Journal of Cleaner Production 328 (2021) 129548

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Spent coffee grounds as a source of smart biochelates to increase Fe and Zn levels in lettuces

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ARTICLE INFO

Handling Editor: Cecilia Maria Villas Bôas de Almeida

Keywords:
Spent coffee grounds
Melanoidins
Bio-fortification
Micronutrients
Lettuce

ABSTRACT

Spent coffee grounds (SCG), an ever-increasing food waste whose annual generation is 15 million tons, have the potential to fulfil many different purposes after being recycled. However, though SCG have been used as soil organic amendment, their utilization is limited by their phytotoxic nature. Although SCG favour the assimilation of nutrients by plants, whether or not they could be used as an agronomical bio-fortifying agent has not been investigated yet. Therefore, we carried out an *in vitro* investigation to analyse the influence of SCG and coffee melanoidins functionalized with Fe and Zn (biochelates) on the agronomic biofortification of lettuce (*Lactuca sativa* var. *longifolia*), one of the most consumed edible plants worldwide. Commercial chelates (EDDHA-Fe and EDTA-Zn) and non-functionalized SCG and melanoidins were used as controls. Total Fe and Zn content in lettuces and available Fe and Zn in soil were measured. Functionalized SCG and melanoidins were able to significantly increase both Fe and Zn levels in lettuces. While Fe levels increased around 28–30%, Zn showed a much larger improvement with levels up to 177–416% higher. According to our results, both SCG and coffee melanoidins could be used as agronomical bio-fortifying agents. Additionally, bio-fortified lettuce would provide up to a 16.3% of the Fe recommended daily intake and up to a 6.6% of that of Zn, reflecting positively in human health.

1. Introduction

Hidden hunger is a form of malnutrition that happens when a diet lacks micronutrients such as iron, zinc, vitamin A or iodine (Kumar et al., 2019), affecting around 2 billion people worldwide (von Grebmer et al., 2014). Specifically, Zn and Fe deficiencies are present in about a third of the world's population, particularly in Asia and Africa, although deficiencies of these two microelements have also been found in Spain (Sánchez et al., 2009). These deficiencies are behind several diseases related to the immune and nervous system and can even hinder intellectual development (Li et al., 2019). Low levels of Fe and Zn in soils are one of the main reasons that can explain such situations of deficit. However, while Zn deficiency in soils is the most commonly encountered (Alloway, 2008), Fe is usually a very abundant element in soils worldwide, classified between macro and microelement (Navarro and Navarro, 2013), although its availability is reduced in alkaline and calcareous soils (Li et al., 2019).

Different solutions have been suggested to address hidden hunger: diet diversification, commercially available fortified foods, supplementation and agronomic-genetic bio-fortification (von Grebmer et al., 2014). Agronomic bio-fortification with commercial chelates (DTPA, EDTA, EDDHA, etc.) and salts is one of the most used tools to address hidden hunger generated by Fe and Zn deficiencies (Kozik et al., 2011; Mohammadi and Khoshgoftarmanesh, 2014; Cakmak and Kutman, 2018). Nevertheless, it has some limitations: salts are very soluble forms that tend to be blocked in soil by means of different mechanisms, whereas chelates could cause toxicity and have other side effects on plants such as impaired nutrient balance (Mohammadi and Khoshgoftarmanesh, 2014).

Lettuce is one of the most consumed leafy vegetables in the world

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https://doi.org/10.1016/j.jclepro.2021.129548
Received 25 July 2021; Received in revised form 14 October 2021; Accepted 28 October 2021
Available online 29 October 2021

(Garg et al., 2018), with a world production in 2017 of 2.6 million tons (Shatilov et al., 2019) and it has been object of study in agronomic and genetic biofortification assays (Kozik et al., 2011; Giordano et al., 2019). It is therefore a good candidate to investigate its possibilities in agronomic biofortification and to combat the hidden hunger.

The use of organic amendments is another alternative to improve the assimilation of micronutrients by plants (Diacono and Montemurro, 2010), since they provide a steadier source of nutrients. For example, the addition of sewage sludge, compost produced from agricultural wastes and municipal solid waste compost increases the available nutrients N, P and K (Alvarenga et al., 2017). Likewise, the use of food waste from the industry as organic amendments is an alternative that has gained much importance in recent years, due to the need to generate processes in the so-called *circular economy* (Velenturf et al., 2019). Accordingly, the use of spent coffee grounds (SCG) as soil organic amendment has been widely studied (Morikawa and Saigusa, 2008; Cruz and Marques dos Santos Cordovil, 2015; Cruz et al., 2015; Hardgrove and Livesley, 2016; Cervera-Mata et al., 2018, 2019a, 2019b; Comino et al., 2020). SCG is a food waste generated in large amounts, specifically 15 million tons annually (Kamil et al., 2019), that still has a high content of macroelements such as C, N, P and K (Cervera-Mata et al., 2018). SCG have also showed positive effects on soil, improving their chemistry, physico-chemistry, as well as their physical and biological properties (Cervera-Mata et al., 2018, 2019a, 2020; Comino et al., 2020). However, the presence of some toxic compounds, mainly phenolics, in SCG can reduce the crop biomass (Cruz and Marques dos Santos Cordovil, 2015; Hardgrove and Livesley, 2016; Cervera-Mata et al., 2018, 2019b). On the other hand, authors have reported that such negative effect could be solved by "direct composting" SCG in the soil (Cruz et al., 2015). Nevertheless, it is a time-consuming and cost-intensive process. Therefore, it could be necessary to look for other sustainable alternatives in order to make a more efficient use of SCG in agriculture.

