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ORIGINAL PAPER

Zinc and Iron Biofortification and Accumulation of Health-Promoting Compounds in Mycorrhizal *Cichorium intybus* L.

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Abstract
The positive impact of arbuscular mycorrhizal symbionts on plant growth and health has been reported for many species, and supports their use as biofertilizers and bioenhancers. Here, the potential role of the arbuscular mycorrhizal symbiont *Funneliformis mosseae* in the improvement of chicory (*Cichorium intybus* L.) nutritional value, in terms of nutrient uptake and accumulation of health-promoting compounds, was studied using an in vivo whole-plant system, allowing both plant and fungal tissue collection. Biomass and nutrient distribution were determined in plant and extraradical mycelium, and photosynthetic pigments and fructooligosaccharide concentrations were evaluated in chicory shoots and roots. Zinc shoot concentration of mycorrhizal chicory was significantly increased, as well as the whole-plant Fe uptake, while root Cu concentration was decreased, compared with uninoculated controls. *F. mosseae* extraradical mycelium accumulated Cu, Zn, Mn, and Fe at high concentrations, compared with those of the host plant tissues, suggesting that it plays a double functional "scavenging-filtering" role, by its ability to balance the uptake of microelements or to limit their translocation depending on plant-soil concentrations. The higher Zn and Fe uptake by mycorrhizal plants was significantly correlated with higher carotenoid, inulin, and fructose levels, suggesting a relationship among the modulation of micronutrient uptake by mycorrhizal symbionts and the biosynthesis of health-promoting molecules by the host. Overall, data from this work may boost the implementation of arbuscular mycorrhizal fungal inoculation aimed at inducing plant biofortification and enhancement of nutritional value of plant-derived food.

Keywords Arbuscular mycorrhizal fungi · Micronutrients · Extraradical mycelium · Biofortification · Inulin · Chicory

1 Introduction

Many soils worldwide are affected by macro- and micronutrient deficiencies which can significantly reduce crop yields (Vanlauwe et al. 2015). Previous studies reported that soil availability in essential micronutrients, such as Zn, Fe, Cu, Mn, Mo, and B, affects the nutritional quality of plant-derived food and feed and, especially when coupled with low total food intake, may cause silent metabolic alterations in humans (hidden hunger) and animals, with retarded growth and development, increased susceptibility to infections, and cognitive impairment (Biesalski and Birner 2018; Gödecke et al. 2018; Koç and Karayiğit 2022). Biofortification, which enhances essential nutrient concentration or bioavailability in food/feed crops, can be achieved by using diverse strategies (Szerement et al. 2022): by manipulating plant gene expression (Koç and Karayiğit 2022), by selecting crop genotypes (Nyiraguhirwa et al. 2022; Swamy et al. 2021) or species able to reduce rhizospheric pH, thus increasing root nutrient uptake (Bouis et al. 2019), or by using fertilizers, lime, or organic manures (Ramzani et al. 2016; White and Broadley 2009).

A further practice of agronomic biofortification is the use of microbial biostimulants (Liu et al. 2021; Verma et al. 2021), among which arbuscular mycorrhizal fungi. These beneficial soil fungi establish mutualistic associations with most land plant species and develop extraradical mycelial

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networks, functional to increase the volume of explored soil and to facilitate the absorption of macro- and micronutrients and their subsequent transfer to plant cells (Fellbaum et al. 2012; Kiers et al. 2011), coupling a "mycorrhizal" uptake pathway with the "direct" pathway, operated by root cells. A reciprocal reward mechanism, providing plant organic carbon to the fungal partner in exchange of mineral nutrients, often results in greater host plant biomass, with higher tissue nutrient concentrations and accumulation of secondary metabolites with both plant defense and human health-promoting activities in mycorrhizal plants, compared with non-mycorrhizal ones (Jacott et al. 2017; Sbrana et al. 2014). Indeed, the stimulation of plant secondary metabolism by arbuscular mycorrhizal symbioses induces the biosynthesis of phytochemicals such as polyphenols, carotenoids, flavonoids, and phytoestrogens, and a higher activity of antioxidant enzymes (Avio et al. 2018; Pedone-Bonfim et al. 2018; Rozpądek et al. 2014). Moreover, some studies on the impact of arbuscular mycorrhizal fungi on essential micronutrient uptake and distribution in edible tissues support their potential use for the optimization of human diet (Hart et al. 2015).

Arbuscular mycorrhizal fungi are important members of the plant microbiome and they influence the plant nutrient economics (Averill et al. 2019; Wang et al. 2017). However, so far most analyses have focused on the effects of arbuscular mycorrhizal fungi on plant nitrogen fixation ability, carbon cycling, and phosphorous acquisition strategies (Cornelissen et al. 2001; Jansa et al. 2011; Schütz et al. 2022), while less is yet known on micronutrients uptake and distribution. Moreover, although the complex architecture of mycorrhizal networks and a possible hyphal nutrients transport system have been described (Giovannetti et al. 2004; Uetake et al. 2002), the determination of micronutrient content inside the extraradical mycelium (ERM) has been rarely performed, due to limitations in hyphal biomass and in the sensitivity of technologies suitable for examining such a fragile structure (Cardini et al. 2021; Chen et al. 2001; Neumann and George 2005; Orłowska et al. 2008).

On the other hand, as a consequence of human industrial, agricultural, and military activities, the levels of some micronutrients, particularly those that are also trace elements or heavy metals, dramatically increased in many local sites, causing direct toxicity to soil organisms and plants and representing a long-term threat to humans when entering the food chain (Beygi and Jalali 2019; Järup 2003). Arbuscular mycorrhizal fungi may also play a role in tolerance of host plants to heavy metals (Lehmann and Rillig 2015; Leyval et al. 2002) either directly, by modulating host plant heavy metal allocation, or indirectly, by modifying root system architecture, thus representing a potential tool in agricultural restoration of contaminated soils (Chen et al. 2007; Göhre and Paszkowski 2006; Mnasri et al. 2017). The positive effects of mycorrhizal symbiosis, combined with the associated mycorrhizospheric microbiota (Devi et al. 2022), on phytoremediation of heavy metal–polluted soils are of great biotechnological interest, because mycorrhizal plants can become as effective at extracting metals such as Cu, Cd, Pb, or Zn as non-mycorrhizal hyperaccumulator plants (Ebbs and Kochian 1998; Leyval et al. 2002), due to heavy metal immobilization in the dense extraradical mycelium (Cornejo et al. 2017; Joner and Leyval 2001). When the heavy metals absorbed are also micronutrients (e.g., Cu, Fe, Zn), the mycorrhizal fungus-plant system can represent a source of biofortified food/feed; otherwise, it behaves as a phytoremediation tool for hazardous pollutants (e.g., Cd, Pb, Hg).

