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EDTA-Assisted Metal Uptake in *Raphanus sativus* L. and *Brassica oleracea* L.: Assessment of Toxicity and Food Safety

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Abstract

A study was conducted to determine the effect of ethylenediaminetetraacetic acid (EDTA) on phytoextraction potential of radish and cabbage. Plant biomass, photosynthetic pigments, proline and phenolics were significantly affected by the accumulation of heavy metals (HM). The metal uptake potential was increased significantly by the application of EDTA. Target hazard quotient (THQ) associated with exposure of these contaminants to food chain was calculated. Agronomic interventions to increase mineral levels in crops often increases the leaf concentrations only, the mineral concentration in edible portions are not increased at desired level due to low mobility of Zn in phloem. Since the leaves of both these crops are edible and a component of staple vegetarian diet, biofortification through Zn present in soil and its solubilization and mobilization through chelators can be implemented. However in no such instance these crops should be consumed when grown on Pb contaminated soil due to associated hazards.

Keywords Heavy metal · Phytoremediation · Hazard quotient · Risk assessment

Heavy metal (HM) contamination of soil through natural and/or anthropogenic activities has threatening effects on quality and functioning of biosphere. While extensive industrialization has added a range of contaminants to soil, rapid urbanization has resulted in shortage of agricultural land. Moreover, the use of urban wastewater for irrigation can be a source of HM exposure in cultivated areas (Sun et al. 2009). Not only HMs are known to exert mutagenic and clastogenic effects on plants (Venkatachalam et al. 2017) but also tend to bioaccumulate in consumers through food chain, hence emergent and imperative measures need to be taken in order to decontaminate soil. While conventional clean-up methods (physical and/or chemical) are costly and labor intensive in nature, phytoremediation offers the advantage of being cost effective and solar driven.

Till now, more than 500 hyperaccumulator plant species comprising of at least 101 families have been reported (Krämer 2010). Among these, around 90 hyperaccumulator plants belong to the family Brassicaceae including model hyperaccumulator species *Alyssum*, *Noccaea*, *Arabidopsis* and *Brassica*. Practically, most hyperaccumulators are not feasible for phytoextraction since they are slow-growing plants with shallow root system and small biomass (Prasad and Freitas 2003). For practical implications, researchers are screening fast-growing and high biomass producing plants for their metal tolerance and accumulation potential. Brassicaceae is a family containing many metal-accumulating species and are possible candidates for phytoremediation. There are several agriculturally important crops among these which are used for human consumption, animal feed, production of biofuels, etc. (Schmidt and Bancroft 2010). The possible use of members from the family Brassicaceae in phytoremediation (mainly phytoextraction) derives from

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their inherent tolerance to HMs and substantial biomass production (Neilson and Rajakaruna 2012).

While continuous phytoextraction involves the use of hyperaccumulators to clean-up soil, chelate-assisted phytoextraction involves the application of chelators to enhance metal uptake potential of plants (Sun et al. 2009) by making precipitated metal species more soluble. In contrast to continuous phytoextraction where uptake and accumulation takes place gradually with time, chelate-assisted phytoextraction is rapid since chelates are applied to the soil to liberate huge quantities of HM. Chelates have been employed to enhance the uptake of a number of HM contaminants including Pb, Cd, Cu, Ni and Zn. EDTA has been found to be the most effective chelating agent considerably enhancing the accumulation of HM (Garba et al. 2012) in plants and *Brassica* species (Lim et al. 2004; Zaier et al. 2010).

Many edible and non edible crops are being tested for their HM accumulating potential, but an account of edibility has not been stated when these crops are grown in HM contaminated soil. There is increasing concern that cultivation of edible plants on contaminated soil can lead to the uptake and accumulation of heavy metals in the edible parts with their concentrations exceeding statutory or advisory limits (e.g., legal limit set by FAO/WHO) (Luo et al. 2011). *Raphanus sativus* L. and *Brassica oleracea* L. are two commonly grown seasonal vegetables, with a small life cycle i.e., 90 and 60 days, respectively from the family Brassicaceae, often showing HM accumulation when grown on wastelands by marginal farmers or irrigated with wastewater. The present study was undertaken to—(i) assess the effect of EDTA on HM uptake potential (ii) study the effect of HMs on plants under study at physiological and biochemical level, and (iii) highlight the risks associated with consumption of such crops upon food chain exposure.

