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1           **Wheat Biological Responses to Stress Caused by Cadmium, Nickel and Lead**

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18

**19 Abstract**

20 Several stressors like different types of heavy metals are found in the soil and can affect the  
21 growth and genomic integrity of wheat grains (*Triticum aestivum* L.). The aim of this study  
22 was to compare the effect of exogenous Cd (30, 60, 120 mg/kg), Ni (50, 100 and 150 mg/kg)  
23 or Pb (100, 200 and 300 mg/kg) on wheat agronomic characteristics through the assessment  
24 of oxidative stress indices at protein and gene expression levels, photosynthetic pigments and  
25 genetic aberrations using RAPD analysis that were studied during two winter seasons  
26 (2015/2016 and 2016/2017). The results showed that all stressors significantly decreased the  
27 vegetative growth parameters, altered the activities of antioxidants enzymes in seedlings  
28 (after 30 days) and grains (after 5 months) and differently affected their expression levels in  
29 seedlings leaves and roots. Pb treated plants showed the poorest agronomic characteristics as  
30 it exhibited the worst affected wheat height, number of tillers, fresh and dry weight, flag leaf  
31 area as well as yield. Pb treatment caused poorest plant performance, it showed the highest  
32 proline content, least protein and chlorophyll contents, thus affects the overall plants growth  
33 followed by Cd and Ni, respectively. Furthermore, high Pb and Cd doses revealed highest  
34 degree of polymorphism and lowest degree of genome stability. Altogether, heavy metals  
35 accumulated mainly in wheat straw and induced genotoxic effect which consequently altered  
36 normal plant metabolism and pigment content which resulted in a significant reduction in  
37 wheat yield and quality. Moreover, Pb induced more genotoxic and phytotoxic effects than  
38 Cd and Ni.

39

40 **Keywords:** Cadmium; Nickel; Lead; Antioxidants; vegetative growth parameters; RAPD

## 41 **1. Introduction**

42 Pollution concerns the deterioration of chemical, physical and biological properties of the  
43 surrounding environment air, water and soil (Ahmad et al., 2015b). Heavy metal pollution is  
44 one of the most global environmental problems worldwide facing humanity. Unlike organic  
45 pollutants that can be easily degraded to harmless small molecules, heavy metals belong to a  
46 group of immutable, non-biodegradable, inorganic chemical constituents and their total  
47 concentration in soils persists for a long time after their introduction (Wuana & Okieimen,  
48 2011; Malar et al., 2014). Despite heavy metals are natural constituents of soils in trace  
49 amount, they become toxic to soil as they decrease soil fertilization which consequently cause  
50 plant cellular damage when their quantity exceeds the normal limit (Al Naggar et al., 2014;  
51 Placek et al., 2016; Jalmi et al., 2018). Moreover, this adverse effect has been extended to  
52 human populations via food chain (Xie et al., 2016).

53 Among heavy metal ions, cadmium (Cd), nickel (Ni) and lead (Pb) contaminants are an  
54 emerging problem all over the world due to anthropogenic, technogenic and geogenic  
55 activities (Wuana & Okieimen, 2011; Rizwan et al., 2016). In agricultural soils, naturally Cd  
56 concentration ranged from 0.01 to 0.7 mg kg<sup>-1</sup> soil (Szolnoki et al., 2013), while the mean  
57 content of Pb is 50 mg kg<sup>-1</sup> soil (Nagajyoti et al., 2010) and Ni concentration, with respect to  
58 the World Health Organization (WHO), is 15-30 mg kg<sup>-1</sup>soil (Kabata-Pendias & Pendias,  
59 2001).

60 Nickel (Ni) is an essential micronutrient for plant growth and development; it has high  
61 biological activity as an integral component of various biomolecules (including  
62 metalloenzymes). However, it becomes toxic at high concentration (Sirhindi et al., 2016). In  
63 contrast, Cd and Pb are non-essential elements and at very low concentration they can cause  
64 morphological, physiological, and biochemical dysfunctions in plants (Shu et al., 2012; Meng  
65 et al., 2017).

66 Wheat (*Triticum aestivum* L.) is the most domesticated-cultivated-food crop and it is  
67 considered the most important human food grain source. As other plants, wheat is sensitive to  
68 various biotic and abiotic factors like, heavy metals (Parlak, 2016; Ali et al., 2018). Heavy  
69 metals have many direct and indirect damaging effects on plants growth (Jalmi et al., 2018).  
70 Heavy metals induced-phytotoxicity is reported to initiate secondary oxidative stress by  
71 importing the formation of harmful reactive oxygen species (ROS) or modification of the  
72 antioxidant defense system as well as replacement of enzyme cofactors and transcription  
73 factors, cellular redox and ionic transport imbalance, DNA damage and protein oxidation,  
74 thus inhibiting growth and grain yield (Sardar et al., 2013; Shahid et al., 2014; Theophanides  
75 & Anastassopoulou, 2017; Jalmi et al., 2018).

76 The present study was conducted to evaluate and compare the effect of different  
77 concentrations of Cd, Ni or Pb (the concentration range for each metal was chosen between  
78 the maximum allowable concentrations in soil and toxic soil concentration for plants) on  
79 wheat agronomic characteristics through studying the biochemical assessments of oxidative  
80 stress indices, detection of the genotoxic effect using RAPD assay and the evidence on  
81 vegetative growth parameters. RAPD technique, is a reliable and sensitive PCR based  
82 technique, has the potential to identify the DNA polymorphisms by detecting DNA damage  
83 (e.g. DNA adducts, DNA breakage) and mutations in plants (Jhang & Shasany, 2012;  
84 Gjorgieva et al., 2013). Differences in RAPD profiles between untreated and treated samples  
85 would be acted as a marker and elucidate the potential genotoxic effects of environmental  
86 hazardous (Atienzar & Jha, 2006; Azimi et al., 2013).

87 Soil samples were collected from one of the famous farms in Egypt which is Al-Gabal Al-  
88 Asfar farm (25 km north east of Cairo). Al-Gabal Al-Asfar farm was irrigated with sewage  
89 water of Cairo city for more than eighty years and there are many field crops cultivated in it  
90 such as wheat, tomato, potato, onion, garlic, maize, eggplant, bean, cauliflower, clover,

91 cabbage and strawberry beside pecan nuts and date palm. Total contents of Cd, Ni, Pb, iron,  
92 zinc and cobalt were found to increase as time goes on in soils irrigated with sewage at El-  
93 Gabal El-Asfer (Omran et al., 1988; Abdel-Sabour et al., 1996; Saber et al., 2016). Therefore,  
94 El-Gabal El-Asfer farm was chosen for the present study.

95

## 96 **2. Materials and methods**

### 97 **2.1. Materials**

98 The soil sample was collected from the surface layer (0-30 cm) of Al-Gabal Al-Asfar  
99 farm. Physiochemical characteristics of soil were illustrated in **Supp 1**. Wheat grains were  
100 obtained from the wheat Research- Crop Research Institute, Ministry of agriculture. Metals  
101 concentrations were selected according to the guideline values for metals concentrations in  
102 Egyptian (Al Naggar et al., 2014; Shokr et al., 2016) and global soils (Sipos & Póka, 2002;  
103 Toth et al., 2016) where the maximum allowable Cd, Ni and Pb concentrations in soil (0.3, 50  
104 and 60 ppm) and toxic concentration in soil for plants (3-8, 100-150 and 100-400 ppm),  
105 respectively.

