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1 **Anthropogenic charcoal-rich soils of the XIX century reveal that biochar leads to enhanced fertility and fodder**
2 **quality of alpine grasslands**

3
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20
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22
23 **Abstract**

24
25 *Background and Aims*

26 Soil incorporation of charcoal (biochar) has been suggested as practice to sequester carbon, improve soil properties and
27 crop yields but most studies have been done in the short term. Old anthropogenic charcoal-rich soils in the Alps enable
28 to explore the long-term impact of charcoal addition to alpine grassland on seed germination, fertility and fodder
29 nutritive value.

30
31 *Methods*

32 A germination test and a growth experiment in pots with *Festuca nigrescens* Lam. and *Trifolium pratense* L. were
33 performed using three different substrates: control soil (i.e. sandy-loam brown acid soils with some podsolization),
34 charcoal hearth soil (i.e. charcoal-enriched anthropogenic soils derived from the carbonization of larch wood on flat
35 terraces) and control soil mixed with a fraction of fresh larch wood charcoal to reach the soil-charcoal ratio of 0.6.

36
37 *Results*

38 Both aged and fresh charcoal improved germination and markedly increased plant growth of the two plant species. The
39 addition of fresh charcoal had an initial detrimental effect that disappeared in the second and third growth cycles. Plant
40 Nitrogen:Phosphorus ratio revealed that growth was N-limited in the anthropogenic soils and P-limited in the control

41 and freshly amended soils demonstrating that biochar aging is critical to obtain a significant growth stimulation. Plant
42 nutrient contents revealed an improved fodder quality in both the charcoal amended soils.

43

44 *Conclusions*

45 Despite the occurrence of limited toxic effects on seedlings, larch wood charcoal appears to have positive effects on
46 fertility and fodder quality of alpine grasslands in the long term.

47

48 **Keywords:**

49 biochar, charcoal, alpine grasslands, fertility, N:P ratio, fodder nutritional value

50

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55 Franchi (Fondazione Edmund Mach) for her important advise on alpine pastures and meadows management and the
56 Italian Biochar Association (ICHAR).

57 **Introduction**

58 Alpine grasslands are considered critical biodiversity hotspots (Väre et al. 2003) providing a series of
59 ecosystem services (Fontana et al. 2013) and sustaining the production of typical food resources with added value
60 (Bovolenta et al. 2011). The persistence of these semi-natural ecosystems is based on ancient and specific management
61 techniques adapted to the local needs ensuring their productivity for thousands of years (Poschlod and Wallisdevries
62 2002) and the accumulation of one of the highest soil carbon stock (Gamper et al. 2007; Ciaia et al. 2010). Traditional
63 management techniques include intense land uses based on different stocking rates (different breeds and grazing period
64 lengths), mowing frequency, organic and inorganic fertilization (Maurer 2005), periodic re-sowing (Tozer et al. 2013)
65 and more rarely irrigation (Riedener et al. 2013). Major threats to these ecosystems arise from climate change, land
66 abandonment and inadequate intensification. In particular, abandonment has substantially reduced the surface area
67 occupied by mountain grasslands throughout Europe (Tasser et al. 2007), mainly leading to the expansion of secondary
68 forests (Tasser and Tappeiner 2002). Inadequate management techniques are instead increasing the risks of soil erosion,
69 landslides and avalanches (Tasser et al. 2003), often causing the diffusion of unpalatable plant species (Krahulec et al.
70 2001), loss of biodiversity (Dullinger et al. 2003), decreasing touristic attractiveness (Hunziker 1995) and, in some
71 instances, the loss of important soil organic carbon stocks (Poepflau and Don 2013). In the context of these changes,
72 appropriate management of mountain pastures and meadows is becoming a societal priority that should be based on
73 novel techniques ensuring the socio-economic viability of mountain communities, the conservation of their beauty as
74 well as their ecosystem services.

75 The use of biochar, a carbon rich co-product of pyrolysis, as soil amendment is currently receiving a lot of
76 attention as it can improve the physico-chemical properties of soils, reduce nutrient leaching (Glaser et al. 2001),
77 increase water infiltration and water holding capacity (Lim et al. 2016; Novak et al. 2016), enhance cation exchange
78 capacity (Liang et al. 2006), soil aeration (Case et al. 2012), thus stimulating biomass yields (Jeffery et al. 2011;
79 Biederman and Harpole 2012). Moreover, because of its substantial recalcitrance to microbial degradation, biochar
80 application to soil is also considered as an effective way to sequester atmospheric carbon (Lehmann et al. 2006; Sohi et
81 al. 2009; Criscuoli et al. 2014; Wang et al. 2015). On the other hand, the use of biochar as a soil amendment entails also
82 some risks which are not yet fully understood (Kuppusamy et al. 2016). In fact, biochar can be a source of toxicants
83 such as polycyclic aromatic hydrocarbon (PAHs) (Kloss et al. 2012), can retain heavy metals, can suppress the efficacy
84 of applied pesticides and may induce changes in soil microbial community composition and structure (Jenkins et al.
85 2016) with possible influence on the microbial-mediated transformation of nutrients. Moreover, its role in offsetting C
86 and the other greenhouse gases' emissions has been questioned because of the possible priming effect on the native soil
87 organic matter (Ventura et al. 2015; Fang et al. 2015), the induced changes in the surface albedo (Genesio et al. 2012)
88 and the possible associated C aerosol emissions (Genesio et al. 2016).

89 The large majority of studies involving biochar applications, including those made on lowland grasslands (Van
90 de Voorde et al. 2014; Schimmelpfennig et al. 2014; Schimmelpfennig et al. 2015), are based on short-term
91 experiments, mostly made immediately after biochar application. Only the work of Hernandez-Soriano et al. (2015)
92 showed that 150 years old charcoal kiln areas located in agricultural fields were still able to increase the productivity of
93 maize (+10%). As biochar properties were shown to change with time of exposure (i.e. ageing), in particular through
94 surface oxidation (Liang et al. 2006), short-term studies may be insufficient to assess its impact on soil fertility in the
95 long-term. The productivity of mountain grassland ecosystems is the result of a complex set of interactions between
96 grazing pressures and nutrients export, plant species composition and the establishment of soil microorganisms and soil

97 micro-fauna communities, which are related to specific functional traits, whose dynamics necessarily require long-term
98 studies (Grigulis et al. 2013).

