Effects of gibberellic acid on the process of organic reserve mobilization in barley grains germinated in the presence of cadmium and molybdenum

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A B S T R A C T

Soil contamination by heavy metals such as cadmium (Cd), molybdenum (Mo), lead, zine and others as a result of industrial and agricultural practices, is a widespread problem in many countries across the world. Despite the fact that Mo is an essential nutrient required by plants in small concentrations, the exposure of crops, including metabolic and enzymatic activities during seed germination, to high concentrations of these metals can have adverse effects on their growth and performance. The current study assesses not only the deleterious effects of Cd and Mo contamination on barley grain germination but also the ability of gibberellic acid (GA3) to alleviate these negative effects. Stress generated by Cd and Mo contamination engendered the accumulation of total soluble proteins and a reduction of free amino acids in the endosperm followed by a decline of soluble proteins in seedling roots. This shows that protein reserves were not successfully mobilized in the endosperm of Cd or Mo-treated seeds, thus inhibiting protein synthesis in the roots. A reduction of soluble sugar content in the endosperm followed by a decrease in the activities of hydrolytic enzymes (α- and β-amylase, acid and alkaline phosphatase) also unveiled inhibited starch degradation caused by these heavy metals. However, the addition of 0.5 μM GA3 to the germination medium significantly alleviated the inhibitory effect of Cd and Mo on the activity of the four hydrolytic enzymes and concomitantly increased the sugar and amino acid content of the endosperm. Thus, GA3 treatment partially restored the mobilization of protein and starch reserves from the endosperm to seedling roots during germination. Alleviation of the phytotoxic effects of heavy metal pollution by GA3 in barley shows that the major effect of Cd and Mo toxicity is in suppressing the production of GA3 or inhibiting its activity in the aleurone tissue of the seed. In the future, barley improvement programs can use this information to devise strategies to enhance plant growth and production output in soils infected by heavy metals.

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1. Introduction

Industrial activity and anthropogenic lifestyles have resulted in a progressive increase in water and soil pollution by heavy metals, including cadmium (Cd) and molybdenum (Mo), which can enter the food chain as a result of their uptake by plants and pose risks to human health (Marichali et al., 2014). Several studies have shown that various heavy metals are essential for plant growth and development. Yet, excessive concentrations can cause deleterious effects on physiological and biochemical parameters of plants such as photosynthesis and mineral nutrition while also significantly decreasing growth and biomass accumulation (Gangwar et al., 2014). Many plant species have the capacity to absorb and accumulate contaminants such as lead, Cd, chromium, and arsenic although survival depends on the balance between the rate at which metal ions are taken up and the efficiency with which they are detoxified within the plant (Hajar et al., 2014). Usually, germination and early seedling growth stages are key steps in a plant’s life and are more sensitive to metal toxicity than fully developed plants, especially when some defense mechanisms have not yet been fully developed (He et al., 2014). Kalai et al. (2013) studied the effect of heavy metals on barley (Hordeum vulgare L.) seed germination, and reported that the exposure of seeds to high concentrations of Cd (100 μM) and Cu (500 μM) for 2 days led to a decrease in the growth of radicals and shoots, a decline in the activities of α-amylase, acid phosphatase and alkaline phosphatase in the endosperm, the accumulation of soluble sugar

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in the endosperm and a significant accumulation of proline, essentially in the radicles. Similarly, Gubrelay et al. (2013) found that treatment of barley seeds with 10, 20 and 30 mM Cd had a toxic effect on germination percentage, germination rate and seedling growth while Cd also inhibited root and shoot growth with the negative effect increasing significantly as Cd concentration increased. However, no research has ever been conducted on barley germination in which heavy metals and gibberellic acid (GA3) or other phytohormones were used.

