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Efeitos de Roundup Transorb no perfil transcriptômico de folhas e na composição de grãos de G*lycine max (L.) Merr.*

Florianópolis
2020

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Dissertação submetida ao Programa de Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do título de mestre em Ciências
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RESUMO

A abordagem atual de avaliação com base somente na equivalência substancial entre Organismos Modificados comparados com sua linhagem isogênica mais próxima, não é considerada suficiente, por muitos cientistas, como método seguro de análise de risco. Assim, o presente trabalho no seu primeiro capítulo apresenta uma análise de expressão de EPSPS transgênica e nativa de soja *(Glycine max),* com o uso da técnica RT-PCR. As análises dos resultados indicaram que a expressão gênica foi regulada igualmente para eventos únicos e estaqueados (*stacking*). Com a interpretação do RNA-seq verificou-se que apesar das variedades compartilharem respostas semelhantes, a variedade simples exibe um comportamento fisiológico esperado como resposta ao estresse, aumento do teor de açúcar, diminuição da taxa de captação de clorofila A e B e comprometimento do balanço celular redox. Por sua vez, a variedade estaqueada apresentou forte regulação nas rotas relacionadas a metabólitos secundários (β-glucosidase, isoflavona 7-O-metiltransferase, bem como, componentes de defesa e mecanismos de sinalização de Ca2+, chaperonas moleculares, proteínas Rboh e WRKY, o que sugere uma resposta ao estresse mais pronunciada. No segundo capítulo, foi obsevado que aplicação no estádio fenológico V2 produziu resíduos de glifosato e AMPA no grão colhido pronto para consumo. A análise dos resíduos permite refletir sobre o atual cenário alimentar, considerando que apenas um herbicida e em dosagens recomendas pela bula foram adotadas. O efeito do HBG sobre alguns componentes também foi testado e foi observado que as amostras não-GM e GM tenderam ao agrupamento independente do tratamento com herbicida nas duas dosagens. É imprescindível conhecer o organismo biológico GM de maneira mais complexa, neste sentido, a utilização de transcriptômica permitiu observar alterações metabólicas oriundas do efeito de HBG. Por exemplo, foram descritas alterações em uma série de genes relacionados ao metabolismo defesa, carbono, homeostase redox e fotossíntese e na variedade estaqueada, chama a atenção a alteração da rota do chiquimato, constatada devido a presença do aminoácido fenilalanina e subprodutos do mesmo.

P**alavras-chave:** Herbicida à base de glifosato, ômicas, equivalência substancial, transgênicos, efeitos inesperados.

ABSTRACT

The current assessment approach based only on the substantial equivalence between Modified Organisms compared to their closest isogenic lineage, is not considered sufficient, by many scientists, as a safe method of risk analysis. Thus, the present work in its first chapter analyzes and presents an analysis of the expression of transgenic and native EPSPS, from soy (Glycine max), using the RT-PCR technique. Through the analysis of the results, it was observed that the gene expression was regulated equally for single and stacked events (staking). Stacking analysis. With the interpretation of RNA-seq it was demonstrated that despite the varieties sharing similar responses, the simple variety exhibits an expected physiological behavior as a response to stress (, increase in sugar content, decrease in the rate of uptake of chlorophyll A and B harvest and impairment of the redox cell balance). On the other hand, the stacked variety, on the other hand, showed a high regulation in the pathways related to secondary metabolites (β-glucosidase, isoflavone 7-O-methyltransferase), as well as defense components and signaling mechanisms of Ca2 +, molecular chaperones, Rboh and WRKY proteins, which suggests a more pronounced stress response. In the second chapter, it was observed that application in the phenological stage V2 produced residues of glyphosate and AMPA in the harvested grain ready for consumption. The analysis of residues allows us to reflect on the current food scenario, considering that only one herbicide and in dosages recommended by the package insert were adopted. The effect of HBG on some components was also tested and it was observed that the non-GM and GM samples tended to group independently of the herbicide treatment at the two dosages. It is essential to know the biological organism GM in a more complex way, in this sense, the use of transcriptomics allowed us to observe metabolic changes arising from the effect of HBG. For example, changes have been described in a series of genes related to defense metabolism, carbon, redox homeostasis, and photosynthesis, and in the staked variety, attention is drawn to the alteration in the route of the shikimate, found due to the presence of the amino acid phenylalanine and its by-product.

Keywords: Glyphosate, omics, substantial equivalence, transgenics, unexpected effects

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LISTA DE ABREVIATURAS E SIGLAS

AMPA - Ácido aminometilfosfônico

ATP - Adenosina trifosfato

BCC - Blocos Completos Casualizados

Bt - *Bacillus thuringiensis*

CDB - Convenção da Diversidade Biológica

CP4 - *Agrobacterium sp*., cepa cp4

Cry – Proteínas cristais

DAS - Dias Após Semeadura

DNA - Ácido desoxirribonucleico

EM BRAPA - Empresa Brasileira de Pesquisa Agropecuária

EPSPS - 5-enolpiruvilshiquimato-3-fosfato sintase

EU - União Europeia

EUA - Estados Unidos da América

FAO - Organização das Nações Unidas para a Alimentação e a Agricultura

FDA - Food and Drug Administration

GM - Geneticamente Modificado

HBG - Herbicida a base de glifosato

INTACTA RR2 PRO® - Cultivares Roundup Ready de segunda geração

i.a - Ingrediente Ativo

IDA - Ingestão Diária Aceitável

LMR - Limite Máximo de Resíduos

não-GM – Não geneticamente modificado

OECD - Organização para a Cooperação e Desenvolvimento Econômico

OGM/OVM – Organismo Geneticamente Modificado/ Organismo Vivo Modificado

PES – Princípio da Equivalência Substancial

pH - potencial Hidrogeniônico

RNA - Ácido ribonucleico

RR – Cultivares Roundup Ready

USDA - Departamento de Agricultura dos Estados Unidos

SUMÁRIO

1 JUSTIFICATIVA E ANTECEDENTES

No mundo, lavouras e campos de agricultura contam com uma crescente presença de plantas geneticamente modificadas (GM). Somente a soja, conta com 50% de adoção o que permite liderança em 95,9 milhões de hectares. Na última década, cultivos GM com mais de uma característica (empilhamento) tornaram-se uma realidade e em 2017 eventos com resistência a insetos e tolerância a herbicidas ocuparam 42% da área global (ISAAA, 2018).

No Brasil a primeira soja estaqueada para resistência a insetos e tolerância a herbicidas foi liberada no ano de 2010, sendo resultante da combinação dos dois eventos de transformação MON87701 e MON89788. O primeiro evento citado possui inserido o gene Cry1Ac recombinante (rCry1Ac), inicialmente islolado da bactéria de solo *Bacillus thuringiensis* (Bt) e posteriormente modificado *in vitro*, que confere resistência a alguns insetos da ordem lepdoptera. Por sua vez, epsps C4, é projetado para tolerância ao glifosato (CTNBIO, 2010). A tolerância aos herbicidas a base de glifosato (HBG) é devido à expressão da enzima resistente EPSPS (5-enolpiruvilchiquimato-3-fosfato sintase) isolado da estirpe CP4 de *Agrobacterium tumefaciens* (WATRUD et al., 2004).

A construção do conhecimento em relação à fluidez do genoma tem permitido expandir o que era até pouco tempo conhecido como concreto - mensagens genéticas no DNA são transcritas em RNA, que então traduzidas para proteínas, cada uma determinada a uma característica específica (HO, 2013). A abordagem do "genoma fluido" surgida em meados da década de 1970 contraria o determinismo genético e pressupõe que outros processos estão envolvidos na regulação gênica e que, portanto, devem ser considerados como fatores de alteração e distúrbios (HO, 1987; HO, 2013). Entre estes processos, os mecanismos de reparo de DNA e emenda alternativa do pré-mRNA, podendo introduzir etapas intermediárias que alteram a informação, a epigenética, as estruturas da cromatina, a metilação do DNA e as modificações nas histonas (SHAPIRO,2009; LUCO et al., 2010). Além desses, tem-se os microRNAs sendo mostradas em OGMs (AGAPITO-TENFEN et al., 2018), o *splicing* de proteínas (LUCO et al., 2010; VOLKMANN; MOOTZ, 2013), bem como os erros ou incompatibilidades entre as sequências de RNA e seu DNA codificador (HAYDEN, 2011) e ainda, efeitos pleiotrópicos de uma nova proteína inserida (KULIKOV, 2005; BAKER et al., 2006).

Alterações, efeitos e desvios que eram desconhecidos no genoma, têm sido subsidiados por um campo emergente da genética que investiga utilizando como abordagem

ferramentas as chamadas ômicas. Estas, são utilizadas como método não direcionado para auxiliar na investigação e mapeamento podendo ser consideradas complementares na avaliação de segurança de culturas transgênicas por serem análises imparciais (DAVIES et al., 2010; JIANG et al., 2013a; SIMÓ et al., 2014; WANG et al., 2018) que permitem auxiliar na compreensão das consequências diretas ou indiretas e efeitos não intencionais da modificação genética, como, efeitos metabólicos não esperados (SÉRALINI et al., 2011) potenciais interações entre genes, RNA, proteínas (DAVIES; BRYAN; TAYLOR, 2008).

Para fins de regulamentação de cultivos GMs, o Critério de Equivalência Substancial (CES) é adotado por alguns países na avaliação de segurança de um novo alimento em relação ao seu equivalente convencional. O objetivo é identificar semelhanças e diferenças, em geral, a partir da análise composicional, o perfil dos principais nutrientes e, em alguns casos, substâncias tóxicas presentes (FAO, 2008).

Neste estudo, utilizamos o Roundup Transorb® (HBG) como substância tóxica com potencial estressor na fisiologia das plantas e capaz de gerar alterações composicionais em grãos. O herbicida utilizado é um formulado comercial composto pelo princípio ativo, glifosato que em degradação produz outra substância, o ácido aminometilfosfônico (AMPA) (RUEPPEL et al., 1977; MYERS et al., 2016). Nas folhas, a captação de HBG é um processo bifásico que envolve uma rápida penetração inicial através da cutícula em direção ao apoplasto, seguindo para o floema que transfere a molécula para o restante da planta, (MONQUERO, CHRISTOFFOLETI, *et al.*, 2004; DILL, SAMMONS, *et al.*, 2010). É característico desse herbicida a rápida absorção e translocação para todas as partes da planta. Após a penetração nas folhas, flui por sítios metabólicos ativos, como os meristemas das raízes e brotações (DELLA-CIOPPA, BAUER, *et al.*, 1986; BHATLA, LAL, 2018).

Na fisiologia de soja GM RR, a aplicação de HBG foi associado a mecanismos de alteração fisiológica, com ação fitotóxica, compromentendo os processos biológicos e favorecendo aparecimento de doenças (ALBRECHT et al., 2012). Efeitos no metabolismo secundário, estado oxidativo, hormonal, alterações na fotossíntese (YU et al., 2007; VIVANCOS et al., 2011; ARRUDA et al., 2013; GOMES et al., 2014) e o acúmulo de carboidratos nos tecidos também foram observados (JIANG et al., 2013b). Em soja GM INTACTA RR2 o glifosato influenciou a captação e translocação de nutrientes fundamentais para o desenvolvimento (ZOBIOLE et al., 2011).

Usamos essas informações para testar a hipótese de que a combinação de transgenes desencadeiam uma resposta diferente pelas plantas de soja devido ao custo de expressão de

um gene e uma (EPSPS e rCry) heteróloga, além disso, para elucidar se rotas metabólicas diretas e indiretas seriam afetadas por HBG.

Neste sentido, no primeiro capítulo foi analisado o perfil transcriptômico de variedades de soja GM de evento único e evento estaqueado quando submetidas à aplicação de um HBG. Nossos dados de transcriptoma demostram que o desvio da via EPSPS insensível ao HBG, conforme determinado pelas alterações no transcriptoma da variedade de soja transgênica empilhada tratada, é notável um efeito em cascata que é traduzido como um forte impacto no sistema de defesa.

No segundo capítulo, são apresentadas as análises dos resíduos de glifosato e AMPA no grão colhido R8 advindos da aplicação Roundup Transorb® no estádio fenológico V2. Além disso, foram analisados alguns componentes nutricionais e com base na quantidade foram observadas alterações para mais e para menos entre os componentes analisados para as amostras não-GM e GM tratadas com Roudup Transorb®.

Esta dissertação teve o objetivo de contribuir na investigação de possíveis efeitos não esperados na fisiologia das plantas de soja GM de evento simples e estaqueado quando submetidas à aplicação de HBG, neste contexto, o presente trabalho é parte de um esforço maior que o grupo de pesquisas em biossegurança da Universidade Federal de Santa Catarina (UFSC) realiza desde 1997 e parte do histórico compilado desta trajetória é demostrada (ver Apêndice).

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2 OBJETIVO

2.1 OBJETIVO GERAL

- Investigar alterações metabólicas em variedades de soja colocar nome científico submetidas à aplicação do herbicida Roundup Transorb®.

2.2 OBJETIVOS ESPECÍFICOS

- Determinar o perfil transcriptômico de variedades de soja NA 5909 RG e BRS 1001 IPRO após aplicação de Roundup Transorb®;
- Verificar efeito diferencial metabólico entre a variedade NA 5909 RG e BRS 1001 IPRO após aplicação de Roundup Transorb®;
- Determinar a expressão do gene EPSPS nas variedades de soja NA 5909 RG e BRS 1001 IPRO após aplicação de Roundup Transorb®;
- Avaliar o nível de resíduo de glifosato e AMPA em grãos maduros (estádio R8) de soja BRS 1001 IPRO;
- Identificar alguns componentes principais do grão de soja BRS 1001 IPRO e não-GM.

3 CAPÍTULO - 1

Manuscrito submetido ao periódico Environmental Sciences Europe em 20/01/2019 (ESEU-D-20-00016).

Stacked genetically modified soybean harboring herbicide resistance and insecticide rCry1Ac shows strong defense and redox homeostasis disturbance after glyphosate-base herbicide application

Abstract

Background: World agricultural production of genetically modified (GM) products in particular, the combination of different traits/genes in the same plant has been intense over the last decade. The stacking of herbicide and insect-resistant transgenic genes can result in fitness costs that rely on the type and strength of the selection pressure exerted by the environment. Here we report the results of transcriptomic analysis comparing the effect of glyphosate-based herbicide (GBH) on various biological processes, metabolic pathways, and main shikimic enzymes in stacked versus single soybean resistant varieties.

Results: Gene expression data were grouped according to treatment, ie the herbicidal factor strongly influenced. Common physiological results between the single and established varieties were mainly in Redox metabolism, energy, and metabolism. Photosynthesis was only found negatively affected in the single variety. The defense components, although present in both varieties, show a more intense presence in stacked pathways, that demonstrated pathways related to up-regulated secondary metabolites biosynthesis, a known response when plants are under various stress conditions. RT-PCR results confirmed that native EPSPS expression was up-regulated at the same level for single and stacked events. However, metabolic differences in expression were observed, suggesting a distinct cascade effect between simple and stacked, triggered by GBH application.

Conclusion: Changes in plant metabolism by glyphosate-based herbicide application have been observed in several pathways, particularly the shiquimate pathway, suggesting that event staking may promote a more intense defensive genetic response. Omics profiling techniques, such as transcriptome, can be considered tools to support risk assessment based on detecting unwanted effects, both on plant physiological changes and on the safety of foods and products from new genetic editing technologies.

Keywords

Roundup; transcriptomic; gene expression; metabolic pathways; shikimate pathway; beta-glucosidase; gluthathione; thealose, Recombinant Cry1Ac.

3.1 Background

The combination of different traits or genes in genetically modified (GM) plants has rapidly emerged in worldwide crop production. In recent years, an increasing number of GM plants with stacked traits reached about 81 million hectares equivalent to 42% of the total 191.7 million hectares planted with transgenic crops worldwide in 2018 [5]. The predominant trait, for both single and stacked crop varieties is herbicide resistance and it is estimated to remain so in the near future [6].

According to the current regulatory practice within the European Union (EU), stacked events are considered new GM organisms, requiring similar risk assessment procedures to those from single events [7]. Whereas in other countries, such as Brazil, stacked events are also considered new GMOs but require simplified risk assessments upon approval of single parental events [8].

Previous studies have shown that stacking herbicide and insect-resistant transgenes can result in fitness costs that are dependent on the type and strength of selection pressure, and could also contribute to changes in plant communities through hitchhiking of unselected traits [9]. In that particular study, one of the tested selective pressure was the spray of glyphosate, which has been shown to adversely affect plant uptake and transport of micronutrients (e.g. Mn, Fe, Cu, and Zn) whose undersupply can reduce disease resistance and plant growth [10] [11].

Glyphosate manufacturers claim that it works by inhibiting the enzyme 5-enolpyruvylshikimate-3- phosphate synthase (EPSPS), which catalyzes the penultimate step of the shikimate pathway leading the conversion of shikimic acid to chorismate, the precursor for aromatic amino acids (tyrosine, phenylalanine, and tryptophan) and other secondary plant metabolites. Glyphosate competes with phosphoenolpyruvate (PEP), a substrate for the EPSPS enzyme, to form a very stable enzyme−herbicide complex that inhibits the product-formation reaction [12].

Despite glyphosate widespread use in global crop production, its precise mode(s)-of-action and cascade metabolic effects in plants remain unclear. After inhibition of EPSPS, many physiological processes were observed to be affected by glyphosate and these could also be associated with glyphosate toxicity [13] It has been also shown that carbon and nitrogen metabolism are affected within hours after glyphosate treatment [14] i.e., total free amino acid content increases, soluble protein content decreases [15] and carbohydrate content accumulates [16].

Application of transcriptomic and proteomic methods have helped to identify the common causes/mechanisms of the effect of glyphosate-based herbicides on the metabolism of resistant plants. The accumulation of glyphosate in single-event transgenic soybean enhanced cellular oxidation, possibly through mechanisms involving stimulation of the photorespiratory pathway [17]. It also indicated that most of the glyphosate-induced genes are homologous to the known expression sequence tags - ESTs induced by abiotic stress factors [18]. *In silico* and *in vivo* studies also showed stress response, i.e. glutathione redox metabolism alteration, in glyphosate-treated resistant soybeans [19]. The photosynthesis metabolism has also been affected by herbicide application in glyphosate resistant GM maize varieties [20].