99 To properly address this critical issue, this paper assesses the effects of 158 years old anthropogenic charcoal
100 rich soils (Criscuoli et al. 2014), derived from carbonization of larch wood (*Larix decidua* L.) on flat terraces
101 (Backmeroff 2013), on germination, growth and nutritive value of a typical grass and mountain leguminous species
102 (*Festuca nigrescens* Lam. and *Trifolium pratense* L. subsp. *nivale* (Koch)) in comparison to native (control) soil and
103 control soil amended with fresh biochar derived from larch wood. The presence of multiple charcoal rich soils gave us
104 the opportunity to work on a fully replicated scheme, to be considered as an analogue of a deliberate centennial time-
105 scale application of biochar to a mountain grassland soil. The hypothesis is that plant productivity and nutritional value
106 of both plant species, and especially of *T. pratense*, are higher in charcoal hearth soils compared to control soils and to
107 soils amended with fresh larch charcoal because of char ageing (Pusceddu et al. 2013). The effects on seeds germination
108 are expected to be less evident.

109

110 **Materials and methods**

111

112 *Study site, soil sampling and charcoal production*

113 Several charcoal-enriched anthropogenic soils (charcoal hearths) derived from the carbonization of larch
114 (*Larix decidua* L.) wood on flat terraces, dating back to between 1500 and 1858 (Backmeroff 2013), were identified in
115 Val di Pejo (Trentino, Northern Italy) at an altitude of 2150 m a.s.l., in a larch alpine grassland grazed in summer. A
116 complete description of the site, soil characteristics and historical charcoal production can be found in Criscuoli et al.
117 (2014). In brief, the charcoal hearths' soils are made of a surface organic horizon (~2 cm) and a thicker black
118 anthropogenic layer (~20 cm) rich in charcoal residues left after the carbonization 158 years ago and today well mixed
119 with the pre-existing soil layer. The control soils are sandy-loam brown acid soils with some podsolization (Lithic
120 Dystrudept and Entic Haplorthod) (Smith and Atkinson 1975). Both control and hearths' soils have a pH of 5.1. The
121 hearths' soils are very rich in carbon ($26.2 \pm 5.3 \text{ kg C m}^{-2}$), 90% of which is contained in the charcoal, and are also
122 richer in nutrients than the surrounding control soils (Tab. 1).

123 Three paired sites (i.e. charcoal hearth and native soil as control) were selected on the basis of common aspect
124 (SE) and conservation state (no significant geo-morphological dynamics or recent anthropogenic disturbances). In
125 September 2014, 20 L of soil were sampled with shovels from the center of the three hearths and in the corresponding
126 adjacent control areas, after removing the top organic layer.

127 Fresh charcoal was produced from larch wood at 450°C, the average temperature occurring in traditional
128 carbonization wood piles (FAO 1987). Fragments of larch wood were carefully wrapped in Aluminum foil and placed
129 in a preheated muffle furnace at a temperature of 450°C for 10 minutes. The carbon content of the freshly produced
130 charcoal was $76 \pm 7 \text{ gC kg}^{-1}$, the nutrient composition is reported in the Tab. 1 and the specific surface area (total BET)
131 was $239.5 \text{ m}^2 \text{ g}^{-1}$.

132

133 *Germination test*

134 Seeds of *Festuca nigrescens* Lam. and *Trifolium pratense* L. subsp. *nivale* (Koch) were obtained from a
135 nursery. Total germination and germination rates for each species were assessed through a test in petri dishes. Three
136 different substrates were considered: control soil (C), charcoal hearth soil (H) and control soil mixed with a fraction of

137 fresh larch wood charcoal (CC) to reach the soil-charcoal ratio of 0.6 equivalent to a charcoal dose of 39 kg m⁻², which
138 was estimated as the initial char input at the three charcoal hearths 158 years ago (Criscuoli et al. 2014). 54 dishes were
139 prepared (2 species x 3 soil types x 3 sampling areas x 3 replicates). In each petri dish, 10 seed of one species were
140 sown and dishes were maintained in the dark. Germination was determined when the coleoptile was ≥ 2 mm for *F.*
141 *nigrescens* and when the two cotyledons were visible and free from the seed coat for *T. pratense*.

142

143 *Growth experiment*

144 The three soil types were also used as substrates for a plant growth experiment in small pots of 135 cm³. Prior
145 to sowing, the pots were left for two months in a greenhouse with regular irrigation in order to allow the seeds
146 contained in the seed bank to germinate spontaneously. Naturally germinated plants were then periodically removed
147 manually. To eliminate possible confounding effects due to differences in the soil temperature, caused by different light
148 reflection/absorbance properties (albedo), the soil surface of each pot was covered with an uniform thin layer of black
149 inert quartz granules of 1.5 mm diameter (Granulati Zandobbio, Bergamo). The plants were grown in monoculture for a
150 total of 144 pots (2 species x 3 soil types x 3 sampling areas x 8 replicates). Seeds were sown at an initial density of 20
151 seeds pot⁻¹ on 7th January 2015 and the pots were stored on trays in a greenhouse, following a fully randomized
152 experimental design. Air temperature and light in the greenhouse were left to fluctuate following external conditions,
153 however temperature never dropped below 15°C by means of an automated heating system, while a minimum day
154 length of 12 hours was ensured using artificial illumination during the winter months. Soil temperature was regularly
155 checked at 3 cm depth using a digital thermal probe (109SS-L, Campbell scientific, Inc.) and no significant difference
156 was observed among the soil treatments.

157 Nebulized irrigation provided water automatically at regular intervals, four times a day. Seven weeks after
158 sowing, plant density was reduced to six plants per pot. Subsequently, three growth cycles were considered with
159 complete aboveground biomass harvest made 17 (1st cycle), 22 (2nd cycle) and 30 weeks (3rd cycle) after sowing. At
160 each harvest, the plants were oven dried at 80°C for 48 hours and weighed. At the end of the 3rd cycle, the roots were
161 manually separated from the soil, washed and dried for 48 hours at 80°C and weighed.

