

Multi-Level Assessment of Environmental Effects on Winter Wheat

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This study aimed to evaluate variability in agronomic performance and physiological and molecular markers indicative of genomic responses in winter wheat (*Triticum aestivum* L.) exposed to a gradient of environmental radiation. Field analyses and plant sampling were conducted at two locations situated 500 m and 1000 m from a nuclear power station (NPS). Parameters assessed included the normalised difference vegetation index (NDVI), plant density, spike number per plant, grain number per spike, thousand grain weight, and grain yield. NDVI values declined from the BBCH 32 stage to BBCH 51 (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie phenological scale) at both sites. A marked increase in NDVI was observed at BBCH 59 and at the onset of flowering (BBCH 61). Vegetation indices and yield-related traits were generally higher at the site located further from the radiation source. Genomic analysis of stress-sensitive markers revealed elevated amplification of miR168-based loci during early developmental stages, regardless of cultivation distance, whereas elevated amplification of miR408-based loci was observed during the transition to flowering and at flowering. These patterns corresponded with agronomic and physiological indicators, as well as background radiation records. Multivariate correlation showed that miR168-based loci of 150 base pairs (bp) and 200 bp, and the miR408-based locus of 150 bp, had moderate to strong non-linear relationships with developmental stage and growth location. These findings suggest that early growth stages of winter wheat are particularly sensitive to environmental stress, as reflected in NDVI metrics and microRNA-based (miRNA) markers.

Keywords: winter wheat, environmental stress, NDVI, miRNA-based markers, grain yield

1 Introduction

Environmental radiation may represent an abiotic stress factor with potential implications for agricultural productivity, particularly in areas influenced by natural background sources, accidental releases, or radiation-related technologies. Understanding crop responses under such conditions is important for assessing potential risks to agricultural production and for developing appropriate monitoring strategies (Nakanishi, 2017). Radiation background is one of the important elements that are strictly controlled and regularly measured. A radiation monitoring network has been established to ensure radiation safety in terms of potential dangers

to the population and the environment (SHMÚ, 2024a). The measured quantity is the absorbed dose rate, from which the equivalent gamma dose rate in air is determined. This parameter combines the amount of absorbed radiation with the health effects of the radiation type in question. The sievert (Sv) is the unit of dose equivalent, but in practice the parameter is measured in nSv·h⁻¹ (SHMÚ, 2024b).

Wheat is among the most important cereal crops worldwide. Winter wheat is a suitable crop for assessing responses to environmental stress because of its widespread cultivation and sensitivity to environmental conditions (Han et al., 2016). It provides higher

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nutritional value than many other cereals and contains most essential nutrients and vitamins (Igrejas & Branlard, 2020). For these reasons, the assessment of yield-forming parameters provides a quantitative basis for evaluating crop responses under radiation-related environmental conditions. These agronomic indicators are directly linked to food quantity and quality, and any disruption caused by radiation may have cascading effects on food systems (Hasanuzzaman et al., 2019).

In addition, physiological indices such as the NDVI provide non-destructive, remote-sensing-based tools for monitoring plant health, stress responses, and photosynthetic activity, thereby enabling early detection of radiation effects (Robles-Zazueta et al., 2021). Globally, there is increasing interest in understanding how environmental radiation, whether from accidental release, natural background sources, or controlled applications, affects agricultural productivity. Identifying trends in crop responses under such conditions is vital for risk assessment, land-use planning, and the development of resilient crop management strategies (Singh et al., 2025).

MicroRNAs (miRNAs) are small regulatory molecules that play a crucial role in controlling gene expression at both genetic and epigenetic levels in plants (Cuperus et al., 2011). They are integral to numerous physiological processes, including growth, development, organ formation, and defence mechanisms. Deeply conserved miRNA families form essential components of regulatory networks that enable plants to adapt to abiotic and biotic stress. By modulating transcriptional and posttranscriptional pathways, stress-responsive miRNAs help plants cope with environmental challenges, and their identification provides valuable insights into plant adaptation strategies (Bej & Basak, 2014; Kruszk et al., 2012).

