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Cadmium accumulation in leaves of leafy vegetables

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ABSTRACT

Leafy vegetables have a relatively high potential for Cd uptake and translocation, and are thus considered Cd accumulators. For this reason, leaves and roots of lettuce (*Lactuca sativa* L.) and endive (*Cichorium endivia* L.) plants, grown on different agricultural soils in Campania region (southern Italy), subjected to different fertilisation treatments (unfertilisation, compost amendment and mineral fertilisation), were analysed for Cd concentrations. Moreover, to clarify if the highest concentrations found are linked to older and inedible or to younger and edible leaves, external and internal endive leaves were separately analysed.

All the leafy vegetables analysed showed on average 2-fold higher Cd concentrations in leaves than in roots. Leaf Cd concentrations in both lettuce and endive plants significantly differed among fertilisation treatments, with values highest in the plants grown on mineral fertilised soils. Apart from the soil fertilisation treatments, however, Cd leaf concentrations were often higher (up to 4-fold) than the threshold deduced by the EU 420/2011 Regulation, although the plants grew on unpolluted soils. Anyway, external leaves of endive plants showed significantly higher concentrations than internal leaves (in some cases the values were 3-fold higher), partly reassuring on the consumption of the younger leaves. Moreover, this study points out two major drawbacks in the Italian and European regulatory frameworks: (1) metal concentration (as total and/or available fraction) limits in agricultural soils are lacking; (2) metal concentration thresholds (currently existing only for Cd and Pb in crops) reported in the EU 420/2011 Regulation, expressed on the fresh weight basis rather than on the dry weight basis, appear not suitable.

1. Introduction

All heavy metals are persistent chemical elements and can not be deleted from the environment. Thus, a problem arises when their bioavailability is high, either in relation to high background levels or to anthropogenic activities (Greger, 2004). Cadmium (Cd), in particular, is a toxic element for all living beings, even at low concentrations (Garate et al., 1993). Anthropogenic activities, such as soil fertilisation, have increased both the total amount of Cd in soils (Huang et al., 2004) and the fraction of this element available to plants (Garate et al., 1993). Consequently, Cd uptake and accumulation in edible plants and its possible effects on human health have received great attention in the last decades (Garate et al., 1993; Akoumianakis et al., 2008), considering also that food is the main source of Cd intake in non-smoking people (Järup and Akesson, 2009). Cd is carcinogenic to human beings, according to the International Agency for Research on Cancer (IARC, 2012), and affects reproductive processes and the embryonic development (Thompson and Bannigan, 2008). Moreover, it is involved in human bone disease, lung edema, liver damage, anaemia and hypertension (Nordberg, 1974; Staessen et al., 1999) and it is the cause of Itai-Itai disease (renal damage and osteomalacia) in individuals chronically exposed to high concentrations through the diet (Stayner et al., 1992). Hence, Cd is one of the metals for which the Food and Agricultural Organization and the World Health Organization have set limits (FAO-WHO, 1978), with a maximum permitted human intake of 70 μg/d.

The process of Cd uptake by plant roots can be either active or passive, depending on Cd concentration in the nutrient solution (Cataldo et al., 1983), and its extent depends on temperature, pH, salinity, organic matter content and nutrient concentrations in the

Abbreviations: CRA, Agricultural Research Council and Analysis of the Agrarian Economy; UNF, unfertilised soil; CMP, compost amended soil; MIN, mineral fertilised soil; BF, bioavailability factor; DTPA, diethylenetriamine-pentaacetic acid

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soil (Bingham et al., 1983; Jackson and Alloway, 1991; McBride, 2002). Anyway, Cd has generally a high soil bioavailability (Baldantoni et al., 2010) and has higher mobility in plants compared to other heavy metals (Greger, 2004; Akoumianakis et al., 2008; Zhang et al., 2013), being easily transported by roots to shoots (Baldantoni et al., 2014).

