Arbuscular mycorrhizal fungi affect total phenolics content and antioxidant activity in leaves of oak leaf lettuce varieties

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Abstract

Plant secondary metabolites are considered key bioactive compounds for a healthy diet. Arbuscular mycorrhizal fungi (AMF) may interact with host plant metabolism, inducing the accumulation of health-promoting phytochemicals and antioxidant molecules. Lettuce is a largely consumed vegetable, which may interact with AMF to alter its content of secondary metabolites and natural antioxidants molecules, as previously shown in cultivars belonging to var. *capitata* or var. *longifolia*. In this study, the effects of red and green leaf *Lactuca sativa* var. *crispa* inoculation with different AMF species, *Rhizoglomus irregulare* and *Funneliformis mosseae*, were investigated, by assessing the total phenolics and anthocyanins content, and the antioxidant activity of leaf tissue. A significant increase of antioxidant activity and of phenolics were observed in plants of both cultivars inoculated with *R. irregulare*, compared to non inoculated plants. Likewise, anthocyanins (in red leaf lettuce) were more abundant in inoculated plants than in controls. Altogether, the results

indicate that *R. irregulare* strain showed a stronger ability than *F. mosseae* in affecting plant metabolism and that mycorrhizal inoculation may be used to enhance concentration of phenolics in leaf type lettuces, provided that a suitable AMF is selected.

Keywords

Mycorrhizal symbiosis; *Lactuca sativa* L. var *crispa*; Secondary compounds; Antioxidant capacity; Anthocyanins

1. Introduction

Fruits and vegetables have been since long considered as healthy food, and recent evidences suggest that they may protect at least against cardiovascular diseases and some cancers (Boeing et al., 2012; Leenders et al., 2013; Wang et al., 2014). Together with other chronic non-communicable diseases (NCDs), they are responsible for more than 55% of deaths worldwide, including low and middle income countries (WHO, 2014), prompting international and national institutions to promote fruits and vegetables consumption (USDHHS and USDA, 2015; WHO, 2000). A key role of bioactive compounds belonging to terpenoids and polyphenols, such as flavonoids and phenolic acids, produced by plant secondary metabolism has been confirmed (Duthie, 2000; Kim et al., 2011; Lazzè et al., 2009; Pandey and Rizvi, 2009; Schaefer et al., 2006). Therefore, since consuming whole food rich in these beneficial substances may be more effective than assuming dietary supplements, there is scope to enhance the nutritional value of fresh products, by exploiting their genetic diversity or environmental plasticity.

It is known that the content of secondary metabolites in plants may change in response to a number of environmental conditions such as nutrient availability, temperature or light intensity (Becatti et al., 2009; Bian et al., 2015; Boo et al., 2011; Coria-Cayupán et al., 2009). While these management conditions usually take benefit from inducing some stresses to plants, which may cause negative effect on biomass production (Sgherri et al., 2008), the use of arbuscular mycorrhizal fungi (AMF)

has gained recently much interest since they may be more effective and ecologically sound, especially in sustainable and/or organic agriculture (Giovannetti et al., 2012; Njeru et al., 2014). Arbuscular mycorrhiza (AM) is the most widely distributed symbiosis between plants and fungi, which, living both inside and outside roots, supply plants with phosphorous and other relatively immobile nutrients, exchanged for plant produced sugars. Most vegetable crops benefit from mycorrhizal symbiosis, which improves their nutrition and increases tolerance to biotic and abiotic stresses, possibly by altering plant secondary metabolism (Bruisson et al., 2016). Thus, AMF may lead to enhanced biosynthesis of health-promoting phytochemicals (polyphenols, carotenoids, flavonoids, phytoestrogens) and to a higher activity of antioxidant enzymes (Sbrana et al., 2014; Schweiger and Müller, 2015). While some researches suggest that food plants with higher contents of carotenoids or mineral nutrients may be obtained through the biotechnological use of mycorrhiza (Castellanos-Morales et al., 2010; Farmer et al., 2007; Giovannetti et al., 2012; Nzanza et al., 2012; Strack and Fester, 2006), contrasting results have been reported for phenolic compounds and antioxidant activities in a number of vegetables (Albrechtova et al., 2012; Castellanos-Morales et al., 2010; Ceccarelli et al., 2010; Giovannetti et al., 2012; Hart et al., 2015; Lee and Scagel, 2009; Nell et al., 2009; Nzanza et al., 2012; Scagel and Lee, 2012). As lettuce is a highly appreciated vegetable, which is largely consumed as fresh or ready-to-eat bagged salads, it is critical to understand its interactions with AMF, since even low increases in secondary metabolites concentrations may affect their total level of intake. Nevertheless, only a few studies focused on AMF and lettuce, with reported results mainly limited to cultivars of two botanical varieties, *longifolia* and *capitata*.

