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Lead effects on *Brassica napus* photosynthetic organs

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ABSTRACT

In this study, effects of lead on ultracellular structure and pigment contents of *Brassica napus* were examined. Pb (II) was added in soluble form to soil prior to sowing. Pb contents were measured in plant organs at the ontogenetic stages of flowering (FL) and physiological maturity (PM). Pigment contents were evaluated through reflectance measurements. Pb content in organs was found to decrease in the order; roots > stems > leaves. Lead content in senescent leaves at FL stage was significantly higher than harvested leaves, strongly suggesting a detoxification mechanism. Leaves and stems harvested at the PM stage showed damage at subcellular level, namely chloroplast disorganization, cell wall damage and presence of osmiophilic bodies. Chlorophyll content increased in the presence of Pb at the FL stage, compared with control; at the PM stage, chlorophyll contents decreased with low Pb concentration but showed no significant differences with control at high Pb soil concentration. The results suggest an increase in antioxidants at low Pb concentration and cell damage at higher lead concentration.

1. Introduction

Lead is a highly toxic metal, which can be harmful even at trace level, if bioaccumulation is allowed for a long time (Wong et al., 2006). The presence of lead in the environment may be due either to natural processes or to a wide variety of mining, industrial and urban human activities (Ferreyroa et al., 2014). Soil is one of the major environmental compartments for accumulation of this and other metals. From soil this pollutant, generally as Pb(II), can be absorbed by plants. Lead presents a wide range of behaviors in terms of absorption, accumulation, location and effects on plants, with great variability in tolerance. In general, plants exposed to high heavy metal levels show toxicity symptoms, reduced height, decreases in root and aerial biomass growth and yield, and even death (Adriano, 2001).

Several authors have reported negative effects on photosynthetic rate in different species grown in media with high concentrations of Pb and other heavy metals (Briat and Lebrun, 1999). This effect is associated with the destruction of the chloroplasts ultrastructure, the decrease in the concentrations of chlorophylls *a* and *b* and lower antioxidant catalase activity (Islam et al., 2008; Ali et al., 2013; Tian et al., 2014).

In the presence of pollutants such as Pb, some higher plant species have developed strategies aimed to metal accumulation in root, in order to avoid the transport to aerial biomass. In other species a high concentration is reached in leaves, affecting the plant, and several detoxification strategies are observed (Dahmani-Muller et al., 2000).

Few studies have shown how plants absorbed heavy metals in different growth stages. Studying metal accumulation during growth of the hyperaccumulator *Thlaspi caerulescens* (Perronnet et al., 2003) it was reported that accumulation was metal dependent and varied with different plant organs and plant age considered. In another study (Dinelli and Lombini, 1996) it was found that metal concentrations in plant organs were higher in early vegetative growth stage along with a relatively high nutrient uptake compared to growth rate and lower when plants flowered.

Reflectance spectroscopy of plant organs, mainly leaves, has been extensively employed for years to assess the state and contents of pigments (Cordon et al., 2010; Gamon et al., 1990; Gausman and Allen, 1973), because leaf reflectance in the UV and visible part of the electromagnetic spectrum is related to the presence of pigments (mainly

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chlorophylls, carotenoids and anthocyanins). On the other hand, reflectance in the near infra-red region (NIR) between 700 and 1300 nm is strongly dependent on leaf structure (Gausman and Allen, 1973). At longer wavelength (1200–2500 nm), water absorption peaks are observed (Kumar et al., 2002). In addition, variations in plant reflectance are related to changes in the physiological state of the plant. These optical signals are powerful indicative tools because of their potential use on the non-destructive diagnosis of plant health and in remote sensing.

Several parameters are obtained from reflectance measurements which are useful in the study of plant physiology and health (Gamon et al., 1992; Yaryura et al., 2009). One of them, of useful and important quality, is the remission function ($F(R)$) which is directly related to the chromophore concentration and may be easily obtained from reflectance data; this function represents the absorption spectrum for an opaque solid and it is directly proportional to the chromophore concentration when no light is transmitted through the sample (Cordon et al., 2010; Cordon and Lagorio, 2007). Another one is the photochemical reflectance index (PRI), an index based on leaf reflectance at 531 nm and 570 nm. The first band at 531 nm is associated to the absorption of carotenoids and chlorophyll; the 570 nm band is considered as a reference band and corresponds to the chlorophyll absorption. On a diurnal time scale, PRI gives a measure of the photosynthetic light-use efficiency, as is related to the xanthophyll cycle activity, which adjusts the energy distribution at the photosynthetic reaction center. At larger time scales, PRI becomes dependent on pigment concentration and may act as an optical indicator of stress (Garbulsky et al., 2011). This index has been found to be suitable for tracking variations in photosynthetic activity from leaf to ecosystem levels (Peñuelas et al., 1995; Filella et al., 2009). Recently, it was shown that canopy PRI positively correlated with the quantum efficiency of photosystem II even when tissues at different ontogenetic stages were present (Cordon et al., 2016).