Although the use of these approaches provides a robust understanding of the genetic regulation of the response of resistant genotypes, a comprehensive picture of the metabolic manifestations of resistant genotypes conferring multiple transgenic traits is still lacking. This is partly because previous untargeted *omics* approaches were limited to the analysis of single transgene resistant plants [18][17][21][20][22].

In order to gain new insights into the response of glyphosate-based herbicide application in soybean harboring two or more transgenic events, the current study compared the effect of glyphosate on several biological processes, metabolic pathways and key enzymes of the shikimate and phenylpropanoid pathway and other cascade pathways in stacked INTACTA RR2 PRO soybean varieties. We hypothesized that transgenic plants with a combination of transgenes respond differently to glyphosate accumulation due to (1) the cost of expressing more than one heterologous protein and (2) synergistic and antagonistic interactions of each transgene cascade pathways to glyphosate direct and indirect target pathways. This study was undertaken in order to characterize the interactions between the shikimate pathway and other unsupervised side-affected pathways as determined by changes in the transcriptome of glyphosate-treated stacked transgenic soybean variety, which express the *Agrobacterium tumefaciens* strain CP4-EPSPS enzyme that is resistant to inhibition by glyphosate. We undertook this work on soybean because it is a major crop species: genetically modified lines currently occupy more than 50% of the acreage planted globally. The data presented here provide new knowledge concerning the influence of recombinant Cry1Ac (rCry1ac) transgene cassette on the defense response and glutathione metabolism, the abundance of beta-glucosidase and oxidoreductase enzymes when glyphosate is applied.

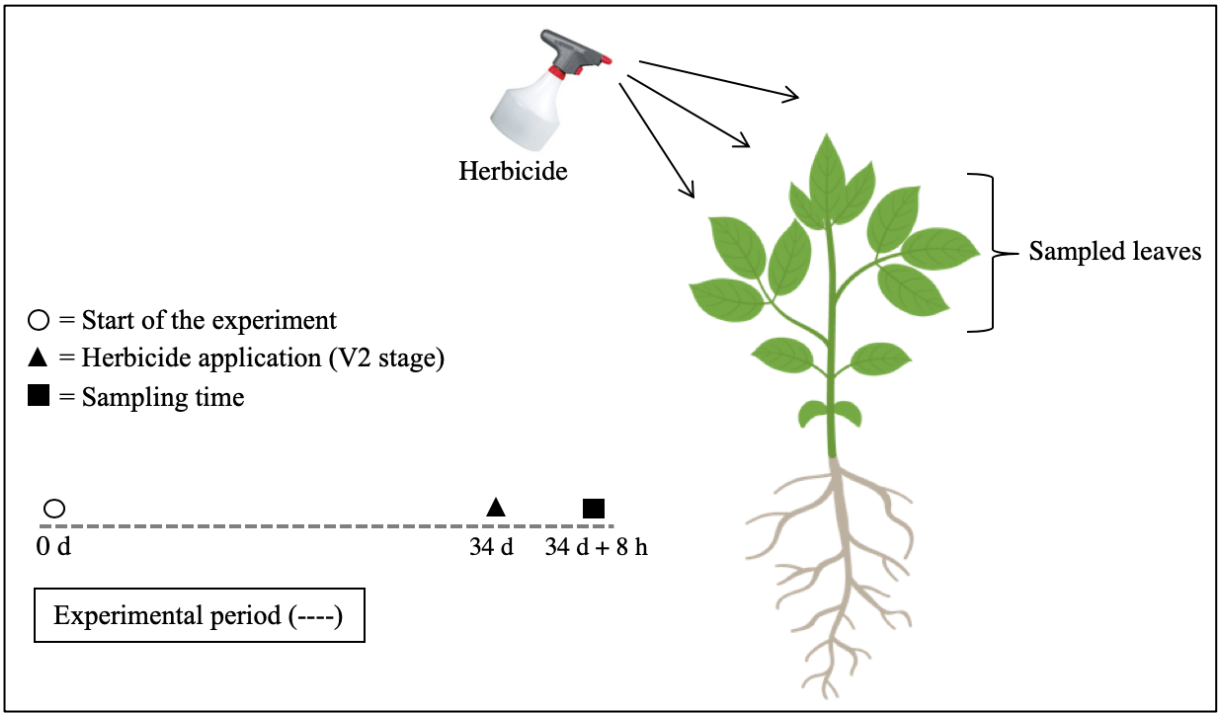
3.2 Methods

Plant material and herbicide treatment

Two soybean (*Glycine max*) varieties were used in this transcriptomic profiling study: NA 5909 RG and BRS 1001 iPRO. The first one, NA 5909 RG, is a single-event variety containing one transgenic event (GTS-40-3-2; unique identifier MON-Ø4Ø32-6) which confers herbicide tolerance (Roundup Ready® technology, Monsanto do Brasil S.A.). The second variety, BRS 1001, is considered as stacked-event variety because it contains two combined transgenic events (MON87701 and MON89788; unique identifier MON-877Ø1-2 x MON-89788-1) conferring GBH tolerance and resistance to lepidopteran species (Intacta RR2 PRO technology, Monsanto do Brasil S.A). Both commercial varieties are widely grown in Santa Catarina state and commonly found in the seed market in southern Brazil.

The experiment was conducted in a full-factorial no-choice experiment in block design with two factors: soybean variety and herbicide treatment. Seeds of each soybean variety were grown in greenhouse under two treatment conditions: herbicide spray application treated group); and no herbicide application (control group). Seeds were grown in 14 L plastic pots filled with a substrate (1/3 clay soil; 1/3 cellulose residue and 1/3 poultry organic residue) with pH corrected to 6.0. There were three plants per pot, and three pots per treatment, disposed in three random blocks. The experiment was watered daily. All plants were kept under the same conditions until they reach V2 stage, approximately 34 days after emergence, when the herbicide treatment was applied to a subset of plants (treated group). Glyphosate treatment was conducted through spray application using glyphosate-based herbicide formulation Roundup Transorb® (Monsanto company) under the general maximum dosage informed by the leaflet (4.5 L / ha; 2.2 kg a. i. / ha) for soybean crops [23]. To minimize spray contamination and drift, all plants were placed outside the greenhouse and separate into plastic barriers. Within each block (three) plants were randomized and border protected. Leaf samples were collected eight hours after herbicide application[16]. For each treatment, three leaves of the fourth trifoliate were collected from the three plants present in each pot, immediately frozen in liquid nitrogen and stored at -80°C. Therefore, each treatment contained three biological pools of three different plants, which were used for the transcriptomic analysis (Figure 1).

Fig 1. A schematic drawing of how the application and collection were performed. At the phenological stage V2, Roundup Transorb® was applied in its commercial formulation with a



manual sprayer (4.5 L / ha; 2.2 kg a.i./ha). Eight hours after application, the middle leaf of the second trifolium of each plant in the pot was collected. The leaves were rolled and stored in cryotubes and immediately placed in liquid nitrogen at -80°C.

Library construction, sequencing and mapping

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of RNA was measured using Nanodrop 2000 (Thermo Scientific), and the integrity of RNA was determined by agarose gel electrophoresis. Samples of 1-2 µg mRNA were used for library construction and sequencing. Libraries were constructed by Novogene Corporation (Sacramento, CA). Briefly, mRNA was enriched from total RNA using oligo (dT) beads. The mRNA was then randomly fragmented and cDNA was synthesized using random hexamers. After cDNA synthesis and library construction (terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment). Library concentration was quantified and then diluted to 1 ng/µl. The libraries were sequenced on the Illumina HiSeq platform (PE150). Raw reads are filtered to get rid of reads containing adapter

or with low quality to get the clean reads for analysis. The Hisat2 v2.0.4 algorithm was used to map the filtered sequenced reads to the reference genome [24].

The mapped transcripts were annotated and counted generating normalized readcount values in terms of fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEGs) between the different treatments were determined using DESeq [25]. Only genes with FDR adjusted p-value (q-value) < 0.05 after Benjamini-Hochberg correction for multiple-testing [26] were considered as significant DEGs.

Functional annotation of DEGs and bioinformatics

The DEGs were annotated for gene ontology (GO) terms using Blast2GO v2.5 [27] and categorized into Molecular Function (MF), Cellular Component (CC), and Biological Process (BP) categories. The DEGs were annotated using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis aiming to identify significantly enriched metabolic pathways or signal transduction pathways affected by the glyphosate treatment in both single and stacked varieties. Pathways with q-value < 0.05 were significantly enriched. A heatmap clustering analysis of the log10(FPKM+1) values was conducted using pheatmap library [28] in R [29] aiming to find gene expression patterns across the different treatments.

EPSPS Expression by qRT-PCR

The qRT-PCR analysis was performed aiming to quantify the expression of native and transgenic EPSPS in both single and stacked soybean varieties. Primers were designed based on the predicted coding sequence of each of the three target EPSPS genes (MON89788-1; MON4032-6; and EPSPS native) by using the online tool PrimerQuest (Integrated DNA Technologies Inc., Skokie, IL, USA) (Table S1). The cDNA was synthesized using 100 ng of total RNA from each biological replicate and the Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Real-time PCR reactions were performed using 10-fold diluted cDNA product and set up using the Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's directions and run on a StepOnePlus Real-Time PCR system (Applied Biosystems). Thermocycling conditions were: 50°C for 2 min; 95°C for 10 min; and 40 cycles at 95°C for 15 s, 60°C for 1 min, with melt curve set at 95°C for 15 s, 60°C for 1 min, 95°C

for 30 s, and 60°C for 1 min. There were three biological replicates (pool of three plants) of each sample; in addition, three technical replicates were run for each biological replicate. To normalize the gene expression, 60S ribosomal protein was selected as housekeeping gene. Relative expression levels (Rq) were calculated, based on the average cycle threshold (Ct) of the technical replicates for each biological replicate, by the ΔΔCt method using the StepOnePlus Software v2.3 (Applied Biosystems). Statistical significance was assessed through t-test ($p < 0.05$) [30].

3.3 Results

Transcriptome assembly and gene expression

An average of 44,921,097 (98.18% > Q20; 43.63% GC content) and 45,172,415 (98.12% > Q20; 44.39% GC content) reads after filtering were generated from treated and control single variety samples, respectively. Similarly, treated stacked samples generated 43,768,427 (98.10% > Q20; 44.39% GC content), while stacked control generated 45,793,340 (98.17% > Q20; 43.38% GC content) clean reads (Table 1). Robust analysis was performed once approximately 96% of the reads were mapped to the soybean reference genome (PRJNA19861, Glycine_max_v2.1) and ∼1.8 to 2.7 million reads were mapped to multiple regions.

Table 1. Summary of RNA-Seq assembly performed for single and stacked soybean varieties under herbicide treatment.

Sample	Clean Reads	> Q20 (%)	GC (%)	Total Mapped (%)
Single Control 1	43,752,142	98.52	43.88	95.48
Single Control 2	44,706,616	98.12	43.82	96.03
Single Control 3	46,304,534	97.90	43.24	95.76
Single Treated 1	49,058,110	98.46	43.90	96.11
Single Treated 2	43,749,124	98.01	44.50	95.86
Single Treated 3	42,710,012	97.91	44.77	94.01
Stacked Control 1	52,509,694	98.50	43.58	96.33
Stacked Control 2	44,592,750	98.06	43.07	96.58
Stacked Control 3	40,277,576	97.96	43.50	96.00
Stacked Treated 1	45,299,654	98.48	44.51	96.42
Stacked Treated 2	42,526,766	97.89	44.49	95.92
Stacked Treated 3	43,478,860	97.93	44.17	96.17
Average	44,913,820	98.14	43.95	95.89

In order to isolate the effect of herbicide and not account for those generated by differences in the genotype and phenotype of the varieties used, we have examined each variety separately. Therefore, differentially expressed genes (DEGs) were examined for single and stacked varieties under herbicide treatment with glyphosate commercial fórmula Roundup Transorb®. A total of 1425 (1024 up-regulated; 401 down-regulated) and 547 (522 up-regulated; 25 down-regulated) were identified as DEGs in response to herbicide treatment for the single and stacked variety respectively, when compared to the control (Figure 2).

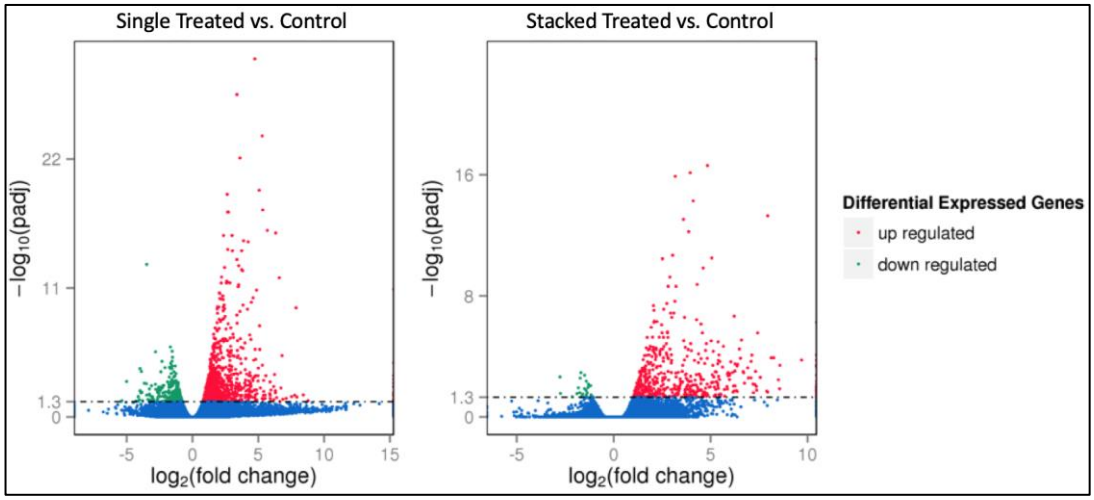


Figure 2. Volcano plot of two varieties single and stacked. The representation show the differentially expressed genes when herbicide is applied. In red were the DEGs with high regulation and in green the DEGs with low regulation. Only the DEGs with a p-adjusted value ≤ 0.05 and |FC| ≥ 1.5 were considered. The X axis represents the change in folds; The Y axis represents significance.

Additionally, we have further explored the data by running a hierarchical clustering analysis of DEGs aiming to find genes with similar expression patterns across the different varieties and treatments. The heatmap showed that gene expression data clustered according to treatment, which means that the factor 'herbicide' resulted in a major effect of variability as compared to the 'variety' factor (Figure 3). Although we have no precise information regarding the genetic background of the two commercial hybrids we have used in this experiment, such result was expected since its already known that soybean genetic diversity in Brazil is relatively low. For instance, most of the Brazilian soybean germplasm is derived from four main genotypes (CNS, S-100, Roanoke and Tokyo), which contributed to more than a half of the genetic base of all commercial cultivars released in Brazil [31] [32].

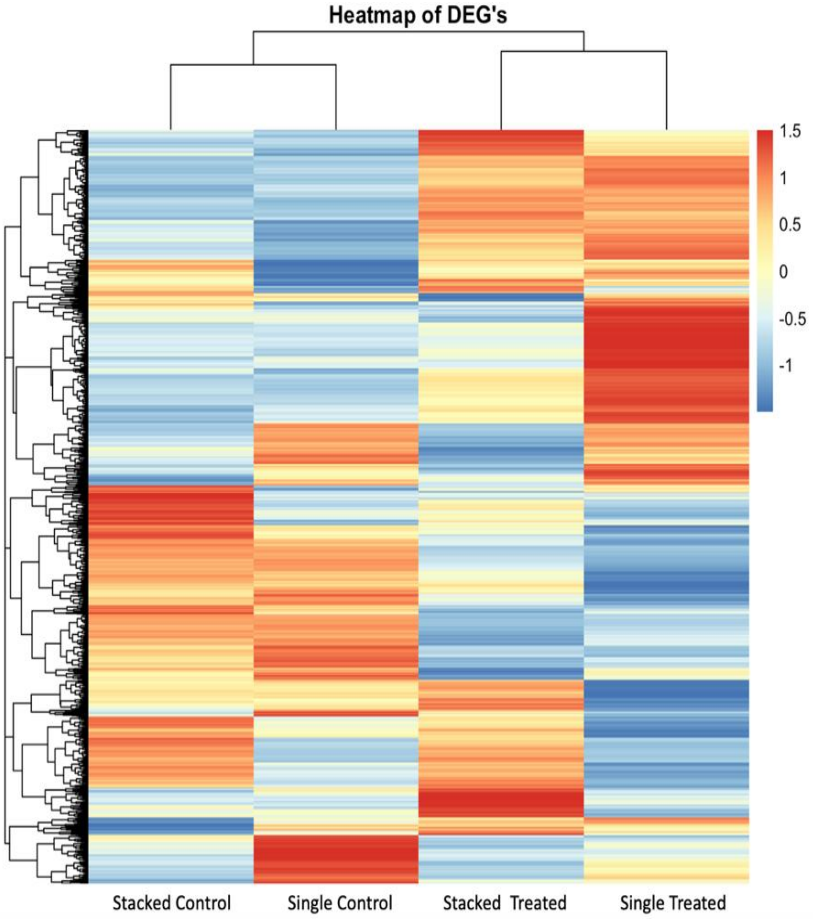
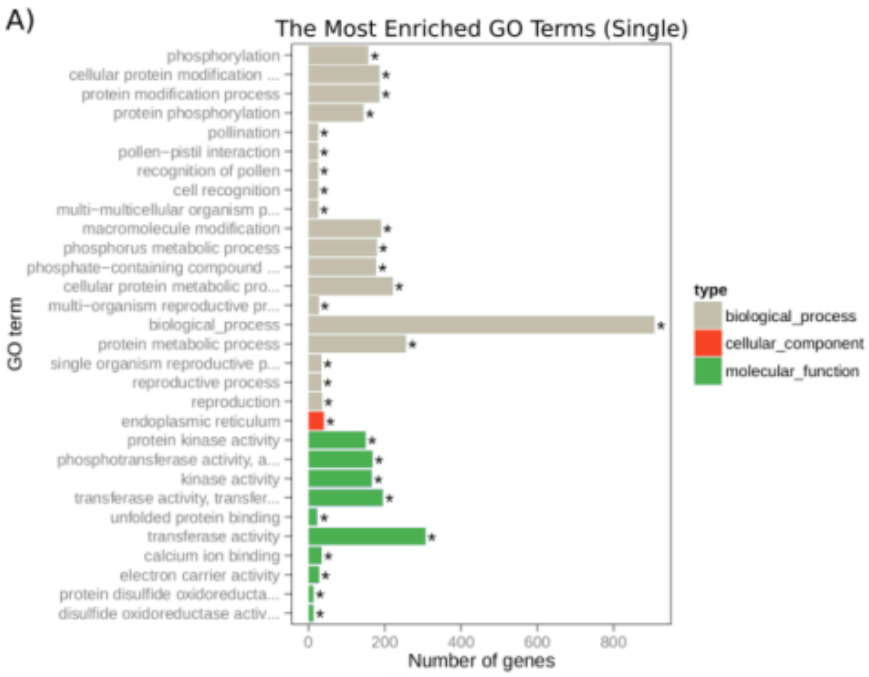


Figure 3. Hierarchical grouping of genes differentially expressed in samples in this study. The DEGs in the stacked control and single control formed a cluster due the alteration was similar, another was formed due to the herbicide factor and maybe observed through cluster Stacked Treated and the Single Treated (p adjusted value ≤ 0.05). Each line of the heat map represents the log2 values transformed with z score (1 + FPKM) of a gene differentially expressed in all samples (blue, low expression; red, high expression).