In addition, some inconsistent responses of plants to AM occurred in lettuces belonging to the var. *longifolia*, which showed significant increases in the concentration of soluble phenolic compounds only in external leaves (Baslam et al., 2011a), while those belonging to var. *capitata* rarely accumulated soluble phenolics (Baslam et al., 2013a). The same authors reported a differential effect due to lettuce cultivars and fungal symbionts (Baslam et al., 2011a).

To investigate whether mycorrhizal inoculation alters the content of health promoting secondary metabolites and natural antioxidants molecules in lettuce, grown in a commercial nursery under organic management, two differently pigmented cultivars of *Lactuca sativa* var. *crispa* were inoculated with the AMF species *Funneliformis mosseae* (formerly *Glomus mosseae* and *Rhizoglomus irregulare* (formerly *Glomus intraradices*) to assess (a) total phenolics content (TPC), (b) anthocyanins content and (c) antioxidant activity, expressed in ORAC units (Oxygen Radical Absorbance Capacity) of lettuce leaf extracts.

2. Materials and Methods

2.1. Fungal material

Two AM fungal isolates were used: *Rhizoglomus irregulare* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl (syn. *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler), isolate IMA6 and *Funneliformis mosseae* (T. H. Nicolson & Gerd.) C. Walker & A. Schüssler, isolate AZ225C. The fungi were maintained for several multiplication cycles under identical growth conditions, at the laboratory of Microbiology, Department of Agriculture, Food and Environment, University of Pisa, Italy. For the experiment, each isolate was reproduced in 8 L pots filled with a sandy loam soil mixed (1:1 v/v) with calcinated clay (OILDRI, Chicago, IL, USA), and steam-sterilized (121°C for 30 min, on two consecutive days) to kill naturally occurring endophytes. Chemical and physical characteristics of the soil used were as follows: $pH_{(H_2O)}$, 8.0; clay, 15.3%; silt, 30.2%; sand, 54.5%; organic matter, 2.2% (Walkley-Black); total N, 1.1 g kg^{-1} (Kjeldahl); extractable P, 17.6 mg kg^{-1} (Olsen). Seeds of *Medicago sativa* L. were sown and plants grown for four months, then shoots were excised and roots were chopped into fragments. The substrate, containing mycorrhizal roots, extraradical mycelium, spores and sporocarps, was air-dried at room temperature and utilized as crude inoculum. A mycorrhizal inoculum potential (MIP) bioassay (Njeru et al., 2017) performed on the inoculum mixtures showed that the AM fungi were active: MIP values, determined using *C. intybus* as test plant, were on average 40% for AZ225C