Conversely, spent coffee grounds can increase the level of nutritionally important mineral elements for human nutrition (Cervera-Mata et al., 2019b), which is attributed to the presence of some chelating compounds such as polyphenols (Morikawa and Saigusa, 2008) and melanoidins (Rufián-Henares and de la Cueva, 2009). The latter are compounds generated during the Maillard reaction (Delgado-Andrade et al., 2006; Rufián-Henares et al., 2006). In this sense, Rufián-Henares and de la Cueva (2009) and Morales et al. (2005) studied the chelating capacity of melanoidins against Fe and found, at a concentration of 2.5 mg/ml, 15% of Fe was chelated in one case and 50 μg of Fe in another.

Various important aspects of SCG must be taken into account to plan their correct agronomic use. i) SCG is a waste from the agri-food industry with a high content of organic carbon. ii) Cultivated soils in most of the world have a large deficit of carbon. iii) Therefore, SCG must be added to the soil and not be used as fuel or other uses. iv) The phytotoxic character of these residues prevents their use in high quantities (such as other organic residues), so it is not a typical organic residue. v) Their composition, rich in polyphenols and melanoidins, makes them susceptible to being tracers and mobilizers of microelements. vi) This last property, both of SCG and of melanoidins extracted from SCG, could be improved through functionalizing with some nutritionally important elements, such as Fe and Zn, converting them into *biochelates*. Therefore, the aim of this project was to determine the effect of biochelates obtained from SCG, within subtoxic levels for the plant, and coffee melanoidins on Fe and Zn agronomic biofortification of lettuces under *in vitro* conditions. Their effect was compared to that of commercial chelates. The agronomic use of melanoidins was studied here for the first time. Finally, it was also assessed how bio-fortified lettuces could contribute to Fe and Zn daily intake in the adult Spanish population.

2. Material and methods

2.1. Soil, SCG and lettuces

A typical Mediterranean agricultural soil from the Vega of Granada (Andalucía, Southern Spain) was selected at a depth of 0–20 cm (arable layer). This soil is classified as Cambic Calcisol (Aric, Ochric) (IUSS Working Group WRB, 2014). Before planting, the soil was air-dried and sieved at 5 mm. The properties of soil are: sand 28.1%, clay 58%, silt 13.9%, moisture 4.1%, pH 8.2, OC 1.36%, total N 0.105%, C/N 13, carbonates as $CaCO_3$ 39%, available K 453 ppm, available P 69 ppm (Cervera-Mata et al., 2021). SCG were obtained from the canteen of the School of Pharmacy (University of Granada). The SCG were spread to air-dry any residual moisture. All SCG have a similar composition (McNutt and He, 2019) and are characterized by the presence of cellulose (8.6–15.3%), hemicellulose (31.7–41.7%), lignin (22.2–33.6%), protein (13.0–17.5%), oil (7–21%) and ash (1.3–2.2%) (Battista et al., 2020). Regarding the chemical and physicochemical properties of SCG, they have been characterized by our research group: C 47.03%, N 1.92%, H 7.66%, fixed carbon 13.89%, volatile matter 85.02%, ash 1.20%, C/N 24, pH 5.76, electrical conductivity (EC_{25}) 2.22 dS/m (Cervera-Mata et al., 2021). Lettuces belonged to the "Little Duende" variety (*Lactuca sativa* var. *longifolia*), commercially available in Southern Spain (Saliplant S.L., Granada). This variety of lettuce has been previously used in assays carried out in a climatic chamber due to its small size (Cervera-Mata et al., 2019b).

2.2. Chelates

Two biochelates were tested: biochelate of SCG and biochelate of melanoidins. Melanoidins were isolated from SCG according to the method described by Rufián-Henares and de la Cueva (2009). Briefly, a coffee brew from SCG was prepared at high pressure (62.5 g SCG per 1000 mL of water). The brew was subjected to two diafiltration steps with deionized water and to a final concentration step using a PilotUnitMulti MF/UF/NF/ROM39/M3.8 filtration unit (Alfa Laval, Nakskov, Denmark). Subsequently, the final concentrated product was subject to spray drying in a Spray Dryer model 4M8-TriX (ProCepT, Zelzate, Belgium). The process was carried out at an inlet temperature of 200 °C and an atomization pressure of the nozzle of 2 bar. The conditions were previously optimized.

Melanoidins were functionalized with Fe and Zn. For this purpose, the following reagents were employed: Iron (II) sulfate heptahydrate $FeSO_4 \bullet 7H_2O$ and Zinc sulfate heptahydrate $ZnSO_4 \bullet 7H_2O$ (Sigma, St. Louis, MO). In each case, 1.5 g of the reagent, 30 g of coffee melanoidins and 300 mL of distilled water were used. Each mixture was prepared and kept under stirring for 2 h and allowed to stand at room temperature in the dark for 24 h until reaching a stable state. Each mixed sample was ultrafiltered using an Amicon 8400 ultrafiltration cell model (Amicon, Beverly, MA), with a 5000 Da molecular mass cut-off membrane. The retained product was filled to 150 mL with distilled water and washed again. This washing procedure (diafiltration) was repeated at least three times. Finally, the samples were freeze-dried and stored.

