

# 1 Using soil microbial inoculations to enhance substrate performance on 2 extensive green roofs

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## 11 Abstract

12 *Green roofs are increasing in popularity in the urban environment for their contribution to*  
13 *green infrastructure; but their role for biodiversity is not often a design priority. Maximising*  
14 *biodiversity will impact positively on ecosystem services and is therefore fundamental for*  
15 *achieving the greatest benefits from green roofs. Extensive green roofs are lightweight*  
16 *systems generally constructed with a specialised growing medium that tends to be biologically*  
17 *limited and as such can be a harsh habitat for plants to thrive in. Thus, this investigation aimed*  
18 *to enhance the soil functioning with inoculations of soil microbes to increase plant diversity,*  
19 *improve vegetation health/performance and maximise access to soil nutrients. Manipulations*  
20 *included the addition of mycorrhizal fungi and a microbial mixture ('compost tea') to green*  
21 *roof rootzones, composed mainly of crushed brick or crushed concrete. The study revealed that*  
22 *growing media type and depth play a vital role in the microbial ecology of green roofs, with*  
23 *complex relationships between depth and type of substrate and the type of microbial inoculant*  
24 *applied, with no clear pattern being observed. For bait plant measurements (heights, leaf*  
25 *numbers, root/shoot biomass, leaf nutrients), a compost tea may have positive effects on plant*  
26 *performance when grown in substrates of shallower depths (5.5 cm), even one year after*  
27 *inoculums are applied. Results from the species richness surveys show that diversity was*  
28 *significantly increased with the application of an AM fungal treatment and that overall, results*  
29 *suggest that brick-based substrate blends are most effective for vegetation performance as*  
30 *are deeper depths (although this varied with time). Microbial inoculations of green roof*  
31 *habitats appeared to be sustainable; they need only be done once for benefits to still be*  
32 *seen in subsequent years where treatments are added independently (not in combination).*  
33 *They seem to be a novel and viable method of enhancing rooftop conditions.*

34

35 **Keywords:** Microbial Communities; Resilience; Substrates; Nutrients; Species Richness; Sustainability.

36 **1. Introduction**

37 Extensive green roofs are those with a shallow rootzone – generally between 5 – 15  
38 cm in depth, and often fall into three main types: *Sedum* systems, wildflower systems  
39 and biodiverse roofs. From an ecological perspective, biodiverse roofs that mimic  
40 brownfield habitat are of great interest and importance in our urban landscapes  
41 (Schadek *et al.*, 2009). With increasing construction in our cities it is vital to create  
42 wildlife spaces to mitigate associated negative effects. Biodiverse green roofs  
43 therefore offer great potential, if designed appropriately (Lundholm, 2015), to offer  
44 regional biodiversity at roof level (Connop *et al.*, 2016). The issue is that many green  
45 roofs are constructed with a lack of knowledge about how to maximise biodiversity  
46 (Kadas 2002). *Sedum* systems are selected by architects for their proven hardiness to  
47 rooftop conditions (Monterusso *et al.*, 2005) and the aesthetic value of instant  
48 greening (Molineux *et al.*, 2009). Biodiverse roofs are becoming more popular in cities  
49 like London, however these are often extremely homogenous – with the same  
50 substrate type and depth (Heim & Lundholm, 2014) over the roofs' entirety. Substrate  
51 type is particularly important (Molineux *et al.*, 2009; Graceson *et al.*, 2014b; Bates *et*  
52 *al.*, 2015; Molineux *et al.*, 2015; Eksi & Rowe 2016), as it is the main green roof  
53 component that will support the vegetation. Previous studies suggest that engineered  
54 substrates may be biologically limited but that microbial inoculants could be used to  
55 enhance the functioning below-ground (Molineux *et al.*, 2014; Ondoño *et al.*, 2014;  
56 Young *et al.*, 2015). Thus a physically engineered substrate, that has considered  
57 biological functionality, will underpin the success of a specified planting scheme.

58         Soil microbial communities at ground level have been well studied in many  
59 habitats. These microscopic organisms, including bacteria and fungi, are vital for  
60 colonization of a substrate by plants (Lavelle *et al.*, 2006). They offer favourable  
61 conditions for plants to extract limited nutrients, either by breaking down and  
62 recycling dead and decaying matter, or by providing access to nutrient pools that can  
63 be unexploitable (Smith and Read, 2010). One group in particular, the arbuscular  
64 mycorrhizal fungi (AMF), facilitate this via hyphal networks in plant root cells (Van der  
65 Heijden *et al.*, 1998) and in doing so also increase root hair surface area allowing  
66 access to water films on soil particles in times of extreme drought stress (Allen, 2009).  
67 AMF comprise of about 150 known fungal species and are said to be associated with  
68 around 80% of all plant species root systems (Hodge, 2000).

69 The microbial ecology of green roof habitats is beginning to receive attention  
70 McGuire *et al.*, 2013, Rumble & Gange, 2013, John, 2014, Buffam *et al.*, 2015, however  
71 little of this research links the effects of microbial communities to plant growth on  
72 green roofs (Young *et al.*, 2015) or their effects on substrate nutrient levels. Green  
73 roofs can be extreme environments for many plant species; thus microbial groups  
74 such as AMF could potentially provide vegetation with a better chance of survival at  
75 roof level (Molineux *et al.*, 2014). This in turn would help maintain ecosystem services,  
76 like building cooling, evapotranspiration and reduction in the urban heat island effect  
77 (Oberndorfer *et al.*, 2007; Lundholm *et al.*, 2010); as well as increased storm water  
78 retention (Connop *et al.*, 2016), carbon sequestration (Parras-Alcántara *et al.*, 2015)  
79 and urban soil security (Anaya-Romero *et al.*, 2015).

80 The aim of the research was to determine how substrate type and depth  
81 effected plant species richness and plant 'health' determined by performance  
82 measurements such as heights, leaf numbers, root and shoot biomass. It also explores  
83 the additions of microbial inoculants to green roof substrates and the effect this had,  
84 not only on the microbial communities themselves (as described in Molineux *et al.*,  
85 2014), but also on the substrate nutrients and bait plant leaf nutrients. The main  
86 research questions regarding the addition of microbial inoculations to various  
87 substrate types and depths (described in methods section) were, did they (i) produce  
88 larger plants (heights, leaf numbers, root and shoot biomass), (ii) increase root  
89 colonisation by beneficial arbuscular mycorrhizal fungi, (iii) effect leaf nutrient levels,  
90 (iv) increase species diversity and (v) increase available soil nutrients?

91

92

## 93 **2. Methods**

### 94 **2.1 Field Site**

95 To study the effects of substrate type and depth, an existing experimental set-up on  
96 the gift shop at London Zoo (Regents Park, London) was utilised and microbial  
97 inoculation treatments were applied. The experimental green roof is approximately  
98 180 m<sup>2</sup> and split into 2m × 2m plots which contain various substrates at five different  
99 depths (further details in Kadas, 2007). Molineux *et al.* (2014) fully describes the  
100 additions of the microbial treatments, but in short: two substrate types (brick-based

101 and concrete-based) at two of the depths (5.5cm and 8cm) were chosen for the  
102 investigation, each replicated 3 times. Substrate properties data can be found in  
103 Appendix I. The existing plots were further divided into quarters, which were then  
104 used for the microbial manipulation experiments. The inoculations were applied three  
105 times over the summer of 2007. The treatments were a commercial arbuscular  
106 mycorrhizal fungal mix (hereafter referred to as 'Fungi'), a live compost tea containing  
107 bacteria and fungi (Tea), a combination of both treatments (Fungi + Tea), and finally  
108 control plots where no inoculants were added (Control). Information on product  
109 content is available at: [http://www.symbio.co.uk/horticulture\\_datasheets.aspx](http://www.symbio.co.uk/horticulture_datasheets.aspx).

110

111

## 112 **2.2 Bait Plants**

113 Before microbial manipulation could begin, bait plants – to be used as indicators for  
114 any changes in plant growth due to the addition of microorganisms – were planted  
115 into the experimental plots. The bait plant species chosen was *Plantago lanceolata*;  
116 as a perennial it retains some leaves over winter and re-sprouts each spring from the  
117 rootstock, making the recording of growth from one year to the next possible. It is  
118 strongly mycorrhizal and is often used as a model plant in field studies (e.g. Walter *et*  
119 *al.* 2016). By growing the *P. lanceolata* in pumice, in a controlled temperature room,  
120 the bait plant roots remained mycorrhizal-free until added to the green roof plots.  
121 Colonisation of the roots could then be analysed in the different treatments, by  
122 removing one bait plant from each treatment plot annually. This also allowed for the  
123 collection of dry shoot and root biomass data whilst leaving the established green roof  
124 *P. lanceolata* population undisturbed by the experiment. Four bait plants of *P.*  
125 *lanceolata* were planted into each of the designated experimental plots in May 2007,  
126 after three months of growth in a control temperature room at Royal Holloway  
127 University. This was to ensure that at least two plants would survive for removal after  
128 treatments were applied. Plants were selected for similarity in size in order for height  
129 comparisons to be made, and to reduce plant phenotypic variability.

130

### 131 2.2.1 Plant Heights & Leaf Numbers

132 Plant heights and leaf numbers for the bait plants of *P. lanceolata* were recorded in  
133 November 2007, following treatments and November 2008, a year after treatments  
134 were first applied. Means taken from three replicates were used to determine any  
135 differences between the underlying substrate types (including depth) and the  
136 microbial treatments.

137 All samples were taken in November, so that seasonal variation in microbial  
138 biomass (Blume *et al.* 2002) was reduced as much as possible, many studies have also  
139 shown microbial biomass is increased under cool and wet conditions, thus November  
140 represented an ideal soil sampling time (Van Gestel *et al.* 1992; Arnold *et al.* 1999;  
141 Papatheodorou *et al.* 2004). November also represented the end of the growing  
142 season on our zoo green roof and therefore the plants were at their largest before the  
143 frost began to restrict their growth.

144

#### 145 2.2.2 Dry Biomass

146 In November 2007, the first batch of bait plants were removed from the green roof  
147 plots. One plant was taken from each sub-plot and taken back to the laboratory where  
148 they were washed, roots stored in 70 % ethanol and leaves transferred to large paper  
149 envelopes. This was then repeated with the last batch of *P. lanceolata* bait plants,  
150 which were removed from the London Zoo green roof plots in November 2008. Plant  
151 leaves were placed into labelled envelopes and dried in an oven at 60 °C for 48-72 h.  
152 Once dried, each sample was placed in a weighing boat and weighed to determine  
153 total dry shoot biomass for each treatment plot. Means taken from three replicates  
154 were used to observe differences between the underlying substrate types (including  
155 depth) and the microbial treatments.

156

#### 157 2.2.3 AMF root colonisation

158 The plant roots stored in 70 % ethanol, were washed in distilled water and put into  
159 5% potassium hydroxide and then rinsed again with distilled water. They were  
160 transferred to 1% HCl for 15mins, then placed in a simple ink stain comprising of Quink  
161 ink, 1% HCl and water in a 0.2:1:50 ratio for 1hr. The samples were then cleaned by  
162 soaking in Destain solution (glycerol:water:1%HCl in the ratio 70:24:1) for 24hrs  
163 before temporary slides could be made for mycorrhizal analysis. This method was

164 modified from (Vierheilig *et al.*, 2005). Mycorrhizal occurrence could be calculated by  
165 slide scanning under the microscope at a magnification x200 as described by  
166 McGonigle *et al.* (1990). Means taken from three replicates were used to determine  
167 any differences between the underlying substrate types (including depth) and the  
168 microbial treatments. Once AMF analysis completed, all the roots (including those on  
169 temporary slides) were collected and subjected to the same drying technique used for  
170 shoot biomass data collection (described in 2.2.2) in order to determine dry root  
171 biomass.