Different species of the *Brassicaceae* family have been identified as heavy metal accumulators, among them Pb. From several years ago, most research had been concentrated on eastern oil crop, namely Indian mustard (*Brassica juncea*) (Blaylock et al., 1997; Liu et al., 2000; Karak et al., 2013). The effect of Pb on rapeseed (*Brassica napus*), the equivalent western oil crop, and its capacity to accumulate it has been studied more recently but to a lesser extent, being also reported as highly tolerant to Pb intake (Solhi et al., 2005; Azimzadeh et al., 2014; Bilal Shakoor et al., 2014; Tian et al., 2014).

In Argentina, lead has been found mainly in field regions surrounding urban areas (Lavado et al., 1998; Bermudez et al., 2010; Rodriguez et al., 2014). Due to this fact and its high toxicity, in this work the effect of Pb accumulation on *Brassica napus* is studied, by analyzing the cellular ultrastructure and the content of photosynthetic pigments in leaves, in the plant ontogenetic stages of flowering (FL) and physiological maturity (PM).

2. Materials and methods

2.1. Soil

The employed soil was a Vertic Argiudol from the Solís town surroundings, Buenos Aires province, Argentina. This soil has a loamy texture, acid pH and high content of organic matter (OM) (Gonzalez et al., 2013). This soil has been characterized elsewhere (Ferreyroa et al., 2014); its main characteristics are presented in Table S1 (Supplementary material). Pb was not detected in this soil (< 10 mg kg^{-1}).

2.2. Experimental setup

The experiment was carried out in 3 L pots under a shelter with environmental conditions of light, humidity and temperature, between the months of June and November (Table S2, Supplementary material).

Pb(II) was added in the form of Pb(NO$_3$)$_2$ solution at the beginning of the experiment so as to reach the desired concentrations. The treatments were control soil (Co) and Pb-polluted soils containing 200 mg kg^{-1} of Pb (Pb-200), typical of polluted soils (Lavado et al., 1998). Additionally, some pots received treatments of 50 and 100 mg kg^{-1} (Pb-50 and Pb-100, respectively), for complementary reflectance measurements.

The spring *Brassica napus*, cultivar Legacy, was employed. The seeds have not received any treatment prior to sowing. The phenological development and ontogenetic stages were described following the scale of Miralles et al. (2003). Four seeds of *Brassica napus* were sown per pot at 1 cm depth approximately; when the seedlings reached four leaves they were thinned, leaving one plant per pot. The crop was irrigated with distilled water, according to plant water demand, keeping approximately constant soil water content at 70–80% of soil field capacity. Two harvests were performed: the first one at a hundred days after sowing, at the ontogenetic stage of flowering, and the second one at a hundred and forty days after sowing, in the ontogenetic stage of physiological maturity.

2.3. Total plant biomass

All plant organs, including roots, were harvested and its dry biomass was determined by drying in oven at 70 °C until constant weight. Results were analyzed by ANOVA (Graphpad Prism 7.02).

2.4. Pb contents in leaves, stems and roots

Pb content was determined in both senescent and functional leaves, stems and roots, of plants corresponding to the Pb-200 treatment at the two stages defined above. Senescent leaves were collected along the full growing period between harvests. Harvest biomass was washed with ultrapure water and then was oven-dried at 70 °C to constant dry weight, smashed, homogeneously mixed and stored until analytical determinations were carried out. The samples were digested following the USEPA 3050B method, and the Pb concentration was measured by flame atomic absorption using a Perkin Elmer 3110 equipment (DL < 0.5 mg kg^{-1}). T-test (Graphpad Prism 7.02) was performed to statistically compare lead content in senescent and functional leaves.

2.5. Transmission electron microscopy (TEM)

TEM observations were performed in the PM stage to study the metal effect over the cellular ultrastructure. Samples of leaves and stems of about 5 mm^2 were taken with a scalpel. These samples were cut in fragments of 1 mm^2, rinsed with distilled water and placed immediately in fixing solution (pH = 7.0, 0.1 M phosphate buffer with 2.5% paraformaldehyde and 2.0% glutaraldehyde) and left standing for 48 h. Afterward, they were cleaned with the phosphate buffer, and then preserved until the final treatment with osmium, resin inclusion and microtome cutting. Observation was performed with a Zeiss EM 109 T TEM, equipped with a digital camera Gatan ES1000W.

2.6. UV–vis reflectance spectroscopy and chlorophyll content

A non-destructive quantification technique was employed to determine the pigment contents in leaves, based in diffuse reflectance spectra measurements (Cordon et al., 2010). Several leaves from one plant for each soil treatment were collected, washed with distilled water and kept frozen. Leaves were placed at room temperature for 20 min, carefully cleaned and dried, and kept in the dark until spectra measurement. That was performed in a Shimadzu UV3101PC spectrophotometer equipped with a Shimadzu ISR-3100 integrating sphere. The diffuse reflectance spectra were obtained from stacked leaves achieving zero transmittance; thus, the diffuse reflectance, R_∞, was recorded. BaSO$_4$ was used as standard to adjust 100% reflectance; the

baseline was recorded with BaSO$_4$ in both sample and reference positions. Between 6 and 9 spectra were recorded for each plant, using different stacks of leaves in each one. The recording range was 360–800 nm. Additional measurements at 1456 nm were performed in order to estimate water content (see below).

