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Variations in Membrane Lipid Metabolism in *Brassica juncea* and *Brassica napus* Leaves as a Response to Cadmium Exposure

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Abstract: This research aims to examine the effect of cadmium on membrane lipid biosynthesis, lipid peroxidation levels and the reduced thiol (-SH) content in leaves of Indian mustard (*Brassica juncea*) and oilseed rape (*Brassica napus*) treated plants. Two weeks-old-plants from each specie are subjected to various $CdCl_2$ concentrations (0, 10, 25 and 50 μM) in the hydroponic medium, for 15 days. Results showed a marked differences between both species. Total lipid content decrease dramatically in *Brassica napus* leaves. Whereas, amount of total lipids was significantly increased in *Brassica juncea* treated plants. Compared to *Brassica juncea*, *Brassica napus* treated plants showed a higher lipid peroxidation level expressed as malondialdehyde production. Studies of the lipid metabolism using radioactive labelling with (1-^{14}C) acetate as a major precursor of lipid biosynthesis, showed that levels of radioactivity incorporation in total lipids of *Brassica napus* leaves were lowered by Cd doses. While they were enhanced in *Brassica juncea* treated plants. Moreover, results led to the conclusion that, under heavy metal stress, considerable changes in polar lipids metabolism were observed in both species. In *Brassica napus* treated plants, cadmium stress provoked an inhibition of the chloroplastic lipids biosynthesis pathway while a stimulation of the extra-chloroplastic lipid metabolism was occurring. However, in *Brassica juncea* treated plants, the both pathways were stimulated under cadmium treatment. Level of reduced thiol content in roots and leaves of *Brassica juncea* treated plants was found more higher at all Cd treatment as compared to *Brassica napus*. The tolerance exhibited by *Brassica juncea* to Cd toxicity may be attributed to the enhanced lipid synthesis leading to a better compartimentalization of Cd ions as Cd-phytochelatin complexes in the cell vacuoles or in the vesicles formation.

Key words: *Brassica juncea*, *Brassica napus*, Cadmium, leaves, lipid biosynthesis, reduced thiol content

INTRODUCTION

Cadmium (Cd), which is termed a heavy metal or a metal trace element, is dispersed in natural and agricultural environments principally through human activities such as mining, refining, municipal waste incinerators and fossil fuel combustion sources (Wagner, 1993), as well as natural rock mineralization processes (Sanità di Toppi and Gabrielli, 1999). Major inputs of Cd into agricultural soils are due to the application of phosphatic fertilizers (Williams and David, 1976; McLaughlin *et al*., 2000), soil amendments with municipal sewage sludges and atmospheric deposition (Wagner, 1993; Weissenhorn and Leyval, 1995). Since Cd poses a serious human health risk (Benoff *et al*., 2000; Satarug *et al*., 2000) and since it can be easily incorporated into the human food chain through uptake by agronomic crops or through grazing of contaminated plants by herbivores. Phytoremediation as an environmental cleanup technology was initially proposed for the remediation of metal-contaminated soil (Chaney, 1983; Baker *et al*., 2000). The identification of metal hyperaccumulators, plants capable of accumulating extraordinarily high metal levels, demonstrates that plants have the genetic potential to clean up contaminated soil. *Brassica juncea* (Indian mustard) can accumulate heavy metals, including cadmium (Kumar *et al*., 1995), from environments polluted by industrial wastes or mining activities.

While some research efforts have been directed at the physiology and chemistry of Cd-accumulating plant species (Sanita di Toppi and Gabrielli, 1999; Clemens, 2001), research on the role of membranes and the

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metabolism of lipids (as major membrane components) is needed for several reasons: (i) cell membranes are major targets of environmental stresses and the alterations in the composition of the plasma membrane may change membrane permeability and, consequently, net metal ion uptake (Cumming and Taylor, 1990); (ii) the level of lipid peroxidation which can alter many metabolic processes in the cell is determined, to an important extent, by the degree of fatty acid unsaturation of membrane lipids. Membrane unsaturation has been shown to be closely related to heavy metal tolerance in a number of higher plants, algae and micro-organisms (Avery *et al*., 1996; Howlett and Avery, 1997a; Maksymiec, 1997) and (iii) the rapid turnover of membrane components may represent a strategy for adaptive modification to metal stress.