105 and 52% for IMA6. In order to prepare the control treatments, aliquots of the crude inoculum were
106 steam-sterilized (121°C for 30 min, on two consecutive days).
107
108 **2.2. Plant material**
109 Two differently pigmented *Lactuca sativa* (L.) var. *crispa* cultivars, a green (Panisse), and a red
110 (Eluarde) oakleaf lettuce, were used. These cultivars are extensively cultivated in greenhouses and
111 highly commercialized in Italy. Panisse is a variety with large, rounded leaves with a bright green
112 colour, whereas Eluarde has soft, well lobed leaves with bright red pigmentation.
113
114 **2.3. Experimental conditions**
115 Two experiments were performed in the greenhouse facilities of the L'ortofruttifero di Pacini Sara
116 S.a.S., a commercial nursery located 5 km NW of Pisa, Italy, latitude 43° 46' N, longitude 10° 22'
117 E. In both eperiments, seeds of the selected lettuce cultivars were germinated, and then transplanted
118 into 9-cell trays in a mixture of peat (Hochmoor Hortus, TERFLOR, Capriolo BS, Italy, containing
119 organic C 46.5%, organic N 1%, organic matter 93% on a dry matter basis) and crude inoculum
120 (1:5 v/v), one plant per cell. As a control, a mock inoculum was set by steam-sterilizing an aliquot
121 of the inoculated peat. All trays received identical volume of a filter paper soil eluate, obtained
122 using AMF inoculum, to ensure a common microbiota to all treatments. According to organic
123 management practices adopted in the nursery, organically produced seeds, and fertilizer and plant
124 protection products allowed in organic agriculture were used: a fluid organic fertilizer (Lysodin®
125 Alga-Fert, CBC Europe, Nova Milanese MB, Italy) was applied at the time of sowing and
126 transplanting, and, for pest control, a commercial preparation of *Bacillus amyloliquefaciens*
127 (AMYLO-X®, CBC Europe) applied once, early in the growing season.
128 A first trial was performed, from April to June 2014, using *R. irregulare* IMA6 in order to assess
129 whether polyphenol concentration and antioxidant activities were affected by the harvest stage.
130 Three replicate trays were harvested at four to five leaf stage (transplant stage) and from other three

trays were selected three plants to be transplanted in 4 L pots, filled with peat based growing substrate (peat, sandy loam soil and calcinated clay, 1:1:1 by volume). These plants were harvested at marketable size, four weeks later.

In the second experiment, germinated seeds of inoculated (with *F. mosseae* AZ225C and *R. irregulare* IMA6) and control lettuce cultivars were transferred in 9-cell trays and all trays received the filter paper soil eluate, obtained using a mixture of the two AMF inocula. For each combination of lettuce cultivar and fungal inoculum, three replicate trays were prepared. Plants were harvested at transplant stage, seven weeks after germination, on December 2014.

2.4. Samples preparation

At harvest, either leaves of plant in pots or pooled plants (9) of each tray, were separated from roots and used for determination of fresh weight. Then, an aliquot (10 g) of a mixture of inner and outer fresh leaves was liquid N-powdered and stored at -80 °C until sample extraction. Roots were used to assess mycorrhizal colonization.

2.5. Determination of arbuscular root colonization

Percentages of AMF colonization were assessed under a dissecting microscope by the gridline intersect method (Giovannetti and Mosse, 1980) after clearing and staining plant roots with Trypan blue in lactic acid (0.05% w/v).

2.6. Determination of antioxidant activity (ORAC assay)

Samples extraction was performed according to Ninfali et al. (2005) with some modifications: 1 g of each sample was suspended (1:10 w/v) in acetone (70:30 v/v) with 5% perchloric acid (v/v), shaken for 3 h in the dark at 4°C, then centrifuged at 5000 x g for 20 min. The extraction was repeated twice and the supernatants were collected and used directly, without evaporation, for ORAC assay, according to Michiels et al. (2012).