SCG was also functionalized with Fe and Zn as melanoidins. Different concentrations of Fe and Zn were previously tested to check the amount chelated by SCG: the final chosen ratio was 9 g of Fe or Zn salt, 50 g SCG and 500 mL of distilled water. Each mixture was prepared and kept under stirring at room temperature in the dark for 24 h; then the sample was decanted and centrifuged. The solid residue obtained was washed with 250 mL of distilled water, decanted and centrifuged. This washing procedure was repeated two more times. Finally, the samples were left in an oven at 50 °C for 24 h to remove the water content.

Two commercial chelates were selected as controls: iron ethylenediamine-*N*,*N*′-bis (EDDHA-Fe, 6% w:w) and zinc ethylenediaminetetraacetate (EDTA-Zn, 14% w:w) supplied by Trade Corporation International S.A.U. (Madrid, Spain). The contents of Fe and Zn of each

commercial chelate were checked as described below.

2.3. Experimental design

The dose of commercial chelates and coffee melanoidins added to each pot was calculated in order to obtain a final concentration of Fe and Zn of 10 mg/kg soil (Morikawa and Saigusa, 2008; Almendros et al., 2015). However, the amount of SCG added was 2 g per pot, since this dose was the minimum necessary to obtain a homogeneous mixture, taking into account that theaverage particle size of SCG ranged between 500 and 250 μm (Comino et al., 2020). Moreover, this was highest dose of SCG that did not cause any phytotoxicity in lettuces. Thus, 2 g of SCG biochelate corresponds to 20.21 mg Fe/Kg soil and 89.61 mg Zn/Kg soil. The commercial chelates (EDDHA-Fe and EDTA Zn) were applied to the soil surface in a single dose along withirrigation water.

$$Available\ reserve\ efficiency\ (\%) = \frac{Extractable\ treatment - Extractable\ control}{Micronutrient\ added} \times 100$$

The assay was carried out with 300 ml pots. A fibreglass mesh was used to avoid the loss of fine particles. Then, the samples were incubated for 40 days in a climatic chamber under controlled conditions of humidity (50–60%) and temperature (22 °C during the day and 18 °C during the night). The photoperiod was 12h/12 h. Basal fertilization was applied with 100 mg N/Kg soil (as HNO_3 and NH_4), 44 mg P/Kg soil (as P_2O_5) and 84 mg K/Kg soil (as K_2O). The water holding capacity was calculated at field capacity and at the permanent wilting point, to maintain the humidity between both potentials. In this way, leaching and water stress are avoided. The pots were watered every three days with distilled water, by weighing. As controls, melanoidins and SCG not treated with Fe and Zn (non-functionalized biochelates) were applied in the same conditions described above.

After incubation time, lettuce leaves were cut, weighed (fresh weight) and dried at 65 °C for 24 h to calculate their dry weight (Cervera-Mata et al., 2019b). For analysis, lettuce samples were homogenized and frozen at −80 °C. The soils were air dried and stored at 5 °C.

2.4. Analytical methods

Lettuces were mineralized using HNO_3 and H_2O_2 of supra-pure quality (Merck, Darmstadt, Germany) in a microwave digester (CEM MARS, XP1500 Plus). The digest was diluted to 50 mL with Milli-Q water in order to obtain the analytical solution.

Soil available Fe and Zn were extracted by the Lindsay and Norvell, 1978. The total content of Fe and Zn in SCG, melanoidins, biochelates and commercial chelates were determined by acidification with H_2SO_4 at 350 °C, catalysed with Se. The total content of Fe and Zn in soil were determined by mineralization with HNO_3, HCl and HF in a microwave digestor (Multiwave 5000, Anton Paar).

The determination of Fe and Zn was performed using an atomic absorption spectrophotometer (Varian SpectraA, Mulgrave, Victoria, Australia). Calibration curves were previously prepared in 1% HNO_3 by the dilution of 1000 mg/L stock solutionsfor the analyzed elements (Merck, Darmstadt, Germany).

Soil pH was measured in 1:2.5 (w/w) soil–water suspension and in 1:5 (w/w) for SCG. Electrical conductivity (EC_{25}) was measured in the extract of the 1:5 (w/w) soil–water and the 1:10 (w/w) SCG–water suspensions.

To value the efficiency of the biochelates in plant and soil, different equations (obtained from the literature) were used. The Fe and Zn utilization efficiency for lettuce with respect to the total amount of Fe and Zn added to soil was calculated according to Zhao et al. (2019):

$$Utilization\ efficiency\ (\%) = \frac{Uptake\ treatment - Uptake\ control}{Micronutrient\ added} \times 100$$

In the same way, the Fe and Zn available reserve efficiency in soil were calculated:

The transfer factor for the micronutrients was also calculated to relate the parameters determined in soil and plant (Almendros et al., 2013):

$$Transfer\ factor = \frac{Total\ concentration\ plant}{Concentration\ extracted\ soil}$$

2.5. Calculations of daily Fe and Zn intake through lettuce consumption

The daily intake of Fe and Zn was calculated as the contribution of lettuces (biofortified or not) taking into account the amount of lettuce per serving and their daily consumption (Mercasa, 2020). Thus, the intake of lettuces was referred to the usual serving size in Spain (Salvador i Castells, 2000).

2.6. Statistical analysis

The homogeneity of variance was assessed using the Levene test and the normal distribution of the samples with the Shapiro-Wilk test. The analysis of variance (ANOVA) combined with the Tukey test was used to analyse parametric data and the Kruskal-Wallis test combined with the Mann-Whitney *U* test to analyse non-parametric data. The significance level was set at 5% ($p < 0.05$) in all tests. SPSS 22.0 for Windows (IBM SPSS Inc., New York, USA) was used for data analyses.

