and Tuteja 2010). Also, plants of hairy fleabane show damaged aspect at 192 HAT, which is more intense in GS plants than in GR (Figure 1). On this context, that visual status of plants could lead the researcher to believe that RNA is not suitable for further studies whether extracted from plants in that circumstance. However, the results of the present work show that until 192 and 288 HAT for GS and GR biotypes, respectively, the glyphosate treatment does not interfere negatively in RIN integrity (Figures 2, 3, and 4-6). Thus, for further molecular analysis such as RNA-Seq and qRT-PCR, the RNA integrity results indicate that studies can be performed in times higher than 24 and 48 HAT (Peng et al., 2010; Hereward et al., 2018) to capture as much as possible the process of glyphosate plant detoxification (Table 1).

Analysis of RNA integrity is frequently used before molecular studies. It is important to ensure that RNA present suitable conditions to be analyzed and to provide reliable results. The inclusion of degraded RNA on molecular analysis has shown significant influence on the bioinformatics and statistical analysis and hence the interpretation of gene expression levels, leading to the conclusion that degraded samples should not be reasonably considered for analysis (Copois et al. 2007; Die and Román 2012).

The results of the present work provide results that will help future molecular studies in hairy fleabane, especially during planning experiments. We demonstrate that RNA can be extracted until 192 HAT in GS biotype and until 288 HAT in GR biotype with suitable results. In GR biotypes, RNA can be explored in higher times than those used in the present study, obviously because those plants are not supposed to die after glyphosate treatment.

Another important point to be considered is the costs for RNA extraction, RIN, and electrophoresis gel analysis. The costs of RNA extraction using the methodology adopted in the present work were US$ 5.10 (US$ 1.00 is equivalent to R$ 3.90) per sample, considering just the reagents. In the present work, 86 RNA samples were extracted with a cost of US$ 438.60. The costs of RIN analysis was US$ 6.40 per sample in a commercial laboratory. The electrophoresis gel cost around US$ 0.60 per sample. As in the present work were analyzed 86 samples in bioanalyzer and 62 samples (some samples were pooled) in an electrophoresis gel, the total cost was US$ 550.40 and US$ 37.20, respectively. The shipping costs of RNA samples to the laboratory were around US$ 200.00, including the price of dry ice, required to keep RNA samples frozen in temperature close to -80 ^{o}C. Thus, the total costs of analysis of RNA integrity were of US$ 1,226.20. If we considered 86 samples, the costs of each one were approximately US$ 14.25. Is important to highlight that costs of laboratory use and workforce were not considered, and if so, probably the costs will be doubled. Thus, RNA integrity analysis provides very important results, however, with an expensive cost.

The results of the present study indicate that glyphosate treatment does not reduce the RNA integrity in glyphosate-sensitive and -resistant hairy fleabane biotypes until 192 h and 288 h after treatment, respectively.

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Table 1. RNA-Seq studies performed for weed glyphosate-resistance mechanisms investigation in the world and times of RNA extraction after glyphosate treatment.

Weed specie	Herbicide Resistance	Enzyme/process inhibition	RNA-Seq performed at (hours)*	Specific resistance mechanism[1]	Country	Reference
Conyza bonariensis	Glyphosate	EPSPS[3]	48	No	Australia	Hereward et al. (2018)
Eleusine indica	Glyphosate	EPSPS	UT[2] and 48	No	China	Chen et al. (2017)
Euphorbia esula	Glyphosate	EPSPS	UT and 1,008	No	USA	Doğramacı et al. (2015)
Kochia scoparia	Glyphosate	EPSPS	No herbicide	No	USA	Wiersma et al. (2015)
Ipomoea purpurea	Glyphosate	EPSPS	UT and 8	No	USA	Leslie and Baucon (2014)
Conyza canadensis	Glyphosate	EPSPS	UT and 24	No	USA	Yuan et al. (2010)
Conyza canadensis	Glyphosate	EPSPS	UT and 24	No	USA	Peng et al. (2010)

*Time of RNA extraction after herbicide treatment for RNA-Sequencing – hours after treatment (HAT); [1] If specific resistance mechanism was understood; [2] Untreated; [3] EPSPS: 5-enolpyruvylshikimate 3-phosphate synthase. Articles searched in: Web of Science, PubMed, NCBI, Scopus. Keywords used: RNA-Seq glyphosate-resistance, Transcriptome weed glyphosate-resistance, Differential expression analysis, RNA sequencing, *de novo* Assembly.

Figure 1. Plants of glyphosate-resistant (B11R) and -sensitive (B17S) biotypes of *Conyza bonariensis* representing their damage caused by glyphosate at 192 hours after treatment (1,480 g ae ha^{-1}). The times 24 h, 96 h, 192 h, and 288 h indicate the number of hours after glyphosate treatment that leaves were collected to RNA extraction in each biotype. In each time, three biological replicates were collected. Experiment 2. In this experiment were collected two completely expanded leaves from apex per plant for RNA extraction.

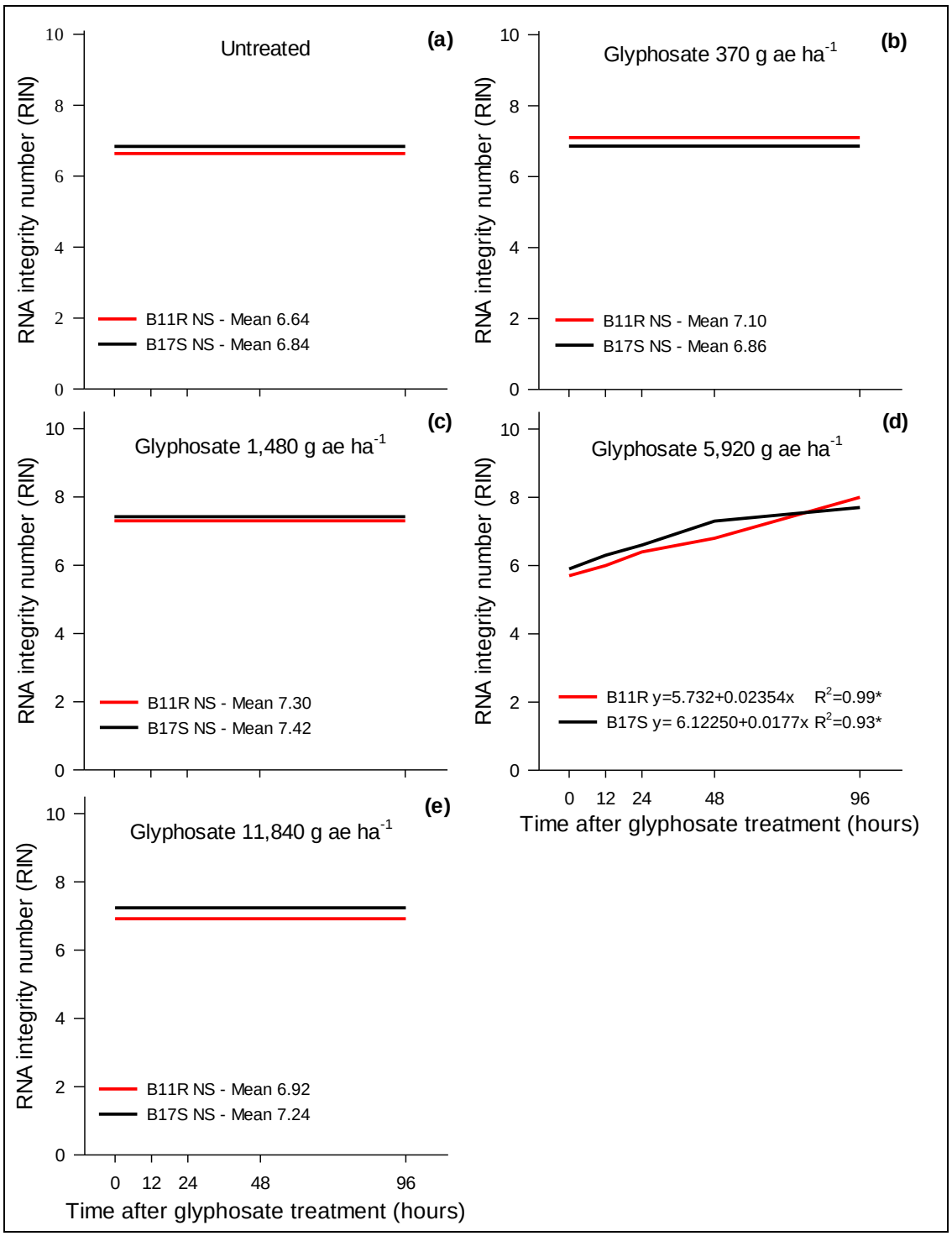

Figure 2. RNA integrity number (RIN) analyzed in glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes at 0, 12, 24, 48, and 96 hours after glyphosate treatment: a) untreated; b) 370 g ae ha^{-1}; c) 1,480 g ae ha^{-1}; d) 5,920 g ae ha^{-1}; and e) 11,840 g ae ha^{-1}. NS: no significant at $p \leq 0.05$. Experiment 1 (E1).

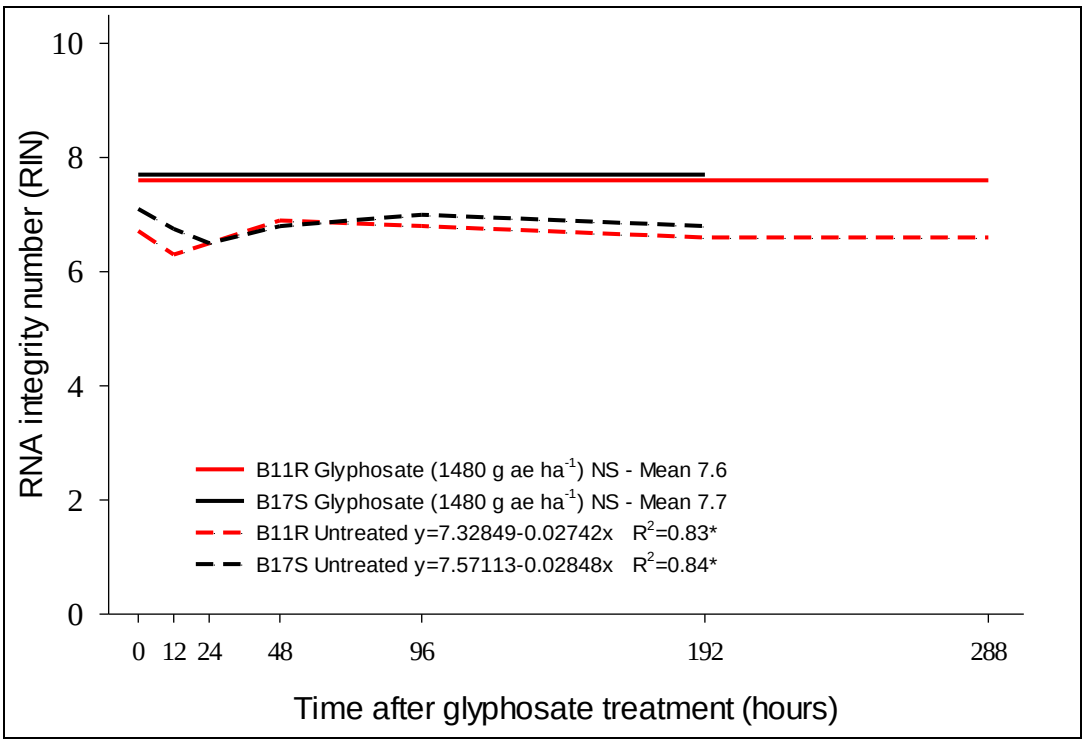

Figure 3. RNA integrity number (RIN) analyzed in glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes at 0, 12, 24, 48, 96, 192, and 288 hours after glyphosate treatment at 0 (untreated) and 1,480 g ae ha^{-1}. NS: no significant at $p \leq 0.05$. Experiment 2 (E2). B17S was evaluated until 192 HAT because after that time plants died.

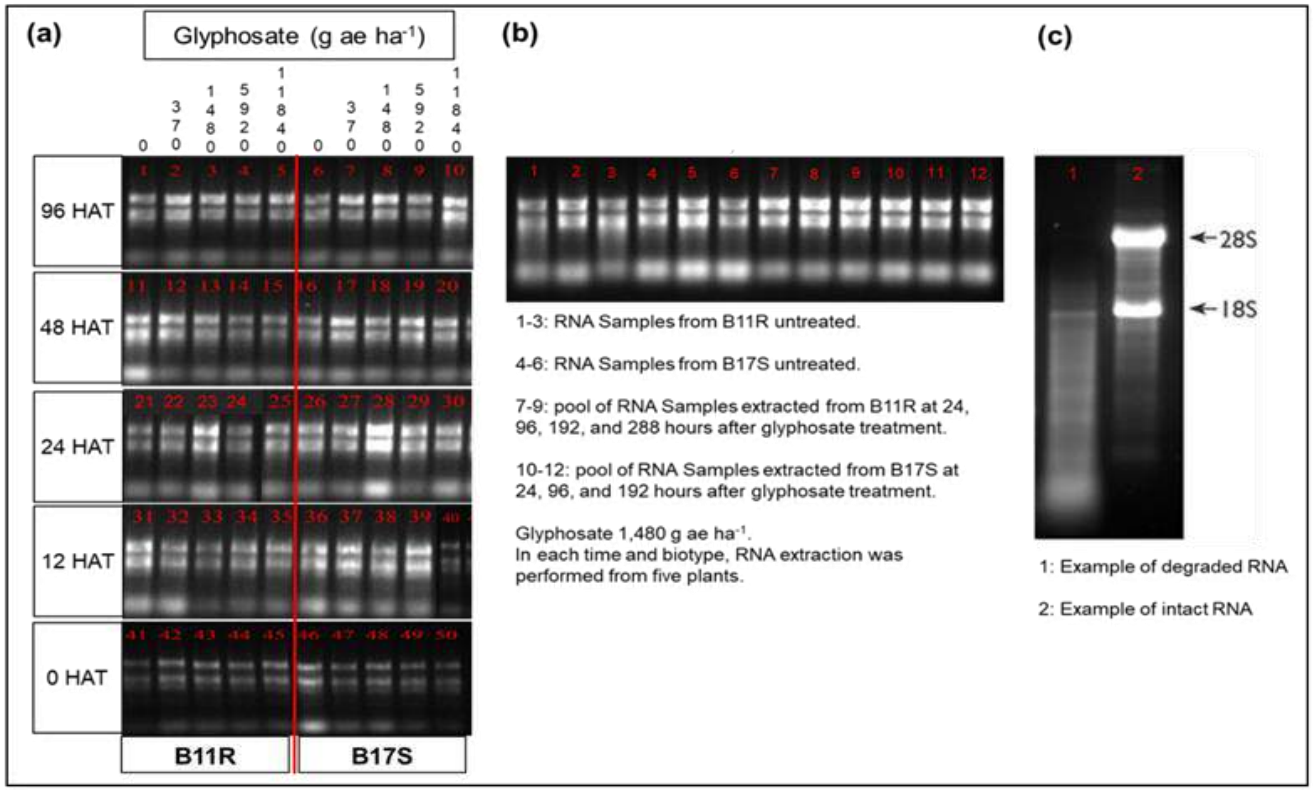

Figure 4. Electrophoresis gel results of the RNA extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes followed by glyphosate treatment. (a) Experiment 1 (E1) – RNA extracted from B11R and B17S at 0, 12, 24, 48, and 96 hours after glyphosate treatment at 0 (untreated), 370, 1,480, 5,920, and 11,840 g ae ha^{-1}. (b) Experiment 2 (E2) – RNA Samples 1-3: B11R untreated; RNA Samples 4-6: B17S untreated; RNA Samples 7-9: pooled RNA extracted from B11R at 24, 96, 192, and 288 hours after glyphosate treatment (1,480 g ae ha^{-1}); RNA Samples 10-12: pooled RNA extracted from B17S at 24, 96, and 192 hours after glyphosate treatment (1,480 g ae ha^{-1}). (c) Examples of degraded RNA – 1; and intact RNA - 2. HAT: hours after glyphosate treatment.

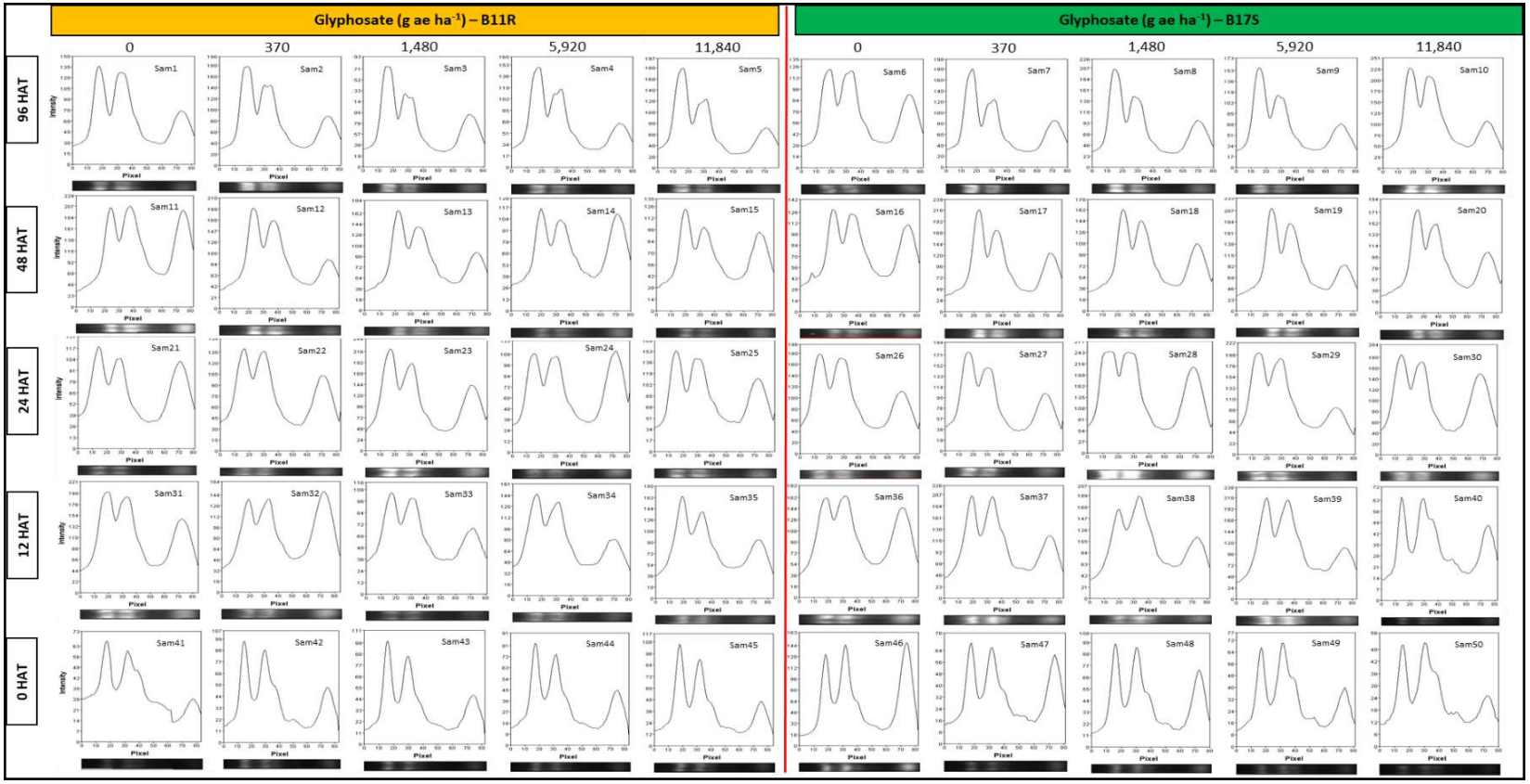

Figure 5. Results of analysis of electrophoresis gel using the software Gel Analyzer. The RNA was extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes, followed by glyphosate treatment. Experiment 1 (E1). RNA extracted from B11R and B17S at 0, 12, 24, 48, and 96 hours after glyphosate treatment (HAT) at 0 (untreated), 370, 1,480, 5,920, and 11,840 g ae ha^{-1}. Sam: indicate the sample number. The samples follow the same order of electrophoresis gel presented in Figure 4 a.