Gene Ontology annotation of DEGs

DEGs profiles for the single and stacked varieties under herbicide application were analyzed by Gene Ontology (GO) and enriched into three main domains: Biological Process (BP); Cellular Component (CG), and Molecular Function (MF) (Figure 4). In the single variety, herbicide application resulted in 16 significant Biological Process terms being up-regulated, in which the most predominant was protein metabolic processes including protein phosphorylation. A total of 6 BP terms were annotated as significantly down-regulated and the most enriched terms were photosynthesis and response to auxin (Fig. 4a). Similarly, the

stacked variety showed 10 BP terms up-regulated, all related to protein metabolism. No significant BP terms were found down-regulated (Fig. 4b). Interestingly, when looking at the Molecular Function domain, DEGs of single and stacked varieties showed different enriched GO terms in response to herbicide application. The single variety presented protein kinase activity term as the most up-regulated enriched MF terms out of 13, and copper ion binding as the only significant down-regulated MF term. Whereas the stacked variety showed a total of 8 MF terms that were significantly up-regulated, in which the most enriched terms were related to catalytic activity (i.e. oxidoreductase and fatty acid) and nucleic acid binding. No terms for down-regulated DEGs were significantly annotated. Under the Cellular Component domain, the single variety showed endoplasmatic reticulum term annotated as up-regulated, while 8 significant down-regulated terms were cellular components related to the Photosystem II. The stacked variety showed nuclear chromosome and extracellular matrix CC terms being up-regulated, and no terms were annotated as significantly down-regulated.



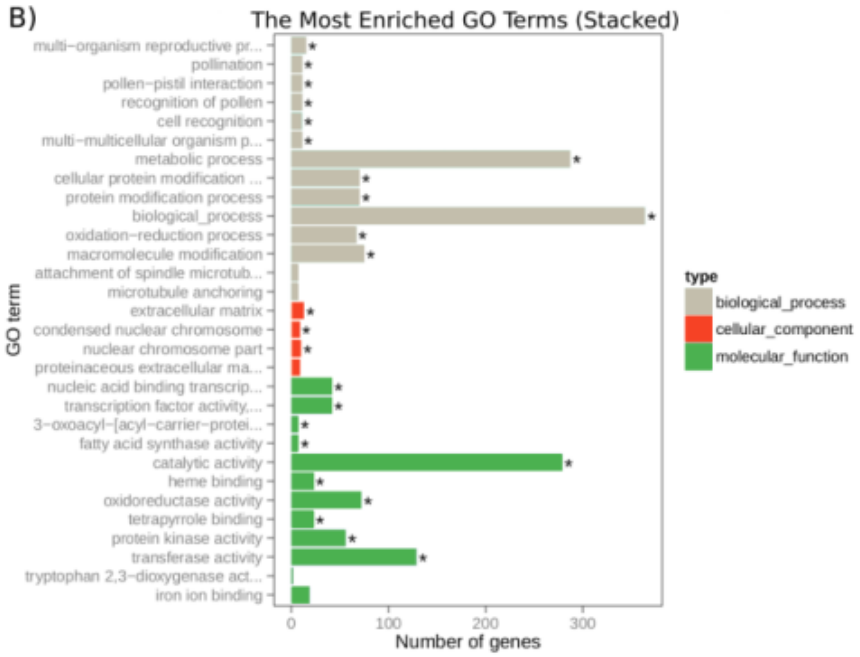


Figure 4. Ontological classification of DEGs for application of glyphosate in (a) simple and (b) stacked soybean varieties). The results are divided into three main categories or domains: biological process, cellular and molecular function. The X axis indicates the main terms of GO that are organized according to the function of genes and genetic products. The Y axis of figure (a) and (b) indicates the number of genes present in each GO term. The GO enrichment bar chart of DEGs presents the number of DEGs enriched in biological process, cellular component and molecular function. The figure shows the 30 most significant enriched terms. For the single transgene variety, the five terms GO with p-value most significative were: reproductive process of multiple organisms (p-value: 0.0027); pollination (p-value: 0.0027); pollen-pistil interaction (p-value: 0.0027); pollen recognition (p value 0.0027); cell recognition (p value: 0.0027). Alread for stacked variety, the five most significative GO terms were: cellular protein modification process (p value 1.06E-05); protein modification process (p value 1.06E-05); phosphotransferase activity, alcohol group as acceptor (p value 4.08E-05); phosphorylation (p value 8.19E-05); protein phosphorylation (p value 1.37E-04).the figure shows the 30 most significant enriched terms. For the single transgene variety, the five terms GO with p-value most significative were: reproductive process of multiple organisms (p-value: 0.0027); pollination (p-value: 0.0027); pollen-pistil interaction (p-value: 0.0027); pollen recognition (p value 0.0027); cell recognition (p value: 0.0027). Alread for stacked variety, the five most significative GO terms were: cellular protein modification process (p value 1.06E-05); protein modification process (p value 1.06E-05); phosphotransferase

activity, alcohol group as acceptor (p value 4.08E-05); phosphorylation (p value 8.19E-05); protein phosphorylation (p value 1.37E-04).

Pathways with differentially expressed genes

Aiming to understand the biological pathways activated in response to herbicide treatment, DEGs were mapped against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We have considered only DEGs with FDR adjusted p-value (q-value) < 0.05 after Benjamin-Hochberg correction, as well as pathways were significantly affected when q-value < 0.05 in the KEGG enrichment analysis. Pathway enrichment analysis of DEGs helped us to investigate whether Roundup Transorb® induced metabolic changes in soybean examining the effect of maximum herbicide dosage (2.2 kg a.i. ha) in each single and stacked variety. When looking at the single variety, the most affected pathways were: protein processing in endoplasmatic reticulum, photosynthesis – antenna proteins, phagosome, protein export, and plant-pathogen interaction; and for the stacked variety: phenylpropanoid biosynthesis, glutathione metabolism, plant-pathogen interaction, as well as protein process in endoplasmatic and plant-pathogen interaction (Figure 5).

For the single variety, the altered components are directly related to defense response mobilization. Notably, the herbicide treatment up-regulated energy and defense-related pathways while down-regulated growth-related pathways (Figure 5). For instance, carbon metabolism (22 DEGs out of 488 genes, 4.5%); N-Glycan biosynthesis (7/74, 9.45%); carbon fixation (9/129, 7%); propanoate metabolism (6/56, 10.7%), protein processing in endoplasmic reticulum (55/375, 14.6%), protein export (13/91, 14.3%), phagosome (13/265, 5,1%), monoterpenoid biosynthesis (4/14, 28.6%), and biosynthesis of amino acids (20/249, 8%) were pathways significantly up-regulated in response to the herbicide stress. Key genes involved in plant defense mobilization across such pathways could be identified: molecular chaperones from the endoplasmatic reticulum, calnexin [100810595 (log2FC = 1.4302); 547851 (log2FC= 1.6079); 100802236 (log2FC = 1.2665)] and calreticulin [100811997 (log2FC = 1.0754); 100037475 (log2FC = 1.1854)]; calmodulin like proteins [100775336 (log2FC = 2.8053); 100791253 (log2FC = 2.333); 100527439 (log2FC = 1.1821)]; WRKY transcription factors [100776837 (log2FC= 1.7459); 100811997 (log2FC = 1.0754)]; as well as glycerol kinases [547750 (log2FC = 1.3814]. Also, important energy-related genes involved in primary metabolism were identified as being up-regulated: fructose-bisphosphate

aldolase (ALDO) [100802732 (log2FC = 1.7658); 100786504 (log2FC = 1.6327)]; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [100789951 (log2FC = 1.3482)]; malate dehydrogenase [100782096 (log2FC = 1.1071)]; alanine aminotransferase [100127413 (log2FC = 0.98378)]; phosphoenolpyruvate (PEP) carboxylase [100807407 (log2FC = 1.0789)] and carboxykinase [100786257 (log2FC = 0.91215)]. On the other hand, photosynthesis and growth-related pathways showed to be significantly down-regulated under the herbicide treatment: photosynthesis - antenna proteins (13/34, 38.2%), photosynthesis (10/120, 8.3%), porphyrin and chlorophyll metabolism (6/85, 7%), pentose and glucuronate interconversion (6/194, 3.1%) (Figure 4). Most genes repressed by the herbicide treatment in the single variety are involved in the light-harvesting chlorophyll complex (LHCa and LHCb proteins) [106797220 (log2FC = -2.1825); 100802138 (log2FC = -0.92404); 100808379 (log2FC = -0.8179); 100798548 (log2FC = -0.80487); 100799813 (log2FC = -2.8689); 547819 (log2FC = -1.7924); 100805310 (log2FC = -1.3478); 100800351 (log2FC = -1.52)] in both Photosystems I and II; as well as key genes encoding for pectinases - polygalacturonase [100776623 (log2FC = -1.3251); 100786608 (log2FC = -1.5456)], pectate lyase [100781845 (log2FC = -3.2966); 100101868 (log2FC = -2.7063); 100808253 (log2FC = -2.4293); 100779940 (log2FC = -2.4338)] and pectinesterase [100776623 (log2FC = -1.3251)] - acting in the cell wall degradation from the pentose and glucuronate interconversion pathway.

Differently, the stacked variety revealed up-regulation of pathways related to the biosynthesis of secondary metabolites under herbicide application, a well-known defense response of plants under a range of stress conditions. Phenylpropanoid biosynthesis (22/332 genes, 6.63%), Glutathione metabolism (15/158, 9.5%), and Plant-pathogen interaction (18/325, 5.54%) were the most enriched up-regulated pathways in response to the herbicide stress, followed by others, such as: Cyanoamino acid metabolism (8/84, 9.5%); Flavonoid (8/86, 9.30%) and Isoflavonoid (4/17 genes, 23.53%) biosynthesis; Protein processing in endoplasmatic reticulum (12/375, 3.2%); Circadian rhythm (6/98, 6.12%); Cysteine and methionine metabolism (7/195, 3,6%); as well as sugar (starch and sucrose) metabolism (11/452, 2.43%). Not expected, Phenylalanine metabolism pathway (5/72, 8.3%), which is responsible for the synthesis of phenylalanine, one of the three aromatic amino acids inhibited by glyphosate in sensitive plants, was also significantly up-regulated for the stacked variety in the presence of glyphosate-based herbicide. More specifically, a group of genes encoding phenylalanine ammonia lyase (PAL) [100787902 (log2FC = 5.04); 100788438 (log2FC =

4.69); 100803857 (log2FC = 1.1305); 100811101 (log2FC = 2.9862)], an enzyme which eliminates ammonia from phenylalanine to form trans-cinnamic acid, a precursor of lignins and flavonoids, represented the main genes being up-regulated in the pathway. Again differently, the stacked variety under herbicide treatment showed repression of only the pathway related to protein processing in endoplasmic reticulum (4/375, 1.1%).

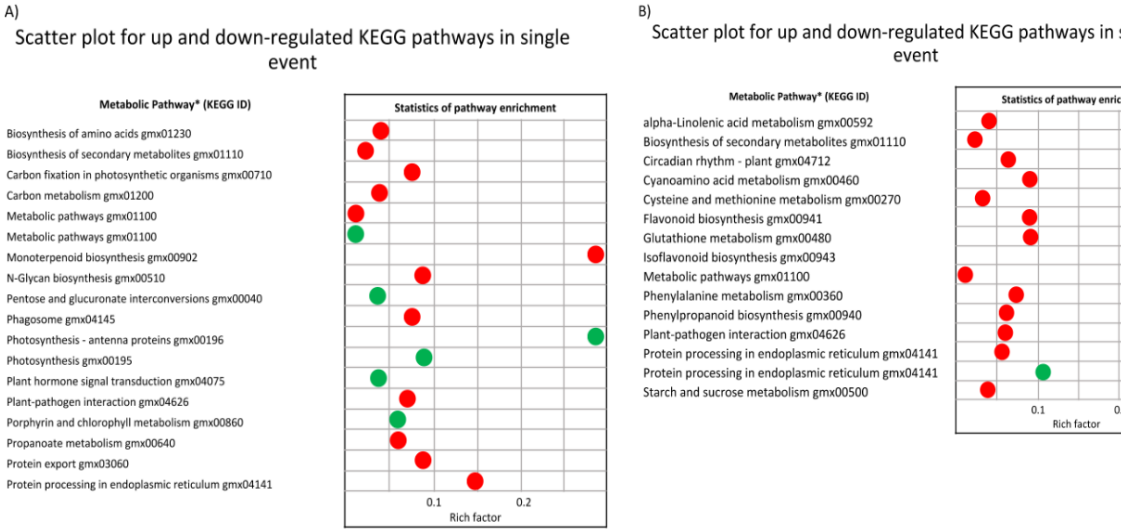


Figure 5. KEGG enrichment scatter plots from DEGs regulated up and down in simple and stacked varieties. Only statistically significant enriched pathways (p-adjusted <0.05). Circles in red are set up and circles in green are set down. Rich factor is the proportion of DEGs counted for this route in the counts of annotated genes. The more the Rich factor, the greater the degree of enrichment in the metabolic pathway.

Native and transgenic EPSPS transcript levels

We analyzed the transcript levels of native and transgenic EPSPS aiming to quantify their relative expression in both single and stacked varieties under glyphosate treatment compared to control. RT-PCR relative expression data showed that native EPSPS was significantly up-regulated in Single Treated (1.49 ± 0.34 Rq; p = 0.001), as well as in Stacked Treated (1.43 ± 0.16 Rq; p = 1.66e-5) samples, compared to their respective controls (Fig. 6a and b). Similar results were found when we looked at expression levels of native EPSPS from the RNA-seq analysis. For the single variety, herbicide treated samples showed significant up-regulation of native EPSPS transcript (27.08 ± 5.84 FPKM) compared to control plants (14.35 ± 2.46

FPKM). The same trend was found for the stacked variety in which samples presented 28.57 ± 1.04 FPKM, against 18.22 ± 4.72 FPKM for control samples.

On the other hand, relative expression of transgenic EPSPS in the single variety (MON4032-6) showed significant down-regulation after herbicide application (0.79 ± 0.16 Rq; p = 9.67e-7) compared to control samples (Fig. 6c). The same pattern was found for the transgenic EPSPS present in the stacked variety (MON89788-1), in which expression levels in Stacked Treated samples was cut by half (0.47 ± 0.36 Rq; p = 5.33e-11) compared to untreated control samples (Fig. 6d).

3.4 Discussion

Impact of glyphosate in the shikimate pathway and cascade effects

Glyphosate affects sensitive plants by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), an enzyme from the shikimate pathway, which leads to prevention of the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan [33]. In transgenic resistant plants, a synthetic low-affinity EPSPS gene sequence (*Agrobacterium* sp. Strain CP4) is inserted into the genome of commercial crop plants, thus making them tolerant to glyphosate [34].

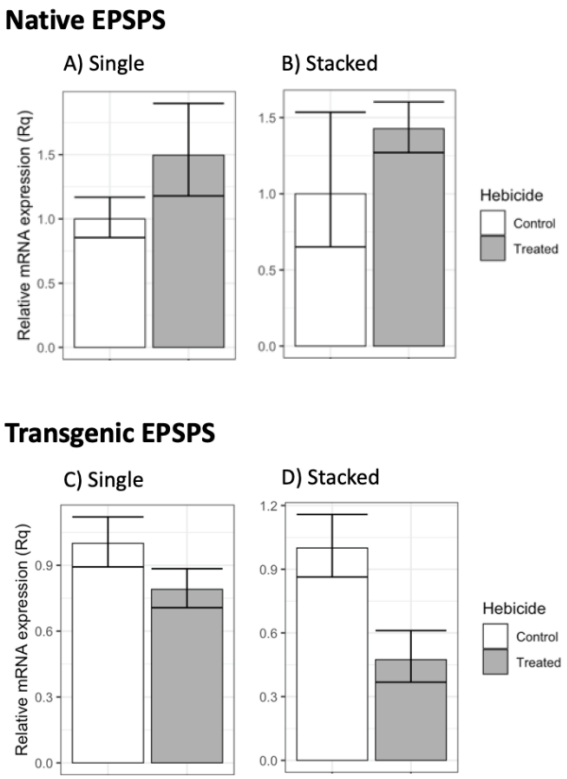


Figure 6. Representation of levels of native and transgenic EPSPS expression found in the simple variety and stacked without application of Roundup and with application. For the native EPSPS the two events (a) and (b) show the same level of expression. Transgenic CP4-EPSPS showed decreased expression when herbicide was applied in both events (c) and (d). The bars in the figures show the standard deviation.

Despite the widespread use of glyphosate-based herbicides in agriculture, major questions remain concerning how this herbicide affects cell metabolism and physiology in glyphosate-resistant plants and, more importantly, if there are antagonistic or synergistic effects in stacked transgenic varieties. In order to address these questions, we have profiled the transcriptomic changes after glyphosate treatment in stacked versus single glyphosate-resistant soybean varieties and characterized the interactions between amino acid metabolism through the shikimate pathway and other unsupervised metabolic pathways, as determined by changes in statistically enriched defense, carbon, cellular redox and photosynthesis related metabolic pathways.