Materials and Methods

The experiment was conducted in pot assay at plant repository, department of Botany, St. John's College, Agra (27°1939′ N latitude and 78°0025′ E longitude). Soil used in the study was sandy-loam with an average pH value (7.2 ± 0.04) and electrical conductivity (EC) (0.55 ± 0.08 dS/m). It was collected from the botanical garden (0–20 cm), sieved (< 5 mm mesh), autoclaved thrice (121° C, 15 lbs pressure for 30 min) and air dried. Each pot was then filled with a homogenous mixture of soil (4 kg for radish and 2 kg for cabbage) and 25, 50 and 100 mg kg^{-1} Pb and Zn separately. Further, a counter set supplemented with EDTA (5 mmol kg^{-1}) was maintained. There are no reported negative effects of EDTA on soil physicochemical properties and flora at such low concentration (Epstein et al. 1999). Pots with no HM treatment served as control. All pots were placed in random block design in a field and three replicates of each treatment were present. The pots were covered and left undisturbed for 15 days in order to stabilize. Simultaneously, certified seeds of radish and cabbage were germinated in trays and 15 days old plantlets were then transplanted to respective pots. The pots were kept under field conditions and watered regularly. The minimum temperature during the course of experiment ranged between 3.2–22.3 °C and the maximum temperature ranged between 16.1–34.6 °C. The humidity and light intensity ranged between 42%–58% and 3.12–6.32 kWh/m^2 /day respectively. The plants were harvested at maturity (i.e., 90 days for radish and 60 days for cabbage). The phytoextraction efficiency differs under field and pot conditions due to unlimited volume of soil in the field. The limited volume of soil in the pots coupled with compact rhizospheric conditions leads to profuse proliferation of root system/unit area, thus providing more surface area to interact with soil treatment thereby better manifestation of plants' potential. In a preliminary study conducted to determine the treatment doses, soil from different depths (0–20 cm, 21–40 cm and 41–60 cm) was collected randomly from the study area to quantify the content of test metals. Results show the maximum quantity of HM content at the depth 0–20 cm which ranged between 22.97 and 103.45 mg kg^{-1} for Pb and 17.45–98.96 mg kg^{-1} for Zn, which gradually declined with the depth. Size of pot was determined by taking into account the root length, rhizospheric area of test plants and volume of soil.

For the estimation of chlorophyll and carotenoids, fresh leaf samples (0.5 g) were ground in 80% (v/v) acetone (10 ml) and centrifuged at 4000 rpm for 5 min at 4 °C. The extract was collected and the optical density was measured spectrophotometrically at 665, 649 and 470 nm according to Lichtenthaler (1987) and expressed in mg g^{-1} FW.

The content of free proline was measured as per Bates et al. (1973). Fresh leaf samples (250 mg) were homogenized in 3% sulphosalicylic acid (5 mL) and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and a 2 mL aliquot was treated with an equal volume of acetic acid and acid ninhydrin reagent. The reaction mix was heated in a water bath at 65 °C for 1 h and the reaction was terminated by keeping in ice bath. Proline was extracted by adding 4 mL of toluene and vortexing the mix. The chromatophore containing toluene layer was separated and its absorbance was measured at 520 nm by a UV–Vis spectrophotometer. Pure toluene was used as blank. The content of proline was estimated using a standard curve obtained after plotting the absorbance of pre-prepared proline concentration stocks (20, 40, 60, 80 and 100 ppm) and expressed in µmol g^{-1}.

Total phenolics content (TPC) was determined using the Folin-ciocalteu assay, as per Bray and Thorpe (1954). Dried leaves (500 mg) were ground and boiled in 80% ethanol (10 mL) using a water bath at 100° C for 3 h. After cooling the solution to room temperature, it was centrifuged at 600 rpm for 15 min. The extraction step was repeated twice and the supernatant was collected after each step. The supernatant was pooled, evaporated to dryness and dissolved in water (10 mL). To 1 mL of alcoholic extract, Folin-ciocalteu reagent (1 mL) and 20% Na_2CO_3 (2 mL) were added. The mixture was heated in a boiling water-bath for 1 min, cooled immediately under water and absorbance was recorded at 650 nm in a UV–Vis spectrophotometer. Standard curve of catechol was prepared using catechol standard solutions. Phenols were quantified using catechol as standard and the results were expressed as mg catechol equivalent gram^{-1} DW.

