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Studying the expression of productivity and immunity genes of chickens under the influence of feed glyphosate using the RNA-seq method

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Abstract. The aim of the study was to study the effect of different concentrations of glyphosate on meat productivity and differential expression of genes for immunity and broiler productivity. Broilers were divided into groups: Control I, who received a diet without the introduction of glyphosate, Experimental II, who received a diet with the addition of glyphosate at a dose of 10 mg/kg of feed (0.5 MPC for food); Experimental III, who received a diet with the addition of glyphosate at a dose of 20 mg/kg of feed (1 MPC); Experimental IV, who received a diet with the addition of glyphosate at a dose of 100 mg/kg of feed (5 MPC). Transcriptome analysis was performed by RNA-seq on the Illumina Miseq platform, using the TruSeq Stranded mRNA kit (Illumina, USA). In the 22-28-day period of broiler rearing, a decrease in live weight gain (83 g less) was observed in Experimental group III compared to Control I ($P \leq 0.05$). It has been shown that glyphosate at the level of 1 and 5 MPC for food causes activation ($P \leq 0.05$) of the proinflammatory response genes (IL15, IL1B, IL34, IL22) and apoptosis (Casp1, Casp2, Casp6, Casp7, Casp8, Casp9) up to 31.1 times, which probably leads to the redistribution of nutrients in in the body towards the immune system, reducing the functions of absorption in the intestine. In parallel, there is an inhibition ($P \leq 0.05$) of the expression of genes that have a direct effect on growth and development, which ultimately leads to a decrease in poultry meat productivity. Exposure to glyphosate is an important but unaccounted-for risk factor for reducing meat productivity in birds, acting through a change in gene expression.

1 Introduction

Preparations based on glyphosate (N-[phosphonomethyl] glycine), such as, for example, Roundup, are the most commonly used herbicides in the world (Solomon et al., 2007). The use of glyphosate in agriculture has significantly expanded due to the development of glyphosate-resistant varieties of GM crops. Data are accumulating on the negative impact of this herbicide on the health of many animal taxa, from invertebrates to vertebrates (Faria MA (2015)). The content of glyphosate has been the subject of regular assessments by national

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and international regulatory authorities (Williams et al., 2000), which, however, have concluded that it has relatively low toxicity to animals and humans. Currently, the safety of glyphosate for farm animals and birds remains a controversial issue, since there is no unambiguous evidence of its toxicity.

Meat productivity is the most important economically valuable property of poultry. The concept of intestinal health includes morphological integrity, physiological functions of the intestinal tract (including digestion and absorption of nutrients), adequate microbiota, tissue metabolism and energy balance, effective immune responses, stable anti-inflammatory balance. The intestine is also the primary barrier that interacts with toxic substances coming from feed. Intestinal damage due to stress factors, including toxic substances, causes the release of large amounts of inflammatory cytokines and chemokines by peripheral immune cells (Jiang et al., 2009). This mediates apoptosis, disorders of the microbial flora, a decrease in the level of digestive enzymes and transporters in the intestine and damage to structural integrity, which leads to impaired digestion, absorption, and, therefore, reduced productivity. In a number of experiments, according to the assessment of the effect of glyphosate on animals, there was no decrease in their productive qualities (Vicini et al., 2019). Nevertheless, a number of experiments have demonstrated the negative effect of residues of a number of other commercial pesticides contained in feed on productive birds (Eid, et al., 2023). To date, the molecular mechanisms of this negative impact are not clear. In recent decades, studies of differentially expressed genes as a transcriptional response of the genome to various environmental stimuli or physiological/pathological conditions have been one of the most modern approaches (Gmoshinsky et al., 2018). The transcriptome of birds has previously been studied in connection with immunity (Wahl, et al., 2004), adaptive potential (Kumar et al., 2020) and meat productivity (Zhang, et al., 2021). However, experiments to study the effect of glyphosate on the transcriptome of living organisms have not been conducted before.

In this regard, the **aim of the study** was to study the chronic effect of different concentrations of feed glyphosate on meat productivity and differential expression of immunity genes and productivity of broiler chickens.

