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Cerium negatively impacts the nutritional status in rapeseed

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HIGHLIGHTS

- Ce fertilization is controversial and Ce movement pathways to roots and shoots are unknown.
- Ce, Ca (Lithosphere) and rapeseed (Biosphere) interaction was assessed in hydroponics.
- Ce was without beneficial effects and already toxic at very low concentrations (2 μM).
- Ce accumulation was inhibited in a non-competitive way by high Ca concentrations in the nutrient solution.
- The use of Ce-containing fertilizers in agriculture should be avoided.

GRAPHICAL ABSTRACT
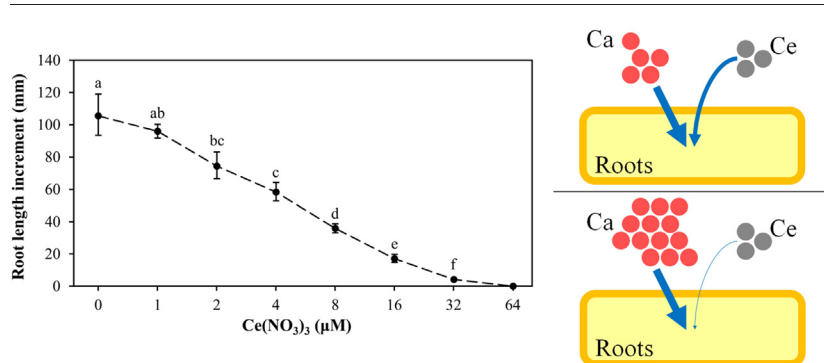
ARTICLE INFO

ABSTRACT

Cerium (Ce) has been reported to be both beneficial and harmful to plants. This contradiction deserves explanation in the light of increased anthropogenic release of Ce in the environment.

Ce tolerance and accumulation were evaluated in hydroponically cultivated *Brassica napus* L. (rapeseed). Ce and other nutrient concentrations were measured with increasing Ce concentration in the nutrient solution. Moreover, Ce and calcium (Ca) accumulation were evaluated at different Ca and Ce concentrations in nutrient solution and a Michaelis-Menten type inhibition model considering Ce and Ca competition was tested. Plants were also sprayed with Ce solution in Ca-deficient media.

Ce decreased the growth and root function, which affected shoot nutritional status. Calcium was the most severely inhibited nutrient in both roots and shoots. High Ca concentrations in the nutrient solution inhibited Ce accumulation in a non-competitive way. Moreover, phosphorus (P) precipitated Ce inside root cells. Ce spraying did not alleviate Ca deficiency symptoms and the results were critically compared to the available literature.

1. Introduction

Despite being classified as a rare earth, Ce is the 25th most abundant element and its presence in the soil ranges approximately from 2 to 150 μg g^{-1}, with mean values of 50–66 μg g^{-1} (Greenwood and Earnshaw, 1984; El-Ramady, 2010; Emsley, 2011). Cerium and other rare earths

(REs) have the potential to accumulate progressively in soil due to their increased use in a variety of modern industries and in the last 30 years their wide use as fertilizers in Chinese agriculture (Hu et al., 2004; Tyler, 2004; El-Ramady, 2010; Emsley, 2011; Ramos et al., 2016). Due to elevated concentrations of REs in phosphate fertilizers, Ce can accumulate in agricultural soils outside China too (Kanazawa and Kamitani, 2006; Ramos et al., 2016). For example, it has been estimated that in Brazilian soils 12,000 t of Ce were added through phosphate fertilizers in 2014 (Ramos et al., 2016). However, the increase of REs in soil is usually small and large amounts are easily washed away

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from upper soil horizons through rain of watering (Tyler, 2004). Nevertheless, REs supplied as fertilizers or those from other anthropogenic sources are more soluble and reactive than REs from the soil pool. Rare earths pollution can therefore be considered an upcoming environmental problem (Tyler, 2004).

In a natural environment Ce bioavailability will be enhanced by low pH, and it is likely that it will be complexed with dissolved organic carbon (Tyler and Olsson, 2002). Instead, Ce bioavailability will decrease in soils with high phosphate, which can precipitate Ce as non-soluble $CePO_4$ (Diatloff et al., 1993; Diatloff et al., 1996). An approximation of Ce solubility in soil would be its concentration in soil solution, which had a range of 0.01–0.51 μM in unamended Australian soils (Diatloff et al., 1996) and of 0.005–0.19 μM in Swedish non-fertilized forest (Tyler and Olsson, 2002). Soluble Ce concentrations in fertilized or contaminated soils could be much higher.

Most plants in unfertilized soils have been reported to avoid REs accumulation, with a plant to soil concentration ratio far lower than unity, and with roots usually having higher concentrations than shoots (Tyler, 2004). However, it has been reported that *Dryopteris erythrosora* contain up to 30 μg g^{-1} (0.2 μmol g^{-1}) of Ce in leaves, which is a factor 10 to 100 higher than in non-accumulator ferns from the same place (Ozaki et al., 2000). Another fern, *Dicranopteris dichotoma* (synonymous with *D. linearis*) has the highest concentrations of total REs (and Ce) in leaves ever reported for a vascular plant thus far (Ichihashi et al., 1992; Wang et al., 1997). Ichihashi et al. (1992) reported that *D. dichotoma* can contain up to 140 μg g^{-1} (1 μmol g^{-1}) of Ce in leaves. This is confirmed by data derived from Wang et al. (1997), which also showed a high Ce root to shoot translocation ratio (>80), with a mean value of c. 508 μg g^{-1} (3.6 μmol g^{-1}) Ce in leaves, which was on average seven times higher than the concentration in the soil. Robinson et al. (1958) also observed a high Ce concentration (on average 172 μg g^{-1} i.e. 1.2 μmol g^{-1}) in leaves of hickory trees (*Carya* sp.). However, in the above-cited papers it is not possible to exclude contamination of leaves by dust containing REs deposited by wind and experimental confirmations are still lacking.

In nutrient solutions with a realistic range of Ce concentrations (0.37–1.31 μM) maize (*Zea mays*) fresh and dry weight and nutritional status were much less affected than those of mungbean (*Vigna radiata*) at ≥0.2 μM (Diatloff et al., 1995a, 1995b; Diatloff et al., 2008). In maize low Ce concentrations appeared to have beneficial effects on root elongation and dry weight (Diatloff et al., 1995b). However, no beneficial effects were observed for shoot and total dry biomass, and Ce inhibited root elongation at concentrations higher than 1.31 μM (Diatloff et al., 1995a). Hu et al. (2002a, 2002b) observed no beneficial effects of Ce on growth of common wheat (*Triticum aestivum*) at 3.6 μM in nutrient solution, while only harmful effects such as decreased root growth and nutrient unbalance were noted at concentrations ≥7.1 μM. Similarly, Wang et al. (2007) observed that Ce induced oxidative stress in *Hydrilla verticillata* already at 10 μM Ce in the nutrient solution. Liu et al. (2012) who cultivated rice (*Oryza sativa*) in agar, and Shyam and Aery (2012) who cultivated cowpea (*Vigna unguiculata*) in Ce-spiked soil, observed a beneficial effect of Ce on plant growth, i.e., at low concentrations (till 100 μM in rice and c. 18 μM in cowpea) Ce improved growth, but plants were negatively affected at higher concentrations. Similar beneficial effects have been reported for fresh and dry weight in *Ginkgo biloba* cell suspensions (Chen et al., 2015). The reasons for these beneficial effects are unknown. Fashui and co-authors demonstrated an increased photosynthetic rate, in particular the photochemical activity of photosystem II, its oxygen evolving rate, and improved growth and chlorophyll content in spinach after submersing seeds in Ce solution before germination, and then spraying the leaves with Ce at c. 15 μM (Fashui et al., 2002) and 81 μM (Fashui et al., 2005). Similar beneficial effects on photosynthesis have also been obtained with Ce concentrations at 5, 10 and 30 μM (Xiaoqing et al., 2007). The authors attributed these effects to a possible replacement of Mg by Ce atoms in their coenzyme sites. Indeed, Chen et al. (2000), working in vitro

