Root distribution responses to three-dimensional soil heterogeneity in experimental mesocosms

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Abstract

Aims Plant root systems respond to local variation in soil conditions, but principles underlying the spatial distribution of roots in soils with different heterogeneity are not well known. This study investigates how root systems react to experimental variation of soil heterogeneity in three dimensions (3D).

Methods We created four levels of soil heterogeneity in mesocosms by alternating nutrient-rich and nutrient-poor substrate in three dimensions. The cell sizes of this soil matrix were 0, 12, 24 or 48 cm. Root distributions of the plant communities establishing on these soils were examined at different scales: mesocosm, substrate type and horizontal layer.

Results Mesocosms with higher soil heterogeneity (smaller cells) had more shoot biomass while root biomass was unaffected, in line with our assumption that greater proximity to nutrient-rich patches allows plants on nutrient-poor patches to invest relatively less in roots. More heterogeneous soils also yielded spatially more heterogeneous root systems, i.e. with root biomass that diverged more between nutrient-poor and nutrient-rich cells. This suggests that plants on nutrient-poor cells can more easily grow into adjacent nutrient-rich cells at higher soil heterogeneity.

Conclusions More realistic yet complex 3D designs can help improve understanding of root spatial distribution as driven by soil configurational heterogeneity.

Keywords: Community · Root spatial heterogeneity · Root/shoot ratio · Scale · Three dimensions

Introduction

The heterogeneous distribution of soil resources such as nutrients, water, etc. is an innate characteristic of soils. According to classical niche theory, more plant species can coexist on heterogeneous soils because more niches are available (Questad et al. 2008; Price et al. 2014). However, species do not merely undergo soil heterogeneity. They also develop different strategies to adapt to it, such as adjusting root architecture (Caldwell 1994; Fitter 1994), root foraging or nutrient uptake kinetics (Wijesinghe et al. 2001; Mommer et al. 2012), or simply by producing more roots (Šmilauerová, 2001; Maestre et al. 2006). In many cases, these responses will modify belowground competition (Casper and Jackson 1997; Hodge et al. 1999; Hutchings et al. 2003) and alter the performance of species (Day et al. 2003; Hutchings and John 2004; Wijesinghe et al. 2005). Measuring root distribution in soils with different heterogeneity could help elucidate these mechanisms, but several factors add complexity. One is that soils are heterogeneous in the horizontal (Williams and Houseman 2014) as well as in the vertical dimension (Maestre et al. 2006; Maestre and Reynolds 2006a), while heterogeneity can also change through time (Fitter et al. 2000; Maestre and Reynolds 2006a). Moreover, the feedback between plants and soil can alter soil heterogeneity (Hendriks et al. 2015; Burns et al. 2017). In nature, it is complicated to uncover the role of soil heterogeneity as many uncontrolled factors (nutrients, microclimate, plant age, etc.) make it more difficult to precisely link cause and effect (De Boeck et al. 2015). Exploring this role is facilitated with controlled approaches where factors other than soil heterogeneity are kept constant as much as possible. Although root placement has been investigated in response to soil heterogeneity with such approaches (Campbell et al. 1991; Wijesinghe et al. 2001), no studies have to our knowledge examined the spatial distribution of roots in soils where heterogeneity is experimentally varied in three dimensions, i.e. horizontally and vertically.

In multi-species systems, complexity is increased further because species interact not only with soil heterogeneity, but also with each other (Ravenek et al. 2016). Additionally, the presence of N-fixing species in a community, such as legumes, may strongly modify heterogeneity itself by locally adding nitrogen to the soil. Yet, community-level experiments on soil heterogeneity are rare (García-Palacios et al. 2012). The few available studies show that finer-grained patchiness may either increase (Gazol et al. 2013) or decrease (Maestre et al. 2005; Wijesinghe et al. 2005; Maestre et al. 2006; Maestre and Reynolds 2007; García-Palacios et al. 2011) community biomass, but the underlying mechanisms remain unclear. A key mechanism at the community level could be that some species can grow relatively better than others on different patches of heterogeneous soils. For example, on resource-rich patches competitive species would be favoured relative to stress-tolerant species as they are stronger competitors for resources, while the balance would tilt more towards stress-tolerants on resource-poor patches. According to the theory of niche complementarity (Loreau et al. 2001), such a partitioning of heterogeneous resources could improve their overall use and increase community biomass.

To improve understanding of how soil heterogeneity fundamentally affects root deployment, we investigate the spatial distribution of roots in multi-species mesocosms where soil heterogeneity is experimentally created in three dimensions using a recently developed technique, while keeping all other factors constant (Liu et al. 2017) (Fig. 1a). In the mesocosms, soil patches (cells) of two different substrates, which mainly differ in nutrients, alternate in all directions. We create four different levels of heterogeneity by varying the cell size, while providing the same seed rain to all mesocosms in order to let species composition develop freely. We tested the following hypotheses (Fig. 1b, c): (1) In mesocosms with higher soil heterogeneity (smaller cell size), plants growing on nutrient-poor cells

have access to resources from neighbouring nutrient-rich cells at shorter distance as compared to mesocosms with lower soil heterogeneity (larger cell size). This allows for less investment in roots and thus lower root/shoot ratios at mesocosm scale. For the same reason, the root biomass of nutrient-poor and nutrient-rich cells will diverge more in more heterogeneous soils, making the spatial distribution of root biomass more heterogeneous as well; (2) Plants growing on nutrient-poor cells in the top layer have to invest more root biomass to acquire the same amount of resources than plants growing on nutrient-rich cells in the top layer, even if deeper cells compensate for this and the total amount of resources in a soil column is the same. This drawback for plants growing on nutrient-poor cells is greater when cells are larger. Differences in root biomass between adjacent columns should therefore decrease when cells are smaller, i.e. with increasing soil heterogeneity.

