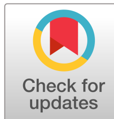

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10 Plasticity in root symbioses following shifts in soil nutrient availability during long-11 term ecosystem development

12

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23 Corresponding author: François P. Teste (francois.teste@uwa.edu.au).**Summary**

24 1. The vast majority of terrestrial plants form root symbioses with arbuscular mycorrhizal
25 (AM) fungi to enhance nutrient (particularly phosphorus, P) acquisition. However, some
26 plant species also form dual symbioses involving ectomycorrhizal (ECM) fungi, with a
27 subset of those also forming triple symbioses also involving dinitrogen (N_2)-fixing bacteria.

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It has been suggested that these plants show plasticity in root symbioses to optimise nutrient acquisition depending on the type and strength of soil nutrient limitation (e.g., N vs. P), yet empirical evidence remains limited. Alternatively, the degree of investment or ‘preference’ in particular root symbioses might simply reflect differences in inoculum potential among soils of contrasting nutrient availability, reflecting adaptations of root symbionts to different edaphic conditions.

2. Here, we grew two co-occurring plant species forming triple (AM / ECM / N_2-fixing; *Acacia rostellifera*) or dual (AM / ECM; *Melaleuca systena*) symbioses in soils of increasing age and contrasting nutrient availability from an Australian long-term soil chronosequence to disentangle the relative importance of abiotic factors (e.g., soil nutrient availability and stoichiometry) and biotic factors (e.g., soil inoculum potential) in determining root colonisation patterns and functional outcomes of these multiple root symbioses.
3. For both plant species, we found clear hump-shaped plant growth patterns along the pedogenesis-driven gradient in soil nutrient availability, with peak growth in intermediate-aged soils, while high levels of mycorrhizal colonisation by the ‘preferred’ root symbionts were maintained across all soils. We found large increases (540%) in foliar manganese concentrations with increasing soil age and declining P availability, suggesting that plants may be relying on the release of carboxylates to help acquire P in the most P-impoverished soils. Finally, we found that soil abiotic properties, such as strong differences in soil nutrient availability, are generally more important than soil inoculum potential in explaining these shifts in our plant and root responses.
4. *Synthesis*. Our study suggests that plants capable of forming multiple root symbioses show plasticity in their nutrient-acquisition strategies following shifts in soil nutrients during long-term ecosystem development, yet maintain a preference for certain root symbionts despite changes in soil microbial inoculum.

Key-words: Mycorrhizal fungi, nutrient-acquisition strategies, root traits, foliar manganese, ecosystem development and retrogression, nitrogen and phosphorus limitation, poor and deficient soil.

Introduction

The majority of plant species form intimate root symbiotic associations with soil biota to improve nutrient acquisition, especially when soil nitrogen (N) and/or phosphorus (P) are in low supply. Mycorrhizal fungi are particularly widespread (Tedersoo *et al.* 2014) and represent a commonly

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61 used nutrient-acquisition strategy that most (~92%) plant species use in almost every terrestrial
62 ecosystem (Brundrett 2009). In fact, arbuscular mycorrhizal (AM) fungi represent an ancient group
63 (Phylum Glomeromycota) that likely contributed to the colonization by plants of terrestrial
64 ecosystems some ~450 million years ago (Remy *et al.* 1994). By contrast, ectomycorrhizal (ECM)
65 fungi (mainly from the Basidiomycota and Ascomycota) have a more recent origin and are
66 restricted to associations with a few families of perennial woody plants, yet are widespread in
67 major temperate and boreal forest biomes (Smith & Read 2008). Both groups of mycorrhizal fungi
68 can enhance the acquisition of P, while ECM fungi is also capable of accessing unavailable forms of
69 N and P such as organic N and sorbed and/or organic P (Read & Perez-Moreno 2003; Hodge,
70 Helgason & Fitter 2010; Plassard & Dell 2010; Cairney 2011). Finally, plant species from the legume
71 (Fabaceae) family can also form root associations with specific groups of bacteria (e.g. *Rhizobia*)
72 capable of fixing atmospheric dinitrogen (N_2) thus reducing their dependence on soils for N
73 acquisition (Lambers *et al.* 2008).
74
75 Although most plant species tend to associate with only one type of mycorrhizal fungi, multiple-
76 root symbioses, such as those found in some *Populus* and *Eucalyptus* tree species (Brundrett
77 2009), may be more widespread than previously thought. For example, there are multiple reports
78 of AM on typically ECM hosts (Cázares & Trappe 1993; Dickie, Koide & Fayish 2001; McGuire *et al.*
79 2008). We do not fully understand under what conditions an individual plant forms more than one
80 mutualistic root symbiosis, and whether or not these interactions depend on environmental
81 context such as soil nutrient availability. Different explanations of dual-AM-ECM associations have
82 been suggested. For example they may: i) be 'relictual' AM associations in primarily ECM plant
83 taxa (Brundrett 2002); or ii) be promoted if they enhance plant growth in nutrient-poor soils by
84 enabling better nutrient scavenging and providing a wider access to different pools of nutrients
85 (Smith & Smith 2011); or iii) represent a 'backup' mechanism when inoculum sources of one type
86 are low (Chen, Brundrett & Dell 2000); iv) and/or be induced by the life stage of a plant (e.g.
87 seedling or mature tree stages, (Chen, Brundrett & Dell 2000; Neville *et al.* 2002; Gange *et al.*
88 2005)). Functional studies on dual-AM-ECM associations have provided some evidence that the
89 ECM symbiosis is more often beneficial for plant growth plant species forming dual mycorrhizal
90 symbioses (Jones, Durall & Tinker 1998; Chen, Brundrett & Dell 2000; Gange *et al.* 2005). On the
91 other hand, there is some evidence that associating with ECM fungi costs more in carbon (C) than
92 AM fungi for host plants (Phillips & Fahey 2006), pointing to potentially important trade-offs.

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93 Therefore, the relative benefits of either type may depend on environmental context, particularly
94 soil nutrient availability (Albornoz *et al.* 2016a).
95
96 Soil nutrient availability may be an important factor influencing the functional outcomes of
97 multiple root symbioses. For example, soils with high levels of organic matter may favour ECM
98 plant species over AM ones, since ECM fungi can improve plant N and P acquisition from organic
99 sources (Read & Perez-Moreno 2003). On the other hand, AM plants might be favoured in mineral
100 soils (Hodge 2017, and references therein). Recent work appears to support the hypotheses that
101 changes in soil nutrient availability or specific changes (e.g. soil organic P concentrations) may lead
102 to a 'switching' from AM to ECM or vice-versa (Albornoz *et al.* 2016a). Similarly, plants that host N-
103 fixing bacteria tend to down-regulate atmospheric N-fixation when growing in soils with high
104 levels of N (Hellsten & Huss-Danell 2000) or in P-deficient soils (Valentine, Kleinert & Benedito
105 2017). What remains unclear, however, is the relative importance of abiotic factors (e.g., soil
106 nutrient availability) vs biotic factors (e.g., soil inocolum potential) in driving these dual or triple
107 root symbioses.
108
109 'Priority-effects' (*sensu* Kennedy 2010) and persistence of ECM fungi in soils may also be a key
110 factor to explain why some plants apparently 'switch' strategies depending on successional or soil
111 nutrient availabilities. For example, if the arrival order of ECM fungal species is faster than AM
112 fungi at a given successional stage; this could explain why some plants predominantly form ECM
113 associations at that stage. Furthermore, there is evidence suggesting that ECM fungal species are
114 adapted to particular edaphic conditions (Peay *et al.* 2015), which could potentially lead to
115 'switching' of AM to ECM along edaphic gradients. Such switching of strategies to acquire
116 nutrients has been shown to be driven by changes in soil nutrient availability (Albornoz *et al.*
117 2016a). We however do not fully understand if the switching of strategies within the same plant
118 host species results primarily from abiotic factors (e.g. soil nutrient availabilities), or whether
119 biotic factors (e.g. symbiont propagule densities) play a more important role. Recent work on soil
120 chronosequences with strong N and P limitations do provide some clues that biotic factors may be
121 important, since both AM and ECM fungal communities shift in composition and both tend to be
122 less diverse in the most severely-impoverished soils (Krüger *et al.* 2015; Martínez-García *et al.*
123 2015; Albornoz *et al.* 2016b).
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125 While root symbionts provide clear benefits to plants, particularly with regard to enhanced
126 nutrient acquisition, they also impose significant C costs (Courty *et al.* 2010). As such, plants that
127 invest in maintaining such nutrient-acquisition strategies in theory should benefit the most when
128 growing in nutrient-poor soils (Smith & Read 2008). On the other hand, some recent studies found
129 lower mycorrhizal fungal biomass and low root colonisation by these fungi in very old, strongly
130 nutrient-impoverished soils, suggesting that the functioning of root symbioses may be hindered if
131 soils are severely nutrient-impoverished (Krüger *et al.* 2015; Albornoz *et al.* 2016b; Teste *et al.*
132 2016). In Australia's ancient and nutrient-impoverished soils several plant species are capable of
133 forming multiple-root symbioses such as dual-mycorrhizal (AM and ECM) associations as seen in
134 the Myrtaceae or multipartite (AM, ECM, and *Rhizobia*) associations as seen with *Acacia* (Zemunik
135 *et al.* 2015; Albornoz *et al.* 2016a; Teste *et al.* 2017). In the case of multipartite symbioses (AM,
136 ECM, and *Rhizobia*) we have some basic understanding of their basic biology (Smith & Read 2008),
137 however there is very little information on the ecology and functioning of such multipartite
138 symbioses, which are widespread in Australia (Brundrett 2009).
139
140 Here we studied the growth of co-occurring plant species in parallel to associated changes of their
141 in root symbiotic associations (AM, ECM, N_2-fixing nodules) and their functioning (extraradical
142 hyphal production, N_2-fixation) across a strong natural soil fertility gradient driven by long-term
143 soil and ecosystem development: the 2-million year Jurien Bay soil chronosequence (Laliberté et
144 al. 2012; Turner and Laliberté 2015). The Jurien Bay chronosequence shows strong decreases in
145 soil P and pH with increasing soil age and clear shifts from N to P limitation of plant growth with
146 soil age (Laliberté *et al.* 2012; Turner & Laliberté 2015). Our main hypothesis was that plants
147 capable of forming multiple root symbioses would preferentially allocate C to different types of
148 symbionts depending on strength and type of nutrient limitation. Our earlier work (Krüger *et al.*
149 2015; Albornoz *et al.* 2016a; Albornoz *et al.* 2016b) pointed us to this hypothesis but could not
150 disentangle effects of soil abiotic properties (e.g. nutrient availability) vs. soil biotic effects (soil
151 inoculum potential). In addition, our earlier work did not explore plant functional outcomes of
152 these root symbioses. Using a glasshouse bioassay experiment, the present study attempts to fill
153 these two gaps in our knowledge. Specifically, we extend previous work (Albornoz *et al.* 2016a) by
154 studying a broader range in soil properties (i.e. five chronosequence stages instead of three), use
155 experimental treatments to disentangle the relative importance of soil abiotic properties vs soil
156 inoculum potential in driving root symbioses, and measured response variables that indicate

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157 functional outcomes (e.g. % N derived from air) to gain a more better understanding of the
158 functioning of root symbioses across edaphic gradients.
159
160 The following specific hypotheses were tested:
161 1. When plants are grown with the same soil biota species pool across all soil ages (i.e. regional
162 species pool) or with specific soil biota communities in a mix of soil ages (i.e. soil-age specific
163 species pool), the soil abiotic effects are more important than biotic effects;
164 2. Variation in the levels of root occupancy by their symbionts (AM, ECM, N_2-fixing nodules) is
165 primarily driven by soil abiotic effects rather than soil biotic effects such as inoculum type or
166 potential;
167 3. The greatest level of extraradical hyphal production occurs in the most-severely P-impoverished
168 soils, since it has been shown that plants allocate more C to mycorrhizal fungi under P limitation.
169 However, we hypothesised that there might be a ´tipping point' where AMF become P limited
170 themselves in the most P-impoverished soils due to extreme P –growth limitations (Teste et al.
171 2016).
172
173 **Materials and methods**
174 *Study area*
175 We used the Jurien Bay >2-million year dune chronosequence, located in south-western Australia
176 (30.29° S, 115.04°111 E; ~200 km north of Perth), to collect soils of contrasting nutrient
177 availability, while minimising variation in other important soil-forming factors (e.g., climate, parent
178 material, topography) (Laliberté *et al.* 2012; Turner & Laliberté 2015). This long-term,
179 retrogressive chronosequence forms an exceptionally strong natural gradient of soil nutrient
180 availability (Laliberté *et al.* 2012; Hayes *et al.* 2014; Turner & Laliberté 2015) and shows the
181 expected shifts from N to P limitation of plant growth with increasing soil age (Laliberté *et al.*
182 2012; Hayes *et al.* 2014). Details on geology, climate, soils, vegetation and site selection along the
183 Jurien Bay chronosequence are given elsewhere (Laliberté *et al.* 2012; Laliberté *et al.* 2013; Hayes
184 *et al.* 2014; Laliberté, Zemunik & Turner 2014; Turner & Laliberté 2015; Zemunik *et al.* 2015). In
185 this study, we selected the same five distinct chronosequence stages, in terms of soil nutrient
186 availability and stoichiometry that were used in Hayes et al. (2014). The five stages selected
187 correspond to stages 1 (with anestimated soil age (ka) of <0.1 ka), 2 (~1 ka), 3 (~7 ka), 4 (~120 ka),
188 and 6 (>2000 ka) in Turner and Laliberté (2015) (Table S1).
189

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190 *Plant species selection*

191 We selected two plant species, *Acacia rostellifera* (Fabaceae) and *Melaleuca systena* (Myrtaceae),
192 that naturally co-occur across the chronosequence (Zemunik *et al.* 2016) to control for plant host
193 identity, thereby allowing us to isolate effects of soil properties and changes in soil inoculum on
194 plant performance and functioning of the associated root symbionts. Both plant species form
195 arbuscular mycorrhizal and ECM associations (Fig. 1) on the same root system, and *A. rostellifera*
196 also produces N_2-fixing nodules (Albornoz *et al.* 2016a).
197

198 *Soil collection and potting mixes*

199 We collected soils from the five chronosequence stages mentioned above for the glasshouse
200 experiment. Within each chronosequence stage, we randomly chose five replicate sites
201 corresponding to permanent 10 m x 10 m vegetation plots (Zemunik *et al.* 2016), for a total of 25
202 sites. At each site, we collected 5.3 l of bulk soil from the top 30 cm layer from three randomly
203 positioned points. In addition, we collected 1.6 l of soil to be used as inocula from the top 20 cm
204 layer of soil at seven randomly-positioned points. Soils were then bagged and brought back to the
205 laboratory for mixing (i.e. within-site). The bulk soil was sterilised via triple-steam pasteurisation at
206 80 °C for two hours per day over seven days, creating two periods where resistant spores would
207 be eliminated. We used steam pasteurisation due to its successful use in previous studies in sandy
208 soils from the same region (Fang, You & Barbetti 2012; Ryan *et al.* 2012; Teste *et al.* 2017).
209

210 We then created three types of soil mixes (see Table 1 in (Albornoz *et al.* 2016b)), hereafter
211 referred as the 'soil inocula' mix or treatment. The 'average inoculum' mix was made up of sterile
212 site-specific soil with a 7 % (v/v) soil inocula mix created from equal mixing of inoculum soil from
213 all sites from all chronosequence stages. This treatment corresponds to plants growing in
214 contrasting soil abiotic properties, but exposed to the same soil biota. The purpose of growing the
215 plants in this mix was to determine the relative importance of abiotic factors in driving the
216 responses such as the mycorrhizal status (type and colonisation levels), while holding soil biota
217 constant. The 'specific inoculum' mix was made up of sterile bulked soil (from all sites mixed in
218 equal proportions by volume) and inoculated with a 7 % (v/v) site-specific inoculum. This
219 corresponds to plants growing under the same soil abiotic properties, but exposed to different soil
220 biota. In the 'specific inoculum' mix we did not attempt to correct for differences in abundance or
221 composition of the soil biota in the 7 % (v/v) inoculums applied to the pots; instead these
222 differences where an integral part of the treatment. While we recognise that a full factorial (i.e.

