Changes in phytohormone contents in chickpea seeds germinating under lead or zinc stress

Ö. ATICI*[1], G. AĞAR* and P. BATTAL**

*Department of Biology, Science & Art Faculty, Atatürk University, 25240 Erzurum, Turkey**
*Department of Biology, Science & Art Faculty, Yüzüncü Yıl University, 65080 Van, Turkey***

Abstract

The present work describes the changes that take place in phytohormone contents in germinating chickpea (*Cicer arietinum* cv. Aziziye-94) seeds in response to heavy metal stress. For this aim, endogenous abscisic acid (ABA), gibberellic acid (GA_3), zeatin (Z) and zeatin riboside (ZR) contents were followed for 24, 48 and 72 h in chickpea seeds germinating at the concentrations of 0.1, 1.0 and 5.0 mM Pb or 0.1, 1.0 and 10 mM Zn. The results showed that Pb and Zn significantly delayed and impeded the germination of chickpea seeds. The negative effect of Pb on germination was higher than that of Zn. Further, Pb increased ABA and Z contents while decreased GA_3 content in the germinating seeds. The high concentrations of Zn (1.0 and 10 mM) decreased contents of Z, ZR and GA_3 while 0.1 mM Zn increased the content of the same hormones. The ABA content was enhanced by Zn in all concentrations used.

Additional key words: abscisic acid, *Cicer arietinum*, cytokinins, germination, gibberellic acid, heavy metal stress, HPLC.

Introduction

Heavy metals in high concentrations inhibit seed germination, the growth and development of plants, and disturb many biochemical and physiological processes, for instance, injure cell membranes, reduce transpiration, cause breakdown of the protein synthesis, damage the photosynthetic apparatus and inhibit photosynthetic rate, affect the activity of several enzymes, raise lipid peroxidation (Foy *et al.* 1978, Sanita di Toppi and Gabbrielli 1999, Talanova *et al.* 2000, Monni *et al.* 2001, Atıcı *et al.* 2003). Heavy metal concentrations in seeds are a function of heavy metal content in environment. When the soil is heavy metal-contaminated, plants will take up metals via root system (Nwosu *et al.* 1995, Xiong 1997, Berkelaar and Hale 2000). Lead (Pb^{2+}) and zinc (Zn^{2+}) heavy metals are among important environmental pollutants, particularly in areas with high anthropogenic pressure (Wierzbicka 1995, Sresty and Rao 1999, Talanova *et al.* 1999, Herrero *et al.* 2003). Their excess in environment can cause serious problems to all organisms (Xiong 1997, Sresty and Rao 1999) and the heavy metals excessive bioaccumulation in the food chain is highly dangerous (Wierzbicka 1995, Sanita di Toppi and Gabbrielli 1999). Pb belongs among nonessential metals for plants and has no known biological function, and probably is one of the most frequently encountered heavy metals in polluted environment (Wickland 1990, Wierzbicka 1995). There is a number of reports on the inhibitory and toxic effects of lead in the germination of seeds of the following species: *Oryza sativa* (Mukherji and Maitra 1977), *Lupinus luteus* (Wozny *et al.* 1982), *Sinapsis alba* (Fargasova 1994), *Sonchus oleraceus* (Xiong 1997), *Brassica pekinensis* (Xiong 1998), and *Pisum sativum* (Wierzbicka and Obidzinska 1998). On the other hand, although certain metals such as Zn are excessively essential for plants and are used as micronutrients (Moraghan and Grafton 1999), they inhibit plant growth and development when get over a critical level and behave like other toxic heavy metals such as Pb and Cd (Ali *et al.* 1999, 2000). Zn at appropriate concentrations is required for structural and catalytic components of proteins and enzymes as cofactors, essential for normal growth and development of plants,

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Abbreviations: ABA - abscisic acid; GA_3 - gibberellic acid; PVPP - polyvinyl polypyrrolidone; Z - zeatin, ZR - zeatin riboside.
[1] Corresponding author; fax: (+442) 236 09 48, e-mail: oatici@atauni.edu.tr

and a vast number of protein sequences containing Zn-binding structural domains (Steffens 1990, Clarke and Berg 1998). However, excessive accumulations of the micronutrient in plants operate as stress factors causing physiological constraints leading to decreased seed vigor and plant growth (Van Assche and Clijsters 1986, 1990, Ali *et al.* 2000).