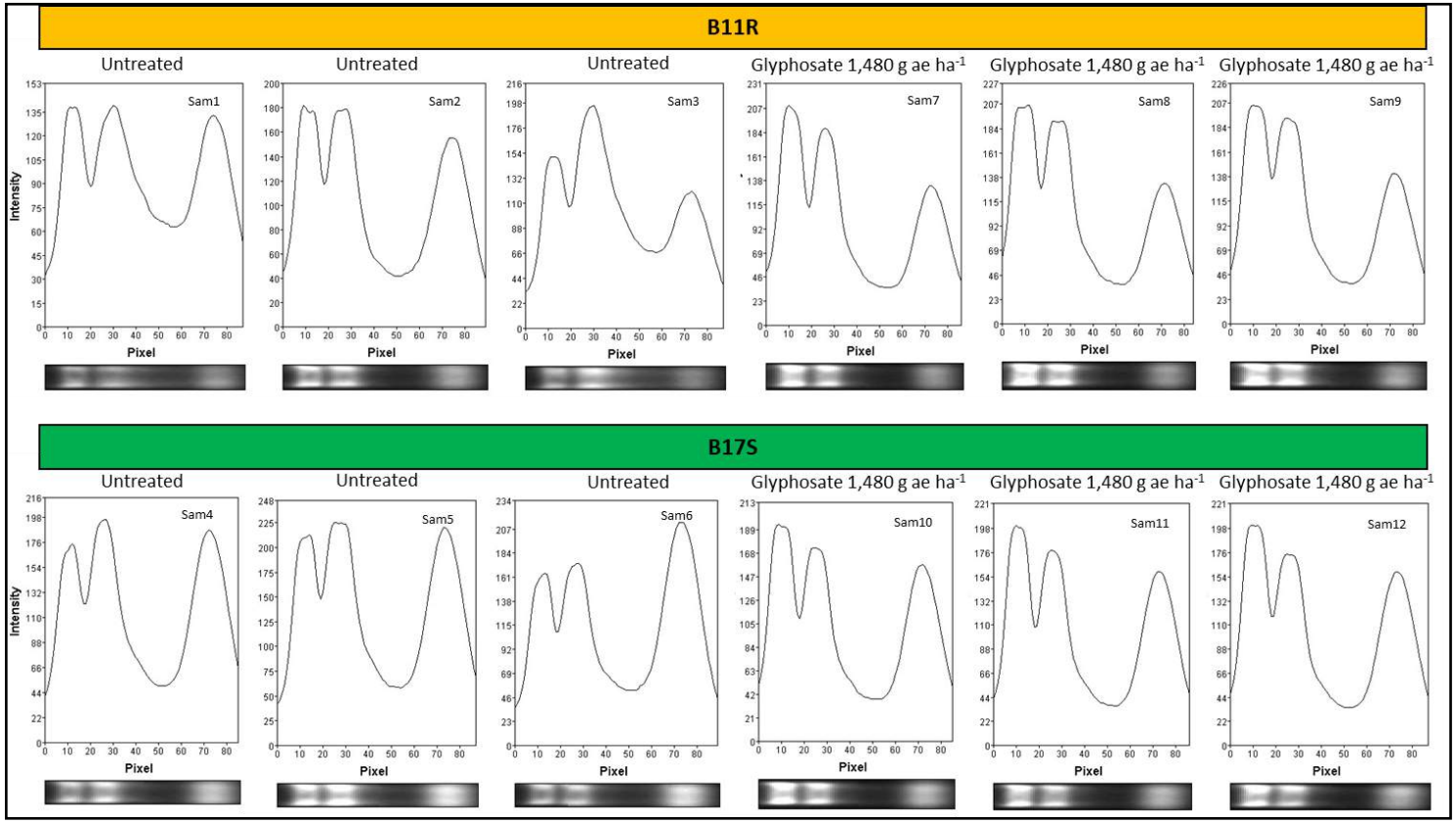

Figure 6. Results of analysis of electrophoresis gel using the software Gel Analyzer. The RNA was extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes, followed by glyphosate treatment. Experiment 2 (E2). RNA Samples 1-3: B11R untreated; RNA Samples 4-6: B17S untreated; RNA Samples 7-9: pooled RNA extracted from B11R at 24, 96, 192, and 288 hours after glyphosate treatment (1,480 g ae ha^{-1}); RNA Samples 10-12: pooled RNA extracted from B17S at 24, 96, and 192 hours after glyphosate treatment (1,480 g ae ha^{-1}). Sam: indicate the sample number. The samples follow the same order of electrophoresis gel presented in Figure 4 b.

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Transcriptomic Analysis Identifies New Non-Target Site Glyphosate-Resistance Genes in *Conyza bonariensis*

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Abstract: *Conyza bonariensis* (hairy fleabane) is one of the most problematic and widespread glyphosate-resistant weeds in the world. This highly competitive weed species significantly interferes with crop growth and substantially decreases crop yield. Despite its agricultural importance, the molecular mechanisms of glyphosate resistance are still unknown. The present RNA-Seq study was performed with the goal of identifying differentially expressed candidate transcripts (genes) related to metabolism-based non-target site glyphosate resistance in *C. bonariensis*. The whole-transcriptome was *de novo* assembled from glyphosate-resistant and -sensitive biotypes of *C. bonariensis* from Southern Brazil. The RNA was extracted from untreated and glyphosate-treated plants at several time points up to 288 h after treatment in both biotypes. The transcriptome assembly produced 90,124 contigs with an average length of 777 bp and N50 of 1118 bp. In response to glyphosate treatment, differential gene expression analysis was performed on glyphosate-resistant and -sensitive biotypes. A total of 9622 genes were differentially expressed as a response to glyphosate treatment in both biotypes, 4297 (44.6%) being up- and 5325 (55.4%) down-regulated. The resistant biotype presented 1770 up- and 2333 down-regulated genes while the sensitive biotype had 2335 and 2800 up- and down-regulated genes, respectively. Among them, 974 up- and 1290 down-regulated genes were co-expressed in both biotypes. In the present work, we identified 41 new candidate target genes from five families related to herbicide transport and metabolism: 19 ABC transporters, 10 CYP450s, one glutathione S-transferase (GST), five glycosyltransferases (GT), and six genes related to antioxidant enzyme catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD). The candidate genes may participate in metabolic-based glyphosate resistance via oxidation, conjugation,

transport, and degradation, plus antioxidation. One or more of these genes might 'rescue' resistant plants from irreversible damage after glyphosate treatment. The 41 target genes we report in the present study may inform further functional genomics studies, including gene editing approaches to elucidate glyphosate-resistance mechanisms in *C. bonariensis*.

Keywords: hairy fleabane; herbicide resistance; herbicide metabolization; non-target-site resistance (NTSR); RNA-Seq; next-generation sequencing; differential gene expression

1. Introduction

Weed interference is among the most significant biotic constraints to crop yield and global food security, causing up to $100 billion in damage annually [1,2,3]. Weed species with widespread occurrence and highly competitive capacity with crops that are difficult to manage are particularly damaging to agricultural security. *Conyza bonariensis* (L.) Cronq., commonly called hairy fleabane, is one of the most threatening and difficult weed species to manage around the world [4,5]. This Asteraceae species is native to the Americas and has a cosmopolitan distribution [4]. Some of its weedy traits include high production and dispersion of seeds, phenotypic plasticity, and tolerance to unfavorable environmental conditions [4,6]. Further, the evolved glyphosate resistance (GR) is a critical factor that complicates controlling weeds in the field [7].

The first report of glyphosate-resistant *C. bonariensis* in Brazil occurred in 2005 [8]. Since then, the evolution of resistance to glyphosate in this species has become a significant concern around the world, and to date, there have been 13 GR reports in several crops and countries [9]. *Conyza* spp. have become one of the world's worst weeds because of their propensity to evolve strong herbicide resistance [7] in both cropland and fallow land [10,11,12].

Glyphosate is a valuable herbicide because of its high efficacy to a broad spectrum of annual and perennial weeds, relatively low cost, and low negative impact on the environment when compared to other herbicides [13,14]. However, the intensive and widespread use of glyphosate has been a factor in evolved resistance [13,15]. The glyphosate target site is the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction converting phosphoenolpyruvate (PEP) and 3-phospho-shikimate into phosphate and 5-enolpyruvylshikimate-3-phosphate [14,16]. Glyphosate interrupts the shikimic acid pathway and disrupts aromatic amino acid biosynthesis, causing alterations in the metabolic stoichiometry of carbon intermediates. The massive physiological disruption in glyphosate-sensitive (GS) plants leads to their death in a matter of days [17]. However, weeds have evolved multiple mechanisms to tolerate glyphosate treatment, reduce its damage, and facilitate weeds' recovery and fitness after exposure.

GR mechanisms can be grouped into two large categories: target site (TS) resistance, i.e., caused by changes in the EPSPS gene and shikimic acid metabolism, and non-target site resistance mechanism (NTSR), which encompasses, essentially, all mechanisms not involving EPSPS-related alterations [18,19,20]. To-date, TS resistance has not been observed in *C. bonariensis*; thus, one or more NTSR mechanisms are to blame [6,16] but have not been elucidated. The best physiological evidence indicates that NTSR in *C. bonariensis* is caused by subcellular glyphosate sequestration, likely to the vacuole, which prevents glyphosate from being translocated to tissues that have not been sprayed [6,16,21,22]. However, the molecular mechanisms are unknown for any GR weed.

Transcriptomic studies have been performed at 24 h and 48 h after glyphosate treatment to understand the genomic basis of glyphosate NTSR in *Conyza canadensis* (United States) [23] and *C. bonariensis* (Australia) [16]. However, the specific mechanism of glyphosate NTSR has not yet been elucidated [6,16,24]. After herbicide treatment, plant damage begins within the first 3 to 8 h [25]. NTSR mechanisms can typically rescue resistant plants before irreversible cellular damage has occurred and must have sufficient persistence to outlast glyphosate activity [25,26,27]. Moreover, stress-responsive genes have circadian regulation, and transcriptome sampling during an off-peak regulation period may thus lead to an incomplete representation of the plant molecular response to herbicide [26,27].

One hypothesis for why published transcriptomic analyses have failed to identify NTSR genes responsible for GR is that RNA has been isolated too soon after glyphosate treatment, which missed gene regulatory changes related to key metabolic steps in GR weeds. Our previous studies using the *C.*

bonariensis biotypes from Brazil, which are also used in the present study, have shown that shikimic acid accumulation in GR hairy fleabane is transient, reaching its peak at 96 h after glyphosate treatment and then declining. Approximately 288 h after treatment, shikimic acid concentrations of GR plants are equivalent to those of untreated plants [28]. Another study reported similar transient shikimic acid accumulation in *C. canadensis* [29]. Thus, shikimic acid content may be an important indicator of weedy plant response to glyphosate treatment. The strategy of assaying gene expression relative to shikimic acid content, which is the first indicative of the EPSPS inhibition after glyphosate treatment, has the potential to help us to better understand the underlying genomics of NTSR biotypes vis-à-vis GR biotypes vs. GS biotypes. This paper describes the results of the *de novo* assembly of the transcriptome (RNA-Seq) of GR regarding the GS *C. bonariensis* over a long period (up to 288 h after glyphosate spray), and then the identification of candidate NTSR genes based on differential gene expression data.

2. Results

The present study aimed to study the molecular changes of *C. bonariensis* in response to glyphosate treatment in the long term after herbicide treatment. The timepoints of RNA extraction to RNA sequencing were determined based on shikimic acid content in the GR biotype from 24 h to 288 h after treatment. In the last time point, the shikimic acid content on treated GR plants did not differ regarding untreated plants. Thus, the gene expression changes were evaluated in GR glyphosate treated and untreated plants in contrast to GS with the same treatments.

2.1. Illumina Sequencing and De Novo Assembly

The twelve cDNA libraries sequenced from *C. bonariensis* leaves produced a high-quality assembled dataset (Table S1). The Phred scores of the *de novo* assembled data were ≥Q30 level (error probability 0.1%) for more than 90% of all raw reads. Transcriptome assembling produced 203,054 (>200 bp) transcripts and 90,124 contigs at the "gene" level (N50 of 1118 bp and the average length of 777 bp) (Table S1). The sequence distribution of transcripts length threshold varied from 0 to 3000 bp (Figure S1). In this way, the large scale and high-quality results provided in the present study will serve as a molecular reference for further *C. bonariensis* studies.

2.2 Functional Annotation of Assembled Contigs

The BLASTx results of sequences indicated that 68.1% of the annotated contigs (90,124) had hits with nucleotide sequences from the NCBI database (Figure S2). Most of the annotated nucleotide sequences of *C. bonariensis* transcriptome corresponded to *Helianthus annuus* (36.9%) and *Cynara cardunculus* var. *scolymus*(18.8%). Further, the contig identity to other plant species was 12.4%, and the number of novel genes without interspecies identity was 31.9% (Figure S3).

The functions of *C. bonariensis* contigs were classified according to gene ontology assignments. A total of 11,877 contigs (13.2%) were attributed to at least one gene ontology term and classified in 30 functional categories using the complete set of GO terms in three main categories: molecular function (n = 3188, 26.9%), biological process (n = 7491, 63.1%), and cellular component (n = 1198, 10.1%) (Figure S4). The largest proportion of genes was represented by a cellular (3.7%) and metabolic process (3.2%) in the biological process; molecular function (4.7%) and binding (3.5%) in molecular function; and a cellular component (4.7%) and cell organelle (4.2%) in cellular component (Figure S4).

The high number of novel genes (31.9%) provided in the present RNA sequencing results shows the importance of characterizing genomes of weeds and also serves to enable follow-on molecular studies in *C. bonariensis* and other weedy species. Further genome sequencing studies in *Conyza* spp. will be helpful to completely characterize the novel genes presented here. Further, the gene ontology indicates that the biological process represented the highest proportion of annotated genes regarding molecular function and cellular component.

2.3 Differentially Expressed Genes

The total of differentially expressed genes (DEGs) (9622 "genes"; 39.9% of total) was found in response to glyphosate treatment in both biotypes: 4297 (44.6%) genes were up-regulated and 5325 (55.4%) were

down-regulated (Figure 1 and Figure S5). Among the DEGs, 1770 and 2333 genes were up- and down-regulated in GR plants, while 2335 and 2800 genes were up- and down-regulated in GS plants, respectively. A total of 974 up- and 1290 down-regulated genes were co-expressed in both biotypes as a response to glyphosate treatment (Figure 1, Figure S5). In GR and GS biotypes without glyphosate treatment (t0), there were around 100 genes each in up- and down-regulation, with no apparent co-expression (Figure 1).

The most abundant GO terms for up-regulated genes were biological process—protein metabolism (87 DEGs from GR plants and 120 DEGs from GS plants) (Figure 2A,B); molecular function—ATP binding (302 DEGs from GR plants and 321 DEGs from GS plants) (Figure 2C,D); cellular component—integral component of membrane (282 DEGs from GR plants) and cytoplasm (376 DEGs from GS plants) (Figure 2E,F). It is interesting to note that GR plants have 45.9% more up-regulated genes in transmembrane transport than GS (37 vs. 17). However, in the GR biotype, this process was classified as the fifth-most prominent and was not included among the top ten up-regulated genes in GS (Figure 2A,B). On the other hand, the GS biotype had 126 (50%) more up-regulated genes than GR (376 vs. 250) in processes located in the cytoplasm (cellular component) (Figure 2E,F). Further, the resistant biotype had 53 (36%) more up-regulated genes that are chloroplast localized (200 vs. 147) than in GS plants (Figure 2E,F).

The attributed GO functions for down-regulated genes show the highest proportions of characterized genes respectively in GR and GS related to biological process—cell wall metabolism (3.8%) and protein metabolism (3.5%) (Figure 3A,B); molecular function—ATP binding for both biotypes (8.1% and 10.1%) (Figure 3C,D); and cellular component—integral component of membrane (9,6%) and chloroplast (13.4%) (Figure 3E,F). In the biological process, 63 genes related to translation were down-regulated in GR while just 16 in GS, which do not figure in the top ten in the sensitive biotype. Further, the transmembrane transport-related contigs were not among the top ten down-regulated DEGs in GR but were so in GS (Figure 3A,B). In molecular function, there were 52 down-regulated genes related to ribosome metabolism in GR while just one in GS (Figure 3C,D). Further, GR presented 40% and 31% less down-regulated genes related to chloroplast and integral component of the membrane than GS (Figure 3E,F).

The high number of DEGs represents the high molecular changes that glyphosate action causes in the *C. bonariensis* transcriptome. Further, the differences on expressed genes according to each category show differences between GR and GS biotypes and might indicate the processes related with resistance to glyphosate.