Moreover, several studies have shown that Cd causes physiological and biochemical deteriorations in treated plants (Baryla *et al*., 2001; Mallick and Mohn, 2003; Sanita di Toppi and Gabrielli, 1999; Poschenrieder and Barcelo, 2004) and many of these effects can be interrelated through a general action on membrane biogenesis and integrity which in turn can occur because lipid metabolism is altered. Indeed, alterations in the activities of enzymes such as fatty acid synthase and oleoyl-ACP desaturase have been noted previously (Jones *et al*., 1987; Jones and Harwood, 1993). Many of the above activities are probably related to the affinity of heavy metals for sulphydryl groups, which results in inhibition of the active sites of enzymes and/or conformational modifications of macromolecules (Cumming and Taylor, 1990).

Based on previous data (Nouairi *et al*., 2005, 2006) it was anticipated that fatty acid composition might be a significant target of heavy metal exposure. It has been shown that lipid changes in *Brassica juncea*, the well known Cd-hyperaccumulator specie, revealed a more stability of its cellular membranes to cadmium-stress as compared to *Brassica napus* (Nouairi *et al*., 2006).

In order to understand how membrane components may be involved in *Brassica juncea* and *Brassica napus* plants to cadmium exposure, the patterns of lipid labelling were studied in the presence or absence of Cadmium. We have also studied the effects of metal on lipid peroxidation and reduced thiol content in treated plants.

MATERIALS AND METHODS

All experiments were conducted during the year 2005, in a controlled greenhouse chamber at the Centre of Biotechnology, Technopark of Borj-Cedria, located in the southern suburbs of Tunis.

Plant material: Seeds of Indian mustard (*Brassica juncea*, accession No. 426308) identified as a metal accumulator (Kumar *et al*., 1995) were obtained from the North Central Regional Plant Introduction Station (Ames, IA) and oilseed rape (*Brassica napus* L., cv Drakkar) were germinated on vermiculite and grown hydroponically for 2 weeks in black containers with 6l of continuously aerated nutrient solution (pH 5.5) contained 136 mg L^{-1} KH_2PO_4, 492.3 mg L^{-1} $Ca(NO_3)_2$, 303 mg L^{-1} KNO_3, 17.4 mg L^{-1} K_2HPO_4, 146 mg L^{-1} $MgSO_4$, 1.98 mg L^{-1} $MnCl_2$, 0.294 mg L^{-1} $CuSO_4$, 0.287 mg L^{-1} $ZnSO_4$, 1.85 mg L^{-1} H_3BO_3, 0.37 mg L^{-1} $(NH_4)MoO_4$ and 20 M Fe-EDTA. The containers were placed in a greenhouse chamber with day/night temperature and humidity regime of 25/20°C and 55/75% Rlative Hmidity (RH), respectively. A 16 h (daily) photoperiod was used. The nutrient solution was changed every 3 days until the experiment was started. Cadmium was added to the medium as $CdCl_2$ in four concentrations: 0, 10, 25 and 50 μM. During treatment with $CdCl_2$, the hydroponic medium was changed daily to avoid depletion of Cd in nutrient solution. After 15 days of heavy metal treatment, samples were harvested and used for chemical analyses.

Lipid extraction and analysis: The lipids were extracted according to the method of Folch *et al*. (1957) modified by Bligh and Dyer (1959) Measurements were carried out on the second pair of leaves grown after Cd treatment of each species. The plant tissues were fixed in boiling water for 5 min to denature phospholipases (Douce, 1964) and then homogenized in chloroform/methanol mixture (2/1, v/v). The homogenate was centrifuged at 3000 g for 15 min. The lower chloroformic phase containing lipids was aspirated and evaporated at 40°C under vacuum using a rotary evaporator or with Nitrogen gas. The residue was immediately redissolved in 2 mL of toluene/ethanol mixture (4/1, v/v) for conservation. Fatty acids from total lipids were methylated by the method of Metcalfe *et al*. (1966). Methyl esters of fatty acids were separated and quantified with a Hewlett-Packard model 4890D gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 μm film thickness fused Silica capillary column (Innowax) coupled to a flame ionisation detector (Column temperature 210°C). Both the injector and detector were maintained at 230 and 250°C, respectively. Nitrogen was used as the carrier gas at 1 mL $^{-1}$ min with split injector system (split ratio 1:100). For measuring the amount of fatty acids, heptadecanoic acid C17:0 was added as internal standard before methylation. Calculation of fatty acid contents was obtained using an integrator.

