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Cadmium-Induced Changes in Germination, Seedlings Growth, and DNA Fingerprinting of *in vitro* Grown *Cichorium pumilum* Jacq.

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Abstract

The aim of this study was to assess the effect of Cd^{2+} on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *Cichorium pumilum.* Results showed that seed germination was highly inhibited by cadmium (down to 47% at 1600 µM $CdCl_2$). In addition, root and shoot growth showed significant decreases in response to $CdCl_2$ level. Analysis of proline content and lipid peroxidation showed that with increasing $CdCl_2$ levels in the growing medium, the amount of proline accumulation and lipid peroxidation increased gradually. Total chlorophyll content was found to increase only at higher tested levels of Cd^{2+} (800 and 1600 µM). The results also show that Cd^{2+} inhibits callus growth at different levels starting from 50 µM $CdCl_2$ compared with the control in the callus growth experiment. Callus growth ceased completely at 200 µM $CdCl_2$ and above. Random amplified polymorphic DNA (RAPD) analysis showed DNA alterations in Cd^{2+}-treated *C. pumilum* microshoots compared with the control. The results of this experiment showed that Cd^{2+} stress affects several physiological, biochemical, and molecular processes in *C. pumilum.*

Keywords: Cadmium, proline, lipid peroxidation, fingerprinting, *Cichorium pumilum*

1. Introduction

Chicory (*Cichorium pumilum* Jacq., Asteraceae), is a bushy perennial herb with blue or lavender flowers which grows as a wild plant on roadsides. Chicory is also known as blue sailors, endive, radicchio, French endive, red endive, sugarloaf, witloof, elit, and coffeeweed. It is a culinary and medicinal plant grown worldwide. In the Middle East, its leaves are widely used in salads after being blanched, as the unblanched leaves taste bitter. In Europe, the root is eaten like a vegetable after being boiled, or it can be roasted then ground for use as a coffee substitute (Robert et al., 2008). Al Khateeb et al. (2012) showed that *C. pumilum* methanolic extracts have high levels of phenolic compounds and showed very strong antioxidant properties. Moreover, they found that methanol and ethanol extracts obtained from *C. pumilum* have antimicrobial effects on 10 different bacterial species.

In the last few decades, a dramatic increase in the contamination of the environment, (including soil, air, and water) has been observed. It appears that anthropogenic activities are the main source of the pollution that is causing the environment contamination (Gratao et al., 2005). Recently, it has been shown that large areas of land have been contaminated with heavy metals as a result of urban activities, agricultural practices, and industry.

Heavy metals are defined as the group of elements that have specific weights higher than about 5 $g \times cm^{-3}$. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients which are required for normal growth and for many metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg, etc.) are potentially highly toxic for plants (Sebastiani et al., 2004). Contamination of soil by heavy metals is a global ecological problem because heavy metals are included in the main category of environmental pollutants which can remain in the environment for long periods. Their accumulation is potentially hazardous to humans, animals, and plants (Benavides et al., 2005).

Agricultural soil contamination can severely affect humans, both directly (through the food web) and indirectly (by damaging environmental health) (Nriagu, 1990). For plants, heavy metals are phytotoxic, causing growth inhibition and eventually plant death through mechanisms that are still not completely understood (Romero-Puertas et al., 1999). The toxic effect of increasing cadmium (Cd) concentration in the environment has

become a major environmental concern (Shriarastava & Singh, 1989). Cadmium accumulation in soils may come from various sources: from air pollutants or through applications of commercial fertilizers, sewage sludge, manure, and lime (Kidd et al., 2007). Also, industrial effluents may contain a wide variety of pollutants depending on the industries involved (Iribar et al., 2000). Cd is generally present in soil as free ions or in different soluble forms, and its mobility is affected by pH and the presence of chelating substances and other cations (Hardiman & Jacoby, 1984). In plants, Cd is accumulated mainly in the edible parts, thus making crop yield a potential hazard for human and animal health. It has been suggested that Cadmium may cause damage even at very low concentrations, and healthy plants may contain Cd levels that are toxic for mammals (Chen et al., 2007). Pinot et al. (2000) showed that the main source of Cd accumulation in food is the Cd uptake by plants.

