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Morphological and physiological characteristics of rapeseed plants regenerated *in vitro* from thin cell layers in the presence of zinc

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Abstract

Phytoremediation offers owners and managers of metal-contaminated sites an innovative and cost-effective option to address recalcitrant environmental contamination. The use of plants to restore or stabilize contaminated sites, known as phytoremediation, takes advantage of the natural abilities of plants to take up, accumulate or store metals. This includes the use of plants that tolerate and accumulate metals at high levels for phytoextraction and the use of plants growing under conditions that are toxic to other plants, for preventing, for example, soil erosion (phytostabilisation). Rapeseed (*Brassica napus* L.) was shown to be able to accumulate substantial amounts of metals combined with high biomass. *Brassica napus* was therefore selected for heavy metal (HM) tolerance and accumulation through *in vitro* selection. A selective pressure applied during the neoformation process from transversal thin cell layers (tTCLs) allowed us to select tolerant cells and tissues. Toxic metals (such as Zn) were added to the culture media in order to select zinc-tolerant plants. Exerting a selective pressure during tTCLs regeneration aimed at selecting plants with exceptional zinc tolerance and/or accumulating capacity. The morphological and physicochemical characteristics of regenerated plants cultivated in greenhouse appeared to depend very significantly on the concentration of $ZnSO_4$ applied during the neoformation process. Plants regenerated in the presence of $ZnSO_4$ at 100 µM exhibited a greater size and a higher biomass together with flowering precocity. The contents of zinc, chlorophyll, and proline were modified in the regenerated plants. Pre-treatment with an excess of $ZnSO_4$ (>500 µM) was responsible for a percentage of tTCLs intolerance above 96%. With lower Zn concentrations (100–250 µM), the survival rates (33–15%) were higher. ***To cite this article: A. Ben Ghnaya et al., C. R. Biologies 330 (2007).***

Résumé

Caractérisation morphologique et physiologique de colzas régénérés *in vitro* à partir de couches cellulaires minces en présence de zinc. La phytoremédiation est l'utilisation de plantes vertes pour éliminer, contenir, ou rendre moins toxiques les

contaminants environnementaux. Cette technique peut faire l'objet de diverses stratégies telles que la phytoextraction, la phytodégradation, la phytostabilisation, la phytovolatilisation et la rhizofiltration. L'amélioration génétique des performances de plantes tolérantes ou accumulatrices de polluants (tels que des métaux toxiques) passe par une meilleure connaissance des voies métaboliques impliquées. Dans ce but, des plantes de colza (*Brassica napus* L.) sélectionnées *in vitro* ont été produites par la technique des couches cellulaires minces transversales (CCMts) couplée avec un crible de sélection. L'idée était que la pression de sélection exercée par un polluant durant la phase de néoformation *in vitro* devait permettre de sélectionner des plantes tolérantes, et éventuellement hyperaccumulatrices, dont on allait étudier les caractéristiques physiologiques et biochimiques. Avec des teneurs de 100 et 250 µM de $ZnSO_4$, la proportion de plantes régénérées reste relativement importante (33 et 15%, respectivement). À partir de 500 µM de $ZnSO_4$, le pourcentage de mortalité des CCMts atteint 96%, mais la pression de sélection est élevée pour les plantes restées vivantes, lesquelles ont pu être néoformées, acclimatées en serre et étudiées. Les caractères morphologiques et physiologiques des plantes acclimatées et cultivées en serre dépendent fortement de la concentration en zinc appliquée au cours de la phase de néoformation *in vitro*. Les plantes régénérées en présence de 100 et 250 µM de $ZnSO_4$ sont caractérisées par une biomasse importante et une floraison précoce. Les teneurs en zinc, chlorophylles et proline des régénérants sont modifiées. ***Pour citer cet article : A. Ben Ghnaya et al., C. R. Biologies 330 (2007).***

1. Introduction

Elemental toxicity in plants is a complex problem, the characteristics of which depend on the species, the element concentration, the form and the soil pH and composition. Soil contamination by heavy metals (HM) has become a critical environmental concern due to potential adverse ecological effects. Such toxic elements are considered as soil pollutants due to their widespread occurrence, and their acute and chronic toxicity. Pollution by metals such as zinc is very common. Zn concentrations in the range of 150 to 300 $mg\,kg^{-1}$ have been measured in polluted soils [1,2].