We were interested in GBH effects at native and transgenic EPSPS protein expression levels and whether changes might have a cascade impact in other metabolic pathways. Therefore, we have quantified EPSPS transcripts through RNA-Seq and real-time qPCR using primers

located at distinct sequences in both EPSPS versions. Our results showed that while native EPSPS expression was up-regulated at the same level for both single and stacked events (approx. 1.5 log2FC); transgenic CP4-EPSPS showed a decrease in transcript accumulation also in both single and stacked varieties (approx. 60% expression level). Although stably integrated into the genome, variable and non-directional levels of CP4 EPSPS was observed with other factors like genetic background, trait stacking, growing region or season [35]. But the extent to which detection protocols can differentiate both versions of EPSPS is unclear in previous studies and might partially explain the deviations. It is uncertain why cp4-EPSPS is down-regulated after GBH spray.

Interestingly, while cp4-EPSPS has been modulated in the same manner in both single and stacked varieties; differences in the metabolism has been observed and thus suggests it is not related to either native or transgenic EPSPS modulation.

The impact of directly related metabolic pathways to shikimate pathway has been observed in both single and stacked variety. However, in the stacked variety the impact on the isoflavonoid, flavonoid, glutathione, cysteine and methionine and phenylalanine metabolism were most prominent (Figure 7). In addition, alteration in the jasmonic acid metabolism was also observed. Increased levels of jasmonic acid have been also observed after glyphosate and drought stress application in NK603 herbicide resistant GM maize [20]. Glyphosate was also shown to interfere with other hormones such as ethylene [36] and abscisic acid (ABA) [16]. Despite its projected resistance to the primary mode of action of glyphosate, it has been shown that impairment of physiological defenses of diseases occurs in some GR cultivars. Some cases demonstrate that the application of glyphosate reduced the absorption content of macro and microelements by GM soy when applied HBGs disrupted by crop nutrition [37] [38] [39], as well as declines in the production of plant biomass [40] and reduction in the number of nodules and reduction in the production of dry mass have been observed [38] [41]. Intermediates in the shikimate pathway also are a starting point for the biosynthesis of by-products, thus, this path is of great importance for the of several compounds [42]. For example, those involved in growth and development, in plant defense, and photosynthesis activities [43]. In our study, the changes observed in figure 7, indicate that HBG is not at all harmless. In the staked soybean variety we observed changes demonstrate that there is a potential for the action of the herbicide not limited to the EPSPS pathway and can trigger the disorder in several metabolic pathways. This finding is also indicated in the review article by Martinez et al. [44]. There are already reports of GM plants that demonstrate vulnerabilities of

physiological defenses after the application of GBH in subaltern doses [45] [10]. In this sense, studies investigate that GM plants submitted to HBG would be more favorable to the appearance and installation of diseases. This is because, the content of lignin is associated with the morphological and functional quality of the organs when plants are deprived of adequate lignin content they are vulnerable to diseases [46]. Zobiole et al. [47] shows that in comparison with the control, in GM soy decreased sharply the lignin content with the increase in the rates of application of glyphosate (isopropylamine salt). It is known that lignin production is controlled by phenylalanine (Phe), a key product of the shikimate pathway (amino acid was altered in this study, including Phe-dependent products were also affected) [47]. It is conventionally understood that GR cultures should not have altered amino acid content after treatment with GBH, due to the main mode of action of HBG in the plant's EPSPS [48]. However, it is also investigated that in addition to phenylalanine, AMPA, a glyphosate degradation product can also influence the amino acid glycine in soy GR due to chemical similarity and, therefore [48][49]. This can have indirect effects on various plant physiological functions, including nutrient absorption and amino acid requirement in biological sites and disturbance in metabolic pathways [44].

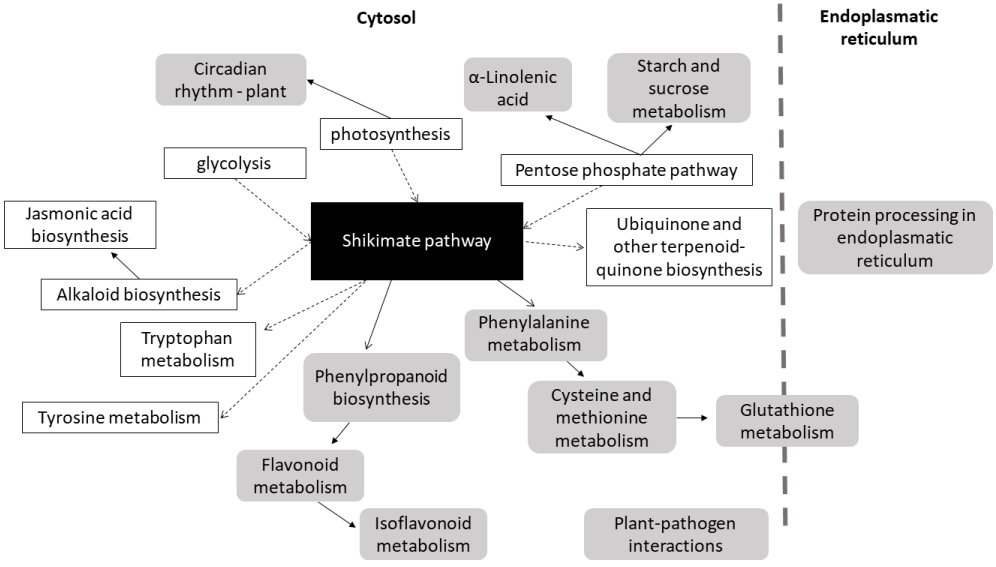


Figure 7. Schematic representation of the response to stress caused by glyphosate-based herbicides (HBG) by the stacked variety. The figure shows that the herbicide triggered a series of changes in the pathways and products related to the biosynthesis of secondary compounds. The gray boxes show the metabolic pathways that were altered in response to the herbicide. The blank boxes are known routes that are reported to be altered by the herbicide but which have not changed for this variety. Filled arrows demonstrate direct relationships

between the metabolic routes affected by herbicide, the dashed lines, show the relationships between the shikimate route and related routes. The action of HBG leads to oxidative stress in plants, a likely side effect of the shikimate pathway. Altered enzymes have been observed in glutathione metabolism. Reactive oxygen species (ROS) have also been observed from differentially expressed genes (DEGs) in the route of plant-pathogen interactions. We cannot conclude that the above impacts will occur in all cases. The environmental conditions, the cultivars, and the individual agricultural practices will influence the result, however, in the study in question, HBG demonstrated to be potential disruptors of the secondary metabolism of stacked soybean varieties.

Strong Defense Response triggered by HBG

Defense imposes a substantial demand for resources that can negatively impact growth and diminish the overall set of energy reserves and/or promote resource diversion for growth, defense, and reduction of photosynthesis [50]. Previous transcriptome studies using microarray technique to investigate the metabolic impact of glyphosate treatment in susceptible and resistant soybean, Arabidopsis and brassica showed that most affected pathways are involved with defense metabolism [18][51][16]. In this study, genes related to defense metabolism were up-regulated, indicating that glyphosate may be related to alterations in gene cascades and unexpected pathways.

To defend themselves from adverse situations, plants need to mobilize a rapid response. Increases in calcium (Ca 2+) rates are essential to coordinate adaptive responses in various species[52][53]. The single and stacked varieties signaled a defense response with increased prominent calcium-related pathways. Calmodulin protein families were found for both single (two altered genes, average 2.10 log2FC) and stacked (five altered genes, average 3.3 log2FC) varieties. Previous studies with glyphosate application in sensitive soybean also observed changes in calcium-related genes regardless of herbicide concentrations and collection time after application (4 and 24 hours) [54][16]. The Ca2+/CaM complex play key roles in plant metabolism as it is a signal transduction pathway involved in turgor regulation[55]. In addition, cytosolic Ca2+ concentration controlled by ion channels in the plasma membrane of guard cells can modulate further cellular responses by promoting stomatal closure [56][57]. Considering calcium as one of the fundamental actors for the full functioning of the stoma, its accumulation may be involved in the imbalance between stomatal opening and closing.

Rapid recognition of injuries by cellular signal transduction pathways occurs through various signaling molecules, including calcium, protein phosphorylation and ROS, which are well-known triggers of stress resistance in plants[58]. Herbicides are considered abiotic stressors that can disrupt the balance between the production and elimination of reactive oxygen species (ROS) [59]. There is a close relationship between calcium-dependent ROS production and a specific group of genes. For example, the respiratory burst oxidase homolog (Rboh) gene family. Activation of this group occurs after the recognition of pathogens and a variety of other processes [60][61]. We observed strong up-regulation of the Rboh group (3.58 log2FC) in the stacked variety. Such oxidases have been reported as key factors in activating innate and mobilized immunity during oxidative stress damage [62].

Another example of defense regulatory circuit was the identification of WRKY transcription factors that is phosphorylated by MAPK and a W-box in the promoter region of Nicotiana tabacum Rboh, interconnecting the phosphorylation events of MAPK in response to pathogen recognition with the accumulation of Rboh protein [61]. Strict regulation and fine-tuning of WRKY proteins are directly linked to plant stress signaling responses [63][64], such as saline stress[65], drought[66][67] and heat stress [68]. We observed up-regulation of WRKY genes in both varieties, with higher expression and number of genes in the stacked variety (five genes with an average of 2.5 fold change). WRKY genes have not yet been found affected by HBG.

The pathogenesis-related proteins (PR), known as an indispensable component of innate immune responses in plants under biotic or abiotic stress conditions were also observed in this study. In the single-variety, we find one gene PR1, up-regulated with a 2.9 log2FC. These proteins are also involved in hypersensitive response or systemic acquired resistance against a variety of plant infections [69] and an important response mechanism to multiple stresses [70]. PR proteins are considered the signature genes of salicylic acid and jasmonic acid pathways in many crop plants [71][72][70][73].

Immune sensors in plants are well-known substrates for heat shock proteins, such as heat shock protein 90 (Hsp90). To recognize potential pathogens, higher eukaryotic organisms use extra- or intracellular sensors as the initial switch in the induction of disease defense responses[74][75]. Beyond defense, the environmentally responsive HSP90 chaperone complex is suggested to be involved in multiple signaling cascades, with the potential to be of great importance for sensing the environment and mediating appropriate phenotypic plasticity [76]. In our study, Hsp90 gene families were found up-regulated in the both single and

stacked varieties. In soybean, Hsp90 gene was induced by heat, salt, and osmotic stresses but the response times and expression abundances were diverse [77].

Many plant defense compounds are used in a non-active glycosylated form suitable for storage in the vacuole and further protection from toxic side-effects as a consequence of its defense system [78]. These are recognized as class of secondary metabolites called phytoanticipins. When plant tissue in which they are present is disrupted, the phytoanticipins are bio-activated by the enzymatic removal of a protecting glucose group by a β-glucosidase. These are binary systems in which two sets of components that, when separated, are relatively inert provide plants with an immediate chemical defense against protruding herbivores and pathogens [79]. Strikingly, the stacked variety up-regulated seven β-glucosidase-related genes with an average of 5 log2FC. We also found a regulated isoflavone 7-O-methyltransferase gene found 8.5 log2FC. This specific change in the metabolism of secondary metabolites has not been reported before in plants treated with glyphosate. Thus, suggesting that the presence of rCry1Ac transgenic cassette has an impact on the defense metabolism when glyphosate is sprayed.

Changes in Carbon Allocation

The insertion of transgenes controlled by strong promoters has been always a concern as to the potential physiological effects on carbon allocation metabolism. In this paper, we applied glyphosate, an inhibitor of the enzyme EPSPS, present in the shikimate pathway as an abiotic stressor at concentrations present in real HR crop fields.

While under normal growth conditions, more than 20% of plant-fixed carbon flows through the shikimate pathway [80] [81]. Under stress, plants mobilize their carbon stocks to transform energy and resist harmful effects on cells. In our study, in the single variety, we observed the up-regulation of enzymes related to energy transformation processes and structural functions, such as lactate dehydrogenase, which participates in the process of transforming glucose into energy formed from pyruvate [82] and N-acetylglucosamine, a cellulose analogous structural polysaccharide, involved in structural roles on the cell surface [83].

The total biomass production of soybean depends on energy supplied by photosynthesis for synthesizing carbon compounds [84]. Alteration in carbon metabolism has been already observed after glyphosate-based herbicide application. This occurs because the inhibition of

EPSPS deregulates the pathway, which results in an uncontrolled flow of carbon and subsequent massive accumulation of shikimate and other acids in metabolic sinks such as leaves and nodules of legumes [85]. Two of the major metabolic checkpoints co-ordinating primary nitrogen and carbon assimilation in leaves are nitrate reductase (NR) and PEPC [86].

The enzyme PEPC has been found up-regulate and is biologically related to maintaining load balance during the upward flow of xylem sap in vegetables [87] [88] and in the supply of substrates for symbiotic organism, developing a central role for biological nitrogen fixation [89]. Our data demonstrate a relationship of up-regulated genes that involve balancing the energy supply to the nodules and retaining sufficient carbon for growth, at a level of change beyond the PECP enzyme. Several PEPC isoforms are controlled by an interaction between allosteric regulation and reversible phosphorylation. In legume leaves and root nodules, these regulatory functions of PEPC are governed primarily by phosphoenolpyruvate carboxykinase (PEPc Kinase) (Also found as altered in this study) [90] reversible protein phosphorylation is likely to be of major importance in controlling legume nodule carbon metabolism and related metabolite transport [91]. PEPc Kinase is a member of the Ca2+/calmodulin-regulated group of protein kinases. However, it lacks the auto-inhibitory region and EF-hands of plant Ca2+-dependent protein kinases [92]. Regulatory mechanisms related to the formation and maintenance of root nodules for biological nitrogen fixation are fundamental for the proper adjustment of metabolic flow between host plants and symbolic organisms [93]. In the cytoplasmic compartment of plants, glucose and fructose-free hexoses (two genes for fructose bisphosphate aldolase were found with an average of 1.7 log2FC) are phosphorylated by glucose or pentose pathways [94][95]. PEPC and malate dehydrogenase (a gene also found in this 1.7 log2FC study) convert the carbon flux of glycolysis to malate [96] which is used as carbon skeletons for N2 amino acid synthesis [97] [98]. Although malate is the main source of energy for symbiotic organisms, high volume malate may inhibit N2 fixation and nitrogen uptake [99].

This leads to the belief that in the single variety there was a change in carbon metabolism relative to storage strategies. Both in the structural form and in the carbon flow demand required by nodular organisms. This may be involved in the change in carbon flow required for growth and development.

In the stock variety, on the other hand, carbon appears to be stored as starch, the carbohydrate used as the energy source for the defense response. We find altered starch and sucrose metabolism, especially gene related to trehalose-phosphate with 3.6 log2FC. Sucrose and

starch management and balance promote optimization of growth rates [100][101]. Trehalose (α-d-glucopyranosyl-1,1-α-d-glucopyranoside) is a nonreducing disaccharide that is found in many organisms and has various functions: osmolyte, storage reserve, transport sugar, and stress protectant [102]. It is also involved in growth and development metabolism [103] with clear links to abscisic acid and auxin signaling [104] as well as to the activation of starch synthesis [105]. The levels of trehalose increase in response to osmotic stress [106] as well as to dehydration stress tolerance [107][106]. Trehalose is one of the most effective osmoregulatory sugars in terms of the minimal concentration required to establish a normal balance [108]. Many plants accumulate substantial starch reserves in their leaves to provide carbon and energy for maintenance and growth [109][110]. Therefore, the accumulation of soluble sugars, such as trehalose, is suggested to be a protective mechanism under oxidative stress conditions [111][112]. Wingler et al.[113] showed a strong accumulation of starch in response to trehalose [113]. In this study, in the stacked variety we suggest that the trehalose transcripts may also be involved in the accumulation of starch, carbohydrate required to cope in the energy balance due to the need for response observed by up-regulation in various secondary metabolites (flavonoids, isoflavonoids, phenylpropanoids).

Altered Cellular Redox Homeostasis

Exposure to glyphosate-based herbicides is directly linked to accumulation of antioxidant enzymes, indicating that glyphosate treatment might result in oxidative stress [114]. Glutathione (GSH) is a key of the complex antioxidant network in plants, acting to control ROS accumulation and facilitating cellular redox homeostasis especially under stress conditions [115]. For instance, GSH plays an important role in herbicide detoxification via the glutathione S-transferase (GST) system [116]. We found evidence for cellular detoxification response through significant up-regulation of GST in both soybean varieties under herbicide stress (Single: average log2FC = 3.1; Stacked: average log2FC = 3.5).

On the other hand, other genes encoding important enzymes related to glutathione metabolism showed to be differently affected in the single and stacked varieties, revealing that both genotypes may respond in a different manner in response to oxidative stress. For instance, we found glucose 6-phosphate dehydrogenase (G6PDH) – an enzyme participating in the first two reactions of oxidative pentose phosphate pathway - being significantly down-regulated in the single variety. Reduced levels of G6PDH is related to glutathione depletion and

consequent high oxidative stress in the cell [117]. It is known that reduced glutathione (GSH) is required to combat oxidative stress and maintain the normal reduced state in the cell, a phenomenon known as the redox homeostasis [114][115][17]. Oxidized glutathione (GSSG) is reduced to GSH by NADPH generated by G6PDH in the pentose phosphate pathway [118]. Complete depletion of glutathione in its reduced form (GSH), or the production of GSSG from GSH, with concomitant accumulation of formaldehyde have already been reported as signs of undergoing oxidative stress in single-event GM soybean varieties as compared to its non-GM isogenic line [21][19]. On the other hand, for the stacked variety, although G6PDH gene expression has not been significantly affected, herbicide treatment up-regulated the expression of 6-phosphogluconate dehydrogenase (6PGDH) gene (log2FC = 1.25). 6PGDH, a second enzyme participating in the OPPP, catalyses the NADP-dependent oxidative decarboxylation of 6-phosphogluconate generating NADPH and ribulose-5-phosphate, a precursor for the synthesis of nucleotides and nucleic acids [119]. We hypothesize that the production of such reducing equivalents is being used in further reductive reactions in stacked plants, such as keeping GSH in its reduced form, aiming at maintaining the cell redox homeostasis.