with Mg-less bathing solutions, found that at low concentrations (≤6 μM) Ce promoted ribulose 1,5-bisphosphate carboxylase (RuBPcase) activity by replacing Mg coenzyme activity. Instead, higher Ce concentrations (≥8 μM) inhibited RuBPcase activity in vitro. Bakou et al. (1992) and Bakou and Ghanotakis (1993) observed inhibition of the oxygen evolution rate in photosystem II (PSII) in vitro, owing to replacement of Ca with REs in plants treated with very high REs concentrations (2 mM and 800 mM). Negative or no effects after spraying Ce at high concentrations have also been observed (Diatloff et al., 1999; Guo et al., 2007). Diatloff et al. (1999), who used commercial fertilizer containing Ce and La, and in a parallel experiment a spray with Ce and La of analytical grade in a ratio of 1 to 0.7, found no beneficial effects on the growth and yield of mungbean and maize after applying a range of high concentrations (c. 0.2–8.8 mM Ce). However, necrotic patches on maize and small necrotic spots on mungbean were observed at the highest application rates (c. 4.4 and 8.8 mM Ce). Guo et al. (2007) reported a significantly decreased content of Ca, K and Mg and a time dependent increase of Ce in horseradish (*Armoracia rusticana*) roots after applying 1000 μg Ce l^{-1} (c. 7 mM) on the leaf surface, suggesting that high Ce concentrations can disturb the homeostasis of these major cation nutrients.

Although Ce is typically 3+ charged and Ca 2+, both elements have similar atomic radii (Ca = 197 pm, Ce = 181.8 pm) and their cations have similar Shannon-Prewitt effective ionic radii (for example for coordination number 6 Ca^{2+} has an ionic radius of 100 pm whereas Ce^{3+} of 101 pm). It can thus be supposed that Ce might partly replace or interact with Ca and its binding sites. Indeed Chao et al. (2008, 2009) and Huang et al. (2008) showed that spraying with 15 μM Ce improved plant growth, photosynthesis, RuBPcase activity, nitrogen metabolism, and the antioxidative response, and alleviated calcium-deficiency symptoms in spinach, grown in Ca-deficient media. However, a mechanistic insight into the effects of Ce and its interaction with Ca metabolism is still lacking.

The objectives of the present research are therefore, (i) to assess the toxicity or any beneficial effects of Ce on rapeseed (a widely cultivated crop for its oil production) in controlled experimental conditions under increasing and realistic Ce concentrations in nutrient solution; (ii) to evaluate the rapeseed response to Ce exposure in terms of Ce accumulation and translocation and its effects on the plant mineral status; (iii) to investigate the nature of Ca/Ce interference regarding their uptake and root-to-shoot translocation.

2. Material and methods

2.1. Plant culture and experimental conditions

Seeds of *Brassica napus* L. var. 'Pulsar' (rapeseed) from University of Udine (Italy) were sown in a garden peat soil (Typical Typ 2 Gebr. Brill Substrate GmbH and Co, Georgsdorf, Germany) and left for ten days in a growth chamber. Seedlings were then transferred to aerated hydroponic culture, in 1-L polyethylene pots (one plant per pot) containing a modified half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 20 μM Fe(Na)-EDTA, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.1 μM $CuSO_4$ and 0.1 μM $(NH_4)_6Mo_7O_{24}$ in demineralised water buffered with 2 mM MES, pH 5.5, adjusted with KOH. Nutrient solutions were renewed after five days and plants were grown in a growth chamber (22: 18 °C, day: night; light intensity 220 μE m^{-2} s^{-1}, 12 h day^{-1}; relative humidity 75%).

2.2. Ce tolerance testing

After ten days of pre-culture, plants were transferred to the test solution, which was of the same background composition as the pre-culture solution, but without $NH_4H_2PO_4$ and Fe(Na)-EDTA, to avoid precipitation of $CePO_4$ and Ce-EDTA complex formation, owing to

displacement of Fe(III). Plants were exposed to a series of Ce (0, 1, 2, 4, 8, 16, 32, 64 μM $Ce(NO_3)_3$) concentrations (one plant per pot, six plants per concentration in a randomised design). Cerium(III) nitrate was chosen because of its high solubility in water (600 g l^{-1} at 20 °C). Before exposure, roots were stained with active carbon powder to facilitate the measurement of root growth (Schat and ten Bookum, 1992). After six days of exposure, the length of the longest unstained root segment was measured and plants were harvested for analysis.

2.3. Root cleansing protocol evaluation

To choose the most efficient and satisfactory cleansing protocol for Ce desorption from the root free space, after ten days of pre-culture, plants were exposed to a 5 μM $Ce(NO_3)_3$ solution with a background composition as the pre-culture solution, but without $NH_4H_2PO_4$ and Fe(Na)-EDTA. After five days of exposure roots were excised and carefully rinsed in one of the following ice-cold solutions (each with 3 replicates): (i) demineralised water for 6 min, (ii) 20 mM (Na$_2$)-EDTA, (iii) 20 mM $NH_4H_2PO_4$, or (iv) 0.65% (141 mM) HNO_3. Solutions ii–iv were applied for 2 or 6 min. After each rinsing procedure roots were further carefully rinsed in demineralised water for 6 min. Separate subsamples were immediately analysed for Ce content without any rinsing—thus representing the full Ce presence on and in the roots. Given the results, for the further analysis it was chosen to rinse roots with ice-cold 0.65% HNO_3 for 6 min, which should not desorb too much Ce from the symplast and endodermis, because of the relatively short duration of the treatment (Fig. 1).

2.4. Testing the effect of Ca supply on Ce accumulation

After ten days of pre-culture, plants were exposed to 0.5 and 6 μM $Ce(NO_3)_3$ in a background solution of the same composition as the pre-culture solution, but without $NH_4H_2PO_4$ and Fe(Na)-EDTA, and Ca concentrations were set at 0.5, 2, or 4 mM $Ca(NO_3)_2$. Six plants (one per pot) per Ce per Ca concentration were exposed for five days and then harvested for analysis.