157 The antioxidant activity of lettuce extracts was evaluated in triplicate by the ORAC assay (Ninfali
158 et al., 2005), with some modifications. Fluorescein sodium salt stock solution (400 µM) and Trolox
159 stock solution (5mM) in 0.075 M K-phosphate buffer, pH 7.4 were stored at -20°C. 2,2'-azobis(2-
160 methylpropionamidine) dihydrochloride (AAPH) 400 mM in 0.075 M K-phosphate buffer pH 7.4
161 was prepared fresh daily.
162 The final reaction mixture of our assay contained 0.04 mM fluorescein sodium salt in 0.075 M
163 phosphate buffer, pH 7.4, at diluted sample or 5 mM Trolox. The control was 0.075 M phosphate
164 buffer, pH 7.4. AAPH was used as peroxyl radicals generator and fluorescein as probe.
165 The assay was carried out using a Victor X3 plate reader fluorimeter (Perkin Elmer Life and
166 Analytical Sciences, Wallac Oy, P.O. Box 10, FIN-20101 Turku, Finland). A calibration curve was
167 previously performed using Trolox as standard antioxidant. Fluorescence decay was read at 485 nm
168 and 514 nm of excitation and emission, respectively, until complete extinction. The ORAC values
169 were calculated according to the formula:

170 $$ORAC = \frac{(A_s - A_b)}{(A_t - A_b)}\text{ka}$$

171 where A_s is the area under curve (AUC) of fluorescence of the sample, A_b is the AUC of the blank,
172 A_t represents the AUC of Trolox, k is the dilution factor, a is the Trolox concentration (µM).
173 The ORAC values were expressed as micromoles (µmol) of Trolox Equivalents (TE) 100 g^{-1} fresh
174 weight.
175

176 **2.7. Determination of total phenolics content**

177 Total phenolics content of leaf extracts was determined according to Folin-Ciocalteu's colorimetric
178 method (Singleton et al., 1999) with some modifications. Extraction was performed according to
179 Michiels et al. (2012) with some modifications: lettuce samples treated with liquid nitrogen were
180 weighted (1 g) and suspended in 80% aqueous methanol (v/v) in the ratio of 1:10 w/v.

Gallic acid was used to obtain the standard calibration curve. Total phenolics content (TPC) was expressed as mg of gallic acid equivalent (GAE) g^{-1} fresh weight.

2.8. Determination of total anthocyanins content

Total monomeric anthocyanins were determined according to the pH differential method described by Giusti and Wrolstad (2001), a spectrophotometric method based on the change in pigmentation pH-dependent of anthocyanins. Absorbance was measured at 510 and 700 nm. The anthocyanin concentration was expressed as μg cyanidin-3-glucoside equivalents (C3GE) g^{-1} fresh weight (C3G, molar extinction coefficient of 26,900 L cm^{-1} mol^{-1}; molecular weight of 449.2 g mol^{-1}).

2.9. Statistical analysis

Student's t-test as well as one-way or two-way Analysis of Variance (ANOVA) with Tukey's post-hoc tests or simple main effect test were used as appropriate, to analyze data on plant growth and metabolic activity. Percentage colonization data were arcsine-transformed before analysis. Pearson correlations were also used to determine whether AMF colonization was positively, negatively or not associated with plant growth response, and whether accumulation of secondary compounds was correlated with antioxidant activity. Statistical tests were performed using SPSS 23.0 software (IBM Corp., Armon, NY Inc., USA).

3. Results

3.1. First experiment

In the first experiment, lettuce plants inoculated with *R. irregulare* showed 18±2 % (mean ± standard error) colonized root length at both early and late harvest stages. At the first harvest, shoot fresh weight values were not significantly different between lettuce varieties (P =0.233), inoculum treatments (P =0.847), and their interaction (P =0.085), ranging from 1.30 to 1.73 mg $plant^{-1}$ in *R. irregulare* IMA6 inoculated plants of Eluarde and Panisse, respectively. Likewise, no significant

differences were found for shoot fresh weight at the final harvest between lettuce varieties (P =0.625), inoculum treatments (P =0.109), and their interaction (P =0.135). Average value of shoot fresh weight was 26.9±1.5 and 25.2±3.8 mg plant^{-1}, for Eluarde and Panisse, respectively.

