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Phytoremediation potential of *Brassica oleracea* varieties through cadmium tolerance gene expression analysis

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ABSTRACT

Background: *Brassica oleracea* var. *acephala*, commonly referred to as kale, is a well-documented plant species, a food crop but well recognized for its capacity to endure and manage the accumulation of heavy metals. In this research, the phytoremediation potential of kale was evaluated based on cadmium intake, utilizing three distinct kale varieties originating from Bosnia and Herzegovina. All kales were grown in controlled conditions, with different concentrations of cadmium (Cd), a known strong pollutant found in small concentrations in soil under normal environmental conditions. After the root length analysis and cadmium atomic spectrometry, we utilized quantitative PCR (qPCR) and cycle threshold (Ct) values to calculate the expression levels of five genes associated with Cd heavy metal response: Mitogen-activated protein kinase 2 ($MAPK_2$), Farnesylated protein 26 and 27 ($HIPP_{26}$, $HIPP_{27}$), Natural resistance-associated macrophage protein 6 ($RAMP_6$), and Heavy metal accumulator 2 (HMA_2).
Results: The atomic reader's analysis of rising cadmium concentrations revealed a proportional decline in the length of kale roots. The gene expression levels corresponded to cadmium stress differently among varieties, but mostly showing notable up-regulations under Cd stress, indicating the strong Cd presence within the plant.
Conclusions: This study demonstrated differences in gene expression behavior among three *B. oleracea* varieties from Bosnia and Herzegovina, indicating and filtering the Cd-resistant kale, and kale varieties suitable for phytoremediation. For the first time, such a study was conducted on kale varieties from Bosnia and Herzegovina, analyzing the impact of cadmium on the growth and resilience of these species.

1. Background

Brassica oleracea, a kale variety from South Asia, renowned for its health benefits, including antioxidants and anti-cancer properties, is rich in vitamin C and soluble fiber, alongside cabbage and broccoli.[1] Its high indole-3-carbinol content aids DNA repair and inhibits cancer cell growth, while the Brassicaceae family's abundance in carotenoids, proteins, and glucosinolates strengthens its defense against fungi and pathogens.[2,3]

B. oleracea species are found everywhere except for Antarctica, but the biggest diversity of these plants is found in the Mediterranean area.[4] In animals, including rats and pigs, this plant abolishes thyroid function as they provide more side effects of iodine (I) deficiency, or sulfadimethoxine (SDM) ingestion.[5] *Brassica oleracea* var. *acephala* is a daily diet food in many countries as it provides many health benefits and all parts of this plant such as stems, and roots are consumed.[2] It is already confirmed that *B. oleracea* species are cadmium-tolerant plants, known to belong to the Brassica genus of phytoremediators,[2] as it can accumulate heavy metals in their tissues, without any visible symptoms.[6,7] In the periodic system of elements, there are 90 natural elements, out of which 53 of them are classified as heavy metals,[8] examples include cadmium (Cd), fluorine (F), and lead (Pb).[9] Specifically, cadmium poses a significant concern due to its propensity for long-range atmospheric dispersion within the Earth's atmosphere and its known toxicity to the human body.[10] Heavy metals cannot be destroyed or degraded as they are present in Earth's crust.[11] In plants, toxic heavy metals and even excess essential heavy metals may result in chlorosis, malnutrition, and diminished development. However, some of these plants and other organisms can accumulate heavy metals, despite heavy metal toxicity.[12] This phenomenon is known as phy-

Abbreviations: Cd, Cadmium; qPCR, Quantitative polymerase chain reaction; BiH, Bosnia and Herzegovina; $MAPK_2$, Mitogen-activated protein kinase 2; $HIPP_{26}$, Farnesylated protein 26; $HIPP_{27}$, Farnesylated protein 27; $RAMP_6$, Natural resistance-associated macrophage protein 6; HMA_2, Heavy metal accumulator 2.