Among different methods used to remediate polluted soils, phytoremediation appears as a cost-effective method to address recalcitrant environmental contamination [3–6]. Phytoremediation includes phytoextraction, phytodegradation, phytostabilisation, phytovolatilisation, and rhizofiltration. Phytoextraction is based on the ability of (hyper)accumulating plants to take up metals from soils, to concentrate them in the roots and to translocate them to the aerial parts [7].

Hyperaccumulation may lead to a possible valorization of pollutants. In that case, the plants are incinerated in order to extract the metals from the ashes and re-use them in metallurgy (phytomining). Ideally, the plants used for phytoextraction should be hyperaccumulators and yield a high biomass. The properties of these plants can be optimised by biotechnology [8].

The objective of this work is to produce variants or mutants of crop plants, regenerated from tissue culture and modified for the main factors controlling the uptake, transport, and accumulation of pollutants. The genotypes selected for HM content (hyper- or hypoaccumulators) would also provide a useful tool to achieve a better understanding of the mechanisms involved in this complex phenomenon [9]. They should finally help to select interesting varieties for phytoremediation of polluted fields or for improved food safety [10]. Rapeseed (*Brassica napus* L.) is known to be able to accumulate substantial amounts of metals; moreover, this plant has a high biomass, various genotypes are easily available and the plant belongs to the Brassicaceae [11], a family in which several hyperaccumulator species exist. The aim of our project was to select hyperaccumulator plants by the technique of transversal Thin Cell Layers (tTCLs) cultivated *in vitro* in the presence of heavy metals. The tTCLs technique has already been used successfully for the micropropagation of a variety of plants, including recalcitrant ones. Furthermore, this technique induced a fast and intensive neoformation process [12,13].

Although we have not found anything of the sort in the literature, we have assumed that exerting a selective pressure during the neoformation process from tTCLs may help to select plants with exceptional zinc tolerance and/or accumulating capacity. We are also going to study the morphological and physiological features of regenerated plants submitted to zinc excess. Previous studies have indicated that HM (particularly zinc) have effects on chlorophyll [14,15] and proline [16,17] contents in plants. Our present work deals with the effects of different concentrations of $ZnSO_4$ applied during the regeneration process from tTCLs, on the size, biomass, growth, and endogenous Zn content of regenerated plants grown in greenhouse. The contents in

chlorophyll, carotenoid, and proline, known as stress markers, will also be evaluated.

2. Material and methods

2.1. Plant material

In this study, rapeseed (*Brassica napus* var. Drakkar) was selected. This genotype was a pure, genetically fixed line and it was obtained by autofertilization. The seeds were disinfected in 5% (w/v) calcium hypochlorite for 30 min and washed three times with sterile distilled water during 10 min. They were sowed in test tubes on Murashige and Skoog's medium (MS) [18] containing 2% of sucrose and 0.65% agar. The treated seeds were incubated under a photoperiod of 12 h light/12 h dark and a thermoperiod of 22/20 °C (light/dark). Explants of hypocotyl and petiole from 15-day-old seedlings were excised transversally (0.5–1 mm in thickness) and disposed in Petri dishes containing MS medium supplemented with 1-naphthaleneacetic acid (NAA: 0.1 to 0.3 mg l^{-1}), 6-benzylaminopurine (BAP: 1 to 3 mg l^{-1}), sucrose (3%) and agar (0.65%). ZnSO$_4$ (100 to 1000 μM) was dissolved in MES monohydrate 2-(*N*-morpholino)ethanesulfonic acid buffer (1 mM, pH: 5.8–5.9) and added to the medium. The frequency of shoot regeneration was determined after two weeks.

