1 **Phenolics content and antioxidant activity in the leaves of two artichoke cultivars are**

2 **differentially affected by six mycorrhizal symbionts.**

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5 Luciano Avio§, Rita Maggini§, Gergely Ujvári, Luca Incrocci, Manuela Giovannetti, Alessandra

6 Turrini*

7

8 *Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124*

9 *Pisa, Italy*

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12 § Both authors contributed equally to this work.

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14 * Corresponding author at: Alessandra Turrini, Department of Agriculture, Food and Environment,

15 University of Pisa, Via del Borghetto 89, 56124 Pisa, Italy

16 E-mail address: alessandra.turrini@unipi.it (Alessandra Turrini)

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19 ABSTRACT

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21 Globe artichoke is cultivated for its flower heads and as the source of pharmaceutical compounds

22 from the leaves, whose extracts contain a high level of health-promoting compounds and show one

23 of the highest antioxidant capacities reported for vegetables. The content and composition of such

24 beneficial plant secondary metabolites may be greatly affected by cultivation variables, including

25 plant genotype and mycorrhizal symbiosis. This study was carried out to gain knowledge on

26 antioxidant activity, total phenols and chlorogenic acid levels in the leaves of two artichoke

cultivars, Romanesco and Tema, as affected by six arbuscular mycorrhizal fungi (AMF) belonging

to diverse genera, species and isolates. The six AMF showed significantly higher percentages of

root colonization in Romanesco (46%) than in Tema (32%), and different colonization dynamics.

The overall pooled data showed that in Romanesco the levels of total phenols increased by 35%,

chlorogenic acid by 67% and antioxidant activity by 43%, compared with Tema. The six AMF

differentially affected such levels, with *Claroideoglomus claroideum* 22W3 as the only isolate

producing significant increases compared with controls, for total phenols and chlorogenic acid, and

with *C. claroideum* 22W3 and *Funneliformis mosseae* IMA1 as the isolates enhancing  antioxidant

activity, compared with controls. Moreover, a strong correlation was found between total phenols

and antioxidant activity in the leaves of both artichoke cultivars. This work, comparing the highest

number of diverse AMF studied so far in relation to their modulation of plant secondary

metabolism, expanded our knowledge on their functional diversity and allowed the detection of the

best performing symbionts to be utilized for obtaining artichoke leaves with enhanced health-

promoting activities.

1. **Introduction**

Globe artichoke (*Cynara cardunculus* L. var. *scolymus* Fiori, Asteraceae) is a robust herbaceous

perennial plant, extensively cultivated along the Mediterranean basin, with Italy, Egypt and Spain

as the main world producers. Artichoke is cultivated not only for its large immature flower heads,

whose edible parts are represented by the tender inner bracts and the receptacle, but also as the

53    source of pharmaceutical compounds primarily extracted from the leaves, which contain a very high

54    level of polyphenols (Rouphael et al., 2016; Petropoulos et al., 2017). Artichoke leaf extracts are

55    utilized for their choleretic, hypocholesterolemic, and antioxidant activity, mainly due to their high

56    contents in chlorogenic acid, cynarine, and luteolin (see Ceccarelli et al., 2010).

57    Chlorogenic acid (5-O-caffeoylquinic acid), 1,5- and 3,4-di-O-caffeoylquinic acids and

58    cynarine (1,3-di-O-caffeoylquinic acid) are artichoke phenolics displaying a strong scavenging

59    activity against reactive oxygen species (ROS) and free radicals, showing a protective activity

60    against oxidative damage (Kono et al., 1997; Pavlica and Gebhardt, 2005). Artichoke leaf extracts

61    were reported to show hypocholesterolemic activity, reducing cholesterol biosynthesis and

62    inhibiting LDL oxidation, through the action of chlorogenic acid and cynarine (Kraft, 1997; Bundy

63    et al., 2008).

64    The content and composition of such beneficial plant secondary metabolites may be greatly

65    affected by cultivation variables, i.e. plant genotype, harvest season, cultivation site and techniques,

66    soil quality, nutrient availability, light intensity, irrigation, use of pesticides and chemical fertilizers,

67    and conventional/organic management (see Sbrana et al., 2014). In artichoke flower heads,

68    significant differences among different varieties were reported for antioxidant activity, total

69    phenolic and chlorogenic acid contents (Curadi et al., 2005; Graifenberg et al 2013), and for

70    phenolic profiles (Lombardo et al. 2012). A recent study investigated total phenolics, flavonoids,

71    and flavonols content and antioxidant activity in artichoke leaves, showing that phenolic profiles

72    highly differed among 19 cultivars, allowing the detection of the best performing cultivars, to be

73    further selected and cultivated for their beneficial properties (Rouphael et al., 2016).

74    One of the most promising agronomic factors affecting the production of health-promoting

75    compounds is represented by arbuscular mycorrhizal (AM) fungi (AMF), a key functional group of

76    beneficial soil microbes, establishing mutualistic symbioses with the roots of about 80% of plant

77    species. AMF are not host specific, and can be associated with the most important agricultural and

78    horticultural crops, such as cereals, legumes, the majority of vegetables, fruit trees including

79    grapevine and olive, medicinal plants and industrially important species, such as sunflower,

80    sugarcane, cotton, tobacco, coffee, tea, cocoa, rubber and cassava (Smith and Read, 2008). As AMF

81    are obligate biotrophs, they obtain carbon from the host plants and facilitate, in exchange, the

82    uptake and transfer of mineral nutrients-phosphorus (P), nitrogen (N), sulfur (S), potassium (K),

83    calcium (Ca), copper (Cu), and zinc (Zn)-from the soil, through an extensive extraradical mycelium

