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Article

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 (RT-qPCR) 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 a.e. ha^{-1} and evaluated in a time-course until 288 hours 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 and 192 hours after glyphosate treatment, respectively. The RNA integrity analysis provides useful results to evaluate the RNA condition for further analysis. However, the costs were around US$ 14.25 per sample, considering only 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 glifosato 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 (RT-qPCR), têm sido empregadas para estudar as respostas moleculares ao tratamento com glifosato em espécies de Conyza. No entanto, o tratamento com glifosato 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 glifosato interfere negativamente na integridade do RNA de biótipos de buva resistente e sensível ao glifosato. Dois experimentos foram realizados*

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*utilizando doses de glifosato 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 glifosato não reduziram os escores do RIN em biótipos de buva resistentes e sensíveis ao glifosato. 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 glifosato não afeta negativamente a integridade do RNA dos biótipos resistentes e sensíveis ao glifosato da buva até 288 e 192 horas após o tratamento com glifosato, respectivamente. A análise da integridade do RNA fornece resultados úteis. Entretanto, os custos são altos e ficaram em torno de US-$ 14,25 por amostra, considerando apenas os reagentes. Esses resultados são úteis para o planejamento de experimentos futuros em **Conyza** spp. após o tratamento com glifosato.*

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 with 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 (VanGessel, 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, 2019). Together, these three species accounts 63 reports of GR around the world (Heap, 2019).

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 real-time quantitative reverse transcriptase (RT-qPCR) (Peng et al., 2010; Hereward et al., 2018). RNA-Seq and RT-qPCR 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 in non-model species and identify candidate differentially expressed genes, and further evaluations are performed using RT-qPCR.

Some articles report 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 that how long time the

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.

plant metabolism system 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 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 score is evaluated in an equipment called 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 degradation of other types of RNA such as mRNA (Schroeder et al., 2006), which is the target nucleic-acid of transcriptome and RT-qPCR studies.

The evaluation of RNA integrity is pre-requisite before molecular analysis as RNA-Seq and RT-qPCR. 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 high tissue damage in a determined time-point after treatment, especially in GS biotype, and it can 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 times 24 h, 96 h, 192 h, and 288 h indicate the hours after glyphosate treatment that leaves were collected to RNA extraction in each biotype. In each time, three biological replicates were used. Experiment 2. In this experiment were collected two completely expanded leaves from apex per plant for RNA extraction.

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 a.e. ha^{-1}).

The plants were cultivated in a greenhouse until 60 days after emergence (DAE) at 25 °C/15 °C day/night (± 3 °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 a.e. 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).

The 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 a.e. ha^{-1} (4 L pc ha^{-1}); the third factor comprised evaluations of RNA integrity in six times-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 a.e. 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 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™ (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.