Radiolabelling protocol: (1-^{14}C)Acetate (1.95 GBq mmoL^{-1}) was purchased from Amersham International p.l.c., (Amersham, Bucks, UK). Radioactive labelling of lipids was achieved by laying (1-^{14}C)acetate microdroplets, on young leaves of either control or treated plants, using a Hamilton syringe. After incubation at 2, 6, 12 and 24 h, leaves were washed in deionized water and total lipids (TL), were extracted. Phospholipids (PL), glycolipids (GL) and neutral lipids (NL) were separated by TLC on silica gel plates 60 (Merck) according to the method of Lepage (1967). After development, bands were located with iodine vapours or spraying the plates with 0.1% Rhodamine 6G in ethanol. Individual lipids were identified by comparison with lipid standards and by specific strains for PL and GL. The TL, GL, PL and NL-associated radioactivity was determined by liquid scintillation counting (Beckman LS 6500 Liquid Scintillation Counter).

Malondialdehyde determination: The amount of lipid peroxidation was assessed using thiobarbituric acid (TBA) assay, in which malondialdehyde (MDA) is quantified as an end product according to Heath and Packer (1968). MDA content was expressed on the basis of initial fresh weight (FW).

Measurement of reduced thiol content: Non-protein thiols (SH) content of *Brassica napus* and *Brassica juncea* leaf samples were determined colorimetrically with 5–5'dithiobis [2-nitrobenzoic acid] (DTNB), essentially as described by De Kok *et al.* (1988).

RESULTS

Fatty acid composition: Table 1 shows the fatty acid composition of total lipids, the fatty acids most subjected to variation in *Brassica napus* treated plants were linoleic (C18:2) and linolenic (C18:3) acids. In leaves of plants treated with high Cd doses, the percentage of C18:3, the abundant fatty acid, decreased and that of C18:2 increased. On the other hand, Cd treatment did not induce significant changes in the fatty acid composition of total membrane lipids and remained unchanged in leaves of *Brassica juncea* plants treated with high metal doses (Table 1).

Lipid biosynthesis and peroxidation: The results given in Fig. 1 indicate a dramatic decrease by 52% of the total lipid content, which is evaluated from the amount of total fatty acids, in the young leaf of *Brassica napus* plants grown at 50 μM Cd for 15 days. While it increased by 19% in *Brassica juncea* leaves in comparison with control plants (0 μM Cd) for each species, respectively.

On the other hand, MDA, which is a degradation product resulting from membrane lipid peroxidation, was accumulated in leaf tissue at $CdCl_2$ concentrations higher than 10 μM. At 50 μM Cd, the level of MDA accumulation represented more than 600% of the control in *Brassica napus* leaves (Fig. 2). However, in *Brassica juncea* leaves, Cd treatment did not significantly affect the MDA content and remained unchanged with the increase of external metal concentration.

In order to monitor rapidly any disturbances to metabolism, radiolabelling from (1-^{14}C)acetate was used. This precursor has been well justified for lipid labelling in plant tissues (Roughan and Slock, 1982). Moreover, this aspect was particularly interesting because heavy metals have often been noted to change fatty acid biosynthesis in plants (Jones *et al*., 1987; Maksymiec *et al*., 1992; Jones and Harwood, 1993). As can be seen from Fig. 3A, radioactivity incorporation in total lipids of *Brassica napus* leaves decreased as compared to that of the control. Moreover, the high cadmium dose (50 μM) depressed radioactivity incorporation for all incubation times. Whereas, in *Brassica juncea* treated plants, there was an increase of the total radioactivity incorporation with increasing Cd concentrations in the nutrient solution at all incubation times (Fig. 3B). This result can explain the increase of non-labelled lipids observed in leaves of *Brassica juncea* treated plants (Fig.1).