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223 specific soil inocula for each chronosequence stage) would have been preferable, doing so was not
224 logistically possible given the resources available. We thus settled on a single 'average' soil. The
225 purpose of growing the plants in this average soil was to determine the relative importance of
226 biotic factors (e.g., mycorrhizal inoculum) vs. abiotic factors (e.g., soil nutrient availability and
227 stoichiometry) in driving root symbioses. Finally, the 'field soil' mix was made up of unsterilised
228 site-specific soil that represented both abiotic and biotic effects. Here, plants were simply grown
229 directly into the field soil as is. Soil mixes were then potted into 150 (5 dunes * 2 species * 3 soil
230 treatments * 5 replicate sites) 1 l pots. Seedlings were then immediately transplanted into pots
231 and watered to 75% field capacity throughout the duration of the experiment.
232
233 *Glasshouse experiment*
234 The 1 l pots were randomly laid out in a glasshouse at the UWA Plant Growth Facilities with an
235 automatic irrigation system. We setup the experiment as a factorial with a 'Soil origin' treatment
236 (5 soil age levels: <0.1 ka, ~1 ka, ~7 ka, ~120 ka, >2000 ka) and a 'soil inocula' treatment (3 levels:
237 field soil, average inocula, specific inocula). The purpose of the soil age treatment was to
238 contextualise the plant responses to different soil nutrient availabilities growing with their soil
239 biotic communities. We germinated *A. rostellifera* and *M. systena* seed in sterile sand at 19 °C with
240 12 hours of light per day. After 20 days, healthy seedlings were transplanted into pots with field
241 soil. At the start of the experiment, we destructively harvested 30 seedlings per species to assess
242 initial growth (length of first true leaf and height) and biomass (dry weight after 48 h at 65 °C). We
243 included five replicates per species per experimental unit.
244
245 *Plant growth*
246 After five (*A. rostellifera*) or seven months (*M. systena*) of growth in the glasshouse, final height
247 and root-collar diameter were measured. Seedlings were then harvested by severing shoots from
248 roots. Using a 2.2 cm-diameter soil corer, we sampled soil across the entire depth of the pot and
249 then removed any root fragments manually. This soil was then immediately frozen at -20 °C for
250 hyphal length. The remaining soil was then carefully washed away from root systems over a 1-mm
251 sieve. We then subsampled and weighed (fresh biomass) a representative set of fine (1 mm
252 diameter cut-off) roots for mycorrhizal colonisation and removed all nodules (for *A. rostellifera*).
253 The remaining roots, shoots, and nodules were oven-dried at 65 °C for 48 h and then weighed.
254 Initial biomass of planted seedlings was estimated through an allometric regression equation
255 based on the length of the longest true leaf (Leaf) and seedling height (Height) based on 30

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256 additional seedlings from each species (*A. rostellifera*: Initial biomass = 0.00605+ 0.0052*Height,
257 R^2=0.80, $P < 0.001$; *M. systena*: Initial biomass = 0.00048 + 0.0038 * Leaf, $R^2 = 0.67$, $P < 0.001$).
258
259 *Foliar nutrient*
260 Oven-dried (65 °C, 72 h) leaves were finely ground using a Teflon-coated stainless steel ball mill. A
261 subsample (~0.5 g) was acid-digested using concentrated HNO_3:$HClO_4$ (3:1) and analysed for Al,
262 Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S and Zn concentrations using inductively coupled plasma
263 optical-emission spectrometry (ICP-OES; ChemCentre, Perth, WA, Australia). Foliar N
264 concentrations were also measured (see *^{15}N analyses*). All digests were first analysed using a
265 simultaneous Varian Vista Pro (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia), radially
266 configured ICP-OES equipment fitted with a charge-coupled device (CCD) detection system and an
267 A.I. Scientific AIM-3600 auto-sampler. Samples with P concentrations close to minimum reporting
268 limit were re-analysed on more sensitive axially-configured ICP-OES equipment (ChemCentre,
269 Perth, WA, Australia).
270
271 *Mycorrhizal colonisation*
272 Mycorrhizal colonisation of both plant species was quantified at the end of the experiment
273 following a vinegar and ink clearing and staining protocol for Australian native plants outlined in
274 Teste *et al.* (2014), except for the following modifications: roots were not cut into fragments prior
275 to clearing and roots were incubated in potassium hydroxide (10% KOH w/v) for 4 h in a water
276 bath at 90 °C, and then bleached in alkaline hydrogen peroxide (0.5% v/v NH_4OH and H_2O_2) for 30
277 min. We determined arbuscular mycorrhizal and ectomycorrhizal colonisation simultaneously with
278 a gridded (14/11 cm grid) Petri dish following the gridline intersect method (Giovannetti & Mosse
279 1980). When needed, confirmation of the presence of arbuscules and Hartig net was done with a
280 compound microscope at X100 and X400 magnification. Because some samples did not have many
281 root tips intersecting the grid in the above method, we also estimated ectomycorrhizal
282 colonisation based on all root tips present in our Petri dish, irrespective of the grid lines.
283 Therefore, all root tips (non-ectomycorrhizal and ectomycorrhizal tips) were counted until 200
284 ectomycorrhizal or 200 non-ectomycorrhizal were attained, thus giving values to calculate percent
285 root colonisation following (Teste *et al.* 2014). In some instances, typically the smallest samples,
286 we counted all of the root tips since we could not reach the 200-root tips criterion.
287
288 *Hyphal length*

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289 We extracted soil hyphae generally following the procedure of Jakobsen, Abbott and Robson
290 (1992) with the some notable modifications for our sandy soil samples. First, 5-g soil samples were
291 incubated in 100 ml of DI water at room temperature for 30 min. We then homogenised the soil
292 solution vigorously with a magnetic stirrer for 1 min. A subsample of 5 ml was then taken 90 s
293 after homogenisation and hyphae were captured with a filtration manifold holding 10 µm filters
294 (Millipore, Billerica, MA, USA). Filters with captured hyphae were then transferred to vials and
295 incubated in a 5 ml of a vinegar-ink solution (5% ink (Black Sheaffer) in white vinegar) at room
296 temperature for 90 min. Vials were then vortexed vigorously for 15 s and decanted on 0.45 µm
297 nitrocellulose gridded filters (Millipore, Billerica, MA, USA) again using the filtration manifold
298 holding with a larger adapter to host large diameter filters. Finally, gridded filters were placed on a
299 microscope slide to dry and then mounted with 50% glycerol under a thin cover slips and clear nail
300 polish. Hyphae were scored under a compound microscope (Olympus BX51, Shinjuku, Tokyo,
301 Japan) at X200 magnification similar to gridline intersect method mentioned above except using
302 the length conversion factor of 0.23568 for 3 mm grids (Tennant 1975). We counted all hyphae
303 seen intersecting the lines, making up the grid, except that we excluded very small pieces of
304 hyphae that were less than half the length of the width of the line seen under X200 magnification.
305 We used hyphal length as a crude approximation for extramatrical mycelium (EMM) biomass
306 produced by AM and ECM fungi (Wallander *et al.* 2013), since we assumed minimal contributions
307 by saprophytic fungi in nutrient-poor, sandy mineral soils used in our relatively short glasshouse
308 experiment. To convert the hyphal length into EMM biomass, we used the conversion factors from
309 (Lodge & Wentworth 1990; Wallander *et al.* 2013).

310

311 EMM biomass = sum of hyphal length * sg * Db * soil volume (1)

312

313 where specific gravity (sg) = 0.22 g cm^{-3} and is from the Fogel and Hunt (1979) study and bulk
314 density (Db) taken from Turner and Laliberté (2015). Since there were large differences in plant
315 biomass we also standardised the EMM biomass (1) by total plant dry weight.

316

317 *^{15}N analyses*

318 After homogenising the dry and ball-milled leaf tissue, another subsample (mean weight: 1.5 mg)
319 was then analysed for ^{15}N and ^{14}N concentrations using an isotope ratio mass spectrometer
320 (Sercon 20-22, Sercon Ltd., Crew, UK) connected with an automated N: C analyser (Sercon, Crewe,
321 UK) in the West Australian Biogeochemistry Centre at The University of Western Australia. All

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322 results were initially expressed as ^{15}N atom fraction in percentage (%^{15}N) (Coplen 2011) following
323 multipoint normalization (Skrzypek 2013) based on three international standards (IAEA305A,
324 IAEA305B, IAEA311) distributed by the International Atomic Energy Agency (IAEA) and laboratory
325 standards. Then, $\delta^{15}N$ (‰) values were calculated from raw ^{15}N atom fractions with an air
326 standard N_2 atmospheric gas (3.6764×10^{-3}) for N. The obtained $\delta^{15}N$ raw values were normalized
327 using the standards and method listed above, and then used to calculate percent of N derived
328 from air (%Ndfa, see below) for *A. rostellifera*, our N_2-fixing plant (Lodge 1989; Giovannetti &
329 Sbrana 1998).
330
331 To determine the proportional contribution of biological N_2-fixation (BNF) to overall N acquisition
332 of *A. rostellifera*, % N derived from air (%Ndfa) was calculated following the formulas described in
333 Boddey *et al.* (2000) and Jones, Durall and Tinker (1998). First, since some plants were small at the
334 end of the experiment, we accounted for the potential influence of seed N, as suggested by Jones,
335 Durall and Tinker (1998), in the estimate of the ^{15}N abundances (‰) of *A. rostellifera* as follows:
336
337 $$\delta^{15}N_{AroL}SC = ((\delta^{15}N_{AroL} * TN_{AroL}) - (\delta^{15}N_{AroS} \times Ps * TN_{AroS})) / (TN_{AroL} - TN_{AroS})$$
338 (2)
339
340 where $\delta^{15}N_{AroL}SC$ is the $\delta^{15}N$ (‰) of *A. rostellifera* corrected for the influence of seed N, Ps is the
341 proportion of the seed N that was assimilated by the plant tissue, and$\delta^{15}N_{AroL}$ and $\delta^{15}N_{AroS}$ are the
342 ^{15}N abundances (‰) of the *A. rostellifera* leaves and seed, respectively, and TN is the total N in
343 these compartments. Since we based %Ndfa on leaf tissue, we was assumed that Ps was 0.5 (i.e.
344 50% of the seed N was incorporated into the aerial tissue) (Jones, Durall & Tinker 1998). From our
345 previous ^{15}N analyses of *A. rostellifera* grown with negligible amounts of soil N (i.e. from very
346 young dunes with 0.04% total N, undetectable dissolved organic N, and N mineralization), we
347 calculated %Ndfa as follows:
348
349 $$\%Ndfa = 100 * (\delta^{15}N_{Ref} - \delta^{15}N_{AroL}SC) / (\delta^{15}N_{Ref} - B) \quad (3)$$
350
351 where $\delta^{15}N_{Ref}$is the $\delta^{15}N$ (‰) of our companion plant (*M. systena*) growing in the same soil at the
352 same time as *A. rostellifera*, the $\delta^{15}N_{AroL}SC$ is the seed-corrected^{15}N abundance of our N_2-fixing
353 plant (*A. rostellifera*), and B is the$\delta^{15}N$ of *A. rostellifera* grown when obtaining all of its N from N_2
354 fixation (Boddey *et al.* 2000). We used *M. systena* since it is an ideal reference plant given it forms

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355 the same nutrient-acquisition strategies as *A. rostellifera* (Albornoz *et al.* 2016a) but without the
356 capacity to fix N_2 (Högberg *et al.* 1994). Nevertheless, we obtained some negative and values >
357 100 % which were converted to zero and 100 %Ndfa, respectively, prior to the statistical analyses.
358
359 *Data analysis*
360 Linear or generalised linear mixed-effect models (Pinheiro & Bates 2000) were used to analyse the
361 plant growth, mycorrhizal colonisation, hyphal length in soil, foliar nutrient concentrations, and
362 %Ndfa with two fixed factors (soil origin and inocula) and one random factor (site). We assessed
363 model assumptions and fit graphically with plots of residuals, likelihood ratio tests, and the Akaike
364 Information Criterion (AIC). If a fixed factor was statistically significant based on an analysis of
365 variance and $P < 0.05$, then we performed *post hoc* Tukey HSD tests and generated 95%
366 confidence intervals in R (R Core Team 2017) using packages 'nlme', 'lme4', 'multcomp', and
367 'effects' (Fox 2003; Hothorn, Bretz & Westfall 2008; Bates *et al.* 2015; Pinheiro *et al.* 2016).
368
369 The mycorrhizal colonisation data was analysed with generalised linear models with the binomial
370 or quasibinomial error distributions. We calculated a mycorrhizal-type preference (MTP) ratio
371 estimated from the ratio of ECM to AM fungal colonisation levels (i.e. % ECM / % AM). The MTP
372 ratio was then transformed using a hyperbolic tangent function to bind the values between 0 and
373 1. This transformation was necessary since the raw MTP ratio had values equal to infinity and
374 standardised MTP ratio allowed for more interpretable results (e.g. MTP > 0.5 indicates a
375 preference for ECM fungi). A beta regression model was used to fit the MTP data. Finally, foliar
376 nutrient concentrations were analysed together as a group using permutational multivariate
377 analysis of variance (PERMANOVA) with the adonis function of the 'vegan' (Oksanen *et al.* 2018)
378 package in R (R Core Team 2017).
379
380 **Results**
381 *Plant growth along a gradient of soil ages*
382 Plant growth differed strongly among soils from different chronosequence stages for both species,
383 and was lowest in the very young (<0.1 ka) and very old soils (>2000 ka) and greatest in young soils
384 (~1 ka) (Fig. 2). This humped-back trend was still apparent but muted in the average inoculum soil
385 treatment (Fig. 2). In the specific inoculum treatment, plant growth did not differ significantly
386 among chronosequence stages except for *M. systena,* where biomass remained greatest when