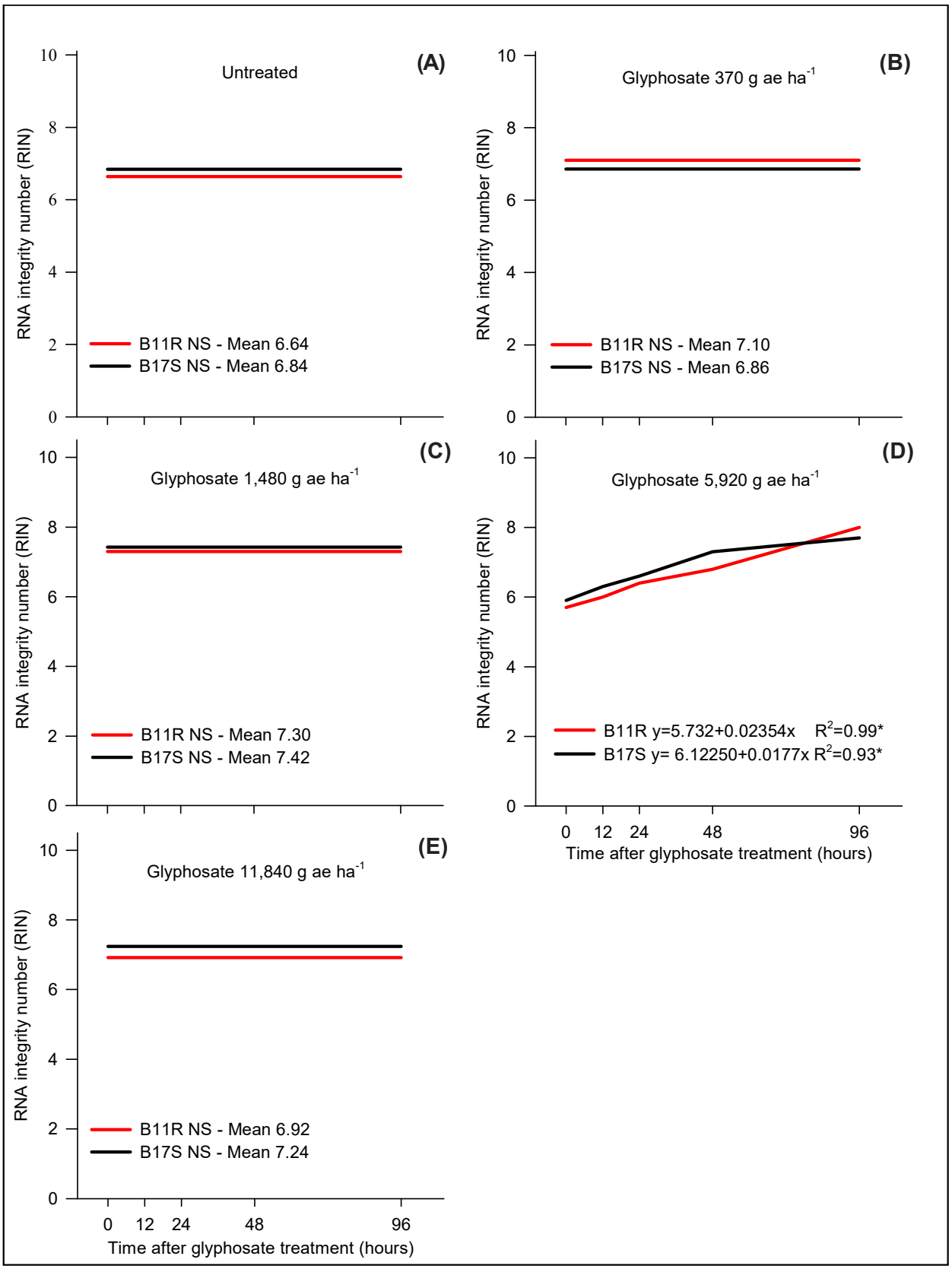
Statistical analyses were performed using the GLM package statement from SAS (version 9.0, SAS Institute Inc, Cary, NC, USA), and results fitted using Sigma Plot®. SAS Proc Univariate was used to test for normality (Shapiro-Wilk's test), and homogeneity of variance (Hartley's test). Proc Mixed was used 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 a.e. ha^{-1} of glyphosate (Figure 2A, B, C, and E). These results indicate that the glyphosate action does not have relation with RNA integrity. However, after treatment of 5,920 g a.e. ha^{-1} of glyphosate, the RIN increased until 96 HAT for both biotypes with no statistical differences between them (Figure 2D). 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 that 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, 5 and 6). For all results, a clear band can be visualized at the 28S and 18S position (Figure 4A, B). These results can be compared with an example of degraded and intact RNA (Figure 4C). 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



NS: non-significant at p≤0.05. Experiment 1 (E1).

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 a.e. ha^{-1}; (C) 1,480 g a.e. ha^{-1}; (D) 5,920 g a.e. ha^{-1}; and (E) 11,840 g a.e. ha^{-1}.

defined two bands at 28S and 18S positions, with no traces through the gel (Figure 4C). 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. The 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, the RNA quality and integrity depend on employed methods during extraction, reagents, and environment conditions (Johnson et al., 2012).

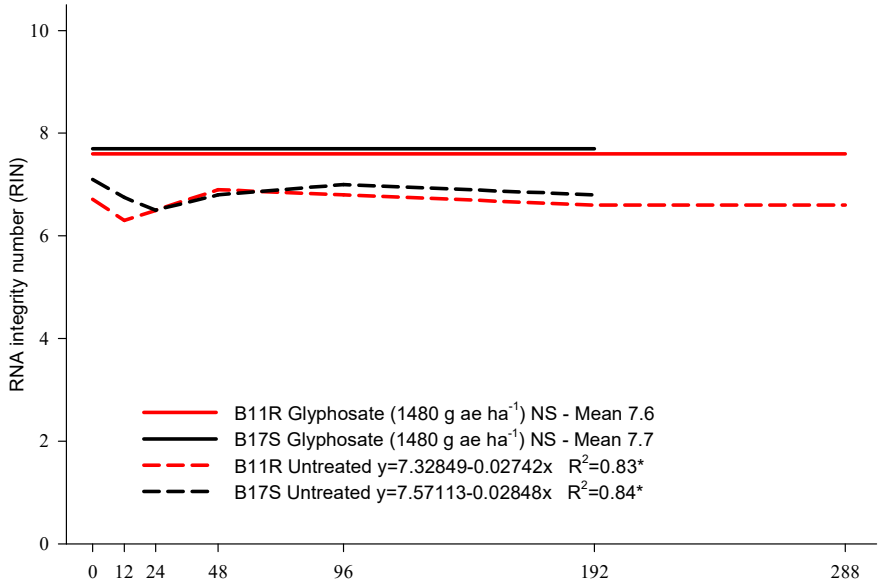
Isolate RNA to transcriptomic experiments with quality (i.e., non-degraded, free of impurities) and yield is a challenge. It is because of RNases, which degrade the RNA rapidly, are present in large scale in nature and laboratories (Johnson et al., 2012). Plant secondary metabolites such as phenols and 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, 4, 5 and 6). Thus, for further molecular analysis such as RNA-Seq and RT-qPCR, 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).

