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Original Scientific Paper

Effect of N-acetyl-L-cysteine (NAC) on soluble sugar and polyamine content in wheat seedlings exposed to heavy metal stress (Cd, Hg and Pb)

Nesrin Colak[1]*, Petr Tarkowski[2,3], and Faik Ahmet Ayaz[1]

1 Department of Biology, Faculty of Science, Karadeniz Technical University, 61080 Trabzon, Turkey
2 Centre of the Haná Region for Biotechnological and Agricultural Research, Central Laboratories and Research Support, Faculty of Science, Palacky University, Šlechtitelů 27, 78371 Olomouc, Czech Republic
3 Centre of the Haná Region for Biotechnological and Agricultural Research, Department of Genetic Resources for Vegetables, Medicinal and Special Plants, Crop Research Institute, Slechtitelu 29, 78371 Olomouc, Czech Republic
* correspondence: ncolak@ktu.edu.tr

ABSTRACT:
Heavy metal stress adversely affects plant growth and productivity worldwide. Alleviating the stress effect through the exogenous use of various chemical substances has become an interesting area of study in the field of plant stress tolerance. As a thiol compound, the cysteine derivative N-acetylcysteine (N-acetyl-L-cysteine, NAC) is the precursor of glutathione synthesis and a potent ROS scavenger with powerful antioxidant and free radical scavenging capabilities. This study investigated the effects of heavy metals (Cd, Hg and Pb, 100 µM) on accumulation of soluble sugars and polyamine content in roots and shoots of wheat seedlings, the water potential and proline content in shoots and the role of NAC in protection against heavy metal toxicity. The addition of 1 mM NAC significantly increased root content of glucose, fructose and sucrose in varying degrees (avg. 1.34-, 1.20- and 1.51-fold, respectively) in comparison with heavy metals alone. The treatments led to a significant reduction of sugar content in shoots. Water potential values were highly correlated with proline and sugar content in wheat seedling shoots. Heavy metal stress significantly reduced polyamine content in both plant parts. The addition of NAC increased polyamine content in seedlings in comparison with heavy metals alone in both roots and shoots. These results suggest that NAC may protect plants from oxidative stress damage in heavy metal stress, and this enhancement of stress tolerance seems to involve soluble sugar and polyamine biosynthesis.

Keywords:
leaf water potential, N-acetyl-L-cysteine, *Triticum aestivum*, osmolytes, stress tolerance

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INTRODUCTION

Although soil naturally contains heavy metals (HMs), increases in the levels of these HMs rising to harmful levels due to anthropogenic activities such as mining, disposal of sewage sludge, smelting and creation of HM pollution have become a major environmental problem for all organisms (Wuana & Okieimen 2011). The consequences of HM toxicity include the inhibition or reduction of photosynthesis, mitochondrial respiration and carbohydrate metabolism; reduced biomass generation; and decrease in the content of grain nutrients. This toxicity threatens not only plants, but also human health (Gill 2014).

Plant cells regulate the accumulation of metabolites such as carbohydrates, polyamines (PAs) and phytohormones, and also the expression of genes involved in the synthesis of these metabolites in order to cope with the adverse impacts of HMs (Gill & Tuteja 2010). Sugars (including sucrose, glucose and trehalose) are low-molecular-weight osmolites and signal compounds (Dhir *et al.* 2012). These compounds contribute to reducing the adverse effects of HMs by maintaining cell turgor and membrane integrity, detoxifying reactive oxygen species (ROS) and playing a precursor role in synthesis of lignin and phenolic compounds (Rosa *et al.* 2009; Dhir *et al.* 2012).

Polyamines are nitrogen-rich phytohormone-like compounds widely present in all organisms. Putrescine (Put), spermidine (Spd) and spermine (Spm) are the major PAs, and they are found in three forms: free (F-PAs), covalently conjugated (CC-PAs) or non-covalently conjugated (NCC-PAs) (Chen *et al.* 2019). These participate in numerous fundamental processes, including cell division, seed germination, fruit growth and morphogenesis, in addition to which they alleviate damage resulting from environmental stresses such as HM stress, salinity and drought by regulating their tolerance mechanisms (Choudhary *et al.* 2010; Gill & Tuteja 2010; Aldesuquy 2016).

N-Acety-L-cysteine (N-acetyl cysteine, NAC, $C_5H_9NO_3S$) is a synthetic compound derived from the amino acid cysteine. It acts as a chelating agent, antioxidant, ROS scavenger and GSH precursor (Sun *et al.* 2014). It is an important compound used in drug production and has been employed for many years to treat diseases such as liver damage and bronchitis, as well as to prevent HM-induced injuries (Sun *et al.* 2014). However, studies concerning the role of NAC in alleviating stress damage are limited. Studies investigating the stress-alleviating effect of NAC have mostly focused on HM toxicity in plants, particularly Cd toxicity and tolerance (Sun *et al.* 2014; Colak *et al.* 2019). Treatment with NAC has recently been reported to improve growth in HM-stressed plants through the coordinated induction of antioxidant defence and phytochelating systems coupled with the plant phenolic pool to mitigate oxidative stress under stress conditions (Colak *et al.* 2019). However, no data have been reported concerning the stress-alleviating effect of NAC in sugars and PAs in plants exposed to HM stress. The present study therefore investigated the stress-alleviating effect of NAC in HM-treated wheat seedlings by determining sugar and PA content and the leaf water potential (Ψw), which are directly related to growth, in plants exposed to Cd, Hg and Pb toxicity.