2.5. Concentration-dependent kinetics of Ce uptake at different Ca concentrations in the nutrient solution and at different temperatures

After ten days of pre-culture, plants were transferred to a 0.5 mM $CaCl_2$ solution buffered with MES, pH 5.5, adjusted with KOH and kept for 20 h. After this adaptation period, plants were transferred to 0.5 mM $CaCl_2$ solution kept at 2 °C, or either 0.5 or 4 mM $CaCl_2$ kept at 22 °C. All the three test solutions were buffered with MES, pH 5.5,
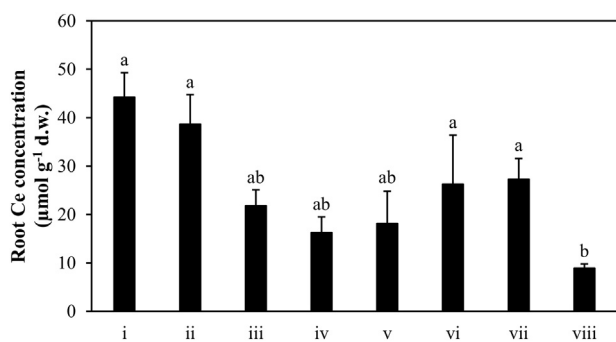

Fig. 1. Root Ce concentrations (μmol g^{-1} d.w.) of *Brassica napus* (median ± SE, n = 3) after exposure to 5 μM Ce for 5 days (i) without desorption of the roots, or after different desorption protocols: (ii) rinsing in demineralised cold water for 6 min; (iii) rinsing in 20 mM cold (Na$_2$)-EDTA for 2 min; (iv) rinsing in 20 mM cold (Na$_2$)-EDTA for 6 min; (v) rinsing in 20 mM cold $NH_4H_2PO_4$ for 2 min; (vi) rinsing in 20 mM cold $NH_4H_2PO_4$ for 6 min; (vii) rinsing in 0.65% (141 mM) cold nitric acid for 2 min; (viii) rinsing in 0.65% cold nitric acid for 6 min. Each of the iii-viii root cleansing protocols included additional rinsing in demineralised water for 6 min. Different letters indicate significant differences between means (p < 0.05, Tukey's test).

adjusted with KOH. For each different Ca concentration and temperature, plants were exposed to a series of $Ce(NO_3)_3$ concentrations (2.78, 3.57, 5, 8.33, 25, 50 μM) (one plant per pot, three plants per Ce concentration for each Ca concentration and temperature, in a randomised design) to study the influx kinetics of Ce. After 40 min (by stopwatch) roots were excised for analysis.

2.6. Element concentrations analysis

Immediately after harvesting plant specimens were divided into shoots and roots and the latter were carefully rinsed with ice-cold 0.65% HNO_3 for 6 min and for a further 6 min in demineralised water to desorb metals from the root free space and then blotted with paper tissue. The plant fractions were then oven-dried for 24 h at 105 °C, except in the case of roots from the concentration-dependent kinetics experiment, where fresh roots were analysed. Five to 10 mg of root material, and 100 to 300 mg of shoot material were digested in 5 ml of a 1 to 4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO_3 in Teflon cylinders for 10 min at 180 °C in a microwave oven (CEM, Mars Xpress). After that the volume was adjusted to 20 ml with milli-Q water and filtrated through 0.45 μm Teflon filters. The solution was further diluted 6 to 10 times, with milli-Q water, and in the case of Ce tolerance testing, total Ca, Ce, Cu, Fe, K, Mg, Mn, P and Zn concentrations were determined (three plants per concentration). In the case of testing the effect of Ca supply on Ce accumulation, Ca and Ce were determined (six plants per concentration) while in the case of concentration-dependent kinetics experiment only Ce in roots was determined (three plants per concentration). Elements were determined by an ICP-AES (Varian Inc., Vista MPX) and the accuracy of the analytical procedure was checked running standards every 20 samples and using certified standard reference material (tomato leaves 1573a from the National Institute of Standards and Technology, USA), which was treated exactly as the samples. The recovery values had a mean of 98%, with RSD ± 3%. Quality control was conducted using Y as the internal standard, reagent blank samples, and triplicates reading for each sample. Detection limits were: 160 μg l^{-1}, 56 μg l^{-1}, 4 μg l^{-1}, 21 μg l^{-1}, 77 μg l^{-1}, 110 μg l^{-1}, 3 μg l^{-1}, 31 μg l^{-1}, and 6 μg l^{-1} for Ca, Ce, Cu, Fe, K, Mg, Mn, P and Zn respectively. For a validation of the results obtained by ICP-AES, 20 samples with low Ce concentrations were re-analysed after further dilution of 1 to 10 with milli-Q water by an ICP-MS (PerkinElmer, NexION 350x). Samples were analysed in triplicates with ^{89}Y as the internal standard. Detection limit in ICP-MS was 0.1 μg l^{-1} for Ce. The correlation among ICP-AES and ICP-MS was high (r = 0.99) with a slope close to 1 in a linear regression analysis.

2.7. Effects of Ce spraying on leaves

In order to evaluate if Ce can mimic Ca in the leaves, seeds of rapeseed were sown in a garden peat soil as above but left for only five days in order to minimize the leaf sink of Ca in the following experiment. Seedlings were then transferred to aerated hydroponic culture, in 1-L polyethylene pots (one plant per pot) with a background composition either as the full pre-culture solution (see above) or as the same solution, but without $Ca(NO_3)_2$ (Ca deficient half-strength Hoagland's solution). After six days in hydroponic culture, leaves were sprayed once with 15 μM $Ce(NO_3)_3$ solution or 15 μM $CaCl_2$ solution. There were 5 groups (each with three plants): (i) half-strength complete Hoagland's solution without spraying, (ii) half-strength complete Hoagland's solution and plants sprayed with 15 μM $Ce(NO_3)_3$, (iii) Ca deficient half-strength Hoagland's solution, (iv) Ca deficient half-strength Hoagland's solution and plants sprayed with 15 μM $Ce(NO_3)_3$, (v) Ca deficient half-strength Hoagland's solution and plants sprayed with 15 μM $CaCl_2$. After five days from the treatment chlorophyll was measured by a SPAD-502 Chlorophyll Meter (Minolta Corp., Ramsey, NJ) in the second and third leaves from the base of each plant (three readings per leaf). The shoots were then excised and their fresh weight measured.

2.8. Statistics

Statistical analysis was performed using one- and two-way ANOVA. A posteriori comparison of individual means was based on the minimum significant difference (MSD) method obtained from the T statistic (Tukey's test) (Sokal and Rohlf, 2010). Data from hydroponics were subjected to logarithmic transformation before analysis, which effectively homogenized the variances (Hartley's F_{max}-Test) and produced approximately normal distributions (Kolmogorov-Smirnov test) (Sokal and Rohlf, 2010). We consequently chose to present the median values instead of the arithmetic means.

The estimated EC_{50} (the concentration that reduces growth to 50% of the maximum rate) was estimated by linearly regressing root growth on the logarithm of the Ce concentration and in order to obtain good linearity ($r^2 = 0.93$), non-toxic concentrations and concentration that completely arrested root growth were excluded from the calculation of the regression line (Schat and ten Bookum, 1992).

The relationships between the concentrations of Ce and those of the other elements were, separately for shoots and for roots, tested using Pearson's product-moment correlation coefficients and, in case of significance, the regression lines and their 95% confidence limits were calculated (Sokal and Rohlf, 2010).

The concentration dependent uptake kinetic curves can show a saturable (hyperbolic) component and a non saturable linear component that can be solved mathematically by the Michaelis-Menten model with an added linear component: $V_{Ce} = (V_{max}[Ce])/(K_m[Ce]) + a[Ce]$. In this equation [Ce] is the concentration of Ce in solution, V_{Ce} is the accumulation rate in roots (nmol g^{-1} min^{-1}) and V_{max}, K_m and a are parameters determined by the curve fitting algorithm (non-linear least-squares fitting Excel Solver Add-In, Kemmer and Keller, 2010).