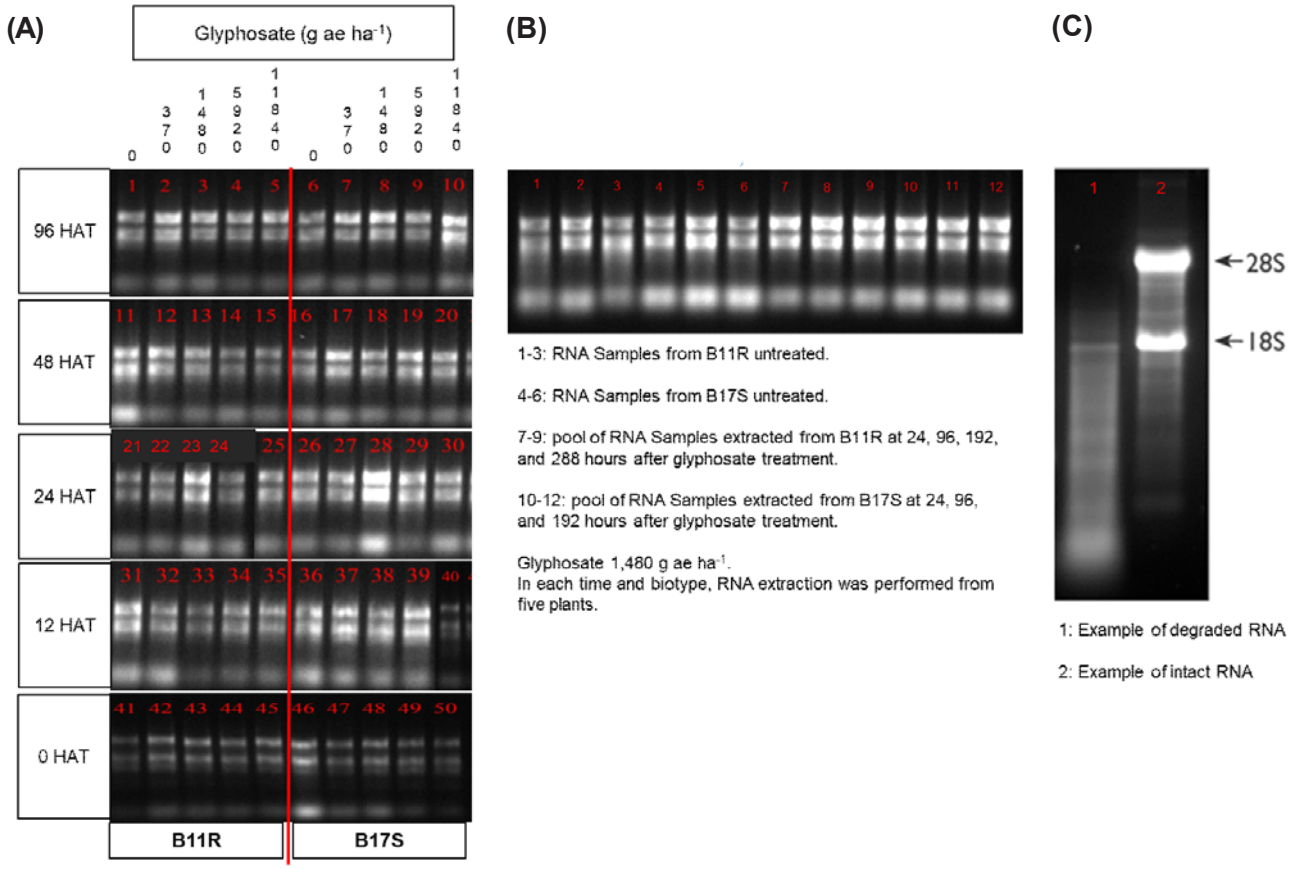
Analysis of RNA integrity is frequently used before molecular studies. It is important to ensure that RNA present suitable conditions to be analyzed and provide reliable results. The inclusion of degraded RNA on molecular analysis has shown significant influence on the bioinformatics and statistical 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.