Materials and Methods

EXPERIMENTAL DESIGN

The experiment was conducted at the Drie Eiken Campus of the University of Antwerp (Belgium, 51°09′41″N, 04°24′29″E) from 19 May to 2 September 2015. This location is characterized by mild winters and cool summers, with average annual air temperature 10.6 °C and rainfall 832 mm, equally distributed throughout the year (Royal Meteorological Institute of Belgium). The four levels of soil heterogeneity were created from nutrient-poor and nutrient-rich substrate (see characteristics in Table 1). These substrates were produced by combining potting soil and Lommel sand, bought from commercial suppliers in Belgium. The nutrient-poor substrate consisted of a 1:4 mixture of potting soil and sand, and the nutrient-rich substrate of a 4:1 mixture. Each substrate was thoroughly homogenized in a cement mixer. Note that the two substrates varied also in water holding capacity, but soil water

content was kept optimal in the experiment (see below), such that heterogeneity was predominantly driven by nutrients. Only configurational heterogeneity was modulated (through varying cell size), while qualitative heterogeneity was constant since the same two substrates were used across all cell sizes (Liu et al. 2017). Compared with the average of local soils in Belgian grasslands (86 mg P L^{-1} and 231 mg K L^{-1}, Janssens et al. 1998, data for N was not available), our nutrient-rich substrate had comparable (101 mg P L^{-1} and 230 mg K L^{-1}) and our nutrient-poor substrate substantially lower (46 mg P L^{-1} and 119 mg K L^{-1}) contents of macro-nutrients (values derived from Table 1). The variation induced in the experiment was consequently broadly in the range of existing values in the field.

The four levels of soil heterogeneity in our experiment were produced in mesocosms of the same size (48 cm × 48 cm × 48 cm) and consisted of cubic cells with edge dimensions of 0, 12, 24 or 48 cm. Large differences in soil quality across short distances, as between our two substrates, have been applied in the previous studies (for example, 10 cm, Kleb and Wilson 1997; 20 cm, Farley and Fitter 1999) and can also be found *in situ*. For example, dung patches can create nutrient-rich spots in an otherwise nutrient-limited ecosystem; tussocks can induce high variation in soil water content, temperature and nutrients in the range of 10-20 cm; and runoff water can increase local resources (e.g. water, nutrients) in depressions of uneven terrain (Jackson and Caldwell 1993; Stark 1994; Huber-Sannwald and Jackson 2001). The mesocosms in our experiment were held in wooden boxes. Cell size was the only difference among the four levels of soil heterogeneity as each level had the same amount of nutrient-poor and nutrient-rich substrate and thus the same average amount of resources. The mesocosms with cell size 48 cm were filled with either the nutrient-poor or nutrient-rich substrate, and can actually be considered to have “infinite” patch size, since there are no surrounding patches of different substrate. This consequently represents the lowest level of heterogeneity. The mesocosms

with cell size 24 and 12 cm were filled with nutrient-rich and nutrient-poor substrate alternating in three dimensions, using the technique of Liu et al. (2017). These treatments with declining cell sizes represent progressively larger heterogeneity as an 'observer' such as a growing plant root or a burrowing soil animal will encounter changes in soil conditions at shorter and shorter distances. The greatest variation in soil conditions, and thus the highest level of soil heterogeneity, is reached when cell size approximates 0 cm. This treatment was created by constructing mesocosms with a perfect mixture of the nutrient-rich and nutrient-poor substrate. We thus adopt the view of Liu et al. (2017) that heterogeneity is inversely related to cell size across all spatial scales. This can be supported by calculating the fractal dimension $D = \log N/\log M$, where N is the number of cubes and M is the magnification factor, the latter in this case being the number of layers in a mesocosm. Resulting fractal dimensions of nutrient-rich and nutrient-poor soils are 1, 2, 2.5 and infinite when cell size decreases from 48 to 24 to 12 and 0, respectively. Other authors have considered perfect mixtures as homogeneous, but this leads to low heterogeneity at both ends of the cell size scale and thus no systematic relationship between cell size and heterogeneity.

Each of the cell sizes 0, 12 and 24 cm was replicated in five mesocosms. Cell size 48 cm was replicated ten times, i.e. five with nutrient-rich and five with nutrient-poor substrate, in order to be able to determine the influences of both these substrate types, but also since they jointly constitute the mesocosm-level response at 48 cm (we consequently lumped them in mesocosm-scale analyses). As all mesocosms had the same depth, those with cell size 12, 24 and 48 cm were composed of four, two and one layer, respectively. To construct the mesocosms, plastic plates with slits were placed into the wooden boxes to separate them into different cells, before adding the substrates. These partitions were removed after filling (layer by layer), such that all cells were connected and roots could grow freely

142 throughout the whole mesocosm. Similar compression was applied when filling the cells to make sure
143 each mesocosm had equal amounts of substrates. Four 12-mm-diameter holes in the bottom of each
144 wooden box ensured drainage of water.

145 Twenty-four perennial herbaceous plant species, naturally occurring in grasslands in Belgium,
146 were used in the experiment. They were classified into two groups, i.e. high (6-8) or low (1-4)
147 preference for nitrogen (N) availability according to Ellenberg's ecological indicator value for N (12
148 species per group, Table 2, Ellenberg et al. 1991), in order to encompass species adapted to nutrient-
149 poor as well as to nutrient-rich substrate. With seeds obtained from commercial suppliers (Herbiseed,
150 Reading, UK and Cruydt-Hoeck, Nijeberkoop, The Netherlands), a seed mixture with equal relative
151 abundances of all the species was composed, corrected for differences in germinability according to a
152 germination test conducted three weeks prior to the actual experiment. Emergence times of the species
153 were similar and did not require correction. Each mesocosm received a uniform seed rain of 423 of the
154 mixed seeds, so that the substrate quality of each patch could determine the local community as
155 described in the Introduction. After sowing, seeds were covered with a few millimetres of the substrate
156 corresponding to that of the cell concerned. During the experiment, all mesocosms were irrigated when
157 needed to account for any shortage in natural rainfall, at the prevailing frequency of rainfall events in
158 the region (every two days). Fungicide was applied twice, one at the end of June and once one week
159 later, to avoid fungal diseases. Weeds were regularly removed.

160

161 SAMPLING AND HARVESTING

162 At the end of the experiment, the shoots and roots were harvested. Shoots were clipped at the soil
163 surface and separated according to the substrate they had grown on (nutrient-poor or -rich) in each

mesocosm, yielding one sample in mesocosms with cell size 0 and 48 cm, and two samples in mesocosms with cell size 12 and 24 cm (one for nutrient-poor and one for nutrient-rich substrate). To collect the roots, the soil of each mesocosm was cut into 64 cubes of 12 cm × 12 cm × 12 cm (16 cubes/layer × 4 layers/mesocosm), corresponding with the cell structure in the mesocosms with the 12-cm cells in order to have the same (high) resolution everywhere. Subsequently, each cube was cut into four subcubes of size 6 cm × 6 cm × 12 cm (12 cm being the height), from which one subcube was randomly selected. Finally, in each horizontal layer of each mesocosm, all the subcubes of the same substrate were grouped into one pooled sample. This yielded four pooled soil samples in mesocosms with cell size 0 and 48 cm (one substrate type × four layers), and eight pooled soil samples in mesocosms with cell size 12 and 24 cm (two substrate types × four layers). The soil samples were carefully washed to separate the roots from their growing soil; separation by species was not possible. Finally, shoot biomass and root biomass were oven-dried at 70 °C for 4 days and weighed.