162

163 *Plant, charcoal and soil physico-chemical analysis*

164 Plant, soil and charcoal samples were dried and finely ground prior to analysis.

165 Isotopic ratios were determined with an Isotope Ratio Mass Spectrometer (Thermo Fischer Scientific, Delta V
166 Plus). Natural abundances of stable carbon (δ¹³C) and nitrogen (δ¹⁵N) isotopes were measured for both plant species and
167 the fine soil fraction (<2 mm) of hearth and control soils as well as for both the ancient and the freshly produced
168 charcoal fragments. The carbon isotope discrimination (Δ) was then calculated according to Farquhar and Richards
169 (1984):

$$170 \quad \Delta = \frac{\delta_a - \delta_p}{1 + \delta_p}$$

171 where δ_a is the δ¹³CO₂ in the air and δ_p is that of plant carbon.

172 Total Ca²⁺, K⁺, Mg²⁺, Na⁺, P, Cu, Fe were determined on oven-dried plant subsamples (105°C for 24 h) from
173 the second harvest, according to the EPA method 3052 (USEPA 1996) using an ICP-OES spectrophotometer (Varian

174 Inc., Vista MPX). Total carbon and nitrogen concentrations were determined via total combustion in an elemental
175 analyzer (EA Flash 1112 ThermoFinnigan).

176 Soil and charcoal pH were measured in a soil-charcoal/distilled water solution (1:4 ratio as reported in Di
177 Lonardo et al. 2013; Vaccari et al. 2015).

178 The surface area of fresh charcoal was calculated by using the BET (Brunauer-Emmet-Teller) method and
179 Langmuir method applied to nitrogen adsorption data in the relative pressure (P/P^0) range of 0.1-0.44.

180

181 *Statistical analyses*

182 Data were analyzed using R (version 2.15.3). The effects of the 3 treatments (C, H and CC) and of the 3 blocks
183 (3 charcoal hearths and 3 control soils) were analyzed by a two-way ANOVA for each plant species. Individual
184 comparisons were based on the Tukey's HSD post-hoc test. The data were checked for normality and homogeneity of
185 variances by inspection of the residuals vs. fitted values and the Normal Q-Q plot. When data did not fulfill these
186 requirements, they were log-transformed or square root-transformed. In the case of negative data ($\delta^{13}\text{C}$), the data were
187 standardized. Germination rates were compared using a binomial test (test for equality of proportions). The p-values of
188 individual comparisons were corrected with the Holm method. All data in the text, figures and tables are reported as
189 mean \pm standard deviation, if not differently stated.

190

191 **Results**

192 *Germination test*

193 Plants started to germinate after 96 hours of incubation. The percentage of germinated seeds after 96 and 120
194 hours from sowing was higher in CC compared to C and H both for *F. nigrescens* and *T. pratense* (Tab. 2), while early
195 germination rates were similar or the same in C and H. Looking at the total number of germinated plants, almost all
196 seeds germinated in all soil types (between 78 and 94%). The plant germination rates at the end of the experiment for
197 both plants species were higher in both CC and H than in C (*F. nigrescens*: CC vs. C +14%, $p=0.08$ and H vs. C +13%,
198 $p=0.09$; *T. pratense*: CC vs. C +13%, $p=0.05$ and H vs. C +12%, $p=0.07$).

199

200 *Plant growth*

201 At the end of the first growth cycle (week 1-17), both plant species grew more in H than in C and CC (Fig. 1)
202 and the lower growth observed in the CC was highly statistically significant if compared to both H and C. The relative
203 changes in plant biomass in comparison to C were +21, +9% for H and -35, -71% for CC, for *T. pratense* and *F.*
204 *nigrescens*, respectively. The mean plant growth rate (MGR) was much lower in CC (*T. pratense*: $1 \text{ mg day}^{-1} \text{ pot}^{-1}$; *F.*
205 *nigrescens*: $1.5 \text{ mg day}^{-1} \text{ pot}^{-1}$) than in C (*T. pratense*: $2.3 \text{ mg day}^{-1} \text{ pot}^{-1}$; *F. nigrescens*: $3.2 \text{ mg day}^{-1} \text{ pot}^{-1}$) and H (*T.*
206 *pratense*: $2.8 \text{ mg day}^{-1} \text{ pot}^{-1}$; *F. nigrescens*: $3.5 \text{ mg day}^{-1} \text{ pot}^{-1}$).

207 At the end of the second growth cycle, biomass was again significantly higher in H than in C and CC for both
208 species (Fig. 1). *T. pratense* and *F. nigrescens* plants grown on H produced almost 3 and 1.5 times more than on C,
209 respectively. Plants grown on CC performed better than on the control soil (both *T. pratense*, *F. nigrescens* = +37%),
210 even though the difference was significant only in the case of *T. pratense* ($p=0.01$). MGR was accelerated compared to
211 the first cycle and rose to 10.7 and $41.1 \text{ mg day}^{-1} \text{ pot}^{-1}$ for *T. pratense* and to 16.6 and $40.7 \text{ mg day}^{-1} \text{ pot}^{-1}$ for *F.*
212 *nigrescens* grown in C and H, respectively.

213 Similarly to the second cycle, at the end of the third cycle plant biomass was significantly higher in H than in
214 the other two treatments (Fig. 1). *F. nigrescens* plants grown on H produced almost 1.5 times more than plants grown
215 on C and *T. pratense* 0.6 times more. Plants grown on CC performed better than on C (*F. nigrescens* = +51%, *T.*
216 *pratense* = +22%) even though the difference was significant only in the case of *F. nigrescens* (p=0.01). The MGR
217 decreased compared to the second cycle to 24.1, 9.9 and 15 mg day⁻¹ pot⁻¹ for *F. nigrescens* and to 19.1, 12.1 and 14.9
218 mg day⁻¹ pot⁻¹ for *T. pratense* grown in H, C and CC, respectively.

219 Overall, the total amount of biomass produced over the three cycles (above and belowground) was 88% and
220 108% higher in H compared to CC (p<0.001) and 114% and 148% higher in H compared to C (p<0.001) for *F.*
221 *nigrescens* and *T. pratense*, respectively (Fig. 2). Total biomass was slightly higher in CC compared to C for both plant
222 species, but the differences were not significant (*F. nigrescens*: p=0.29, *T. pratense*: p=0.25).

223 The root:shoot ratio, was significantly higher for *T. pratense* plants grown on H than C (p=0.02), while for *F.*
224 *nigrescens* no differences in the ratio were detected among treatments (Fig. 3).