2 Materials and methods

The experiment was conducted in the vivarium of BIOTROF+ LLC in May-June 2022 on broiler chickens of the Ross 308 cross. The feeding and maintenance conditions met the requirements for the broiler cross (Methodology of scientific ..., 2013). Up to the age of 28 days, full-fledged compound feed PK5-1G-1101 was used for broilers aged from 1 to 4 weeks (produced by Gatchina Feed Mill JSC, Russia). From 28 to 35 days of growing chickens were fed with full-fledged compound feed PK-6-G-1102 (produced by Gatchina Feed Mill JSC, Russia) (Supplementary Table 1). In addition, the bird additionally received a vitamin and mineral biologically active supplement (Supplementary Table 2). The birds were divided into groups of 65 heads in each group: Control I – control, who received a diet without the introduction of glyphosate, Experimental II – experimental, who received a diet with the addition of glyphosate at a dose of 10 mg/kg of feed, which corresponded to 0.5 MPC for food (SanPiN 1.2.3685-21); Experimental III – experimental, who received a diet with the addition of glyphosate at a dose of 20 mg/kg of feed, which corresponded to 1 MPC for food; Experimental IV – experimental, who received a diet with the addition of glyphosate at a dose of 100 mg/kg of feed, which corresponded to 5 MPC for food. The duration of bird rearing is 35 days. The mode of watering, lighting and humidity corresponded to the guidelines for the maintenance of broiler livestock of the Ross 308 cross. The experimental and control young were kept in three-tiered cages consisting of blocks (BB-1 manufactured by Scientific Production Association Stimul-INK, Russia).

For the production experiment, glyphosate was used as part of the preparation Agrokiller (CJSC firm "August", Russia), containing 500g/l of acid glyphosate (isopropylamine salt). To do this, a working solution was prepared from the preparation Agrokiller, the working solution was applied to compound feed by spraying. Mixing was carried out mechanically in compliance with the safety requirements of personnel. After the introduction of glyphosate, its concentration in the feed was monitored by enzyme immunoassay (ELISA). In addition, the broiler diet practically did not contain background amounts of glyphosate, which indicates the purity of the experiment. To analyze the content of glyphosates by the ELISA method in feed and nutrient media, the STAT FAX 303+ strip enzyme immunoassay analyzer (Awareness Technology, LLC, USA) and the Glyphosate ELISA Microtiter Plate test system (Abraxis, USA) were used. The test is based on a direct competitive enzyme immunoassay between glyphosate, which is present in the sample, and an enzyme labeled with glyphosate to bind rabbit antibodies to glyphosate and goat antibodies to rabbit immobilized in microwells. After the enzyme immunoassay in the wells, the intensity of the color signal of the solution is inversely proportional to the concentration of glyphosate present in the samples.

Zootechnical analysis was carried out according to the recommendations (Methodology of scientific ..., 2004). The analysis of live weight gain was carried out weekly, individually.

To determine the expression of genes at the end of the experiment, the cecum tissues were taken from three broilers. The samples were stabilized using the RNAlater reagent (Thermo Fisher Scientific, Inc., USA) and immediately sent to the molecular genetic laboratory of the BIOTROF+ Research and production company for RNA isolation. To obtain RNA, the tissues were mixed with liquid nitrogen and homogenized. Total RNA was isolated using a mini-set of AurumTM Total RNA (Bio-Rad, USA), following the manufacturer's instructions. The reverse transcription reaction was performed to obtain cDNA on an RNA matrix using iScriptTM Reverse Transcription Supermix (Bio-Rad, USA).

To prepare samples for RNA-seq analysis (full-transcriptome sequencing) on the Illumina Miseq platform, the TruSeq Stranded mRNA (Illumina) kit was used. The MiSeq Reagent Kit v3 - 150 (Illumina) was used to sequence the resulting libraries. The quality of the data obtained as a result of sequencing, in fastq format, was determined using the Fastqc program. To quantify the RNA sequencing data and annotate the sequencing results, we used Salmon software (a program for obtaining high-precision quantitative estimates at the transcript level based on RNA sequencing data). The Gallus gallus reference genome was used to quantify the abundance at the transcript level in Salmon (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_016699485.2/). Next, an index was built on the reference genome (a structure that is used for quasi-mapping of RNA sequences during quantitative determination). Then the sequences obtained by us as a result of sequencing were mapped to the reference genome.

