Title: The potential of native species as bioenergy crops on trace-element contaminated Mediterranean lands

Short title: Potential of Mediterranean species for energy crops in contaminated lands

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Abstract

The establishment of energy crops could be an option for the management of degraded and contaminated lands, where they would not compete with food production for land use. Here, we aimed to explore the potential of certain native Mediterranean species for the revegetation of contaminated lands for energy production purposes. A field survey was conducted in a trace-element (TE) contaminated area from SW Spain, where the patterns of biomass production, (TE) accumulation and the calorific value of some thistle species were analyzed along a soil contamination gradient. In a greenhouse experiment the response of two thistle species (*Cynara cardunculus* and *Silybum marianum*) and the shrub *Dittrichia viscosa* to soil contamination was assessed, as well as the effects of these species on some

soil microbial parameters involved in nutrient cycling (enzyme activities and arbuscular mycorrhizal colonization in roots). *Silybum marianum* was able to colonize highly contaminated soils. Its aboveground biomass accumulated Cd and had a relatively high calorific value; this value was similar in biomass obtained from both heavily and moderately contaminated soils. Greenhouse experiment confirmed that *S. marianum* biomass production and calorific value is scarcely affected by soil contamination. In addition, some soil enzyme activities were clearly enhanced in the *S. marianum* rhizosphere. *Diitrichia viscosa* is another promising species, given its capacity to produce a high biomass with appreciable calorific value in acid contaminated soils. Germination of both species was hampered in the acid contaminated soil, and therefore pH soil correction would have to be accomplished before establishing these species on extremely acid soils. Further assessment of the risk of transfer of Cd and other TE to the food chain would be needed to confirm the suitability of these species for the revegetation of contaminated lands with energy production purposes.

Key words: biofuel, soil pollution, *Silybum marianum*, *Cynara cardunculus*, soil enzymes

Highlights

- Some Mediterranean plant species were assessed as bioenergy crops for degraded lands
- In a contaminated land *Silybum marianum* produced high biomass with a high calorific value
- A greenhouse experiment confirmed its potential for biomass production in contaminated soils

- *Dittrichia viscosa* also produced a high biomass with high calorific value in highly degraded soils
- Possible problems associated to Cd accumulation into biomass must be further assessed

1. Introduction

Soil contamination is one of the main causes of land degradation at the European level (Panagos et al., 2013). In the last decades, the concern about the extent of soil contamination by trace elements (TE) has led to high investments in technologies to address the recovery or decontamination of soils, among which phytoremediation has received special attention. This technology involves the combined use of plants and amendments, either to extract (phytoextraction) or stabilize (phytostabilization) TE in the soil-plant system, promoting the establishment of a plant cover that minimizes erosion risks and consequently the spread of pollutants to surrounding areas (Mendez & Maier, 2008; Robinson et al., 2009).

The environmental benefits of phytoremediation of a contaminated area are undeniable (Mendez & Maier, 2008). However, the accumulation of potentially toxic elements in the established vegetation often prevents an agricultural or livestock use of these areas (Madejón et al., 2009). It is therefore necessary to explore alternative uses of the vegetation in contaminated areas that do not only pose environmental, but also direct economic benefits to the social partners involved (Robinson et al., 2009; Conesa et al., 2012). The

establishment of energy crops could be an option for the management of contaminated lands. Since agricultural production for food purposes is not allowed or recommended in these lands, the establishment of energy crops would not compete with food production for land use. Using these marginal lands for biofuel production would contribute to the attainment of national renewable energy targets as suggested by the European Renewable Energies Directive, which highlights the need for a sustainable production of biofuels that do not compete with food production.

In addition to the benefits derived from energy generation, the plantation of fast-growing species might also promote the sustainability of the degraded land by improving soil quality, in particular by increasing soil C stocks in the long-term (Baumert et al., 2014; Singh et al., 2006; Singh et al. 2015). At shorter time scales, the presence of the fast-growing crop species usually stimulates microbial biomass and extracellular enzyme activities in organic-matter poor or degraded soils (Cotton et al., 2013; Dou et al., 2013). Different crop species, however, might promote different changes in the soil microbial community, due to contrasted patterns of root exudation and litter quality (Mao et al., 2013).

Currently, examples of the use of fast-growing species for the revegetation of contaminated areas for energy production purposes are very limited (Ruttens, 2011; Witters et al., 2012; Madejón et al., 2016). Given the particularities of the Mediterranean climate (irregular rainfall, low water availability, and frequently low soil organic matter and nutrient content), many of the most common energy crops (*Miscanthus sp.*, *Panicum sp.*, *Jatropha sp.*) are not suitable for degraded Mediterranean areas (Robledo & Correal, 2013). Several works

94 have shown that thistle species in the *Silybum* and *Cynara* genus could be adequate for
95 solid fuel production in Mediterranean lands, due to their physiological adaptations to dry
96 environments (Fernandez & Curt, 2005), and their high dry matter content and calorific
97 value (Fernandez et al., 2006; Angelini et al 2009a; Oliveira et al, 2012; Ledda et al, 2013).
98 In addition, biodiesel can be obtained from their oily seeds, with fuel properties comparable
99 to those of mineral diesel, and in agreement with biodiesel standards (Encinar et al., 2002;
100 Ullah et al., 2015). *Dittrichia viscosa* (L.) Greuter has also been identified as a promising
101 bioenergy crop species for Mediterranean lands (Robledo & Correal, 2013), because of its
102 ability to produce a high biomass in highly degraded soils, including soils contaminated by
103 TE (Martínez-Sánchez et al., 2012; Gómez-Ros et al., 2013).

