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Plant perception of soil heterogeneity in the field

Vnímání heterogenity půd rostlinami v polopřirozených podmínkách

Diploma thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Nutrients are usually patchily distributed in natural soils. Plants are often able to respond to nutrient heterogeneity in artificial conditions by active plastic changes of root system morphology. The occurrence or magnitude of a foraging response can be altered by the presence of competition. However, it is unclear to what extent root foraging takes place in the field. I conducted a field experiment in order to determine the effect of an artificial nutrient patch on fine belowground biomass of (a) an established community and (b) model plants. The study array consisted of a grid of 30×30 cm plots with model plants located in the centre. Half of the plots contained the artificial patch located 5.5 cm from the model plant.

Fertilizer patch treatment did not increase mean plot fine underground biomass. Instead, fine underground biomass was higher in places of greater soil moisture estimated from mean plot EIVs. Neither total model plant root biomass nor proportion of roots in the enriched quarter increased in the fertilizer treatment. Competition was probably higher in fertilized than in control plots judging by a 2-fold increase in death rate of model plants. However, greater proportion of model plants flowered in the treatment plots. Possible causes include a plastic response to the patch as well as strengthened aboveground competition. The results suggest that for species with small root systems, it is the distance from the nutrient source rather than foraging abilities that affects their survival. On a community level, smaller peaks of nutrient concentration are unlikely to cause shifts in fine underground biomass allocation.

Keywords: soil heterogeneity, root foraging, morphological plasticity, roots, resource competition, field conditions, fertilizer, belowground biomass, plant community

Abstrakt

V přirozených ekosystémech jsou živiny v půdě rozmístěny nerovnoměrně, ve formě tzv. půdních kapes. Rostliny v umělých podmínkách často vykazují schopnost na takovou heterogenitu půdy reagovat pomocí morfologické plasticity kořenového systému. Ukazuje se, že přítomnost kompetice může mít vliv na to, zda a v jaké míře plastická odpověď proběhne. Míra uplatnění plastických úprav morfologie kořenů v přirozených podmínkách je však dosud neobjasněná. Ve světle toho jsem provedl terénní experiment, jehož cílem bylo posouzení vlivu uměle vytvořené půdní kapsy bohaté na živiny na podzemní biomasu (a) přítomného společenstva a (b) modelových rostlin. Pokusný prostor sestával z mřížky čtverců (30×30 cm) s modelovou rostlinou uprostřed. V polovině čtverců byla vytvořena umělá půdní kapsa bohatá na živiny, vzdálená 5,5 cm od modelové rostliny.

Přítomnost umělé půdní kapsy neměla za následek zvýšení jemné podzemní biomasy ve čtverci. Tato však přibývala se zvyšující se půdní vlhkostí, odhadnutou pomocí průměrných Ellenbergových indikačních hodnot ve čtvercích. Podíl kořenů modelových rostlin v obohacené čtvrtině ani celková biomasa kořenů modelových rostlin nevzrostly ve skupině ošetřených rostlin oproti kontrolní skupině. Kompetice ve čtvercích s umělou půdní kapsou byla pravděpodobně vyšší, o čemž svědčí dvojnásobná úmrtnost modelových rostlin oproti kontrolním čtvercům. I přesto v ošetřených čtvercích vykvetlo větší procento modelových rostlin než v kontrolních. To mohlo být způsobeno např. plastickou reakcí kořenového systému nebo zvýšenou nadzemní kompeticí. Výsledky experimentu naznačují, že pro druhy s malými kořenovými systémy je zásadnější vzdálenost od bohaté půdní kapsy než schopnost plasticky reagovat na podzemní heterogenitu. Zároveň se zdá, že na úrovni společenstev se na podzemní biomase výrazně neprojeví menší výkyvy v koncentracích živin v půdě.

Klíčová slova: půdní heterogenita, *root foraging*, morfologická plasticita, kořeny, kompetice o zdroje, polopřirozené podmínky, hnojivo, podzemní biomasa, rostlinné společenstvo

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1 Introduction

Mineral nutrients in natural soils are usually neither randomly nor homogeneously distributed, rather they are concentrated in *patches* (Ball & Williams, 1968; Jackson & Caldwell, 1993a). Nutrient concentrations in soil can differ greatly even at very small scales (centimetres) and this heterogeneity can change rapidly in time (Bell & Lechowicz, 1991; Březina et al., 2019; Farley & Fitter, 1999a; Jackson & Caldwell, 1993b). However, for the heterogeneity to be relevant to a plant individual, the size, quality and duration of a patch need to fall into a certain range (Hutchings et al., 2003). Only then can the plant perceive the contrast between *background* and *patch* and respond to this by morphological and/or physiological modifications of its root system. Such perceivable heterogeneity might be relatively rare in natural conditions (Březina et al., 2019; Herben et al., 2018). However, many plant species are able to respond to it under artificial conditions (Hodge, 2004; Hutchings & de Kroon, 1994; Kembel & Cahill Jr., 2005). This ability to locate nutrient-rich patches and inside them proliferate roots or enhance nutrient uptake (without the change of morphology) is called root foraging (Bray, 1954; Grime, 1979; Grime et al., 1986).

The distribution of roots in soil is influenced by several environmental factors, both biotic and abiotic, and by developmental instability. The stochastic process called developmental instability describes the potential of every two plants, even genetically identical, to grow roots in a different manner (Forde, 2009). Because of these intra-genotypic differences, developmental instability has been referred to as the third source of phenotypic variability, alongside genotype and environment (Lajus et al., 2003).

The abiotic factors stand primarily for mineral nutrients and water. Plants generally tend to respond to the pattern of water and nutrient distribution, favouring the patches with higher nutrient and water availability than background soil (for a review see e.g. Hodge, 2009, 2006, 2004; Hutchings & de Kroon, 1994; Kembel & Cahill Jr., 2005). This behaviour, called foraging precision, was reported to reflect phylogeny – on average, eudicots were found to be more precise in their morphologically plastic responses than monocots (Kembel & Cahill Jr., 2005). However, the monocot species used in their analysis were mostly clonal grasses, and so the observed signal might have in fact been a result of clonal status rather than phylogeny (Weiser et al., 2016). Furthermore, other studies show that substantial differences in foraging abilities can be found within a family (Keser et al., 2014) or even a genus (Keser et al., 2015). Depending on the pattern of heterogeneity, plants can use either

physiological (Cui & Caldwell, 1997) or both physiological and morphological plasticity (Wang et al., 2006). Foraging precision can also be achieved by changes in root demography – – decreasing mortality of existing roots in a patch (rather than increasing root growth) is another means of nutrient uptake optimization (Gross et al., 1993). The fact that many plants respond to nutrient patchiness under artificial conditions suggests that root foraging might have some adaptive value (Hodge et al., 1999; Keser et al., 2015; Robinson et al., 1999; but see e.g. Dong et al., 2015).

The biotic factors entail mainly interactions with other plants, mycorrhizal fungi, herbivores and soil microbial community (Cahill & McNickle, 2011). Plant-plant underground competition can – due to other intrinsic or environmental factors – lead to three different behavioural responses of roots: aggregation, segregation or no response (Litav & Harper, 1967). Aggregation describes a situation where a plant increases root growth upon detection of neighbour roots. This behaviour has been documented repeatedly (Gersani et al., 2001; Mommer et al., 2010; O'Brien et al., 2005), and although some of the studies were questioned later due to potential bias (Hess & de Kroon, 2007), it is probable that plants often do respond in such a manner (Frank et al., 2015; Herben et al., 2020). Another possible outcome of encountering neighbour roots is avoidance, which results in smaller overlap of root systems than found under random root distribution (Cahill & McNickle, 2011). This response is also likely to take place in the field (Brisson & Reynolds, 1994; Schenk et al., 1999; Semchenko et al., 2007), although its role in maintaining species coexistence may be limited (Frank et al., 2010) and dependent on overall availability of resources (Frank et al., 2015; Schenk et al., 1999). Absence of response was also reported several times (Frank et al., 2010; Litav & Harper, 1967; McNickle & Brown, 2014), meaning the only effect of neighbours was the limitation of nutrient availability.