The R_∞ values were converted to remission function, $F(R)$, following the Kubelka-Munk theory and the plate stack model (Cordon et al., 2010; Cordon and Lagorio, 2007):

$$F(R) = \frac{(1 - R_\infty)^2}{2R_\infty} \tag{1}$$

The contents (mmol cm^{-2}) of chlorophyll-a (Chl-a), chlorophyll-b (Chl-b) and total chlorophyll (Chl-t) were estimated from the reflectance at 550 nm ($R_{\infty,550}$) using the correlations given by Cordon (Cordon, 2009):

$$Chl-a = -7.5 \times 10^{-7} + 1.1 \times 10^{-5}F(R_{\infty,550}) \quad (r^2 = 0.920) \tag{2}$$

$$Chl-b = 1.1 \times 10^{-6} + 4.6 \times 10^{-6}F(R_{\infty,550}) \quad (r^2 = 0.921) \tag{3}$$

$$Chl-t = 3.6 \times 10^{-7} + 1.6 \times 10^{-5}F(R_{\infty,550}) \quad (r^2 = 0.926) \tag{4}$$

Additionally, in order to analyze possible dilution or concentration effects, the water contents (Cw) was estimated from the reflectance at 1456 nm using (Cordon et al., 2010):

$$Cw = 2.0 \times 10^{-4} + 3.0 \times 10^{-4}F(R_{\infty,1456}) \quad (r^2 = 0.938) \tag{5}$$

The PRI index was obtained following Peñuelas et al. (1995) as:

$$PRI = \frac{R_{\infty,531} - R_{\infty,REF}}{R_{\infty,531} + R_{\infty,REF}} \tag{6}$$

where REF is a suitable reference wavelength, here 570 nm, based on the fact that short-term reversible changes in xanthophylls concentrations, due to plant stress, affect reflectance at 531 nm but not reflectance at 570 nm. This index also gives information on changes in chlorophyll/carotenoids ratio as chlorophyll absorbs both in the blue and red region and carotenoids only in the blue region of the electromagnetic spectrum.

3. Results

3.1. Plant biomass

Plant biomass results for all treatments are presented in Table S3 (Supplementary material). At FL stage, total plan biomass ranges between 6 and 7 g for the different treatments; in the PM stages plant biomass was around 10 g in all cases. No significant differences were found by ANOVA analysis in any case. Also, visual inspection of the aerial organs during growth did not revealed differences between treatments, nor between plants for the same treatments.

3.2. Lead contents

Table 1 shows the Pb contents found in leaves, both senescent and functional, and in harvested stems and roots, both in Co and Pb-200 treatments. At FL ontogenetic stage, lead concentration in organs follows the order: roots > stems > functional leaves; whereas at PM ontogenetic stage, the lead concentration in organs increase according to the following order: roots > functional leaves ≈ stems. At FL stage in Pb-200 treatment, lead concentration was significantly higher in senescent leaves than in the functional leaves (p < 0.05).

3.3. Effects of Pb in cell ultrastructure

Micrographs of ultrastructure leaf cells, obtained with transmission electron microscope, are shown in Fig. 1. For control treatment (Co) an

Table 1
Pb mean contents ± SE in *B. napus* organs (mg kg^{-1} of dry mass) (N = 3 unless otherwise noted).

Treatment	Ontogenetic stage	Roots	Stems	Functional leaves	Senescent leaves
Co	**Average**	6 ± 1[b]	4 ± 1	3 ± 1	ND
Pb-200	**FL**	15.3 ± 0.8[a]	9.3 ± 0.2	4.1 ± 0.9[*]	13 ± 2[b,*]
	PM	16 ± 3	4.9 ± 4.4	6.8 ± 0.3	8 ± 1[b]

ND: not determined.
[*] Indicates significant differences (p < 0.05).
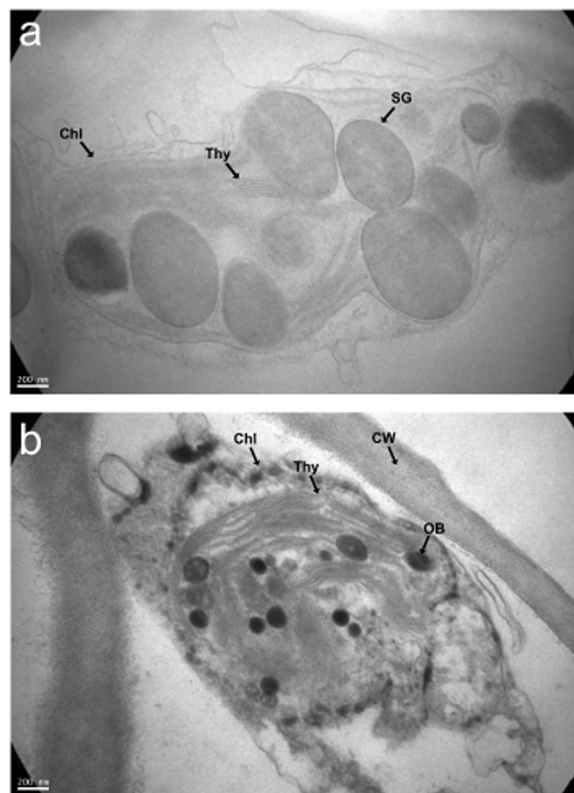[a] N = 1; instrumental error reported.
[b] N = 2.