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387 plants were grown with soil incolum from the young soils (~1 ka) compared to older soils (~120 ka)
388 (Fig. 2).
389
390 *Responses of root systems with dual arbuscular and ectomycorrhizal fungal colonisation*
391 In the 'Field soil', arbuscular mycorrhizal colonisation in *A. rostellifera*, a dual-mycorrhizal host that
392 prefers AM fungi (Fig. 4), gradually increased with soil age (Fig. 3; 39% increase from the youngest
393 to the oldest soil). Similarly, ECM colonisation in *M. systena*, a dual-mycorrhizal host that prefers
394 ECM fungi (Fig. 4), increased gradually with soil age and was greatest in the oldest, most-
395 impoverished soils (Fig. 3, Table S1). When *A. rostellifera* was exposed to the average inoculum,
396 ECM colonisation (i.e. the non-preferred mycorrhizal type) rose steadily and was significantly
397 greater in the two oldest soils (~120 ka & >2000 ka) compared to the two youngest soils (<0.1k &
398 ~1 ka) (Fig. 3). Similarly, when *M. systena* grew exposed to the average inoculum, the non-
399 preferred mycorrhizal type (i.e. AM in this case) was generally greater in the oldest soils compared
400 to the young soils (Fig. 3). The AM fungal colonisation (preferred type) of *A. rostellifera* and to
401 some extent the ECM fungal colonisation (preferred type) of *M. systena* was significantly lower in
402 the 'specific inoculum' compared to the 'average inoclum' treatment (Fig. 3).
403
404 *Nitrogen-fixing nodule biomass and % Ndfa*
405 A sharp decline (97.5 %) in the production of N_2-fixing nodules with increasing soil age (from the
406 youngest to the oldest soil) were found on the roots of *A. rostellifera* in the field soil (Fig. 5). This
407 strong pattern was also observed in the average inoculum, but not in the specific inoculum, where
408 differences between soil ages did not differ significantly (Fig. 5). Although in the field soil
409 treatment we found the lowest nodule biomass in the oldest soils (Fig. 5, Fig. S1), we estimated
410 that *A. rostellifera* plants growing these old severely impoverished soils obtained the greatest
411 proportion of their overall N uptake as atmospheric N_2, compared to all other soil ages (Fig. 5).
412 The N-derived from air in the other soil treatments were not significantly different (Fig. 5).
413
414 *Foliar nutrients*
415 Foliar manganese (Mn) levels in *A. rostellifera* greatly increased with soil age and was significantly
416 greater in the two oldest soils (~120 and >2000 ka) compared to the two youngest soils (<0.1 and
417 ~1 ka) (Fig. 6). This pattern was found in the average and specific inoculum soil treatments (Fig. 6).
418 Other key foliar nutrients such as P did not differ significantly across soil ages (Fig. S7), however
419 foliar nutrient concentrations analysed together as a group were significantly affected by soil age

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420 (Table S3). By contrast, *M. systena* showed significant declines in foliar [P] with increasing soil age;
421 the greatest concentrations were found in the very young soils (<0.1 ka) and both the oldest soils
422 (~120 ka & >2000 ka) had the lowest concentrations (Fig. S8). Similar to other plant responses, the
423 trend found in the field soil remained similar in the average inoculum soil treatment but the trend
424 was more muted (Fig. S8). Foliar Mn levels in *M. systena* were similar across soil ages (Fig. 6).
425
426 *Hyphal length in soils*
427 For *M. systena* growing in the specific inoculum soil treatment, hyphal length strongly increased
428 with soil age (Fig. S2, Fig. S3). In this case, hyphal length was significantly greater in the two oldest
429 soils (~120 ka & >2000 ka) compared to the other three young and medium-aged soils (<0.1k, ~1k,
430 and ~7 ka) (Fig. S2). On a per root biomass basis this trend was still apparent (Fig. 7), providing
431 evidence for mycorrhizal fungi present in the oldest soil (>2000 ka) likely had an 'explorative'
432 hyphal strategy compared to the younger soil ages (<0.1 ka, ~1 ka, and ~7 ka), thus suggesting a
433 larger investment in extraradical hyphae for P scavenging. In the field and average inoculum soils
434 where either *A. rostellifera* or *M. systena* grew, hyphal length did not differ significantly across soil
435 ages (Fig. S2).
436
437 **Discussion**
438 In this study, we investigated the effects of strong soil nutrient limitations (in particular N and P)
439 on the growth of plants forming multiple root symbioses, root occupancy levels of different types
440 of symbionts, and associated functional responses. Importantly, we also aimed to disentangle the
441 effects of soil abiotic properties (soil nutrient availabilities) vs soil biotic effects (priority and
442 propagule density effects). Finally, we aimed to determine if shifts in mycorrhizal type 'mycorrhizal
443 switching' were more driven by plant-selection processes or to differences in inoculum potential.
444 Plants capable of forming multiple root symbioses had clear hump-shaped growth patterns along
445 the soil nutrient availability gradient while maintaining relatively high levels of mycorrhizal
446 colonisation by their 'preferred' root symbionts. We found clear increases in foliar Mn
447 concentrations with increasing soil age with *A. rostellifera* suggesting that these plants might also
448 rely on the release of carboxylates to help acquire P in the most impoverished soils (Lambers *et al.*
449 2015). Our results also suggest that soil abiotic properties, such as strong differences in soil
450 nutrient availability, are more important than soil biotic properties (e.g., inoculum potential) in
451 explaining these shifts in our plant and root responses.
452

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453 *Abiotic vs biotic factors in explaining the responses of plants with multiple root symbioses during*
454 *ecosystem development*
455 *Plant growth responses*
456 We included three soil inocula treatments as opposed to only the 'field soil' with the first three
457 youngest soil ages used in Albornoz *et al.* (2016a). We found evidence for increased plant growth
458 in the most severely-impoverished soil when host plants were exposed to the full root symbiont
459 species pool (i.e. average inoculum vs. field soil). This finding suggests that plant abundance in
460 these nutrient-poor soils is to some degree constrained by the absence of optimal root symbionts.
461 The improved growth of *M. systena* in the oldest soils, when exposed to full soil biota species pool,
462 was particularly clear, suggesting that soil biota from the young to medium-old soils are more
463 optimal and beneficial for plant growth compared to specific soil biota from the oldest soils.
464 Nevertheless, the improved growth of *A. rostellifera* and *M. systena* in the 'average inoculum'
465 relative to 'field soil' in the oldest most nutrient poor soils may also be due to a small fertilisation
466 effect from the inoculum itself (see Supporting Information). Even though only 7% v/v of inoculum
467 was used, we cannot rule out the possibility that small amounts of young soil (from <0.1 ka and ~1
468 ka) increased the levels of P availability in the older soils of the 'average inoculum' treatment.
469 Future experiments should aim to circumvent this caveat by isolating soil-age specific mycorrhizal
470 fungi (e.g. ECM fungi from the youngest soils) and then inoculate the host plant before growing in
471 the oldest most nutrient-poor soils.
472 For the 'specific inoculum' soil treatment, in which plants were exposed to soil-age specific
473 inocula, overall we did not find marked plant growth patterns between the soil ages. In the case of
474 *A. rostellifera* this soil treatment muted the plant growth responses with soil age, suggesting that
475 this plant species: i) associates with a broad range of root symbiotic species; or ii) beneficial root
476 symbionts such as AM and ECM are not critical for nutrient-acquisition. *M. systena*, on the other
477 hand, did better in 'specific inoculum' in the very young soils compared to the young soils in the
478 other soil inoculation treatments ('field soil' and 'average inoculum'), thus suggesting that average
479 soil abiotic conditions such as higher N availability, notably improve the growth of this plant
480 species.
481 *Root functional responses*
482 *A. rostellifera* generally had more root growth compared to shoots (i.e. higher root:shoot ratio)
483 when grown in very nutrient-poor soils (Fig. S4). This response is in line with earlier studies (Hodge
484 2004; Hodge 2009). Furthermore, when *A. rostellifera* grew in the 'average inoculum' soil,
485 interestingly the ECM fungal colonisation levels significantly increased with increasing soil age. Our

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486 results show that when exposed to the entire ECM fungal species pool, *A. rostellifera* colonisation
487 by ECM fungi is higher in the poorest soils. Results from the 'specific inoculum' soil only provided
488 evidence towards a shift toward mycorrhizal fungal species that invest more in extraradical hyphal
489 biomass with increasing soil age only with *M. systena* (Fig. S2). The increase in AM fungal
490 colonisation in *A. rostellifera* is the opposite of what we previously found (Albornoz *et al.* 2016a).
491 One possible explanation for this discrepancy is that we grew the plants from seed and applied the
492 soil treatments early on as opposed to transplanting commercial nursery-grown seedlings into the
493 potted field soil. Overall, the results from our glasshouse experiment complement field data from
494 vegetation studies at the Jurien Bay chronosequence. In these previous studies, we found that the
495 abundance of non-mycorrhizal plants increased with soil age along the Jurien Bay
496 chronosequence, at the expense of mycorrhizal plants (Zemunik et al. 2015). By contrast, our
497 results here show a general increase in mycorrhizal colonization (AM or ECM) within individual
498 plant species. However, changes in nutrient-acquisition strategies at the community level are
499 strongly driven by changes in plant community composition, and much less so by plastic responses
500 within individual species, because there is very high species turnover across the chronosequence
501 (Zemunik et al. 2016).
502
503 The benefits in having more than one root-associated mutualistic associations is not well known,
504 yet we show that very low soil P levels could be a key driving factor. Generally the mycorrhizal
505 symbiosis is considered beneficial to plants since they improve nutrient uptake at a modest C cost
506 and non-beneficial or producing negative effects when C costs exceed the benefits of improved
507 nutrient uptake (Johnson, Graham & Smith 1997; Tuomi, Kytöviita & Härdling 2001; Jones & Smith
508 2004). That C cost is likely small compared to producing more fine roots since it only involves
509 expending C for the growth and maintenance of fungal hyphae. However, C costs of forming AM vs
510 ECM associations are different, ECM fungi receive more C from their host plants than AM fungi
511 (Leake *et al.* 2004; Gehring, Mueller & Whitham 2006; Orwin *et al.* 2011). As such, a plant capable
512 of forming both types presumably selects the most favourable type based on cost-efficiency
513 altogether driven by soil nutrient availability levels. However, there are conflicting results from
514 studies comparing the cost-efficiency between mycorrhizal and non-mycorrhizal plants (Jones,
515 Durall & Tinker 1991; Jones, Durall & Tinker 1998; Tuomi, Kytöviita & Härdling 2001) that appear
516 due to the level of P or N limitations (Colpaert, Van Laere & Van Assche 1996; Jones, Durall &
517 Tinker 1998). Furthermore, a recent comprehensive study showed that C is not a major "cost" and
518 mycorrhization depends more on the increase or not in nutrient acquisition that a plant will

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519 experience (Correa *et al.* 2012). Similarly, we propose that in dual-mycorrhizal plants, the
520 mycorrhizal status shifts depending on the nutrient availability in soil, where very low levels of
521 growth-limiting nutrients such as P and N regulate the 'switching' and preference.
522
523 *'Mycorrhizal switching' among plants capable of forming multiple root symbioses*
524 We demonstrate dual-mycorrhizal symbioses for *A. rostellifera* and *M. systena* and suggest that
525 there might be more of these dual-mycorrhizal plant species (Teste et al. 2017; Zemunik et al.
526 2015) than previously thought (Brundrett 2009). We show that plants forming associations with
527 more than one group of mycorrhizal fungi, retain a certain level of preference for AM or ECM fungi
528 across a strong nutrient-availability gradient. Our results provide further support for the
529 hypothesis that these plants can switch symbionts across natural soil availability gradients, which
530 is presumably linked to differences in the strength and type of nutrient limitation (i.e. N vs. P
531 limitation of plant growth as soils age; Laliberté et al. 2012). *A. rostellifera* showed a preference
532 for AM fungi in the poorest soils, while *M. systena* was more strongly colonised by ECM fungi
533 across all chronosequence stages. Furthermore, on the basis of percent root colonisation, *A.*
534 *rostellifera* had greater AM fungal colonisation of roots in the oldest soil (~2 Ma), that had the
535 lowest levels of P, compared to the more fertile soils younger soils. In the case of *M. systena*, it
536 had greater levels of ECM fungal colonisation in the oldest soils only compared to the youngest
537 (<0.1k). Still, AM fungal colonisation in *M. systena* tended to be greater in the oldest soils but only
538 in some cases (~120 ka and ~2000 ka greater than ~1 ka old soil; Fig. 3). We expected that plants
539 'switch' to mycorrhizal types that possess more complex morphological structures than AM fungi
540 (e.g. ECM fungi have rhizomorphs and/or elaborate mantles with prolific extraradical hyphae) in
541 the most nutrient-impoverished soils. The latest mycorrhizal fungal community studies partly
542 support our 'mycorrhizal switching' hypothesis since both AM and ECM fungal community
543 diversity significantly falls in the oldest poorest soils (Krüger *et al.* 2015; Albornoz *et al.* 2016b).
544 This decline in mycorrhizal fungal species diversity might be associated with a decrease in fungal
545 inoculum density in the poorest soils. Therefore, these declining fungal responses (i.e. diversity
546 and density) would likely not favour greater levels of mycorrhizal colonisation (Baar *et al.* 1999;
547 Taylor & Bruns 1999; Teste *et al.* 2016), yet we noted an increase in mycorrhizal colonisation in
548 our AM and ECM host plants along with soil age, supporting our 'mycorrhizal switching'
549 hypothesis.
550
551 *High foliar manganese and adaptive strategies for severely impoverished soils*

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552 *A. rostellifera* foliar Mn increased with soil age in all soil treatments, and this trend was
553 particularly strong in the 'field soil' as soil P declined. Increase in foliar Mn is consistent with
554 greater reliance on carboxylates for P acquisition in P-poor soils (Lambers *et al.* 2015; Abrahao *et*
555 *al.* 2018; Pang *et al.* in press). Interestingly, this trend remained in the average and specific
556 inoculum soil treatments suggesting that specific fungi may have led to increased carboxylate
557 exudation. However, *A. rostellifera* AM fungal colonisation also increased with soil age. *M. systena*
558 foliar Mn also increased with soil age but due to large variation in the last two soil ages (~120 ka
559 and ~2Ma years) differences were not significant. Altogether, our findings support the view that
560 plants capable of forming multiple root symbioses are deploying multiple nutrient-acquisition
561 strategies in response to very nutrient-impoverished soils.
562
563 Lambers *et al.* (2015) proposed that Mn accumulation in foliage might reflect increased
564 transpiration to get more P via mass flow in these P-poor sandy soils, where mass flow could
565 perhaps make an important contribution. If so we would expect a more depleted foliar $\delta^{13}C$
566 signature in older soils, however our foliar $\delta^{13}C$ data did not support this contention (Figs. S6-S9).
567 Nevertheless, our foliar P data partly supported the contention, since the P concentrations in the
568 leaves of *M. systena* were significantly lower in older soils, but not *A. rostellifera* (Figs. S6-S9).
569
570 Our findings suggest that plants associating with multiple root symbionts partly rely on multiple
571 nutrient-acquisition strategies, such as the carboxylate-releasing strategy (Lambers et al. 2015), to
572 grow in the most nutrient-poor soils. Furthermore, we also propose that plants capable of
573 associating with multiple root symbionts are functioning with fewer species of mycorrhizal fungi
574 (Krüger *et al.* 2015; Albornoz *et al.* 2016b) yet in a more efficient way. For example, in the case of
575 *A. rostellifera*'s *rhizobia* nodules responsible for the N_2-fixing, plant N derived from air was the
576 greatest in the oldest most impoverished soils with very little nodule biomass. The sharp decline in
577 nodule biomass with increasing soil age was also observed in the average inoculum treatment
578 suggesting a strong and general pattern. As for *M. systena*, ECM extraradical hyphal density was
579 greatest in the most impoverished soil of the 'specific inoculum' treatment; while ECM root
580 colonisation was invariant, suggesting adaptive symbiotic responses to severe P limitation.
581 However, our earlier work suggested that plants are not investing more C in a particular
582 mycorrhizal symbiosis or its structures such as extraradical hyphae under severe P limitation
583 (Teste *et al.* 2016), it does not exclude the idea that mycorrhizal plants are simultaneously