It was hypothesised that proximity to the Jaslovské Bohunice NPS affects the agronomic performance, NDVI-based physiological status, and miRNA-based molecular responses of winter wheat. The study aimed to assess these parameters under field conditions at two distances from the NPS.

2 Material and Methods

2.1 Characteristics of the Object of Investigation

Winter wheat (*Triticum aestivum* L.) sampling and analysis were conducted in the vicinity of the Jaslovské Bohunice NPS. Two cultivation areas (variants) were established within a 50.36-hectare plot of selected agricultural land. The first area was located 500 m from the generating unit of the NPS, while the second area, situated 1000 m away, served as the control site. Sampling and analyses were performed at selected developmental stages according to the BBCH scale described by Meier (2001), as shown in Table 1.

The experimental field is located in a warm, very dry lowland region. The soil profile is deep and non-skeletal (with a skeletal fraction of less than 10% to a depth of 0.6 m), and its texture corresponds to a medium-heavy loam. The soil is classified as typical chernozem and brown chernozem developed on loess, with a site-specific production potential rating of 83. The terrain is flat and shows no evidence of sheet water erosion (BPEJ, 2019). The farm on which the experiment was conducted has a total utilizable agricultural area of 1,299.32 hectares. Crop production is primarily focused on cereals, oilseed crops, sugar beet, and forage crops intended for silage.

2.2 Crop Characteristics

In this study, the winter wheat variety Suffolk CS was analysed. This winter variety is characterised by very early maturity and high forage quality. It exhibits good baking quality and tolerance to chlorotoluron. Suffolk CS is also drought-tolerant and winter-hardy. The thousand grain weight (TGW) is approximately 46 g, and plant height is medium to tall, at approximately 88 cm (RWA Slovakia, 2024). Regarding cultivation practices, the forecrop was sugar beet, and winter wheat was grown within a conventional tillage system.

2.3 Agronomic Analyses

Sampling for agronomic analyses was performed at the full maturity stage of winter wheat. At both sites, samples were collected in three replicates from different

Table 1 Sample identification and developmental stage of sampling

Sampling	BBCH
1	32 – node 2 at least 2 cm above node 1
2	39 – flag leaf stage: flag leaf fully unrolled, ligule just visible
3	51 – beginning of heading: tip of inflorescence emerged from sheath, first spikelet visible
4	59 – end of heading: inflorescence fully emerged
5	61 – beginning of flowering: first anthers visible
6	90 – fully ripe: grain hard, difficult to divide with thumbnail

locations while maintaining a consistent distance from the reactor unit of the NPS. The collected samples were analysed and yield-forming parameters were determined, including number of plants per m², number of spikes per plant, number of grains per spike, and thousand grain weight (TGW). TGW was measured using a PCB-3500-2 laboratory scale manufactured by Kern & Sohn GmbH (Balingen, Germany). Sampling of plant material for agronomic analyses and grain yield estimation followed the methodology described by Satorre & Slafer (1999).

2.4 Physiological Analyses

At the experimental sites, the NDVI was measured using a PlantPen NDVI 300 device (Photon Systems Instruments, Brno, Czech Republic). During wheat sampling at BBCH growth stages 32 – 61, 10 plants were randomly selected at each site. The uppermost leaf of each plant was measured, with ten repeated measurements taken per leaf. NDVI values were recorded and evaluated according to methodologies described by Jones et al. (2009).

2.5 Molecular Analyses

For the miRNA-based markers assay, the uppermost leaves of wheat were sampled, on which the measurement of the vegetation index NDVI was carried out. After harvesting, leaves were kept chilled in a portable refrigerator and then stored at -20 °C until further analyses. Prior to deoxyribonucleic acid (DNA) isolation, the pooled samples of leaves from 10 plants were pulverised in liquid nitrogen and genomic DNA was then isolated using a commercial isolation kit Isolate II DNA kit (Bioline Meridian Bioscience, Cincinnati, Ohio, USA) according to manufacturer's recommendations. A spectrophotometer NanoPhotometer P-Class P 360 (Implen GmbH, Munich, Germany) was used to measure the quality and quantity of the isolated DNA which was diluted to 70 ng·µl⁻¹.