These three behavioural types are not necessarily conserved – in fact, the same species may respond differently under varying circumstances (Litav & Harper, 1967). Furthermore, the actual responses may not be apparent at first sight. What may appear as avoidance, might in fact be a result of allelopathy, even though the resulting root distributions are similar (Mahall & Callaway, 1991). The scale of an observation can be decisive for the results. Herben et al. (2020) found both aggregation and segregation of roots in the field at a scale of centimetres. Also, the ratio of these responses changed with increasing soil depth. None

of this would be possible to detect without taking the fine structure of underground environment into account. The shape of a root system may also be affected by other interactions, such as with mycorrhizal fungi or soil microbiota: for example, in some cases, presence of mycorrhiza can alter the morphological response to nutrient heterogeneity (Hoepfner et al., 2015; Šmilauerová, 2001). Importantly, the effects of competition and nutrient heterogeneity may interact, resulting in various behavioural outcomes (Casper et al., 2000; Hodge et al., 1999; Mommer et al., 2012; Zhang et al., 2016; see Caldwell et al., 1996, for a field study).

Soil characteristics and other environmental parameters of a site can be either directly measured or estimated. One approach to the estimation of natural conditions are species indicator values, among which Ellenberg's indicator values (EIVs, Ellenberg, 1992) are the most used in Central Europe. These values represent ecological optima of species (Ellenberg, 1992) and therefore contain information integrated over time (Ellenberg, 1992; Schaffers & Sýkora, 2000). Various environmental conditions of different areas have been successfully predicted based on EIVs (Diekmann, 2003; Hill et al., 2000; Hill & Carey, 1997; Nieppola, 1993). Because of their integrative nature, EIVs are not suitable for estimating small-scale temporal soil heterogeneity; analyses of soil nutrient concentrations are the preferred tool in that case. With long term heterogeneity the situation might be different, provided the spatial scale of the patchiness is neither too small nor too large (Hutchings et al., 2003). Here, fine scale vegetation sampling could provide clues about differences in soil qualities. It is common for species coexistence data to be collected on large scales relative to the extent of the root system of the majority of species (Chytrý & Otýpková, 2003). Such data are unlikely to reveal the described heterogeneity, should there be any. Although they may be less common, places with long-term differences in soil properties over short distances do occur naturally. For example, in a sandstone region in Northern Bohemia, several small-sized areas where basiphytic flora prevails can be found in close proximity of species-poor, acidophytic flora (Sádlo et al., 2011). There, vegetation sampling on a small scale could be quite efficient in estimating the pattern of soil calcium carbonate accumulation. Another example of a vegetation response to a long-term mosaic determined by soil parameters can be seen in a study by Silvertown et al. (1999). They found that the community composition reflects fine-scale differences in soil hydrology even at a site without any marked topographic variation.

I chose a temperate grassland as a study system for its species diversity sufficient for the purpose of the study and non-extreme environmental conditions. Intermediate productivity of the system suggests that (a) the prevailing selection pressure should be competitive abilities, as opposed to stress and/or disturbance tolerance; and (b) both belowground and aboveground competition are likely to shape the plant community structure there (DeMalach et al., 2016; Lamb et al., 2009). Under field conditions, plant reactions to abiotic as well as biotic factors interplay (Cahill & McNickle, 2011; Hutchings et al., 2003). The natural presence of competition, together with common environmental conditions, could allow for a more realistic outcome of the study compared to greenhouse experiments.

The aim of this study is to examine spatial variation in plant underground biomass with respect to artificial nutrient-rich patches and inherent environmental variation of the site. Using data from the field experiment I intend to answer the following questions:

1) How did an artificial nutrient-rich patch affect fine underground biomass?

The nutrient-enriched patch is likely to be colonized by adjacent root systems. However, whether this will result in an increase in fine underground biomass is unsure. One possibility is that higher nutrient levels will support larger amounts of roots. Having said that, other abiotic conditions such as moisture or pH might alter the distribution of fine underground biomass by affecting nutrient availability. An estimation of these conditions (i.e., EIVs) can be obtained based on the data on species composition of the vegetation.

2) What was the impact of an artificial nutrient-rich patch on model plants?

The soil around model plant individuals might exhibit some degree of both spatial and temporal heterogeneity. Nevertheless, the duration and quality of naturally occurring patches are unlikely to exceed those of a patch created by the fertilizer. Thus, upon contact with this enriched patch, model plants may be able to assess its quality and respond by investing their root biomass in the corresponding direction. In addition, information on whether they were able to reach the patch and proliferate in it could be acquired if the method for soil removal without horizontal shift of model plant roots proves itself

applicable. The design of the experiment together with species indicator value data could allow us to separate the effect of the artificial patch from that of environmental gradients. Irrespective of the reaction to the patch by model plants, I aim to compare their survivorship in treated and control plots during the experiment. The data on the development of vitality might help assess the relevance of the effect of the treatment.

2 Materials and methods

2.1 Model species

Rumex acetosa L. is a clonal, perennial herb native to mostly temperate areas (Europe, continental Asia, northern Africa) (Blamey & Fitter, 2003; Kubát et al., 2002; Meusel et al., 1965). This species was chosen for several reasons, including: (a) lack of lateral spread, as the experimental design was unsuitable for clonal plants with notable vegetative spread; (b) perenniality, enabling the experiment to run longer than one season; (c) it rarely forms mycorrhizal symbiosis (therefore any observed response can be attributed to the plant itself); (d) it is a species native to Bohemia and can be found at the study site, eliminating the risk of observing the effects of non-natural coexistence; (e) it has been previously shown to be quite effective at root foraging (Keser 2015); (f) it does not form a tap root and (g) the colour of its roots ranges from bright yellow (middle and distal root parts) to orange or red (typically at the root base), enabling easy distinction from other species' roots. There were two other species, *Senecio aquaticus* Hill and *Holcus lanatus* L., that met the criteria to an extent and were also available at a local seed company. However, in the end the seeds of *Rumex acetosa* exhibited the best germination performance and this species was used for the experiment.

2.2 Model plants' preparation

The pre-cultivation of model plants was conducted in spring 2019 on the premises of the Botanical Garden of the Faculty of Science, Charles University in Prague (GPS: 50°4'16.612"N, 14°25'13.675"E), and consisted of pre-cultivating 160 seedlings of *Rumex acetosa*. The seeds were obtained from Planta Naturalis company. The quantity of the seedlings was twice the needed amount – this was to account for seedling mortality before planting and/or need for re-establishment of the experiment in case of large seedling mortality in the field. The seeds were placed on a wet sand on March 26 and watered every day, the germination became apparent 6 days later (April 1). Three weeks later (April 24), each of the seedlings were put in the centre of a rectangular pot (7×7 cm at the top, 3.5×3.5 cm at the bottom, 12 cm deep) filled with a mixture of peat and soil (1:1) and buried approximately 1 cm under the surface. At first, the pots were put into a greenhouse with a temperature range of 18°C (night) to 24°C (day) and were supplied with tap water

every day. Two weeks later the pots were transferred outside [temperature range of approx. 16°C (night) to 22°C (day)] and watered every day for another 7 weeks. The cultivation lasted 9 weeks in total.

2.3 Study site

The second part of the experiment was conducted on a temperate grassland located in central Bohemia, district Příbram, near the settlement Placy, about 7 km ESE from the town Příbram (GPS: 49°40'23.956"N, 14°6'16.246"E). Despite its small area, the grassland is quite heterogeneous both in environmental conditions and vegetation composition. The mean annual precipitation at the site is 550 mm (average of total annual precipitation in years 2005–2019; ČHMÚ, 2021), vegetational season is from March to October (personal observation). From the phytosociological perspective, the site falls mostly into the *Mollinion caeruleae* alliance in the *Mollinio-Arrhenatheretea* class. The area belongs to a nearby shooting range and is used occasionally for training purposes. The management consists of mowing once or twice a year. The meadow is surrounded by a semi-natural mixed forest and the closest road is about 300 metres away.