Plant species, but also genotypes, vary in their capacity for Cd uptake, transport and accumulation (Grant et al., 1999; Greger, 2004; Zhang et al., 2013). In addition, heavy metal accumulation in plants differs greatly not only among species and cultivars, but also among organs of the same plant (Oliver, 1997). As a general rule, metal concentrations are normally higher in roots than in shoots (Garate et al., 1993; Kabata-Pendias, 2011). However, in several plants (Baldantoni et al., 2014), and also in leafy vegetables grown on unpolluted soils (Kabata-Pendias, 2011), Cd is primarily allocated in leaves. Since leafy vegetables, such as lettuces, endives and similar horticultural crops have a relatively high potential for Cd uptake and translocation (Peijnenburg et al., 2000), they are considered Cd accumulators (FAO, 1983). This, coupled with the importance of leafy vegetables in human diet, makes them an important source of Cd intake for people.

It was estimated that vegetable consumption can contribute to Cd exposure from 70% (Sarwar et al. 2010) up to more than 90% (Swartjes et al. 2007) of total Cd intake by human beings. Lettuce (*Lactuca sativa* L.) and endive (*Cichorium endivia* L.) are two important salad crops of the Mediterranean diet and are available worldwide, so their consumption may represent an effective risk for human health. The present study aims to evaluate Cd concentrations in leafy vegetables grown on different agricultural soils underwent to different fertilisation practices, and to clarify if the highest concentrations occur in the external (older and inedible) or the internal (younger and edible) leaves. To this end, the research was carried out in two years: the first targeting Cd accumulation in leaves and roots, and the second focusing on Cd partitioning between edible and inedible leaves.

2. Materials and methods

2.1. Experimental design

Leafy vegetables were cultivated in three experimental stations of the Agricultural Research Council and Analysis of the Agrarian Economy (CRA), namely the Research Units of Pontecagnano (40°38' N, 14°53' E, 48 ma.s.l.), Battipaglia (40°34' N, 14°58' E, 65 ma.s.l.) and Scafati (40°44' N, 14°30' E, 9 ma.s.l.), in southern Italy (Campania Region), all characterized by a Mediterranean climate (Baldantoni et al., 2010; Baldantoni et al., 2015). During 2005, lettuce (*L. sativa* cv Arcadia) at Pontecagnano and endive (*C. endivia* cv Crispum Hegi) at Battipaglia, were cultivated in greenhouse and in open-field, respectively. During 2009, endive (*C. endivia* cv Cuartana) was cultivated in open-field, side by side at Scafati and Battipaglia. Soils at the three sites (Table 1) differed in their physical and chemical properties (Pagano et al., 2008; Baldantoni et al., 2015).

In both years, the study was carried out in plots (three and four replicated for each soil treatment in 2005 and 2009, respectively) subjected to different soil treatments, performed according to a randomized-block design. The treatments were (1) unfertilised soil (UNF), (2) compost amended soil (CMP), and (3) mineral fertilised soil (MIN). High quality compost (Legislative Decree 75, 29 April 2010), obtained from the organic fraction of municipal solid waste and the urban yard trimmings (1:1 = w:w.), was homogeneously spread on the soil surface at the dose of 30 t/ha on dry weight basis and then incorporated by rotovating to a depth of about 20 cm. Mineral fertilizers (N, P, K) were applied two times during growing season, based on soil nutrient availabilities.

Table 1
Physical and chemical properties of the soils (0–20 cm) of Pontecagnano and Battipaglia (from Pagano et al., 2008) as well as Scafati (from Baldantoni et al., 2015) experimental fields; n.a.: not available datum.

	Pontecagnano	Battipaglia	Scafati
Classification (WRB-FAO, 2014)	Sandy Loam Calcaric Cambisol	Clay Gleyc Luvisol	Vitric Calcaric Andosol
Sand (0.02-2.00 mm) (%)	43.0	31.0	45.0
Silt (0.002-0.020 mm) (%)	39.0	29.2	50.0
clay (< 0.002 mm) (%)	18.0	39.8	5.0
pH $_{H2O\ (1:2.5)}$	7.9	7.6	8.4
Organic matter (% d.w.)	4.4	1.3	2.2
Total carbonates (%)	59.1	Traces	3.4
Electrical conductivity (dS/m)	0.22	0.12	0.34
Cation exchange capacity ($cmol_{(+)}$/kg)	22.7	16.7	n.a.
Total nitrogen (g/kg d.w.)	2.25	0.80	1.50
Bioavailable phosphorus (mg/kg d.w.)	45.0	45.0	71.0
Exchangeable potassium (mg/kg d.w.)	458.0	394.0	726.0

2.2. Sampling

Soil samples from each plot were collected in the 0–20 cm layer. At each sampling, six sub-samples were collected from each plot and mixed to obtain one representative sample per plot for the laboratory analyses. From the same plots, three–four lettuce or endive plants were picked up and divided in leaves and roots. Endive cv Cuartana leaves were separated in external (older) and internal (younger) ones. Care was taken in all sampling and in following analyses to avoid metal contamination.