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toremediation, and it usually happens when plants and microorganisms eliminate and degrade heavy metals. Phytoremediation is not always effective but at least it is less harmful than using chemical treatments such as membrane filtration, soil washing, and physical treatments.[13]

To date, multiple genes are associated with heavy metals, demonstrating correlations with exposure to cadmium. Most commonly, the gene known as Heavy Metal Accumulator 2 (HMA_2), is responsible for encoding the Heavy Metal Accumulator 2 protein and regulating the homeostasis of zinc (Zn) and cadmium (Cd) ions.[14] Additionally, there is evidence of HMA_2 demonstrating a strong correlation with the expression of the Antioxidant 1 (ATX_1) and $HIPP_{26}$ (also known as Farnesylated protein 6 – FP6) genes, as revealed through an *in silico* investigation conducted by Šutković et al. in 2016.[2] Subsequently, the same researcher showcased the viability of an *in silico* prediction by validating the predicted proteins *in vivo*, establishing a connection between YSL_3 and Cd stress.[7] Mitogen-activated protein kinase 2 ($MAPK_2$), alternatively recognized as extracellular signal-regulated kinase 2 (ERK_2), has been demonstrated to play a role in numerous cellular pathways, including cell proliferation and its activation occurs in response to various stimuli such as hormones and growth factors (GF).[15] The participation of Farnesylated protein 26 ($HIPP_{26}$) in cadmium homeostasis within *B. oleracea* cultivar has been previously demonstrated.[16] Additionally, both $HIPP_{26}$ and $HIPP_{27}$ exhibit upregulation in A*rabidopsis thaliana* under conditions of cadmium and zinc stress,[17] as well observed in *Brassica Juncea*.[18] Similarly, in *Brassica napus L*, $HIPP_{27}$ has been identified as a gene associated with cadmium tolerance.[19]

Natural resistance-associated macrophage protein 6 ($RAMP_6$) is part of metal transporter proteins that are capable of transporting bivalent metal ions such as Fe^{2+} and Mn^{2+} into cytoplasm and are present in many organisms. It is found that $RAMP_6$ transports excess Cd ions intracellularly in *A. thaliana* and it enables plants to develop normally with inadequate nutrients such as Mn^{2+}.[20,21] *B. oleracea* species is widely employed as a plant model organism due to its ease of rapid growth, requiring minimal equipment.[22]

In light of the previously mentioned information, recognizing that specific kale varieties excel as phytoremediators, it becomes crucial to distinguish species with lower accumulation potential from those without. Essentially, some varieties acknowledged for phytoremediation may pose risks to human health, underscoring the importance of identifying such species.

Therefore, the goal of this study is to assess the capacity of domestic kale varieties (*Brassica oleracea* var. *acephala*) to withstand cadmium stress and identify the most tolerant and accumulative varieties to cadmium. In that sense, this study explores the phytoremediation potential of two local kale varieties and compares it to that of a hybrid kale from Bosnia and Herzegovina.

2. Methods

2.1. Seed germination and root analysis

The domestic seeds of *Brassica oleracea* var. *acephala* varieties utilized in this study were procured from the Herzegovina region, while the hybrid variety was acquired from a local agricultural market, as shown in Table 1. The seedlings were grown using Tap of paper method. Up to 30 seeds were treated with different concentrations of cadmium (50, 100, 200, and 500 μM) and incubated for 5 days at 27 °C in a growth chamber (NUVE GC400) where they were exposed to light for 16 h a day. When plants reached their sufficient growth they were removed carefully from the paper and root length was measured with a ruler. After the root length measurement, the numbers, indicated in centimeters, were entered for statistical analysis. All samples were stored at −80 °C prior RNA isolation.[23] In addition to the seeds cultivated in petri dishes, a larger quantity of plant material was requisite for atomic absorption spectrophotometry (AAS). Consequently, the seeds were also cultivated in soil under distinct cadmium concentrations (control – distilled water, 100 μM, 250 μM, 500 μM, and 1000 μM of $CdCl_2$) within a growth chamber, with 10 seeds per pot. This incubation period extended for 10 days at a temperature of 27 °C, with daily exposure to light for 16 h.

Table 1
List of variants from geographical region.

Name of the variant	Geographical coordinates	Label
Mostar, Blagaj	43°25 North, 17°88 East, BiH	KM
Stolac, Dubrava	44°82 North, 18°57 East, BiH	KS1
Brassica oleracea L. – Bonanza F1	Italy	K23

Note: BIH (Bosnia and Herzegovina).