225

226 *Soil and plant nutrients*

227 The content of Ca²⁺, Mg²⁺, Na⁺ and P (mg g⁻¹) in the plants tissues (Tab. 3) was the highest in *F. nigrescens*
228 and *T. pratense* grown on H followed, in most cases, by plants grown on CC. The differences in the nutrient content of
229 *T. pratense* between H and C were significant at p=0.10 for all the nutrients, while no significant differences between H
230 and CC in *T. pratense* and among all treatments for *F. nigrescens* were detected (Tab. 3). The K⁺ content of *T. pratense*
231 was higher in plants grown on H (H vs. CC: p=0.18; H vs. C: p=0.06), but for *F. nigrescens* plants it was higher when
232 plants were grown on CC, even though not significantly. An opposite trend was observed for N, which was higher for
233 plants grown on C for both species, followed by CC and H. Fe content showed a very high variability in plants grown
234 on the same soil type, while soil Cu content was very similar for all the three treatments. The nutrient content of *T.*
235 *pratense* was usually higher than that of *F. nigrescens* with the exception of P that was higher for *F. nigrescens* plants
236 when grown on H and C.

237 The N:P ratio of *F. nigrescens* and *T. pratense* plants grown on H was 10.4 and 10.7, respectively, while plants
238 grown on C showed a ratio of 18.3 and 21.5 and plants grown on CC soils 23.3 and 16.3 (Fig. 4).

239 Soil nutrient content data were taken from Criscuoli et al. (2014) and are here reported in the Tab. 1. The soil
240 N:P ratio in soil was 13.3, 5.7 and 11 in C, H and CC, respectively (Fig. 4).

241 Plant P concentration increased with increasing soil concentration. The highest values were observed for the H
242 soil and in the plants grown on it, while soil and plants grown on CC and C gave very similar results (Fig. 5). Plant K⁺
243 concentration correlated positively with the soil nutrient contents. Concentrations of both soils and plants decreased in
244 the order H>CC>C (Fig. 5). We observed a similar behavior for Ca²⁺ in the case of *T. pratense*, while the content of *F.*
245 *nigrescens* was insensitive to the soil content variations (Fig. 5). Na⁺ content was the lowest for H soils. The
246 concentration of the plants tissues decreased for *T. pratense* with increasing soil concentrations, while values observed
247 for *F. nigrescens* did not reflect the Na⁺ soil content (Fig. 5). Mg²⁺ concentration of the soil was not reflected in the
248 content of plants for both species. The Mg²⁺ content of *T. pratense* was double compared to *F. nigrescens* (Fig 5, Tab. 1
249 and Tab. 3).

250

251 *Isotopic signature*

252 The ^{13}C discrimination (Δ) at the end of the first growth cycle was significantly higher for *F. nigrescens* and *T.*
253 *pratense* plants grown on CC (24.5 and 23.6‰, respectively) than on C (21.8‰ with $p=0.0006$ and 22.1‰ with
254 $p=0.051$) and H (21.5‰ with $p=0.0004$ and 22.1‰ with $p=0.051$).

255 At the end of the second growth cycle, the Δ of both plant species grown on H and C slightly increased in
256 comparison with the first one (*T. pratense*: H +0.74‰, C +0.42‰; *F. nigrescens*: H +1.02‰, C +0.56‰), while, when
257 they were grown on CC, the Δ decreased (*T. pratense*: -0.56‰; *F. nigrescens*: -1.55‰) to similar values for both
258 species (22.99‰) and to the other two soil treatments. At the third harvest, plants showed smaller Δ values and no
259 differences among treatments (Fig. 6).

260 At the end of the first growth cycle, the $\delta^{15}\text{N}$ was very similar for *T. pratense* and *F. nigrescens* plants grown
261 on C soils (6.9 and 7‰, respectively). The isotopic signature of plants grown on H was lower and very similar for the
262 two plant species as well (*T. pratense*: 4.9‰; *F. nigrescens*: 5.1‰). Similar data were observed at the end of the second
263 growth cycle (*T. pratense* C: 6.5‰, H: 4.8‰; *F. nigrescens* C: 6.4‰, H: 5.8‰), while, at the third harvest, the isotopic
264 signature rose in both species and became slightly higher for plants grown on H as compared to those grown on C (*T.*
265 *pratense* C: 7.2‰, H: 7.7‰; *F. nigrescens* C: 7.1, H: 7.3‰; Fig. 7). A very different trend was observed for plants
266 grown on CC where, at the end of the first growth cycle, *T. pratense* plants showed a $\delta^{15}\text{N}$ of -0.07‰, much lower than
267 the other treatments, especially compared to C (CC vs. H $p=0.19$; CC vs. C $p=0.08$). *F. nigrescens* had a $\delta^{15}\text{N}$ equal to
268 3.3‰ (CC vs. H $p=0.24$; CC vs. C $p=0.04$). After the second growth cycle, the isotopic signature of both *T. pratense*
269 and *F. nigrescens* increased (1.4‰ and 4.8‰, respectively), but did not equal the values observed for the other two soil
270 treatments (*T. pratense*: CC vs. H $p=0.06$, CC vs. C $p=0.02$; *F. nigrescens*: CC vs. H $p=0.25$, CC vs. C $p=0.07$). The
271 $\delta^{15}\text{N}$ of both species increased also during the third growth cycle (*T. pratense*: 5.3‰ and *F. nigrescens*: 5.8‰), getting
272 closer to the signature measured in the other treatments (ranging between 7.1 and 7.7‰; Fig. 7).

273 The control soil had a $\delta^{15}\text{N}$ equal to $3.9\pm 1.3\%$, markedly higher than H ($2.1\pm 1.1\%$), while the ancient and the
274 freshly produced charcoal had very similar $\delta^{15}\text{N}$ signatures ($1.1\pm 1.6\%$ and $0.9\pm 0.2\%$, respectively).