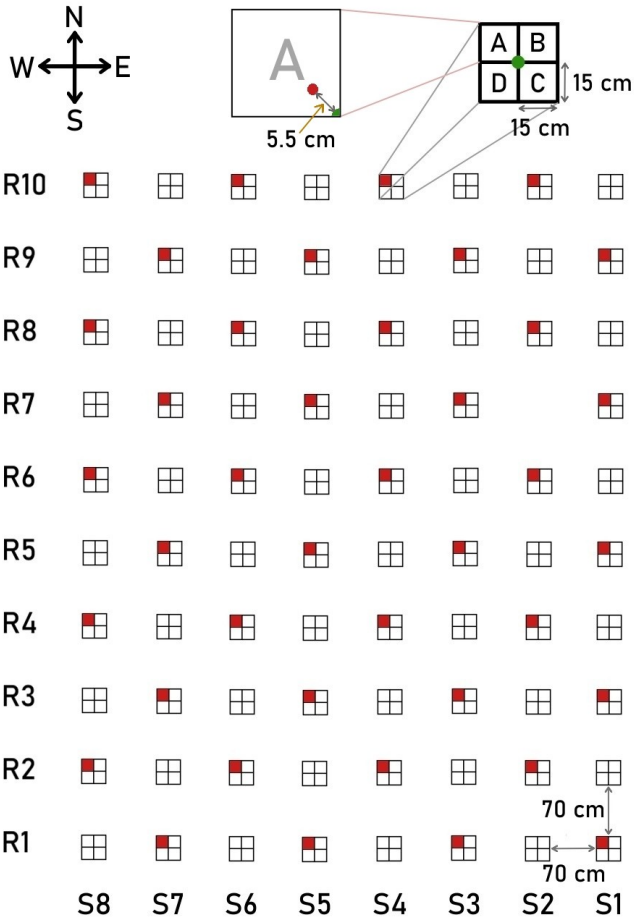


Figure 1: Study array. The plots (rectangles) were arranged in a grid with each two nearest neighbours' centres spaced 1 metre apart. Every plot was divided into 4 quarters (A, B, C and D clockwise from top left). The green point represents the position of a model plant. The red quarters represent the fertilized quarters. The red dot in the A quarter zoom-in represents the position of fertilizer application. Note the empty position [column S2, row R7] – no plot was established there because of a local terrain disturbance.

2.4 Experimental setup

At the site, a grid of 79 rectangular plots 30×30 cm (8 columns and 10 rows; see Figure 1) was established between May 30 and June 2, 2019. The centres of neighbouring plots were spaced 100 cm apart, and consequently their edges were spaced 70 cm apart. Each plot was divided into four quarters (15×15 cm; named A, B, C and D clockwise from top left – see Figure 1) and data on vascular plant species presence were collected in all the quarters. After the sampling, most of the aboveground biomass inside the quarters was removed using scissors and stored in paper bags, leaving approx. 1-1.5 cm high plants. Then, a 4.5 cm diameter hole was dug in the middle of each plot using a custom made corer and a seedling of *Rumex acetosa* was transferred into the hole with tips of the roots torn off for accelerated rooting. Only healthy looking plants of approximately the same size were used in the field experiment.

2.5 Fertilizer application

On June 2, 2019, in half of the plots, 1 g of N-P-K slow release fertilizer granules was inserted into the A quarter of the plot, 5.5 cm from the model plant shoot base and approx. 6 cm deep (see Figure 1 or Figure 5). The fertilizer treatment and control plots were designed to form a checkerboard pattern (see Figure 1) in order to avoid any spatially autocorrelated phenomenon confounding the results. On November 12, 2019, the same plots (treatment) were fertilized once again, this time using 3 g of N-P-K slow release fertilizer granules. The contents of individual elements of the fertilizer are listed in Table 1.

Table 1: Mixed fertilizer N-P-K (17-11-11 + 2 MgO). Combined percentage of all forms of a given element is shown in bold.

name	*formula*	*content*
Total Nitrogen	**N**	**17%**
Nitrate Nitrogen	NO_3	5.4%
Ammonium Nitrogen	NH_4	7.4%
Urea Nitrogen	NH_2	4.4%
Phosphorus pentoxide (soluble in ammonium citrate / water)	**P_2O_5**	**11%**
Phosphorus pentoxide (water soluble)	P_2O_5	9.9%
Potassium oxide (water soluble)	**K_2O**	**11%**
Manganese oxide (water soluble)	MgO	2,0%
trace elements	B, Cu, Fe, Mn, Mo, Zn	<1 %

2.6 Survivorship evaluation

After the planting of seedlings in the field the site was repeatedly visited in order to (a) water the plants to prevent their mortality and support growth and (b) collect data on plants' growth performance. The plants were watered immediately after their planting on July 2, 2019, then again 5 days later and after that every time the site was visited in the rest of 2019 (August 1, October 10, October 17, November 12). The performance of the plants was assessed by visual inspection during three visits in 2019 (August 1, October 10, November 12) and one in 2020 (June 17). Every plant was assigned a rank on a relative scale from 0 to 6:

0 – dead plant/not found

1 – plant with one leaf, often very small

2 through 4 – plant with more leaves, larger

5 – healthy looking plant with a rather large leaf rosette

6 – flowering plant

After the last check in June 2020 the data were adjusted to account for possible mistakes in either of the first three observations: all zero values that were followed by a non-zero value were changed to 1, as these zero values meant “not found” rather than “dead”. Such corrections were only applied in 3 cases, while 7 other plants of rank 0 were confirmed dead in the last observation.

2.7 Obtaining the roots

Prior to the excavation of soil monoliths, two different methods were tested out – digging the monolith out with no special equipment, and digging it out using a custom made tool (later on referred to as “the nailboard”).

In the first method, a slightly larger monolith than needed (approximately 50×50×15 cm) was dug out with a spade. After that, the marginal (redundant) parts were cut off with a knife and the remaining block (30×30×10 cm) was cut into four pieces (A, B, C and D), each sized 15×15×10 cm. The depth of 10 cm was chosen based on the fact that most plant roots in grasslands tend to be concentrated in the uppermost 10 cm of soil (Hejduk & Hrabě, 2003; Herben et al., 2018; Jackson et al., 1996; Mamolos et al., 1995), as confirmed by personal observation in the field. The pieces were wrapped in non-woven fabric and transported into a basin filled with water. After at least a day of soaking, the pieces were taken out and the roots and rhizomes were washed out using a bucket and several sieves of various aperture size ranging from 0.1 mm to 2 mm.

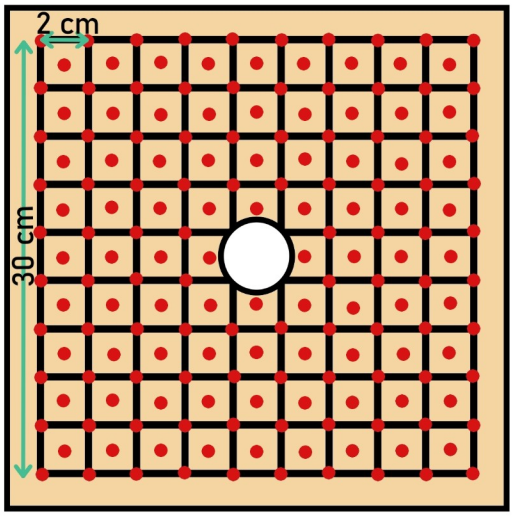


Figure 2: Scheme of the nailboard. The red dots represent nails, the black grid is only for illustration. In the middle, a circle-shaped hole was left for the above-ground part of the model plant.

The second method used a nailboard consisting of a 35×35 cm wooden board and approximately 400–450 nails (15 cm long, 5.4 mm in diameter) nailed into the board in a specific grid pattern (see Figure 2). The middle part of the board was cut out creating a circle-shaped hole (5 cm in diameter) allowing for precise orientation of the board with respect to the focal plant. In the field, a nailboard was hammered into the ground as deep as possible, so that only the board remained visible. Following this, a monolith slightly larger than the board (approximately 55×55×20 cm) was dug out using a spade, turned upside down and the soil outside the nail grid was discarded by hand or knife. The monolith was then transported to a water source and a strong water stream was used to wash out the soil. The nails were supposed to prevent the roots from changing their horizontal position, which they did reasonably well. The vertical shift of roots was an expected drawback of the method, but such loss of information was considered an acceptable cost. Although a significant amount of soil was successfully washed out, the rest could not be removed due to the high density of fine roots in the topsoil layer. Therefore, the nailboard method turned out to be inefficient.