84    (ERM), which spreads from colonized roots into the surrounding soil and increases the root

85    absorbing surface, up to 40 times (Smith and Read, 2008). Beyond improving plant growth and

86    nutrition, AMF promote plant performance and health, by increasing plant tolerance to biotic and

87    abiotic stresses, reducing the need of chemical fertilizers and pesticides, and providing

88    multifunctional agroecosystem services (Gianinazzi et al., 2010; Rouphael et al., 2015). Several

89    studies reported that AMF may induce changes in plant secondary metabolism, enhancing the

90    production of antioxidant enzymes and phytochemical compounds with health-promoting activities

91    (see Sbrana et al., 2014; Avio et al., 2018). Previous data showed that mycorrhizal colonization

92    increased total phenolic content and antioxidant activity in artichoke leaves and flower heads

93    (Wang et al., 2003; Ceccarelli et al., 2010), the content of leaf chlorogenic acid (Romani et al.,

94    2006) and of flower heads total polyphenols (Rouphael et al., 2017). Interestingly, a large variation

95    in phenolic profiles, total polyphenols and antioxidant activity of artichoke leaves was reported

96    among 19 artichoke cultivars (Rouphael et al., 2016).

97         The data available so far were obtained comparing only few species or strains ~~number~~ of

98    AMF, thus limiting the complete understanding of their extensive physiological and functional

99    diversity.

100   The main aim of this work was to acquire knowledge on the differential levels of antioxidant

101   activity and health-promoting phytochemicals in two artichoke cultivars, Romanesco and Tema,

102   inoculated with six AMF belonging to different genera, species and isolates and originating from

103   various geographical areas: three isolates of the species *Funneliformis mosseae* from UK, USA and

104   Italy, one isolate of *Rhizoglomus irregulare* from France, one isolate of *Claroideoglomus*

105  *claroideum* and one of *Glomus* sp. 14W1, isolated from the soil of the experimental plot 14 in a hot-

106  spot field near Pisa (Turrini et al., 2018). To this aim we assessed (1) the establishment of

107  mycorrhizal colonization on a time-course basis, (2) plant growth parameters and mineral nutrient

108  uptake, (3) antioxidant capacity, total phenols and chlorogenic acid contents.

109

110  **2.  Materials and methods**

111

112  *2.1. Plant and fungal material*

113  Two cultivars of artichoke were used in this study: Romanesco C3 Italy and Violetto Tema

114  (hereafter, Romanesco and Tema). Micropropagated artichoke plantlets were purchased from

115  Microplant (Cesena, Italy).

116     The six AMF isolates belonged to the following species: *Funneliformis mossea*e (T.H.

117  Nicolson & Gerd.) C. Walker & A. Schüßler, *Rhizoglomus irregulare* (Błaszk., Wubet, Renker &

118  Buscot) Sieverd., G.A. Silva & Oehl, *Claroideoglomus claroideum*, (N.C. Schenck & G.S. Sm.) C.

119  Walker & A. Schüssler and an undescribed *Glomus* sp. The geographical origin, biome and

120  suppliers of each isolate are listed in Table 1. AMF inocula were obtained from pot-cultures

121  maintained in the collection of the Microbiology Laboratories of the Department of Agriculture,

122  Food and Environment (DAFE), University of Pisa.

123     The pot cultures, containing a mixture (1:1, v/v) of soil and a calcinated clay, were

124  inoculated with a crude inoculum containing mycorrhizal roots, spores and extra-radical mycelium

125  of each AMF isolate, sown with *Medicago sativa* and maintained for 6 months. At harvest, the

126  shoots were excised and discarded, while the substrate and the roots, cut in ca. 1-cm fragments,

127  were mixed to form a homogenous crude inoculum mixture, to be used for artichoke inoculation.

128

129  *2.2. Experimental conditions*

130  A completely randomized 2x2 factorial experiment was set up with two artichoke cultivars and 7

131 AMF treatments (6 AMF inocula and 1 mock inoculum, representing the control). A total of 196

132 microcosms were established, 98 per each cultivar, with 14 replication units (one plant per pot).

133   At transplanting, each artichoke plant was inoculated with 15% (w/w) of crude inoculum,

134 whose mycorrhizal potential was comparable among the different AMF (18-21%). Control pots

135 (mock inoculum) received the same amount of sterile crude inoculum, and all pots received 50 ml

136 of a filtrate, obtained by sieving a mixture of all mycorrhizal inocula through a 50 μm pore diameter

137 sieve and a Whatman paper no. 1 (Whatman International Ltd, Maidstone, Kent, UK), to ensure a

138 common microbiota for all treatments.

139   Artichoke plantlets were grown in 13-cm diameter (1 L) plastic pots (one plant/pot)

140 containing a mixture (1:1, v/v) of soil and calcinated clay (OILDRI, Chicago, IL, USA), which was

141 steam-sterilised (121°C for 40 min) to kill naturally occurring AMF. Chemical and physical

142 characteristics of the soil were as follows: pH (water) 7.5, total N 1.7 g/kg, available P ($NaHCO_3$

143 soluble P, Olsen method) 5.6 mg/kg, organic matter 2.7%, clay 15.4%, silt 15.3%, sand 69.3%.

