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CDDP Profiles and Selected WRKY Genes Expression Response to Zinc Dioxide Nanoparticles Foliar Application in *Glycine max* L.

Jana Žiarovská[1], Adam Kováčik[1]

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ABSTRACT

Background: Zinc is a vital trace element required by plants for numerous cellular activities. Application of nano-fertilizers in the form of Zn-O nanoparticles was evaluated in this study to describe the changes in CDDP fingerprints and selected WRKY genes expression in two *Glycine max* L. varieties.

Methods: Foliar dispersions of ZnO nanoparticles were applied by handheld sprayer with the nanoparticle concentrations 1.4 mg×L^{-1}, 14 mg×L^{-1} and 140 mg×L^{-1} plus control plants. Four primer combinations of conserved parts of plant WRKY genes were used in CDDP fingerprinting and four WRKY genes were evaluated for their expression changes.

Result: Polymorphic CDDP profiles were generated for three primer combination. No unique amplicons were obtained, but the generated fingerprint profiles differ in control plants of both varieties. The ability of used primer combinations to detect polymorphism was comparable only for combinations F1R2b and F1R3a. No significant changes of expression were obtained in the case of 1.4 mg×L^{-1} foliar application of ZnO nanoparticles for WRKY11, WRKY106 and WRKY149 genes. The highest applied concentration of ZnO nanoparticles resulted in the relevant upregulation of all the analysed WRKY genes with the values from 5 times (Adelfia, WRKY 90) up to the 80 times (Mentor, WRKY 106).

Key words: Expression changes, Fingerprints, Foliar application, Zn nanoparticles.

INTRODUCTION

Glycine max L. (soybean) belongs to crops with the highest economic impact worldwide with strong relevancy for human and animal feeding, chemical and food industry of biofuel production (Usha and Dadlani, 2015; Jassal and Singh, 2020. In the last decade, global production of this legume has expanded 8.4 times as a result of genetic improvements and advanced production techniques, while productivity has doubled (Delele *et al.*, 2022). Based on this, soybean is the object of different research fields connected to its ability to response stress or interaction with different substances. Application of nano-fertilizers in the form of Zn-O nanoparticles was evaluated for its effect on environmental important soil properties with soybean cultivation under field agronomic conditions previously (Ernst *et al.*, 2024) and no adverse effect was concluded on selected soil properties together with positive stimulation of pollen viability.

Zinc is a vital trace element required by plants for numerous cellular activities. In soybean, zinc deficiency can lead to severe physiological and morphological changes, affecting productivity. Understanding the functions of zinc in *Glycine max* can help improve nutrient management strategies and enhance crop yield (Broadley *et al.*, 2007; Alloway, 2008; Gonyane and Sebetha, 2021). In crops, Zn toxicity at concentrations above 3-10 ppm in nutrient solutions significantly inhibited growth, with susceptible crops like spinach showing a 50% yield reduction at lower thresholds (Saboor *et al.*, 2021). Different physiological and biochemical functions of zinc were reported for soybean. It is involved in enzyme activation, where zinc acts as a cofactor for various enzymes such as superoxide dismutase, carbonic anhydrase and RNA polymerase, essential for cellular metabolism and stress responses (Marschner, 2011). Zinc is a part of protein and nucleic acid synthesis, where it is crucial for the proper functioning of ribosomes and the synthesis of proteins and nucleic acids, which are essential for cell division and growth (Broadley *et al.*, 2007). Zinc influences auxin synthesis and stability, affecting root elongation and shoot growth (Cakmak, 2000), stabilizes cell membranes by reducing oxidative stress and lipid peroxidation, enhancing

[1]Institute of Plant and Environmental Sciences, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 02, Slovak Republic.

Corresponding Author: Jana Žiarovská, Institute of Plant and Environmental Sciences, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 02, Slovak Republic. Email: jana.ziarovska@uniag.sk
ORCIDs: 0000-0002-0005-9729, 0009-0004-7474-6056

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overall plant health (Alloway, 2008). Even zinc is important micronutrient, its overdoses result in stress for plants, is phytotoxic, cause structural as well as functional abnormalities an weakening the plant performance, but these responses vary with the type of plant species and their developmental stages.