275

276 Discussion and conclusions

277 The three substrates had differential effects on germination. For both plant species, early germination rates
278 were higher in the control soils amended with fresh charcoal (CC) than in hearths soils (H) and control soils (C).
279 Moreover, the total number of germinated seeds 10 days after sowing was stimulated for both species by the presence of
280 old (H) and fresh charcoal (CC) amendment (Tab.2). Previous research on *T. pratense* by Van de Voorde et al. (2014)
281 found no effect of biochar addition to the soil substrate on the germination and Solaiman et al. (2011) found a dose-
282 dependent negative germination effect on another *Trifolium* species. In contrast, we did not observe any inhibitory
283 effect on seeds germination, but a tendency to an increase in germination in the presence of both fresh and old charcoal.
284 These positive effects could be related to the presence of specific compounds such as karrikins, a family of butenolides,
285 which are known to be contained in charcoal (Flematti et al. 2008; Nelson et al. 2012). Karrikins act as germination
286 stimulants and have been recently isolated, identified (Flematti et al. 2004) and successfully synthesized (Flematti et al.
287 2011) even though the mechanisms by which they might trigger seed germination are far from being completely
288 described. Another possible mechanism explaining the higher germination rates in CC and H could be related to the
289 ability of charcoal to adsorb biogenic phyto-toxins eventually present into the soil (Garnett et al. 2004; Hille and Den
290 Ouden 2005).

291 In the pot experiment, at the end of the first growth cycle, both species grew better on H than on C and CC
292 (Fig. 1). Reduced growth on CC was remarkable and attributable to a stress effect caused by the addition of fresh char.
293 Such a stress caused a decrease in the photosynthetic capacity of plant leaves, which was reflected in an increase in their
294 $\Delta^{13}\text{C}$ (Fig. 6) (Brugnoli et al. 1989). A possible source of stress affecting plant growth on CC could have been the
295 difference in the pH between the fresh larch charcoal (pH=7.1) and the control soil to which it was added to (pH=5.1).
296 Soil alkalization is known to have detrimental effects on plants as high pH generally causes metal ions to precipitate,
297 thus affecting the absorption of inorganic ions and disrupting the ionic balance of tissues (Chen et al. 2009). In our case,
298 the bulk soil pH of CC rose to 5.8 and we cannot exclude that plant roots were in direct contact with charcoal fragments
299 with a higher pH. However, 7.1 is a value close to neutrality and, moreover, Gell et al. (2011) did not find a clear
300 relationship between pH and short-term phyto-toxicity on lettuce, radish and wheat plants. Thus, the toxic effect
301 observed in the first growth cycle is very likely not to be linked to a pH stress. On the contrary, there could be a direct
302 phytotoxic effect of charcoal on plant growth, both on germination and root and shoot growth as observed in previous
303 studies that related the phyto-toxicity to the presence of chemicals in the biochar. For example, Rombolà et al. (2015)
304 made the hypothesis that the phyto-toxicity was due to NH_3 , Volatile Fatty Acids and Benzoic acids contained in the
305 charcoal, while Gell et al. (2011) associated the toxic effect of different kinds of biochar with their high water-soluble
306 salts content, such as Chloride and Sodium, and possibly aliphatic and aromatic hydrocarbons such as phenols.

307 Observations made during the first growth cycle have important implications regarding the possibility of using
308 biochar as a soil amendment in the framework of mountain pastures and meadows management and restoration. The
309 data showed that while long-term exposure of pyrogenic carbon in soil, as occurred in H, buffers any possible toxic
310 effect, the sudden addition of biochar could have detrimental effects on plant growth. Even if the exact temporal
311 dynamics of the transition between toxic and non-toxic conditions following biochar incorporation remains unknown,
312 these data already suggest that the negative effect of charcoal incorporation into the soil is transitory as better
313 environmental conditions certainly develop during biochar ageing. Moreover, transitory toxic effects can be avoided if
314 is a) washed with water or an organic solvent, b) degraded via a biological activity through composting or mixing it
315 with activated sludge (Bargmann et al. 2013; Rombolà et al. 2015) or c) dried at temperatures between 100 and 300°C
316 for 24 hours (Kotowski and Oleszczuk 2015) before being amended to soil. It is also likely that lower doses than used
317 in the present experiment, could reduce detrimental effects on the plant growth, even in the very early stages.

318 The hypothesis of a transitory toxic effect of biochar on plant growth is also confirmed when looking at
319 biomass production during the second and the third growth cycles (Fig.1). In fact, at the end of the second cycle,
320 biomass was again significantly higher for plants grown on H compared to those grown on C. *T. pratense* plants grown
321 on H produced almost threefold if compared to C and *F. nigrescens* 1.5-times more. On the other hand, conversely to
322 what was observed in the first cycle, plants grown on CC performed better than those grown on C (both *T. pratense* and
323 *F. nigrescens*= +37%). The recovery of both species grown in CC was also confirmed by the $\Delta^{13}\text{C}$, which was not
324 significantly different from the other two treatments (Fig. 6). The mean plant growth rate measured for plants grown on
325 H was 4 and 3 times higher than those of plants grown on C for *T. pratense* and *F. nigrescens* and 2 and 3 times higher
326 compared to those grown on CC, respectively, indicating a clearly higher fertility in charcoal hearths soils than in their
327 natural counterparts and soils with fresh biochar. Similar results were obtained by Naisse et al. (2014), who observed
328 much higher (micro-)biological activity in charcoal hearths as compared to control soils without ancient charcoal. The
329 increase in the charcoal hearth soils' fertility of our study site can be explained by the accumulation over time of soil

330 nutrients, their higher plant availability as well as improved physical characteristics of charcoal hearth soils compared to
331 control (Criscuoli et al., 2014, Tab. 1).

332 Similarly to the second cycle, also at the third harvest, plant biomass was significantly higher for H than for the
333 other two treatments (Fig. 1). However, the biomass measured for *T. pratense* grown on H at the end of the third cycle
334 was lower in comparison to the second one. This could be due to the higher temperatures registered during the third
335 growth cycle (summer) compared to the second one (spring), which caused the spread of necrotic tissues in the
336 leguminous species (data not shown).

337 Overall, the total plant biomass produced over the three cycles (above and belowground) was significantly
338 higher when plants were grown on H compared to CC and C both for *F. nigrescens* and *T. pratense* (Fig. 2). No
339 significant differences were detected between plants grown on CC and C, making the positive impact of charcoal on the
340 long term productivity of alpine grasslands even more evident than the results of one single growth cycle.