### 106 **2.2. Growth conditions and treatments**

107 To study the effect of either Cd, Ni or Pb polluted soils on wheat agronomic characteristics,  
108 two pot experiments were conducted under natural conditions at Sakha Agricultural Research  
109 Station, Kafr El-Sheikh Governorate (31°5' 35.93" latitude N and 30°56' 54.33" longitude E)  
110 during winter seasons of (2015/2016) and (2016/ 2017). Control soil was a pot (30 cm width  
111 and 35 cm height) filled with 9 kg of soil without any treatment, while treatments were  
112 prepared by using aqueous solution of either Cd (30, 60 and 120 ppm) using Cadmium sulfate  
113 octahydrate ( $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ ) or Ni (50, 100 and 150 ppm) using Nickel chloride ( $\text{NiCl}_2$ ,  
114 anhydrous) or Pb solution (100, 200 and 300 ppm) using Lead acetate trihydrate  
115 ( $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ ) and applied to respective pots with four replicates. Seeds of wheat

116 were then uniformly sown in pots. After harvesting of wheat plants, another seed of wheat  
117 was grown in the same pot without fresh treatment of heavy metals. All plants were regularly  
118 irrigated and basal fertilizers (NPK) were applied in liquid forms for all pots. Nitrogen (N) as  
119 urea ( $91.575 \text{ mg kg}^{-1}$  dry soil) was splatted in three doses, the first active dose (20%) directly  
120 at the time of sowing of wheat and the remaining dose (80%) was applied in two equal splits  
121 at tillering and flower initiation stages, phosphorus (P) as calcium superphosphate ( $18.315$   
122  $\text{mg kg}^{-1}$  dry soil) and potassium (K) as potassium sulphate ( $29.304 \text{ mg kg}^{-1}$  dry soil) applied  
123 at the time of sowing of wheat.

### 124 **2.3. Plant sampling**

125 Plant sampling and analysis are summarized in **Figure 1**. First plant samples were collected  
126 **one month** after sowing (on December 14<sup>th</sup>, 2015). Roots were carefully separated from the  
127 soils, washed with tap water and the whole plants (seedlings) were rinsed in distilled water.  
128 Plants were separated into roots, stems and leaves. Each part was separately homogenized in  
129 9 volumes phosphate buffer (0.1 M, pH 7.4), centrifuged at 3000 rpm for 10 minutes and the  
130 supernatant was collected for biochemical investigation. Glutathione peroxidase (GPx) and  
131 glutathione-S-transferase (GST) activities as well as proline content were estimated in whole  
132 seedling. Total protein content and the expression level of superoxide dismutase (SOD), GPx  
133 and catalase (CAT) were estimated in seedling roots and leaves. DNA stability was also  
134 estimated in seedling roots.

135 At flowering stage (**after two months**) in the first and second seasons (on January 19<sup>th</sup>, 2016  
136 and 2017), the plant pigment (chlorophyll a, chlorophyll b and total chlorophyll) and flag leaf  
137 area ( $\text{cm}^2$ ) were determined using the method of Quarrie and Jones (1979), where:

$$138 \text{ Leaf area} = \text{Maximum length} \times \text{Maximum width} \times 0.75.$$

139 Where 0.75 is a coefficient factor for wheat

140 At full maturity of wheat (**harvesting after five months**) in the first and second seasons (on  
141 21, April, 2016 and 2017), the plants were picked out and straw and spike (grains) were  
142 separated. The mean of two seasons was recorded for some agronomic traits (fresh and dry  
143 weight of plant, plant height, weight and number of spikes and weight of 100 grains as well  
144 as number of tillers per plant). Finally, Cd, Ni or Pb contents were estimated in straw, grains  
145 and post-harvest soil samples.

#### 146 **2.4. Biochemical and molecular analysis**

147 **The activity of GPx** was measured following the method of Paglia and Valentine (1967).  
148 GPx catalyzes the oxidation of GSH by cumene hydroperoxide. GPx activity was determined  
149 by subtracting the excess GSH after enzymatic reaction from the total GSH in absence of the  
150 enzyme. GSH reacts with DTNB to form yellow colored chromophore which was measured  
151 at 412 nm spectrophotometrically.

152 **The GST activity** was determined according to the method of Habig et al. (1974). Ten  
153 microliters of p-nitrobenzyl chloride (1 mM) was added to 1.365 mL phosphate buffer (0.1  
154 M, pH 6.5) and mixed well. 25  $\mu$ l of either plant supernatant (test) or phosphate buffer, pH  
155 7.4 (blank) were added and incubated at 37 °C for 5 min, then 100  $\mu$ l GSH (5 mM) was added  
156 to start the reaction and incubated for 20 min at room temperature. The absorbance of  
157 samples was read against blank at 310 nm using UV spectrophotometer.

158 **Proline content** was estimated by using the method given by Bates et al. (1973). Ninhydrin  
159 can form a red product with proline at acidic pH. The plant material (100 mg) was added to 2  
160 mL aqueous sulfosalicylic acid (3%), grounded and centrifuged for 5 min at room  
161 temperature. The reaction mixture was prepared (100  $\mu$ L of 3% sulfosalicylic acid, 200  $\mu$ L  
162 glacial acetic acid and 200  $\mu$ L acidic ninhydrin) and 100  $\mu$ L of the plant extract supernatant  
163 was added, mixed well, incubated at 96 °C for 60 min and the reaction was terminated on ice.  
164 The samples were extracted with 1 mL toluene then vortexed for 20 s, and left for 5 min to

165 allow the separation of the organic and water phases. The chromophore containing toluene  
166 was removed and the absorbance was measured at 520 nm using toluene as a reference. The  
167 proline concentration can be determined using a standard curve of L-Proline (20-100 µg/ml)  
168 and calculated on fresh weight basis and expressed as micromole per gram FW (µmol/g FW).  
169 **The total protein level** was measured using the method of Gornall et al. (1949) that depends on  
170 the formation of colored complex when protein reacts with cupric ions in alkaline medium  
171 and the absorbance of samples were recorded at 546 nm.

### 172 **Reverse transcriptase Polymerase chain reaction**

173 The total RNA was isolated from different plant parts (leaves and roots) using Easy-RED™  
174 solution. One microgram of isolated RNA was reverse transcribed into complementary DNA  
175 (cDNA) using reverse transcriptase (Maxime RT PreMix kit, iNtRON Biotechnology,  
176 Korea). The resulting cDNA was used as templates for subsequent PCR amplification using  
177 specific primers for SOD, GPx, CAT and G3PDH as internal control using PCR thermal  
178 cyclers (ESCO, Swift MiniPro Thermal Cycler, USA). Primer sequences and PCR conditions  
179 were summarized in **Supp 2**. Semi-quantitative PCR analysis was performed using 2X PCR  
180 Master mix (i-Taq™) with standard buffer (iNtRON Biotechnology, Korea). The PCR  
181 product was resolved on 1.5% Agarose electrophoresis and stained with ethidium bromide.  
182 DNA bands were revealed by UV light and the band intensity was analyzed using UVitec  
183 software. The expression levels were determined by calculating the relative density of each  
184 SOD, GPx and CAT band to GAPDH.

