Potential Phytoextraction with in-vitro regenerated plantlets of Brassica juncea (L.) Czern. in presence of CdCl₂: Cadmium accumulation and physiological parameter measurement.

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Heavy metal contamination of agricultural land is partly responsible for limiting crop productivity. Cd²⁺ is known as a non-essentiel HM that can be harmful to plants even at low concentrations. Brassica juncea (L.) is able to accumulate more than 400 µg.g⁻¹ D.W. in the shoot, a physiological trait which may be exploited for the phytoremediation of contaminated soils and waters.

In our study, we have subjected regenerated plantlets obtained by the means of transverse thin cell layer technology from hypocotyl of B. juncea in the presence of 150 µM CdCl₂ to a second stress of CdCl₂ (75 µM), applied hydroponically for 3 days, to show whether the in-vitro plantlets were able to accumulate more Cd than wild type (plantlets obtained in the absence of 150 µM CdCl₂, and hence argue about their potential use in the phytoextraction process. The application of 75 µM CdCl₂ for three days does not show any effect in the B. juncea growth parameters (F.W. and D.W.) whatever the type of plantlets. This application decreases also the contents of chlorophyll a, carotenoids and Chl a/b ratio (2.26) for plantlets regenerated in the absence of CdCl₂ but not those of plantlets regenerated in its presence. Moreover, the amounts of MDA were increased in all plantlets however even more in those obtained in the presence of CdCl₂. Cd contents were higher in the plantlets regenerated in the absence of 75 µM CdCl₂ than those in its presence. Roots have the highest contents (3071; 1544 µg.g⁻¹ D.W.) followed by stems (850; 687 µg.g⁻¹ D.W.) and leaves (463; 264 µg.g⁻¹ D.W.) respectively.

In our conditions, we suggest that the low accumulation in the plantlets regenerated in the presence of CdCl₂ by the means of in-vitro regeneration technology is still beneficial, to some extent, for the phytoextraction process and seems to be an interesting technology that allows the cultivation of these plantlets in contaminated soils with low accumulation of metal in their shoots and probably in their seeds used in many food technologies. This results in decreasing the health risk due to the consumption of the spinoff products.

Keywords Brassica juncea, phytoextraction, regeneration, transverse thin cell layer (tTCL), CdCl₂

Abbreviations D.W. Dry weight, F.W. Fresh weight, Heavy metals HM, MDA Malondialdehyde, MES 2-(N-morpholino ethane sulfonic) acid, MS Murashige and Skoog medium (1962), tTCL(s) transverse Thin Cell Layer(s), S.D. Standard Deviation.

I. INTRODUCTION

Heavy metal (HM) contamination of soils has become a serious problem in areas of intense industry and agriculture. Increasing of HM concentrations in polluted soils pose a serious health hazard to man, animals, plants as well as soil micro-organisms, vitally important for soil health and fertility, especially those which are particularly sensitive to HM stress (Dahlin et al. 1997) and their biological diversity in the soil is reduced by HM contamination (Giller et al. 1998).

The remediation of contaminated soils by conventional technologies (e.g. excavation, physical stabilization or washing) used for small areas of heavily contaminated soils is not economically feasible. Phytoextraction, the use of plants for extraction of HM from contaminated sites, has emerged as an economically viable and socially acceptable decontamination for remediation of low to medium polluted soil (Salt et al. 1995a).

Cadmium is an HM naturally occurring in soils at low concentration (Traina, 1999). It is a toxic metal that can be harmful to animals and plants. Plants are able to absorb Cd via roots, to translocate it to leaves and thus introduce

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it into the food chain (Hart et al. 1998; Cakmak et al. 2000).

In the recent past decades, Brassica juncea has attracted researchers because of its high biomass production with added economical value and its high capacity to translocate and accumulate many metals and metalloids as As, Cd, Cu, Pb, Se, and Zn from polluted soils and therefore, can be considered as a good candidate in phytoextraction process (Kumar et al. 1995; Salt et al. 1995b; Blaylock et al. 1997; Raskin et al. 1997).