MATERIALS AND METHODS

Plant material and growth conditions. Seeds of bread wheat (*Triticum aestivum* L. 'Ceyhan-99') were obtained from the Eastern Mediterranean Agricultural Research Institute (Adana, Turkey). Germination of seeds and growth conditions of the seedlings were almost identical to those described in our previous paper (Colak *et al.* 2019). Briefly, the seeds were sown in steam-autoclaved glass Petri dishes (15 cm in diameter), and one-day-old germinated seedlings were transferred to 1.5-L plastic pots containing half-strength aerated Hoagland's solution (Hoagland & Arnon 1950). The seedlings were grown for four days at 25°C and 60% humidity in a growth chamber (BINDER, KBWF 720, Germany) in the dark in order to avoid the effect of photosynthesis and numerous enzyme activities on sugar and PA metabolism. The investigation included a control and seedlings exposed to 1 mM NAC, 100 μM $CdCl_2$, 100 μM $HgCl_2$, 100 μM $PbNO_3$, 100 μM $CdCl_2$ + NAC, 100 μM $HgCl_2$ + NAC, and 100 μM $PbNO_3$ + NAC. On the fifth day, 30 seedlings from each experimental group were randomly selected from the medium. These were harvested and divided into shoots and roots, which were then treated with liquid nitrogen (-195.79°C) and stored at -80°C until analysis. All experiments and extractions were performed in triplicate (n = 6).

Determination of the leaf water potential in shoots of wheat seedlings. The leaf water potential (Ψ_w) in shoots of the wheat seedlings was measured using a psychrometer (PSΨPRO, Wescor, USA) equipped with C-52 sample chambers. Measurements were performed on leaf discs (0.3 cm in diameter) cut from the middle part of six separate shoots (n = 6).

Determination of proline content in shoots of wheat seedlings. Shoot samples (0.1 g dw) were homogenised in 10 ml of a sulphosalicyclic acid ($C_7H_6O_6S$, 3% w/v) solution at room temperature. The homogenate was then centrifuged at 7000*g* for 15 min at room temperature to obtain a clear supernatant. One milliliter of the supernatant was added to 1 ml of glacial acetic acid and ninhydrin (1 ml, 3% w/v). The reaction mixture was allowed to stand for one hour at 100°C, after which the reaction was terminated by placing the test tubes in an ice bath for 10 min. A measured volume (3 ml) of toluene was then used to extract the sample in triplicate. The mixture was left to stand in order to allow phase separation. Proline content of the organic phase was determined according to Bates *et al.* (1973) using a UV-visible spectrophotometer at 520 nm (Thermo e-201, England). The concentration of proline was determined using a five-point calibration curve. Values were expressed as μg of proline per gram of dry weight (dw).

Extraction and determination of soluble sugars in roots and shoots of wheat seedlings. Soluble sugars were extracted and quantified using the method described by Ayaz *et al.* (2015). Fresh weighed root and shoot samples (2 g) in triplicate were pulverised with liquid nitrogen (-195.79°C) and homogenised using aqueous methanol (80%, v/v, 10 ml). The homogenates were centrifuged (HermleZ 326 K, Germany) at 8000*g* for 30 min, and the supernatants were separated and evaporated using a rotary evaporator (Heidolph, Hei-VAP Expert-Standard, Germany). The samples were further fractioned by a solid-phase extraction (SPE) column (Agilent C-18, 3 ml, 500 mg of packed bed, USA) activated with 2 × 5 ml methanol, and sugars were eluted with 10 ml deionised water (aqueous fraction).

An Agilent 1100 HPLC system (Palo Alto, USA) equipped with a refractive index detector (RID), quaternary HPLC pump, micro-vacuum degasser (MVD), thermostatic column compartment (TCC) and multivariable wavelength detector (MWD) was used to determine the soluble sugar content. Sugar separations were performed on a Thermo Hypersil GOLD Amino carbohydrate analytical column (250 × 4.6 mm, 5 µm particle size) with a column temperature of 30°C. The mobile phase was acetonitrile:water (79:21, v/v) for isocratic elution at a constant flow rate of 1 mL min^{-1}. The results were expressed as mg /100 g of fresh weight (fw).

Extraction and determination of PAs. Extraction of PAs and determination of free PA content were performed as described by Colak *et al.* (2017). Accordingly, 1 g of wheat root and shoot samples was extracted with trichloroacetic acid (TCA, 5% v/v) and subjected to benzoyl chloride derivatisation. Different concentrations of Put, Spd, Spm, cadaverine (Cad) and 1,3-diaminopropane standard solutions were prepared to produce calibration curves. Benzylated derivates of PAs from the samples and standards were extracted using 3 × 10 mL diethyl ether and evaporated under nitrogen gas (99.99%). Both derivatives were dissolved in 100 µL of the initial mobile phase and analysed using an HPLC device (Knauer Smartline-Manager) equipped with a Pump 1000, a PDA detector 2800, an autosampler and a GraceSmart™ RP 18 column (5 µm particles; 4.6 × 250 mm). The mobile phase contained water (solvent A) and methanol (solvent B), and the flow rate was 0.8 ml/min throughout the analysis. The following gradient program was used for determination of PAs -55-70% B for 20 min, isocratic 70% B for 5 min, 70-55% B for 1 min and finally 10 min of 55-70% B. The column was kept at a constant temperature (40°C), and individual PAs were identified at a UV detection wavelength of 227 nm. The results were expressed as µmol per gram of dw (µmol/g dw).

Statistical analysis. One-way ANOVA and Pearson correlation (*r*) analysis were carried out on IBM SPSS Statistics Ver. 22.0 software. Principal Component Analysis (PCA) was performed on a statistical software package (Addinsoft 2019, XLSTAT statistical and data analysis solution, Long Island, NY, USA).

RESULTS AND DISCUSSION

Seedling growth during the heterotrophic stage of germination depends mainly on storage substances (e.g., sugars) mobilised from storage seed tissues and transmitted to various organs such as shoots and roots during growth and development. Since sugars exhibit osmoprotectant and antioxidant properties, they play a central role in the cellular redox balance (Rosa *et al.* 2009). In addition, sugars are a precursor for a diverse group of phenolic compounds and are one of the most promising candidates for protecting plants from the adverse effects of environmental stresses (Aoki *et al.* 2006; Rosa *et al.* 2009).