### 185 **DNA extraction and RAPD procedures**

186 Total genomic DNA was extracted from root tips of seedlings (100 mg) by chloroform-  
187 phenol method. Chloroform-phenol mixture was used to precipitate proteins while DNA is  
188 precipitated by ethanol and dissolved in Tris-EDTA buffer. The PCR amplification was  
189 carried out with ten primers (10- base pair each, Biolegio, Korea, **Supp 3**) and genomic DNA

190 as a template. PCR was performed in a reaction mixture of 25 µl containing approximately 1  
 191 µl primer, 1 µl template DNA, 12.5 µl master mix and 10.5 µl nuclease free water. After PCR  
 192 cycle, 10 µl of PCR product was resolved on 1% Agarose electrophoresis at 80 mA for 20  
 193 minutes. DNA bands were revealed by UV light. DNA polymorphism represented in RAPD  
 194 profile included the change in number of bands, presence or absence of bands compared with  
 195 the control profile. Polymorphism was calculated according to Gjorgieva et al. (2013) as  
 196 following:

$$197 \quad P (\%) = [(a+ d)/c]* 100$$

198 Where, a is the number of new bands detected in samples, d is the number of disappeared  
 199 bands and c is the total number of bands in control. In addition, genomic template stability  
 200 (GTS, %) was calculated as following:

$$201 \quad GTS (\%) = (1 - p / n) \times 100$$

202 Where, p is the total number of RAPD polymorphic bands detected in each metal treated  
 203 sample and n the number of total bands in the control (Gjorgieva et al., 2013; Mohamed et  
 204 al., 2017).

205 **Chlorophyll a, b and total chlorophyll contents** were determined as described by Moran  
 206 (1982). One cm<sup>2</sup> of fresh flag leaf of plant was taken and placed into tubes containing 5 mL  
 207 of N,N-dimethylformamide. The tubes were kept at 25 °C in darkness for 24 h and shaken 15  
 208 s every 12 h. Samples were filtered and immediately measured for light absorption at 664  
 209 (Abs<sub>664</sub>) and 647 (Abs<sub>647</sub>) nm using spectrophotometer. Chlorophyll contents (mg/cm<sup>2</sup>) were  
 210 calculated based on the equations:

$$211 \quad \text{Chlorophyll a} = (12.64 \times \text{Abs}_{664}) - (2.99 \times \text{Abs}_{647})$$

$$212 \quad \text{Chlorophyll b} = (23.26 \times \text{Abs}_{647}) - (5.6 \times \text{Abs}_{664})$$

$$213 \quad \text{Total Chlorophyll} = (7.04 \times \text{Abs}_{664}) + (20.27 \times \text{Abs}_{647})$$

214 **Heavy Metals content**

215 Heavy metals (Cd, Ni or Pb) contents in wheat plants and post-harvesting soils were  
216 measured as suggested by Chapman and Pratt (1961) and Lindsay and Norvell (1978),  
217 respectively. For the mature-grown, straw and grains from each concentration were separated,  
218 dried in oven at 72 °C for 48 hours and powdered. 0.5 g dry powder (straw or grains) was  
219 added to 8 ml concentrated H<sub>2</sub>SO<sub>4</sub> and left to next day. The mixture was heated and the  
220 temperature was raised gradually to 370 °C. When sample color changed to pale brown,  
221 sample cold, 0.5-1 ml of 70 % perchloric acid was added and the mixture was further boiled  
222 until the release of dense white fumes. After cooling, 20 ml distilled water was added and  
223 heated until a clear solution was obtained. At room temperature, the mixture was filtered and  
224 transferred quantitatively to a 50 ml volumetric flask by adding double-distilled water. While,  
225 the soil sample (20g) were mixed with 40 ml diethylenetriaminepentaacetic acid (DTPA) and  
226 secured upright on shaker for 2 hours. Then the suspensions were filtered. The obtained  
227 samples' solutions were analyzed through atomic absorption spectrophotometer, PERKIN  
228 ELMER 3300.

## 229 **2.5. Statistical analysis**

230 Data were analyzed using IBM SPSS software package version 20.0. The Kolmogorov-  
231 Smirnov test was used to verify the normality of distribution. Quantitative data was described  
232 as mean  $\pm$  standard deviation (mean  $\pm$  SD). Data were analyzed by using one-way ANOVA  
233 to compare between more than two groups and Post Hoc test (Tukey) for pairwise  
234 comparisons. Significance of the obtained results was judged at the 5% level. The marked  
235 changes observed in RAPD profiles (disappearance and/or appearance of bands compared  
236 with control) were evaluated (Gjorgieva et al., 2013).

## 237 **3.Results**

### 238 **3.1Activities and relative gene expression of antioxidant enzymes**

239 **After 30 days**, most Cd, Ni or Pb contaminated soils significantly elevated **GPx and GST**  
240 **activities** in seedlings compared to control (**Table 1**). A non-significant elevation in GPx  
241 activity was observed at Cd 60, 120 ppm and Pb at 300 ppm and in GST activity at Cd 120  
242 ppm and Ni 100 ppm. While, lowest Ni concentration (50 ppm) showed a significant  
243 suppression in GST activity compared to control.

244 In addition, the expression level of GPx was significantly up-regulated in seedlings leaves at  
245 different treatments except Pb at 200 ppm compared to control. While seedlings roots were  
246 diversely affected by metals contamination where Cd (30 and 60 ppm) showed non-  
247 significant increase and Ni (150 ppm) showed a significant up-regulation and other metals  
248 showed a significant reduction in GPx expression level compared to control (**Supp 4 and**  
249 **Table 2**). Cd, Ni or Pb stressed soils were significantly up-regulated the expression level of  
250 CAT in seedlings leaves and roots except Cd significantly down-regulated CAT expression  
251 level in seedlings roots compared to control. This down regulation was peaked at lower Cd  
252 concentration and declined at higher Cd concentration. The expression level of SOD was  
253 significantly down-regulated in both seedlings leaves and roots compared to control.  
254 Moreover, 200 and 300 ppm Pb treatment resulted in maximum down-regulation of SOD  
255 expression about 52 and 58 %, respectively.

256 After 5 months, GST activity in grains showed a non- significant change compared to control  
257 except Cd 30 ppm showed a significant elevation. In addition, Cd 60 ppm, Ni 50 ppm and Pb  
258 100, 200 and 300 ppm showed a non- significant elevation in grain GPx activity.

259 Notably, in seedlings and grains the activities of GPx and GST were maximum at lower Cd  
260 concentration followed by a decline at higher Cd concentrations compared to control (**Table**  
261 **1**). GPx and GST activities of seedlings increased about 61.9 and 76.9 %, while this increase  
262 was around 16.6 and 14.6 % for grains at 30 ppm Pb compared to the control. GPx activity  
263 was lower in grains than that in seedlings and vice versa in GST.

### 264 **3.2 Proline and total protein contents in seedling parts**

265 Proline content was significantly increased in either Cd, Ni or Pb- stressed wheat plants  
266 (**Table 3**). It was found to increase in a dose dependent manner, showing highest elevation at  
267 Pd 300 ppm (about 363%).