3. Results and discussion

3.1. Chelating capacity of SCG and melanoidins

The chelating capacity of SCG and melanoidins had a similar behavior for Fe and Zn (Table 1). The treatment increased SCG's Fe content 108-fold and Zn's 18,000-fold. Melanoidins content in Fe was increase 156-fold whereas their Zn content was increased 32-fold. Therefore, SCG showed higher affinity for Zn whereas melanoidins showed it for Fe. The chelating capacity of SCG and melanoidins has been studied previously (Takenaka et al., 2005), although such chelating capacity was never considered for agronomic purposes. Only the research group of Morikawa and Saigusa (2008) generated a coffee waste "compost" functionalized with Fe by mixing 800 g SCG with 200 g

Table 1
Total Fe and Zn content in SCG and melanoidins biochelates.

Sample	Fe (ppm)	Zn (ppm)
Soil	38,034	79
SCG	37	ND
SCG-Fe	4025	Nd
SCG-Zn	nd	17,924
Mel	205	77
Mel-Fe	6495	Nd
Mel-Zn	nd	11,984

SCG: spent coffee grounds, Mel: melanoidins, ND: not detected, nd: not determined.

$FeSO_4$, at high temperatures (60 °C) for a long-time (60 days). The resulting coffee waste "compost" had a final concentration of 40,000 mg Fe/Kg, 10-fold higher than our SCG-Fe (Table 1), due to the methodology used by these authors. Other studies that have demonstrated the chelating capacity of SCG, by using this food by-product to decontaminate polluted water with heavy metals (Mohamed and Yee, 2019).

The chelating capacity of coffee melanoidins could be attributed to their anionic charge (Rufián-Henares and de la Cueva, 2009), which is also the responsible for their antimicrobial activity (Rufián-Henares and Morales, 2008; Delgado-Andrade et al., 2017). Similarly, Wen et al., 2004, Takenaka et al. (2005) also described a polymer extracted from coffee brew (possibly melanoidins), with metal chelating properties against Fe, Zn and Cu.

Spent coffee grounds are rich in polysaccharides, lignin, and proteins. In addition to these nutritional components, SCG also contains melanoidins and polyphenols (specially chlorogenic acid) (Rufián-Henares and Pastoriza, 2015). Therefore, SCG metal chelating activity could be attributed to both melanoidins (Cervera-Mata et al., 2019b) and polyphenols (Morikawa and Saigusa, 2008). These results are in line with the those previously stated by Shen et al. (2019) who affirmed that the metal chelating activity of bread melanoidins increased with the amount of amino acids, specially alanine. Additionally, Mohammadi and Khoshgoftarmanesh (2014) obtained a Zn biochelate with amino acids, since these compounds can capture the Zn ion in their amine and carboxylate groups. In summary, molecules with functional groups, such as amino, hydroxyl and carboxyl groups, appear to be able to retain microelements. Accordingly, it must be taken into account that most of the components of SCG and melanoidins do not have a defined composition, but a mixture of amino acids, polyphenols and other compounds with these functional groups (Rufián-Henares and Pastoriza, 2015).

Biochelates retained lower amounts of Fe and Zn than their commercial counterparts (Table 1). SCG contained 0.4% Fe and 1.8% Zn, while melanoidins had 0.64% Fe and 1.2% Zn (calculated from data of Table 1). According to the specifications of commercial chelates, EDDHA-Fe contains 6% Fe and EDTA-Zn contains 14% Zn, as stated previously.

3.2. Effects of biochelates on soil-plant Fe

Iron levels in lettuces ranged between 0.669 mg/100 g (Table 2) in lettuces cultivated with only NPK (regular agriculture practice) to 1.085 mg/100 g in EDDHA-Fe lettuces ($p < 0.05$). Both commercial chelates and biochelates increased the content of Fe in lettuces. The addition of EDDHA-Fe induced an increase of Fe concentration in lettuces leaves by 62% with respect to the NPK sample, while the addition of SCG-Fe and Mel-Fe improved Fe levels ($p < 0.05$) in a lower extent (by 28 and 30%, respectively). Fig. 1A corroborates the data stated above: the highest Fe utilization efficiency was obtained with EDDHA, followed by coffee melanoidins. The utilization efficiency of SCG-Fe was very low, even obtaining values lower than non-functionalized melanoidins. This means that the amount of Fe supplied by SCG-Fe was high (Table 1), but very little Fe was absorbed by lettuces. This could be explainedby iron ions bounded to molecules embedded into the solid mass of SCG and therefore, in order to become available, iron needs to be released from the solid mass by degradation, which would need more time. On the contrary, in the case of melanoidins or commercial chelates, Fe ions are part of soluble molecules. Morikawa and Saigusa (2008) obtained a final concentration of 100 mg/Kgdry matter in radish after the addition of "composted" coffee waste with Fe salts. In our case, bio-fortification with SCG-Fe generated lettuces with 0.857 mg Fe per 100 g of fresh matter, which corresponds to 137 mg/kg dry matter. This amount is higher than that obtained by the previously cited authors, although their composted coffee waste contained 10 times more Fe than the SCG-Fe bioquelate. In this case, iron could be even more embedded within the solid mass of the coffee compost which could explain such differences with our study.

Table 2
Fe and Zn contents in lettuces (fresh weight).