The aim of our study is to investigate the potential use of B. juncea plantlets regenerated from hypocotyl tTCl explants in the presence of CdCl$_2$ in the phytoextraction process.

II. MATERIAL AND METHODS

Plant material

Brassica juncea AB79/1 was used in our study. This cultivar is pure spring line, genetically fixed and was obtained by autofertilization.

Culture condition and regeneration of plants

Seeds of Brassica juncea were decontaminated in 70% ethanol for 30 sec, followed by immersion in calcium hypochlorite (5%, w/v) added with two drops of Tween-20 for 10 min. The seeds were rinsed twice for 5 min with sterile water upon sterilization and sown in test tubes on MS medium containing sucrose 2% (w/v) and solidified with agar at 6 g.l$^{-1}$ (Kalys, HP 696). They were incubated later on under a photoperiod of 12h (60 $\mu$mol photon.m$^{-2}$s$^{-1}$) provided by cool white fluorescent lamps, with a 22/20$^\circ$C thermoperiod (light/dark).

tTCLs (400-500 $\mu$m) were excised from hypocotyls of 7 day-old Brassica juncea seedlings and cultivated in a Petri dishes containing MS medium (25 ml) (15 tTCLs per Petri dish).

MS medium (comprising macronutrients, micronutrients and vitamins of Murashige and Skoog, 1962) supplemented with BAP (26.6 $\mu$M), NAA (3.22 $\mu$M), Sucrose (20 g.l$^{-1}$), AgNO$_3$ (10 $\mu$M) and CdCl$_2$ (0 or 150 $\mu$M). All media were solidified with agar (0.6 %, w/v), adjusted to pH 5.8 by 0.1 N NaOH and sterilized by autoclaving at 120$^\circ$C for 20 min.

All cultures were incubated in the same conditions as previously described. After 6 weeks, shoots were separated and transferred to test tubes containing MS medium (10 ml) without either, CdCl$_2$ and PGRs to induce rooting. The small plantlets were transferred to pots containing sterile vermiculite (EFISOL, VERMEX M) in a naturally-lighted greenhouse, watered daily and fertilized with half strength Hoagland solution (Hoagland and Arnon, 1950).

Hydroponic application of CdCl$_2$

Indian mustard (Brassica juncea) plantlets were grown on humidified vermiculite at 22$^\circ$C. When seedlings have 18 days, they are extracted from vermiculite and then roots are washed carefully under water flow. Then, 75 $\mu$M CdCl$_2$ was applied hydroponically in a half strength Hoagland solution (Hoagland and Arnon, 1950) for 3 days in presence of MES buffer (1 mM). All experiments were conducted in a growth chamber under a photoperiod of 16h (60 $\mu$mol photon.m$^{-2}$s$^{-1}$) provided by cool white fluorescent lamps, with a 22/20$^\circ$C thermoperiod (light/dark). The final pH in all culture medium, was 6.60 ± 0.05.

Determination of pigment contents

The contents of chlorophyll a and b and carotenoids are determined by Lichtenthaler procedure (Lichtenthaler, 1987). Approximately 1g of limb fresh weight is extracted by acetone 100 % (25 ml), and then the mixture is centrifuged at 5000 rpm for 10 min before reading the wavelength at 470, 662 and 645nm respectively using a Shimadzu UV-visible spectrophotometer (UV-1605).

