International Research Journal of Biotechnology (ISSN: 2141-5153) Vol. 2(3) pp. 062-071, March, 2011
Available online http://www.interesjournals.org/IRJOB

Full Length Research Paper

Modulation of Zn-induced oxidative stress, polyamine content and metal accumulation in rapeseed (*Brassica napus* cv Jumbo) regenerated from transversal thin cell layers in the presence of zinc.

Asma Ben Ghnaya [A,B], Annick Hourmant [A,B,C], Michel Couderchet[d], Michel Branchard[A,B], Gilbert Charles [a,b,e]

[a] Université Européenne de Bretagne, France
[b] Université de Bretagne Occidentale, Laboratoire de Biotechnologie et Physiologie Végétales, ESMISAB, Technopôle Brest-Iroise, 29280 Plouzané, France.
[c] Université de Bretagne Occidentale, Laboratoire de Toxicologie Alimentaire et Cellulaire, UFR Sciences et Techniques, C.S. 93 837, 29238 Brest Cedex 3, France.
[d] Université de Reims Champagne-Ardenne, Laboratoire Plantes Pesticides et Développement Durable, URVVC-SE, BP 1039, 51687 Reims, France.
[e] Université de Bretagne Occidentale, Laboratoire d'Ecophysiologie et Biotechnologie des Halophytes et Algues Marines (LEBHAM), IUEM, Technopole Brest Iroise, Place Nicolas Copernic, 29280 Plouzané, France.

Accepted 11 March, 2011

The present work was designed to select hyperaccumulator plants by *in vitro* breeding from transversal thin cell layers. The thin layer explants of *Brassica napus* cv. Jumbo were cultivated directly in the presence of Zn at different concentrations (0.1-1 mM). Regenerated shoots were transferred into pots, acclimatized, and cultured in the greenhouse. After 3 weeks, the regenerants were treated with 2 mM of Zn during 7 days. Growth parameters, guaiacol peroxidase activity (GPOX), malondialdehyde (MDA), polyamines (PAs) and pigment contents were evaluated. Zn applied during the regeneration process influenced significantly both physiological and biochemical characteristics of regenerated plants. Particularly, an increase of Zn level in the tissue culture induced an increase in MDA content and GPOX activity in the leaves and a decrease in pigment contents. Exposure to 2 mM Zn induced 112 % increase in free polyamines in leaves and roots, while this increase was as high as 399 % for plants regenerated in the presence of the metal.

KEYWORDS: phytoremediation, hyperaccumulator, *in vitro*, polyamines, malondialdehyde, guaiacol peroxidase, Zn stress.

INTRODUCTION

Soil contamination by heavy metals is a consequence of industrial activities and phytoremediation appears as cheap and environmentally friendly method to restore soil quality (Pilon-Smits, 2005). However, the efficacy of this technique could be improved if plants were better adapted to grow on sites rich in pollutants. A number of research works are being carried out aiming at increasing the ability of plants to satisfy this application, including biotechnology and genetic engineering (Rugh *et al*., 1996; Pilon-Smits, 2005). In this respect, *in vitro* culture and selective pressure were proposed to develop more

*corresponding author Email: benghnaya_asma@yahoo.fr

Abbreviations:

BAP: 6-benzylamino-purine; GPOX: guaiacol peroxidase; MDA: malondialdehyde; MS: Murashige and Skoog's medium (1962); NAA: α-naphthaleneacetic acid; PAs: Polyamines; Put: putrescine; ROS: reactive oxygen species; Spd: spermidine; Spm: spermine, tTCL(s): transverse thin cell layer(s).

efficient phytoremediation system through the selection of hyperaccumulator plants to remediate polluted soils (Ben Ghnaya *et al*., 2007). In this earlier study, plants regenerated on metal rich media were selected and their ability to accumulate metals and some morphological and physiological characteristics were described. However, mechanisms by which these regenerated plants could overcome metal excess effects were not studied.

Heavy metals such as Zn are generally essential for plant growth and development since they are constituents of many enzymes and other proteins. However, high concentrations of both essential and non essential heavy metals in soils can induce toxicity symptoms and growth inhibition in most plants (Hall, 2002). Heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, often resulting in oxidative stress (Ferrat *et al.,* 2003). The antioxidative responses of plants exposed to metals and other stress factors have been reviewed (Sharma and Dietz, 2009). In resistant forms, stress conditions may enhance protective processes such as accumulation of compatible solutes and increase detoxifying enzymes activities. Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage, both in plants and animals (Ohkawa *et al.,* 1979; Wood *et al.,* 2006; Zhang *et al*., 2008). Besides, plant antioxidative defense response can be evaluated by analyzing the activity of antioxidant enzymes such as guaiacol peroxidase [GPOX, EC 1.11.1.7] or cell antioxidant contents (Iannone *et al.,* 2010). Among substances able to protect plant cell from oxidative attack, a specific role has been attributed to polyamines (PAs) in preventing photo-oxidative damages (Franchin *et al*., 2007). The antioxidative effect of polyamines is due to a combination of their anion and cation-binding properties involving a radical scavenging function (Bors *et al*., 1989) and capability to inhibit both lipid peroxidation (Kitada *et al*., 1979) and metal-catalysed oxidative reactions (Tadolini, 1988). Moreover, polyamine catabolism produces hydrogen peroxide that can enter the stress signal transduction chain, promoting the antioxidative defense response activation (Agazio and Zacchini, 2001).

In the present study, we characterize how rapeseed plants, obtained, as described earlier (Ben Ghnaya *et al*., 2007), cope with Zn exposure. We especially analyze oxidative stress markers in response to subsequent metal exposure. The influence of high Zn concentration (up to 1 mM) during regeneration of *Brassica napus* cv Jumbo and of subsequent Zn application (2 mM) have been presented on accumulation of the metal and on antioxidative defense responses such as PAs and MDA contents, GPOX activity as well as chlorophyll and carotenoid contents.