3. Results

3.1. Effects of Ce on root growth

As estimated from the root growth response, rapeseed already showed significant growth reduction at 2 μM Ce treatment ($p < 0.05$) (Fig. 2). No beneficial effect at the lowest (1 μM) Ce treatment was observed and roots already developed very hairy lateral roots. Complete growth inhibition was observed at the highest (64 μM) treatment level (Fig. 2). The estimated EC_{50} was 4.2 μM, with 3.0 and 5.9 μM lower and upper 95%-confidence limits, respectively.

When plants were exposed to Ce in a geometric concentration series with common ratio of 2 in half-strength Hoagland's solution with P and Fe(Na)-EDTA, the roots only showed a slightly decreased root elongation at 640 μM treatment, compared to the control (data not shown).
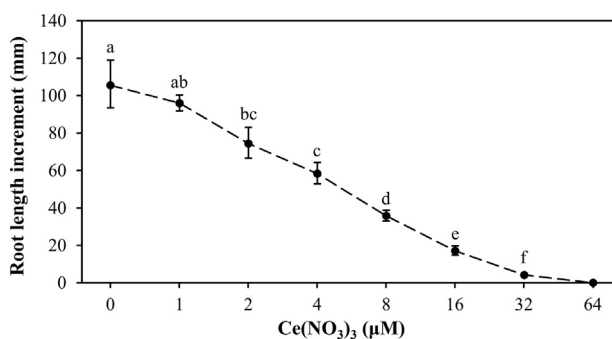
3.2. Root washing solution evaluation

An effective protocol for metal desorption from the root free space is required whenever studying kinetics or generally metal uptake by roots (Harrison et al., 1979). It is visible from our results that deionised water is not an efficient desorption solution for Ce (Fig. 1). The most efficient desorbing agent was 0.65% (141 mM) cold HNO_3 for 6 min. However, the effect of the 141-mM HNO_3 desorption treatment was not significantly different from that of 20 mM (Na_2)-EDTA for 2 or 6 min of rinsing, or that of 20 mM $NH_4H_2PO_4$ for 2 min. Surprisingly, the Ce presence in roots increased after prolonged rinsing in $NH_4H_2PO_4$, which could be due to precipitation of $CePO_4$ in the root apoplast.

3.3. Ce and nutrients accumulation in plants under hydroponics

The root Ce concentrations increased gradually with increasing Ce concentration in nutrient solution up to the 16-μM treatment and more sharply at higher concentrations (Fig. 3a). Plants from the highest (64 μM) treatment were not included in the analysis, because their roots were dead (Fig. 2).

The Ce concentrations in shoots showed a different pattern from that in roots (Fig. 3b). Already after the lowest (1 μM) treatment the shoot Ce concentration levelled off, but significantly increased again in the 32-μM treatment (Fig. 3b).The shoot to root Ce concentration ratios varied between treatments, decreasing with the Ce exposure level from 0.012 at 1 μM to 0.003 at 32 μM.

Root Ca concentrations were already significantly decreased at the 2-μM Ce exposure level and continued to decrease under higher Ce exposure, although only significantly in the two highest (16 and 32 μM) Ce treatments (Table 1). Regressing root Ca on root Ce yielded a significant ($R^2 = 0.7$) negative slope (Fig. 4). On the other hand, regressing root P on root Ce, yielded a significant ($R^2 = 0.8$) positive slope, although the mean root P concentrations in the control up to the 8-μM Ce treatment were not significantly different among each other (Table 1). The other macro- and micro-nutrients in roots were not significantly different between Ce treatments, remaining in the range of control treatment although with some fluctuations (Table 1). Their regression slopes on the Ce concentrations were not significant too (Fig. 4).
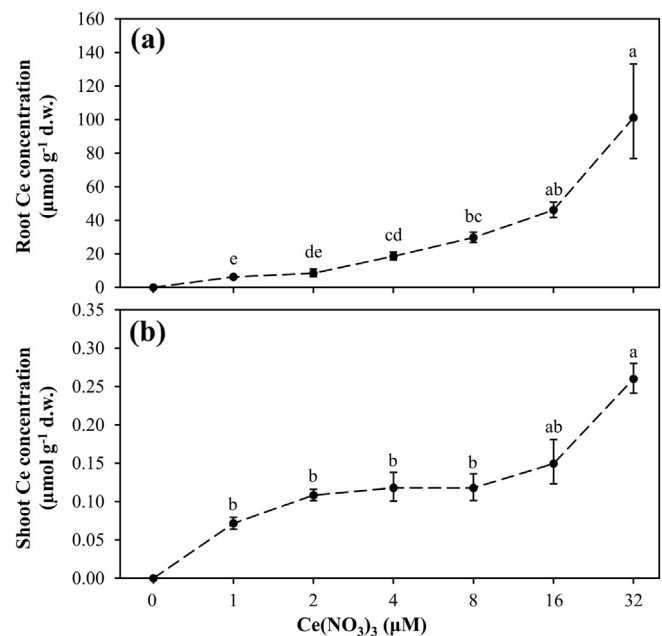

Fig. 3. Ce concentrations (μmol g^{-1} d.w.) of *Brassica napus* (median ± SE, n = 3) in roots (a) and shoots (b) after exposure to increasing Ce concentrations (μM) for 6 days. Different letters indicate significant differences between treatments separately for roots and shoots ($p < 0.05$, Tukey's test).

Fig. 2. Ce-imposed root growth inhibition (mm) of *Brassica napus* (median ± SE, n = 6) after exposure to increasing Ce concentrations (μM) for 6 days. Different letters indicate significant differences between treatments ($p < 0.05$, Tukey's test).

Table 1
Root and shoot macro- and micro-nutrient concentrations ($\mu mol\ g^{-1}$ d.w.) of *Brassica napus* (median ± SE, n = 3) after exposure to increasing Ce concentrations (μM) for 6 days.

Plant fraction	Ce treatment	Ca	K	Mg	P	Cu	Fe	Mn	Zn
Roots	0 (control)	934 ± 90 a[a]	128 ± 22 a	67 ± 10 a	129 ± 0.3 c	0.67 ± 0.26 a	4.81 ± 3.55 a	0.41 ± 0.14 a	1.93 ± 0.22 a
	1	457 ± 44 ab	189 ± 66 a	40 ± 8 a	135 ± 3 c	0.70 ± 0.24 a	11.81 ± 3.45 a	0.61 ± 0.02 a	4.23 ± 0.75 a
	2	384 ± 108 b	64 ± 17 a	29 ± 10 a	142 ± 2 c	0.61 ± 0.08 a	4.51 ± 1.00 a	0.31 ± 0.08 a	3.13 ± 1.37 a
	4	325 ± 23 b	46 ± 13 a	27 ± 3 a	144 ± 6 c	0.82 ± 0.10 a	4.58 ± 2.47 a	0.22 ± 0.06 a	2.02 ± 0.40 a
	8	250 ± 29 bc	44 ± 19 a	33 ± 8 a	163 ± 7 bc	0.45 ± 0.22 a	3.79 ± 1.13 a	0.18 ± 0.03 a	1.39 ± 0.23 a
	16	148 ± 38 c	106 ± 16 a	34 ± 10 a	210 ± 22 ba	0.65 ± 0.14 a	8.27 ± 3.31 a	0.46 ± 0.19 a	1.59 ± 0.42 a
	32	177 ± 12 c	110 ± 56 a	36 ± 12 a	266 ± 42 a	1.08 ± 0.40 a	8.86 ± 3.36 a	0.56 ± 0.13 a	3.00 ± 1.13 a
Shoots	0 (control)	293 ± 8 a	730 ± 34 a	149 ± 2 a	97 ± 3 a	0.22 ± 0.02 a	1.18 ± 0.28 a	5.99 ± 0.65 a	4.30 ± 0.28 a
	1	296 ± 11 a	808 ± 144 a	149 ± 6 a	93 ± 2 a	0.20 ± 0.05 a	1.23 ± 0.17 a	5.33 ± 0.31 a	3.59 ± 0.08 a
	2	278 ± 16 ab	879 ± 54 a	151 ± 17 a	91 ± 4 a	0.28 ± 0.03 a	1.21 ± 0.06 a	5.31 ± 1.40 a	5.01 ± 0.99 a
	4	243 ± 17 ab	893 ± 39 a	146 ± 15 ab	92 ± 1 a	0.20 ± 0.05 a	1.10 ± 0.21 a	4.86 ± 0.38 a	3.88 ± 1.46 a
	8	238 ± 17 ab	768 ± 45 a	107 ± 8 abc	86 ± 8 a	0.11 ± 0.01 a	0.69 ± 0.05 a	1.55 ± 0.24 b	0.81 ± 0.13 b
	16	232 ± 9 b	791 ± 54 a	101 ± 5bc	92 ± 10 a	0.13 ± 0.01 a	1.14 ± 0.20 a	1.59 ± 0.11 b	0.95 ± 0.21 b
	32	228 ± 8 b	751 ± 64a	96 ± 10 c	82 ± 5 a	0.17 ± 0.03 a	0.71 ± 0.05 a	1.84 ± 0.16 b	0.65 ± 0.01 b