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584 deploying more than one type root symbiosis and releasing more carboxylates in response to
585 strong shifts in nutrient availability.
586
587 *Conclusions*
588 The large shifts in soil nutrient availability found along this long-term soil chronosequence
589 imposed strong growth limitations to co-occurring plants that host multiple root symbioses. Soil
590 abiotic properties (i.e. nutrient availabilites in particular) were more important than effects of soil
591 biotic properties such as mycorrhizal inoculum potential in explaining the shifts in our plant and
592 root responses. We found that *A. rostellifera and M. systena* plants maintained high levels of root
593 colonisation by their 'preferred' mycorrhizal fungal groups (AM for *A. rostellifera* or ECM for *M.*
594 *systena*) in the oldest most nutrient-impoverished soils. The lower colonization levels of the
595 'preferred' mycorrhizal fungal types in both plant species observed in the 'specific inoculum' is
596 difficult to explain. One possibility is that the bulk soil that was used to create this soil treatment
597 had sufficient P availability (i.e. resembled a ~7k old soil in terms of soil P levels) for the plants to
598 down-regulate investment in mycorrhizas. This finding thus lends some support for the idea that
599 mycorrhizal symbioses provide more energetically-viable benefits in nutrient-poor soils (Smith &
600 Read 2008). Other root symbionts such as *rhizobia* on *Acacia* appeared to show more variable
601 responses; drastic decrease (97.5%) in nodule biomass yet maintenance of high levels of N_2-
602 fixation in the most impoverished soils. We also found that *A. rostellifera* had high foliar Mn
603 concentrations indicating some reliance on the release of carboxylates as another strategy to cope
604 with severe P-limitation. Our findings support the hypothesis that shifts in the root occupancy of
605 symbionts is primarily determined by soil nutrient availability. We further hypothesize that dual-
606 mycorrhizal plants may have an advantage in severely nutrient-impoverished soils within semi-arid
607 ecosystems because they benefit from hosting more than one nutrient-acquisition strategy and
608 remain competitive in diverse plant communities. Our study provides evidence that better plant
609 growth in the most nutrient-impoverished soils is achieved when plants are exposed to the all soil
610 biota species from the regional species pool, suggesting that plant abundance may be constrained
611 by optimal root symbiont species. Finally, in the most nutrient-impoverished soil, mycorrhizal
612 plants likely remain competitive and relatively abundant since they not only benefit from
613 associating with multiple root symbionts, but also by deploying other nutrient-acquisition
614 strategies such as the carboxylate-releasing strategy.
615
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625

626 *Authors' contributions*

627 All authors contributed equally to the conception and design of the study and interpretation of the
628 data. FPT gathered most of the data and led the analyses. All authors contributed critically to the
629 drafts and gave final approval for publication.
630

631 *Data accessibility*

632 Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.q3fv55j (Teste &
633 Laliberté, 2018)

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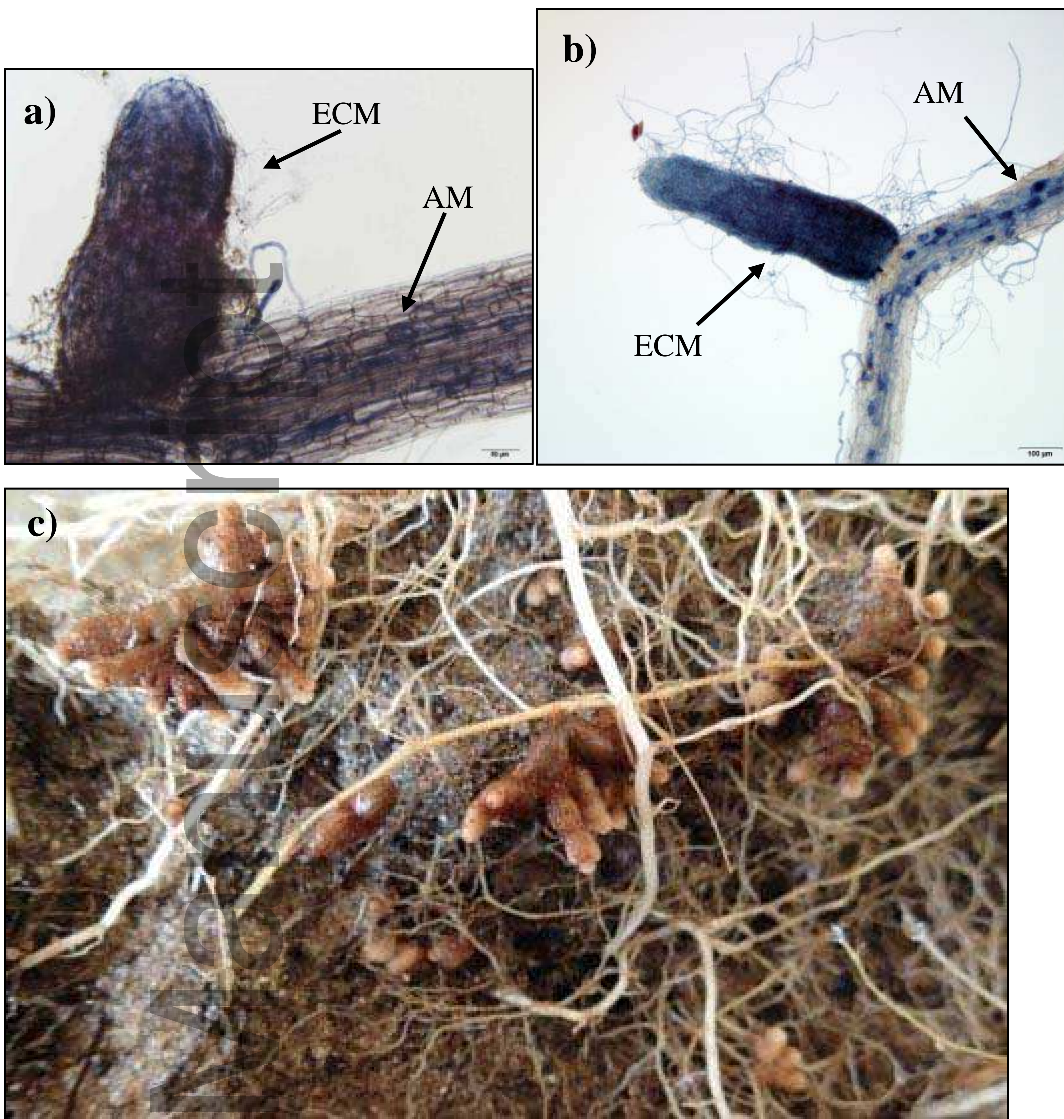

Fig. 1 Multiple root symbioses found on *Acacia rostellifera* and *Melaleuca systena*. Dual mycorrhizal symbioses, such as these ones, are commonly observed on many species from the Fabaceae and Myrtaceae in Western Australia. Shown here are **a)** *A. rostellifera* and **b)** *M. systena* hosting ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi on the same root fragment. Arrows are pointing to characteristic features of ECM and AM fungi, such as the mantle and arbuscules, respectively. Shown in **c)** *A. rostellifera*'s root system with root nodules formed by dinitrogen (N_2)-fixing bacteria in *A. rostellifera*. Photo credits: F.P.Teste.

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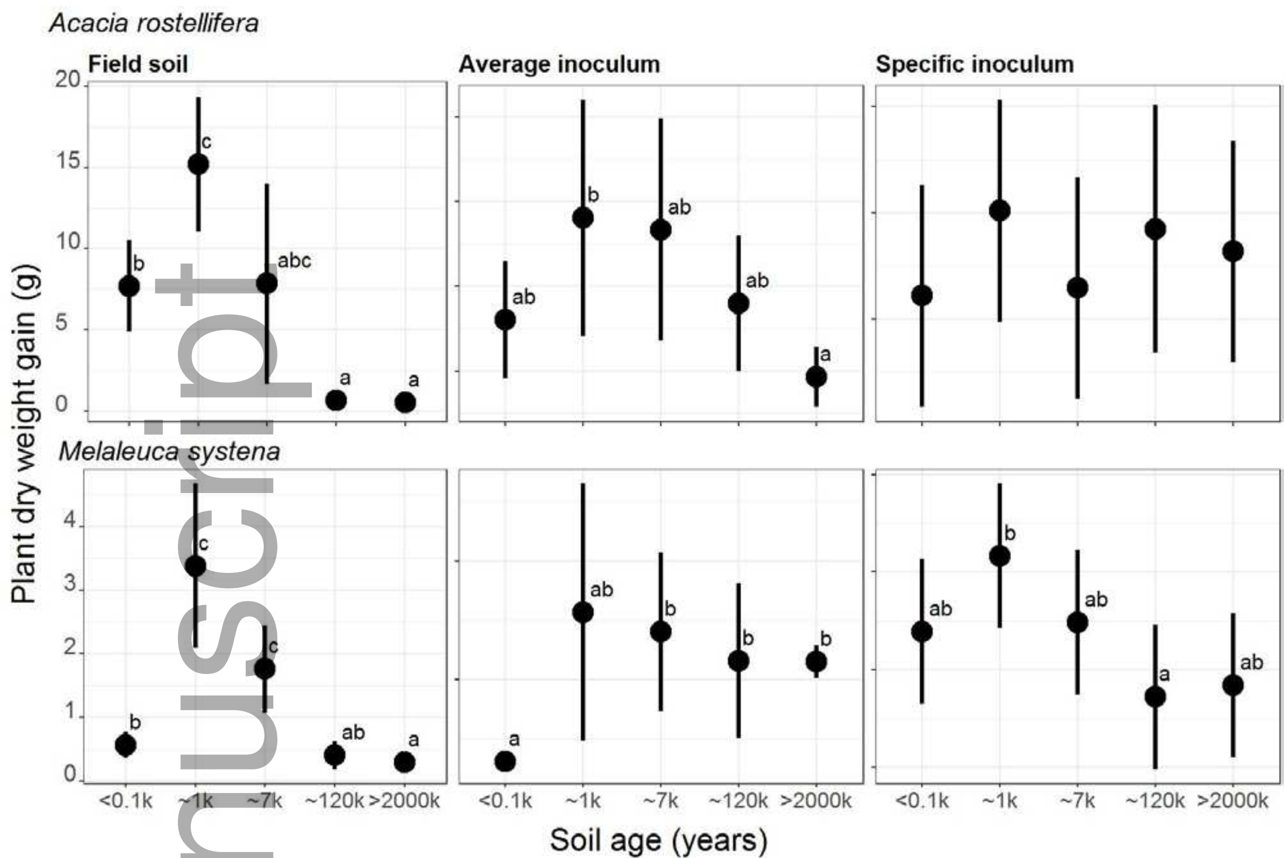

841

842 **Fig. 2** Total dry weight gain of *Acacia rostellifera* and *Melaleuca systena* seedlings grown in soils of
843 different ages and with different types of soil inocula. Means with 95% confidence intervals are
844 shown. Also shown are different letters, which indicate statistical significance ($P \leq 0.05$), within
845 individual panels, based on linear-mixed effects models (Table S2a) and a Tukey HSD test
846 restricted to the soil age treatment within each soil inocula treatment. Interactions between soil
847 age and the soil inocula treatment were found in some cases (see Table S2a for details). Field soil:
848 seedlings grown directly in soil of different ages as is, Average inoculum: seedlings grown in sterile
849 soil of the different ages with a 7 % (v/v) average inocula gathered from all soil ages, Specific
850 inoculum: seedlings grown in sterile soil mixture from all soil ages with a 7 % (v/v) soil-age specific
851 inocula. Root to shoot ratio results are provided in **Fig. S4**.

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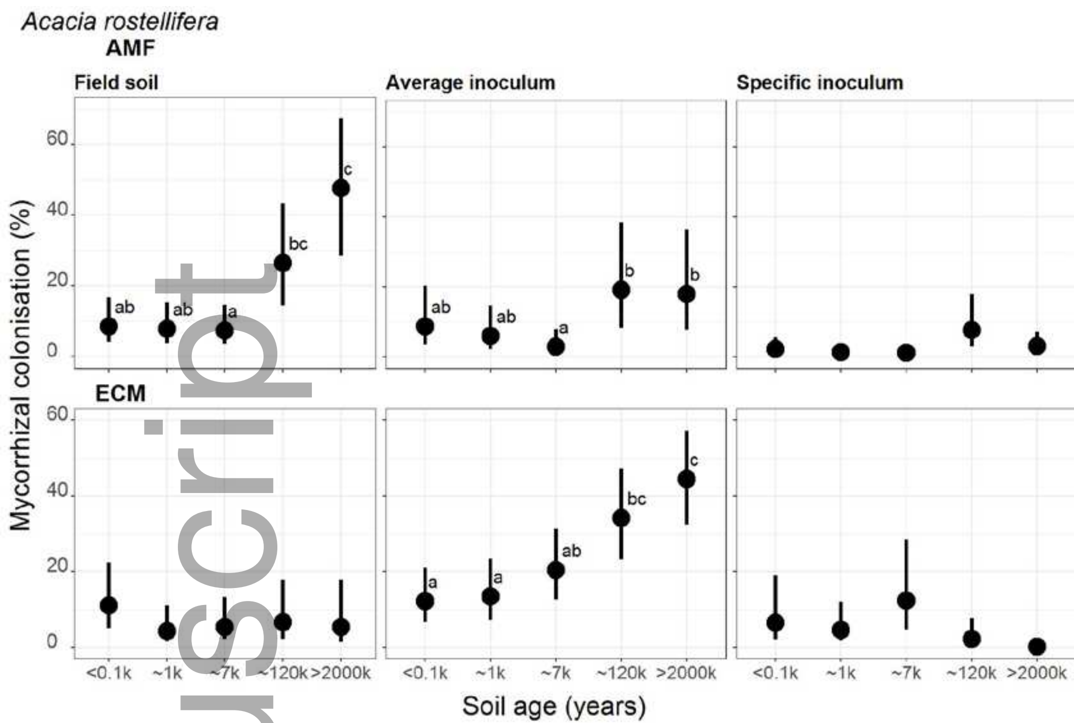