MATERIALS AND METHODS

Plant material and zinc treatments

Experiments were carried out on rapeseed (*Brassica napus* cv. Jumbo) provided by National Institute of Agronomic Research (INRA), Rennes (France). This genotype was a pure, genetically fixed line and was obtained by autofertilization.

Seeds, sterilized as previously described (Ben Ghnaya *et al*., 2007), were sown in test tubes on MS medium (Murashige and Skoog, 1962) supplemented with 2% (w/v) sucrose and 0.65% (w/v) agar at pH 5.8. Seeds were allowed to germinate for 2 weeks under conditions detailed earlier (Ben Ghnaya *et al.,* 2008). Then, transverse thin cell layers (tTCLs to 0.3–0.5 mm) were excised from hypocotyls and petioles and placed in petri dishes on MS medium containing 0.3 mg L^{-1} of 1-naphthaleneacetic acid (NAA), 3 mg L^{-1} of 6-benzylaminopurine (BAP), sucrose (3%) and agar (0.65%), supplemented with various concentrations of Zn (0 to 1 mM final concentration) dissolved in MES-buffer (MES=2-morpholinoethanesulfonic acid), pH 5.8.

The plants regenerated from tTCLs, without Zn (control) or in the presence of Zn, were acclimatized under a 16-h photoperiod, at 22 ± 1°C. The surviving plants were transferred to 750 mL pots containing sterile compost and cultured in a greenhouse. During three weeks, each pot was watered twice a week, alternating water and Hoagland's nutrient solution (Hoagland and Arnon, 1950). Subsequently, the three-week regenerants were treated or not every day with 2 mM Zn supplied to the roots (150 mL per pot). After 7 days, the plants were harvested, washed and sampled for analysis. The chemical assays were repeated three times.

Determination of growth and zinc accumulation

Fresh weight of leaves, roots and stems of the control and treated plants were measured after harvest and dry weight was estimated by heating tissues at 70 °C for three days. Two hundreds milligrams of dried plant tissues were ground up and digested by a mixture of HNO_3, HF and H_2O_2 (4/3/1, v/v/v) using a microwave digestion as described by Weiss *et al*. (1999). Zn content was measured by electrothermal atomic absorption spectrometer (Perkin-Elmer SIMAA 6100, USA) operated in the single-element mode. All solutions were prepared with high-purity water from a MilliQ-system (Millipore, Milford, MA, USA).

Pigment content

The pigment content was determined using the method of Lichtenthaler (1987). Leaves samples (1g fresh weight) from each plant were ground in a mortar in 100% acetone. The extracts were centrifuged at 5000 rpm for 10 min at 4°C before reading the absorbance at 470, 663, and 645 nm.

Lipid peroxidation (MDA)

The level of lipid peroxidation was determined using malondialdehyde (MDA) as a marker. Its content was measured according to Minotti and Aust (1987) and Iturbe-Ormaetxe *et al*. (1998). About 1g leaf fresh tissue was homogenized with 6 mL of metaphosphoric acid (5%, w/v) and 120 µL of 2% butyl hydroxytoluene (in ethanol). After 30 min at 4 °C, homogenate was centrifuged at 5000 rpm for 20 min. The chromogen was formed by mixing 4 mL supernatant with 400 µl 2% butyl hydroxytoluene, 2 mL of 1% (w/v) thiobarbituric acid (in 50 mM NaOH) and 2 mL of 25 % HCl. The reaction mixture was heated for 30 min at 95 °C and then

Table 1. Effect of $ZnSO_4$ (2 mM) treatments on *B. napus* (cv. Jumbo) growth and accumulation of zinc in roots (R), stems (S) and leaves (L) of plants regenerated in the presence of (0.1-1mM) or absence of $ZnSO_4$ and cultivated in greenhouse.

$ZnSO_4$ treatments (mM)		Plant growth						Zn content		
During *in vitro* regeneration	After regeneration	Fresh weight (g)			Dry weight (g)			($\mu g.g^{-1}$ DW)		
		L	S	R	L	S	R	L	S	R
0.00	0.00	7.88^{a}	4.63^{b}	1.25^{d}	0.60^{ab}	0.21^{b}	0.14^{b}	268.00^{e}	222.33^{f}	171.33^{f}
0.00	2.00	6.47^{b}	3.91^{c}	1.70^{b}	0.52^{b}	0.22^{b}	0.22^{a}	429.67^{d}	693.00^{d}	1821.33^{d}
0.10	2.00	7.95^{a}	4.92^{a}	1.49^{c}	0.68^{a}	0.30^{a}	0.19^{a}	519.67^{d}	545.33^{e}	751.00^{e}
0.25	2.00	7.80^{a}	4.73^{b}	1.38^{d}	0.61^{ab}	0.27^{a}	0.20^{a}	912.67^{c}	929.00^{c}	2168.33^{c}
0.50	2.00	5.03^{c}	5.08^{a}	0.90^{e}	0.37^{c}	0.29^{a}	0.21^{a}	1069.00^{b}	2215.33^{b}	5368.00^{b}
1.00	2.00	4.67^{c}	2.19^{d}	1.90^{a}	0.53^{b}	0.20^{b}	0.12^{b}	1947.67^{a}	4466.67^{a}	9089.00^{a}

Data are means from three independent experiments. In each column, the values with different letters are significantly different according to ANOVA and Duncan's test at the 5% level.

rapidly cooled in ice. Chromogen was extracted by adding 1.5 mL of 1-butanol. After 30 s vortexing, organic phase was separated by centrifugation (5000 rpm, 5 min) and the thiobarbituric acid reactive-substances (TBARS) determined by measuring absorbance at 532 nm. TBARS concentration was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Guaiacol peroxidase activity (GPOX, EC 1.11.1.7)

Fresh plant leaves, stems or roots (500 mg) were homogenized in 6 mL of cold 100 mM potassium phosphate buffer (pH 6.5) and centrifuged at 5000 g for 10 min. Guaiacol peroxidase activity was assayed in a mixture containing 2.7 mL of 0.1 M K-phosphate buffer (pH 6.0), 100 µL of 0.18 M guaiacol and 100 µL of 0.03 M hydrogen peroxide (MacAdam, *et al.,* 1992). Reaction was initiated by adding 50 µL of supernatant, and increase in A_{470} min^{-1} was measured. The amount of oxidized guaiacol was calculated from the extinction coefficient of 26.6 mM^{-1} cm^{-1}. Enzyme activity was expressed as µmol of min^{-1} g^{-1} DW.