2.4 RNA-Seq Validation by qRT-PCR Analysis

The average expression for 19 contigs with relative expression performed in a time course indicates an amplitude of (log fold change—logFC) from 0.01 to 1990, while the transcriptome results for the same contigs ranged from logFC 0.1 to 3625 (Figure 4 and Figure S6). The transcriptome dataset and qRT-PCR presented a significant correlation (r) of 0.9 for transcriptional results of GR and GS biotypes (Figure S6). On the other hand, the results of the time course qRT-PCR experiments show that gene expression response to glyphosate treatment varies according to gene-related function, to biotype, and to time after herbicide exposure (Figure 4). In general, the results of the evaluated genes in a time course experiment suggest that the highest expression levels occur between 96 and 192 h after treatment (HAT) (Figure 4). The differences of gene expression in time might be a very good reference for further studies involving glyphosate resistance; between 96 and 192 HAT is the best time-window to capture molecular responses to glyphosate treatment in *C. bonariensis.*

2.5 EPSPS Sequence Analysis

The BLASTn analysis identified, with an E-value threshold of zero and score bits >600, one single copy of each the three EPSPS sequences assembled in each one of the individual *C. bonariensis* transcriptomes of GR and GS. There was no amino-acid substitution at Thr 102 and Pro 106 codons from EPSPS sequences alignment (Figure S7A–C). On the other hand, the basal EPSPS transcription varied among the three copies but did not between biotypes. In general, EPSPS2 presented the lowest level of expression among the three copies. In response to glyphosate treatment, the EPSPS2 and EPSPS3 had increased transcription; however, there were no differences between biotypes. EPSPS1 had low variation in expression (Figure S7D). These results indicate that the glyphosate-resistance process in GR plants is due to a NTSR.

2.6 Candidate NTSR Genes Related to Putative Functions of Glyphosate Transport and Metabolism

We identified a total of 41 candidates differentially expressed genes associated to the herbicide metabolism phases of oxidation (CYP450), conjugation (GST and GT), transport (ABC transporters), and protection/compensation (antioxidant enzyme—CAT, POD, and SOD) [20,30]. Two ABC transporters and one GT target gene were chosen for validation by qRT-PCR. From these results, the ABC transporters' transcript abundance varied between biotypes in response to glyphosate treatment and in time after herbicide exposure. The expression of ABC_12 showed the highest expression in GR rather than GS at 24 h after treatment (HAT) (fold change 29.6), whereas ABC_16 at 192 HAT (fold change 3.4). The GT_5 highest expression in GR about GS occurred at 192 HAT with a fold change of 2.8 (Figure 4). Further, eight genes had their highest expression in GS plants, with a more significant increase in expression between 96 and 192 HAT. Among these eight genes, we highlighted the steroid 5-beta-reductase (fold change 11.1—96 HAT) and aspartyl protease (fold change 281.5—192 HAT) (Figure 4).

We identified two copies of each ABC transporters M10 and M11 gene (M10_g1 and M10_g2; M11_g1 and M11_g2) in the *C. bonariensis* transcriptome. Except for M10_g2, all other genes had increased transcript abundance from glyphosate treated plants. The GS biotype presented higher M10 and M11 expression levels than the resistant biotype (Figure S8). Because of that, M10 and M11 genes were not considered glyphosate-resistance candidate genes in the present work.

Here, we report 19 new ABC transporter candidate genes associated with glyphosate resistance in *C. bonariensis*. In the differential expression analysis, these genes had a higher response to glyphosate treatment in GR than in GS with different levels of transcripts abundance (Table 1, Figure 5). Fourteen ABC transporter genes (ABC_2-4, ABC_7-9, ABC_12-19) responded to treatment in both biotypes, but with higher transcription in the resistant biotype. However, two genes (ABC_5 and ABC_10) appeared to have reduced expression in GS plants but increased expression in the GR biotype; and three genes (ABC_1, ABC_6, and ABC_11) had increased transcription only in GR plants with a fold change of 3.1, 3.7, and 8, respectively, by comparing GR- and GS treated biotypes (Figure 5, Table 1). In general, the ABC_2, ABC_5, ABC_7, and ABC_10-12 genes presented the highest expression ratio in the resistant biotype, which varied from 6- to 8-fold (Table 1).

Among the 10 CYP450 genes we report in the present work in response to glyphosate treatment, four (CYP450_1, CYP450_3, CYP450_4, and CYP450_7) had expression suppressed in the GS biotype and increased in GR; three (CYP450_8-10) increased the transcription in both biotypes, although with higher levels in GR; and three (CYP450_2, CYP450_5, and CYP450_6) showed higher expression only in the resistant biotype with a fold change of 3.3, 5.9, and 4.5, respectively, in comparison between biotypes with treatment (Table 2; Figure 6). The CYP450_1 and CYP450_4 presented the highest differences in GR rather than GS in response to treatment, which was 11.5- and 27-fold, respectively (Table 2).

The transcription level of the glutathione-related gene increased only in the GR biotype in response to treatment with a ratio between biotypes of 4.6 (Table 3, Figure 7). The basal GST transcription level in the GR biotype was around 50% less than in GS (Figure 7). The expression levels for the five glycosyltransferase genes increased in both biotypes as a response to glyphosate treatment, although with a higher response in the GR biotype. In this way, the ratio between biotypes was almost doubled in GR rather than GS, except GT_4, which was of 4.8 (Figure 7, Table 3). GT_5 was evaluated in qRT-PCR in a time-course experiment and presented the highest response to treatment at 192 HAT in the GR biotype, while in GS, the expression levels had low variations (Figure 4).

The two catalases had the highest expression after glyphosate treatment in both biotypes among all 41 candidate genes. The CAT_1 gene had >60,000 TPM, while CAT_2 was >7000. Glyphosate-treated GR plants had CAT_1, and CAT_2 increased transcription by 4.8- and 8.5-fold, whereas the GS biotype had increases of 1.2- and 2.3-fold, respectively (Figure 8A,B, Table 4). The comparison between treated biotypes indicates the higher expression levels of CAT_1 and CAT_2 in the resistant biotype at a ratio of 4- and 3.7-fold, respectively (Table 4). With regard to the two differentially expressed peroxidase genes, POD_1 presented a high response to glyphosate treatment in both biotypes, whereas POD_2 had increased expression in the GR biotype and was suppressed in GS plants (Figure 8C,D, Table 4). After glyphosate treatment, POD_1 increased the expression in GR biotype at 11.9-fold, while 4.6-fold in GS (ratio of 2.6). POD_2 expression increased 4.5-fold in GR and reduced at 0.2-fold in GS, presenting a ratio of 22.5 (Table 4). The gene expression related to SOD was higher in the GR biotype than in GS (Figure 8E,F, Table 4). SOD_2 presented

high basal expression in the GR biotype, which reduced ~28% after glyphosate treatment; however, there was 2.7 times higher expression than in GS plants (Figure 8F).

In summary, the candidate gene results indicate that glyphosate resistance is a very complex process of plant defense against glyphosate action involving several molecular changes that allow GR plant survival. The new 41 candidate genes presented in the present study comprise a four-phase of a well-known process of herbicide resistance (oxidation; conjugation; transport; and degradation, detoxification, and protection).

3. Discussion

3.1 The C. bonariensis Transcriptome

The transcriptomic profiles performed up to 288 h after treatment (HAT) will assist in untangling the molecular processes underlying the glyphosate NTSR mechanisms in *C. bonariensis*. The current transcriptome assembly produced a large dataset, which likely includes the major candidate genes involved in glyphosate resistance in *Conyza*. The number of assembled contigs at the gene level in our study is similar in scope to those obtained in another *C. bonariensis* transcriptome study (~81,000) [16].

The gene ontology assignments of the differentially expressed genes (DEGs) showed interesting differences between biotypes. The GR biotype had a higher number of up-regulated genes related to transmembrane transport (biological process) and chloroplast (cellular component) than GS. On the other hand, the GS biotype presented a higher number of down-regulated genes related to these processes than GR (Figure 2; Figure 3). In our study, we focus on up-regulated transcripts in HR hairy fleabane, which indicate the putative importance of the transmembrane transport process mediated by ABC transporters in conferring glyphosate resistance. Upon application, glyphosate first impacts chloroplast biology [31]; therefore, the increase in transcription of genes related to chloroplast function in the GR biotype suggests the plant may be mounting a potential defense to protect plastids against glyphosate's action. These results are in accordance with a study with proteomic analysis that identified chloroplast proteins differentially expressed as the primary sites of GR in *C. canadensis* [32]. Thus, the results of the present study suggest that transmembrane transport and chloroplast proteins might act in association to reduce damages caused by glyphosate action and allow the GR biotype to survive.

The substantially higher number of down-regulated genes related to translation (63 DEGs in GR vs. 16 in GS) and ribosome metabolism (52 DEGs in GR vs. 1 in GS) in GR suggest that in response to glyphosate action, the protein production was reduced in GR (Figure 3). Thus, one coping mechanism evolved in the GR biotype may be induced metabolic slowdown, which ameliorates the plant′ defense against glyphosate movement and damage.

The EPSPS sequence analysis revealed no nucleotide substitutions at Thr 102 and Pro 106 codons and no increase in expression and number of copies in GR plants (Figure S7). Therefore, we conclude glyphosate resistance in the GR biotype is NTSR. Currently, few mutations that confer resistance to glyphosate have been observed in weeds [19]; ergo, glyphosate resistance across weedy plants is also NTSR. Few peptide changes in EPSPS are tolerated without loss of function because of most mutations are lethal [14,16,19]. Our results are in accordance with those related by Hereward et al. (2018) [16].

3.2 Candidate Genes Involved in Glyphosate Resistance in C. bonariensis

A group of well-established NTSR gene families is the cytochrome (CYP450) monooxygenases, glutathione S-transferases (GSTs), and glycosyltransferases (GTs) [20,25,31,33]. These enzyme classes have versatile and multifunctional activities and might catalyze the oxidation and herbicide conjugation to variable substrates [20]. Further, a piece of emerging knowledge about the antioxidant system complementing the glyphosate resistance process has been described [34]. Metabolic-based herbicide resistance, in general, follows a four-phase process: I—oxidation; II—conjugation; III—transport; and IV—degradation, detoxification, and protection [14,20,30,35]. In the present work, we report transcriptional increases of the genes belonging to these four processes in a GR biotype. This increase in transcription of the enzyme-coding gene indicates a potential enhancement of activity, and subsequently glyphosate metabolization to allow GR plants to survive after herbicide exposure. To our knowledge, this is the first report that associated the CYP450, ABC transporters, GT, GST, and antioxidant enzyme differential gene expression to glyphosate resistance in *C. bonariensis*.

In phase I, CYP450 acts to oxidize the herbicide molecules and expose certain functional groups to phase II enzymes [20,25,36]. The P450s catalyze several reactions in plant metabolism, and their role in herbicide conversion is through hydroxylation or dealkylation [14,37,38]. P450s use electrons from NADPH (NADPH-P450 reductase) to insert an oxygen atom in the herbicide molecule, producing a more suitable product to be conjugated to glucose and transported to the vacuole [36]. The CYP450 enzyme has been reported to be involved in herbicide resistance to herbicides belonging to groups of acetanilide, aryloxyphenoxy-propanoate, imidazolinone, phenoxyalkanoic-acid, phenylurea, sulfonamide, sulfonylurea, bentazon, and clomazone herbicides [14,33,37,38]. However, to our knowledge, there is no report associating CYP450 to glyphosate resistance. In the present work, we report the up-regulation of 10 CYP450 genes in the GR biotype followed by glyphosate treatment showing the potential to be involved with GR in *C. bonariensis* (Figure 6, Table 2). Further, the annotated families of the 10 CYP450 contigs in the present work (Table 2) were not described by other authors conferring resistance to herbicide [33,36,37]. According to Powles and Yu (2010) [14], identifying the P450s conferring herbicide resistance in weeds is a vast research frontier, and this group of enzymes may indeed play significant roles in resistance owing to their ability to affect several herbicides modes of action.

In phase II, GSTs and GTs detoxify herbicides through direct conjugation or conjugate it to a wide range of substrates [20,35,39]. GSTs are found in the cytoplasm at high concentrations and work by catalyzing the glutathione conjugation to several substrates, producing a polar product [20,39,40]. In this way, the up-regulation of GST and GT genes in the GR biotype followed by glyphosate treatment in the *C. bonariensis* might indicate the increase in activity of these enzymes to cope with glyphosate (Figure 7). GTs are a huge gene family in which proteins conjugate a sugar to a large variety of lipophilic molecules and herbicides [20,41]. GTs are in the cytoplasm and act by transferring sugar to lipophilic molecules enabling access to membrane transporters, including ABC transporters [41].

In phase III, ABC transporters may direct herbicides to be compartmentalized in the vacuoles or extracellular spaces after the action of CYP450, GSTs, and GTs and thus contribute to herbicide resistance process. ABC transporters are membrane-targeted proteins that require ATP for active transport of substrates across membranes [20,30]. Studies have been reported that ABC transporters are involved in glyphosate resistance in horseweed [23,24,42]. After analysis of the M10 and M11 ABC transporters genes previously reported in the literature as being involved in GR in *C. canadensis* [23], the results of the present study indicate that M10 and M11 genes do not appear to be differentially regulated in hairy fleabane and do not play a role in glyphosate resistance, which is consistent with other studies [16,21,32].

On the other hand, we present 19 new candidate target genes that might be involved in NTSR GR (Figure 5, Table 1). The relatively high number of ABC transporter genes with higher expression in the resistant biotype relative to the sensitive biotype indicate that this gene family may play an important role in the glyphosate-resistance process. Some of them were responsive to treatment in both biotypes, although with higher transcription in the GR biotype. The most interesting ABC genes are ABC_1, ABC_6, and ABC_11, which were only responsive to glyphosate treatment in the resistant biotype with a fold change ratio between biotypes of 3.1, 3.7, and 8, respectively (Figure 5, Table 1). Further, the time-course qRT-PCR results for two ABC transporter contigs indicates that the stimulus for an increase in expression levels might vary among members of this family of the gene at different times after herbicide exposure. For example, the ABC_12 had the highest expression at 24 HAT, while that for ABC_16 occurred at 192 HAT (Figure 4). These results clearly show that the gene transcription responses to glyphosate treatment do not occur simultaneously. A recent study involving ABC transporters genes in horseweed showed the importance of the timing of gene overexpression initiation for the resistance mechanism itself [42]. The same authors described that early overexpression of ABC transporters genes in a short time followed by glyphosate treatment could be more useful to sequester the herbicide to vacuoles or extracellular space. In this case, the delayed overexpression could not offer enough protection. Another transcriptional study with ABC transporter genes showed the influence of the environmental conditions such as glyphosate dosage and time after treatment, light, and temperature on gene induction [43]. Thus, several ABC transporters contigs seem to be gene stress-responsive because of the increase in the gene expression in both GR and GS biotypes. However, some of them presented higher regulation in the GR biotype (ABC_1, ABC_6, and ABC_11) and can be considered as related to the glyphosate-resistance mechanism in *C. bonariensis*.

If HR-associated ABC transporters sequester glyphosate into vacuoles or apoplasts [44], glyphosate would have mitigated early damage to sprayed tissues than in non-sequestered cells, and even more important, this would prevent effective transport to non-sprayed tissues. Indeed, this is likely the major basis of NTSR to glyphosate. In addition, if the transporters can also act in different secondary metabolites produced after glyphosate treatment, their early and late activity might help to ameliorate secondary stress.

In phase IV, the oxidized herbicides by the CYP450 action and subsequently conjugated molecule are degraded in the vacuole or extracellular space, resulting in less phytotoxic molecules [20]. Further, in this stage, the antioxidant system works to scavenge reactive oxygen species (ROS) produced by the glyphosate action.

It is well known that glyphosate inhibits EPSPS to short-circuit the shikimic acid pathway, which results in decreased aromatic amino acid (phenylalanine, tyrosine, and tryptophan) biosynthesis. In so doing, shikimic acid accumulates, which ultimately leads to oxidative stress via ROS production [45]. ROS are highly reactive toxic molecules causing damage to cellular structures and cell death [46]. Further, the lack of tyrosine inhibits the synthesis of plastoquinone, which is an electron acceptor in the photosynthetic electron transport chain in photosystem two (PSII). The non-regeneration of plastoquinone interrupts the electron transport in PSII. Therefore, GS plants die from numerous factors beyond amino acid shortages [31].

Plants have an efficient enzymatic and non-enzymatic antioxidant system in response to ROS production [46,47]. The antioxidant enzyme-coding genes with differential expression in the present study were SOD, CAT, and POD (Figure 8). SOD removes the superoxide ($O_2^{\bullet -}$) through catalyzing its dismutation and providing the first line of defense against ROS [46]. CAT can directly dismutase H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions [47,48]. PODs play an essential role in scavenging ROS and protecting cells in higher plants through scavenging H_2O_2 in water–water and ascorbate-reduced glutathione cycles and utilize ascorbate as the electron donor [47]. The increase in the transcription of the antioxidant enzyme-coding genes SOD, CAT, and POD in resistant plants are in accordance with the previous study we reported for these enzymes' activities; that study was performed using the same *C. bonariensis* as that used in this study [28]. In that study, we demonstrated the higher antioxidant enzyme activity in GR than in GS and less cellular damage after glyphosate treatment. Further, other studies of glyphosate-resistance mechanisms performed in *A. trifida* and *A. palmeri*concluded that antioxidant enzymes have the potential to play a role in the resistance processes [34,49]. Indeed, in the described antioxidant enzyme action, GSTs can also cope with ROS. GSTs are isozymes known to protect the cells against chemical-induced toxicity. These enzymes catalyze the conjugation of GSH to a variety of electrophilic and hydrophobic substrate [50]. Accordingly, the action of the cellular antioxidant machinery is essential to control excess ROS to protect plant cells from oxidative damage and to restore the redox homeostasis.

4. Materials and Methods

4.1 Plant Accessions, Experimental Treatments, and RNA Isolation

Experiments were performed on GR and GS *C. bonariensis* biotypes from Pelotas, the Rio Grande do Sul State, Brazil, 32°04′05.91″ S, 52°52′59.14″ W. The resistance factor was twice determined to be 18.4 [28]. Seeds of each biotype were germinated in plastic trays containing sterilized soil and substrate (Mac plant—Mec Prec, Brazil) 3:1 and watered daily in a greenhouse at 30 °C (day) /20 °C (night) (±4 °C) with a 12-h photoperiod. Thirty days after emergence (30 DAE), seedlings of each biotype were transplanted to plastic pots of 3 L containing soil–substrate mix and kept in the same greenhouse and conditions. Thirty days after transplant (60 DAE; rosette stage—plants 6 to 8 cm in diameter), plants were treated with glyphosate (1480 g ae ha^{-1}—Roundup Original DI 370 g ae L^{-1}; Monsanto) with a CO_2 sprayer and 150 L ha^{-1} of spray volume.