Chicory (*Cichorium intybus* L.) is a perennial, deep-rooting herb that can be found as a wild plant in natural grasslands, where it represents a useful indicator for toxic metal contamination (Simon et al. 1996). Many selected varieties of *C. intybus* are cultivated as leafy vegetable crops for human consumption (fresh salad or cooked) and for animal feeding, and for their roots, which can be used for the production of inulin-type fructans and as coffee substitute. In recent years, chicory has also received more attention for its bioactive secondary metabolites, such as inulin, sesquiterpene lactones, coumarins, and flavonoids, whose accumulation was reported to be modulated by mycorrhizal symbiosis (Rozpądek et al. 2014), although the involvement of arbuscular mycorrhizal symbionts in chicory nutritional and nutraceutical traits has yet to be unravelled.

In order to gain information on the ability of arbuscular mycorrhizal fungi to facilitate the transfer of key micronutrients to the host plant, analyses of the distribution patterns of some micro- and macronutrients in plant and fungal tissues of *C. intybus* in symbiosis with the mycorrhizal symbiont *Funneliformis mosseae* were carried out. As chicory accumulates components with therapeutic and nutraceutical properties, the ability of *F. mosseae* to enhance health-promoting plant metabolites was also assessed. Overall, data from this work may be useful to implement the use of mycorrhizal inoculants aimed at improving food/feed plant nutritional value.

2 Materials and Methods

2.1 Fungal and Plant Material

Cichorium intybus seeds ('Zuccherina di Trieste' green chicory) were surface-sterilized, germinated, and grown for 10 days in sterile quartz grit (aquarium gravel, mean diameter size 2 mm) and then inoculated with either living (mycorrhizal treatment) or autoclaved (non-mycorrhizal mock treatment, hereafter control) spores or sporocarps, mycelium, and colonized roots obtained from pot-culture

soil of *Funneliformis mosseae* (isolate code IMA1), after wet sieving through a 100-µm-mesh size sieve.

2.2 Experiment Design

All plants were individually grown in 5-cm diameter pots disinfected by chlorination, filled with the same sterile quartz grit, and placed into sun-transparent bags (Merck, Milano, Italy) in a growth chamber at 25 °C, with 25 °C day and 21 °C night temperature (16 h of light per day, photon flux density of 350 µmol m^{-2} s^{-1}). The main characteristics of plant seeds and substrate used are described in Online Resource 1. After 4 weeks' growth, grit was washed from roots, spores and sporocarps adhering to plant roots were carefully removed with forceps under a Leica M 205C dissecting microscope (Leica, Milano, Italy), and plant root systems were placed between two semicircular 13-cm diameter Millipore™ membranes. Plants were then transferred into 14-cm diameter Petri dishes containing moist sterile quartz grit, with the root-containing lower half of plates wrapped into aluminum foil and the plant shoot developing out of the plate (whole-plant system; Sbrana et al. 2020). Before sealing the plate with parafilm, each plant was fertilized with 15 mL of Long Ashton nutrient solution (modified by Hewitt 1966), containing 108 µg L^{-1} Cu, 78.5 µg L^{-1} Zn, 571.4 µg L^{-1} Mn, and 16.8 µg L^{-1} Fe. As water loss of this growth system was limited by Petri dish parafilm sealing and bagging, moisture was maintained by the addition of 5 mL of the same solution to each plate, after 3 weeks of culture. For each treatment (control and mycorrhizal), 108 whole-plant system plates were prepared.

After 4 weeks of culture in the growth chamber, the root sandwiches described above were opened in ice-cold sterile water and the extraradical mycelium (ERM) spreading on membranes containing mycorrhizal plants (Fig. 1a) was harvested in the ice-cold sterile water using a rubber cell scraper. Collected mycelium was stored in Eppendorf tubes at − 80 °C, after blotting on a filter paper to remove excess water. In order to obtain the biomass needed for nutrient determination (at least 10 mg of dry weight (DW)), the extraradical mycelium collected from roots of 36 mycorrhizal plants for each treatment was pooled, to obtain three replicate tubes for further analyses.

Before preparing plant samples for analytical procedures, mycorrhizal status of *F. mosseae*– and mock-inoculated plants was assessed. Each plant root system was observed under blue light by using an inverted microscope (Leica DM IRB model, Milano, Italy) equipped with epifluorescence (Jabaji-Hare et al. 1984), with the aim of ascertaining the occurrence of arbuscular colonization in mycorrhizal