Measurement of Cd in the different organs
The determination of Cd concentrations in the different digested solutions is conducted with electrothermal atomic absorption spectrometry. A Perkin-Elmer SIMAA 6100 working in single element monochromator mode was used for all atomic absorption measurements. At harvest (84 h of treatment), leaves, stems were weighed and then oven-dried for 4 days at 80°C, however, roots were extensively washed in distilled water for 10 min, then weighed before oven-dried for 4 days at 80°C. For the preparation of all solutions, high-purity water from a MilliQ-system (Millipore, Milford, MA, USA) was used. Sample aliquots of approximately 200 mg were transferred into the Teflon vessels. After addition of acid mixture: nitric acid, hydrogen peroxide and hydrofluoric acid to the powders in the ratios (4/3/1, v/v/v), the vessels were closed and exposed to microwaves digestion as described in detail elsewhere (Weiss et al. 1999).

Estimation of lipid peroxidation

The level of lipid peroxidation was determined as malondialdehyde (MDA) content able to react with thiobarbituric acid and was measured according to Minotti and Aust (1987) and Iturbe-Ormaetxe et al. (1998). Approximately 1 g limb fresh weight was extracted by 6 ml of a mixture of meta-phosphoric acid (5 %, w/v) and butylhydroxy-toluene (50/1, v/v) the homogenate was centrifuged at 5000 rpm for 30 min and then 4 ml of supernatant were homogenized with 200 µL of butyhydroxytoluene (2 %, w/v), 1 mL of HCl (25 %, v/v) and 1 mL of 2-thiobarbituric acid (1 %, w/v) prepared in NaOH (50 mM). The homogenate was incubated at 95°C for 30 min followed by rapid cooling in an ice bath to stop the reaction. Finally, we added 3 ml of 1-butanol on the homogenate then centrifuged at 500 rpm for 5 min before reading the absorbance at 532 nm. MDA contents were calculated using the extinction coefficient of MDA (α = 155 mmol L−1 cm−1).

Data analysis

Each experiment was repeated 3 times with 3 independent runs. For all parameters, the values were compared by analysis of variance (ANOVA) and the differences among means (5% level of significance) were tested by the LSD test using StatGraphics Plus 5.1.

III. RESULTS

Cadmium accumulation in B. juncea regenernted plantlets

Regenerated plantlets, from hypocotyl tTCLs cultivated in the presence of 150 µM CdCl₂ for 6 weeks, showed an accumulation of 154.8 and 46.3 µg Cd.g⁻¹ D.W. in their root and stem tissues respectively, but not in their leaves (Table 1). When the plantlets were subjected to 75 µM CdCl₂ for 3 days, the accumulation of Cd (µg.g⁻¹ D.W.) was enhanced in the order: Roots (3070.84) >> stem (849.34) > Leaves (463.17). However, plantlets regenerated from hypocotyl tTCLs in the presence of 150 µM CdCl₂ showed a decrease of the Cd accumulation when they were subjected to 75 µM CdCl₂; indeed, the accumulation of Cd decrease 1.98 and 1.75 in root and leaf tissues respectively (Table 1).

Effect of CdCl₂ on growth

The presence of 150 µM CdCl₂ in the culture medium of tTCLs induces the inhibition of growth of neoformed buds; this inhibition was observed in plantlets after their transfer to growth chamber for 5 weeks in the absence of CdCl₂ and resulting from a significant decrease of fresh and dry weight of leaves (56 and 48 %), stem (64 and 66 %) and roots (54 and 61 %). However, when regeneratd plantlets in the presence or not of 150 µM CdCl₂ were subjected hydroponically to 75 µM CdCl₂ for 3 days, their growth (fresh and dry weight) was not significantly modified per comparison with their respective plantlet controls (Table 2 and 3).

Effect of CdCl₂ on the pigment contents

The plantlets regenerated from hypocotyl tTCLs in the presence or absence of 150 µM CdCl₂ did not show any significant changes in their pigment contents (Figure 1) and the Chl a / b ratios were 2.95 for control and 3.24. However, the application of 75 µM CdCl₂ did not decrease pigment contents of plantlets regenerated in the presence of 150 µM CdCl₂, but decrease the Chl a and carotenoid contents by 33 % of plantlets regenrated in its presence per comparison to control plants. we observed also the decrease of Chl a / b ratio to the value of 2.26. Moreover,
whatever the treatment, Chl b contents did not produce any significant effect (Figure 1).