172

#### 173 2.2.4 Leaf Nutrient Analysis

174 Following the collection of dry shoot biomass data (as described in 2.2.2), the dried  
175 bait plant leaves were ground into a fine powder using a pestle and mortar for leaf  
176 nutrient analysis. Approximately 2 µg of leaf material was used for total carbon and  
177 total nitrogen analysis using a Nitrogen and Carbon Soil Analyser (Flash EA1112 Series)  
178 equipped with a Carbon, Hydrogen, Nitrogen and Sulphur configuration. The leaf  
179 material was placed into individual tin containers and dropped by an autosampler into  
180 the furnace, where total N and total C could be calculated for each plant collected.  
181 Means were found for plants in each microbial treatment, with respect to underlying  
182 substrate type and depth.

183

184

### 185 **2.3 Species Diversity**

186 The London Zoo gift shop green roof plots were monitored for plant species diversity  
187 where both species type and individual numbers were recorded, using Blamey *et al.*  
188 (2003). Surveys took place in July 2007, after microbial treatments were added and  
189 May 2008, one year after treatments applied.

190

191

### 192 **2.4 Substrate Analysis**

193 Substrate/soil samples were also taken from each treatment plot on London Zoo gift  
194 shop green roof to determine the quantity of available nitrates from nitrogen and  
195 ammonia, potassium and phosphorus in the sub-plots. These nutrients are essential

196 for effective plant growth, so it was important to assess if the microbial treatments  
197 had altered these properties of the substrate. Approximately 100 g of soil was  
198 removed in November 2006 (before manipulations), November 2007 (after  
199 manipulations) and November 2008 (one year following manipulations) and stored at  
200 -20 °C until needed. A segmented flow analyser – Skalar Ltd, UK – comprised of SA1050  
201 random access autosampler, chemistry unit SA4000, SA 853 SFA interface with a  
202 digital photometer head and Flowaccess software was used to analyze all but  
203 potassium nutrients. For all nutrients analysed each sample was replicated three times  
204 to give a representative mean.

205

#### 206 2.4.1 Nitrates

207 Substrate nitrogen was determined using a hydrazine reduction method (modified  
208 from Henriksen & Selmer-Olsen, 1970) for nitrates and nitrites; and a Berthelot  
209 method (modified from Rhine *et al.* 1998) for ammonia.

210 For nitrates and nitrites, 1 g substrate samples were added to 1M potassium  
211 chloride in 100 ml conical flasks and placed on a shaker rack for 30 minutes. Three  
212 samples of just the KCL reagent were used as control blanks. After this time each  
213 sample was filtered through Whatman 25 mm GF/C paper directly into acid washed  
214 tubes. These were then capped and stored at 5 °C in a fridge until needed. Reagents  
215 for the Skalar SFA were also prepared ready for analysis. These included a buffer  
216 solution containing potassium sodium tartrate, tri-sodium citrate and Brij 35, sodium  
217 hydroxide, hydrazinium sulphate and a colour reagent containing sulphanilamide and  
218 1-naphthylethylenediamine dihydrochloride. Standards were also produced to give 1,  
219 2, 3, 4 and 5 ppm of sodium nitrate solution. For analysis, each sample was transferred  
220 to Skalar vials and placed into an autosampler. The determination of nitrate and nitrite  
221 is based on the hydrazine reduction method; which forms a highly coloured azo dye  
222 measured at 540 nm.

223 Ammonia was also extracted from substrate samples as above, however  
224 different Skalar reagents were used for analysis. These included sodium salicylate,  
225 sodium nitroprusside, sodium dichloroisocyanurate and the same buffer solution as  
226 above. The standards were 0.4, 0.8, 1.2, 1.6 and 2 ppm of ammonium chloride solution.  
227 For analysis, each sample was transferred to Skalar vials and placed into an

228 autosampler as with the nitrates. The procedure for the determination of ammonia is  
229 based on the modified Berthelot reaction; after oxidation and oxidative coupling a  
230 green coloured complex is formed and absorption measured photometrically at  
231 660nm.

232

#### 233 2.4.2 Phosphates

234 For phosphates, Olsen's Extractable Phosphorus in soil\_method was followed  
235 (modified from Watanabe & Olsen 1965), whereby 2.5 g of soil was added to 50 ml  
236 Olsen's reagent in 100 ml conical flasks. The Olsen's extractant is a 0.5 M sodium  
237 bicarbonate solution with pH of 8.5. The samples were placed on a shaker rack for 30  
238 minutes along with three blanks of just the Olsen's reagent as control samples. After  
239 this time each sample was filtered through Whatman 25 mm GF/C paper directly into  
240 acid washed tubes. These were then capped and stored at 5 °C in a fridge until needed.  
241 To determine phosphorous content, the following reagents were also prepared:  
242 ammonium molybdate (1.2 % m/V), ascorbic acid solution and 1.5 M sulphuric acid  
243 along with standards of 0, 1, 2, 4, 6 and 8 ppm potassium dihydrogen orthophosphate.  
244 Before analysis, 2.5 ml samples were combined with 0.5 ml sulphuric acid, 10 ml  
245 ammonium molybdate and 2.5 ml ascorbic acid solutions and allowed to stand for 30  
246 minutes. The automated procedure is based on a reaction that produces an intensely  
247 blue coloured complex, with absorbency read at 880 nm.

248

#### 249 2.4.3 Potassium

250 Finally potassium was extracted from substrates based on the Ammonium Acetate (pH  
251 7.0) method (modified from Simard, 1993); whereby 2.5 g of soil was added to 63 ml  
252 of ammonium acetate (pH 7) solution. Three blanks to be used as controls containing  
253 only the ammonium acetate were also produced. Samples were then placed onto a  
254 shaker for 1h then filtered as described above. They were stored at 5 °C in a fridge  
255 until needed. Potassium was analysed using a flame photometer with standards of 2,  
256 4, 6, 8 and 10 ppm of the potassium stock solution.

257

258

### 259 **2.5 Statistical Analysis**



260 Plant performance measurements and leaf and soil nutrients analysis were examined  
261 using a split-plot multiple analysis of variance (ANOVA) (Zar, 2005) to determine  
262 differences between the factors: substrate type, substrate depth and microbial  
263 treatment in the years 2007 and 2008. This analysis allowed for interactions between  
264 treatments and underlying substrate types and depths to be explored. Data that were  
265 not normally distributed were transformed with square roots or logarithms. Means  
266 were separated with a Tukey's HSD post hoc test (Fowler et al., 1998). All analyses  
267 were conducted using the statistical package UNISTAT®.

268

269

### 270 **3. Results**

#### 271 **3.1 Bait Plants**

272 The following data obtained for bait plant performance have been displayed in  
273 relation to statistically significant results. Where the microbial treatments did not  
274 have an effect on a particular plant measurement, data has been graphed according  
275 to underlying variables, such as substrate type and substrate depth irrespective of  
276 treatment. Data are displayed in respect to 2007, after microbial treatments applied  
277 and 2008, one year after treatments were first added.

278

##### 279 3.1.1 Plant Heights

280 Figure 1a shows the effect of substrate type and depth (irrespective of treatment) on  
281 plant heights over the study period. *Plantago lanceolata* bait plants on London Zoo  
282 gift shop green roof were considerably taller in 2007 than they were in 2008 ( $F_{1,66} =$   
283  $36.98$ ,  $P < 0.01$ ). Substrate depth was also a significant factor affecting how tall plants  
284 grew ( $F_{1,66} = 9.77$ ,  $P < 0.01$ ), and there were interactions between the substrate type  
285 and depth ( $F_{1,66} = 4.56$ ,  $P < 0.05$ ). Plants in concrete-based substrate at 5.5cm depth  
286 were similar in height over the two years whilst those in brick-based substrate at 8 cm  
287 depth were considerably taller in 2007 and remained so in 2008 ( $F_{1,66} = 5.66$ ,  $P < 0.05$ ).  
288 These interactions mean that the choice of substrate composition for a green roof is  
289 vital, as plant performance can change with varying depths.

290 Figure 1b shows that in 2007 the addition of AM fungi produced the largest  
291 increase in heights ( $F_{1,66} = 4.20$ ,  $P < 0.05$ ). However by 2008, a year after inoculations

292 took place, all heights were reduced to similar levels with no significance found  
293 between treatments. Furthermore, the AM fungi treatment and the compost tea  
294 treatment were not additive as predicted, instead there was a significant interaction  
295 between the two products used in combination ( $F_{1,66} = 3.82$ ,  $P < 0.05$ ). This is shown  
296 by fungi + tea bars being similar in size to all other treatments.

297

### 298 3.1.2 Leaf Numbers

299 As with the data for plant heights, there were decreased leaf numbers from *P.*  
300 *lanceolata* bait plants in 2008 ( $F_{1,66} = 7.39$ ,  $P < 0.05$ ) following one year without any  
301 microbial treatments, Figure 1c & 1d. Figure 1c shows leaf numbers were affected by  
302 substrate depth ( $F_{1,66} = 8.99$ ,  $P < 0.01$ ), where plants in concrete-based substrate at 5.5  
303 cm depths had almost twice as many leaves as those in 8 cm plots in 2008.

304 The addition of treatments (Figure 1d) AM fungi and compost tea, appeared  
305 to increase leaf numbers compared to controls but this was not statistically significant.  
306 Likewise there was no additive benefit when the two treatments were used in  
307 combination, instead there was a significant interaction between AM fungi and  
308 compost tea products ( $F_{1,66} = 6.68$ ,  $P < 0.01$ ), suggesting antagonism between the  
309 microbial species applied.

310

### 311 3.1.3 Root & Shoot Biomass

312 Dry shoot biomass (Figure 2a) and dry root biomass (Figure 2b) of *P. lanceolata* plants  
313 were both lower in 2008 compared to 2007 ( $F_{1,66} = 5.71$ ,  $P = 0.07$  and  $F_{1,66} = 11.09$ ,  $P$   
314  $< 0.05$  respectively). Yet, the addition of the AM fungi treatment appeared to increased  
315 root biomass slightly ( $F_{1,66} = 3.32$ ,  $P = 0.07$ ) compared to other treatment plots and  
316 control, regardless of year. Root biomass was also affected by underlying substrate  
317 depth, where overall 8 cm plots allowed roots to become larger, thus increasing  
318 biomass ( $F_{1,66} = 4.58$ ,  $P < 0.05$ ). In 2007 (Figure 2c) the tea treated plots showed the  
319 opposite trend, where substrates that were 5.5 cm deep, contained plants with a  
320 larger total plant biomass compared to plots that were 8 cm deep. However by 2008  
321 (Figure 2d), there was little difference in biomass between either substrate depths  
322 where the tea treatment was applied.

323

#### 324 3.1.4 AMF root colonisation

325 Figure 3 shows the levels of colonisation in relation to treatments applied in both years,  
326 as well as the percentage of vesicles and arbuscules encountered. From 2007 to 2008  
327 there was a considerable ( $F_{1,58} = 8.46$ ,  $P < 0.05$ ) increase in arbuscular occurrence in  
328 bait plant roots. In 2007, plants from tea treated plots contained approximately four  
329 times more AM fungal root colonisation compared to plants from control plots, where  
330 both arbuscules ( $F_{1,58} = 6.69$ ,  $P < 0.01$ ) and vesicles ( $F_{1,58} = 11.88$ ,  $P < 0.001$ ) were  
331 significantly increased. The ratios of arbuscules and vesicles observed also shifted  
332 from 2007 to 2008. In 2007 most plots contained more vesicles than arbuscules,  
333 except for the tea treated plots, which contained even amounts of each. Yet in 2008  
334 the opposite was true, ratios were more in favour of vesicles where treated with  
335 compost tea; for all other treatments, there was an even divide between the vesicle  
336 and arbuscular structures.

