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### ► To cite this version:

Marine Fernandez, Philippe Malagoli, Christiane Gallet, Catherine Fernandez, Antoine Vernay, et al.. Investigating the role of root exudates in the interaction between oak seedlings and purple moor grass in temperate forest. *Forest Ecology and Management*, 2021, 491 (119175), 10.1016/j.foreco.2021.119175 . hal-03196931

**HAL Id: hal-03196931**

**<https://amu.hal.science/hal-03196931v1>**

Submitted on 15 Apr 2021

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# Forest Ecology and Management

## Investigating the role of root exudates in the interaction between oak seedlings and purple moor grass in temperate forest --Manuscript Draft--

<b>Manuscript Number:</b>	FORECO-D-20-01535R2
<b>Article Type:</b>	Full Length Article
<b>Keywords:</b>	interaction; competition; interference; allelopathy; forest regeneration
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<b>Abstract:</b>	<p>Oak regeneration in temperate forests often fails in the presence of understory grass. Competition by resource exploitation between plants has been extensively studied. By contrast, competition by interference, especially chemical interference (allelopathy), has been much less thoroughly examined and its relative importance remains unclear. We investigated the influence of allelopathic interaction on plant performance (biomass production) in a pot experiment with sessile oak ( <i>Quercus petraea</i> ) and purple moor grass ( <i>Molinia caerulea</i> ), either sole- or mixed-grown. Plants were watered with either <i>Quercus</i> root exudates or <i>Molinia</i> root exudates. After 6 months of growth, oak biomass increment was significantly lowered by <i>Molinia</i> root exudates. The oak's root system was more strongly affected than its aerial part. <i>Quercus</i> root exudates favoured oak growth but did not affect moor grass. Conversely, <i>Molinia</i> root exudates had a small depressive effect on its own growth, but its biomass was favoured by the presence of oak grown in the same pot. Allelopathy reduced oak biomass by about 17%, and resource exploitation by an additional 33%, totalling 50%, assuming the two processes are strictly additive, which is debatable. Although untargeted metabolomic analysis by UHPLC failed to identify any potentially allelopathic substances involved, our study demonstrates a lower but critical contribution of chemical interference on oak seedling-moor grass competition compared to exploitation processes. To ensure oak regeneration, management of forest ecosystems should thus first focus on reducing moor grass close to oak seedlings to help decrease its allelopathic effect and ease resource competition.</p>
<b>Suggested Reviewers:</b>	Marie-Charlotte Nilsson marie-charlotte.nilsson@slu.se Expertise in N cycling within tree-graminoïds  Lluís Coll lluis.coll@udl.cat Expertise in competition for soil nutrient root uptake into tree-graminoïds system  Leslie Weston lewiston@csu.edu.au Expertise in belowground allelopathy  Manuel Reigosa mreigosa@uvigo.es Expertise in plant physiology response to allelochemicals

1 **Highlights**

- 2 - Oak-moor grass interaction also relies on chemical interference
- 3 - Moor grass roots emit allelopathic compounds reducing oak root growth
- 4 - Moor grass interference critically contributes to reduce oak growth
- 5 - Oak regeneration management should first focus on reducing moor grass density

29 sessile oak (*Quercus petraea*) and purple moor grass (*Molinia caerulea*), either sole- or mixed-  
30 grown. Plants were watered with either *Quercus* root exudates or *Molinia* root exudates. After  
31 6 months of growth, oak biomass increment was significantly lowered by *Molinia* root  
32 exudates. The oak's root system was more strongly affected than its aerial part. *Quercus* root  
33 exudates favoured oak growth but did not affect moor grass. Conversely, *Molinia* root exudates  
34 had a small depressive effect on its own growth, but its biomass was favoured by the presence  
35 of oak grown in the same pot. Resource exploitation had a more detrimental effect than  
36 allelopathy and both processes together decreasing oak biomass by 50%. Although untargeted  
37 metabolomic analysis by UHPLC failed to identify any potentially allelopathic substances  
38 involved, our study demonstrates a lower but critical contribution of chemical interference on  
39 oak seedling-moor grass competition compared to exploitation processes. To ensure oak  
40 regeneration, management of forest ecosystems should thus first focus on reducing moor grass  
41 close to oak seedlings to help decrease its allelopathic effect and ease resource competition.

42

## 43 Introduction

44 Identifying the balance of processes involved in plant-plant interactions has drawn much  
45 attention. Numerous studies have focused on competition for light and soil resources, but the  
46 mechanisms involved in soil resource competition (intrinsic root uptake capacity, monitoring  
47 of resources uptake dynamic, roles of root exudation, etc.) are seldom investigated, especially  
48 in the context of forest tree regeneration. Root competition (intra or inter-specific) has often  
49 been described as one of the main interactions driving plant establishment in communities  
50 (Casper and Jackson, 1997; Coomes and Grubb, 2000; Schenk, 2006). Two types of root  
51 competition have been identified, based on two plant strategies, termed “scramble” and  
52 “contest” by Schenk (2006). So far, most studies have focused on “scramble”, which is  
53 competition by resource exploitation (Grime, 1974; Tilman, 1990; Wilson and Tilman, 1993).  
54 This is when two species capture the same resources in the same finite space. Each species then  
55 depletes the resource reservoir to the detriment of the other. “Contest”, or competition by  
56 interference, includes mechanisms by which one species inhibits belowground resource access  
57 to another species by active or passive release of chemicals from either roots or shoots. Plant  
58 ecologists often use the term allelopathy to describe “contest” competition, but interference also  
59 include other non-resource interactions, such as autotoxicity (Guretzky, 2020), space  
60 competition (Casper and Jackson, 1997; Schenk, 2006), litter composition and thickness  
61 (Foster, 1999; Michalet et al., 2017). Schenk (2006) defines allelopathy narrowly in terms of  
62 resource access, but it can include all processes of chemically-mediated interference that alter  
63 germination, growth, survival or reproduction (Reigosa et al., 1999; Rice, 1984).  
64 Allelochemicals produced by plant organs are released into the environment through (i) release  
65 of volatile organic compounds (Effah et al., 2019; Penuelas and Llusia, 1998; Santonja et al.,  
66 2019), (ii) decomposition of fallen leaves and needles (Fernandez et al., 2016; Hashoum et al.,  
67 2017; Nilsson, 1994), (iii) living root exudates (van Dam and Bouwmeester, 2016), and  
68 (iv) decomposition of dead roots (Bertin et al., 2003; Fernandez et al., 2016; Mallik et al., 2016;  
69 Tsunoda and van Dam, 2017). Phytotoxic substances in root exudates have been identified in  
70 various species such as *Secale cereale* (Pérez and Ormeno-Nuñez, 1991), *Avena fatua* (Pérez  
71 and Ormeño-Nuñez, 1991), *Cucumis sativus* (Yu et al., 2003; Yu and Matsui, 1994), *Oryza*  
72 *sativa* (Kato-Noguchi, 2004), *Chenopodium murale* (Batish et al., 2007) and *Peperomia*  
73 *argyreia* (Hao et al., 2010). Allelochemicals can alter neighbouring plant growth and  
74 functioning either directly (e.g. by interfering with root system growth and development, root  
75 nutrient uptake, or physiological processes) or indirectly through changes in belowground soil

76 properties, such as physicochemical conditions (pH, ions availability...) (Huang et al., 2013;  
77 Xuan et al., 2005; Zeng, 2014) or soil community diversity, including N<sub>2</sub>-fixing bacteria and  
78 mycorrhizal associations). Seed germination and seedling establishment have been reported as  
79 life stages and processes often targeted by allelochemicals (Gallet and Pellissier, 2002).  
80 Allelopathy and competition by exploitation can act simultaneously. This makes determining  
81 the contributions of the two processes a challenge, especially under field conditions (Fernandez  
82 et al., 2016; Nilsson, 1994; Viard-Crétat et al., 2012). Evidence of allelopathic potential can be  
83 obtained by removing donor plants or organs, but this is not possible with roots (Fernandez et  
84 al., 2016; Fuerst and Putnam, 1983; Inderjit and Mallik, 2002; Olofsdotter et al., 1999; Ridenour  
85 and Callaway, 2001).

86 In most boreal and temperate forests, understory vegetation has been shown to affect  
87 the establishment and growth of tree regeneration (Balandier et al., 2006; Davis et al., 1998;  
88 Pagès and Michalet, 2003). Nilsson (1994) sought to separate and quantify contributions of  
89 exploitation competition and allelopathic effects of *Empetrum hermaphroditum* shoots on Scots  
90 pine (*Pinus sylvestris*) seedling growth. These authors pointed out both competition types were  
91 involved: *E. hermaphroditum* led to soil resource depletion but also exerted chemical inhibition  
92 on Scots pine seedling growth. Significant studies have been carried out on conifer regeneration  
93 failures in the presence of an ericaceous community (Mallik, 2003), underlining the potential  
94 role of allelochemicals released by ericaceous species that alter conifer seedling growth (Inderjit  
95 and Mallik, 2002, 1996; Mallik et al., 2016). More generally, interactions involving allelopathic  
96 substances in woodland have been identified in different tree species regeneration failures  
97 (Fisher, 1987) but are still poorly understood, especially in temperate deciduous forests.