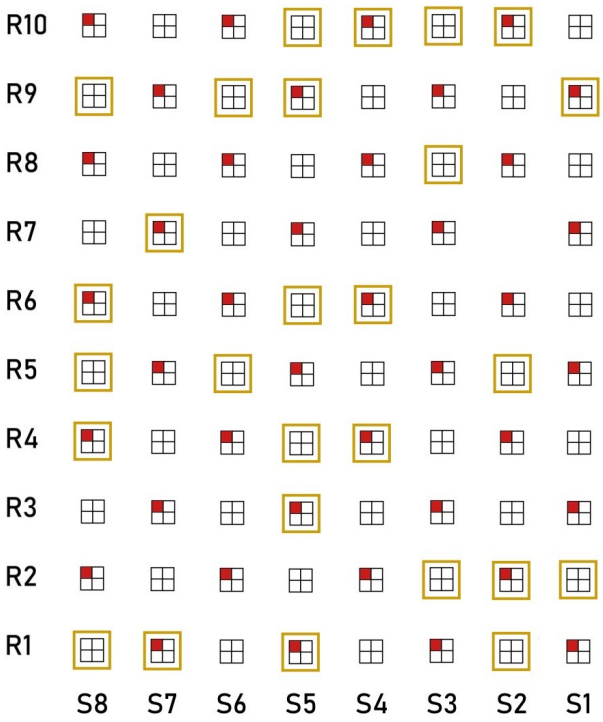


Figure 3: Position of excavated soil monoliths. The monoliths were taken from the highlighted plots. See text for criteria for choosing the plots.

Eventually, only the first method was used for obtaining the roots and rhizomes. The total amount of monoliths was 26 (14 control and 12 treatment; see Figure 3) and their position was chosen according to these criteria: (a) every row and every column was to be represented at least once;

(b) the model plant needed to be alive with a minimum rank of 3 (see chapter 2.6 Survivorship evaluation for details) and (c) treatment and control plots were to be represented similarly. In four cases, plots with plants of rank 2 were chosen in order to achieve a more even cover of the array (see Figure 4 for rank distribution). Aboveground biomass was unfortunately not collected before the digging because the study site was mown a few weeks earlier.

The monoliths were dug out, cut into quarters and transported on June 22 and July 1, 2020. Before further handling, the quarters were stored in an outdoor tank of cold water for 1–4 days. The soil removal consisted of two phases. In the first one, most of the soil was washed out and the roots/rhizomes with remaining soil and parts of shoots were put in a ziplock bag and stored in a refrigerator at 4–6°C. In the second one, finer soil particles were washed away, and shoots (if present) were cut off, leaving soil-free underground plant parts only. After that, the roots and rhizomes were divided into three categories: FR (fine, less than 4 mm in diameter), CR (coarse, 4 mm and more in diameter) and MR (roots of the model plant – *Rumex acetosa*). Fine roots are usually defined as those smaller than 2 mm in diameter (Keser et al., 2015; Wang et al., 2009). In the case of this experiment, the threshold between FR and CR was set to 4 mm in diameter. This was due to the large amount of roots/rhizomes with diameters between ca 2 mm and ca 3.5 mm in the test blocks, implying it would be very complicated and labour-consuming to separate the roots/rhizomes with a diameter of 2–4 mm. Roots and rhizomes with a diameter very close to 4 mm were divided into FR and CR categories based also on their lignification, as the more lignified parts (CR) play a minor role in water and nutrient uptake. The roots of *Rumex acetosa* were separable thanks to their bright yellow to orange colour, which was not encountered in any other roots handled

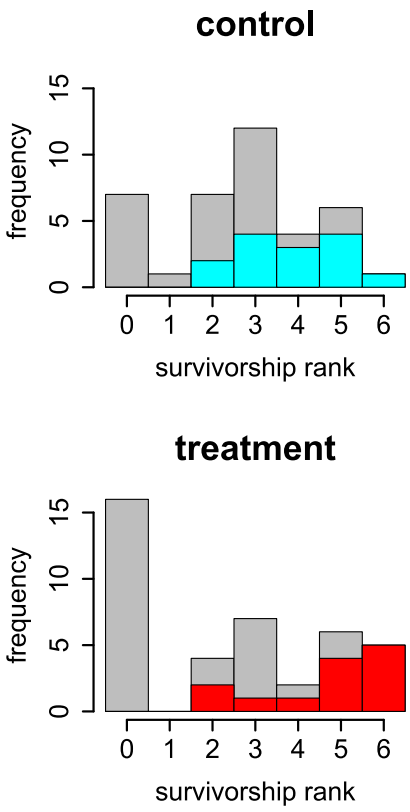


Figure 4: Observed vitality ranks of model plants in excavated plots. The grey bars show the number of all plants of a given rank at the end of the experiment. The blue and red bars show the number of plants of a given rank in plots chosen for excavation.

within the experiment. The only exception to this were dark yellow to brownish or dark red rhizomes which were encountered with low to intermediate frequency. The distinction between these rhizomes and *Rumex acetosa* roots was clear on closer examination. In the MR category, the roots were not divided into subcategories by diameter. Instead, they were only separated from the rhizome and shoot part. The rhizomes were discarded, as they function as storage organs and do not contribute to nutrient uptake. Following the separation of the root/rhizome material into FR, CR and MR categories, the dry weights were determined after drying at 60°C for a minimum of 48 hours.

2.8 Phosphorus content determination

On October 17, 2019, 26 plots (13 control / 13 fertilizer treatment) from different parts of the grid were chosen and cylinders (6 cm high, 4.5 cm in diameter) of the upper layer of soil were removed in the A and C quarters of the plots (see Figure 5). The positions where soil cores were removed from the model plant were equidistant from the model plant. The soil was then dried at room temperature (20°C) for at least 48 hours, sieved (2 mm aperture mesh) and homogenized. From each soil sample, a minimum of 1 g of very fine soil particles (<0.1 mm) was taken and stored in a plastic container. The rest of the analysis was carried out in the Laboratory of environmental chemistry and soil analyses of the Department of Environmental studies at the Faculty of Science, Charles University in Prague. First, water extracts from the soil samples were prepared using Mehlich-3 solution as the extractant. Available phosphorus content was then determined by optical emission spectroscopy (OES). OES is an extensively used technique based on vaporizing the sample material and exciting it into emission of radiation, which is then dispersed into its spectral components. Radiation intensity is measured and used to calculate the concentration of an element in the sample.

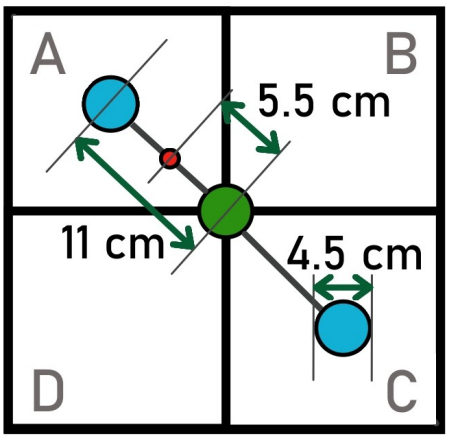


Figure 5: Soil sample removal for phosphorus content analysis. The green circle shows the position of the model plant. The red circle represents the position of fertilizer application. The blue circles show the positions where soil samples were taken for analysis.

Phosphorus was chosen for the analysis in order to determine the distribution of less mobile ions. The analysis of available phosphorus content revealed no effect of treatment nor any spatial pattern inside the study array. Most of the values ranged between 2 and 15 mg/kg

of soil, with a few exceptions reaching as high as 25 mg/kg. These peaks in phosphorus concentration (recorded at the end of season 2019) were correlated with neither total FR biomass nor MR biomass (data obtained in early summer 2020). There was no sign of relationship between FR biomass or any of the EIVs and available phosphorus content. After the evaluation of the analysis, second batch of fertilizer was applied in the treatment plots. After the second application of fertilizer, no more element content analyses were carried out due to government restrictions concerning the spread of COVID-19, which took place at a time of previously planned sample collection and analysis.

2.9 Data analysis

All of the statistical analyses were computed using R statistical software (version 3.6.1; R Core Team, 2019). The fine (FR) and coarse (CR) underground biomass data were ln or square root transformed if necessary to meet the assumption of normality. Whole-plot averages were used in most cases, except for *FR:CR biomass* ratio. This was calculated on the individual quarters' level as the majority of variance in CR biomass was within plots. The Ellenberg indicator values (EIVs) for moisture (M), nutrients (N) and soil reaction (R) were obtained from the Pladias database (Chytrý et al., 2021; Chytry et al., 2018). R and N values had a positively skewed distribution, M values had a distribution closer to normal. All of the EIVs were square root transformed prior to analyses. The EIV data turned out to be quite limited: even though the number of species with assigned EIVs was intermediate (23 for M, 15 for R and 20 for N), the quarter means were usually calculated from very few values, partly due to low small-scale species diversity. Therefore, only plot means of EIVs were used in further analyses, providing greater explanatory power.