Mathematical and statistical processing of the results was carried out by the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio v. 1.1.453 (https://rstudio.com). The results are presented as mean (M) and standard errors of mean (±SEM). The reliability of the differences was established by the Student's t-criterion, the differences were considered statistically significant at $P \leq 0.05$. The mean values were compared using the significantly significant Tukey Difference (HSD) test and the TukeyHSD function in the R Stats Package.

The research was conducted in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 1986). The research was approved by the bioethical commission of the L.K. Ernst Federal Research Center for Animal Husbandry (protocol №2021–02/1, dated Feb 01, 2021) and performed in accordance with Russian

Federation ethics legislation with respect to the Russian Federal Law No 498-FZ on Responsible Treatment of Animals.

3 Results of the study

The results of changes in the absolute live weight gain of broilers of the Ross 308 cross by the periods of the experiment are presented in Table 1. In the 22-28-day period of broiler rearing, a decrease in live weight gain (83 g less) was noted in Experimental group III (1 MPC of glyphosate for food products) compared with Control I (P≤0.05). Whereas in the Experimental group IV (5 MPC of glyphosate), there was no difference in this indicator with Control I. The data obtained may be related, among other things, to different levels of expression of avian genes against the background of different dosages of glyphosate. The absence of an effect in group IV in the presence of a negative impact in group III can be explained by the increased activation of adaptive potential genes in response to an increased concentration of glyphosate in feed.

Table 1. Change in absolute live weight gain of Ross 308 cross broilers by experiment periods (1-7 days, 8-14 days, 15-21 days, 22-28 days, 29-35 days) in response to glyphosate feeding (M±m, n=65).

The period of growing birds, day.	Control I	Experimental II	Experimental III	Experimental IV
1-7	112.2±5.74	112.1±6.53	115.1±6.11	110.4±5.90
8-14	240.5±13.20	233.9±12.80	250.2±14.45	237.9±12.14
15-21	442.5±24.51	425.1±21.49	413.2±25.36	437.8±28.45
22-28	615.6±30.15	584.6±26.70	532.6±28.21	577±32.83
29-35	731±38.88	721.5±36.52	747.3±32.50*	756.7±28.44

Note: * - significant differences from the control group at P≤0.05

Next, we conducted an RNA-seq analysis of the cecum tissues of broilers against the background of different dosages of glyphosate. As a result of the analysis, the following number of unprocessed readings was obtained: for Control group I – 2663216 bps, for Experimental group II – 2662336 bps, for Experimental group III – 3086139 bps and for Experimental group IV - 2852829 bps. After filtering for the presence of genes with low expression, 33,060 genes were identified in the total pool of sequenced RNA of all groups.

Figure 1 shows a heat map showing the results of hierarchical clustering of samples from different groups according to the level of differential gene expression. It can be seen that Control group I and Experimental group II (in which birds received glyphosate at 0.5 MPC) fell into one cluster, while birds from Experimental groups III and IV (where broilers received glyphosate at 1 and 5 MPC, respectively) fell into another. That is, the expression level of group II samples was similar to Control I, which means that the effect of glyphosate at the level of 0.5 MPC was practically not expressed. While the impact of this herbicide at the level of 1 and 5 MPC is significant.

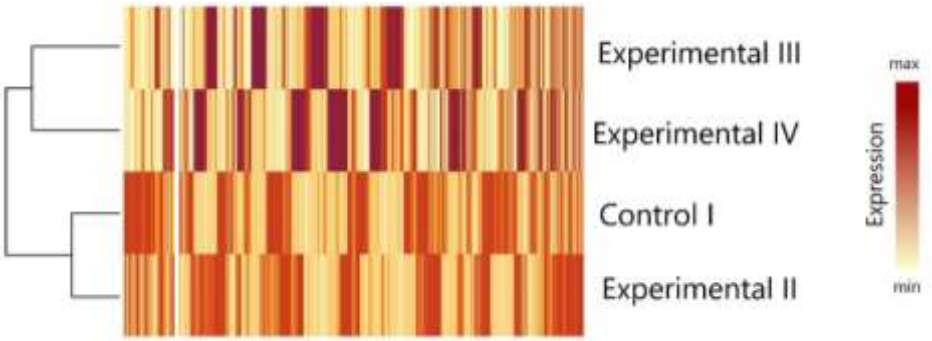

Fig. 1. Hierarchical clustering of differential gene expression in the cecum tissues of Ross 308 cross broilers under the influence of different dosages of glyphosate, obtained based on the results of RNA-seq analysis. Each row represents a sample of the Control and Experimental groups, each column represents the differential expression of genes. The color scale of the heat map indicates a change in the normalized expression level in this cell.