98 Tree seedlings often show significant susceptibility to the presence of herbaceous  
99 neighbours. In particular, monopolistic perennial grasses, with a rapid vegetative development  
100 and a dense fasciculate root system, rapidly absorb soil resources such as inorganic nitrogen,  
101 competing by exploitation to the detriment of tree seedling growth and biomass (Balandier et  
102 al., 2006; Coll et al., 2003; Davies, 1987; Vernay et al., 2018a, 2016). At the opposite, with  
103 erectophile leaves (angle  $\geq 65^\circ$ , e.g. Balandier et al., 2006), perennial grasses generally induce  
104 not so strong competition for light (Gaudio et al., 2011; Picon-Cochard et al., 2006; Vernay et  
105 al., 2016). A common case is the regeneration of valuable oak species such as *Quercus petraea*  
106 in temperate forests, which is often jeopardized by the presence of interfering understory  
107 species such as *Molinia caerulea*, a widespread monopolistic grass (Taylor et al., 2001). For  
108 instance, competition by exploitation for N-resources in favour of moor grass was described as

109 a major reason for oak regeneration failure (Vernay et al., 2018a, 2018b). However, the  
110 respective importance of allelopathy in oak-grass interactions remains to be unravelled (Becker  
111 and Lévy, 1982; Timbal et al., 1990). Some studies have demonstrated the allelopathic potential  
112 of some oak species (*Q. robur*, *Q. pubescens*, *Q. coccifera*) exerted both on model and  
113 cultivated species (lettuce, wheat, barley, lentil, chickpea, faba bean and flax) and native  
114 herbaceous species (Alrababah et al., 2009; Hashoum et al., 2017; Souto et al., 1994).  
115 Conversely, *Quercus* seedling susceptibility to tree allelochemicals has been demonstrated in  
116 very few studies (Fernandez et al. 2016; *Q. pubescens* and *Pinus halepensis*).

117 Studies generally focus on “one-way” allelopathic interaction, analysing effects of  
118 species A on species B but rarely testing effects of root exudates of species B on A, so that the  
119 resultant interaction between the two plants is often inaccurately assessed. Moreover,  
120 allelochemicals can have intraspecific allelopathic effects, also called auto-allelopathy or  
121 autotoxicity (Böttger et al., 2018; Friedman and Waller, 1985; Singh et al., 1999). Various  
122 grasses or crops are known to exert an allelopathic effect on themselves, such as wheat (Wu et  
123 al., 2007) or barley (Ben-Hammouda et al., 2002), which can influence the community  
124 dynamics. In an interspecific context, it is not known whether *Quercus* root exudates contain  
125 secondary metabolites, in addition to the nitrogen released in the soil, that can improve moor  
126 grass growth (Fernandez et al., 2020). Allelopathic potential of root exudates has received much  
127 attention but little is known about their facilitative potential (Li et al., 2016). Fernandez et al.  
128 (2020) concomitantly observed a negative effect of the presence of moor grass on oak seedling  
129 growth and a positive reverse effect largely due to rapid nitrogen transfers from tree seedling  
130 to grass. Interactions simultaneously involving both a competition effect of plant A on plant B  
131 and a facilitation effect of plant B on plant A are called antagonistic (Michalet et al., 2011;  
132 Pistón et al., 2018; Schöb et al., 2014, 2013; Zélé et al., 2018) and have been scantily described.

133 Based on two complementary experiments, the “two-way” allelopathic interaction  
134 between sessile oak seedling and purple moor grass was investigated and extended by a more  
135 specific focus on the “one-way” interaction to characterize the allelopathic effect of *Molinia*  
136 exudates on oak seedlings specifically on shoot and root biomass. In the “two-way” experiment,  
137 we hypothesized (i) that *Molinia* root exudates might contain chemicals adverse to oak seedling  
138 growth and (ii) oak seedling root exudates might contain chemicals facilitating moor grass  
139 growth. We also applied root exudates of *Quercus* and *Molinia* on oak and moor grass,  
140 respectively, to test inhibitory or facilitative potential in the context of intraspecific interaction.

## 141 **Materials and Methods**

142 Two experiments were conducted in pots under outdoor conditions in Clermont-Ferrand  
143 (Auvergne, France, 45°45'N 3°07'E, altitude 394 m a.s.l, temperate climate). Using pots  
144 allowed a better controlled irrigation and harvesting of the whole root system. A “two-way”  
145 experiment was managed to study both *Molinia* allelopathic effect on oak seedling and on  
146 conspecific *Molinia*, and the reciprocal, *Quercus* allelopathic effect on *Molinia* and conspecific  
147 oaks. This was done watering oak or *Molinia* grown alone with a water solution of root extracts  
148 of either oak or *Molinia*. In this experiment, we also considered the exploitative competition  
149 effects on *Molinia* and *Quercus* at the plant scale (total biomass), by growing the two species  
150 together in a same pot. In a “one-way” experiment, we only tested the allelopathic effect of  
151 *Molinia* on oak growth: here oak's shoot and root compartments were separately measured to  
152 focus on oak organs response to interference competition. One-year-old oak seedlings were  
153 sourced from a local nursery. *Molinia caerulea* (L.) Moench was collected in a local forest at  
154 Paray-le-Frésil (Auvergne Rhône-Alpes, France; 46°39'N 3°36'E). Oaks and moor grasses  
155 were planted in pots filled with natural forest soil (typical luvisol redoxisol pseudogley, sandy  
156 loam) collected in the same forest as the moor grasses. To avoid water stress, pots were irrigated  
157 to field capacity throughout the experiment. Humidity probes set up in some pots monitored  
158 soil water content in the middle of the pot. No fertilizer was added to the pot during the  
159 experiment.

#### 160 “Two-way” experiment (**Figure 1A**)

161 The experiment lasted from March to September 2018. In March 2018, 30 1-year-old  
162 bare-root oak seedlings (*Quercus petraea* (Matt.) Liebl.) and 30 moor grass tufts were planted  
163 separately in 5 L plastic pots or together in 10 L plastic pots. Oaks were  $51.7 \pm 4.0$  cm in height  
164 (mean  $\pm$  SE), and  $7.6 \pm 1.3$  mm in diameter on average. Moor grasses were  $10.1 \pm 1.0$  g fresh  
165 weight. To prepare solutions of fresh root aqueous maceration, 12 donor oaks and 12 donor  
166 moor grasses planted in 5 L pots were collected at the rate of one plant per week (one solution  
167 of root exudate supply per week for 12 weeks). Intact fresh roots were washed, weighed and  
168 consistently macerated in distilled water for 48 h in darkness at room temperature. The  
169 concentration of these root exudates was 2% (root dry weight/water volume), which is the range  
170 commonly reported in literature (Fernandez et al., 2006: 2.5 and 5%; Hashoum et al., 2017: 1  
171 and 2.5%; Nilsson et al., 1993: 0, 0.5, 1.5, 2.5 and 3.75%). The final volume of aqueous  
172 exudates was split and immediately poured into the pots containing the target oak seedling or  
173 moor grass. Concomitantly, sole-growing targets received an equivalent volume of distilled



174 water (control). Root exudates of *Quercus* and *Molinia* were then applied on soil immediately  
175 after maceration. The experimental design was as follows (Fig. 1):

176 (i) Six recipient oaks and six recipient moor grasses were planted alone in 5 L pots without  
177 addition of root exudates (“sole-grown”, SG), they received an equivalent volume of distilled  
178 water (control)

179 (ii) Six sole-growing recipient oaks received *Quercus* root exudates (“SG + *Quercus*  
180 exudates”) and six others received *Molinia* root exudates (“SG + *Molinia* exudates”),

181 (iii) Six sole-growing recipient-moor grasses received *Quercus* root exudates (“SG + *Quercus*  
182 exudates”) and six others received *Molinia* root exudates (“SG + *Molinia* exudates”),

183 (iv) One oak and one moor grass were planted in the same 10 L pot (“mixed-grown”) to analyse  
184 competition through physical interaction without addition of root exudates ( $n = 6$ ).

185 Oak seedlings used in conditions (ii) and (iii) were on average  $44.3 \pm 13.6$  g fresh weight. Oaks  
186 used in (i) and (iv) were  $33.4 \pm 10.5$  g fresh weight. Because of this unintentional initial biomass  
187 difference, we could not compare final biomass of shoot and root between the different pot  
188 conditions. Alternatively, we compared response in total dry biomass by relative growth  
189 increment, i.e. considering the initial difference, in the four experimental conditions. Relative  
190 increment (RI) was calculated by the expression (South, 1995):

191 
$$RI = \frac{DW_F - DW_I}{DW_I},$$

192 where  $DW_I$  was the initial dry weight of oak and moor grass when potting and  $DW_F$  was the  
193 final dry weight at harvest.

194 Plants were harvested in September 2018, i.e. after 6 months of growth. Shoots and roots were  
195 collected, washed and dried at 60 °C for 48 h before weighing.

#### 196 *One-way experiment (Figure 1B)*

197 The experiment lasted from April 2017 to August 2017. In April 2017, 16 one-year-old  
198 bare-root oak seedlings (*Quercus petraea*) and 12 moor grass tufts were planted separately in  
199 5 L plastic pots. In this experiment, moor grasses were used only as ‘donors’ to produce root  
200 exudates, and oaks were only targets. Oaks were  $19.9 \pm 6.7$  g (mean  $\pm$  SE) fresh weight,  $40.8 \pm$   
201  $4.2$  cm in height, and  $4.9 \pm 0.8$  mm in diameter on average. Moor grasses were  $10.3 \pm 1.5$  g  
202 fresh weight. In this experiment, special attention was paid to height and weight

203 homogenization of oaks to compare shoot and root biomass of controls and *Molinia* exudate  
204 receivers.

205 One donor moor grass tuft was harvested every week for 12 weeks from June 2017. Root  
206 exudates were obtained by the same method as described in the “two-way” experiment. The  
207 final volume of aqueous exudates was split weekly and immediately poured into the eight pots  
208 containing one target oak seedling each. Concomitantly, eight sole-growing target oak seedlings  
209 received an equivalent volume of distilled water (control).

210 Receiver oak seedlings were harvested in October 2017, *i.e.* after 6 months of growth. Oak  
211 shoots and roots were collected, roots were carefully separated from soil by washing, dried at  
212 60 °C for 48 h, and weighed. Organ biomass was used as an integrated proxy of oak growth  
213 throughout the season.

#### 214 *Data analysis*

215 Statistical analysis was performed using R software (Version 3.4.1.).

216 In the two-way experiment, data were means of  $n = 6$  biological replicates. We used a linear  
217 model (lm function) to determine the response of relative dry biomass increment, the root/shoot  
218 (R/S) dry biomass ratio to competition (SG vs MG) and exudate exposure (SG + *Quercus*  
219 exudates, SG + *Molinia* exudates). A pairwise comparison was then applied to the different  
220 treatment combinations to assess the significant differences ( $\alpha = 0.05$ ). Data were tested for  
221 normality using the Shapiro-Wilk test and for homoscedasticity using the Levene test before  
222 statistical analyses. Data were analysed using one-way ANOVA with Tukey’s *post hoc* test.