In such plants, total phenolics concentration and ORAC values at the two harvest times tested were highly correlated (Pearson's r = 0.778, P = 0.003 and r = 0.714, P= 0.009 for total phenolics concentration and ORAC, respectively).

Moreover, it was observed that independently on harvest time and plant cultivar, AMF inoculum treatments were effective in modulating concentration of total phenolics and antioxidant activities of lettuce plants (Fig. 1). The red lettuce cultivar (Eluarde) achieved higher values than the green cultivar (Panisse), as expected (P<0.001 for both variables), as well as AMF colonized plants compared to uncolonized plants (P<0.05 for both variables).

On the basis of such results, the main experiment was planned to harvest plants at an early stage, and to test, besides the already utilized AM fungus, an additional isolate, AZ225C, belonging to the species *F. mosseae*.

3.2. Second experiment

3.2.1. Arbuscular root colonization

Eluarde and Panisse lettuce cultivars showed similar root colonization by both AMF isolates: *R. irregulare* IMA6 produced a higher level of root colonization, 31.7% and 31.5%, than *F. mosseae* AZ225C, 10.0% and 14.3%, in Eluarde and Panisse, respectively (Tab.1). Student t-tests, performed separately for each cultivar, showed that the differences in colonization between fungal isolates were statistically significant (P <0.001 for Panisse, P=0.028 for Eluarde). AMF root colonization was absent in all Mock inoculated plants, as expected.

3.2.2. Growth response

232 Lettuce shoot fresh weight values were very similar, ranging from 1.99 mg plant^{-1} (in *R. irregulare*
233 IMA6 inoculated Eluarde) to 2.87 mg plant^{-1} (in Mock inoculated Panisse) (Tab.1), and two-way
234 ANOVA confirmed that such variable was not affected by fungal inoculum or plant cultivar (Tab.
235 2). In addition, neither of the experimental factors significantly affected water content that averaged
236 96.2 % of shoot fresh weight in both Eluarde and Panisse.
237
238 **3.2.3. Antioxidant activity (ORAC assay)**
239 Antioxidant activity was higher in plants belonging to cultivar Eluarde than in Panisse, with the
240 highest values found in plants inoculated with *R. irregulare* IMA6, while Mock inoculated plants
241 provided the lowest values (Fig. 2a). However, analysis of data showed the occurrence of a
242 significant interaction (Tab. 2), linked to a stronger increase of activity in leaves of *R. irregulare*
243 IMA6 inoculated Panisse (+52%) than in Eluarde (+36.5%), when compared with Mock plants.
244 Additionally, *F. mosseae* AZ225C inoculated plants showed only a marginal increase of antioxidant
245 activity over the Mock plants, compared with *R. irregulare* IMA6 inoculated plants.
246
247 **3.2.4. Total phenolics content**
248 Total phenolics content was affected by both plant cultivar and inoculum treatments (Tab. 2), but,
249 as with antioxidant activity, a significant interaction (P=0.047) was observed. Eluarde plants
250 showed consistently higher values (0.57 mg g^{-1} FW averaged over inoculum treatments) than
251 Panisse plants (0.50 mg g^{-1} FW), and, within each cultivar, *R. irregulare* IMA6 inoculated plants
252 performed better than Mock inoculated plants. Total phenolics content was higher in leaves of *R.*
253 *irregulare* IMA6 inoculated Eluarde (+22%) than in Panisse (+12%) leaves, when compared with
254 Mock inoculated plants. On the contrary, values for *F. mosseae* AZ225C treated plants were only
255 marginally different from those of Mock treatment, either with Eluarde (+2.5%) or Panisse (-7.0%)
256 lettuce varieties (Fig. 2b). A high correlation coefficient was found between the antioxidant activity
257 and phenolics content (r=0.818, P <0.001).