341 The root:shoot ratio, a widely used indicator of plants health, gave differential results according to the species.
342 It was significantly higher for *T. pratense* plants grown on H than for those grown on C, while for *F. nigrescens* no
343 difference in the ratio was detected among treatments (Fig. 3). The different behavior between the two species is related
344 to the high interspecific variation in root:shoot ratios as reported by Koerner & Renhardt (1987). The results observed
345 for *T. pratense* seem to be in contrast with previous literature. In fact, for another species of clover (Solaiman et al.
346 2012), inconsistent effects of biochar on root:shoot ratio were observed. At high application rates charcoal had a
347 negative effect. Moreover, it is known that the development of the root compartment may be smaller compared to
348 aboveground biomass in nutrient rich soils (Agren and Franklin 2003), but according to our results *T. pratense* had a
349 much higher root to shoot ratio in H soil, which shows the highest nutrient contents, both total and available (Criscuoli
350 et al., 2014, Tab. 1). A possible explanation of high root to shoot ratios for *T. pratense* grown on H may be that roots
351 development was more driven by hormonal factors rather than nutrients concentration. In fact, an increase in ethylene
352 production, a plant hormone with important implication for plant growth and development, has been previously
353 observed from biochar and biochar-amended soil (Spokas et al. 2010).

354 Soil and plants nutrient contents provided useful information to further examine and interpret our results. The
355 idea that the ratios of N, P and K in plant tissues provides an indication of the relative availability of these nutrients in
356 the soil has often been discussed since the Von Liebig's Law of the Minimum (Von Liebig 1840). Koerselman &
357 Meuleman (1996), among others, assumed that the N:P ratio of plant tissues is a reliable indicator of soil fertility and
358 proposed, on the basis of a meta-analysis of experimental data, that N:P thresholds of plant tissues can be used as
359 indicators of N-limitations ($N:P < 14$) or P limitations ($N:P > 16$) or their co-limitation ($14 < N:P < 16$) to plant growth.
360 Accordingly, plants of both *F. nigrescens* and *T. pratense* species grown on H soils can be considered mostly N-limited
361 (leaves N:P ratios of 10.4 and 10.7, respectively), while they were P-limited when grown on C and CC soils (N:P ratios
362 > 16 ; Tab. 3 and Fig. 4). Thus, the lower plant biomass production in C and CC could be related to a P-limitation. In
363 fact, total and available P-concentrations of these soils were similar and about one third compared to that of H soils
364 (Criscuoli et al., 2014; Tab. 1). The soil P concentration for C and CC was reflected in the plants concentration which
365 was similar (Tab. 3 and Fig. 5), showing that P added via charcoal was available for plants. This has been previously
366 demonstrated specifically for larch wood (*Larix gmelinii* Rupr.) charcoal produced at 400°C (available P: 42.7 mg kg⁻¹
367 biochar) and for the larch seedlings grown on a mixture of sand larch biochar which had higher foliar P concentrations
368 in the presence of higher rates of char (Makoto et al., 2011). Thus, the higher P content observed in the H soils and
369 plants growing on it have to be explained via other processes taking place in the long term other than the initial P input

370 via charcoal addition. It has been shown that P availability in charcoal amended soils is enhanced also via an increased
371 mycorrhizal colonization and a change in soil P fractionation (Graber et al., 2015). Moreover, charcoal hearth soils
372 might have accumulated the P atmospheric depositions due to desert dust transport and atmospheric pollution
373 (Bergametti et al. 1992) or dung released from cattle over the 158 years of exposure in soil.

374 The higher plant N:P ratio observed in CC soils for *F. nigrescens* compared to *T. pratense* (Fig. 4) is due to the
375 higher N content in *T. pratense* (19.2 vs. 14.2 mg g⁻¹ in *F. nigrescens*), while P content is almost the same in the two
376 species (0.9 vs. 0.8 mg g⁻¹, respectively; Tab. 3). The higher N content of the leguminous species might be explained by
377 its nitrogen fixing capacity and this is also confirmed by the lower $\delta^{15}\text{N}$ that we observed for plants grown on CC in the
378 first and second growth cycle, much closer to the atmospheric value (0 ‰) as compared to *F. nigrescens* (Fig. 7). These
379 results are in line with several previous studies reporting an increase in nitrogen fixation in biochar amended soils
380 (Rondon et al. 2006; Ogawa and Okimori 2010; Güereña et al. 2015; Van Zwieten et al. 2015) which has been mainly
381 related to a greater B, Mo and P availability. The $\delta^{15}\text{N}$ signature of *F. nigrescens* plants grown on CC was higher than
382 the signature of *T. pratense* grown on the same soil, but was lower than the signature of plants of the same species
383 grown on the other soil types along the three growth cycles. This might be explained by the uptake of nitrogen
384 contained in the fresh charcoal fragments (De la Rosa and Knicker, 2011), as the nitrogen isotopic signature of charcoal
385 fragments is close to 1‰, while control soils have a signature of 3.9 ± 1.3 ‰.

386 Similar to P, the concentration of K⁺ in tissues of both plant species and of Ca²⁺ in *T. pratense* plants correlated
387 positively with soil nutrient content (Fig. 5), with higher concentrations observed in the case of higher biomass
388 production (H>CC>C). Similar observations were made by Schimmelpfennig et al. (2015). Even if the Ca²⁺ content is
389 much lower in C compared to H and CC, it cannot be considered as limiting factor as its concentration is well above the
390 critical deficiency levels (0.5-1.5 cmol_c kg⁻¹) reported by Kopittke and Menzies (2007). The concentration of Ca²⁺ was
391 doubled in the leguminous species compared to grass, in agreement with literature results (Juknevičius and Sabiené
392 2007).

393 Na⁺ content of the plants did not reflect the concentration of the nutrient in the soil (Fig. 5). Sodium is toxic at
394 high concentrations for most of the plant species (Greenway and Munns 1980), so the adsorption through the plant
395 roots' cell is limited as much as possible by mechanisms of selective uptake and ion exclusions (Schachtman and Liu
396 1999). The behavior of Na⁺ is opposite to what was observed for K⁺. The two ions are very similar for their ionic radius
397 and ion hydration energies, factors determining their movement through the cell's membrane (Hille 1992), but the
398 competition between the two ions in soils which are not saline is usually in favor of K⁺ because it is not toxic. On the
399 contrary, it is fundamental for plant growth of all species and it is the cation with the highest concentration in plant
400 tissues (Mäser et al. 2002). As for Ca²⁺, the concentration of *T. pratense* plants is double, or more, compared to *F.*
401 *nigrescens* even if *T. pratense* is less tolerant to salinity than *F. nigrescens* (FAO 2002). However Na⁺ concentration in
402 the leaves of *T. pratense* did not reach toxic levels, in fact no detrimental effects were observed on the plant growth.