337 Furthermore, interactions occurred for arbuscules ( $F_{1,58} = 6.16$ ,  $P < 0.01$ ) and  
338 vesicles ( $F_{1,58} = 5.14$ ,  $P < 0.05$ ) where compost tea and AM fungi treatments were  
339 added together (irrespective of year), resulting in an antagonistic effect rather than  
340 the additive one that would have been expected.

341

#### 342 3.1.5 Leaf Nutrient Analysis

343 Figure 4 shows the nutrient content of bait plant shoots after microbial treatments  
344 were applied to London Zoo green roof experimental plots in 2007. Data from 2008  
345 have not been displayed as they were very similar to 2007 and year was not a  
346 significant factor affecting either leaf nitrogen or leaf carbon.

347 Figure 4a shows the nitrogen percentage content of shoots. The combination  
348 of the fungi and tea treatments increased nitrogen content in the brick-based  
349 substrate (additive effect), but reduced nitrogen content in shoots from the concrete-  
350 based substrate (antagonistic effect). Therefore there was a significant three-way  
351 interaction ( $F_{1,63} = 6.16$ ,  $P < 0.01$ ) between the substrate type and the fungi and tea  
352 treatments; whilst individually the fungi treatment ( $F_{1,63} = 0.40$ ,  $P = 0.52$ ) and tea  
353 treatment ( $F_{1,63} = 2.11$ ,  $P = 0.15$ ) were not significant factors effecting nitrogen in  
354 leaves.

355 Figure 4b shows leaf carbon in bait plants taken from the London Zoo  
356 experimental plots in 2007. There was a significant three-way interaction ( $F_{1,63} = 3.71$ ,  
357  $P < 0.05$ ) between substrate depth and the fungal and tea inoculants; meaning that  
358 where treatments were applied to deeper substrate plots (8 cm), plants contained  
359 larger quantities of leaf carbon compared to plants grown in shallower plots (5.5 cm).

360

361

### 362 **3.2 Species Diversity**

363 The plant surveys conducted in the summer months of 2007 and 2008 indicated that  
364 there was increased plant species diversity ( $F_{1,66} = 4.91$ ,  $P < 0.05$ ) with the addition of  
365 the AM fungi treatment (Figure 5a). Figure 5b shows differences between species  
366 richness in the substrate types over the three years where treatments (sub-plots) have  
367 been combined to give means for each experimental plot. Results have also shown  
368 that the type and depth of substrate play an important role in determining how many  
369 plant species are supported on a green roof. Brick-based substrates supported more  
370 plant species than the concrete-based substrate ( $F_{1,66} = 4.91$ ,  $P < 0.05$ ) whilst there was  
371 also an interaction between the year and substrate depth ( $F_{1,66} = 12.66$ ,  $P < 0.001$ ). In  
372 2007, deeper substrates contained more plant species, whilst in 2008 the reverse was  
373 true, with shallower substrates (depths of 5.5 cm) becoming more species rich.

374

375

### 376 **3.3 Substrate Analysis**

#### 377 3.3.1 Nitrates

378 The nitrate and ammonium levels in substrate samples from London Zoo experimental  
379 site were combined to give the total amount of nitrogen available in the soil for plant  
380 acquisition (Table 1). There was a significant interaction between substrate type and  
381 year ( $F_{1,51} = 4.51$ ,  $P < 0.05$ ); where brick-based substrates contained larger quantities  
382 of available nitrogen in 2007 compared to concrete-based substrates in the same year.  
383 By 2008, there was little difference in available N levels between the two substrate  
384 types. Interestingly however, there were no significant effects observed with the  
385 addition of microbial treatments (Appendix II in supplementary material).

386

### 387 3.3.2 Phosphates

388 The substrate phosphorus levels (Table 1) were higher in 2007 than in 2008,  $F_{1,51} =$   
389 26.08,  $P < 0.01$ , and this was particularly affected by underlying substrate type,  $F_{1,51} =$   
390 4.90,  $P < 0.05$ . In 2007, brick-based substrates contained more phosphates than  
391 concrete-based substrates, however by 2008, it was these substrate that held more  
392 soil phosphorus. The compost tea inoculum increased quantities of available  
393 phosphates in 2007, compared to 2008 ( $F_{1,51} = 5.07$ ,  $P < 0.05$ ). There were also  
394 increased levels found in brick-based substrates where this treatment was applied  
395 ( $F_{1,51} = 4.45$ ,  $P < 0.05$ ). Therefore there was a significant three-way interaction between  
396 the year, substrate type and compost tea treatment ( $F_{1,51} = 4.68$ ,  $P < 0.05$ ); implying  
397 that in certain substrate types, the addition of compost tea may increase available  
398 phosphates to plants in the year of application, but that this is not sustained unless  
399 subsequent treatments are carried out.

400

### 401 3.3.3 Potassium

402 Finally substrate potassium levels (Table 1) were analysed from the green roof  
403 experimental plots. Potassium content was significantly increased in 2008 compared  
404 to 2007,  $F_{1,51} = 54.47$ ,  $P < 0.01$  and thus it seems that the addition of microbial  
405 treatments had a negative effect on the substrate's potassium. Furthermore, brick-  
406 based substrates contained slightly larger quantities of potassium in 2008 compared  
407 to 2007 – where both substrate types were similar in levels. The application of  
408 compost tea increased potassium in 2008 at the deepest depth of brick-based  
409 substrates but decreased this in the 8 cm concrete-based substrate plots. Despite  
410 microbial treatments, in 2008, levels of potassium returned to similar levels as those  
411 found in the baseline data (around 17-20 mg/kg soil).

412

## 413 4. Discussion

414 The use of bait plants on London Zoo green roof demonstrated the possible effects of  
415 microbial inoculations on general plant performance over time. *Plantago lanceolata*  
416 appeared well suited to the green roof environment, with all planted seedlings  
417 surviving the course of the study. As a single species, it could not represent every plant  
418 response in the green roof community, however it is considered a good model to

419 measure microbial effect in other field studies (Walter *et al.*, 2016). Results showed  
420 inconsistent patterns of microbial treatment benefit, varying with underlying  
421 substrate type and depth. Generally, *P. lanceolata* plants increased in height from  
422 plots where the AM fungi treatment was applied. As arbuscular mycorrhizal fungi have  
423 been shown to significantly increase the survival, establishment and growth of plants  
424 with colonised roots (Koske & Gemma, 1997; Bakker *et al.*, 2013; Miransari, 2016) and  
425 are said to be key elements in nutrient-unbalanced and xeric environments (Roldan-  
426 Fajardo, 1994; Requena *et al.*, 1996; Peña-Becerril *et al.*, 2016); results suggest that  
427 the fungal treatment effectively increased AMF root colonisation compared to  
428 controls. Despite this, all plants were reduced in size by 2008. Substrates containing  
429 75 % crushed brick at depths of 8 cm, produced plants that were considerably taller  
430 than the 5.5 cm plots and any plot containing the concrete-based substrate. This was  
431 probably because deeper plots retained more rainwater than shallower ones,  
432 providing plants with increased access to water – essential for survival and growth  
433 (Kramer & Boyer, 1995). Interestingly, plants grown in 75 % crushed concrete at 5.5  
434 cm depths remained unchanged in height from 2007 to 2008, perhaps due to better  
435 water storage capacity or less efficient drainage at shallower depths than the brick-  
436 based substrate.

437 Leaf numbers on *P. lanceolata* plants showed similar patterns to heights,  
438 where decreases were seen from 2007 to 2008. Average rainfall (from MetOffice data)  
439 in 2006 was 101.2 mm, 86.9 mm in 2007 and 67.0 mm in 2008. This suggests that the  
440 application of microbial treatments were successful in increasing plant size and leaf  
441 numbers in 2007 but by 2008 - when numbers decreased for all plants - reduced water  
442 availability may have been a reason for these changes. Appendix I (in supplementary  
443 materials) also shows that mean maximum and minimum temperatures as well as  
444 average sunlight hours decreased from 2007 to 2008. Thus weather conditions in 2008  
445 were colder, drier and less sunny which would account for reduced growth rates  
446 overall. The interesting findings were where significant interactions between  
447 underlying substrate type and depth were observed and often this produced the  
448 largest changes in leaf numbers. In 2008, concrete-based substrate contained bait  
449 plants with twice as many leaves when grown at 5.5 cm depths compared to those in  
450 8 cm plots. Furthermore, in 2007 *P. lanceolata* plants in 5.5 cm plots had up to six

451 more leaves when treated with the compost tea, compared to those in 8 cm  
452 substrates.

453 Overall *P. lanceolata* biomass – root and shoot – was decreased in 2008  
454 compared to 2007 (as generally seen with all *P. lanceolata* performance data). In 2007  
455 the total biomass of plants grown in 5.5 cm deep substrates were significant larger  
456 where the compost tea treatment was added and in 8 cm deep substrates where the  
457 AM fungi treatment was applied. By 2008 however, there was little difference  
458 between the total biomass in plants from either substrate depths or with microbial  
459 inoculation. The reduction in 2008, as with bait plant heights and leaf numbers, was  
460 therefore likely due to abiotic factors as discussed above. The soil nutrients could also  
461 have been a contributing factor, which is also addressed further on.

462 Bait plant root colonisation by arbuscular mycorrhizal fungi increased  
463 significantly from 2007 to 2008. After microbial inoculants were applied, experimental  
464 plots treated with compost tea increased in mycorrhizal occurrence from 5 %  
465 colonisation (in control plots) to approximately 25 % colonisation. However by 2008,  
466 colonisation levels in the control plots had increased to over 20 % whilst the fungi and  
467 tea treated areas were noticeably higher at over 30 % colonisation. Controls seem to  
468 have naturally increased in the substrates at this time, perhaps due to natural  
469 processes The structures of AM fungi found within plant roots are important in  
470 determining how it is functioning within the substratum (Klironomos *et al.*, 2004). In  
471 2007, vesicles were observed more frequently than arbuscules in control plots and  
472 fungi treated plots. Vesicles are storage structures whilst arbuscules are sites of  
473 symbiotic nutrient exchange, and as such are thought to be more indicative of active  
474 functioning (Klironomos *et al.*, 2004). Therefore these results imply that the  
475 mycorrhiza may have been stressed and not that active within the host bait plants  
476 (Duckmanton & Widden, 1994; Titus & Leps, 2000; Wearn, 2006) until 2008, where  
477 there was an increase in arbuscules.

478 Even though colonisation increases were recorded, the microbial treatments  
479 often had small effects on plant performance measurements, with other parameters  
480 such as underlying substrate type and depth being the most significant variables.  
481 Therefore it appears that plants are not exploiting the usually beneficial root AMF.  
482 Reasons for this could be because nutrients such as phosphorus (Koide, 1991), are so

483 limiting on a green roof, that the fungi are not helping plants gain any more than they  
484 could without the symbionts. It has been said that optimal phosphorus levels, for AMF  
485 to produce the greatest benefits to host plants is approximately 50 ppm (Swift *et al.*,  
486 1979; Schubert & Hayman, 1986; Smith & Read, 1997) but the exceedingly low (< 5  
487 ppm) plant phosphates from this study (see Table 1) suggest that green roof  
488 substrates are extremely P limited. This probably means that, regardless of increased  
489 AM fungi colonisation, mycorrhizae are ineffective in these environments. The use of  
490 alternative aggregates in green roof growing media could provide more favourable  
491 conditions for both plants and AM fungi. Molineux *et al.*, (2009) found that clay pellet  
492 substrates contained five times more phosphorus pentoxide – a common form of P in  
493 many fertilizers (Bridger *et al.*, 1953) – compared to red brick (contained in the  
494 substrates on London Zoo green roof). This suggests that aggregates produced from  
495 recycled waste materials, such as sewage sludge (Debosz *et al.*, 2002), may provide a  
496 source of potential phosphates that could be released in rainwater leachates.