To estimate the aboveground species composition, one 12 cm × 12 cm sample was randomly selected from each mesocosm with cell size 0 and 48 cm, and two 12 cm × 12 cm samples from each mesocosm with cell size 12 and 24 cm (one from nutrient-poor and one from nutrient-rich substrate). Shoots were separated by species, oven dried at 70 °C for 4 days and weighed. This extracted biomass was included in the shoot biomass totals per mesocosm referred to above.

DATA ANALYSIS AND STATISTICS

Shoot and root biomass were calculated and analysed at different scales: mesocosm, substrate and horizontal layer (the latter only for root). Shoot biomass at mesocosm scale refers to the total shoot biomass in each mesocosm, converted to g m^{-2} by dividing by the total mesocosm surface area (0.48 m

× 0.48 m). Shoot biomass at substrate scale was calculated by dividing the biomass of plants growing on each top layer substrate by the surface area of that substrate, i.e. (0.48 m × 0.48 m)/2 for mesocosms with cell size 12 and 24 cm, and 0.48 m × 0.48 m for mesocosms with cell size 0 and 48 cm.

Root biomass at mesocosm scale refers to the root biomass in all the subcubes in a mesocosm (i.e. 64 subcubes/mesocosm = 16 subcubes/layer × 4 layers/mesocosm), converted to g m^{-2} by dividing by the theoretical mesocosm surface area under which these subcubes were collected (0.06 m × 0.06 m × 16 subcubes/layer as the 4 layers share the same mesocosm surface area). The resulting expression per unit surface area of the mesocosms allows for comparison with the shoot biomass at mesocosm scale, and for calculation of the root/shoot ratio. We do not calculate root biomass expressed per unit volume, i.e. root density, because all mesocosms have the same volume, so identical response patterns to the treatments would emerge.

Root biomass at substrate scale refers to the root biomass of all the subcubes of a given substrate in a mesocosm, converted to g m^{-2} mesocosm surface area by dividing by the area of those subcubes. In mesocosms with cell size 12 and 24 cm, the biomass was thus pooled of 32 subcubes (= 8 subcubes/layer × 4 layers/mesocosm) and divided by (0.06 m × 0.06 m × 8 subcubes/layer), whereas in mesocosms with cell size 0 and 48 cm the biomass was pooled of 64 subcubes (= 16 subcubes/layer × 4 layers/mesocosm) and divided by (0.06 m × 0.06 m × 16 subcubes/layer). These calculations, as they are expressed per unit surface area of the mesocosms, again take into account that the 4 layers share the same surface area. Similar to above, we do not calculate root biomass per unit volume for a given substrate, as it will show the same response pattern to the treatments.

Root biomass at layer scale refers to the total root biomass of all 16 subcubes in a given layer

208 within a mesocosm, converted to g m^{-2} surface area by dividing by the area of those subcubes (0.06 m
209 × 0.06 m × 16 subcubes/layer). Finally, root biomass at layer scale within a mesocosm was also
210 calculated separately for nutrient-poor and nutrient-rich substrate (here the reference area equals 0.06
211 m × 0.06 m × 8).

212 Total plant biomass and root/shoot ratio (R/S) of a mesocosm were calculated as shoot biomass +
213 root biomass and root biomass / shoot biomass, respectively. We also calculated the fraction of the
214 total root biomass present in nutrient-rich patches, both at mesocosm and at layer scale. The
215 heterogeneity of the root biomass in three dimensions was expressed as the coefficient of variation
216 (CV) of all the pooled root biomass samples across substrate types and layers, i.e. 8 pooled samples (4
217 in nutrient-rich and 4 in nutrient-poor) for mesocosms with cell size 12, 24 and 48; CV of the root
218 biomass for mesocosms with cell size 0 was also calculated, obviously only across the four layers as
219 the substrates were not discernible. Note that root biomass heterogeneity calculated in this way is a
220 proxy as subcubes of the same substrate were pooled within each layer.

221 Aboveground species composition (proportion of total shoot biomass by species) was calculated
222 at substrate scale by combining the five randomly selected 0.12 m × 0.12 m samples taken in nutrient-
223 poor or nutrient-rich substrate for each level of soil heterogeneity, and making the relative abundance
224 diagram.

225 At mesocosm scale, one-way ANOVA was used to investigate the effect of cell size on the root
226 biomass, shoot biomass, total biomass and R/S in mesocosms with cell size 0, 12, 24 and 48 cm. As
227 post-hoc analyses (pairwise comparisons with Fisher' LSD) were not significant but the data showed a
228 contrast between the lowest heterogeneity level and all others for some of the response variables (see
229 Results), we also verified with Student's *t* tests whether root biomass, shoot biomass, total biomass

and R/S were different between cell size 48 cm and cell sizes 0, 12 and 24 cm combined. At substrate scale, we applied generalized linear mixed models (GLMMs) to test effects of cell size and substrate type on shoot biomass and on root biomass in mesocosms with cell size 12, 24 and 48 cm, excluding mesocosms with cell size 0 where substrate types could not be distinguished. Mesocosm identity was a random factor in these tests, and non-significant explanatory variables were excluded stepwise. At layer scale, four separate analyses were done. First, GLMMs to test the effect of cell size, substrate type and layer and all interactions on the root biomass in mesocosms with cell size 12, 24 and 48 cm. Mesocosm identity was again the random factor, non-significant explanatory variables were likewise excluded stepwise, and post-hoc analyses (pairwise comparisons with Fisher's LSD) were conducted when differences among treatments were significant. Second, GLMM and associated post-hoc analysis tested the effect of layer on the root biomass in mesocosms with cell size zero. Third, GLMM investigated the effect of cell size and top substrate type on the total root biomass in soil columns of mesocosms with cell size 12 and 24 cm, to assess possible differences between columns with the same amount of resources but different access to them in the vertical profile. Fourth, GLMM was conducted to explore the effect of cell size and layer on the fraction of the total root biomass present in nutrient-rich patches in mesocosms with cell size 12, 24 and 48 cm. Also in these last three analyses, mesocosm identity was the random factor.