A comparative analysis of gene expression profiles in the cecum tissues of broilers exposed to glyphosate at 0.5 MPC (group II) revealed only 23 gene transcripts whose expression levels were statistically significantly different from Control I ($P \leq 0.05$) (Fig. 2). Significantly more pronounced were changes in the expression profile when using glyphosate at higher dosages – 1 and 5 MPC: in groups III and IV, the expression of 20168 and 21888 genes changed, respectively, compared with the control ($P \leq 0.05$). At the same time, in group III, the expression of 9125 genes increased compared to the control, 9328 genes decreased ($P \leq 0.05$). In group IV, the expression of 11043 genes increased compared to the control, 12560 genes decreased ($P \leq 0.05$).

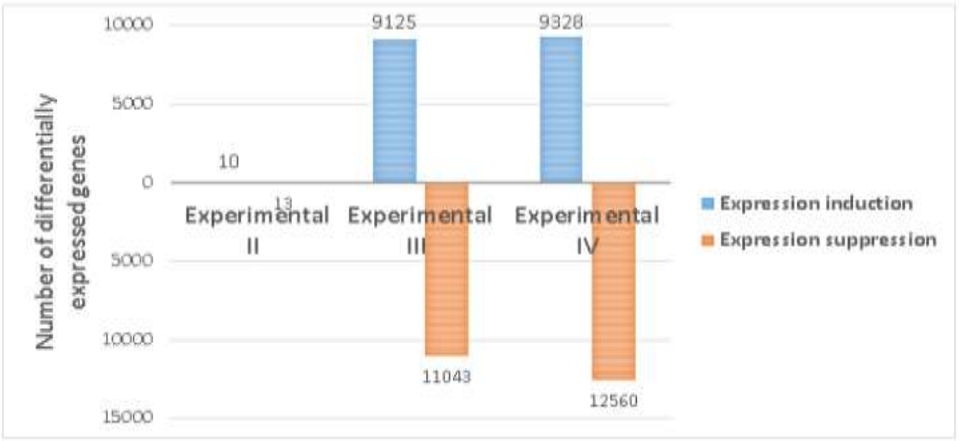

Fig. 2. The number of differentially expressed genes in the cecum tissues of Ross 308 cross broilers under the influence of different dosages of glyphosate in samples of Experimental groups II-IV compared with Control I, $P \leq 0.05$ (obtained based on the results of RNA-seq analysis).

Annotation of the obtained transcripts allowed us to determine their distribution by categories: biological processes, molecular functions, cellular components. Due to the phenotypic change in the level of meat productivity in birds, at this stage of the study, the collected and annotated transcriptome was used to search for genes potentially encoding proteins that have an immunoregulatory effect on the body, which can affect productivity indirectly, as well as genes directly related to the formation of productive qualities.