497         An alternative explanation for these results may be that once the mycorrhiza  
498 from the inoculation experiments have colonised plant roots, they could be having  
499 deleterious effects on their hosts, as shown in more recent microbial studies by  
500 Gadhav *et al.* (2016) and L. Jin *et al.* (2016). These studies highlight that AM Fungi  
501 can cause growth depressions in plants (Johansen, 1993), particularly when growing  
502 conditions are poor (i.e. in low nutrients, during drought periods). L. Jin *et al.* (2016)  
503 propose that for AM fungal structures to grow, such as vesicles, the fungus needs to  
504 obtain more photosynthetic products from the host plant, resulting in plant growth  
505 depression. This would help explain why, in general, all bait plant performance  
506 measurements in this investigation were reduced in 2008 compared to 2007 despite  
507 the observed increase in AMF colonisation from 2007 to 2008. Furthermore, vesicles  
508 were increased due to non-favourable conditions for the fungus, which would account  
509 for the negative relationship between plant performance and AMF root colonisation.

510         Results from bait plant leaf nutrients have shown significant interactions  
511 between the substrate type, depth and microbial treatments. For leaf nitrogen, there  
512 were significantly higher levels found in plants from substrate composed of 75 % brick  
513 compared to those that were 75 % concrete, where both the fungi and tea treatments  
514 were added. Leaf carbon was also increased with the combination of AM fungi and



515 compost tea treatments, but only in the deepest substrate plots (8 cm). Increased root  
516 biomass as well as higher nitrogen and carbon content of shoots, points to an  
517 increased photosynthetic capacity by plants (Field & Mooney, 1986). This heightened  
518 rate of photosynthesis implies that microbial treatments enhanced plant access to soil  
519 nutrients, such as nitrogen and phosphorus – vital constituents of the photosynthetic  
520 process (Blevins, 1999) – leading to improved plant fitness. Leaf nitrogen analysis  
521 indicated that, in brick dominated media, the two microbial treatments were additive,  
522 meaning that the fungi and tea treatments together resulted in higher concentrations  
523 of leaf nitrogen than those from plots where just AM fungi or just compost tea was  
524 applied. Conversely, in the concrete-based substrate, the two treatments were  
525 competitive resulting in decreased concentrations of leaf nitrogen compared to plots  
526 that were treated with just the AM fungi or just the compost tea. Possible reasons for  
527 this could be substrate N and P content. As already seen, soil phosphates can vary  
528 considerably with different aggregate types, and this is probably the same for soil  
529 nitrogen. In the London Zoo plots, crushed brick dominated substrates may contain  
530 higher N and P levels than the predominately crushed concrete ones. The applications  
531 of the treatments together may have increased microbial mobilisation of phosphorus  
532 and nitrogen for plant availability in the brick dominated substrates, because more  
533 nutrients pools were present for symbiotic benefits to be exploited (Koide, 1991).  
534 Previous microbial inoculation experiments by Requena *et al.*, (1996) showed that leaf  
535 nitrogen was increased with AMF root colonisation, and suggested this was due to an  
536 increased exploration of soil nitrogen pools (Ames *et al.*, 1984; Barea *et al.*, 1991;  
537 Azcon-Aguilar *et al.*, 1993; Johansen *et al.*, 1993). However, they also showed that  
538 interactions between AMF and certain bacteria could lead to decreased shoot  
539 nitrogen, indicating that limited P in soils could lead to antagonism between the  
540 microbial groups due to resource competition. This may help explain the reduced leaf  
541 nitrogen results from the concrete-based substrates.

542 The London Zoo green roof experimental site was originally seeded with a  
543 wildflower mix but since then, natural colonisation of the plots has occurred with the  
544 effect of increasing plant coverage and diversity (Kadas, 2007). Results from the  
545 species richness surveys showed that in 2007, the 8 cm plots supported the most plant  
546 species, correlating with previous research showing that deeper green roof substrates

547 are far more biodiverse than shallower ones (Brenneisen, 2006; Dunnett *et al.*, 2008).  
548 However, by 2008 the 5.5 cm plots became more species rich. In addition, the brick-  
549 based substrate was also more effective at supporting increased diversity than the  
550 concrete dominated media. The applications of compost tea did not affect plant  
551 diversity in the green roof plots, however the use of the AM fungi treatment  
552 significantly increased species numbers where added. Many studies have shown  
553 similar positive effects on plant species diversity with the presence of AMF (Grime *et*  
554 *al.*, 1987; Gange *et al.*, 1993; Klironomos *et al.*, 2000); proposing that AM fungi provide  
555 hyphal links between plants allowing a more even distribution of soil nutrients –  
556 reducing competition by strong plant species that usually monopolise resources.

557         Soil nutrient analyses have shown that for both nitrogen and phosphates,  
558 levels were higher in brick-based substrates in 2007, whereas potassium levels were  
559 not increased in this substrate until 2008. For soil P, further increases were found with  
560 the applications of compost tea. This supports the discussion above, where increased  
561 substrate nitrogen and phosphates would account for increases in leaf nitrogen  
562 content. Overall, the levels of total available nitrogen and phosphates were similar  
563 below 5 ppm, and potassium was found at levels of around 20 ppm (Table 1). These  
564 levels are extremely low compared to other habitats. Wearn, (2006) found levels of  
565 nitrogen and potassium in field soil (grassland area on the Royal Holloway campus) to  
566 be approximately 32 ppm and 80 ppm respectively and phosphates to be found on  
567 average at 20 ppm. These were considered to be very low levels (Allen, 1989; Edwards  
568 *et al.*, 1999); in fact Swift *et al.*, (1979) stated that phosphorus levels can reach above  
569 150 ppm in grasslands/pastures. Phosphates are one of the most limiting nutrients to  
570 plants in soils, especially in habitats like brownfield sites (Schadek *et al.*, 2009), shingle  
571 beaches (Scott, 1960; Lee *et al.*, 1983) and xeric Mediterranean ecosystems (Azcon-  
572 Aguilar *et al.*, 1993; Requena *et al.*, 1996). The extremely low levels found in the  
573 London Zoo green roof plots would be a significant factor affecting floral growth  
574 (Hinsinger, 2001).

575         Statistical analysis of data from *Plantago lanceolata* heights, leaf numbers and  
576 AMF root colonisation identified significant interactions between the arbuscular  
577 mycorrhizal fungi treatment and the compost tea treatment. When combined and  
578 applied to the green roof plots, there was not always an additive effect as would be

579 expected, instead there was frequently competition between the two. Recent work  
580 by Gadhave *et al.* (2016) has explored possible reasons for commercial inoculants  
581 competing against each other when used in combination and there is evidence of  
582 antagonism in other studies looking at the interactions between plant growth  
583 promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (Bethlenfalvay &  
584 Linderman, 1992); as well as specific interactions between AM fungi and other soil  
585 microbes (Vosatka *et al.*, 1992; Requena *et al.*, 1996; Saison *et al.*, 2006; Ondoño *et*  
586 *al.*, 2014). These authors suggest that competition arises due to soil nutrient  
587 availability – especially the phosphorus content, supporting the nutrient analysis of  
588 the London Zoo experimental plots previously discussed.

589

590

## 591 **5. Conclusion**

592 The results indicate that the addition of microbial treatments to London Zoo green  
593 roof were variable in terms of having an effect on vegetation compared to controls.  
594 The interactions between the AM fungi and compost tea applications and the different  
595 substrate types and varying substrate depths produced significant changes in plant  
596 heights, leaf numbers, species richness, and leaf/soil nutrient contents. Yet there were  
597 inconsistent patterns with regards to the ‘best’ substrate type and the ‘most  
598 appropriate’ substrate depth; generally speaking brick-based media at 8 cm depths  
599 were more favourable but this did vary with time as well as microbial treatment.  
600 However, what was clear from most results was that 2007 data were significantly  
601 different from post-treatment data from 2008. This seemed to be due to a  
602 combination of variables including the microbial inoculations, soil N and P and abiotic  
603 factors such as the amount of rainfall (water), mean max. and min. temperatures and  
604 sunlight hours. From previously published work, the treatments do seem to have  
605 long-lasting effects on the microbial communities themselves, but more research is  
606 needed to determine how much benefit they provide to the green roof plants over  
607 time. This short-term study shows that enhancement of soil microbial functioning can  
608 have positive impacts on some plant health/performance measurements on extensive  
609 biodiverse roofs and, with the right substrate, also increase plant species diversity.  
610 Green roofs need to be considered as habitats, albeit those with harsh conditions for

611 their flora and fauna; and should therefore be engineered, not only mechanically, but  
612 biologically as well. The introduction of microbial communities through various  
613 inoculations can help to improve green roof biodiversity and future research should  
614 look at how this then boosts their role in urban green infrastructure; particularly as a  
615 provision for ecosystem services and in respect to climate change mitigation.

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618

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## 627 **7. References**

628 Ames, R.N., C.P.P. Reid, and E.R. Ingham (1984). Rhizosphere bacterial population  
629 responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. **New**  
630 **Phytology**, 96:555 – 563.

631

632 Allen, S. E. (1989). **Chemical analysis of ecological analysis of ecological materials**. In:  
633 Allen, S.E. (Editor), Blackwell Scientific Publication.

634

635 Allen, M. F. (2009). **Water relations in the mycorrhizosphere**. In: Luttge, U.,  
636 Beyschlag, W., Budel, B., Francis, D. (Eds.), Progress in Botany, vol. 70. Springer,  
637 Berlin, Germany, 257–276.

638

639 Anaya-Romero, M., S. K. Abd-Elmabod, M. Muñoz-Rojas, G. Castellano, C. J. Ceacero,  
640 S. Alvarez, M. Méndez, and D. De la Rosa (2015). Evaluating Soil Threats Under Climate  
641 Change Scenarios in the Andalusia Region, Southern Spain. **Land Degradation and**  
642 **Development**, 26 (5): 441-449. doi:10.1002/ldr.2363.

643

644 Azcón-Aguilar C., C. Alba, M. Montilla and J. M. Barea (1993). Isotopic (<sup>15</sup>N) evidence  
645 of the use of less available N forms by VA mycorrhizas. **Symbiosis**, 15:39 – 48.

646

647 Bakker, P. A, R. L. Berendsen, R. F. Doornbos, P. C. Wintermans and C. M. Pieterse  
648 (2013). The rhizosphere revisited: root microbiomics. **Frontiers in Plant Science**, 4:165.  
649 doi:10.3389/fpls.2013.00165.

650

651 Barea, J. M., C. Azcón-Aguilar and R. Azcón, (1991). The role of vesicular-arbuscular  
652 mycorrhizae in improving plant N acquisition from soil as assessed with <sup>15</sup>N. In: IAEA-  
653 SM 313/67 ed. **Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental**  
654 **Studies**, Vienna: International Atomic Energy Agency, 209 – 216.

655

656 Bates, A., J. Sadler, R. Greswell, and R. Mackay (2015). Effects of recycled aggregate  
657 growth substrate on green roof vegetation development: a six year experiment.  
658 **Landscape and Urban Planning**, 135: 22–31.

659

660 Bethlenfalvay, G. J and R. G. Linderman (1992). **Mycorrhizae in sustainable agriculture**.  
661 Madison: ASA Special Publication.