In order to evaluate specific effects of heavy metal on lipid metabolism, lipids were separated into neutral (NL, neutral lipids) and polar (GL, glycolipids and PL, phospholipids) classes. The kinetics of (1-^{14}C)acetate incorporation in lipid classes showed that radiolabelling in GL, the main lipids of plant leaves and NL decreased

Table 1: Fatty acid composition in total lipids of *Brassica napus* and *Brassica juncea* leaves from plants treated with different Cd concentrations

	Cd (μM)	C16:0	C16:1c	C16:1t	C16:3	C18:0	C18:1	C18:2	C18:3
Brassica bapus	0	17.30^{b}	0.90^{a}	1.30^{a}	13.60^{a}	2.11^{c}	5.11^{c}	13.32^{a}	44.90^{a}
	10	16.75^{b}	0.73^{ab}	1.12^{a}	5.19^{c}	4.90^{b}	9.09^{a}	20.12^{b}	36.86^{b}
	25	20.91^{a}	0.63^{b}	1.00^{a}	9.01^{b}	4.10^{b}	6.25^{b}	17.99^{b}	38.37^{b}
	50	18.21^{b}	0.31^{c}	0.66^{c}	5.80^{c}	7.02^{a}	7.09^{ba}	28.15^{a}	32.91^{c}
Brassica juncea	0	16.70^{b}	0.51^{a}	2.77^{a}	14.60^{a}	1.61^{b}	1.90^{b}	12.70	49.00^{a}
	10	18.45^{a}	0.50^{a}	2.31^{a}	10.81^{b}	2.80^{a}	2.80^{a}	17.32^{a}	44.50^{a}
	25	18.90^{a}	0.50^{a}	2.20^{a}	11.40^{b}	2.70^{a}	2.20^{ab}	16.70^{a}	45.90^{a}
	50	17.71^{ba}	0.42^{b}	2.30^{a}	11.81^{b}	2.84^{a}	1.90^{b}	15.91^{a}	46.50^{a}

Data's are means of three replications. For comparisons among means an analysis of variance was used. For each treatment means in rows followed by different letters are significantly different at p = 0.05, c: cis, t: trans

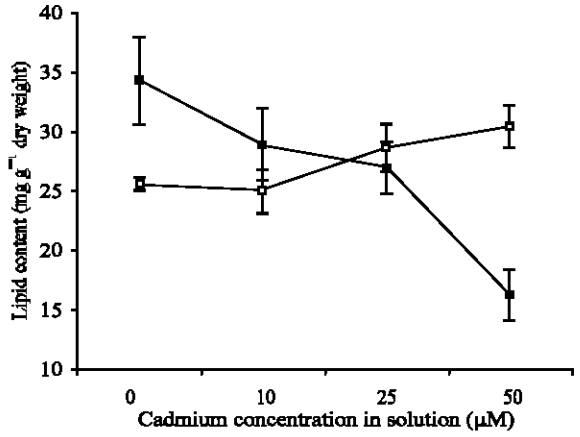

Fig. 1: Total lipid content of young leaves of *B. juncea* (□) and *B. napus* (■) exposed to various Cd concentrations in the nutrient solution for 15 days. Lipid content was estimated from the amounts of fatty acids. Data represent the means±SD of three independent experiments

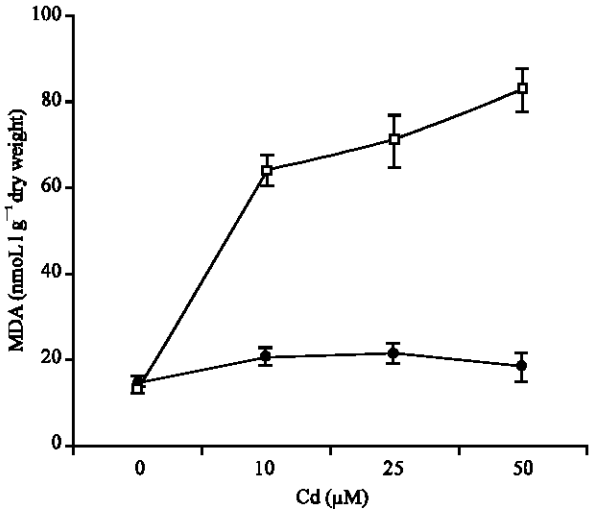

Fig. 2: MDA content of young leaves of *Brassica juncea* (●) and *Brassica napus* (□) exposed to various Cd concentrations in the nutrient solution for 15 days. Data represent the means±SD of three independent experiments

for all times of incubation in *Brassica napus* treated plants. So, the amount of radioactivity incorporation decreased by 50 and 45% in GL and NL, respectively at the higher Cd dose (50 µM). While radioactivity