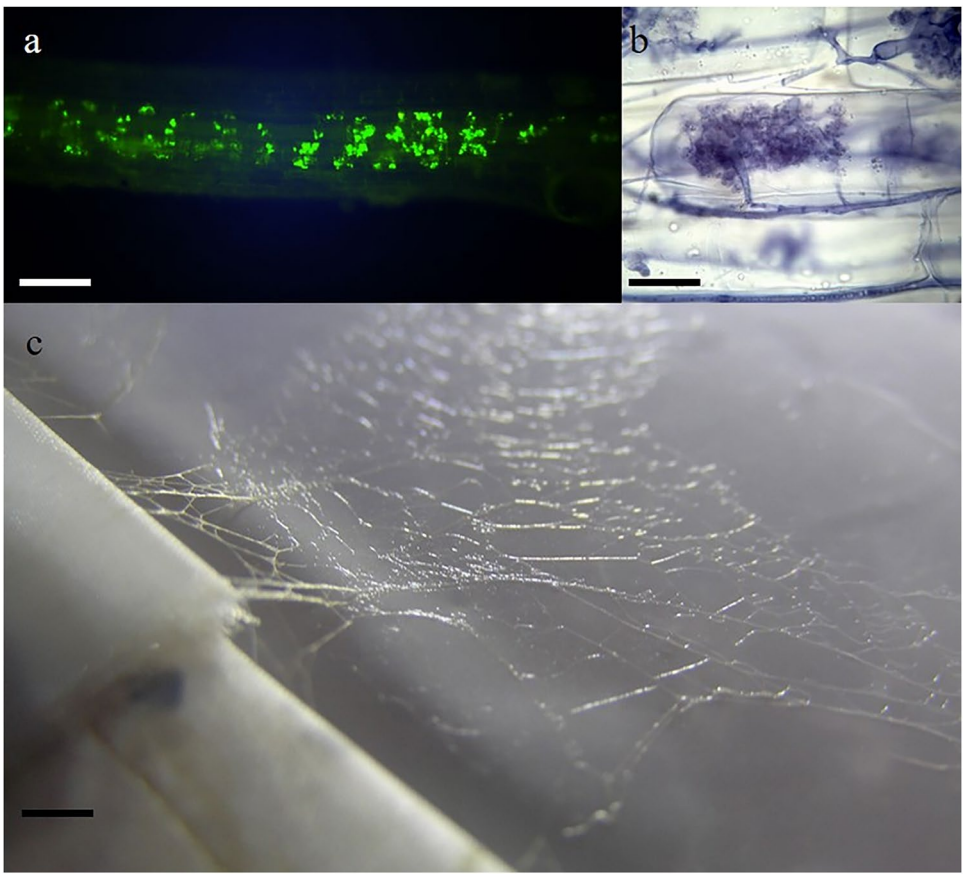

Fig. 1 Pictures showing extraradical mycelium (ERM) and roots obtained from the whole-plant growth system where *Cichorium intybus* grew in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae*. **a** Autofluorescence of intraradical fungal structures in chicory roots observed under blue light. Scale bar = 120 µm. **b** Intercellular hyphae and arbuscules developed by *F. mosseae* within chicory roots, after Trypan blue staining. Scale bar = 50 µm. **c** ERM spreading on the membrane surface outside the nylon net which encloses the chicory root system. Scale bar = 0.3 cm

treatment and the absence of any root colonization in the mock treatment. In order to quantify root colonization, five random replica plants from each treatment were selected for root clearing and staining with Trypan blue, using lactic acid instead of lactophenol (Phillips and Hayman 1970). Total and colonized lengths of stained root systems were measured under the dissecting microscope using the grid-line intersect method (Giovannetti and Mosse 1980).

2.3 Plant Growth Analysis

Chicory stems were severed using a stainless-steel razor blade perpendicularly to the stem axis to separate shoots from roots, and leaf number (LN), leaf area (LA), and fresh weight (FW) were immediately determined. The LA was measured on 20 randomly sampled plants for each treatment, using an imaging analysis software (ImageJ, IJ 1.46r, http://imagej.nih.gov/ij/). The maximum root length was also measured to calculate the root length mass ratio (RLMR), m g^{-1}. Root and shoot samples were oven-dried at 60 °C until their weights remained constant to determine the DW and nutrient contents. Some growth analysis parameters or indices such as the leaf mass per area (LMA), in g m^{-2}, shoot and root mass ratio (SMR and RMR, respectively), in g g^{-1}, and RLMR were determined or calculated as described in Di Baccio et al. (2009), or otherwise described.

2.4 Nutrient Determination

In order to collect the dry biomass needed for nutrient determination, single dry roots or shoots were grouped into the three replicate pools, each composed by tissues originating from the same 36 plant samples previously identified to pool extraradical mycelium. Shoot and root pools were then grinded to a powder in an analytical steel mill (Foss Tecator 1093 Cyclotec Sample Mill, Sweden).

The percentages of carbon and nitrogen in shoots and root pooled samples were determined by an elemental analyzer system with autosampler (Carlo Erba model EA1108) by using atropine sulfate as a standard for instrument calibration. Samples (about 6 mg dry material from each pooled replicate) were put into a tin capsule (3.5 × 5 mm) closed leaving out the air and analyzed. Each capsule falls into the combustion column where it reaches a temperature of 1060 °C, under a constant flow of helium (He, carrier) and in the presence of catalysts and excess of oxygen. The flow of combustion products is injected into a packed chromatographic column (length: 2 m) for the separation of the elements to be analyzed.

For the determination of iron, copper, zinc, and manganese, aliquots of pooled dry shoot or roots (0.25–0.30 g) were used for residual water determination at 105 °C. Such material was digested in concentrated nitric acid (HNO_3), ultrapure water, and hydrogen peroxide (4:3:2, v:v:v) in a microwave oven (Excel, PreeKem Scientific Instruments Co., China) and analyzed by atomic absorption spectrophotometry (Varian model SpectrAA 220FS, Australia) equipped with appropriate lamps for each element to be analyzed. Chemical analyses were validated by blanks and reference materials. The concentration of micronutrients in shoot and root samples was expressed as µg per g of dry matter (µg g^{-1} or ppm of DW), and the micronutrient content (uptake) as µg per plant tissue.

The pooled extraradical mycelial samples were oven-dried (60 °C) and ashed in a porcelain crucible in a muffle furnace (550 °C). After cooling down, the ash was boiled for few minutes in diluted HNO_3; the residue was filtered through a membrane filter with pore size of 0.45 µm. The contents of Fe, Cu, Zn, and Mn were determined by atomic absorption spectrometry with graphite furnace (Varian model SpectrAA 220G; limit of detection (LOD): 0.5 µg L^{-1}, limit of quantification (LOQ): 1 µg L^{-1}) or by inductively coupled plasma optical emission spectrometry (Varian model 720 ICP OES; LOD: 5–20 µg L^{-1}, LOQ: 20–40 µg L^{-1}), depending on interference effects. The standard methods used followed the procedures described by APAT (Agenzia per la protezione dell'Ambiente e per i servizi tecnici), in APAT IRSA-CNR (2003): the method 3250 B Man 29 was used for the determinations by atomic absorption with graphite furnace, and the method 3020 Man 29 was used for the optical ICP determinations. The concentration of micronutrients in fungal biomass was expressed as µg per g of dry matter (µg g^{-1} or ppm of DW), and the micronutrient content (uptake) as ng per individual plant network.

2.5 Pigment, Fructose, and Inulin Contents

Chlorophylls (*a* and *b*) and total carotenoids were measured on 5 fresh leaf disk replicates of known area (0.785 cm^2, about 50 mg in weight), randomly selected among plants belonging to mycorrhizal and control treatments, frozen in aluminum sheets in liquid nitrogen, and then stored at − 80 °C. Subsequently, the samples were homogenized in 80% (w/v) cold acetone and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was filtered (0.2 µm) by Lab Filtration Process (Sartorius Stedim Biotech, Göttingen, Germany) and spectrophotometrically analyzed for photosynthetic pigments. The absorbance was measured at 663.2, 646.8, and 470.0 nm against the blank with an UV–Vis spectrophotometer (Shimadzu UV-1800, Shimadzu, Italy), and the concentrations calculated following the method of Wellburn (1994).