Our results also showed protein processing in endoplasmatic reticulum (ER) as one of the most up-regulated pathways in both, single and stacked varieties when glyphosate is applied. Glutathione homeostasis in response to oxidative stress has been also described as active in the ER [120]. A diverse range of genes encoding important molecular chaperones guiding secretory folding proteins, as well as ubiquitin-proteasomes responsible for exporting and degradation of misfolded proteins, were shown to be significantly up-regulated in the presence of glyphosate. Interestingly, the ER protein processing-related genes was much higher in terms of number of genes and expression levels in the single variety when compared to the stacked one. For instance, chaperones/folding enzymes [i.e. calreticulin, protein disulfide-isomerase (PDI), and glucose regulated protein 94 (GRP94)], enzymes involved in the cytosol-to-ER and ER-to-cytosol transport of glutathione [i.e. endoplasmic reticulum oxidoreductin-1 (Ero1), binding immunoglobulin protein (BiP), B-cell receptor associated protein 31 (Bap31), and protein transport Sec 61] were exclusively up-regulated in the single variety. In agreement, ER was annotated as the most up-regulated cellular component term under the GO enrichment analysis for the single variety. The only ER genes substantially affected in the stacked variety were those encoding for ER-associated degradation (ERAD)

enzymes, such as derlin, Hsp40, Hsp70, Hsp90, and small heat shock factors (sHSF), which were also affected in the single variety.

Abiotic stress causes significant increase in protein unfolding metabolism, leading to the accumulation of misfolded proteins in the ER [121]. Such accumulation triggers the increase in degradation capacity of ERAD system aiming to maintain ER homeostasis. Over time, this process can lead to a variety of cellular signaling pathways which determine the state and fate of cell, which can include autophagy, apoptosis, inflammation, and even activation of cell death under severe conditions [122]. In our study, the metabolic responses to the oxidative stress caused by HBG seems to be highly correlated to ER-related genes; most probably due to GSH depletion or elevated production of GSSG as already suggested by previous studies [17] [19]. Since glutathione is oxidized, transport proteins must export GSSG from the ER to the cytosol aiming to reach an ideal glutathione homeostasis [120]. Conversely, the stacked variety showed evidence of oxidative stress responses due to the up-regulation of cytosolic glutathione genes (GST log2FC = 3.5; 6PGDH log2FC = 1.25), while only genes encoding ERAD enzymes were significantly up-regulated in ER. Vivancos et al. [17] have also found effects of herbicide on cellular redox homeostasis of single event glyphosate-resistant soybean variety. More specifically, the authors reported that accumulation of high levels of glyphosate in GM tolerant plants have enhanced cellular oxidation, possibly through mechanisms involving increasing of photorespiratory pathway [17]. Moreover, a recent integrative in silico model of C1 metabolism in single event glyphosate-resistant GM soybean, predicted complete depletion of glutathione and accumulation of formaldehyde as a result of oxidative stress compared to its non-GM counterpart. The authors alert on how a single event modification can potentially create a large perturbation to molecular system equilibria [21]. According to our findings, single and stacked GM soybean showed oxidative stress at different levels and cellular components.

Photosynthesis imbalance

HBG has been shown to have detrimental effects on many plant physiological and biochemical processes, which reduce photosynthesis efficiency and inhibits chlorophyll function [123][41][49]. Chlorophyll is essential in photosynthesis and provides matter and energy for plant growth [124]. The concentration of total chlorophyll is the sum of chlorophyll A and chlorophyll B [125][126]. In our study, the single variety showed a

decrease in the light-harvesting chlorophyll A and B contend (complex I of class LhcA 2,3 and 4 with four genes involved, and the complex II of class LhcB 1,2,3 and 6 with nine genes involved). These findings are supported by Li et al.[127], who also observed a decline in the content of chlorophyll A and B in GM and conventional soybean varieties under glyphosate treatment [127].

After light excitation of chlorophyll molecules in the light-harvesting complexes, the energy is transferred to the reaction centers of photosystems I (PSI) and II (PSII) [128]. The electron transfer chain, which mediates the transmembrane charge separation, is the functionally most important part of photosystem [129]. Ferredoxins (FDXs) in chloroplasts are electron transfer proteins that deliver reducing equivalents from PSI in photosynthetic organisms [130]. Electrons from reduced FDXs are accepted by FDX-NADPH-oxidoreductase to generate NADPH, which is required for carbon assimilation in the Calvin cycle [131]. Limited capacity of electron transport after glyphosate exposure was shown by [123][17]. In this study, we find two genes down-regulated related to putative FDX. The amount of FDX is also decreased in tobacco under various stresses, including those from herbicide treatment [132].

In general, we have observed, down-regulation of genes involved in photosynthesis and this is in agreement with previous studies when GBH application is performed. Iquebal et al. [133] observed that genes involved in the photosynthetic pathway were deregulated after exposure to herbicides in resistant chickpea variety [133]. In Lolium perene sensitive plants, chlorophyll fluorescence was also affected by glyphosate [134].

Relevance to risk assessment of stacked GM crops

Worldwide, a growing number of GM crops with stacked transgenic traits are being developed to confer resistance to herbicide active ingredients and some insect species. For most varieties, the single events might never reach market and pre-market risk assessment. Therefore, an assessment of the risks of a stacked GM plant to cause combinatorial and cumulative effects should be considered in the context of the closely related non-modified recipient organism in the receiving environment.

Omics profiling analysis can contribute to the identification of combinatorial effects that may occur due to interactions among the proteins and metabolites produced by the transgenes or endogenous genes of a stacked GM plant. In addition, interactions between the stacked transgenes or their products, or interactions among the physiological pathways in which the

transgenes are involved, taking into account the possibility that these interactions could result in potentially harmful substances, such as anti-nutritional factors, some of which may persist or accumulate in the environment should be also considered.

Stacked GM plants can be produced through different approaches. In addition to the cross-breeding of two GM plants, multiple traits can be also achieved by the natural cross of transgenic lines that have been found in crop field boundaries [135][136], such as feral transgenic canola outside of cultivation [137][138].

Accordingly, it is reasonable to anticipate future occurrence of stacked traits within ruderal and wild populations. Despite the potential for the formation of feral populations with multiple transgenes, we have little understanding of how these traits could migrate, evolve or influence native and naturalized plant communities. Thus, such profiling studies could generate useful information to assist risk assessment of stacked GM crops and potential feral populations.

3.5 Conclusions

In conclusions, the RT-PCR results showed that native and transgenic EPSPS expression was regulated for single and stacked events. Although modulated in the same way, differences in metabolism were observed and therefore suggest a cascade of effects of various metabolic pathways resulting from glyphosate application. Our RNA-seq data supports this information. We show that although the varieties tested have similar responses in some ways when we analyze route by route, it is possible to suggest that the single-variety exhibited similar overall behavior to susceptible plants when glyphosate-based herbicide is applied, which is expected as a physiological response when plants are subjected to stress (increased sugar content, significant decrease in light harvest of chlorophyll A and B and impairment of redox cell balance). On the other hand, the stacked variety showed strong up-regulation of secondary metabolite accumulation as well as defense components and redox equilibrium mechanisms. We found Ca2 + signaling responses and several up-regulated molecular chaperones in both varieties. However, distinct stress responses were found among the varieties. The stacked variety showed a more pronounced stress response (activation of specific stress defense proteins (Rboh, WRKY) and secondary compounds (β-glucosidase, isoflavone 7-O-methyltransferase).

Both varieties were energetically impacted, the single showed changes in carbon metabolism

about storage and accumulation strategies, while the stacked variety showed starch accumulation we have found a regulated response to trehalose and suggest that this carbohydrate is being stored to help regulate energy balance due to the regulation of some secondary metabolites (flavonoids, isoflavonoids, monoterpenoids). There were responses to cellular detoxification through significant up-regulation of GST in both varieties, but in the stacked, we hypothesized that the production of some reducing equivalents is being used in additional reductive reactions as a way to keep GST low to maintain cellular redox homeostasis.

Similar results to ours for the single variety were also found using single GM with glyphosate-based herbicide application and omic approaches. However, this is one of the first studies to use the RNA-seq approach on bet varieties to track transcriptomic profiles. Profile approaches that allow unbiased comparisons contribute to the knowledge of biological processes that regulate plant composition and the interactions between genes, RNA, proteins, and metabolites of a stacked GM plant. In addition, they have been shown to have biological relevance to the possible safety implications of such changes in genetically modified plants.

List of abbreviations

GM: genetically modified; EU: Europe Union; GMOs: Organism genetically modified; EPSPS: 5-enolpyruvylshikimate-3-phosphate-synthase; PEPC: phosphoenolpyruvate carboxylase; PEPc Kinase: phosphoenolpyruvate carboxykinase; CP4-EPSPS: Agrobacterium tumefaciens strain CP4 5-enolpyruvylshikimate-3-phosphate-synthase; CRY: Crystal Baccilus turigiensis; RNA: ribonucleic acid; mRNA: messenger ribinucleic acid; cDNA: complementary deoxyribonucleic acid; rtPCR: reverse transcription polymerase chain reactions; FPKM: fragments per kilobase of transcript per million mapped reads; DEGs: Differentially expressed genes; GO: gene ontology; MF: Molecular Function; CC: Cellular Component; BP: Biological Process; KEGG: Kyoto Encyclopedia of Genes and Genomes; Rq: Relative expression levels; Ct: cycle threshold; PAL: Phenylalanine ammonia lyase; CaMCML: calcium-related pathways/ Calmodulin protein families; Ca 2+: calcium-related genes; ROS: reactive oxygen species; Rboh: respiratory burst oxidase homolog; NADPH oxidase: Reduced nicotinamide adenine dinucleotide phosphate oxidase; WRKY: transcription factors; HR: hypersensitive response; SAR: systemic acquired resistance; SA: salicylic acid; JA: jasmonic acid; PR: pathogenesis-related proteins; LDH: Lactate dehydrogenase; MDH: Malate dehydrogenase; Nitrate reductase (NR); GSH: Glutathione;

GST: Glutathione S-transferase; OPPP: Reactions of oxidative pentose phosphate pathway; G6PDH: Glucose 6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase ; FDR: False discovery rate; FLNC: Full-length non-chimeric reads; RNA-Seq: RNA sequencing, RT-qPCR: Quantitative reverse transcription PCR, SRA: Sequence Read Archive.

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4 CAPÍTULO - 2

Análise de resíduo de subprodutos de Roundup Transorb® e componentes nos grãos de G*lycine max (L.) Merr.*

4.1 INTRODUÇÃO

A crença de que o Critério da Equivalência Substancial (CES) é um conceito do ponto de vista científico é controverso (MILLSTONE; BRUNNER; MAYER, 1999; ZATERKA, 2019). Na medida em que se adota o CES, a discussão limita-se a uma análise quantitativa e probabilistica de alguns parâmetros composicionais previamente estabelecidos (PELAEZ, 2004). Em termos de genoma, uma planta transgênica e sua convencional não são equivalentes nem iguais. A construção genética inserida em uma planta GM contém elementos distintos daqueles encontrados naturalmente nas plantas, efeitos de diversas ordens são desconsiderados (NODARI; GUERRA, 2001).

No início dos anos 90, o termo foi estabelecido pela Organização de Alimentos e Agricultura das Nações Unidas (FAO) e Organização para Cooperação e Desenvolvimento Econômico (OCDE) e se restringe a comparação direta entre a cultura melhorada através da utilização de técnicas de biotecnologia e uma planta controle, o mais isogênica possível, com base nos níveis dos principais componentes e antinutrientes (FAO; WHO, 1991). O termo foi amplamente adotado pelos pesquisadores das indústrias e agências regulatórias de alguns países (ex: Estados Unidos, Argentina e Brasil) que se utilizam desse critério para liberação e comercialização de produtos oriundos de organismos geneticamente modificados (OGM).

Em 1996, a soja GM Roundup Ready (RR) é liberada pelo Departamento de Agricultura dos Estados Unidos (USDA-EUA) e pela Administração de Alimentos e Medicamentos (FDA) sob a justificativa de que a proteína recém-introduzida é segura e de que a modificação genética não alterou a segurança alimentar do grão, uma vez que a soja transgênica foi considerada substancialmene equivalente à soja convencional. Em seguida, a soja RR também foi liberada por agências reguladoras em outros países com a mesma justificativa (MONSANTO, 1996). No Brasil, muito embora o CES não esteja previsto na legislação, os parceiros econômicos exigem a aplicação do CES para que os alimentos GM sejam globalmente aceitos, como descritos no documento de consenso da OECD (OECD, 2001; COSTA; MARIN, 2011).

Estudos independentes que abordam análises comparativas entre o material vegetal tolerante ao glifosato e o material não-modificado quase-isogênico são escassos. Em artigo de revisão, CUHRA (2015) compilou estudos que tratam sobre composição de soja, milho e canola GM. Foram analisados 15 estudos, nos quais a contraparte não-GM era equivalente em termos de composição à variedade GM. Todavia, em nenhum estudo, os resíduos de glifosato

foram quantificados, ou seja, não é possível verificar se houve aplicação de herbicida e acumulação. Ainda, as análises realizadas se restringiram aos componentes estipulados pela OECD (análise composicional). No artigo referido, os autores mencionaram que um único estudo encontrou diferenças significativas na composição da soja GM versus não-GM; neste, a quantidade de glifosato e seu metabólito AMPA foram quantificados, indicando a aplicação do herbicida e sua acumulação no grão.

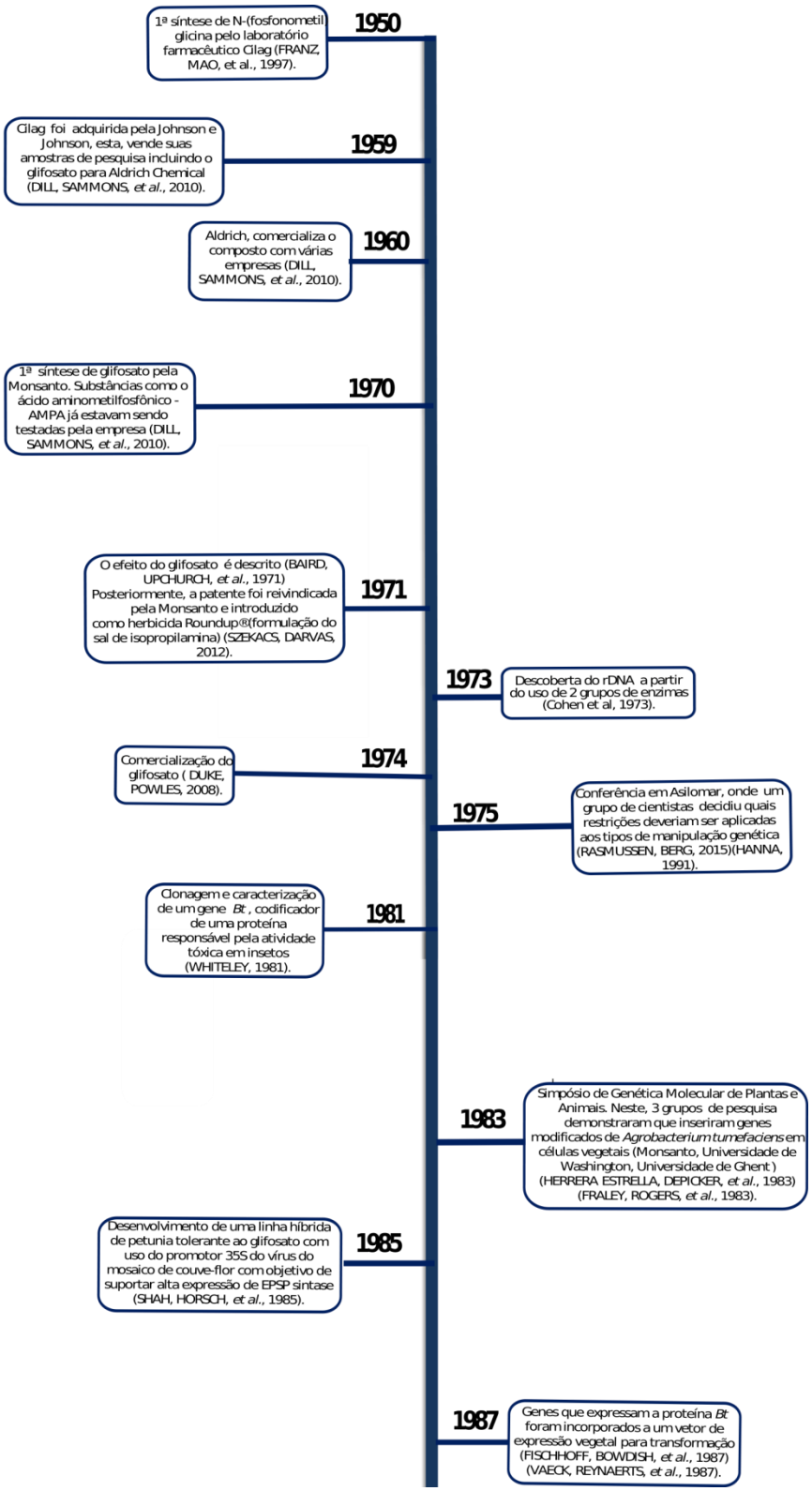
A análise de resísuos de glifosato e seus sub-produtos, tais como o AMPA, são particularmente relevantes uma vez que alterações composicionais, além de serem atribuídas a intervenções genéticas, já foram associadas a interações com herbicidas que podem alterar a composição nutricional das plantas (GELINSKY; HILBECK, 2018). A relevância para incluir análises de resíduos de herbicidas nas avaliações de risco é apoiada pelo cenário atual de produção. Apenas em 2018, a produção de soja GM alcançou 95,9 milhões de hectares no mundo inteiro (ISAAA, 2018). Considerando a pressão de seleção sobre as plantas espontâneas que desenvolveram resistência ao glifosato, há demanda maior de herbicida para o sistema de produção da lavoura transgênica. Atualmente, mais da metade dos agrotóxicos usados no Brasil são herbicidas a base de glifosato (IBAMA, 2018). Por consequência, alimentos e rações derivados dessas plantas podem apresentar padrões específicos de resíduos químicos e composição nutricional alterada (MIYAZAKI et al., 2019).