[a] Different letters indicate significant differences between treatments separately for each element and plant fraction ($p < 0.05$, Tukey's test).
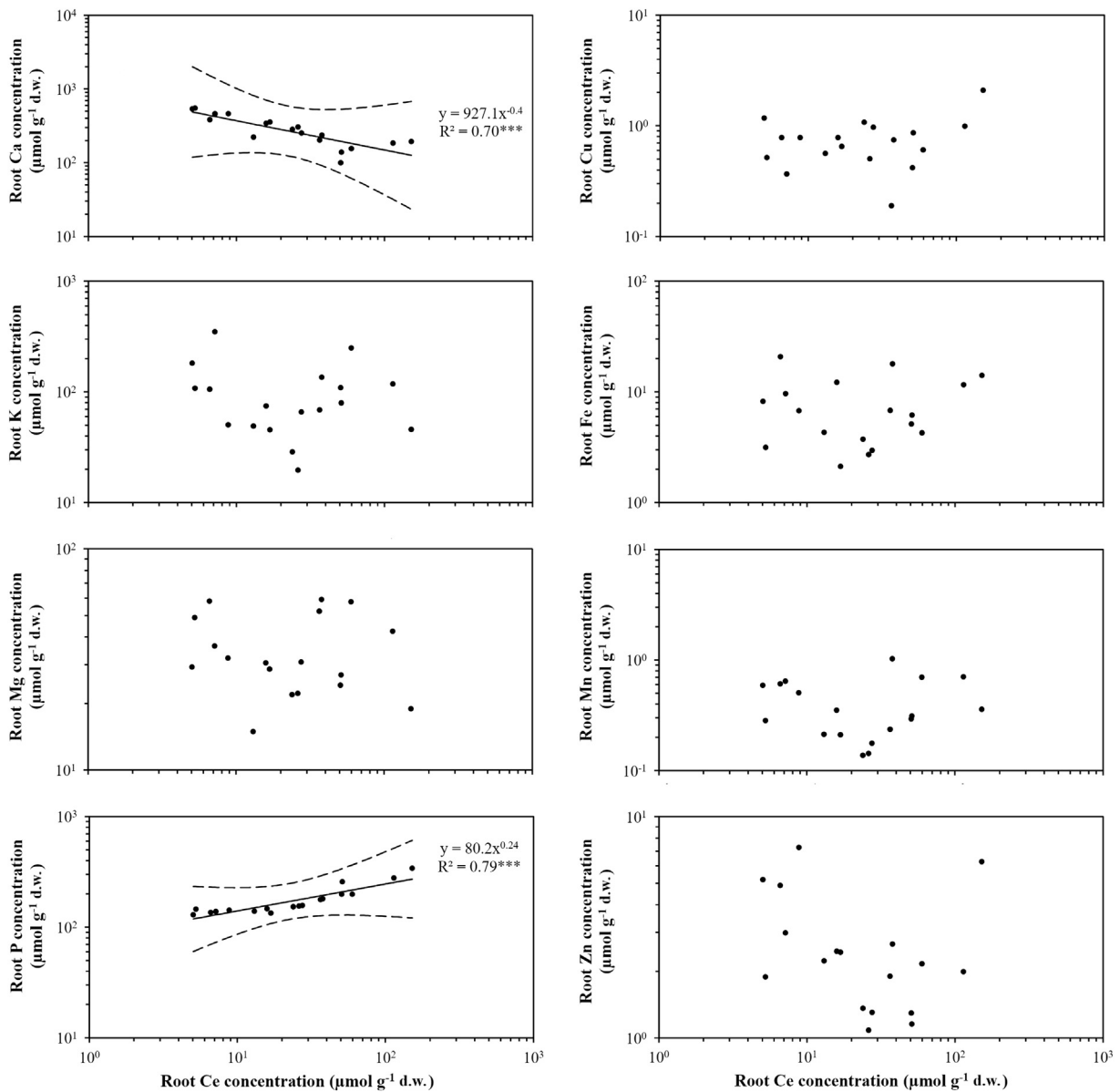

Fig. 4. Relationships between the concentrations ($\mu mol\ g^{-1}$ d.w.) of nutrients and Ce in roots of *Brassica napus* after exposure to increasing Ce concentrations for 6 days. Roots from all treatments except the control were analysed (n = 18). The regression lines (power function) and their coefficients are provided only in case of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). 95% confidence limits are shown as broken lines.

In shoots the regression slopes were significantly negative for Ca and Mg, as well as for the micronutrients Fe, Mn and Zn (Fig. 5). Although significant, the slopes and coefficients of determination were relative low (Fig. 5). Comparing means between treatments, at the two highest (16 and 32 μM) Ce treatments Ca and Mg concentrations in shoots were significantly decreased in comparison with control (Table 1).

Ca uptake in six days, expressed as the total amount of plant Ca per g root d.w. (Fig. 6), was reduced at concentrations higher than 1 μM Ce although significantly only at concentrations higher than 4 μM Ce ($p < 0.05$).

Among the micronutrients, Mn and Zn concentrations in shoots were significantly lowered at treatments ranging from 8 to 32 μM Ce. The shoot K, P, Cu and Fe concentrations were not significantly lowered by any of the Ce treatments (Table 1).