For the data presentation, derived from the root assay analysis, a descriptive statistics method was employed, resulting in a bar graph. Utilizing Tukey's multiple comparisons test, it was demonstrated that there is a noteworthy distinction in the mean root length across all concentrations examined in the study ($p < 0.05$), as illustrated in Fig. 1.

2.2. Atomic absorption spectroscopy (AAS)

For the detection of cadmium concentrations in kales, different wavelengths of electromagnetic (EM) radiation were utilized,[24] using a Shimadzu Flame atomic absorption spectrophotometer (model AA7000F), based on the protocol explained by Girgin et al. in 2022.[25]

2.3. RNA isolation and quality control

RNA isolation and DNase treatment were performed with Monarch® Total RNA Miniprep Kit protocol (NEB #T2010). According to the kit manufacturer, two main steps were performed. The first step was that the whole plant tissues were lysed, and the second step was that RNA was purified in which DNase treatment was performed leaving no traces of DNA.[26] The RNA concentration, quality and purity were calculated using a Thermofisher Multiskan GO μDrop spectrophotometer. In addition, agarose gel electrophoresis was used to evaluate the integrity and quantity of RNA samples as the RNA fragments are separated based on their size.[27]

2.4. Primer design and quantitative real-time PCR (qPCR)

The primer sequences were designed according to the protocol published in 2020, by Šutković et al.[7] The housekeeping gene used was Ubiquitin 2 (UBQ_2), according to the study conducted by Brulle et al. in 2014.[28] All primer sequences are presented in Table 2.

The qPCR amplification was performed on the Step One Plus system by Applied Biosystems®. The reaction was prepared using the Luna®Universal Probe One-Step RT-qPCR Kit and cycling conditions were set according to the Luna Kit protocol.[29] Only the results whose melt curve generated a single peak were used, which means that only one product (gene of interest) was produced in the reaction.

Melt curve analysis was performed to validate the specificity of the reaction by checking for primer dimers or nonspecific amplifications. Data are presented graphically where the Y-axis in graphs represents $^{2\text{-}\Delta\Delta}$ Ct which analyzes the relative changes in gene expression.[30,31]

3. Results

The average root length of each kale is observed in Fig. 1. The length of the root becomes shorter with the increase of $CdCl_2$ concentrations. In the control group, the length ranged from 2.54 cm to

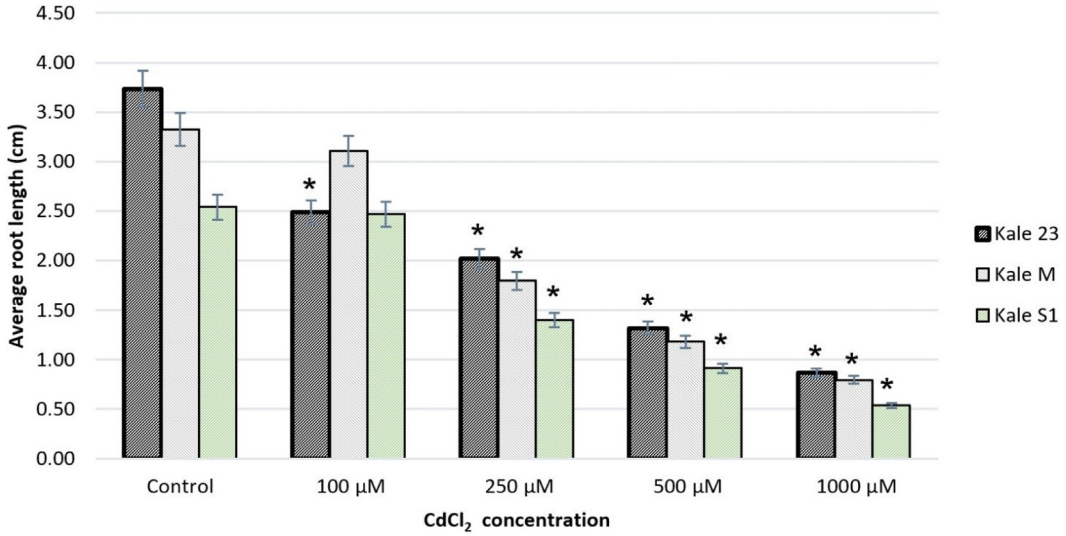

Fig. 1. Comparison between the average root of domestic and hybrid kales grown with different $CdCl_2$ concentrations. Asterisks indicate the mean values that are significantly different between the treatments and control (*$p < 0.05$).