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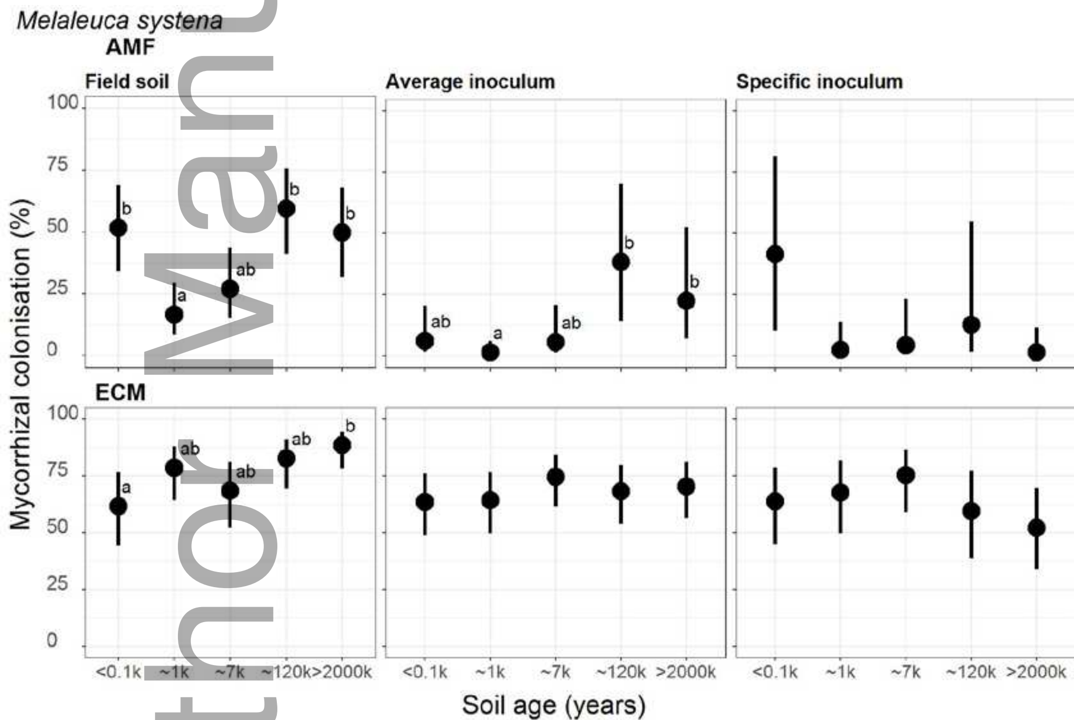

853

854 **Fig. 3** Arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungal colonisation levels on the
855 roots of *Acacia rostellifera* and *Melaleuca systena* seedlings grown in soils of different ages and
856 with different types of soil inocula. Means with 95 % confidence intervals are shown. Also shown
857 are different letters, which indicate statistical significance ($P \leq 0.05$), within individual panels,
858 based on a generalised linear-mixed effects model (binomial distribution) and Tukey HSD tests
859 restricted to the soil age treatment within each soil inocula treatment (Table S2b). Field soil:
860 seedlings grown directly in soil of different ages as is, Average inoculum: seedlings grown in sterile
861 soil of the different ages with a 7 % (v/v) average inocula gathered from all soil ages, Specific

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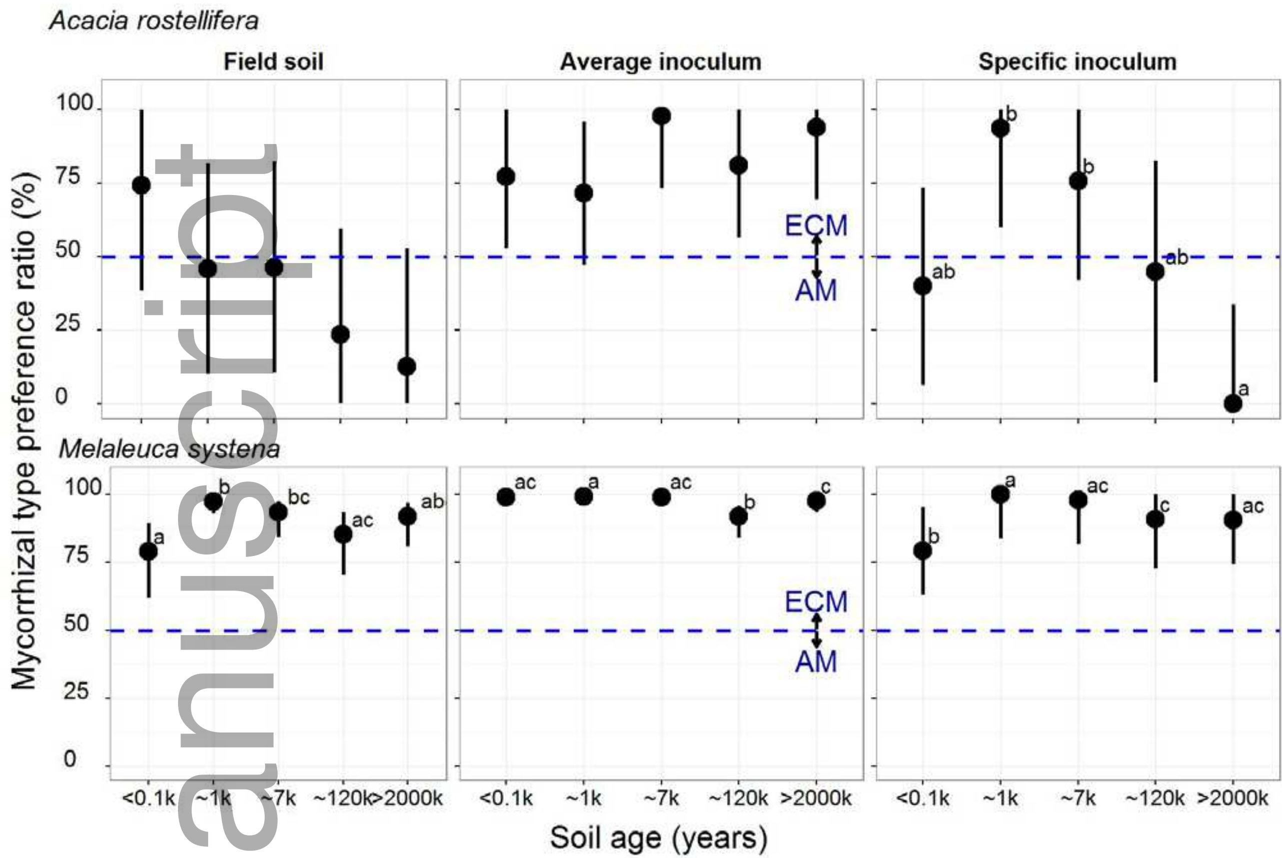
862 inoculum: seedlings grown in sterile soil mixture from all soil ages with a 7 % (v/v) soil-age specific

863 inocula.



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865

866 **Fig. 4** Mycorrhizal-type preference (MTP) ratio estimated as the ratio of ectomycorrhizal (ECM) to

867 arbuscularmycorrhizal (AM) fungal colonisation levels (% ECM/ % AM). The MTP ratio indicates if

868 'switching' between ECM and AM fungi occurs on the roots of *Acacia rostellifera* or *Melaleuca*

869 *systena* seedlings grown in soils of different ages and with different types of soil inocula. Means

870 with 95 % confidence intervals are shown and were based on a standardisation of the MTP ratio

871 using a hyperbolic tangent function to bind the values between 0 and 1. This transformation was

872 necessary since the raw MTP ratio had values equal to infinity (due to 0 in the denominator in

873 some cases) and standardised MTP ratio allowed for more interpretable results (e.g. MTP > 0.5

874 indicates a preference for ECM fungi). A linear-mixed effects model with a transformation (square

875 root or arcsin) was used to fit the data and test for treatment effects (Table S2h). Also shown are

876 different letters, which indicate statistical significance ($P \leq 0.05$) based on Bonferroni adjusted *P*-

877 values from all treatment level contrasts. Confidence intervals that overlap the 0.5 MTP dashed

878 line indicate there was no preference for either type of mycorrhizas. Field soil: seedlings grown

879 directly in soil of different ages as is, Average inoculum: seedlings grown in sterile soil of the

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880 different ages with a 7 % (v/v) average inocula gathered from all soil ages, Specific inoculum:
881 seedlings grown in sterile soil mixture from all soil ages with a 7 % (v/v) soil-age specific inocula.
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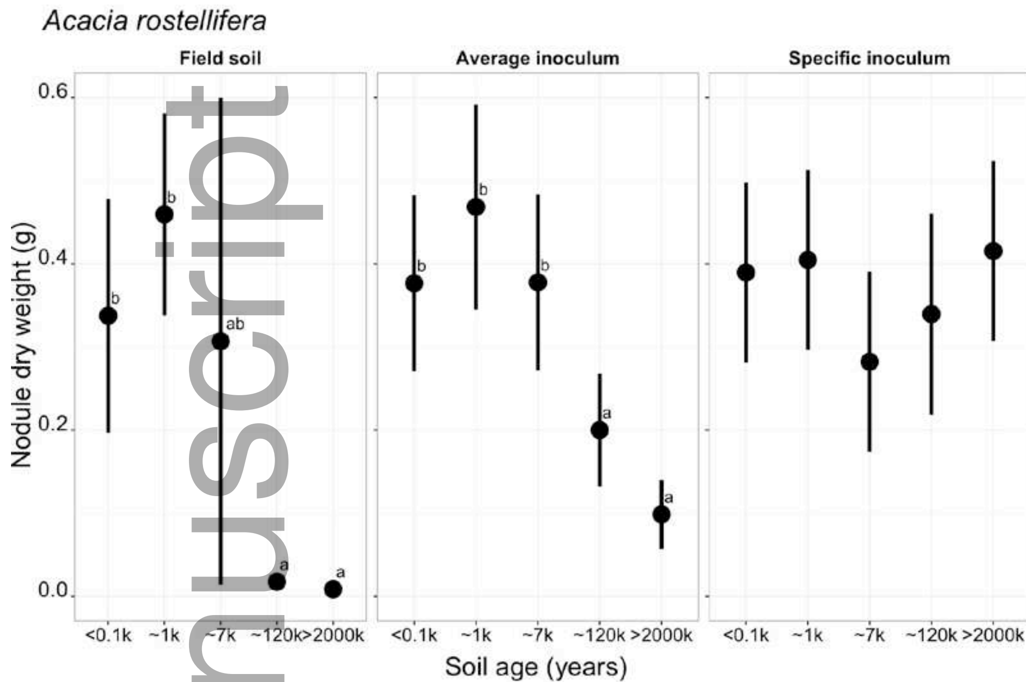

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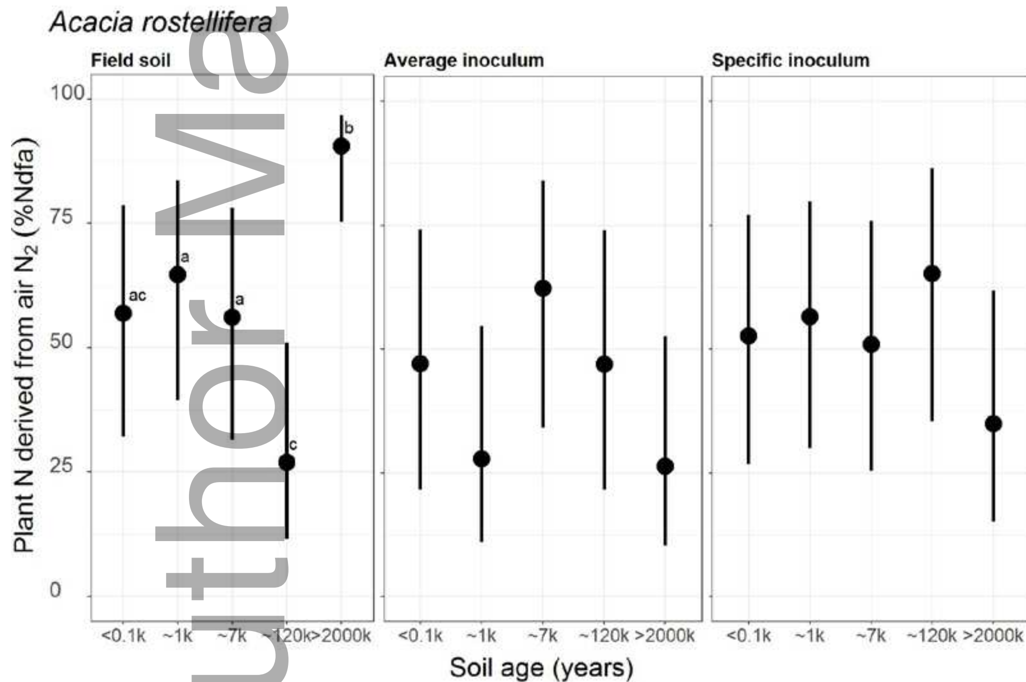

884

885 **Fig. 5** Total dry weight of *Acacia rostellifera* N_2-fixing nodules and Nitrogen-derived from air (Ndfa)
886 via N_2-fixation on seedling roots grown in soils of different ages and with different types of soil
887 inocula. Means with 95% confidence intervals are shown. Also shown are different letters, which
888 indicate statistical significance ($P \leq 0.05$), within individual panels, based on linear-mixed effects
889 models (Table S2c,d) and a Tukey HSD test restricted to the soil age treatment within each soil
890 inocula treatment. Interactions between soil age and the soil inocula treatment were found in