223 In the one-way experiment, data were the means of  $n = 8$  biological replicates. We used a linear  
224 model (lm function) to determine the response of oak dry weight and oak relative dry biomass  
225 increment to the different pot conditions: SG and SG + *Molinia* exudates. A *t* test was used to  
226 examine the difference in shoot and root dry weight and RI between “sole-grown” and “SG +  
227 *Molinia* exudates”. Here, results were analysed with one level of significance ( $\alpha = 0.05$ ).

228

## 229 **Results**

### 230 *Response of relative biomass increment and root/shoot ratio to root exudate application (two-* 231 *way experiment)*

232 In the two-way experiment, *Molinia* root exudates (SG + *Molinia* exudates) had no  
233 significant effect on oak relative dry weight relative increment (RI) in comparison with SG ( $p$

234 = 0.46, **Figure 2A, Tables S1 and S2**). Oak biomass was significantly lower in MG than in SG  
235 treatment ( $p = 0.04$ ), and SG + *Molinia* exudate treatment was marginally higher than MG ( $p =$   
236 0.09). Globally, oak RI was ordered SG > SG + *Molinia* exudates > MG. Application of  
237 *Quercus* root exudates marginally increased oak RI compared to the sole-grown condition ( $p =$   
238 0.08).

239 For moor grasses, a marginally lower relative dry weight increment was recorded in the  
240 presence of its own root exudates compared to control ( $p = 0.08$ , **Figure 2B, Tables S1 and**  
241 **S2**), whereas neither *Quercus* root exudates (“SG + *Quercus* exudates”) nor oak physical  
242 presence (“mixed-grown”) had any effect on *Molinia* RI. However, a significantly higher  
243 *Molinia* RI was estimated between SG + *Molinia* exudates and MG ( $p = 0.04$ ).

244 Oak root/shoot ratio (R/S) was unaffected by application of either root exudate (**Figure**  
245 **3, Table S3 and S4**). By contrast, moor grass R/S ratio was dramatically reduced when mixed-  
246 grown with oak ( $p = 0.02$ , **Figure 3, Table S3 and S4**).

247 *Response of oak root and shoot biomass, relative biomass increment and root/shoot ratio to*  
248 *Molinia root exudate application (one-way experiment)*

249 In the one-way experiment, root dry weight of oak seedlings was significantly lower  
250 after weekly supply of *Molinia* root exudates ( $p = 0.03$ , **Figure 4 and Table S5**), whereas shoot  
251 dry weight reduction in response to *Molinia* root exudates was only marginally significant ( $p =$   
252 0.06, **Figure 4 and Table S5**). Root dry weight was reduced by 50% and shoot dry weight by  
253 44% by *Molinia* exudates. No significant difference was observed in relative biomass increment  
254 (**Figure 5 and Table S5**) or in R/S ratio (**Figure 6 and Table S5**).

## 255 **Discussion**

256 Our results show that oak-moor grass interactions are more complex than hitherto  
257 described. Competition by exploitation is not the only explanation for the deleterious effect of  
258 moor grass on oak growth. *Molinia* root exudates generally had a negative effect on oak growth,  
259 especially on roots, suggesting involvement of allelochemicals. Another interesting result was  
260 the strong decrease in the moor grass R/S ratio in the presence of oak, revealing a change in  
261 biomass allocation. A higher biomass allocation in shoot purple moor grass without necessity  
262 to invest in the root compartment suggested that oak facilitated grass growth. Conversely, root  
263 exudates of *Quercus* had no significant effect on moor grass but they tended to have a positive  
264 intraspecific effect.

265 As reported by numerous studies in natural conditions, light competition occurs  
266 simultaneously with belowground resource and interference competition. Even if light  
267 competition by perennial grasses is often limited as highlighted in the introduction, we  
268 recognize that the net outcome of competition *in situ* would be the integration of light and  
269 belowground competition processes. However, the study and the following discussion focused  
270 on the allelopathic effect. Accordingly, light competition was removed from the experimental  
271 design to understand in more details the belowground allelopathic effect. Our aim was not to  
272 reproduce natural conditions but rather to lean on belowground processes. A more suitable  
273 design would be necessary to disentangle the combined contribution of light, belowground  
274 resource competition and allelopathy in the field.

275 The two-way experiment showed a consistent lower oak RI when mixed-grown with  
276 moor grass, and to a lesser extent after application of *Molinia* exudate solution. This is  
277 consistent with the simultaneous expression of the two types of competition, namely resource  
278 exploitation and chemical interference, respectively. When mixed-grown with moor grass, oak  
279 underwent both processes, whereas when sole-grown and watered with the *Molinia* exudate  
280 solution, oak was subjected only to allelopathic effects. On the assumption, which remains to  
281 be thoroughly tested, that effects of interference and competition by resource exploitation do  
282 not interact and can be distinguished from each other (Inderjit and Callaway, 2003; Inderjit and  
283 Del Moral, 1997; Uddin and Robinson, 2017; Weidenhamer, 2006), the difference between oak  
284 SG, SG + *Molinia* exudates and MG gave the relative contributions of the two competition  
285 types. Mixed-grown oak RI showed a 50% decrease compared to sole-grown, whereas  
286 allelopathy (SG + *Molinia* exudates) was responsible for a 17% decrease, although not  
287 statistically significant. Considering that allelopathy and resource competition are independent  
288 of each other, have additive effects, and the nature and quantity of root exudates were the same  
289 than in maceration, this means that resource competition could be responsible for about a  
290  $50\% - 17\% = 33\%$  decrease. This result suggested that in our pot experiment, resource  
291 exploitation had a greater effect than the allelopathic pathway. These results were consistent  
292 with other studies working on interactions between *P. sylvestris* and *E. hermaphroditum*  
293 (Nilsson et al., 1993) or between *Q. pubescens* and *P. halepensis* (Fernandez et al., 2016). They  
294 demonstrated that *P. sylvestris* and *Q. pubescens* biomass were more strongly reduced by a  
295 combination of allelopathy and competition than allelopathy alone. Moreover, treatments  
296 altered biomass allocation of *Quercus pubescens* with a lower R/S ratio in the case of  
297 allelopathy (Fernandez et al., 2016). Our results did not support this effect, oak R/S being non-

298 significantly affected by the treatment although the root system was more strongly altered than  
299 the shoot system, probably owing to a high variability of responses.

300 *Root exudates of oak favour its own growth, but exudates of Molinia inhibit its own growth*

301 *Quercus* root exudates tended to favour oak biomass but had no effect on moor grass  
302 biomass. This result was surprising because previous studies using different species  
303 compositions demonstrated a facilitating effect in interspecific interactions and not in  
304 intraspecific interactions. For example, Li et al. (2016) evidenced that root exudates of maize  
305 promoted N<sub>2</sub> fixation of faba bean (*Vicia faba* L.), whereas to our knowledge, whether root  
306 exudates can facilitate growth of conspecific species is still not known. Further studies should  
307 analyse the effects of species physical presence on the nature and intensity of root exudates  
308 rather than root exudates of sole-grown species. By contrast, in the present study, moor grass  
309 biomass was not significantly affected by *Quercus* root exudates. This is at variance with some  
310 studies reporting an allelopathic effect of *Quercus* sp from different regions on understorey  
311 species (Li et al., 2007). Callaway et al. (1991) identified an allelopathic property of adult  
312 *Quercus* root exudates that inhibited understorey productivity in woodlands. Alrababah et al.  
313 (2009) and Hashoum et al. (2017) demonstrated that aqueous extract of green and senescent  
314 mature *Q. coccifera* and *Q. pubescens* leaves and litter contained allelochemicals that reduced  
315 seed germination and controlled the dynamics of the herbaceous species. In our study, we were  
316 interested in the regeneration phase of the oak. At this stage young seedlings are characterized  
317 by small below- and aboveground biomasses, making them more vulnerable to grass  
318 understorey, and their allelochemical profile is probably different from that of a mature tree.  
319 *Molinia* own root exudates tended to decrease its total relative dry weight increment. Auto-  
320 allelopathy is a recognised mechanism in various plant species (Jamshidi et al., 2011; Mafeo  
321 and Mashela, 2010; Yu et al., 2003) that can lead to community stability by tempering the  
322 establishment of a single species. Specificity and intensity of plant interactions seem difficult  
323 to predict and probably depend on the species and on its biotic and abiotic environment.

324 As reported elsewhere (Vernay et al., 2018a, 2018b) grass dry weight was higher when  
325 mixed-grown with oak, although the difference was marginally significant in the present study.  
326 The most remarkable effect was recorded on the moor grass R/S ratio in the mixed-grown  
327 condition, which was significantly lower. Preferential biomass allocation to shoots can be  
328 interpreted as a strategy of a fast-growing species associated with a need to meet its C  
329 requirements when N availability allows it (Mardanov et al., 1998). Sufficient N availability

330 could be attributed to oak rhizodeposition, which provided nitrogen and facilitates moor grass  
331 shoot growth (Fernandez et al., 2020).

### 332 *Molinia* root exudates inhibit oak growth

333 Biomass analysis in our “one-way” experiment suggested that *Molinia* root exudates  
334 might contain chemicals that inhibit oak seedling growth. Biomass analysis of both root and  
335 shoot highlighted that *Molinia* root exudates had a greater effect on oak roots than shoots, as  
336 also observed in Nilsson's study (1994). This decrease was also observed in the relative total  
337 dry weight increment and R/S ratio, though not significantly, probably because of wide  
338 variabilities. However, the allelopathy effect on oak revealed a temporal variability, decreased  
339 growth appearing more marginal in our “two-way experiment” in 2018 than in our “one-way  
340 experiment” in 2017.

341 The one-way experiment focusing specifically on each organ allowed a better  
342 understanding of the action mechanisms of *Molinia* root exudates: inhibiting oak root growth,  
343 root exudates participated in strengthening competition for resources in favour of moor grass.  
344 Such inhibition of the oak root system is likely to have a negative impact on shoot growth in  
345 the following spring.