104

105 Here, we present two studies aimed to explore the potential of some thistle species and *D.*
106 *viscosa* for the revegetation of contaminated Mediterranean lands for bioenergy production
107 purposes. In the first study, a field survey was conducted in a TE contaminated area in SW
108 Spain (Guadamar River Valley), where we explored the patterns of TE accumulation and
109 the capacity for energy production through combustion (calorific value) of *D. viscosa* and
110 some thistle species that spontaneously colonized the contaminated soils. In the second
111 study the response of two thistle species (*Cynara cardunculus* L. and *Silybum marianum*
112 (L.) Gaertn) and *D. viscosa* to soil contamination was analyzed under controlled conditions.
113 Although *C. cardunculus* is a cultivated species, and does not usually colonize altered soils
114 like *S. marianun* and *D. viscosa* do, the interest of including *C. carducunlus* in the
115 greenhouse experiment relies on its well-known potential as bioenergy crop for
116 Mediterranean lands (Fernández & Curt, 2005; Fernández et al., 2006; Angelini et al.,
117 2009a; Oliveira et al., 2012). However, its tolerance to soil trace element contamination is

still poorly characterized. Given that the interest of establishing these species on contaminated soils would not be limited to the production of solid fuel, but also to the improvement of soil quality in these degraded soils, the effects of the plantation of these species on some microbial parameters involved in soil nutrient cycling and C sequestration were also analyzed, namely, soil extracellular enzyme activities, which are usually used as soil quality indicators in degraded soils (Pajares et al., 2011; Paz-Ferreiro & Fu, 2016), and the degree of root colonization by arbuscular mycorrhizal (AM) fungi, which might facilitate plant establishment in degraded soils (Camprubi et al., 2015).

2. Materials and methods

2.1. Field study

Sampling design

Field survey was conducted in the Guadianar River Valley (SW of Spain), an area affected by a large pollution episode in 1998, when a mining accident produced the release of ca. 6 hm³ of trace element-polluted waters and sludge into the Guadianar River (Grimalt et al., 1999; Domínguez et al., 2008). As a result, soils were polluted by several TE, in particular by As, Cu, Cd, Pb and Zn (Cabrera et al., 1999).

Nine locations were selected along a gradient of soil contamination in the Guadianar River Valley. These locations include one site unaffected by the mining accident (site 1), seven sites affected by the accident in which remediation actions were undertaken (sludge removal, amendment addition and revegetation with woody species, sites 2-5 and 7-9), and

one site affected by the accident in which no remediation activity was conducted, and therefore sludge remained on the soil surface (site 6). Details about the remediation activities conducted in the area can be found in Domínguez et al. (2008).

Biomass and soil sampling took place during May 2015, when thistle species had completely developed the floral scape and maximal biomass was reached. In each of these 9 sites 3 subplots of 10 x 10 m were established, and the presence of the focal species was recorded (*D. viscosa*, and thistle and related species -belonging to the Carduoideae subfamily in the Asteraceae family-). The objective of this survey was to assess TE accumulation and potential energy production from biomass combustion of the focal species (thistle species and *D. viscosa*) 17 years after the pollution episode, and not to conduct a detailed analysis of the plant richness and the drivers of plant diversity in the area. Thus, data of plant richness and relative abundances of the whole plant community were not recorded. Species were identified following the nomenclature by Valdés et al. (1987). In each of these subplots three 1 m² square were delimited, and biomass of each of the present focal species was harvested separately. A subsample of the harvested biomass of each species was weighed immediately to determine fresh weight. Samples of surface soil (0-15 cm) were taken using a cylindrical auger from each of the 1 m² quadrats and combined in a composite sample for each of the three subplots delimited at each site (3 soil samples per site), except at site 1 (non-contaminated) where a single composite soil sample of the three subplots was analyzed.

Sample preparation and analysis

A subsample of each plant sample was washed with deionized water for chemical analysis. The rest of the harvested biomass was directly oven dried at 70 °C during at least two days and weighed. Washed subsamples were dried, ground, passed through a 500-µm stainless-steel and sieved. Then, they were digested by wet oxidation with concentrated HNO₃ (65%, trace analysis grade) in a DigiPrep-MS block (SCP Science). Determination of As, Cd, Cu, Pb, S and Zn in the extracts was performed in a Varian ICP 720-ES (simultaneous ICP-OES with axially viewed plasma). Accuracy of the analytical methods was assessed through analysis of a reference plant sample (INCT-TL-1, Tea leaves). Recovery values were 93 % for As, 104 % for Cd, 100 % for Cu, 95 % for S, and 98.4 % for Zn. Recovery values for Pb were low (70 %) and therefore Pb concentrations in plants are not reported.

The Gross Calorific Value (GCV) of the three thistle species identified at the most contaminated site (*Carduus tenuiflorus* Curtis, *Scolymus maculatus* L. and *Silybum marianum*) was determined in the biomass obtained from this site (site 6), as well as in the biomass obtained from those sites where maximal level of biomass of each of these species were recorded, with the objective to study variability in GCV of plants growing with contrasted soil conditions. Three samples per plant species and site were analyzed, using a Parr 6300 Automatic Isoperibol Calorimeter and according to “CEN/TS 14918:2005 (E) Solid biofuels—Method for the determination of calorific value” and UNE 164001 EX standards.

184

185 Soil samples were air-dried, ground and sieved to < 2 mm. A subsample was ground to <
186 60 µm for S and TE determination. The < 2 mm subsample was analyzed for pH
187 potentiometrically in a 1:2.5 soil-water suspension. Organic matter content was analyzed
188 according to the Walkley & Black (1934) method. The < 60 µm soil subsamples were
189 digested with ‘aqua regia’ (1:3 concentration HNO₃: HCl) in the DigiPrep-MS block, and
190 As, Cu, Cd, Fe, Pb and Zn were determined by ICP-OES. ‘Aqua regia’ digestion does not
191 extract those TE associated to silicates (Reimann & Caritat, 1998). Nevertheless, this
192 method is widely accepted to characterize trace element concentrations in soil pollution
193 studies, because the silicate-binding metals have less environmental implications. Such
194 concentrations are referred to as ‘pseudo-total’ concentrations. Soil CaCl₂ soluble
195 concentrations were determined in 1:10 soil: 0.01 M CaCl₂ extracts (Houba et al., 2000)
196 using ICP-OES.

197

198 2.2. Greenhouse experiment

199

200 *Plant species*

201

202 *Cynara cardunculus* (cardoon), *S. marianum* (milk thistle) and *D. viscosa* are
203 Mediterranean native species belonging to the Asteraceae family. *C. cardunculus* and *S.*
204 *marianum* have an annual growth cycle, drying up the leaves and scapes in the summer. *C.*
205 *cardunculus* is a perennial plant, while *S. marianum* is an annual or biannual plant. Both

206 plant species show basal rosette leaves of around 30 cm length (up to 80 cm in the case of
207 *C. cardunculus*). *D. viscosa* is an evergreen perennial plant with a remarkable pioneer and
208 ubiquitous character, classified as a nanophanerophyte or sometimes as a chamaephyte with
209 a woody base (Parolin et al., 2014). It develops many branches and a very dense canopy of
210 up to 150 cm of height.