Polyamine Analysis

Extraction and dansylation of free polyamines were performed using the procedure described by Flores and Galston (1982) and slightly modified by Féray *et al*. (1992). Tissues were extracted in 5 % (v/v) cold $HClO_4$. The mixture was kept at 4°C for 1 h and then pelleted at 5000 g for 15 min. Aliquots (200 µL) of the supernatant were mixed with 200 µL saturated sodium carbonate and 200 µL dansyl chloride in acetone (5 mg. mL^{-1}). The samples were vortexed (30 s) and incubated overnight at room temperature. Dansylated polyamines were extracted in 2 x 500 µL benzene, 200 µL were evaporated and re-dissolved in 500 µL methanol for analysis. Standards were treated in the same way. The HPLC system consisted of two solvent pumps (Kontron 422) coupled to a high performance mixer, 7125 Rheodyne injection valve fitted with 20-µL loop and stainless precolumn spheri ODS 5 mm (Brownlee) (30 x 4.6 mm) in conjunction with stainless steel analytic column (Ultrasphere). The mobile phase consisted of 0.01 M NaH_2PO_4, pH 4.4 (A) and methanol/ acetonitrile (50/50, v/v) (B) mixed at ambient temperature to produce a gradient flow rate of 1 mL. min^{-1} with 5min B, 80%; 2 min B, 80-89 %; 5 min B, 89-100 %; 3 min B, 100-80 %; 10 min B, 80 %. The gradient and data analysis were controlled by a microcomputer data system (Kontron Inst.). SFM 25 spectrofluorimeter (Kontron) was used to detect with excitation at 360 nm and emission at 510 nm.

Statistical analysis

The experiments were conducted according to randomized block design. The values in the tables and figures were compared by analysis of variance (ANOVA) and the differences among means (5 % level of significance) were tested by the Duncan's Multiple Range Test using StatGraphics Plus 5.1.

RESULTS

Plant growth and biomass

Control plants that were not exposed to Zn (neither during nor after regeneration) showed the highest leaf fresh and dry weight and lowest root weight (Table 1). The 2 mM of Zn greenhouse treatment of plants regenerated in the absence of Zn induced a significant decrease in fresh weight of aerial parts and increase in root fresh weight (Table 1). Dry weight was increased in roots and not affected in leaves and stems (Table 1). The aerial parts of plants regenerated in the presence of 0.1 or 0.25 mM of Zn and treated with 2 mM of Zn in the greenhouse revealed significantly higher fresh and dry weights than those regenerated without Zn (Table 1), whereas in roots, the fresh weight was decreased and the dry weight remained unchanged.

In contrast, the leaves of plants regenerated in the presence of higher Zn concentrations (0.5 or 1 mM) showed significant decrease of leaves fresh, but for dry weight we observed the significant decrease only with 0.5 mM. The other organs were less affected (Table 1).

Zinc accumulation

The lowest content of Zn in control plants was observed in roots (171 µg g^{-1} DW) while the highest (268 µg g^{-1} DW) was in the leaves (Table 1). Exposing the plants to 2mM of Zn in the greenhouse resulted in an increase in Zn content by ten folds in roots, three folds in stems, and only one and a half fold in leaves. The plants regenerated *in vitro* in the presence of 0.1 mM of Zn resulted in an accumulation of Zn lower than in plants that had been regenerated in the absence of Zn. In these plants, Zn content in roots (751 µg. g^{-1} DW) was less than half the content of plants regenerated without Zn (1821 µg g^{-1} DW). When the thin layers were submitted *in vitro* to the presence of higher Zn concentrations (0.25, 0.5 and 1 mM), the Zn content of regenerated plants was significantly higher and proportional to Zn concentration in regeneration medium and was most pronounced in the roots where it reached 9089 µg. g^{-1} DW. The concentration of Zn in stems and roots relative to the one of the leaves of plants that had been regenerated with 0.5 or 1 mM Zn is comparable to that of plants regenerated in the absence of the metal, the roots containing from 424 to 502 % of the amount in leaves. In contrast, when plants were regenerated with low Zn concentration, the relative distribution of Zn in the tissue was comparable to that observed in control plants (Figure 1).

Chlorophyll and carotenoid contents

Exposure of plants regenerated in the absence of Zn to 2 mM Zn resulted in a decrease in chlorophyll a and carotenoid contents (Table 2). By contrast, when plants were regenerated *in vitro* in the presence of 0.1 mM and 0.25 mM of Zn. Chlorophyll a, b and carotenoid contents were similar or higher than in plants regenerated without the metal. Increasing Zn concentration (0.5 and 1 mM) in the regeneration medium caused a significant decrease in chlorophyll a and in carotenoid contents in regenerated plants leaves (Table 2).

Lipid peroxidation (MDA)

The occurrence of oxidative stress induced by Zn treatment was monitored by analyzing membrane damage through measurement of MDA levels: exposure of plants regenerated in the absence of Zn to 2 mM of Zn in the greenhouse resulted in a significant increase of MDA, whose content has doubled (Table 3). This increase was even more important when the plants were regenerated *in vitro* in the presence of Zn (0.1-1 mM), the maximum (39.92 nmol.g^{-1} fresh weight) being for plants regenerated with 1 mM Zn, approximately 4 times more than the untreated control (Table 3).