The second and third leaves (from the apex) from untreated and glyphosate-treated plants were collected in a range of time points: GR—untreated, 24, 96, 192, and 288 h after treatment; GS—untreated, 24, 96, and 192 h after treatment (Figure 9). Leaves from three biological replicates were collected at each time from a total of 27 plants (Figure 9). After collection, leaves were immediately frozen in liquid nitrogen and stored at −80 °C. The times to leaf collection and RNA extraction were determined based on a shikimic acid transient accumulation in the GR biotype, which increased substantially at 24 h after treatment, peaked at

96 h, and at 288 h presented a similar content to that of the untreated plants [28] (Figure S9). By contrast, the GS biotype had similar initial shikimic acid levels as GR plants at 24 h and 96 h, but it peaked at 192 h after treatment and died after that timepoint.

Total RNA was extracted from leaves from 27 plant samples using Trizol reagent (Invitrogen, Carlsbad, Calif, USA) in accordance with the manufacturer's protocol (Figure 9). In the total RNA, an additional treatment with RNase-free DNase I (Invitrogen) was performed to remove residual genomic DNA. The RNA samples from each respective biotype and treatment (untreated and glyphosate treated) were pooled to form three technical replicates per treatment totaling 12 samples, i.e., GR untreated (GR t0), GS untreated (GS t0), GR treated (GR t1), and GS treated (GS t1) (Figure 9).

4.2 cDNA Library Construction and Illumina Sequencing

Before cDNA construction, the RNA integrity number (RIN) was measured in Bioanalyzer (Agilent Bioanalyzer 2100 system, Agilent Technologies, Santa Clara, Calif, USA). The library preparation of 12 cDNA samples and Illumina sequencing were performed at the Laboratory of Functional Genomics Applied to Agriculture and Agri-Energy, University of São Paulo (USP), São Paulo, SP, Brazil. The Illumina TruSeq Stranded mRNA LT Sample Prep Protocol was used to cDNA libraries construction. The 12 libraries were sequenced using the HiSeq Flow Cell v4, with the Illumina HiSeq 2500, producing 125 bp paired-end reads (2×).

4.3 De Novo Assembly and Functional Annotation

Raw data were filtered using FastQC (https://www.bioinformatics.babraham.ac.uk/) and trimmed adapter by Trimmomatic [51]. A single *de novo* assembly was generated using the reads from all treatments (12 libraries) with Trinity for further differential expression analysis [52]. For differential expression analysis, it is essential that all treatments be included in the transcriptome assembly [53,54]. Further, a single *de novo* assembly for each biotype (GR and GS) was generated for EPSPS sequence alignment. Trinity was executed using default settings in all assemblies and additional assembly analysis performed using the BioPython package [55]. The assembled transcripts generated by Trinity were aligned to the UniProt-trEMBL [56] database using Diamond [57], and only those with hits on plants (E-value threshold = 1×10^{-10}) were selected for further analysis.

The protein prediction was performed based on amino acid sequence using the Trinotate pipeline (https://trinotate.github.io/), which included the identification of protein families from the Pfam [57] and UniProt-SwissProt database, the identification of signal peptides and transmembrane proteins by SignalP and TMHMM, respectively [58], and rRNA prediction by RNAmmer [59]. The gene ontology functional categorization was performed by the BLASTx hits [60,61] with an E-value threshold of $<10^{-5}$ from the non-redundant database according to molecular function, biological process, and cellular component ontologies.

4.4 Differential Expression Analysis

For differential expression analysis sections, we termed the assembled contig as "gene". After data normalization, gene expression levels were calculated using transcript reads per million mapped reads (TPM) ≥1. For each treatment, gene-level expression was estimated using the Kallisto method [62] implemented in the Trinity accessory, which was used to generate an expression matrix. The matrix was processed in the edgeR mode [63] to perform the differential gene expression analysis and GO binning and enrichment was performed [63].

Differentially expressed genes (DEGs) were analyzed within each biotype with (t1) and without (t0) glyphosate treatment. DEGs were also filtered based on the false discovery rate (FDR) and *p*-value threshold at ≤0.001. After that, lists of all filtered DEGs were exported for each comparison and MA plots produced. In that case, DEGs with a log fold change ≥2 was considered up-regulated and ≤–2 as down-regulated. The up- and down-regulated genes of both biotypes for t1 and t0 treatments were categorized according to their GO functions using the same methods as those described above.

4.5 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Analysis to Validate RNA-Seq Results

The same RNA samples from above were as converted into cDNA using RevertAid First Strand cDNA Synthesis (Thermo Fisher Scientific™), following the manufacturer's protocol. The cDNA was pooled according to each biotype and time of collection after glyphosate treatment, resulting in 9 cDNA samples, and the qRT-PCR experiments performed using three technical replicates in a QuantStudio6™ (Applied Biosystems™). The reaction mixture of 15 µL contained 7.5 µL SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific™), 1 µL of 1:3 diluted cDNA, 0.3 µL of each primer (forward and reverse) (10 µM), and the final volume was adjusted with appropriate amounts of RNAse free water. The QuantStudio6™ thermal method was a multi-step of 10 min at 95 °C for pre-denaturation, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C (2×). Data were collected during the extension step, and threshold-cycle (CT) values were calculated for each reaction using the Second Derivative Maximum method in the QuantStudio Real-Time PCR software (Applied Biosystems™).

For control reactions, no cDNA sample was added to the reaction mix of each studied primer. The sequence annotated as hexosyltransferase was used as an internal control (Contig: Trinity_DN_28781—F: CAATGGCCAGTCAAAACCAT; R: CCAGGCTCCATCCTATCGT A). The hexosyltransferase presented the best stability results among eleven candidate internal control genes tested, which included heat shock–protein and actin previously reported for *C. bonariensis* and *C. canadensis*, respectively [21,23].

Nineteen contigs with relative expression were selected from a transcriptome dataset based on the results of TPM of treated rather than untreated biotypes (GR—t1/t0; GS—t1/t0). In this case, in each respective replicate, TPM values >2 were considered as relative expression and ≤2 as absolute. Contigs with absolute expression were not considered for further analysis. Contigs with a low, medium and high expression based on log fold change (logFC—t1/t0) results were selected from differential expression analysis to further analysis in qRT-PCR. The relative expression was calculated using the equation QR = $2^{-(\Delta\Delta CT)}$ [64]. The fold change results from transcriptome dataset were correlated to the time-course average of those from qRT-PCR using the Pearson model (r). The real-time results were subjected to ANOVA and averages compared by orthogonal contrasts at $p \leq 0.05$ [64].

4.6 EPSPS Transcript Sequence Analysis

The sequences of the three EPSPS gene copies for *C. bonariensis* were obtained from GenBank (EPSPS1–EF200070; EPSPS2–EF200069; EPSPS3–EF200074) and mapped into the individual assembled transcriptomes of GR and GS using BLASTn command-line to identify their respective contigs. Trinity-assembled and GenBank sequences of EPSPS copies were translated to amino acids and aligned into BioEdit (http://www.mbio.ncsu.edu/BioEdit/) using the ClustalW multiple alignment functions with default settings. The alignment was performed to verify the occurrence of amino acid substitution at Threonine 102 and Proline 106, commonly altered reported regions to confer resistance to glyphosate [16,19]. Hence, the assembled EPSPS contigs were analyzed for transcription levels (TPM) to verify the overexpression occurrence in GR and GS in response to glyphosate treatment and copy number.

4.7 Selection of Similar Contig to Transport and Metabolic Glyphosate-Resistance Coding Gene

DEGs were selected by their UniProt/SwissProt assignment to gene families related to known roles in transportation and metabolic herbicide resistance [20,30]. We selected gene families of ABC transporters that were hypothesized to be involved in NTSR in *C. canadensis* [23], and cytochrome P450 (CYP450), glutathione (GST), glycosyltransferase (GT), related to be involved in herbicide metabolism [20,30,33]. Further, we selected DEGs annotated as antioxidant enzymes catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) reported with differential activities and to be involved in the reactive oxygen species (ROS) scavenging after glyphosate exposure in the same biotypes of *C. bonariensis* of the present study [28]. The antioxidant enzymes were also described as being involved in glyphosate resistance in *Amaranthus palmeri* and *Ambrosia trifida* [34,49].

The candidate genes were chosen according to the Venn diagram results from up-regulated genes in GR t0 (81 genes) and t1 (763 genes) and co-expressed in GR and GS plants (974 genes) in response to glyphosate treatment (Figure 1). The query DEGs were selected based on expression differences between

GR (t1/t0) and GS (t1/t0) biotypes in response to glyphosate treatment (fold change ratio GR/GS). In this case, DEGs with a GR (t1/t0)/GS (t1/t0) ratio ≥2 were selected, except for the two SOD genes because they had a much higher basal expression in GR relative to GS plants. Finally, each gene sequence was searched against the UniProt/SwissProt database (www.uniprot.org/blast/) to assign a putative function.

We obtained from Peng et al. (2010) [23] the sequences of ABC transporters M10 and M11 that were hypothesized to be implicated in NTSR to glyphosate in horseweed. These sequences were mapped in our whole *C. bonariensis* transcriptome using BLASTn, and after, their expression levels were evaluated using the TPM results for GR and GS plants in response to glyphosate treatment.

5. Conclusions

The present transcriptomic study revealed 41 new candidate NTSR genes that are annotated to be related to transport and metabolism in herbicide resistance. Among these candidates, there were 19 ABC transporters, 10 CYP450, one glutathione, and five glycosyltransferases. In addition, we also report the transcription results of two genes coding for antioxidant enzyme catalase, peroxidase, and superoxide dismutase. Thus, all target genes from different groups with a transcriptional increase in the glyphosate-resistant biotype might be acting in association to confer resistance to glyphosate in *C. bonariensis*. Further, these results indicate that gene expression in *C. bonariensis* varies among gene groups and within the same group, between biotypes, in response to glyphosate treatment and is dependent on the time after herbicide exposure. The present transcriptome study is the first report that associates various CYP450, ABC transporters, GT, GST, and antioxidant enzyme differential gene expression to glyphosate resistance in *C. bonariensis.* The results of the present work will serve as a data resource for further studies on the molecular mechanisms of glyphosate resistance in *C. bonariensis.* Further studies will involve functional genomic analysis using the protoplasts and gene editing approaches.

Supplementary Materials

The following are available online at https://www.mdpi.com/2223-7747/8/6/157/s1, Figure S1. Length distribution of transcripts assembled from transcriptome libraries of *C. bonariensis*. Figure S2. Sequence comparison to other organisms from the distribution of BLASTx hits (e-value < 1×10^{-10}) against the non-redundant protein database of the National Center for Biotechnology Information. Figure S3. Sequence comparison to other plants (hit ≥1%) from the distribution of BLASTx hits (e-value < 1×10^{-10}) against the non-redundant protein database of the National Center for Biotechnology Information (NCBI). Figure S4. Top ten Gene Ontology (GO) terms identified in the *C. bonariensis* transcriptome assembly summarized in three main categories: (A) biological process, (B) molecular function, and (C) cellular component. Figure S5. MA plot of differential expression analysis generated by EdgeR from transcriptome study performed in *C. bonariensis* glyphosate-resistant (GR) and -sensitive (GS) biotypes in response to glyphosate treatment. Dots above zero are up-regulated, and dots below are down-regulated. Red dots indicate significant expression at an adjusted *p*-value and false discovery rate (FDR) threshold set at ≤0.001, and log2FC ≥ 2 (up-regulated) or ≤log2FC (down-regulated). Plots for each contig its log2FC (fold change) (A, Y-axis) vs. its counts (mean of normalized counts) (M, X-axis). t0 = without glyphosate treatment; t1 = with glyphosate treatment. GR t1: RNA sampled at 24, 96, 192, and 288 h after treatment and pooled; GS t1: RNA sampled at 24, 96, and 192 h after treatment and pooled. Figure S6. Correlation of transcriptomic and qRT-PCR (average of time-course) expression levels results of 19 genes of glyphosate-resistant (GR) and -sensitive (GS) *C. bonariensis* biotypes. Figure S7. Partial sequence alignment of the EPSPS transcripts and amino acid sequence assembled of glyphosate-resistant (GR) and -sensitive (GS), and a sensitive *C. bonariensis* sequence from GenBank. (A) EPSPS1 (GenBank—accession number EF200070); (B) EPSPS2 (GenBank—accession number EF200069); (C) EPSPS3 (GenBank—accession number EF200074). The red boxed amino acids show no substitution at positions Threonine 102 and Proline 106. (D) Transcriptome expression levels (transcript reads per million mapped reads—TPM) of the three EPSPS copies in GR and GS in response to glyphosate treatment and expression difference (Fold change). GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Figure S8. *C. bonariensis* transcriptome expression analysis (transcript reads per million mapped reads—TPM) of the M10 and M11 ABC transporters reported being involved in glyphosate resistance in *C. canadensis* by Peng et al. (2010). M10_c1: Score (Bits) 2682, *E*-value zero; M10_c2: Score 608, *E*-value 1×10^{-72}; M11_c1: Score 2669, *E*-value zero;

M11_c2:1232, *E*-value zero. GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Contigs were filtered according to the *p*-value and false discovery rate (FDR) threshold set at ≤0.001. Intervals indicate the standard error. Figure S9. Responses of *C. bonariensis* glyphosate-resistant (GR) and -sensitive (GS) biotypes to glyphosate treatment (1480 g ae ha^{-1}). (A) The shikimic acid content in a time-course experiment; and (B) plants status at 288 h after glyphosate treatment (12 days). GS plants died 192 h after treatment, whereas GR plants survived. Leaf samples were collected for RNA extraction based on the shikimic acid content curve in GR biotype. Then RNA was extracted from untreated plants in GR and GS biotypes and treated GR plants at 24, 96, 192, and 288 h after treatment, and at 24, 96, and 192 h in the GS plants, which died 192 h after treatment. Figure S10. Evolutionary analysis of the candidate contigs sequences similarity to be related to glyphosate-resistance in *C. bonariensis* obtained from a transcriptome. (A) ABC transporters; (B) cytochrome P450 (CYP450). The number in front of the contig description indicate the order presented in each respective table 1 and 2. ABCC5A *Arabidopsis*: ABC Transporter full sequence obtained from GenBank *Arabidopsis thaliana*; M10 and M11 ABC: sequences of ABC transporters obtained from Peng et al. (2010). The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Supplementary Sequences: .docx file.

Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

The RNA-Seq data generated in this study have been uploaded into the NCBI-SRA database under bio project PRJNA436902 and the accession numbers SRA: SRS3018834-SRS3018845. Available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA436902/.

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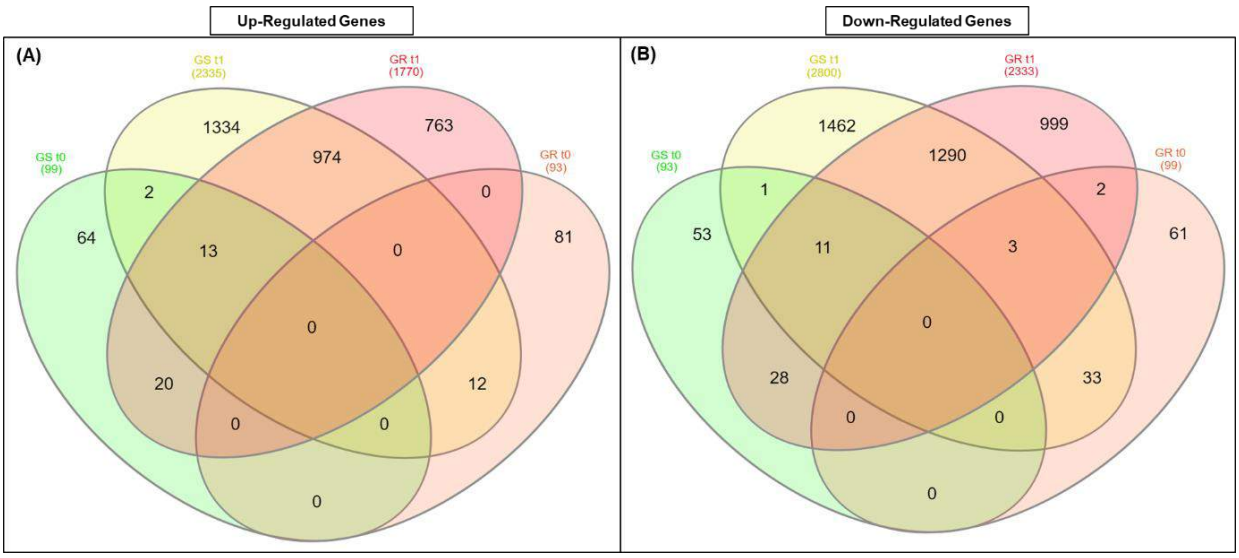

Figure 1. The differential expression results in a Venn diagram from transcriptome analysis in glyphosate-resistant (GR) and -sensitive (GS) biotypes of *C. bonariensis* in response to glyphosate treatment. (A) Up-regulated; (B) Down-regulated. t0: without glyphosate treatment; t1: with glyphosate treatment. Genes were filtered according to relative expression (counts number), *p-value* and false discovery rate (FDR) set at ≤ 0.001.