662

663 Blamey, M., Fitter, S. R., & Fitter, A. (2003). **Wild Flowers of Britain & Ireland**. A. & C.  
664 Black.

665

666 Blume, E., M. Bischoff, J. M. Reichert, T. Moorman, A. Konopka, and R. F. Turco (2002).  
667 Surface and subsurface microbial biomass, community structure and metabolic  
668 activity as a function of soil depth and season. **Applied Soil Ecology**, 20(3): 171-181.

669

670 Blevins, D. G. (1999). Why plants need phosphorous. **Better crops**, 83(2):29 – 30.

671

672 Buffam, A. P. D. I and M. E. Mitchell (2015) **Nutrient cycling in green roof ecosystems**.  
673 In: Sutton R (ed) Green roof ecosystems, 1st edn. Springer International Publishing,  
674 New York, pp. 107–137

675

676 Bridger, G. L., D. R. Boylan, and J. W. Markey (1953). Colorimetric determination of  
677 phosphorus pentoxide in fertilizers using standard calibration plot. **Analytical**  
678 **Chemistry**, 25(2): 336-338.

679

680 Brenneisen S (2006) Space for urban wildlife: designing green roofs as habitats in  
681 Switzerland. **Urban Habitats**, 4:10

682

683 Connop, S., Vandergert, P., Eisenberg, B., Collier, M. J., Nash, C., Clough, J., & Newport,  
684 D. (2016). Renaturing cities using a regionally-focused biodiversity-led multifunctional  
685 benefits approach to urban green infrastructure. **Environmental Science & Policy**, 62,  
686 99-111.

687

688 Debosz, Kasia, Søren O. Petersen, Liv K. Kure, and Per Ambus (2002). Evaluating effects  
689 of sewage sludge and household compost on soil physical, chemical and  
690 microbiological properties. **Applied Soil Ecology**, 19(3): 237-248.

691

692 Duckmanton, L. and P. Widden (1994). Effect of ozone on the development of  
693 vesicular-arbuscular mycorrhizae in sugar maple saplings. **Mycologia**, 181-186.

694

695 Dunnett, N., Nagase, A., Booth, R., and Grime, P. (2008). Influence of vegetation  
696 composition on runoff in two simulated green roof experiments. **Urban Ecosystems**  
697 11:385-398.

698

699 Edwards, G. R, M. J Crawley and M. S. Heard (1999). Factors affecting molehill  
700 distribution in grassland: implications for controlling the damage caused by molehills.  
701 **Journal of Applied Ecology**, 36:434 – 442.

702

703 Eksi, M. & D. B. Rowe (2016). Green roof substrates: Effect of recycled crushed  
704 porcelain and foamed glass on plant growth and water retention. **Urban Forestry &**  
705 **Urban Greening**, 20 :81-88.

706

707 Field, C. and H. A. Mooney (1986). The photosynthesis-nitrogen relationship in wild  
708 plants. In: Givnish, T. J. (ed). **On the economy of plant form and function**. Cambridge  
709 University Press, Cambridge, 25 – 55.  
710

711 Fowler, J., Cohen, L., & Jarvis, P. (1998) **Practical Statistics for Field Biology**, second  
712 edition. John Wiley & Sons.  
713

714 Gadhave, K. R., J. E. Hourston & A. C. Gange (2016). Developing Soil Microbial  
715 Inoculants for Pest Management: Can One Have Too Much of a Good Thing? **Journal**  
716 **of chemical ecology**, 42(4): 348-356.  
717

718 Gange, A. C. (1993) Translocation of mycorrhizal fungi by earthworms during early  
719 succession. **Soil Biology and Biochemistry**, 25, 1021 – 1026.  
720

721 Graceson, A., J. Monaghan, N. Hall, and M. Hare (2014b). Plant growth responses to  
722 different growing media for green roofs. **Ecological Engineering**, 69:196–200.  
723

724 Grime, J. P., J. M. L. Mackey, S. H. Hillier and D. J. Read (1987). Floristic diversity in a  
725 model system using experimental microcosms. **Nature**, 328:3.

726 Heim, A., and J. Lundholm (2014). The effects of substrate depth heterogeneity on  
727 plant species coexistence on an extensive green roof. **Ecological Engineering**, 68:184-  
728 188.  
729

730 Henriksen, A. A., and A. R. Selmer-Olsen (1970). Automatic methods for determining  
731 nitrate and nitrite in water and soil extracts. **Analyst**, 95(1130): 514-518.  
732

733 Hinsinger, P. (2001). Bioavailability of soil inorganic P in the rhizosphere as affected  
734 by root-induced chemical changes: a review. **Plant and Soil**, 237:173 – 195.  
735

736 Hodge, A. (2000) Microbial ecology of the arbuscular mycorrhiza. **FEMS Microbiology**  
737 **Ecology**, 32, 91 – 96.  
738

739 Johansen, A., I. Jakobsen and E. S. Jensen (1993). External hyphae of vesicular-  
740 arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 3. Hyphal  
741 transport of <sup>32</sup>P and <sup>15</sup>N. **New Phytologist**, 124:61 – 68.

742

743 John, J., J. Lundholm and G. Kernaghan (2014). Colonization of green roof plants by  
744 mycorrhizal and root endophytic fungi. **Ecological Engineering**, 71:651-659.

745

746 Kadas, G. (2002). **Study of invertebrates on green roofs: How roof design can**  
747 **maximise biodiversity in an urban environment**. Master of science thesis. University  
748 College, London.

749

750 Kadas, G. (2007) **Can Green Roofs provide a habitat for invertebrates in an urban**  
751 **environment?** Unpublished PhD thesis. Royal Holloway University of London.

752

753 Klironomos, J.N., McCune, J., Hart, M., Neville, J., (2000) The influence of arbuscular  
754 mycorrhizae on the relationship between plant diversity and productivity. **Ecology**  
755 **Letters**, 3:137–141

756

757 Klironomos, J. N., J. McCune and P. Moutoglis (2004). Species of arbuscular  
758 mycorrhizal fungi affect mycorrhizal responses to simulated herbivory. **Applied Soil**  
759 **Ecology**, 26(2):133-141.

760

761 Koide, R. T. (1991). Nutrient supply, nutrient demand and plant response to  
762 mycorrhizal infection. **New Phytologist**, 117:365 – 386.

763

764 Koske, R. E., and J. N. Gemma, (1997) Mycorrhizae and succession in plantings of  
765 beachgrass in sand dunes. **American Journal of Botany**, 84, 118 – 130.

766

767 Kramer, P. J. and J. S. Boyer (1995). **Water Relations of Plants and Soils**. Academic  
768 Press, San Diego, USA.

769

770 Lavelle, P., Decaens, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P.,



771 Mora, P. and J. P. Rossi, (2006) Soil invertebrates and ecosystem services. **European**  
772 **Journal of Soil Biology**, 42, S3 – S15.  
773

774 Lee, J. A., R. Harmer and R. Ignaciuk (1983). Nitrogen as a limiting factor in plant  
775 communities. **Nitrogen as an Ecological Factor** (eds J. A. Lee, S. McNeil & I. H. Rorison),  
776 Blackwell Scientific Publications, Oxford, 95 – 112.  
777

778 Lundholm, J. T. (2015). Green roof plant species diversity improves ecosystem  
779 multifunctionality. **Journal of Applied Ecology**, 52(3):726-734.  
780

781 Lundholm, J., J. S. MacIvor, Z. MacDougall, M. Ranalli (2010). Plant species and  
782 functional group combinations affect green roof ecosystem functions. **PLoS ONE**, 5  
783 (3):e9677.  
784

785 Jin, L., Q. Wang, Q. Wang, X., Wang, and A. C. Gange (2016). Mycorrhizal-induced  
786 growth depression in plants. **Symbiosis**, 1-8.  
787

788 McGonigle, T. P., M. H. Miller, D. G. Evans, G. L. Fairchild, and J. A. Swan (1990). A new  
789 method which gives an objective measure of colonization of roots by vesicular—  
790 arbuscular mycorrhizal fungi. **New phytologist**, 115(3): 495-501.  
791

792 McGuire, K.L., S. G. Payne, M. I. Palmer, C. M. Gillikin, D. Keefe, S. J. Kim, S. M.  
793 Gedalovich, J. Discenza, R. Rangamannar, J. A. Koshner, and A. L. Massmann (2013).  
794 Digging the New York City skyline: soil fungal communities in green roofs and city parks.  
795 **PloS one**, 8(3):58020.  
796

797 Miransari, M. (2016). Stress and Mycorrhizal Plant. In: **Recent Advances on**  
798 **Mycorrhizal Fungi**, 63-79. Springer International Publishing.  
799

800 Molineux, C. J., Fentiman, C. H., & Gange, A. C. (2009) Characterising alternative  
801 recycled waste materials for use as green roof growing media in the U.K. **Ecological**  
802 **Engineering**, 35, 1507 – 1513.

803

804 Molineux, C. J., S. P. Connop, and A. C. Gange. (2014). Manipulating soil microbial  
805 communities in extensive green roof substrates. **Science of the Total Environment**,  
806 493:632-638.

807

808 Molineux, C. J., A. C. Gange, S. P. Connop and D. J. Newport (2015). Using recycled  
809 aggregates in green roof substrates for plant diversity. **Ecological Engineering**, 82:596-  
810 604.

811

812 Monterusso, M. A, D. B. Rowe and C. L Rugh (2005). Establishment and persistence of  
813 *Sedum* spp. and native taxa for green roof applications. **HortScience** 40: 391–396.

814

815 Oberndorfer, E., Lundholm, J., Bass, B., Coffman, R., Doshi, H., Dunnett, N., Gaffin, S.,  
816 Köhler, M., Rowe, B., (2007) Green Roofs as urban ecosystems: ecological structures,  
817 functions and services. **Bioscience**, 57, 823 – 833.

818

819 Ondoño, S., Bastida, F., and J. L. Moreno (2014). Microbiological and biochemical  
820 properties of artificial substrates: A preliminary study of its application as Technosols  
821 or as a basis in Green Roof Systems. **Ecological Engineering**, 70: 189-199.

822

823 Papatheodorou, E. M., M. D. Argyropoulou, S. J. Grayston, C. D. Campbell, R. D.  
824 Bardgett, J. L. Mawdsley, C. D. Clegg, H. Ferris, R. C. Venette and K. M. Scow (2004).  
825 Soil management to enhance bacterivore and fungivore nematode populations and  
826 their nitrogen mineralisation function: The effects of large-and small-scale differences  
827 in soil temperature and moisture on bacterial functional diversity and the community  
828 of bacterivorous nematodes. **Transport**, 25(1).

829

830 Parras-Alcántara, L., B. Lozano-García, E. C. Brevik, and A. Cerdá (2015). Soil Organic  
831 Carbon Stocks Assessment in Mediterranean Natural Areas: A Comparison of Entire  
832 Soil Profiles and Soil Control Sections. **Journal of Environmental Management**, 155:  
833 219-228. doi:10.1016/j.jenvman.2015.03.039.

834

835 Peña-Becerril, J. C., A. Monroy-Ata, M. S. Orozco-Almanza, and E. M. García-Amador  
836 (2016). Establishment of catclaw plants (*Mimosa biuncifera* Benth.) inoculated with  
837 arbuscular mycorrhizal fungi in greenhouse and field drought conditions. **Revista de**  
838 **Biología Tropical/International Journal of Tropical Biology and Conservation**, 64(2):  
839 791-803.

840

841 Requena, N., P. Jeffries and J. M. Barea (1996). Assessment of natural mycorrhizal  
842 potential in a desertified semiarid ecosystem. **Applied and Environmental**  
843 **Microbiology**, 62:842 – 847.