The determination of fructose and inulin from shoot and root tissues of chicory was performed using the method of Kumari et al. (2007), with some modifications for the extraction and analysis on the basis of Gibson et al. (1995) and

McRary and Slattery (1945) methods. Three aliquots per treatment, each containing 10 mg of dry material from the pooled replicate samples, were extracted in 1.5 mL of 80% ethanol for 6 h. Aliquots (0.5 mL) of extracts were directly used for the colorimetric reaction with alcoholic resorcinol solution (0.1%); from the same extracts, 0.5 mL aliquots were hydrolyzed in HCl in a water bath at 80 °C, and then added to alcoholic resorcinol solution. Both sample aliquots were read spectrophotometrically at 490 nm. D(-)Fructose (F2793 analytical standard, Merck, Italy) and inulin (inulin from chicory, Merck, Italy) were treated as above and used in calibration curves covering the ranges of 0–3 mg and 0–2 mg, respectively.

2.6 Data Analyses

Data of shoots and roots dry weight and of leaf area of individual plants were analyzed by comparing mycorrhizal treatment and control on the whole dataset ($n = 108$). Concentrations and contents of micronutrients, C and N percentages and contents, inulin, and fructose, obtained from homogenized dry tissues of the three plant pools, were analyzed with three replicate data for each treatment, while 5 replicate data were obtained from pigment analyses carried out on fresh leaf tissues.

Percentage data were subjected to arcsine transformation before carrying out statistical analyses. All datasets were checked for fulfilment of ANOVA assumptions (robust Levene's test of homogeneity of variances) and submitted to one-way analyses. Data showing unequal variances were analyzed by using Welch's test. Pearson correlation and/or linear regression coefficients were calculated to reveal relationships among the different variables related to concentrations or contents of plant nutrients and nutraceutical compounds. All statistical analyses were carried out with IBM SPSS Statistics version 23. Principal component analysis (PCA) was performed in Canoco ver. 5.

3 Results

3.1 Mycorrhizal Colonization and Development of Chicory Plants

After 4 weeks of culture in the whole-plant system with standard Long Ashton fertilization, all root systems of *F. mosseae*–inoculated chicory plants observed under blue light consistently showed autofluorescent arbuscular colonization (Fig. 1a), confirmed by selected sample staining with Trypan blue (Fig. 1b). The colonized root length percentages of stained root systems were variable among replicates, ranging between 46.2 and 62.8% (Table 1), while control plants were not mycorrhizal.

Plant biomass production was significantly higher (+69.6%) in mycorrhizal plants than in controls (Table 1). This was due to a two-fold and 1.5-fold increase of root

Table 1 Mycorrhizal status and growth traits of *Cichorium intybus* plants, in symbiosis (mycorrhizal) or not (controls) with the arbuscular mycorrhizal fungus *Funneliformis mosseae*, grown in a whole-plant experimental system. In rows, means (± standard error of the mean) followed by the same letter do not differ significantly at $P \leq 0.05$ by one-way ANOVA (homogeneous variances) or Welch's test (nonhomogeneous variances)

Plant growth traits	Mycorrhizal plants	Control plants	ANOVA *F*	Welch's *F*	*P*
Mycorrhizal root length (%)	53.03 ± 2.92	Not detected			
Root DW (mg)	89.52 ± 3.81 a	45.24 ± 3.52 b		45.62	< 0.001
Root FW/DW	7.07 ± 0.22 a	7.84 ± 0.47 a		2.32	0.137
RMR (g g^{-1})	0.46 ± 0.01 a	0.43 ± 0.01 a	3.17		0.076
Maximum root length (cm)	22.41 ± 0.42 a	23.83 ± 0.61 a	4.16		0.111
RLMR (m g^{-1})	3.90 ± 0.26 b	5.48 ± 0.34 a		8.25	0.005
Shoot DW (mg)	93.94 ± 3.03 a	63.03 ± 4.51 b		29.00	< 0.001
Shoot FW/DW	7.87 ± 0.20 a	6.94 ± 0.25 b	8.08		0.005
LN	6.73 ± 0.12 a	6.12 ± 0.11 b	4.89		0.092
LA (cm^2)	40.93 ± 1.38 a	25.65 ± 1.24 b	11.15		0.002
MLA (cm^2)	5.96 ± 0.20 a	4.28 ± 0.22 b	7.91		0.008
LMA (g m^{-2})	24.80 ± 0.44 b	32.08 ± 0.95 a	5.47		0.025
SMR (g g^{-1})	0.54 ± 0.01 a	0.57 ± 0.01 a	3.11		0.079
Plant DW (mg)	183.42 ± 6.12 a	108.24 ± 7.71 b	44.36		< 0.001
Plant FW/DW	7.32 ± 0.18 a	7.21 ± 0.26 a	0.10		0.748
Root/shoot DW	0.95 ± 0.03 a	0.82 ± 0.03 b		4.38	0.038

DW, dry weight; *FW*, fresh weight; *RMR*, root mass ratio (root DW/plant DW); *RLMR*, root length mass ratio (root maximum length/root DW); *LN*, leaf number; *LA*, leaf area; *MLA*, mean leaf area; *LMA*, leaf mass per area (leaf mass/leaf area); *SMR*, shoot mass ratio (shoot DW/plant DW). DW, FW, and derived variables ($n = 108$); LA and derived variables ($n = 20$)

and shoot biomass, respectively. The shoots FW/DW, LN, and LA were also enhanced by the symbiotic status (+13.4, 9.8, and 59.6%, respectively; Table 1), as like the root/shoot ratio, which was 1.2-fold higher compared to controls. On the contrary, the LMA decreased (−22.7%) in mycorrhizal plants compared to controls.

Mycorrhizal colonization did not affect the root maximum length or hydration status (FW/DW), although the RLMR, indicating the root biomass partitioning for length or root density, decreased in mycorrhizal plants compared to controls (−29%).

3.2 Accumulation of Mineral Nutrients, Pigments, and Fructooligosaccharides in Chicory Plants

Chicory root and shoot C concentrations and contents did not reveal any difference among treatments, although the C content in the whole plant was at the limit of significance level ($P=0.056$) with an increasing trend (+28%) in mycorrhizal plants. On the contrary, N concentration decreased in root and shoot (−25 and −14%, respectively) of mycorrhizal plants, although such variations did not impact N contents (Table 2). The C/N ratio was higher in mycorrhizal roots and whole plants (+40 and 27%, respectively) in comparison with controls (Table 2).