It has been shown that Cadmium can inhibit plant growth and photosynthesis, reduce chlorophyll content, and induce oxidative stress (Schill et al., 2003). In addition, the genotoxicity of Cd is directly related to its effect on the structure and function of DNA. Therefore, Cd toxicity not increases the mortality rate in the exposed organisms, but may also result in the modification of the population dynamics and the species biological diversity (Theodorakis et al., 2006). Furthermore, it has been shown that cadmium generates oxidative stress through the formation of reactive oxygen species (ROS). The formation of ROS by cadmium suggests that DNA can also be taken into account as a potential target of this metal (Błasiak, 2001).

It has been suggested that proline plays a role in protecting plants from heavy metal toxicity. Siripornadulsil et al. (2002) reported that proline reduces cadmium stress not by sequestering cadmium but by reducing cadmium-induced free radical damage, thus maintaining a more reducing environment in the cell. Xu et al. (2009) found that proline pre-treatment of *Solanum nigrum* reduces the reactive oxygen species levels and protects the plasma membrane integrity of callus under cadmium stress.

The objectives of this study were to study the effects of Cd on germination, seedling and callus growth, biochemical properties and DNA fingerprint of *in vitro* grown *C. pumilum*.

2. Materials and Methods

2.1 Plant Material, Seed Germination and Proliferation

Ripe fruits of *Cichorium pumilum* Jacq were collected from Irbid/Jordan during the summer of 2011. Seeds were surface sterilized with 2% sodium hypochlorite solution for 10 min, then washed with 70% ethanol for 30 s, followed by three rinses in sterile distilled water. Seeds were inoculated into Petri dishes containing germination medium of half strength Murashige and Skoog (MS) salts, 2% sucrose, and 0.8% Difco-Bacto agar in addition to different levels of $CdC1_2$. Plates were incubated in the dark at 24 ± 2 °C for 7 days, and then germination percentages, hypocotyl and root lengths were recorded.

Shoot tips were excised from in vitro grown seedlings and cultured on MS medium supplemented with 1 µM *Benzyl adenine* (BA) and 0.5 µM naphthaleneacetic acid (NAA) for shoot proliferation (Al Khateeb et al., 2012). The new microshoots were subcultured after 6 weeks on fresh medium containing different levels of $CdC1_2$ (50, 100, 200, 400, 800, and 1600 µM). Cultures were placed in a growth chamber (24 ± 2 °C and 16 h light in cool white fluorescent light) for further growth. After 6 weeks, the fresh weights of the shoots were recorded.

2.2 Callus Growth

Al Khateeb et al. (2012) protocol was used for callus induction. Three-week-old callus was divided into parts of 0.5 g. Then, these parts were subcultured onto the same medium supplemented with different $CdC1_2$ levels. Cultures were maintained at 24 ± 2 °C and 16 h light in cool white fluorescent light. Fresh weights were taken every week for a period of 6 weeks.

2.3 Chlorophyll Analysis

The effect of different concentrations of Cd^{2+} on chlorophyll content was tested. Microshoots grown on MS medium supplemented with different levels of $CdC1_2$ were extracted with 80% acetone overnight, the A_{645} and A_{663} were determined using spectrophotometer and chlorophyll content was calculated according to the method of Mackinney (1941).

2.4 Proline Analysis

500 mg of plant tissues from microshoots grown on MS medium supplemented with different levels of $CdC1_2$ were homogenized in 10 mL of aqueous solution of sulfosalicylic acid. The solution was then filtered rapidly through a Buchner funnel using Whatman filter paper Nº 2. 2 mL of the filtrate was transferred to a test tube in addition to 2 mL of ninhydric acid and 2 mL of glacial acetic acid, followed by one hour incubation at 100 °C. The reaction was then stopped in an ice bath. Afterwards, 4 mL of toluene was added and the contents of the tube

were inverted for 20 seconds. After this, the toluene phase was separated by centrifugation at 13,000 g for 10 minutes. Finally, the absorbance was measured at 520 nm with a visible light spectrophotometer. The concentration of proline was determined from the calibration curve.