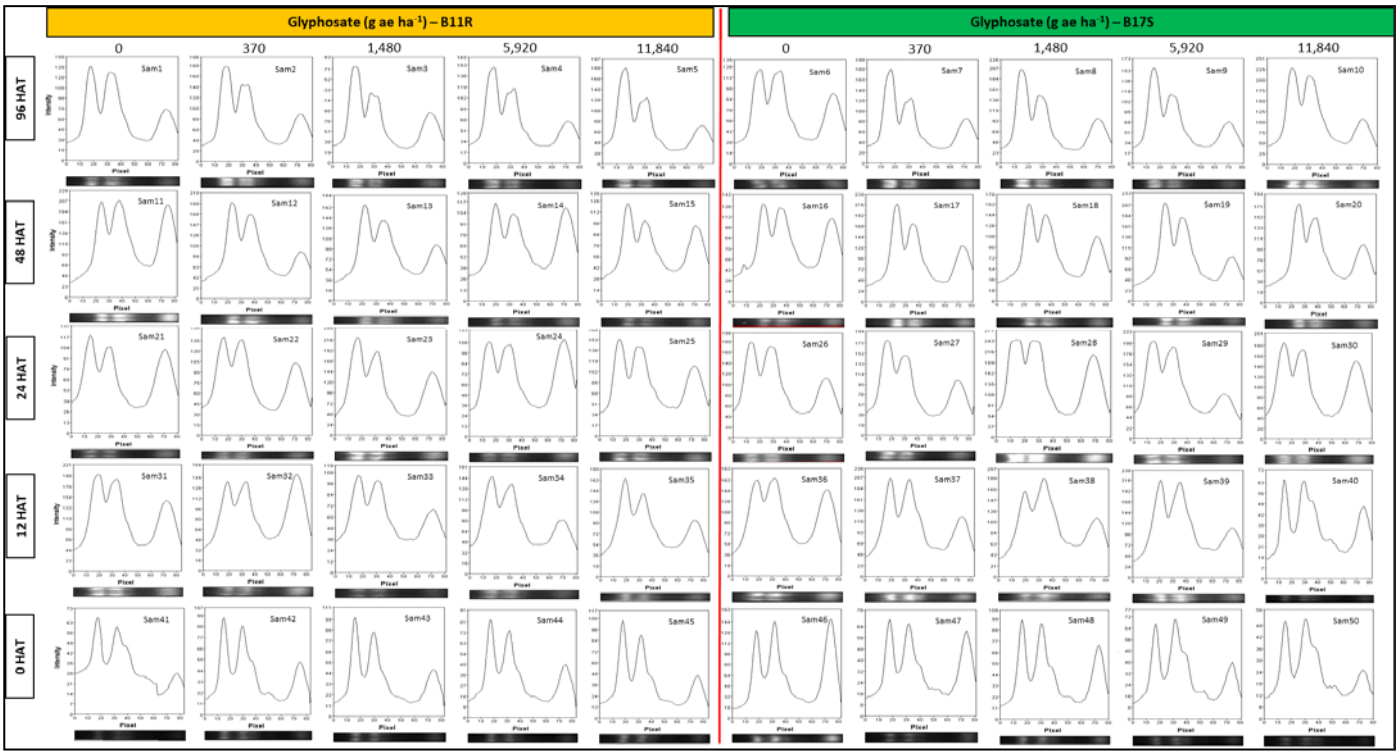
NS: non-significant at p≤0.05. Experiment 2 (E2). B17S was evaluated until 192 HAT because after that time plants died.

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 a.e. ha^{-1}.