2.3. Laboratory analyses

Soil, sieved through a 2.0-mm mesh screen, and plants were dried at 75 °C up to constant weight. For the determination of total Cd concentration in soil and plants, samples were pulverized in a planetary ball mill (PM4, Retsch, Haan, Germany) and digested by an acid mixture (65% HNO_3 and 50% HF, 2:1 = v:v) in a microwave oven (Ethos, Milestone, Shelton, CT, USA). The Cd bioavailable fraction was extracted from the dried soil samples with a DTPA (diethylenetriamine-pentaacetic acid) solution (0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA, pH 7.3) at room temperature in continuous agitation for two hours (Lindsay and Norvell, 1978). Further details on these methods are reported in Baldantoni et al. (2009). Cadmium concentrations were determined by an atomic absorption spectrophotometer (AAnalyst 100, PerkinElmer, Wellesley, MA, USA), via graphite atomizer. Standard reference materials, namely calcareous loam soil BCR CRM 141R (European Commission, 1996) and *Olea europea* leaves BCR 62 (Commission of the European Communities, 1982) were also analyzed in order to verify the accuracy of soil total and leaf Cd determinations.

2.4. Data processing

Cadmium bioavailability factors (BFs) in the analysed soils were calculated as the percentage of Cd available fractions compared to the total Cd concentrations.

For each experimental field, on the normalised data set through logarithmic transformation, (1) one-way ANOVA was performed in order to check for differences in soil Cd bioavailable concentrations as well as in BFs among soil treatments, and (2) two-way ANOVA, with the plant organ and the soil treatment as fixed factors, was performed in order to evaluate differences in leaf and root Cd concentrations and among soil soil treatments. The ANOVA tests were followed by the *post hoc* tests of Tukey. Normality was assessed using the Kolmogorov–Smirnov test and homoscedasticity using the Levene Median test. The paired Student *t*-test was performed to evaluate the significance of the differences between Cd concentrations in the external and internal leaves of endive cv Cuartana grown on the three differently treated soils of Scafati and Battipaglia experimental fields.

Correlations between total and bioavailable Cd concentrations in soil, between root and leaf Cd concentrations, between soil bioavailable and root Cd concentrations, as well as between soil bioavailable and leaf Cd concentrations were evaluated by the Spearman's test, according to the non-normal distribution of the data, assessed using the Shapiro–Wilk test.

The statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc.).

3. Results

Soils of the three experimental fields differed in their physical and chemical properties measured at the beginning of the experimentation (Table 1). At the end of the crop cycle, the average soil total Cd concentrations were equal to 0.31 ± 0.03 μg/g d.w. at Pontecagnano, 0.51 ± 0.01 μg/g d.w. at Battipaglia, and 0.48 ± 0.01 μg/g d.w. at Scafati, whereas the average Cd bioavailable fractions (DTPA-extractable) were equal to 0.12 ± 0.01 μg/g d. w. at Pontecagnano, 0.13 ± 0.01 μg/g d.w. at Battipaglia, and 0.15 ± 0.02 μg/g d.w. at Scafati.

The soils of both Pontecagnano (Fig. 1a) and Scafati (Fig. 1c) differed in their Cd bioavailable fractions among fertilisation treatments ($F=7.153$, $P<0.05$ and $F=5.344$, $P<0.05$, respectively), with values higher in C30 than in UNF and MIN plots at Pontecagnano and with values higher in C30 than in UNF plots at Scafati. Cd bioavailability factors (BFs) varied among the three experimental fields (Table 2), but did not differ ($\alpha=0.05$) in relation to soil fertilisation treatments.