2.2. Plant regeneration and sampling of experimental specimens

The regenerated shoots were acclimatized under a photoperiod of 16 h light/8 h dark at 22 ± 1 °C. The surviving plants were transferred to pots containing a sterile soil and grown in a greenhouse. Each pot was watered twice a week, altering water and a modified Hoagland solution. After three weeks, the regenerants were soaked each day into 2000 μM of ZnSO$_4$ for one week. The plants were finally harvested and subdivided in limbs (L), stems and petioles (S + P) and roots (R). These different parts of plants were thoroughly rinsed with distilled water, oven dried at 80 °C for 72 h and used for ZnSO$_4$ analysis. The chlorophyll and carotenoid contents of the leaves were measured colorimetrically in 80% acetone. The proline content of the different organs fixed with liquid nitrogen was measured after the completion of the experiments. The chemical assays were repeated three times. At harvest, the fresh weight of different organs was measured. In addition, to determine dry weight, the tissues were heated at 70 °C for three days.

2.3. Zinc analysis

After digestion of 200 mg of dried plant material by a mixture of HNO$_3$, HF and H$_2$O$_2$ (4 ml/1 ml/3 ml) until dryness at 100 °C, followed by 3 h at 80 °C in 4 ml HNO$_3$, the tissue Zn content was measured by Electrothermal Atomic Absorption Spectrometry using a SIMAA 6100 Atomic Absorption Spectrometer (PerkinElmer) and the programme recommended by the manufacturer.

2.4. Chlorophyll and carotenoid analyses

The pigments were extracted from leaf discs in 80% acetone. The chlorophyll and carotenoid contents were determined by spectrophotometry according to the procedure described by Lichtenthaler [19] and using the following equations for the determination of the concentration of total chlorophyll and carotenoid in the leaf pigments:

$$C_a = 12.25A_{663} - 2.79A_{645}$$
$$C_b = 21.50A_{645} - 5.10A_{663}$$
$$C_{a+b} = 7.15A_{663} + 18.71A_{645}$$
$$C_{x+c} = \frac{1000A_{470} - 1.82C_a - 85.02C_b}{198}$$

where C_a = chlorophyll *a*; C_b = chlorophyll *b*; C_{a+b} = total chlorophyll; C_{x+c} = carotenoid, A_λ = absorbance at λ (nm).

2.5. Determination of proline content

Proline was estimated spectrophotometrically according to the methods described by Bates et al. [20], on the one hand, and Lee and Takahashi [21], on the other hand.

2.6. Statistical analysis

All our experiments were done in triplicate and repeated at least once. The data were compared by variance analysis (ANOVA) and the differences among means (5% level of significance) were tested by Duncan's Multiple Range Test using the software StatGraphics Plus 5.1.

3. Results

3.1. Caulogenesis induction

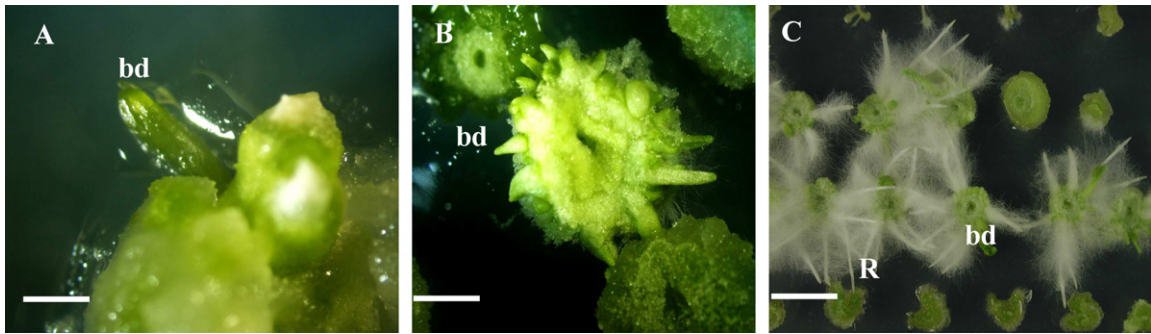The best medium for shoot regeneration from different tTCL sources of *Brassica napus* L. was the MS