For HM analysis, harvested plants were washed with distilled water and dried in hot air oven at 65 °C for 24 h to remove moisture content. The dried samples were ground into a fine powder and 0.5 g of powdered sample was digested with 10 mL HNO_3 at 80 °C, till the solution became transparent. The resulting solution was passed through Whatman No. 1 filter paper and the filtrate was analyzed for each metal(loid) by flame atomic absorption spectrophotometer (AAnalyst100, Perkin Elmer, USA) using an air-acetylene flame. The recovery rates for metals analyzed were 71% for Pb and 95% for Zn.

Since the efficiency of phytoextraction depends on the biomass of plants to a great extent, remediation factor (RF), which is defined as the ratio of metal content in plant to that in soil (Sun et al. 2009) (modified), was calculated as follows:

$$RF\% = \frac{M_{plant} \times W_{plant}}{M_{soil} \times W_{soil}} \times 100\%$$

where, M_{plant} is the content of HM accumulated in plant (mg kg^{-1}); W_{plant} is the plant dry biomass (g); M_{soil} is the content of HM in soil (mg kg^{-1}) and W_{soil} is the amount of soil in the pot (g).

Target Hazard Quotient (THQ) method stated by the United States Environmental Protection Agency (Zhou et al. 2016), was used to quantify the risk associated with the consumption of vegetables grown on HM contaminated soil as follows:

$$THQ = \frac{C \times F_{IR} \times E_F \times E_D}{R_F D \times W_{AB} \times T_A} \times 10^{-3}$$

where, C is the concentration of HM in the edible part of vegetable; F_{IR} is the food ingestion rate (recommended average consumption value of vegetable for adult men and women is 300 g person^{-1} day^{-1}, respectively) (National Institute of Nutrition 2011); E_F is the exposure frequency 350 days/year (USEPA 2011); E_D is the exposure duration (68.35 years, equivalent to the average lifetime of the Indian population as per World Bank Statistics 2015); $R_F D$ is the oral reference dose for Pb and Zn (0.0035 and 0.3 mg kg^{-1} day^{-1}, respectively) (USEPA 2003); W_{AB} is the average body weight of Indian population (60 kg for men and 55 kg for women); and T_A is the average exposure time for non-carcinogens (E_D * 365 days year^{-1}).

The experiment was carried in triplicate and means were recorded. The data was subjected to one-way ANOVA using Student–Newman–Keuls (SNK) test for comparisons between groups to analyze significant differences in treatments ($p \leq 0.05$).

Results and Discussion

Photosynthesis is the principle metabolic process channeled by a number of molecular and cellular entities, susceptible to HMs. HMs are not only known to alter the structure and composition of proteins involved in photosynthesis, but chlorophyll and carotenoids, the constituents of LHCs (light harvesting complexes) also show a decrease in content upon encounter with HMs (Kuzminov et al. 2013). In the present study, plants showed a dose dependent decrease in the concentration of pigments in response to HM stress (Fig. 1a, b). Radish and cabbage plants under Pb stress showed up to 42% and 34.6% decrease in the concentration of chlorophyll and up to 40.65% and 52.38% reduction in the concentration of carotenoids, respectively. However, lower concentration of Zn was found to affect the concentration of photosynthetic pigments positively. At 25 mg kg^{-1} concentration, radish and cabbage plants showed up to 0.58% and 0.59% increase in chlorophyll and 1.29% and 1.58% increase in carotenoids, respectively. Zinc, though a HM, is an essential micronutrient required as a cofactor for many enzymes participating in photosynthesis (Shanmugam et al. 2011).

