Shoot regeneration seedlings from transverse thin cell layer explants excised from cotyledon, petiole, hypocotyl of *Brassica juncea* L. Czern. in the presence of CdCl₂

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The present work describes a novel neoformation process of *Brassica juncea* (L.) Czern. seedlings from transverse thin cell layers in the presence of CdCl₂. In order to investigate the regeneration ability of this crop, the effect of CdCl₂ on shoot regeneration (frequency of regeneration and bud number per tTCL) was examined. The tTCL explants were excised from cotyledon, petiole and hypocotyl of 7 day-old *B. juncea* seedlings and cultivated on a solid basal MS medium supplemented with α-naphtalenacetic acid (NAA : 3.22 µM), 6-benzylamino-purine (BAP : 26.6 µM), sucrose (2 %, w/v), silver nitrate (AgNO₃ : 10 µM) and various concentrations of CdCl₂ (0 - 250 µM).

A concentration of CdCl₂ of 5 µM is enough to reduce significantly the percentage of regenerated tTCL from 95-100 % to 77-86 % for all organs tested. In addition, 5 µM of CdCl₂ reduces the bud number from 4.5 to 2.98 and 3.2 to 2.02 of hypocotyl and petiole tTCLs, but not for cotyledon tTCLs. Besides, 250 µM of CdCl₂ is lethal for all tTCLs whatever the organ, and 200 µM is lethal only for cotyledon and petiole tTCLs but not for hypocotyl explants which had 3.6 % of frequency of shoot regeneration. Plantlets regenerated from all shoots, whatever the treatment, developed and flowered normally 6 weeks after the transfer to pots. The regenerated plants were fertile and identical to source plants.

**Keywords** Buds, neoformation, regeneration, transverse thin cell layer (tTCL), CdCl₂

**Abbreviations** BAP 6-benzylamino-purine; NAA α-naphtalenacetic acid; MS Murashige and Skoog’s medium (1962); PGRs Plant growth regulators; tTCL(s) Transverse thin cell layer(s)

I. INTRODUCTION

*Brassica* oilseed crops, *Brassica juncea*, *B. napus* and *B. rapa*, cover more than 11 million hectares of the world’s agricultural land and provide over 8% of the major oil when grown under a variety of climatic conditions (Downey, 1990). The annual production of *Brassica juncea* in India reaches alone five million tons (FAO, 2003).

In the recent past decades, *Brassica juncea* has drawn the attention of researchers because of its high biomass production with added economical value and its high capacity to translocate and cumulate 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 phytoremediation processes (Kumar et al. 1995; Salt et al. 1995).

Research focused mainly on the transgenic approach in order to increase the capacity of *Brassica juncea* or other plants to cumulate high metal levels (Lasat, 2002; Pilon-Smits et Pilon, 2002). However, to the best of our knowledge, there is no report on the *in vitro* selection of *Brassica juncea* using tTCLs excised from cotyledons, petioles and hypocotyls of young plants in the presence of Cd²⁺ which can result into a novel approach to obtain a regenerant cumulating more Cd than the source plants and use in many phytoremediation processes.

Cadmium like other metals such as Al, Cu, Pb and Zn is toxic for humans, animals and plants and is a widespread contaminant with a long biological half-life (Wagner, 1993; Das et al. 1997). Cd, that is easily absorbed by plants, has a major toxic effect on their photosynthesis and their growth (Prasad, 1995; Briat and Lebrun, 1999). The growth inhibition has been mainly studied on whole plants originating from bean seeds (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. 1995).
However, to the best of our knowledge, there is no report on the effect of Cd on plant regeneration by *in vitro* culture.

In the present paper, the effect of CdCl₂ on shoot regeneration (frequency of shoot regeneration and number of bud per explant) from tTCLs is investigated in Indian mustard.

**II. MATERIAL AND METHODS**

**Plant material**

*Brassica juncea* AB79/1 was used to evaluate shoot regeneration in presence or absence of CdCl₂. This cultivar is pure spring line, genetically fixed and was obtained by autofertilization.

**A. 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 20 g.l⁻¹ of sucrose and solidified with agar at 6 g.l⁻¹ (Kalys, HP 696). They were incubated later on under a photoperiod of 12h (60 µmol photon.m⁻²s⁻¹) provided by cool white fluorescent lamps, with a 22/20°C thermoperiod (light/dark).

tTCLs (400-500 µm) were excised from cotyledons, petioles and hypocotyls of 7 day-old *Brassica juncea* seedlings and cultivated in a Petri dishes containing MS medium (25 ml) (15 tTCLs per Petri dish). Cotyledon, petiole and hypocotyl tTCLs were cultivated on MS medium (comprising macronutrients, micronutrients and vitamins of Murashige and Skoog, 1962) supplemented with BAP (26.6 µM), NAA (3.22 µM), Sucrose (20 g.l⁻¹), AgNO₃ (10 µM) and various concentrations of CdCl₂ (0-250 µM). 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°C for 20 min.