268 Total protein content was significantly elevated in roots and leaves after both Cd and Ni  
269 treatments than corresponding untreated control parts, except Cd at 120 ppm showed a non-  
270 significant decrease by only 3% in roots (**Table 3**). On the other hand, in Pb stressed plants  
271 the total protein content was significantly decreased in roots while it was significantly  
272 elevated in leaves except 100 ppm showed a non-significant change.

### 273 **3.3 Chlorophyll content and flag leaf area**

274 Cd, Ni and Pb treatments adversely affected the plant pigment system. A significant decline  
275 in chlorophyll a, chlorophyll b, total chlorophyll and flag leaf area was observed in metal-  
276 stressed plants and the magnitude of decline was in a dose dependent manner (**Table 3**). The  
277 concentration of chlorophyll a was found to be higher than chlorophyll b in plant leaves. Pd  
278 treatment especially at 300 ppm showed the highest decrease in chlorophyll a, chlorophyll b  
279 and total chlorophyll by about 36 % and flag leaf area was decreased by about 46%.

### 280 **3.4 Vegetative growth parameters**

281 After 5 months, Cd, Ni and Pb ions had negatively impacted the plant growth parameters.  
282 This inhibition was in a dose dependent manner and the highest metals concentration  
283 produced the shortest plant and the least number of tillers/plant compared to control (**Table**  
284 **4**). A significant reduction in wheat height by 21.67, 16.11, 21.11 % and the number of  
285 tillers/plant by about 29 % was observed in Cd (120 ppm), Ni (150 ppm) and Pb (300 ppm)  
286 stressed soils, respectively. In addition, fresh and dry weight of wheat were clearly affected  
287 by Cd, Ni and Pb treatments, reaching lowest values at 300 ppm Pb, 150 ppm Ni and 120  
288 ppm Cd, respectively. Moreover, spike weight, number of spikes/pot and weight of 100

289 grains were remarkably reduced by applying different concentrations of Cd, Ni and Pb  
290 (**Table 4**). Interestingly, the highest Pb concentration (300 ppm) showed the maximum  
291 inhibitory effect on plant growth parameters. On the contrary, Ni 50 ppm resulted in no  
292 statistically change in wheat height, dry weight, spike weight and number of spikes/ pot  
293 compared to control.

### 294 **3.5DNA polymorphism profile**

295 RAPD profiles generated following the metal exposure were altered and different from those  
296 of control DNA as indicated in **Figure 2** and **Table 5**. Generally, in this study, heavy metals  
297 treatment leads to normal fragment pattern disappearance as the total number of disappeared  
298 fragments was higher than the number of appeared bands. A variation in band intensities in  
299 comparison with control profiles was also observed. The maximum change in RAPD profiles  
300 was obtained at 120 ppm Cd and 300 ppm Pb concentrations (P= 100 % and GTS= 0 %), as  
301 compared with control profile. The percentages of polymorphism are in the range of 73.1-  
302 100% and the values of GTS ranged from 26.9% to zero %. Samples treated with Cd, showed  
303 an increase in the percentages of polymorphism and a decrease in GTS with increasing the  
304 concentration, although the pattern of Ni and Pb induced genotoxicity did not follow the  
305 concentration gradient.

### 306 **3.6Heavy metals content in wheat straw and grains as well as in post-harvest soils** 307 **(after 5 months)**

308 In the present study, heavy metals (Cd, Ni and Pb) accumulation in different wheat parts  
309 (straw and grains) was directly proportional with metals concentrations in soil (**Table 6**).  
310 Metals were strongly accumulated in straw more than in grains. The highest Cd concentration  
311 (120 ppm) showed the maximum accumulation in straw and grains by about 14 and 16 fold,  
312 respectively than control (P<0.05). As well as, Ni (150 ppm) and Pb (300 ppm) treated soils

313 were found to increase Ni and Pb accumulation in straw and grains by lower levels than Cd  
314 compared to control.

315 The chemical analysis of Cd, Ni and Pb treated-soils indicated that the use of different  
316 quantities of heavy metals significantly increased its availability to 27.2, 4.08, 1.44 fold in  
317 first season and 12.5, 7.1, 1.56 fold in second season, respectively, compared to control  
318 **(Table 6)**.

#### 319 **4.Discussion**

320 Detecting and evaluating biological changes that result from chemical contamination are  
321 essential steps to determine the significance of such contamination (Stegemann et al., 2018).  
322 High concentration of heavy metals becomes toxic to biota and humans and significantly reduces  
323 plant production. The present study revealed a significant decrease in vegetative growth  
324 parameters by Cd, Ni or Pb toxicity. Moreover, Pb treated plants showed the poorest growth as  
325 indicated by the highest reduction in wheat height, number of tillers, fresh and dry weight, flag  
326 leaf area as well as yield components. Pb intoxication also resulted in the highest proline content,  
327 least protein and chlorophyll contents, thus affected the overall plants growth followed by Cd  
328 and Ni, respectively. Furthermore, high Pb and Cd doses revealed highest degree of  
329 polymorphism and lowest degree of genome stability.

330 Heavy metals induce irreparable physiological and biochemical dysfunctions in plants by  
331 inducing the rapid generation of ROS and may cause oxidative stress and cell death (Shu et al.,  
332 2012; Shahid et al., 2014; Jalmi et al., 2018). In 2015, studies reported that the non-enzymatic  
333 and enzymatic antioxidants like SOD, peroxidase (POD), ascorbate peroxidase (APX) and CAT  
334 showed up or down regulation to cope and repair the damage and this confer the stress tolerance  
335 of plants (Ahmad et al., 2015a; Sofu et al., 2015).

336 Shahabivand et al. (2016) reported that Cd toxicity significantly altered the activities and the  
337 transcription levels of CAT (decreased), APX and GST (increased) in leaf samples. Also Aydin  
338 et al. (2016), illustrated that the gene expression pattern of CAT under different Cd  
339 concentrations revealed a positive correlation, and there is no correlation found between the  
340 mRNA level and CAT enzyme activity in tomato leaves. Shu et al. (2012) stated that Pb  
341 treatment had a stimulating effect on SOD activity at low concentration and an inhibitory effect

342 at higher concentration in cuttings while in seedlings SOD activity was increased by increasing  
343 Pb concentration. In addition, 2 mM Ni solution, increased the expression levels of Fe-SOD,  
344 CAT, POD, and APX in soybean seedlings than the control levels (Sirhindi et al., 2016).

345 In the present study, the activities of GPx and GST in wheat seedlings (after 30 days) and grains  
346 (after 5 months) were increased by Cd, Ni or Pb- stress in an irregular manner not concentration  
347 dependent. Interestingly, the highest Cd (120 ppm), Pb (300 ppm) and Ni (150 ppm)  
348 concentrations showed the lowest GPx activities in seedlings while it altered in grains. Li et al.  
349 (2012) reported that GPx may be considered as an intrinsic defense against Pb-induced oxidative  
350 damage in perennial ryegrass and he also stated that the antioxidant enzymes (including; GPx,  
351 SOD, CAT and POD) responded to Pb exposure at an early stage. Genes encoding SOD and GPx  
352 were upregulated within a few hours after Pb treatment and after 24 or 48 h, their expression  
353 levels were returned to the levels of control or below and after 7 d the SOD activity was below  
354 that in untreated control (Li et al., 2012). Liu et al. (2009) also reported that the SOD gene  
355 expression was substantially increased within 3 h of Pb treatment and then gradually declined to  
356 a stable level in *A. thaliana* seedlings. In agreement with these findings, the activities of GPx and  
357 GST after 30 days (in wheat seedlings) more than that after 5 months (in grains). Therefore, the  
358 antioxidant enzymes might be responsible for the early stage defense to heavy metals- induced  
359 oxidative stress and may play an important role in eliminating the metals toxicity.