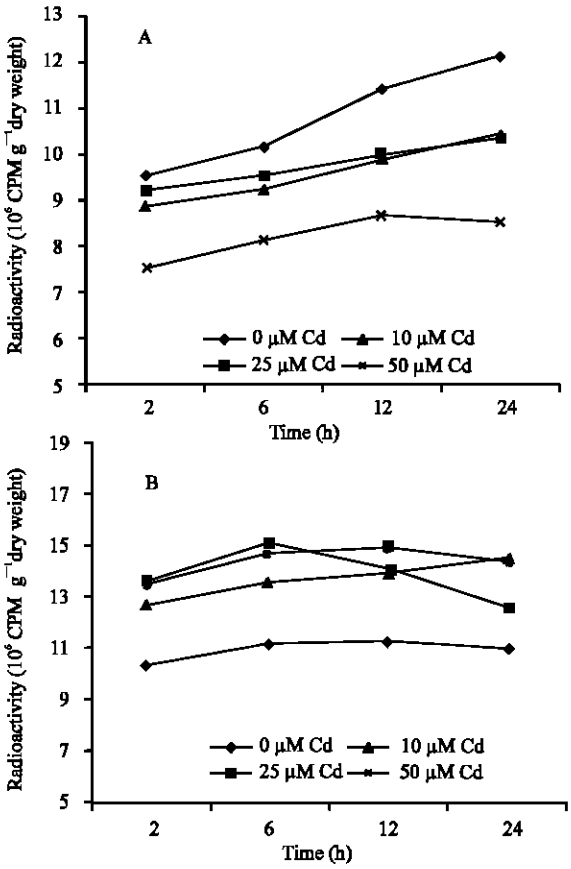

Fig. 3: Radioactivity incorporation in total lipids of *Brassica napus* (A) and *Brassica juncea* (B) leaves exposed to various Cd concentrations in the nutrient solution for 15 days. Data represent the means of three independent experiments (results expressed in Counts Per Minute, CPM)

incorporation in phospholipids increased slightly in radiolabelled *Brassica napus* young leaves (Fig. 4).

In contrast to *Brassica napus*, the incorporation of (1-^{14}C) acetate into glycolipids, phospholipids and neutral lipids of *Brassica juncea* was stimulated in cadmium treated plants for all times of incubation (Fig. 5) as compared with controls.

Reduced thiol content: The reduced thiol content in shoots and roots of *Brassica juncea* treated plants increased after Cd treatments but it was low and unchanged in *Brassica napus* tissues (Fig. 6A and B). The level of reduced thiol content in the shoots and roots of *Brassica juncea*, reached, respectively a value about 19