Guaiacol peroxidase activity

GPOX activity was significantly higher in all plant parts after a 2 mM Zn treatment in the greenhouse than in those of untreated plants (Table 3). This activity was further increased in the leaves of plants regenerated in the presence of 0.1 or 0.25 mM Zn (reaching 202 % of untreated control activity for 0.25 mM) and recovered the levels of normal untreated plants with 0.5 and 1 mM Zn. In the shoots, the GPOX activity was reduced proportionally to the Zn level and with 1 mM, the activity was similar to the one of unstressed plants. The highest activity was observed in roots and the selection *in vitro* (0.1 to 0.5 mM Zn) produced plants with normal activity (similar to unstressed plants), but it was strongly reduced with 1 mm Zn during regeneration. This stimulation was less important in stems and roots of plants regenerated *in vitro* in the presence of Zn. In these plant organs, the higher the Zn concentration the lower the GPOX activity was for 1 mM Zn the activity was down to untreated control activity in stems and 56 % less than untreated control activity in roots. In the case of leaves, activity of GPOX in response to 2 mM Zn was further stimulated when the plants had been regenerated with 0.1 and 0.25 mM Zn reaching 202 % of untreated control activity for 0.25 mM. Beyond that, GPOX activity in plants regenerated in the presence of 0.5 and 1 mM of Zn was no longer stimulated by a treatment with 2 mM of Zn in the greenhouse (Table 3).

Free polyamines

Non metal treated plants (neither during nor after regeneration) presented a gradation in the distribution of free polyamines, concentration increasing from leaves to roots.The richest organs contained 163.82 nmol. g^{-1} DW PAs (Table 4). In all organs, spermidine (Spd) was the predominant polyamine whereas spermidine (Spm) was the less abundant (Figure 2).

Watering the control plants in the greenhouse with Zn (2 mM) induced a significant increase in the levels of the 3 PAs in leaves and roots (respectively + 115 and + 100 %) but not in stem fraction (Table 4, Figure 2).

When the plants were regenerated *in vitro* in the presence of Zn, the total concentration of PAs in the leaves was significantly increased (Table 4). This increase was observed for all the plants treated *in vitro*, with the maximum for those exposed to 0.1 mM during regeneration (+ 402 % *vs*. treated control). This increase of global PAs levels resulted from higher levels of Put, Spd and Spm (Figure 2A).

The analyses of stems showed that greenhouse exposure to the metal induced no significant change in the level of total (Table 4) and individual PAs (Figure 2B). Nevertheless, the addition of 0.25 mM and 1 mM of Zn, in the regeneration medium induced an increase in the total

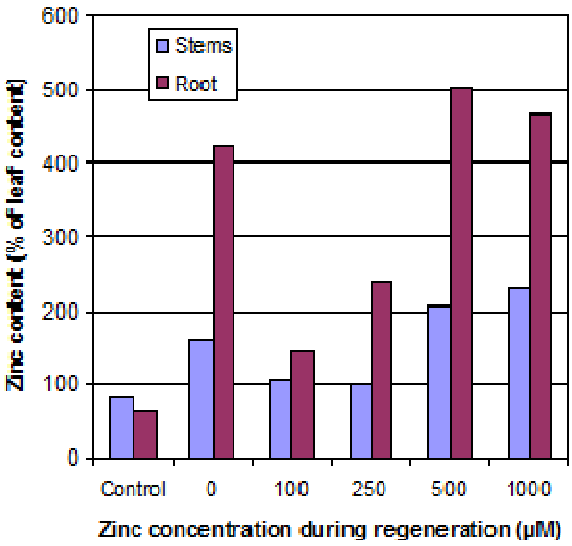

Figure 1. Effect of $ZnSO_4$ (2 mM) treatments on zinc content in roots (R) and stems (% of leaf content) of plants regenerated in the presence of (0.1-1mM) or absence of (0 mM) $ZnSO_4$ and cultivated in greenhouse. Control plants were neither treated with $ZnSO_4$ nor regenerated with the metal.

Table 2. Effect of $ZnSO_4$ (2 mM) treatments on leaf pigment contents (Chlorophyll and Carotenoid) of *B. napus* (cv. Jumbo) regenerated in the presence (0.1-1 mM) or absence of $ZnSO_4$.

$ZnSO_4$ treatments (mM)		Leaf pigments (µg g^{-1} FW)			
During *in vitro* regeneration	After regeneration	Chlorophyll			Carotenoid
		C_a*	C_b	C_{a+b}	C_{x+c}
0.00	0.00	196.22^a	59.16^b	255.39^a	56.31^a
0.00	2.00	163.57^b	62.33^b	225.90^b	50.87^b
0.10	2.00	207.13^a	75.87^a	282.99^a	62.89^a
0.25	2.00	195.50^a	78.20^a	273.67^a	60.83^a
0.50	2.00	114.25^c	51.28^b	165.53^c	40.79^c
1.00	2.00	93.07^d	68.42^a	161.49^c	35.67^c

*C_a= Chlorophyll a, C_b = Chlorophyll b, C_{a+b} = Total chlorophyll and C_{x+c} = Carotenoids. Data are means of 3 independent experiments. In each column, the values with different letters are significantly different according to ANOVA and Duncan's test at the 5% level.

Table 3. Effect of $ZnSO_4$ treatments (2 mM) on GPOX activity of leaves (L), stems (S), and roots (R) and on MDA content of *B. napus* cv Jumbo regenerated in the presence (0.1-1 mM) or in the absence of $ZnSO_4$.

$ZnSO_4$ treatments (mM)		GPOX (µmol min^{-1} g^{-1}DW)			MDA (nmol g^{-1} FW)
During *in vitro* regeneration	After regeneration	L	S	R	L
0.00	0.0	3.12^{c}	6.89^{d}	34.30^{b}	9.77^{e}
0.00	2.00	5.02^{b}	10.28^{a}	40.88^{a}	18.16^{d}
0.10	2.00	5.69^{a}	9.11^{b}	34.63^{b}	25.27^{c}
0.25	2.00	6.29^{a}	8.71^{c}	29.51^{b}	32.70^{b}
0.50	2.00	3.85^{c}	7.50^{c}	30.37^{b}	34.79^{b}
1.00	2.00	2.92^{c}	5.95^{d}	15.17^{c}	39.92^{a}

The data are means from three independent experiments. In each column, the values with different letters are significantly different according to ANOVA and Duncan's test at the 5% level.