2.5 Lipid Peroxidation

Lipid peroxidation was estimated based on measuring the malondialdehyde (MDA) content. The MDA content in microshoots grown on MS medium supplemented with different levels of $CdC1_2$ was analyzed following Heath and Packer (1968). This assay is based on the reaction with thiobarbituric acid. Fresh microshoots (0.5 g) were ground in 20 mL of 0.1% tri-chloroacetic acid (w/v) then centrifuged for 15 min at 13,000 *g*. One mL of the supernatant was reacted with 5 mL of 20% TCA solution containing 0.5% thiobarbituric acid (w/v). After that, the mixture was heated for 45 min. at 95 °C and then cooled immediately in an ice bath. Next, the mixture was centrifuged for 5 min at 13,000 *g*, and finally the absorbance of the supernatant was measured using spectrophotometer at 532 and 600 nm. MDA content was calculated using the extinction coefficient of 155/(mM/cm) (Soltani et al., 2006).

2.6 DNA Extraction and RAPD Analysis

DNA was extracted from *C. pumilum* microshoots using modified CTAB (cetyltrimethylammonium bromide) method (Porebski et al., 1997). DNA concentration was measured spectrophotometrically at 260 nm. The RAPD reaction was performed in a total volume of 50 µL containing 5 µL template DNA, 10 × PCR buffer, 5 mM $MgCl_2$, 250 µM deoxynucleoside triphosphates, 1.5 U of Taq DNA polymerase and 1 µM of each primer. Primers were obtained from commercially available kits (OPA, OPC, and OPG) (Operon Technologies, CA and USA) (Table 1). DNA amplification program in the thermal cycler was as follows; 40 cycles of 94 °C for 1 min, 52 °C for 45 sec and 72 °C for 30 s. A final extension step was also used at 72 °C for 5 min. PCR products were loaded on agarose gel (1.5% agarose) and run with Tris-borate-EDTA (TBE) buffer for 90 min.

Table 1. Sequence information of RAPD primers used for *C. pumilum* fingerprinting

RAPD Primers	Sequence Information
OPC	GTCCCGACGA
OPG03	GAGCCCTCCA
OPG05	CTGAGACGGA
PM5	CGACGCCCTG
PM6	GCGTCGAGGG
OPAB 14	AAGTGCGACC
OPO 08	GCTCCAGTGT
OPAH 15	CTACAGCGAG
OPAO 01	AAGACGACGG
OPAP 20	CCCGGATACA
CRC	GCGAACCTCG
CRA22	CCGCAGCCAA

2.7 Statistical Analysis

Statistical significance was confirmed by analysis of variance (ANOVA) using SPSS for Windows (version 16.0). Results were expressed as mean ± standard error. Means were separated by using Tukey t-test at 0.05 level of probability. All experiments were repeated at least three times.

3. Results

Analysis of variance (ANOVA) showed that cadmium affects germination, hypocotyl and root length, fresh weights of shoots, chlorophyll and proline content, lipid peroxidation level, and callus growth of *Cichorium pumilum* significantly at 0.05 level of probability.

3.1 Effect of Cadmium on Germination Percentage

In general, the germination percentage of *Cichorium pumilum* decreased as Cd^{2+} level increased (Figure 1. A). The highest germination percentage (100%) was found in the control. A sharp reduction in germination percentage was observed when the seeds were treated with 50 µM $CdCl_2$ (80%). No significant differences were observed between 100, 200 and 400 µM $CdCl_2$ levels. The lowest germination percentage was observed when *C. pumilum* seeds were treated with the highest $CdCl_2$ concentration (1600 µM) which resulted in only 50% germination.

3.2 Effect of Cadmium on Hypocotyl and Root Length

Results show that the hypocotyl length of *C. pumilum* decreased with increasing $CdCl_2$ concentration (Figure 1. B). No significant difference was observed for hypocotyls length between control and the lowest levels of $CdCl_2$. No significant differences in hypocotyls length was observed between 100, 200, 400 and 800 µM $CdCl_2$ levels. Treating *C. pumilum* seedlings with 1600 µM $CdCl_2$ resulted in the highest reduction of the hypocotyls length.

A clear trend for the effect of $CdCl_2$ on root length was observed: root length of *C. pumilum* decreased when increasing $CdCl_2$ concentration (Figure 1. C&D). Root length was moderately affected by 50 µM $CdCl_2$, while higher levels (100 and 200 µM) decreased root length significantly to 50% of the control. Furthermore, seedlings grown on 1600 µM $CdCl_2$ had the shortest roots.