O cenário exposto nos permite refletir sobre quatro aspectos: 1) se análises composicionais são suficientes e se mais análises tenderiam a comprovar o mesmo, que GM e não-GM são equivalentes; 2) se a falta de clareza do conceito de Equivalência Substancial reflete em uma análise de risco fraca, pois não considera todas as hipóteses científicas de causa de dano; 3) passados 30 anos da adoção do termo – considerando que ele é suficiente como norteador da análise de risco - se o mesmo continua atual tendo em vista a evolução e o aprimoramento das técnicas analíticas moleculares e 4) se a análise dos componentes nutricionais, que atualmente norteia a equivalência substancial, é capaz de detectar alterações metabólicas, inclusive as pleiotrópicas, nas novas variedades que passaram a possuir novas inserções transgênicas tanto para tolerância a herbicidas quanto para resistência a insetos.

Assim, neste trabalho, foram examinados o resíduo de glifosato e AMPA e alguns dos componentes nutricionais principais do quadro recomendado pela OCDE (2001). O objetivo foi avaliar e fornecer estratégias adicionais para a identificação de efeitos não-intencionais, o que vem ao encontro do que é previsto pelo Protocolo de Cartagena sobre Biossegurança, particularmente também previsto pela Convenção sobre Diversidade

Biológica: “contribuir para assegurar um nível adequado de proteção no campo da transferência, da manipulação e do uso seguros dos OVM/OGM resultantes da biotecnologia moderna que possam ter efeitos adversos na conservação e no uso sustentável da diversidade biológica, levando em conta os riscos para a saúde humana” (CDB, 2000).

Figura 1. Marcos históricos que permitiram o desenvolvimento das variedades de soja GM utilizadas neste estudo



*Parte 1, Elaborado pela autora

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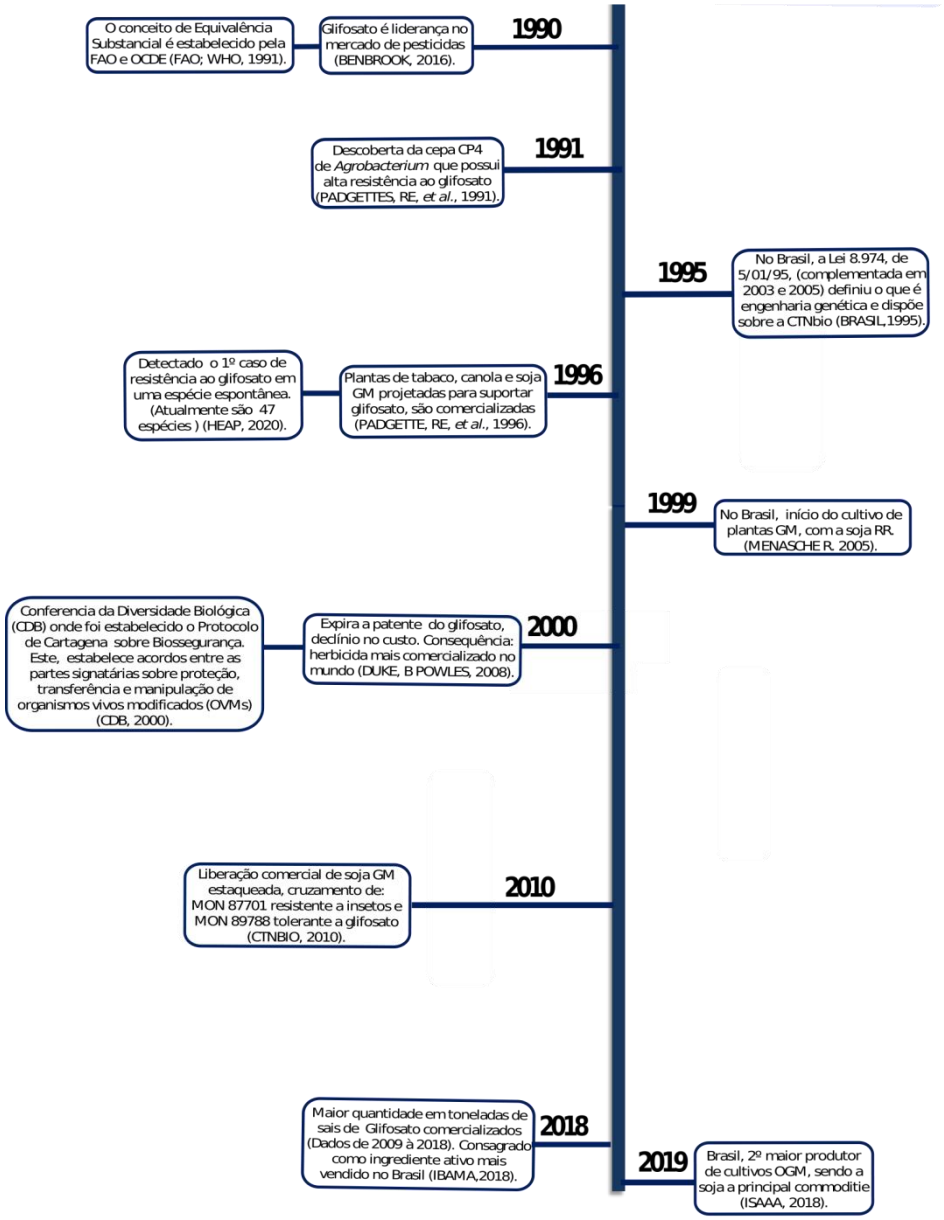

*Parte 2, Elaborado pela autora

4.2 MATERIAL E MÉTODOS

4.2.1 Material vegetal e design experimental

O material utilizado para este estudo foi coletado do mesmo experimento do capítulo anterior. Em suma, o experimento foi implantado (09/11/2018) em casa de vegetação na Fazenda da Ressacada, da Universidade Federal de Santa Catarina, em blocos completos casualizados (BCC) cujas parcelas estavam espaçadas 15 cm entre plantas e 45 cm entre linhas (CHOI et al., 2016). Os tratamentos foram casualizados em cada um dos três blocos, composto por uma repetição de cada tratamento. Anterior à semeadura, foi feita a análise química e física do substrato (1/3 de argila; 1/3 de resíduo de celulose e 1/3 de resíduo orgânico de aves). Com base no Manual de Adubção e Calagem, não foi necessário realizar adubação química, mas apenas a correção do pH para 6.0 (CQFSRS/SC, 2004). Foram utilizadas quatro variedades de soja, duas convencionais (BRS 284 e BRS 283) e duas GM (BRS 1001 IPRO e BRS 1010 IPRO, também denominadas de INTACTA RR2), que se caracterizam por compartilharem algumas linhagens parentais com duas variedades convencioinais, respectivamente. As sementes de todas as variedades receberam tratamento com fungicida Derosal® Plus (Carboxin + Thiram), seguindo as orientações prescritas na bula e, posteriormente, foram tratadas com inoculante Masterfix® *Bradyrhizobium japonicum* (250 g para 50 kg de sementes) e microelementos cobalto (Co) e molibdênio (Mo). A semeadura foi realizada em vasos cilíndricos com volume de 14 L (ZHANG et al., 2016). Em 22 dias após a semeadura (DAS) foi feito o desbaste para três plantas por vaso, que compuseram uma amostra biológica. Todos os vasos receberam água recomendada para a cultura através de irrigação por fita gotejadora.

4.2.2 Ensaio de aplicação de Roundup Transorb®

Neste estudo, o Roundup Transorb®, formulado comercial que contém o ingrediente ativo glifosato, que foi aplicado no estádio fenológico V2 (34 DAS), nas concentrações de 2,77 L/ha; 1,3 kg i.a/ha (média geral) e (4,5 L/ha; 2,2 kg i.a ha (máxima geral). Estas dosagens foram calculadas de acordo com a média e máxima do produto indicado na ficha técnica para cada espécie de planta espontânea prevista em lavouras de soja GM (AGROFIT, 2017). Assim, para o cálculo de aplicação foram consideradas cada uma das duas dosagens e um estande de 280.000.00 plantas/ha para chegar ao valor de produto por planta. O valor de produto por planta foi multiplicado ao número de plantas por vaso, número de vasos por bloco

que recebeu o tratamento e número de blocos para obtenção da quantidade de produto a ser aplicada no experimento. Então, a partir da quantidade de produto, foi realizado o cálculo de calda a ser aplicada considerando as recomendações da ficha do produto. Assim, o valor de calda total foi dividido pelo número de vasos que receberiam o tratamento, gerando o valor de calda por vaso, este valor foi dividido pelas três plantas presentes no vaso, indicando que cada planta deveria receber 0,7 ml de calda. Anterior a aplicação, foi considerado a deriva como um possível fator de contaminação. Desta forma, as duas janelas da casa de vegetação e as cortinas de lona laterais foram fechadas. Todas as plantas correspondentes aos tratamentos com herbicida a base de glifosato foram retiradas da casa de vegetação retornando após tratamento.

4.2.3 Colheita dos grãos e análise de resíduo de herbicida

Ao final do ciclo de cultivo, 119 DAS após a aplicação de Roundup Transorb (estádio R8; Figura 2), os grãos foram colhidos (154 DAS, dia 18/03/2019) e cada amostra biológica (pool de três plantas/vaso) foi misturada por repetição (três/bloco), variedade (GM e não-GM) e tratamento (sem Roundup e com Roundup nas dosagens média e máxima, referidos como GMsr, GMcmd e GMcmx, respectivamente). Cada conjunto de amostra foi armazenado a 4°C, e no dia 13/11/19 os mesmos, foram enviados para a empresa Eurofins Scientific (Noruega) a qual realizou as análises. Os dados da Eurofins são aceitos pela Autoridade Européia de Segurança Alimentar (*European Food Safety Authority* – EFSA). A quantificação de glifosato e AMPA foi realizada pelo laboratório Eurofins SOFIA Berlin (Rudower Chaussee) (EUDEBE2). Já os componentes, cálcio e fósforo foram determinados pela Eurofins WEJ Contaminants de Hamburgo/Alemanha; ácidos graxos pela Eurofins Food & Feed na Súecia, ácido fítico e inibidor de tripsina pela Eurofins Nutrition Scientific em Des Moines/EUA e o teor de isoflavonas totais foi determinado por (HPLC) na Eurofins Scientific Inc. Petaluma/EUA.

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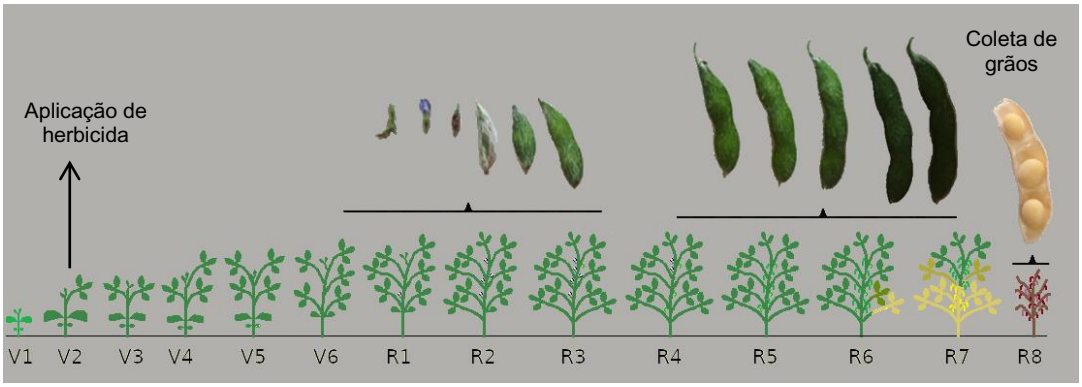
Figura 2. Representação dos estágios fenológicos da soja, no estádio vegetativo V2, o herbicida Roundup Transorb foi aplicado nos tratamentos com dosagem média e máxima. No estádio reprodutivo R8 os grãos foram colhidos. No momento em que o grão atingiu 15% de umidade, as amostras foram armazenada e enviadas para EUROFINS- Noruega, empresa prestadora de serviço.



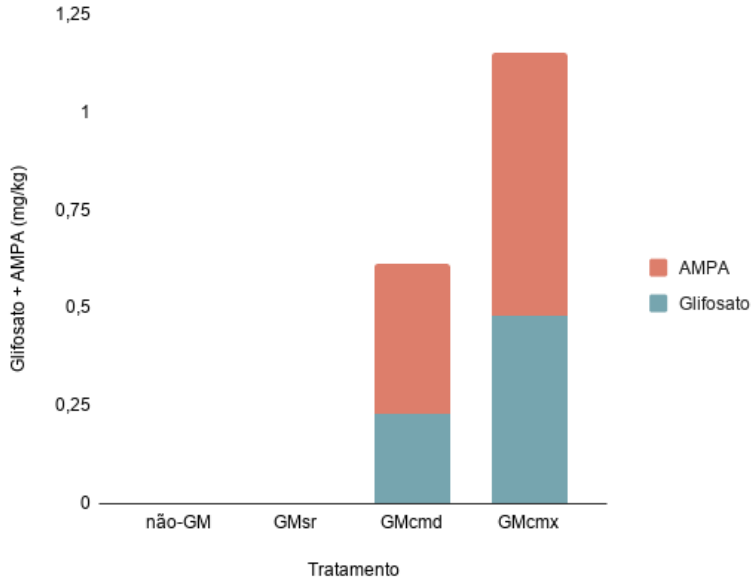
Elaborado pela autora

5.0 RESULTADOS E DISCUSSÃO

5.1 ANÁLISE DE RESÍDUOS DE HERBICIDA

No presente estudo, as amostras das variedades pulverizadas com Roundup Transorb® nos dois regimes de tratamentos [(dosagem média de 1,3 kg i.a/ha (ingrediente ativo/hectare) e dosagem máxima de 2,2 kg i.a/ha)] durante o estádio fenológico V2, apresentaram resíduos de glifosato e AMPA nos grãos. Nos grãos do tratamento com dosagem média, a concentração de AMPA (0,38 mg/kg) foi superior à de glifosato (0,23 mg/kg), assim como nos grãos do tratamento com dosagem máxima, no qual a concentração de AMPA (0,67 mg / kg) superou a do glifosato (0,48 mg/kg). Os resultados confirmam a estreita associação entre quantidade aplicada e resíduo nas folhas (estádio V2) e residuos nos grãos (estágio R8). A concentração da dose aqui denominada de máxima contém 70% a mais de glifosato, o que causou um aumento de 76% na quantidade de AMPA e de 108% de glifosato comparativamente ao tratamento sem herbicida. (Figura 3).

Figura 3. Concentração do ingrediente ativo glifosato e de seu metabólito AMPA em grãos de soja colhidos de plantas que não foram ou foram pulverizadas em estádio V2. Na figura os termos significam respectivamente: não-GM (amostra não modificada geneticamente); GMsr (amostra geneticamente modificado, sem aplicação de Roundup Transorb®); GMcmd (amostra geneticamente modificado, com aplicação de Roundup Transorb® na dosagem média); e GMcmx (amostra com aplicação de Roundup Transorb® nas dosagem máxima).



Elaborado pela autora

A pulverização em V2 resultou em grãos contaminados na colheita. Este fato, que é totalmente ignorado pela agência regulatória brasileira, a CTNBio, alerta para questões que vão além de um herbicida e de uma única pulverização. Em experimento na Argentina em 1998, utilizando a dosagem recomendada de Roundup Original para a soja, foram observadas concentrações mais altas de glifosato e AMPA no grão de soja quando o glifosato foi pulverizado repetidas vezes durante o ciclo e quando a aplicação foi próxima do estágio de floração. A aplicação da dosagem de 0,96 kg i.a/ha em dois momentos do ciclo da cultura resultou em 1,6 mg/kg de glifosato e 0,4 mg/kg de AMPA. No mesmo estudo, três aplicações na mesma dosagem produziram 1,8 mg/kg de glifosato e 0,9 mg/kg de AMPA nos grãos (ARREGUI, LENARDON, et al., 2003). Ao comparar esses dados com os resultados obtidos em nosso estudo, é possível verificar que, dependendo da formulação do herbicida a base de glifosato, diferentes níveis de resíduo são encontrados. Em estudos de campo no Mississippi e

Missouri, aplicações de Roundup® em diferentes concentrações e estágios de desenvolvimento da soja também foram testados. Entretanto, a maior quantidade de resíduo foi constatada quando foram aplicados 1,26 kg i.a/ha de glifosato em plena floração (8 semanas após o plantio) (DUKE, 2011). No Mississipi/ EUA, o conteúdo de resíduos nos grãos foi de 2,18 mg/kg de glifosato e 7,27 mg/kg de AMPA. No Missouri/ EUA, os grãos apresentaram 3,08 mg/kg de glifosato e 25,0 mg/kg de AMPA. Em um outro estudo realizado no Brasil, a mesma dosagem do estudo anterior (0,96 kg ai/ha), a aplicação feita 28 dias após plantio gerou um acúmulo de resíduo de 15 mg/kg de glifosato e 16,33 mg/kg de AMPA nos grãos (BOHM, ROMBALDI, et al., 2015). Os autores brasileiros comprovaram ainda que com duas aplicações, 28 e 56 dias após o plantio, foram encontradas concentrações de 20,67 mg/kg de glifosato e 24,33 mg/kg de AMPA. Essas maiores concentrações de resíduos em estudos mais recentes pode ser fruto de uma evolução da capacidade analítica das empresas que aumentou a confiabilidade dos testes, assim como seus limites de detecção, gerando resultados mais robustos. (SZEKACS; DARVAS, 2012).

Ainda que esses estudos tenham testado concentrações que chegam a 2,2 kg ai/ha, existem fortes evidências de que o uso de herbicidas a base de glifosato de 2000 até 2012, podem ter chegado a 9 kg i.a/ha de aplicação no Brasil. Ou seja, quatro vezes maior do que a dose máxima testada no presente estudo. O aumento da quantidade total de glifosato aplicado (kg/ha) está estritamente relacionado à adoção da soja GM no Brasil. Após a adoção de variedades GM, o uso de herbicidas a base de glifosato aumentou 1,6 vezes, exceto para a soja, que chegou a 3 vezes mais (ALMEIDA et al., 2012). De fato, em duas fazendas comerciais do Rio Grande do Sul no ano de 2015, foram encontradas concentrações de 12 mg/kg de glifosato e 21 mg/kg de AMPA e 19 mg/kg de glifosato e 25 mg/kg de AMPA em Sananduva e Pelotas, respectivamente (BOHM, ROMBALDI, et al., 2015). Nos EUA, no estado de Iowa, foram encontrados altos índices de resíduos de glifosato e AMPA (média de 3,26 mg/kg e 5,7 mg/kg, respectivamente) em 31 lotes de soja GM coletados para análise (BØHN, CUHRA, et al., 2014).