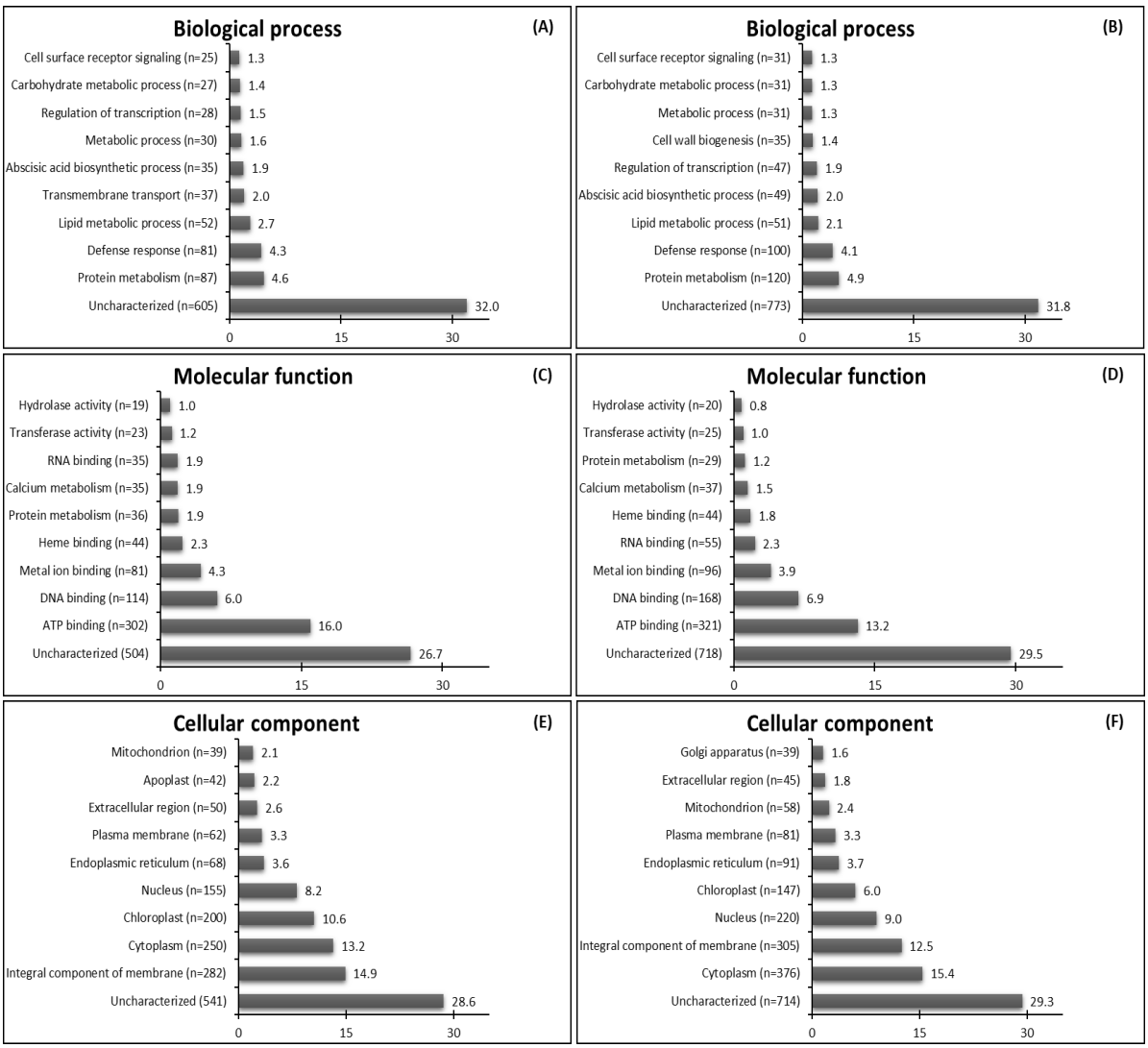

Figure 2. Summary of top ten gene ontology (GO) terms identified as up-regulated genes (DEGs-up) in glyphosate-resistant and -sensitive *C. bonariensis* biotypes. Annotated sequences were classified into the biological process, molecular function, and cellular component. (A, C, and E) GR – up-regulated DEGs, n = 1,770; (B, D, and F) GS – up-regulated DEGs, n = 2,335.

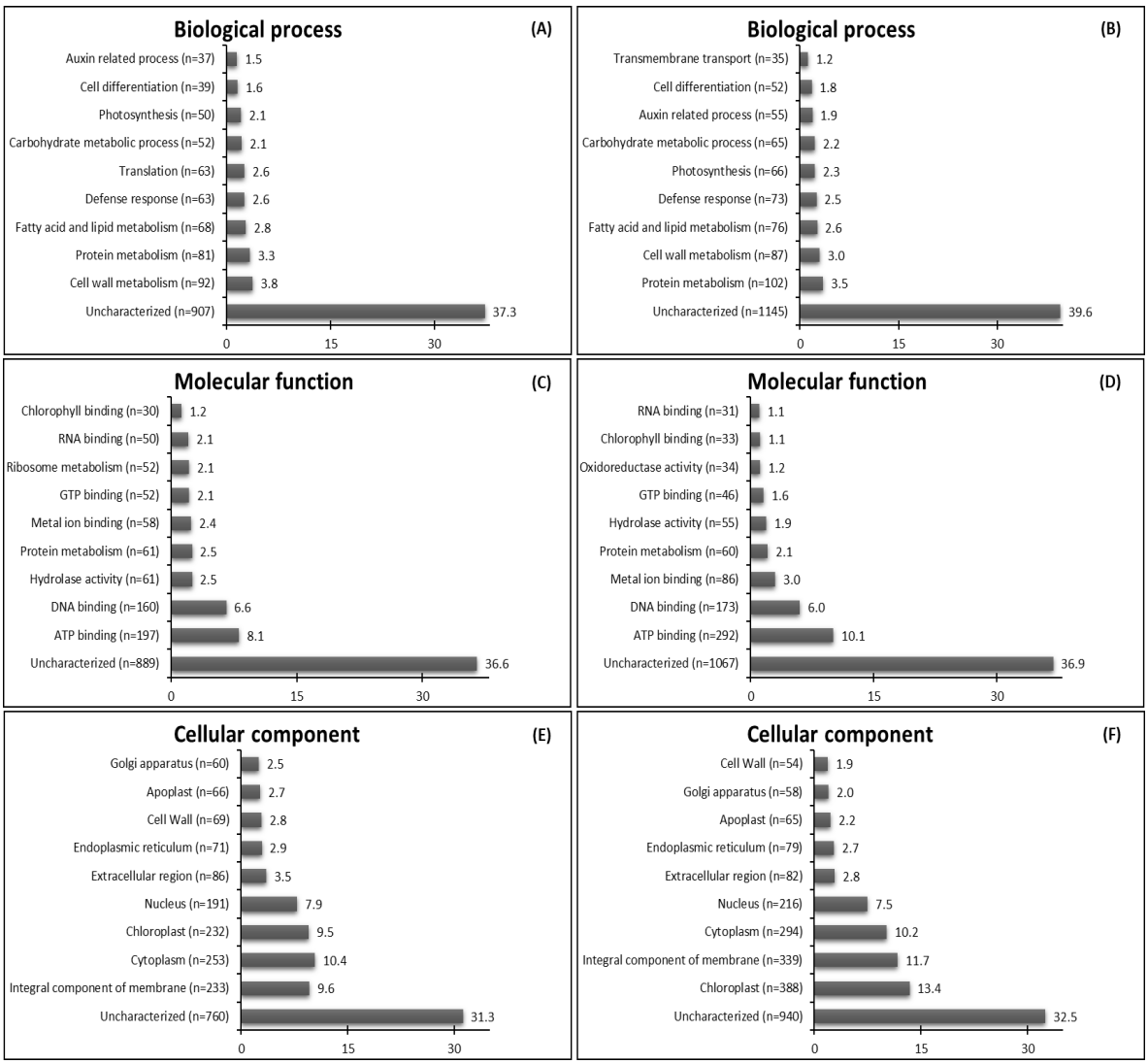

Figure 3. Summary of top ten gene ontology (GO) terms identified as down-regulated genes (DEGs-down) in glyphosate-resistant and -sensitive *C. bonariensis* biotypes. Annotated sequences were classified into the biological process, molecular function, and cellular component. (A, C, and E) GR – down-regulated DEGs, n = 2,333; (B, D, and F) GS – down-regulated DEGs, n = 2,800.

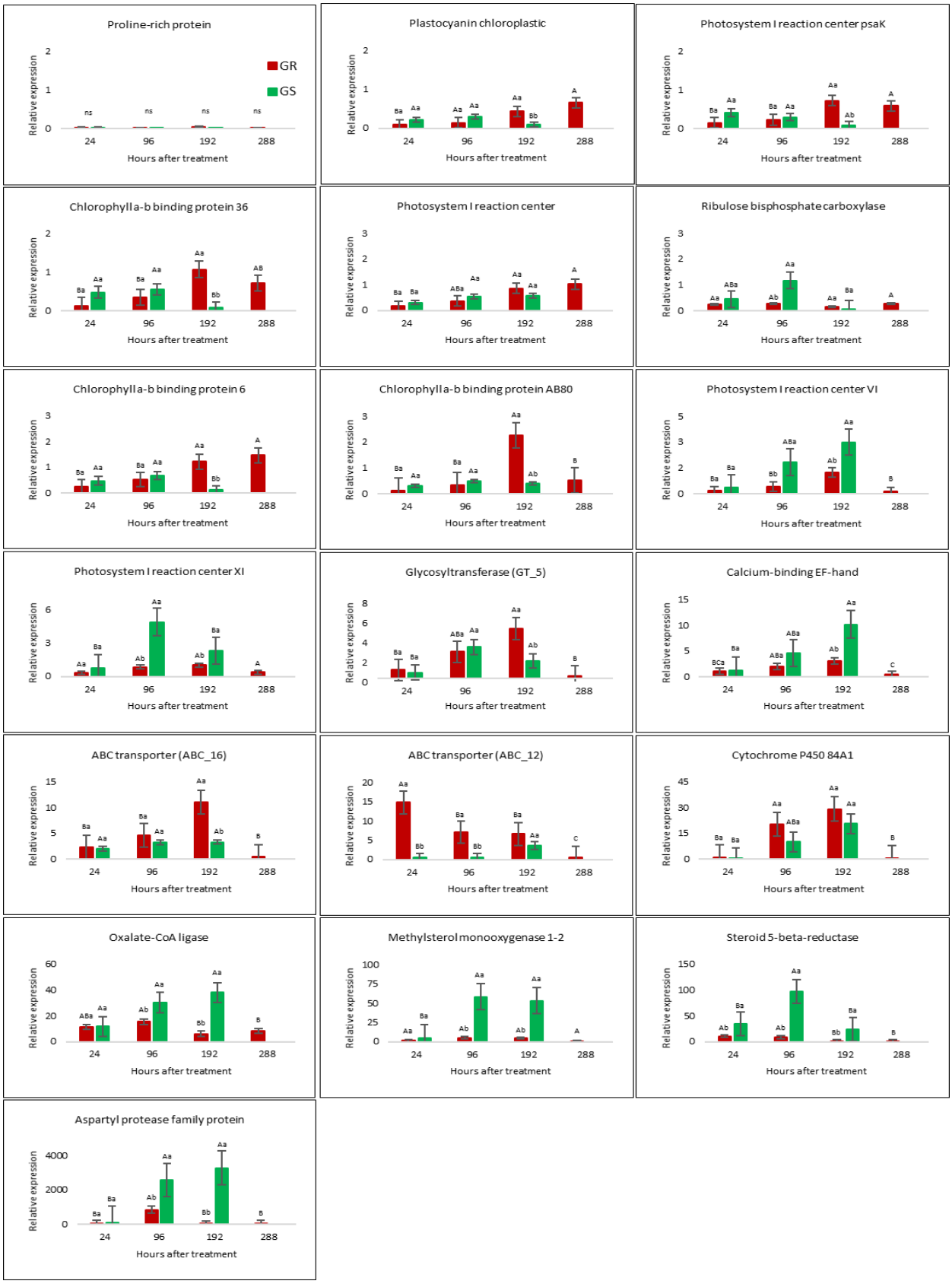

Figure 4. Relative expression levels of 19 selected genes in leaves of *C. bonariensis* glyphosate-resistant (GR – red bars) and -sensitive (GS – green bars) biotypes in a time-course experiment after glyphosate treatment relative to an internal control hexosyltransferase gene expression in the untreated plants using real-time qRT-PCR ($2^{-\Delta\Delta Ct}$). Results were compared by contrasts ($p \leq 0.05$), and upper-case letters indicate comparisons of each biotype in different times and lower-case letters between biotypes in each respective time.

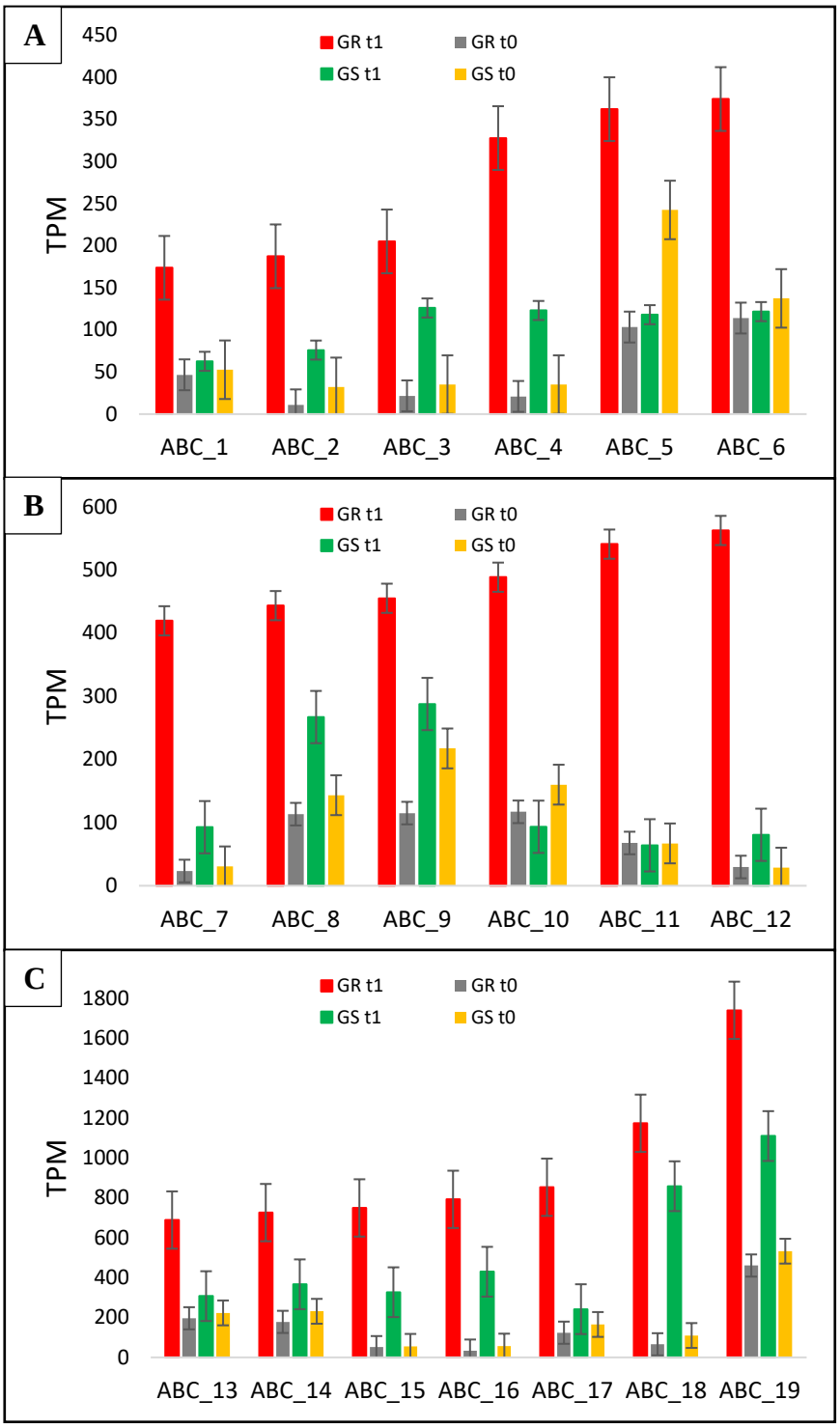

Figure 5. *C. bonariensis* transcriptome expression analysis (transcript reads per million mapped reads - TPM) of the 19 ABC transporters genes (1 to 19). GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Intervals indicate the standard error. In ABC_12 (ABC transporter C family member 14) and ABC_14 (ABC transporter C family member 9) was performed the qRT-PCR analysis (see Figure 4).

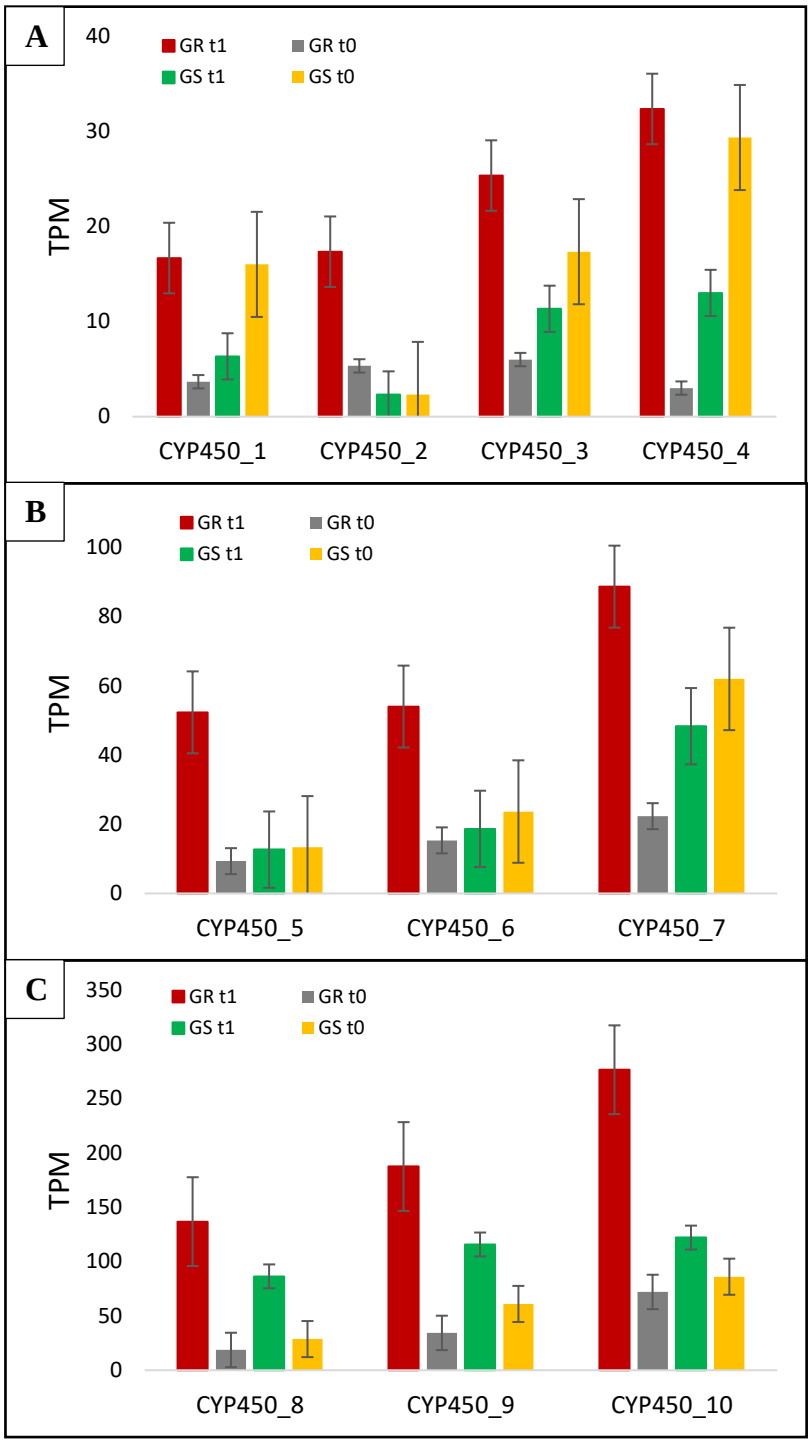

Figure 6. *C. bonariensis* transcriptome expression analysis (transcript reads per million mapped reads - TPM) of the 10 CYP450 genes (1 to 10). GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Intervals indicate the standard error.