By common convention (Jackson & Caldwell, 1993b, 1993a), only distances up to half of the maximum distance within study array are generally used for calculating spatial autocorrelation. The threshold would be 5.7 metres in this experiment. Such restriction caused substantial loss of information during preliminary analyses, and so, after consideration, I decided to move this threshold to 8 metres (out of the maximum distance of 11.4 metres). The number of observations was for most distances (with only one exception) higher than 40 and therefore these data still carried enough information.

The effect of treatment on aboveground, FR, CR and MR biomass patterns was tested using analysis of variance (function *aov*). Building on exploratory analyses' outcomes, either linear or non-linear regression models were fitted using functions *lm* or *gam* (package *mgcv* version 1.8-33; Wood, 2011) when at least one predictor was a continuous variable. In the event of two or more similar models (e.g., with an either transformed or untransformed variable), function *AICc* (penalized Akaike's Information Criterion) from the package *MuMIn* (version 1.43.17; Barton, 2020) was used for comparison and the model with the lowest value was used. Within-plot variation of FR biomass was assessed using the coefficient of variation (CV; calculated as CV = standard deviation / mean). When testing the spatial autocorrelation of EIVs, both linear and quadratic models were fitted using *lm*. After that, the models were compared and the one with the lowest *p*-value (or *AICc* value) was chosen. EIV distribution inside the study array was visualised using packages *ContourFunctions* (version 0.1.1; Erickson, 2019) and *mlegp* (version 3.1.8; Dancik & Dorman, 2008). Differences in survivorship of model plants in control and treatment groups were compared using a binomial test designed to compare two proportions (function *prop.test*). For the assessment of relationships between individual EIVs and aboveground biomass, principal component analysis (PCA; a method of indirect ordination) was carried out using function *rda* from the package *vegan* (version 2.5-6; Oksanen et al., 2019). The data were standardized prior to the ordination using *decostand* function (method "standardize" – every variable was scaled to zero mean and unit variance).

In the case of a negative adjusted coefficient of determination (*Adj.* R^2) of a model, only the *p*-value and number of observations (*n*) are shown. When describing a regression slope or differences between categories of a variable, standard errors (*s.e.*) are given; correlation coefficients that are presented result from Pearson's correlation test. Some models used residuals of another model as a response variable to account for initial differences caused by other variables. There, *F* statistics and *p* values given in the text are related to the net effect of the explanatory variable on the (initial) response variable – i.e., without the effects of other variables. In the caption of a graph, the statistics of the final model (i.e., with another model's residuals as a response variable) are shown.

In some comparisons, one treatment plot was omitted [column S7, row R1] due to the extreme value of FR biomass in the A quarter. No similarly high values were recorded elsewhere in the array and therefore the extreme value is probably a result of either very specific conditions or an error during data collection. At the end of the first season, one plot [column S7, row R2] was used for excavation trial and it was not used in any further analyses.

All of the underground biomass data were collected at two points in time. Therefore, the effect of the time of excavation on FR, CR and MR biomass was tested using *aov*. Also, the effect of the removal of small soil samples (for the phosphorus analysis) on FR, CR and MR biomass was tested using *aov*. The analyses revealed no effect of the time of excavation nor phosphorus analysis sample collection. Similarly, including these factors as a covariate did not significantly increase variance explained by a model, and therefore all models use combined datasets.

3 Results

3.1 Aboveground biomass

Total plot aboveground biomass was on average 2.25 g higher (*s.e.* = 0.84) in control plots ($F_{1,71} = 7.202$, $p < 0{,}01$; Figure 6) compared to treatment plots. After accounting for this difference, the outcome of further analyses (concerning other than aboveground biomass data) did not change.

There was a positive relationship between aboveground (collected in 2019) and fine underground (collected in 2020) biomass (correlation coefficient 0.385, p=0.057; Figure 7). The aboveground biomass increased by 0.9 g (*s.e.* = 0.046) with a 10 g elevation of FR biomass (model statistics: *Adj.* $R^2 = 0.2018$, $F_{2,22} = 4.034$, $p = 0.0322$;). The slopes were not significantly different between control and treatment groups.

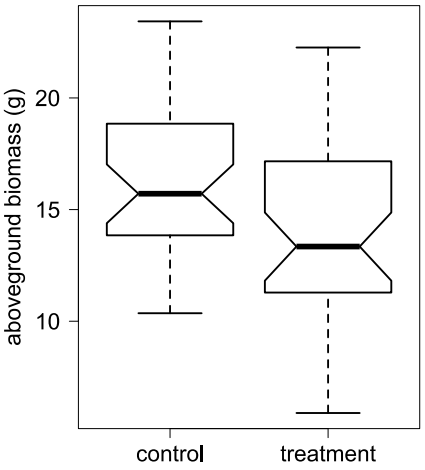


Figure 6: Total aboveground biomass in control vs. treatment plots. The graph shows conditions prior to the beginning of the experiment. Model statistics: *Adj.* $R^2 = 0.0793$, $F_{1,71} = 7.202$, $p = 0.0091$, $n = 73$

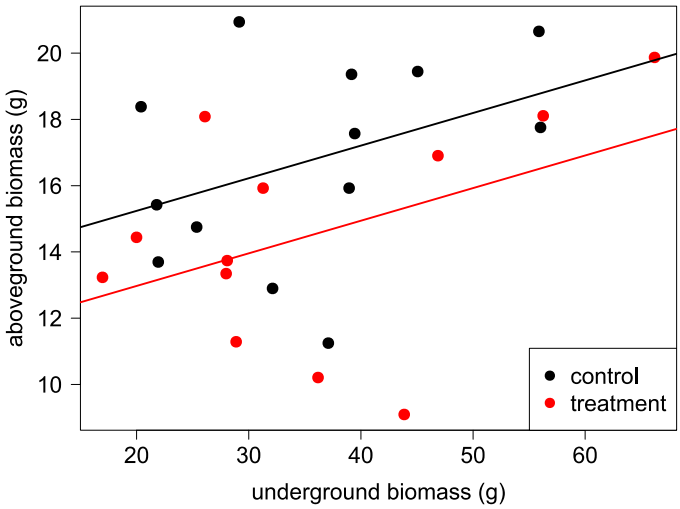


Figure 7: Total aboveground and FR biomass relationship. Black colour represents control plots, red colour represents treatment plots. The effects of FR biomass and treatment were marginally significant ($p = 0.0452$ and $p = 0.0708$, respectively). Model statistics: *Adj.* $R^2 = 0.2018$, *slope* = 0.0985, $F_{2,22} = 4.034$, $p = 0.0322$, $n = 25$

3.2 Model plants' survivorship

At the beginning of summer 2019, most plants from both treatment and control group were either in very good condition (rank 5) or small with no signs of damage (rank 2 and 1). In the late summer 2019, the number of plants in good or very good condition (rank 3 to 5) increased in both groups. In autumn 2019, the state of most plants worsened irrespective of group, although the smaller plants were in better shape in the treatment group (*rank 1:rank 2* ratio was 0.92 and 2.34 in treatment and control group, respectively). In early summer 2020, some differences occurred between groups: only one plant (2.6 %) in control group flowered [compared to 5 plants (12.5 %) in treatment group; *prop.test* p-value < 0.001], on the other hand, only 7 plants (18.4 %) were dead [as opposed to 16 plants (40 %) in treatment group; *prop.test* p-value < 0.001; see Figure 4]. During the first season of the experiment, several cases of foliar damage by high solar radiation were observed in the model plants. However, model plants were often able to recover from such condition. Notable shifts in the observed vitality were recorded repeatedly (see Figure 8), with several plants being able to regrow from poor condition in autumn 2019 to their previous (higher) rank in early summer 2020. The overall development showed no distinct spatial pattern, although the plants in the rows R1 and R2 seemed to exhibit lower overall changes than the rest of the array (Figure 8; Figure S1 in Appendix 1).