Regulation of the plant response to abiotic stress is under different metabolic pathways (Shiade *et al.*, 2024). WRKY transcription factors play a crucial role in the regulatory networks associated with stress responses in plants and are reported to comprise one of the largest families of regulatory proteins in plants (Eulgem and Somssich, 2007). They are involved in pathways that govern the expression of defense-related genes, thus contributing to the plant's ability to withstand various stressors, including those induced by nanoparticle (Lei *et al.*, 2024; Li *et al.*, 2025). The WRKY family, characterized by their conserved WRKY domain, binds to specific W-box elements in the promoters of target genes, activating or repressing their expression in response to external stimuli, including nanoparticles (Zhang *et al.*, 2022). Recent studies have indicated that the application of nanoparticles can significantly alter the expression levels of WRKY genes. Some nanoparticles have been shown to induce the expression of specific WRKY TFs, which are implicated in enhancing plant resistance to pathogens and abiotic stresses such as drought and salinity. The expression of WRKY genes like WRKY3 and WRKY4 has been particularly noted to increase in response to nanoparticle treatment, suggesting that these factors may mediate the plant's defense mechanisms against nanoparticle-induced stress (Liu *et al.*, 2024). In soy plants, different expression patterns of WRKY genes were obtained for drought tolerant and susceptible genotypes in water stress conditions and their involving in signaling, gene expression control, ion/proton transporter and reactive oxygen scavenger were hypothetized. Additionally, WRKY genes such as GmWRKY27 have been shown to enhance stress tolerance by interacting with other transcription factors like GmMYB174, which modulates the expression of downstream genes involved in stress responses (Wang *et al.*, 2015). WRKY transcription factors are integral to soybean's response to both biotic and abiotic stresses. GmWRKY40 has been identified as a positive regulator in response to *Phytophthora sojae* infection, enhancing resistance through modulation of reactive oxygen species and jasmonic acid signaling pathways (Cui *et al.*, 2019). Similarly, WRKY genes are involved in the response to low phosphorus conditions, with specific genes showing altered expression patterns that contribute to stress tolerance (Kurt and Filiz, 2020).

WRKY genes variability at genomic level is a background of DNA based marker technique - Conserved DNA-Derived Polymorphism (CDDP) (Collard and Mackill, 2009). This technique uses a well characterized plant WRKY genes involved in response to abiotic and biotic stresses to generate DNA markers, where different aminoacid motifs were transponed into primers that generate polymorphic profiles. Up today, CDDP was used for different plant species to characterized their variability as well as markering the stress answer (Haffar *et al.*, 2022; Aziz and Tahir, 2023).

In this study, experimental approach was used to analyse the selected WRKY gene expression changes and CDDP fingerprint polymorphism for soybean plants under the foliar treatment of ZnO-nanoparticles.

MATERIALS AND MATHODS

Plant material

Two *Glycine max* L. varieties were used, Mentor and Adelfia. Mentor is the most cultivated and versatile mid-early variety in Central Europe that is suitable for growing in corn and beet fields. This medium-tall variety is non-recumbent and has excellent health status. The first pods are placed at a height of 11-12 cm. It ripens evenly, its seed is large, with a light navel and has a high protein and oil content. It is suitable for use for special food purposes, tofu preparing. Adelfia is an early variety with purple flower color. It has rapid initial development and higher growth with a very high yield. It is also suitable for sowing in wider rows. It is semi-determinant-partially branching variety. It reliably closes the stands quickly and thus competes with weeds. A very good state of health guarantees high yields. Pods and seeds with a light navel have good resistance to spontaneous shedding (Saatbau, 2024). The experiment was carried out at the Slovak University of Agriculture in Nitra, on the experimental plots of the Field Crops Sample Garden - Demonstration Garden of the SPU in Nitra, which is part of the set of sample gardens of the University Agricultural Enterprise of the SPU in Nitra. The area is located in a very warm and dry lowland region. The soil type was classified as Fluvisol and according to the classification of agricultural soils, the soils in this area are very heavy. The climate of the area is characterized as warm and dry, with a long-term average temperature of 10.2°C and an annual rainfall of 539 mm.