For the touchdown polymerase chain reaction (PCR), the following were used: primers designed according to the sequences of the microRNA molecules derived from miRBase (University of Manchester, Manchester, United Kingdom) miR168 and miR408 (Generi Biotech s.r.o., Hradec Králové, Czech Republic) (Table 2), and a PCR

Master Mix 2× DreamTaq Hot Start Green (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The microRNA-based marker assay was performed according to Ražná et al. (2020).

2.6 Background Radiation Analyses

During sampling, the radiation background was measured at the locations with a dosimeter Radiameter FH 40 G [E] (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After arriving at the station, the dosimeter was stabilized for 5 minutes. After stabilization of the dosimeter, the average function was used for 5 minutes with 3 repetitions. The data were measured in units of batch power equivalent of ionizing radiation – nSv·h⁻¹ (nanosievert per hour).

2.7 Data Processing

The data of individual analyses were processed in the Microsoft Excel program (Microsoft Corporation, Redmond, WA, USA), the functions of averages and sums were used. The GeneTool software (Syngene International Ltd., Bengaluru, India) was used to evaluate electrophorograms. The agronomic and physiological data were analysed using analysis of variance (ANOVA), and Fisher's least significant difference (LSD) test was applied at a 95% significance level. All analyses were performed using Statistica 14.0.1 (TIBCO Software Inc., Palo Alto, California, USA). The Point-Biserial Correlation analysis was applied for testing the relationship between miRNA-based DNA fragments polymorphism and growth stage or experimental sites. Decision Tree Regression was applied for multivariate correlation analysis.

3 Results and Discussion

3.1 Agronomic Analyses

The analyses of yield-forming parameters and grain yield indicated numerical differences between the two experimental locations, whereas a statistically significant difference was observed only for thousand grain weight (TGW). Results are presented as averages from three replicates of plant material. At the closer site to the NPS (500 m), plant density averaged 174.7 plants per m², which was 12% higher than the 156 plants per m² recorded at the more distant site (1,000 m).

In contrast, the number of spikes per plant was higher at the distant site, averaging 3.5 spikes compared to 2.7 spikes at the closer site, representing a 22.9% reduction at the nearer location. Similarly, the number of grains per spike was higher at the distant site, with 35 grains versus 32.3 grains at the closer site, corresponding to a 7.7% decrease. TGW, an important determinant of grain yield,

Table 2 Sequences of primers for amplification of miRNA loci

Primer	Sequences 5' → 3'
miR168_F	CACGCATCGCTTGCTGCAGGT
miR168_R	CCAGTGCAGGGTCCGAGGTA
miR408_F	GGCTGGGAACAGACAGAGCATGGA
miR408_R	GGGAAAAAGGCCAGGGAAGAGG

F – forward, R – reverse

was statistically significantly higher at the closer site, measuring 39.8 g compared to 36.1 g at the distant site, an increase of 10.3%.

Considering overall grain yield as a comprehensive measure of productivity, the crop at the closer site produced 5.9 t·ha⁻¹, which was 0.9 t·ha⁻¹ lower than the 6.8 t·ha⁻¹ recorded at the distant site, amounting to a 13.2% decrease, as summarised in Table 3. Although grain yield was numerically lower at the 500 m site, the difference was not statistically significant. Therefore, this trend should be interpreted cautiously and in relation to the other physiological and molecular indicators.