211

212 *Experimental design*

213

214 The greenhouse experiment was conducted at La Hampa farm (Coria del Rio, Seville).
215 Containers filled with four different soils were installed. Three of these soils were collected
216 from some of the sites selected for the field survey, and had contrasted pH and trace
217 element concentrations. Soil 1 was an acid contaminated soil from site 3, with low pH, high
218 solubility of TE, low organic matter content and a sandy-loam texture. Soil 2 was a neutral
219 contaminated soil from site 7, with higher carbonate content, lower trace element
220 availability and a loam texture. Soil 3 was an acid, non-contaminated soil, collected outside
221 the spill affected area close to site 1, with low concentrations of soluble TE, higher level of
222 organic matter than soils 1 and 2 and a sandy-loam texture. Soil 4 was a neutral, non-
223 contaminated soil from the experimental farm, with a sandy loam texture and low organic
224 matter content. All the soils are classified as Calcic Fluvisols (IUSS Working Group WRB,
225 2007). At each location, soils were collected from the top 25 cm and transported to the
226 experimental farm, where they were manually homogenized before distribution of the soils
227 into the containers. Stones were removed before distribution into containers. For each

228 combination of species and soil type 15 containers (15 kg capacity) were filled with about
229 10 kg of soil plus 2.5 kg of gravel placed in the bottom to facilitate drainage. The
230 containers were placed outdoors, randomly arranged. Table 1 shows the main
231 characteristics of these soils at the time of see sowing.

232

233 Seeds were sown in November 2014. For *S. marianum* and *C. cardunculus* 10 seeds were
234 sown in each container, while for *D. viscosa* an amount of 0.05 g, equivalent to 60 seeds,
235 was used. The containers were kept outdoors until March 2015 to study germination under
236 ambient weather conditions. Periodic monitoring of the number of germinated seeds per
237 container was conducted during January and February.

238

239 In March 2015, 8 containers of each combination of plant and soil type were moved into a
240 greenhouse where the plants were grown with irrigation. One of the emerged seedlings per
241 container was left, and the rest removed. In the case of the acid contaminated soil (Soil 1),
242 in which almost no seeds were able to germinate, sugar beet lime was applied as a soil
243 amendment at a dose equivalent to 10 T ha⁻¹, and one previously germinated seedling was
244 planted per container. These previously germinated seedlings were also sown in November
245 2014, at the same time as the seeds into the containers, and were kept at the same
246 conditions as the containers until their use. Before transferring these seedlings to the
247 containers, topsoil (0-10 cm) was homogenized to facilitate the amendment incorporation.
248 After lime application topsoil (0-10 cm) pH was raised to 6.8, on average.

249

250 From March to April plants were irrigated twice weekly with an amount of water
251 equivalent to 4 L m⁻². In May, due to the increased evapotranspiration in the greenhouse,
252 sprinkler irrigation was applied, at a dose of 2.8 L m⁻² day⁻¹ per container. The plants were
253 grown until the end of June 2015 (*S. marianum*, when it had completely dried-out) and the
254 end of July 2015 (*C. cardunculus* and *D. viscosa*). Daily average temperatures ranged from
255 21.5 to 26.8 °C during the growth period; photoperiod was set from 8:00 to 19:00, with
256 minimum illuminances of 6.8-8.8 klux at 8:00 and maximums of 19.6-23.1 klux at 16:00.

257

258 *Sample preparation and analysis*

259

260 Aboveground biomass was weighed fresh at the time of harvest in all containers, then dried
261 and weighed. The root system of each plant was carefully removed from the container and a
262 sample of the rhizospheric soil of each plant was taken. Then, root biomass was removed
263 from rhizospheric soil by washing, and then dried and weighed. A subsample of fine roots
264 (< 2 mm diameter) of 5 plants per treatment (combination of soil type and species) was
265 washed immediately after plant harvest, and kept in 70 % (v/v) ethanol for subsequent
266 analysis of arbuscular mycorrhizal fungi (AMF) colonization. Roots were cleared with 10%
267 (wt/vol) KOH at 85°C for 15 min, rinsed several times with tap water, and heated in a 5%
268 ink-vinegar solution (3 min, 85°C). Before keeping the roots in lactoglycerol, the excess of
269 ink was eliminated by rinsing in tap water acidified with a few drops of vinegar for 2 h.
270 AMF colonization was measured in 44 fragments of 1 cm per sample. Total colonization

(%) and colonization by fungal structures (percentage of hyphae, vesicles and arbuscules from total colonization) were estimated according to Hernández-Cuevas et al. (2008).

Sulphur and TE concentrations in plant shoots were analyzed in a subset of three plants per species and soil type, using the method described above. Gross calorific value was determined as described above in six samples of each species, from which three corresponded to plants that had grown in the acid contaminated soil, and the other three to plants that had grown in the neutral contaminated soil, for which the greatest biomass production values were recorded.

Soil samples were divided into 2 subsamples, one for chemical analysis and another one for biological analysis. Soil pH and organic matter content were determined as described above. Subsamples for biological analysis were sieved (2 mm) and stored at 4 °C until analysis. Dehydrogenase activity was determined according to Trevors (1984), β -glucosidase activity was measured as suggested by Eivazi & Tabatabai (1988), and urease activity was determined according to Kandeler & Geber (1988).

2.3 Data analysis

With the field data, estimates of the potential energy yield obtained by combustion of *C. tenuiflorus*, *S. maculatus* and *S. marianum* biomass at the most contaminated site (site 6)

and the most productive site (site 8) were calculated from biomass data, using the average GCVs for each of the three species at each of the two sites. For each species, differences in GCV and potential energy yield between sites were explored by applying one-way ANOVA. Previously, potential energy yield data were log-transformed to meet normality and homoscedasticity assumptions.

For each of the species tested in the greenhouse experiment, and in order to test whether biomass production in each species was affected by the type of soil, one-way ANOVAs and Tukey post-hoc tests were applied to analyze differences in total biomass production, aboveground biomass, shoot:root ratio and dry matter content among soil types. Root biomass data was previously log-transform to meet the normality assumption. Also, one-way ANOVA was applied to test for differences in the GCV of the biomass obtained from Soil 1 and Soil 2, respectively, from which contrasted levels of biomass production were recorded. To analyze whether soil enzyme activities at the time of biomass harvest differed among plant species, one-way ANOVAs were applied for each soil type to enzyme data, with plant species as categorical factor. Finally, two-way ANOVAs were applied to test for differences in shoot concentrations of As, Cd, Cu, Fe and, as well as in root colonization by AMF, with soil type and plant species as categorical factors. A principal component analysis was applied to all the soil microbial variables (including root AMF colonization) with plant species as a grouping factor to explore trends in soil microbial activity as affected by the plantation of the different species.