Seed germination is a highly complex process as an inert quiescent seed is transformed into a vigorously metabolizing system (Dow and Schwintzer 1999). The germination of all seeds begins with the imbibition of water, and after an interval, enzymatic proteins are synthesized and activated. Germination is regulated by hormonal interactions and environmental factors (Paleg 1960, Iglesias and Babiano 1997) and occurs only when conditions become favourable. Many factors, both exogenous and endogenous, affect this process. Among such intrinsic factors, plant growth regulators are one of the most important. Gibberellic acid plays an important role in the germination of seeds and the embryo is a source of gibberellic acid during germination. Also, the hormone gibberellin affects a number of processes during plant development including seed germination, leaf expansion, stem elongation, flower initiation, and flower and fruit development (Salisbury and Ross 1992, Dewar *et al.* 1998, Hamman *et al.* 2003). Abscisic acid has been related to different types of environmental stress (Colorado *et al.* 1994, Monni *et al.* 2001, Pospíšilová 2003) and also it was increased dramatically when seeds were subjected to stressful conditions (Daie and Cambell 1981, Cohen *et al.* 1991, Curry *et al.* 1991). Cytokinins that control plant growth and development were first identified as a factor that induces cell division in the presence of auxin (Miller *et al.* 1955). Seed tissues were also the source for isolation of the first naturally occurring cytokinins (Miller 1961, Van Staden *et al.* 1982, Letham and Palni 1983, Letham 1994). Zeatin and zeatin riboside were shown to be the most active of these compounds. Their levels have been found to change significantly in plants under a variety of stress conditions including water, salt, temperature, heavy metal and viral infection (Itai *et al.* 1973, Atanassova *et al.* 1996, Pospíšilová *et al.* 2000, Atıcı *et al.* 2003, Pospíšilová 2003).

Although seed germination represents an initial and crucial phase in the life cycle of plants, virtually little information is available on the impact of heavy metals on the metabolism of endogenous plant hormones in germinating seeds. On the other hand, acute effect of heavy metals in high doses and effect of gradually increasing concentrations can be also different on plant growth and development (Sobolev *et al.* 1982, Ali *et al.* 1999, 2000, Talanova *et al.* 2000). Recently, a negative correlation between gibberellic acid and cytokinins was found in chickpea seeds germinating at low Cd concentrations, but not at high concentrations (Atıcı *et al.* 2003). The aim of our study was to elucidate the action of gradually increasing concentrations of Pb and Zn heavy metals, essential and nonessential for plants, on the changes of abscisic acid, gibberellic acid, zeatin and zeatin riboside hormones in germinating chickpea seeds.

Materials and methods

Plants and germination conditions: Chickpea (*Cicer arietinum* L. cv. Aziziye-94*)* seeds were surface-sterilized with 1 % sodium hypochloride for 5 min and thoroughly rinsed with distilled water. Then, the seeds were placed to germinate in Petri dishes on two filter-paper discs. The Petri dishes contained 12 cm^3 of 0.1, 1.0 and 5.0 mM Pb^{2+} from $Pb(NO_3)_2$ or 0.1, 1.0 and 10 mM Zn^{2+} from $ZnCl_2$. The seeds were germinated for 24, 48 and 72 h in an incubator (*Sanyo Co.,* Osaka, Japan) at 25 °C and 50 % air humidity.

Extraction, purification and determination of phytohormones: The analysis of cytokinins was performed according to Cakmak *et al.* (1989), Kuraishi *et al.* (1991) and Zaffari *et al.* (1998) with little modifications. The frozen samples (2 g) were powdered in liquid nitrogen and cold methanol was added. They were stored at 4 °C for 24 h in the dark, homogenized (*Ultrasonic Processor, Jenway Ltd*., Essex, UK) and then filtered through a filter paper (*Whatman No. 1*). The residues were treated in the same way, and the former and latter filtrates were combined. These filtrates were filtered through PTFE filter (0.45 μm) (Cutting 1991, Battal and Tilekliоğlu 2001). After evaporation of the samples at 35 °C, the extracts were re-dissolved in 0.1 M KH_2PO_4 buffer (pH 8) and centrifuged at 10 000 *g* for 1 h at 4 °C. Then, the supernatants were placed in flask (50 cm^3), each containing 1 g polyvinyl polypyrrolidone (PVPP, *Sigma Chemical Co*, Dorset, UK), mixed and filtered through *Whatman No. 1* (Mooney and Van Staden 1984). The filtrates were passed through *Sep-Pak* C_{18} (*Waters Hichrom Ltd.,* Berkshire, UK) cartridges (Machackova *et al.* 1993). Cartridge adsorbing hormones were eluated with 80 % methanol and the extracts were collected in vials. The hormone extracts were injected into HPLC to detect zeatin and zeatine riboside contents.