As shown in Fig. 1A, shoots of the control plants (-0.70 MPa) and those treated with NAC alone (-0.72 MPa) exhibited very similar Ψw values. The leaf Ψw value was reduced by HM treatment alone, while NAC in combination with the three HMs significantly increased Ψw values ($P < 0.05$). All treatments with HMs alone induced an increase in proline content (in the range of 3.13 – 5.23 µmol g^{-1} dw) in comparison with the control seedlings (2.67 µmol g^{-1} dw). The enhanced proline content in shoots was significantly reduced by NAC in combination with HMs (2.47 – 2.72 µmol g^{-1} dw), particularly in the case of treatment with Hg alone (Fig. 1B). These results are in agreement with those reported by Yusuf *et al.* (2011) indicating reduced Ψw values in *Triticum aestivum* L. leaves and by Schat *et al.* (1997) indicating increased proline content in *Silene vulgaris* (Moench) Garcke in response to HM stress. The leaf water potential is known to be an important indicator of a plant´s water status (Rucińska-Sobkowiak 2016), and plants that successfully deal with lower water potentials can cope with limited water availability either by maintaining high internal water concentrations or by shrinking water-requiring root structures and accumulating compatible solutes that keep osmotic potentials low (Lee *et al.* 2008; Rucińska-Sobkowiak 2016).

The effects of NAC alone and in combination with HMs (Cd, Hg and Pb) on the content of soluble sugars (glucose, fructose and sucrose mg 100 g^{-1} fw) in roots and shoots of wheat seedlings are summarised in Table 1. These findings revealed that NAC was effective when combined with HM treatment. As shown in Table 1, NAC combined with the three HMs significantly ($P < 0.05$) stimulated accumulation of soluble sugars in the roots of seedlings as compared with treatment with HMs alone. Glucose content increased significantly compared to HM treatments alone. In addition, wheat roots treated with NAC in combination with the three HMs had greater fructose and sucrose accumulation compared to the results of HM treatments alone. In contrast, NAC in combination with the HMs significantly reduced accumulation of the three soluble sugars in shoots (Table 1).

Sugar content of both shoots and roots increased significantly in seedlings treated with HMs alone and in combination with NAC in comparison with the controls. In particular, this increase was more apparent in the case of fructose content after Cd treatment, with 1.8-, 2.2- and 2.4-fold increases, than after Pb and Hg treatments. Similarly, the levels of the sugars increased

Table 1. Effects of different heavy metals and NAC on soluble sugar content (mg 100 g^{-1} fw) in seedlings of the 'Ceyhan-99' wheat cultivar.

Treatments	**Roots**			
	Glucose	**Fructose**	**Sucrose**	**Total Sugar**
Control	355.66 ± 20.20^{a}	404.80 ± 1.41^{a}	219.86 ± 33.48^{a}	1347.78
NAC	553.83 ± 28.10^{a}	466.87 ± 27.40^{a}	327.08 ± 21.33^{b}	959.06
Cd	639.85 ± 10.79^{c}	906.06 ± 56.03^{d}	786.88 ± 32.91^{f}	2332.79
Hg	511.84 ± 23.88^{b}	619.06 ± 17.18^{b}	414.37 ± 13.48^{c}	1545.26
Pb	616.53 ± 11.55^{c}	612.28 ± 13.20^{b}	547.00 ± 25.66^{d}	1779.15
Cd + NAC	724.89 ± 21.09^{d}	1051.56 ± 39.58^{e}	893.14 ± 17.68^{g}	2669.59
Hg + NAC	870.02 ± 24.55^{e}	797.89 ± 26.08^{c}	864.31 ± 32.94^{g}	2532.22
Pb + NAC	727.11 ± 6.08^{d}	819.49 ± 18.75^{c}	606.60 ± 6.75^{e}	2153.20
	Shoots			
Control	2135.48 ± 37.36^{c}	617.02 ± 6.35^{b}	616.56 ± 6.75^{b}	3369.06
NAC	1924.26 ± 3.58^{a}	571.84 ± 9.23^{a}	449.47 ± 5.42^{a}	2945.56
Cd	2742.43 ± 41.92^{g}	1197.79 ± 17.91^{g}	975.62 ± 9.82^{f}	4915.84
Hg	2593.07 ± 51.43^{f}	1150.65 ± 26.68^{f}	1070.12 ± 17.54^{g}	4813.84
Pb	2404.22 ± 62.23^{e}	1295.89 ± 12.88^{h}	1121.64 ± 45.80^{h}	4821.75
Cd + NAC	2251.18 ± 39.52^{d}	1034.82 ± 10.94^{d}	807.30 ± 0.24^{d}	4093.30
Hg + NAC	2041.97 ± 41.03^{b}	964.81 ± 21.64^{c}	678.70 ± 27.99^{c}	3685.48
Pb + NAC	2027.83 ± 53.69^{b}	1076.60 ± 37.44^{e}	843.99 ± 6.97^{e}	3948.42

*Data are expressed as means ± standard deviation (SD) of at least three independent experiments and extractions. Analysis of variance (SPSS version 22, one-way ANOVA) was used for comparison between means. The same letters at superscript within each column are not significantly different at $P < 0.05$.

approximately single-fold in shoots, particularly in the case of fructose content, with 1.9-, 1.9- and 2.1-fold increases for each HM treatment (Cd, Hg and Pb, respectively) in comparison with the control seedlings. Treatment with NAC alone yielded varying relative increases of sugar content in roots (1.6-, 1.2- and 1.5-fold, respectively) in comparison with the control seedlings, while the sugar content of shoots was reduced by such treatment (1.1-, 1.1- and 1.4-fold).