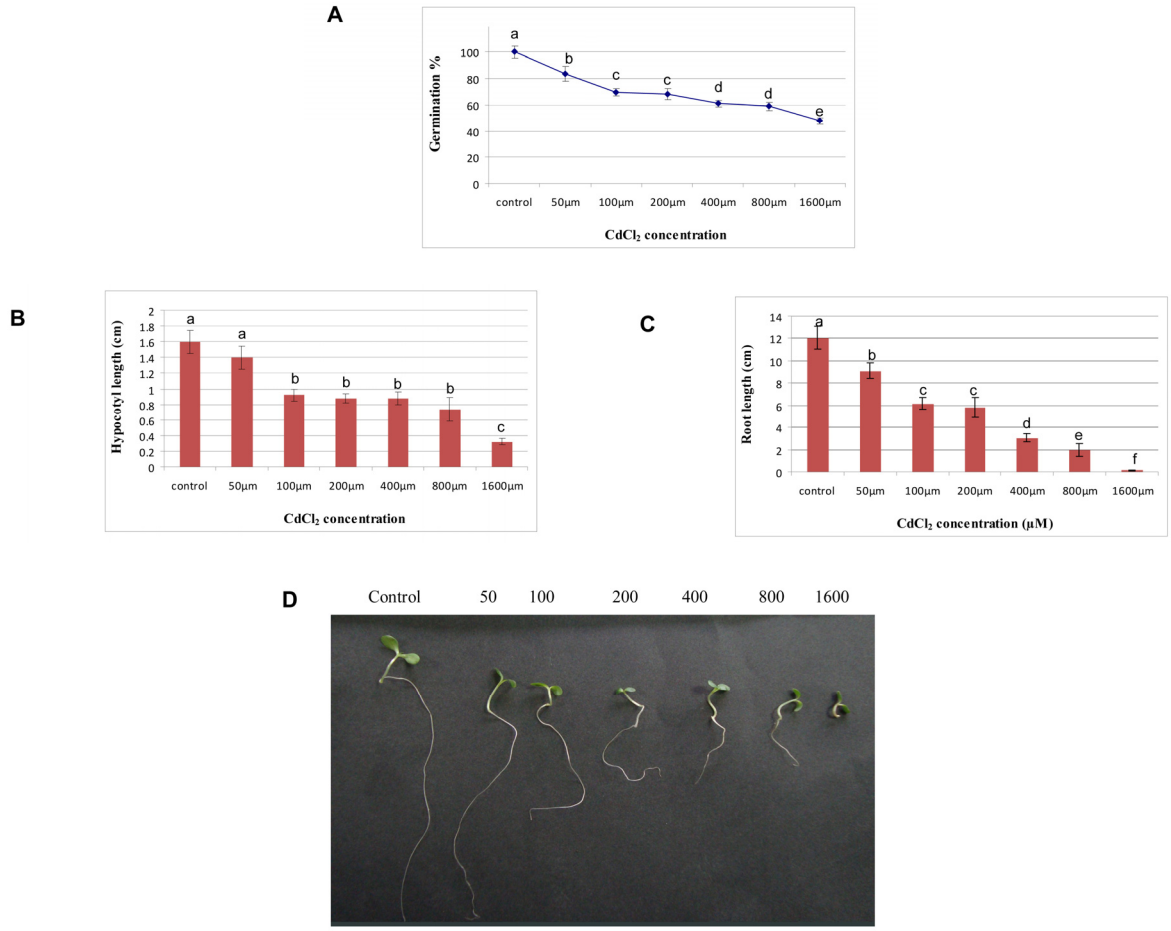

Figure 1. Germination percentage, hypocotyls length and root length of *C. pumilum* grown under different levels of $CdCl_2$. Data represents mean values ± standard error of ten replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.3 Effect of Cadmium on Shoot Fresh Weight

Shoot fresh weight of *C. pumilum* was measured after 6 weeks of *in vitro* growth under different levels of $CdCl_2$.

Shoot fresh weight was affected adversely with increasing $CdCl_2$ concentration (Figure 2. A). Microshoots grown on MS medium supplemented with 50 µM $CdCl_2$ resulted in a significant reduction in shoot fresh weight with 7.6 g compared to 8.9 g in the control. Higher levels of $CdCl_2$ reduced shoot fresh weight severely. No significant difference for shoot fresh weight was observed between 100 and 200 µM $CdCl_2$. Sharp significant decreases in shoot fresh weights were observed for *C. pumilum* exposed to 800 and 1600 µM $CdCl_2$ which resulted in more than tenfold reduction compared to the control.