3. Results and Discussion

3.1. Field survey

Biomass production along the soil contamination gradient

The nine sampled sites showed contrasted levels of TE contamination (Table 2). Site 6 (with no remediation activity) showed the highest level; soil As and Pb pseudo-total concentrations were up to 800 and 2500 mg kg⁻¹ respectively, well above the normal ranges in soils (0.1 - 40 mg kg⁻¹ for As and 2 - 300 mg kg⁻¹ for Pb; Bowen, 1975), and also above those levels reported to cause toxicity to plants (20 mg kg⁻¹ for As and 100 - 400 for Pb; Singh and Steinnes, 1994). In general, CaCl₂-extracted TE concentrations were relatively low, even at site 6, where concentrations for the most soluble element (Zn) were lower than 3 mg kg⁻¹. In the vegetation survey four thistle species with a high occurrence across sites and with a significant biomass production were identified. These species were *Carduus tenuiflorus*, *Galactites tomentosa* Moench, *Scolymus maculatus* and *Silybum marianum*, being present at 56%, 78 %, 44 % and 78 % of the sampled sites, respectively. Another two related species (*Centaurea pullata* L. and *Cynara baetica* (Spreng.) Pau) were also present in the area, but at a lower abundance (11 % and 22 %, respectively). From those four species, all of them except *G. tomentosa* were present at the most severely contaminated site (site 6, Fig. 1). *D. viscosa* was not observed at any site, despite its well-known ability to colonize highly disturbed soils, including those affected by mining activities (Martínez-Sánchez et al., 2012; Gómez-Ros et al., 2013). The absence of *D. viscosa* in our survey, however, does not necessarily mean that this species could not be established in the area as a crop, as other variables, such as propagule availability in the soil seed bank or in the

surrounding plant communities, might be the factors limiting its presence in the current plant community in the study area. Indeed, in the greenhouse experiment, *D. viscosa* showed a capacity to produce a high biomass in contaminated soils collected from the same area (see below).

Thistle plant biomass ranged from 0.34 T ha⁻¹ (site 1), to 3.08 T ha⁻¹ (site 8). Interestingly, the lowest levels of thistle biomass were observed at the non-contaminated site. At the contaminated sites, variability in thistle biomass production was remarkably high, as indicated by high standard deviation values (Fig. 1). This is likely related to the patchy distribution of the remnants of sludge in the soils affected by the mining spill, especially in the Northern zone of the Guadamar valley. In this zone, 7 % of the land surface consists of spots of bare soil, where vegetation had not established 16 years after the mining accident that resulted in soil contamination, due to the low soil pH and the high soluble trace element concentrations (Martín-Peinado et al., 2015). In addition, the irregular application of soil amendments resulted in a patchy distribution of soil pH and nutrient content at the remediated sites (Domínguez et al., 2016).

Among the identified thistle species *S. marianum* showed, by far, the highest level of standing aboveground biomass. On average, aboveground biomass of this species was 140g m⁻² (equivalent to 1.4 T ha⁻¹, Fig. 1). This value is lower (although of the same order of magnitude) than biomass production reported for *C. cardunculus* bioenergy crops under Mediterranean climate during years of low rainfall (6.5- 7.5 T ha⁻¹, Fernández & Curt, 2005), and ten times lower than the value of 15 T ha⁻¹ reported by Sulas et al. (2008), who assessed the suitability of *S. marianum* as a bioenergy crop species under Mediterranean

climate. Growing conditions in the study by Sulas et al., (2008), however, were clearly more favorable for *S. marianum* growth than the field conditions in our study, since it included a sowing density of 2 kg seed ha⁻¹ and soil fertilization in an agricultural, non-contaminated land. In any case, the growing season of year 2015 was particularly dry (170 mm of rainfall from December 2014 to May 2015, 43 % lower than the average for the 1981-2010 period for the Seville Province; AEMET, 2016), therefore it is likely that higher *S. marianum* biomass productions can be obtained during wetter years, given that rainfall is the main driver of biomass production in rainfed cardoon bioenergy crops under Mediterranean climate (Fernández & Curt 2005, Angelini et al 2009a). This study confirms the previously reported ability of *S. marianum* to tolerate relatively high concentrations of TE in soils, being able to colonize highly contaminated soils where other herbaceous plants fail to establish (Brunetti et al., 2009; Perrino et al. 2014).

For *G. tomentosa*, *C. tenuiflorus* and *S. marianum* dry matter content values ranged between 30 % and 40 % (Supplementary Material, Fig. S1), which are typical of cardoon stalks and heads in bioenergy crops under Mediterranean climate (Ierna et al., 2012), and similar to the value reported by Sulas et al. (2008) for *S. marianum* plants at the same phenological stage, after complete development of inflorescences but before shoot dieback. *S. maculatus* showed the lowest dry matter content.

Trace element accumulation in biomass

386 Accumulation of TE into the aboveground biomass of the studied species was highly
387 dependent on the sampling site (Fig. 2). As expected, maximum concentrations were found
388 at site 6, where As accumulation in *C. tenuiflorus* and *S. marianum* were above the normal
389 limits for higher plants (Chaney et al., 1989). Interestingly, the studied species tended to
390 accumulate high levels of Cd into their aboveground biomass, particularly *C. tenuiflorus*
391 and *S. maculatus*. In 4 out of 9 sampling sites Cd levels in these thistle species were higher
392 than the normal limits for plants (1 mg kg^{-1} , Fig. 2), and above the maximum level tolerable
393 for livestock (Adriano et al., 2001). The ability of some Asteraceae species to accumulate
394 Cd had been reported in a previous study in the same area that detected high Cd
395 concentrations in pastures dominated by Asteraceae species (Madejón et al., 2009).