Table 4. Effect of $ZnSO_4$ greenhouse treatments (2 mM) on total polyamine content (PAs) of leaves (L), stem (S), and roots (R) of *B. napus* cv Jumbo regenerated in the presence (0.1-1 mM) or in the absence of $ZnSO_4$.

$ZnSO_4$ treatments (mM)		PAs (nmoles g^{-1} DW)		
During *in vitro* regeneration	After regeneration	L	S	R
0.00	0.0	84.55^{f}	101.64^{c}	163.82^{f}
0.00	2.00	181.57^{e}	105.52^{c}	328.31^{c}
0.10	2.00	424.35^{a}	104.29^{c}	390.77^{b}
0.25	2.00	339.44^{c}	130.22^{b}	511.79^{a}
0.50	2.00	274.44^{d}	104.66^{c}	209.98^{e}
1.00	2.00	365.82^{b}	170.21^{a}	243.25^{d}

The data are means from three independent experiments. In each column, the values with different letters are significantly different according to ANOVA and Duncan's test at the 5% level.

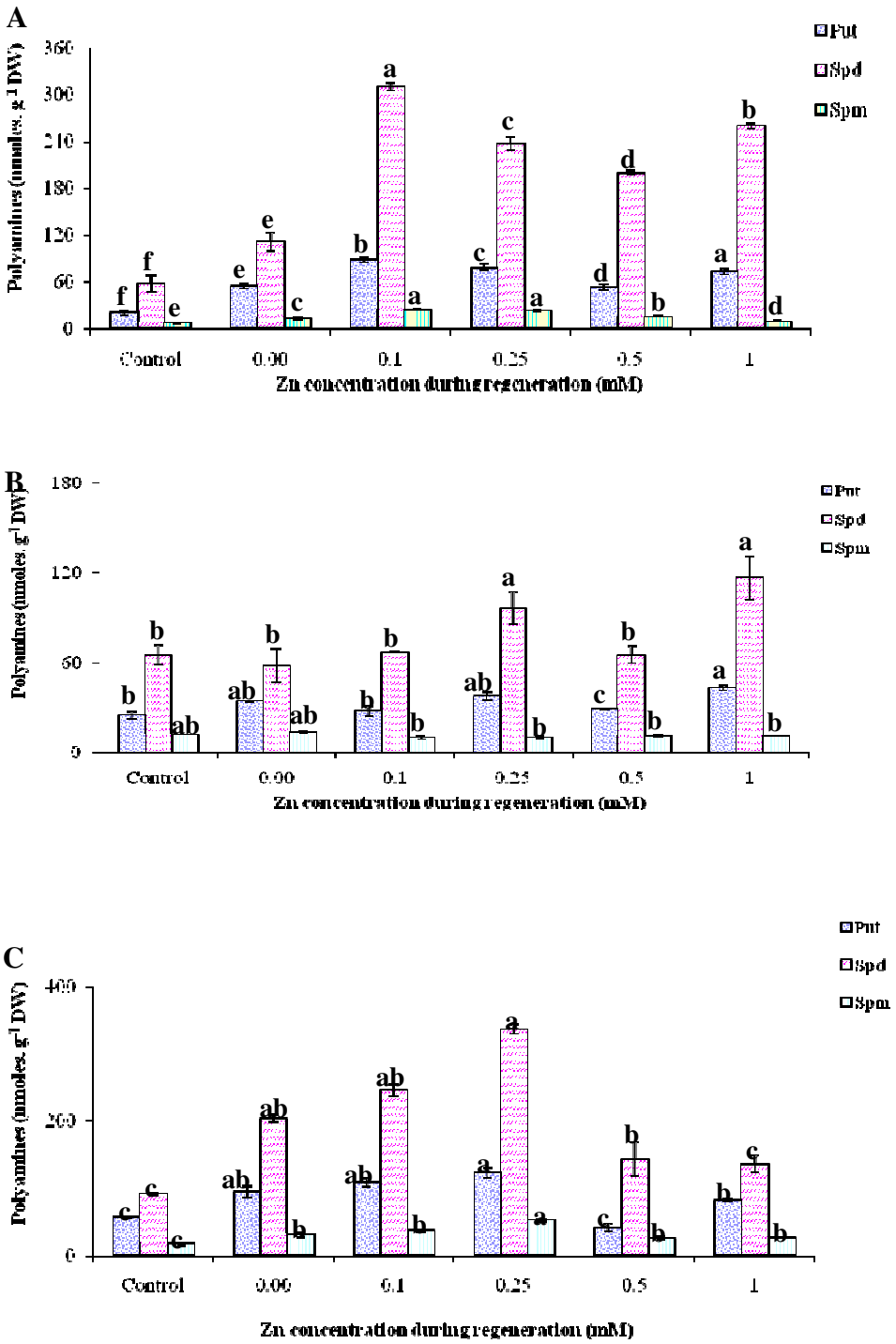

Figure 2. Effect of $ZnSO_4$ greenhouse treatment (2 mM) on free polyamine (nmoles. g^{-1} DW) of *B. napus* cv Jumbo plants regenerated in the presence (0.1-1 mM) or in the absence of $ZnSO_4$. (A) leaves, (B) stems, (C) roots. Put: putrescine, Spd: spermidine, Spm: spermine. The data are means from three independent experiments. In each column, the values with different letters are significantly different according to ANOVA and Duncan's test at the 5% level.

PAs due to a shift in Spd content (+ 28 and 49 %, respectively) (Figure 2B).

In the roots of plants regenerated in the presence of 0.1 or 0.25 mM Zn a further increase in the level of total and

individual Pas was observed (Table 4). This stimulating effect of the in vitro selection was maximum for 0.25 mM of Zn , but inversely the PAs content was diminished for 0.5 and 1 mM Zn (+ 28 and + 48 %, respectively). Furthermore, for high concentrations of 0.5 and 1 mM of Zn in the regeneration medium, the increase of PAs contents in response to the subsequent Zn treatment was lower than observed for control plants treated in the greenhouse with the metal (-36 and 26 %, Table 4). This was mainly due to a reduction in Put and Spd (Figure 2C

DISCUSSION

In this study, some effects of Zn treatment on plants regenerated *in vitro* from tTCLs with or without the metal were investigated. The results indicate that rapeseed metabolism was strongly perturbed when the plants were exposed to Zn treatment during regeneration and the responses were dependent on the Zn concentration.