Experimental design

ZnO nanoparticles were obtained as dispersion commercially (Sigma-Aldrich). Visualization, crystallinity, structure and colloidal properties were verified (Kolenèík *et al.*, 2019, 2022; Ernst *et al.*, 2024). A true experimental design was set up during the growing season of 2024. Control variant and variants with ZnO-NPs and TiO2-NPs were randomly organized in perpendicularly selected blocks. Foliar dispersions of ZnO nanoparticles were applied by handheld sprayer, Gamma 5 (Mythos di Martino) with experimental concentration: nanoparticles free control and variants with the nanoparticle concentrations 1.4 $mg \times L^{-1}$, 14 $mg \times L^{-1}$ and 140 $mg \times L^{-1}$ based on previous experimental results (Ernst *et al.*, 2024) in the growth phase BBCH 30

(the end of formation of end shoots). Samples of leaves were collected in biological triplicates day after the treatment.

Nucleic acid extraction

Total genomic DNA was extracted using the GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific) following the instructions of manufacturer. RNA was extracted by GeneAll® Ribospin™ Plant Kit (GeneAll) according to the manufacture´s protocol. Quality and quantity of extracted nucleic acids was analysed by Nanophotometer (Implen). Reverse transcription was performed from 2620 ng by Tetro cDNA Synthesis kit using the oligo (dT) primers following the manufacturer's protocol.

CDDP analysis

Conserved DNA-Derived Polymorphism (CDDP) technique was used to marker a length polymorphism of a total of three primer combinations of WRKY genes: WRKY-F1 TGGCGSAAGTACGGCCAG (GC 67%); WRKY-R1 GTGG-TTGTGCTTGCC TGGCGSAAGTACGGCCAG (GC 60%); WRKY-R2b TGSTGSATGCTCCCGTG GCGSAAGTACGGCCAG (GC 67%); WRKY-R3 GCASGTGTGCTCGCC TGGCGSAA-GTACGGCCAG (GC 73%) and WRKY-R3b CCGCT-CGTGTGSACGTGGCGSAAGTACGGCCAG (GC 63%) (Collard and Mackill, 2009). The reaction conditions for time and temperature were: initial denaturation at 95°C for 15 min; than 40 cycles at 95°C for 45 s, 54°C for 45 s, 72°C for 90 s and for the final extension, 72°C for 10 min. Amplicons were evaluated in 1.5% agarose gels with 1× TBE buffer and the DNA fragments were stained using GelRed® Nucleic Acid Gel Stain (Biotium). For visualisation, BDAdigital system 30 (Analytik Jena) was used.

qRT-PCR analysis

A two-step real-time PCR protocol was applied for expression analysis. WRKY genes of *Glycine max* L were screened for their induction in abiotic stress conditions from the Soybean Genome Project Database (http://bioinfo03.ibi.unicamp.br/soja/) and four genes were selected with following access number from Phytozome - Glyma01g31921 (WRKY11), Glyma05g29310 (WRKY 90), Glyma07g02630 (WRKY106) and Glyma15g11680 (WRKY149) to analyse their expression. Actin gene (Glyma18g52780) was used as housekeeping gene. 5x Hot FirePol EvaGreen (Solis BioDyne) was used in amplification and the analysis was performed by in CFX thermocycler (Biorad) under the following reaction conditions: initial denaturation at 95°C for 2 min, then 40 cycles of 95°C for 10 s and 62°C for 40 s followed by final analysis of amplicon dissociation curves.

Data analysis

The separated CDDP amplicons were transformed to binary matrices based on the presence or absence of the band. Based on binary matrixes calculated by Jaccard coefficient of genetic similarity (Jaccard and Paul, 1908). UPGMA dendrograms were constructed in Statgraphics software. A qPCR analysis with the biological triplicates was used in the study and the relative expression values were calculated by the delta-delta Ct method in MS Excel (Livak and Schmittgen, 2001) when the expression of individual WRKY genes was determined as the number of amplification cycles obtained in the reaching of the threshold during the exponential phase of the PCR.

RESULTS AND DISCUSSION

CDDP variability

Different fingerprints were obtained for individual primer combinations. The primer combination F1R3b generated monomorphic profiles for all of the analysed accessions. The other used degenerate WRKY primers amplified a total of 34 consistent band lines, of which 22 were polymorphic. Fragment sizes ranged from 50 bp up to the 1200 bp when compared to 100 bp ladder (Bioline). A total of 258 bands were amplified in the set of control plants and six analysed variants of ZnO treatments and the polymorphism generated by individual primer pair ranged from 18% up to the 88% (Table 1). No unique amplicons were obtained, but the generated fingerprint profiles differ in control plants of both varieties (Fig 1).