Fig. 1. Electron transmission micrograph of leaf mesophyll cells of *B. napus*. A: Control (0 mg kg^{-1} Pb) treatment. B: Pb-200 (200 mg kg^{-1} Pb). Chl: chloroplast, Thy: thylakoid, SG: starch grain, CW: cell wall, OB: osmiophilic body.

intact chloroplast is observed, with a clear disposition of the thylakoids and the presence of large starch grains (Fig. 1a); however, cell membrane and cell wall could not be clearly observed. For Pb-200 treatment, a degraded chloroplast can be observed, with disorganized grana and thylakoids and the presence of osmiophilic bodies (Fig. 1b); starch grains are not visible.

Fig. 2 shows micrographs of ultrastructure of stem cells. A similar trend to that found on leaf cells is observed for Co (Fig. 2a and b) and Pb-200 (Fig. 2c and d) treatments. In the presence of Pb, chloroplast membranes are disorganized and osmiophilic bodies (attributed to accumulation of Pb) are observed nearby cell walls as electron dense granules.

3.4. Reflectance spectra

Remission function spectra for leaves in the FL ontogenetic stage for Co, Pb-50, Pb-100 and Pb-200 treatments are shown in Fig. 3. In the ranges 530–570 nm and 600–700 nm, $F(R)$ values for plants grown on lead contaminated soils were consistently higher than those grown in control soil.
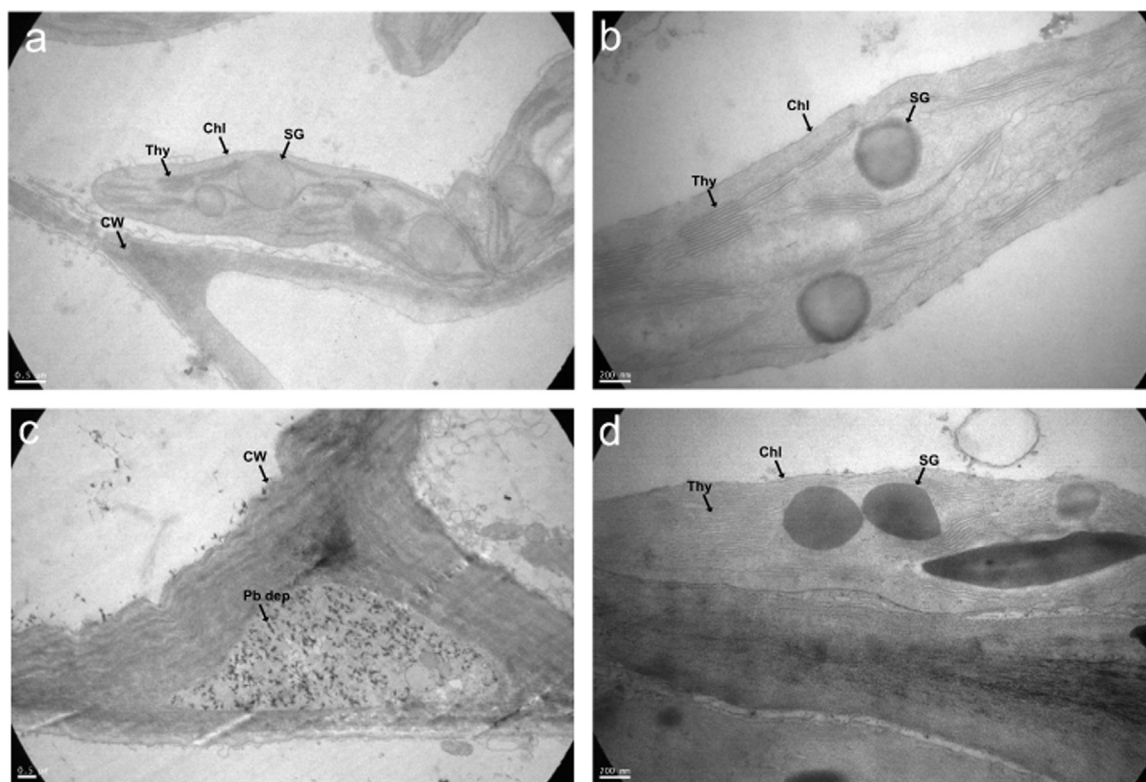
Fig. 2. Electron transmission micrographs of stem cells of *B. napus*. A, B: Co (0 mg kg^{-1} Pb); C, D: Pb-200 (200 mg kg^{-1} Pb). Chl: chloroplast, Thy: thylakoid, SG: starch grain, CW: cell wall, Pb dep.: Pb depositions.

Remission function spectra for leaves from Co, Pb-50 and Pb-200 treatments at the PM stage are shown in Fig. 4. With an opposite behavior to that for the plants at the FL stage, $F(R)$ values for Pb-treated plants at the PM stage were consistently lower than those for controls in the entire visible spectrum.