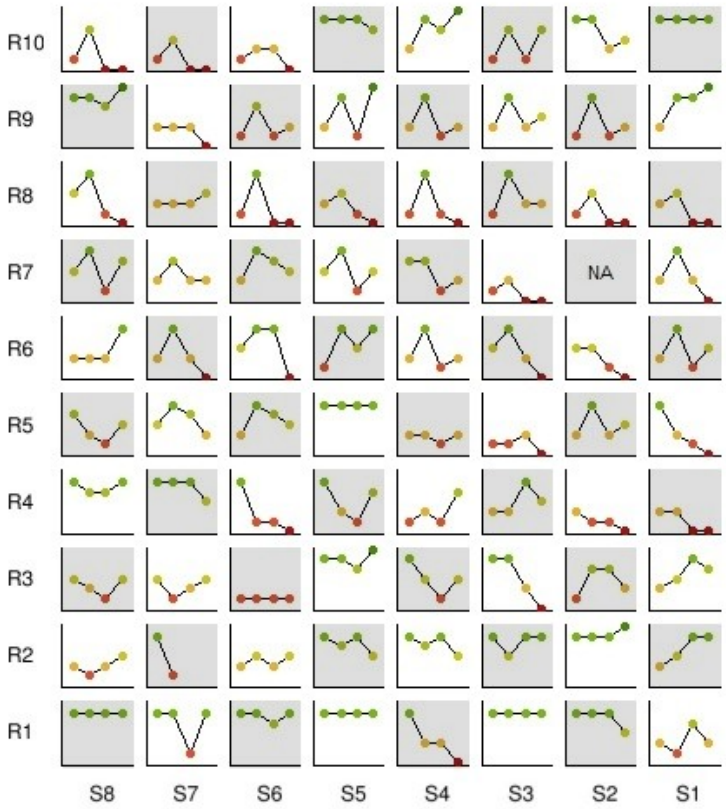


Figure 8: Survivorship changes of model plants in time – – individual plants' development. Each small graph shows the development in one plot. White background represents treatment plots, grey background represents control plots. In each small graph, the horizontal axis shows time of survivorship evaluation and the vertical axis shows observed vitality on a relative scale from 0 to 6. Also, each dot is colour coded by observed vitality: rank **0** – dead plant (●), rank **1** (●), rank **2** (●), rank **3** (●), rank **4** (●), rank **5** (●), rank **6** – flowering plant (●).

3.3 Underground biomass patterns

Mean plot FR biomass did not differ significantly across treatments [after accounting for aboveground and M EIV differences, the FR biomass in treatment plots was on average 0.016 g higher (*s.e.* = 0.146) than in control plots; $F_{1,21}$=0.1143, p = 0.7386; Figure 10]. Within plot variation of FR biomass was similar in both control and treatment plots [treatment group values were on average 0.033 lower (*s.e.* = 0.044) than control group values; $F_{1,22}$ = 0.548, p = 0.467; Figure 11]. When only the difference between the A quarter (fertilized in treatment plots) and the C quarter (far from fertilizer in treatment plots) was taken into consideration, no significant distinction was found between treatments [difference in treatment group on average 6.416 g higher (*s.e.* = 4.897) than control group; $F_{1,23}$ = 1.716, p = 0.2031; Figure 9].

Mean plot FR biomass was positively correlated with average plot EIV for moisture, increasing at a rate of approx. 14.24 g (*s.e.* = 1.41) with a unit increase of M ($F_{1,22}$ = 10.11, p = 0.004; Figure 12). There was no significant relationship between fine underground biomass and N EIV. The data on CR biomass were more scarce and the values were not affected by treatment or correlated with FR biomass. Also, the ratio of FR and CR biomass did not change with treatment [the value of mean quarter FR:CR ratio was on average 1.31 lower (*s.e.* = 0.425) in treatment plots than in control plots; $F_{1,52}$ = 0.403, p = 0.528; Figure 13].

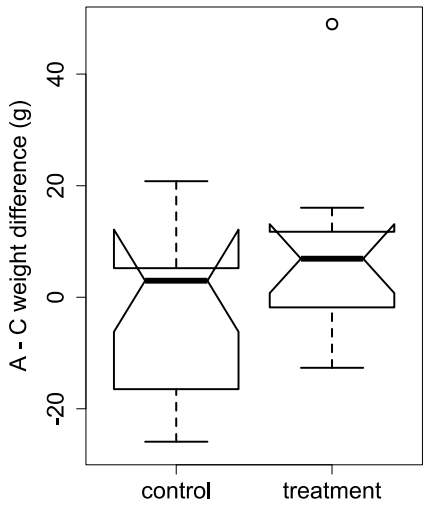


Figure 9: A – C FR biomass difference across treatments. The difference was calculated as:

y = FR(A quarter) – FR(C quarter)

Two models were fitted: one accounting for the initial difference in aboveground biomass between treatments and another ignoring the effect of aboveground biomass. Both models produced similar results, and only the second one is shown. The outlier point in the treatment group was excluded from the models. Model statistics: *Adj.* R^2 = 0.029, $F_{1,23}$ = 1.716, p = 0.2031, n = 25

Figure 10: Mean plot FR biomass across treatments. The vertical axis shows model residuals after removing of aboveground biomass and M EIV effects. Model statistics: $p = 0.535$, $n = 25$

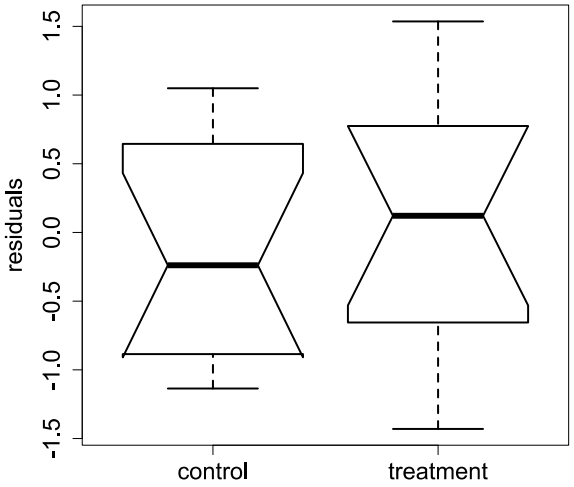
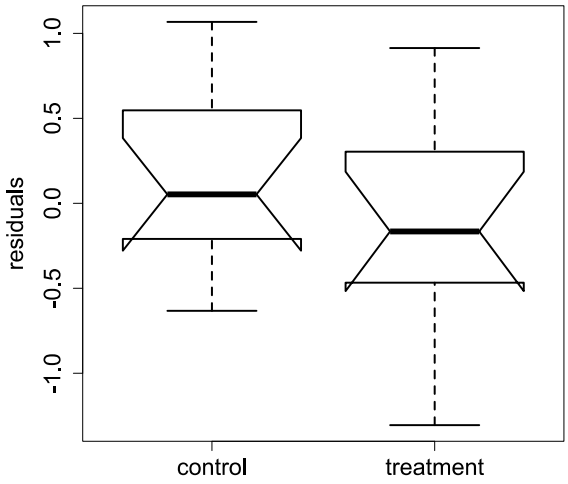


Figure 11: Within-plot FR variation in control and treatment groups. The vertical axis shows model residuals after accounting for initial difference in aboveground biomass. Coefficient of variation data were ln-transformed prior to the analysis. Model statistics: $p = 0.391$, $n = 25$



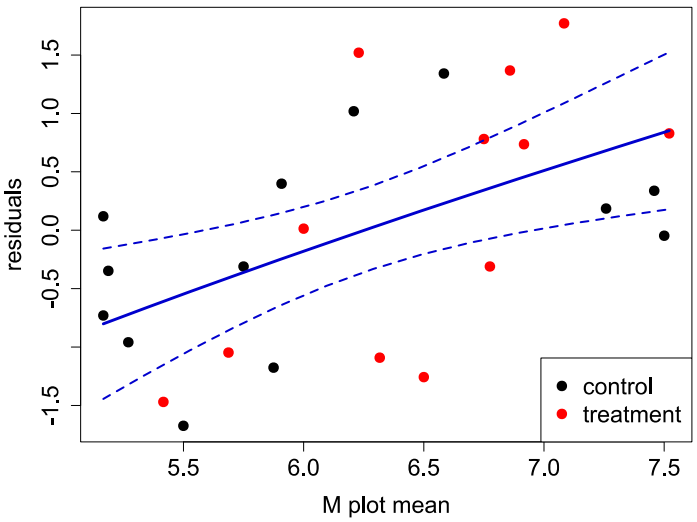


Figure 12: Mean plot FR biomass and M EIV relationship. The vertical axis shows model residuals after accounting for initial difference in aboveground biomass between treatments. The M values were square root transformed for the purpose of the model. The graph shows the relationship with back-transformed M values. Dashed lines represent 95% confidence interval of the regression. Model statistics: *Adj.* $R^2 = 0.263$, $F_{1,23} = 9.568$, $p = 0.0051$, $n = 25$