The ability of used primer combinations to detect polymorphism was comparable only for combinations F1R2b and F1R3a, in the case of primer combination F1R1, the PIC value was much lower-0.08. The highest discrimination power was obtained for primer combination F1R2b, what correspond to the most distinct fingerprints for individual analysed accessions.

Constructed dendrogram for CDDP (Fig 2) was able to distinguish all analysed acces-sions. A total of two main branches were separated, distinctive fingerprints were obtained for control plants of both analysed soybean varieties with the Jaccard coefficient of genetic similarity 0.38. These two accessions produced specific fingerprints for all the primer combinations used in this study.

WRKY genes expression

Selected WRKY genes were subjected to compare its expression in leaves of ZnO foliar application treated plants compared to control ones as they represent the functional

Table 1: Characteristics of CDDP fingerprints by individual primer combinations for analysed varieties.

Marker	Polymorphism	PIC	N	MI	DI	D
F1R1	18%	0.08	12.75	0.01	0.09	1
F1R2b	81%	0.4	9	0.04	0.68	8.5
F1R3	88%	0.3	10,5	0.04	0.44	7.5

AN- Average number of amplified alleles per accession; MI- Marker index; DI- Diversity index; D- Resolving power.

part of CDDP technique used in this study. Two different soybean varieties were used for this purpose. Effectivity of the real-time PCR for individual analysed WRKY genes was 0.97 for WRKY11, 0.90 for WRKY90, 0.96 for WRKY106 and 0.94 for WRKY149. The average Cts for individual WRKY genes were 31.7 for WRKY11, 33.1 for WRKY90, 31.8 for WRKY106 and 35.2 for WRKY149. Both analysed varieties were similar in the expression changes except of the WRKY90, where different patterns were obtained (Fig 3). No significant changes of expression were in the case of 1.4 mg×L^{-1} foliar application of ZnO nanoparticles for WRKY11, WRKY106 and WRKY149 genes. The highest applied concentration of ZnO nanoparticles resulted in the relevant upregulation of all the analysed WRKY genes with the values from 5 times (Adelfia, WRKY 90) up to the 80 times (Mentor, WRKY 106). In none of analysed variants, downregulation of analysed genes was obtained.

Expression profiles of WRKY 11 gene were the most similar in both of analysed soybean varieties. WRKY90

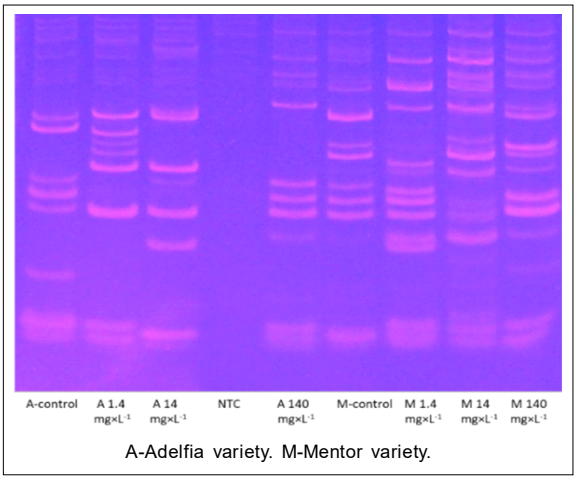


Fig 1: CDDP profile obtained for F1R2b primer combinations.

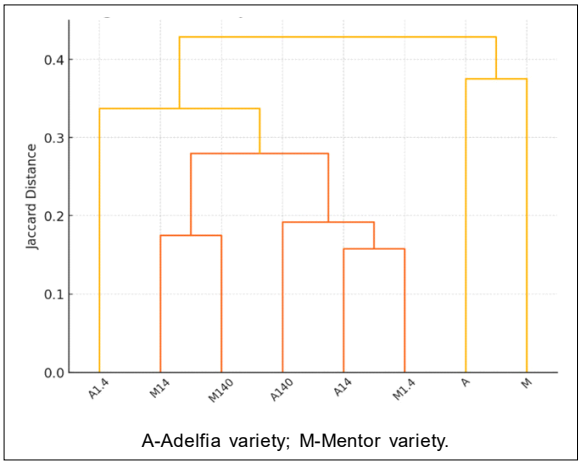


Fig 2: UPGMA dendrogram of Jaccard genetic similarity values among analysed control and treated plants of soybeans varieties Mentor and Adelfia for CDDP technique.