Application of EDTA resulted to an increase in photosynthetic pigments of control plants which indicate that in the absence of HM, it leads to mobilization of micronutrients efficiently and benefits the plants. In plants under Pb stress, it lead to up to 45.63% and 38.71% reduction in chlorophyll and up to 47.09% and 53.97% reduction in carotenoids in radish and cabbage plants, respectively. Similarly, in plants exposed to Zn stress, application of EDTA lead to a decrease up to 42.73% and 24.92% in chlorophyll and 40.64% and 50.79% in carotenoids in radish and cabbage plants, respectively. However in plants under 25 mg kg^{-1} Zn treatment, application of EDTA lead to an increase up to 1.02% and 2.05% in the concentration of chlorophyll and 2.58% and 3.97% in the concentration of carotenoids in radish and cabbage plants, respectively.

Under HM stress conditions, higher proline content along with enhanced HM tolerance, indicate its protective, osmoregulative and antioxidative properties (Huang and Wang 2010). Besides being an osmoprotectant and ROS

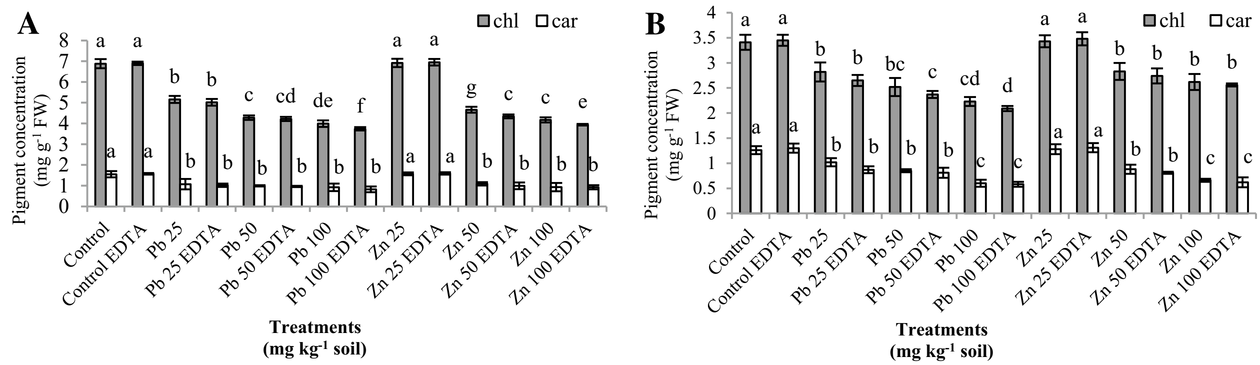

Fig. 1 Effect of Pb and Zn on photosynthetic pigments (*Chl* chlorophyll, *car* carotenoids) of (**A**) radish and (**B**) cabbage plants. Values (means ± SD of triplicates) followed by same letters (a–c) were not significantly different from each other at $p \leq 0.05$ (SNK test)

(reactive oxygen species) quencher, proline is also known to be a HM chelator and thus alleviates stress (Hayat et al. 2012). In all the treatments, plants experienced an increase in proline levels (Fig. 2a). While radish and cabbage plants under Pb stress encountered up to 29.05% and 49.68% increase in proline level, up to 19.59% and 38.85% increase was observed under Zn stress, respectively. Since EDTA treatment increased the metal availability for plants, an increase in proline content (up to 39.86% and 56.05% under Pb treatments and up to 25.67% and 42.68% under Zn treatments) was seen in radish and cabbage plants, respectively.

A dose dependent increase in TPC was encountered in all the treatments (Fig. 2b). Radish and cabbage plants experienced up to 50.13% and 47.04% increase in TPC under Pb stress and up to 33.42% and 34.23% increase under Zn stress, respectively. In the presence of EDTA, the increase was up to 58.62% and 53.45% in Pb treated plants whereas up to 40.05% and 43.35% in Zn treated plants. Both proline and phenols are non-enzymatic antioxidants that play an important role in defence strategies. Like proline, plant phenolics are also metal chelators along with being antioxidants. The efficient survival of radish and cabbage plants under HM stress along with an increase in the contents of both non-enzymatic antioxidants suggests the importance of these molecules in imparting protection against ROS injuries.