144 Plants were grown in greenhouse under natural light, at DAFE, University of Pisa. The average air

145 temperature inside the greenhouse was 24.4°C in spring and 31.3°C in summer. During the

146 experiment, started on March 29th 2018 and ended on September 5th 2018, the minimum air

147 temperature was kept at 11°C by an air heating system, and ventilation air temperature was set to

148 25°C. Drip irrigation was carried out (one minute twice a week in spring; daily in summer) using a

149 modified Hoagland solution with the phosphorus level of 0.5 mM and the following composition of

150 the other macroelements (mM): 0.5 $HCO_3^-$; 14.0 $N-NO_3^-$; 1.0 $N-NH_4^+$; 6.0 K; 5.0 Ca, 2.0 Mg; 3.0 S-

151 $SO_4^{2-}$. The concentrations of trace elements (μM) were: 50.0 Fe; 45.0 B; 0.3 Cu; 0.8 Zn; 9.0 Mn;

152 0.1 Mo. The electrical conductivity of the solution was 2.5 dS/m, and the pH was adjusted to 5.8

153 with sulphuric acid.

154

155   *2.3. Sample preparation for biochemical analyses and mycorrhizal colonization*

156 Seven and 12 weeks after inoculation, four plants per AMF treatment and artichoke cultivar were

157 harvested. Twenty-three weeks after inoculation, a non-destructive sampling of roots and leaves

158 was carried out on four plants per treatment (to be utilized in further investigations in the field),

159 while two plants were destructively harvested for dry weight (DW) analysis.

160 Each plant root system was carefully washed with tap water, and cleared and stained with

161 0.05 % Trypan blue in lactic acid. Percentage of colonised root length was assessed on each root

162 sample by using the gridline intersect method (Giovannetti and Mosse, 1980). Mycorrhizal roots

163 were mounted on microscope slides and observed under a Reichert-Jung (Wien, Austria) Polyvar

164 light microscope to detect intraradical fungal structures.

165 From plants sampled 12 and 23 weeks after inoculation approximately 7 g fresh weight of

166 leaves were picked up to obtain two distinct samples: one to be extracted for the biochemical assays

167 (1 g), which was wrapped in aluminium foil and frozen at -80°C; one, to be analyzed for mineral

168 content and for the determination of the DW percentage, which was dried in a ventilated oven at

169 75°C until constant weight. The fresh weight of each sample was 6.7±0.3 g and 6.1±0.2 g in Tema

170 and 4.7±0.2 g and 5.8±0.1 g in Romanesco, at 12 and 23 weeks, respectively. Four distinct

171 replicates were sampled for each treatment. The frozen leaf samples were immersed in liquid

172 nitrogen, finely ground with mortar and pestle, and transferred into plastic test tubes with 5 ml

173 methanol. The tubes were sonicated four times in ice bath for 15 minutes and stored at -20°C

174 overnight. After 5 minutes centrifugation at 2700 $g$ and separation of the supernatant, the pellet was

175 extracted again with 5 ml fresh methanol. For each sample, the two extract aliquots were pooled,

176 filtered with 0.45 μm polyester membrane syringe filter, and used for the determination of

177 antioxidant capacity and the concentrations of chlorogenic acid, total phenols and chlorophylls. The

178 dried leaf samples were weighed for dry matter determination and ground to a fine powder. A 200

179 mg aliquot was mixed with nitric (5 ml) and perchloric (2 ml) acids and mineralized at 220°C for

180 two hours using a heating digester. The residue was diluted with 40 ml of double distilled water and

181 analysed by atomic absorption spectroscopy or by spectrophotometry.

182

### 2.4. Chemicals and apparatus

HPLC grade solvents, chlorogenic acid and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased

from Sigma–Aldrich (Milan, Italy). Reagent grade chemicals were purchased from Carlo Erba

Reagents (Cornaredo, Milan, Italy). All the spectrophotometric assays were performed by an UV-

1204 spectrophotometer (Shimadzu, Tokyo, Japan). The mineral content of the samples was

determined with a Varian AA240FS fast sequential atomic absorption spectrometer (Agilent, Santa

Clara, California, USA). The HPLC apparatus (Jasco, Tokyo, Japan) used for the determination of

chlorogenic acid was equipped with a PU-2089 four-solvent low-pressure gradient pump and a MD-

4010 diode array detector. The HPLC separation was performed using a C18 250/4.6 Nucleodur®

100-5 Isis column (Macherey–Nagel, Düren, Germany).

### 2.5. Analysis of chlorogenic acid

Chlorogenic acid was determined by HPLC, using acetonitrile (solvent A) and 0.1% phosphoric

acid (solvent B) for elution, with the following gradient: 0-0.4 min, A 5%; 0.4-0.5 min, A 5-15%;

0.5-7 min, A 15-20%; 7-8 min, A 20-95%; 8-11 min, A 95%; 11-12 min. A 95-5%, followed by 3

min equilibration (A 5%). The analyses were performed in the wavelength range 220 - 420 nm,

with 1 ml/min flow rate, after 1:10 dilution of the extracts with methanol. Injection volume was 20

µl. Chlorogenic acid in the concentration range 25 - 100 mg/l was used for calibration.

### 2.6. Analysis of total phenolic compounds

The determination of total phenols was carried out using the Folin-Ciocalteu reagent, as reported by

Maggini et al. (2018). The plant extract (100 µl) was mixed with 2.0 ml distilled water and 300 µl

Folin-Ciocalteu reagent in a spectrophotometric cuvette. After four minutes, 7.5% sodium

carbonate (1.6 ml) was added, and the solutions were kept for 2 hours in the dark at room

temperature prior to absorbance readings at 765 nm. Standard solutions of gallic acid (0 – 500 mg/l)

208    were used for calibration, and the results were expressed as milligrams of gallic acid equivalents

209    (GAE) per gram of DW (mg GAE/g DW).