Figure 2A presents the PCA loading plot of treatments and soluble sugar content in roots and shoots of the wheat seedlings. The first two PCA components explained 93.85% of the variance: specifically, principal components PC1 (χ-axis) and PC2 (γ-axis) account for 65.90 and 27.04% of the total variance, respectively. The alleviating effect of NAC against HM stress can be explained by PC1. The contents of all three soluble sugars in shoots on the upper right plane were associated with HMs alone (Hg, Pb and Cd) with positive loadings and correlated significantly with sucrose and total soluble sugar (TSS) content (range: $r = 0.976 - 0.971$, $P < 0.05$) and insignificantly with fructose and glucose levels (range: $r = 0.026 - 0.688$, $P < 0.05$). However, NAC in combination with the HMs (Hg + NAC, Pb + NAC and Cd + NAC) on the lower right plane was closely associated with positive loadings and correlated with soluble sugar content in roots (range: $r = 0.822 - 0.986$, $P < 0.05$). On the other hand, sugar content in the control and treatment with NAC alone exhibited negative scores on PC2 and were not associated with the other treatments due to their relatively low sugar content. Factor analysis was performed on the basis of the matrix correlation coefficients using PCA factor analysis to identify and characterise possible associations due to sugar content among the treatments (Table 2). The matrix of dominant rotated factor loadings revealed two factors: Factor 1 exhibited the largest association, composed of shoot fructose, root fructose and total soluble sugar content. Factor

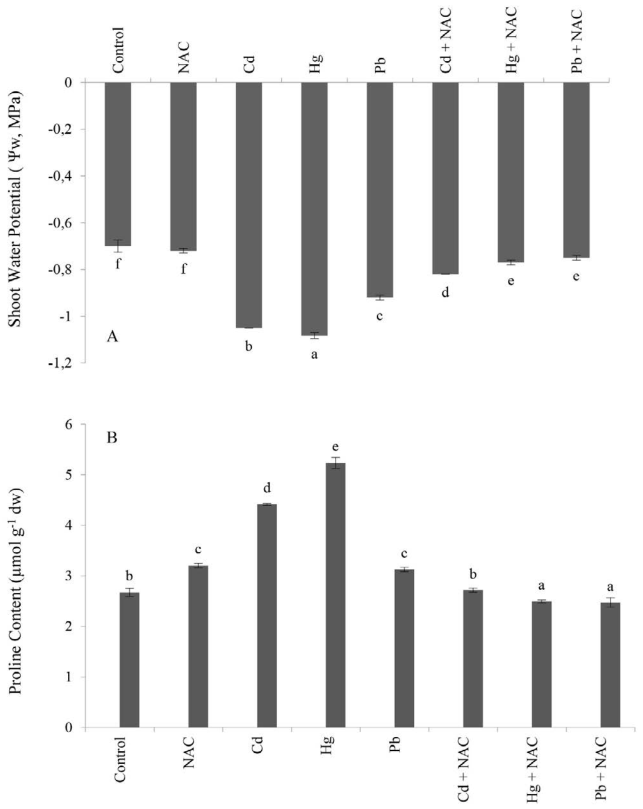

Fig. 1. Effects of different heavy metals and NAC on **(A)** water potential (MPa) and **(B)** proline content in wheat shoots (Ceyhan-99). Data are expressed as means ± standard deviation (SD) of at least three independent experiments and extractions. Analysis of variance (SPSS version 22, one-way ANOVA) was used for comparison among means. The same letters within each column are not significantly different at P < 0.05.

2, which refers to shoot glucose content, was moderately significant (Table 2B, Fig. 2A). With respect to PCA, the first two components obtained explained 93.85% of total variability of the original data, with 65.91% being assigned to the first factor (F1) and 27.94% to the second (F2) (Table 2). The matrix of dominant rotated factor loadings of treatments (observation) revealed two factors: Factor 1 exhibited the largest association, composed of NAC treatment alone followed by the control and Cd treatment; and Factor 2, which refers to Hg treatment alone and in combination with NAC (Table 2B, Fig. 2).

These results showed that the sugar metabolism of wheat seedlings was significantly altered by HM stress, and that the addition of NAC to the growth medium caused a marked increase of in sugar content in an attempt to overcome the adverse stress effect by maintaining a balanced water status in the plant. One recent study also showed an alleviating effect of NAC in HM stress, which was demonstrated using the same three HMs (plus Cu) at concentrations of 100 µm and the same wheat cultivar (Ceyhan) (Colak *et al.* 2019). The authors of