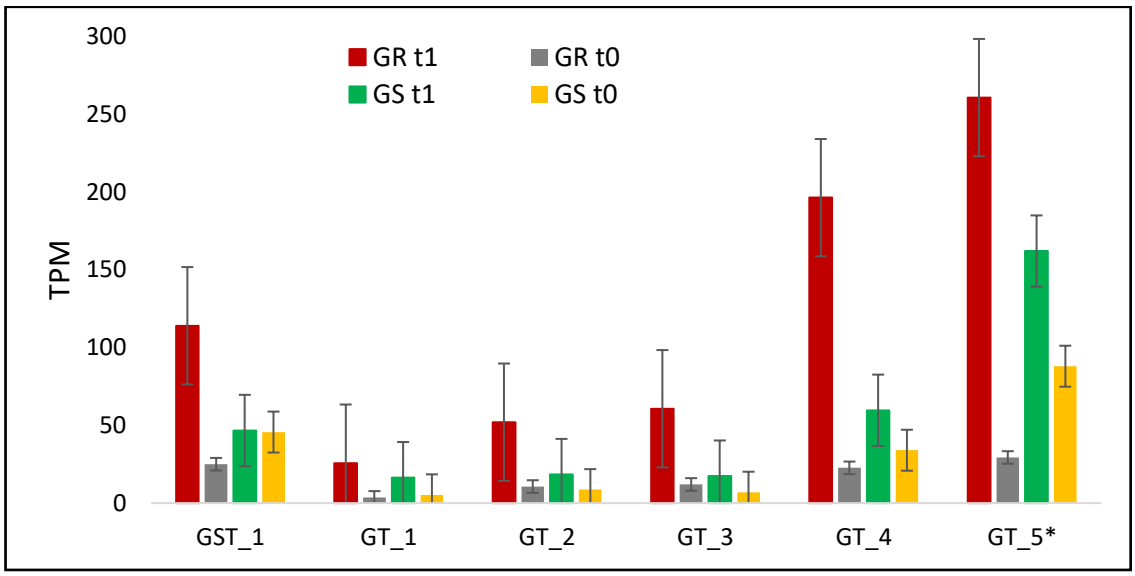

Figure 7. *C. bonariensis* transcriptome expression analysis (transcript reads per million mapped reads - TPM) of glutathione (GST) and five glycosyltransferases (GT) genes. GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. *Indicate that was performed qRT-PCR analysis in that specific contig (See Figure 4). Intervals indicate the standard error.

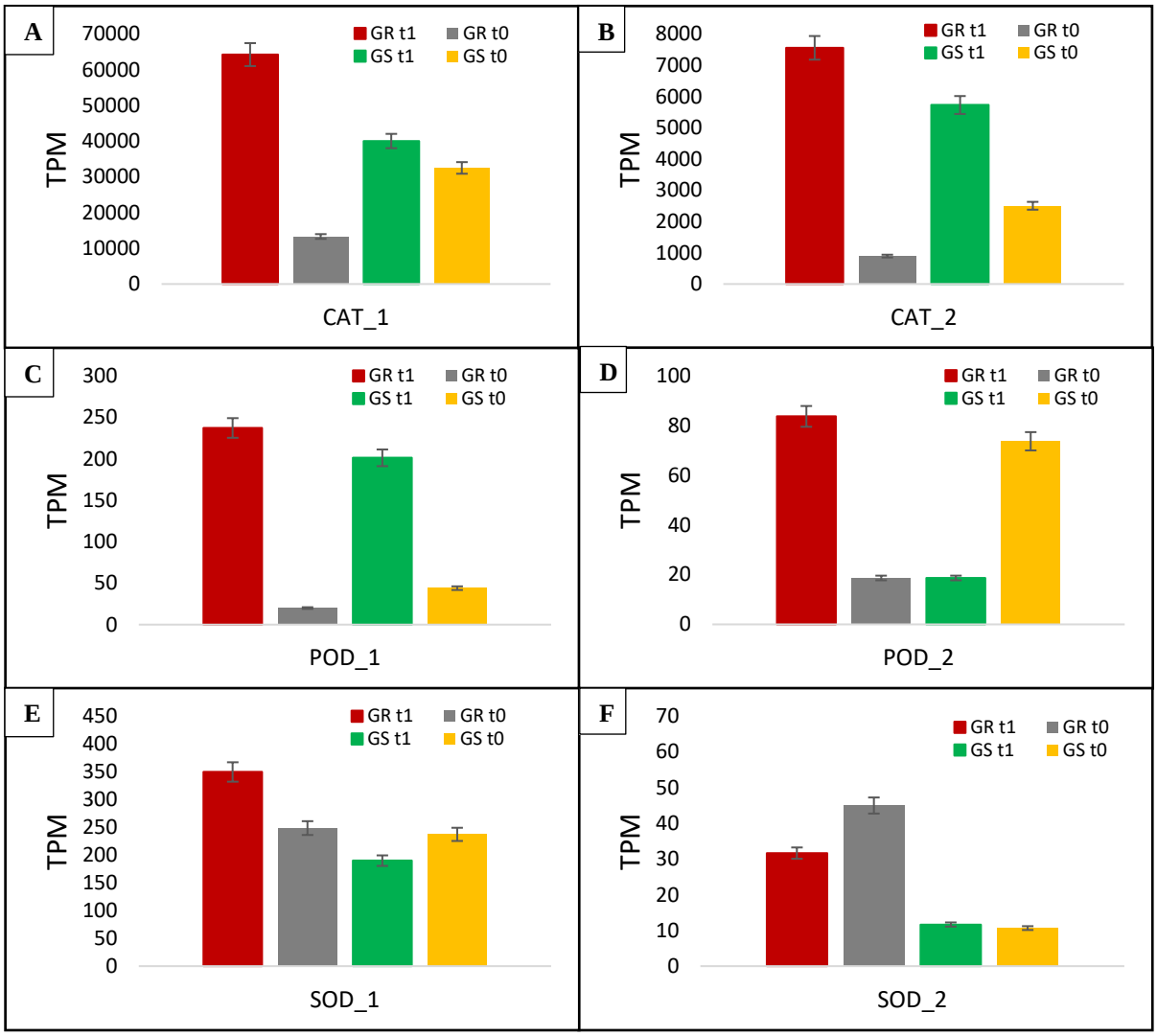

Figure 8. *C. bonariensis* transcriptome expression analysis (transcript reads per million mapped reads - TPM) of a two catalase (CAT – A and B), Peroxidase (POD – C and D), and superoxide dismutase (SOD – E, and F) genes. GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Intervals indicate the standard error.

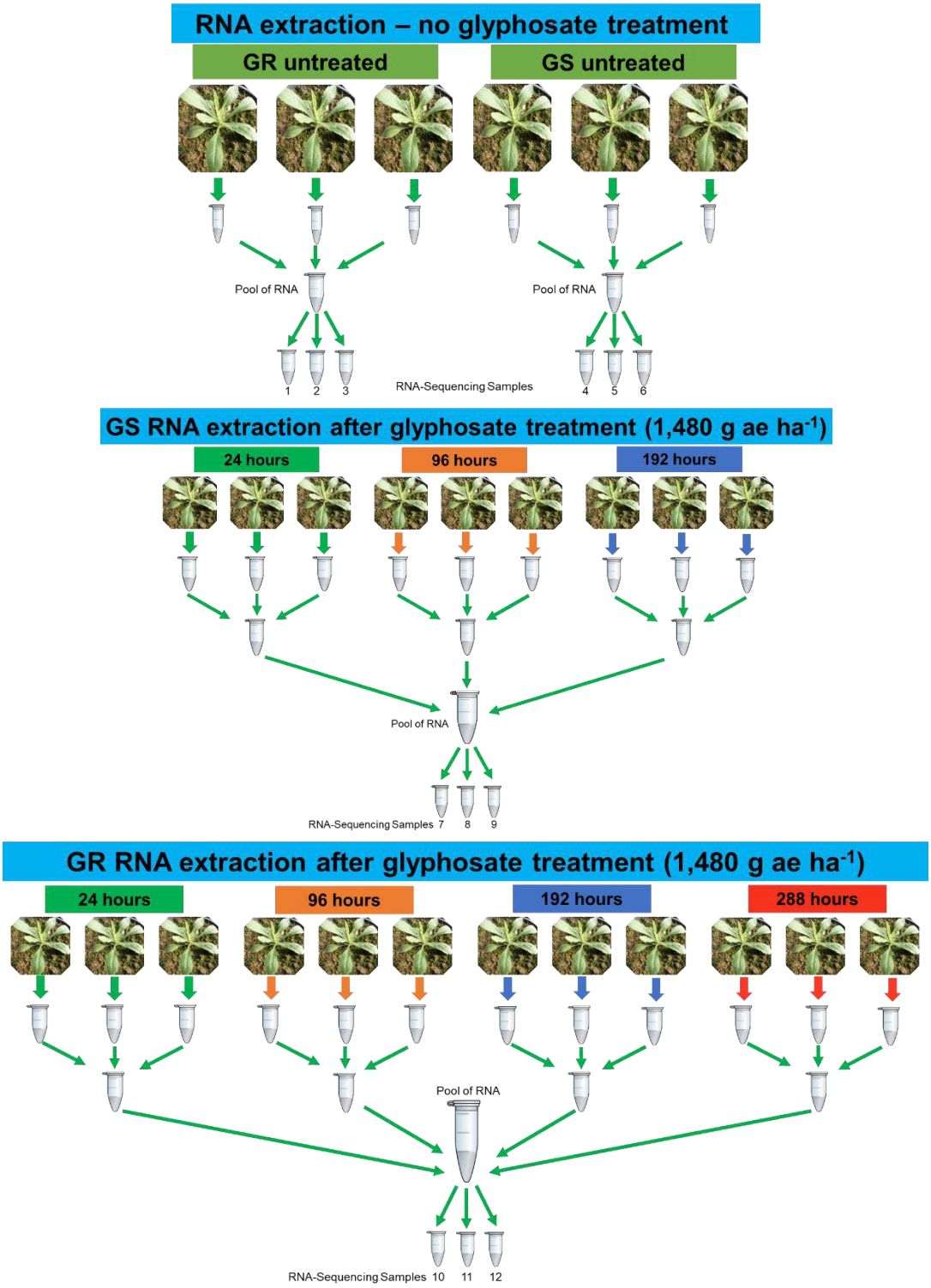

Figure 9. Experimental design for RNA extraction and RNA-Seq experiment in Conyza bonariensis glyphosate-resistant (GR) and -sensitive (GS) biotypes with (t1) and without (t0) glyphosate treatment. RNA samples 1-3: GR t0 - untreated; RNA samples 4-6: GS t0 - untreated; RNA samples 7-9: GS t1 – RNA samples extracted at 24, 96, and 192 hours after glyphosate treatment; RNA samples 10-12: GR t1 – RNA extracted at 24, 96, 192, and 288 hours after glyphosate treatment. RNA was extracted from a total of 27 plants and pooled according to each treatment. Three technical replicates were sequenced from each treatment (GR t0, GS t0, GS t1, and GR t1 - a total of 12 libraries). Glyphosate rate of 1,480 g ae ha^{-1}

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Table 1. Differential expression of ABC transporter genes via RNA-Seq analysis of glyphosate-resistant (GR) and –sensitive (GS) *Conyza bonariensis* biotypes in response to glyphosate treatment up to 288 hours after treatment. The transcriptional fold change data for the GR and GS biotypes are each expressed as the ratio of the ABC transporter gene expression from glyphosate treated to untreated individuals. Therefore, a value of 1 means the gene was neither induced nor repressed. The rightmost column represents the ratio of ratios; i.e., the magnitude of the effect of being glyphosate resistant.

Nº	Contig ID	UniProt ID	Functional Category	Transcriptional Fold Change		Ratio
				GR	GS	GR/GS
ABC_1	TRINITY_DN38339_c0_g1	Q9SKX0	ABC transporter C family member 13	3.7	1.2	3.1
ABC_2	TRINITY_DN41040_c1_g2	Q8LGU1	ABC transporter C family member 8	17	2.4	7.1
ABC_3	TRINITY_DN27993_c1_g1	Q7DM58	ABC transporter C family member 4	9.5	3.6	2.6
ABC_4	TRINITY_DN37018_c0_g1	Q9C8H0	ABC transporter C family member 12	15.6	3.5	4.5
ABC_5	TRINITY_DN25809_c0_g1	Q8LGU1	ABC transporter C family member 8	3.5	0.5	7
ABC_6	TRINITY_DN38603_c0_g1	Q9ZR72	ABC transporter B family member 1	3.3	0.9	3.7
ABC_7	TRINITY_DN41040_c1_g1	Q8LGU1	ABC transporter C family member 8	18	3	6
ABC_8	TRINITY_DN42180_c1_g3	Q7DM58	ABC transporter C family member 4	3.9	1.9	2
ABC_9	TRINITY_DN41654_c6_g1	Q9ZU35	ABC transporter G family member 7	4	1.3	3.1
ABC_10	TRINITY_DN23566_c0_g1	Q8LGU1	ABC transporter C family member 8	4.2	0.6	7
ABC_11	TRINITY_DN42180_c1_g1	Q7DM58	ABC transporter C family member 4	8	1	8
ABC_12	TRINITY_DN32993_c0_g1	Q7DM58	ABC transporter C family member 14**	19	2.8	6.8
ABC_13	TRINITY_DN41851_c0_g1	Q9ZR72	ABC transporter B family member 1	3.5	1.4	2.5
ABC_14	TRINITY_DN37243_c0_g1	Q9C9W0	ABC transporter I family member 17	4.1	1.6	2.6
ABC_15	TRINITY_DN37746_c1_g1	Q9FNU2	ABC transporter B family member 27	14.6	5.9	2.5
ABC_16	TRINITY_DN41980_c0_g1	Q9M1C7	ABC transporter C family member 9**	23.3	7.6	3.1
ABC_17	TRINITY_DN29514_c0_g1	Q9FNU2	ABC transporter B family member 25	6.9	1.5	4.6
ABC_18	TRINITY_DN41405_c0_g1	Q9LYS2	ABC transporter C family member 10	17.9	7.8	2.3
ABC_19	TRINITY_DN42119_c0_g1	Q42093	ABC transporter C family member 2	3.8	1	3.8

Differentially expressed genes were selected using a *p-value* and FDR threshold set at ≤ 0.001; **indicates that the gene was subjected to qRT-PCR analysis (See Figure 4).

Table 2. Differential expression of Cytochrome P450 (CYP450) genes via RNA-Seq analysis of glyphosate-resistant (GR) and –sensitive (GS) *Conyza bonariensis* biotypes in response to glyphosate treatment up to 288 hours after treatment. The transcriptional fold change data for the GR and GS biotypes are each expressed as the ratio of the ABC transporter gene expression from glyphosate treated to untreated individuals. Therefore, a value of 1 means the gene was neither induced nor repressed. The rightmost column represents the ratio of ratios; i.e., the magnitude of the effect of being glyphosate resistant.

Nº	Contig ID	UniProt ID	Functional Annotation	Transcriptional Fold Change		Ratio
				GR	GS	GR/GS
CYP450_1	TRINITY_DN41388_c1_g1	O49858	Cytochrome P450 82A3	4.5	0.4	11.5
CYP450_2	TRINITY_DN27559_c0_g1	Q9STK7	Cytochrome P450 71A26	3.3	1.0	3.3
CYP450_3	TRINITY_DN22805_c0_g1	A0A103YCT3	Cytochrome P450	4.2	0.7	6.0
CYP450_4	TRINITY_DN35527_c1_g1	A0A103XYM0	Cytochrome P450 78A5	10.8	0.4	27
CYP450_5	TRINITY_DN15853_c0_g1	A0A251TZE7	Cytochrome P450 71B7	5.6	1.0	5.6
CYP450_6	TRINITY_DN34846_c0_g1	Q9LMX7	Cytochrome P450 78A5	3.5	0.8	4.5
CYP450_7	TRINITY_DN35866_c0_g1	A0A251SVC5	Cytochrome P450 76C2	4.0	0.8	5.0
CYP450_8	TRINITY_DN25141_c0_g1	O81973	Cytochrome P450 93A3	7.3	3.0	2.4
CYP450_9	TRINITY_DN31873_c0_g1	A0A103YMI2	Cytochrome P450 94A1	5.5	1.9	2.9
CYP450_10	TRINITY_DN73328_c0_g1	A0A251RP29	Cytochrome P450 82A4	3.8	1.4	2.7

Differentially expressed genes were selected using a *p-value* and FDR threshold set at ≤ 0.001.

Table 3. Differential expression of Glutathione (GST) and Glycosyltransferase (GT) genes via RNA-Seq analysis of glyphosate-resistant (GR) and –sensitive (GS) *Conyza bonariensis* biotypes in response to glyphosate treatment up to 288 hours after treatment. The transcriptional fold change data for the GR and GS biotypes are each expressed as the ratio of the ABC transporter gene expression from glyphosate treated to untreated individuals. Therefore, a value of 1 means the gene was neither induced nor repressed. The rightmost column represents the ratio of ratios; i.e., the magnitude of the effect of being glyphosate resistant.