During the germination of a cereal grain, the aleurome layer is a secretory tissue that produces enzymes to hydrolyze the starchy endosperm (Aoki et al., 2014). The synthesis and secretion of hydrolytic enzymes are induced by gibberellins synthesized in embryos and that diffuse to the starchy endosperm where they catalyze the degradation of starch, protein, cell wall components and other storage compounds (Murray et al., 2006). GA3 has an important role in seed germination: it stimulates the synthesis and translation of mRNA specific for α-amylase, a hydrolytic enzyme responsible for the digestion of reserves within the seed (Muralikrishna and Nirmala, 2005). After it has been synthesized, α-amylase diffuses into the endosperm and produces sugars that are necessary for embryo growth (O’Brien et al., 2010).

Barley belongs to the oldest and economically most important cereals (Bolechova et al., 2015). Hence, in approximately 75% of the world, barley production is used for animal feed, 20% is malted for use in alcoholic and non-alcoholic beverages while 5% is employed as an ingredient in food products (El Halal et al., 2015). In Tunisia, barley is an important cereal crop where it is mainly used for human and animal nutrition and has been the topic of ample research investigations (Bettaieb Ben Kaâb et al., 2005; Bchini et al., 2013). Contamination of agricultural soils by heavy metals has become a concern of scientific interest because the uptake of heavy metals by crops affects food quality and security (Qi et al., 2015). Tunisia is one of the largest phosphate producers in the world (production was 8 million tons in 2007) and this industry produces emissions rich in toxic elements of Cd and Mo toxicity in barley can be very useful to devise strategies for alleviating its negative effects on crop performance.

The two key objectives of this study were as follows: (1) to investigate the effects of Cd and Mo on the organic reserves of barley grains after 96 h of germination and on the mobilization of these reserves by assaying amylase and phosphatase enzymes given their important role in starch metabolism in developing as well as germinating seeds; and (2) to study the effectiveness of GA3 in reversing the inhibitory effects of Cd and Mo on organic reserve mobilization during barley seed germination.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MI, USA) and were of the highest purity available.

2.2. Plant material

All experiments were conducted on seeds of a widely adapted barley cultivar ‘Manel’, which is an important part of the cereal production system in Tunisia. ‘Manel’ was developed by the National Agricultural Research Institute of Tunis (INRAT) in 1996 and is known for its high yield potential and disease resistance, especially in sub-humid areas (Deghais et al., 1999). The experiments were conducted in November 2014 at the laboratory of the Biology Department, Faculty of Sciences of Tunis, Tunisia, as part of a research project on ‘Plant Nutrition, Nitrogenous Metabolism and Proteins of Stress during heavy metal stress’ (UR/13ES-29).

2.3. Germination conditions and heavy metal treatment

‘Manel’ seeds (300 in total) were surface disinfected with 2% sodium hypochlorite for 10 min, and then rinsed thoroughly with distilled water. For each treatment, three replicates of 30 seeds each were placed on two layers of Whatman filter paper (0.5 mm; General Electric Co. Healthcare Life Sciences, Buckinghamshire, UK) in 15-cm diameter glass Petri dishes to which distilled water (control), 150 μM CdCl₂ or 100 μM (NH₄)₂MoO₄ were added. One set each of control and heavy metal-treated seeds (i.e., GA3 + Cd and GA3 + Mo) were allowed to germinate for 96 h in the dark at 25 °C in the presence of a 0.5 μM solution of GA3 while another set of seeds received no GA3 treatment. The 0.5 μM GA3 concentration was selected based on its ability to maximize the germination rate of barley seeds at all time points up to 96 h without inhibiting germination (Table 1). Furthermore, an analysis of variance (ANOVA) for germination (%) as a function of GA3 concentration and incubation time (H) (Table 2a and b) was achieved by using the Proc GLM (general model) implemented in the statistical software SAS version 9.1. This analysis showed that germination (%) varied significantly as a function of incubation period and GA3 concentration. Thus, based on this ANOVA analysis, GA3 = 0.5 μM and 96 h incubation time were selected for the remainder of the experiments.