expression profiles showed higher expression in 1.4 mg×L^{-1} foliar application of ZnO nanoparticles when compared to all other evaluated WRKY genes and for Adelfia variety, 14 mg×L^{-1} foliar application of ZnO nanoparticles showed 8 times higher expression, what was unique result.

The narrow gap between Zn essentiality and toxicity in plants exist (Kaur and Garg, 2021), in sweet potato a total of 17.945 differentially expressed genes were identified in the two genotypes under zinc stress by transcriptomic analysis (Meng *et al*., 2023). Conversely, Zn is involved in the defence mechanism of plants as well, such as Zn finger WRKY transcription factors control various plant processes including systemic acquired resistance (SAR) (Rushton *et al*., 2010).

The mechanisms by which nanoparticles influence WRKY gene expression may involve several pathways. One potential pathway includes the activation of signalling cascades that lead to the phosphorylation of WRKY proteins, thus modulating their activity and stability (Liu *et al*., 2024; Ma and Hu, 2024). Additionally, the involvement of reactive oxygen species (ROS) generated by nanoparticles could further stimulate the expression of WRKY genes, as ROS are known to act as signalling molecules in stress response pathways (Li *et al*., 2025). The interaction of WRKY proteins with other regulatory elements and proteins, such as plant resistance (R) proteins, may also enhance their role in mediating responses to nanoparticles (Liu *et al*., 2024; Ma and Hu, 2024).

WRKY11 transcription factors in *Glycine max* L. have variable evolutionary conservation and functional importance in different roles. They express across tissues and WRKY11-1, WRKY11-2 and WRKY11-3 have been identified as being ubiquitously expressed across soybean tissues, indicating a broad regulatory role (Mohanta *et al*., 2016). They are active in response to low phosphorus, as WRKY genes in soybean, including WRKY11 orthologs, show significant transcriptional responses to phosphorus deficiency, which is crucial for developing phosphorus-efficient soybean cultivars (Kurt and Filiz, 2020). Their role in stress response in soybean was described with its co-expression networks suggesting involvement in tolerance to environmental challenges (Mohanta *et al*., 2016). The expression pattern of WRKY genes in response to water deficit was found to be different in drought tolerant and drought susceptible genotypes (Dias *et al*., 2016) and a concordance was for this and our study for WRKY106 and WRKY149 genes.

CDDP uses long primers that target plants' genes responsive to abiotic and biotic stress (Collard and Mackill, 2009). To our knowledge, in this study, it is the first time CDDP markers were used to characterize these genetic fingerprints to analyse plant response to nanoparticles foliar application. The typical fragment size range for

CDDP markers is between 200 and 1,500 bp stress (Collard and Mackill, 2009). The results presented in our study contain a total of 258 amplified fragments in the range that not fully corresponding to the expected length, such as

shortest amplicon started with the length of 50 bp, what should be specific for soybean genomic characteristics of WRKY genome-conserved sections of CDDP markers. When examining the averages of fragment sizes and their deviations, it was observed a distinctive pattern in the fingerprint of control plants of varieties Adelfia and Mentor. The CDDP was reported previously to be able revealed the same grouping pattern for genotypes of stressed plants (El-Mogy *et al*., 2022) and for tomato, CDDP-genotypic and visual phenotypic assortment permitted the selection of two contrasting heat-tolerant and heat-sensitive cultivars (Werghi *et al*., 2021). CDDP markers was proved to be used to determine the genetic variations among nanoparticles affected plant accessions.