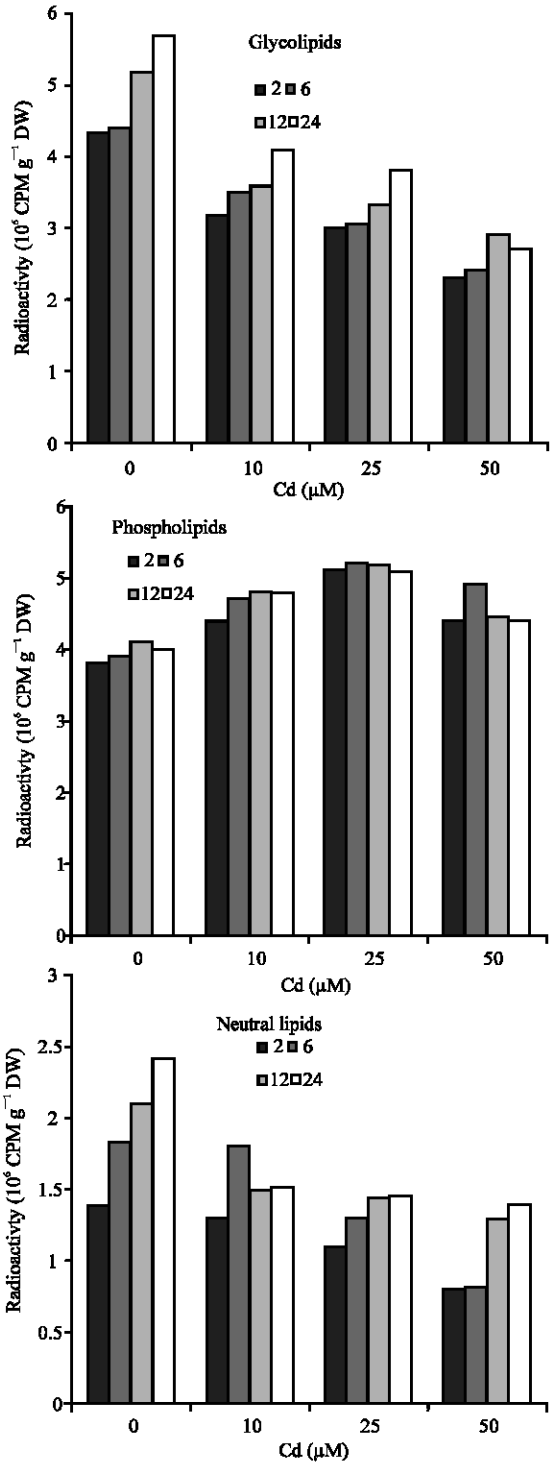

Fig. 4: Radioactivity incorporation in glycolipids (GL), phospholipids (PL) and neutral lipids (NL) of *Brassica napus* leaves exposed to various Cd concentrations in the nutrient solution for 15 days. Data represent the means of three independent experiments. DW: Dry Weight