Finally, one-way ANOVA was used to test the effect of cell size on the coefficient of variation (CV) of the root biomass, and on the fraction of root biomass present in nutrient-rich patches, in both cases at mesocosm scale, so all substrates and layers combined, for cell sizes 12, 24 and 48 cm. All statistics were done with SPSS 23.0 (IBM Corp., 2015).

Results

At mesocosm scale (Table 3, Fig. 2), there was no overall effect of cell size on root biomass, shoot biomass, total biomass or R/S. However, the average shoot biomass of the mesocosms with the three smaller cell sizes (0, 12 and 24 cm combined) was significantly greater ($P = 0.015$, 14% increase) than the shoot biomass of the mesocosms with cell size 48 cm. Also, the combined mesocosms with cell sizes 0, 12 and 24 cm had a marginally lower R/S ($P = 0.076$, 22% decrease) than the mesocosms with cell size 48 cm. Across all levels of soil heterogeneity (mesocosms with cell size 0 cm not included), plants invested on average 55% of their total root biomass in nutrient-rich patches. This fraction did not depend on cell size ($P = 0.230$).

At substrate scale (Table 4, Fig. 3), more shoot and root biomass was found in nutrient-rich than in nutrient-poor substrate. For shoot biomass, the increase depended on cell size (cell size × substrate type interaction), and amounted to 25, 11 and 109% at cell size 12, 24 and 48 cm, respectively. Fig. 3 reveals that the aforementioned decline in mesocosm shoot biomass at 48 cm cell size (relative to the other cell sizes) originates from a sharp drop in biomass specifically on the 48-cm nutrient-poor patches. Species compositions at the different substrates and cell sizes can be found in the supplementary material (S1). For root biomass, the overall pattern of greater values in nutrient-rich substrate (Table 4) seemed to be absent at cell size 48 cm (Fig. 3b). A separate Student's *t* test indeed showed no significant difference ($P = 0.824$).

At layer scale, in the first analysis on mesocosms with cell size 12, 24 and 48 cm, root biomass varied with layer, substrate type, cell size × substrate type interaction, and layer × substrate type interaction (Table 5, Fig. 4). As a single factor, cell size had little influence on the vertical distribution of roots. Yet, in nutrient-rich substrate, plants grew more roots in 24-cm-cell than in 48-cm-cell

mesocosms, while in nutrient-poor substrate they grew less roots in 12-cm-cell than in 48-cm-cell mesocosms (Fig. 4). This cell size × substrate type interaction seems to have caused the convergence of the root biomass in nutrient-rich and in nutrient-poor soil towards cell size 48 cm in Fig. 3b. The layer × substrate type interaction is visible in Fig. 4 as more slowly decreasing root biomass with depth in nutrient-rich than in nutrient-poor substrate (compare right with left profile in layers 2-3-4). This is in line with expected deeper root growth when the top soil consists of nutrient-poor substrate.

In the second analysis at layer scale, on mesocosms with cell size 0, layer significantly affected root biomass ($P < 0.001$). More roots were found in the top layer than in the three layers below. In the third analysis, neither cell size ($P = 0.131$) nor substrate type of the top layer ($P = 0.516$) or their interaction ($P = 0.445$) influenced the root biomass of a column in mesocosms with cell size 12 and 24 cm (Fig. 5a). In the fourth analysis, conversely to the lack of effect at mesocosm scale, both cell size and layer affected the fraction of total root biomass present in nutrient-rich patches. This fraction was higher in mesocosms with smaller cells ($P < 0.001$) and higher in deeper layers ($P = 0.004$) (Fig. 4b).

Finally, the coefficient of variation of the root biomass of all samples across substrate types and layers in a mesocosm was not affected by cell size ($P = 0.642$, Fig. 5b).

Discussion

Our first hypothesis stated that plants growing on nutrient-poor substrate can explore nutrients from neighbouring or deeper nutrient-rich substrate more easily when soil heterogeneity is high, i.e. when cell size is small. Such easier access to nearby resources allows these plants to invest relatively less in roots and thus have lower root/shoot ratios, compared with lower heterogeneity. This was partly supported by our findings at mesocosm level of marginally higher R/S, significantly lower shoot

biomass, and similar root biomass at cell size 48 cm relative to the smaller cell sizes, as well as by our finding at layer scale that the fraction of the total root biomass occurring in nutrient-rich patches declined with increasing cell size. Apparently, roots that were forced to forage more than on average 12 cm towards a patch of nutrient-rich substrate (actually between 0 and 24 cm, which is the distance to the next nutrient-rich patch at cell size 24) exceeded a cost/benefit threshold, above which shoot biomass can no longer be maintained with the same amount of roots (Fitter 1994). This corresponds to studies on root foraging that found negative relationships between individual biomass and distance to a nutrient patch (Maestre and Reynolds 2006b). Further studies may investigate whether this acts as a selective force, driving the species composition towards species that can forage further when heterogeneity is lower. Note, however, that the lack of difference in biomass at mesocosm level between cell sizes 0, 12 and 24 can be explained by another mechanism, notably that, towards smaller cell size, reduced root biomass in nutrient-poor substrate was compensated by increased root biomass in nutrient-rich substrate (cf. Fig. 3b). This increase may originate from invading roots of neighbouring plants growing in nutrient-poor substrate, but also from stimulated root growth in the plants growing in rich substrate themselves owing to more intense root competition with these invading roots.

The easier ingrowth of roots from plants established on nutrient-poor substrate into nearby nutrient-rich substrate when soil heterogeneity is higher, as conjectured under the first hypothesis, would also lead to more divergent root biomass between nutrient-poor and nutrient-rich cells, and thus a more heterogeneous root biomass distribution. Yet, there was no significant effect of cell size on the CV of root biomass at mesocosm scale. Possibly, the effect was not properly picked up with our proxy for the real CV, as we pooled the individual root samples (subcubes) by substrate in each layer. However, as mentioned above, Fig 3b does provide support for a more heterogeneous root distribution

318 at higher levels of soil heterogeneity, since the nearly equal root biomass of nutrient-poor and nutrient-
319 rich substrate observed at cell size 48 cm diverged at smaller cell sizes, which is further confirmed by
320 the layer analysis of Table 5 and Fig 4. Coupling of soil and root heterogeneity results in a more
321 efficient nutrient acquisition when a fixed amount of nutrients is spatially clumped, as plants tend to
322 grow roots in favourable patches (Jackson and Caldwell 1996; Maestre et al. 2005). The current study
323 demonstrates that this coupling occurs in multi-species communities growing across a gradient of soil
324 heterogeneity applied in all directions.