Sample	Fe (mg/100 g)	Zn (mg/100 g)	Fresh weight (g)
NPK	0.669 ± 0.090^{a}	0.094 ± 0.005^{a}	35.3 ± 0.7^{c}
SCG	0.795 ± 0.076^{ab}	0.106 ± 0.015^{a}	29.0 ± 1.7^{a}
Mel	0.765 ± 0.040^{ab}	0.095 ± 0.008^{a}	34.2 ± 0.5^{bc}
SCG-Fe	0.857 ± 0.089^{b}	nd	30.4 ± 0.7^{ab}
SCG-Zn	nd	0.485 ± 0.030^{c}	29.0 ± 0.2^{a}
Mel-Fe	0.868 ± 0.058^{b}	nd	35.6 ± 1.9^{c}
Mel-Zn	nd	0.260 ± 0.010^{b}	32.3 ± 1.6^{abc}
EDDHA-Fe	1.085 ± 0.025^{c}	nd	35.4 ± 1.9^{c}
EDTA-Zn	nd	0.560 ± 0.014^{d}	32.0 ± 2.2^{abc}

SCG: spent coffee grounds; Mel: melanoidin; nd: not determined. Significant differences are indicated by different letters ($p < 0.05$). Mean values ± standard deviation.

Bio-fortification of lettuces using different synthetic chelates has been previously studied (Kozik et al., 2011; Giordano et al., 2019). According to these authors, the bio-fortified lettuces had a content (of what, Fe or Zn) ranging from 80 to 400 mg/kg DM (dry matter), being lettuce genotype able to affect the mineral composition (Giordano et al., 2019). The values of Fe obtained by us were similar. Thus, the iron levels were as follows: 137.8 mg/kg DM with SCG-Fe, 127.3 mg/kg DM with Mel-Fe and 177.5 mg/kg DM with EDDHA-Fe. The maximum values of 400 mg/kg DM obtained by Kozik et al. (2011) correspond to the addition of approximately 200 ppm of iron (10-fold more than in our assay) generating a significant reduction of lettuce yield.

Regarding soils, the addition of SCG-Fe showed a significantly increase in available Fe ($p < 0.05$), followed by EDDHA- Fe and Mel-Fe (Table 3). SCG-Fe increased the content of Fe in soil by 78%, although its availability was lower than that of the achieved with EDDHA-Fe (Fig. 1B), due to the same reason stated above for the plant; SCG-Fe supplied twice the amount of iron than the commercial chelate, although the former seems to be less available or extractable by the lettuce. This fact is consistent with transfer factor values (Fig. 2). SCG-Fe was the treatment with the lowest transfer factor, but the addition of Mel-Fe achieves values of transfer factor very close to those obtained with EDDHA-Fe. The fact that iron-functionalized melanoidins presented high transfer factors is very remarkable in calcareous soils, where Fe is normally blocked (Navarro and Navarro, 2013). In the same way, Morikawa and Saigusa (2008) also found an increase in extractable Fe in a calcareous soil by means of the addition of coffee waste "compost" functionalized with iron.

3.3. Effects of biochelates on soil-plant Zn

Zn levels in lettuces ranged from 0.094 to 0.560 mg/100 g, being statistically higher ($p < 0.05$) in the lettuces cultivated with EDTA-Zn (Table 2). The biochelates SCG-Zn and Mel-Zn also showed a significant ($p < 0.05$) increase in the concentration of this micronutrient in lettuce leaves (416% and 177%, respectively) behaving as biofortifying agents. Despite of the large Zn increase achieved with both commercial chelates and biochelates, the total Zn threshold values in leaf stablished by Alloway (2008) of 150–200 mg/kg dry matter (in sensitive plant species) was never exceeded. In our assay, the values attained in lettuce leaves, expressed as mg Zn per Kg of dry matter, were 63 for SCG-Zn, 48 for Mel-Zn and 93 for EDTA-Zn. Kozik et al. (2011) found that the application of 10 mg/kg of Zn in the form of Zn (II) DEDTA + Zn (II) DTPA in a calcareous soil can increase the Zn levels in lettuce leaves to about 63 mg/KgDM (consistency). Navarro and Navarro (2013) obtained lettuce leaves with 100 mg Zn/Kg DM using synthesized zinc-amino chelates (Zn-arginine, Zn glycine and Zn-glutamine). This result is quite interesting because it corroborated that amino acids could indeed act as chelating agents. This could also explain why SCG could be a good bio-fortifying agent, since they have amino acids in its