844

845 Rhine, E. D., R. L. Mulvaney, E. J. Pratt, and G. K. Sims (1998). Improving the Berthelot  
846 reaction for determining ammonium in soil extracts and water. **Soil Science Society of**  
847 **America Journal**, 62(2): 473-480.

848

849 Roldan-Fajardo, B. E. (1994). Effect of indigenous arbuscular mycorrhizal endophytes  
850 on the development of six wild plants colonizing a semiarid area in south-east Spain.  
851 **New Phytologist**, 127:115 – 121.

852

853 Rumble, H., & A. C. Gange (2013). Soil microarthropod community dynamics in  
854 extensive green roofs. **Ecological engineering**, 57, 197-204.

855

856 Saison, C., V. Degrange, R. Oliver, P. Millard, C. Commeaux, D. Montange, and X. Le  
857 Roux (2006). Alteration and resilience of the soil microbial community following  
858 compost amendment: effects of compost level and compost-borne microbial  
859 community. **Environmental Microbiology**, 8(2):247-257.

860

861 Schadek U., B. Strauss, R. Biedermann and M. Kleyer (2009) Plant species richness,  
862 vegetation structure and soil resources of urban brownfield sites linked to  
863 successional age. **Urban Ecosystems**, 12:115–126

864

865 Schubert, A., and D. S. Hayman (1986). Plant growth responses to vesicular -  
866 arbuscular mycorrhiza. **New Phytologist**, 103(1): 79-90.

867

868 Scott, G. A. M. (1960). **The biology of shingle beach plants with special reference to**  
869 **the ecology of selected species**. PhD thesis, University of Wales.

870

871 Simard, R. R. (1993). Ammonium acetate-extractable elements. **Soil sampling and**  
872 **methods of analysis**, 39-42.

873

874 Smith, S. E., and D. J. Read (1997). Mycorrhizal symbiosis. **Mycorrhizal symbiosis**.  
875 Edition 2.

876

877 Swift, M. J., O. W. Heal and J. M. Anderson (1979). **Decomposition in terrestrial**  
878 **ecosystems**, Blackwell Scientific, Oxford.

879

880 Titus, J.H. and J. Lepš (2000). The response of arbuscular mycorrhizae to fertilization,  
881 mowing, and removal of dominant species in a diverse oligotrophic wet meadow.  
882 **American Journal of Botany**, 87(3):392-401.

883

884 Van der Heijden M. G. A, J. N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel,  
885 and T. Boller (1998). Mycorrhizal fungal diversity determines plant biodiversity,  
886 ecosystem variability and productivity. **Nature**, 396:72–75

887

888 Van Gestel, M., J. N. Ladd, and M. Amato (1992). Microbial biomass responses to  
889 seasonal change and imposed drying regimes at increasing depths of undisturbed  
890 topsoil profiles. **Soil Biology and Biochemistry**, 24(2): 103-111.

891

892 Vierheilig, H., P. Schweiger, and M. Brundrett (2005). An overview of methods for the  
893 detection and observation of arbuscular mycorrhizal fungi in roots. **Physiologia**  
894 **Plantarum** 125:393-404.

895

896 Vosatka, M., M. Gryndler and Z. Prikryl (1992). Effect of the rhizosphere bacterium  
897 *Pseudomonas putida*, arbuscular mycorrhizal fungi and substrate composition on the  
898 growth of strawberry. **Agronomie**, 12:859–863.

899

900 Walter, J., J. Kreyling, B.K. Singh and A. Jentsch (2016). Effects of extreme weather  
901 events and legume presence on mycorrhization of *Plantago lanceolata* and *Holcus*  
902 *lanatus* in the field. **Plant Biology** 18: 262-270.

903

904 Watanabe, F. S., and S. R. Olsen (1965). Test of an ascorbic acid method for  
905 determining phosphorus in water and NaHCO<sub>3</sub> extracts from soil. **Soil Science Society**  
906 **of America Journal**, 29 (6): 677-678.

907

908 Wearn, J. A. (2006). **Effects of above-ground herbivory on rhizosphere community**  
909 **dynamics in lowland grasslands**. PhD Thesis. Royal Holloway University of London.

910

911 Young, T., Cameron, D.D., Phoenix, G.K (2015). Using AMF inoculum to improve the  
912 nutritional status of *Prunella vulgaris* plants in green roof substrate during  
913 establishment. **Urban Forestry and Urban Greening**, 14 (4): 959-967.

914

915 Zar, J. H. (2005) **Biostatistical Analysis**, Pearson Education, Upper Saddle River, NJ.

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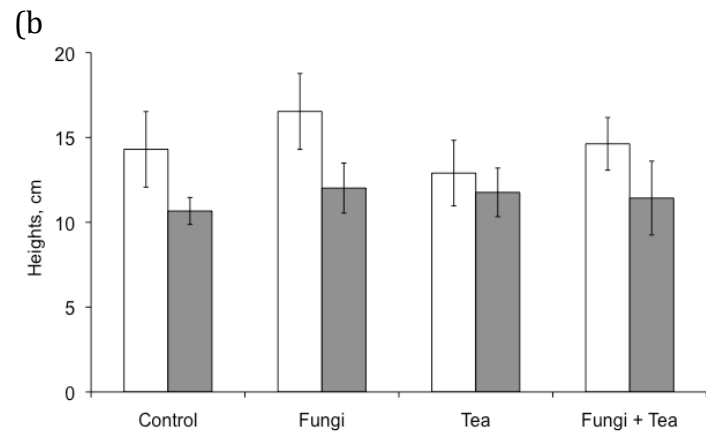
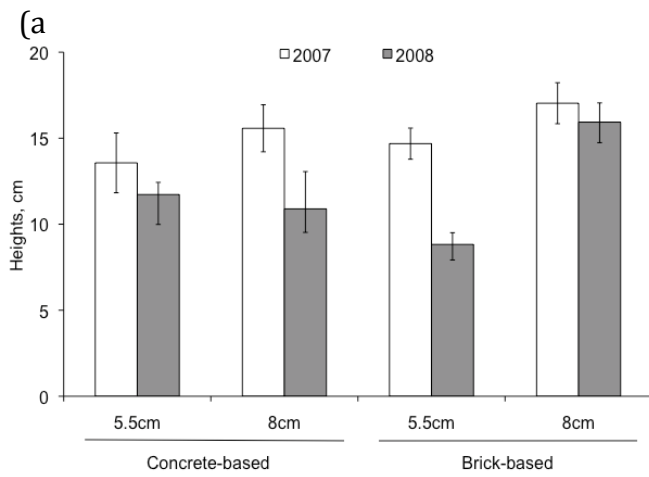
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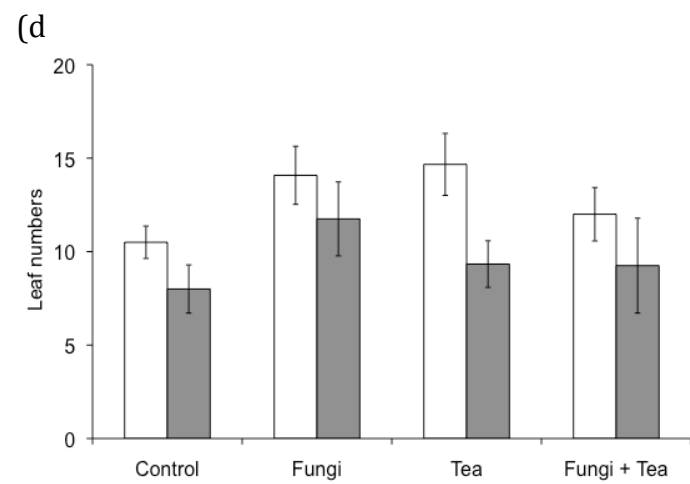
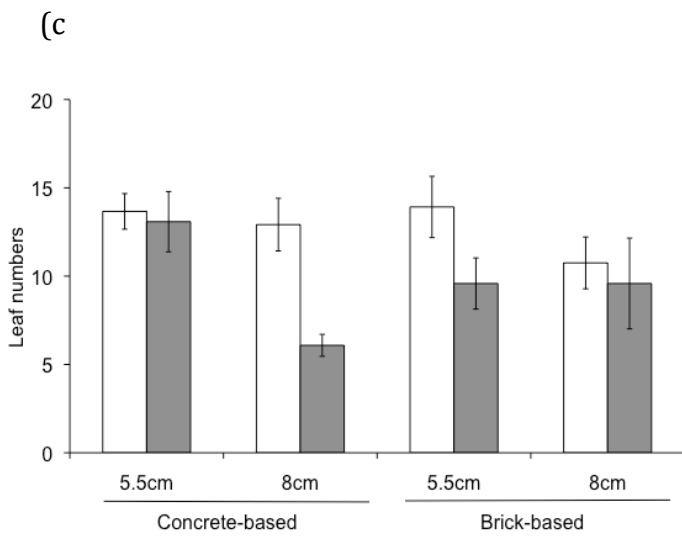
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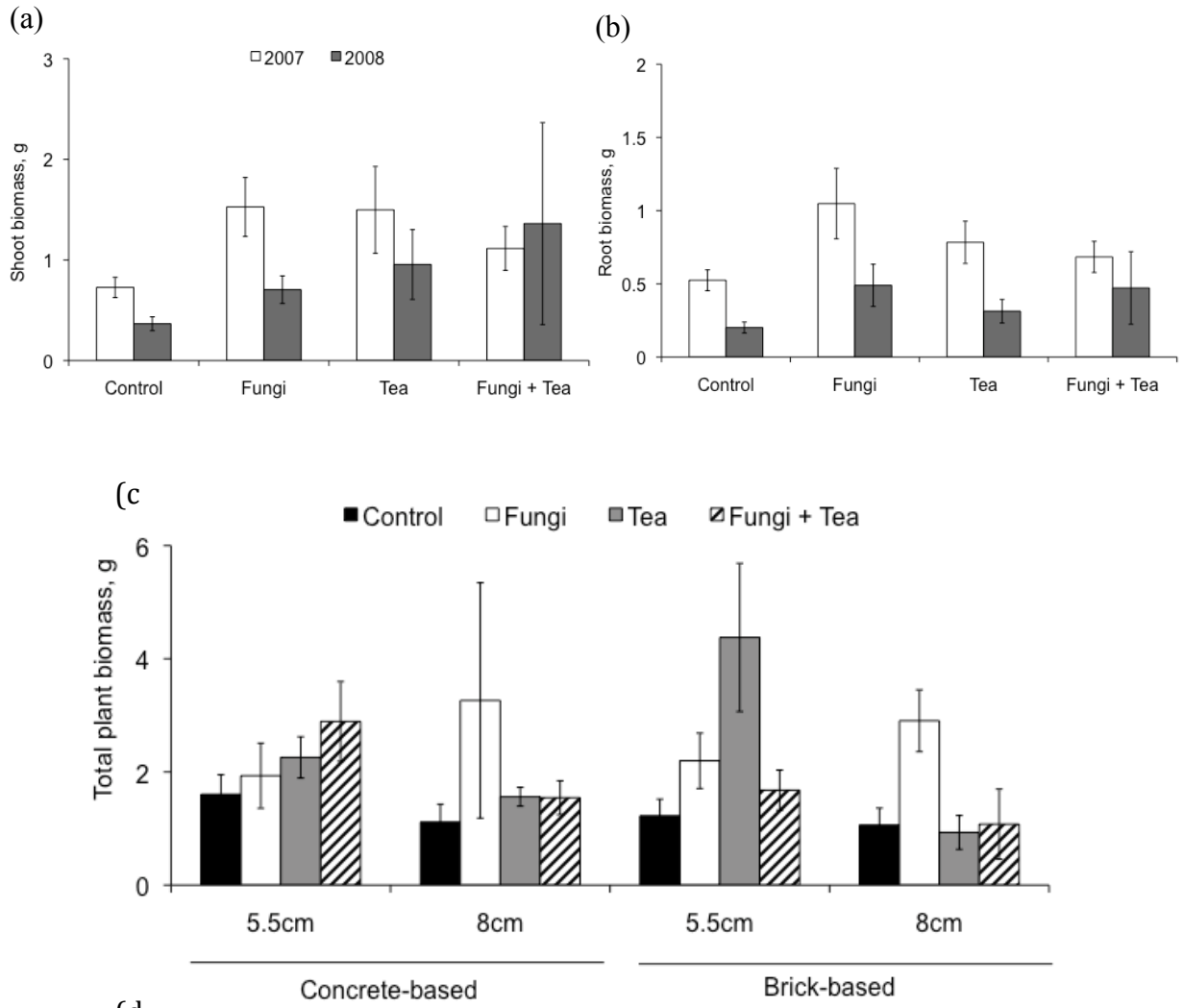