Toxic effect of Zn was evident from curtailed growth and reduced biomass. When Zn (2 mM) was applied to plants regenerated in the absence of Zn and cultivated in the greenhouse, reduction in biomass was drastic in leaves (Table 1). Growth inhibition was consistent with data reported for Zn in Brassicacae and other species **(**Alia and Saradhi, 1995; Gisbert *et al*., 2006, Ben Ghnaya *et al.,* 2009). Biomass distribution between roots and leaves may be a plant strategy to tolerate high soil metal contents (Audet and Charest, 2008). However, the treatment was applied to plants regenerated *in vitro* in the presence of 0.1 or 0.25 mM of Zn, the growth reduction was totally alleviated. This alleviation could not be explained by reduced Zn accumulation in plant organs except for roots of plants regenerated with 0.1 mM Zn.

When Zn was applied to plants regenerated in the absence of Zn, a significant increase in the Zn content in the whole plant was observed. Accumulation of the metal was maximal in roots (+ 963 %). Below this value, stems (+ 212 %) accumulated more Zn than leaves (+ 60 %). Such metal-partition in plants is generally observed and is considered a way to avoid the metal toxicity (Audet and Charest, 2008). Although it is not possible to exclude that Zn has mainly accumulated in root apoplasm, it should be noted that this high Zn concentration in the roots (1821 $\mu g.g^{-1}$ DW) was associated with a negligible , indicating a relative tolerance of this cultivar (cv. Jumbo) to Zn treatment.

When $ZnSO_4$ was applied to plants regenerated *in vitro* in the presence of 0.1 mM of Zn, a significant decrease of Zn accumulation was observed, mainly in roots and also in stems, but not in leaves.

Nevertheless, for plants regenerated in presence of 0.25 mM of Zn and more, accumulation of Zn increased with the increase of the Zn concentration in the regeneration medium. A considerable elevation of Zn content was observed in the different organs but particularly in roots. An exclusion mechanism may have been positively regulated as a consequence of the presence of low Zn concentrations during regeneration. It could also be proposed that plants habituation to low Zn during regeneration resulted in a partition of biomass and metal different from that observed without Zn habituation and modelized by Audet and Charest (2008) in case of high soil contamination. At higher concentration in vitro, the further accumulation of Zn is no more alleviated but clearly enhanced.

The treatment of plants with 2 mM of Zn in the greenhouse diminished significantly chlorophyll and carotenoid contents as reported in other plant systems for Zn (Monnet *et al*., 2001; Singh and Sinha, 2005) and other metals (Chatterjee and Chatterjee, 2000; Zengin and Munzuroglu, 2005; Groppa *et al*., 2007). Chlorophyll content's reduction may be attributed to inhibition of their synthetic enzymes (Stobart *et al*., 1985). As discussed below, since Zn induced an oxidative stress in plants, it is also very likely that this stress was in turn responsible for pigment destruction (Singh and Sinha, 2005).

The regeneration *in vitro* in the presence of 0.1 mM and 0.25 mM of Zn induced a significant increase in chlorophyll and carotenoid contents. This finding is consistent with previous studies (Ferrat *et al.,* 2003; Ben Ghnaya *et al*., 2007) showing higher carotenoid content in plants subjected to Zn and various stresses. Carotenoid, as non enzymatic antioxidant, would play a vital role in the protection of plants from the adverse impact of reactive oxygen species (Halliwell, 1987). Similarly, stress of low intensity were already found to result in higher chlorophyll content (Teisseire *et al.,* 1999) and formation of shade type chloroplasts (Lichtenthaler, 1984). That this increase was observed in case of substances inhibiting non-cyclic electron transport during photosynthesis would confirm an effect of Zn on this process as observed for *Lolium perene* (Monnet *et al*., 2001).

However, increasing Zn concentration (0.5 and 1 mM) in the regeneration medium was responsible of significant inhibition in chlorophyll and carotenoid contents in leaves from regenerated plants (Table 2). This strong reduction of pigments may be the result of partial destruction due to a strong production of ROS as suggested by GPOX activity and MDA contents.

Indeed, MDA accumulated in response to Zn treatment and was further accumulated in a concentration dependent manner when Zn was present during regeneration. MDA is a major cytotoxic product of lipid peroxidation and as such it is an indicator of free radical production (Ohkawa *et al.,* 1979; Ferrat *et al.,* 2003). In the regenerated plants, the higher production of MDA, that increased with Zn concentration applied *in vitro* during regeneration, indicate an enhanced level of lipid peroxidation and a higher ROS production, which are also in accordance with the metal accumulation observed in the leaves.

Antioxidants and antioxidative enzymes are considered to play an important role in detoxification of oxygen species generated in the presence of metal ions. Among enzymes allowing the destruction of these ROS are peroxidases, particularly guaïacol peroxydase (GPOX). A significant increase of GPOX activity in aerial parts of plants in response to watering with Zn was observed. This increase was even higher in plants regenerated in the presence of 0.1-0.25 mM of Zn. Increase in GPOX activity may be attributed to its role in the elimination of H_2O_2 which is produced in excess, the increase in H_2O_2 leading to a higher GPOX activity. Many studies suggested the implication of GPOX in the oxidative stress could be correlated with the amount of the accumulated metal (Cuypers *et al.,* 2002; Sharma and Dubey, 2007). However, at higher concentrations of Zn applied *in vitro* (1 mM) Zn concentration in the leaves increased (Table 1), and a decrease of GPOX activity was observed. The decrease in GPOX activity suggests a stress too severe for the defense capacity of these plants (Siedlecka and Krupa, 2002). Zn treatment did not induce any change in roots GPOX activity despite a strong Zn accumulation in this organ. It then could be that the main effects of Zn are ROS production in green tissues upon illumination leaving roots unaffected by the 9089 µg g^{-1} DW of metal accumulated.