396

397 In a previous study, Pb and Zn accumulation in *S. marianum* biomass was reported in a pot
398 experiment, and the use of this species for the remediation of contaminated soils with
399 phytoextraction purposes was proposed (Río-Celestino et al., 2006). Such levels of TE
400 accumulation usually imply the existence of several physiological mechanisms for trace
401 element detoxification in the aboveground biomass (Monferrán & Wunderlin, 2013), and
402 therefore a relatively high tolerance to contamination of soils by TE. On the one hand, this
403 Cd accumulation capacity might be considered as a positive feature of these species, given
404 that the harvest of their Cd-enriched biomass for energy production could lead to a slow but
405 progressive removal of Cd from soils, which poses a secondary benefit to the establishment
406 of this species in contaminated soils with energy production purposes. On the other hand,
407 the accumulation of Cd into the aboveground biomass of these species could pose a risk of
408 transfer of this element to herbivores. Thistles are not consumed by many large herbivores
409 because of their spiny leaves and stalks, but are an important food source for a wide range

of invertebrates (Goeden et al., 1986). The risk of transfer of Cd to these organisms should be further analyzed for a full assessment of the feasibility of *S. marianum* crops in contaminated lands. In addition, metal-enriched ash could be produced during energy conversion processes, which may reduce the environmental sustainability of the bioenergy crop system; ideally ash resulting from biofuel combustion should be returned to land as fertilizers, but excessive amounts of metal in ash might prevent this option or obligate to subsequent operations to remove metals from ash (Ljund & Nordin, 1997; Vervaeke et al., 2006), which would reduce the net energy output of the system.

Calorific value and potential energy yield

Gross Calorific Values of *S. marianum*, *S. maculatus* and *C. tenuiflorus* biomass ranged between 15 and 16.8 MJ kg⁻¹ (Fig. 3a). For each of these species, GCV of plants growing in the heavily contaminated site (site 6), where no soil remediation actions were undertaken, was not significantly different than the GCV of plants growing in contiguous, remediated soils (moderately contaminated), where maximal biomass production values for each species were recorded. The measured GCV are slightly higher than those reported for some *Cynara cardunculus* crops under rainfed conditions (Angelini et al., 2009a), but slightly lower than the calorific value of other herbaceous bioenergy crop species cultivated in Mediterranean environments, such as *Arundo donax* and *Miscanthus x giganteus*, usually ranging between 17.6 and 18 MJ kg⁻¹ (Angelini et al., 2005, 2009b).

In these two sites with contrasted biomass productivity, potential energy yield obtained from the combustion of the biomass of these species was estimated (Fig. 3b). The results

clearly show that the potential of *S. marianum* for energy production is greater than the potential energy yield of *C. tenuiflorus* and *S. maculatus*, due to the combination of a high biomass production and a reasonably high GCV, less variable between sites than *C. tenuiflorus* or *S. maculatus* GCVs. For *S. maculatus*, the potential energy yield was significantly different between the two sites, while for *S. marianum* and *C. tenuiflorus* differences between sites were not statistically significant. The estimated energy yield was considerably lower than those values reported in other studies with different bioenergy crops (*C. cardunculus*, *A. donax* and *M. x giganteus*) in Mediterranean conditions (Angelini et al., 2009a, b), due to the lower levels of biomass production. In those studies, however, conditions were much more favorable for a high biomass production and, consequently, for greater energy yields, since they included soil preparation and fertilization, high planting density, and the use of selected cultivars with high growth rates. Considering the conditions of our field study (plants spontaneously colonizing a TE contaminated soil, without any soil preparation or fertilization treatment), our results show a potential for the revalorization of the vegetation established in this contaminated area (specially for *S. marianum* biomass) through energy production from biomass combustion, which could probably be enhanced by an adequate land management (soil preparation and sowing).

3.2. Greenhouse experiment

Seed germination and biomass production

Seed germination was clearly inhibited in Soil 1 (Fig. 4), likely due to the concurrence of low pH and relatively high Cd and Zn soluble concentrations (Table 1). With these soil

conditions, pH correction before sowing seems to be an essential step in the establishment of these species in contaminated lands. In general, germination was very low for *D. viscosa*, likely because, as it is naturally adapted to open sites with a high radiation incidence, its seeds would have required higher temperatures to germinate (Parolin et al., 2014).

Among the three species *D. viscosa* showed the highest levels of both total and aboveground biomass (Fig. 5a, b), while the lowest total biomass records were observed for *S. marianum* (Fig. 5a). Similar levels of root biomass were found for *D. viscosa* and *C. cardunculus*, although *D. viscosa* developed a deeper root system. For the revegetation of contaminated soils, the development of a large root system is a positive feature, given that roots can improve soil structure minimizing the spread of contaminants through soil erosion, and that TE can get stabilized in the rhizosphere through precipitation and adsorption onto root surfaces (Mendez & Maier, 2008; Domínguez et al., 2009; Conesa et al., 2012).

Biomass produced by *D. viscosa* and *S. marianum* in this study was higher than that reported by Martínez-Fernández et al. (2014), who investigated the effects of the addition of different composts and fertilizers to contaminated soils on the growth of these species in a similar greenhouse experiment. Dry matter content observed for *C. cardunculus* was 20 %, considerably lower than those values reported for *C. cardunculus* in long-term crops under field conditions (Angelini et al., 2009a; Ierna et al., 2012). Probably, production of this perennial species was not at its optimum in our short-term study, and a greater plant yield can be obtained few years after establishment, as found in experimental *C. cardunculus* crops (Angelini et al., 2009a).

482

483 Significant differences in biomass production or dry matter content among soil types were
484 found for the three species (Supplementary Information, Table S1). The most important
485 finding was the constant biomass production of *S. marianum* plants growing in the four
486 soils tested (Fig. 5), once that soil pH in Soil 1 was corrected through sugar beet lime
487 application. *D. viscosa* root and aboveground biomass varied among soil types, but was not
488 particularly decreased in the two contaminated soils (Soil 1 and Soil 2). In contrast, *C.*
489 *cardunculus* root and aboveground biomass was considerably reduced in the acid
490 contaminated soil (Soil 1; Fig. 5b, c). These results might be related to different ranges of
491 optimum pH conditions for growth among species. *S. marianum* is tolerant to a wide range
492 of pH, but grows well in soils with a pH of 5.5–7.6 (Karkanis et al., 2011). *D. viscosa*,
493 might also be tolerant to a wide range of soil pH, as it has the ability to colonize highly
494 disturbed soils, such as those affected by mining activities (which are often acidic due to
495 acid drainage), as well as ultramaphic sites, where pH is often above 7 (Parolin et al.,
496 2014). *C. cardunculus*, however, seems to have an optimal growth in basic soils (Fernández
497 & Curt, 2005).