The analysis of micronutrient concentration in chicory tissues showed significant differences between mycorrhizal and control plants for Cu in roots ($F_{1,4}=12.8$, $P=0.023$) and Zn in shoots ($F_{1,4}=47.1$, $P=0.002$) (Fig. 2a, b). Compared to controls, mycorrhizal plants' Cu concentration was reduced by 60% in roots, while shoot Zn concentration was enhanced by 38%. Data computed for micronutrient content confirmed the higher Zn uptake in shoot of mycorrhizal plants, compared to controls, with an enhanced Zn accumulation in the whole plants (Table 3), while they did not reveal significant differences between treatments in root Cu uptake. The root of plants grown in symbiosis with *F. mosseae* showed a higher Fe content (1.3-fold) than control plants (Table 3). Interestingly, both Zn and Fe contents in the whole mycorrhizal plants were significantly higher than those of controls (+38 and +34%, respectively; Table 3). In chicory plants, independently on the inoculation treatment, a significant positive correlation was detected between root Zn and Fe concentrations (Pearson's $r=0.87$, $P=0.026$).

Carotenoids and carotenoids to total chlorophyll ratio were significantly higher (about twofold) in leaves of plants in symbiosis with *F. mosseae*, compared to controls, while chlorophyll *a* and *b* concentrations did not differ between treatments (Table 4).

Concentrations of both fructose and inulin did not differ in shoots while they were significantly higher in roots of mycorrhizal plants than in those of controls, with 57 and 48% average increases, respectively ($F_{1,4}=13.25$, $P=0.022$ for fructose and $F_{1,4}=11.99$, $P=0.026$ for inulin; Fig. 3).

Regression analyses, carried out independently on the inoculation treatment, highlighted the significant positive regression between shoot Zn concentration and root fructose and inulin ones ($R=0.83$; $F=9.0$ and 8.8, respectively; $P=0.04$; R^2 and regression equations in Fig. 4a) and the negative relation between root Cu concentration and those of fructose and inulin ($R=0.92$ and 0.90; $F=21.2$ and 17.9, respectively; $P=0.01$; R^2 and regression equations in Fig. 4b).

A consistent relationship among Zn, Fe, carotenoid, inulin, and fructose accumulation and mycorrhizal plants is supported by PCA, which also highlights the opposite behavior of control and mycorrhizal plants regarding Cu accumulation (Fig. 5).

Table 2 Carbon (C) and nitrogen (N) concentration and content in tissues of mycorrhizal plants of *Cichorium intybus*, in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae*, and of non-mycorrhizal plants (controls), grown in a whole-plant experimental system. In rows, means (±standard error of the mean, $n=3$) followed by the same letter do not differ significantly at $P\leq0.05$ by one-way ANOVA (homogeneous variances) or Welch's test (nonhomogeneous variances)

		Mycorrhizal plants	Control plants	ANOVA *F*	Welch's	*P*
C concentration (%)	Root	40.21 ± 0.37 a	38.79 ± 0.91 a	2.08		0.223
	Shoot	36.92 ± 0.59 a	37.58 ± 0.14 a		1.16	0.384
C content (mg per plant)	Root	36.03 ± 5.31 a	16.95 ± 5.74 a	5.58		0.078
	Shoot	34.72 ± 3.47 a	23.22 ± 9.20 a	0.47		0.532
	Plant	70.75 ± 5.37 a	40.17 ± 14.91 a	7.12		0.056
N concentration (%)	Root	0.80 ± 0.08 b	1.06 ± 0.03 a	9.62		0.036
	Shoot	1.69 ± 0.06 b	1.97 ± 0.06 a	9.97		0.034
N content (mg per plant)	Root	0.70 ± 0.05 a	0.47 ± 0.16 a	1.62		0.272
	Shoot	1.58 ± 0.08 a	1.22 ± 0.48 a	1.83		0.248
	Plant	2.27 ± 0.07 a	1.69 ± 0.64 a		0.82	0.458
C/N ratio	Root	51.36 ± 4.74 a	36.69 ± 1.09 b	9.10		0.040
	Shoot	21.93 ± 1.12 a	19.10 ± 0.52 a	5.23		0.084
	Plant	31.04 ± 1.48 a	24.47 ± 1.15 b	12.25		0.025

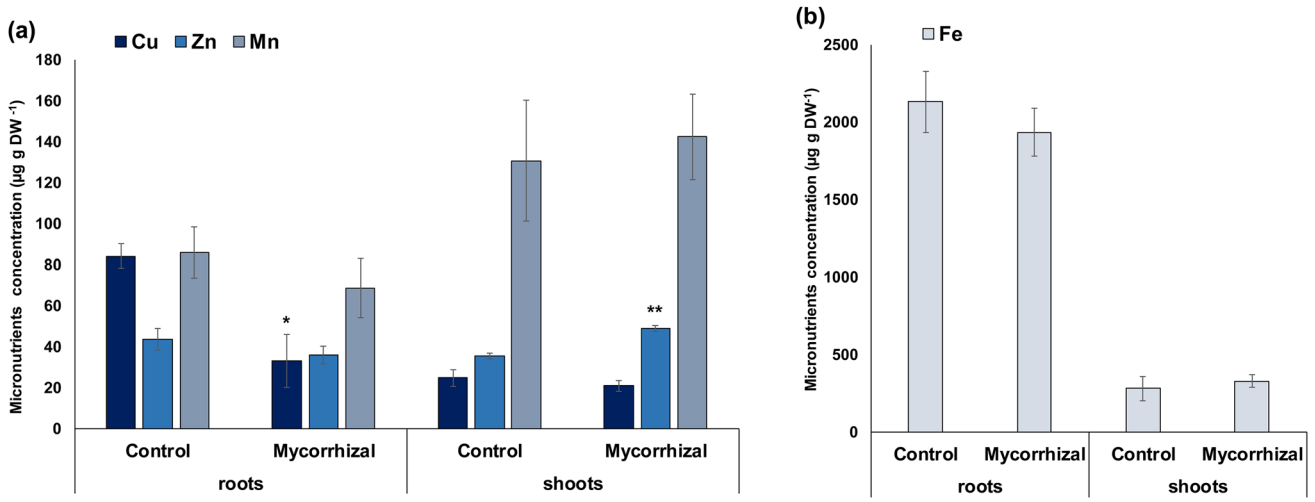

Fig. 2 Mean values (± standard error of means) of micronutrient (**a** Cu, Zn, and Mn; **b** Fe) concentration in roots and shoots of *Cichorium intybus* plants in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae* (mycorrhizal) and of non-mycorrhizal controls, grown in a whole-plant experimental system. Asterisks indicate significant differences between mycorrhizal and control plants by one-way ANOVA: roots Cu $F_{1,4}=12.82$, $P=0.023$ (*); shoots Zn $F_{1,4}=47.14$, $P=0.002$ (**)

3.3 Fungal Micronutrient Accumulation

F. mosseae extraradical mycelium (ERM; Fig. 1c) showed very high micronutrient concentrations, particularly Fe, which exceeded 3000 µg g^{-1} dry mycelium (Table 5). The concentration of Cu, Zn, Mn, and Fe was higher in mycelium than in shoot and root chicory tissues (Fig. 2a, b; Table 5). Calculated contents showed that the average contents of micronutrients in each individual *F. mosseae* network, originating from a single chicory plant, ranged from 22.7 (Zn) to 904 ng (Fe), depending on the element (Table 5).