The importance of oxidative stress in plants upon heavy metal exposure has been repeatedly shown (Cuypers *et al.,* 1999; 2001; 2002). Zn is one of the metals that stimulate reactive oxygen species (ROS) production and antioxidative defense system (Cakmak, 2000). Our study revealed the importance of oxidative stress effects and antioxidative defense mechanisms after application of an environmentally realistic toxic Zn concentration (2 mM), i.e. the concentration similar to what is measured in Zn highly polluted soils.

As far as polyamines were concerned, Zn treatment in greenhouse caused an important increase in Put, Spd and Spm contents in leaves and roots. When plants were regenerated *in vitro* in the presence of 0.1 or 0.25 mM of Zn, the further increase of the PAs contents observed was significant in leaves and roots but (Table 4).

Accumulation of PAs in plants exposed to oxidative stress caused by heavy metals such as Cu, Cd, and Zn has been previously reported by several authors (Wettlaufer *et al.,* 1991; Groppa *et al.,* 2003; Franchin *et al*., 2007). Particularly, a protective role for polyamines in plant cells exposed to this stress has been described by many authors who showed that these substances may act as radical scavengers (Bors *et al.,* 1989; Sharma and Dietz, 2006) and as inhibitor of lipid peroxidation (Velikova *et al.,* 2000; Groppa *et al.,* 2001). This protective role for polyamines was recently confirmed by the increase of tolerance to copper and cadmium created by an exogenous supply of polyamines (Groppa *et al.,* 2001; Rhee *et al.,* 2007; Wang *et al*., 2007). When Zn treatment was applied to plants regenerated *in vitro* in the presence of 0.5 or 1 mM of $ZnSO_4$, total PAs contents in leaves was significantly higher than in untreated plants, but clearly diminished in roots (Table 4). The relationship between Zn increase of oxidative stress that would in turn lead to a PAs contents increase was observed in most cases, however, this does not explain the increase of PAs contents in the roots despite no increase of GPOX activity, further investigations are required to clarified this point.

In this study, it was clearly demonstrated that deep changes occurred in the stress response (antioxidant system and metal accumulation) of plants regenerated from cells submitted *in vitro* to Zn, in a dose-dependent manner.

Plants regenerated in the presence of Zn showed a higher capacity of Zn accumulation in roots and aerials parts. This accumulation increased with Zn concentration during the regeneration phase and this selective pressure applied during the regeneration process may have induced a physiological adaptation (Hall, 2002). Although it is unlikely, mutation which affected major genes responsible for Zn absorption or accumulation cannot be excluded. The present investigation showed that application of selective pressure during regeneration of rapessed from tTCLs can improve Zn tolerance capacity and accumulation in aerial parts (x 5) for more efficient phytoextraction and this strategy may complement plant genetic engineering that is being developed for enhanced phytoremediation.

ACKNOWLEDGEMENTS

The authors are grateful to the French government for providing financial support to the first author via the French Embassy in Tunisia.

REFERENCES

Agazio M, Zacchini M (2001). Dimethylthiourea, a hydrogen peroxide trap, partially prevents stress effects and ascorbate peroxidase increase in spermidine-treated maize roots. Plant Cell Environ. 24: 237-244.

Alia VSK, Saradhi PP (1995). Effect of zinc on free radicals and proline in *Brassica* and *Cajanus*. *Phytochemistry*. 39: 45-47.

Audet P, Charest C (2008). Allocation plasticity and plant-metal partitioning: Meta-analytical perspectives in phytoremediation. *Environ. Pollut.* 156: 290-296.

Ben Ghnaya A, Charles G, Hourmant A, Ben Hamida J, Branchard M (2007). Morphological and physiological characteristics of rapeseed plants regenerated *in vitro* from thin cell layers in the presence of zinc. *C. R. Biol.* 330: 728-734.

Ben Ghnaya A, Charles G, Branchard M (2008). Rapid shoot regeneration from thin cell layers excised from petioles and hypocotyls explants in four cultivars of *Brassica napus* L. *Plant Cell, Tiss. Organ Cult.* 92: 25-30.

Ben Ghnaya A, Charles G, Hourmant A, Ben Hamida J, Branchard M (2009). Physiological behaviour of four rapeseed cultivars (*Brassica napus* L.) submitted to metal stress. *C. R. Biol.* 332: 363-370.