403 Similarly to Na⁺, higher soil concentrations of Mg²⁺ did not correspond to any increase in the magnesium
404 content in plant tissues. The concentrations of Na⁺ and Mg²⁺ were lower (H<CC<C) in the soils with elevated K⁺ and
405 Ca²⁺ concentrations (H>CC>C). This may be related to the competition between cations given a certain cation exchange
406 capacity of the soil. The fact that lower Mg²⁺ soil content was not reflected by a lower concentration in plants tissues
407 might be seen as the evidence of a proper cations balance in the soil. As for Na⁺ and Ca²⁺, Mg²⁺ content of *T. pratense*
408 plants is twice than that of *F. nigrescens* and this is in line with previous works on these two plant species (Juknevičius
409 and Sabiené, 2007).

410 The micro- and macro-nutrient contents of pasture and meadow plants are fundamental for the quality of the
411 fodder. The German Society of Nutritional Physiology, among others, provides specific recommendations at this regard
412 (Flachowsky et al. 2001, Online Resource Tab. 1). In our experiment the P content of fodder improves when plants
413 were grown on H compared to those grown on C and CC but did not fulfill the cattle requirements.

414 K^+ content of plants increased for both charcoal treatments compared to C where both plant species had lower
415 concentrations than recommended. K^+ content in *F. nigrescens* was in line with recommendations when grown on H
416 and CC, while K^+ concentrations in *T. pratense* plants exceeded the recommendations when grown on H. However, the
417 value did not reach the limit of $\geq 35 \text{ g kg}^{-1}$ dry biomass, which is considered to be the cause of the so called “grass
418 tetany”, a Mg^{2+} deficiency in ruminants which implies cattle to be fed with supplementary magnesium (Kessler, 2001).

419 Ca^{2+} and Mg^{2+} concentrations were very high for both plant species grown on all soil types compared to the
420 recommendations, especially for those grown on soils amended with charcoal and for *T. pratense* plants. But an
421 adequate nutritive balance between K^+ , Ca^{2+} and Mg^{2+} was guaranteed, as the ratio $K:(Ca+Mg)$ was lower than 2.2
422 (Reid and Horvath 1980).

423 Fe measurements showed a large variability in our experiment so they have to be considered with caution, but
424 it is clear that Fe content of plant tissues exceeded the recommendation in all treatments and species, with highest
425 values for those grown on C. It is very common for pastures and meadows plants to exceed the iron supply
426 recommendations for cattle and values up to 500 mg kg^{-1} are usually tolerated (Flachowsky et al. 2001). This threshold
427 is only exceeded in the case of *F. nigrescens* plants grown on C, showing that charcoal application has been beneficial.

428 Na^+ content was higher than recommended for *T. pratense*, especially when grown on soils amended with
429 charcoal, but these concentrations are not considered harmful for cattle. The concentrations we found were lower than
430 2.5 g kg^{-1} Dry Matter (DM) (Tab. 3), while the maximum level of sodium tolerated in forage for dairy cattle is 15.73 g
431 $Na \text{ kg}^{-1}$ DM (Johansson 2008)

432 Cu was higher than recommended only for *T. pratense* plants but with no difference among the treatments.
433 Chronic copper poisoning is possible in cattle with a dietary concentration of 40 mg kg^{-1} (National Research Council
434 2001), but this threshold was far from being reached in our samples with values around 17 mg kg^{-1} (Tab. 3).

435 Charcoal application to the alpine grasslands in this study, both in the short and in the long term (CC and H),
436 overall improved fodder quality in terms of P, K^+ and Fe content compared to the control soils. Ca^{2+} , Mg^{2+} , Na^+ and Cu
437 contents were found to be higher than recommendations in all treatments, including control, but they did not reach
438 harmful levels, thus guaranteeing cattle health.

439 The positive results observed for charcoal amended soils in terms of germination, plant growth, root:shoot ratio
440 and fodder quality, both directly after application and after ageing, support the idea that larch wood charcoal is a soil
441 amendment suitable for alpine pastures and meadows even at an application rate of 390 t ha^{-1} . From a management
442 point of view, biochar incorporation into soil implies ploughing, a technique which is very rarely used in alpine
443 management because of multiple factors: the soil layer is thin to bedrock, with very irregular profiles (Stanchi et al.
444 2012); ploughing will increase soil erosion which is already a main problem in mountain soils because of slope, soil
445 depth, climate and soil low resilience which makes them almost no renewable (Tasser et al. 2003; Stanchi et al. 2012);
446 most of the grasslands are located in areas very difficult to reach with machines that is needed for biochar spreading and
447 incorporation into soil. Thus, biochar cannot be used for standard management, but can be helpful in the framework of
448 grassland restoration and rehabilitation, as defined by Aronson et al. (1993). The restoration and rehabilitation of
449 abandoned pastures and meadows in the Alps is usually based on the manual removal of trees and invasive species,

450 sometimes applying herbicides locally, and very rarely destroying the soil layer because of the reasons listed above
451 (Belleri 2014). For these reasons, biochar application is worth considering in cases of severely damaged grasslands (ski
452 tracks openings) or where the modifications to the environment have been so profound that the reconstitution of the
453 former ecosystem is no longer possible (roads or dumps construction) (Muller et al. 1998). However, possible impacts
454 on plant biodiversity remain to be explored and a deeper examination of nutrient cycles, microbial biodiversity and the
455 role played by hormones should be a research priority for next experiments in this unique environment to better
456 understand and quantify the overall impact of biochar application on nature conservation and the important ecosystem
457 services that mountain grasslands provide.