3.4 Effect of Cadmium on Chlorophyll Content

Chlorophyll content for *C. pumilum* microshoots at 50 and 100 µM $CdCl_2$ was found to be similar to control microshoots (Figure 2. B). In contrast, microshoots grown in MS medium supplemented with 200, 400, 800 and 1600 µM $CdCl_2$ showed higher levels of chlorophyll content than those grown in the control medium and lower levels of $CdCl_2$. No significant differences in chlorophyll content were observed between shoots grown on medium supplemented with 400, 800 and 1600 µM $CdCl_2$.

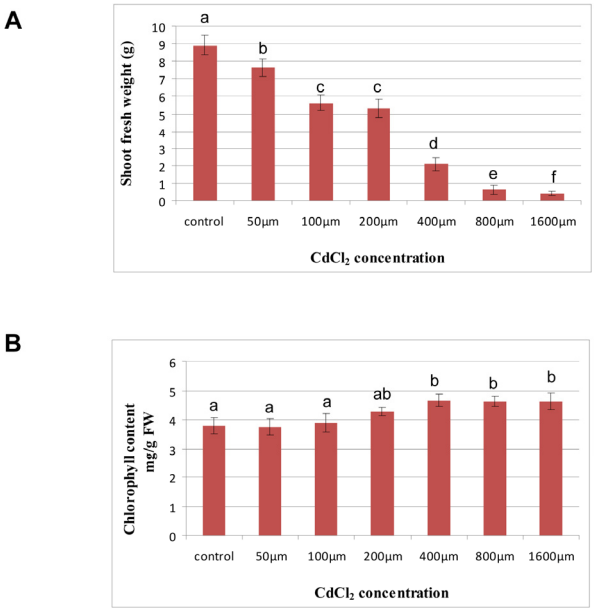

Figure 2. Shoot fresh weight and chlorophyll content of *in vitro* grown *C. pumilum* under different levels of $CdCl_2$. Data represents mean values ± standard error of eight replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.5 Effect of Cadmium on Callus Growth

Different callus growth rates were observed based on fresh weight gain between the different levels of $CdCl_2$ (Figure 3). Calli grown on control medium showed the highest growth rate during the six weeks reaching a final fresh weight of 11.3 g. On the other hand, calli grown on MS medium supplemented with 50 µM $CdCl_2$ showed growth inhibition compared with the control and resulted in a final fresh weight of only 3.2 g. Calli grown on medium supplemented with 100 µM $CdCl_2$ showed more inhibition than that grown on 50 µM $CdCl_2$. Higher levels of $CdCl_2$ appear to be lethal for callus growth.

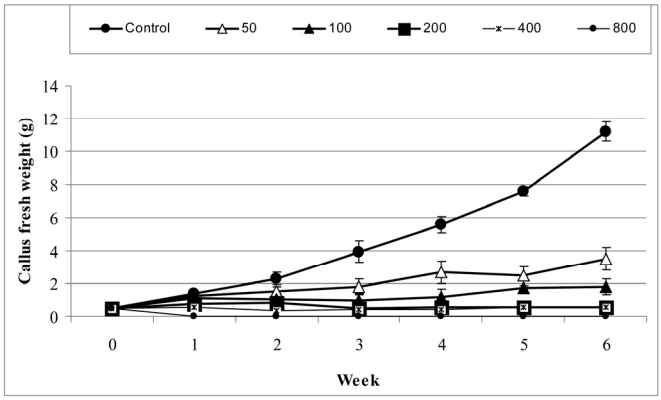

Figure 3. Callus growth curve of of *C. pumilum* grown under different levels of $CdCl_2$. Data represents mean values ± standard error of ten replicates and the whole experiment was repeated three times

3.6 Effect of Cadmium on Proline and Lipid Peroxidation Level

Proline content for *C. pumilum* microshoots was examined under different levels of $CdCl_2$. Results showed that proline content increased gradually and significantly as $CdCl_2$ level increased in the growth medium (Figure 4. A). The highest proline content was obtained from microshoots grown on the MS medium supplemented with the highest level of $CdCl_2$ (400 µM). Results showed that growing microshoots on 400 µM $CdCl_2$ increased proline content by more than tenfold.