360 Furthermore, the expression level of GPx in leaves was significantly up-regulated (except in Pb  
361 at 200 ppm) after heavy metals stress, while in roots Cd (30 and 60 ppm) showed non-significant  
362 changes, Ni (150 ppm) showed a significantly up-regulation and other metals showed a  
363 significant reduction in GPx expression level. Cd, Ni or Pb stressed soils were significantly up-  
364 regulated the expression level of CAT in seedlings leaves and roots (except Cd treatments in

365 roots). A protective mechanism was suggested by the increased expression of specific genes  
366 encoding antioxidant enzymes (Baek & Skinner, 2003). On the other hand, the expression level  
367 of SOD was significantly down-regulated in both seedlings leaves and roots. This indicates the  
368 consumption of antioxidant enzymes to protect the membrane stability and to scavenge oxidative  
369 damage ( $O_2^-$  and  $H_2O_2$  production) induced by heavy metals (Shahabivand et al., 2016; Sirhindi  
370 et al., 2016). The altered enzymatic levels indicate the tolerance of wheat plants against heavy  
371 metals stress.

372 In this study, total protein content was progressively increased in leaves stressed by Cd, Ni or Pb  
373 and in roots stressed by Cd (30, 60 ppm) and Ni, while it was decreased in roots stressed by Cd  
374 (120 ppm) and Pb. The increase in total protein may be due to the induction of stress proteins  
375 that mainly involved in detoxification, antioxidant processes and the activation of genes for  
376 synthesis of specific proteins associated with stress such as some heat shock proteins that  
377 maintain the membrane proteins and the plant cell structures (Shu et al., 2012; Alayat et al.,  
378 2014). On the other hand, the inhibitory action of Cd (120 ppm) and Pb on roots protein content  
379 may be due to metals toxicity that reduces the protein synthesis and elevates protease activity,  
380 causing proteins degradation. Nevertheless, heavy metal stress generates ROS that in turn  
381 damages proteins, also the binding of metals to protein-SH groups can cause deleterious effect in  
382 the normal protein structure (Sirhindi et al., 2016; Natarajan et al., 2018). This decrease agrees  
383 with the decrease in GPx expression level in roots concerning Cd (120 ppm) and Pb (100, 200 &  
384 300 ppm) toxicity.

385 Proline is a pivotal and multifunctional amino acid, plays an essential role not just in plant  
386 developmental processes (including; chlorophyll reconstruction, Krebs cycle activation and an  
387 energy source) but also in reducing environmental stress (Trovato et al., 2008). Proline

388 accumulation under heavy metal stress has been reported to be a potential indicator of stress  
389 tolerance (Parlak, 2016; Sirhindi et al., 2016). This accumulation may be due to the reduction of  
390 its degradation as it acts as an osmoprotectant, macromolecules stabilizer, metal chelator and  
391 inhibitor of lipid peroxidation as well as free radical scavenger (Ahmad et al., 2015a; Ahmad et  
392 al., 2015b). In consistent with these findings, proline content shows a positive association with  
393 increasing the three metals concentrations in the soil and Pb proved to be the strongest inducer  
394 for proline accumulation.

395 Toxic Metal ions directly or indirectly influence DNA synthesis and the replication process  
396 (Morales et al., 2016; Theophanides & Anastassopoulou, 2017). Metal ions can interact with  
397 DNA through hydrogen bonding of the metal-coordinating water molecules resulting in the  
398 disruption of base pair hydrogen bonding and cause changes in the structure of the DNA double  
399 helix (Anastassopoulou, 2003; Theophanides & Anastassopoulou, 2017). ROS generated by  
400 metal-catalyzed reactions can modify DNA bases; three base substitutions, G/ C, G/ T and C/ T,  
401 can occur as a result of oxidative damage by metal (Al-Qurainy, 2009).

402 The genotoxic effect of Cd, Ni or Pb contamination was investigated in the present study through  
403 the analysis of ten different RAPD primers in wheat seedlings roots. The results showed  
404 obviously different RAPD amplification products in metals treated samples compared to control  
405 sample. According to our results, low metals concentrations may lead to the lower level of  
406 genotoxic effects of wheat seedlings than higher concentrations as indicated by higher values of  
407 GTS at Cd 30 ppm, Ni 50 ppm and Pb 100, 200 ppm. In addition, the number of missing bands  
408 was found more than that of newly appeared bands. These results are in agreement with the study  
409 of Gjorgieva et al. (2013) however, their results showed that Ni treatment at concentrations of  
410 150 and 350 ppm showed the highest number of lost bands in bean seedlings than Cd and Pb at

411 the same concentrations, but in the present study Cd 60 ppm and Pb 100, 300 showed the highest  
412 number of missing bands (15 band/ 10 primers). The disappearance of normal DNA bands may  
413 be resulted from DNA damage (e.g. single- and double-strand breaks, bulky adducts, modified or  
414 oxidized bases) and point mutation that may be induced by heavy metals treatments (Atienzar &  
415 Jha, 2006). New RAPD amplification products (extra bands) may be attributed to a change in  
416 some oligonucleotide priming sites due to mutations (new annealing events), homologous  
417 recombination and/or large deletions (bringing to pre-existing annealing site closer) (Atienzar et  
418 al., 1999). The highest number of extra bands (12 band/ 10 primers) was observed in Cd 120  
419 ppm and Ni 100 ppm treated samples suggests that this metal probably cause mutations on  
420 genomic level in high doses. Cd and Pb at highest concentrations were detected for the highest  
421 level of DNA damage (GTS 0% and P 100%), and for Ni, 100 ppm showed highest genotoxicity  
422 (GTS 3.8% and P 96.2%) than other concentrations. There is correlation between results obtain  
423 for Cd 300 ppm in the present study (highest GPx and SOD expression level, highest number of  
424 missing bands in RAPD profile and lowest GTS; comparing to ether metals treated samples) with  
425 a study from 2013 (Gjorgieva et al., 2013) and 2010 (Wan-Ibrahim et al., 2010) where author's  
426 deduced the correlation between genotoxicity and high antioxidant level. In (2017) Meng et al.  
427 provided the molecular mechanisms that investigated Cd toxicity on DNA and protein  
428 metabolism at protein levels. Cd toxicity was found to inhibit DNA glycosylase, ligation  
429 enzymes expressions and gap-filling, therefore, decreasing DNA repair capacity. At the protein  
430 level, Cd induced the heat shock protein response, initiation of protein refolding as well as  
431 degradation by ubiquitin proteasome pathway. In addition, excess Cd also induced antioxidant  
432 responses (Meng et al., 2017).