3.5. Chlorophyll and water contents

Contents of chlorophyll *a, b* and *total* were obtained from Eqs. (2–4) and the corresponding results are shown in Fig. 5. At the flowering ontogenetic stage, the content of *Chl-a, Chl-b* and *Chl-t* expressed as mmol of pigment per cm^2 of fresh leaf were higher for Pb-treated plants compared to Co. A slight decrease in chlorophylls content was observed, however, when Pb content in soil was increased from 100 to 200 mg kg^{-1}. The opposite behavior was found at the PM stage. In this stage, chlorophyll contents are lower than their corresponding values at FL stage and they decreased in the Pb-50 treatment. Nevertheless, when increasing Pb content in soil from 50 to 200 mg kg^{-1}, a slight increment in chlorophylls content was observed.

Water contents (Fig. 6), computed from Eq. (5) for leaves from different treatments, did not show differences, except for Pb-200 leaves in the FL stage, which presented the highest water concentration.

3.6. PRI index

Fig. 7 shows the results obtained for the photochemical reflectance index obtained from Eq. (6). At the FL stage, the PRI is positive for Co, Pb-50 and Pb-100 treatments, but it becomes negative for Pb-200 treatment. At the PM stage, there is an increase in PRI from Co to Pb-50 followed by a net decrease at Pb-200, reaching also a negative value, similar to the FL stage.

4. Discussion

Lead is a potent toxic metal. It is absorbed and accumulated by plant roots, and it can be translocated to the aerial biomass. Pb inhibits activity of many enzymes and disturb mineral nutrition, with the subsequent effects on plant growth, cell ultrastructure and photosynthetic attributes (Sharma and Dubey, 2005). The Pb treated plants showed clearly higher Pb concentration in their tissues. On senescent leaves a higher content of lead respect to the functional ones was detected in the FL stage (Pb-200), whereas in the PM stage no significant differences were found. The loss of leaves is a detoxification mechanism employed by several species in order to reduce the toxic chemical accumulated in plant tissues (Dahmani-Muller et al., 2000). In the present case, the high concentrations of Pb in some leaves and the subsequent loss of them can be attributed to a detoxification strategy of *B. napus*. The differences between stages can be rationalized taking into account the behavior of Pb in the soil. In a previous study (Ferreyroa et al., 2014) it was found that, when Pb(II) is incorporated to soil, it evolves from labile forms (soluble and exchangeable) presumably bioavailable, to more stable forms (organic and mineral bound) which are expected to be of lower bioavailability, if any, in approximately 60 days time. Thus, the major Pb intake will take place in the first stages of the plant growth; this is consistent with senescence as a detoxification mechanism taking place when the plant undergoes high Pb intake.

TEM images indicated lead depositions on cellular spaces and showed damages at the subcellular level, in the physiological maturity ontogenetic stage. On leaves and stems of treated plants, osmiophilic bodies were observed around the chloroplasts, inside the vacuoles, and in the cytoplasm nearby to the wall cell. Also, damages on inner and outer chloroplast membranes was observed. These observations are consistent with literature reports. A total damage on ultrastructure of mesophyll cells has been observed in *B. napus* (Tian et al., 2014) after 15 days of growth in solutions with high concentration of Pb; TEM observation revealed fused cellular wall and membrane, damage on
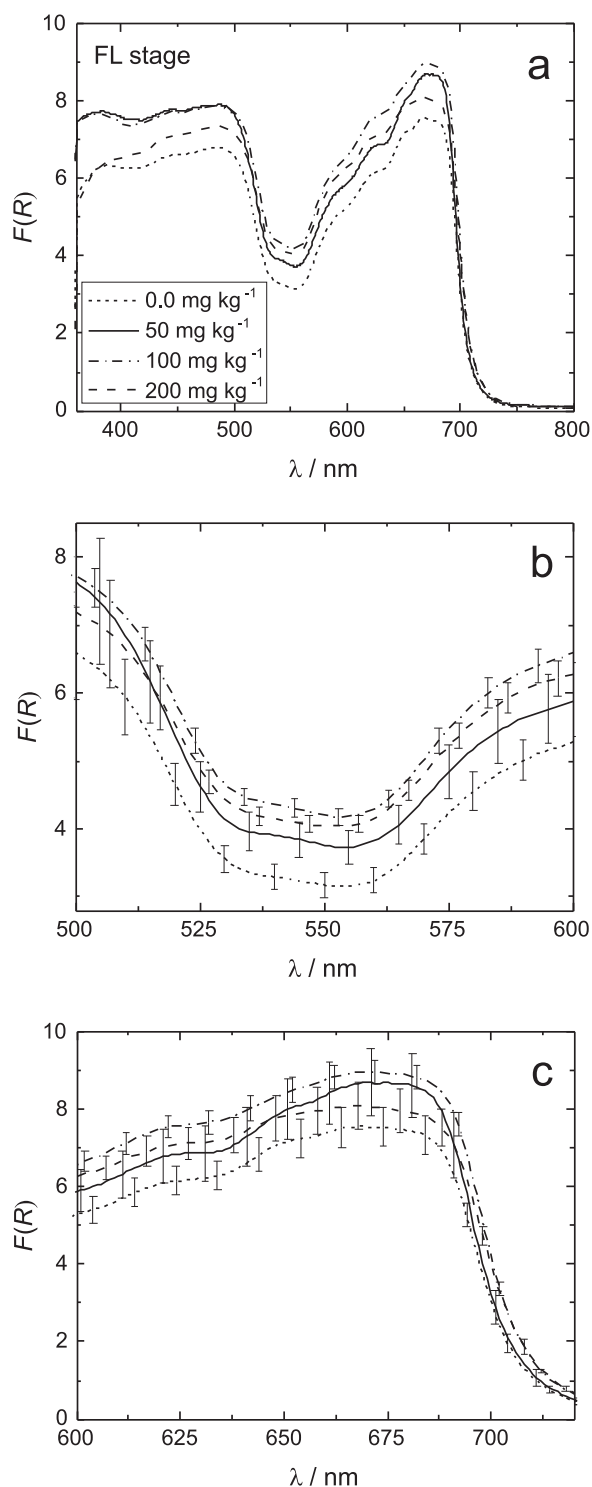
Fig. 3. (a) UV–vis Remission function spectra of *B. napus* leaves of plants grown in soils with different Pb treatments, in the FL stage. Average values measured for stacks of leaves are shown. (b) Detail of the 500–600 nm region, including the standard errors of the averages (only bars for selected points are shown for clarity) (c) Idem (b) but for the 600–720 nm region.