210

211    *2.7. Analysis of antioxidant activity.*

212    The antioxidant capacity was determined by the ferric reducing antioxidant power (FRAP),

213    according to the procedure of Benzie and Strain (1996) with some modifications. The following

214    solutions were mixed in a spectrophotometric cuvette: 0.25 M acetate buffer pH 3.6 (2.0 ml); FRAP

215    reagent (900µl) containing 2mM ferric chloride and 1 mM TPTZ; properly diluted methanol extract

216    (100 µl). A calibration curve was prepared with standard solutions of ferrous ammonium sulphate

217    up to 1000 µM concentration. The absorbance was detected at 593 nm and the results were

218    expressed as µmol Fe(II)/g DW.

219

220    *2.8. Analysis of chlorophyll content*

221    Chlorophyll concentrations were determined spectrophotometrically, based on known values of

222    molar absorptivity (Lichtentahler and Buschmann, 2001), using properly diluted methanol extracts.

223    The absorbance was measured at 665.2, and 652.4 nm, and the concentrations were expressed as

224    µg/g DW. The percentage of DW of the samples was 17.4±0.2 and 10.2±0.3 in Tema, and 19.5±0.2

225    and 11.0±0.2 in Romanesco, at 12 and 23 weeks, respectively.

226

227    *2.9. Mineral content*

228    The solution obtained from the mineralization of leaf tissues was used for the determination of K,

229    Na, Ca, Mg, Cu, Mn, Fe and Zn by atomic absorption spectroscopy, while an aliquot of the same

230    solution was used for spectrophotometric determination of phosphorus as phosphomolibdate (Olsen

231    and Sommers, 1982). For nitrate determination, 100 mg of the powdered dry tissue were dispersed

232    in 20 ml distilled water and stirred for 2 hours by an orbital shaker. The water extract was analysed

233 spectrophotometrically for the determination of nitrate by salicylic acid nitration (Cataldo et al.,

234 1975).

235

236 *2.10.      Statistical analysis*

237 The data were subjected to two-way ANOVA with the cultivar and AMF inoculum as the sources

238 of variation, followed by Bonferroni post tests for means separation. When a significant interaction

239 was found, the simple effects were analysed using the Sidak correction for multiple comparisons,

240 using SPSS statistical package v. 23.0. The Prism 5 software (GraphPad Software, San Diego,

241 California, USA) was used for the remaining statistical analyses.

242

243

244 **3.  Results**

245

246 *3.1. Mycorrhizal colonization of artichoke plants*

247 Percentages of mycorrhizal root length were variable among AMF, sampling time and cultivars,

248 across the time-course assessment. As three ways ANOVA revealed an interaction of AMF and

249 cultivars with harvest time, the data of each harvest time were analysed separately. At the final

250 sampling, 23 weeks after inoculation, two ways ANOVA showed significant differences among the

251 different AMF isolates and between the two artichoke cultivars. Overall, the six AMF isolates

252 reached significantly higher percentages of colonized root length in Romanesco (46%) than in

253 Tema (32%) ($p < 0.001$) (Table 2).

254       Two groups of low and high colonizers were detected within the inoculated AMF: *C.*

255 *claroideum* 22W3, *F. mosseae* IN101C and *Glomus* 14W1, whose colonization percentages never

256 exceeded 24% and *F. mosseae*, IMA1, *F. mosseae* 2W3 and *R. irregulare* IMA6, whose

257 colonization percentages were always higher than 42% ($p < 0.05$).

258    *R. irregulare* IMA6 was the most infective symbiont, reaching 82 and 66% of colonized root

259    length, in Romanesco and Tema, respectively (Table 2). Such values, together with those of *F.*

260    *mosseae* IMA1, were significantly different (p<0.05) from those detected in all the other AMF

261    treatments. Two isolates of *F. mosseae*, IMA1 and 2W3, showed high percentages of mycorrhizal

262    colonization, 72 and 56% in Romanesco, and 42 and 54% in Tema respectively, which were

263    statistically different from the remaining AMF. *F. mosseae* isolate IN101C, *C. claroideum* 22W3

264    and *Glomus* 14W1 were less infective in both artichoke cultivars, although the percentages of

265    colonized root length ranged from 18 to 24% in Romanesco and from 8.8 to 12.5% in Tema (Table

266    2).

267            Interestingly, the dynamics of mycorrhizal colonization by the three high colonizers AMF

268    was different but followed the same trend in the two cultivars: *R. irregulare* IMA6 and *F. mosseae*

269    IMA1 were the fastest colonizers, spreading rapidly in the roots, with values of 32-42%, 7 weeks

270    after inoculation, which progressively increased during the experiment, while *F. mosseae* 2W3 was

271    a slow colonizer, as its colonization values were 11%, 14-20% and 54-56% after 7, 12 and 23

272    weeks, respectively (Table 2).

273

274    *3.2. Dry weight, chlorophyll and mineral content of artichoke leaves*

275    The dry weights of mycorrhizal artichoke plants did not differ from the controls (2.4±0.1 and

276    49.0±0.8 g, mean±SE, 7 and 23 weeks after inoculation, respectively) (Fig. S1). Chlorophyll

277    contents were not affected by plant cultivars and mycorrhizal treatments at both samplings (2.3-5.8

278    and 6.0-11.1 $\mu$g g$^{-1}$ DW, 12 and 23 weeks after inoculation, respectively). There were no clear

279    effects of artichoke cultivars and AMF on mineral element concentrations in the leaves. Twenty-

280    three weeks after inoculation, a significant interaction (p<0.001) was found in nitrate concentration,

281    as AMF treatments did not show any difference in Romanesco, while significant differences were

282    detected in Tema. Control plants showed the highest levels of nitrates (1.03% $NO_3^-$-N on a DW

283     basis), which in mycorrhizal plants decreased highly, showing the lowest values with *C. claroideum*

284     22W3 and *Glomus* 14W1 (0.27 and 0.05 %, respectively) (SM, Table S1).