433

434 In present study, chlorophyll a, b and total chlorophyll contents were drastically reduced  
435 in wheat flag leaf with increasing the concentrations of externally supplied metals and Pb had the  
436 least contents followed by Ni and Cd, respectively. This reduction may be occurred due to (i) the  
437 disturbance in the uptake of  $Mg^{2+}$  and essential elements (such as Mn and Fe) (Parlak, 2016), (ii)  
438 the inhibition of chlorophyll biosynthesis due to the interaction of heavy metals with –SH group  
439 of enzymes involved in that process (Shu et al., 2012) and (iii) the increase in chlorophyllase (the  
440 first enzyme of the chlorophyll catabolic pathway) activity, which resulted in chlorophyll  
441 degradation (Liu et al., 2008). The reduction in chlorophyll content and consequent inhibition of  
442 photosynthesis are indicating symptoms of metals toxicity and generally reduce plants growth  
443 (Ci et al., 2010; Deswal & Laura, 2018; Marzilli et al., 2018). In this study, Cd, Ni or Pb at  
444 different concentrations showed a significant reduction in wheat height, number of tillers, fresh  
445 and dry weight, flag leaf area as well as yield components (weight and number of spikes and  
446 weight of 100 grains) and the highest reduction was observed in Pb, these results are consistent  
447 with who reported that metal stress created disorders in metabolic system of plants and quickly  
448 inhibited the cell division and cell elongation process leading to reduction of leaf blade area, leaf  
449 density and plant stem growth with accompanying decrease in numbers of flowers and fruits as  
450 well as shoot and root growth.

451 In the present study Cd, Ni and Pb were accumulated more in straw (leaves+ stem) than  
452 grains of wheat. Note also, the content of metal in straw and grains was increased in a dose  
453 dependent manner corresponding to metal concentration in the soil and Cd showed the highest  
454 accumulation in straw and grains. These results corroborate with the findings of Dang et al.  
455 (1990); Jafarnejadi et al. (2011) and Ali et al. (2018) who reported that metal uptake by grains  
456 was directly related to the applied heavy metal in the soil and accumulation of metal in plants

457 differed greatly among organs or tissues and was found to follow soil > root > shoot > grain.  
458 Moreover, the DTPA extractable elements (Cd, Ni or Pb) from soil were increased with  
459 increasing levels of applied elements. In addition, the metals levels were found to be higher than  
460 the maximum allowable limits for Cd (0.3 ppm), Ni (50 ppm) and Pb (60 ppm) in soils (Al  
461 Naggar et al., 2014; Shokr et al., 2016; Toth et al., 2016). Sardar et al. (2013) and Placek et al.  
462 (2016) reported that removal of heavy metals through leaching or uptake by the plants is much  
463 lower than the rate of their entry into the soil, leading to gradual accumulation of elements in the  
464 soil. The studies of Dang et al. (1990) and Deswal and Laura (2018) deduced that the  
465 phytotoxicities and metals accumulation in soil were found to be in the order:  $Cd^{2+} > Ni^{2+} > Pb^{2+}$   
466 in different crop species by using the same concentrations of heavy metals. In the present study,  
467 similar trend was observed for metals accumulation in soil, however, the phytotoxicities was  
468 found to be in the order:  $Pb^{2+} > Cd^{2+} > Ni^{2+}$  where metals concentrations were selected according  
469 to guideline values for metals in soils.

470

## 471 **Conclusions**

472 The studied heavy metals (i.e. Cd, Ni and Pb) have a negative impact on plant growth and  
473 development. They are readily taken up and accumulated in plant parts causing proportionate  
474 changes in the biochemical responses (concerning the contents of protein, proline, chlorophyll,  
475 antioxidant system and DNA stability), reduction in vegetative growth parameters (concentration  
476 dependent) and finally led to a significant reduction in wheat yield and quality. The changes  
477 occurred in RAPD profiles, can be successfully used as a sensitive marker for detecting metal  
478 induced DNA damage and showed the potential as a reliable assay for genotoxicity. Finally, we  
479 concluded the importance of increasing the public awareness of the threat of heavy metals

480 pollution and also keep the soil, water and fertilizers away from heavy metals contamination,  
 481 especially cadmium, nickel and lead.

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485

486 **References:**

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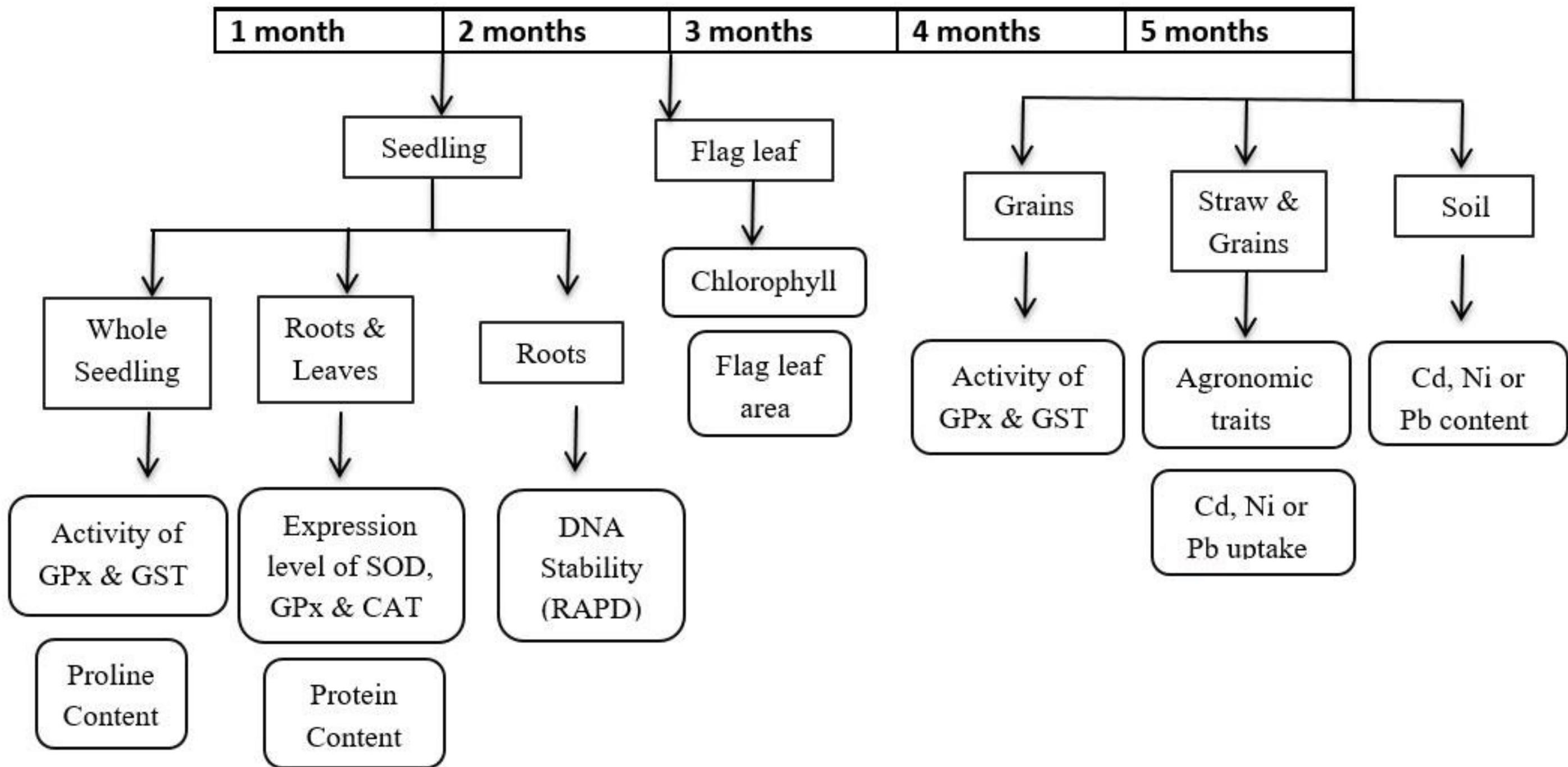
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681 **Figure Legend**