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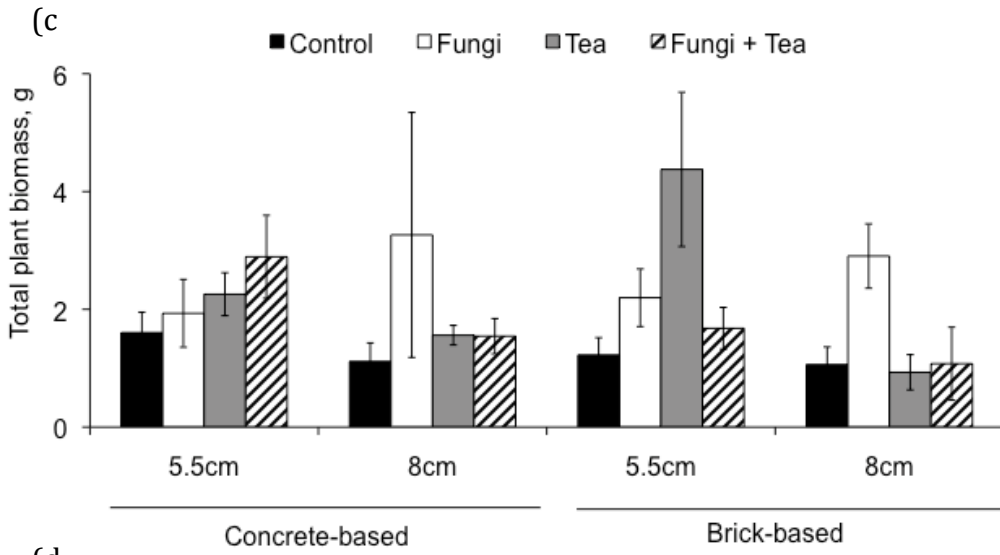
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946 **Figure 1.** (a) Bait plant heights and (c) bait plant leaf numbers, with regards to underlying  
 947 substrate type and depth; and (b) bait plant heights and (d) bait plant leaf numbers, with  
 948 microbial treatments on London Zoo green roof experimental site, where: 2007 = after  
 949 treatments and 2008 = one year after treatments applied. Bars represent means  $\pm$  S.E.



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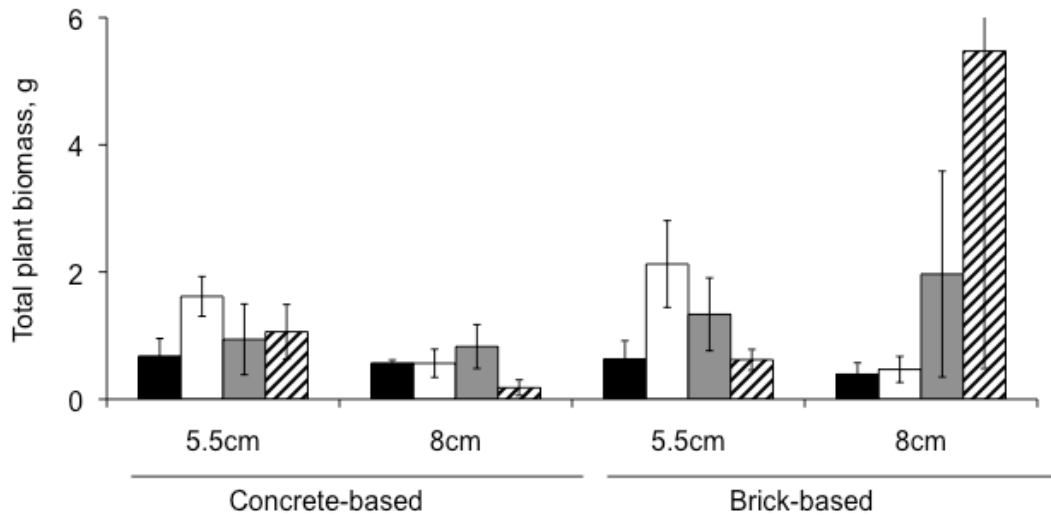
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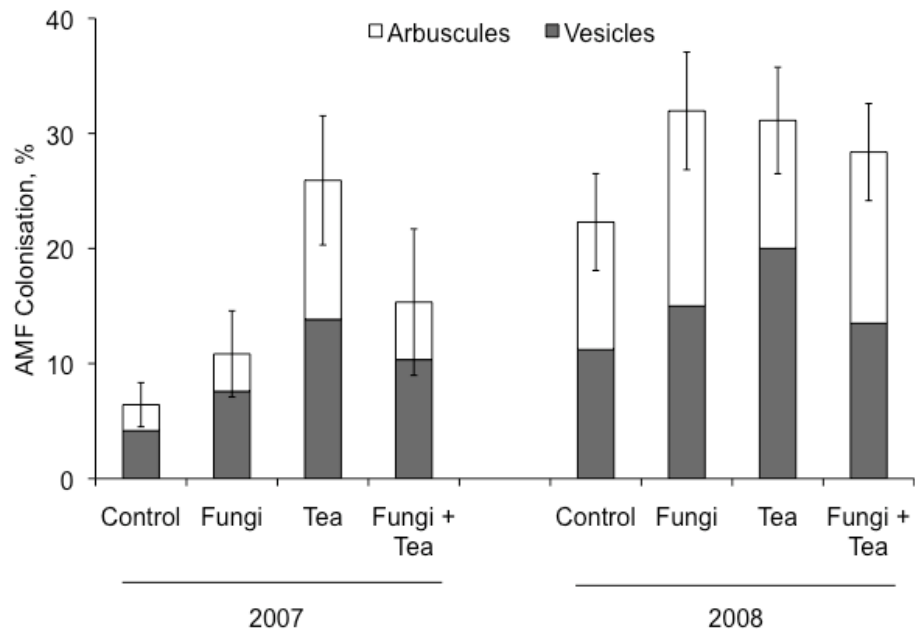
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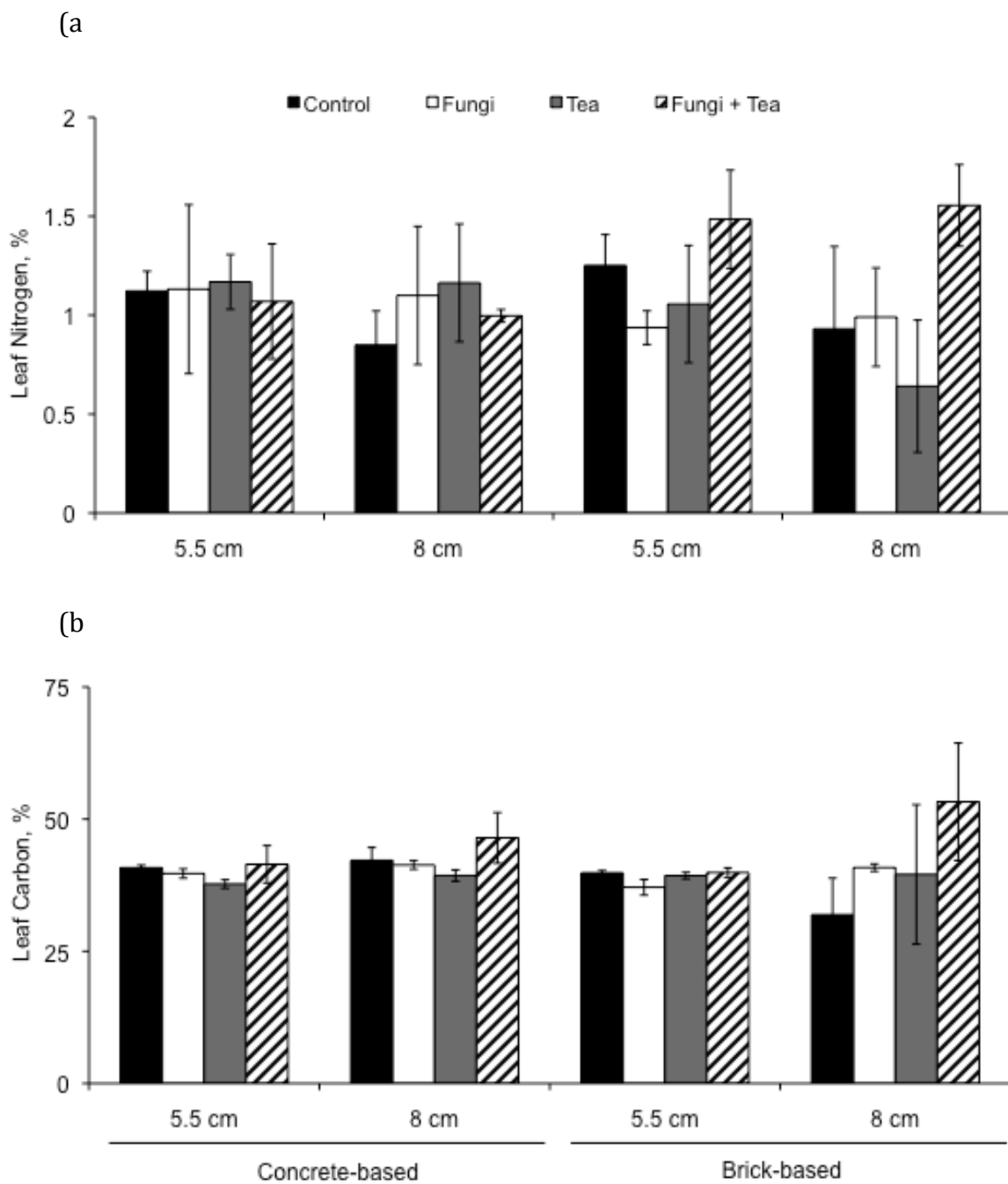
**Figure 2.** Bait plant from the treated plots on London Zoo green roof experimental site, where (a) shoot biomass and (b) root biomass in grams from 2007 = after treatments and 2008 = one year after treatments applied, means from 12 replicates per year; and total bait plant biomass with respect to underlying substrate type/depth in (c) 2007 and (d) 2008, means from three replicates. Bars represent means  $\pm$  S.E.





**Figure 3.** Bait plant root colonisation with AM fungi, from the treated plots on London Zoo experimental site in 2007 and 2008. Bars represent both arbuscule and vesicle colonisation means  $\pm$  S.E. (of total AMF colonisation).