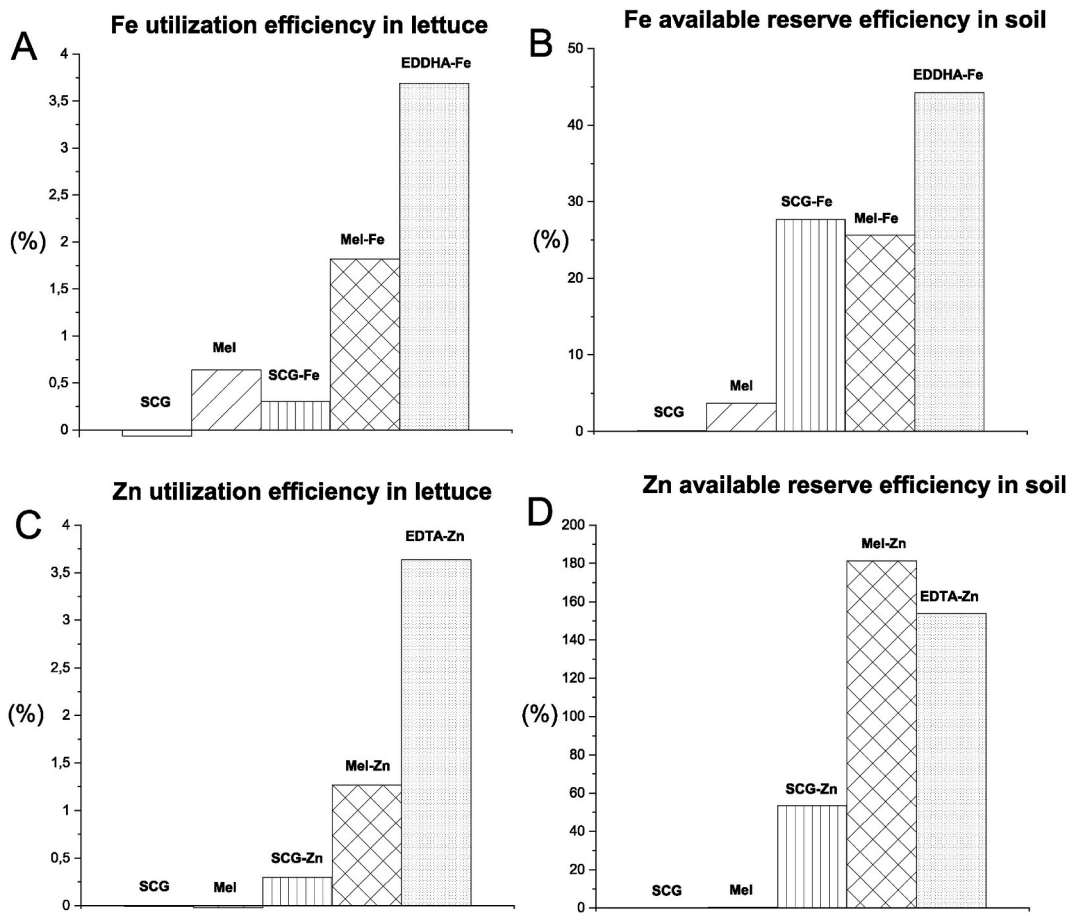

Fig. 1. Fe (panel A) and Zn (panel C) utilization efficiency of biochelates and chelates in lettuces. Fe (panel B) and Zn (panel D) available reserve efficiency of biochelates and chelates in soil.

Table 3
Available Fe and Zn, pH and electrical conductivity (EC_{25}) in soil.

Sample	Av. Fe (ppm)	Av. Zn (ppm)	pH	EC_{25} (dS/m)
Native soil[a]	6.860	1.35	8.2	1.3
NPK	6.695 ± 0.404^{a}	1.256 ± 0.056^{a}	8.0 ± 0.1^{a}	1.774 ± 0.147^{a}
SCG	6.716 ± 0.176^{a}	1.247 ± 0.044^{a}	7.7 ± 0.6^{a}	1.666 ± 0.109^{a}
Mel	7.066 ± 0.057^{b}	1.301 ± 0.095^{a}	8.0 ± 0.0^{a}	1.831 ± 0.072^{a}
SCG-Fe	12.231 ± 0.180^{d}	nd	8.0 ± 0.0^{a}	1.868 ± 0.048^{a}
SCG-Zn	nd	49.003 ± 1.681^{c}	7.9 ± 0.0^{a}	2.067 ± 0.086^{a}
Mel-Fe	9.258 ± 0.570^{c}	nd	8.0 ± 0.0^{a}	1.721 ± 0.100^{a}
Mel-Zn	nd	6.699 ± 0.859^{b}	8.1 ± 0.0^{a}	1.708 ± 0.009^{a}
EDDHA-Fe	11.117 ± 0.313^{e}	nd	8.1 ± 0.0^{a}	1.816 ± 0.088^{a}
EDTA-Zn	nd	5.872 ± 0.237^{b}	8.1 ± 0.0^{a}	1.843 ± 0.359^{a}

[a] Soil sample before the assay; Av: available; SCG: spent coffee grounds; Mel: melanoidin; EC_{25}: electrical conductivity measured at 25 °C; nd: not determined. Significant differences are indicated by different letters ($p < 0.05$). Mean values ± standard deviation.

composition, as previously mentioned.

Regarding utilization efficiency, Zn chelates behaved seemingly to Fe (Fig. 1C). SCG-Zn showed the lowest utilization efficiency (0.30%), which could be explained as in the case of Fe. On the contrary, EDTA-Zn was the chelate that showed the largest utilization efficiency, reaching a 3.70%; the efficiency of melanoidins was in between (1.27%). Zhao et al. (2019) also used EDTA-Zn (4 mg/kg) to enrich wheat (which is the crop on which more bio-fortifying tests are performed) and utilization efficiency was about 2%. Almendros et al. (2013) found that 10 mg/kg Zn-EDTA had a utilization efficiency of 2.58% in flax crop. However, those same authors performed another study in onions finding a utilization efficiency of 0.20% after adding 10 mg/kg Zn-EDTA. These authors stated that this rate depends on the chelate applied, the rate of application and the soil tested. Moreover, according to Broadley et al. (2007), tissue Zn concentrations in different plant species can differ considerably when grown under comparable conditions.