285

286     *3.3. Total phenols, chlorogenic acid and antioxidant activity*

287     Data collected 12 weeks after inoculation were very variable and not predictive of AMF impact on

288     total phenolics and chlorogenic acid production at the final harvest, while antioxidant activity was

289     already significantly higher (+21%, $p < 0.01$) in Romanesco than in Tema (Table 3).

290     At the final harvest, 23 weeks after inoculation, the two-way ANOVA showed that there

291     was no significant interaction between the two factors. Thus, AMF treatment and artichoke cultivar

292     were analysed separately as main factors. The overall pooled data from all AMF treatments showed

293     that artichoke leaves of Romanesco and Tema differed significantly for their levels of total phenols,

294     chlorogenic acid and antioxidant activity. Indeed, total phenols increased by 35%, chlorogenic acid

295     by 67% and antioxidant activity by 43% in Romanesco, compared with Tema (Figure 1, left).

296     Mycorrhizal inoculation increased the levels of total phenols, chlorogenic acid and

297     antioxidant activity in artichoke leaves. The pooled data from the two plant cultivars showed that

298     the different AMF improved such levels following the same response pattern, with *C. claroideum*

299     22W3 as the best performing isolate, with increases, significantly different from control, of 39, 48

300     and 48% for total phenols, chlorogenic acid and antioxidant activity, respectively (Fig. 1, right).

301     Moreover, the inoculation with *F. mosseae* IMA1 significantly improved antioxidant activity levels,

302     by 44%.

303     A strong correlation was found between total phenols and antioxidant activity in the leaves

304     of both artichoke cultivars (Pearson correlation: r=0.812 for Romanesco, r=0.972 for Tema,

305     p≤0.001).

306

307

308     **4. Discussion**

309

310   This is the first work reporting differential increases of total phenols, chlorogenic acid and

311   antioxidant activity in the leaves of two artichoke cultivars inoculated with six AMF isolates

312   belonging to diverse genera and species, allowing the detection of the best performing symbionts to

313   be utilized for producing plants with improved content of health-promoting compounds.

314

315   *4.1. Mycorrhizal colonization, plant growth and mineral content*

316   Artichoke plants established mycorrhizal symbioses with the six AMF isolates, confirming the high

317   mycotrophy of plant species belonging to the family Asteraceae, in natural and non-natural

318   conditions (Warcup and McGee, 1983; Turrini et al., 2018). For example, percentage of colonized

319   root length reached values as high as 79 and 91% in cultivars and wild accessions of *Helianthus*

320   *annuus*, respectively (Turrini et al., 2016), 54 and 87% in *Berkheya coddii* and *Senecio coronatus*,

321   respectively (Turnau and Mesjasz-Przybylowicz, 2003), and 70 and 83% in *Cynara cardunculus*

322   (wild cardoon) and *Cynara cardunculus* var. *scolymus*, respectively (Marin et al., 2002; Ceccarelli

323   et al., 2010).

324   Here, the percentage of colonized root length was higher in Romanesco than in Tema,

325   whatever the AMF symbiont inoculated, consistently with previous findings on the variable

326   susceptibility to mycorrhizal colonization by different plant genotypes of the same species (Turrini

327   et al., 2016; De Vita et al., 2018). Accordingly, also in artichoke mycorrhizal colonization varied

328   largely among three different cultivars, Madrigal (82%), Opal (69%) and Concerto (58%)

329   (Campanelli et al., 2011), in nursery experiments. Other data, obtained using two seed propagated

330   cultivars, Romolo and Istar, showed lower colonization values (21 and 15%, respectively)

331   (Rouphael et al., 2017), to be ascribed to the different experimental conditions, as the plants were

332   cultivated in open field after seed coating inoculation, and to the different isolates used.

333   In this work, *R. irregulare* IMA6 was the most infective symbiont, reaching 82 and 66% of

334   colonized root length, confirming previous data obtained on artichoke, where the percentage of

335 colonized root length was 83% (Ceccarelli et al., 2010). The isolate of *F. mosseae* IMA1 showed

336 intermediate colonization levels, in agreement with the quoted study. As to the *F. mosseae* isolates

337 2W3 and IN101C, *C. claroideum* 22W3 and *Glomus* 14W1, there are no previous data on their

338 infectivity.

339       The mycorrhizal colonization pattern detected in the two artichoke cultivars indicated a

340 distinctive host preference for particular AMF symbionts, at species and intraspecific level, or,

341 otherwise, a differential affinity of diverse AMF species and intraspecific isolates for artichoke.

342 Such suggestions need to be confirmed using a broader range of artichoke cultivars and AMF

343 species and isolates.

344       The three most infective AMF, *R. irregulare* IMA6, *F. mosseae* IMA1 and *F. mosseae* 2W3,

345 displayed different root colonization patterns. Actually, while the first two AMF rapidly reached

346 high levels of colonized root length, as early as 7 weeks after inoculation, *F. mosseae* 2W3 was a

347 much slower colonizer, as it showed 10 and 20% and 10 and 12% colonization at the first and

348 second sampling, in Romanesco and Tema, respectively. Such a behaviour represents a negative

349 trait for an AMF inoculant, which should be able to compete with native endophytes by a rapid and

350 extensive root colonization. Further investigations may improve our understanding of the most

351 important AMF infectivity traits, with the aim of selecting the best performing isolates for field

352 inoculation.

353       Whatever the AMF inoculum, mycorrhizal inoculation did not produce significant increases

354 of total dry weight and leaf mineral content, compared with controls, in the two artichoke cultivars.