Table 2
Primers used in this study.

Primer name	Sequences (5′->3′)
F-HMA2	TTCTGTCATCGTGCCGTCAA
R-HMA2	GAGTGTTGCTCCCACGGTTA
F-MAPK2	GGGCTGCCAAAGGACTTACT
R-MAKP2	GTCTTGTCACCGGTAGGACC
F-RAMP6	GCGATATCTCTCCTCGGTGC
R-RAMP6	AAGCTTCCTTGATGCCGGTT
F- HIPP26	TCTTTACACCTCCACTTTCCCT
R- HIPP26	CCACCGTCTGCAACTGTTTG
F-HIPP27	GTTCCAGGCACTCTTCTCCC
R-HIPP27	CGGTGACTTTGCTGACTCCT
F-UBQ2	TATTCGTGAAGACGCTGACG
R-UBQ2	TATTCGTGAAGACGCTGACG

3.73 cm, while in 1000 µM $CdCl_2$ it ranged from 0.54 cm to 0.86 cm. All kales were treated with Cd five times. The results also indicate that there was no significant difference in root growth between the analyzed kales because the p-values are not greater than 0.05 (data not shown). However, in all kales, based on Tukeys multiple comparisons test ($p < 0.05$), there was a significant difference in the mean root length between controls and the treated 250 µM, 500 µM and 1000 µM $CdCl_2$ concentrations.

There is clear increase in plant Cd levels with the increasing $CdCl_2$ concentrations, in all kale varieties, as seen in Fig. 2. However, there is a slight exception, as the concentration of 1000 µM Kale S1 is lower than the concentration of 500 µM Kale S1.

The qPCR analysis, involving 3–5 replicates, assessed the expression levels of five specific genes: HMA_2, $RAMP_6$, $MAPK_2$, and $HIPP_{26}$, $HIPP_{27}$, The results are presented in a column with $2^{-\Delta\Delta ct}$ located in Y axis, indicating exact results of all genes being expressed, as seen in Figs. 3, 4 and 5. In most of the cases there was a noticeable elevation in gene expression levels with increasing concentrations of Cd with all genes, whereas after 500 µM concentration, a decrease in gene expression was observed. For example, HMA_2 exhibited a decline in expression, starting at 500 µM and finishing at 1000 µM $CdCl_2$ concentrations in Kale S and Kale M, whereas in Kale 23 HMA_2 exhibited an increase in expression from 100-1000 µM, as seen in Fig. 3. The expression of the Mitogen-activated protein kinase 2 gene (MAPK2) in Kale M and Kale S1 showed up-regulation with the increase of $CdCl_2$, however, in Kale 23 it was a different case. A reduction in the expression of MAPK2 in Kale 23 is visible, with relative expression levels decreasing from 1.4 in the control group to 0.34 in the presence of 500 µM $CdCl_2$. The expression of $RAMP_6$ in all kale varieties exhibited a consistent pattern of increase in response to rising $CdCl_2$ concentrations. However, there is a slightly lower expression of $RAMP_6$ in Kale 23, at a concentration of 1000 µM $CdCl_2$ it is lower if compared to 500 µM $CdCl_2$. The expression of HIPP26 exhibited distinct patterns in domestic varieties compared to the hybrid Kale 23. Specifically, at 250 µM $CdCl_2$, there was an average increase in relative expression of 0.6 compared to the control. However, this increase rapidly declined at concentrations of 500 µM and 1000 µM, with Kale M showing a slightly less steep decrease.

$HIPP_{27}$ displayed a rise in expression as the $CdCl_2$ concentrations increased (up to 250 µM), followed by a decrease at 500 µM and 1000 µM $CdCl_2$ concentrations. However, in Kale 23, the expression of $HIPP_{27}$ showed variability: it initially decreased at 100 µM, then experienced a sudden increase at 250 µM and 500 µM, followed by a further decline at 1000 µM compared to the control.