Fig. 1. Shoot neoformation from *Brassica napus* (var Drakkar) tTCLs cultured on MS + 3 mg l^{-1} BAP + 0.3 mg l^{-1} ANA + 30 g l^{-1} sucrose + 100 μM of ZnSO$_4$. (**A**) A light-green callus proliferation and the development of young shoot buds occurred on tTCLs originating from petioles after 8 days of culture (bar: 1 mm). (**B**) A crown of buds and multiple shoots formed on tTCLs originating from hypocotyls after two weeks (bar: 1.25 mm, bd = bud). (**C**) Multiple shoots formed on tTCLs from hypocotyls and intense rhizogenesis after two weeks of culture on MS + 3 mg l^{-1} BAP + 0.3 mg l^{-1} ANA + 30 g l^{-1} sucrose + 250 μM of ZnSO$_4$ (bar: 10 mm, R = roots).
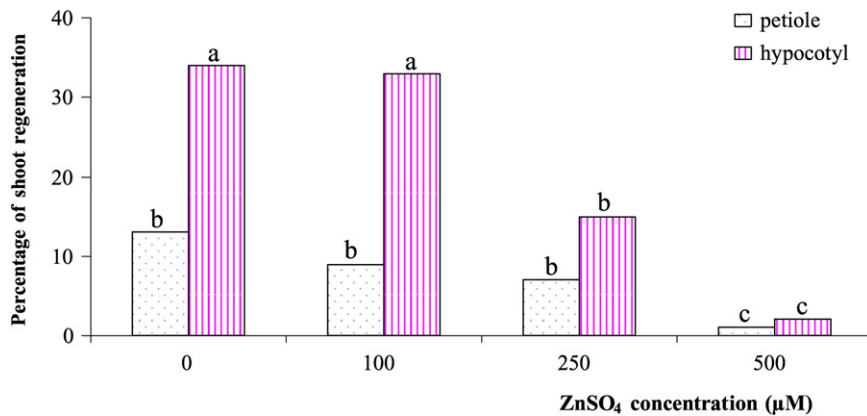

Fig. 2. Effect of the concentration of ZnSO$_4$ on the frequency of shoot regeneration with tTCLs from hypocotyl and petioles of *Brassica napus* (var Drakkar). The data were calculated from three replicated experiments, each with 50 tTCL explants. In each column, the values with different letters (a, b, c) are significantly different from one another according to ANOVA and the Duncan's test at the level of 5%.

medium supplemented with 3% sucrose, 3 mg l^{-1} BAP and 0.3 mg l^{-1} NAA in the conditions of our study. The size of the tTCL explants of *Brassica napus* L. was significantly increased after 5–6 days of culture. The growth was greater in the explants isolated from the hypocotyl than in those taken from the petiole. After 8–10 days, a small amount of light-green callus was observed on the subepidermal surface (Fig. 1A). When regeneration from tTCL explants occurred, these explants appeared to be green in colour and forming a crown of buds (Fig. 1B); then shoot buds could be detected after 15–20 days. Bud neoformation was usually associated with an intense rhizogenesis (Fig. 1C). Roots were formed on both regenerating and non-regenerating shoot bud explants. Shoot-regeneration ability was strongly influenced by the donor-organ: the evaluation of the regeneration potential of the plants revealed that tTCLs from hypocotyls had a higher fre-

quency of shoot regeneration than those from petioles in the presence of ZnSO$_4$ (Fig. 2). For tTCLs from both hypocotyl and petiole, shoot regeneration was not significantly affected by ZnSO$_4$ concentrations up to 100 μM. At higher Zn concentrations, the shoot regeneration frequency decreased; however, some plants succeeded to continue to grow at Zn concentrations up to 500 μM (Fig. 2). After being transferred into test tubes, all the buds obtained in the presence of the different concentrations of ZnSO$_4$ exhibited a spontaneous rooting ability and a rapid development (Fig. 3A and B). Just after rooting, the plantlets transferred to pots in greenhouse exhibited a normal development (Fig. 3C).

3.2. Morphology and plant growth

The concentration of ZnSO$_4$ applied during the neoformation process had a strong effect on the morpho-
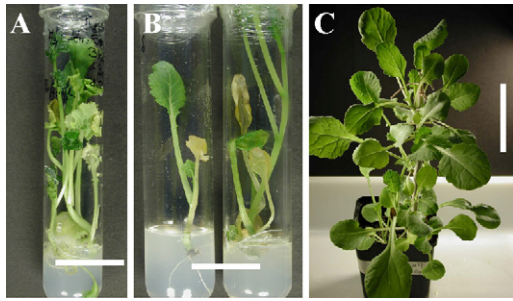
Fig. 3. Plants regenerated from hypocotyl tTCLs (**A**) in the presence of $ZnSO_4$ (250 µM) (bar: 26 mm). (**B**) $ZnSO_4$ (500 µM) transferred into test tubes on MS for development and rooting (bar: 23 mm). (**C**) Rooted plants regenerated from hypocotyl tTCLs in the presence of $ZnSO_4$ (250 µM) transplanted in pots (bar: 10 cm).

logical characteristics of plants cultivated in greenhouse and treated with 2000 µM of $ZnSO_4$. Plants regenerated in the presence of $ZnSO_4$ at 100 and 250 µM exhibited a greater size combined with a great precocity in flowering, compared with those grown in the presence of larger Zn concentrations (Fig. 4).