Bors W, Langebartels C, Michel C, Sandermann JH (1989). Polyamines

as radical scavengers and protectants against ozone damage. *Phytochemistry*. 28: 1589-1595.
Cakmak I (2000). Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. *New Phytol.* 146: 185-205.
Chatterjee J, Chatterjee C (2000). Phytotoxicity of cobalt, chromium and copper in cauliflower. *Environ. Pollut.* 109: 69-74.
Cuypers A, Vangronsveld J, Clijsters H (1999). The chemical behaviour of heavy metals plays a prominent role in the induction of oxidative stress. *Free Radical Res.* 31: 39-43.
Cuypers A, Vangronsveld J, Clijsters H (2001). The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. *Plant Physiol. Biochem.* 39: 657-664.
Cuypers A, Vangronsveld J, Clijsters H (2002). Peroxidases in roots and primary leaves of *Phaseolus vulgaris*; copper and zinc phytotoxicity: a comparison. *J. Plant Physiol.* 159: 69-876.
Féray A, Hourmant A, Penot M, Moisan-Cann C, Caroff J (1992). Effects of interaction between polyamines and benzyladenine on the betacyanin synthesis in *Amaranthus* seedlings. *J. Plant Physiol.* 139: 680-684.
Ferrat L, Pergent-Martini C, Roméo M (2003). Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. *Aquat. Toxicol.* 65: 187-204.
Flores HE, Galston AW (1982). Analysis of polyamines in higher plants by high performance liquid chromatography. *Plant Physiol.* 69 : 701-706.
Franchin C, Fossati T, Pasquini E, Lingua G, Castiglione S, Torrigiani P, Biondi S **(**2007). High concentrations of zinc and copper induce differential polyamine responses in micropropagated white poplar (*Populus alba*). *Physiol. Plant.* 130: 77-90.
Gisbert C, Clemente R, Navarro-Avino J, Baixauli C, Ginér A, Serrano R, Walker DJ, Bernal MP (2006). Tolerance and accumulation of heavy metals by *Brassicaceae* species grown in contaminated soils from Mediterranean regions of Spain. *Environ. Exp. Bot.* 205:19-27.
Groppa M D, Benavides M P, Tomaro M L (2003). Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. *Plant Sci.* 164: 293-299.
Groppa M D, Ianuzzo M P, Tomaro ML, Benavides MP (2007). Polyamine metabolism in sunflower plants under long-term cadmium or copper stress. *Amino Acids*. 32: 265-275.
Groppa M D, Tomaro M L, Benavides M P (2001). Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs. *Plant Sci.* 161: 481-488.
Hall J L (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot*., 53: 1-11.
Halliwell B (1987). Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chem. Phys. Lipids*. 44: 327-340.
Hoagland D R, Arnon D I (1950). The water-culture for growing plants without soil. *Cal. Agric. Exp. Sta* Cir. 347: 1-32.
Iannone MF, Rosales EP, Groppa MD, Benavides MP (2010). Reactive oxygen species formation and cell death in catalse deficient tobacco leaf disks exposed to cadmium. Protoplasma. 245:15-27.
Iturbe-Ormaetxe I, Escuredo PR, Arrese-Igor C, Becana M (1998). Oxidative damage in pea plants exposed to Water deficit or Paraquat. *Plant Physiol.* 116: 173-181.
Kitada M, Igarashi K, Hirose S, Kitagawa H (1979). Inhibition by polyamines of lipid peroxide formation in rat liver microsomes. *Biochem. Bioph. Res. Co.* 87: 388-394.
Lichtenthaler H K (1984). Chlroplast biogenesis, its inhibition and modification by new herbicide compounds. Z. *Naturforsch*. 39c: 492-499.
Lichtenthaler H K (1987). Chlorophylls and carotenoïds: Pigments of photosynthetic biomembrane (Colowick, S.P., Kaplan, N. O., Dones, R. et al. Eds.). *Methods Enzymol*. 148: 350-381.
MacAdam J W, Nelson C J, Sharpe R E (1992). Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiol*. 99: 872-878.
Minotti G, Aust S D (1987). The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. *J. Biol. Chem.* 262: 1098-1104.
Monnet F, Vaillant N, Vernay P, Coudret A, Sallanon H, Hitmi A (2001). Relationship between PSII activity, CO_2 fixation, and Zn, Mn and Mg contents of *Lolium perenne* under zinc stress. *J. Plant Physiol*. 158: 1137-1144.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiol*, 15: 473-497.
Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351-358.
Pilon-Smits E (2005). Phytoremediation. *Annu. Rev. Plant Biol.* 56: 15-39.
Rhee H J, Kim E J, Lee JK (2007). Physiological polyamines: simple primordial stress molecules. *J. Cell. Mol. Med.* 11: 685-703.
Rugh C L, Wilde H D, Stack NM, Thompson D M, Summers AO, Meagher RB (1996). *Proceedings of the National Academy Sciences*. 93: 3182-3187.
Sharma P, Dubey R S (2007). Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum. *Plant Cell Rep.* 26: 2027-2038.
Sharma S S, Dietz K J (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot.* 57, 711-726.
Sharma, S. S., Dietz, K. J. 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci*. 14: 43-50.
Siedlecka A, Krupa Z (2002). Functions of enzymes in heavy metal treated plants. In: Physiology and biochemistry of metal toxicity and tolerance in plants. (Prasad, M. N. V., and Strzalka, K., Eds). Kluwer. *Academic Publishers* pp, 303-324.
Singh S, Sinha S (2005). Accumulation of metals and its effects in *Brassica juncea* (L.) Czern. (cv Rohini) grown on various amendments of tannery waste. *Ecotoxicol. Environ. Saf.* 62: 118-127.
Stobart A K, Griffiths W T, Bukhari I A, Sherwood R P (1985). The effect of cadmium on the biosynthesis of chlorophyll in leaves of barley. *Physiol. Plant.* 63: 293-298.
Tadolini B (1988). Polyamine inhibition of lipid peroxidation. *Biochem. J.* 249: 33-36.
Teisseire H, Couderchet M, Vernet G (1999). Phytotoxicity of diuron alone and in combination with copper or folpet on duckweed (*Lemna minor*). *Environ. Pollut*. 106: 39-45.
Velikova V, Yordanov I, Edreva A (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Sci.* 151: 59-66.
Wang X, Shi G, Xu Q, Hu J (2007). Exogenous polyamines enhance copper tolerance of *Nymphoides peltatum*. *J. Plant Physiol*. 164: 1062-1070.
Weiss DJ, Shotyk W, Schafer J, Loyall U, Grollimund E, Gloor M (1999). Microwave digestion of ancient peat and lead determination by voltammetry. Fresenius. *J. Anal. Chem*. 363: 300-305.
Wettlaufer S H, Osmeloski J, Weinstein LH (1991). Response of polyamines to heavy metal stress in oat seedlings. *Environ. Toxicol. Chem.* 10: 1083-1088.
Wood L G, Gibson P G, Garg M L (2006). A review of the methodology for assessing *in vivo* antioxidant capacity. *J. Sci. Food Agr.* 86: 2057-2066.
Zengin FK, Munzuroglu O (2005). Effects of some heavy metals on content of chlorophyll, proline and some antioxidant Chemicals in Bean (*Phaseolus vulgaris* L.) Seedlings. *Acta Biol. Cracov. Bot.* 47: 157-164.
Zhang GW, Liu ZL, Zhou JG, Zhu YL (2008). Effects of $Ca(NO_3)_2$ stress on oxidative damage, antioxidant enzymes activities and polyamine contents in roots of grafted and non-grafted tomato plants. Plant Growth Regul. 56: 7-19.