3.4. Testing the effect of Ca on Ce accumulation

As root length growth did not change significantly when Ca was supplied at 0.5 or 4 mM, instead of the standard 2 mM (data not shown) we
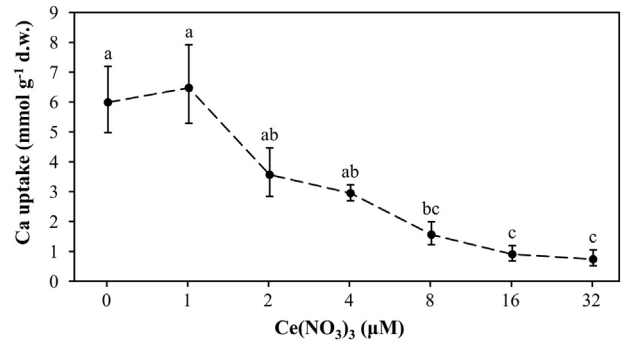

Fig. 6. Calcium uptake (mmol g^{-1} d.w.) of *Brassica napus* (median ± SE, n = 3) after exposure to increasing Ce concentrations (μM) for 6 days. Different letters indicate significant differences between treatments ($p < 0.05$, Tukey's test).

analysed data on Ce and Ca concentrations in roots and shoots using two-way ANOVA. Root Ce concentrations were significantly affected by the concentrations of both Ca and Ce in the nutrient solution (both
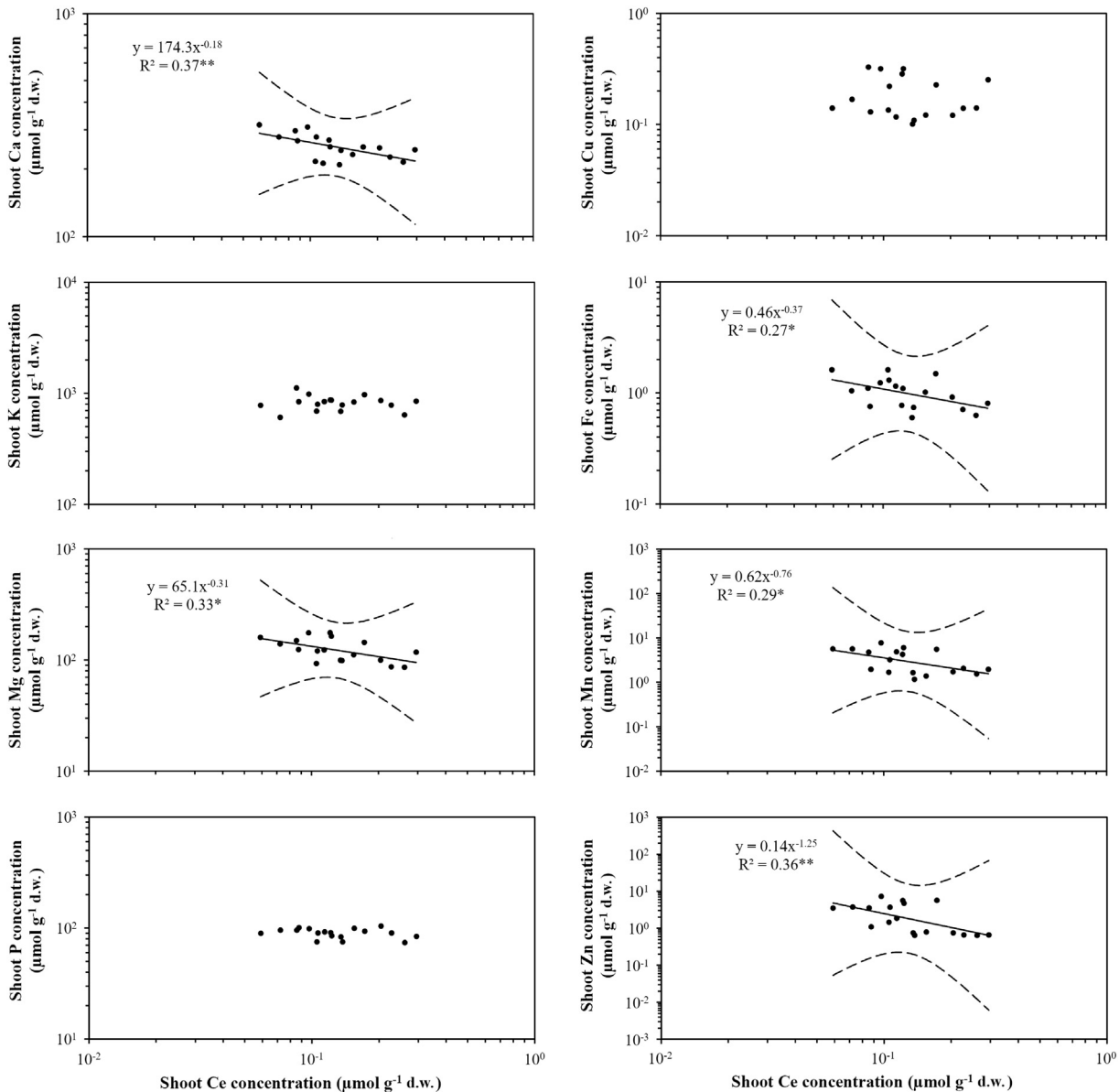

Fig. 5. Relationships between the concentrations (μmol g^{-1} d.w.) of nutrients and Ce in shoots of *Brassica napus* after exposure to increasing Ce concentrations for 6 days. Shoots from all treatments except the control were analysed (n = 18). The regression lines (power function) and their coefficients are provided only in case of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). 95% confidence limits are shown as broken lines.

main effects $p \ll 0.001$). Shoot Ce concentrations were unaffected by Ca and Ce concentrations in the nutrient solution and the Ce × Ca interaction was not significant in either roots or shoots (Table 2). Thus, independently of the Ce concentration, at increasing Ca concentrations in the nutrient solution root Ce concentrations were significantly reduced while shoot Ce concentrations remained unaltered (Table 2).

Root Ca concentrations were significantly affected both by the Ca ($p < 0.001$) and Ce ($p \ll 0.001$) concentrations in the nutrient solution. On the contrary, shoot Ca concentrations were significantly affected only by the Ce concentration in the nutrient solution ($p \ll 0.001$). Shoot Ca concentrations were unaffected by Ca concentrations in the nutrient solution and the Ce × Ca interaction was not significant in either roots or shoots (Table 2). For each level of Ca supply and at increasing Ce concentrations in the nutrient solution the Ca root and shoot concentrations were significantly lowered (Table 2).

The ratio of Ce to Ca in roots was linearly and positively related to that in nutrient solution at $p \ll 0.001$ ($r = 0.97$ and $r = 0.99$ at 0.5 μM and 6 μM Ce respectively). Conversely the ratio of Ce to Ca in shoots was not significantly correlated to that in nutrient solution.

Ca uptake in five days, expressed as the total amount of plant Ca per g root d.w. (Table 3), was significantly reduced only by Ce concentration in the nutrient solution ($p \ll 0.001$). On the other hand, Ca uptake was not significantly affected by the Ca concentration in the nutrient solution and the Ce × Ca interaction, although at 4 mM Ca the mean Ca uptake seemed to increase.

3.5. Effect of Ca and temperature on concentration-dependent uptake kinetics of Ce

To test whether Ce uptake is passive (apoplastic pathway) or active (symplastic pathway), we analysed the concentration-dependent uptake kinetics at 2 °C and at 22 °C. At cold temperatures, uptake by the symplastic pathway should be minimal so the apparent uptake after desorption can be mainly attributed to passive adsorption via the apoplastic pathway. The concentration-dependent uptake kinetics for Ce in 0.5 mM Ca at 2 °C could not be described by the Michaelis-Menten model and only a linear component was observed (Fig. 7 and Table 4).

To test if Ce can interact with Ca uptake in roots we analysed the concentration-dependent uptake kinetics at 22 °C at two different Ca concentrations (0.5 and 4 mM) in solution. The curves of the concentration-dependent uptake kinetics for Ce with either 0.5 mM or 4 mM Ca at 22 °C showed a saturable (hyperbolic) component and a Michaelis-Menten model was sufficient to describe them (Fig. 7). An additional linear component to the Michaelis-Menten model did not satisfactorily improve the fit. Saturable Ce influxes were characterized by

Table 3
Ca uptake (mmol g^{-1} d.w.) of *Brassica napus* (median ± SE, n = 6) after exposure to different Ca and Ce concentrations in hydroponics for 5 days.