498

499 *Trace element concentration in biomass*

500

501 Concentrations of As and Cu in shoots were not significantly different among species
502 (Supplementary Information Table S2; Table S3). Copper concentrations in the three
503 species were slightly above the normal ranges for plants reported by Chaney et al., (1989)
504 in all the tested soils (20 mg kg⁻¹; Table S2), which suggests that these species have a high

capacity for Cu uptake from soil and translocation and accumulation into aboveground biomass.

Cadmium and Zn concentrations varied among species and soil types, with a significant species $\times$ soil interaction (Supplementary Information, Table S3). In general, *D. viscosa* and *S. marianum* accumulated more Cd into their shoots, in comparison to *C. cardunculus*. (Fig. 6a). For the three species, plants growing in Soil 1 and Soil 2 showed Cd concentrations that were above the normal levels for plants (0.1- 1 mg kg⁻¹) reported by Chaney (1989). In the case of *D. viscosa* and *S. marianum*, these values were also above the maximum levels tolerable by livestock (0.5 mg kg⁻¹; Chaney et al., 1989). In comparison to the field results, Cd accumulation in the shoots of *S. marianum* plants growing in the contaminated soils was slightly higher.

In the two soils with acid pH (Soil 1 and Soil 3), *S. marianum* plants showed higher concentrations of Zn than the other two species (Fig. 6b). These Zn concentrations were also above the normal ranges for plants (15-150 mg kg⁻¹). Interestingly, the highest Zn concentrations in *S. marianum* shoots were found in those plants growing in Soil 3. This soil was obtained from a location in the Guadiamar Valley outside the spill-affected area, but likely exposed to diffuse TE contamination due to its proximity to the open-cast mine, as indicated by the large soil Fe concentration in this soil (Table 1). In this soil Zn concentrations in shoots of the three species were higher than those found in plants growing in the Soil 1, despite the latter soil having higher pseudo-total soil Zn concentrations (Table 1). This might indicate a high stabilization of Zn in Soil 1 after the addition of the amendment to correct soil pH.

529

530 The results suggest a significant accumulation of TE into the aboveground biomass of the
531 studied species, particularly in *D. viscosa* and *S. marianum*. The capacity of *C. cardunculus*
532 to accumulate Cd into foliage had already being described in different pot experiments
533 (Henández-Allica, 2008; Papazoglou et al., 2011; Lugany et al., 2012). Similarly, the
534 capacity of *D. viscosa* to accumulate Cd into leaves *in-situ* had also been observed in some
535 Mediterranean sites affected by long-term mining activities (Gómez-Ros et al., 2013). As
536 previously discussed, the ecological and technical implications of such TE accumulation
537 into the biomass of these two species should be further assessed in order to fully
538 characterize the potential of these species as a bioenergy crop for similar Mediterranean
539 contaminated areas.

540

541 *Biomass gross calorific value*

542

543 Gross calorific values were 15.1 ± 0.8 MJ kg⁻¹ for *C. cardunculus*, 17.2 ± 0.2 MJ kg⁻¹ for
544 *D. viscosa*, and 16.2 ± 0.7 MJ kg⁻¹ for *S. marianum* (plants growing in Soil 1 and Soil 2
545 pooled, mean $\pm$ standard deviation). For each species, GCVs in plants growing in Soil 2 and
546 Soil 1 (which showed the maximum and minimum biomass production, respectively) were
547 not statistically significant (one-way ANOVAs, p-values > 0.05). For *C. cardunculus* and *S.*
548 *marianum*, values of GCV were similar to those reported for some experimental cardoon
549 crops under Mediterranean conditions (Angelini et al., 2009a). *Dittrichia viscosa* GCV was
550 higher than that of the thistle species, and only slightly lower than the calorific value of

other common bioenergy crop such as *A. donax* and *M. x giganteus* (17.6 -18 MJ kg⁻¹; Angelini et al., 2005, 2009b).

Soil pH, organic matter and microbial activity after plant establishment

Analysis of the rhizospheric soils at biomass harvest showed a limited effect of the species on chemical soil variables. As expected for this relatively short-term (seven months) experiment, species identity did not influence soil organic matter content, and significant differences were limited to those differences in background organic C content among soil types (Supplementary Material, Fig. S2a). For pH, there was a significant influence of species identity in the Soil 1. In this soil, pH in the rhizosphere of *D. viscosa* plants was significantly lower than in the rhizosphere of the other two species (Supplementary Material, Fig. S2b). This is likely due to the differences among plant species in root biomass production and distribution through the soil profile. *D. viscosa* roots penetrated lower into the soil profile, where soil pH conditions were less modified by the sugar beet lime added to amend soil pH before planting. Despite topsoil at each container was homogenized after amendment application to promote its incorporation, the buffering effect of this amendment on soil pH seems to have been limited to the top 10 cm of soils, where most of *C. cardunculus* and *S. marianum* roots develop, while *D. viscosa* roots might have been exposed to lower pH conditions. Thus, the application of lime to Soil 1 containers might have introduced an uncontrolled gradient of chemical conditions in the pots filled with this type of soil. This situation, although not ideal for a controlled experiment, it is likely to be representative of field situations where amendment application and incorporation is restricted to the topsoil, plants with deep root systems being exposed to

less favourable chemical conditions than plants with shallower roots. The results suggest that *D. viscosa* roots are highly tolerant to those acid conditions in the rhizosphere that promote TE solubility, and reinforce its interest for the revegetation of similarly degraded soils.

For soil enzyme activities, there were some differences among species (Supplementary material, Table S4). Acid phosphatase and β -glucosidase activities varied among soil types, being considerably higher in the Soil 3 and the Soil 2, respectively (Fig. 7), which are the soils with the highest levels of organic matter content. This is in agreement with other works with remediated soils that showed that the activity of these two enzymes is mainly determined by the availability of organic C substrates (Caravaca et al., 2003; Madejón et al., 2014). In general, there was a trend for higher enzyme activities in the rhizosphere of *S. marianum* than in *C. cardunculus* or *D. viscosa* rhizospheres (Fig. 7). Some works have indicated that *S. marianum* produces high amounts of root exudates in response to defence signalling molecules (Badri et al., 2009), and as inhibitors of the germination of other species (Syed et al., 2014). Thus, it is possible that a high release of exudates by *S. marianum* roots could stimulate the activity of its rhizospheric microbial community.