4 Discussion

Data obtained in this work showed that the mycorrhizal symbiont *F. mosseae* is able to facilitate biofortification of Zn in chicory leaves and Fe in the whole plant, even at an early plant growth stage, suitable for the consumption as ready to eat "baby leaf." Interestingly, the fungal symbiont also induced young plant leaves to accumulate carotenoids, important health-promoting compounds, and enhanced root storage of inulin, a bioactive compound with prebiotic, hypocholesterolemic, and hypoglycemic properties.

The use of mycorrhizal symbionts as plant biofertilizers and biostimulants, with the aim of increasing yield and nutrient levels in plant-derived food, is supported by studies showing that concentrations of both mineral elements and important macromolecules may be enhanced in mycorrhizal plant tissues (Kaur and Suseela 2020; Noceto et al. 2021).

In the present study, chicory plants in symbiosis with *F. mosseae* showed larger shoot and root biomass, and leaf number and area, confirming general issues on the ability of arbuscular mycorrhizal fungi to boost host growth. Interestingly, a recent work found that both leaf area index and the fraction of intercepted radiation were enhanced in chicory by *R. irregulare* inoculation (Langeroodi et al. 2020).

Here, Zn and, at a lesser extent, Fe uptake were enhanced in mycorrhizal chicory, leading to their accumulation in shoots. Compared with non-mycorrhizal controls, larger Fe concentration in both shoots and roots of sorghum plants inoculated with multiple species of arbuscular mycorrhizal fungi (Prity et al. 2020), and higher concentration of Mn, Cu, and Fe in lettuce leaves produced by plants inoculated with *Rhizophagus intraradices* and *F. mosseae* (Baslam et al. 2013) were reported. Moreover, wheat and barley in symbiosis with *Rhizoglomus irregulare* accumulated more Zn and Fe in grain (Coccina et al. 2019; Watts-Williams and Cavagnaro 2018), various micronutrients showed increased concentration in zucchini fruits and leaves when plants were treated with commercial mycorrhizal inoculum (Cardarelli et al. 2010), and tomato plants in symbiosis with *R. irregulare* showed higher levels of Zn in fruits (Giovannetti et al. 2012). The significant effect of the inoculation with mycorrhizal fungi on host Zn and Fe accumulation in various tissues has been confirmed by meta-analyses carried out on data from 263 and 233 experiments, respectively (Lehmann et al. 2014; Lehmann and Rillig 2015). Here, notwithstanding the early plant growth stage, both concentration and content of Zn in shoots of mycorrhizal plants were enhanced,

Table 3 Mean values (±standard error of means) of micronutrient content in roots and shoots of *Cichorium intybus* plants, in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae* (mycorrhizal) and non-mycorrhizal controls, grown in a whole-plant experimental system. In columns, means (±standard error of the mean, $n = 3$) followed by the same letter do not differ significantly at $P \leq 0.05$ by one-way ANOVA (homogeneous variances) or Welch's test (nonhomogeneous variances)

	Cu content (µg per plant)			Zn content (µg per plant)			Mn content (µg per plant)			Fe content (µg per plant)		
	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant
Mycorrhizal plants	1.94 ± 0.10a	2.76 ± 0.75a	4.71 ± 0.78a	4.62 ± 0.41a	3.14 ± 0.14a	7.76 ± 0.48a	13.69 ± 3.13a	5.80 ± 0.47a	19.48 ± 3.46a	30.90 ± 4.35a	170.53 ± 17.22a	201.44 ± 12.95a
Control plants	2.13 ± 0.36a	5.05 ± 0.60a	7.18 ± 0.54a	3.06 ± 0.13b	2.57 ± 0.18a	5.64 ± 0.18b	11.33 ± 2.82a	5.18 ± 0.96a	16.52 ± 3.36a	24.44 ± 6.86a	125.71 ± 5.14a	150.15 ± 7.19b
ANOVA $F_{1,4}$	0.24	5.72	6.80	13.27		17.20	0.31	0.33	0.38	0.63	6.22	11.99
Welch's					8.35							
P	0.647	0.075	0.059	0.022	0.102	0.014	0.606	0.598	0.572	0.471	0.067	0.026

suggesting that the symbiotic Zn uptake efficiency overcomes the known "dilution effect," due to mycorrhizal plant growth increase (Baslam et al. 2011). Although Zn and Fe content is high in most agricultural soils, these elements are often not phyto-available due to high soil pH and physico-chemical characteristics (White et al. 2012). The resulting plant deficiencies can severely reduce growth and yield, due to the role played by these trace elements in key metabolic pathways and enzymatic activities.

At the establishment of mycorrhizal symbioses, the down-regulation of plant genes involved in direct nutrient uptake (Handa et al. 2015; Tian et al. 2017; Vangelisti et al. 2018) is balanced by the fungal uptake from soil of both P, the main element translocated by arbuscular mycorrhizal symbionts to their hosts, and other nutrients, among which Zn and Fe, through the activity of extraradical networks. This wide hyphal network is able to actively intake phosphorus, through specific fungal phosphate transporters, and metal elements, through the expression of metal transporters and of genes putatively involved in metallophore-metal uptake and in metallophore synthesis (Tamayo et al. 2014). Previous studies have also shown that P uptake, positively related with the interconnectedness of extraradical mycelium and with the density of fungal appressoria on host roots (Avio et al. 2006; Pepe et al. 2020), increases mycelial acquisition and translocation of other metal minerals, as the negative charges of polyP synthesized in hyphae may be balanced by the active absorption of di- and monovalent species from the soil solution (Bücking and Shachar-Hill 2005; Kikuchi et al. 2014). Moreover, the mycorrhizal mycelium hosts a wide diversity of associated microorganisms, among which members of phosphate-solubilizing and nitrogen-fixing bacteria, whose activity may favor nutrient absorption by the fungal partner (De Novais et al. 2020; Emmett et al. 2021; Jiang et al. 2021; Rawat et al. 2021; Sbrana et al. 2022; Scheublin et al. 2010).