Afterward, the seed integument and plumule of the embryonic axis of each germinated seed were excised, and the seeds were dissected using a razor blade into radicles and endosperm samples. The bulked radicle and endosperm samples of each replicate were then frozen by liquid nitrogen separately, before grinding by a mortar into a powder, and then stored at −80 °C in liquid nitrogen for up to 3 days to analyze enzymatic activities. To determine the total soluble sugar content, the radicles and endosperm samples were vacuum dried (Memmert, Schwabach, Germany) at 70 °C for at least 24 h before grinding. All experimental treatments were replicated three times.

2.4. Biochemical analyses

2.4.1. Soluble sugar assay

Total soluble sugars were assayed in the endosperm following the method of Yemm and Willis (1954). Soluble sugars were extracted after macerating 25 mg of dry endosperm powder in 5 ml of 80% ethanol in a test tube and then stirring in a water bath at 70 °C for 30 min. The homogenate was centrifuged at 6000 × g for 15 min at 4 °C and 25 μl of supernatant for each sample was added to 5 ml of anthrone. After shaking, the test tubes, they were placed into a preheated water bath at 100 °C for 10 min then cooled in the dark for 30 min to prevent the oxidation of sugars. Total soluble sugar concentration was estimated colorimetrically at 640 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Norwalk, CT, USA) and expressed in mg/g dry mass (DM) based on a glucose standard curve generated using the same protocol.

2.4.2. Total soluble proteins assay

Total soluble proteins were assayed following the Bradford and Marion (1976) method using bovine serum albumin as the protein

<table>
<thead>
<tr>
<th>GA3 treatment (μM)</th>
<th>Germination (%) at 24 h</th>
<th>Germination (%) at 48 h</th>
<th>Germination (%) at 72 h</th>
<th>Germination (%) at 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>80 ± 1.5</td>
<td>88 ± 1.2</td>
<td>88 ± 1.2</td>
<td>92 ± 1.2</td>
</tr>
<tr>
<td>0.1</td>
<td>84 ± 2.2</td>
<td>90 ± 2.2</td>
<td>94 ± 2.2</td>
<td>96 ± 3.9</td>
</tr>
<tr>
<td>0.2</td>
<td>84 ± 2.2</td>
<td>90 ± 2.2</td>
<td>94 ± 2.2</td>
<td>96 ± 3.9</td>
</tr>
<tr>
<td>0.3</td>
<td>86 ± 2.2</td>
<td>92 ± 2.2</td>
<td>94 ± 2.2</td>
<td>96 ± 3.9</td>
</tr>
<tr>
<td>0.4</td>
<td>86 ± 2.2</td>
<td>94 ± 2.2</td>
<td>96 ± 2.2</td>
<td>98 ± 2.2</td>
</tr>
<tr>
<td>0.5</td>
<td>92 ± 2.2</td>
<td>96 ± 2.2</td>
<td>96 ± 3.9</td>
<td>98 ± 2.2</td>
</tr>
</tbody>
</table>

Percentage values based on three replications.

Table 1 Germination (%) of barley seeds as a function of time (h) and as a function of different GA3 concentrations (μM).
standard. The radicles and endosperms preserved in liquid nitrogen were powdered separately. Then 100 mg fresh material was extracted with 600 μl of extraction buffer consisting of 25 mM Tris–HCl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA-Na, 14 mM β-mercaptoethanol, and 5 mg PVP. The mixture was centrifuged for 20 min at 5000 × g. After centrifugation, 975 μl of Bradford reagent was added to 25 μl of supernatant and photometric absorbance was read at 595 nm using a UV–vis spectrometer (Lambda 25, PerkinElmer).

2.4.3. Free amino acids assay

Free amino acids were assayed in fresh endosperm and radicles by using ninhydrin reagent according to the Moore and Stein (1954) method. Absorbance was read at 570 nm.