3.3. Zn accumulation

The results are presented in Table 1. The quantity of Zn accumulated in the stems and petioles of plants regenerated *in vitro* in the presence of 500 µM of $ZnSO_4$ and treated in the greenhouse with 2000 µM of $ZnSO_4$ was 177 $\mu g\,g^{-1}$ DW. This value differed significantly from the Zn distribution in both control (61.6 $\mu g\,g^{-1}$ DW) and the clone regenerated in the presence of 100 µM of $ZnSO_4$ (70.7 $\mu g\,g^{-1}$ DW).

3.4. Pigment content

Increasing the Zn concentration in the growth medium was responsible for an increase in the total chloro-

Fig. 4. Effects of $ZnSO_4$ on the morphological characteristics of plants cultivated in greenhouse and treated with 2000 µM of zinc. Bar: 18 cm, C = control (Zn 0) = shoots regenerated in the absence of $ZnSO_4$, Zn 100 = shoots regenerated in the presence of 100 µM of $ZnSO_4$, Zn 250 = shoots regenerated in the presence of 250 µM of $ZnSO_4$, Zn 500 = shoots regenerated in the presence of 500 µM of $ZnSO_4$.

phyll content in the leaves of the regenerated plants (Table 1). By contrast, the carotenoid content of the leaves decreased with increasing zinc concentration during the neoformation process (Table 1).

3.5. Proline accumulation

A significant increase of the tissue content of proline was observed in the different parts of plants regenerated *in vitro* from tTCLs exposed to different concentrations of Zn during the neoformation process and after them being transferred into pots in the greenhouse (Table 1). Proline accumulation in the test plants varies as a function of Zn concentration.

Table 1
The influence of $ZnSO_4$ on different biochemical and growth parameters of regenerated plants

$ZnSO_4$ content during the neoformation process (µM)	Plant growth						Zn accumulation ($\mu g\,g^{-1}$ DW)			Pigments limbs (mg g^{-1} FW)				Proline (mg g^{-1} DW)		
	Fresh weight (g)			Dry weight (g)						Chlorophyll			Carotenoid			
	L	S + P	R	L	S + P	R	L	S + P	R	C_a	C_b	C_{a+b}	C_{x+c}	L	S + P	R
0	48.56[a]	44.85[c]	15.97[a]	8.98[a]	6.12[c]	2.61[a]	109[b]	61.6[c]	82.4[d]	0.558[c]	0.27[c]	0.83[d]	84.5[a]	1.44[b]	1.59[d]	1.0[c]
100	39.17[b]	70.74[a]	14.26[b]	6.04[b]	21.18[a]	2.225[b]	88[b]	70.7[c]	296.2[c]	0.89[b]	0.35[b]	1.24[b]	72.9[b]	2.16[b]	3.33[c]	1.13[c]
250	30.49[c]	58.63[b]	10.88[c]	4.34[c]	9.28[b]	1.815[b]	86[b]	108[b]	331.4[b]	0.83[b]	0.33[b]	1.16[c]	68.4[c]	1.69[b]	4.12[b]	1.71[b]
500	14.99[d]	42.67[c]	8.76[d]	1.30[d]	5.83[c]	1.26[b]	134[a]	177.1[a]	398.1[a]	1.066[a]	0.73[a]	1.80[a]	45.3[d]	5.37[a]	7.78[a]	3.34[a]

The data were calculated from three replicated experiments. In each column, the values with different letters (a, b, c, and d) are significantly different according to ANOVA and the Duncan's test at the level of 5%. L = limbs, S + P = stems and petioles, R = roots, C_a = chlorophyll *a*, C_b = chlorophyll *b*, C_{a+b} = total chlorophyll, C_{x+c} = carotenoids.