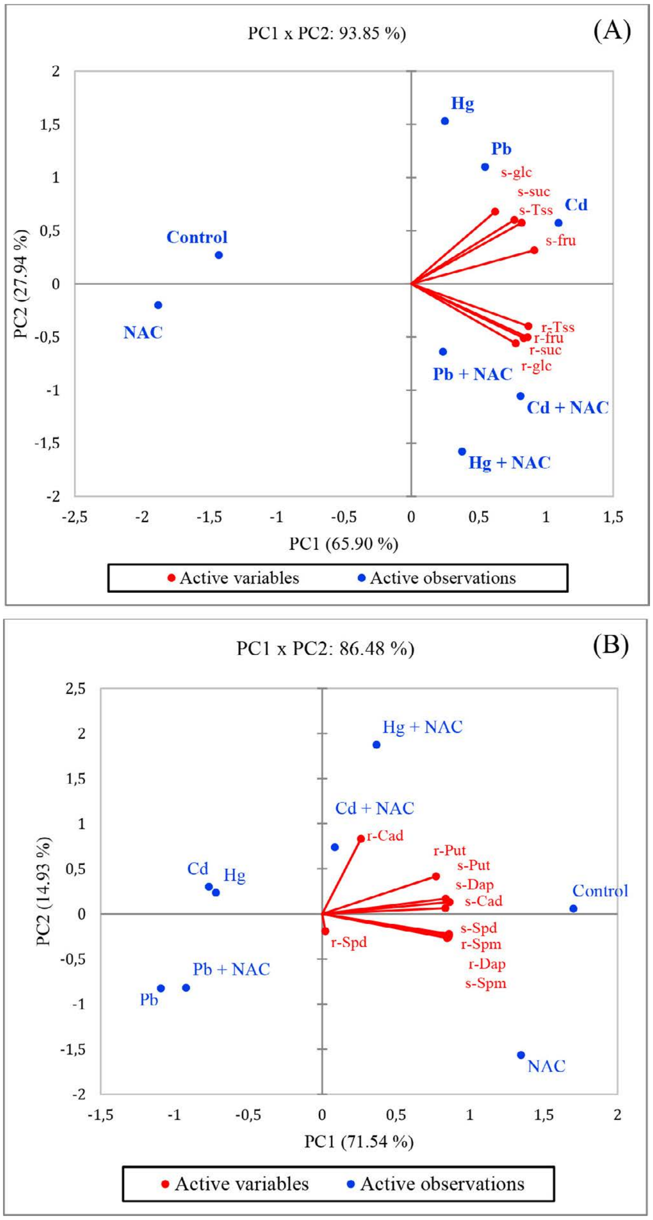

Fig. 2. Biplot (PC1 × PC2) of scores and loadings for the PCA of all data for soluble sugar (A) and polyamine (B) content in shoot and root of wheat seedlings exposed to heavy metal stress. s: shoot, r: root, Tss: total soluble sugar, glc: glucose, fru: fructose, suc: sucrose, Put: putrescine, Spd: spermidine, Spm: spermine, Cad: cadaverin. Values in bold differ from 0 at a significance level of α = 0.05.

that study reported significant retardation of increase in root length, fresh weight and dry weight, in addition to a severe oxidative burst that induced lipid peroxidation and H_2O_2 accumulation. They also indicated that NAC exerts its stress-alleviating effect by inducing increases or decreases in the activity of antioxidant enzymes or in the content of non-enzymatic antioxidant compounds in the roots through coordinated induction of pheno-

Table 2. Matrix of correlation coefficients (*r*) of variables based on sugar content (A) and the matrix of dominant rotated factor loadings on sugar content (B) in roots and shoots of wheat seedlings exposed to HMs alone (Cd, Hg, Pb) and to NAC in combination with HMs.

(A) Correlation coefficients (*r*)

Variables	root-glc	root -fru	root -suc	root -Tss	shoot-glc	shoot-fru	shoot-suc	shoot-Tss
root-glc	**1**	**0.822**	**0.901**	**0.933**	0.026	0.589	0.296	0.317
root-fru		**1**	**0.934**	**0.963**	0.321	0.627	0.397	0.479
root-suc			**1**	**0.986**	0.236	0.560	0.298	0.390
root-Tss				**1**	0.218	0.614	0.346	0.417
shoot-glc					**1**	0.688	**0.796**	**0.898**
shoot-fru						**1**	**0.935**	**0.930**
shoot-suc							**1**	**0.971**
shoot-Tss								**1**

Values in bold differ from 0 at a significance level of α = 0.05.

(B) Factors:

Variables	F1	F2	F3	F4	F5	Observations	F1	F2	F3	F4	F5
root-glc	**0.601**	0314	0.063	0.018	0.004	Control	**0.964**	0.014	0.016	0.000	0.004
root-fru	**0.756**	0.158	0.038	0.047	0.001	NAC	**0.993**	0.005	0.000	0.000	0.001
root-suc	**0.697**	0.261	0.024	0010	0.009	Cd	**0.785**	0.091	0.118	0.004	0.002
root-Tss	**0.744**	0.252	0.004	0.000	0.000	Hg	0.058	**0.934**	0.001	0.000	0.003
shoot-glc	0.387	**0.461**	0.139	0.012	0.002	Pb	0.308	**0.529**	0.152	0.002	0.007
shoot-fru	**0.834**	0.099	0.061	0.001	0.001	Cd + NAC	**0.534**	0.383	0.053	0.027	0.003
shoot-suc	**0.584**	0.361	0.043	0.004	0.005	Hg + NAC	0.111	**0.823**	0.017	0.049	0.000
shoot-Tss	**0.670**	0.330	0.000	0.000	0.000	Pb + NAC	0.146	**0.459**	0.300	0.087	0.008

Values in bold correspond to the factor with the largest squared cosine for each variable.
Abbreviations: glc: glucose, fru: fructose, suc: sucrose, Tss: Total soluble sugar content (sum of invidual sugar content)

Table 3. Pearson correlation (*r*) of water potential compared with proline and soluble sugar content in roots and shoots of wheat seedlings exposed to HMs alone (Cd, Hg, Pb) and NAC in combination with HMs.

	Proline	**Glucose**	**Fructose**	**Sucrose**	**Total Soluble Sugar Content**
	R				
Water Potential (Ψw)	-0.887**	-0.940**	-0.730*	-0.816*	-0.896**

*Significant at P < 0.05
** Significant at P < 0.01

lic compound pools and components of the antioxidant defence system (Colak *et al.* 2019). In agreement with that research, another study reported that non-essential HMs exhibited toxic effects even in minimal quantities by generating oxidative stress, binding protein structures, changing sugar source-sink partitioning, altering primary carbohydrate metabolism and inhibiting plant growth (Lin *et al.* 2007; Gill 2014). Solutes such

Table 4. Effects of different heavy metals and NAC on polyamine content (µmol g^{-1} dw) in seedlings of the 'Ceyhan-99' wheat cultivar.