All the leafy vegetables analysed showed significantly higher Cd concentrations in leaves than in roots (Fig. 1): in particular $P<0.001$ ($F=59.657$) for lettuce cv Arcadia at Pontecagnano, $P<0.01$ ($F=34.369$) for endive cv Crispum Hegi at Battipaglia, $P<0.05$ ($F=6.614$) for endive cv Cuartana at Scafati, and $P<0.001$ ($F=57.130$) for endive cv Cuartana at Battipaglia. Cd concentrations in lettuce cv Arcadia (Fig. 1a) and in endive cv Crispum Hegi (Fig. 1b) significantly differed among fertilisation treatments ($F=6.453$, $P<0.05$ and $F=5.137$, $P<0.05$, respectively), with values highest in the plants grown on MIN soils. In particular, leaf Cd concentrations in lettuce were higher ($P<0.05$) in plants grown on MIN plots compared to those grown on both UNF and CMP plots, which did not significantly differ, and leaf Cd concentrations in endive were higher ($P<0.05$) in plants grown on MIN plots compared to those grown on CMP plots. External leaves of endive cv Cuartana grown at Scafati and Battipaglia showed significantly ($t=7.605$, $P<0.001$) higher concentrations compared to the internal ones, apart from the soil fertilisation treatments. In some cases, external leaves showed three fold higher values than those found in internal leaves (Table 3).

Spearman correlations among Cd concentrations in the different matrices pointed out no significant relationships between soil total and bioavailable fractions (Fig. 2a), positive relationships between roots and leaves (Fig. 2b), and negative relationships between soil bioavailable fractions and roots (Fig. 2c), as well as soil bioavailable fractions and leaves (Fig. 2d).

Table 2
Mean values ± s.e. of Cd bioavailability factors in the analysed soils (UNF: unfertilised, CMP: compost amended, MIN: mineral fertilised).

	UNF	CMP	MIN
Pontecagnano-*L. sativa* cv Arcadia	42.13 ± 5.07	30.98 ± 0.29	44.88 ± 2.39
Battipaglia-*C. endivia* cv Crispum Hegi	19.33 ± 0.15	22.99 ± 1.55	19.75 ± 0.70
Scafati-*C. endivia* cv Cuartana	25.38 ± 2.54	39.86 ± 3.73	29.54 ± 4.54
Battipaglia-*C. endivia* cv Cuartana	24.40 ± 1.67	34.85 ± 7.23	32.40 ± 2.48