3.2.5. Total anthocyanins content

Anthocyanins content was assessed only in Eluarde lettuce plants which were positively affected by inoculation with both AMF isolates, since their content increased, as compared to Mock inoculated plants (19.3±1.8 µg C3GE g^{-1} FW), more than twofold in *F. mosseae* AZ225C inoculated plants (51.2±1.3 µg C3GE g^{-1} FW) and more than threefold in *R. irregulare* IMA6 inoculated plants (78.3±1.4 µg C3GE g^{-1} FW). Anthocyanins content was highly correlated with both antioxidant activity ($r = 0.943$, $P<0.001$) and phenolics content ($r= 0.863$, $P =0.003$).

4. Discussion

In the present study, an increased antioxidant activity and a higher content of phenolics were detected in green and red leaf mycorrhizal lettuce plants, under organic management system in a commercial nursery. In addition, a differential effectiveness of two different arbuscular mycorrhizal symbionts in modulating lettuce secondary metabolism was observed.

This is the first report on mycorrhizal effects in lettuce cultivars belonging to var. *crispa*, which are commonly considered as having a higher antioxidant activity and phenolics content than other lettuce cultivars of the crisphead and butterhead types, belonging to *capitata* subgroup (Liu et al., 2007; Kim et al., 2016). Previous researches reported that mycorrhizal inoculation could be used to obtain lettuce plants enriched in vitamins, chlorophylls and carotenoids (Baslam et al., 2013a), but total phenolics concentrations were usually not affected (Baslam et al., 2013b) except in water stressed plants (Baslam and Goicoechea, 2012), or in external leaves of a romaine type (Baslam et al., 2011a).

In this study, total phenolics concentration was comparable with those found in works which utilized other red leaf lettuce cultivars and the same detection method (Baslam et al., 2013b; Ordidge et al., 2010; Son and Oh, 2013). Moreover, anthocyanin concentrations, which were in the

range of values found in cultivars belonging to var. *crispa* (Kim et al., 2016), represented in mycorrhizal plants about one tenth of total phenolics, according to Llorach et al. (2008) and Luna et al. (2013).

Both the present study and other involving different AMF-colonized food plants, e.g. *Fragaria vesca*, *Allium cepa* and *Vitis vinifera*, showed enhanced phenolics content and antioxidant capacity (Lingua et al., 2013; Rozpądek et al., 2016; Torres et al., 2016). Here, the content of phenolics was paralleled by the antioxidant activity, as shown by the high correlation coefficient between the two variables. Similarly high (García-Macías et al., 2007), low (Apostolou et al., 2013) or no correlation between antioxidant activity and total phenolics were previously detected, depending on the antioxidant activity assay method (Liu et al., 2007; Mampholo et al., 2016, Viacava et al., 2014). Interestingly, Nicolle et al. (2004) showed that total phenolics accounted for more than 60% of the antioxidant capacity in six lettuce cultivars.

Since no growth increase was observed between inoculated and non inoculated plants, an indirect effect of mycorrhization on secondary metabolism through better nutrition status could be ruled out, contrary to previous findings (Baslam et al., 2011a, b; Bruisson et al., 2016; Giovannetti et al., 2012).

The results of this study showed that *F. mosseae* isolate AZ225C was less effective than *R. irregulare* IMA6 in increasing both phenolics concentration and antioxidant activity, and, in red leaf cultivar, anthocyanin concentration as well. This is in agreement with many observations, which indicate how important the fungal strain may be in affecting changes in the production of plant secondary metabolites (Ceccarelli et al., 2010; Larose et al., 2002; Zubek et al., 2012). In particular, present results support those previously obtained in *Cynara cardunculus* L. var. *scolymus* inoculated with the same fungal isolates (Ceccarelli et al., 2010). In such a study, although plants were grown in different greenhouse conditions, phenolics content in leaves at 60 d after transplant were lower in artichokes inoculated with *F. mosseae* than in those colonized by *R. irregulare*.