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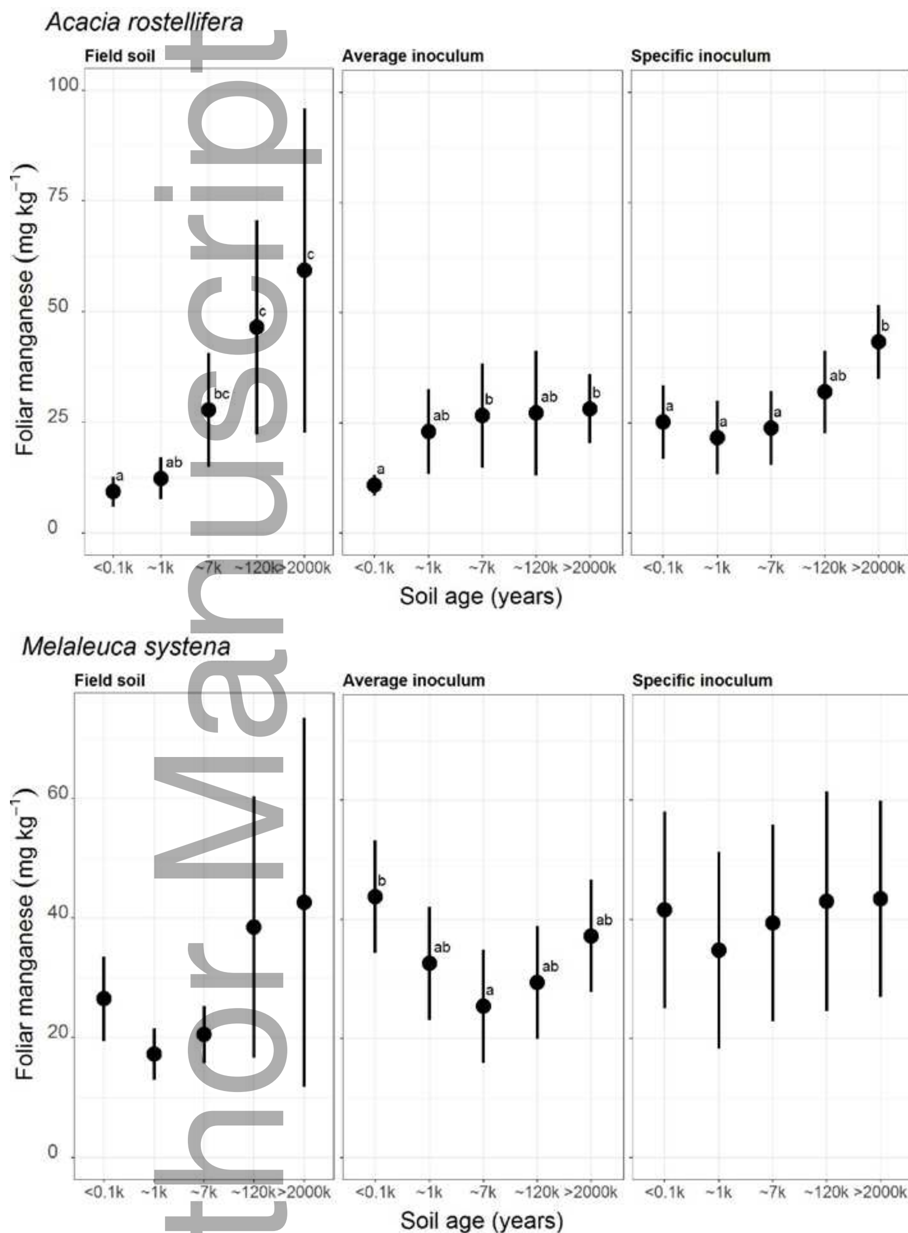
891 some cases (Table S2c,d). Field soil: seedlings grown directly in soil of different ages as is, Average
892 inoculum: seedlings grown in sterile soil of the different ages with a 7 % (v/v) average inocula
893 gathered from all soil ages, Specific inoculum: seedlings grown in sterile soil mixture from all soil
894 ages with a 7 % (v/v) soil-age specific inocula.



895

896

897 **Fig. 6** Foliar manganese (Mn) concentrations of *Acacia rostellifera* and *Melaleuca systena* seedlings
898 grown in soils of different ages and with different types of soil inocula. Means with 95 %
899 confidence intervals are shown. Also shown are different letters, which indicate statistical
900 significance ($P \leq 0.05$), based on linear-mixed effects models (Table S2e,f) and a Tukey HSD test.
901 Field soil: seedlings grown directly in soil of different ages as is, Average inoculum: seedlings
902 grown in sterile soil of the different ages with a 7 % (v/v) average inocula gathered from all soil
903 ages, Specific inoculum: seedlings grown in sterile soil mixture from all soil ages with a 7 % (v/v)
904 soil-age specific inocula.

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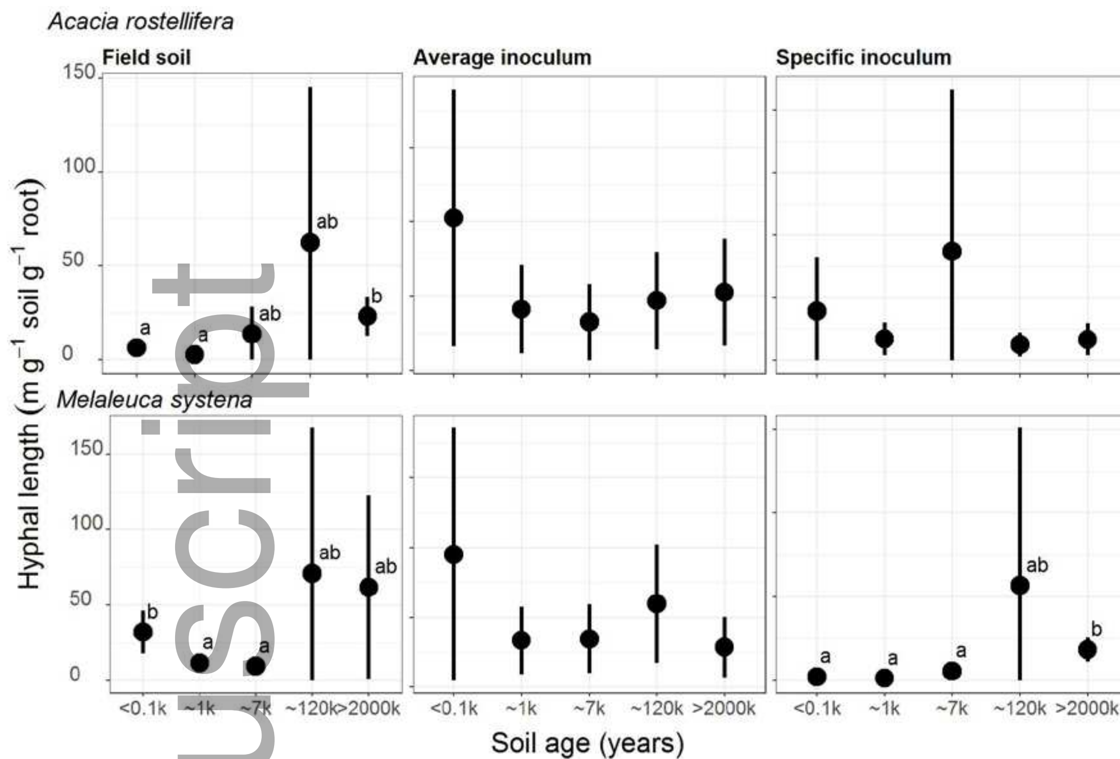

905

906 **Fig. 7** Total fungal hyphal lengths (on a per root biomass basis) found in soils around the roots of

907 *Acacia rostellifera* and *Melaleuca systena* seedlings grown in soils with different types of soil

908 inocula. Means with 95 % confidence intervals are shown. Also shown are different letters, which

909 indicate statistical significance ($P \leq 0.05$), based on linear-mixed effects models (Table S2g) and a

910 Tukey HSD test. Field soil: seedlings grown directly in soil of different ages as is, Average inoculum:

911 seedlings grown in sterile soil of the different ages with a 7 % (v/v) average inocula gathered from

912 all soil ages, Specific inoculum: seedlings grown in sterile soil mixture from all soil ages with a 7 %

913 (v/v) soil-age specific inocula.

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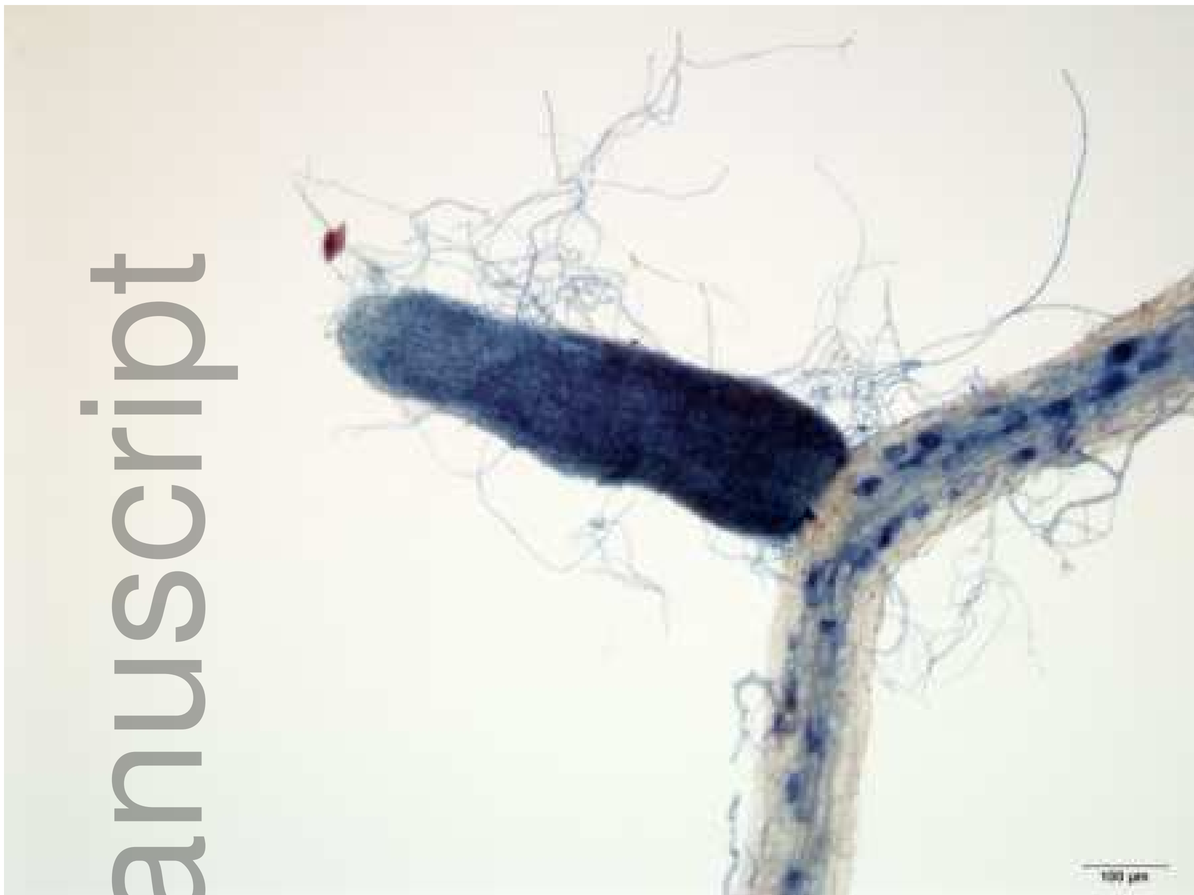

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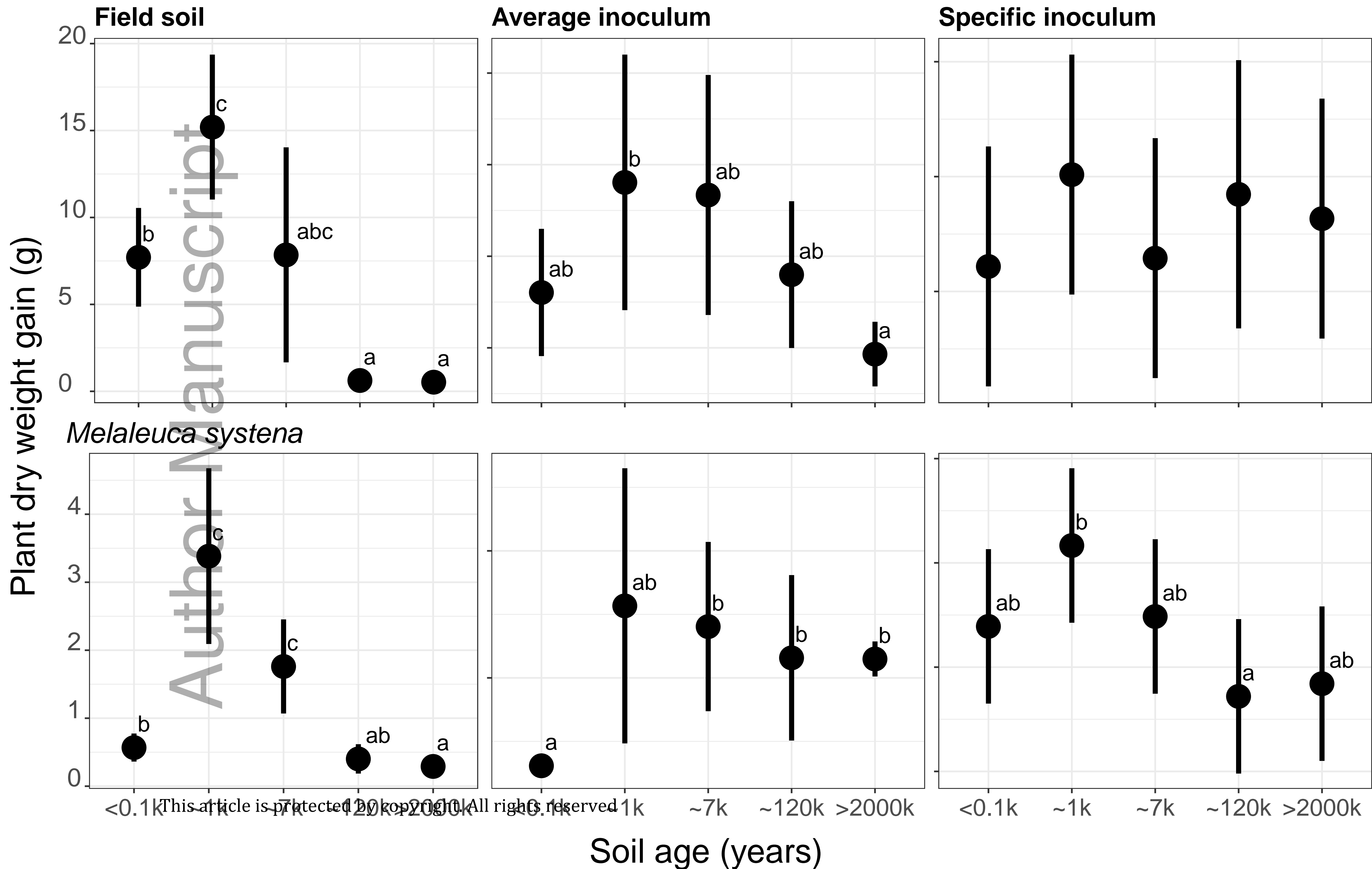

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Acacia rostellifera

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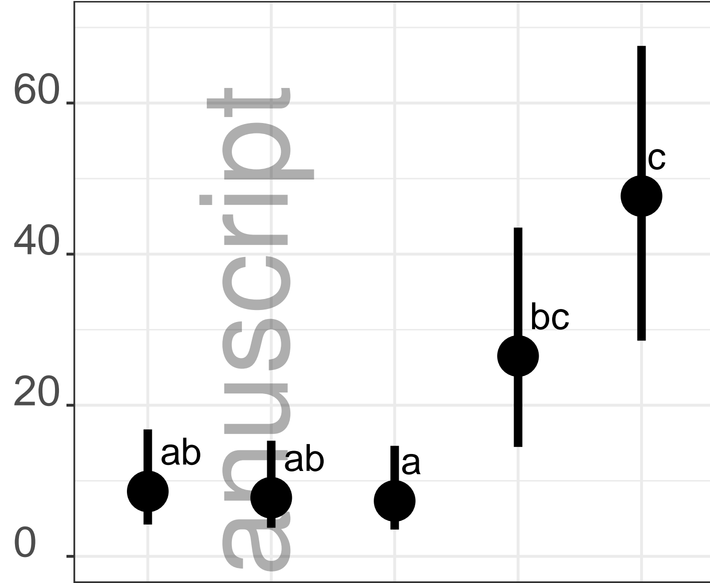