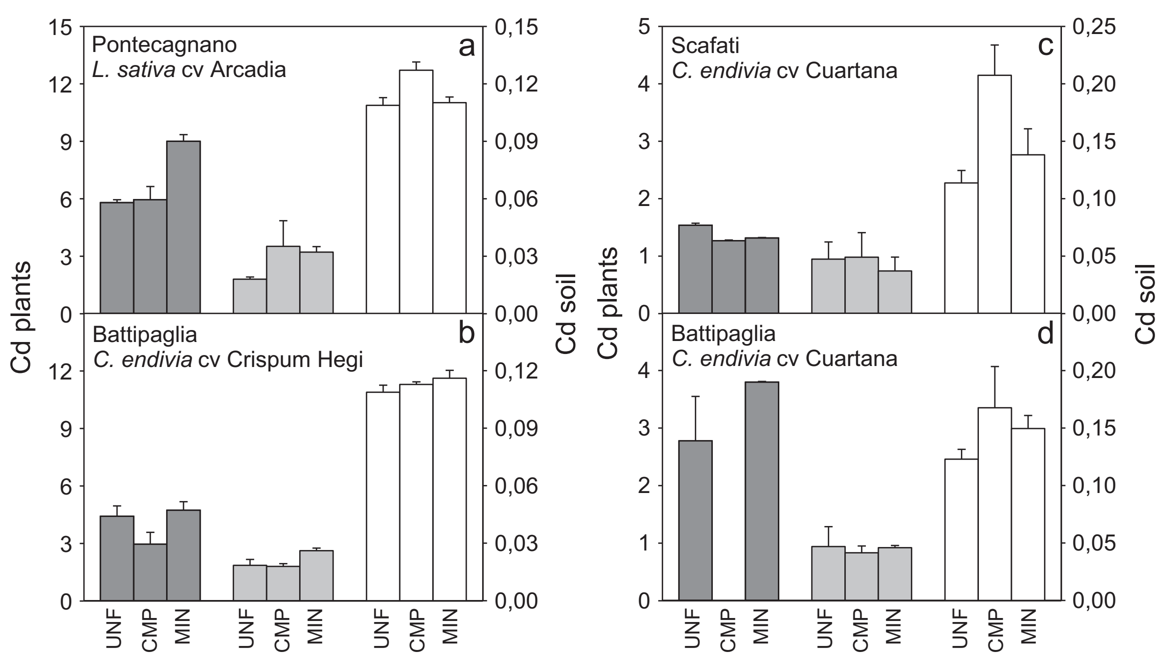

Fig. 1. Mean Cd concentrations in the leaves (dark grey bars) and in the roots (light grey bars) of (a) *L. sativa* cv Arcadia (Pontecagnano), (b) *C. endivia* cv Crispum Hegi (Battipaglia), (c) *C. endivia* cv Cuartana (Scafati) and (d) *C. endivia* cv Cuartana (Battipaglia) grown on the three differently treated soils (UNF: unfertilised, CMP: compost amended, MIN: mineral fertilised). Cd bioavailable fractions (white bars) in the soils are also reported. The scales on y axes for plant and soil data (μg/g d.w.) are in the ratio 100:1 for graphs a and b, and in the ratio 20:1 for graphs c and d; the error bars represent the standard errors of the means. The lack of a bar in the graph d is linked to a not available datum.

Table 3
Mean values ± s.e. of Cd concentrations (μg/g d.w.) in external and in internal leaves of *Cichorium endivia* cv Cuartana grown on the three different soils (UNF: unfertilised, CMP: compost amended, MIN: mineral fertilised) at Scafati and Battipaglia experimental fields; n.a.: not available datum.

	Scafati		Battipaglia	
	external leaves	internal leaves	external leaves	internal leaves
UNF	2.04 ± 0.07	1.04 ± 0.02	2.78 ± 0.77	3.12 ± 0.31
CMP	1.93 ± 0.01	0.61 ± 0.01	n.a.	2.03 ± 0.14
MIN	1.79 ± 0.03	0.84 ± 0.02	3.80 ± 0.01	1.87 ± 0.13

4. Discussion

In our research, the soils of the three experimental fields greatly differed in their texture, pH, organic matter content, total carbonates, electrical conductivity, cation exchange capacity and total nitrogen. The total Cd concentrations also differed among the soils, whereas Cd bioavailable fractions were similar and, as a consequence, bioavailability factors differed. The estimation of total metal concentrations, alone, can not provide information on the chemically extractable fraction of the elements or on their effective bioavailability to the plant. Such an occurrence was also found in our study, where no relationships between soil total and bioavailable Cd concentrations were observed. The bioavailable fraction of a metal, rather than its total concentration, might thus be considered a better synthetic indicator of the suitability of an agricultural soil for crop growth (Huang et al., 2004; Baldantoni et al., 2010; Bello et al., 2014). Indeed, the bioavailable fraction of a metal, depends not only on its total concentration, but also on complex interactions among chemical, physical and biological soil properties. In addition, the interactions between plant and soil, at the rhizosphere level, play an important role in determining the effective bioavailabiliy of the element, which could differ from the chemical extractability (Jackson and Alloway, 1991; Greger, 2004). Indeed, negative correlations between Cd bioavailable fractions and both root and leaf Cd concentrations were found. Conversely, the fraction of Cd translocated to the leaves was positively related to the root concentration. Chang et al. (1987) reported that soil temperature is one of the main factors accounting for variations in metal accumulation by crops, apart from the effective metal bioavailability. Different fertilization practices might have differently influenced soil temperature, leading to diverse Cd accumulations in both leaves and roots, notwithstanding the comparable Cd bioavailability. As a matter of fact, Movahedi Naeini and Cook (2000) demonstrated that soil compost incorporation modifies soil temperature. Moreover, Greger (2004) reported that metal accumulation in plants does not necessarily correlate positively with the bioavailable metal concentration, especially when different crop species or cultivars are considered. For lettuce, in particular, it