All cultures were incubated in the same conditions as previously described. The number of explants with shoot buds was recorded after 4 weeks culture and the number of adventitious shoots per tTCL were counted. After 4 weeks, shoots were separated and transferred to test tubes containing MS medium (10 ml) without any 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). All transferred plants flowered normally and were identical to source plants.

**III. MEASUREMENT OF Cd IN THE NEOFORMED SHOOTS**

The determination of cadmium concentrations in the different digested solutions was conducted by electrothermal atomic absorption spectrometry. A Perkin-Elmer SIMAA 6100 working in the single element monochromator mode was used for all atomic absorption measurements. At harvest (27 days after the initiation of tTCL culture), shoots were weighed and then 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).

**A. Data analysis**

The frequency of shoot regeneration and the number of shoots par tTCL was recorded from 5 replicates, each with 15 tTCLs per Petri dish. Each experiment was repeated 3 times with 3 independent runs. 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.

B. Results

Shoot regeneration occurred from tTCL explants 10 days after the tTCL initiation culture when the tTCLs were cultured in the absence of CdCl₂ whatever the source explants, but slightly later when this metal was applied. This inhibition of tTCL initiation culture was function of the metal concentration levels (Table 1). The largest number of occurrence was obtained at day-27 for all organ tested and whatever the final concentration of CdCl₂; the number of occurrence unchanged thereafter. When the optimal conditions of culture were present, we obtained the maximal frequency of tTCL forming buds (100, 98.87 and 93.78 % for cotyledon, petiole and hypocotyl tTCLs respectively) (Table 1). Optimal conditions of culture were obtained when MS solidified medium was supplemented with NAA (3.22 µM), BAP (26.6 µM), sucrose (2%, w/v) and AgNO₃ (10 µM) (unpublished data obtained in our laboratory). Furthermore, in the same conditions, the origin of tTCL explants affected the number of buds per explant. Indeed, with the hypocotyl tTCLs, we obtained the best number of buds, followed by petiole then cotyledon tTCLs (3.20 and 2.40 respectively).

Besides, 5 µM of CdCl₂ was enough to reduce significantly, the rate of shoot regeneration and the number of buds per tTCL explant except the number of buds per cotyledon tTCLs which stayed unchanged (Table 1). When the concentration of CdCl₂ increased, the frequency of shoot regeneration and the number of buds per explant decreased (Table 1). High Cd concentration (250 µM) in the culture medium was lethal for callus culture and blocked shoot regeneration. Depending on source explants, hypocotyl tTCLs were more tolerant to CdCl₂ than cotyledon and petiole tTCLs. Our results show that only hypocotyl tTCLs were able to regenerate in the presence of 200 µM of CdCl₂ (3.56% and 0.07 bud per explant) (Table 1).

In addition, Cd contents were estimated in shoot buds. We observed that the latter increased in shoots with the increase of metal concentrations in the culture medium (Table 2). The highest content was observed with 150 µM of CdCl₂. However, there is no difference between organs for Cd cumulation in neoformed buds (Table 2). Regenerated plantlets in the presence or the absence of CdCl₂, whatever its concentration, were able to grow and flower normally 6 weeks after their transfer in the greenhouse (Figures 1 and 2).

C. Discussion and conclusion

Based on the efficiency of the thin cell layer technology for the propagation of various plant species, even for the more recalcitrant ones, this study was undertaken to evaluate the effect of CdCl₂ on the frequency of shoot regeneration and bud numbers per tTCLs in *B. juncea* L. Czern. from tTCL explants excised transversally from young axenic plants.

In our experiments, we showed a maximal frequency of regeneration in MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), sucrose (2%, w/v) and AgNO₃ (10 µM). There is no significant difference in the frequency of shoot regeneration between the 3 organs tested. However, we observed a significant difference of bud numbers per explants between organs (Table 1). Indeed, for all organs, hypocotyl tTCLs exhibited the highest bud number per explant, followed by petiole and cotyledon tTCLs respectively. The effect of explant source on shoot regeneration was previously reported in traditional leaf and cotyledon explants in *B. juncea* var. Tsatsai (Guo et al. 2005), longitudinal thin cell layers (tTCLs) from stems (Klimaszewska and Keller, 1985; Nhut et al. 2003) and cotyledon explants (Ono et al. 1994) and more recently, in hypocotyl and petiolar transversal thin cell layers (tTCLs) in *B. napus* L. (Ben Ghnaya et al. 2008).

The presence of CdCl₂ in the culture medium showed a negative impact on, both, the frequency of shoot regeneration and the number of buds per tTCL, even when the concentration of CdCl₂ was low (e.g. 5 µM). High concentrations of CdCl₂ (200 and 250 µM) were critical for the survival and the neoformation process of tTCL explants except hypocotyl tTCLs which were able to regenerate at 200 µM of CdCl₂ (Table 1). Therefore, these results showed source explant variation in response to this metal. To the best of our knowledge, there is no report on this explant variation response to CdCl₂ in *B. juncea* L. Czern.
It was known that cadmium is not essential for plant growth and development, and it was shown to inhibit the growth of many plant 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). However, all these studies on Cd toxicity were performed in plants which came directly from seeds. We believe, as well as Gladkov (2007), are the first authors who obtained regenerated plants in the presence of Cd. Indeed, Gladkov (2007) showed that, the inhibitory effect of Cd on Agrostis stolonifera and red fescue (Festuca rubra) callus cultures, was observed at Cd$^{2+}$ concentration of 7 mg.l$^{-1}$; at 20 and 30 mg.l$^{-1}$, considerable proportions of callus cells darkened and died, and a concentration of 60 mg.l$^{-1}$ was lethal to the cultures. He obtained some regenrated plants (30 from A. stolonifera and 4 from F. rubra).