Exposure of agricultural crops to the given stressor can lead to a reduction in their productive potential through radiation-induced alterations in key physiological processes, ultimately resulting in modifications of plant morphology and, consequently, yield-forming parameters. It has been reported that the percentage germination of plants decreases under higher stressor influence, which may negatively affect plant density per m² (Klubicová et al., 2012; Gudkov et al., 2019). However, this phenomenon was not observed at the closer site. The overall morphology of the spike may also be affected, thereby influencing the number of grains per spike, while TGW remains a crucial parameter for yield assessment. Exposure to the stressor can negatively impact grain size and weight, leading to reduced TGW values (Klubicová et al., 2012). No such reduction was recorded at the closer site, similar to the plant density per m².

Grain yield, however, serves as a comprehensive indicator of the impact of environmental stress, with values decreasing under the influence of the stressor (Klubicová et al., 2012; Gudkov et al., 2019). Based on these observations, the lower grain yield at the closer site (500 m) suggests a higher level of environmental stress.

In general, the values of yield-forming parameters interact with each other when calculating grain yield, which corresponds with common agronomic practice. Higher plant density per m², potentially resulting from

increased germination and emergence, can restrict tillering capacity, ultimately leading to a lower number of spikes per plant. A similar relationship is observed for the number of grains per spike: an increase in grain number per spike often results in lower TGW, likely due to the reduced allocation of nutrients to individual grains within a spike (Satorre & Slafer, 1999).

3.2 Physiological Analyses

Physiological measurements of NDVI revealed a general declining trend at both experimental sites, beginning from the second node stage (BBCH 32), through the flag leaf stage (BBCH 39), and continuing up to the beginning of heading (BBCH 51). NDVI values obtained with the PlantPen NDVI 300 device are expressed on a relative scale; therefore slightly negative values may occur depending on calibration conditions. This pattern suggests that plants are more sensitive to the applied stressor during early developmental stages. Notably, at the end of heading (BBCH 59), NDVI values at the closer site exhibited a continuous decline compared to the distant site. However, during the beginning of flowering (BBCH 61), a marked increase in NDVI was observed at both sites. On average, NDVI values of winter wheat were higher at the distant site (1000 m), with a mean of -0.0636 (Table 3), whereas the closer site averaged -0.0702, corresponding to approximately 9% lower values. The temporal course of NDVI values at both sites during the vegetation period is illustrated in Figure 1.

According to Gudkov et al. (2019), it is well established that chlorophyll content decreases under higher environmental stress, which may result from radiation-induced alterations in the organized structure of grana and thylakoid stroma within chloroplasts. Exposure to the stressor can also lead to changes in lipid and protein synthesis and degradation, accompanied by the generation of reactive oxygen species (ROS), ultimately resulting in pigment oxidation and suppression of chlorophyll synthesis (Agarwal et al., 2008). Consequently, it can be inferred that the crop at the closer site (500 m),

Table 3 Monitored experimental parameters of winter wheat at two experimental sites located at different distances from a NPS (Fisher's LSD test; $\alpha = 0.05$)

Parameter	Distance (variant)	
	500 m	1,000 m
Number of plants per m ²	175.00 ± 32.00a	156.00 ± 12.00a
Number of spikes per plant	2.70 ± 0.63a	3.49 ± 0.32a
Number of grains per spike	32.33 ± 0.70a	35.00 ± 4.04a
TGW (g)	39.76 ± 0.33a	36.05 ± 1.94b
Grain yield (t·ha ⁻¹)	5.90 ± 0.31a	6.83 ± 0.87a
NDVI	-0.0702 ± 0.0042a	-0.0636 ± 0.0050a

Different lowercase letters indicate statistically significant differences (Fisher's LSD test, $\alpha = 0.05$)