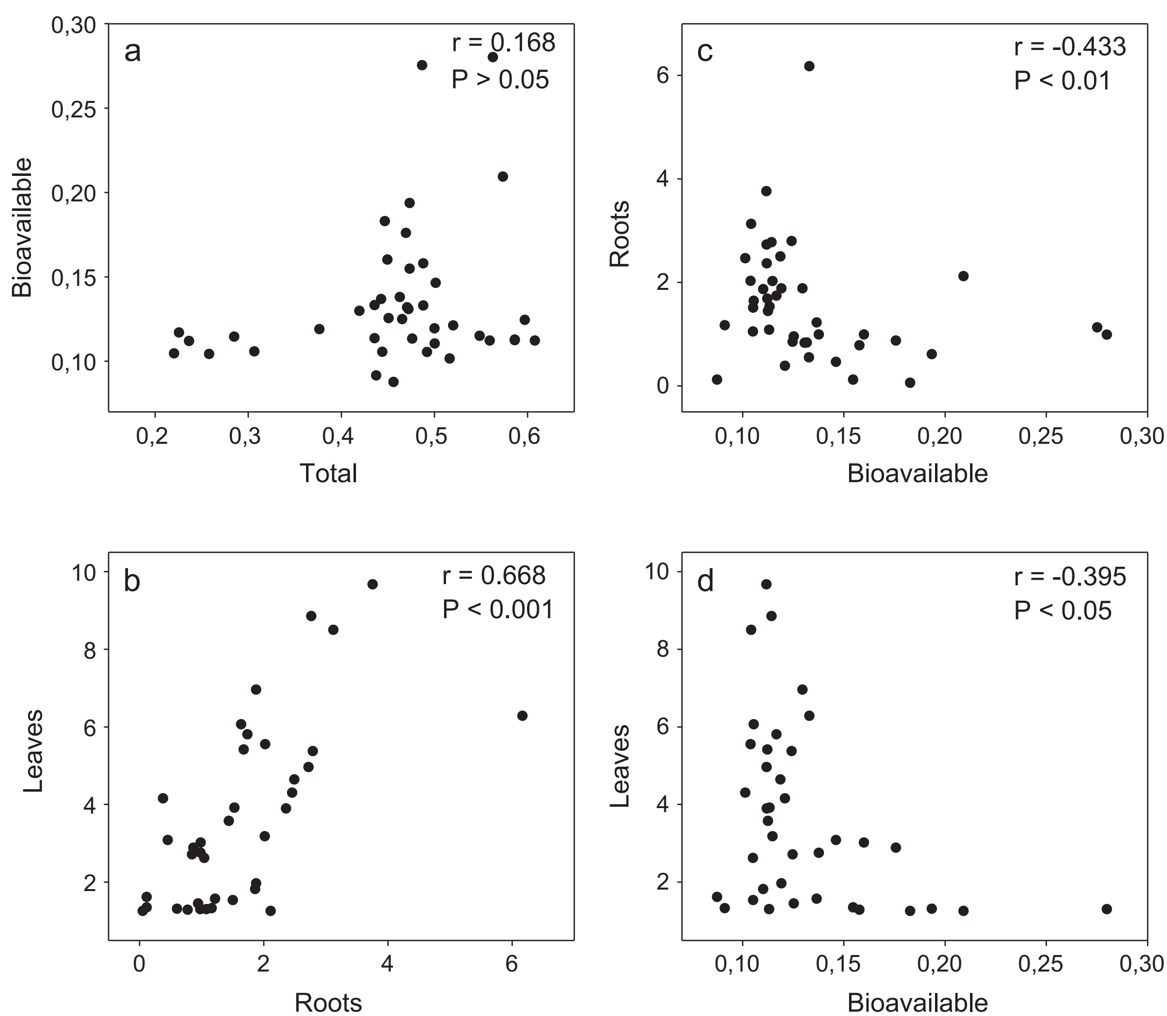

Fig. 2. Relationships between (a) total and bioavailable Cd concentrations in soil, (b) root and leaf Cd concentrations, (c) bioavailable and root Cd concentrations, (d) bioavailable and leaf Cd concentrations. All values are expressed in μg/g d.w. The Spearman correlation coefficients (*r*) and the *P* values are also reported.

has been demonstrated that Cd accumulation in shoots is cultivar-dependent (John and van Laerhoven, 1976; Zhang et al., 2013).