4. Discussion and conclusion

Plants are known for their ability to resist under various types of stress (hydric deficiency, salt, heavy metals...). For plant physiologists, both the mechanisms of the effects of specific stress factors and the general consequences of the integrated effect of these factors are significant [22]. With regard to plant resistance to metallic stress, it was shown that this stress causes variations in the oxidative metabolism. Our data have shown that the exposure to $ZnSO_4$ during the *in vitro* neoformation process from tTCLs affected significantly the zinc tolerance of plants. Many parameters were affected: morphology, growth, Zn accumulation, and the contents of total chlorophyll, carotenoid, and proline.

The toxic effect of HMs on plant growth is well known [23,24]. Our present findings have also revealed that the vegetative growth of plants regenerated in the presence of $ZnSO_4$ was increased in the presence of 100 µM Zn, while it was decreased in the presence of higher Zn concentration (500 µM).

Plants regenerated *in vitro* from tTCLs in the presence of 500 µM $ZnSO_4$ showed a tremendous capability to accumulate Zn in their stems and petioles. Such a Zn accumulation is significantly different from the Zn content and distribution in the non-Zn-treated controls and in the plants regenerated in the presence of 100 µM $ZnSO_4$. This observation may be of particular importance for practical applications to phytoextraction.

The chlorophyll content is often used to assess the impact of environmental stresses in plants. The reason is that changes in pigment contents are linked to visual symptoms of plant illness and photosynthetic productivity [25]. Decreased chlorophyll contents have been observed in various plant species to be due to HM [26]. HMs (especially zinc) often inhibit metabolic processes by inhibiting the action of enzymes. Decreased chlorophyll contents associated with heavy metal stress may be the result of the inhibition of enzymes responsible for chlorophyll biosynthesis [27]. Here we have shown that increasing the Zn concentration in the growth medium tended to increase the content in total chlorophyll of the leaves of regenerated plants. Exposure to zinc during the *in vitro* neoformation process thus may cause an increase in the total chlorophyll content of plants acclimatized and transferred into the greenhouse. By contrast, the carotenoid content decreased in the leaves of plants regenerated in the presence of 500 µM Zn. As regards the effect of elevated concentration of HM on the synthesis and accumulation of photosynthetic pigments, contradictory data have been reported: some researchers reported decreased pigment content, whereas others found it unchanged or even increased [25,27].

Accumulation of free proline in response to HM exposure seems to be widespread among plants [28]. Proline increases the stress tolerance of plants through mechanisms such as osmoregulation, protection of enzymes against denaturing, and stabilization of protein synthesis [29]. An accumulation of free proline in response to Cu, Cd, and Zn was observed in non-tolerant and metal-tolerant *Silene vulgaris* (Moench) plants; the constitutive proline concentration in leaves was 5 to 6 times higher in the metal-tolerant than in the non-tolerant ecotype [30]. Our results have shown that a quantitative relationship exists between proline accumulation and zinc stress during the neoformation process, i.e. plants increased their proline content in response to zinc treatment. Proline accumulation in rapeseed regenerated in the presence of different concentration, of zinc is comparable to the response to water deficit [31], salinity [32], high-temperature [33] and low-temperature [34] stresses.

The results of the present work show that all plants regenerated *in vitro* from tTCLs in the presence of high Zn concentrations (250–500 µM) were able to accumulate significantly more Zn in their stems and petioles than non-Zn-treated controls and plants regenerated in the presence of low Zn concentrations (100 µM). These results are of significance, since this is the first report to our knowledge that plants regenerated *in vitro* from tTCLs may have an enhanced phytoextraction potential in the field. Moreover, it is noteworthy that Zn accumulation in our case occurred during a relatively short period (2 weeks *in vitro* and 4 weeks in the greenhouse).

The outcome of the study encourages us to undertake further researches in the use of *in vitro*-regenerated plants to create innovative lines of hyperaccumulator plants. Their aptitude to accumulate Zn will be compared with that of normal plants cultivated in the presence of different Zn concentrations. The present investigation may also be extended to other new treatments such as mutagenesis and to the study of the effect of various heavy metals on various plant species.

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