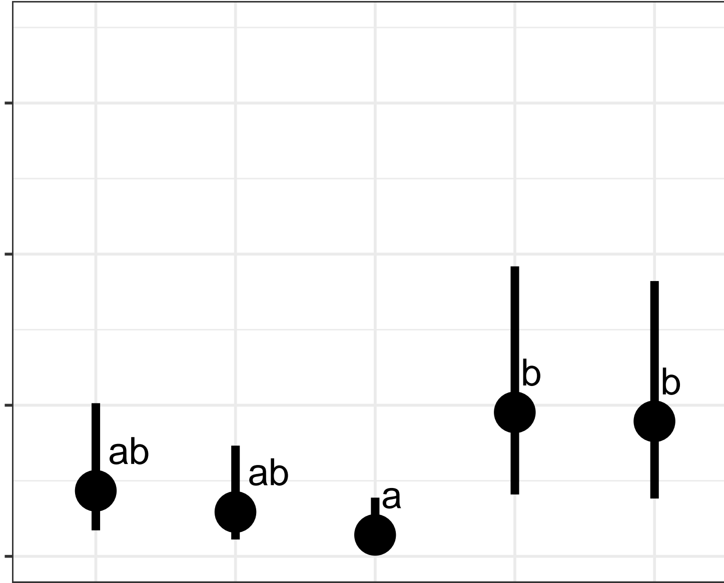

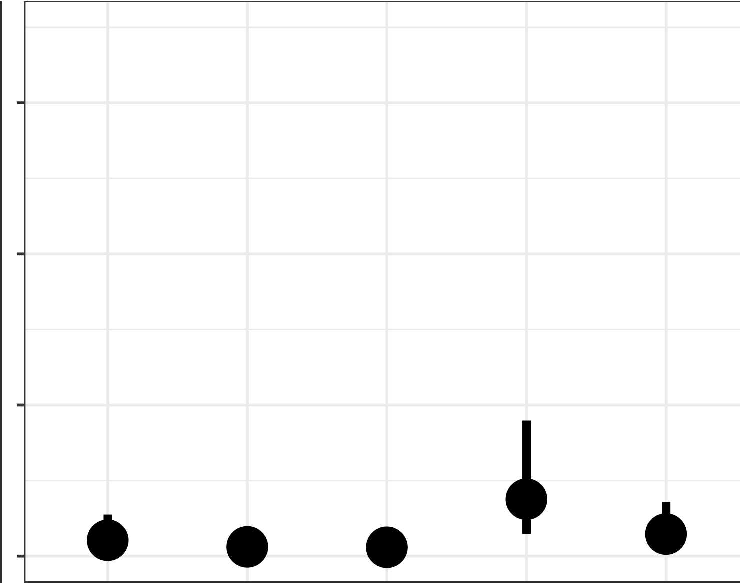

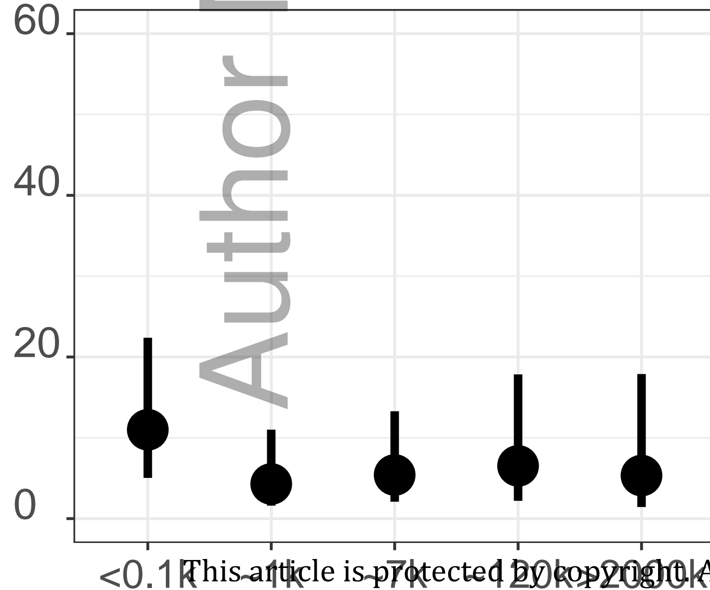

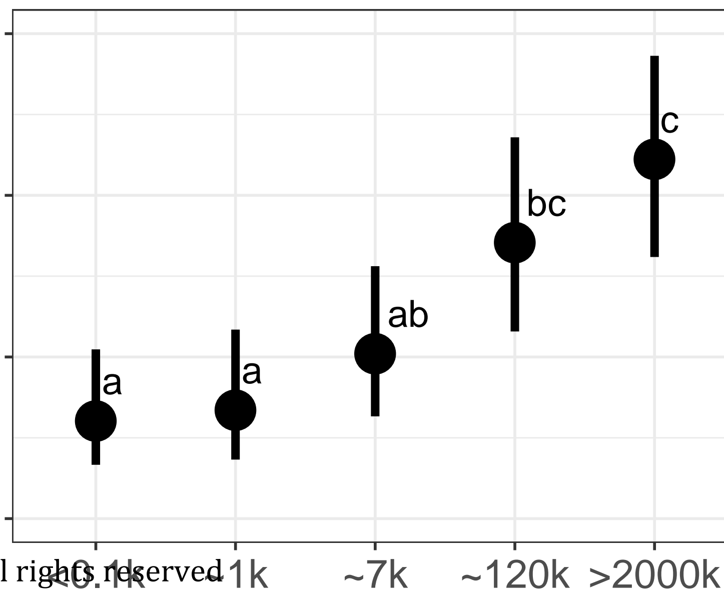

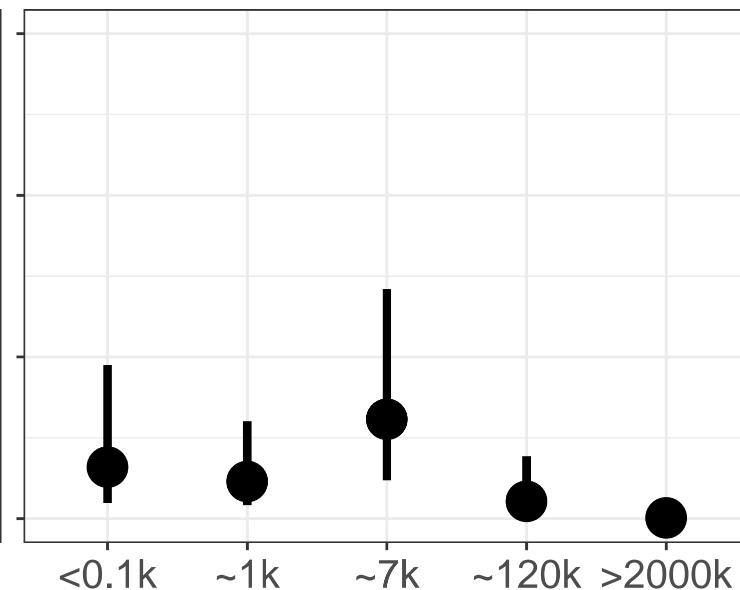

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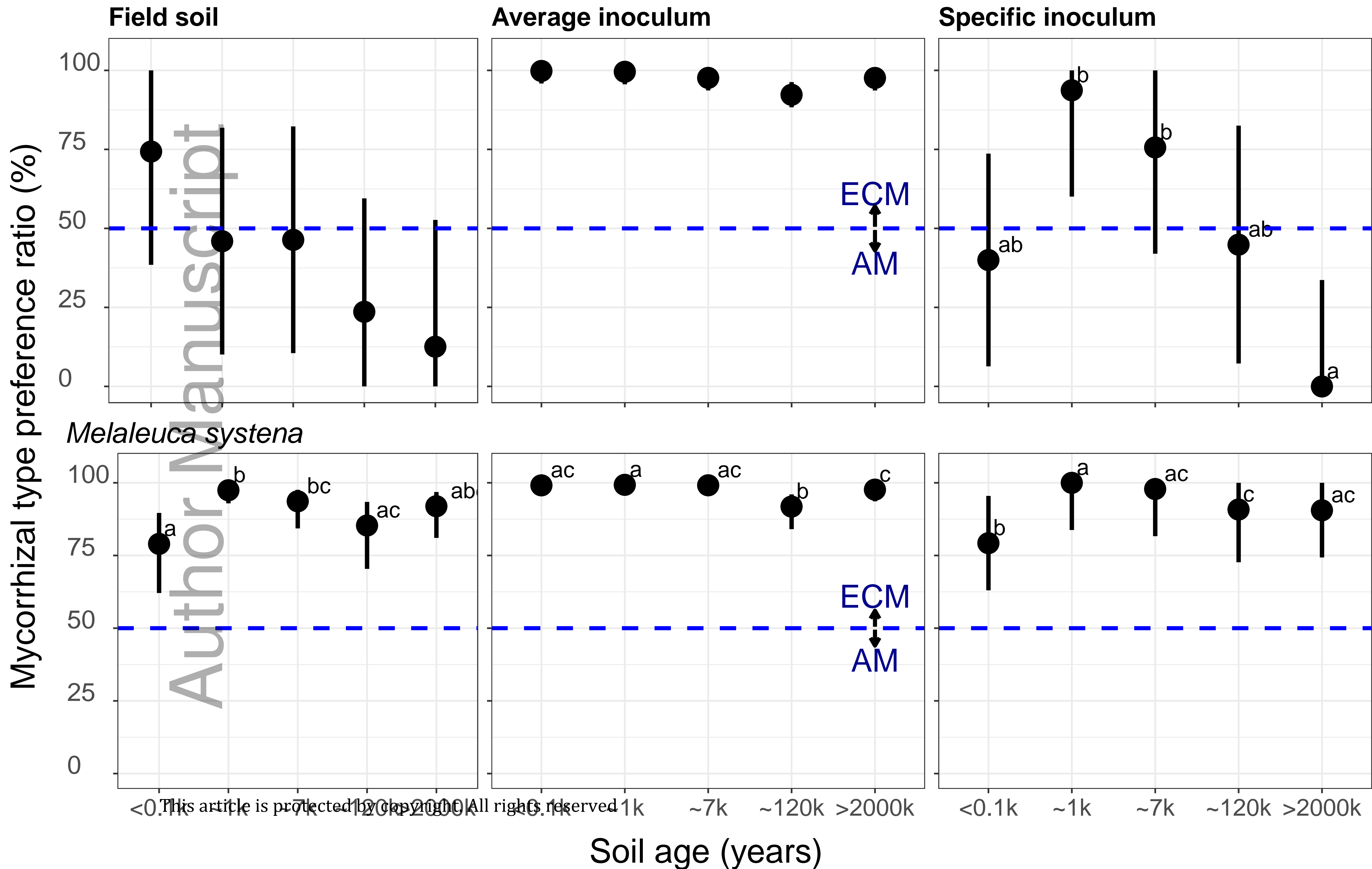

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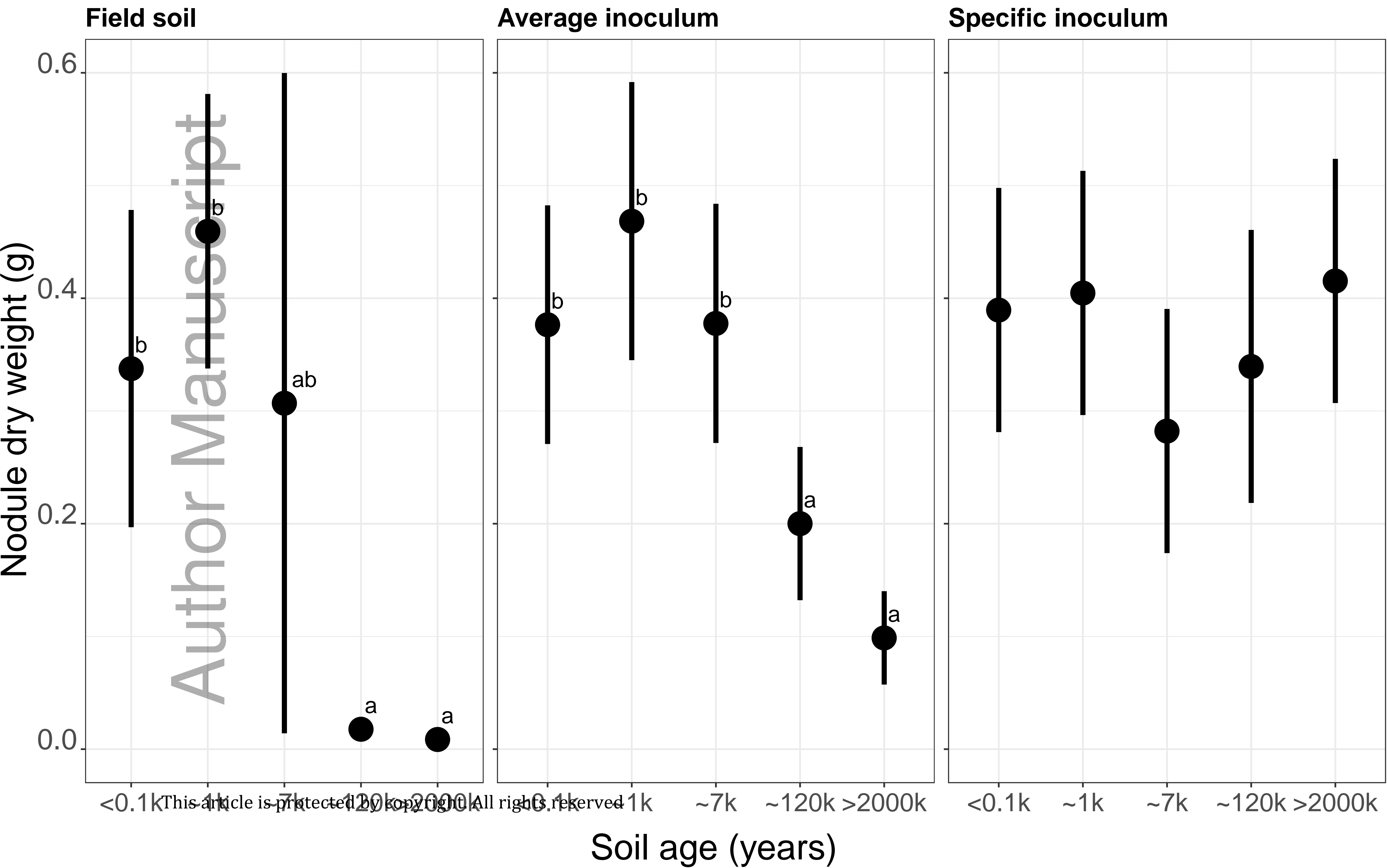