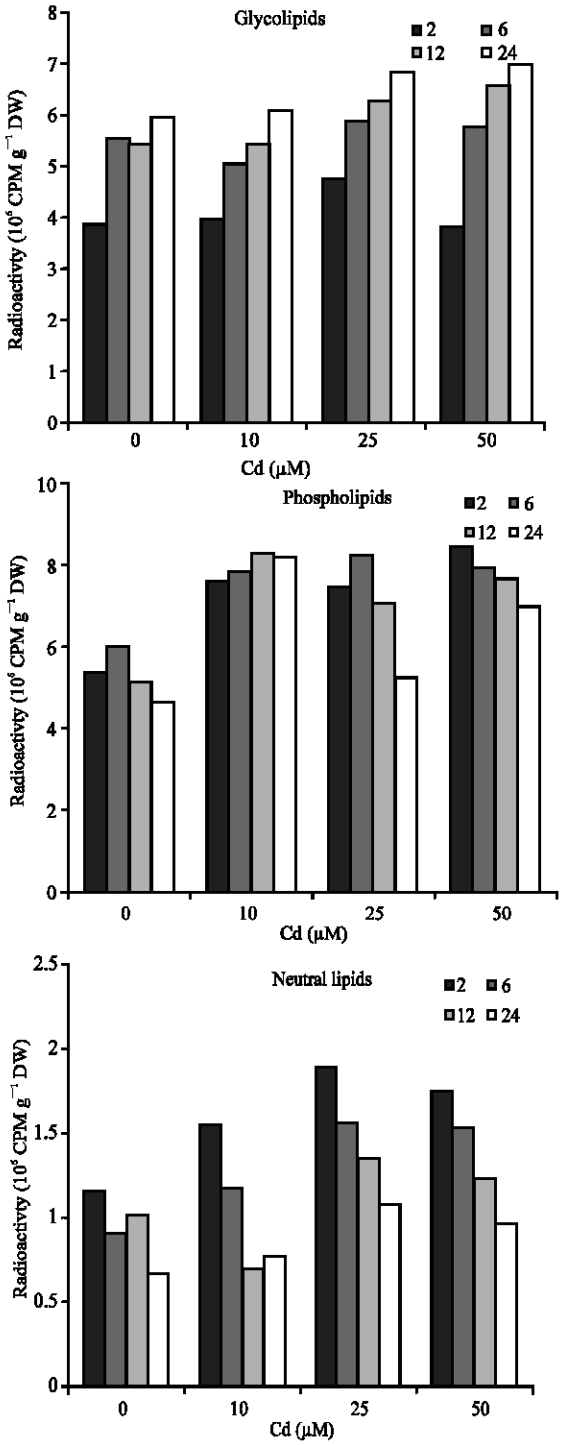

Fig. 5: Radioactivity incorporation in glycolipids (GL), phospholipids (PL) and neutral lipids (NL) of *Brassica juncea* leaves exposed to various Cd concentrations in the nutrient solution for 15 days. Data represent the means of three independent experiments

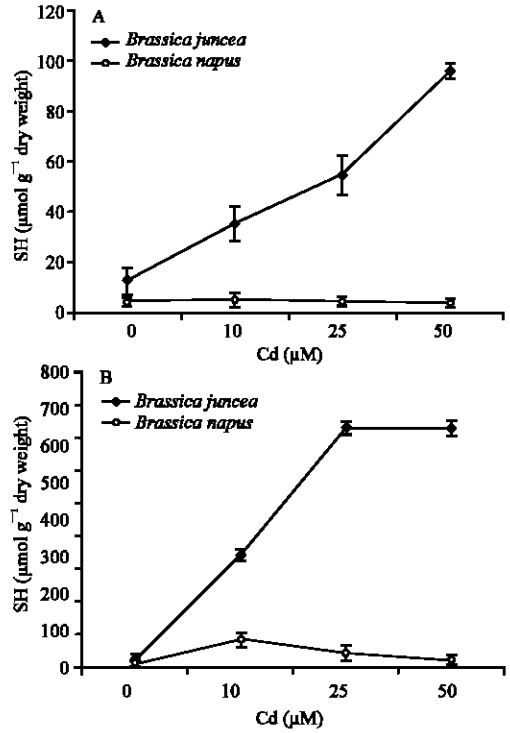

Fig. 6: Changes in reduced thiol concentrations in leaves (A) and roots (B) of *Brassica juncea* and *Brassica napus* plants after 15 days of treatment with different concentrations of cadmium. Data represent the means±SD of three independent experiments

and 36-fold higher than that in *Brassica napus* at the higher Cd concentration (50 µM).

DISCUSSION

Plant cell membranes are dynamic in behaviour, with a lipid composition changing with variations in the environment and may be considered as the first « living » structure that is target for heavy metal toxicity (Harwood, 1995). In previous study, a direct comparison of *Brassica juncea* and *Brassica napus* in hydroponic solution containing Cd (0, 10, 25 and 50 µM) showed that *Brassica juncea* appeared to accumulate three times more Cd in the shoots than *Brassica napus* (values for 15 days exposure: 1450±245 and 555±120 µg Cd g^{-1} dry weight, respectively when plants were exposed to 100 µM Cd) (Nouairi *et al*., 2006). For this reason, *Brassica juncea* may be useful in some phytoremediation strategies in regions with excessive cadmium accumulation and can be defined as an hyperaccumulator plant as it accumulates at least 1000 ppm metal in the shoots (Ernst, 1993).

In this study we have found that total fatty acid composition was changed in treated plants and was remarkably dependent on metal doses and plant species. In leaves of *Brassica napus* plants treated with high Cd doses, the percentage of C18:3, the abundant fatty acid, decreased. This agreed with the reported effects of heavy metals in a variety of plant species, where they reduced formation of polyunsaturated fatty acids (Harwood, 1998). The decrease of polyunsaturated fatty acid (C18:3) level, might be related to direct reaction of oxygen free radicals with unsaturated lipids. However, the observed accumulation of C18:2 rather than C18:3 in leaves of *Brassica napus* treated plants does not exclude an alteration of fatty acid desaturase activity. The same tendency was reported in leaves of some sensitive halophytes plants to Cd stress (Nouairi *et al*., 2005). On the other hand, Cd treatment did not induce significant changes in the fatty acid composition of total membrane lipids and remained unchanged in leaves of *Brassica juncea* plants treated with Cd (Table 1). A similar result has been seen for the halophyte *Sesuvium portulacastrum* under Cd stress (Nouairi *et al*., 2005). This stability of total fatty acid composition observed in *Brassica juncea* leaves showed to be closely related to heavy metal tolerance in plants (Howlett and Avery, 1997).