The most visible outcome of HM toxicity in plants is a reduction in plant growth, others being chlorosis, restricted nutrient uptake and decline in yield (Guala et al. 2010). While plants under HM stress showed a dose dependent inhibition of vegetative growth which was also statistically significant, the application of EDTA further decreased the biomass but this difference was statistically not significant (Table 2). Radish and cabbage control plants showed up to 3.93% and 0.45% increase in vegetative growth upon application of EDTA, suggesting the ability of EDTA to chelate and mobilize micronutrients. While radish plants showed up to 32.86% and 20.32% decrease; cabbage plants showed up to 20.22% and 16.21% decrease in vegetative growth under Pb and Zn stress, respectively. It is noteworthy that Zn at 25 mg kg^{-1} lead to an increase in biomass of cabbage. Overall, Pb was found to affect growth more adversely than Zn in both the plants.

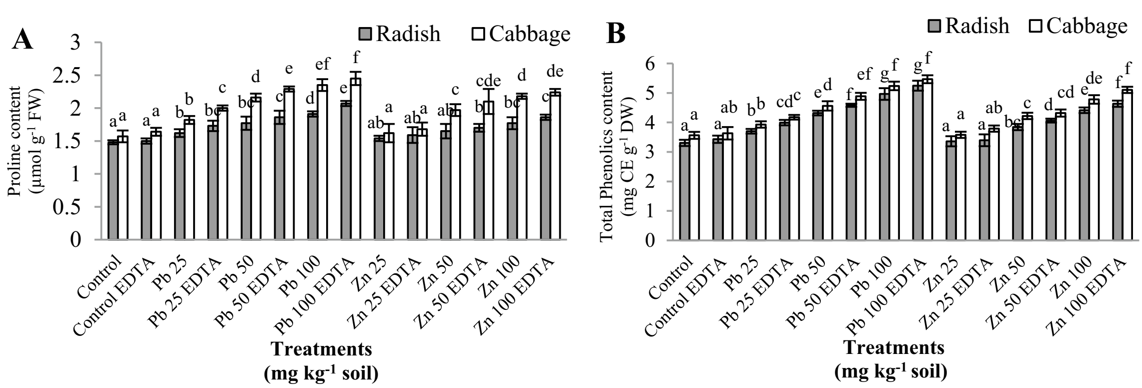

Fig. 2 Effect of Pb and Zn on **A** Proline content; **B** TPC of radish and cabbage plants. Values (means ± SD of triplicates) followed by same letters (a–g) were not significantly different from each other at $p \leq 0.05$ (SNK test)

Both the plants showed dose dependent uptake and accumulation of HM which was significantly increased upon the application of EDTA (Table 1). RF values increased on application of EDTA for all treatments in both the plants, except in the treatment with highest concentration in cabbage. This suggests that EDTA affected the uptake of HM by cabbage plants up to certain level, beyond which the uptake and accumulation remained unaffected probably due to exclusion strategies developed by the plants.

The THQ was found to be > 1 for both the crops when grown on Pb contaminated soil (Table 2). This highlights the risk associated with cultivation of edible crops on such sites. Lead is a common contaminant that enters soil through industrial effluents, municipal sewage sludge use of fertilizers and pesticides. For the level of toxicity, Pb has been ranked first in the list of US Agency for Toxic Substances and Disease Registry (ATSDR), on the basis of its prevalence and severity of toxicity.

The content of Zn was far below being hazardous and this concentration of Zn can be used for biofortification in crop plants. More than 30% of the world's population has been reported to be Zn deficient and biofortification has been proposed to be a promising way to address mineral malnutrition (White and Broadley 2011).

In the present study, the addition of EDTA effectively increased the accumulation of HM in both the plants. Although the application of EDTA increases the efficiency of phytoextraction by enhancing the HM uptake, the decrease in biomass needs to be taken into account. Since the rate of phytoextraction is directly proportional to rate of plant growth, and the amount of phytoextraction is correlated to total plant biomass, selection of high biomass crops with suitable agronomic practices can be a promising strategy (Evangelou et al. 2007). In the above study, no significant decrease in biomass was observed upon EDTA application and hence these crops can be used as potential candidates for phytoextraction. Further, no visible symptoms