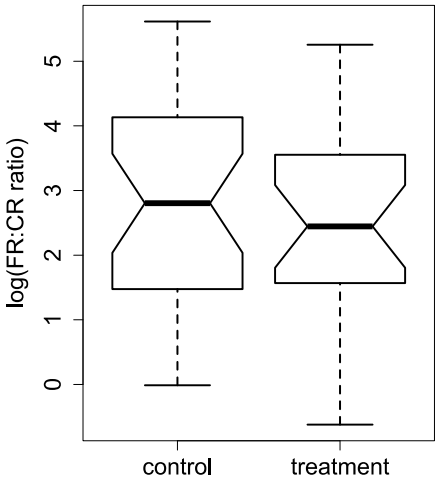


Figure 13: Mean quarter fine and coarse underground biomass ratio between treatments. The vertical axis shows logarithm of *mean quarter FR:mean quarter CR biomass* ratio. Model statistics: $p = 0.528$, $n = 54$

3.4 Model plant root distribution patterns

The technique used for obtaining the roots of model plants turned out to be ineffective in distinguishing between the quarters and therefore all the results are based on sums of root biomass from all four quarters of a plot. The whole-plot *Rumex acetosa* root biomass did not differ significantly based on treatment [values in treatment group were on average 0.157 g higher (*s.e.* = 0.134) than in control group; $F_{1,24} = 1.369$, $p = 0.253$; Figure 14]. Total plot MR biomass increased on average by 0.111 g (*s.e.* = 0.05) with a one-rank increase of the observed vitality at the end of the experiment ($F_{1,23}$=4.779, $p = 0.039$; see Figure S2 in Appendix 1). Total plot MR biomass was not found to be connected to mean plot FR biomass (correlation coefficient 0.023, p = 0.91). Furthermore, it did not seem to be affected by mean plot EIVs (correlation coefficients for M, R and N values: 0.124, -0.116 and 0.114, respectively; *p* values 0.55, 0.58 and 0.57, respectively).

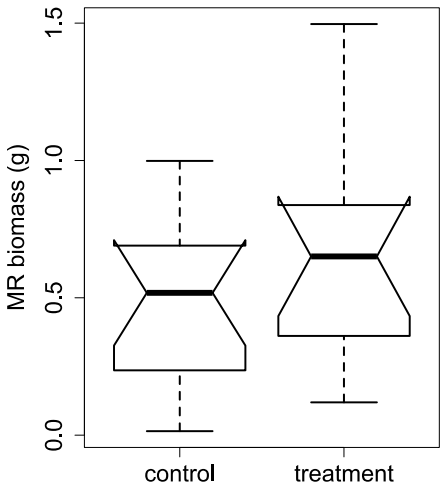


Figure 14: Total plot MR biomass. Two models were fitted: one accounting for the initial difference in aboveground biomass between treatments and another ignoring the effect of aboveground biomass. Both models produced similar results, and only the second one is shown. Model statistics: *Adj.* $R^2 = 0.0146$, $F_{1,24} = 1.369$, $p = 0.253$, $n = 26$

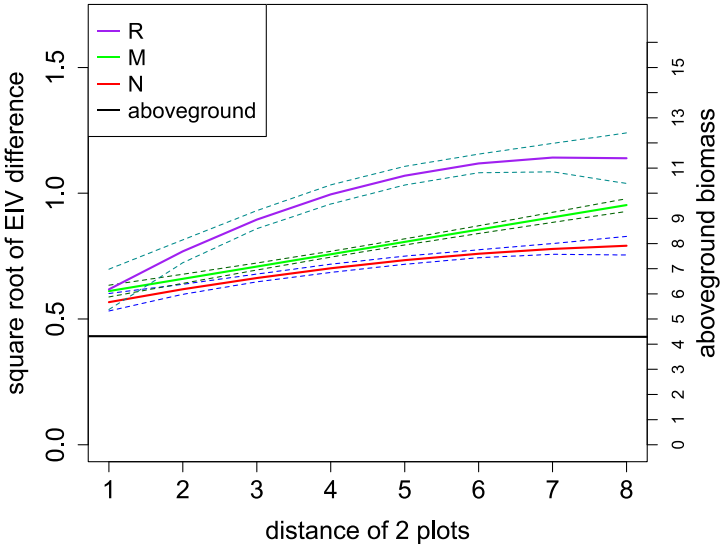


Figure 15: Spatial autocorrelation of EIVs and aboveground biomass. The vertical axis shows square roots of EIV differences. Depending on the fit of the model, either linear or quadratic regression was used. Dashed lines indicate 95 % confidence intervals. Model statistics: **N:** *Adj.* $R^2 = 0.03756$, $F_{2,2681} = 56.87$, $p < 0.00001$, $n = 2864$; **M:** *Adj.* $R^2 = 0.08044$, $F_{1,2782}$=244.5, p<0.00001, n=2784; **R:** *Adj.* $R^2 = 0.04328$, $F_{2,2711} = 62.36$; $p < 0.00001$, $n = 2714$, **AB:** $p = 0.912$, $n = 2455$

3.5 Vegetation and EIV distribution patterns

Vegetation sampling of the array revealed intermediate plant species diversity (30 species altogether). Within plots, the number of species ranged between 10 and 24 with a median of 16. At the study site, 42 plant species were previously recorded on a 120-metre transect

with sampling plots spaced 5 metres apart (Hrouda, unpubl. data). Species diversity as well as EIV variation inside the array were lower than on the whole-site scale.

The study array did not show signs of notable changes of any of the EIVs (M, R, N) on a small scale (distance between 30 cm and 1 m). M, R and N values were all spatially autocorrelated (Figure 15). However, the scale of these autocorrelations differed between the EIVs. Both R and N values exhibited lower *range*[1] than M values, resulting in quadratic relationship as opposed to a linear one (Figure 15) at distances of 1–8 metres. R values had the lowest *nugget* and the highest *sill* from the three EIVs. N values showed the lowest *sill*, but their *range* was estimated to be only a little over 8 m. M values experienced the highest *range*, although it is estimated not to be too different from the other EIVs based on a limited set of data from plot pair distances between 8 and 11.4 m. The aboveground biomass exhibited no spatial autocorrelation within the study array (Figure 15).

On the whole-array scale, the distribution of the EIVs showed some spatial patterns (Figure 17). M values tended to increase with decreasing row number (actually, towards the south-western corner of the array; *Adj.* $R^2 = 0.5271$, $F_{1,77} = 87.93$, $p < 0.00001$; Figures 16 and 17). R values increased in the opposite direction, towards the north-eastern corner of the array, culminating around the empty plot (column S2, row R7). N values were generally quite low with a few exceptions localized in plots of higher R values and lower M values.

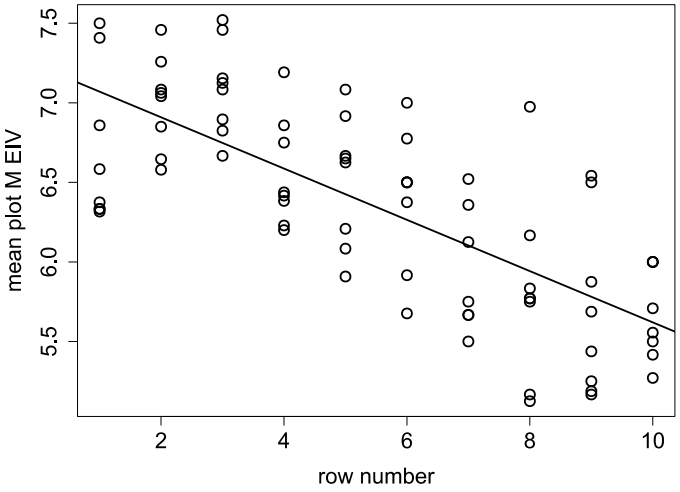


Figure 16: Mean plot M EIV changes across array rows. Model statistics: *Adj.* $R^2 = 0.5271$, $F_{1,77} = 87.93$, $p < 0.00001$, $n = 79$

1 In the spatial autocorrelation graph (variogram), *nugget* stands for small-scale variation (including any error caused by the measurement), *sill* is a value of y at $x \rightarrow \infty$ and *range* presents a threshold of autocorrelation: beyond this value the data are no longer autocorrelated.