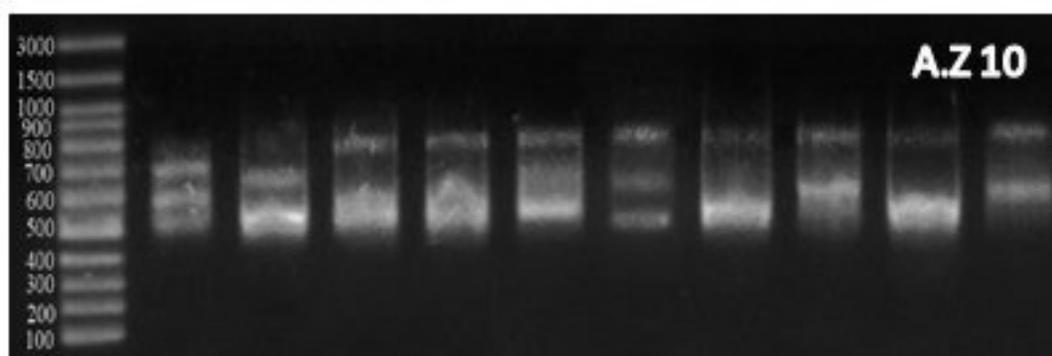
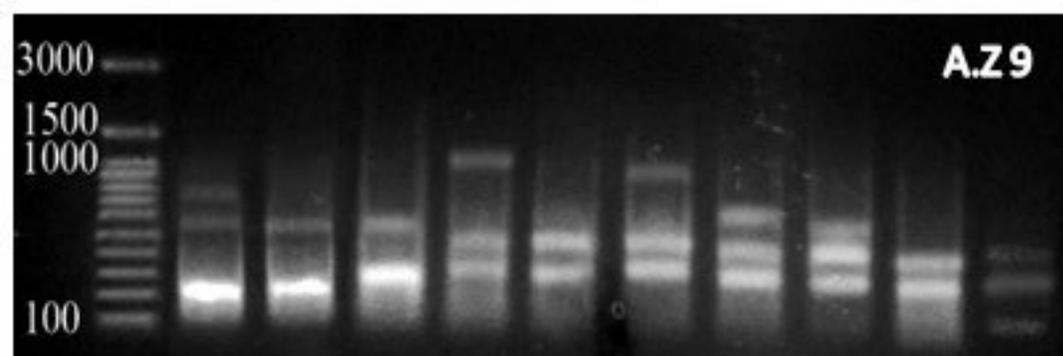
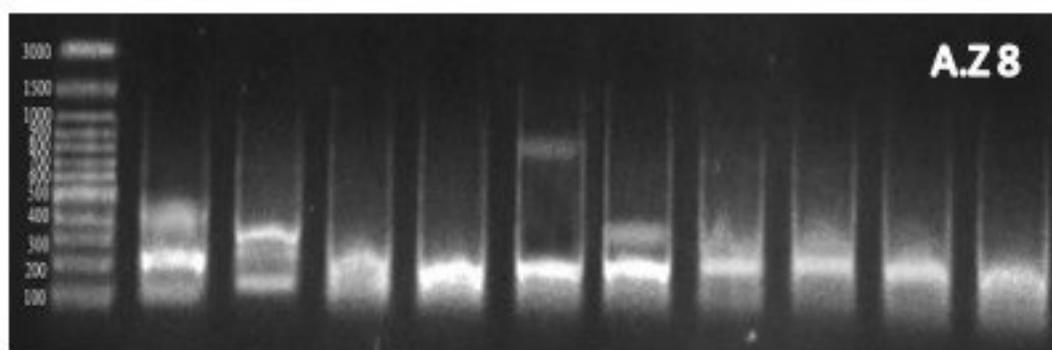
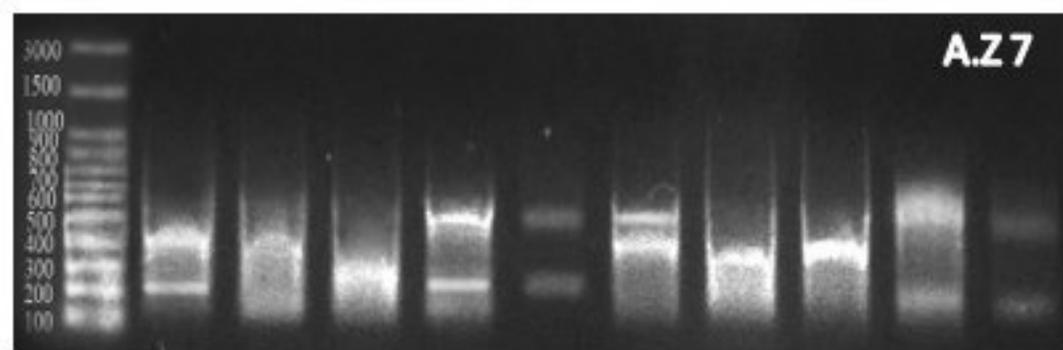
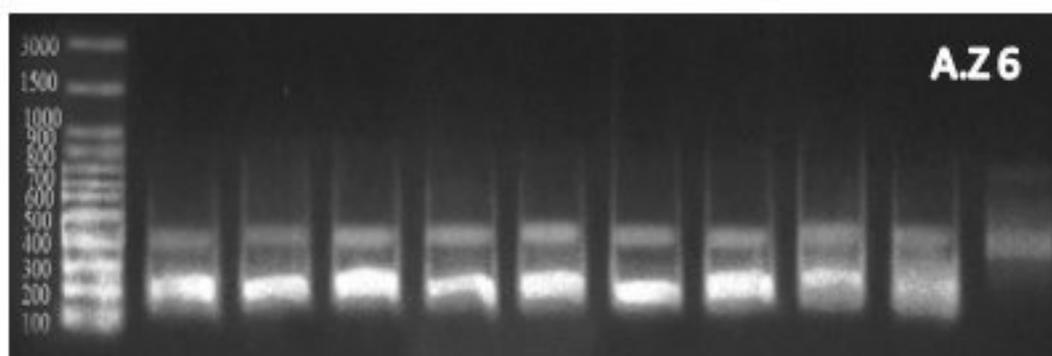
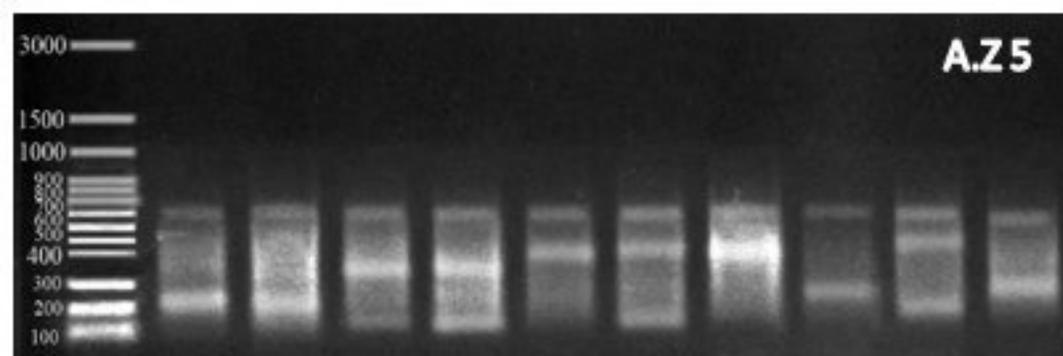
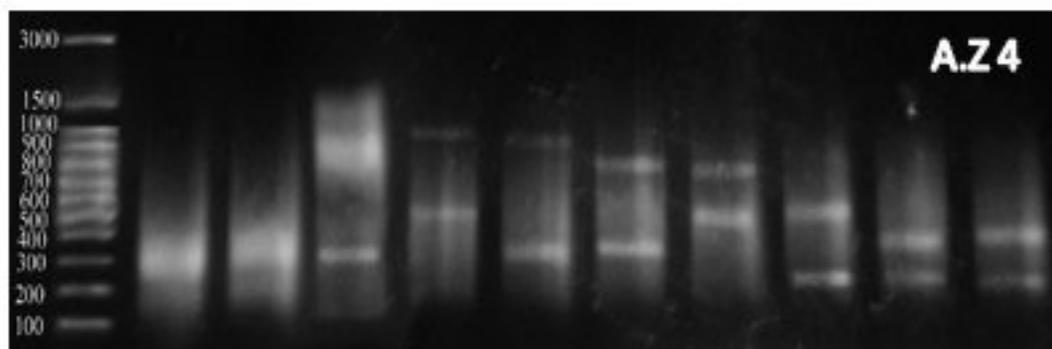
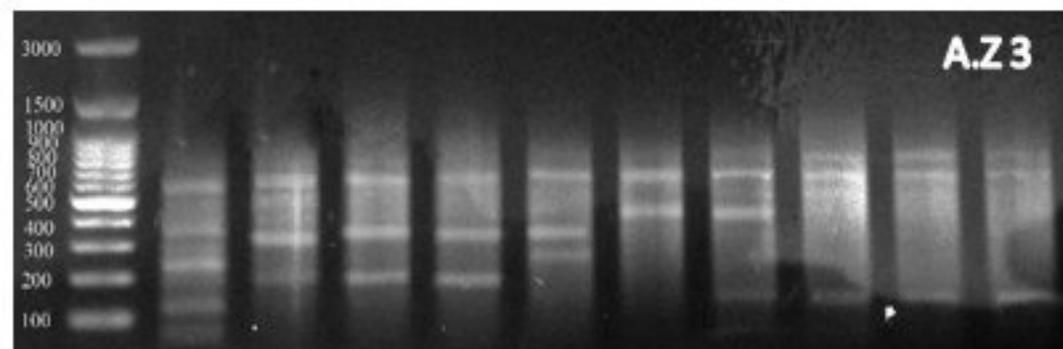
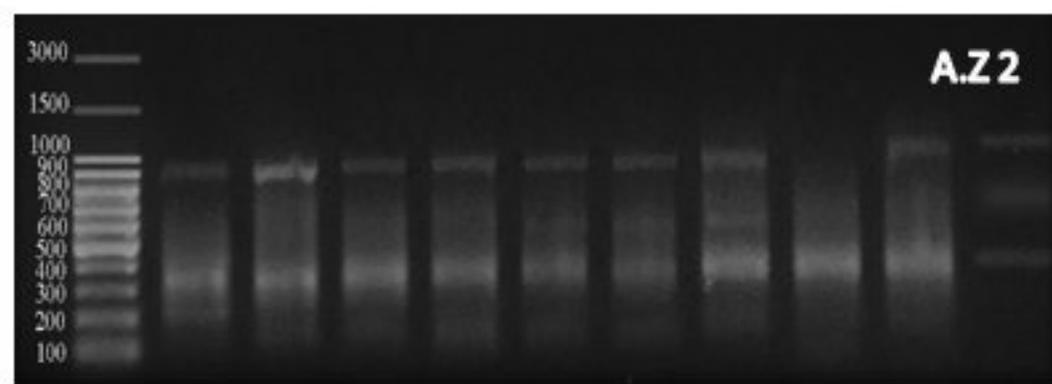
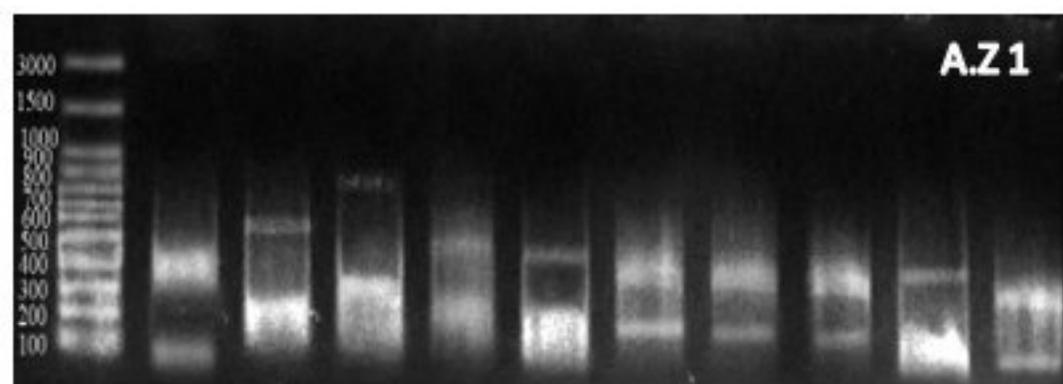
682 **Figure 1. Experimental protocol (Plant sampling and analysis).**