The analysis of abscisic acid was performed according to Zaffari *et al.* (1998) and Unyayar *et al.* (1996) with some modifications. The freeze-dried tissue sample (1 g) was ground to powder in liquid nitrogen and homogenized in 3 cm^3 of 100 % methanol. The homogenate was stirred at 4 °C for 24 h in darkness.

PVPP (0.5 g) was added to homogenate and thoroughly mixed. The mixture was filtered through *Whatman No. 1* and methanol was removed under reduced pressure at 35 °C. The residue was dissolved with 1.5 cm^3 of 0.5 M potassium buffer (pH 8.3). The combined organic phases were partitioned three times against hexane and three times against ethyl acetate at pH 3. The ethyl acetate of the combined organic fractions was removed under reduced pressure at 35 °C. The residue was dissolved in 100 % methanol and loaded onto *Bondasil DEA* (*Waters Hichrom*) column. After the column was washed with 100 % methanol, absorbed hormone was eluted with methanol containing 0.5 % acetic acid and collected in vial. The hormone extracts were injected into HPLC to detect ABA levels.

The analysis of gibberellic acid equivalents was performed according to Fujioka *et al.* (1986), Cakmak *et al.* (1989) and Wang *et al.* (1992) with some modifications. The freeze-dried tissue samples (1 g) were ground to powder in liquid nitrogen and homogenized in 3 cm^3 of 100 % methanol. The homogenate was stirred in 80 % methanol (4 °C) for overnight and then filtered. The residue was re-extracted with 80 % methanol for 4 h, re-filtered and combined with former supernatant. Methanol was removed from combined filtrates under reduced pressure at 35 °C and the aqueous residue was adjusted to pH 2.5 (2 M HCl). This solution was then partitioned three times with equal volumes of ethyl acetate and the combined organic phases were partitioned with 5 % (m/v) sodium bicarbonate (3 × 1/5 volume) and separated gibberellic acid-equvalents were injected into HPLC.

For HPLC analysis, isocreatic system was used. The extracts in the vials were injected into HPLC system equipped with *Waters Hichrom 6000 A* pumps, ultraviolet detector (*Unicam Analytical Systems,* Cambridge, UK) and *Bondapak* C_{18} column (*Waters Hichrom*) using acetonitrile (12.00 %; pH 4.98) as the mobile phase. The flow rate, pressure and wavelength were 2 cm^3 min^{-1}, 13.8 MPa, and 265 nm, respectively. Under these conditions, the retention times of GA_3, Z, ZR, IAA and ABA were determined to be 2.85, 3.88, 5.14, 7.17 and 22.21 min for standards, respectively.

Statistical analysis: Each experiment was repeated at least three times. Analysis of variance was conducted using one-way *ANOVA* test using *SPSS 9.0* for *Microsoft Windows* and means were compared by Duncan or Dunnett tests at the 0.05 level of confidence.

Results

Planning this study, we aimed to work with Pb and Zn gradually increasing concentrations inhibiting about 25, 50, and 75 % the germination of chickpea seeds (*Cicer arietinum* L. cv. Aziziye-94). In our preliminary experiments we determined that the germination of chickpea seeds at 5th day was inhibited by about 25, 50, and 75 % at 0.1, 1.0 and 5.0 mM Pb and at 0.1, 1.0 and 10 mM Zn solutions, respectively. The given Pb and Zn concentrations were therefore used in this work. The chickpea seeds were also found to complete generally germination at 72 h. After that time, the amount of the germinated seeds was lower (Table 1). Therefore, contents of the plant hormones during germination of the seeds were followed for 24, 48 and 72 h.