458 TABLES
459

Element	Control soil	Charcoal hearth soil	Fresh char	Control soil + fresh char
Ca	993±233* (278±61)*	3301±321* (1006±274)*	5909±86	2870±150
K	1604±201* (147±52)	2464±120* (279±208)	2906±54	2100±130
Mg	2739±128 (80±21)*	2379±665 (245±7)*	1522±56	2274±82
Na	298±88* (34±2)	93±15* (33±45)	210±4	264±54
P	321±13* (7±3)*	921±357* (12±7)*	308±1	316±8
N	4307±1402	5189±1756	2139±340	3480±870

460

461 **Tab. 1** Total nutrient content (mg kg⁻¹) in control soil, charcoal hearth soil, fresh char and control soil + fresh char at the
462 beginning of the experiment. In parenthesis, data on available nutrient concentration (mg kg⁻¹) for control and charcoal
463 hearths soils are reported. Data for hearths, control soils and fresh charcoal are taken from Criscuoli et al. (2014), while
464 data for the control soil + fresh charcoal are calculated. Asterisks indicate significant differences between control and
465 charcoal hearth soils at p≤0.05.

466

plant species	time	C	CC	H
<i>T. pratense</i>	96 hours	2a	7a	2a
	120 hours	18ab	29a	16b
	10 days	83a	94b	93b
<i>F. nigrescens</i>	96 hours	6a	13a	6a
	120 hours	11a	19a	12a
	10 days	78a	90b	89b

467

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470

Tab. 2 Germinated seeds (%) of *T. pratense* and *F. nigrescens* after 96 and 120 hours and 10 days from sowing in control (C), control + fresh charcoal (CC) and charcoal hearth (H) soils. Different letters indicate significant differences among soil treatments within each plant species and time frame at $p \leq 0.10$.

<i>Trifolium pratense</i>			
Element (mg g ⁻¹)	C	CC	H
Cu	0.017 ± 0.002a	0.016 ± 0.001ab	0.017 ± 0.001b
Fe	0.4 ± 0.3a	0.3 ± 0.3a	0.2 ± 0.3a
Ca ²⁺	23.9 ± 3.3ab	26.7 ± 1.8a	28.6 ± 2.1b
K ⁺	8.4 ± 0.5ab	10.1 ± 1.9a	13.6 ± 3.5a
Mg ²⁺	5.8 ± 0.7a	4.8 ± 0.3b	6.6 ± 0.7b
Na ⁺	1.6 ± 0.2ab	0.2 ± 0.3a	2.5 ± 0.7b
P	0.9 ± 0.1ab	0.9 ± 0.3a	1.4 ± 0.2b
N	19.6 ± 0.3a	19.2 ± 3.7a	15.1 ± 2a

<i>Festuca nigrescens</i>			
Element (mg g ⁻¹)	C	CC	H
Cu	0.008 ± 0.0002a	0.007 ± 0.0a	0.008 ± 0.002a
Fe	0.5 ± 0.6a	0.2 ± 0.0a	0.2 ± 0.1a
Ca ²⁺	12.8 ± 0.3a	13.2 ± 0.1a	15.2 ± 0.1a
K ⁺	7.6 ± 2.5a	10.6 ± 1.6a	10.1 ± 2.5a
Mg ²⁺	2.6 ± 0.2a	2.7 ± 0.2a	2.9 ± 0.4a
Na ⁺	1.2 ± 0.2a	1.2 ± 0.05a	1.3 ± 0.2a
P	1.1 ± 0.3a	0.8 ± 0.2a	1.5 ± 0.5a
N	18.5 ± 0.9a	14.2 ± 2.5b	14.1 ± 2.1b

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475

Tab. 3 Nutrient content (mg g⁻¹) in *T. pratense* (top) and *F. nigrescens* plants (bottom) at the end of the second growth cycle in control (C), control + fresh charcoal (CC) and charcoal hearth (H) soils. Different letters indicate significant differences among soil treatments at p≤0.10.

476 **FIGURES**

477

478 **Fig. 1** Aboveground biomass (g pot^{-1}) at the end of the three growth cycles for *T. pratense* (plot a) and *F. nigrescens*
479 (plot b) in control (C; light grey), control + fresh charcoal (CC; grey), and charcoal hearths (H; black) soils. Different
480 letters indicate significant differences among soil treatments within each plant species.

481

482 **Fig. 2** Total belowground (black) and aboveground biomass (grey) per pot accumulated over the three growth cycles for
483 *T. pratense* and *F. nigrescens* plants grown on control (C), control + fresh charcoal (CC) and charcoal hearth (H) soils.
484 Different letters indicate significant differences among soil treatments within each plant species.

485

486 **Fig. 3** Root:shoot ratio of total biomass accumulated over the three growth cycles for *T. pratense* and *F. nigrescens*
487 grown on C (light grey), CC (grey) and H (black) soils. Different letters indicate significant differences among soil
488 treatments within each plant species.

489

490 **Fig. 4** N:P ratio of soils (plot a) and of *T. pratense* and *F. nigrescens* (plot b) grown in C (light grey), CC (grey) and H
491 (black) soils. The two lines correspond to N:P ratios of 14 (dashed) and 16 (continuous). Different letters indicate
492 significant differences among soil treatments.

493

494 **Fig. 5** Relationship between plant and soil P, K^+ , Ca^{2+} , Na^+ , Mg^{2+} content (mg g^{-1}) (plot a, b, c, d and e, respectively) in
495 *T. pratense* (circles) and *F. nigrescens* (triangles) grown in C (white), CC (grey) and H (black) soils.

496

497 **Fig. 6** ^{13}C discrimination (Δ) of *T. pratense* and *F. nigrescens* plants after the first (a), second (b) and third growth cycle
498 (c) in C (light grey), CC (grey) and H (black) soils. Different letters indicate significant differences among soil
499 treatments within each plant species.

500

501 **Fig. 7** Isotopic signature ($\delta^{15}\text{N}$) of *F. nigrescens* and *T. pratense* plants after the first (a), second (b) and third growth
502 cycle (c) in C (light grey), CC (grey) and H (black) soils. P-values of the individual comparisons are reported in the two
503 tables. Different letters indicate significant differences among soil treatments within each plant species.

504

505 **Online Resource**

506

507 **Tab. 1** Recommended nutrients supply of a dairy cow with a mean performance of 30 kg milk day⁻¹ and a daily intake
508 of 20 kg biomass dry matter (Flachowsky et al. 2001).

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514

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516

517 **References**

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