Segundo a FAO (2005) o resíduo de agrotóxicos é a combinação do princípio ativo e seus metabólitos. Assim, no caso do glifosato, deve-se calcular como a soma de glifosato+1,5 × AMPA (FAO, 2005). A partir da aplicação desta fórmula, os resultados do presente estudo indicam a presença de 0,8 mg/kg de 'equivalentes de glifosato' de para dosagem média (1,3 kg i.a/ha) e 1,48 mg/kg para a dosagem máxima (2,2 kg i.a/ha), resultantes de uma única aplicação. Dados do material coletado em Iowa nos EUA, apresentaram em média 11,9 mg/kg

de equivalentes de glifosato (BØHN, CUHRA, et al., 2014). Na Argentina, onze amostras de soja GM coletadas em três distritos apresentaram o nível de equivalentes de glifosato entre 5,34 e 75,36 mg/kg (TESTBIOTECH, 2013). De acordo com os dados obtidos no presente estudo para equivalentes de glifosato, os resultados estariam dentro do aceito e regulamentado como Limite Máximo de Resíduo permitido pela agência brasileira ANVISA. Todavia, é possível que a dosagem aplicada no estudo seja muito inferior àquela utilizada pelos agricultores brasileiros. Tomando como exemplo os dados de níveis de glifosato e AMPA encontrados em lavouras de produção de soja do estudo de Bohm, Rombaldi e colaboradores, os equivalentes de glifosato foram de 43,5 mg/kg para o município de Sananduva e 56,5 mg/kg para o município de Pelotas (BOHM, ROMBALDI, et al., 2015). Esses valores são muito superiores aos Limites Máximos de Resíduos (LMR) permitidos pelas agências regulatórias para HBG em grãos de soja seca para consumo (Tabela 1). Segundo o Codex Alimentarius, (2019) o LMR é o nível de resíduo máximo legalmente tolerado de algum agrotóxico nos alimentos ou em alimentos para animais, também é considerado como ferramenta de monitoramento que auxilia contra o uso excessivo (FAO/CODEX ALIMENTARIUS, 2019).

Tabela 1. Principais agências ou instituições que realizam atividades de regulação dos Limites Máximos de Resíduos (LMR) de herbicidas à base de glifosato (HBG) (glifosato + AMPA) permitidos em soja *(Glycine max)* seca para consumo. Na tabela, está também o ano do último registro das diretrizes que estabelecem os valores a serem seguidos por cada agência/país, juntamente com a citação bibliográfica para outras informações.

Agência regulatória/País	*Limite máximo de resíduo (LMR)*	*Ano*	*Citação*
ANVISA (Brasil)	10 mg/kg	2007	(ANVISA, 2007)
European Food Safety Authority (Países europeus membros)	20 mg/kg	2013	(EU MRLs, 2020)
FAO/OMS	20 mg/kg	2005	(FAO,2005)
Environmental Protection Agency (Estados Unidos da América	20 mg/kg	Oct. 1, 1980	(GOVERNMENT U.S, 2020)

Pest Control Products Act (Canadá)	20 mg/kg	2011	(HEALTH CANADA, 2011)
SENASA (Argentina)	5 mg/kg	2008	(SENASA, 2008)
Food Standards Code (Australia/Nova Zelândia)	20 mg/kg	2020	(APVMA, 2020)

Elaborado pela autora

Apesar da crescente adoção de plantas GM combinadas ao uso de herbicidas (ISAAA, 2018), a avaliação de segurança é realizada separadamente para plantas e para os herbicidas. No caso dos herbicidas, a avaliação é apenas realizada com o ingrediente ativo, sendo os adjuvantes das formulações comerciais regulados de maneira diferente. Assim, observa-se um limbo regulatório sobre os efeitos tóxicos em longo prazo da combinação desses compostos químicos com as variedades geneticamente modificados (MESNAGE; ANTONIOU, 2018). Assim, pode-se esperar que derivados de plantas GM pulverizadas apresentem diversos resíduos químicos e, possivelmente, uma composição nutricional alterada desencadeada pelos efeitos de todos os componentes presentes na formulação comercial (BORGGAARD; GIMSING, 2008; KLETER; UNSWORTH; HARRIS, 2011; MIYAZAKI et al., 2019). Idealmente, a avaliação de herbicidas deveria ser realizada conjuntamente com as plantas transformadas, uma vez que os mesmos passam a ser parte constitutiva destas plantas; podendo afetar o metabolismo da planta ou ainda gerar propriedades tóxicas no produto final (BOHN et al., 2014).

Na fisiologia das plantas, por exemplo, efeitos deletérios de HBG sobre processos celulares e crescimento têm sido observados. Como um quelante de metal, o glifosato pode influenciar na fisiologia das plantas privando-as de importantes nutrientes, alterando a fotossíntese, o estado oxidativo e hormonal (GOMES et al., 2014), além de prejudicar a absorção de nutrientes causando efeitos fitotóxicos (MATEOS-NARANJO; PEREZ-MARTIN, 2013). Correia e colaboradores (2013) observaram a redução da atividade nodular, alteração da atividade fotossintética e consequente diminuição da biomassa em plantas de soja GM pulverizadas com 4,8 kg i.a./ha de glifosato (CORREIA, 2013). Em experimento com plantas de milho (*Zea Mays*) que foi testado a irrigação com água contaminada por glifosato (Pestanal) em concentrações superiores ou igual a 25 mg l-, foi constatado que houve acúmulo de glifosato (bem como seu metabolito, ácido aminometilfosfônico) nos tecidos das plantas. Avaliações feitas no estágio V5 revelaram que o glifosato diminuiu a fotossíntese e induziu

alterações na anatomia foliar e nas propriedades biofísicas do caule o que pode desencadear a diminuição da produção de grãos. A presença de produtos químicos é uma preocupação potencial toxicológica, principalmente devido ao acúmulo de resíduos na cadeia alimentar (GOMES et al., 2020). No âmbito da saúde, a associação entre a exposição e alimentação de produtos com resíduos de glifosato já foi relatada. Rodrigues et al. (2018), identificaram resíduos de glifosato e de AMPA nas formulações comerciais de alimentos infantis à base de soja no Brasil (RODRIGUES; PAULA; SOUZA, 2018). Niemann et al. (2015) chamam a atenção para a exposição crônica pela ingestão de alimentos ou de produtos contaminados que contém resíduos de glifosato e AMPA como potenciais fatores de risco à saúde (NIEMANN et al., 2015). Thongprakaisang et al. (2013) relataram que Roundup apresentou efeitos estrogênicos e/ou antiestrogênicos em células de câncer de mama humanas, o que implica que o uso de derivados de soja (que possui fitoestrogênios naturalmente) contaminados, pode representar um risco de câncer de mama devido à sua potencial estrogenicidade aditiva (THONGPRAKAISANG et al., 2013). O Glifosato, i.a do Roundup atua sob os níveis de manganês (Mn) na fisiologia das plantas e investiga-se que também possa estar associado com doenças observadas em animais. Isto porque, a deficiência deste micronutriente, necessário em muitas funções celulares, favorece as neuropatias como o autismo e déficit de atenção. Além disso, pode desencadear quadro de osteoporose e osteomalácia (SAMSEL; SENEFF, 2015). A exposição pré-natal ao glifosato teve associação positiva com o transtorno do espectro do autismo (TEA) em estudo de caso-controle (VON EHRENSTEIN et al., 2019). Os efeitos toxicológicos de Roundup também têm sido investigados em linhas celulares e associados à diversos distúrbios metabólicos (VER, AGOSTINI et al., 2020). Entretanto efeitos crônicos associados à exposição em longo prazo, ainda necessitam ser investigados dado o uso continuado deste tóxico. Assim, estudos epidemiológicos abrangentes se configuram como fundamentais para confirmar a segurança e fornecer recomendações e diretrizes para regular seu uso (AGOSTINI et al., 2020).

5.2 ANÁLISE DE COMPONENTES NUTRICIONAIS

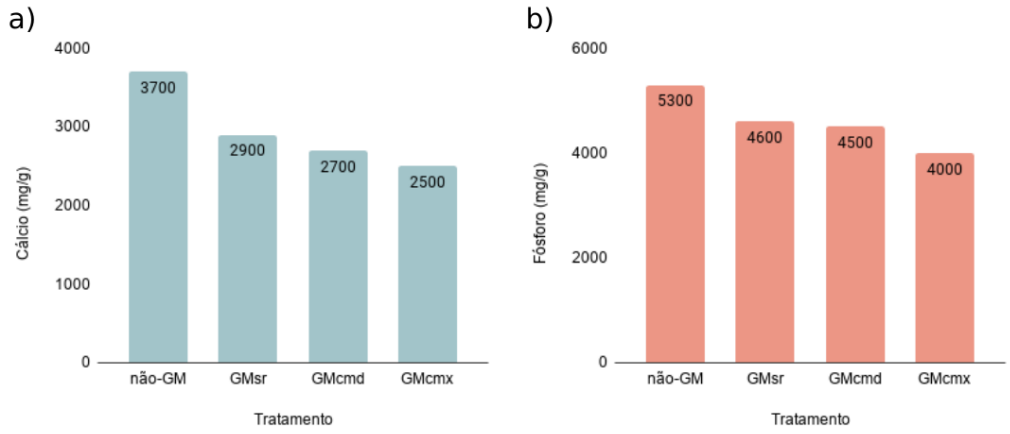
5.2.1 Cálcio e Fósforo

A composição dos grãos de soja, determinado pelo conteúdo de proteínas, minerais e ácidos graxos, são análises fundamentais para a aprovação das variedades transgênicas no Brasil e no mundo (BELLALOUI et al., 2009; BØHN et al., 2014). Tal composição é

diretamente afetada por efeitos no estado fisiológico das plantas ao longo dos seu ciclo de vida. Estudos prévios mostram tendências crescentes e decrescentes com relação ao conteúdo de proteínas, macro e micronutrientes, ácidos graxos e açúcares em soja GM, devido a aplicação de Roundup® (ZOBIOLE et al., 2012; BØHN et al., 2014). Efeitos negativos no estado nutricional de plantas de soja GM também já foram atribuídos à aplicação de HBG (JOHAL; HUBER, 2009; ROSOLEM et al., 2010; MARTINEZ; LOENING; GRAHAM, 2018). Além disso, a soja naturalmente contém vários compostos bioativos, alguns considerados anti-nutrientes, tais como as isoflavonas, o ácido fítico, lipídios, fitoalexinas, lectinas, vitaminas, entre outros ainda desconhecidos, que podem influenciar a sua composição nutricional (POTTER, 1995).

No presente trabalho, as amostras de grãos das diferentes dosagens e dos diferentes tratamentos foram analisadas quantitativamente. A composição dos nutrientes minerais cálcio (Ca) e fósforo (P) apresentaram tendências de agrupamento por variedade (não-GM versus GM) em detrimento ao tratamento com glifosato nas duas dosagens (GMsr, GMcmd e GMcmx) (Figura 4).

Figura 4. Concentração (mg/kg) de cálcio e fósforo presentes nos grãos de soja não-GM (amostra não modificada geneticamente); GMsr (amostra geneticamente modificado, sem aplicação de Roundup Transorb®); GMcmd (amostra geneticamente modificado, com aplicação de Roundup Transorb® na dosagem média); e GMcmx (amostra com aplicação de Roundup Transorb® nas dosagem máxima).



Os resultados representam a média de três plantas/vaso, amostradas em conjunto nas repetições por cada tratamento em cada bloco. Elaborado pela autora.

No presente estudo, os teores de cálcio mostraram níveis (mg/kg) semelhantes entre as amostras transgênicas (GMsr, GMcmd e GMcmx) e distindo da amostra convencional (Figura 4a). Para o fósforo, o mesmo padrão foi observado (Figura 4b). O fósforo desempenha um papel significativo no metabolismo de moléculas-chave, tais como ácidos nucléicos, fosfolipídios e ATP. Além de ser o segundo macronutriente mais limitante para o crescimento das plantas (SCHACHTMAN; REID; AYLING, 1998). Alteração nas concentrações de P e Ca também já foram observadas em grãos de soja GM com aplicação de Roundup, que segudo os autores, foi decorrente dos efeitos quelantes de glifosato-cátion (ZOBIOLE et al., 2010). Qin e colaboradores (2017) também observaram diferenças estatisticamente significativas nos níveis de cálcio e fósforo entre as linhagens transgênicas de soja e suas contrapartes não-transgênicas. Porém, segundo os autores, a diferença observada estaria dentro dos limites de variação aceitos pela OCDE, o que implicaria dizer que, para este macronutriente, ambas amostrassão equivalentes (QIN; OH, 2017). Por consequência, este alimento GM é considerado tão seguro e nutritivo quanto a sua contraparte convencional.

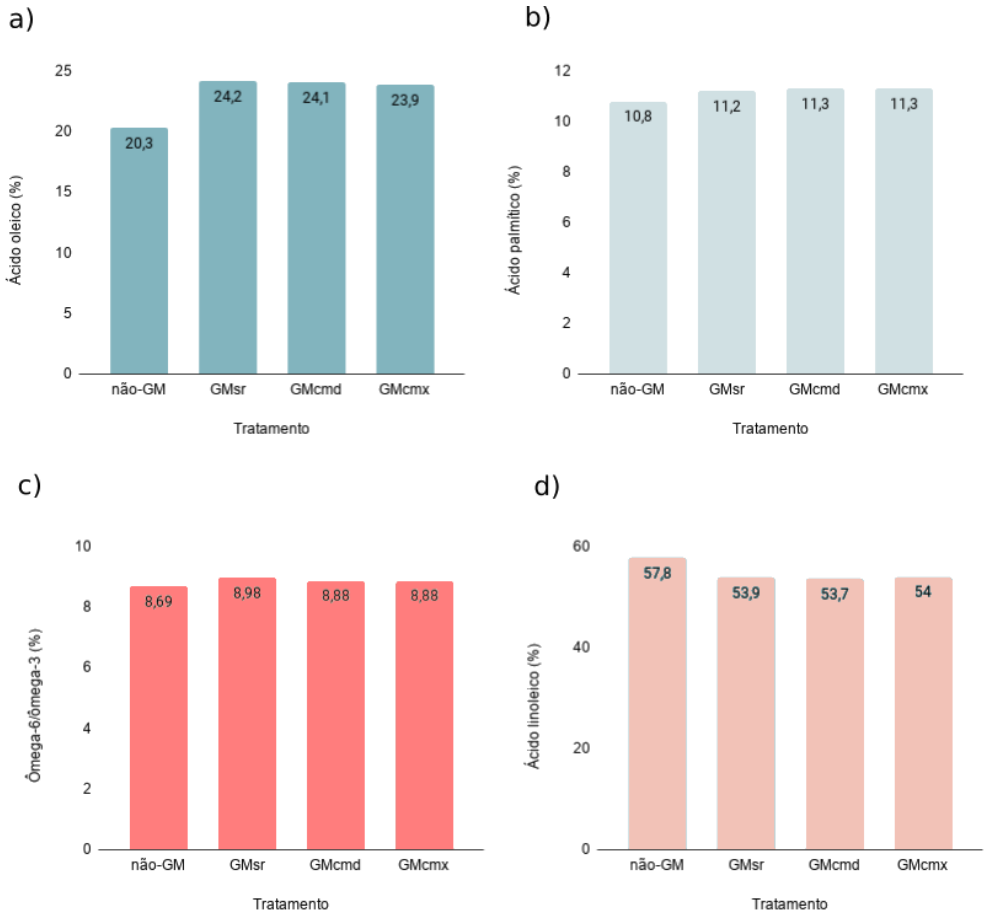
Em geral, a não-significância do valor-p é usada para comprovar a segurança alimentar de um OGM segundo o CES (OBERDOERFER, 2006). Deste modo, informações de um teste de significância podem ser limitadas, especialmente quando reduzidas à afirmação de que um efeito é estatisticamente significativo sem considerar a relevância biológica do efeito (LOVELL, 2013). Segundo a EFSA, (2011) muitos pesquisadores concluem incorretamente que qualquer efeito estatisticamente significativo é biologicamente relevante, uma vez que é suportado pela matemática (EFSA, 2011). Consequentemente, conclusões baseadas na equivalência substancial, devem ser tomadas com cautela, uma vez que a diferença estatística (valor de p de 0,049 ou 0,051) pode converter um resultado em uma conclusão positiva ou negativa binária (LOVELL, 2013). Do ponto de vista científico, é preciso questionar se a ausência de evidência entre GM e não-GM, com base na abordagem estatística de significância suportado pelo CES, deve ser tomada como é justificativa válida na avaliação de risco desses produtos (ALTMAN; BLAND, 1995).

5.2.2 Ácidos graxos

Os ácidos graxos são obtidos por meio da dieta e são fundamentais para uma série de funções no organismo humano (MARTIN et al., 2006; WANTEN; CALDER, 2007). A soja, em grãos ou farelo, é uma ótima fonte de ácidos graxos essenciais e apresenta um perfil

bastante amplo, sendo reconhecidamente utilizada como óleo vegetal (MESSINA, 2016). No presente estudo, quatro ácidos graxos foram selecionados para ilustrar a análise do efeito do herbicida a base de glifosato nas composições de ácidos graxos. De maneira geral, o padrão do perfil de ácidos graxos foi mais influenciado pelo tipo de variedade (não-GM e GM) do que pelo tratamento (com ou sem Roundup®).

Figura 5. Porcentagem de ácidos graxos presentes no grão de soja não-GM (amostra não modificada geneticamente); GMsr (amostra geneticamente modificado, sem aplicação de Roundup Transorb®); GMcmd (amostra geneticamente modificado, com aplicação de Roundup Transorb® na dosagem média); e GMcmx (amostra com aplicação de Roundup Transorb® na dosagem máxima).



Os resultados representam a média de três plantas/vaso, amostradas em conjunto nas repetições por cada tratamento em cada bloco. Elaborado pela autora.