Nº	Contig ID	UniProt ID	Functional Annotation	Transcription Fold Change		Ratio
				GR	GS	GR/GS
GST_1	TRINITY_DN34237_c0_g1	Q9M0G0	Glutathione hydrolase 3	4.6	1	4.6
GT_1	TRINITY_DN32482_c0_g1	Q6VAA9	Glycosyltransferase 73E1 UDP	7	3.1	2.2
GT_2	TRINITY_DN30716_c0_g1	Q9C768	Glycosyltransferase 76B1 UDP	4.9	2.1	2.3
GT_3	TRINITY_DN44583_c0_g1	Q6VAB0	Glycosyltransferase 85C2 UDP	5.1	2.5	2
GT_4	TRINITY_DN30626_c0_g1	Q9LZD8	Glycosyltransferase 89A UDP	8.7	1.8	4.8
GT_5	TRINITY_DN23238_c0_g1	Q6VAA4	Glycosyltransferase 85C1 UDP**	13.4	6.8	2

Differentially expressed genes were selected using a *p-value* and FDR threshold set at ≤ 0.001; t0 = without glyphosate treatment; t1= with glyphosate treatment; GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. **indicates that the gene was subjected to qRT-PCR analysis (See Figure 4).

Table 4. Differential expression of Catalase (CAT), Peroxidase (POD), and Superoxide Dismutase (SOD) genes via RNA-Seq analysis of glyphosate-resistant (GR) and –sensitive (GS) *Conyza bonariensis* biotypes in response to glyphosate treatment up to 288 hours after treatment. The transcriptional fold change data for the GR and GS biotypes are each expressed as the ratio of the ABC transporter gene expression from glyphosate treated to untreated individuals. Therefore, a value of 1 means the gene was neither induced nor repressed. The rightmost column represents the ratio of ratios; i.e., the magnitude of the effect of being glyphosate resistant.

Nº	Contig ID	UniProt ID	Functional Annotation	Transcription Fold Change		Ratio
				GR	GS	GR/GS
CAT_1	TRINITY_DN41650_c0_g1	P45739	Catalase	4.8	1.2	4
CAT_2	TRINITY_DN32938_c3_g1	P29756	Catalase-1/2	8.5	2.3	3.7
POD_1	TRINITY_DN35984_c0_g1	Q9SZB9	Peroxidase 47	11.9	4.6	2.6
POD_2	TRINITY_DN34083_c0_g1	Q9SJZ2	Peroxidase 17	4.5	0.2	22.5
SOD_1	TRINITY_DN36049_c1_g1	Q9FMX0	Superoxide dismutase 3	1.4	0.8	1.7
SOD_2	TRINITY_DN32389_c0_g1	O04996	Superoxide dismutase	0.7	1.1	0.6

Differentially expressed genes were selected using a *p-value* and FDR threshold set at ≤ 0.001; t0 = without glyphosate treatment; t1= with glyphosate treatment; GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype.

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Supplementary Material

Transcriptomic Analysis Identifies New Non-Target Site Glyphosate-Resistance Genes in Conyza bonariensis

Cristiano Piasecki [1,2,*], Yongil Yang [2], Daiane P. Benemann [1], Frederico S. Kremer [3], Vanessa Galli [3], Reginald J. Millwood [2], Joanei Cechin [1], Dirceu Agostinetto [1], Luciano C. Maia [4], Leandro Vargas [5] and C. Neal Stewart Jr. [2,*]

Table 1. Summary of the Illumina sequencing and *de novo* assembly statistics of *Conyza bonariensis* transcriptome.

Description	Assembly Stats*
Total assembled bases	157,784,873
Total of paired-end reads	23,383,488
Total of transcripts	203,054
Total of contigs "gene" level	90,124
Contig N50	1,118
Average contig length (bp)	777.06
GC (%)	40.23

*Assembled from all biotypes and treatments, a total of 12 libraries

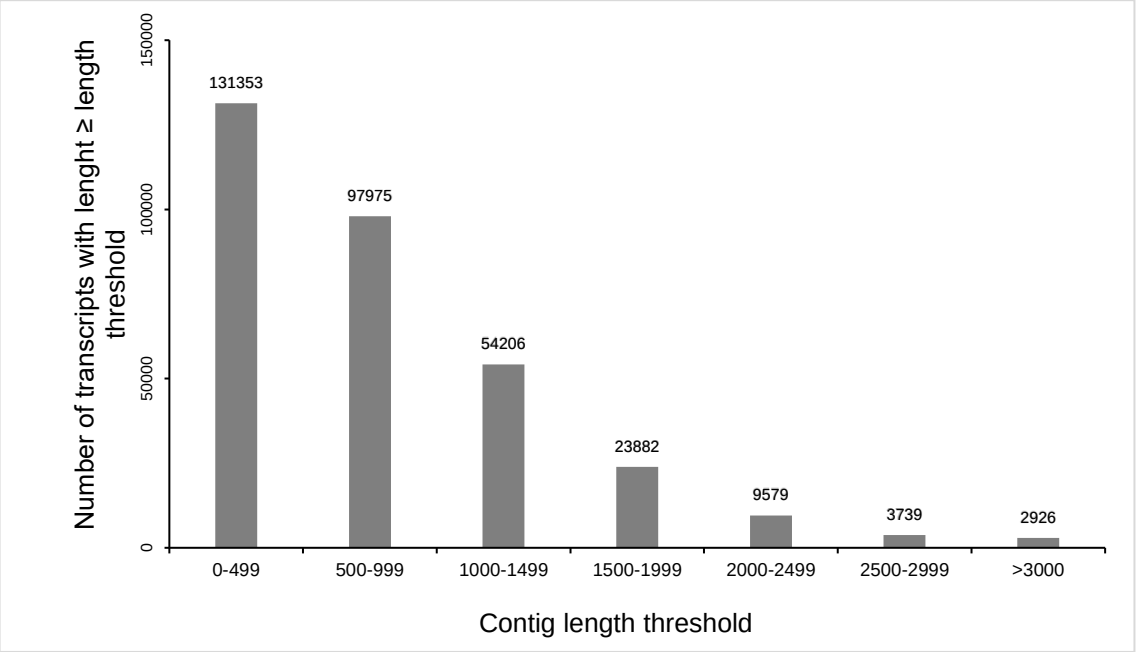

Figure 1. Length distribution of transcripts assembled from transcriptome libraries of hairy fleabane.

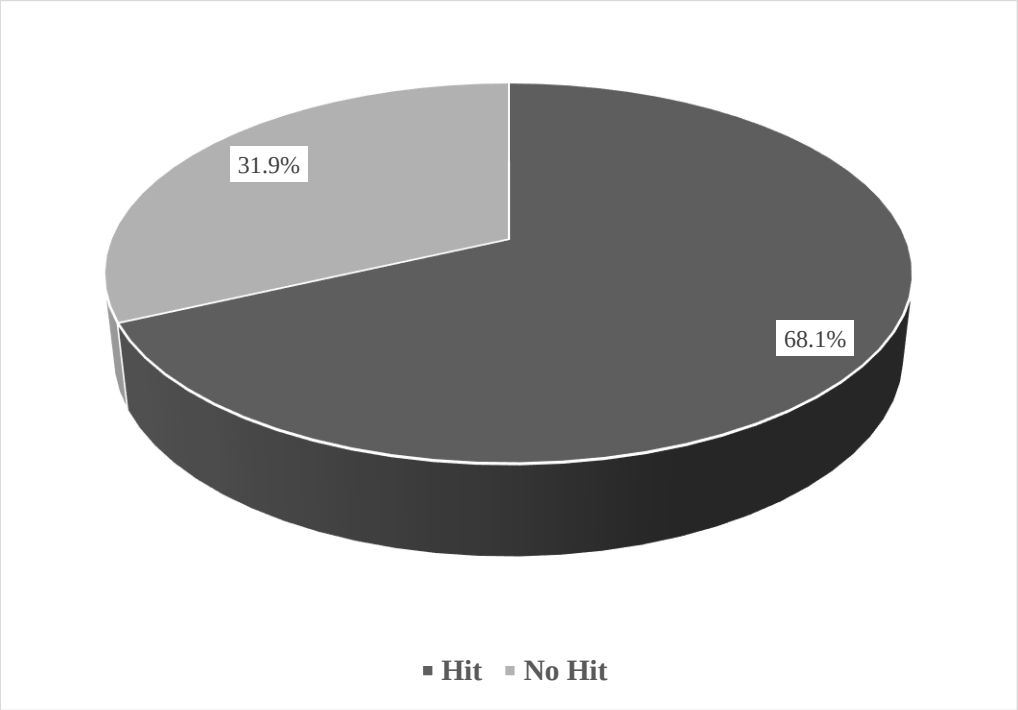

Figure 2. Sequence comparison to other organisms from the distribution of BLASTx hits (e-value < 1e-10) against the non-redundant protein database of the National Center for Biotechnology Information.

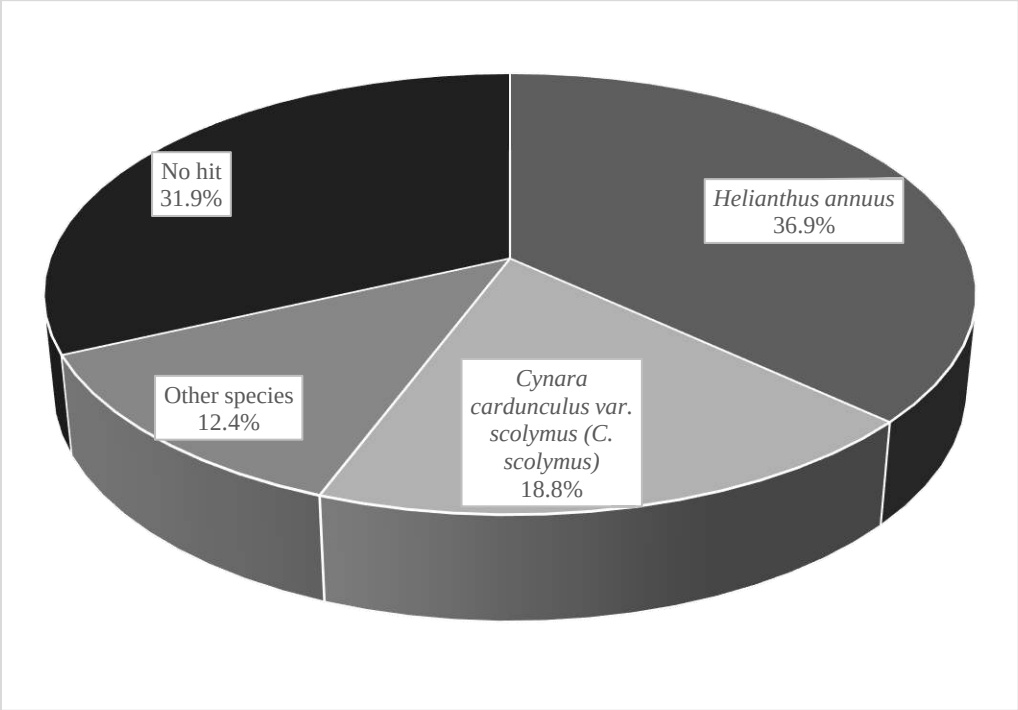

Figure 3. Sequence comparison to other plants (hit ≥ 1%) from the distribution of BLASTx hits (e-value < 1e-10) against the non-redundant protein database of the National Center for Biotechnology Information (NCBI).

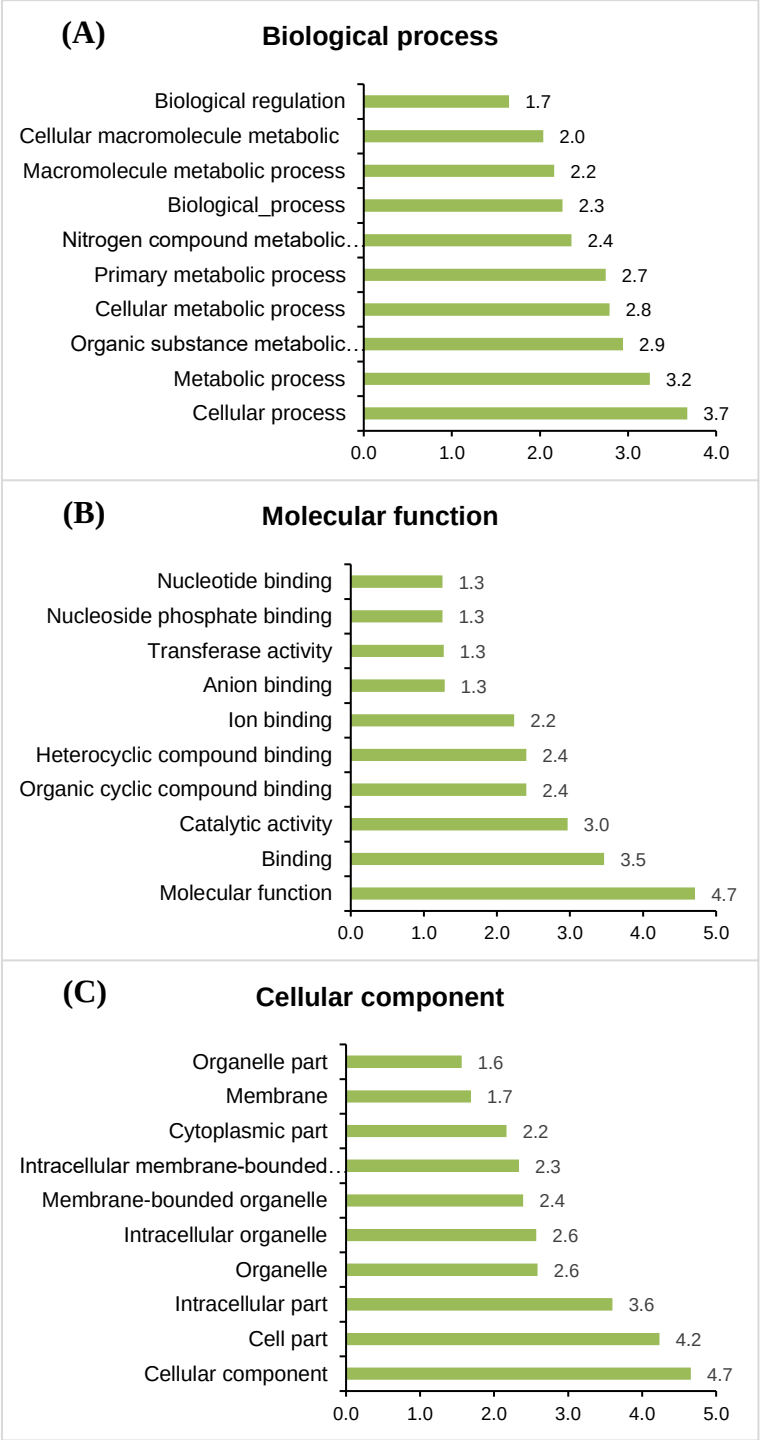

Figure 4. Top ten Gene Ontology (GO) terms identified in the hairy fleabane transcriptome assembly summarized in three main categories: (A) biological process, (B) molecular function, and (C) cellular component.

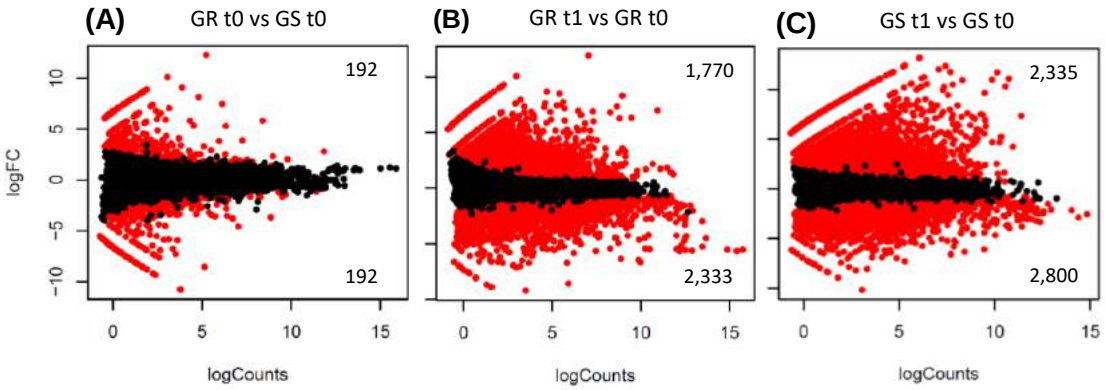

Figure 5. MA plot of differential expression analysis generated by EdgeR from transcriptome study performed in *Conyza bonariensis* glyphosate-resistant (GR) and -sensitive (GS) biotypes in response to glyphosate treatment. Dots above zero are up-regulated, and dots below are down-regulated. Red dots indicate significant expression at an adjusted *p-value* and false discovery rate (FDR) threshold set at ≤ 0.001, and log2FC ≥ 2 (up-regulated) or ≤ log2FC (down-regulated). Plots for each contig its log2FC (fold change) (A, Y-axis) vs. its counts (mean of normalized counts) (M, X-axis). t0 = without glyphosate treatment; t1 = with glyphosate treatment. GR t1: RNA sampled at 24, 96, 192, and 288 hours after treatment and pooled; GS t1: RNA sampled at 24, 96, and 192 hours after treatment and pooled.

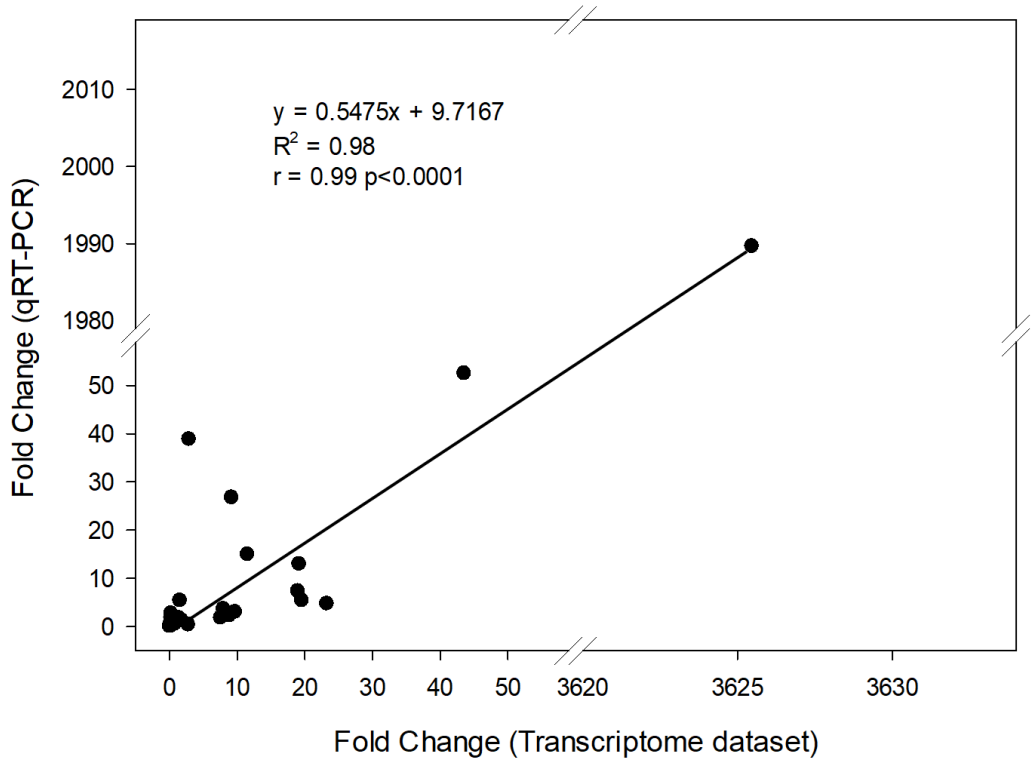

Figure 6. Correlation of transcriptomic and qRT-PCR (average of time-course) expression levels results of 19 genes of glyphosate-resistant (GR) and -sensitive (GS) *Conyza bonariensis* biotypes.