Abscisic acid (ABA) content of control seeds gradually decreased throughout the germination time studied (Fig. 1). A minimum level of ABA was detected at 72 h of germination. Chickpea seeds almost completed their germination by that time (90 %) (Table 1). ABA contents in the seeds subjected to the different Pb or Zn concentrations during germination were significantly ($P < 0.05$) higher than those of control seeds (Fig. 1). As compared with control value, the increases in ABA content at 0.1 and 1.0 mM Zn were lower than those at the same concentrations of Pb. ABA content at the first 24 h of germination, for instance, increased by 102 and 126 % at 0.1 and 1.0 mM Pb while by 7 and 81 % at the same concentrations of Zn, as compared with control value. Similarly to the control, ABA content in the seeds exposed the heavy metals also gradually decreased from 24 to 72 h, but these decreases were not as sharp as in control seeds (Fig. 1).

Gibberellic acid (GA_3), content in the control gradually increased throughout germination (Fig. 2). A maximum content of GA_3 was detected at 72 h of germination [8.65 μg g^{-1} (f.m.)]. The GA_3 content in the seeds exposed to Pb decreased with increasing Pb concentrations (Fig. 2*A*). GA_3 content in the seeds germinated for 72 h at 0.1, 1.0 and 5.0 mM Pb, for instance, was lower by 16, 46, and 56 % than that

Table 1. Effect of Pb and Zn on germination of chickpea seeds. Mean values with different letter are statistically different ($P < 0.05$) according to Duncan's Multiple Range Test. Germination criterion is 0.5 mm radicula (nd - non-detected).

Metal	Concentration [mM]	Germination [%] 1 d	2 d	3 d	4 d	5 d
Pb	0.0 (control)	11	52	90	96	96 a
	0.1	4	20	35	48	48 b
	1.0	1	18	22	28	35 c
	5.0	nd	6	15	19	26 d
Zn	0.1	8	45	70	74	74 b
	1.0	nd	36	52	52	58 c
	10.0	nd	22	28	28	34 d

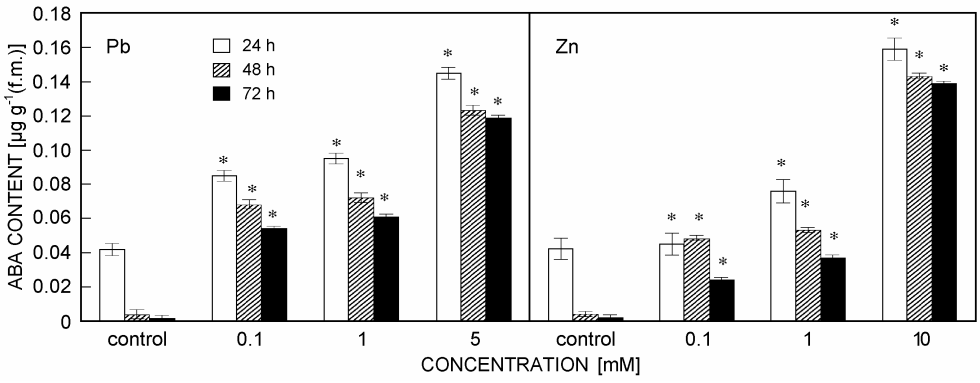

Fig. 1. The content of endogenous ABA in chickpea seeds germinating for 24, 48 and 72 h at various Pb or Zn concentrations. Values are means ± SE. An *asterisk* indicates that this value is statistically different from control ($P < 0.05$).