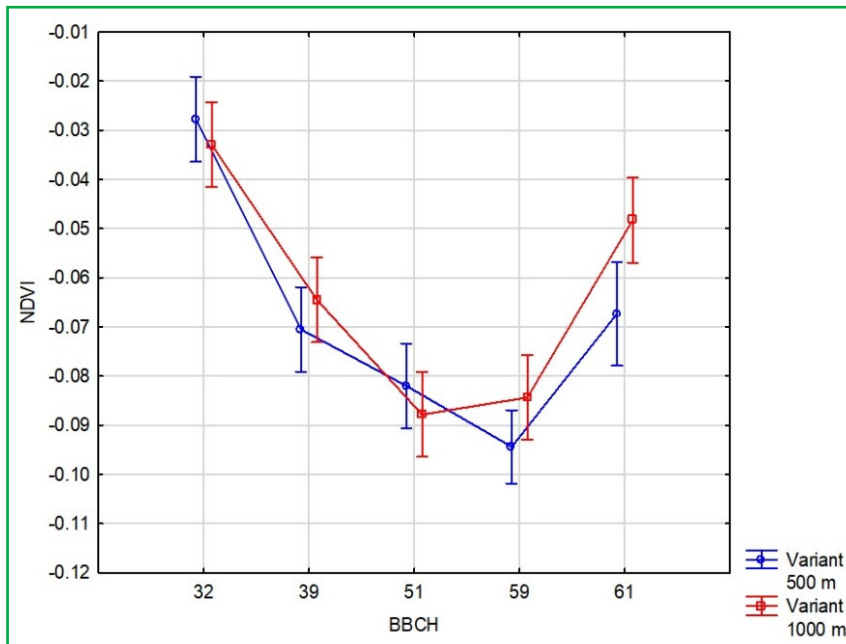


Figure 1 Course of NDVI during the ontogenesis of winter wheat at two experimental sites located at different distances from a NPS ($\alpha = 0.05$; $P = 0.04$)

which exhibited lower chlorophyll content and photosynthetic activity according to NDVI measurements compared to the distant site, was subjected to higher environmental stress.

3.3 Genomic Analyses

Genomic DNA isolated from a pooled sample of leaves of ten

plants in individual developmental stages was analysed using markers derived from sequences of stress-sensitive microRNA (miRNA) molecules. These molecules are involved in the regulation of various developmental and biological processes of plants and regulate plant reactions to stressful stimuli (Zhang et al., 2006; Bej & Basak,

2014). For our analyses, two types of markers miR168 and miR408 were selected, the functionality of which was verified in several experiments of the workplace (Ražná & Hlavačková, 2017; Ražná et al., 2018; Žiarovská et al., 2023). Genomic analyses were repeated in two biological and two technical replicates with each marker to confirm the presence and length in base pairs (bp) of amplified miRNA loci (Figure 2).

Markers based on miRNA molecules represent an efficient, stable, reproducible, low-cost and protocol-portable procedure for polymorphism detection. The indicated type of markers correspond to the genomic sequences of miRNAs. Primers designed by miRNA sequences can bind to different places of occurrence within the loop structure of molecules and can thus amplify fragments of different sizes. High primer annealing temperatures ($> 55^\circ\text{C}$) during the PCR amplification step increase the reproducibility and specificity of miRNA markers. Consequently, the observed polymorphism reflects sequence changes of miRNA loci that may be associated with the changes regulation of target genes (Yadav et al., 2014).