325 Our second hypothesis was that plants growing on nutrient-poor cells in the top layer have to
326 invest more root biomass to acquire the same amount of resources than plants growing on nutrient-rich
327 cells in the top layer. This required greater investment should diminish as cells get smaller, causing
328 differences in total root biomass between adjacent soil columns to fade. At the higher heterogeneity
329 level of cell size 12, the root biomass in the whole soil column was indeed similar for nutrient-rich and
330 nutrient-poor on top, indicating that 12 cm of poor soil in the top layer could be overcome without
331 substantial additional investment in roots. Surprisingly, the root biomass of nutrient-rich and nutrient-
332 poor on top were also similar at cell size 24 cm, where roots had to grow to a depth of 24 cm to find
333 more resources. One mechanism could be that a greater allocation to roots reduced the plants' overall
334 productivity, counterbalancing the initial increase of root biomass associated with this greater root
335 allocation (Drew 1975). However, Fig. 3a does not support this as shoot biomass was not lower on 24-
336 cm than on 12-cm nutrient-poor patches. Likewise, Maestre and Reynolds (2006a) reported no
337 significant difference in total root biomass when a nutrient patch was located in the lower or in the
338 upper half of mesocosms. Most likely, plant roots also proliferate further down even with nutrient-rich
339 patches on top, and to the same extent at cell size 12 and 24, equalizing the root biomass in the

different column types and cell sizes. Based on these findings, soil heterogeneity does not seem to increase total root productivity through the vertical alternation of favourable and unfavourable substrate.

Plants integrate local cues and systemic signals to adjust root and whole-plant growth (De Kroon et al. 2009; Rellán-Álvarez et al. 2016; Xuan et al. 2017). In early experiments with split-root designs (Drew et al. 1973; Drew 1975), lateral root growth was promoted in local nutrient-rich patches. Later research revealed that systemic signals can override such effects of local cues (Zhang and Forde 1998; Zhang et al. 1999; Forde 2002). In the current experiment, the systemic signal in the four levels of soil heterogeneity can be considered identical since these levels contained the same total amount of nutrients, hence the differences among them were caused by the scale of the local cue, i.e. cell size. To our knowledge, scale effects on the local cue have so far only been examined in 2-D (Wijesinghe and Hutchings 1999; Einsmann et al. 1999). Opposite to our experiment, Wijesinghe and Hutchings (1999) measured more root biomass in a clonal herb in large-patch than in small-patch mesocosms, while shoot biomass was not affected. Einsmann et al. (1999) observed positive effects of the spatial distribution of nutrients on whole-plant biomass in some species, but no effect in others (the species were grown separately). We conclude that, compared at constant systemic signal, plant biomass responds to the scale of the local cue. The different findings between our experiment and previous studies may originate from the different dimensions of soil heterogeneity (i.e. 3-D vs. 2-D), but also from the different composition (i.e. mixed community vs. single species).

To our knowledge, we present the first study to explore community root distribution in soil that is heterogeneous in three dimensions. Analogous to previous studies on soil heterogeneity that endeavoured greater realism and explanatory power, we added a large number of species by means of

362 a uniform seed rain and compared multiple levels of heterogeneous nutrient supply (Wijesinghe et al.
363 2005; Gazol et al. 2013). Despite the complexity of the combined 3D and community-scale design,
364 clear and explicable patterns emerged, demonstrating that more realism in soil heterogeneity research
365 is possible without loss of explanatory power. Yet, we recognize that our study was limited to detecting
366 general patterns of the root distribution, as the large number of species and the complex soil system
367 (3D) do not allow for the separation of species by morphological traits (Wijesinghe et al. 2005), unlike
368 heterogeneity studies with few species (Janeček et al 2004; Mommer et al 2012; Robinson et al 1999).
369 The behaviour of individual species under both soil heterogeneity and competition has been explored
370 in a limited number of – albeit small-scale – studies, suggesting that species-specific root distributions
371 are not only determined by soil heterogeneity but also by competition in a nonadditive way (Cahill et
372 al. 2010; Mommer et al 2010; Mommer et al. 2012; Padilla et al. 2013). The capacity to proliferate
373 roots into small yet favourable patches may confer a competitive advantage, changing the relative
374 competitive ability of individual species and consequently further intensifying competition in these
375 nutrient-rich cells (Robinson 1994; Robinson et al. 1999; Fransen et al. 1998, 2001; Day et al. 2003;
376 Janeček et al 2004). Conversely, more intense competition in nutrient-rich patches may result in
377 preferential avoidance of these locations by other species through the selective growth of roots in the
378 competitor-free nutrient-poor patches (Mommer et al. 2012). Future research may therefore focus on
379 how different functional types, or separate species, perform belowground within diverse communities
380 growing in soils where heterogeneity is varied in three dimensions. Two techniques can be used to
381 further explore their performance (Cahill and McNickle 2011). On the one hand, molecular-based
382 essays are able to quantify the relative contribution of different species to root biomass, either after
383 collection of root samples (Mommer et al. 2008; Mommer et al. 2010), or directly in the soil (Haling et

384 al. 2011). On the other hand, the addition of an isotopic tracer to a predetermined layer and/or
385 substrate type, either by injection in shallow soil systems (Reynolds et al. 1997; Mommer et al. 2012)
386 or through the addition of isotopically enriched organic matter during the mesocosm construction
387 process (Maestre et al. 2005), has already proven useful in elucidating root responses to two-
388 dimensional heterogeneity in a competitive environment.
389
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Table 1 Characteristics of the two substrates tested at the beginning (a) and end of the experiment (b)

(a)

Substrate type	pH	C (%)	NaCl (mg L^{-1})	NO_3^--N (kg ha^{-1})	NH_4^+-N (kg ha^{-1})	P_2O_5 (mg L^{-1})	K_2O (mg L^{-1})	MgO (mg L^{-1})	CaO (mg L^{-1})	Na_2O (mg L^{-1})
Nutrient-poor	5.5	1.1	555	142	11	32	118	253	467	18
Nutrient-rich	5.3	8.7	1264	420	12	188	228	1252	1700	81

(b)

Substrate type	NO_3^--N (kg ha^{-1})	NH_4^+-N (kg ha^{-1})
Nutrient-poor	2	28
Nutrient-rich	2	34

608 **Table 2** Plant species used in the experiment and their Ellenberg nitrogen (N) values