**Effect of CdCl₂ on lipid peroxidation and malondialdehyde (MDA) levels**

The levels of malondialdehyde was used as an indicator of lipid peroxidation and they were measured at leaf levels. The application of 75 µM CdCl₂ for 3 days on plantlets regenerated in the absence of CdCl₂ enhanced significantly the MDA formation (+ 55 %) in comparison to control plants (figure 2).

The regeneration of plantlets in the presence of 150 µM CdCl₂ did not show any significant difference in the lipoperoxidation in comparison to control, but this one was enhanced significantly after 3 days application of 75 µM CdCl₂; indeed, the MDA production was 2.82 fold more than the control plants (Figure 2).

**IV. DISCUSSION AND CONCLUSIONS**

*Brassica juncea* has been identified as a high biomass-producing crop with the capacity to take up and accumulate many HM and metalloids (Salt et al. 1995b; Blaylock et al. 1997; Raskin et al. 1997). This study on *B. juncea* provides the first comprehensive analysis of Cd accumulation in in-vitro regenerated plants.

Significant differences were found between the type of regenerant. Indeed, plantlets regenerated in the presence of 150 µM CdCl₂ accumulated less Cd than plantlets regenerated in its absence when all these plantlets were subjected to 75 µM CdCl₂ applied hydroponically. This decrease was observed for all organ of plantlets (Table 1).

Interestingly, the maximum Cd concentration found in our study (463.17 µg Cd g⁻¹ D.W.) was similar to the concentration found in *B. juncea* plants exposed to Cd for the same time exposure (Haag-Kerwer et al. 1999) and to much lower solution concentrations (Salt et al. 1995b, 1997; Haag-Kerwer et al. 1999), indicating an overall limitation of Cd uptake by the leaf tissues in *B. juncea* plants, irrespective of the Cd concentration administered to the plant in the medium. Possible reasons could be limitations of root to shoot translocation, and/or a saturable capacity for intracellular detoxification of Cd (Haag-Kerwer et al. 1999).

In our result, this partial exclusion of Cd seems to result more probably from the inhibition of transporters or ionic channels implicated in the root absorption as demonstrated by Cseh (2002) and Ann Peer et al. (2003) or to a change in the capacity of cell wall to bind metal or to an enhanced excretion of chelated substances as discussed in excluder plants by Ghosh and Singh (2005) and Kirkham (2006). Indeed, whether the Cd amount in roots were reduced by 50 % per comparison to the alone application of 75 µM CdCl₂, the amounts of aerial parts were reduced only by 28%.

The Cd-stress (150 µM) was found to adversely affect plant growth of regenerated plantlets but not 75 µM CdCl₂ applied hydroponically for 3 days. Our results show that the presence of 150 µM CdCl₂ in the culture medium of hypocotyl tTCL explants induced the growth reduction of neoformed plantlets (Table 2).

Indeed, although the plantlets regenerated in the presence of 150 µM CdCl₂ were cultivated after, with absence of this metal for 5 weeks, they showed a reduction of fresh weight of leaves (56 %), stem (64 %) and roots (53 %). At the same time, the dry weight of all organ was reduced, with the less observed effect at foliar level and the most one at root level (Table 3).

Growth reduction in response to Cd-stress was also reported for many species such as bean (Poschenrieder et al. 1989); willow, poplar (Lunackova et al. 2003; Cosio et al. 2005), rice (Aina et al. 2007), sunflower (Groppa et al. 2007), and some Brassica species such as Brassica napus (Larsson et al. 1998) and Brassica juncea (Haag-Kerwer et al. 1999); Cd inhibition was function of time exposure and concentration of Cd.