As stated above, SCG-Zn provided 89.61 mg/kg to the soil representing the largest increased in available Zn in soils in comparison to control samples, achieving 49 ppm of extractable zinc (Table 3). Moreover, if we compare against Mel-Zn and EDTA-Zn, SCG-Zn increased 7-fold and 10-fold the bioavailable Zn respectively (Table 3). However, considering the results of available Zn reserve efficiency in soil (Fig. 1D), Mel-Zn was the biochelate with the highest available reserve efficiency. Thus, in relation to the amount added, Mel-Zn was the biochelate that more available reserve of Zn added to the soil. This parameter could gain importance in successive harvests, which should

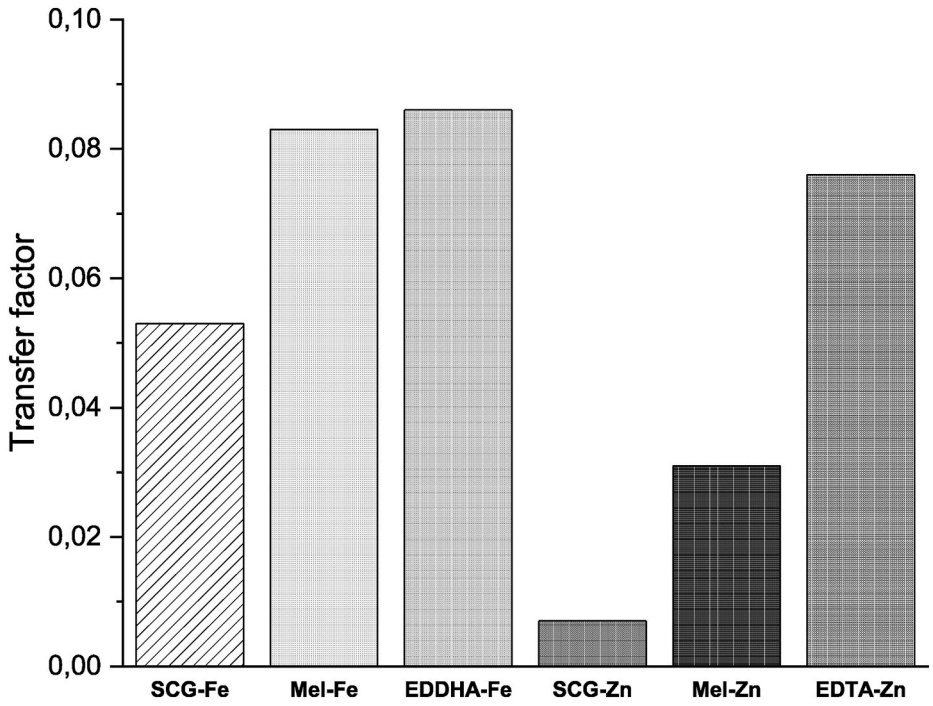

Fig. 2. Transfer factor values (total concentration in plant/concentration extracted from soil) of biochelates and commercial chelates.

be studied in future experiments. Zn concentration in plants belonging to the second or third crop would make for a better representative of this efficiency. This is the first time that this parameter has been calculated, therefore, it is not possible to compare our data with those obtained by other studies.

The Zn transfer factor followed this order: EDTA-Zn (0.076) > Mel-Zn (0.031) > SCG-Zn (0.007) (Fig. 2). Almendros et al. (2013, 2015) also calculated the transfer factor for different commercial synthetic chelates and natural organic Zn complexes. These authors reported transfer factors values of approximately 1.8 for 10 mg/kg of EDTA-Zn in flax crop (Almendros et al., 2013) and transfer factor values of approximately 0.15 for 10 mg/kg of EDTA-Zn in onion (Almendros et al., 2015), which were higher than those obtained in our assay.

3.4. Effects of biochelates on other soil-plant properties

The soil pH was also analyzed (Table 3) due to the influence of this physicochemical parameter on the mobilization of microelements (Navarro and Navarro, 2013). No treatment had a significant influence on the pH, due to the buffering capacity of Vega soil (Cervera-Mata et al., 2018). Therefore, Fe and Zn levels found in lettuces were not affected by soil pH.

Regarding the effect of the different treatments on the EC_{25}, there were no significant changes, despite of the high EC_{25} of SCG (4.5 dS/m, Cervera-Mata et al., 2018). All treatments increased salinity with respect to the native soil (Table 3). However, there were not significant differences ($p < 0.05$) between different treatments. Since soil salinity does not increase the absorption of nutrients (Cakmak and Kutman, 2018), quite the opposite, the increase in Fe and Zn in lettuces could not be attributed to soil salinity.

According to Storksdieck and Harrell (2007), biofortification should not affect crop biomass. In this sense, there were not significant differences between treatments (Table 2). Fresh weight of lettuce leaves ranged from 29.0 to 35.6 g with the highest values ($p < 0.05$) achieved by lettuces cultivated with Mel-Fe. Thus, functionalized coffee melanoidins could be a smart biochelating agent for lettuces. On the other hand, the addition of SCG, in any form, did not cause a significant inhibition of the plant growth, achieving weights of approximately 30 g (Table 2). This constitutes a positive and novel point of this research showing how SCG can be used without any further transformation, such as composting, mixture with inorganic fertilizers, biocharization, etc. (Cruz and Marques dos Santos Cordovil, 2015; Hardgrove and Livesley, 2016; Cervera-Mata et al., 2018, 2019b), and still not causing harm to the plant. Therefore, it could be claimed that the utilization of bio-residues in small amounts together with an inorganic fertilizer (NPK) could be an alternative to synthetic biochelates in order to improve the nutritional value (mineral composition) of cultivars without generating toxicity problems in the crops.

Table 4
Contribution of lettuce consumption to the daily Fe and Zn intake in the Spanish diet.