4. Discussion

The significant decrease of root length with the increase of $CdCl_2$ concentrations is presented in Fig. 1. These findings are in line with the results reported by Waheed et al. in 2022 [32], where the accumulation of heavy metals in *Eruca sativa* leaves are leading to reduced root and shoot growth. This phenomenon can be attributed to increased radical generation and a decreased rate of photosynthesis. Similarly, Qadir et al. noted in their 2014 study [33] that cadmium (Cd) negatively impacts photosynthesis rates, nutrient transport, and promotes radical formation, posing potential harm to plant health. Additionally, similar results were also reported in the plant *Pisum sativum L* [34]. Compared to most organs in plants, roots are mostly affected as they are directly in contact with heavy metals. Heavy metals start disrupting the plants' growth and metabolism by changing many important processes including disrupted water and nutrient balance and reduction in many active enzymes and chlorophyll.[7] In all kale varieties the Cd levels increase in parallel to the increasing concentrations of $CdCl_2$. However, an exception is notable in the concentration of 1000 µM in Kale S, dropping from 81.8 to 52.2 mg/kg Cd. This behavior indicated an inhibition of Cd uptake at higher concentrations in Kale S1, where a lower cadmium accumulation can be caused by lower phytostabilization, and the plant goes into the senescence phase.[13]

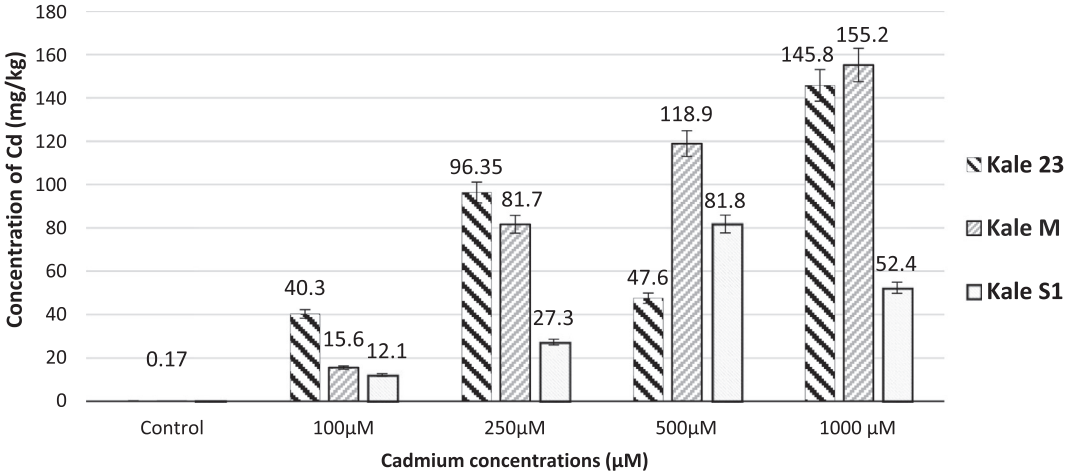

Fig. 2. Atomic analysis Cd concentrations. Vertical lines represent the standard deviation of three replicates of independent experiments.