In our study, we observed that neoformed buds were able to accumulate Cd$^{2+}$ in their tissues. This accumulation was function of Cd concentration. Therefore, an increase of Cd amounts is correlated to an increase of concentration of Cd in the culture medium. These results suggest a translocation of Cd from medium to calli and then to the buds.

Regenerated plantlets were able to grow and flower normally 6 weeks after their transfer in the greenhouse and that whatever the concentration of CdCl$_2$ and their identical to source plants (Figures 1 and 2).

To conclude, our model of shoot regeneration in the presence of Cd should be considered as a novel approach to in vitro selection of tolerant regenerants which should be used in different phytoremediation processes to test their ability in the depollution of contaminated soils and their capacity to cumulate heavy metals. We suggest that in vitro selection process using tTCL technology in presence of heavy metals is a novel interesting alternative which, in the near futur, can be used as the transgenic approach for improving the capacity of B. juncea or other hyperaccumulator plants to tolerate, cumulate and translocate more Cd or other heavy metals than the original wild type.

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### TABLE I: Effect of CdCl$_2$ on *in vitro* organogenesis from tTCLs of cotyledons, petioles and hypocotyls.

| CdCl$_2$ Concentration (µM) | Cotyledons Shoot regeneration frequency (%) | Petioles Hypocotyls | Cotyledons Petioles Hypocotyls | Number of buds per regenerating tTCL |
|-----------------------------|--------------------------------------------|---------------------|---------------------------------|-------------------------------------|
| 0                           | 100*a                                     | 98.87*b            | 93.78*c                         | 2.40*d                              |
| 5                           | 86.22*b                          | 80.89*c            | 77.33*d                         | 2.36*c                              |
| 25                          | 85.78*b                          | 67.56*c            | 67.11*c                         | 1.73*d                              |
| 50                          | 70.22*b                          | 63.56*c            | 66.67*c                         | 0.97*e                              |
| 100                         | 22.20*b                          | 31.11*d            | 35.56*d                         | 0.29*g                              |
| 150                         | 0.44*a                           | 21.30*e            | 23.30*e                         | 0.04*h                              |
| 200                         | 0.49*h                           | 0.49*h             | 0.49*h                          | 0.07*h                              |
| 250                         | 0.49*h                           | 0.49*h             | 0.49*h                          | 0.07*h                              |

Data (shoot regeneration frequency and number of buds per regenerating tTCL) were collected after 4 weeks of culture on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2% (w/v) and AgNO$_3$ (10 µM) and various concentrations of CdCl$_2$ (0-250 µM).

The results were calculated from three independent experiments, each with, at least, five dishes with 15 tTCLs per dish. For each parameter, the values with different letters are significantly different at $p = 0.05$ (LSD test).

Data (Cd contents in regenerated shoots) were collected after 27 days of culture on MS basal medium supplemented with NAA (3.22 µM), BAP (26.6 µM), Sucrose 2% (w/v), AgNO$_3$ (10 µM) and various concentrations of CdCl$_2$ (0-150 µM).

The results were calculated from three independent experiments, each with, at least, five dishes with 15 tTCLs per dish. For Cd contents, the values with different letters are significantly different at $p = 0.05$ (LSD test).
TABLE II: Cadmium contents in regenerated shoots obtained from 27 day-old tTCLs.

| CdCl₂(µM) | Cd content per bud (µg.g⁻¹ D.W.) |
|-----------|----------------------------------|
|           | Cotyledons | Petioles | Hypocotyls |
| 0         | 0          | 0        | 0          |
| 25        | 29.23d     | 30.43d   | 33.01d     |
| 50        | 76.45c     | 78.57c   | 82.84c     |
| 100       | 166.32b    | 168.45b  | 172.34b    |
| 150       | 278.87a    | 281.56a  | 285.87a    |

FIG. 1: Shoot regeneration from tTCLs of Indian mustard in the absence of CdCl₂. (A) : cotyledon tTCLs ; (B) : petiole tTCLs ; (C) : hypocotyl tTCLs ; (D) : regenerated plants grown and flowered in the greenhouse 6 weeks after the transfer in pot.
FIG. 2: Regenerated plants grown and flowered normally in the greenhouse after 6 weeks after transfer in pot. (A) : Shoot regeneration from cotyledon tTCLs in the presence of 150 $\mu$M of CdCl$_2$ ; (B) : Shoot regeneration from petiole tTCLs in the presence of 150 $\mu$M of CdCl$_2$ ; (C) : Shoot regeneration from hypocotyl tTCLs in the presence of 200 $\mu$M of CdCl$_2$. (bar : 10 cm)