Although the tested AMF species revealed different lettuce root colonization extents, these had no effect on the phytochemical analyzed, as shown by using these values as covariates in the ANOVA model. The absence of correlation between extent of mycorrhizal colonization and secondary metabolites in plants has frequently been reported (Toussaint et al., 2007), whereas a positive correlation was found between percent root colonization and the content of castanospermine in *Castanospermum australe* inoculated with a *Glomus intraradices* isolate (Abu-Zeyad et al., 1999). Interestingly, recent papers comparing plants colonized with similar extent by *F. mosseae* and *R. irregulare* isolates reported poorer performance of *F. mosseae*: in *Hypericum perforatum* L. *F. mosseae* BEG12 proved to be less effective than *R. irregulare* BEG 140 (formerly *R. intraradices*) in the enhancement of hypericin concentration (Zubek et al., 2012), and in *Moringa oleifera* it was more detrimental in decreasing carotenoids (Cosme et al., 2014). By contrast, phenolic acids such as rosmarinic and caffeic acids were enhanced more by *F. mosseae* NBR 1–2 than by *R. irregulare* BEG159 (formerly *R. intraradices*) (Toussaint et al., 2007).

5. Conclusions

While genetically controlled, the content of secondary metabolites is highly reliant on different environmental and agronomical factors. Arbuscular mycorrhizal inoculation can be an environment friendly tool to manage the quality of vegetables, after careful tuning of the symbionts involved to maximize the yield of a given product. Our results show that mycorrhizal inoculation with suitable AM fungal strains may be used to enhance concentration of phenolics in leaf type lettuces, used as minimally processed “ready to eat” salads, raising at the same time leaves antioxidant activity. Thus, recurring reports of differences in the ability of specific fungal isolates to activate primary or secondary metabolite pathways and in metabolome profiles of host plants suggest that case-to-case studies are needed to select the best symbiont to enhance the nutraceutical value in the various food plant species and varieties.

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519 Figure captions

520 Fig.1 Antioxidant activity (a) and concentrations of total phenolics (mg 100 g^{-1} of FW) (b) in leaves
521 of lettuces Eluarde and Panisse, inoculated with the arbuscular mycorrhizal fungus *Rhizoglomus*
522 *irregulare* IMA6 (full bars), or not inoculated (open bars) at early (I) and late (II) harvest of the first
523 experiment. Within each harvest time, two-way ANOVA (inoculum treatment × variety) yields a
524 main effect of inoculum treatment, $P = 0.012$; $P < 0.001$ at early and late harvest, respectively,
525 indicated by asterisks; and of variety, $P < 0.001$ at both harvests, indicated by different letters.
526 Error bars refer to standard errors of the means (n = 3).

527

528 Fig.2 Antioxidant activity (a) and concentrations of total phenolics (mg 100 g^{-1} of FW) (b) in leaves
529 of lettuces Eluarde and Panisse, inoculated with isolates of arbuscular mycorrhizal fungi
530 (*Funneliformis mosseae* AZ225C or *Rhizoglomus irregulare* IMA6), or not inoculated (MOCK).
531 Two-way ANOVA yields a significant inoculum treatment × variety interaction effect on
532 antioxidant activity and concentrations of total phenolics ($P = 0.037$; $P= 0.047$ for antioxidant
533 activity and total phenolics, respectively). Different letters within each variety indicate significant
534 differences between inoculum treatments after simple main effects tests. Error bars refer to standard
535 error of the means (n = 3).

536

Table 1. Mycorrhizal colonization and shoot fresh weights of *Lactuca sativa* plants inoculated with isolates of arbuscular mycorrhizal fungi (*Funneliformis mosseae* AZ225C or *Rhizoglomus irregulare* IMA6), and or inoculated (MOCK). Plants were grown for seven weeks. Mean values ± standard errors, n=3.