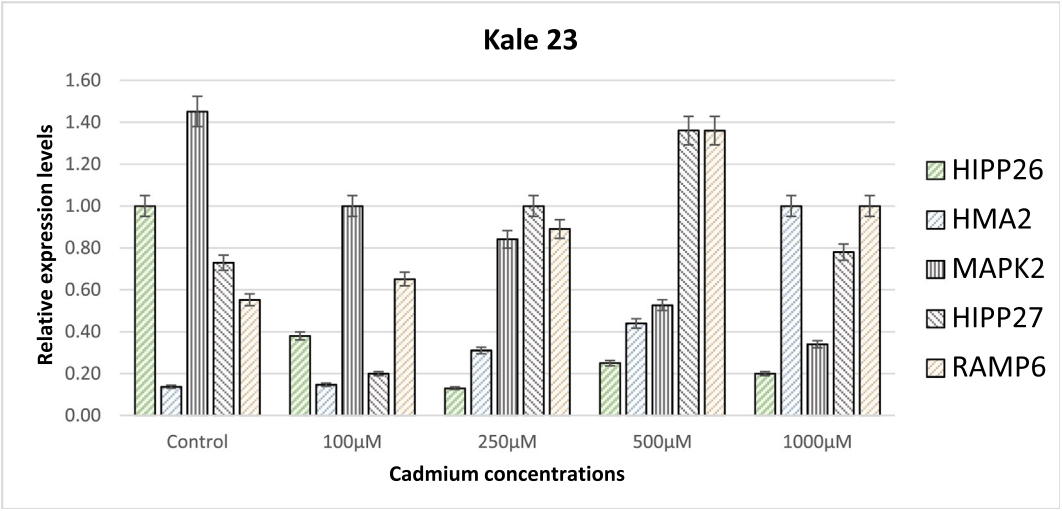

Fig. 3. The effect of Cd on relative expression levels of $HIPP_{26,}$ HMA_2 $MAPK_2$, $HIPP_{27}$ and $RAMP_6$, and HMA_2 genes in Kale 23. The gene was quantified by qPCR and normalized with the housekeeping gene Ubiquitin transcript. Vertical lines represent the standard error of three biological replicates from relative expression values of independent experiments.

The expression levels of five specific genes were evaluated: HMA_2, $RAMP_6$, $MAPK_2$, as well as $HIPP_{26}$ and $HIPP_{27}$. The findings are illustrated in Figs. 3, 4, and 5. An increase in genetic expression as the $CdCl_2$ concentrations increased is observed, although in some cases, the expression of certain genes decreased significantly (if compared to the control) at the 500 µM and 1000 µM concentrations. For example, the expression of HMA_2 decreased at 1000 µM $CdCl_2$, possibly due to the plants reaching a critical mortality threshold at this concentration, as elucidated by Shahid et al. in 2017 [35]. Further, HMA_2 was found to drive heavy metals out of cytoplasm in yeast and *A. thaliana* by removing oxidants from plant roots [36,37].

In the current research, HMA_2 served primarily as a positive control, since HMA_2 up-regulation was previously strongly correlated to Cd stress in *Brassica oleracea* var. *acephala* [23]. Similar findings where observed in *Brassica juncea* in 2021, where an up-regulation HMA_2 was significantly noted [38].

In Kale M and Kale S1, the expression of the Mitogen-activated protein kinase 2 gene ($MAPK_2$) was up-regulated with increasing $CdCl_2$ concentrations, but Kale 23 exhibited a different pattern. We observed a consistent decrease in the expression of $MAPK_2$, where relative expression levels declined from 1.4 in the control group to 0.34 when exposed to 500 µM $CdCl_2$ concentrations. This shift in expression could potentially be attributed to mutations that might have arisen in the $MAPK_2$ primers during the qPCR reaction. In a study involving *A. thaliana* and yeast cells, the L157P mutation introduced during PCR impacted the results of Cd transportation, leading to down-regulation [39]. Additionally, there is a potential indication that exposure to elevated concentrations of $CdCl_2$ may trigger a cell death phase in Kale 23, as shown in *Arabidopsis thaliana* [40]. Research has demonstrated that MAPK genes play a role in regulating both biotic and abiotic stresses [41,42]. Natural resistance-associated macrophage protein 6 ($RAMP_6$), belonging to the metal transporter protein family, is known for transporting Fe^{2+} and Mn^{2+} into the cytoplasm and Cd ions intracellularly in *A. thaliana* [20,21,43]. Based on our research findings, all kale varieties consistently displayed an increasing expression pattern of $RAMP_6$ in response to rising concentrations of $CdCl_2$. This implies a noticeable involvement of Natural resistance-associated macrophage protein 6 in the cadmium (Cd) metabolism of *Brassica oleracea*. However, it's important to note, based on our examination of existing literature, that there hasn't been direct confirmation of $RAMP_6$'s role as a Cd transporter in *Brassica oleracea*. In the context of the Brassicaceae family, our results align with observations in *Brassica rapa*, where the RAMP protein family is associated with responses to Cd stress, showing up-regulation in the roots when exposed to cadmium [44].