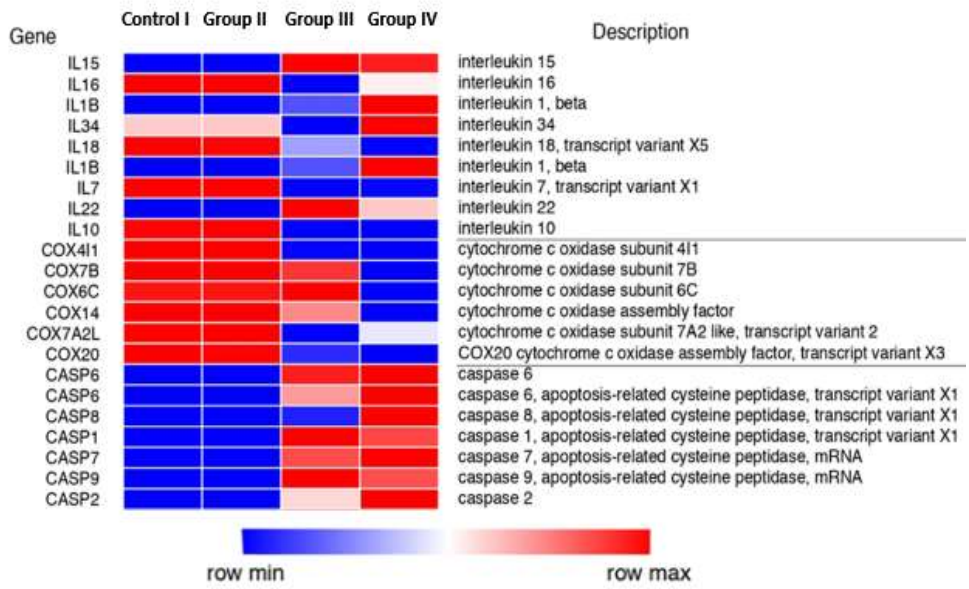

Fig. 3. The level of differential expression of genes associated with inflammatory immune reactions and apoptosis in the cecum tissues of Ross 308 cross broilers under the influence of different dosages of glyphosate in samples of Experimental groups II-IV compared with Control I, P≤0.05 (obtained based on the results of RNA-seq analysis).

As can be seen from Figure 3, glyphosate in dosage at the level of 1 and 5 MPC (Experimental groups III and IV) caused activation (up to 31.1 times) of apoptosis genes, such as *Casp1, Casp2, Casp6, Casp7, Casp8, Casp9*, and pro-inflammatory interleukins, such as *IL15, IL1B, IL34, IL22* (P≤0.05). Thus, the expression level of mRNA interleukin 15 (*IL15*) in groups I and II did not differ, (P0.05), and in groups III and IV it increased by 4.4 and 4.3 times, respectively, compared with Control I (P≤0.05).

Apoptosis of epithelial cells can reduce the level of digestive enzymes and transporters in the intestine, damage the structural integrity of the epithelium, which leads to digestive and absorption disorders (Jiang et al., 2009) and has a negative impact on the level of meat productivity. On the other hand, damage to the intestinal epithelium leads to the penetration of pathogens, their waste products and toxins into the bloodstream.

In addition to inhibiting digestion and absorption, nutrient redistribution may also play a role in the processes leading to a decrease in poultry meat productivity (Spurlock, 1997). Probably under the influence of glyphosate, the body redirects nutrients originally used for muscle synthesis and growth to maintain a highly activated immune system (which is expressed in the activation of interleukin genes and apoptosis) (Bai et al., 2019). The immune response requires a lot of aerobic energy and, consequently, oxygen, which creates an aerobic environment that leads to the conversion of glucose into lactic acid (Cangiano et al., 2019). In addition, the secretion of inflammatory cytokines negatively affects the process of energy storage (Klasing, 1988). This is confirmed by the fact that in birds with a high feed load of glyphosates (groups III and IV), the transcription level of cytochrome-c-oxidase subunit 1 (*COX4I1)* decreased from 1820 to 199 compared to Control I (P≤0.05) (Fig. 3). At a low concentration (group II), glyphosate had no significant effect (P>0.05). Cytochrome-c-oxidase, a terminal enzyme of the electron transfer chain, is an integral part of the mitochondrial mechanism necessary for the production of ATP in cells (Richter OM, Ludwig B., 2003). It is also known that a number of inflammatory factors can inhibit the synthesis of fatty acids

in adipose tissue (Klasing et al., 1997), accelerate the oxidation of fatty acids, which reduces the rate of fat deposition in the body (Li et al., 2021).