The influence of Cd^{2+} on the lipid peroxidation rate of *C. pumilum* shoots was estimated by measuring MDA content, which is the product of lipid peroxidation. Lipid peroxidation rate in *C. pumilum* microshoots increased with increasing $CdCl_2$ level (Figure 4. B). Growing microshoots on MS medium supplemented with 50 µM $CdCl_2$ enhanced lipid peroxidation rate by more than twofold (compared with control). The highest lipid peroxidation rate was achieved in microshoots grown in MS medium supplemented with 400 µM $CdCl_2$.

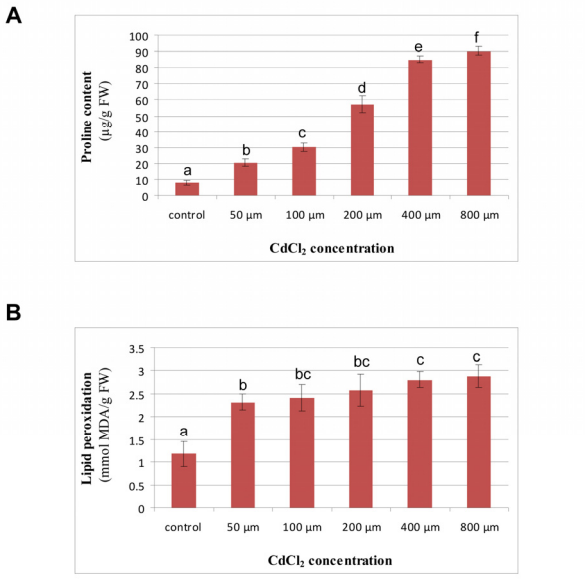

Figure 4. Proline content and lipid peroxidation rate of *C. pumilum* microshoots grown under different levels of $CdCl_2$ Data represents mean values ± standard error of eight replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.7 DNA Fingerprinting Using RAPD Analysis

Genomic DNA was extracted from plants grown for 4 weeks on MS medium supplemented with different levels

of Cd^{2+}. Twelve primers were used in this experiment (Table 1). Amplified profiles resulting from these primers showed variation between untreated and treated plants in terms of number and size of DNA bands. Figureure 5 shows RAPD profiles of treated and untreated samples of *C. pumilum* microshoots obtained from primer OPAP 20 as a representative for the other primers. The RAPD profiles obtained showed bands between 300 and 1800 bp in length. A total of 184 bands scored, only 36 were found to be polymorphic. Figure 5 shows the appearance or absence of bands at 200 and 400 μM $CdCl_2$.

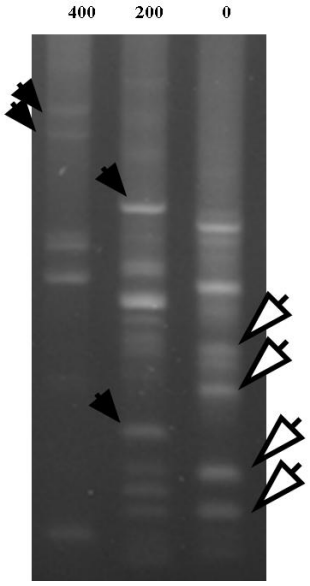

Figure 5. RAPD profiles (using primer OPAP 20) of genomic DNA extracted from *C. pumilum* microshoots grown under 0, 200 and 400 μM $CdCl_2$. Black arrows represent appearance of new bands and white arrows represent absence of bands relative to control (0)

4. Discussion

Plant growth and development under stress conditions are generally negatively affected. One of these stress conditions that affect plants is heavy metals. Recently, heavy metals have become a hot topic of research for many researchers around the world, mostly due to their detrimental effects on many organisms including plants.

Much research has been conducted on the effect of Cd^{2+} on crops and other agricultural plants. However, little information is available on the toxicity of Cd^{2+} on medicinal plants. Thus, the aim of this study was to assess the effect of cadmium on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *C. pumilum*. Here, *in vitro* culture was used which is a convenient system for the study of mechanism of metal toxicity, as it eliminates the interfering processes of translocation and organ-specific trapping of metal ions.