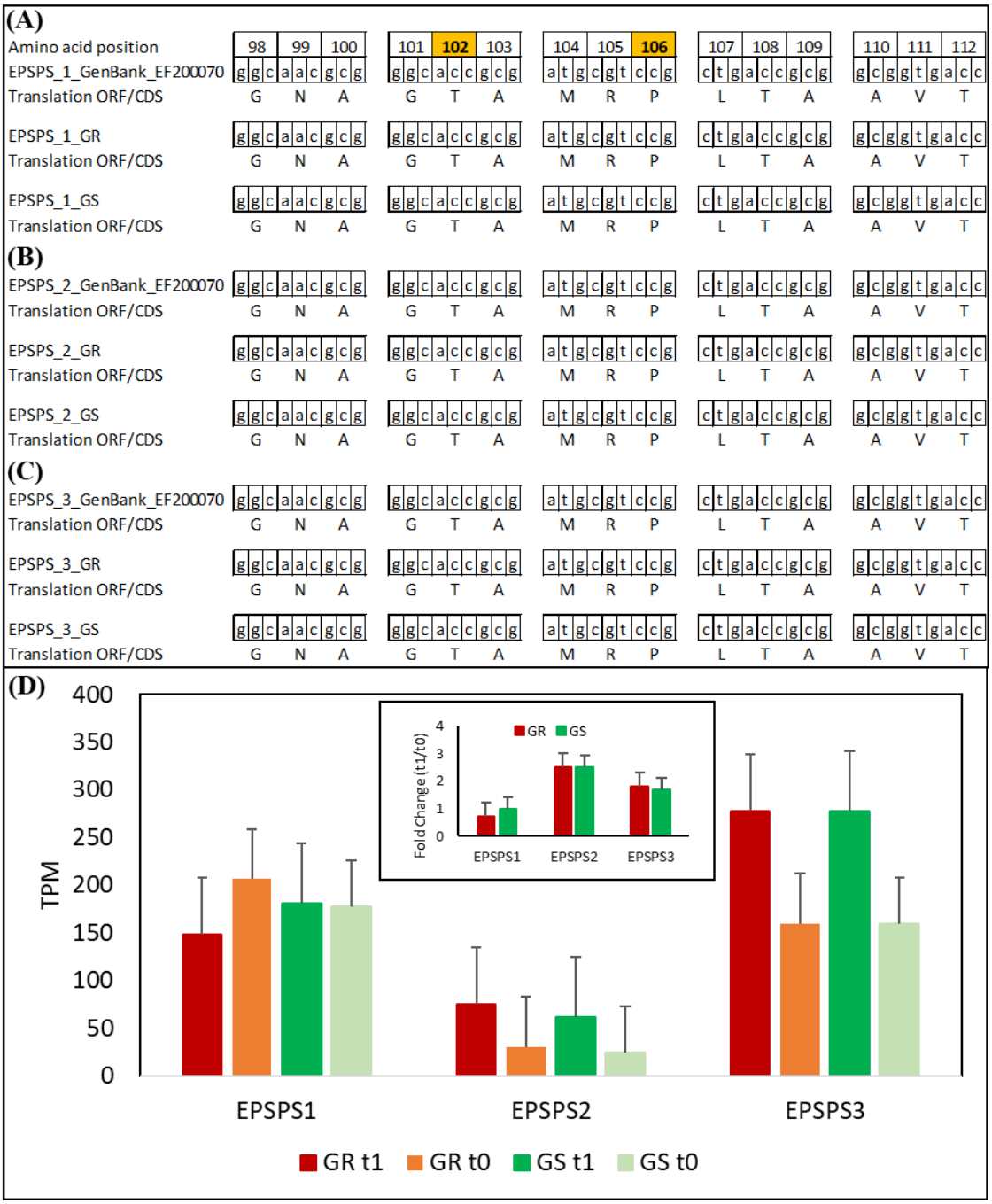

Figure 7. Partial sequence alignment of the EPSPS transcripts and amino acid sequence assembled of glyphosate-resistant (GR) and -sensitive (GS), and a sensitive *C. bonariensis* sequence from GenBank. (A) EPSPS1 (GenBank - accession number EF200070); (B) EPSPS2 (GenBank accession number EF200069); (C) EPSPS3 (GenBank accession number EF200074). The red boxed amino acids show no substitution at positions Threonine 102 and Proline 106. (D) Transcriptome expression levels (transcript reads per million mapped reads - TPM) of the three EPSPS copies in GR and GS in response to glyphosate treatment and expression difference (Fold change). GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment.

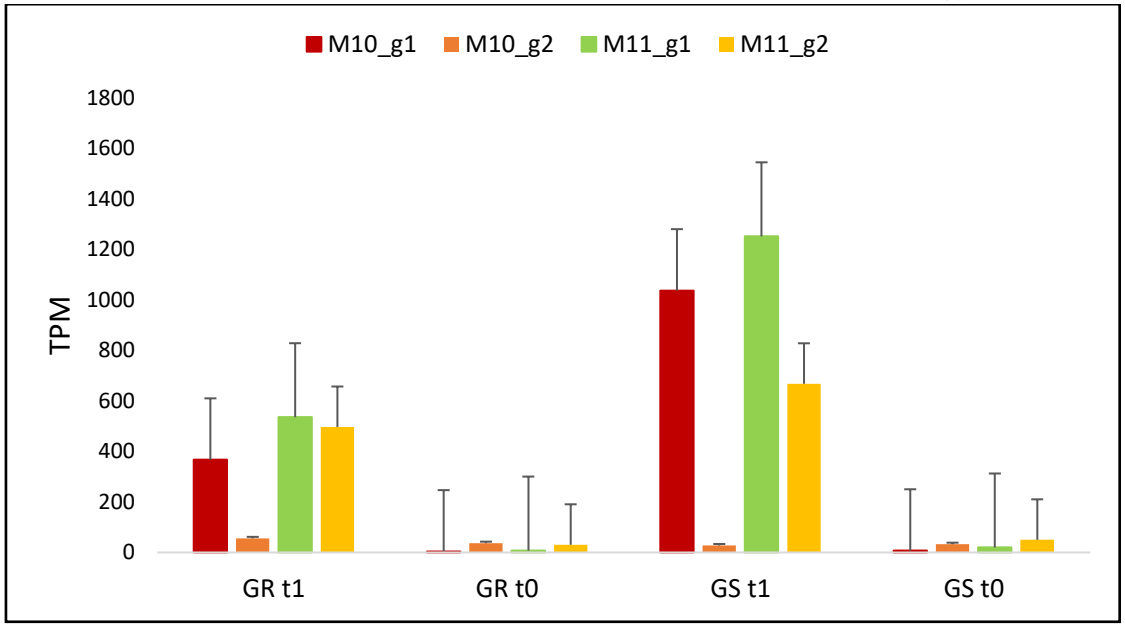

Figure 8. *Conyza bonariensis* transcriptome expression analysis (transcript reads per million mapped reads - TPM) of the M10 and M11 ABC Transporters reported being involved in glyphosate resistance in *C. canadensis* by Peng et al. (2010). M10_c1: Score (Bits) 2,682, *E-value* zero; M10_c2: Score 608, *E-value* 1e-72; M11_c1: Score 2,669, *E-value* zero; M11_c2:1,232, *E-value* zero. GR: glyphosate-resistant biotype; GS: glyphosate-sensitive biotype. t0: without glyphosate treatment; t1: with glyphosate treatment. Contigs were filtered according to the *p-value* and false discovery rate (FDR) threshold set at ≤ 0.001. Intervals indicate the standard error.

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MDPI OPEN ACCESS

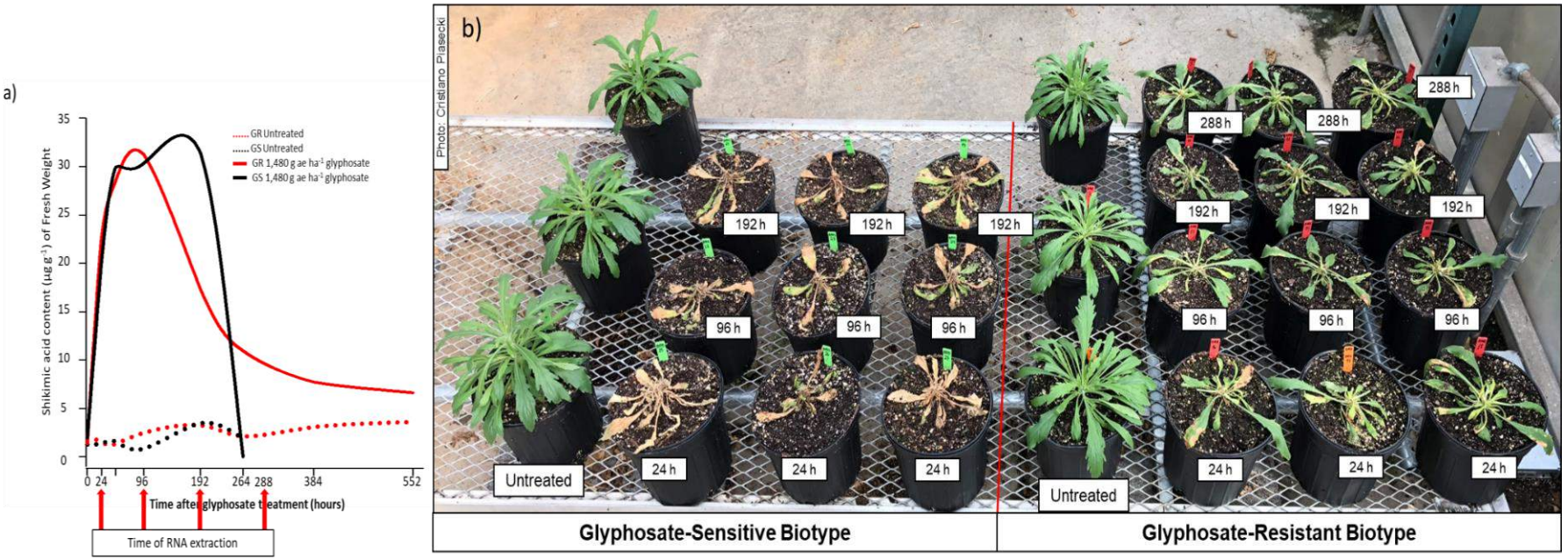

Figure 9. Responses of *Conyza bonariensis* glyphosate-resistant (GR) and -sensitive (GS) biotypes to glyphosate treatment (1,480 g ae ha^{-1}). a) The shikimic acid content in a time-course experiment; and b) Plants status at 288 hours after glyphosate treatment (12 days). GS plants died after 192 h after treatment whereas GR plants survived. Leaf samples were collected for RNA extraction based on a shikimic acid content curve in GR biotype. Then RNA was extracted from untreated plants in GR and GS biotypes and treated GR plants at 24, 96, 192, and 288 h after treatment, and at 24, 96, and 192 h in the GS plants which died after 192 h after treatment.

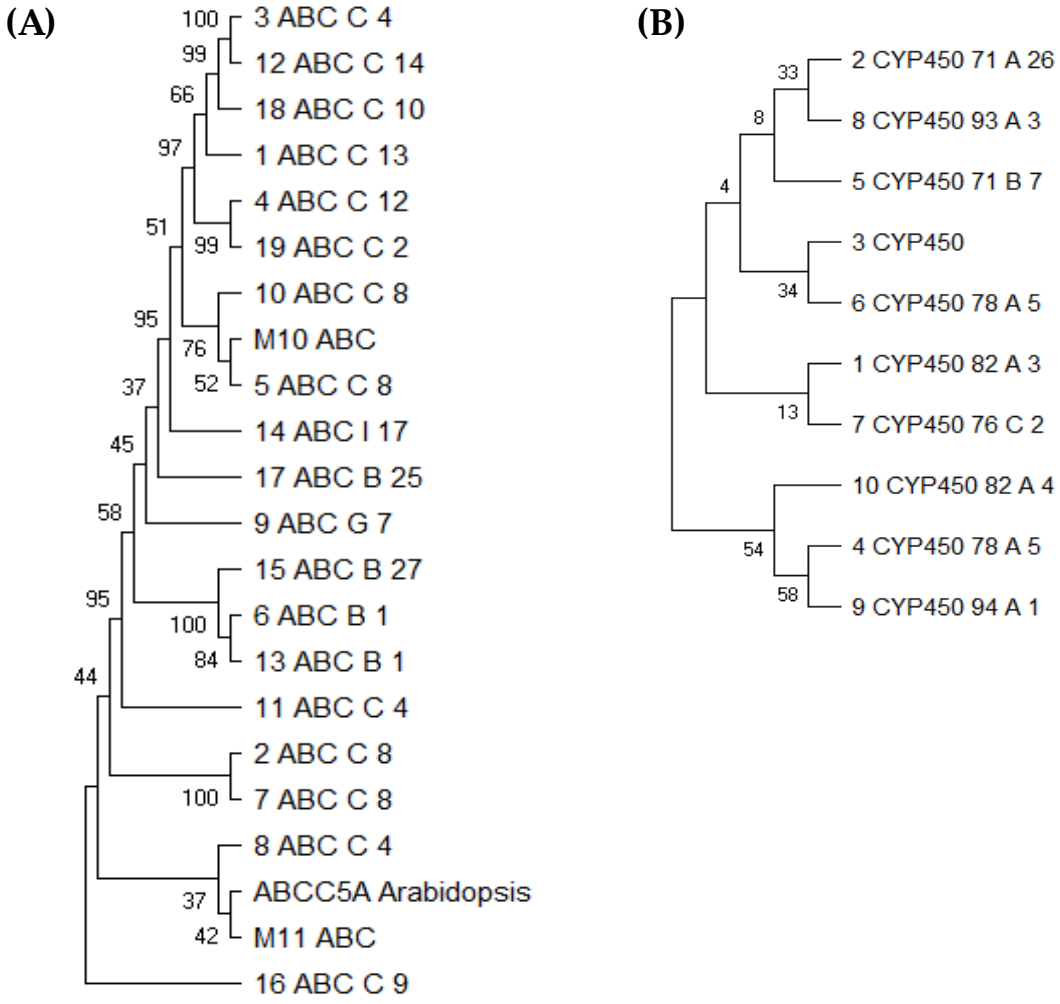

Figure 10. Evolutionary analysis of the candidate contigs sequences similarity to be related to glyphosate-resistance in *C. bonariensis* obtained from a transcriptome. A) ABC transporters; B) cytochrome P450 (CYP450). The number in front of the contig description indicate the order presented in each respective table 1 and 2. ABCC5A *Arabidopsis*: ABC Transporter full sequence obtained from GenBank *Arabidopsis thaliana*; M10 and M11 ABC: sequences of ABC transporters obtained from Peng et al. (2010). The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

Supplementary Sequences

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Plants 2019, 8, 157; doi:10.3390/plants8060157

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Plants 2019, 8, 157; doi:10.3390/plants8060157

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Concluding Remarks

- Glyphosate-resistant *Conyza bonariensis* biotype has transitory shikimic acid accumulation, increasing substantially at 24 h after treatment, peaked at 96 h, and at 288 h presented similar content as the untreated plants. In contrast, the glyphosate-sensitive biotype had similar initial shikimic acid levels as glyphosate-resistant plants at 24 h and 96 h, but it peaked at 192 h and died after that time-point.
- The glyphosate treatment causes the production of reactive oxygen species and oxidative stress in *Conyza bonariensis*. The ROS production and oxidative stress levels were higher in glyphosate-sensitive biotype. In contrast, the glyphosate-resistant biotype had higher antioxidant enzyme activities than -sensitive.
- RNA integrity was not negatively affected by glyphosate treatment in both biotypes.
- The RNA-Seq and qRT-PCR studies can be performed in a time course experiment up to 288 h after glyphosate treatment.
- The assembled transcriptome produced a large dataset with quantitative and qualitative data.
- The gene expression in *Conyza bonariensis* varies among gene groups and within the same group, between biotypes, is responsive to glyphosate treatment and time after treatment.
- The highest gene expression in response to glyphosate treatment occurred between 96 h to 192 h after treatment.
- The glyphosate-resistance in *Conyza bonariensis* is due to a non-target site resistance.

- The RNA-Seq study revealed 41 candidate non-target site glyphosate-resistance genes related to herbicide transport and metabolism in *Conyza bonariensis*. The candidate genes are 19 ABC transporters, 10 CYP450, one glutathione, five glycosyltransferases, two genes coding for each antioxidant enzyme catalase, peroxidase, and superoxide dismutase.
- The mechanisms of glyphosate-resistance in *Conyza bonariensis* involve a complex system of plant defense against stress caused by glyphosate action. This defense system comprises oxidation process (CYP450), conjugation (glycosyltransferases and glutathione), transport (ABC transporters), and antioxidant system (catalase, superoxide dismutase, peroxidase, glutathione).
- The high number of differential expressed genes has difficulted the identification of specific gene group related to particular metabolite produced in glyphosate-resistant biotype suitable to be detected by a portable tool. Further large-scale metabolomics studies could be performed to achieve that goal.
- The results of the present work will serve as a data resource for further studies on the molecular mechanisms of glyphosate resistance in *C. bonariensis.* Further studies will involve functional genomics analysis using protoplasts and gene editing approaches.

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