Species	Family	Group	N value
Achillea ptarmica L.	Asteraceae	1	2
Agrostis capillaris L.	Gramineae	1	4
Berteroa incana (L.) DC.	Brassicaceae	1	4
Briza media L.	Poaceae	1	2
Festuca ovina L.	Poaceae	1	1
Hypericum perforatum L.	Hypericaceae	1	4
Koeleria macrantha (Ledeb.) Schult.	Poaceae	1	2
Leucanthemum vulgare Lam.	Asteraceae	1	3
Nardus stricta L.	Poaceae	1	2
Poa compressa L.	Poaceae	1	3
Rumex acetosella L.	Polygonaceae	1	2
Vulpia myuros (L.) C.C.Gmel	Poaceae	1	1

Species	Family	Group	N value
Brachypodium sylvaticum (Huds.) Beauv.	Poaceae	2	6
Dactylis glomerata L.	Poaceae	2	6
Epilobium hirsutum L.	Onagraceae	2	8
Festuca gigantea (L.) Vill.	Poaceae	2	6
Festuca pratensis Huds.	Poaceae	2	6
Geranium robertianum L.	Geraniaceae	2	7
Lolium perenne L.	Poaceae	2	7
Nepeta cataria L.	Lamiaceae	2	7
Poa pratensis L.	Poaceae	2	6
Poa trivialis L.	Poaceae	2	7
Silene dioica (L.) Clairv.	Caryophyllaceae	2	8
Taraxacum officinale F.H.Wigg	Asteraceae	2	8

Table 3 Effect of cell size in one-way ANOVAs of shoot biomass, root biomass, total biomass and root/shoot ratio (R/S) measured in mesocosms with cell sizes 0, 12, 24 and 48 cm. F-values, *P*-values and degrees of freedom ($df_{between\text{-}groups}$, $df_{within\text{-}groups}$) are given

Source	Shoot biomass			Root biomass			Total biomass			R/S		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Cell size	3, 21	0.041	0.989	3, 21	0.177	0.910	3, 21	0.051	0.984	3, 21	0.460	0.713

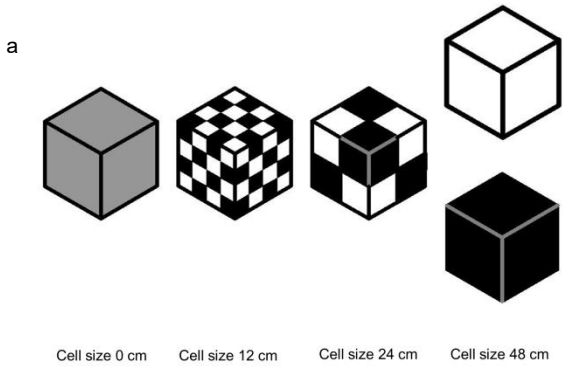
612 **Table 4** Effects of cell size, substrate type and their interaction in GLMMs of shoot and root biomass
613 measured in mesocosms with cell sizes 12, 24 and 48 cm. F-values, *P*-values and degrees of freedom
614 ($df_{between\text{-}groups}$, $df_{within\text{-}groups}$) are given, with significant results ($P < 0.05$) in bold. Nonsignificant factors
615 were removed stepwise from the final model

Source	Shoot biomass			Root biomass		
	df	*F*	*P*	Df	*F*	*P*
Cell size	2, 27	1.035	0.371			
Substrate type	1, 28	17.194	**< 0.001**	1, 28	6.143	**0.019**
Cell size × Substrate type	2, 24	4.202	**0.027**			

Table 5 Effects of cell size, substrate type, soil layer and their interactions in GLMMs of root biomass measured in mesocosms with cell size 12, 24 and 48 cm. F-values, *P*-values and degrees of freedom ($df_{between\text{-}groups}$, $df_{within\text{-}groups}$) are given, with significant results ($P < 0.05$) in bold. Nonsignificant factors were removed stepwise from the final model

Source	Root biomass		
	df	*F*	*P*
Cell size	2, 117	2.278	0.107
Substrate type	1, 118	41.784	**< 0.001**
Layer	3, 116	113.123	**< 0.001**
Cell size × Substrate type	2, 114	12.309	**< 0.001**
Layer × Substrate type	3, 112	6.883	**< 0.001**

620 **Figure 1** (a) 3D view of the mesocosms with the two substrates, i.e. nutrient-rich (black) and nutrient-
621 poor (white), used in the experiment. Configurational heterogeneity decreases from left to right, from
622 fine (small cells) to coarse (large cells) distribution of resources. The cell size of the full mixture of the
623 two substrates on the left can be considered as approximately zero. (b) Predicted pattern of root/shoot
624 ratio (R/S) at mesocosm scale (top view, Hypothesis 1) and (c) predicted pattern for the difference
625 between root biomass of soil columns with nutrient-rich cells on top vs. nutrient-poor cells on top
626 (lateral view, Hypothesis 2), in different levels of soil heterogeneity



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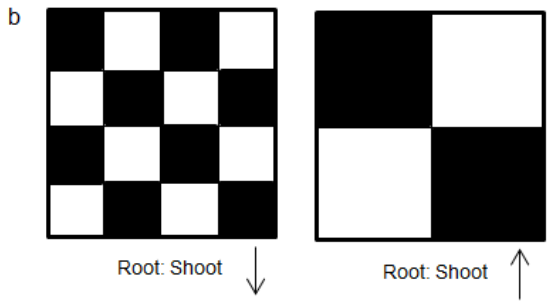

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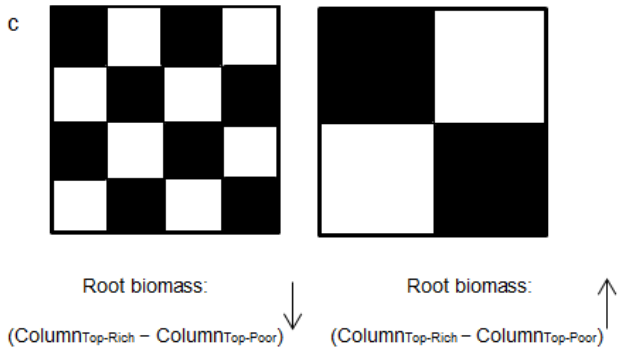
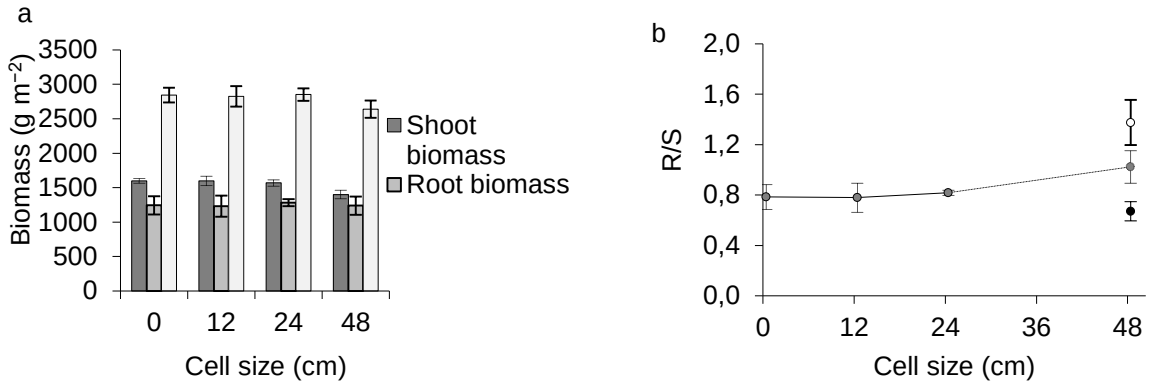


