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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.

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.,

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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: $pH_{(H2O)}$, 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 *Cichorium intybus* as test plant, were on average 40% for AZ225C and 52% for IMA6. In order to prepare the control treatments, aliquots of the crude inoculum were steam-sterilized (121 °C for 30 min, on two consecutive days).

2.2. Plant material

Two differently pigmented *Lactuca sativa* (L.) var. *crispa* cultivars, a green (Panisse), and a red (Eluarde) oakleaf lettuce, were used. These cultivars are extensively cultivated in greenhouses and highly commercialized in Italy. Panisse is a variety with large, rounded leaves with a bright green colour, whereas Eluarde has soft, well lobed leaves with bright red pigmentation.

2.3. Experimental conditions

Two experiments were performed in the greenhouse facilities of the L'ortofruttifero di Pacini Sara S.a.S., a commercial nursery located 5 km NW of Pisa, Italy, latitude 43° 46′ N, longitude 10° 22′ E. In both eperiments, seeds of the selected lettuce cultivars were germinated, and then transplanted into 9-cell trays in a mixture of peat (Hochmoor Hortus, TERFLOR, Capriolo BS, Italy, containing organic C 46.5%, organic N 1%, organic matter 93% on a dry matter basis) and crude inoculum (1:5 v/v), one plant per cell. As a control, a mock inoculum was set by steam-sterilizing an aliquot of the inoculated peat. All trays received identical volume of a filter paper soil eluate, obtained using AMF inoculum, to ensure a common microbiota to all treatments. According to organic management practices adopted in the nursery, organically produced seeds, and fertilizer and plant protection products allowed in organic agriculture were used: a fluid organic fertilizer (Lysodin® Alga-Fert, CBC Europe, Nova Milanese MB, Italy) was applied at the time of sowing and transplanting, and, for pest control, a commercial preparation of *Bacillus amyloliquefaciens* (AMYLO-X®, CBC Europe) applied once, early in the growing season.

A first trial was performed, from April to June 2014, using *R. irregulare* IMA6 in order to assess whether polyphenol concentration and antioxidant activities were affected by the harvest stage. 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).

The antioxidant activity of lettuce extracts was evaluated in triplicate by the ORAC assay (Ninfali et al., 2005), with some modifications. Fluorescein sodium salt stock solution (400 μM) and Trolox stock solution (5 mM) in 0.075 M K-phosphate buffer, pH 7.4 were stored at