2.4.4. Amylase activity assay

The maximum activity of amylase must be found in the endosperm because amylases are specific to the digestion of starch reserves which are abundant in the endosperm. Amylase activity was measured in the endosperms. Proteins were extracted at 4 °C in 0.1 M sodium acetate buffer, at pH 5.0 (α-amylase) and pH 3.4 (β-amylase). The activity of α- and β-amylases were measured following the method of Dure (1960).

2.4.5. Phosphatase activity assay

Alkaline and acid phosphatase activity was assayed from endosperms using the methods of Torriani (1967) and Ikawa et al. (1964). The extraction of both enzymes was carried using 0.1 M acetate buffer (pH 5.0) and the reaction was stopped by the addition of 0.2 M NaOH for acid phosphatase while the reaction for alkaline phosphatase was stopped by the addition of 0.2 M Na₂HPO₄.

2.5. Statistical design and analysis

A data point for an assay represented 30 seeds of a biological replicate, and was obtained by averaging the results of three technical repetitions of the assay. The data set representing three biological replicates of each treatment represented a complete randomized design. Statistical analysis of this dataset was conducted using Statistix 8, version 2.0 (Analytical Software, 2003). ANOVA was used to estimate the significance in differences between the means of different treatments, and the Least Significant Difference (LSD) test was used to separate significant means. P values < 0.05 were considered statistically significant.

3. Results

In our study, the level of soluble sugars decreased significantly after the treatment of the endosperm with Cd or Mo (60% and 22% decrease, respectively relative to the control) (Fig. 1). When GA₃ was added to the germination medium (i.e., GA₃ + Cd and GA₃ + Mo), it corrected the repressive effect of Cd and Mo, regaining 71% and 60%, compared to the grains treated only with Cd or Mo.

Soluble protein content increased significantly in the endosperm compared to the control after treatment with Cd and Mo (29% and 34%, respectively; Fig. 2A) but there was a significant decline in radicles (28% and 33%, respectively; Fig. 2B).

The addition of GA₃ to the germination medium reduced the effect of Cd and Mo and restored the mobilization of soluble proteins in the endosperm. Thus, we noticed a significant reduction of soluble proteins in the endosperm (35% and 45%, respectively, for the grains treated with GA₃ and Cd and GA₃ and Mo compared to the grains treated only with Cd or Mo; Fig. 2A). Treatment with GA₃ restored wild type levels of soluble proteins in the endosperm of Cd- and Mo-treated seeds.

The level of soluble proteins in radicles increased significantly by 37% for grains treated with GA₃ and Cd and 52% for grains treated with GA₃ and Mo compared to grains treated only with Mo or Cd (Fig. 2B).

A decrease in the content of free amino acids was observed in the endosperms after the grains were treated with Cd or Mo (37% and 27% reduction, respectively, relative to the control). This decrease was not significant (Fig. 3A). However, the addition of GA₃ stimulated the mobilization of free amino acids in the endosperms. There was a significant increase (60%) in the level of amino acids for the grains treated with GA₃ and Cd and 55% for the grains treated with GA₃ and Mo compared to the grains treated only with Mo or Cd (Fig. 3A).

In the radicals, the amino acid contents increased after treatment of barley seeds with Cd or Mo. This increase was significant after 96 h of germination (6% and 9% compared to the control values, respectively) following treatment with Cd or Mo (Fig. 3B).

Table 2

Analysis of variance (ANOVA) for germination (%) as a function of GA₃ and time variations.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
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<td>11</td>
<td>1508.70000</td>
<td>137.154545</td>
<td>14.94</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>60</td>
<td>550.80000</td>
<td>9.180000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>71</td>
<td>2059.50000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DF = degrees of freedom; F = Fischer; Pr: probability. Data are the mean ± SEM of at least three independent experiments. Significance of variations was tested at P < 0.01.