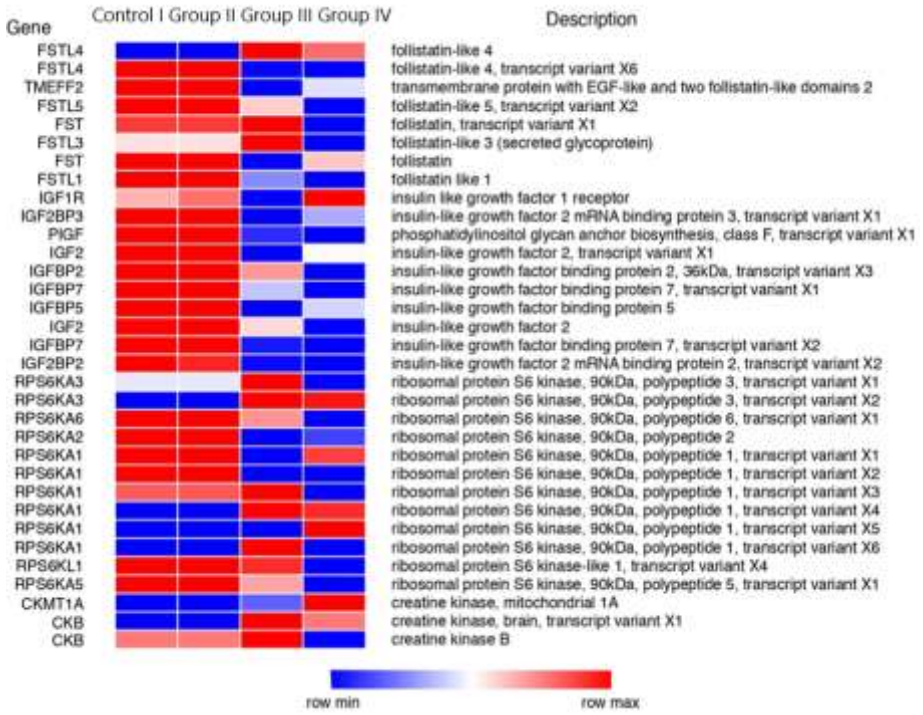

Fig. 4. The level of differential expression of productivity-related genes in the cecum tissues of Ross 308 cross broilers under the influence of different dosages of glyphosate in samples of Experimental groups II-IV in comparison with Control I, $P \leq 0.05$ (obtained based on the results of RNA-seq analysis).

As can be seen from Figure 4, glyphosate at concentrations at the level of 1 and 5 MPC (groups III and IV) sharply inhibited, compared with Control I and Experimental group II ($P<0.05$), the expression of genes related to productivity: a number of protein genes related to the growth factors of the follistatin family, insulin-like growth factors (such as *IGF2, IGFBP2, IGFBP7, IGFBP5, IGF2BP2*), ribosomal protein S6 protein kinase, etc. Expression of these genes is observed in many organs and tissues, including intestinal epithelial tissues (Tortoriello et al., 2001). Follistatin (FST), a member of the beta transforming growth factor superfamily, regulates body growth in chickens by inhibiting the binding of myostatin (a growth inhibitor) to its receptor. Its expression is associated with an increase in muscle mass (Dushyanth K, et al., 2022). Insulin-like growth factors play a vital role in the regulation of growth, metabolism and homeostasis of various organs, including the intestine (Troike et al., 2017), including the rate of mitosis, absorption and secretin production (Dorchester JE, Haist RE, 1953). Numerous studies have demonstrated that physiological levels of insulin-like growth factors maintain intestinal integrity (e.g., reduce intestinal permeability and bacterial translocation) and improve intestinal function (e.g., macronutrient uptake and immune function) (Young et al., 2019).

4 Conclusions

Exposure to glyphosate is an important but unaccounted-for risk factor for reducing meat productivity in birds, acting through a change in gene expression. Transcriptomic analysis

revealed that glyphosate at the level of 1 and 5 MPC for food (which is significantly lower than the MPC in feed) triggers a pro-inflammatory response and apoptosis, which probably leads to a redistribution of nutrients in the body towards the immune system, a decrease in the functions of digestion and absorption in the intestine. In parallel, genes that have a direct effect on growth and development are inhibited, which ultimately leads to a decrease in poultry meat productivity.

Therefore, we believe that assessing the problems associated with the large-scale and intensive use of glyphosate and other pesticides is a much more difficult task than originally anticipated by regulatory authorities.

The pilot data obtained by us need further verification, nevertheless, today they are of undoubted interest for further studies of the molecular and genetic mechanisms of the effect of glyphosate feeds on agricultural poultry, as well as for the development of effective therapeutic and preventive measures. Since the exact causes of differential sensitivity to glyphosate in macroorganisms are unknown, this also needs to be clarified in the future.

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