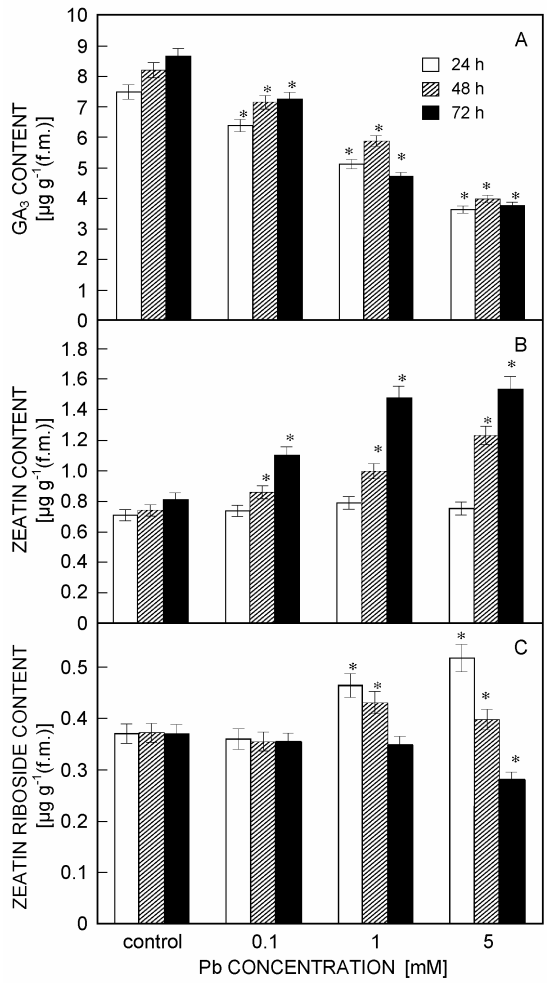

Fig. 2. The contents of endogenous GA_3 (*A*), Z (*B*) and ZR (*C*) in chickpea seeds germinating for 24, 48 and 72 h at various Pb concentrations. Values are means ± SE. An *asterisk* indicates that this value is statistically different from control ($P < 0.05$).

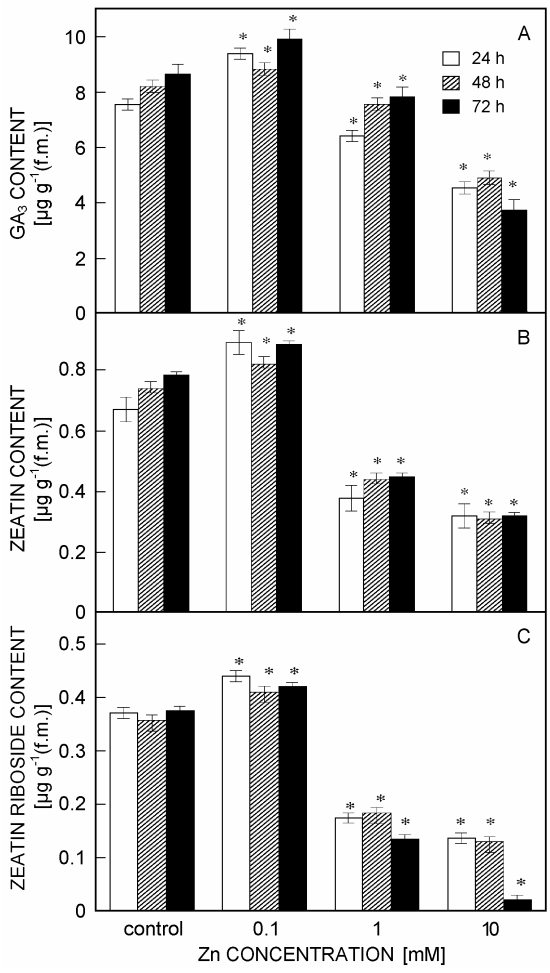

Fig. 3. The contents of endogenous GA_3 (*A*), Z (*B*) and ZR (*C*) in chickpea seeds germinating for 24, 48 and 72 h at various Zn concentrations. Values are means ± SE. An *asterisk* indicates that this value is statistically different from control ($P < 0.05$).

of control, respectively. On the other hand, Z content in the control seeds slightly increased until 72 h of germination (Figs. 2*B*, 3*B*). In the seeds exposed to Pb, Z content was higher at 48 and 72 h of germination than that of control, but was not significantly increased at 24 h (Fig. 2*B*). Z content in the seeds germinated for 72 h at

0.1, 1.0 and 5.0 mM Pb was higher by 38, 85, and 93 % than that of control, respectively. The contents of ZR in control and 0.1 mM Pb did not significantly ($P > 0.05$) change during germination (Fig. 2*B*). However, ZR contents in the seeds germinated at 1.0 and 5.0 mM Pb was higher at 24 and 48 h of germination while lower at 72 h, as compared with control values (Fig. 2*C*).