Fig. 4. (a) UV–vis Remission function spectra of *B. napus* leaves of plants grown in soils with different Pb treatments, in the PM stage. Average values measured for stacks of leaves are shown. (b) Detail of the 500–600 nm region, including the standard errors of the averages (only bars for selected points are shown for clarity) (c) Idem (b) but for the 600–720 nm region.

thylakoids membranes and absence of starch granules, among other effects. A similar study with cadmium (Ali et al., 2013) resulted in chloroplast disorganization, general cell deterioration and presence of metallic deposits on mesophyll cells in *B. napus* leaves; unlike the effect of Pb, it has been an increment in the starch grains in chloroplasts. Notwithstanding, these studies reported the effects of lead on *B. napus* at the early stages of development and on hydroponic cultures. To the
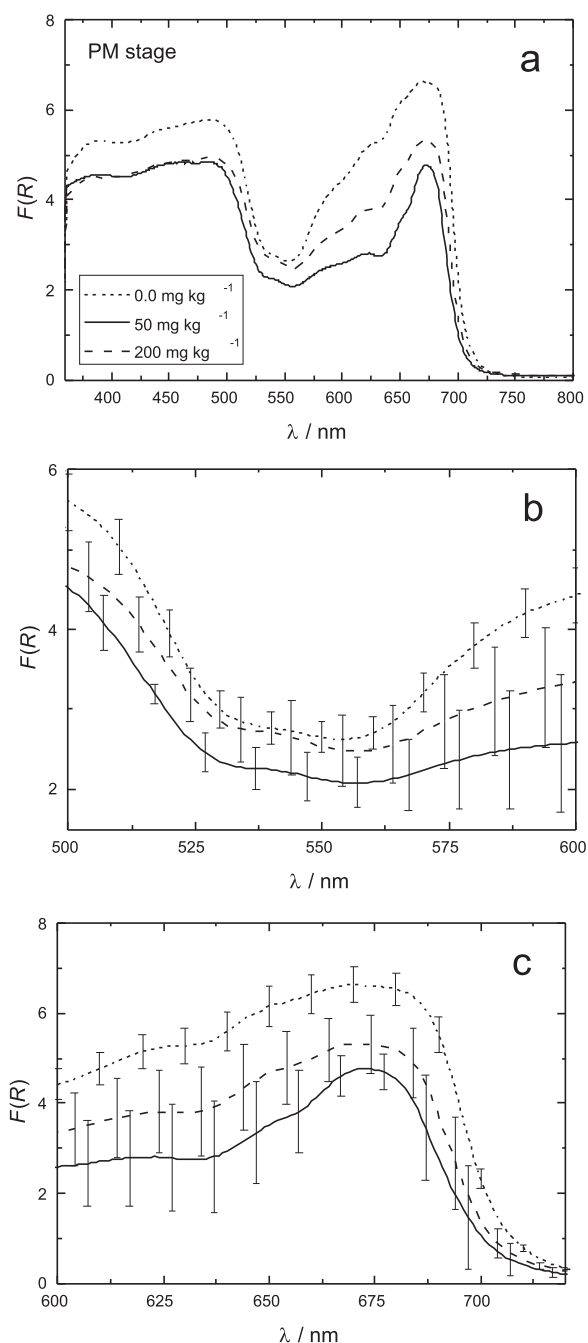
best of our knowledge, there are no previous reports on effects of lead at subcellular level on plants of *B. napus* cultured on contaminated soils, at the ontogenetic stage of physiological maturity.