Sample	Mineral element	Daily intake[a] (mg/day)	Contribution to DRIs[b] (%)	Serving intake[c] (mg/serving)	Contribution to DRIs[b] (%)
NPK	*Fe*	0.032	0.40	1.004	12.55
	Zn	0.005	0.05	0.141	1.28
SCG	*Fe*	0.038	0.48	1.193	14.91
	Zn	0.005	0.05	0.159	1.45
SCG-Fe	*Fe*	0.041	0.51	1.286	16.08
SCG-Zn	*Zn*	0.023	0.21	0.728	6.62
Mel	*Fe*	0.037	0.46	1.148	14.35
	Zn	0.005	0.05	0.143	1.30
Mel-Fe	*Fe*	0.041	0.51	1.302	16.28
Mel-Zn	*Zn*	0.012	0.11	0.390	3.55
EDDHA-Fe	*Fe*	0.052	0.65	1.628	20.35
EDTA-Zn	*Zn*	0.027	0.25	0.840	7.27

[a] Considering lettuce consumption for a whole year.
[b] Dietary Reference Intake (DRIs) for an adult men (19–50 years/old) per day.
[c] Consideing the complete serving ingested a particular day.

3.5. Nutritional implications of bioforfied lettuce consumption

As previously stated, the use of different coffee by-products could

significantly increase the levels of Fe and Zn in lettuces (Table 2). Therefore, it could be possible to calculate the contribution of lettuce consumption to the daily intake of Fe and Zn in the Spanish diet (Table 4). The mean intake of lettuce in Spain is 1.74 kg/inhabitant/year (Mercasa, 2020) which means a daily intake of 4.77 g. With such intake, lettuce contribution to the daily intake of Fe and Zn is rather low (from 0.05 to 0.65%; Table 4). However, the picture is completely different if we make the estimation considering the lettuce serving size (150 g; Salvador i Castells, 2000). Regarding iron, a regular lettuce serving grown with NPK would provide 1.004 mg Fe (Table 4). However, these levels would increase up to 1.286 or 1.302 mg Fe/serving if biochelates derived from coffee by-products (SGC-Fe and Mel-Fe) were used to grown lettuces. Thus, the contribution to the dietary reference intake of iron (DRIs) would increase from 12.55% (for regular lettuces) up to 16.26% for those lettuces grown with the organic Mel-Zn biochelate (Institute of Medicine, 2005). These data could be very important taking into account the prevalence of iron-deficiency anemia (24.8%) in the world population (WHO, 2008).

Finally, regarding Zn, the contribution of lettuce to the daily intake of Zn are lower than those of iron, although they should not be underestimated. For example, a serving size of regular lettuce (grown with NPK fertilization) would provide 0.141 mg Zn, which covers around 1.28% of the DRIs. However, if a Mel-Zn biofortified lettuce is consumed, the contribution to the DRIs increases up to a 3.55% (0.390 mg Zn) and even up to a 6.62% (0.728 mg Zn) in the case of a SCG-Zn biofortified lettuce (Table 4). These are important results since low levels of Zn are closely related to hidden hunger which in turn has been linked to an increased frequency of acute infections and diarrhea (Brown et al., 2004).

3.6. Final considerations

Overall, commercial chelates EDDHA-Fe and EDTA-Zn achieved better evaluation rates (utilization efficiency, available reserve efficiency and transfer factor). Nevertheless, from efficiency point of view, biochelates of functionalized melanoidins were closer to commercial chelates than SCG biochelates. This could be taking into consideration that commercial chelates and functionalized melanoidins supply Fe and Zn in a soluble form, which allows for a much faster mobilization. However, in the case of SCG biochelates, they leave a greater reserve in the soil after cultivation. We could then hypothesize than the SCG biochelate could act as a medium-long term source of iron or zinc to ensure a steady supply to subsequent crops, behaving like a slow-delivery pharmaceutical drug system. Thus, a combination of functionalized SCG and melanoidins could provide a high biofortification rate from the first crop, while improving other soil characteristics such as physical (Cervera-Mata et al., 2019a), physicochemical and biological properties (Cervera-Mata et al., 2018), making this residue a good choice in agricultural purposes. This is very important since SCG is a food waste generated in huge amounts (Murthy and Madhava Naidu, 2012) that is usually just discarded having a negative impact on the environment. Recycling SCG for the purpose described would not only help the environment and save money but also improve crops, especially in those countries where hidden hunger is more prevalent. On the other hand, commercial chelates also present toxicity and nutrient balance problems in crops (Mohammadi and Khoshgoftarmanesh, 2014), as stated above.

CRediT authorship contribution statement

Ana Cervera-Mata: Investigation, Methodology, Formal analysis, Writing – original draft. **Alejandro Fernández-Arteaga:** Investigation, Methodology, Writing – original draft, Formal analysis, Investigation. **Daniel Hinojosa:** Investigation, Methodology, Formal analysis. **Silvia Pastoriza:** Conceptualization, Supervision, Writing – review & editing. **Gabriel Delgado:** Investigation, Supervision, Writing – original draft. **José Ángel Rufián-Henares:** Validation, Formal analysis, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by project PY20_00585 from Junta de Andalucía, Consejería de Economía, Conocimiento, Empresas y Universidad and by the Plan Propio de Investigación y Transferencia of the University of Granada under the program "Intensificación de la Investigación, modalidad B". This paper will form part of Ana Cervera-Mata's doctoral thesis, which is being developed within the context of the "Nutrition and Food Sciences Programme" at the University of Granada.

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