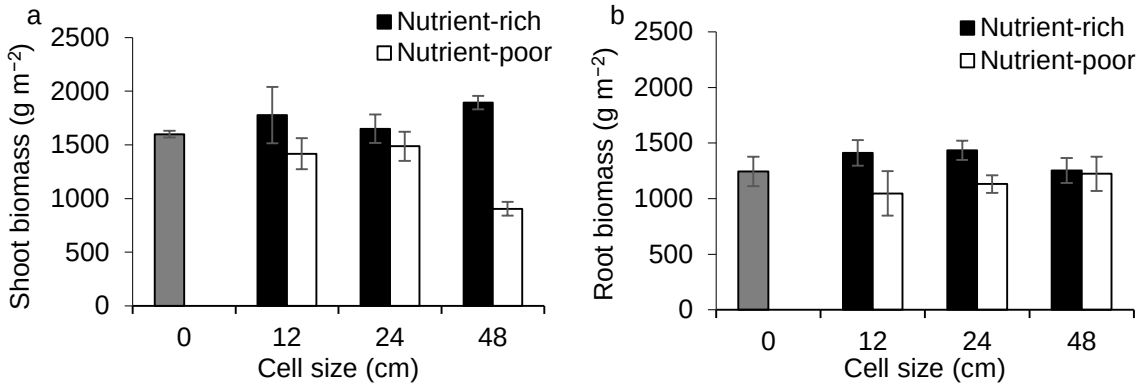
629

Figure 2 Mean ± SE of shoot, root and total biomass (a) and root/shoot ratio (R/S) (b) at mesocosm scale as a function of varying cell size. The gray R/S symbol at 48 cm represents the average of the measurements on nutrient-rich (black symbol) and nutrient-poor (white symbol) mesocosms



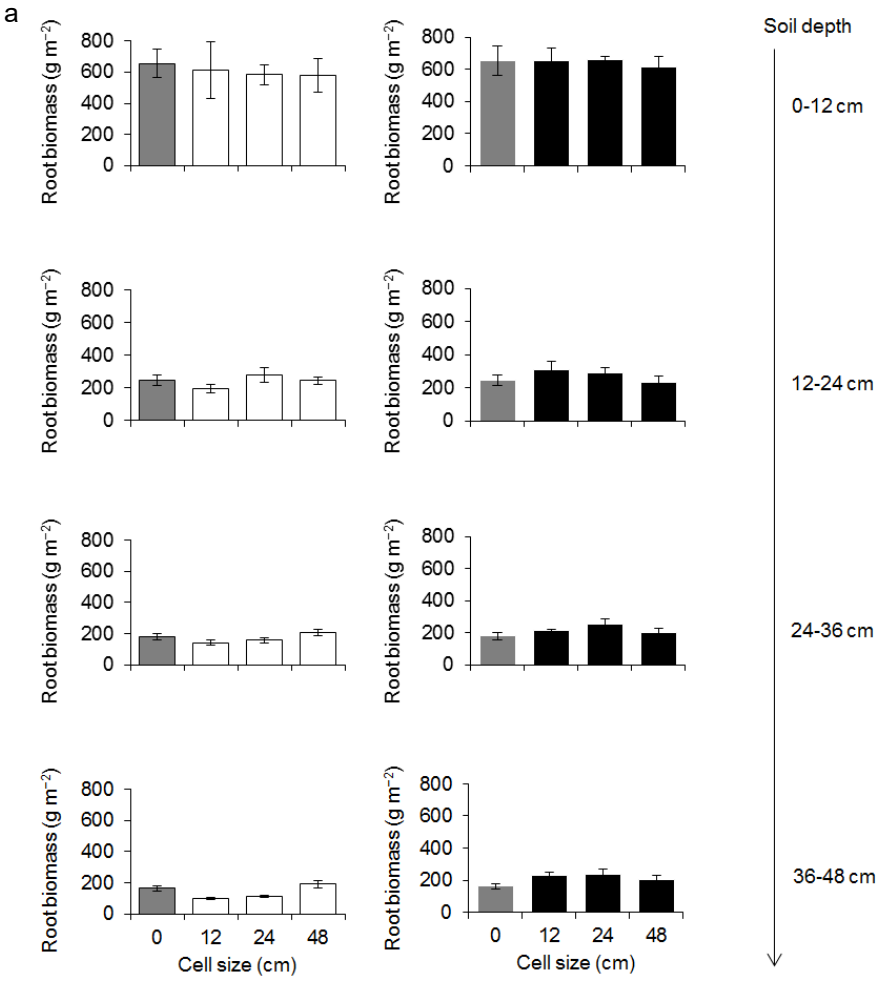
634 **Figure 3** Mean ± SE of shoot biomass (a) and root biomass (b) at substrate scale (nutrient-rich vs.