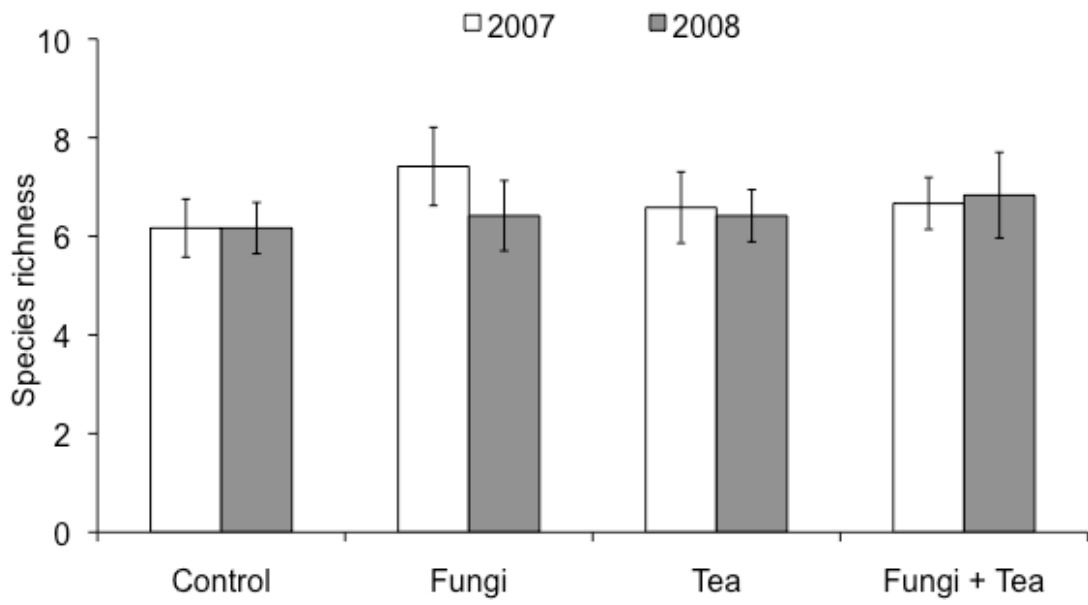
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1010 **Figure 4.** Leaf nitrogen (a) and leaf carbon (b), % content in bait plant shoots from each  
 1011 microbial treatment in 2007. Means from three replicates, bars represent means  $\pm$  S.E.  
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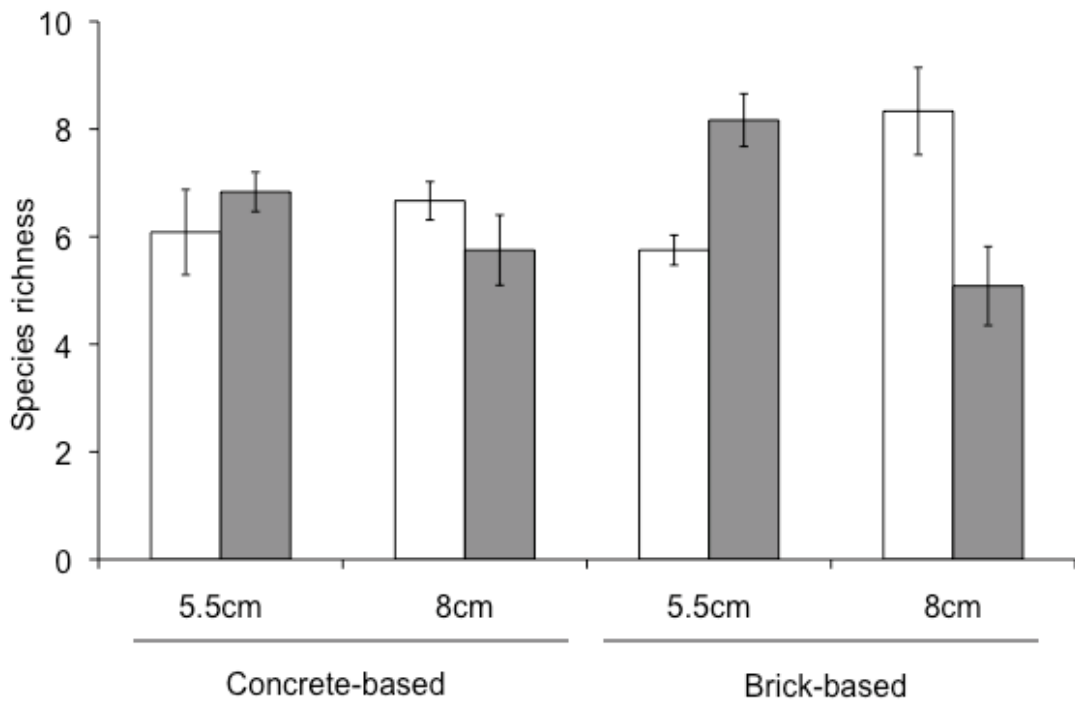
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**Figure 5.** Species richness in (a) the four treatment types irrespective of underlying substrate and (b) in different substrate types, irrespective of treatment where: 2007 = after treatments and 2008 = one year after treatments. Bars represent means ± S.E.

	Baseline				2007				2008			
	Concrete-based		Brick-based		Concrete-based		Brick-based		Concrete-based		Brick-based	
	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	8 cm	5.5 cm	
<b>Treatment</b>												
<i>Control</i>	2.14	2.95	0.29	2.21	0.81	0.69	1.45	2.05	0.85	0.86	0.75	
<i>Fungi</i>					1.67	0.88	0.93	0.75	0.98	0.85	0.72	
<i>Tea</i>					0.41	0.84	4.96	1.00	0.97	1.33	0.73	
<i>Fungi + Tea</i>					0.99	1.57	3.40	2.70	0.65	1.01	0.65	
<i>Control</i>	1.51	0.63	0.66	1.59	1.14	1.62	1.40	1.79	0.94	1.23	0.76	
<i>Fungi</i>					1.18	1.44	0.78	0.78	1.10	1.14	0.84	
<i>Tea</i>					1.58	0.82	2.77	2.41	1.15	1.09	0.77	
<i>Fungi + Tea</i>					1.53	0.95	1.39	3.13	0.59	0.98	0.57	
<i>Control</i>	15.79	16.88	15.15	24.60	5.27	9.08	0.01	0.01	18.42	14.44	18.70	
<i>Fungi</i>					7.10	12.23	6.84	12.11	17.16	13.85	18.00	
<i>Tea</i>					12.12	4.63	6.65	17.13	14.92	11.13	18.03	
<i>Fungi + Tea</i>					10.43	11.00	11.12	4.97	16.78	11.61	17.61	

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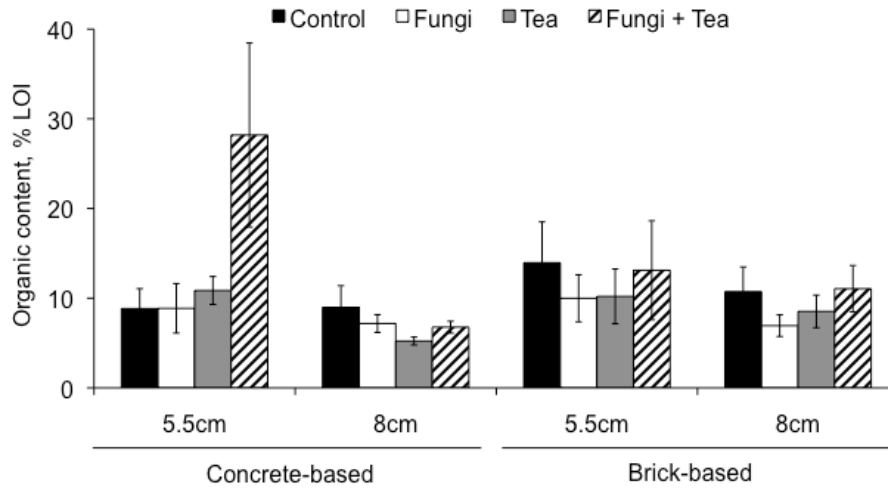
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**Table 1.** Substrate nutrients analysis, with regards to microbial treatment and underlying substrate type and depth on London Zoo green roof experimental site, where: Baseline = before microbial treatments added, 2007 = after treatments and 2008 = one year after treatments applied.

**Appendix I  
London Zoo Substrate Properties**

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**Appendix Figure 1.** Soil organic matter (as % weight loss on ignition) in the different microbial treatments and substrate types in 2007. Means from three replicates and bars represent means  $\pm$  S.E.

Characteristic	2007	2008
Substrate Water Content (%)	34.8	32.7
Mean rainfall (mm) *	86.9	67.0
Max Temperature (°C)	15.8	15.2
Min Temperature (°C)	8.1	7.6
Sun (hours)	127.4	117.5

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**Appendix Table 1.** London Zoo substrate characteristics. Means taken from 48 experimental plots.

\* From Heathrow weather station, 51.479, -0.449, available from Met Office data records.

**Appendix II: Statistical Results – ANOVA Table**

Main effects & interactions	Nitrates		Phosphates		Potassium	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Year	1.31	0.33	<b>26.0</b>	<b>&lt;0.0</b>	<b>54.4</b>	<b>&lt;0.0</b>
Substrate type	2.03	0.16	<b>8</b>	<b>1</b>	<b>7</b>	<b>1</b>
Substrate depth	0.12	0.72	0.90	0.34	0.02	0.87
Fungi	0.22	0.63	2.16	0.14	0.61	0.43
Tea	1.33	0.25	1.06	0.30	1.01	0.31
			1.31	0.25	0.50	0.48
Year x Substrate type	<b>4.1</b>	<b>&lt;0.0</b>	<b>4.90</b>	<b>&lt;0.0</b>	<b>3.82</b>	<b>=</b>
Year x Substrate depth	<b>5</b>	<b>5</b>	0.13	<b>5</b>	1.30	<b>0.05</b>
Year x Fungi treatment	1.71	0.19	0.70	0.71	3.30	0.25
	0.42	0.52		0.40		0.07
Year x Tea treatment	1.09	0.30	<b>5.07</b>	<b>&lt;0.0</b>	2.52	0.11
Substrate depth x Substrate type	0.06	0.80	1.44	<b>5</b>	0.04	0.83
Substrate depth x Fungi treatment	0.19	0.66	0.84	0.23	0.04	0.82
Substrate depth x Tea treatment	0.21	0.64	0.04	0.36	1.26	0.26
Substrate type x Fungi treatment	0.43	0.51	0.12	0.82	0.08	0.77
Substrate type x Tea treatment	1.10	0.29	<b>4.45</b>	0.72	1.16	0.28
Fungi treatment x Tea treatment	0.11	0.73	0.01	<b>&lt;0.0</b>	0.60	0.43
				<b>5</b>		
				0.95		
Year x Substrate type x Substrate depth	1.20	0.27	0.50	0.48	0.01	0.92
	2.17	0.14	1.70	0.19	1.17	0.28
Year x Substrate type x Fungi treatment						
Year x Substrate type x Tea treatment	2.09	0.15	<b>4.68</b>	<b>&lt;0.0</b>	0.49	0.48
	0.01	0.99	0.18	<b>5</b>	0.01	0.94
Year x Substrate depth x Fungi treatment	0.19	0.66	0.37	0.66	<b>4.44</b>	<b>&lt;0.0</b>
	0.94	0.33	0.64	0.54	2.48	<b>5</b>
Year x Substrate depth x Tea treatment				0.42		0.12
Year x Fungi treatment x Tea treatment						
Substrate Type x Substrate Depth x Fungi treatment	0.74	0.39	0.54	0.46	1.19	0.28
Substrate type x Substrate depth x Tea treatment	2.18	0.15	1.57	0.21	0.53	0.46
Substrate type x Fungi treatment x Tea treatment	0.60	0.44	0.43	0.51	3.21	0.07
	0.97	0.32	2.84	0.09	0.33	0.56
Substrate depth x Fungi treatment x Tea treatment						

1102 **Appendix Table 2.** ANOVA results for main effects and interactions with London  
1103 Zoo substrate nutrients. Showing the *F* statistic and probability value. Degrees of  
1104 freedom = 1, 51. Significant results highlighted in **bold**.  
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