346 Zhang et al. (2020) demonstrated through a recent meta-analysis that allelopathy can  
347 reduce plant performance by 25% on average. In the “one-way” experiment, oak shoot and root  
348 biomasses were diminished by 47% and 50%, respectively, with *Molinia* root exudates. RI was  
349 reduced by 31% in the “one-way” experiment and to a lesser extent by 17% in the “two-way”  
350 experiment. Also, R/S ratio was decreased by 9.7% and 3.8% in the “one-way” and “two-way”  
351 experiments, respectively. In our study, we observed an annual variability of the reducing effect  
352 and a different intensity of the allelopathic effect in the considered organ. However, the mean  
353 of all these reductions due to allelopathy was 26%, which lies in the value range reviewed by  
354 Zhang et al. (2020)

### 355 *Nature of root exudates*

356 No specialized metabolite was evidenced in the aqueous exudates, although the  
357 difference in chemical composition of *Molinia* and *Quercus* was confirmed (Figure S1). To our  
358 knowledge, studies on root exudates are rare, as extraction from root extracts obtained after root  
359 grinding are commonly favoured. However, this did not account for what was actually released  
360 by the roots, since all root substances were supplied, including those that would not necessarily

361 be released into the soil. Root exudates were obtained by an artificial method (maceration)  
362 giving a proxy of root exudates, but this is a reliable method if root exudates could not be  
363 extracted from the soil (Oburger and Jones, 2018).

364 Despite a low concentration of root exudates in the analysed solution, continuous supply  
365 interestingly resulted in an inhibition of growth in oak along the monitored period. This striking  
366 result suggested a strong allelopathic effect of *Molinia* roots (SG + *Molinia* exudates).  
367 Moreover, the swift response suggested that allelochemicals likely originated from root  
368 exudation by moor grass roots rather than from release by moor grass root decomposition.

### 369 *Perspectives*

370 Overall, it is noteworthy that different chemicals originating from root aqueous  
371 maceration of different species can have positive or negative effects depending on that species  
372 and on the target species. Contrasting effects of root exudates of *Quercus* and *Molinia* on each  
373 other highlighted the absolute necessity to consider the “two-way” analysis in plant interactions,  
374 as well as the intraspecific interactions. Our experiment was conducted in pots so that we could  
375 test such effects *in vivo*. Although many substances are present in the root exudates, we did not  
376 identify them in this study. Whereas untargeted metabolomics has been successfully applied to  
377 profile root exudates of several species (van Dam and Bouwmeester, 2016), only very few  
378 compounds were identified (often less than 10%) and most of these were primary compounds  
379 (such as sugars or amino acids). Identifying specialized compounds in our root exudates would  
380 require a comprehensive study focused on the composition of exudates, needing a combination  
381 of platforms to succeed. Substances indicated by statistical analysis could be further identified,  
382 either by applying MS/MS on peaks in the extract, or by isolating the substance for additional  
383 structural analysis using NMR (Oburger and Jones, 2018; van Dam and Bouwmeester, 2016).

384 Another important point to consider are soil altering allelopathic effect *via* modifications  
385 of biotic and abiotic soil parameters. Root exudates are known to influence nutrient availability  
386 and physicochemical soil conditions and reciprocally (Mohammadkhani and Servati, 2018;  
387 Zeng, 2014). By altering the soil properties, it is possible that moor grass allelochemicals may  
388 had indirectly affected oak growth. We could not exclude this effect, but results suggested that  
389 moor grass exudate supply to SG moor grass did not significantly reduce moor grass RI.

390 Our study highlights the important role of interference processes in oak-grass  
391 interaction. Further research should pursue the characterization of the nature and quantity of

392 exudates (Oburger and Jones, 2018), given their critical role in plant-plant interaction dynamics  
393 (Ehlers et al., 2020).

394 From a forest management perspective, and specifically in relation to sessile oak  
395 regeneration, this study confirms that forest perennial grasses are detrimental to seedling  
396 establishment. The allelopathic pathway is one of the processes involved in this inhibition but  
397 it seems less important than competition by resource exploitation during the period of growth.  
398 Nevertheless, production of allelochemicals contributes to reduced oak performance (growth,  
399 soil exploration, efficiency in nutrient acquisition), accentuating competition for resources.  
400 Targets of allelopathic substances could be microbial communities associated with oak roots  
401 including mycorrhizae, but extensive studies are still needed to address this question. The  
402 density and proximity of moor grass around young oak should be reduced by various means to  
403 avoid allelopathic effects, which will also reduce resource exploitation. One solution would be  
404 to act on light availability in the understorey to modulate the presence and intensity of the  
405 competitive processes of moor grass (Gaudio et al., 2011). The decoupling of exploitation *vs*  
406 interference interaction may not be straightforward (Inderjit and Del Moral, 1997) and the  
407 potential indirect facilitative effect (Michalet et al., 2015; Siemann and Rogers, 2003) from  
408 mature trees could increase resource availability for seedlings and mitigate the production of  
409 allelopathic substances from understorey species.

#### 410 **Acknowledgements**

411 UHPLC-MS was performed on the IMBE Support Service on Chemical Ecology  
412 and Metabolomics (funded by the CNRS, the Provence Alpes Côte d'Azur Region, the TOTAL  
413 Foundation and ANR) particularly the MALLABAR platform. The authors thank Stéphane  
414 Greff for its valuable help in metabolite extraction and chemical analyses of root exudates.

415



416 **Appendix 1**

417 *Metabolite extraction*

418 *Molinia* root exudate composition was determined with an UHPLC instrument (Dionex  
419 Ultimate 3000 equipped with an RS Pump, an autosampler, a thermostated column  
420 compartment and a UV diode array, Thermo Scientific, USA) coupled to an accurate mass  
421 spectrometer (qToF) equipped with an ESI source (Impact II, Bruker Daltonics, Germany).  
422 UHPLC separation was done on an Acclaim C18 column (2.1 mm × 150 mm, 2.2 μm, Thermo  
423 Scientific, USA). Elution rate was set to 0.5 mL min<sup>-1</sup> at a constant temperature of 45°C. A  
424 pooled sample combining 10 μL of each sample was used to determine the chromatographic  
425 method and the injection volume. This pooled sample was also used as a quality control.

426 Injection was set to 10 μL after twofold dilution of all exudates with the same solvent as that  
427 used for extraction. Chromatographic solvents were composed of A: water with 0.1% formic  
428 acid and B: acetonitrile with the same additive. The chromatographic gradient was set as  
429 follows: 2% of B for 2 min, then increase in the proportion of solvent B to reach 100% at  
430 14 min. Each analysis was followed by a column cleaning phase with 100% B for 3 min, and  
431 column equilibration for 3 min, giving a total runtime of 20 min. Samples of each condition  
432 were randomly injected to integrate time-dependent changes in UHPLC-MS chromatographic  
433 fingerprints. Pooled samples, injected at the beginning, at the end and every six samples, were  
434 used for ion intensity normalization. Blanks were also injected to remove background signals.

435 MS parameters were set as follows: nebulizer gas, N<sub>2</sub> at 51 psi; dry gas, N<sub>2</sub> at 12 L min<sup>-1</sup>,  
436 capillary temperature 200°C and voltage 3000 V. The mass spectrometer was systematically  
437 calibrated with formate/acetate solution forming clusters on the studied mass range before a full  
438 set of analyses. The same calibration solution was automatically injected before each sample  
439 for internal mass calibration. For the mass spectra, tests were performed in both negative and  
440 positive modes. Mass spectra were in negative ionization mode in full scan mode from 50 to  
441 1200 amu at 2 Hz. DDA-MS<sup>2</sup> analyses were performed on three major ions detected at each  
442 scan on a pooled sample for metabolite annotation.

443 *Root exudates*

444 Analyses were automatically recalibrated using internal calibration, ensuring a precision of *m/z*  
445 below 2 ppm on the mass range, before exporting data in netCDF files (centroid mode) using  
446 Bruker Compass DataAnalysis 4.3. Analysis files were then processed using the XCMS

447 package (Smith et al. 2006) of R software, using the different steps necessary to generate the  
448 final data matrix: (i) peak picking for detection of different features, (ii) retention time  
449 correction ("obiwarp" method), (iii) grouping, (iv) filling of peaks to integrate portions where  
450 peaks were initially absent and (v) report and data matrix generation transferral to Excel. Each  
451 individual ion of each analysis was then normalized according to the injection order as  
452 described by Van Der Kloet et al. (2009). After the data set normalization, around 6000 features  
453 were kept before the filtering steps. To ensure data quality and remove redundant signals, three  
454 successive filtering steps were applied to pre-processed data using an in-house script on R. The  
455 first was based on the signal/noise (S/N) ratio to remove signals observed in blanks (S/N set at  
456 10 for features matching between pooled samples and blanks). The second allowed suppression  
457 of signals based on the value of the coefficient of variation of ion intensities in pooled samples  
458 (threshold at 0.4). The last step consisted in the deletion of all auto-correlated ions (threshold  
459 at 0.8) to remove isotopes and adducts.

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701



702 **Figure 1.** Experimental design of “two-way” experiment (A) and “one-way” experiment (B)  
703 with the different treatments tested on oak seedlings and *Molinia* tufts: sole-grown (SG), SG +  
704 *Quercus* exudates, SG + *Molinia* exudates, and mixed-grown (competition by exploitation +  
705 allelopathy).

706 **Figure 2.** Oak (a) and purple moor grass (b) relative dry biomass increment in the “two-way”  
707 experiment for the four different treatments: sole-grown (white bars), SG + *Quercus* exudates  
708 (light blue bars), SG + *Molinia* exudates (blue bars) and mixed-grown (dark blue bars). Values  
709 are reported as means  $\pm$  SE (standard error,  $n = 6$ ). Different letters indicate a significant  
710 difference between treatments.

711 **Figure 3.** Oak (a) and purple moor grass (b) root/shoot dry biomass ratio in the “two-way”  
712 experiment for the four different treatments: sole-grown (white bars), SG + *Quercus* exudates  
713 (light blue bars), SG + *Molinia* exudates (blue bars) and mixed-grown (dark blue bars). Values  
714 are reported as means  $\pm$  SE ( $n = 6$ ). Different letters indicate a significant difference between  
715 treatments.