683 **Figure 2. RAPD profiles of genomic DNA from root-tips of wheat seedlings exposed to**  
684 **varying Cd, Ni and Pb concentrations.** Lanes M: DNA ladder; 1: control; 2: Cd 30  
685 ppm; 3: Cd 60 ppm; 4: Cd 120 ppm; 5: Ni 50 ppm; 6: Ni 100 ppm; 7: Ni 150 ppm; 8: Pb  
686 100 ppm; 9: Pb 200 ppm and 10: Pb 300 ppm.



M 1 2 3 4 5 6 7 8 9 10

M 1 2 3 4 5 6 7 8 9 10



Soil samples from Al-Gabal Al-Asfar farm were collected and treated with different concentrations of Cd, Ni and Pb

Wheat grains were sown in

### Biochemical Parameters after 2,3 & 5 months

Activity and Expression level of antioxidants

Proline and protein Contents

DNA Stability (RAPD)

Agronomic characteristics

Cd, Ni or Pb content in Straw, Grains and soil

### Results

Altered antioxidants enzymes.

Pb showed the highest proline, least protein and chlorophyll contents

High Pb and Cd doses revealed highest polymorphism and lowest genome stability

Heavy metals accumulated mainly in wheat straw and induced genotoxic effect.

Pb toxicity showed poorest agronomic characteristics and induce more genotoxic and phytotoxic effects followed by Cd and Ni.