Ca in solution (mM)	0.5 μM Ce in solution	6 μM Ce in solution	Tukey's between Ce treatments
0.5	1.90 ± 0.40 a[a]	0.89 ± 0.13 a	*[b]
2	2.13 ± 0.34 a	0.88 ± 0.22 a	*
4	3.90 ± 0.44 a	1.59 ± 0.11 a	*

[a] Different letters indicate significant differences for Ca levels for each Ce treatment ($p < 0.05$, Tukey's test).
[b] ns, not significant.
* $p < 0.05$.

similar K_m values but very different maximal influxes (V_{max}) (Table 4). The value of V_{max} for influxes at 4 mM Ca was almost two-fold lower than that at 0.5 mM Ca. It can therefore be concluded that Ce is actively taken up into root cells and that Ca acts as a non-competitive inhibitor of Ce influx.

3.6. Growth of rapeseed sprayed with Ce under Ca deficiency

Shoot fresh weight was severely decreased in Ca-deficient solution and spraying (with either Ce(NO$_3$)$_3$, or CaCl$_2$) did not improve it (Fig. 8). Plants in Ca deficient solution did not develop fully opened leaves and small yellowish patches were visible on leaves. On the contrary, the SPAD index was neither significantly affected by Ca deficiency, nor Ce(NO$_3$)$_3$ or CaCl$_2$ spraying (Fig. 8).

4. Discussion

Rapeseed already showed a negative growth response at low (2 μM) Ce concentration in nutrient solution. This concentration is similar to the lowest inhibiting concentration for root elongation in mungbean cultivated in hydroponics (1 μM, Diatloff et al., 1995a). However, these concentrations are lower than those reported for maize (4 μM, Diatloff et al., 1995a) and common wheat (14.3 μM, Hu et al., 2002a, 2002b). Interestingly, Liu et al. (2012) reported an extremely high concentration (100 μM) of Ce in agar, which did not negatively affect the growth of rice, while Wang et al. (2012) reported a strong growth inhibition of *Arabidopsis thaliana* at 50 μM Ce in agar. Other data available in the literature are questionable because of high P concentrations (0.31 mM) in the nutrient solutions that supposedly precipitated Ce (He and Loh, 2000). Thus, although it is difficult to compare results from hydroponic and agar cultures and there are not yet many data available, it seems that monocots are much more tolerant to Ce toxicity than eudicots. The higher Ce tolerance in monocots can be tentatively explained by

Table 2
Root and shoot Ce and Ca concentrations (μmol g^{-1} d.w.) of *Brassica napus* (median ± SE, n = 6) after exposure to different Ca and Ce concentrations in hydroponics for 5 days.

Analysed element	Plant fraction	Ca in solution (mM)	0.5 μM Ce in solution	6 μM Ce in solution	Tukey's between Ce treatments
Ce	Root	0.5	11.3 ± 0.6 a[a]	24.2 ± 1.0 a	**[b]
		2	5.8 ± 0.4 b	13.1 ± 0.8 b	**
		4	3.8 ± 0.4 c	9.0 ± 0.3 c	**
	Shoot	0.5	0.0573 ± 0.0032 a	0.0498 ± 0.0027 a	ns
		2	0.0554 ± 0.0075 a	0.0544 ± 0.0125 a	ns
		4	0.0639 ± 0.0040 a	0.0640 ± 0.0105 a	ns
Ca	Root	0.5	310 ± 13 b	237 ± 12 b	*
		2	386 ± 12 ab	303 ± 22 a	*
		4	398 ± 28 a	295 ± 16 ab	**
	Shoot	0.5	292 ± 7 a	208 ± 12a	**
		2	301 ± 2 a	225 ± 11 a	**
		4	304 ± 6 a	229 ± 6 a	**

[a] Different letters indicate significant differences for Ca levels for each Ce treatment and separately for roots and shoots ($p < 0.05$, Tukey's test).
[b] ns, not significant.
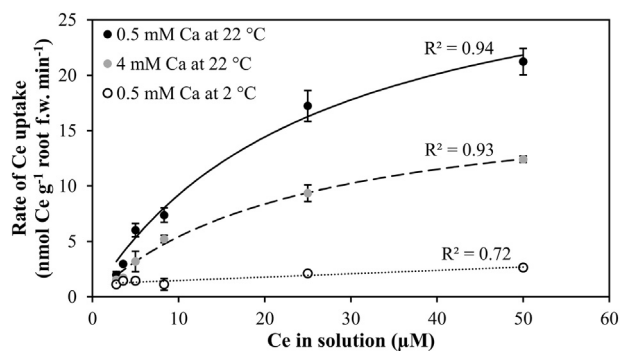* $p < 0.05$.
** $p < 0.01$.

Fig. 7. Ce uptake in *Brassica napus* roots (mean ± SE, n = 3) over 40 min under 0.5 mM Ca at 2 °C (dotted line) or 22 °C (solid line) and under 4 mM Ca at 22 °C (dashed line).
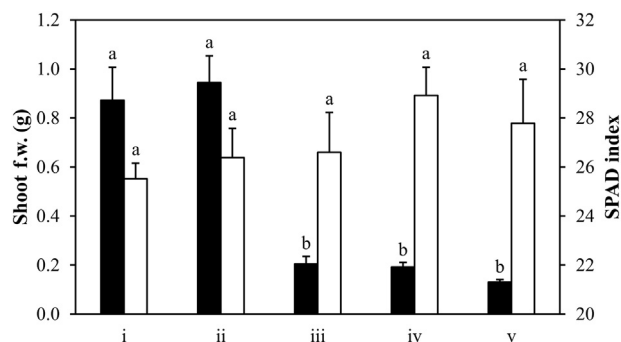

Fig. 8. Effects of foliar spraying of 15 μM $Ce(NO_3)_3$ or $CaCl_2$ on *Brassica napus* shoot fresh weight (black columns, left ordinate) and on the SPAD index (white columns, right ordinate) (median ± SE, n = 3). Plants were cultivated in (i) half-strength Hoagland's solution and not sprayed, (ii) half-strength Hoagland's solution and sprayed with 15 μM $Ce(NO_3)_3$, (iii) Ca deficient half-strength Hoagland's solution and not sprayed, (iv) Ca deficient half-strength Hoagland's solution and sprayed with 15 μM $Ce(NO_3)_3$, (v) Ca deficient half-strength Hoagland's solution and sprayed with 15 μM $CaCl_2$. Different letters indicate significant differences between treatments for each parameter separately ($p < 0.05$, Tukey's test).

their higher efficiency in restricting Ce translocation from roots to shoots and a more efficient antioxidant mechanism compared to eudicots (Liu et al., 2012; Wang et al., 2012).

In any case, both eudicots and monocots tend to exclude Ce from shoots (Diatloff et al., 1995b, 2008; Hu et al., 2002a, 2002b; Liu et al., 2012; Wang et al., 2012), in agreement with our results (Fig. 3), which demonstrate that rapeseed has a typical shoot excluder strategy (Baker, 1981). This is true at least in the range of concentrations where rapeseed is still able to cope with Ce toxicity. At higher (>16 μM) Ce concentrations an indication of a saturation of the metal homeostasis machinery is visible, as root growth was severely affected and Ce in shoots started to increase rapidly (Figs. 2 and 3). Anyway, our data suggest an active mechanism for Ce uptake, as there is very little uptake in roots under cold conditions (Fig. 7).