716 **Figure 4.** Above- and belowground dry weights (g) of oak in the “one-way” experiment for the  
717 two different treatments: sole-grown (white bars) and SG + *Molinia* exudates (blue bars).  
718 Values are reported as means  $\pm$  SE ( $n = 8$ ). \* corresponds to  $p < 0.05$ .

719 **Figure 5.** Oak relative dry biomass increment in the “one-way” experiment for the two different  
720 treatments: sole-grown (white bars) and SG + *Molinia* exudates (blue bars). Values are reported  
721 as means  $\pm$  SE ( $n = 8$ ).

722 **Figure 6.** Oak root/shoot dry biomass ratio in the “one-way” experiment for the two different  
723 treatments: sole-grown (white bars) and SG + *Molinia* exudates (blue bars). Values are reported  
724 as means  $\pm$  SE ( $n = 8$ ).

725

726

727 Appendix

728

729

## Statistical tables

### Abbreviations

<b>Ctrl</b>	Control
<b>ME</b>	<i>Molinia</i> root exudates
<b>OE</b>	Oak root exudates
<b>Comp</b>	Competition (exploitation competition + allelopathy)

**Table S1. (Figure 2).** Means  $\pm$  SE of oak and moor grass relative dry biomass increment (two-way experiment) in sole-grown (SG), *Quercus* root exudates (SG + *Quercus* exudates), *Molinia* root exudates (SG + *Molinia* exudates) and mixed-grown (MG) treatment ( $n = 6$ ).

	Oak				<i>Molinia</i>			
	SG	SG + <i>Q</i>	SG + <i>M</i>	MG	SG	SG + <i>Q</i>	SG + <i>M</i>	MG
Mean $\pm$ SE	1.71 $\pm$ 0.29	3.06 $\pm$ 0.60	1.42 $\pm$ 0.24	0.85 $\pm$ 0.19	30.47 $\pm$ 2.23	28.94 $\pm$ 5.25	23.54 $\pm$ 2.68	34.37 $\pm$ 3.68

**Table S2. (Figure 2).** Test statistic ( $F$ -value), statistical significance ( $p$ -value), and degrees of freedom (DF) assessing the effect of **treatment** on oak and moor grass relative dry biomass increment ( $n = 6$ ).

		SG- SG+ <i>Q</i>	SG- SG+ <i>M</i>	SG- MG	SG+ <i>Q</i> - SG+ <i>M</i>	SG+ <i>Q</i> - MG	SG+ <i>M</i> - MG
Oak	$p$ -value	0.08	0.46	0.04	0.04	0.01	0.09
	$F$ -stat	6.61					
	DF	21					
<i>Molinia</i>	$p$ -value	0.79	0.08	0.39	0.38	0.42	0.04
	$F$ -stat	1.28					
	DF	20					

**Table S3. (Figure 3).** Means  $\pm$  SE of oak and moor grass R/S ratio (two-way experiment) in sole-grown (SG), *Quercus* root exudates (SG + *Q*), *Molinia* root exudates (SG + *M*) and mixed-grown (MG) treatment ( $n = 6$ ).

	Oak				Purple moor grass			
	SG	SG + <i>Q</i>	SG + <i>M</i>	MG	SG	SG + <i>Q</i>	SG + <i>M</i>	MG
Mean $\pm$ SE	0.80 $\pm$ 0.09	0.94 $\pm$ 0.06	0.77 $\pm$ 0.09	0.86 $\pm$ 0.14	1.58 $\pm$ 0.33	1.70 $\pm$ 0.56	1.64 $\pm$ 0.39	0.45 $\pm$ 0.06

**Table S4. (Figure 3).** Test statistic ( $F$ -value), statistical significance ( $p$ -value), and degrees of freedom (DF) assessing the effect of **treatment** on oak and moor grass R/S ratio ( $n = 6$ ).

		SG- SG+ <i>Q</i>	SG- SG+ <i>M</i>	SG- MG	SG+ <i>Q</i> - SG+ <i>M</i>	SG+ <i>Q</i> - MG	SG+ <i>M</i> - MG
Oak	$p$ -value	0.22	0.80	0.72	0.13	0.59	0.58
	$F$ -stat	2.35					
	DF	21					
<i>Molinia</i>	$p$ -value	0.86	0.91	0.02	0.93	0.07	0.02
	$F$ -stat	2.35					
	DF	21					

**Table S5. (Figure 4, 5 and 6).** Means  $\pm$  SE (g) of oak shoot and root dry weight (one-way experiment) in sole-grown (SG) and *Molinia* root exudates (SG + *M*) treatment. Test statistic (*t*-value), statistical significance (*p*-value), and degrees of freedom (DF) assessing the effect of **treatment** on shoot and root dry weight of oak (*n* = 8).

		Biomass		Relative increment		Root/shoot ratio	
		SG	SG+ <i>M</i>	SG	SG+ <i>M</i>	SG	SG+ <i>M</i>
Shoot	Mean	16.36 $\pm$ 2.78	9.37 $\pm$ 1.79	3.34 $\pm$ 1.58	2.29 $\pm$ 1.31	1.75 $\pm$ 0.16	1.58 $\pm$ 0.09
	<i>p</i> -value		0.06		0.17		0.35
	<i>t</i> -value		-2.11		1.44		-0.98
	DF		10.22		13.54		9.39
Root	Mean	28.84 $\pm$ 4.84	14.48 $\pm$ 2.43				
	<i>p</i> -value		0.03				
	<i>t</i> -value		-2.65				
	DF		8.84				