The association between roots and AMF is an important factor during the initiation of cropping systems, since AMF increase the volume of soil explored by the root-mycorrhizae system, enhancing nutrient and water uptake (Jeffries et al. 2003). Analysis of the root colonization by AM fungi revealed differences among soils and plant species, as well as a significant soil $\times$ plant species interaction for the relative abundance of hyphal and

arbuscular structures (two-way ANOVA, Supplementary Information Table S5). In general, mycorrhizal colonization of *D. viscosa* roots was lower than that of *C. cardunculus* and *S. marianum* roots (Fig 8a) in all soils, and showed a higher relative abundance of vesicles than the other two species (Fig. 8b). This could be interpreted as an adaptation to soil contamination: this species produced higher biomass in Soil 1 than *C. cardunculus* or *S. marianum*, even though it showed a much lower degree of mycorrhization, which enhances nutrient and water uptake and protects the plant against the oxidative stress induced by TE toxicity (Firmin et al., 2015). The high abundance of vesicular structures in *D. viscosa* also suggests an adaptation of this species to TE contaminated soils, as vesicles enable the storage of TE, reducing the stress to which plants are subjected (Hildebrandt et al., 2007). The principal component analysis applied to soil microbial variables across all soil types confirmed the trend towards greater enzyme activities in the soils planted with *S. marianum*, and towards a higher abundance of arbuscular structures in the roots of this species, in comparison to the other two species (Supplementary Material, Fig. S3).

In summary, the results suggest a trend towards a greater stimulation of microbial activity by *S. marianum*, although enzyme activities were largely determined by the background properties of each soil type, particularly by OM content. This suggests, as found in other bioenergy cropping systems, that the legacy of the previous land use shapes the microbial response during bioenergy crop conversion (Kallenbach & Grandy, 2015).

4. Conclusions

620

621 Our field and greenhouse studies show that there is a potential for the use of some thistle
622 species (specially *S. marianum*) as bioenergy crops in Mediterranean soils contaminated by
623 TE. In the field, *S. marianum* was able to colonize highly contaminated soils. The
624 greenhouse experiment using soils collected from the same field sites confirmed that
625 biomass production in this species is less affected by soil contamination than *C.*
626 *cardunculus* biomass, and that the calorific value of its biomass is not influenced by the
627 presence of high concentrations of soluble TE in the growing media. In addition, some soil
628 enzyme activities were clearly enhanced in the *S. marianum* rhizosphere. The presence of
629 *D. viscosa* was not detected in the field survey, however this does not necessarily mean that
630 this species is not able to colonize highly contaminated soils, as other factors such as
631 propagule availability in the soil seed bank or in the surroundings might be limiting its
632 establishment in the contaminated area. Indeed, in the greenhouse experiment, *D. viscosa*
633 showed a capacity to produce a high biomass in soils collected from the same area with
634 high concentrations of soluble TE. Given the high calorific value of its biomass and the
635 large extension of its root system, which could contribute to the stabilization of soil
636 contaminants, this species could be considered as another promising bioenergy crop.
637 Germination of both *S. marianum* and *D. viscosa*, however, were hampered in the soil with
638 pH < 4, therefore pH soil correction would have to be accomplished before establishing
639 these species on extremely acid soils. Accumulation of Cd and other TE into the
640 aboveground biomass of these species was relatively high; field-scale experimental studies
641 that include an assessment of the net energy balance of these potential crops and the risk of
642 transfer of Cd and other TE to the food chain would be needed to confirm the suitability of

these species for the revegetation of TE contaminated lands with energy production purposes.

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Tables

Table 1. Soil organic matter (OM), pH and pseudo-total and soluble trace element concentrations in the experimental soils (mean $\pm$ standard deviation, N = 4) at the time of seed sowing. CaCl₂ extractable concentrations of As and Pb were below the detection limits.

Variable	Soil Type			
	Soil 1	Soil 2	Soil 3	Soil 4
OM (%)	1.12 $\pm$ 0.10	1.73 $\pm$ 0.16	4.19 $\pm$ 0.32	0.65 $\pm$ 0.04
pH	3.1 $\pm$ 0.1	6.4 $\pm$ 0.1	5.0 $\pm$ 0.1	6.8 $\pm$ 0.1
Pseudo-total TE (mg kg ⁻¹)				
As	136.6 $\pm$ 1.9	77.4 $\pm$ 1.31	18.1 $\pm$ 0.4	3.6 $\pm$ 0.1
Cd	0.56 $\pm$ 0.02	1.59 $\pm$ 0.02	0.24 $\pm$ 0.01	0.09 $\pm$ 0.01
Cu	159.0 $\pm$ 1.8	104.9 $\pm$ 0.9	37.8 $\pm$ 0.3	23.2 $\pm$ 0.4
Fe	32282 $\pm$ 928	23490 $\pm$ 429	28005 $\pm$ 1018	8364 $\pm$ 169
Pb	189.8 $\pm$ 5.4	156.0 $\pm$ 2.7	189.1 $\pm$ 4.8	11.6 $\pm$ 2.0
Zn	169 $\pm$ 2.6	484.3 $\pm$ 3.0	100.5 $\pm$ 0.68	15.6 $\pm$ 0.15
CaCl ₂ -soluble TE (mg kg ⁻¹)				
As	<0.005	<0.005	<0.005	<0.005
Cd	0.148 $\pm$ 0.005	<0.005	0.008 $\pm$ 0.002	<0.005
Cu	9.321 $\pm$ 0.24	0.151 $\pm$ 0.005	0.074 $\pm$ 0.006	0.079 $\pm$ 0.004
Fe	3.17 $\pm$ 0.97	1.16 $\pm$ 0.19	0.88 $\pm$ 0.14	1.07 $\pm$ 0.05
Pb	<0.005	<0.005	<0.005	<0.005
Zn	22.180 $\pm$ 0.534	0.105 $\pm$ 0.010	2.031 $\pm$ 0.134	0.089 $\pm$ 0.013

897 Table 2. Organic matter (OM), electrical conductivity (EC) and pseudo-total and CaCl₂-soluble trace element (TE) concentrations in
898 soils (0-10 cm depth) from the sites included in the field survey (mean $\pm$ standard deviation, N = 3 per site, except for site 1, N= 1).
899 CaCl₂ extractable concentrations of As were below the detection limits.