Inoculum type	Shoot fresh weight	Root colonization (%)
	g plant^{-1}	
L. sativa cv. ELUARDE		
AZ225C	2.03 ± 0.10	10.0 ± 0.6 a
IMA6	1.99 ± 0.24	31.7 ± 4.8 b
MOCK	2.16 ± 0.32	-
L. sativa cv. PANISSE		
AZ225C	2.03 ± 0.06	14.3 ± 1.2 a
IMA6	2.19 ± 0.32	31.5 ± 0.8 b
MOCK	2.87 ± 0.41	-

Different letters indicate significant differences according to Student's t-test (P <0.001 for Panisse, P=0.028 for Eluarde)

537

538

Table 2. Summary of two-way ANOVA results testing the effects of inoculum type and lettuce cultivar on shoot fresh weight (SFW), antioxidant activity (ORAC) and total phenolics content.

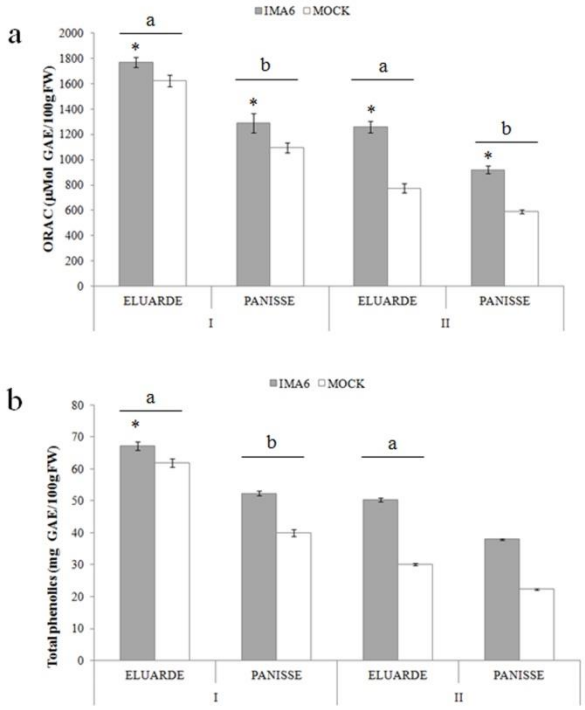
	SFW		ORAC		Total phenolics	
	F	P	F	P	F	P
Inoculum	1.90	0.193	238.78	<0.001	49.69	<0.001
Cultivar	1.81	0.204	56.97	<0.001	53.55	<0.001
Interaction	0.90	0.431	4.40	0.037	3.99	0.047

539

540

541

542 Fig.1



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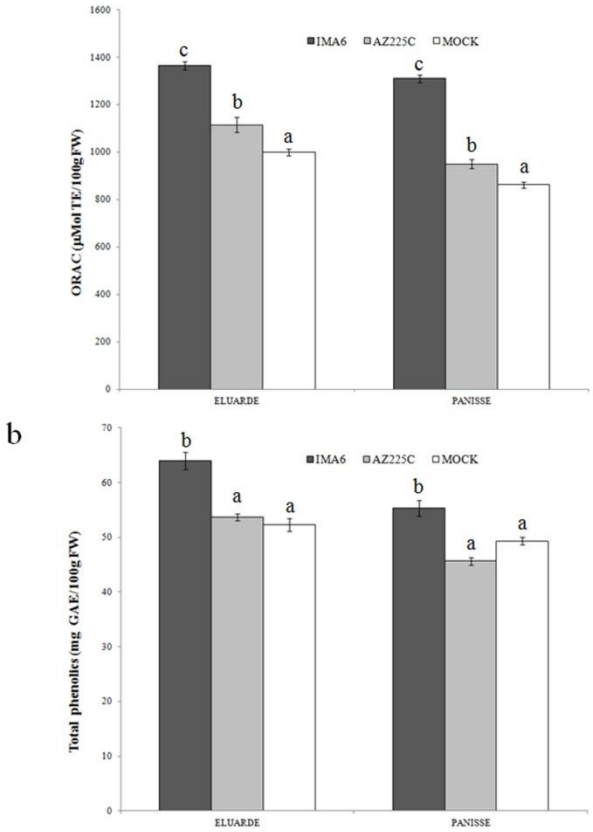
545 Fig.2



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