O ácido oleico, conhecido por ser um ácido graxo insaturado (HUERTAS., 2010), apresentou uma menor quantidade (%g/g de amostra) na amostra não-GM

comparativamente às amostras GMs em seus tratamentos (Figura 5a). Qin et al. (2017) ao compararem soja GM com a sua contraparte não-GM, também observaram um aumento significativo nos níves de ácido oleico. Para a análise do ácido palmítico foi constatado novamente o mesmo padrão, soja não GM com porcentagem inferior às amostras GM (Figura 5b). Bohn et al. (2014) também observaram que os teores de ácido palmítico foram estatisticamente inferiores na variedade convencional quando contrastados com a variedade GM.

Para a razão de ômega-6/ômega-3, as concentrações dos quatro tratamentos foram muito similares, sendo que a variedade não-GM revelou uma quantidade (%) levemente menor que as demais (Figura 4c). Esta tendência também foi encontrada em outro estudo, uma vez que os níveis na relação ômega-6/ômega-3 foram significativamente menores em soja chinesa convencional comparada a variedade GM, o que indicaria um valor nutricional superior para a convencional (XIA et al., 2019). Razões de ômegas-3 e 6 também foram afetados apresentando uma proporção maior para a variedade GM tratada com glifosato (ZOBIOLE et al., 2010). No que diz respeito à importância destes ácidos graxos na dieta humana, é reconhecido que altas relações estão correlacionadas a um aumento significativo na prevalência de sobrepeso e obesidade em adultos (SIMOPOULOS, 2016).

Os ácidos graxos linolênicos são denominados essenciais por não serem sintetizados pelo organismo, ao mesmo tempo em que são necessários para manter as funções cerebrais sob condições normais e por influenciarem diretamente na síntese da hemoglobina (WANTEN; CALDER, 2007). No presente estudo, ao contrário dos demais ácidos graxos, o ácido linolenico apresentou uma porcentagem superior nas amostras não-GM em relação às amostras GM que demostraram semelhantes teores entre si (Figura 5d). Conteúdos de ácido linolênico também foram encontrados em concentrações superiores em variedade convencional comparativamente a variedade GM (BÖHN et al., 2014). A diminuição de ácido linoleico em soja GM já foi observada após aplicação de Roundup (BELLALOUI et al., 2008).

5.2.3 Compostos bioativos e antinutrientes

As três categorias de amostras também foram analisadas quanto à presença de compostos bioativos e substâncias consideradas antinutrientes: isoflavonas, ácido fítico e inibidor de tripsina (Tabela 1). Antinutrintes são aqueles nutrientes que possuem baixa digestibilidade e reduzem a utilização máxima de nutrientes (RABOY, 2009).

Tabela 2. Porcentagem de isoflavonas totais, ácidos fíticos e unidades de inibidor de tripsina/g encontrados nos grãos não-GM (amostra não modificada geneticamente); GMsr (amostra geneticamente modificado, sem aplicação de Roundup Transorb®); GMcmd (amostra geneticamente modificado, com aplicação de Roundup Transorb® na dosagem média); e GMcmx (amostra com aplicação de Roundup Transorb® nas dosagem máxima).

Compostos bioativos	***não-GM***	***GMsr***	***GMcmd***	***GMcmx***
Isoflavonas totais (%)	0.207	0.162	0,139	0.157
Ácido fítico (%)	1.40	1.20	1,27	1.24
Inibidor de tripsina TIU/g)	37500	35500	34800	35200

As médias amostras de três plantas/vaso, amostradas em conjunto nas repetições por cada tratamento em cada bloco. Elaborado pela autora.

Ao examinar a porção de fitato nos grãos foi constatado que as amostras não-GM apresentaram uma quantidade maior de ácidos fíticos em relação as amostras GMsr e GMcr, estas duas últimas amostras com teores foram similares. Teores de fitato são responsáveis por até 80% da concentração total de P nas sementes (COELHO et al., 2002), o que pode explicar em parte, a quantidade superior de P encontrada nos grãos das amostras não-GM observado (Figura 4b). Assim como o ácido fítico, quantidades de inibidor de tripsina também seguiram o mesmo padrão (Tabela 2). No presente estudo, o teor total de isoflavonas foi maior nas amostras não-GM em comparação com os dados colhidos para as amostras GMsr e GMcmd e GMcmx que apresentam resultados semelhantes entre si. A síntese natural de isoflavonas decorre da rota bioquímica do ácido chiquimico, o que é particularmente interessante, uma vez que, a enzima 5-enolpiruvilshiquimato-3-fosfato (EPSP) inserida no cassete transgênico para tolerância ao glifosato também participa desta rota (TAYLOR et al., 1999). Lappé et al. (1999) identificaram que linhagens de soja não-GM também continham níveis maiores de isoflavonas do que as linhas de soja GM. Os autores sugerem que os produtos oriundos de modificação genética podem ser menos potentes na dieta como fonte de fitoestrogênios (LAPPÉ et al., 1999). Em outro estudo, entretanto, não foram encontradas diferenças estatísticas de isoflavonas totais entre soja GM e não-GM, sendo que os teores podem variar de alto a baixo na variedade GM comparada a não-GM (WEI, JONE, & FANG, 2004). A interferência do background genético das variedades de soja

GM e não-GM também foi atribuída às alterações nos teores de isoflavonas em outro estudo, fato que levou os autores a considerarem necessários outros ensaios para discriminar o fator transgênese (BOHM et al., 2008).

6 CONCLUSÕES E PERSPECTIVAS FUTURAS

No presente capítulo informações relevantes sobre os resíduos de glifosato e AMPA nos grãos colhidos das amostras de soja GM tratadas com herbicida a base de glifosato foram obtidas. As análises de resíduo de herbicida e dos componentes foram realizadas sem que os dados brutos das replicatas nos fossem disponibilizados, o que impediu a realização de um teste estatístico de separação de médias. Entretanto, de acordo com os dados quantitativos obtidos, foi possível observar uma tendência nos dados indicando que há maior efeito do fator 'genótipo', seguido do efeito do fator 'tratamento com herbicida' nos níveis dos componentes testados. Estas informações são um auxílio na compreensão do efeito residual de HBG quanto este é utilizado nas dosagens recomendas em lavouras de soja. Permite também, verificar a conversão entre dosagem aplicada no estádio fenológico V2 e teor em mg/kg de resíduo presente no grão colhido em R8 que será o produto para consumo.

A utilização de dosagens prescritas como potencial estressor pode ter limitado a visualização do efeito de alterações composicionais no grão. Assim, sugere-se que para próximos experimentos doses agudas sejam testadas, além disso, ensaios que compreendam os herbicidas que representam o coquetel utilizado por agricultores podem contribuir para compreensão do que de fato é o que é consumido em termos de resíduos e componentes nutricionais. Propomos que, as abordagens que utilizam unicamente o Critério de Equivalência Substancial devam no mínimo incorporar a análise de herbicidas e subprodutos como parte da análise de risco.

Por fim, os resultados do presente estudo nos Capítulo 1 e Capítulo 2, possibilitam discutir o atual processo de a avaliação de risco de OGMs. Abordagens com maior cobertura e mais profundidade como as de perfil ômico podem subsidiar tanto a avaliação nos grãos por análise proteômica e metabolômica, quanto auxiliar na identificação das interações das plantas transformadas com meio agroecológico no qual estão inseridas, gerando dados que podem ser relevantes nos ensaios de segurança ambiental.

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APÊNDICE - Linha do tempo do grupo de pesquisa em biossegurança em plantas Geneticamente Modificadas da Universidade Federal de Santa Catarina.

Período	Acontecimentos/ Publicações marco	Descrição	Envolvidos na atividade
1997-1998	Palestras e resumos expandidos	Implicações dos transgênicos na sustentabilidade ambiental e agrícola	Rubens Nodari, Miguel Guerra
1997-2020	21 Artigos e 15 Capítulos de livro	Análise de Biorriscos ou Biossegurança de OGM ou Transgênicos	Co-autores Rubens Nodari, Miguel Guerra
1997-2020	166 Conferências ou Palestras	Análise de Biorriscos ou Biossegurança de OGM ou Transgênicos	Rubens Nodari
1997-2020	19 Participações em eventos ou Palestras*	Análise de Biorriscos ou Biossegurança de OGM ou Transgênicos	Miguel Guerra
2001	Artigo	Avaliação de riscos ambientais de plantas transgênicas, publicado na Revista Cadernos de Ciência e Tecnologia da Embrapa	Rubens Nodari, Miguel Guerra
2003- 2008	Cargo	Gerência de Recursos Genéticos do Ministério do Meio Ambiente	Rubens Nodari
2004- 2012	Cargo	Comissão Nacional da Biodiversidade - CONABIO	Miguel Pedro Guerra
2007- 2012	Cargo	Conselheiro da Sociedade Brasileira para o Progresso da Ciência	Miguel Pedro Guerra
2007	Estágio de Conclusão de Curso	Biosafety Forecast Service (Prof Jack Heinemann, Nova Zelândia)	Sarah Zanon Agapito-Tenfen/ Rubens Nodari
2007	Parecer submetido à CTNBio	Análise de risco do milho Liberty Link da Bayer	Sarah Zanon Agapito-Tenfen/ Rubens Nodari
2008-2012	Tese de Doutorado (Pós-graduação em Saúde Pública - Fundação Oswaldo Cruz)	Legislativo e Executivo: O que fazem o Congresso Nacional e a CTNBio com relação aos alimentos transgênicos?	Maria Clara C. Camara.
2009	Parecer	Análise de risco do Arroz	Sarah Zanon Agapito-

	submetido à CTNBio	Liberty Link da Bayer	Tenfen/ Rubens Nodari
2009	Curso de Capacitação	Specialist Course in Hazard Identification and Risk Assessment of Transgene Flow (Noruega)	Sarah Zanon Agapito-Tenfen/ Rubens Nodari
2009	Reuniões e Convênios	Embaixada da Noruega, EMBRAPA e UFSC. Memorandum of Understanding Genok-UFSC, Início do programa de pesquisa na UFSC.	Rubens Nodari, Miguel Guerra, Afonso Orth, Paulo Emilio Lovato, Malva Hernandez
2009	Pesquisa	Início da execução dos Projetos de Pesquisa; Programa de intercâmbio FK	Sarah Zanon Agapito-Tenfen, Flor Rivera López, Elena Rocca, Hanna Bjørgaas
2009-2011	Dissertação de Mestrado	Caracterização proteômica de possíveis efeitos pleiotrópicos em milho geneticamente modificado (MON810)	Sarah Zanon Agapito-Tenfen/Rubens Nodari
2009-2011	Dissertação de Mestrado	Relação entre a presença de proteínas recombinantes de milho OGM e a frequência de fenótipos anormais nas variedades de milho nativo, na região Vales Centrais, Oaxaca, México.	Flor Rivera López/Rubens Nodari
2010	Curso de Biossegurança e Conferência	Reunião Anual da Sociedade Brasileira para o Progresso da Ciência em Manaus / INPA Palestras convidadas em Florianópolis, Manaus, São Paulo	Rubens Nodari e Paulo Kageyama
2010	Curso de Biossegurança	Curso de Biossegurança em Florianópolis para representantes dos países da America Latina (Holistic Foundations for Assessment and Regulation of Genetic Engineering and Genetically Modified Organisms)	Sarah Zanon Agapito-Tenfen, Renata Calixto, Elena Rocca, Rubens Nodari e alunos
2010-2011	Intercâmbio	Intercâmbio com Genok – Noruega	Daniel Ferreira Holderbaum.

2010-2012	Dissertação de Mestrado	Efeitos crônicos de milho transgênico (MON810) na dieta de Daphnia magna	Daniel Holderbaum/ Afonso Inacio Orth – Rubens Onofre Nodari
2011	Curso de Capacitação	Special topics in biosafety training: insects, vacines and stress-tolerant plants (Noruega)	Sarah Zanon Agapito-Tenfen
2011	Parecer submetido à CTNBio	Análise de risco do feijão transgênico da EMBRAPA	Sarah Zanon Agapito-Tenfen/ Rubens Nodari
2011	Reunião	Parceria entre a ex-ministra do Meio Ambiente do Brasil e Noruega	Marina Silva e Erik Solheim
2011	Reunião	Reuniões específicas do projeto	Sarah Zanon Agapito-Tenfen, Elena Rocca
2011-2014	Tese de Doutorado	Detecção de alterações no proteoma de plantas geneticamente modificadas oriundas de interações sinérgicas e antagonistas da integração e expressão de transgenes	Sarah Zanon Agapito-Tenfen/Rubens Nodari
2011-2013	Detecção de OGM	Gene flow from gene modified Zea maize to local land races. Case study in Santa Catarina, Brazil	Hanna Bjørgaas/Rubens Nodari
2011-2015	Tese de Doutorado	Interferência do pólen de milho geneticamente modificado em colônias de Apis mellifera e detecção da ocorrência de proteínas transgênicas em mel	Lucilene de Abreu/Afonso Inacio Orth
2012-Presente	Nomeação Representação Brasileira	Âmbito da Convenção da ONU da Diversidade Biológica e Protocolo de Cartagena de Biossegurança	Sarah Zanon Agapito-Tenfen
2012	Reunião	Encontro com o Ministério do Meio Ambiente	
2012	Reunião	Encontro com Instituto Carlos Chagas / Fiocruz PR (Imuno-ecologia / epidemiologia)	Claudia Nunes Duarte dos Santos, Terje Tavick, Rubens Nodari
2012 e 2013	Intercâmbio	FK Exchange- Genok - Noruega	Vinicius Vilperte

2012 e 2013	Curso	Curso de detecção de OMG	UFSC
2012-2014	Dissertação de Mestrado	Avaliação dos impactos do pólen de milho geneticamente modificado (Bt) sobre colônias de *Apis Mellifera* L.	Leon Bizzocchi/Afonso Inacio Orth - Rubens Onofre Nodari
2012-2016	Tese de Doutorado	Efeitos do milho transgênico sobre aspectos morfofisiológicos da associação micorrízica e sobre a diversidade dos fungos micorrízicos arbusculares	Diana Marcela Morales Londoño /Paulo Emílio Lovato
2012-2016	Tese de Doutorado	Besouros indicadores (Coleoptera, Scarabaeinae) na avaliação de alteração ambiental em fragmentos de Mata Atlântica contíguos a cultivos de milho convencional e transgênico	Renata Calixto Campos/ Malva Isabel Medina Hernández
2012-2014	Dissertação de Mestrado	Caracterização da proteína Cry1Ab do milho geneticamente modificado mon810 e detecção das possíveis interações com proteínas endógenas de milho	Diana Karina Diaz Canova/Rubens Nodari
2012 e 2013	Intercâmbio	FK Exchange- Genok – Africa do Sul	Rafael Benevenuto e Leon Bizzocchi
2012 e 2013	Intercâmbio	FK Exchange- Genok – Noruega	Diana Karina Diaz Canova
2013-2018	Tese de Doutorado	Um modelo in vitro para identificação de efeitos não-intencionais da transformação genética de plantas	Daniel Holderbaum/Miguel Guerra
2013-2015	Dissertação de Mestrado	Análises bioquímicas e fisiológicas do milho transgênico tolerante ao Roundup Ready (evento Nk603) submetido ao déficit hídrico e aplicação de herbicida	Rafael Benevenuto/ Rubens Nodari
2013	Curso de Capacitação	Curso Latino Americano de biosseguraqnca de OGMs (40 participantes)	GenØk/UFSC

2013 e 2014	Workshop	Biosafety Research Hub Latin America	Genok/UFSC
2014	Curso de Capacitação	Training Course in Risk Assessement of GMOs (Suazilândia)	Sarah Zanon Agapito-Tenfen
2014- 2015	Intercâmbio	FK Exchange- Genok – Noruega- Daphnia magna ecotoxic model	Carina Macagnan Rover
2014-Presente	Cargo de Pesquisadora	Contratada pelo GenØk - Pesquisadora em genética de plantas	Sarah Zanon Agapito-Tenfen
2014-2016	Dissertação de Mestrado	Transcriptômica e mirnômica comparativa de variedades transgênicas de milho	Vinicius Vilperte/Rubens Nodari
2014-2016	Dissertação de Mestrado	Transcriptômica e mirnômica comparativa de variedades transgênicas de milho	Vinicius Vilperte/Rubens Nodari
2014-2018	Tese de Doutorado	Efeitos do herbicida Roundup® na sobrevivência e dinâmica de colônias de *Apis mellifera*.	Márcia Regina Faita/Afonso Inacio Orth
2015	Apresentação de trabalho em evento internacional	Transgene flow in Mexico (10º Simpósio de Recursos Genéticos Vegetais para a América Latina e Caribe – Brasil)	Sarah Zanon Agapito-Tenfen
2015	Curso de Capacitação	Synthetic Biology – biosafety and contribution to addressing societal challenges (Indonesia)	Sarah Zanon Agapito-Tenfen
2015-2017	Dissertação de Mestrado	Impactos do pólen de soja geneticamente modificada (Intacta RR2 PRO®) e do herbicida Roundap sobre colônias de *Apis mellifera* L.	Mayara Martins Cardozo Martins/Afonso Inácio Orth
2015-2017	Iniciação científica	Metodologias de detecção de transgenes em variedades crioulas de milho	Caroline Bedin Zanatta/ Rubens Nodari
2016	Curso de Capacitação	Synthetic Biology – biosafety and contribution to addressing societal challenges (Africa do Sul)	Sarah Zanon Agapito-Tenfen

2018-2020	Dissertação de Mestrado	Efeitos de Roundup Transorb no perfil transcriptômico de folhas e na composição de grãos de *Glycine max* (L.) Merr	Caroline Bedin Zanatta/ Rubens Nodari – Sarah Zanon Agapito-Tenfen
2019	Parecer submetido ao Ministério Público Federal	Parecer Técnico sobre Resolução Normativa 16/2018 da CTNBio	Sarah Zanon Agapito-Tenfen
2020	Curso de Capacitação	Knowledge and information sharing on second generation GM Technologies in Africa (Africa do Sul)	Sarah Zanon Agapito-Tenfen

Elaborado pela autora com base nas informações fornecidas por Rubens Onofre Nodari e Sarah Zanon Agapito-Tenfen.

*Informações retiradas do Currículo Lattes