Furthermore, by UV spectrometry analysis, differences were detected in leaf pigment contents between treated and control plants. At FL stage, Pb presence up to $100 \, \mathrm{mg \, kg^{-1}}$ induces an increment in chlorophylls content that is not attributable to variations in the water content of leaves. On the other hand, the increase in water content for Pb-200 plants may explain the apparent reduction in chlorophylls contents observed in this case. At PM stage a different trend is observed: chlorophylls content on treated plants showed a decreasing tendency in Pb-50 treatment, but no differences were found in th Pb-200 treatment.
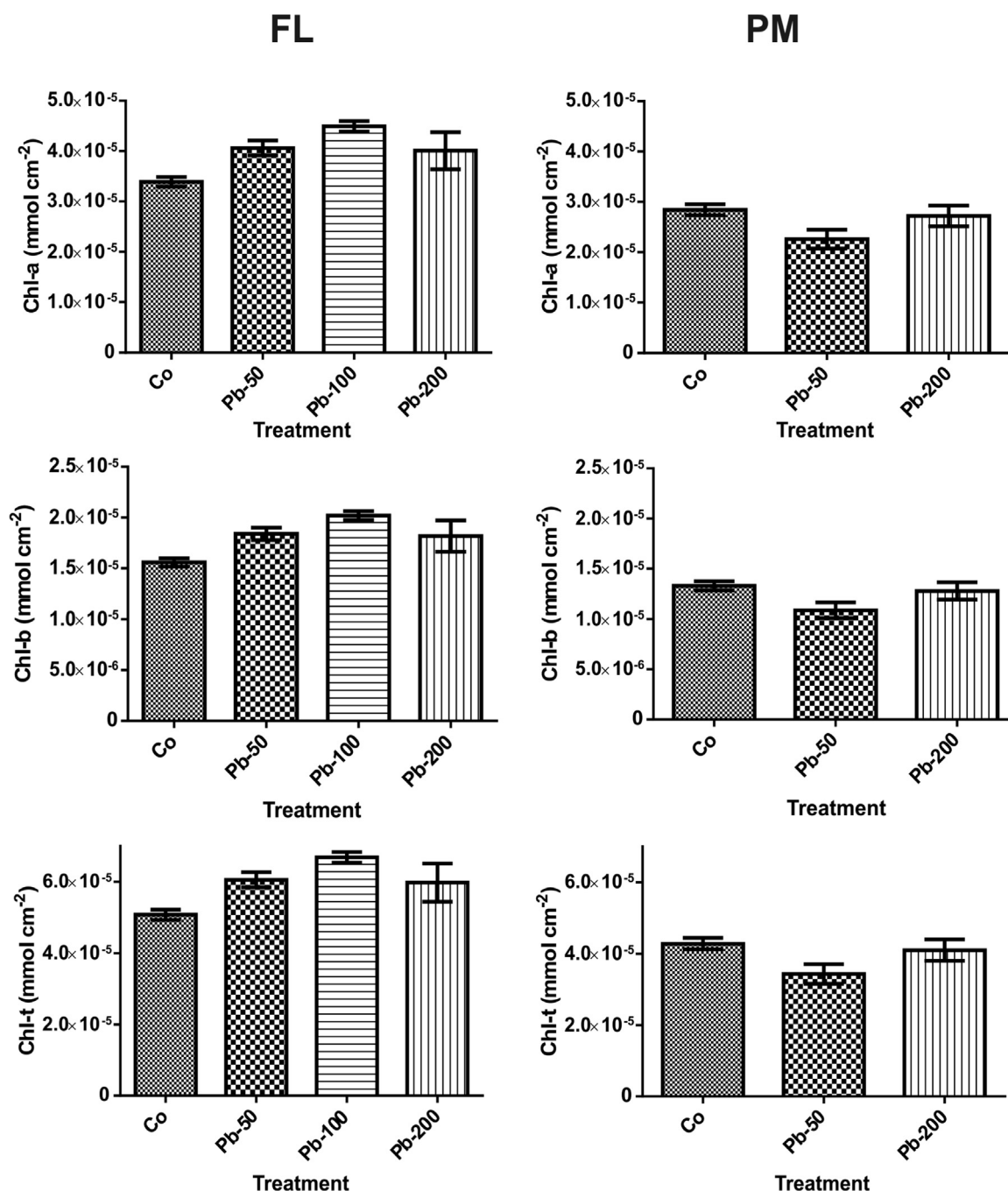
Fig. 5. Mean chlorophyll contents (mmol cm^{-2}) with standard errors for chlorophyll-*a* (top), chlorophyll-*b* (mid) and *total* chlorophyll (bottom) in leaves of *B. napus* at the ontogenetic stages FL (left) and PM (right) obtained with Eqs. (2)–(4) and values from Figs. 3 and 4.

However, chlorophylls content in control and treated plants at PM stage were lower than at FL stage, with a larger difference in treated plants. These results are consistent with reports from other authors. For *Brassica juncea*, it was found that after 60 days of farming in soils polluted with Pb, Cu and Cd, chlorophylls *a* and *b* contents in leaves were higher than in leaves of plants grown in control soils, whereas at 90 days these contents decreased markedly compared with control (Karak et al., 2013). These authors proposed that the chlorophyll increase was related to the increase in antioxidant concentration, which would allow plant metal tolerance via complexation; the pigment decrease at longer times was explained by an increment in free radical concentration, inhibiting chlorophyll synthesis. It is noteworthy that these results were obtained prior to fruiting stages. In the case of *Brasica napus*, Tian et al. (2014) and Bilal Shakoor et al. (2014) reported that

plants grown in solutions with high Pb concentrations had a decrease in chlorophyll *a* and *b* concentration. These authors proposed that the observed damages were due to Pb stress and oxidative response, because in the presence of complexing antioxidants they observed a damage diminution. According to this, the variations of chlorophyll content observed in the present study can be attributed to the accumulation of Pb on the leaf cells; initially as an oxidative response, but at later stages related to the subcellular ultrastructural damage.