Only the treatment with 0.1 mM Zn increased GA_3, Z and ZR contents, but 1.0 and 10 mM Zn significantly ($P < 0.05$) decreased the content of the same hormones compared to their controls (Fig. 3*A,B,C*). The decreases of the hormone contents were higher at 10 mM Zn than at 1.0 mM Zn. At 72 h of germination 0.1 mM Zn increased the GA_3, Z and ZR contents by 15, 14, and 14 %, respectively, while 1 mM Zn decreased them by 10, 43, and 64 % and 10 mM Zn decreased them by 56, 59, and 94 % as compared with their controls.

Discussion

Plants and their seeds can accumulate elements, especially heavy metals, in their tissues due do their great ability to adapt to variable chemical effects of environment. Thus, plants are intermediate reservoirs for elements originating from the lithosphere, hydrosphere or the atmosphere. In order to give an overall picture about the effect of different heavy metals on the plants, it is necessary to have a wide knowledge of various factors concerning the type of the plant and the concentration, the chemical form, the availability, the essentiality and the toxicity of chemical elements. According published results regarding these effects, however, each element has different influence on plants (Kabata-Pendias and Pendias 1984, Varga *et al.* 1999, Shu *et al.* 2002). One of the most fruitful ways to obtain valuable information about the effect of heavy metals is the quantitative determination of endogenous chemical compounds including hormones in plants growing under controlled environmental conditions in presence and absence of heavy metals (Varga *et al.* 1999). For this aim, we investigated the changes in ABA, GA_3, Z and ZR hormones of chickpea seeds germinating under Pb or Zn heavy metals exposure. The role of endogenous plant growth regulators in heavy metal stress is not clearly identified, especially during germination.

In our study, the concentrations of Pb (0.1, 1.0 and 5.0 mM) and Zn (0.1, 1.0 and 10 mM) decreased germination of chickpea seeds (Table 1). Some researchers also observed that the heavy metals decreased germination of seeds (Wierzbicka 1995, Wierzbicka and Obidzinska 1998, Ali *et al.* 2000, Herrero *et al.* 2003). These decreases at germination and growth can be due to the interference of heavy metals with metabolic processes associated with normal development (Van Assche and Clijsters 1990, Wierzbicka and Obidzinska 1998, Gadallah and El-Enany 1999). In our experiments, Pb at 5 mM and Zn at 10 mM inhibited germination by 75 %. Atıcı *et al.* (2003) showed that 5 mM Cd (toxic and nonessential for plants) also inhibited by about 75 % germination of chickpea seeds. According to these data, it can be concluded that nonessential metals can be more effective than essentials in inhibition of the seed germination.

Effect of Pb and Zn on ABA content: Heavy metal-induced inhibitory effects are reported to be concomitant with an increase in endogenous ABA levels in plant tissues indicating the possibility of this phytohormone mediating a part of the metal-imposed phytotoxicity (Sharma and Kumar 2002). Also, ABA level was higher in plants growing under salinity stress and in the pollution source containing heavy metals (Degenhardt *et al.* 2000, Monni *et al.* 2001, Sharma and Kumar 2002, Hsu and Kao 2003). After treatment with Cd, ABA content rapidly increased in the leaves and roots of rice (Hsu and Kao 2003). In addition, an increase in ABA content was observed during water stress (Ober and Setter 1992, Lin *et al.* 1999, Pospíšilová 2003). Our results also clearly showed that ABA contents in the chickpea seeds germinated at all of the Pb or Zn concentrations were increased, but they were decreased in control seeds during germination (Fig. 1). It can be concluded that both Pb and Zn caused the increase of ABA content in the germinating chickpea seeds. Further, ABA content at the each of Pb and Zn concentrations was gradually decreased until 72 h of germination. This appears that ABA content in the presence of the heavy metals is high at the beginning of germination, but is gradually decreased in the course of germination in control seeds as well as in seeds under heavy metal stress. In addition, as compared with control value, Pb was more effective than Zn in the increase of ABA content. This result can show that nonessential metals for plant have more important role than essential ones to form heavy metal stress.