355 This result was not unexpected, given the uniform fertilization levels provided: actually, Hoagland

356 solution was distributed twice a week in spring and daily in summer as drip irrigation, and it was

357 modified to provide plants with P levels compatible with AMF root colonization. Such an approach

358 allowed us to rule out an indirect effect of the mycorrhizal symbiosis on secondary metabolism

359 through a better nutritional status and gave results consistent with previous findings obtained when

360 plant nutrient concentrations were adjusted in order to match in AMF mycorrhizal and control

361 plants (Toussaint et al., 2007; Battini et al., 2016; Avio et al., 2017). The lower concentrations of

362 $NO_3^-$ detected in mycorrhizal Tema suggest a possible role of the symbiosis leading to a lower level

363 of dietary nitrates, which, after reduction to nitrite and N-nitroso compounds, are known for their

364 carcinogenicity (Song et al., 2015).

365

366    *4.2. Total phenols, chlorogenic acid and antioxidant activity*

367 In artichoke leaves the levels of total phenols, chlorogenic acid and antioxidant activity revealed

368 significant differences between the two cultivars Romanesco and Tema. The analysis of the pooled

369 data from all the treatments confirmed findings on the differential content of specific

370 phytochemicals in different varieties of the same plant species (Avio et al., 2018). Previous studies

371 reported similar findings in artichoke. For example, among three artichoke cultivars, Imperial Star

372 and Green Globe showed significantly higher levels of total phenols and antioxidant activity in the

373 leaves, compared with the cultivar Violet (Wang et al., 2003). The content of chlorogenic acid in

374 the leaves was higher in the artichoke cultivar Terom than in Violetto di Toscana (Romani et al.,

375 2006), while the two seed-propagated hybrids Romolo and Istar showed different levels of total

376 polyphenol content in artichoke heads (Rouphael et al., 2017). Large variations in phenolic profiles,

377 total polyphenols and antioxidant activity of artichoke leaves were largely different in a collection

378 of 19 cultivars (Rouphael et al., 2016).

379    Previous works reported that the qualitative and quantitative differences in phenolic contents

380 and antioxidant activity in artichoke leaves of different cultivars can be influenced not only by the

381 genotype, but also by environmental conditions during growth (Lombardo et al., 2010). In this

382 study, the levels of total phenols, chlorogenic acid and antioxidant activity of artichoke leaves at the

383 final harvest were differentially affected by the six different AMF isolates. The consistency of

384 responses obtained by the two artichoke cultivars allowed us to detect the two best performing

385 symbionts, *C. claroideum* 22W3, which triggered significant increases in total phenols, chlorogenic

386 acid and antioxidant activity (by 39, 48 and 48%, respectively), and *F. mosseae* IMA1, which

387 significantly improved antioxidant activity (by 44%). These results are consistent with findings

388 showing that in artichoke leaves the enhanced phenolic content and antiradical power were affected

389 by inoculum composition (Ceccarelli et al., 2010). Moreover, significant differential increases in

390 total phenolic content and chlorogenic acid were detected in the leaves of artichoke plants

391 inoculated with two different commercial inocula (Palermo et al., 2013), confirming that AMF

392 identity plays a key role in the differential production of healthy secondary metabolites. It is worth

393 noting that the two quoted works utilized the AMF species *R. irregulare* and *F. mosseae*, which are

394 widely used worldwide in experimental studies. By contrast, here the best performing AMF was

395 represented by a symbiont, *C. claroideum* 22W3, which was recently isolated in pure culture from a

396 site described as a global "hot spot" of AMF species richness, within the UNESCO Man and

397 Biosphere Reserve, Selva Pisana (Njeru et al. 2015; Turrini et al., 2018). Indeed, the very low

398 number of AMF species utilized so far in nutraceutical studies, only 24 over 300, hindered the

399 acquisition of knowledge on the wide AMF functional diversity and their experimental assessment,

400 not only at the species level, but also at the level of isolates, which show different physiological and

401 functional activities (Avio et al., 2018).

402

403 **5. Conclusions**

404 This work revealed that mycorrhizal inoculation differentially modulated plant secondary

405 metabolism in artichoke, depending on the different AMF symbionts, which strongly influenced

406 the levels of total phenols, chlorogenic acid and antioxidant activity in the leaves of the two

407 cultivars Romanesco and Tema. Our findings pave the way for further studies on environmental,

408 agronomic and biotechnological variables affecting AMF ability to improve artichoke

409 secondary metabolites. Such studies could lead to the detection of appropriate criteria for the

410 selection of the best performing symbionts, to be utilized as sustainable biotechnological tools

411 for the production of plants with enhanced health-promoting activity. Moreover, further works

412 are needed in order to implement artichoke AMF inoculation in the field and produce flower

413     heads, which are already considered a functional food, showing improved concentrations of

414     beneficial phytochemicals. To this aim, a comprehensive approach should be applied,

415     investigating not only plant biochemical responses to root symbioses, but also the main factors

416     affecting the persistence of mycorrhizal colonization by the inoculated AMF in the field, their

417     optimal combinations and their interactions with native symbionts.