	Putrescine	Spermidine	Spermine	Cadaverine	Dap**
Root					
Control	127.98 ± 1.68^{g}	2.19 ± 0.22^{c}	314.42 ± 17.50^{e}	7.70 ± 0.06^{e}	264.76 ± 0.13^{e}
NAC	91.75 ± 1.99^{e}	3.67 ± 0.44^{d}	342.37 ± 10.90^{f}	3.30 ± 0.01^{a}	292.07 ± 2.85^{f}
Cd	70.62 ± 3.95^{d}	0.79 ± 0.05^{a}	47.99 ± 2.28^{b}	6.38 ± 0.36^{d}	65.17 ± 65.17^{c}
Hg	64.03 ± 3.17^{c}	0.44 ± 0.04^{e}	8.39 ± 0.49^{a}	5.44 ± 0.81^{c}	33.12 ± 1.25^{ab}
Pb	17.72 ± 2.02^{a}	1.36 ± 0.27^{b}	7.75 ± 0.21^{a}	2.71 ± 0.33^{a}	28.70 ± 2.30^{a}
Cd + NAC	91.93 ± 4.66^{e}	1.41 ± 0.07^{b}	90.83 ± 0.61^{d}	7.89 ± 0.37^{e}	103.97 ± 5.51^{d}
Hg + NAC	109.23 ± 1.36^{f}	4.24 ± 0.37^{e}	76.15 ± 4.88^{c}	13.08 ± 0.87^{f}	103.60 ± 5.41^{d}
Pb + NAC	28.08 ± 1.11^{b}	8.12 ± 0.01^{f}	10.56 ± 0.32^{a}	4.31 ± 0.20^{b}	34.61 ± 1.14^{b}
Shoot					
Control	107.58 ± 3.17^{d}	250.67 ± 8.47^{e}	296.99 ± 10.31^{c}	1.54 ± 0.02^{g}	14.34 ± 0.01^{g}
NAC	108.65 ± 7.44^{d}	259.36 ± 12.25^{f}	335.11 ± 18.22^{d}	1.12 ± 0.06^{f}	8.32 ± 1.04^{f}
Cd	15.51 ± 0.41^{a}	6.04 ± 0.05^{a}	7.02 ± 0.07^{a}	0.18 ± 0.00^{a}	1.18 ± 0.07^{b}
Hg	22.06 ± 1.35^{b}	7.68 ± 1.22^{a}	8.62 ± 0.63^{a}	0.66 ± 0.03^{c}	1.79 ± 0.19^{c}
Pb	17.51 ± 1.20^{a}	11.37 ± 0.91^{a}	7.53 ± 0.16^{a}	0.17 ± 0.01^{a}	0.52 ± 0.02^{a}
Cd + NAC	69.14 ± 5.91^{c}	60.78 ± 2.06^{c}	79.41 ± 0.96^{b}	0.97 ± 0.05^{e}	5.14 ± 0.52^{d}
Hg + NAC	105.66 ± 0.45^{d}	85.62 ± 1.19^{d}	86.98 ± 0.41^{b}	0.75 ± 0.04^{d}	7.49 ± 0.31^{e}
Pb + NAC	23.62 ± 0.40^{b}	24.21 ± 0.41^{b}	9.38 ± 0.97^{a}	0.33 ± 0.03^{b}	0.83 ± 0.04^{ab}

*Data are expressed as means ± standard deviation (SD) of at least three independent experiments and extractions. Analysis of variance (SPSS version 22, one-way ANOVA) was used for comparison between means. The same letters in superscript within each column are not significantly different at $P < 0.05$.

**Dap: 1,3-diaminopropane

as starch and lipids accumulate during germination and the early stage of seedling growth, resulting in changes of sugar content, and these sugars are then transported to the sites where they are required (Gill *et al.* 2003). The results obtained in the present study are in agreement with those studies, which also indicated that accumulation and transport of sugars under stress conditions are linked to an increase in lignification and cell wall biosynthesis, as well as with their functioning as osmolytes and antioxidant compounds (Gill *et al.* 2003; Abersheim *et al.* 2011; Colak *et al.* 2019) and an oxidative burst due to severe HM stress, which alters the nature of sugar metabolism in plants (Rosa *et al.* 2009).

Heavy metal stress has deleterious effects on membrane permeability, aquaporin conductivity, water relations, the transpiration rate, mineral translocation and metabolism of osmolytes in various plant organs (Rucińska-Subkowiak 2016). High uptake and translocation of HMs have been reported to induce a decline in leaf Ψw as a result of high accumulation of osmolytes (Yadav 2010; Rucińska-Subkowiak 2016). Likewise, a high correlation was determined between water potential values, sugar content and proline content (range: $r = -0.730 - -0.940$, $P < 0.01$ or 0.05, Table 3) in the present study. Antioxidant defence and signal systems, in particular sugars (e.g., glucose, sucrose), also act as osmotic potential regulators in plant cells under stress conditions. Rucińska-Subkowiak (2016) reported that HM stress stimulated cell wall thickening by increasing levels of lignin, callose, pectin, cellulose, etc., and caused reduced water conductivity in the cell wall. In the present study, addition of NAC to the growth medium significantly reduced HM levels in different parts of wheat seedlings by chelating HMs in intracellular regions or in the growth medium, as indicated previously by Colak *et al.* (2019). It may therefore be suggested that any decrease in membrane damage and cell wall thickening by NAC treatment will provide easy water flow and main-

Table 5. Matrix of correlation coefficients (*r*) of variables based on polyamine content (A) and the matrix of dominant rotated factor loadings on polyamine content (B) in roots and shoots of wheat seedlings exposed to HMs alone (Cd, Hg, Pb) and NAC combination with HMs.

(A) Correlation coefficients (*r*):

Variables	root-Put	root-Spd	root-Spm	root-Dap	root-Cad	shoot-Put	shoot-Spd	shoot-Spm	shoot-Dap	shoot-Cad
root-Put	**1**	-0.168	0.696	**0.729**	0.668	**0.851**	0.697	0.688	**0.883**	**0.834**
root-Spd		**1**	0.016	0.028	0.009	0.145	0.111	0.062	0.001	-0.060
root-Spm			**1**	**0.998**	-0.008	**0.809**	**0.987**	**0.993**	**0.860**	**0.836**
root-Dap				**1**	0.049	**0.843**	**0.988**	**0.994**	**0.873**	**0.846**
root-Cad					**1**	0.493	0.048	0.010	0.415	0.294
shoot-Put						**1**	**0.859**	**0.844**	**0.908**	**0.850**
shoot-Spd							**1**	**0.996**	**0.894**	**0.859**
shoot-Spm								**1**	**0.870**	**0.853**
shoot-Dap									**1**	**0.932**
shoot-Cad										**1**

Values in bold differ from 0 at a significance level of α = 0.05.