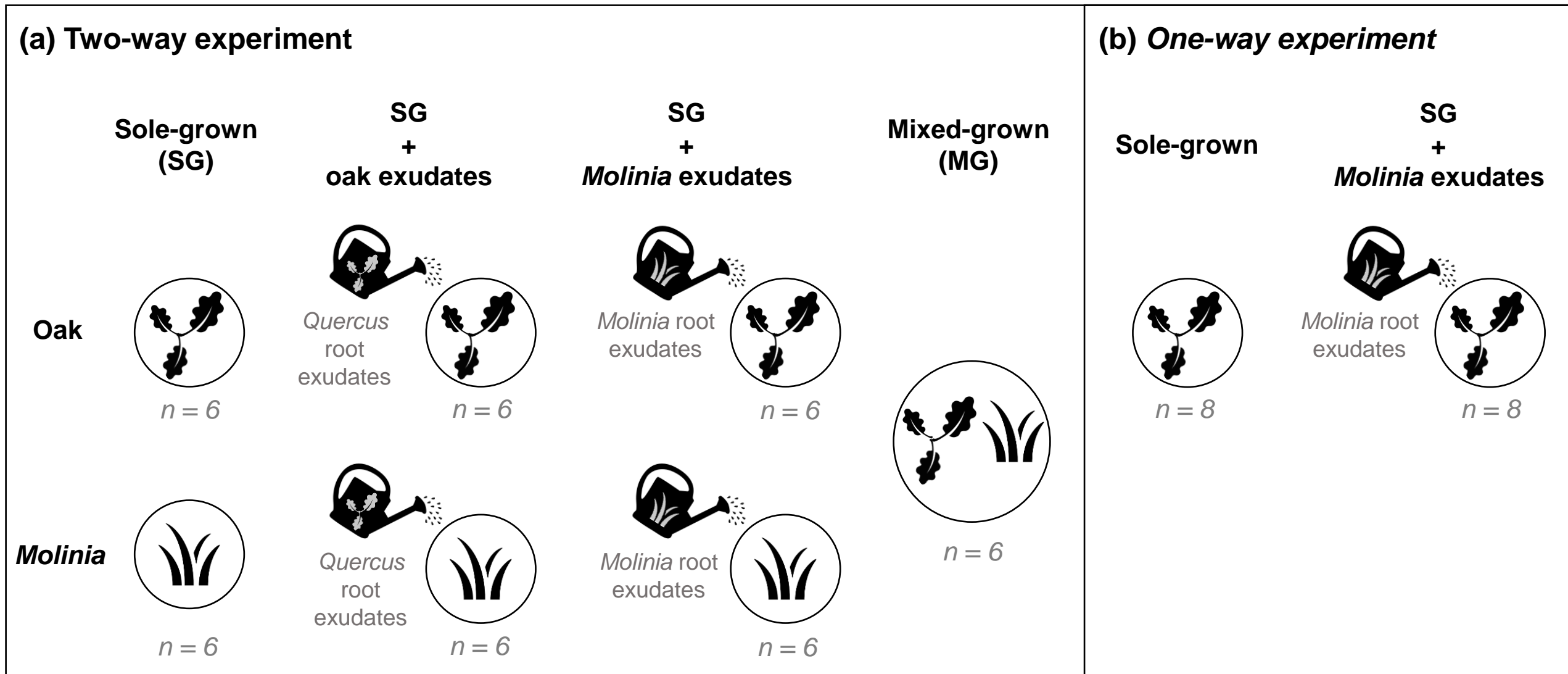
**Figure 1**

Figure 2. Two-way experiment

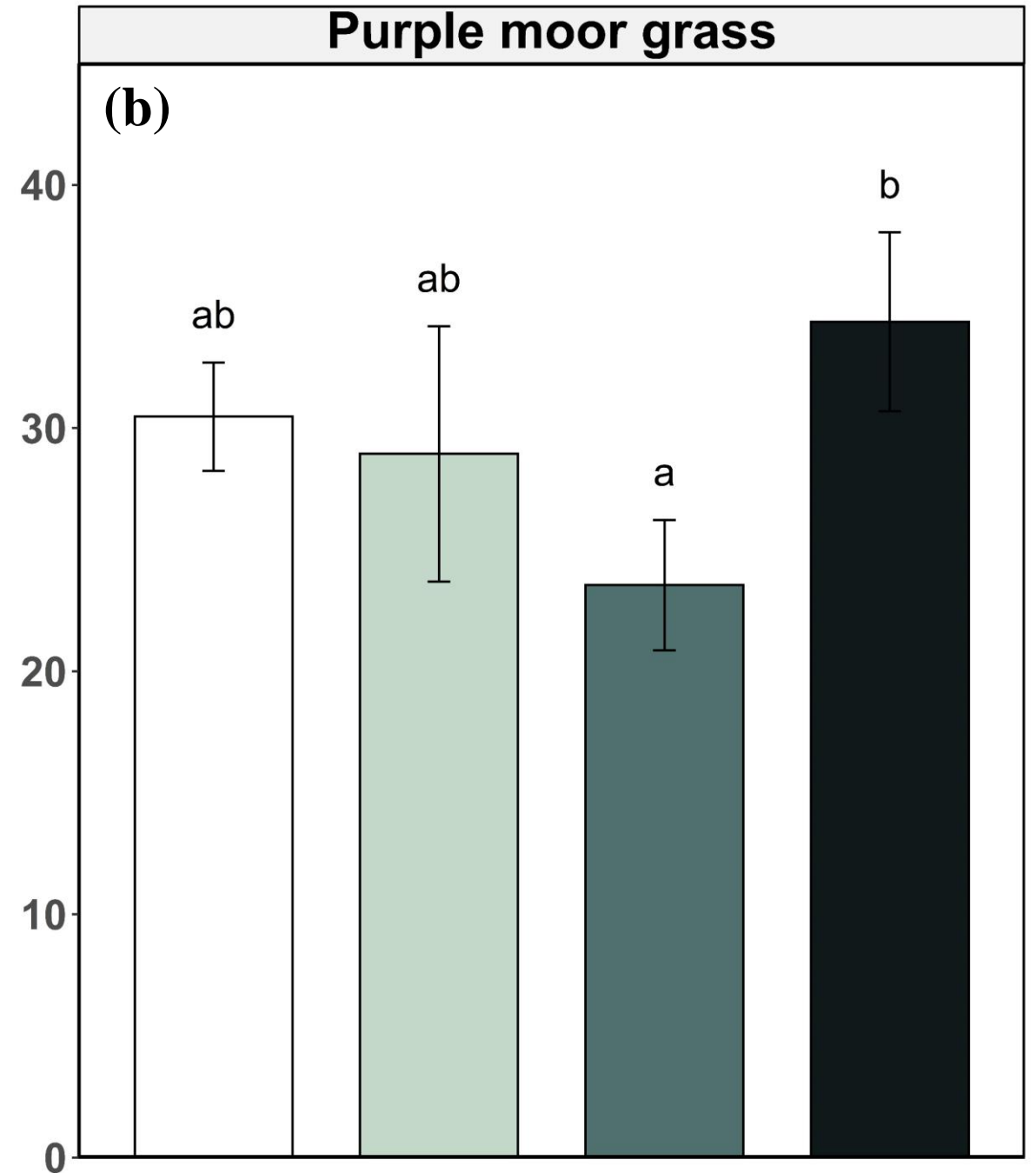
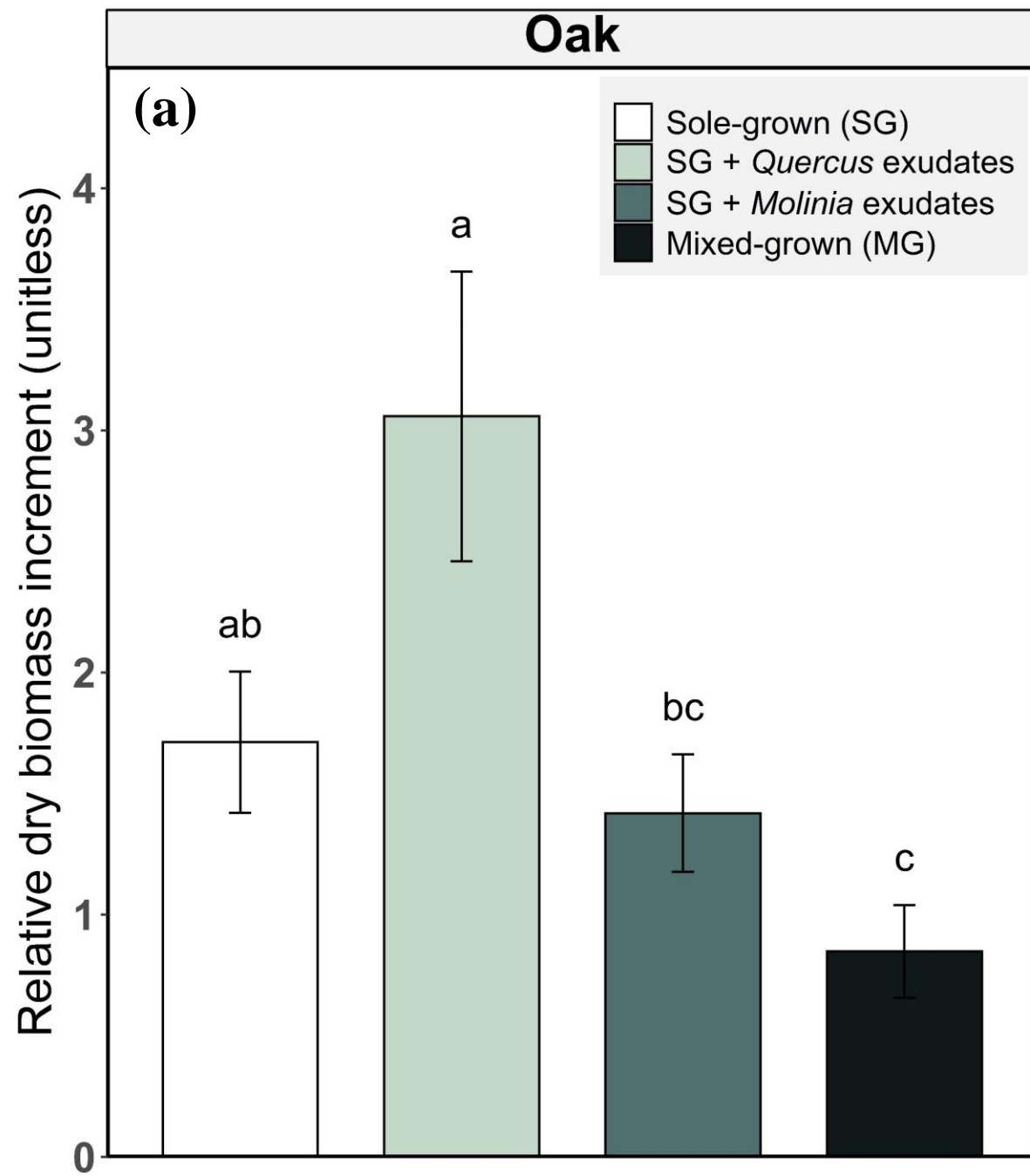