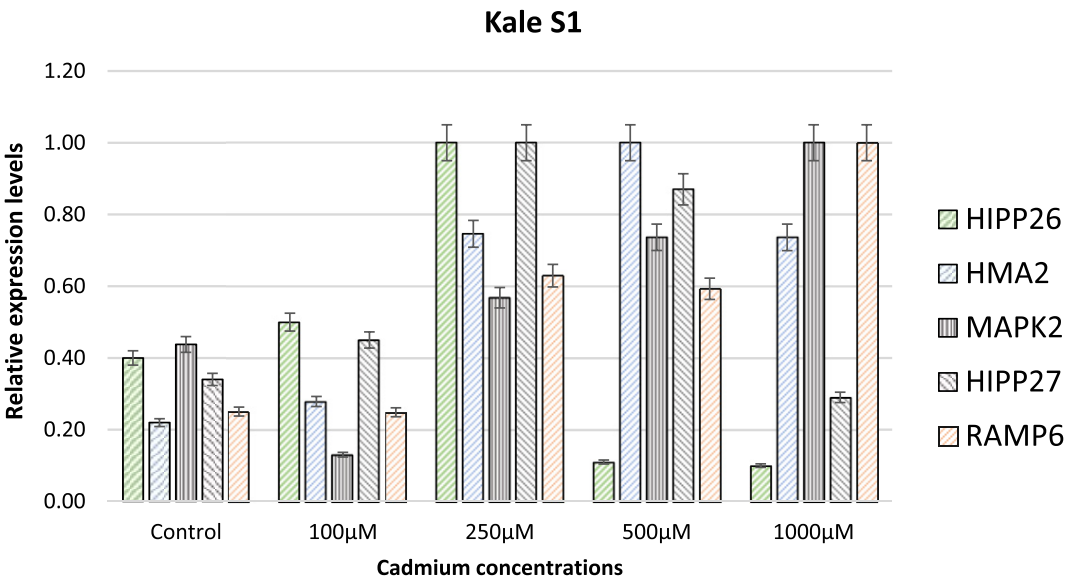

Fig. 4. The effect of Cd on relative expression levels of $HIPP_{26,}$ HMA_2 $MAPK_2$, $HIPP_{27}$ and $RAMP_6$, and HMA_2 genes in Kale S1. The gene was quantified by qPCR and normalized with the housekeeping gene Ubiquitin transcript. Vertical lines represent the standard error of three biological replicates from relative expression values of independent experiments.

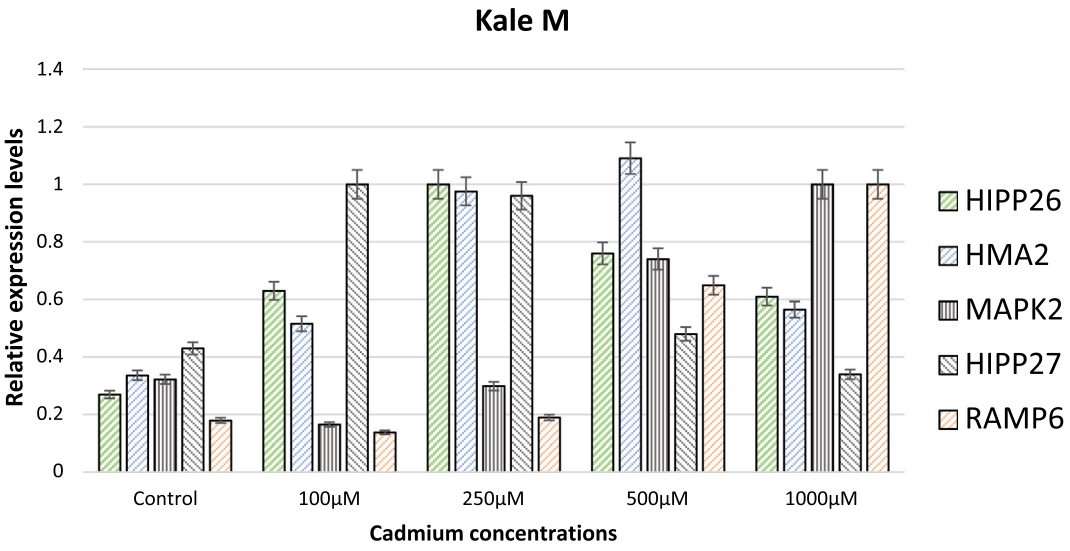

Fig. 5. The effect of Cd on relative expression levels of $HIPP_{26,}$ HMA_2 $MAPK_2$, $HIPP_{27}$ and $RAMP_6$, and HMA_2 genes in Kale M. The gene was quantified by qPCR and normalized with the housekeeping gene Ubiquitin transcript. Vertical lines represent the standard error of three biological replicates from relative expression values of independent experiments.