Table 1 Metal content, biomass and RF values of Radish and Cabbage plants

Treatment	Radish				Cabbage		
	HM content in shoot (mg kg^{-1})	HM content in root (mg kg^{-1})	Biomass (g)	RF%	HM content in head (mg kg^{-1})	Biomass (g)	RF%
Control	ND	ND	11.96 ± 0.42 a	–	ND	13.2 ± 0.20a	–
Control EDTA	ND	ND	12.43 ± 0.25 a	–	ND	13.26 ± 0.35ab	–
Pb 25	19.82 ± 0.79a	41.25 ± 0.61a	11.03 ± 0.32b	0.34	6.08 ± 0.24a	12.73 ± 0.21bc	0.15
Pb 25 EDTA	23.46 ± 1.08b	46.76 ± 0.64b	10.93 ± 0.25b	0.38	6.62 ± 0.22b	12.4 ± 0.26c	0.16
Pb 50	54.63 ± 0.78c	73.57 ± 0.61c	10.30 ± 0.36c	0.33	12.24 ± 0.47c	11.83 ± 0.25de	0.14
Pb 50 EDTA	68.43 ± 0.90d	92.05 ± 0.86d	10.06 ± 0.38c	0.40	12.85 ± 0.62c	11.66 ± 0.21d	0.15
Pb 100	143.16 ± 0.57e	181.38 ± 0.60e	8.26 ± 0.32d	0.34	19.57 ± 0.93d	10.90 ± 0.26f	0.11
Pb 100 EDTA	163.25 ± 0.90f	209.34 ± 0.71f	8.03 ± 0.40d	0.37	20.62 ± 0.69d	10.53 ± 0.15f	0.11
Zn 25	18.3 ± 0.83g	16.83 ± 0.73g	11.90 ± 0.26a	0.21	19.2 ± 0.72e	13.36 ± 0.21a	0.51
Zn 25 EDTA	20.86 ± 0.61h	17.87 ± 0.59g	11.76 ± 0.32a	0.23	21.83 ± 0.66f	12.90 ± 0.20ab	0.56
Zn 50	28.84 ± 0.82i	20.85 ± 0.70h	10.86 ± 0.30b	0.13	35.66 ± 0.67g	12.53 ± 0.21bc	0.45
Zn 50 EDTA	30.53 ± 0.62j	24.36 ± 0.74i	10.53 ± 0.25b	0.14	38.24 ± 0.62h	12.06 ± 0.15e	0.46
Zn 100	34.18 ± 0.61k	32.33 ± 0.65j	9.80 ± 0.30e	0.08	41.07 ± 0.58i	11.46 ± 0.21d	0.24
Zn 100 EDTA	45.33 ± 0.44l	32.63 ± 0.96j	9.53 ± 0.21e	0.09	42.58 ± 0.68j	11.06 ± 0.15f	0.24

ND not detected

Each value is a mean ± SD of triplicates. Values followed by same letters (a–l) are not significantly different as determined by Student–Newman–Keuls test ($p \leq 0.05$)

Table 2 THQ values radish and cabbage

Metal	R_fD (mg kg^{-1} day^{-1})	Radish		Cabbage	
		Range (mg kg^{-1})	THQ	Range (mg kg^{-1})	THQ
Pb	0.0035	30.53–186.23	6.27–38.26 (men) 6.84–41.74 (women)	6.08–20.62	1.25–4.23 (men) 1.36–4.62 (women)
Zn	0.3	17.57–39.12	0.04–0.09 (men) 0.05–0.1 (women)	19.20–42.57	0.05–0.1 (men) 0.04–0.11 (women)

If the THQ value is > 1, the exposure is likely to cause obvious adverse effects of toxicity were seen in due course of experiment suggesting the development of underlying mechanisms imparting defence in both these crops under HM stress. On one hand, a caution needs to be exercised when these crops are grown on Pb contaminated soil; cultivation on Zn contaminated soil was found to be safe for consumption. From phytoextraction point of view, radish emerged as a better and potential crop for remediation of Pb contaminated soil in comparison to cabbage.

One of the current limitations of phytoremediation is disposal of employed plants. The harvested biomass can be processed in order to be converted as a bio-ore for the test metals depending upon the content. Besides phytomining, brassicaceae members particularly are naturally enriched with S-containing organic compounds known as glucosinolates (GLS), which upon enzymatic degradation mainly yield isothiocyanates—compounds exhibiting biocidal properties suitable to produce biopreparate for crop protection by biofumigation (Szczygłowska et al. 2011). Additionally, these species are also possible candidates for raising transgenics by addition of responsible genes to improve their phytoremediation potential.

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