In this work, elemental analysis showed very high concentrations and contents—calculated with respect to the biomass of single plant mycelial networks—of microelements, and particularly Fe, in mycelium growing from chicory roots. Interestingly, the concentrations of Cu, Zn, Mn, and Fe in the extraradical network were higher than those of root and shoot of the host plant. Previous studies showed high microelement binding capacity of extraradical networks produced by *F. mosseae*, *Glomus claroideum*, and *Rhizoglomus* (formerly *Glomus*) *intraradices* (Gonzalez-Chavez et al. 2002; González-Guerrero et al. 2008). Larger Zn and Cu concentrations were found in extraradical hyphae of an unidentified mycorrhizal fungus, compared with plant root cells (Orłowska et al. 2008), and high Fe and Zn concentrations were reported for *F. mosseae* and *Diversispora epigaea* (formerly *Glomus versiforme*) mycelium produced in symbiosis with maize and clover (Chen et al. 2001). In our study, the Zn concentration of mycorrhizal chicory was higher in

Table 4 Mean values (±standard error of means, $n=5$) of photosynthetic pigment concentration in leaf disks of *Cichorium intybus* plants in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae* (mycorrhizal) and non-mycorrhizal controls, grown in a whole-plant experimental system. In rows, means followed by the same letter do not differ significantly at $P \leq 0.05$ by one-way ANOVA (homogeneous variances) or Welch's test (nonhomogeneous variances). Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; Car, total carotenoids; Chl tot, total chlorophyll

	Control plants	Mycorrhizal plants	ANOVA *F*	Welch's	*P*
Chl *a* (μg g^{-1} DW)	86.07 ± 27.64 a	95.41 ± 21.37 a	0.07		0.796
Chl *b* (μg g^{-1} DW)	91.56 ± 36.87 a	79.28 ± 19.08 a	0.09		0.775
Chl *a* / *b* (μg g^{-1} DW)	1.03 ± 0.09 a	1.28 ± 0.19 a		1.32	0.296
Carotenoids (μg g^{-1} DW)	12.49 ± 0.89 b	28.84 ± 4.78 a		11.29	0.026
Car/Chl tot (μg g^{-1} DW)	0.09 ± 0.02 b	0.18 ± 0.03 a	6.28		0.036

shoot than in root, while Cu concentration was maintained unaltered in shoot; this supports the role of *F. mosseae* in modulating element absorption through the promotion of Zn and the limitation of Cu translocation from root to shoot. The occurrence of genes encoding putative transport proteins, mediating the uptake of Cu, Fe, and Zn and their compartmentalization in vacuoles, has been detected in *R. irregularis* (González-Guerrero et al. 2005, 2010; Tisserant et al. 2013; Tamayo et al. 2014). Moreover, variable heavy metal chelating activity, depending on fungal identity and growth conditions, was reported for the insoluble glycoprotein glomalin extracted from extraradical mycelium of arbuscular mycorrhizal fungi, with up to 28 mg Cu g^{-1} of glomalin for *Gigaspora rosea* (Gonzalez-Chavez et al. 2004). Data obtained from this and previous studies suggest that the mycorrhizal mycelium represents a powerful functional element of the symbiosis, playing a "scavenging-filtering" double role, by its ability to balance the uptake of microelements depending on their soil concentrations: it facilitates plant uptake in low-nutrient availability regimes and reduces the risks of toxicity by limiting the excess of element translocation from below- to aboveground tissues, particularly in heavy metal–contaminated soils. Interestingly, the significantly lower Cu concentration found here in roots of mycorrhizal chicory may be partly explained by a "dilution effect," due to the two-fold larger biomass of mycorrhizal roots compared with controls, though it could also be argued that Cu accumulation in extraradical networks may limit

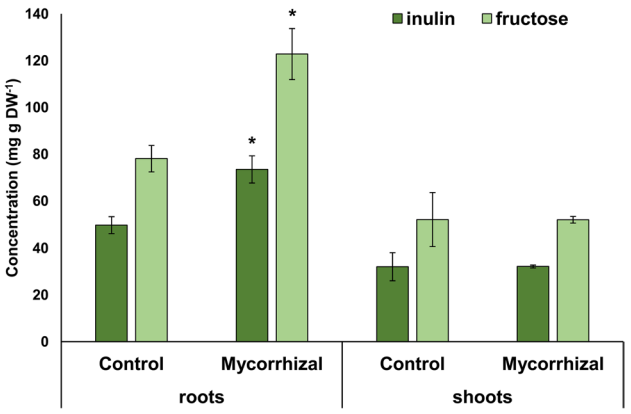

Fig. 3 Mean values (±standard error of means) of fructose and inulin concentrations in roots and shoots of *Cichorium intybus* plants in symbiosis with the arbuscular mycorrhizal fungus *Funneliformis mosseae* (mycorrhizal) and non-mycorrhizal controls, grown in a whole-plant experimental system. Asterisks indicate significant differences between mycorrhizal and control plants by one-way ANOVA: root fructose $F_{1,4}=13.25$, $P=0.022$ (*); root inulin $F_{1,4}=11.99$, $P=0.026$ (*)

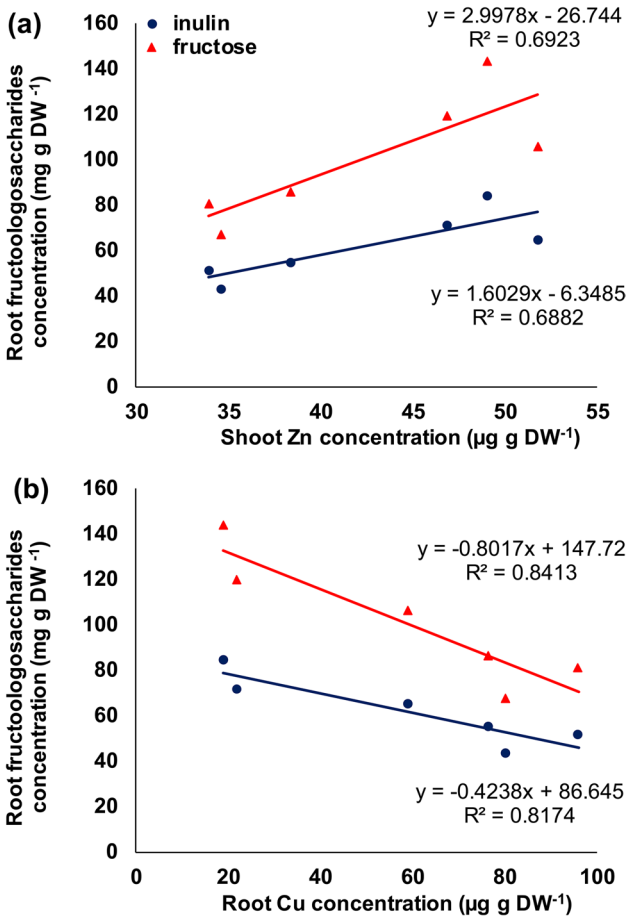

Fig. 4 Regression curves showing the relationships, independently on the mycorrhizal status, among **a** Zn or **b** Cu concentrations in plant tissues and fructooligosaccharide concentration in roots of *Cichorium intybus* plants grown in a whole-plant experimental system