(B) Factors:

Variables	F1	F2	F3	F4	F5	Observations	F1	F2	F3	F4	F5
root-Put	**0.742**	0.218	0.010	0.002	0.020	Control	**0.977**	0.000	0.006	0.008	0.003
root-Spd	0.001	0.045	**0.948**	0.002	0.003	NAC	**0.768**	0.216	0.000	0.011	0.001
root-Spm	**0.894**	0.089	0.005	0.008	0.004	Cd	**0.741**	0.024	0.123	0.068	0.045
root-Dap	**0.920**	0.062	0.002	0.012	0.002	Hg	**0.726**	0.016	0.207	0.044	0.000
root-Cad	0.086	**0.863**	0.044	0.006	0.000	Pb	**0.830**	0.099	0.042	0.001	0.015
shoot-Put	**0.875**	0.035	0.034	0.008	0.047	Cd + NAC	0.039	**0.612**	0.172	0.080	0.020
shoot-Spd	**0.924**	0.070	0.001	0.001	0.000	Hg + NAC	0.125	**0.675**	0.174	0.018	0.007
shoot-Spm	**0.911**	0.085	0.000	0.003	0.001	Pb + NAC	**0.518**	0.085	0.384	0.008	0.004
shoot-Dap	**0.932**	0.021	0.000	0.013	0.001						
shoot-Cad	**0.870**	0.005	0.007	0.110	0.001						

Values in bold correspond to the factor with the largest squared cosine for each variable.
Abbreviations: Put: Putrescine, Spd: Spermidine, Spm: Spermine, Cad: Cadaverine, Dap: 1,3-diaminopropane

tain balanced, compatible osmolyte accumulation to alleviate the diverse effects of HM stress.

Effect of NAC alone or in combination with HMs (Cd, Hg and Pb) on PA content in wheat seedlings. Although PAs function as plant hormones, they also exhibit antioxidant properties and can act as metal chelators. Effects of the three HMs and NAC alone or in combination on free PAs (Put, Spd, Spm and Cad) and 1,3-diaminopropane (Dap), the end-product of Spd and Spm metabolism, in roots and shoots of wheat seedlings are summarised in Table 4. Spermine was the major PA in roots (314.42 µmol/g dw) and shoots (296.99 µmol/g dw) of the control seedlings following treatments with HMs and NAC alone. Treatment with NAC alone led to a significant decrease in the contents of Put and Cad in roots, and in Cad and Dap content in shoots, in comparison with the control seedlings. Treatment with the three HMs alone induced significant decreases in levels of all PAs and the product Dap in both roots and shoots compared to NAC in combination with the HMs. Treatments with the three HMs caused notable decreases in content of Put (avg. 3.7-fold), Spd (avg. 3.1-fold), Spm (avg. 28.2-fold), Cad (avg. 1.8-fold) and Dap (avg. 7.1-fold) in roots in comparison with their respective controls. The addition of NAC in the present study prevented HM-induced reduction of PA content in wheat seedlings. Exogenous NAC application in combination with the three HMs significantly increased the content of PAs and Dap in varying degrees (Put: 1.3 – 1.7-fold, Spd: 1.8 – 9.6-fold, Spm: 1.4 – 9.1-fold, Cad: 1.2– 2.4-fold and Dap: 1.2 – 3.1-fold) in roots compared to HM treatment alone. As shown in Table 4, these reductions of PA concentrations in shoots were mitigated by treatment with NAC in combination with the HMs (Put: 1.4 - 4.8-fold, Spd: 2.1 - 11.2-fold, Spm: 1.3 - 11.3-fold, Cad: 1.1 - 5.4-fold and Dap: 1.6 - 4.4-fold) compared to HM treatment alone.

Principal component analysis was also employed to study the variation of PA content in roots and shoots of wheat seedlings under the same experimental conditions. The PCA extracted from the data shown in Table 4 and Fig. 2B explained 86.48% of the total variance. Among the treatments, only Hg + NAC and Cd + NAC on the upper right plane, control along the axis, and NAC alone on the lower right plane on PC1 (86.48%) were closely associated and strongly correlated with the PA levels of wheat seedling shoots and roots (range: r = 0.729 – 0.998, P < 0.05), except in the cases of Cad, Spd and Put content in roots (range: r = 0.001 – 0.696, P < 0.05, insignificantly). This means that NAC in combination with two HMs (Hg + NAC and Cd + NAC) relatively alleviated the effect of HM stress in the seedlings and induced a marked increase of PA levels, including Pb + NAC, even if this was negatively loaded on the lower left plane on PC2 (14.93%). The three treatments with HMs alone were not associated and significantly correlated with PA content in shoots and roots of the seedlings. Viewed overall, the PA levels in seedlings treated with NAC alone and in the control plants are unique, as are those in seedlings of the Hg + NAC and Cd + NAC variants, which share common characteristics with the Pb + NAC variant. Table 5 shows the factor analysis performed on the basis of matrix correlation coefficients using PCA factor analysis to identify and characterise possible associations of PA levels among the treatments. The dominant rotated factor loading matrix revealed three factors: Factor 1 showed the largest association, composed of shoot-Dap content and followed by shoot-Spd content, which were in strong positive correlation. Factors 2 and 3, which refer to root-Cad content and root-Spd content, respectively, were significantly strongly correlated (Table 5B, Fig. 2B). With respect to PCA, the first three components obtained explained 97% of total variability of the original data: 71.54% was assigned to the first factor (F1), 14.94% to the second (F2) and 10.52% to the third (Fig. 2B). The matrix of dominant rotated factor loadings of treatments (observations) revealed two factors: Factor 1 showed the largest associations, composed of the control, followed by Pb, NAC and Cd treatments. Factor 2 refers to treatment with Hg and Cd in combination with NAC (Hg + NAC and Cd + NAC) (Table 5B, Fig. 2B).