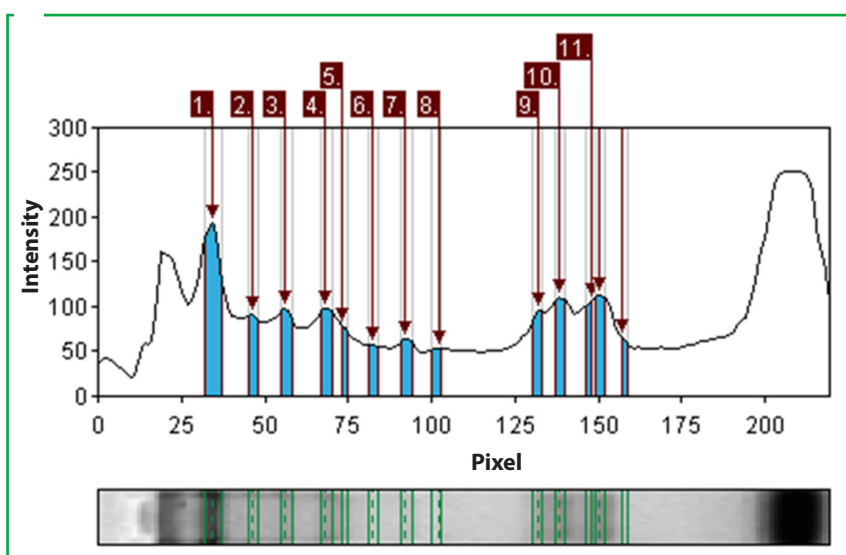


Figure 2 Position and size analysis of individual loci using GeneTool software. The sample comes from miR168 marker amplification, collected at BBCH 32 stage, from an experimental site 500 m away from the generating unit

An increasing trend of amplification of miR168-based loci was observed in earlier stages of development growth (BBCH32 to BBCH 51, for explanation see Table 1). In contrast, increased amplification of miR408-based loci was observed in later stages (BBCH 59 and BBCH 61). The miR168 molecule has an important regulatory function in defense or adaptation processes of plants under the action of abiotic stress. It is considered a potential biomarker of environmental stress (Bej & Basak, 2014). Based on the Point-Biserial Correlation analysis can be observed that the relationship between growth stage and miR168-based loci parameters (position,

size and number) are weak and statistically insignificant. Correlation coefficients range from approximately -0.28 to +0.07, indicating very weak linear associations in both directions. On the other hand, a miR408-based loci fragment of 150 bp stands out with a strong positive correlation of approximately 0.69 and a highly significant p-value ($p \approx 6.7 \times 10^{-7}$), indicating a statistically strong linear relationship: with the transition of plants to later developmental stages, the likelihood of the 150 bp loci amplification increases significantly. This observed fragment length corresponds to the size of stem-loop structures of miRNA precursors in plant species (Yadav et al., 2014).

The total number of miR168-based amplified loci was higher compared to miR408-based amplified loci, regardless of the distance of the cultivation location from the NPS. The results show that most correlations between distance (location of wheat growth) and the amplified miRNA-based alleles are weak and not statistically significant, with correlation coefficients ranging from approximately -0.40 to +0.40 (miR168) or -0.22 to +0.30 (miR408). The miR168-based fragment with the strongest observed relationship is 200 bp long, showing a moderate positive correlation of 0.40. This suggests that greater distances might be associated with a higher likelihood of amplification of this size of fragment. However, the p-value is 0.0805, which is not quite statistically significant at the conventional 0.05 level, but it does hint at a possible trend worth further exploration. Similar trend was observed for miR408 locus of 200 bp or 300 bp, respectively. In summary, no fragment shows a statistically significant correlation with distance, though 200 bp locus comes closest, with a moderate effect size and a borderline p-value. These results suggest that linear relationships between distance and individual fragment size are generally weak.

Multivariate correlation by Decision Tree Regression of growth stage and growth location (distance) with amplified fragments showed for miR168-based loci of 150 bp or 200 bp, respectively the highest levels of accuracy (83% or 67%) indicating that the decision tree can reliably distinguish between presence and absence

of markers 150 bp or 200 bp using just growth stage and distance variables. The miR408-based marker of 150 bp showed accuracy of 83.33%, which implies a moderate to strong non-linear relationship between the variables and the marker 150 bp.

Pearson and Spearman correlation analyses were used to evaluate relationships between amplified miRNA-based loci and growth stage or cultivation distance (Table 4). Correlation analyses indicated no significant relationship for miR168-based loci, whereas miR408-based loci showed a significant association with developmental stage. No statistically significant relationship with cultivation distance was confirmed.