The second application of 75 µM CdCl₂ hydroponically did not affect significantly the growth parameters (F.W. and D.W.) of different organ of plantlets regenerated from hypocotyl tTCL explants. Chlorophyll contents were usually used to evaluate the impact of many environmental stresses on plant health. In our investigation, we showed that the regeneration in the presence of 150 µM CdCl₂ did not modify the chlorophyll and carotenoid contents in 5 week-old neoformed plantlets. It seems that the growth retardation due to the Cd presence in the culture medium of
tTCL explants and which persists for 5 weeks did not affect the biosynthesis of pigments.

However, the hydroponically application of 75 µM CdCl$_2$ induced a reduction of chlorophyll a and carotenoid contents according to many works that reported a decrease of chlorophyll contents under Cd-stress (Padmaja et al. 1990; Larsson et al. 1998; Groppa et al. 2007a, b) but also in the presence of other metals (Chatterjee and Chatterjee, 2000; Mysliwa-Kurdziel and Strzalka, 2002; Lei et al. 2007). The decrease of chlorophyll contents was one of primary events in plants subjected to metal stress and resulted from the inhibition of enzymes responsible to the biosynthesis of pigments (Stobart et al. 1985; Mysliwa-Kurdziel and Strzalka, 2002). As described by Mysliwa-Kurdziel and Strzalka (2002), Cd affected 2 pathways in this biosynthesis: it inhibited the aminolevulinic acid synthesis and the reduction of protochlorophyllid to chlorophyllid.

Moreover, hydroponically applied Cd in B. juncea plants in our study, induced a decrease of Chl a / b ratio agreed with the investigations of Baszynski et al. (1980) and Larsson et al. (1998). The Chl b contents being not affected, it is likely that reduction of Chl a contents may result from more sensitivity to Cd toxicity and then weaker synthesis or a faster degradation of this pigment.

The Cd-stress induce also a reduction of carotenoid contents agreeing with many investigations for many plant species (Baszynski et al. 1980; Larsson et al. 1998; Mysliwa-Kurdziel and Strzalka, 2002). The significant accumulation of Cd in leaves, probably responsible of free radical production, testified by the significant production of MDA. Indeed, this production may lead to the destruction of Chl a and the antioxidant carotenoids.

The application of 75 µM CdCl$_2$ hydroponically shows an enhanced production of MDA and an enhanced lipid peroxidation. It is known that the peroxidation of polyinsaturated fatty acids of cellular membrane disturb their functions, especially by the reduction of membrane fluidity and the inhibition of receptors and enzymes located in the cellular membrane (Lagadic et al. 1997).

Summarizing our results, we conclude that plantlets regenerated in the presence of CdCl$_2$ by the means of tTCL technology while beneficial, to some extent, for the phytoextraction process seems to be an interesting technology for the cultivation of plantlets in a contaminated soils with a low accumulation ability of HM in their tissues. Ultimately, this decreases health risks due to the consumption of their derivative products.

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TABLE I: Cd accumulation (µg Cd.g⁻¹ D.W. ± S.D.) in plantlets regenerated in the presence or not of CdCl₂ (150 µM) after the application of CdCl₂ (75 µM) hydroponically.

| CdCl₂ (µM) | 0     | 150    |
|------------|-------|--------|
|            | Leaves|        |        |
| 0          | 21.14 ± 1.44<sup>a</sup> | 20.01 ± 0.34<sup>b</sup> |
| Stems      | 25.00 ± 1.83<sup>g</sup> | 46.34 ± 2.90<sup>h</sup> |
| Roots      | 22.83 ± 2.09<sup>j</sup> | 154.83 ± 6.12<sup>g</sup> |
|            |        | Leaves|        |
| 75         | 463.17 ± 41.62<sup>e</sup> | 264.43 ± 19.03<sup>f</sup> |
| Stems      | 849.34 ± 56.20<sup>e</sup> | 686.67 ± 10.43<sup>d</sup> |
| Roots      | 3070.84 ± 257.55<sup>a</sup> | 1544.01 ± 38.75<sup>b</sup> |

The results were calculated from three independent experiments repeated 3 times. For Cd levels, the values with different letters are the means ± S.D. and are significantly different at p = 0.05 (ANOVA and LSD test).