423

424

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**Table 1.** List of arbuscular mycorrhizal fungal isolates studied in the present work.

| Fungal species | Isolate code | Geographic origin | Biome | Original inoculum supplier[a] |
|---|---|---|---|---|
| *F. mosseae* | IMA1 [b] | Kent, UK | Unknown | Rothamsted Research, UK |
| *F. mosseae* | IN101C | Indiana, USA | Temperate grassland | INVAM, WV, USA |
| *F. mosseae* | 2W3 | Selva Pisana, I | Agricultural soil | IMA Collection, Italy |
| *G. irregulare* | IMA6 | Burgundy, F | Temperate agriculture | V. Gianinazzi-Pearson |
| *C. claroideum* | 22W3 | Selva Pisana, I | Agricultural soil | IMA Collection, Italy |
| *Glomus* sp. | 14W1 | Selva Pisana, I | Agricultural soil | IMA Collection, Italy |

535    [a] Abbreviations: INVAM, International Culture Collection of (Vesicular) Arbuscular Mycorrhizal
536    Fungi. IMA, International Microbial Archives.
537
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**Table 2.** Mycorrhizal colonization (% of root length) of *Cynara cardunculus* var. *scolymus* plants inoculated with six isolates of arbuscular mycorrhizal fungi (AMF), 7, 12 and 23 weeks after inoculation. Mean values ± standard errors, n=4. 14W1= *Glomus* sp., 2W3 =*Funneliformis mosseae*, 22W3= *Claroideoglomus claroideum*, IMA1 =*Funneliformis mosseae*, IN101C =*Funneliformis mosseae*, IMA6 =*Rhizoglomus irregulare*.

| Cultivar | AMF | 7 weeks | | 12 weeks | | 23 weeks | |
|---|---|---|---|---|---|---|---|
| | | Mean | SE | Mean | SE | Mean | SE |
| Romanesco | 14W1 | 18.8 | ± 2.4b | 30.0 | ± 2.0bcd | 18.0 | ± 2.0a |
| | 2W3 | 11.3 | ± 3.1a | 13.8 | ± 2.4a | 56.0 | ± 7.5b |
| | 22W3 | 11.3 | ± 3.1a | 15.0 | ± 2.0ab | 24.0 | ± 5.1a |
| | IMA1 | 42.5 | ± 4.8b | 32.5 | ± 4.8cd | 72.0 | ± 3.7b |
| | IN101C | 6.3 | ± 1.3a | 20.0 | ± 5.8abc | 22.5 | ± 4.8a |
| | IMA6 | 40.0 | ± 9.1b | 43.3 | ± 6.7d | 82.0 | ± 2.0c |
| | | | | | | | |
| Tema | 14W1 | 31.3 | ± 7.2b | 20.0 | ± 4.1a | 8.8 | ± 1.3a |
| | 2W3 | 11.3 | ± 3.1a | 20.0 | ± 4.1a | 54.0 | ± 2.4b |
| | 22W3 | 11.3 | ± 1.3a | 12.5 | ± 2.5a | 10.0 | ± 1.6a |
| | IMA1 | 37.5 | ± 6.3b | 45.0 | ± 2.9b | 42.0 | ± 3.7b |
| | IN101C | 6.3 | ± 1.3a | 8.8 | ± 2.4a | 12.5 | ± 2.5a |
| | IMA6 | 32.5 | ± 6.3b | 52.5 | ± 2.5b | 66.0 | ± 4.0c |

| Summary of two-way ANOVA results | | | | | |
|---|---|---|---|---|---|
| *Interaction* | ns | | *** | | ns |
| *AMF* | *** | | *** | | *** |
| *Cultivar* | ns | | ns | | *** |

Within each column and plant cultivar, different letters indicate significant differences according to Bonferroni procedure (p=0.05). When a significant interaction was found (12 weeks), the simple effects were analysed using the Sidak correction for multiple comparisons. Asterisks: significant at $P < 0.001$ (***); ns: not significant.

**Table 3.** First harvest data (12 weeks after mycorrhizal inoculation) on the concentrations of chlorogenic acid and total phenols, and antioxidant activity in leaves of *Cynara cardunculus* var. *scolymus* plants inoculated with six isolates of arbuscular mycorrhizal fungi (AMF). Mean values ± standard errors, n=4. CON= Control, 14W1= *Glomus* sp., 2W3 =*Funneliformis mosseae*, 22W3= *Claroideoglomus claroideum*, IMA1 =*Funneliformis mosseae*, IN101C =*Funneliformis mosseae*, IMA6 =*Rhizoglomus irregulare*.

| Cultivar | AMF | Chlorogenic acid mg g$^{-1}$ DW | | | Total phenols mg GAE g$^{-1}$ DW | | | Antioxidant activity μmol Fe(II) g$^{-1}$ DW | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | | SE | Mean | | SE | Mean | | SE |
| Romanesco | CON | 17.95 | ± | 1.51ab | 24.67 | ± | 1.59 | 274.50 | ± | 24.30 |
| | 14W1 | 16.44 | ± | 0.19ab | 26.40 | ± | 2.36 | 314.50 | ± | 39.82 |
| | 2W3 | 14.42 | ± | 1.17a | 24.90 | ± | 3.30 | 263.50 | ± | 21.28 |
| | 22W3 | 17.22 | ± | 1.16ab | 26.55 | ± | 2.86 | 327.50 | ± | 39.02 |
| | IMA1 | 24.47 | ± | 1.74b | 27.45 | ± | 3.05 | 390.00 | ± | 43.36 |
| | IN101C | 19.47 | ± | 1.24ab | 28.15 | ± | 4.71 | 305.75 | ± | 32.88 |
| | IMA6 | 17.94 | ± | 1.62ab | 25.68 | ± | 1.53 | 287.00 | ± | 8.93 |
| | | | | | | | | | | |
| Tema | CON | 20.95 | ± | 1.58 | 25.45 | ± | 2.37 | 264.50 | ± | 28.87 |
| | 14W1 | 18.27 | ± | 3.32 | 26.80 | ± | 3.87 | 296.00 | ± | 48.70 |
| | 2W3 | 18.69 | ± | 2.13 | 22.55 | ± | 3.94 | 214.25 | ± | 43.51 |
| | 22W3 | 23.69 | ± | 1.48 | 27.03 | ± | 1.61 | 260.50 | ± | 19.08 |
| | IMA1 | 15.88 | ± | 2.01 | 22.40 | ± | 3.44 | 207.25 | ± | 23.46 |
| | IN101C | 16.50 | ± | 0.75 | 25.78 | ± | 2.29 | 258.00 | ± | 34.23 |
| | IMA6 | 17.46 | ± | 1.45 | 26.03 | ± | 4.14 | 283.00 | ± | 58.17 |
| Summary of two-way ANOVA results | | | | | | | | | | |
| *Interaction* | | ** | | | ns | | | ns | | |
| *AMF* | | ns | | | ns | | | ns | | |
| *Cultivar* | | ns | | | ns | | | *** | | |

Different letters indicate significant differences according to simple effects analysis using Sidak correction for multiple comparisons.