Figure 3. *Two-way experiment*

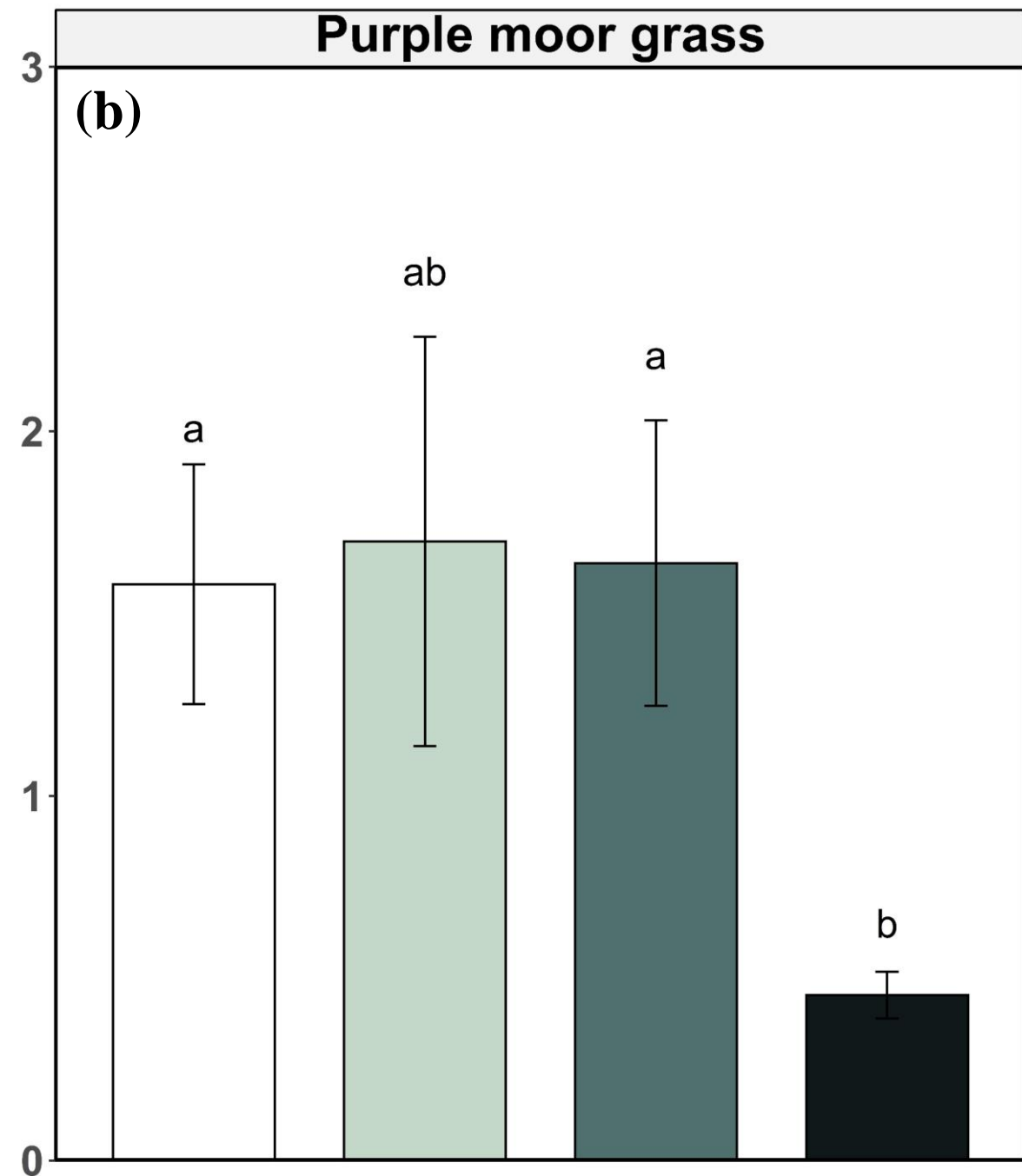
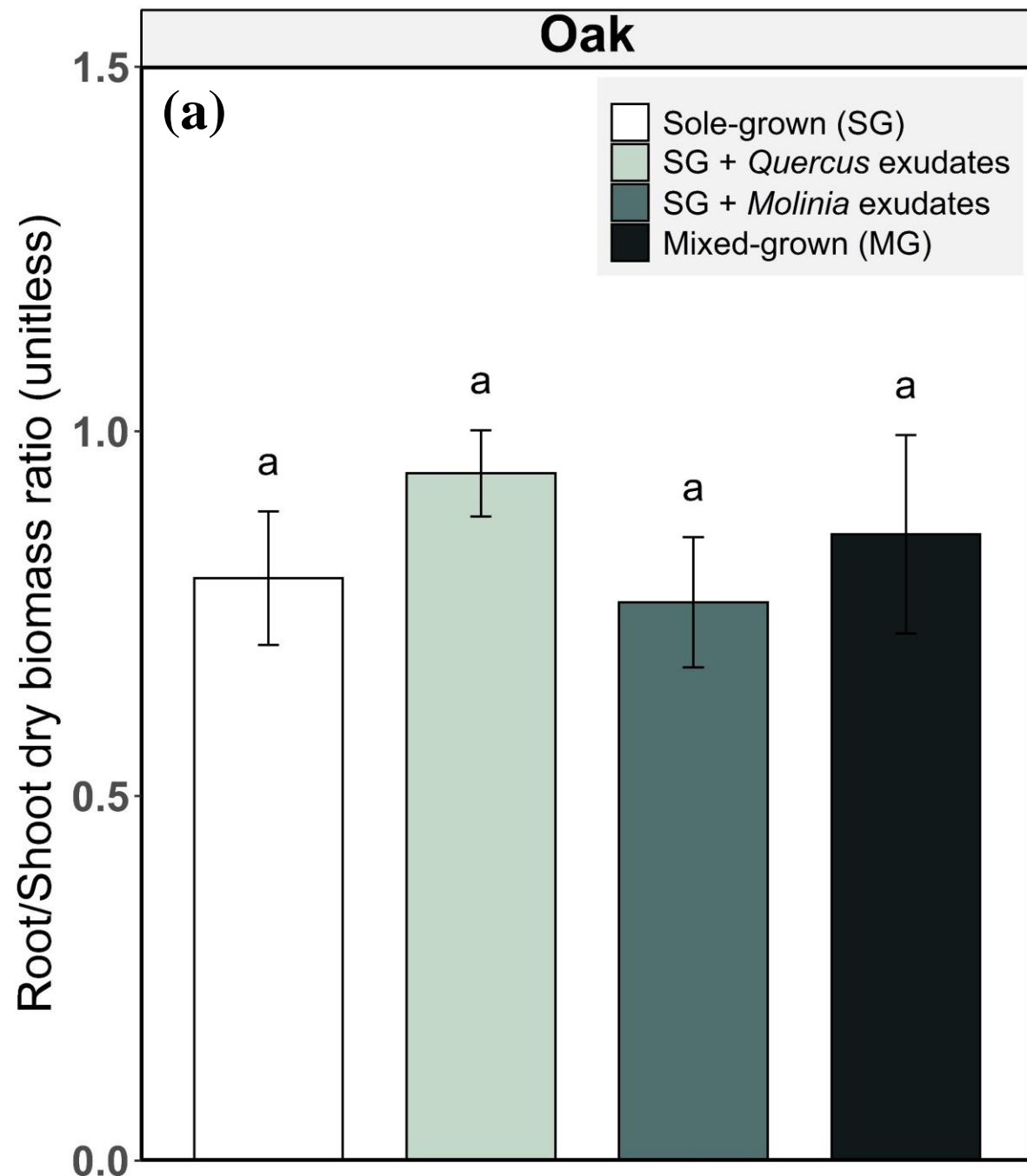
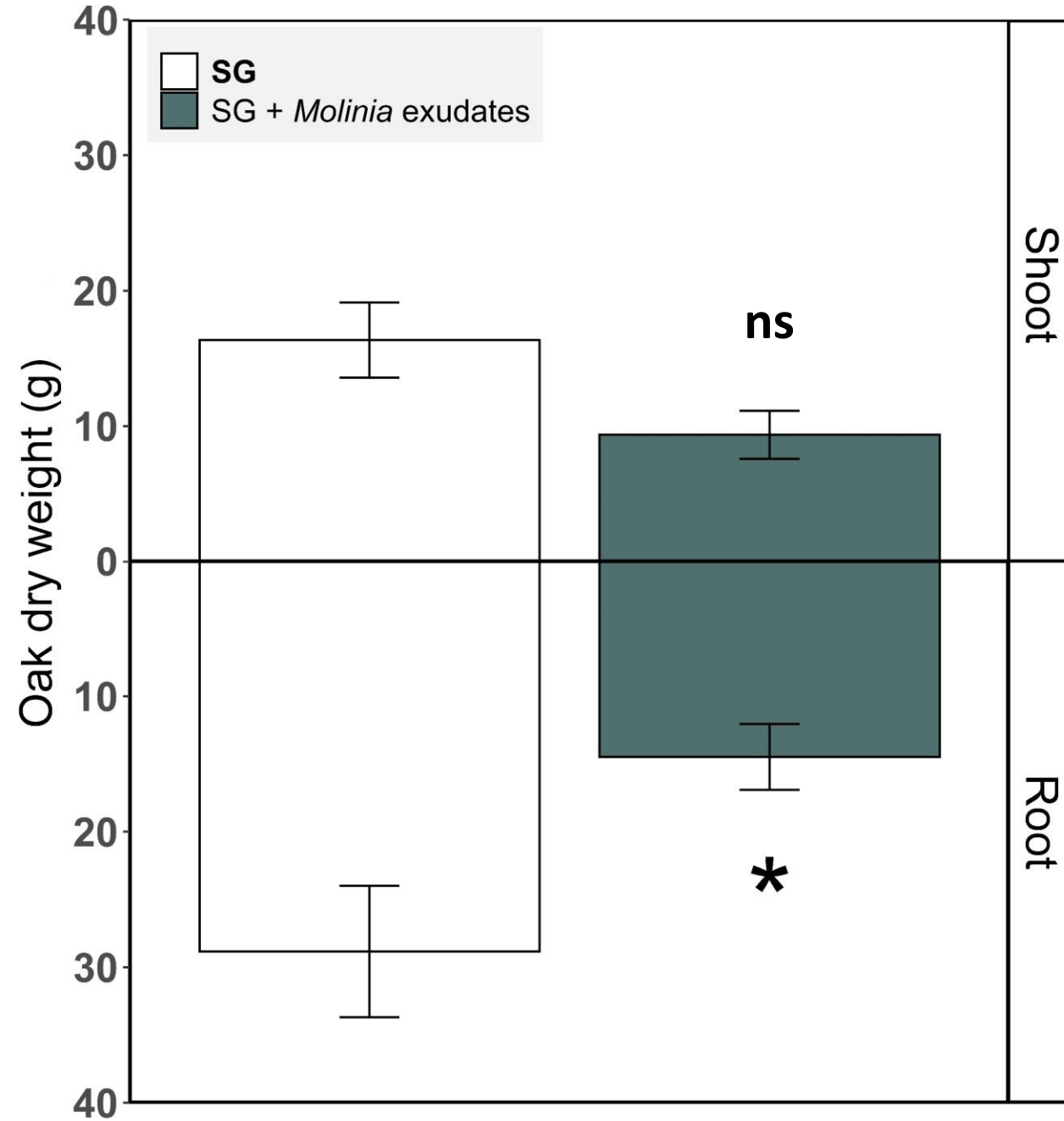
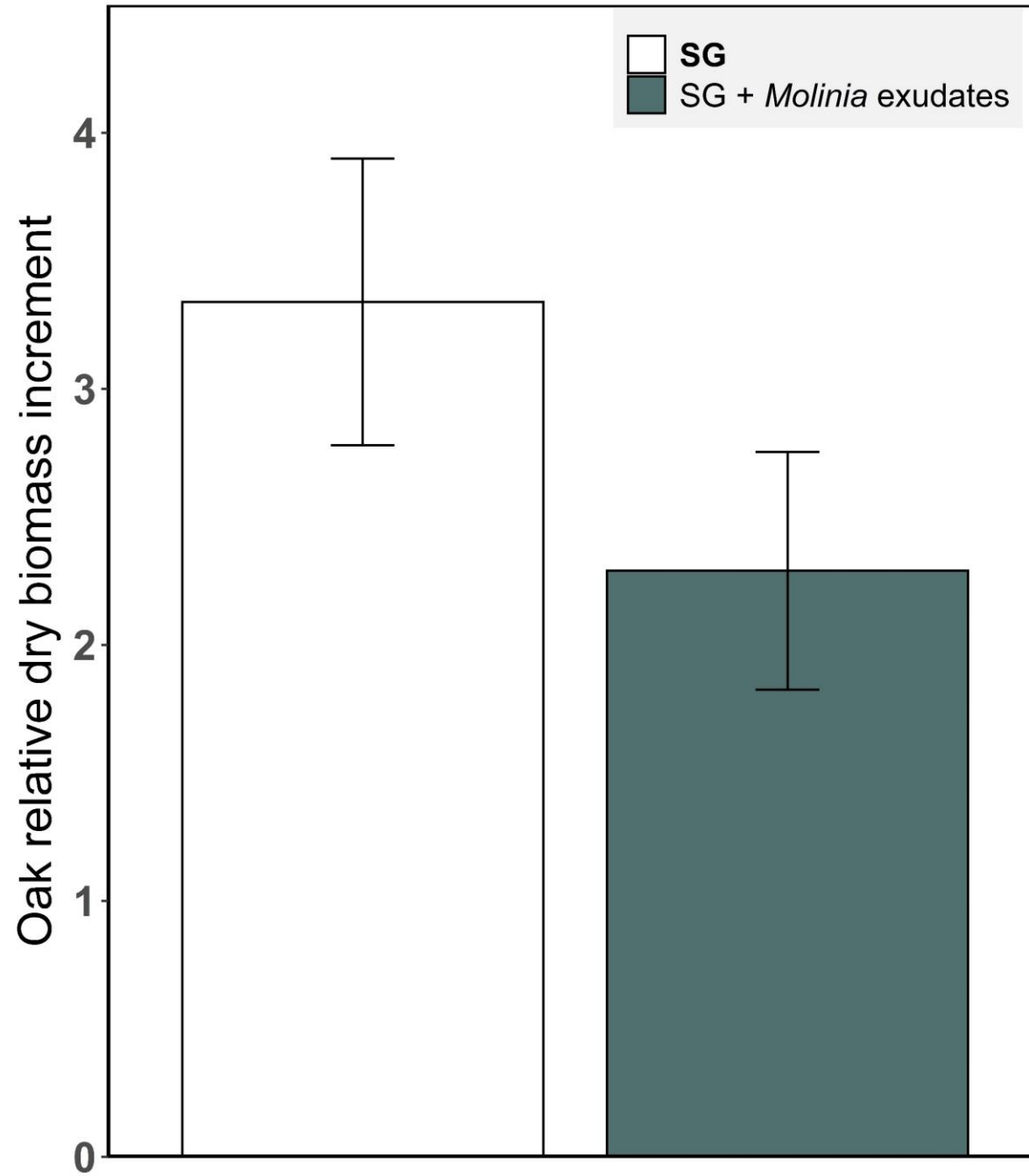