Variations in PRI values may be connected with changes in carotenoids. In fact, Filella et al. (2009) found an inverse relation between PRI and carotenoids content. Analogously, Cordon et al. (2016) recently found a positive correlation between leaf PRI and the ratio chlorophyll/carotenoids along senescence process. In this work, the behavior of PRI values does not have a defined trend during the
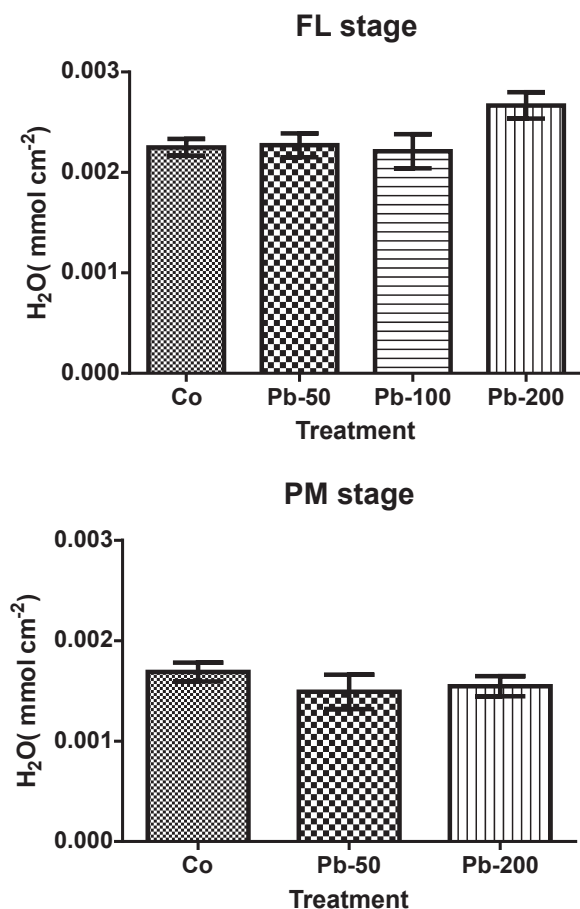
FL stage

PM stage

Fig. 6. Mean water contents (mmol cm^{-2}) with standard errors in leaves of *B. napus* for both ontogenetic stages obtained with Eq. (5) and average reflectance values at 1456 nm.
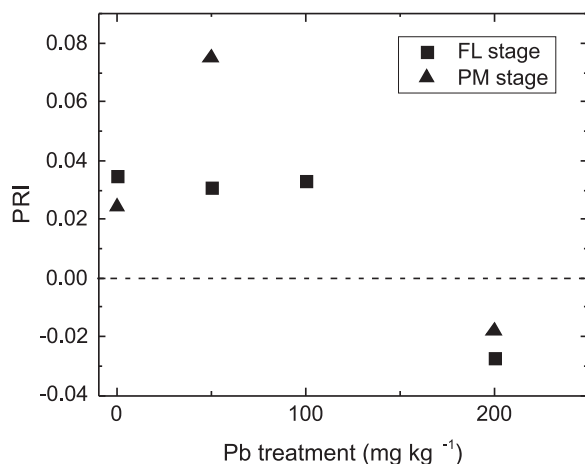

Fig. 7. PRI index as a function of treatment (Pb concentration) for the FL and PM ontogenetic stages.

treatments with the lowest lead contents. Just at concentrations of 200 mg kg^{-1} a net decrease in PRI is observed for both ontogenetic stages studied, indicating a decrease in chlorophyll/carotenoid ratio. This result, taking into account that chlorophyll contents only show small changes, would be presumably connected with an increase in carotenoids content as a response against lead toxicity and a consequent decrease in the Chlorophyll/ Carotenoids ratio.

Albeit Pb ions are generally found to be poorly mobile in plant tissues, there are reports were translocation to aerial organs is found; Lavado et al. (2007) found Pb in grains and shoots of maize (*Zea mays*)

and wheat (*Triticum aestivum*) grown in biosolid treated soils. In the case of *B. napus*, Azimzadeh et al. (2014) reported an important translocation of Pb from roots to shoots, reporting a translocation factor from roots to shots (the ratio of a metal concentration in shoots to that of the roots) of the order of unity for plants harvested 85 d after sown; this can be approximately compared with plants harvested at the flowering stage in the present study (100 d), resulting from the data in Table 1 in a roots/stems translocation factor of about 0.6 which can be considered consistent with the results of Azimzadeh et al. Thus, it can be concluded that rapeseed is a Pb accumulator with translocation to aerial organs.

5. Conclusions

The experimental results show that *Brassica napus* accumulates lead and translocates this metal to aerial organs, having important toxic effects on plant cell morphology and on photosynthetic attributes, at early and later ontogenetic stages. Several effects were observed in *B. napus* due to Pb intake from soil:

- Cell ultrastructure alteration with direct effect on the chloroplast thylakoids.
- Increase in Chlorophyll contents in the flowering ontogenetic stage.
- The results suggest that at high Pb concentration in soils the cell ultrastructure is affected, decreasing pigment synthesis.
- A significantly higher Pb contents in senescent leaves is observed during the FL stage, strongly suggesting a detoxification mechanism via senescence.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.02.031.

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