Effect of Pb and Zn on GA_3 content: GA_3 content in the control seeds was gradually increased during germination (Figs. 2*A*, 3*A*). Previous works also showed that GA_3 level increased at a seed germination (Nambara *et al.* 1991, Debeaujon and Koornneef 2000, Atıcı *et al.* 2003). However, all Pb and 1.0 to 10 mM Zn concentrations decreased GA_3 content in the germinating seed (Figs. 2*A*, 3*A*). These results agree with data of Atıcı *et al.* (2003) who determined that Cd had negative effect on GA_3 content in germinating chickpea seeds. In addition, we interestingly found that Zn at a low concentration

(0.1 mM) increased GA_3 content (Fig. 3*A*). Zn is essential element for plant growth and development including seed germination (Moraghan and Grafton 1999, Clemens 2001). Also, this ion at appropriate concentrations is required for structural and catalytic components of proteins and enzymes, and as cofactors essential for normal growth and development of plants (Steffens 1990, Van Assche and Clijsters 1990, Clemens 2001). But, excessive accumulations of the micronutrient in plants operate as stress factors causing physiological constraints leading to decreased seed vigour and plant growth including seed germination (Van Assche and Clijsters 1990, Ali *et al.* 2000, Clemens 2001). Therefore, it can be suggested that the low concentrations (0.1 mM) of Zn could cause the increase of GA_3 content by directly or indirectly inducing GA_3 biosynthesis in the seed studied, but its higher concentrations in germination environments act as a stress factor.

Effect of Pb and Zn on Z and ZR contents: Cytokinins (CKs) occur as a bound form in the tRNA of most organisms including plants, but plants also possess significant amounts of free cytokinins (Letham 1994). Letham (1994) summarized evidence that supported roots as a site of CK biosynthesis. However, roots are very unlikely the only source of CK, and developing seeds, cambial tissues, and the shoot apex have also been implicated (Letham 1994). The content of cytokinin-type hormones is a good indicator of the resistance of plants to some environmental stresses (Székács *et al.* 2000). For instance, transgenic tobacco and tomato lines (Székács *et al.* 2000), wheat seedlings (Farkhutdinov *et al.* 1997) and pea (Atanassova *et al.* 1996) showing tolerance to several stress factors had high content of CKs. Recently, it was presented that CKs may also play an important role in processes related at heavy metal stress (Atıcı *et al.* 2003). According to results presented here, in the seeds exposed to Pb, gradually increasing concentrations of Pb have opposite effect on Z and ZR contents during germination. Z content was low in the beginning of germination and then was gradually increased, but ZR content was high in the beginning of germination and then gradually decreased (Fig. 2*B*,*C*). From this result it can be concluded that Z is involved by synthesizing *de novo* in response of the seed germination under Pb stress or that Z may be more responsible than ZR for the seed germination under Pb stress. On the other hand, both Z and ZR contents were also increased by 0.1 mM Zn while were decreased by its higher concentration (Fig. 3*B*,*C*). This can be elucidated by the fact that Zn is an essential metal for plants. Further, both hormones behaved in a different manner under Pb and Zn exposure; they interestingly increased at Pb treatments while dramatically decreased in the seeds exposed to Zn treatments (1 mM and 10 mM). It was reported that Zn could become toxic at higher concentrations than Cd (Van Assche and Clijsters 1986, Ali *et al.* 1999) and its uptake and translocation in plants could be greater than those of Pb and Cd (Chakravarty and Srivastava 1997). Thus, Zn at high concentrations (1.0 and 10 mM) could probably cause the more substantial decreases of Z and ZR hormone contents, but this status was not seen in ABA and GA_3 contents. This result may show that an essential heavy metal, uptake and translocation of which is easy, can have stronger effects on cytokinin metabolism than a nonessential heavy metal at high concentrations.

In conclusion, Pb generally increased ABA, Z and ZR contents while decreased germination rate and GA_3 content in germinating chickpea seeds. High concentrations of Zn decreased contents of Z, ZR and GA_3 while only its 0.1 mM concentration increased the contents of the same hormones. It is concluded that the hormonal response of a germinating seed to different heavy metals (essential or non-essential for plant) can be different. This is the first study describing interactions between the endogenous plant hormones and the heavy metals toxicity during germination of chickpea seeds. Since data concerning this phenomenon in the literature are relatively scarce, the causal relationships require further investigations.

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