The drastic alteration in lipid content of *Brassica napus* leaves can be explained by Cd inducing disturbance of the membrane lipid turnover. Indeed, when the polar lipid classes were examined, greater effects were found following cadmium exposure. *Brassica napus*, which had grown under cadmium treatment, showed decreased labeling of typical chloroplast lipid components (e.g., glycolipids: Harwood, 1980) which could, perhaps, be due to inhibited turn-over of components of the thylakoid membranes caused by metal stress. In fact, chloroplast metabolism and function has been shown to be sensitive to heavy metal pollution in a variety of lower and higher plants species (Krupa and Baszynski, 1989; Maksymiec *et al*., 1992; Stefanov *et al*., 1995; Wells and Brown, 1995). However, we have showed that phospholipids (as major components of the extrachloroplastic compartment) remained more stable in rape leaves (Fig. 3A). It can be concluded that the extraplastidic compartment, is rather resistant to cadmium stress. This agreed with previous works reported that the main chloroplastic glycerolipids, namely galactolipids, showed a reduced content, but a phospholipids such as phosphatidylcholine (PC) could either be more (Chetal *et al*., 1980) or less abundant (Dakhama *et al*., 1995). These divergent observations could be linked to

the presence of two distinct pathways of the biosynthesis of glycerolipids (Benhassaine-Kesri *et al*., 2002). In other hand, it has been established that cadmium metal enhanced lipoxygenase activity, which is responsible for catalysing lipid peroxidation by using membrane lipid components as substrates, particularly unsaturated fatty acids (Howlett and Avery, 1997; Quartacci *et al*., 2000), which can explain the high level of MDA (as a major cytotoxic product of lipid peroxidation and acts as an indicator of free radical production) contents in *Brassica napus* treated leaves (Fig. 2). This finding suggested that cadmium treatment caused a lipid biosynthesis inhibition (Fig. 3A and 4) as well as a lipid peroxidation in *Brassica napus* leaves.

In contrast to *Brassica napus*, cadmium treatment induced an enhancement in lipid biosynthesis (Fig. 3B and 5), which explain the increase in non-labelled total lipid content (Fig. 3) and did not significantly affect the MDA content (Fig. 2) of *Brassica juncea* treated plants. The Cd-induced increase in total lipid levels might stimulate membrane biosynthesis, which is essential to vesicles formation involved in compartmentalization and vacuolar storage of Cd-phytochelatin complexes (Vögeli-Lange and Wagner, 1990). Moreover, results showed that the intensity of protection via thiolate formation is higher in *Brassica juncea* treated plants (Fig. 5A and B). The reduced thiol content is much more increased in *Brassica juncea* roots and shoots showing a greater induction of phytochelatins (Sanità di Toppi *et al*., 1999). These results concerning the thiol content suggest the existence, in *Brassica juncea* plants, of a defense strategy relies more on thiol induction and metal binding leading to heavy metal avoidance. Indeed, earlier studies showed that the vacuole is the site for the accumulation of a number of heavy metals including Zn and Cd (Cobbett and Goldsbrough, 2002; Heiss *et al*., 2003), this way can be considered as a very important mechanism of heavy metal tolerance and several studies indicate that heavy metal resistance is one of the prerequisites of metal chelation and sequestration in plants (Krämer *et al*., 1997; Raskin *et al*., 1997). The increase in total lipid content and the enhancement of chloroplastidic and extra-chloroplastidic lipid synthesis was in good correlation with the induction of the reduced thiol content in *Brassica juncea* treated plants. These results, may be considered as a defence strategy developed by the plant to tolerate metal.

CONCLUSIONS

The results observed in *Brassica juncea* plants, showed that this species may have an efficient defence strategies which can be related to a better endocellular compartmentalization of the chemical pollutant and may be an efficient antioxidative mechanisms against oxidative stress.

These results indicate that *Brassica juncea*, as compared to *Brassica napus* plant, have an extraordinary ability to tolerate and take up Cd from the polluted soils and may be useful for phytoextraction of metal in polluted area.

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