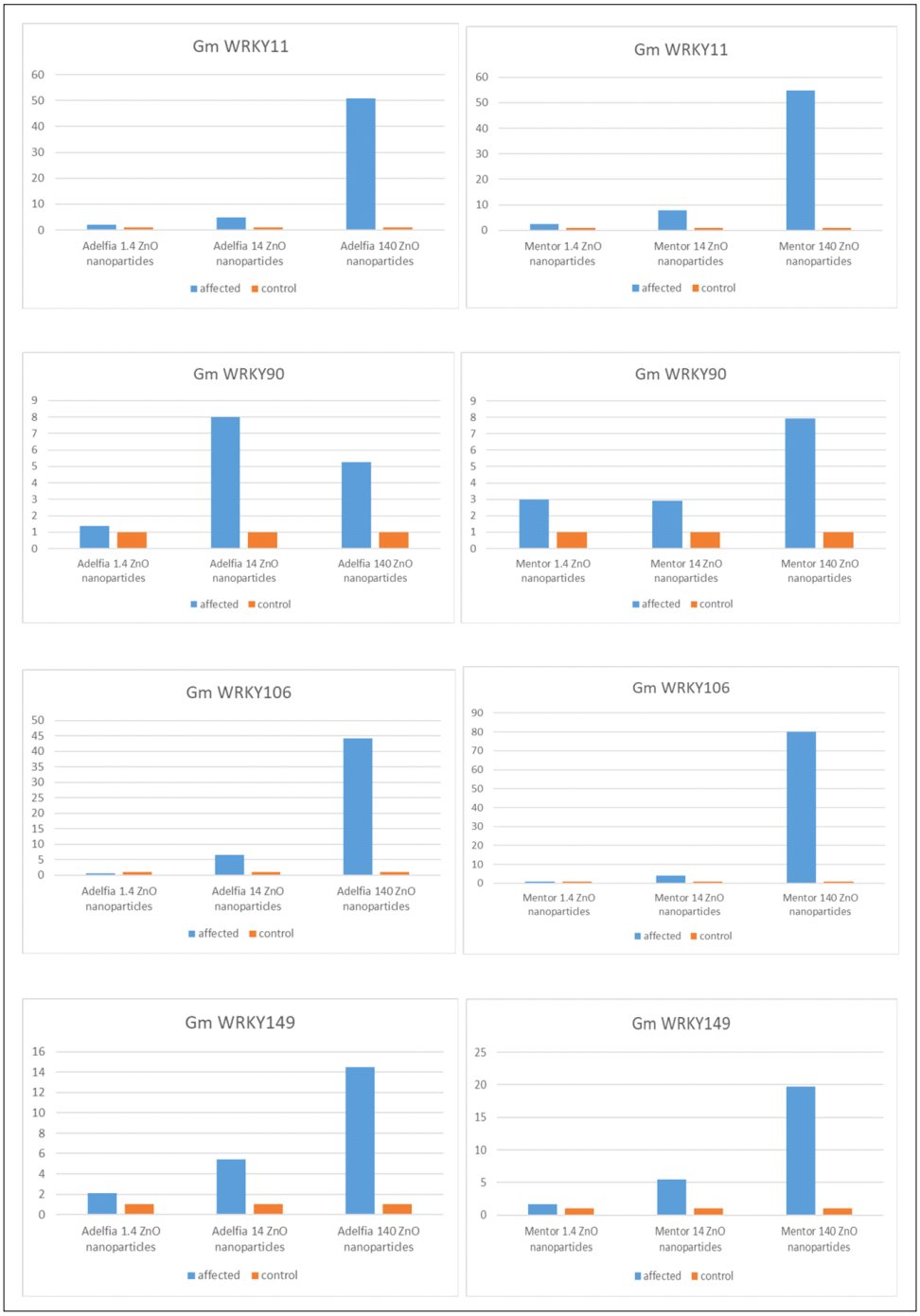


Fig 3: Comparison of WRKY expression levels in leaves between two analysed *Glycine max* varieties in response to ZnO nanoparticles foliar application. Actine reference gene was used as internal control.

CONCLUSION

In this study, the fingerprint profile of WRKY based DNA markers and transcriptional profile of four WRKY genes in response to foliar application of ZnO nanoparticles was outlined for two soybean varieties. The distinctive CDDP fingerprint patterns were obtained for control plants of both analysed varieties when comparing to all the affected variants. The higher expression of three from four analysed WRKY genes for highest concentration of ZnO nanoparticles may be a result of their stronger determinative role in zinc overload in *Glycine max* L., that is, why doses under 100 $mg \times L^{-1}$ seem to have no suppressing effect.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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