The different regulatory function of miR408 in plant organisms has also been observed in several studies. Studies focused on researching the regulatory function of miR408 show that the activity of miR408 affects different stages of development and promotes intensive plant growth and seed yield by increasing the efficiency of photosynthesis (Pan et al., 2018). At the same time, it is also involved in the regulation of drought stress, and the increased level of miR408 is important for drought tolerance (Hajyzadeh et al., 2015). Kantar et al. (2010) found that miR408 in barley responds to dehydration stress. Studies have shown that the response of biomarkers to stress conditions is not only tissue-specific and species-specific (Kruszka et al., 2012), but also in the case of the marker miR408 may be a cultivar-specific response (Melnikova et al., 2016).

Based on the summary of the information obtained, the following conclusions can be drawn: The winter wheat plants of the Suffolk CS genotype reacted more sensitively to the environmental load during the early stages of development which was declared by declining trend of physiological parameters and higher amplification rate of stress-sensitive miR168-based loci.

Monitored agronomic parameters highlighted clear differences between two experimental localities, where at the closer locality (500 m from the NPS) were observed lower yield-forming parameters and grain yield, as well as lower NDVI values.

Table 4 Pearson and Spearman correlation between amplified loci with growth stage and growth locality (distance)

Variable	Pearson correlation	P-value	Spearman correlation	P-value
Lus-miR168				
Growth stage	-0.456	0.185	-0.317	0.371
Distance	-0.215	0.551	-0.251	0.483
Lus-miR408				
Growth stage	0.624	0.054	0.639	0.047
Distance	0.392	0.262	0.414	0.234

Table 5 Background radiation values (nSv·h⁻¹)

Sample identification	Distance 500 m	Distance 1,000 m
1	158	221
2	174	185.3
3	156.6	154
4	166	157
5	167.3	153

Sample identification corresponds to information in Table 1

Higher NDVI values, agronomic and yield parameters at a more distant location (1,000 m) correspond to lower background radiation measurements (see Table 5) and a greater number of miR408-based loci, which also play a regulatory role in photosynthesis (Pan et al., 2018).

3.4 Background Radiation Analyses

Based on the analyses of radiation background measurements during sampling, it can be said that the power input values of the dose equivalent of ionizing radiation, measured in units of nSv·h⁻¹, were during sampling no. 1 and 2 above at a more distant site (1,000 m). During sampling no. 3, 4 and 5, the measured values were higher at a closer location (500 m), (Table 5).

However, the values of both sites did not show significant differences during sampling, they did not exceed the signal level – 400 nSv·h⁻¹, and during the period from sowing to harvesting, there were also no significant differences recorded in the area of the NPS, thus they coincide with the values of the normal and safe level of radiation of the natural radiation background (SHMÚ, 2024c).

4 Conclusions

This study indicated that cultivation distance from the NPS was associated with differences in selected agronomic, physiological and molecular indicators of winter wheat performance. Plants grown at the closer site (500 m) showed numerically lower grain yield and lower NDVI values than those grown at the more distant site (1,000 m), whereas TGW was significantly higher at the closer site. Since most yield-forming parameters did not differ significantly between sites, these results should be interpreted as trends supported by physiological and molecular observations. NDVI patterns suggested greater sensitivity of winter wheat during early developmental stages. Molecular analysis showed higher amplification of miR168-based loci during early growth stages and increased amplification of miR408-based loci during heading and flowering. Correlation analyses did not confirm significant relationships for miR168-based loci, whereas miR408-based loci showed a significant

association with developmental stage. No statistically significant relationship with cultivation distance was confirmed. Overall, the integration of agronomic traits, NDVI measurements and miRNA-based markers may represent a useful approach for monitoring winter wheat responses to environmental stress gradients under field conditions.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Katarína Ražná: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft. Dávid Ernst: conceptualization, formal analysis, investigation, methodology, project administration, resources, supervision, validation, writing – original draft. Dominik Selnekovič: investigation, methodology, resources. Matúš Kučka: investigation, writing – review & editing. Simona Čerteková: investigation, writing – review & editing. Jit Mukherjee: formal analysis, software, writing – review & editing.

AI and AI-assisted technologies use declaration

No generative AI tools/AI-assisted technologies were used during the preparation of the manuscript.

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