Figure 4. *One-way experiment*





**Figure 5.**  
*One-way experiment*



**Figure 6.**  
*One-way experiment*

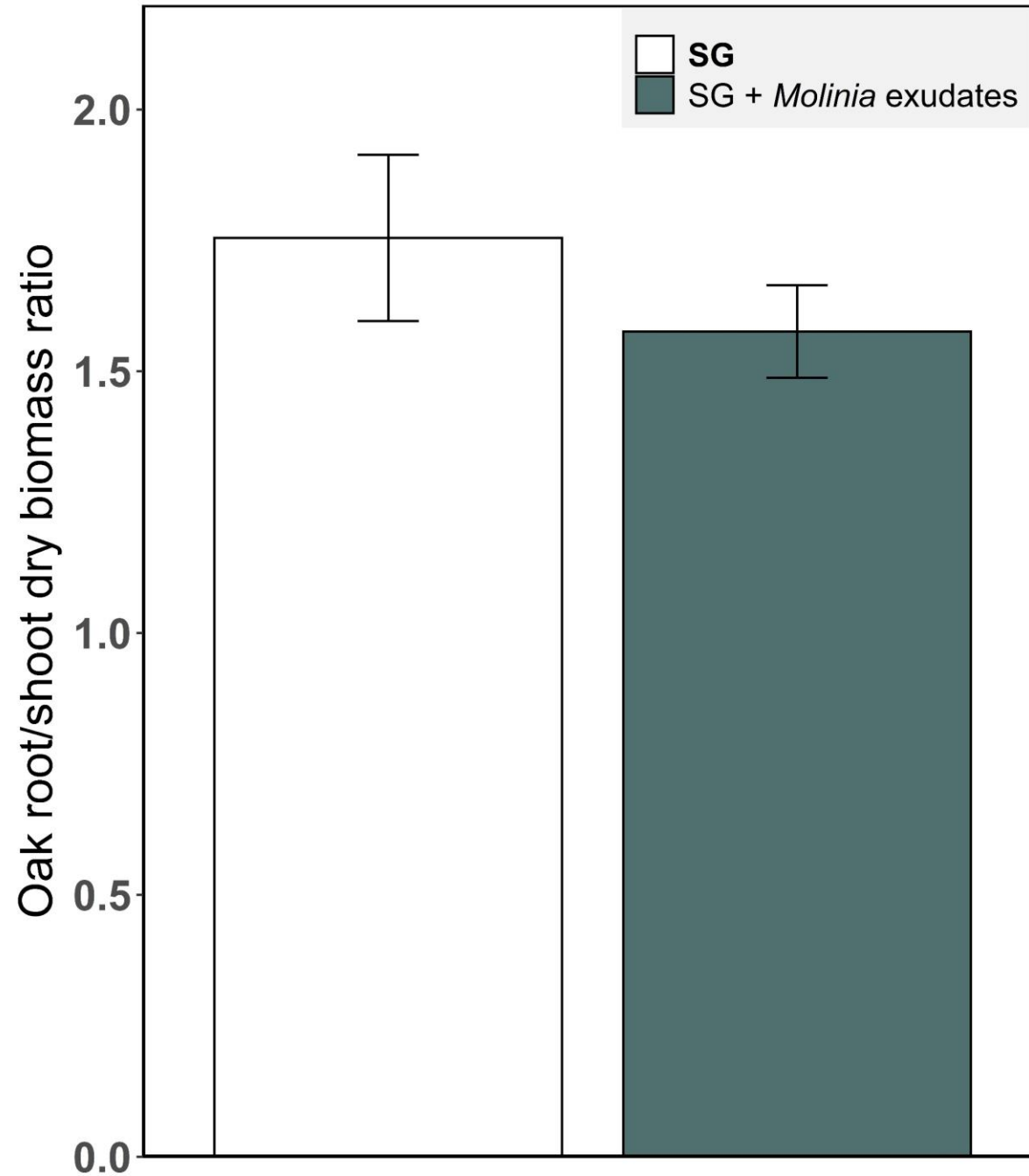
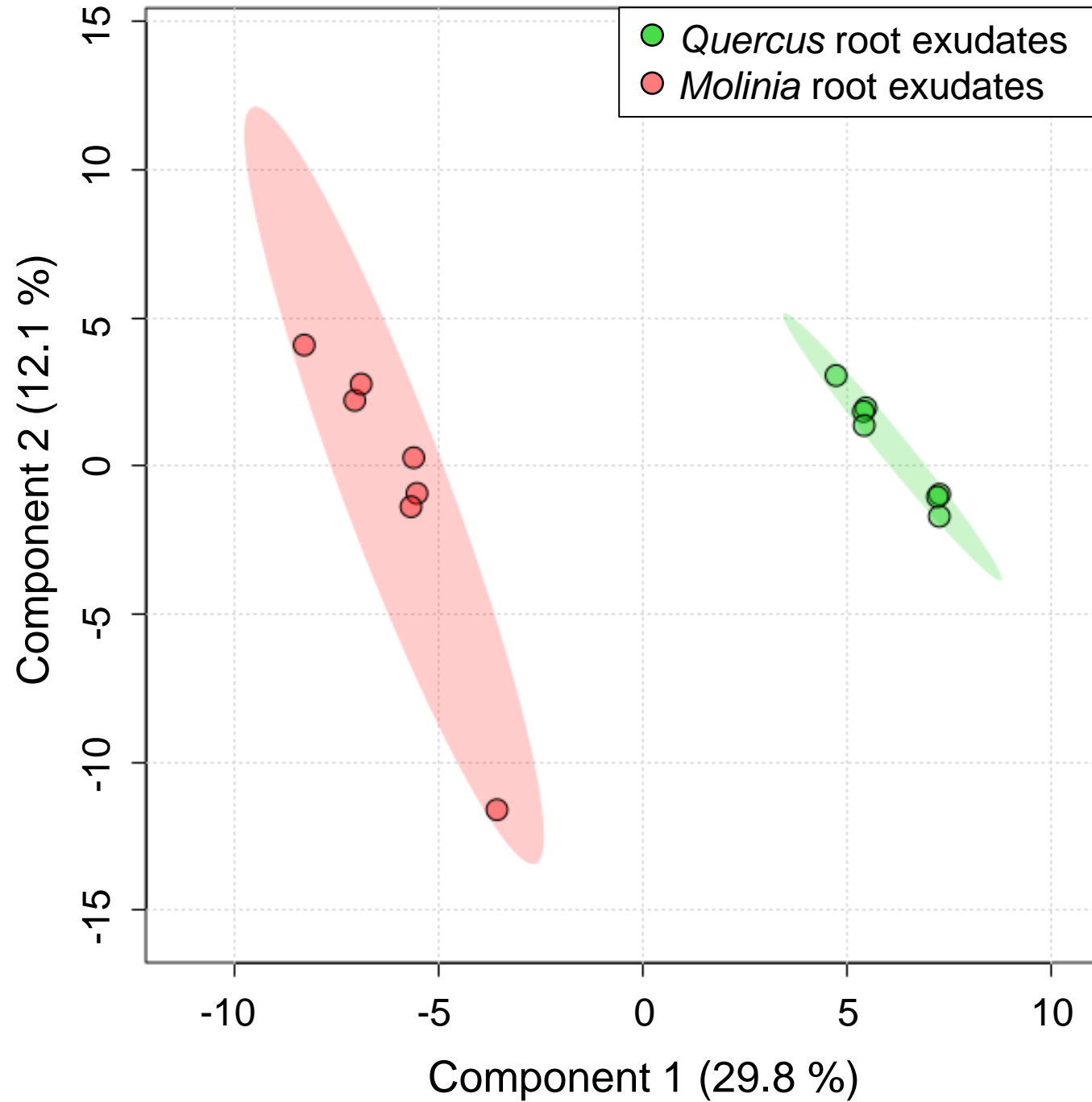


Figure S1



**Declaration of conflict of interest**

None.

MF: Conceptualization; Data curation; Formal analysis; Methodology; Writing - original draft; review & editing.

PM, TA, PB: Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Supervision; Validation; Writing - review & editing.

CF, CG, AV: Formal analysis; Methodology; Software; Validation; Writing - review & editing.