Table 3

Analysis of variance (ANOVA) for free amino acids as a function of GA₃ concentration and time variations.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat</td>
<td>5</td>
<td>568.500000</td>
<td>113.700000</td>
<td>12.39</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Time*Treat</td>
<td>6</td>
<td>940.200000</td>
<td>156.700000</td>
<td>17.07</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Treat: treatment; Time: time (h); DF = degrees of freedom; F = Fischer; Pr: probability; SS = sum of squares. Data are the mean ± SEM of at least three independent experiments. Significance of variations was tested at P < 0.01.

Fig. 1. Soluble sugar content in the endosperm of the barley seeds 96 h after germination. Control (no treatment with GA₃), GA₃ treatment (0.5 μM); Cd (150 μM); GA₃ + Cd; Mo (100 μM); GA₃ + Mo. Data are the mean ± SEM of at least three independent experiments. The different letters indicate significant differences from control values (LSD test; P < 0.05). DW: dry weight.
effect of Cd seems to be more pronounced than that of Mo on the phosphatase activities of the endosperm. Our results show a significant increase (52%) in the α-amylase activity for the grains treated with GA3 and Cd and 59% for the grains treated with GA3 and Mo compared to the grains treated only with Cd or Mo. In addition, there was a significant increase in the β-amylase activity (70% and 61%, respectively) for the grains treated with GA3 and Cd, and GA3 and Mo.

For phosphatases (Fig. 5A, B), treatment with Cd and Mo reduced the activities of acid and alkaline phosphatase. For acid phosphatase, compared to the control treatment, the reduction was 35% and 30%, respectively for grains treated with Cd or Mo. This reduction was significant. For alkaline phosphatase, there was a 30% and 28% reduction, respectively, for grains treated with Cd or Mo compared to the control treatment but this reduction was not significant (Fig. 5B). The inhibitory effect of Cd seems to be more pronounced than that of Mo on the phosphatase and amylase activities.

The addition of GA3 stimulates the activity of amylases in the barley endosperm. Acid phosphatase increased significantly by 38% and 43% when the grains were treated with GA3 + Cd and GA3 + Mo, respectively compared to the grains treated only with Cd or Mo. On the other hand, the alkaline phosphatase activity of the grains treated with either Cd or Mo increased by 36% and 40%, respectively, compared to the grains treated only with Cd or Mo, regardless of the GA treatment. However, this increase was not statistically significant (Fig. 5B).

4. Discussion

Seed germination is a sequence of events that involves hydration of the dried seed, activation of cellular metabolism, followed by synthesis, secretion, and relocation of hydrolytic enzymes and degradation of seed macromolecules by newly synthesized and stored hydrolases (Schmitt et al., 2013).

The germination of barley seeds, including the subsequent growth and development of the embryo, is accompanied by the synthesis of a suite of enzymes by the aleurone cells (Murray et al., 2006). These enzymes are predominantly hydrolytic, and are synthesized in response to gibberellin (GA) originating in the embryo and released by the aleurone cells into the starchy endosperm where they catalyze the degradation of starch, protein, cell wall components and other storage compounds (Murray et al., 2006).

Thus, the contents of protein, free amino acids and soluble sugars are highly dependent on hydrolytic enzymes and protease activity. The application of metal stress in germination medium by either Cd or Mo causes a decrease of these hydrolytic activities at the level of the endosperm, which engenders a reduction in the contents of the soluble sugars and blocks the degradation of soluble proteins. The drop in soluble protein content in the endosperm after grains were treated with Cd and Mo might be caused by a decrease in the activity of proteases in barley grains, particularly in the endosperm, which tends to accumulate proteins (Mihoub et al., 2005). The decrease in soluble proteins in the radicles could be an indirect effect of the same phenomenon, which is
caused by the lack of availability of amino acids translocated from the endosperm. Kalai et al. (2013) found a similar significant accumulation of total soluble proteins in the endosperm and a decrease in radicles (31% and 12% more than the control, respectively) after the barley grains were germinated for 48 h and treated with 500 μM Cd and 100 μM Cu. Proteases are known to be expressed in the endosperm during seed development and germination to facilitate hydrolysis and mobilization of storage proteins to supply the growing embryonic axis (Maheshwari and Dubey, 2008). Mihoub et al. (2005) suggested that the inhibition of protease activity was the event primer leading to the liberation of amino acids. It is likely that Cd and Mo directly and/or indirectly inhibit proteolysis. The inhibition of hydrolytic metabolism by heavy metals during germination explains the low levels of amino acids after 96 h of germination.