The results of this study showed that Cd^{2+} levels affected all of the studied parameters in *C. pumilum* by different magnitudes. A significant reduction in percentages of seed germination, hypocotyl and root lengths of *C. pumilum* was observed here. This is in agreement with many published reports that studied the effects of Cd^{2+} on other plant species. Mathur et al. (1987) found that higher levels of cadmium inhibited germination percentage and the growth of the early seedlings of *Allium cepa.* Similarly, He et al. (2008) found that cadmium stress significantly inhibits germination index and shoot and root growth of rice. Pasquale et al. (1995) studied the influence of cadmium on the growth and biological activity of the medicinal plant *Coriandrum sativum* L. They found that growing plants under cadmium stress significantly reduced shoot and root lengths, resulting in leaf yellowing and in a major alteration in the essential oil quality and quantity. It has been shown that cadmium stress causes many problems in plants including growth and photosynthesis inhibition, alteration in nutrients and formation of free radicals (Sahu et al., 2007). Seed germination reduction due to heavy metals stress could be attributed to higher levels of seeds stored nutrients breakdown and/or change in permeability characteristics of the cell membrane (Shafiq et al., 2008). In peanuts, it has been shown that root and shoot growth and the initiation of lateral roots decreased with the increase in cadmium levels (Renjini & Janardhanan, 1989). The

reason for reduced seedling length in metal treatments could be due to the reduction in meristematic cells present in this region and of some enzymes contained in the cotyledon and endosperms.

It has been shown that another species of Chicory (*Cichorium intybus* L.) showed a potential to be used as heavy metal bioindicator, *C. intybus* plants grown in nutrient solution supplemented with 0.5-50 μM cadmium showed high levels of Cd, in their shoots and roots (Simon et al., 1996). Another study (Kostantinos et al., 2008) showed that the fresh and dry weights of *Cichorium endivia* L. were not affected when grown on soil supplemented with different levels of Cd. Furthermore, they found that no toxicity symptoms were observed on *Cichorium endivia* plants.

Moreover, the result of this study showed that Cd^{2+} treatment significantly increased proline accumulation in *C. pumilum*. Proline accumulation is used as an indicator of stress conditions, including heavy metals. It has been shown that proline acts as a Cd^{2+} chelator in plants and forms a non-toxic complex with Cd^{2+} (Sharma et al., 1998). Similarly, Dinakar et al. (2009) found that proline content increased under cadmium stress in *Arachis hypogaea* L. It has been shown that plants subjected to $CdSO_4$ stress in the presence of proline showed a lower amount of reactive oxygen species compared to plants without proline. (Xu et al., 2009).

In addition to proline accumulation, the amount of lipid peroxidation also increased in response to Cd^{2+} stress. This is in agreement with Shah et al. (2001) who found an increase in malondialdehyde (MDA) levels (enhancement of lipid peroxidation) in rice seedlings after $Cd(NO_3)_2$ exposure. Similarly, Soltani et al. (2006) found that cadmium stress increased lipid peroxidation levels in *Brassica napus* plants. Lipid peroxidation is the main sign of free radical elevation. Plants may have two classes of antioxidative systems against the perceived oxidative stress: enzymatic antioxidants (such as superoxide dismutase (SOD)) and non-enzymatic low molecular weight antioxidants (such as proline, ascorbic acid, and glutathione) that can directly detoxify free oxygen radicals.

Different classical genotoxic assays have been used to examine the effect of heavy metals on plants including the comet assay and the micronucleus assay (Cambier et al., 2010). Recently, DNA fingerprinting has been successfully applied to test the effect of such stresses at the molecular level in different species (Korpe & Aras, 2011; Liu et al., 2012). The ability of cadmium to induce DNA mutations and/or damage has been shown previously (Gichner et al., 2004; Liu et al., 2012). Insertions and deletions, point mutations, base substitutions, single/double-strand breaks are examples of the effect of cadmium stress on DNA (Castano & Becerril, 2004). Here, RAPD profile shows different changes in the DNA fingerprint indicating that Cd^{2+} affects the genome integrity. Not all primers showed variations in the RAPD profile between treated and non-treated plants, which could be explained by the variation in genome sensitivity to heavy metals stress between different regions. Also, some genome areas could be protected from external damage (Liu et al., 2012).

In conclusion, the results of this study showed that Cd^{2+} had a toxic effect on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *C. pumilum*. A reduction in hypocotyl and root length and shoot fresh weight was observed in seedlings grown under Cd^{2+} stress. A gradual increase in proline content and lipid peroxidation along with increasing Cd^{2+} concentration was also observed. The variation that occurred in the RAPD profiles of microshoots following Cd^{2+} treatment can be efficiently used as a sensitive tool to detect DNA damage and genotoxicity.

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