Recently, a transcriptome analysis data from both leaves and roots in *Brassica juncea,* revealed that RAMP expression varies with different concentrations of Cd treatments, showing tissue-specific patterns but predominantly it is up-regulated in the roots [45].

Research examining the gene expression of $HIPP_{26}$ and $HIPP_{27}$ in *Brassica oleracea* var. *acephala* is presently absent. In this study, $HIPP_{26}$ is observed to undergo up-regulation in response to Cd stress in domestic kale varieties (Kale S1 and Kale M). Similar studies conducted with *A. thaliana* have indicated that $HIPP_{26}$ expression rises as the intake of Cd ions increases [46,47]. *B. oleracea* and *A. thaliana* species are recognized for sharing orthologous genes [48,49]. In this regard, our findings can indicate the potential involvement of $HIPP_{26}$ in the Cd influx process of *Brassica oleracea* var. *acephala*..In line with our findings, Zhao et al. (2023) documented that $HIPP_{27}$ interacts with Ubiquitin-specific protease 16 (UBP_{16}), a recognized Cd regulator, and plays a crucial role in cadmium detoxification [50]. In our study, the expression of $HIPP_{27}$ displayed variability within the hybrid kale, although it exhibited comparable patterns to $HIPP_{26}$ in domestic kale varieties.

Furthermore, it is crucial to highlight that this investigation affirmed the greater resistance of domestic *Brassica oleracea L. var. acephala* kale variants to cadmium stress compared to hybrid ones. These results contribute to additional insights into the molecular mechanisms involved in the processing of heavy metals by *B. oleracea L. var. acephala,* affirming its potential capacity for phytoremediation.

5. Conclusion

This research investigation focused on the assessment of three distinct kale cultivars, comprising two indigenous varieties and one hybrid strain. Kale exhibits notable resilience to adverse environmental factors such as atmospheric, aquatic, and soil pollution. Through

the utilization of atomic spectrometry measurements, it was discerned that Kale S1, characterized as an indigenous wild type kale variety, exhibits the highest level of resistance to cadmium (Cd) stress. Experimental evidence substantiates that Kale S1 accumulates the least amount of Cd. Additionally, the outcomes from quantitative polymerase chain reaction (qPCR) analyses revealed that a majority of the target genes under Cd stress exhibited the lowest levels of gene expressions in both Kale S1 and Kale M.

The hybrid Kale 23, embodies the highest cadmium (Cd) toxicity among the examined kale varieties, as substantiated by the consistent findings across all conducted experiments, which indicate its highest Cd accumulation. Therefore, Kale 23 emerges as a prime candidate for phytoremediation purposes, given its potential for efficiently removing Cd from contaminated environments. In contrast, Kale S1 emerges as the most favorable kale choice for human consumption due to its proven resilience to Cd stress and minimal Cd accumulation. The methodologies employed in this study can be applied to evaluate plants with phytoremediation potential. This research is significant as it represents the first comprehensive evaluation of three kale cultivars regarding their resistance to $CdCl_2$. Consequently, the presented approach offers an opportunity to identify and distinguish cabbage varieties that display enhanced resistance to cadmium, as well as those with potential utility as phytoremediation plants. Nevertheless, in order to verify whether the examined kale varieties truly exhibit strong phytoremediation capabilities, additional testing with other prevalent heavy metals should be conducted.

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CRediT authorship contribution statement

Jasmin Šutković: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Annissa Van Wieren:** Writing – original draft, Visualization, Methodology. **Ensar Peljto:** Visualization, Project administration, Methodology. **Ahmet Yildirim:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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