Variable	Sampling Site								
	1	2	3	4	5	6	7	8	9
OM (%)	2.2	3.12 $\pm$ 0.60	3.15 $\pm$ 0.49	3.14 $\pm$ 0.09	3.55 $\pm$ 0.54	2.32 $\pm$ 0.67	2.62 $\pm$ 0.37	3.98 $\pm$ 1.08	3.12 $\pm$ 0.04
pH	5	4.7 $\pm$ 1.8	4.4 $\pm$ 0.8	6.8 $\pm$ 0.2	6.7 $\pm$ 0.1	5.3 $\pm$ 0.6	6.9 $\pm$ 0.3	6.0 $\pm$ 0.1	6.8 $\pm$ 0.4
EC (μ S cm ⁻¹)	35	58 $\pm$ 36	208 $\pm$ 137	152 $\pm$ 11	152 $\pm$ 49	1850 $\pm$ 10	118 $\pm$ 7	128 $\pm$ 7	105 $\pm$ 3
Pseudo-total TE (mg kg ⁻¹)									
As	15.3	78.9 $\pm$ 14.6	138.9 $\pm$ 63.6	105.0 $\pm$ 49.3	102.6 $\pm$ 8.7	799.0 $\pm$ 175.6	31.2 $\pm$ 11.8	75.6 $\pm$ 12.9	60.08 $\pm$ 33.3
Cd	0.28	0.39 $\pm$ 0.13	0.58 $\pm$ 0.17	0.61 $\pm$ 0.30	0.77 $\pm$ 0.25	0.79 $\pm$ 0.83	0.54 $\pm$ 0.24	1.14 $\pm$ 0.36	0.45 $\pm$ 0.21
Cu	33.4	72.0 $\pm$ 6.5	226.1 $\pm$ 14.0	119.4 $\pm$ 26.5	115.9 $\pm$ 7.3	158.9 $\pm$ 73.8	62.1 $\pm$ 10.6	124.5 $\pm$ 18.3	43.8 $\pm$ 12.5
Fe	29036	36729 $\pm$ 667	40810 $\pm$ 4122	32728 $\pm$ 254	33605 $\pm$ 2523	60834 $\pm$ 9402	21750 $\pm$ 21.5	31710 $\pm$ 71	27090 $\pm$ 4192
Pb	46.7	167.6 $\pm$ 0.4	295.4 $\pm$ 111.5	220.9 $\pm$ 92.0	225.8 $\pm$ 27.6	2495.6 $\pm$ 1777.2	75.1 $\pm$ 21.7	153.6 $\pm$ 25.6	137.8 $\pm$ 77.7
Zn	91.5	154 $\pm$ 47	251 $\pm$ 64	267 $\pm$ 109	319 $\pm$ 53	394 $\pm$ 255	206 $\pm$ 58	402 $\pm$ 114	209 $\pm$ 93
Soluble TE (mg kg ⁻¹)									
As	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Cd	0.037	0.034 $\pm$ 0.034	0.080 $\pm$ 0.050	0.007 $\pm$ 0.011	0.004 $\pm$ 0.001	0.034 $\pm$ 0.031	0.004 $\pm$ 0.003	0.003 $\pm$ 0.003	0.004 $\pm$ 0.004
Cu	0.16	0.21 $\pm$ 0.01	0.30 $\pm$ 0.12	0.30 $\pm$ 0.05	0.26 $\pm$ 0.09	0.19 $\pm$ 0.02	0.25 $\pm$ 0.07	0.28 $\pm$ 0.07	0.19 $\pm$ 0.03
Fe	0.09	0.24 $\pm$ 0.01	0.44 $\pm$ 0.02	0.25 $\pm$ 0.11	0.15 $\pm$ 0.05	0.31 $\pm$ 0.12	0.29 $\pm$ 0.08	0.16 $\pm$ 0.05	0.14 $\pm$ 0.08
Pb	0.1107	0.011 $\pm$ 0.033	0.042 $\pm$ 0.045	<0.005	<0.005	0.408 $\pm$ 0.50	<0.005	0.003 $\pm$ 0.027	<0.005
Zn	2.1	3.60 $\pm$ 4.23	6.47 $\pm$ 5.27	0.52 $\pm$ 0.22	0.42 $\pm$ 0.12	2.60 $\pm$ 2.13	0.40 $\pm$ 0.13	0.46 $\pm$ 0.11	0.31 $\pm$ 0.04

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Figure captions

Fig. 1. Biomass of the main thistle species (total thistle biomass, and biomass of each species) growing in the Guadiamar river Valley across the gradient of soil contamination (mean + standard deviation).

Fig. 2. Trace element accumulation in the aboveground biomass of the main thistle species collected across the soil contamination gradient at the Guadiamar River Valley (mean + standard deviation). Dashed lines indicate the upper limit of the range of normal levels in plants (Chaney et al., 1989).

Fig. 3. a) Gross Calorific Value of the biomass of the three most abundant thistle species in the Guadiamar River Valley, at two sites with contrasted levels of biomass production (site 6 -heavily contaminated- and site 8 -moderately contaminated-, mean + standard deviation), b) estimates of potential energy yield at these two contrasted sites, calculated from gross calorific values and biomass production (mean + standard deviation). For each species, values with different letters indicate significant differences between sites (one-way ANOVA, significance level fixed to $p < 0.05$).

Fig. 4. Seed germination (% from total number of seeds per container) in the experimental soils.

Fig. 5. Total biomass (a), aboveground biomass (b), root biomass (c), shoot:root ratio (d) and dry matter content (e) of the studied species growing in the experimental soils (mean + standard deviation). For each species, values with different letters indicate significant differences among experimental soils (one-way ANOVAs and Tukey post hoc comparison tests applied to each species with soil type as categorical factor, significance level fixed to $p < 0.05$). ANOVA results are shown in Supplementary Information Table S1.

Fig. 6. Concentrations of Cd (a) and Zn (b) in the shoots of the three species growing in the greenhouse experiment. Results of the two-way ANOVA applied to test for differences among soil types and species are shown in Supplementary Information Table S3.

Fig. 7. Extracellular enzyme activities in the experimental soils at the time of biomass harvest (mean + standard deviation). For those variables with a significant variation among plant species, values with different letters indicate significant differences among species (one-way ANOVAs and Tukey post-hoc comparison tests applied to each soil type, with plant species as categorical factor, significance level fixed to $p < 0.05$). Results of two-way ANOVA applied to test for differences among soil types and species are shown in Supplementary Information Table S4.

944 Fig. 8. Arbuscular mycorrhizal colonization (mean + standard deviation) of roots in the
945 experimental soils: (a) degree of mycorrhizal colonization (% of root length), and relative
946 abundance of (b) vesicles and (c) arbuscules (% of total mycorrhizal structures).