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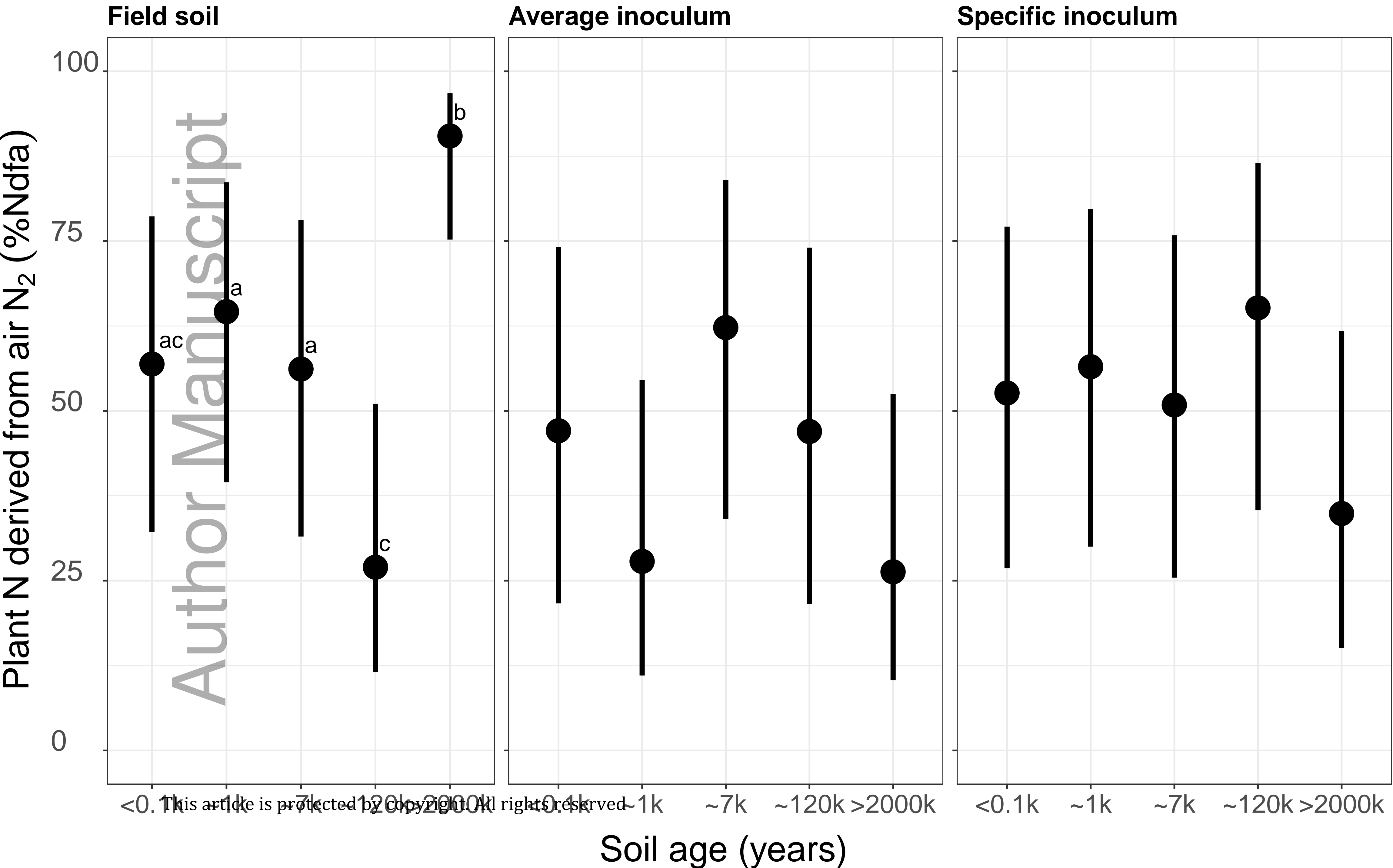

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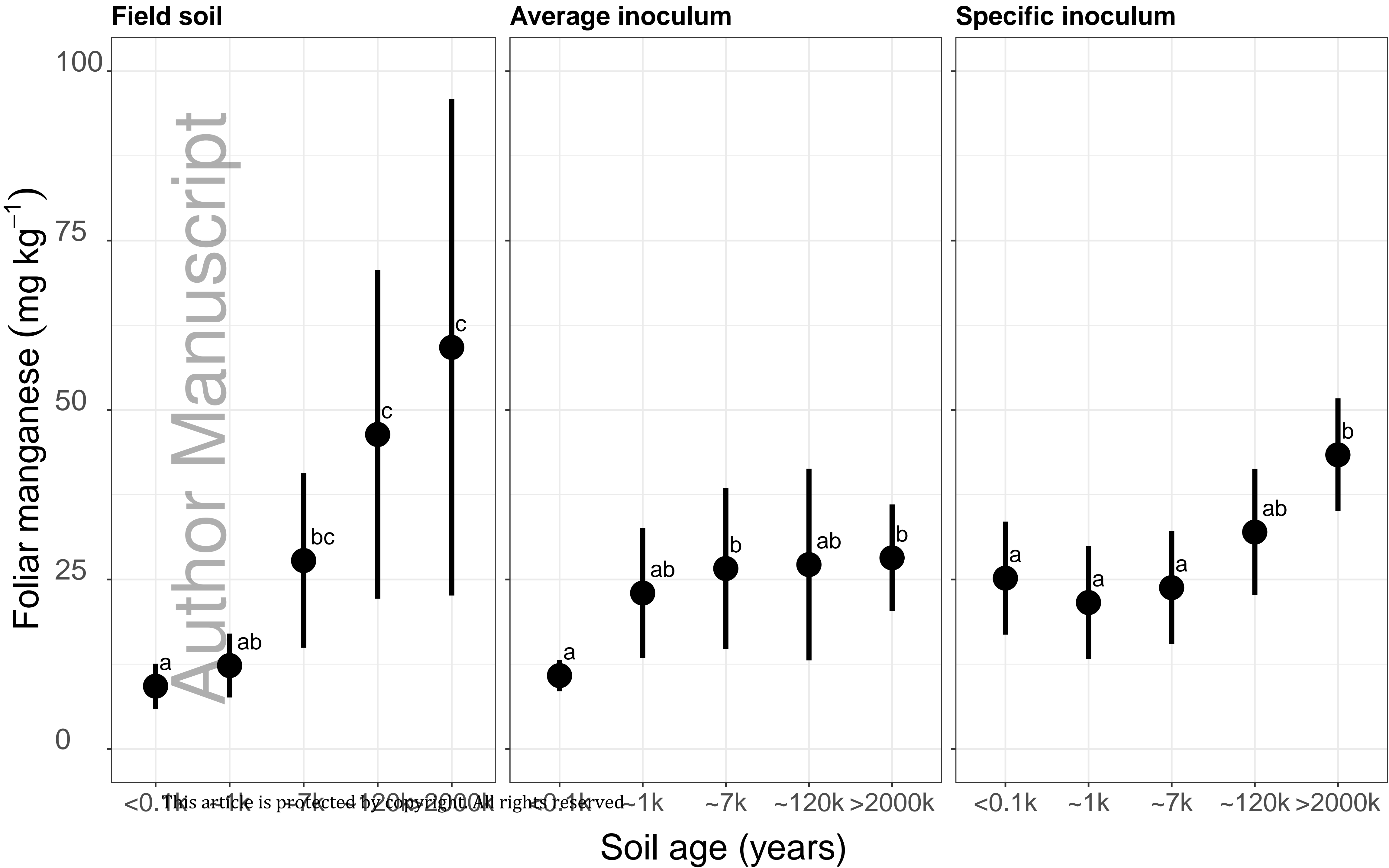

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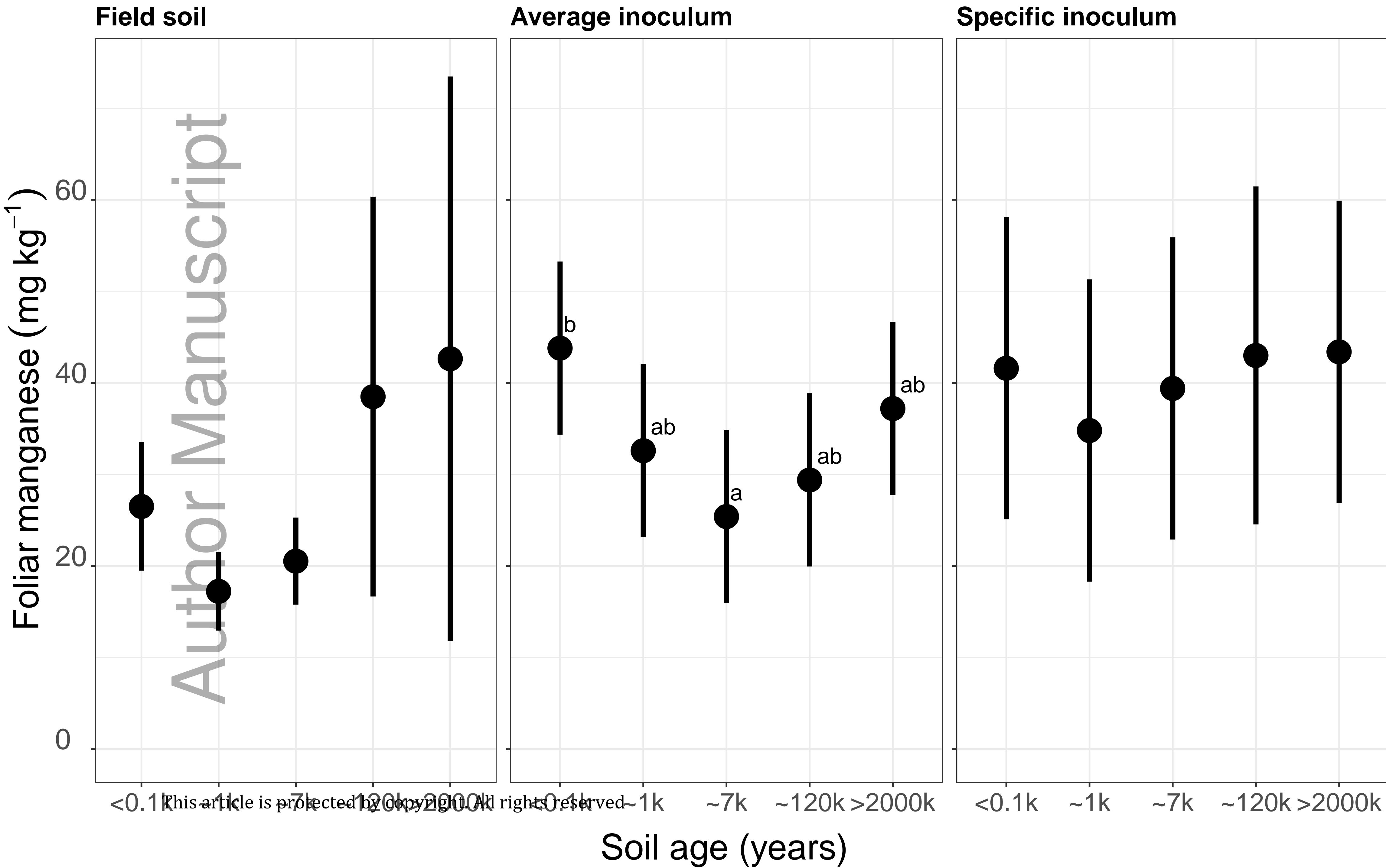

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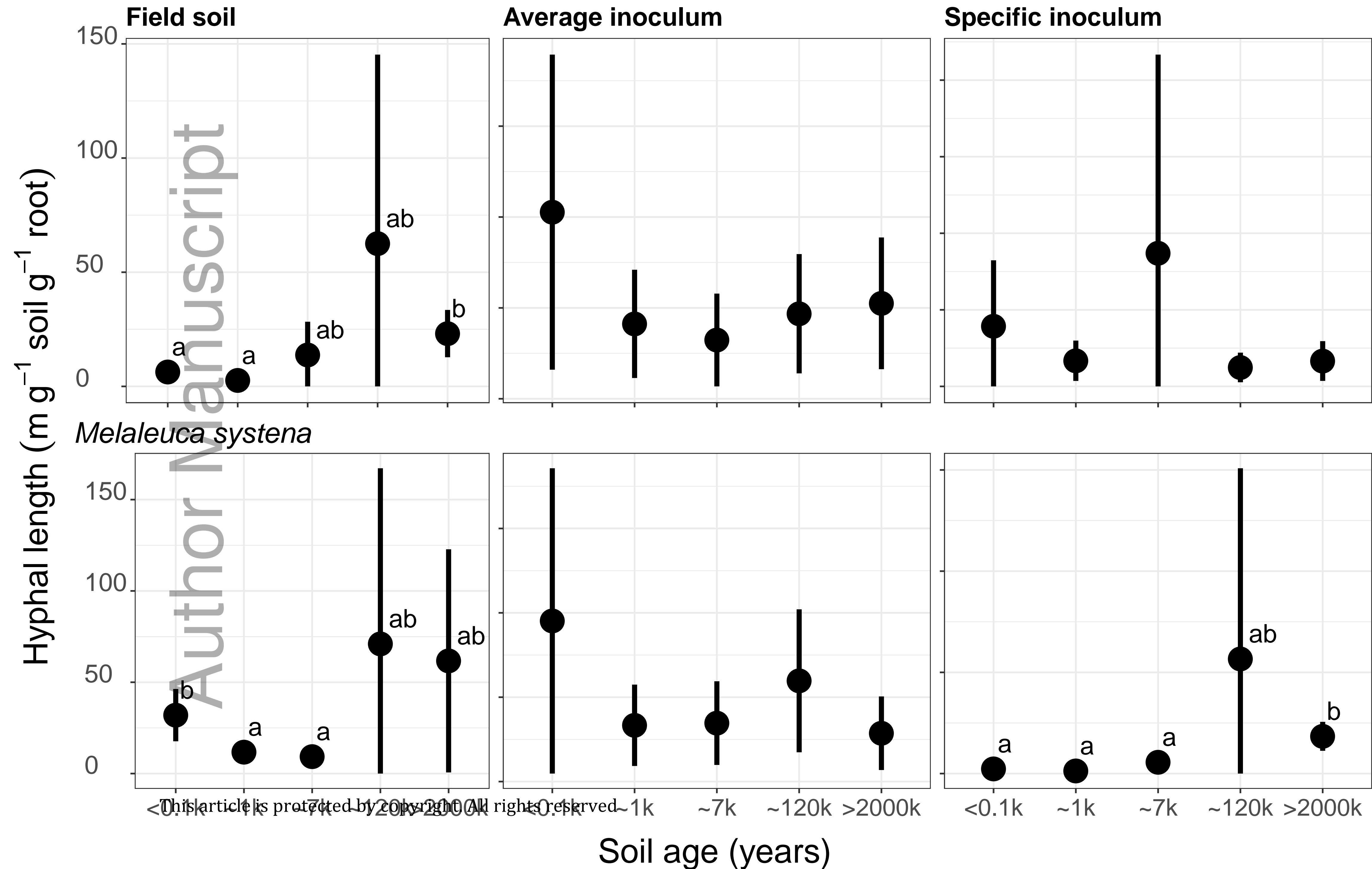

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