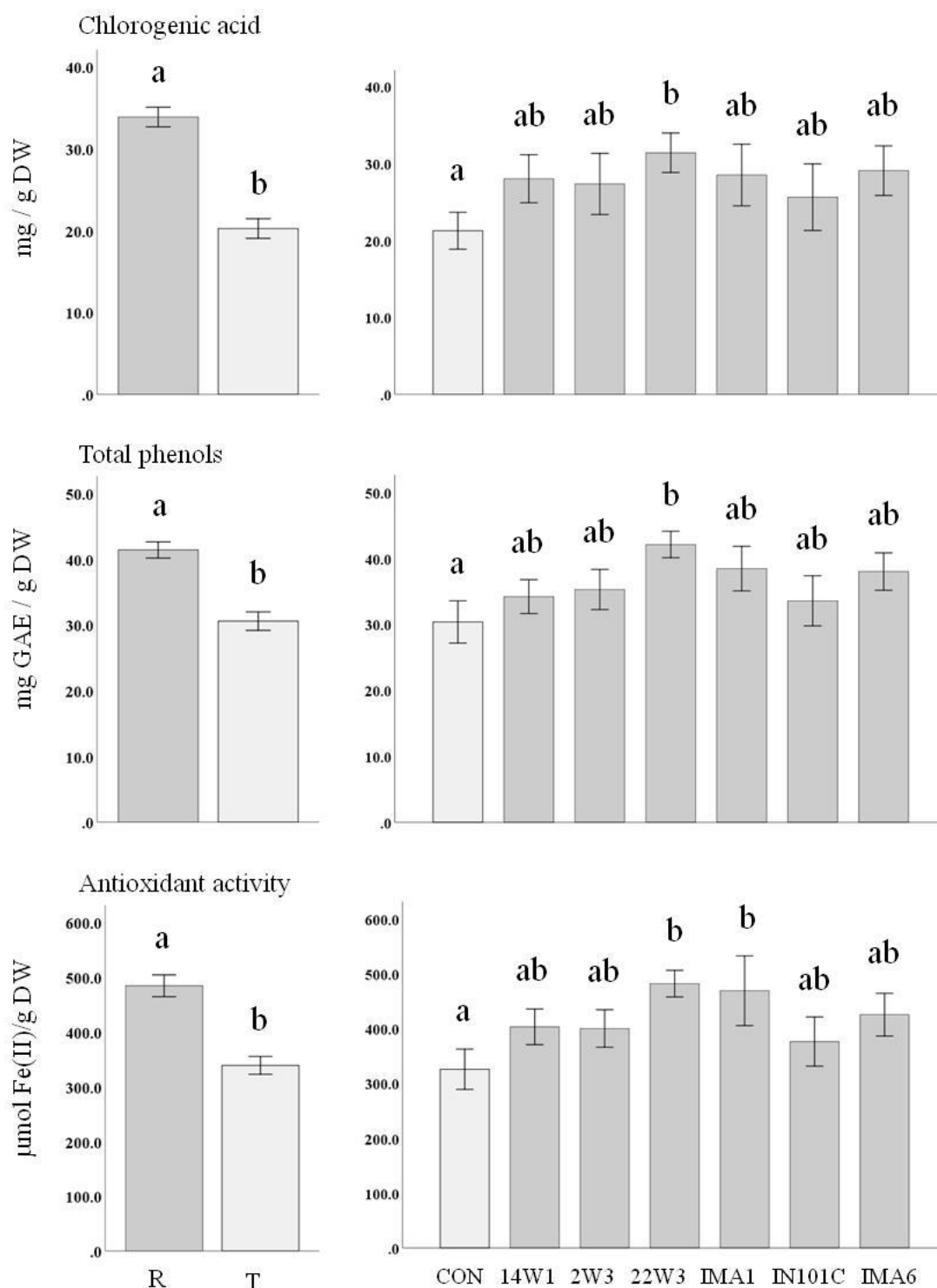Asterisks: significant at P < 0.01 (**) or P < 0.001 (***); ns: not significant.

**Figure 1.** Final harvest data (23 weeks after mycorrhizal inoculation) on the concentrations of chlorogenic acid (mg/g DW) and total phenols (mg GAE/g DW), and antioxidant activity (μmol Fe(II)/g DW) in leaves of two cultivars of *Cynara cardunculus* var. *scolymus* plants inoculated with six isolates of arbuscular mycorrhizal fungi (AMF), as affected by cultivar (left) and mycorrhizal symbiont (right). R= cv Romanesco, T= cv Tema; CON= Control, 14W1= *Glomus* sp., 2W3 =*Funneliformis mosseae*, 22W3= *Claroideoglomus claroideum*, IMA1 =*Funneliformis mosseae*, IN101C =*Funneliformis mosseae*, IMA6 =*Rhizoglomus irregulare*. Within each bar chart, different letters indicate significant differences among treatments. Error bars refer to standard error of the means (n = 4).