He et al. (2008) studied the effect of Cd on the activities of amylase enzymes in two rice varieties. They showed that α-amylase activities under 1, 5, 10, 25, 75, and 200 μM Cd treatments decreased by 5.5%, 21.5%, 32.0%, 50.8%, 70.4% and 85.2% for Xiushui 11 (first variety) and by 2.2%, 7.1%, 16.9%, 24%, 31.7% and 47.5% for Xiushui 110 (second variety), suggesting that α- and β-amylases decreased as Cd concentration increased, even at low concentrations. Kuriakose and Prasad (2007) reported that a high concentration of Cd (3 mM) induced a significant decrease in the activities of hydrolyzing enzymes such as acid phosphatase and α-amylase in sorghum seeds; they also showed that Cd inhibits starch mobilization in the endosperm which suggests an inhibition of the hydrolysis of the carbohydrate reserve and translocation of hydrolyzed sugars which ultimately leads to inhibited germination and impaired growth.

The addition of 0.5 μM GA3 to germination medium corrected the suppressive effect of Cd and Mo. At the level of the endosperm, the sugar content increased compared to grains treated only by Cd or Mo. The level of soluble proteins in the endosperm decreased after treatment with GA3 compared to the grains imbibed in water (control). On the other hand, the level of soluble proteins reached higher levels in the radicles compared to the control. Therefore, we suggest that GA3 facilitated the mobilization of protein reserves from the endosperm to the roots. There was a sharp increase in the free amino acid content of the endosperm in GA3-treated seeds compared to the control, even in the presence of Cd and Mo. Thus, the addition of GA3 to germination medium stimulated protease activity in the endosperm by degrading proteins into free amino acids. However, the stimulatory effect of GA3 seemed to be more significant for grains treated with Mo than those treated with Cd. The treatment of the barley grains with GA3 significantly increased the levels of α- and β-amylase activities compared to the control even in the presence of Cd and Mo. A similar result was observed in the levels of acid and phosphatase activities. However, the stimulatory effect of GA3 was more pronounced for the grains treated with Mo than Cd.

In Arabidopsis thaliana, Zhu et al. (2012) found that GA3 alleviated Cd toxicity by reducing nitric oxide accumulation and expression of a Cd transporter gene IRT1, and finally resulted in less Cd accumulation and an increase in Cd resistance.

Hydrolitic enzymes are directly connected with organic and mineral reserves during germination. Qi et al. (2006) showed that β-amylase activity was positively correlated with grain protein and hordein contents. Thus, the high contents of soluble sugars and amino acids found in the endosperm compared to the control after treatment with GA3 might be a result of high levels of amylase and phosphatase activities recorded after imbibition of GA3.

The molecular mechanisms of α-amylase synthesis and regulation have been a major area of research in cereal science over the past decades, especially in barley. The site of amylase synthesis was reported...
to be either in the aleurone layer or the scutellum, and the synthesis of RNA_α, specifically for α-amylase, was enhanced by GA_3 (Muralikrishna and Nirmala, 2005). In addition, it may increase the efficiency of translation. GA_3 is responsible for the stimulation of α-amylases in the endosperm (Muralikrishna and Nirmala, 2005). Villasuso et al. (2013) reported that in barley aleurone layers, the synthesis of hydrolytic enzymes (primarily amylase) was induced by GA, providing resources for seed germination and early seedling growth.

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References