The nutrient most negatively affected by Ce treatments was Ca in both roots and shoots (Table 1, Figs. 4 and 5), which is remarkable because growth inhibition owing to toxicity usually increases, rather than decreases Ca concentrations, particularly in the root (Manara, 2012). This is in accordance with observations in maize, mungbean, *A. thaliana* and rice (Diatloff et al., 1995b, 2008; Hu et al., 2002a, 2002b; Liu et al., 2012; Wang et al., 2012). There are some indications that the decreased Ca uptake is a specific consequence of Ce toxicity (Fig. 6 and Table 2). However, to further confirm this hypothesis, kinetics of Ca uptake at different Ce concentrations should be done. The reduced Ca uptake can be due to displacement of Ca by Ce in the root apoplast (Diatloff et al., 2008), leading to reduced apoplastic Ca movement to the xylem (White, 2001). However, considering the enormous effect of low micro-molar concentrations of Ce on the uptake of Ca (Fig. 6), which is present at orders-of-magnitude higher concentrations in the nutrient solution, it is likely that Ce, just like the related rare earth, lanthanium (La), acts as a blocker of Ca channels (Huang et al., 1994; Marshall et al., 1994). On the other hand, Ca also blocks Ce uptake, although much less effectively then the other way around, and seemingly in a non-competitive way, because the K_m for Ce is not affected by the Ca concentration (Table 4).

Although Ca clearly inhibits Ce uptake into the root, it does not significantly affect Ce translocation to the shoot, while Ce does inhibit

the translocation of Ca to the shoot, albeit less effectively then its uptake into the root (Table 2). The reason for this is not clear, but it might be that the loading of Ce into the xylem, which is extremely slow, is entirely passive and apoplastic (White, 2001). Alternatively, Ce uptake and xylem loading could be mediated by different transporters with a different responsiveness to Ca.

Another noteworthy result was the observation of an increase of the P concentration in roots in the highest Ce treatments, and the positive correlation between P and Ce concentrations in roots (Table 1, Fig. 4). This could result from non-metabolic $CePO_4$ precipitation in the root cells, which would be expected to reduce the translocation of both P and Ce. An observed increase of P in maize roots and a relative decrease in shoots was also observed by Diatloff et al. (2008), in treatments with relatively low Ce concentrations in nutrient solutions. However, in the same experiment, the P status was not affected in mungbean, which could be due to a lower demand for P. Nagahashi et al. (1974) and Leonard et al. (1975), hypothesised a non-metabolic apoplastic precipitation of La with P in roots and suggested that this is the reason why La does not cross the endodermis. However, our data suggest that Ce precipitation can also occur not just in the apoplast, but probably also inside the cells, because we desorbed the roots in appropriate cleansing solution and we tested the plants in a P-free nutrient solution. P will precipitate Ce even in the solution, as demonstrated by our observation that the EC_{50} for root growth in a complete half-strength Hoagland's is >640 μM, in comparison with 4.2 μM in a half-strength Hoagland's without P and Fe-EDTA. Thus the contrasting observations by Nagahashi et al. (1974) and Leonard et al. (1975) could simply be due to presence of P (0.25 mM) in the test solutions, so there was no chance for La to enter the root cells, let alone to pass the endodermis. However, further confirmation would be required with TEM-EDAX analysis to see if there are any $CePO_4$ precipitates inside root cells.

Under Ce exposure also the shoot concentrations of nutrients other than Ca, such as Mg, Fe, Zn, and Mn, tend to decrease, suggesting that Ce also has non-specific effects on nutrient homeostasis, owing to impaired root functioning (Table 1, Fig. 5). In agreement with this, also Guo et al. (2007) showed disturbed macronutrient homeostasis after applying Ce to leaves, albeit that they used an unrealistically high Ce concentration (7 mM). It has also been reported that addition of 20 μM Ce to a Mn or Mg deficient nutrient solution can partially alleviate Mn and Mg deficiency symptoms in maize (Gong et al., 2011; Zhao et al., 2012). However, these results are questionable, because the authors used a nutrient solution with a high P concentration (0.2 mM), which is sufficient to precipitate all of the Ce added.

Table 4
Parameters of the Michaelis-Menten model (mean ± SE, n = 3) for Ce uptake kinetics in *Brassica napus* roots over 40 min under 0.5 mM Ca at 2 °C or 22 °C and under 4 mM Ca at 22 °C.

Solution		V_{max} (nmol g^{-1} root f.w. min^{-1})	K_m (μM)	a (nmol g^{-1} root f.w. min^{-1} μM^{-1})
Ca (mM)	Temperature (°C)			
0.5	2	1.2 ± 0.1	–[a]	0.03 ± 0.01
0.5	22	33.2 ± 1.8	26.1 ± 5.8	–
4	22	18.4 ± 1.1	23.8 ± 5.9	–

[a] Omitted from the model.

Finally, we could expect that Ce toxicity in shoots would be due to displacement of other weakly bound divalent metal ions from enzymes. Although our data suggest a decrease in Mg concentrations in the shoots this is hardly due to its replacement by Ce in chlorophyll as we did not observe any morphological disorders in leaves and even the relative chlorophyll content (SPAD index) did not change at increasing Ce concentrations in the nutrient solution. In general, in contrast with previous studies (Chao et al., 2008, 2009; Huang et al., 2008), we did not observe any beneficial effect of spraying leaves with Ce. Therefore, we can safely conclude that spraying with REs will not improve the yield of rapeseed. Even in Ca deficient nutrient solution spraying with Ce did not have any significant effect. This could be due to the low Ce concentration in the spray (15 μM), or the short duration of the experiment. Only the slight and statistically insignificant increase of the SPAD index could indicate some potential for Ce to improve the photosynthetic yield as suggested by Huang et al. (2008). In any case, in our experiment this improvement was not greater than that produced by CaCl₂ spraying, in contrast to the results reported by Huang et al. (2008). How Ce interacts in long-term experiments with rapeseed has to be investigated yet.

Remarkably, many studies in the Chinese literature from the past 30 years have suggested that fertilizers with low concentration of REs promote growth and yield of several crops (see reviews by Hu et al., 2004; Tyler, 2004 and El-Ramady, 2010 for details). However, the majority of these papers are unavailable to the international public as they are written in Chinese and it is difficult to discover details of experimental design, methods and statistical analysis. Therefore, Tyler (2004) suggested that it is possible that these REs fertilizers may have contained urea and other micronutrients such as Ca and nitrates (see also Hu et al., 2004). In fact in REs fertilizers analysed by Asher et al. (1990) and Diatloff et al. (1999) significant amounts of nitrate (48.7 and 45.3% respectively) and Ca (2.2 and 0.74% respectively) were found (see also El-Ramady, 2010).

5. Conclusions

Our results showed that rapeseed growth and nutrition in hydroponics is strongly inhibited by Ce, showing that Ce is a highly toxic rare earth. It seems unlikely that Ce can have beneficial effects on crop yields. For these reasons fertilizers containing Ce should be avoided in agriculture. Our work can also serve as a basis for future evaluation of Ce oxide nanoparticles (CeO₂) toxicity, which is a recent concern (OECD, 2010). There have been no attempts to link nanoparticles toxicity and their ability to release soluble ions. Given our results it seems likely that many effects of CeO₂ nanoparticles are simply owing to dissolved Ce.

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