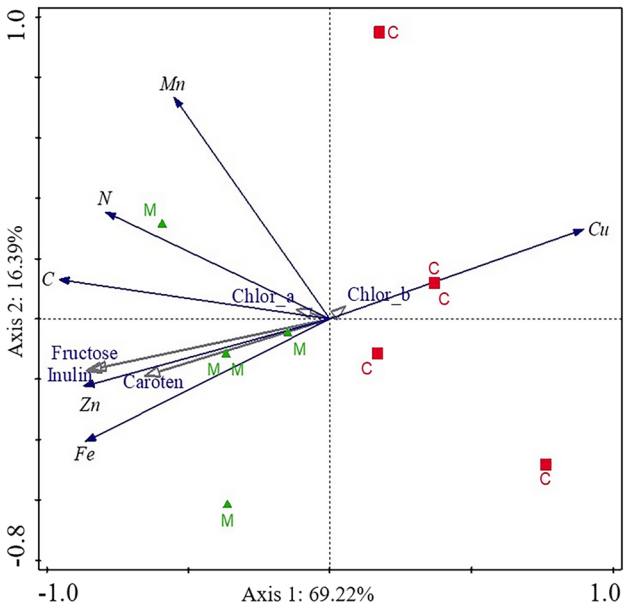

Fig. 5 Principal component analysis (PCA) biplot, summarizing the variability of plant macro- (C and N) and micronutrient (Fe, Cu, Mn, Zn) concentration values in *Funneliformis mosseae*–inoculated (M) and non-inoculated control (C) plants of *Cichorium intybus* grown in a whole-plant experimental system. The concentrations of inulin, fructose, carotenoids (caroten), and chlorophyll *a* (Chlor a) and *b* (Chlor b) have been used as supplementary variables. The first and second axis explain 85.61% of total variance

metal translocation to roots, as increasing concentrations of Cu in fungal mycelium corresponded to decreasing ones in mycorrhizal roots, while shoot concentrations were constant. This represents an important tolerance strategy for mycorrhizal plants growing in heavy metal–contaminated soils, as Cu is fundamental as a catalytic cofactor for all primary metabolic pathways, including respiration (Kim et al. 2008), but when high concentrations are reached it becomes toxic by inhibiting protein activity and inducing the formation of free radicals and reactive oxygen species (Halliwell 1989).

Table 5 Mean values (± standard error of the mean, $n = 3$) of micronutrient concentration and content in dried extraradical mycelium (ERM) produced by *Funneliformis mosseae* in symbiosis with *Cichorium intybus* plants, grown in a whole-plant experimental system

	ERM nutrient concentration (μg g^{-1})	ERM nutrient content (ng per individual plant network)
Cu	121.44 ± 4.41	34.89 ± 0.51
Zn	79.61 ± 8.36	22.69 ± 0.58
Mn	163.33 ± 44.78	46.34 ± 5.02
Fe	3149.90 ± 183.13	903.86 ± 8.42

Growth enhancement of mycorrhizal chicory was here accompanied by an increase in root fructose and inulin concentrations, compared with controls, according to the enhanced photosynthetic carbon (C) flux towards belowground tissues due to the greater sink strength of mycorrhizal roots. Moreover, the potential intensification of C flux and photosynthesis in mycorrhizal plants were consistent with the higher chicory leaf amounts of the photosynthetic pigment carotenoids, which can play important roles in human health due to their provitamin A activity and antioxidant potential. It is known that a side effect of AM symbioses is represented by the modulation of genes encoding for key enzymes of both primary and secondary plant metabolism (Handa et al. 2015; Liu et al. 2007), often leading to an increase in the accumulation of compounds with nutritional and health-promoting activities in plant roots and edible parts: sugars, phenolics, anthocyanins, carotenoids, chlorophylls, and vitamins were enhanced in mycorrhizal lettuce leaves (Baslam et al. 2013; Avio et al. 2017); phenolic acids, anthocyanins, and flavonols were accumulated in mycorrhizal strawberry fruits (Castellanos-Morales et al. 2010) and higher glucose, fructose, β-carotene, lycopene, and lutein contents and larger antioxidant capacity were found in tomato fruits produced by mycorrhizal plants (Copetta et al. 2011; Giovannetti et al. 2012; Hart et al. 2015). Leaf of chicory represents a multiple source of health-promoting and therapeutic compounds such as terpenoids (e.g., lactucin-like sesquiterpene lactones) and phenolic compounds (e.g., flavonoids and hydroxycinnamates) (Atta et al. 2010; Ahmed and Rashid 2019), whose contents vary depending on plant genotype and culture systems (Ferioli et al. 2015; Migliorini et al. 2019; Sinkovič et al. 2015; Spina et al. 2008). Previous studies reported higher concentrations of antioxidant compounds and hydroxycinnamates and enhanced activity of detoxifying enzymes (SOD, CAT, POX) in leaves of mycorrhizal chicory, which also showed improved photochemical efficiency (Langeroodi et al. 2020; Rozpądek et al. 2014; Wazny et al. 2014).

5 Conclusions

This study suggests that high-quality and safe fresh products, either immature leaves (baby leaf) or full-size rosettes, and inulin-rich root material for industrial extraction may be obtained in controlled conditions by inoculation of arbuscular mycorrhizal symbionts. The potential application to field cultures of selected mycorrhizal isolates or consortia should be assessed by studying the impact of pre-inoculated symbionts and their interactions with indigenous microbial communities on the development and nutritional contents at harvest of field-transplanted chicory plants. Interestingly,

the largest inulin accumulation was related to the relatively low root Cu and high shoot Zn concentrations in inoculated plants, indicating the need of further studies unravelling the relationships among the modulation of micronutrient uptake by mycorrhizal symbionts and the biosynthesis of health-promoting molecules by the host. Overall, data from this work may be useful to implement the use of mycorrhizal inocula aimed at improving plant nutrition and resilience and the derived food nutritional value.

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Author Contribution CS, DDB, MG, and AP designed the research; CS, DDB, EM, and AP performed the research; CS, AP, and DDB contributed to data collection, analysis, and interpretation; CS and DDB wrote the manuscript and all authors contributed to its revision.

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Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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