TABLE II: Effect of CdCl₂ (75 µM) hydroponically applied for 3 days on the growth (F.W.) of plantlets regenerated from hypocotyl tTCL explants.

| CdCl₂ (µM) | 0     | 150    |
|------------|-------|--------|
|            | Leaves|        |        |
| 0          | 2670.45 ± 150.3<sup>a</sup> | 1170.23 ± 50.4<sup>b</sup> |
| Stems      | 390.34 ± 20.4<sup>c</sup> | 140.43 ± 10.3<sup>d</sup> |
| Roots      | 130.12 ± 10.12<sup>d</sup> | 60.23 ± 3.02<sup>e</sup> |
|            |        | Leaves|        |
| 75         | 2620.23 ± 190<sup>a</sup> | 1165.16 ± 40.7<sup>b</sup> |
| Stems      | 400.13 ± 20.3<sup>e</sup> | 142.13 ± 10.2<sup>d</sup> |
| Roots      | 130.21 ± 5.2<sup>d</sup> | 56.34 ± 4.1<sup>e</sup> |

Hypocotyl tTCLs were cultivated for 6 weeks on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2 % (w/v), AgNO₃ (10 µM) and CdCl₂ (0 or 150 µM).

The results were calculated from 3 independent experiments repeated 3 times. For F.W., the values with different letters are the means ± S.D. and are significantly different at p = 0.05 (ANOVA and LSD test).

TABLE III: Effect of CdCl₂ (75 µM) hydroponically applied for 3 days on the growth (D.W.) of plantlets regenerated from hypocotyl tTCL explants.

| CdCl₂ (µM) | 0     | 150    |
|------------|-------|--------|
|            | Leaves|        |        |
| 0          | 248.23 ± 6.51<sup>c</sup> | 128.24 ± 3.67<sup>b</sup> |
| Stems      | 42.80 ± 1.92<sup>c</sup> | 14.61 ± 0.17<sup>c</sup> |
| Roots      | 27.91 ± 0.26<sup>d</sup> | 11.01 ± 0.10<sup>c</sup> |
|            |        | Leaves|        |
| 75         | 251.21 ± 11.81<sup>a</sup> | 129.27 ± 2.72<sup>b</sup> |
| Stems      | 43.31 ± 1.64<sup>c</sup> | 14.85 ± 0.35<sup>c</sup> |
| Roots      | 27.86 ± 0.53<sup>d</sup> | 10.86 ± 0.09<sup>c</sup> |

Hypocotyl tTCLs were cultivated for 6 weeks on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2 % (w/v), AgNO₃ (10 µM) and CdCl₂ (0 or 150 µM).

The results were calculated from 3 independent experiments repeated 3 times. For F.W., the values with different letters are the means ± S.D. and are significantly different at p = 0.05 (ANOVA and LSD test).
FIG. 1: 75 µM of CdCl₂ (hydroponically applied for 3 days) effect on pigment contents of Brassica juncea plantlets regenerated on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2 % (w/v), AgNO₃ (10 µM) and CdCl₂ (0 or 150 µM). Results calculated from three independent experiments, repeated 3 times are means ± S.D. Different letters indicated significant difference at $p = 0.05$ (One way ANOVA and LSD test).
FIG. 2: 75 µM of CdCl₂ (hydroponically applied for 3 days) effect on MDA contents of Brassica juncea plantlets regenerated on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2 % (w/v), AgNO₃ (10 µM) and CdCl₂ (0 or 150 µM). Results calculated from three independent experiments, repeated 3 times are means ± S.D. Different letters indicated significant difference at $p = 0.05$ (One way ANOVA and LSD test).