635 nutrient-poor) as a function of cell size. Mesocosms with cell size 0 are indicated in gray



636

637 **Figure 4** Mean ± SE of root biomass at layer scale, separated into nutrient-poor (white, left) and
638 nutrient-rich (black, right) substrate (a) and fraction ± SE of the total root biomass in each layer that
639 occurs in nutrient-rich cells (b), both as a function of cell size. In (a) the full mixture of nutrient-rich
640 and nutrient-poor substrate (cell size 0) is indicated in gray



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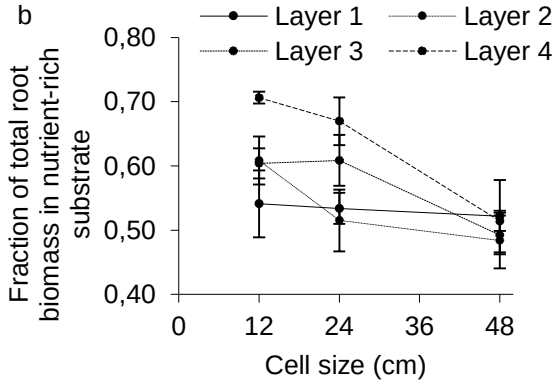
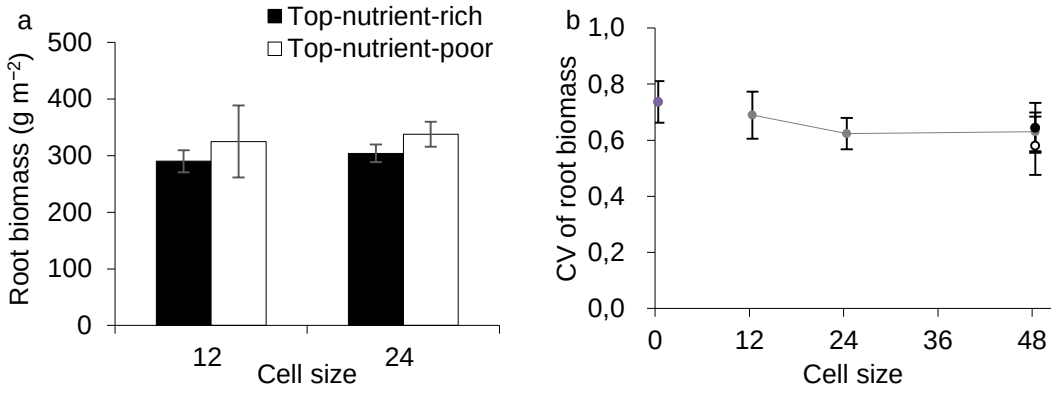


642

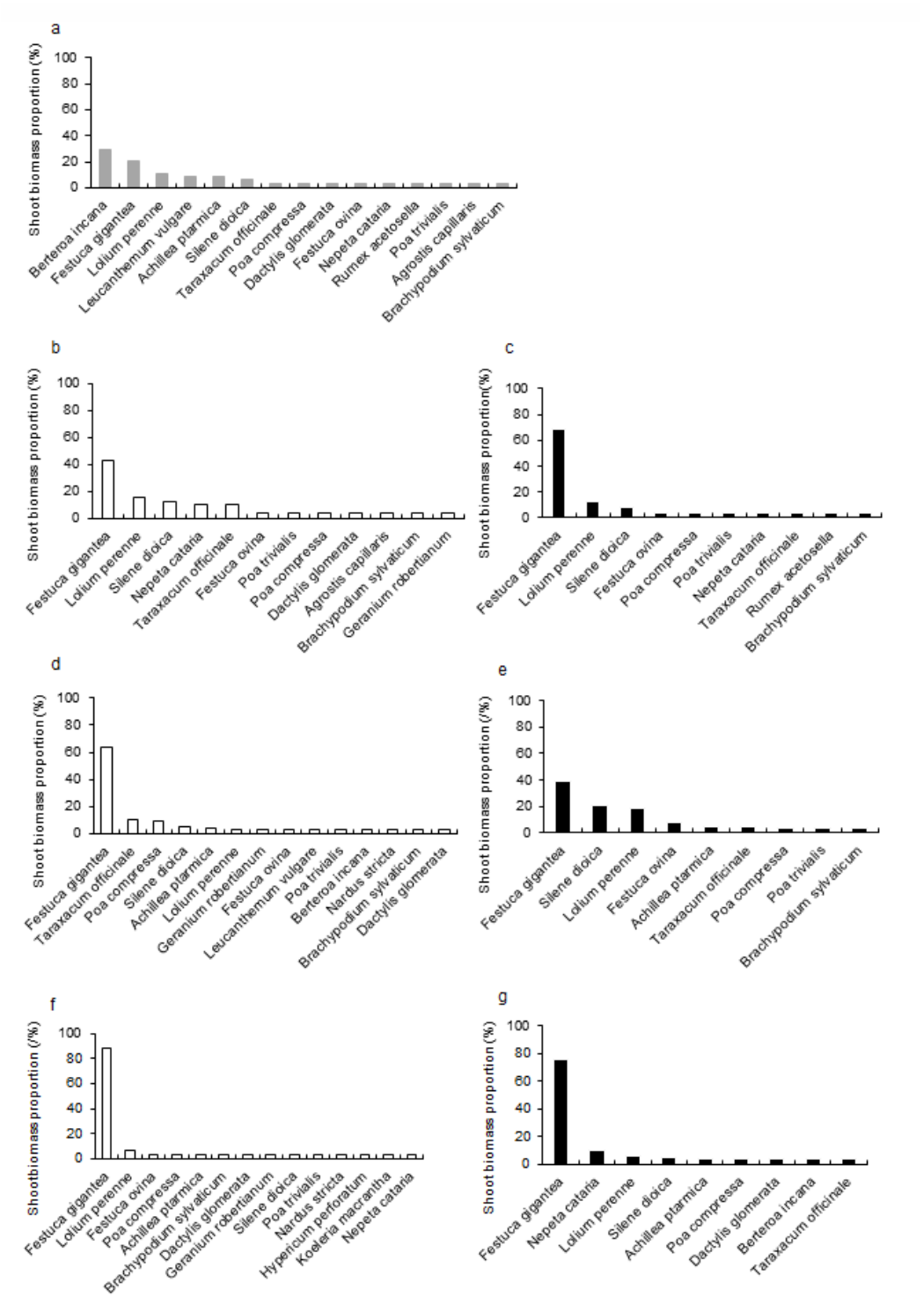
643 **Figure 5** Mean ± SE of root biomass in soil columns with nutrient-rich substrate (black) in the top
644 layer and nutrient-poor (white) substrate in the top layer, in mesocosms with cell size 12 and 24 cm
645 (a); and coefficient of variation (CV) of root biomass among substrates and layers as a function of
646 varying cell size (b). The gray CV symbol at 48 cm represents the average of the measurements on
647 nutrient-rich (black symbol) and nutrient-poor (white symbol) mesocosms



648

649 **Appendix**

650 **S1** Species composition (shoot biomass proportion) in mesocosms with cell size 0 (a), cell
651 size 12 cm (b-c), cell size 24 cm (d-e) and cell size 48 cm (f-g), separated into nutrient-poor
652 (white) and nutrient-rich (black) substrates. Mesocosms with cell size 0 in gray



653