Weinstein *et al.* (1986) recorded that Cd significantly increased Put content in the leaves of oat and bean plants. Similarly, enhancement of Put content by treatment with Cd and Cu in wheat leaf segments was reported by Groppa *et al.* (2007). Our findings are in good agreement with those reported by Groppa *et al.* (2001), who recorded a significant decrease of Put and Spd contents in sunflower leaf discs and Spm content in wheat leaves treated with Cd and Cu (Groppa *et al.* 2001). In addition, Choudhary *et al.* (2010) reported a marked decrease of Cad content in radish seedlings treated with Cu and Cr. These results for PA levels from different plant sources clearly indicate that environmental conditions affect PA levels and their distributions, not only in one plant and its different parts, but also between species, depending on stress severity and tolerance (Gill & Tuteja 2010). Overall, it can be concluded that HM stress significantly affects PA levels in plants (Groppa *et al.* 2001, 2007; Choudhary *et al.* 2010). Similarly, based on the findings of the present study, it can be suggested that NAC in combination with HM treatment increases levels of PAs by promoting the expression of PA-related genes controlling enzyme activity.

Polyamines play an important role in the response of plants environmental factors through regulation of their concentrations, thus allowing plants to enhance their stress resistance (Chen *et al.* 2019). However, in the present study, HM toxicity caused a decrease in PA content. This may be due to down regulation of *ADC*, *SAMDC*, *SPDS* and *SPMS*, the inducing action of amine oxidases or inhibition of PA-synthesising enzymes such as arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) (Chen *et al.* 2018, 2019). With its ability to chelate heavy metals, NAC chelated HMs (Cu, Cd, Hg and Pb) present in the growth medium and intracellular spaces in seedlings of the same wheat cultivar (Ceyhan 99) used in the present study. Chelation resulted in marked decreases of HM content in tissues and stimulation of growth parameters in the plant (Colak *et al.* 2019). It can be suggested that the adverse effect of HMs on PA levels might be reduced by the addition of NAC, with potential improvement of tolerance to HM stress in wheat seedlings. Moreover, as a signal compound and transcription factor, NAC may promote the expression of PA-related genes and enzyme activities.

CONCLUSION

The present study is the first to investigate the content of soluble sugars (glucose, fructose and sucrose) and PAs in roots and shoots of wheat seedlings of the 'Ceyhan 99' cultivar exposed to HMs alone and to NAC in combination with HMs compared to the control seedlings. The major sugar in the wheat seedlings was fructose in the roots, followed by glucose in the shoots. In the case of PAs, roots of the seedlings exhibited high Put and Spm content, while the shoots showed high levels of Put, Spd and Spm. The obtained results indicate that when administered alone, the employed HMs (Cd, Hg and Pb) increased the content of soluble sugars and reduced PA levels in the seedlings, while the addition of NAC to the growth medium further increased sugar content. Treatment with the HMs alone led to an increase in values of the shoot water potential and increase of proline content. In contrast to this, NAC in combination with HMs reduced the water potential values and proline content in shoots. It can be suggested that the effect of HM stress can be reduced by the addition of NAC, thus encouraging potential tolerance mechanisms to maintain a well-balanced water status by increasing the content of compatible osmolytes (e.g., sugars and proline) through coordinated induction of sugar or proline synthesis to overcome the adverse effects of such severe stress. As indicated earlier, the application of NAC, an inexpensive chemical notably less toxic to the ecosystem, to agricultural farmlands contaminated with HMs may be beneficial in terms of developing sustainable agricultural policies ensuring high yields and environmentally friendly and safe food production.

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REZIME

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SERBICA

Efekat N-acetilcisteina (NAC) na sadržaj rastvorljivog šećera i poliamina u klijancima pšenice izloženim stresu teških metala (Cd, Hg i Pb)

Nesrin Colak, Petr Tarkowski i Faik Ahmet Ayaz

Stres izazvan teškim metalima negativno utiče na rast i produktivnost biljaka širom sveta. Ublažavanje efekta stresa egzogenom upotrebom različitih hemijskih supstanci postalo je zanimljivo područje proučavanja u oblasti tolerancije biljaka prema stresu. Kao tiol jedinjenje, derivat cisteina N-acetilcistein (N-acetil-L-cistein, NAC) je prekurzor sinteze glutationa i moćan čistač ROS sa snažnim sposobnostima uklanjanja antioksidanata i slobodnih radikala. Ova studija je istraživala uticaje teških metala (Cd, Hg i Pb, 100 µM) na akumulaciju rastvorljivih šećera i sadržaja poliamina u korenima i izdancima pšenice, kao i na vodni potencijal i sadržaj prolina u izdancima, zajedno sa ulogom NAC-a protiv toksičnosti teških metala. Dodavanje 1 mM NAC značajno je povećalo sadržaj glukoze, fruktoze i saharoze u različitom stepenu (prosečno 1,34 puta, 1,20 puta, odnosno 1,51 puta) u korenovima u odnosu na samostalno delovanje teških metala. Tretmani su doveli do značajnog smanjenja sadržaja šećera u izdancima. Vrednosti vodnih potencijala bile su visoko korelisane sa sadržajem prolina i šećera u izdancima pšenice. Stres izazvan teškim metalima značajno je smanjio sadržaj poliamina u oba dela biljke. Dodavanje NAC-a povećalo je sadržaj poliamina u sadnici u poređenju sa samostalnim delovanjem teških metala, kako u korenu, tako i u izdanku. Ovi rezultati sugerišu da NAC može zaštititi biljke od oksidativnih oštećenja pri stresu izazvanim teškim metalima, a čini se da ovo povećanje tolerancije na stres uključuje biosintezu rastvorljivog šećera i poliamina.

Ključne reči: vodni potencijal listova, N-acetil L-cistein, *Triticum aestivum*, osmoliti, tolerancija na stres