Differently from many other horticultural plants, comprising spinach (Farooq et al., 2008; Singh et al., 2012), the studied leafy vegetables accumulated Cd into the leaves, where, to a different extent in relation to the species, the cultivar or the soil fertilisation, the concentrations were up to 4-fold higher than those detected in the roots. Leaf concentrations, 3-fold higher than root concentrations, were also observed in lettuce by Farooq et al. (2008).

Different soil fertilisations caused a different Cd accumulation in leaves of the studied leafy vegetables, as those collected from the plants grown on mineral fertilised soils were characterised by the highest Cd concentrations. This is not surprising, considering that several mineral fertilisers contain traces of Cd (Huang et al., 2003). Leaf accumulation, however, did not strictly depend on soil Cd bioavailability, that was generally highest in compost amended plots, but rather by a number of abiotic and biotic factors, as discussed above. As a consequence, the metal quantification in the crop edible parts appears to be very important, regardless of agronomic applied techniques and the soil characteristics and properties. For this reason, laws regulating several metals in a high number of vegetables are desirable. In the present study, in fact, Cd leaf concentrations were often higher than the maximum level deduced by the EU 420/2011 Regulation (Regolamento UE n. 420/2011). This rule relative to maximum Cd concentrations in food establishes a limit value for leafy vegetables equal to 0.2 μg/g f.w. Since the leaves of the analysed horticultural crops showed, on average, a water content equal to 90%, they mostly highlighted concentrations (up to 4-fold) higher than the maximum permitted value. In addition, leaf Cd concentrations reached values up to 30- and 20-fold higher than the upper limits reported by Allen (1989) and by Markert (1992) for plant matrices (0.3 and 0.5 μg/g d.w., respectively). These observations heavily worries, also considering that the leafy vegetables here studied grew on unpolluted agricultural soils. Indeed, even if in the Italian and European laws legal limits for heavy metals in agricultural soils have not yet been established, the analyzed leafy vegetables grew on Cd uncontaminated soils, considering 1.0 μg/g d.w. the upper limit of non polluted soils (Greger, 2004). Such considerations confirm these vegetables as Cd accumulators: on average leaves of the lettuce and endive studied cultivars showed Cd concentrations more than one order of magnitude higher than soil.

The analysis of external and internal leaves of endive highlighted very high Cd concentrations in the older and inedible leaves, as a result of an age-dependent accumulation, but also of defence or tolerance mechanisms of the plant to avoid toxic levels in physiologically most active younger organs (De Maria et al., 2013). McKenna et al. (1993) also reported that Cd was greatly accumulated in old leaves respect to the young ones of both lettuce and spinach grown in hydroponic solutions at different Cd concentrations. Likewise, Moustakas et al. (2001) observed that Cd accumulation occurred especially in external than internal leaves of lettuce and Garate et al. (1993) reported higher concentrations in older than in younger leaves of endive after 35 days of exposure to Cd solutions. These observations partly reassure on the consumption of the younger and edible endive leaves, that in only one case (plants grown on unfertilised soil of Battipaglia experimental station) showed values exceeding the threshold.

5. Conclusions

The results of this study highlight worrying Cd concentrations in leaves of the studied leafy vegetables, with concentrations often higher than the threshold deduced by the EU 420/2011 Regulation. The accumulation degree of a such metal depends on both species and cultivar, and it may be affected by the soil fertilisation adopted, since the highest Cd concentrations were found in leaves of leafy vegetables grown on mineral fertilised soils. In relation to leaf age, the highest values were found in the old and inedible leaves, partly reassuring on the consumption of the young and edible leaves. Moreover, our study points out two major drawbacks in Italian and European regulatory frameworks: (1) metal concentration limits, as total and/or available fraction, in agricultural soils are lacking; (2) metal concentration thresholds in edible crops, established only for Cd and Pb, and reported in the EU 420/2011 Regulation, appear not exhaustive and not well suitable since they are expressed on the fresh weight (a parameter highly variable), rather than on the dry weight.

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