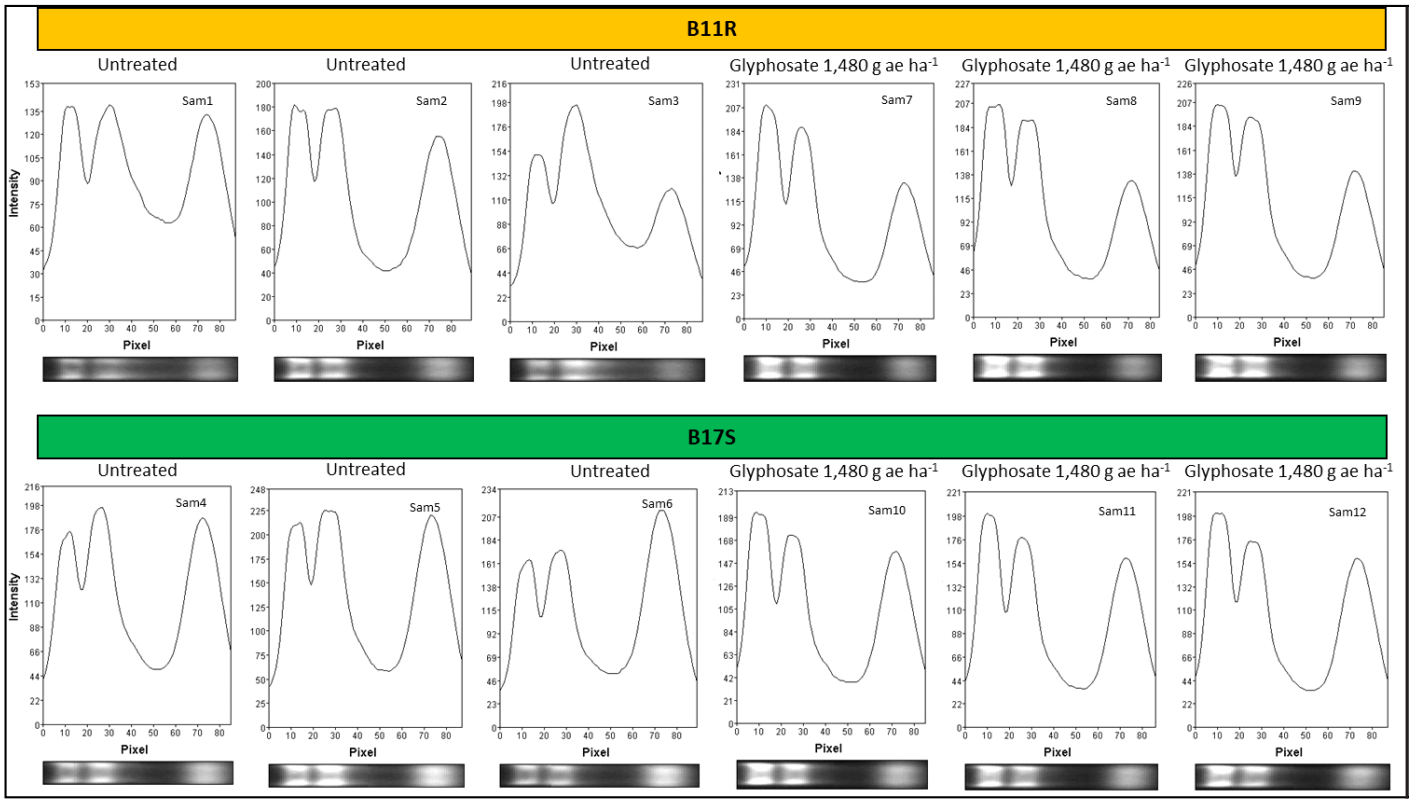
(A) Experiment 1 (E1) – RNA extracted from B11R and B17S at 0, 12, 24, 48, and 96 hours after glyphosate treatment at 0 (untreated), 370, 1,480, 5,920, and 11,840 g a.e. ha^{-1}. (B) Experiment 2 (E2) – RNA Samples 1-3: B11R untreated; RNA Samples 4-6: B17S untreated; RNA Samples 7-9: pooled RNA extracted from B11R at 24, 96, 192, and 288 hours after glyphosate treatment (1,480 g a.e. ha^{-1}); RNA Samples 10-12: pooled RNA extracted from B17S at 24, 96, and 192 hours after glyphosate treatment (1,480 g a.e. ha^{-1}). (C) Examples of degraded RNA – 1; and intact RNA - 2. HAT: hours after glyphosate treatment.

Figure 4 - Electrophoresis gel results of the RNA extracted from glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes followed by glyphosate treatment.



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 a.e. ha^{-1}. Sam: indicate the sample number. The samples follow the same order of electrophoresis gel presented in Figure 4A.

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 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 a.e. ha^{-1}); RNA Samples 10-12: pooled RNA extracted from B17S at 24, 96, and 192 hours after glyphosate treatment (1,480 g a.e. ha^{-1}). Sam: indicate the sample number. The samples follow the same order of electrophoresis gel presented in Figure 4B.

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).

Another important point to be considered 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 only 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 °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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