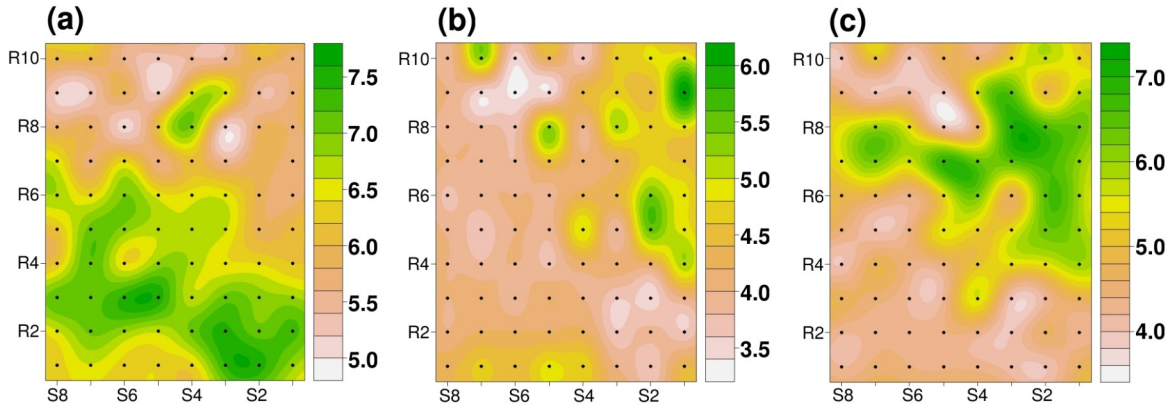


Figure 17: EIV spatial patterns inside the study array. The three graphs show spatial variation of (a) moisture, (b) nitrogen and (c) soil reaction EIVs inside the array. Note the different scales to the right of each graph: the colours represent relative variation of the respective EIVs but they do not stand for the same values across all graphs. The values in the space between plots (dots) were extrapolated using *ContourFunctions* and *mlegp* packages.

The relationships between the EIVs and aboveground biomass were explored using PCA (Figure 18). The first axis seems to reflect the interactions between EIVs, while the second axis covers mainly the aboveground biomass difference between treatments (Figure 18).

Interestingly, N values on the quarter level increased with aboveground biomass (correlation coefficient 0.155, $p = 0.009$; model statistics: $Adj.\ R^2 = 0.021$, $F_{1,280} = 7.083$, $p = 0.01$), while their relationship with FR biomass was not significant (correlation coefficient -0.039, $p = 0.849$; model statistics: $F_{2,99} = 0.037$, $p = 0.849$).

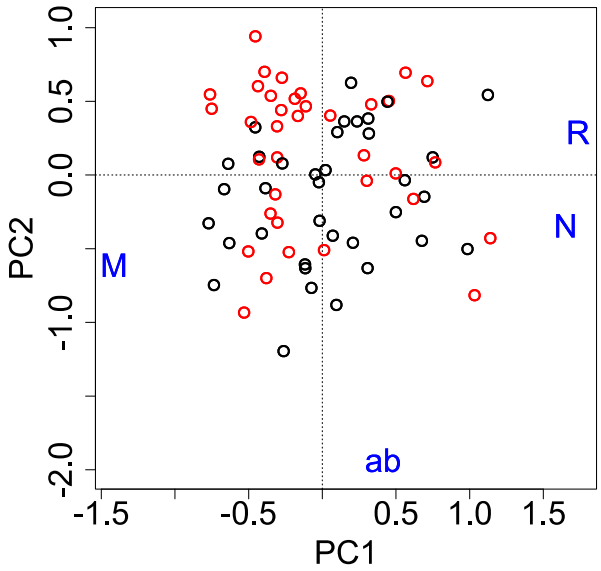


Figure 18: PCA visualisation. The black dots represent control plots, the red dots represent treatment plots. The letters **M**, **R** and **N** represent the respective EIVs, **ab** stands for aboveground biomass. The first (PC1) axis accounts for 46.89 % and the second (PC2) axis for 25.66 % of the overall variance (combined proportion of variance explained by 1st and 2nd PCA axes: 72.55 %).

4 Discussion

4.1 Overall response to artificial patch

Total fine underground (FR) biomass did not change significantly based on the treatment, neither at the plot level nor at the level of quarters. This lack of response might have originated from (a) an inappropriate amount or position of fertilizer, (b) insufficient water availability, (c) the structure of the underground space, (d) fast nutrient uptake by existing roots, (e) the combination of two or more factors or (f) the fact that a response did in fact occur, yet was impossible to observe due to lack of additional data, for example on root demography or final aboveground biomass. Lastly, it is also possible that FR in the studied community do not respond to nutrient heterogeneity and their spatial distribution depends solely on other factors, such as soil pH, functional group composition (e.g., proportion of graminoids, forbs, woody plants etc.) or competitive abilities of species.

Fertilization has been shown to increase root (Campbell et al., 1977; Cougnon et al., 2017; Dong et al., 2002; Tomaškin et al., 2013) and underground (roots & rhizomes) biomass (Hejduk & Hrabě, 2003). However, the amount of fertilizer used in my experiment might have been too low and/or the patch created by the fertilizer too small for a response to occur. The type of fertilizer (controlled-release fertilizer, CRF) used in this study has been shown to work better under warm temperatures (optimum 20-25° C) (Kochba et al., 1990). The site temperature was frequently above 20° C during the first season, possibly increasing the efficiency of nutrient release from the CRF. On the other hand, the precipitation during the first season was generally low, which might have affected soil moisture, perhaps causing the fertilizer to be less efficient (Haase et al., 2007; Kochba et al., 1990). In fact, it is possible that the effect of suboptimal soil moisture itself exceeded that of the fertilizer. The second application of fertilizer (November 2019) might have added only a limited proportion of the total amount of nutrients due to cold conditions during winter 2019/2020. Finally, the release of nutrients might have been slowed due to the application of the fertilizer ca 6 cm below ground as opposed to application 0-1 cm under the surface (Cabrera, 1997).

Another reason for the lack of biomass change might have been the underground structure, i.e. high and relatively homogeneous root densities in the topsoil layer (reported also by Herben et al., 2020). McConnaughay & Bazzaz (1992) showed that fragmentation of soil space by neighbour roots results in reduced plant biomass. This pattern was not observed

at high nitrogen levels, yet the high amount of nitrogen provided during their 2-month experiment was similar to the potential maximum amount supplied during the first 6 months of this experiment. Hence, it is probable that the fertilizer treatment was insufficient in eliciting FR biomass gain. Also, the limitation of soil volume availability simulated in McConnaughay & Bazzaz's (1992) experiment was very low compared to rooting densities found in natural grasslands (Fitter, 1982).

Even if the space had not been limiting to the growth of new roots, it is possible that the contrast between CRF-induced patch and background soil declined rapidly due to changes in the nutrient uptake capacity of adjacent roots. That being said, there is a possibility that a response to the patch did occur, perhaps by changes in root demography or increased aboveground biomass (with no increase in belowground biomass) in close proximity to the fertilizer patch. Gross et al. (1993) found that plants in their experiment reacted to a nutrient-rich patch by changes in root birth and death rates, rather than changes in root biomass. Dong et al. (2002) observed no response to a nutrient-rich half of the tray in either fine or coarse roots in a heterogeneous (HL) treatment despite a significant increase in root biomass between homogeneously nutrient-poor (LL) and homogeneously nutrient-rich (HH) treatment. The only trait that underwent a significant increase in the heterogeneous treatment (HL) was the number of shoot ramets. This may suggest that plants in my experiment could have responded by changes in aboveground rather than underground traits.

In order to better understand the perspective of plants close to vs. far from the patch, additional measurements of soil nutrient availability would need to be carried out. These measurements should preferably: (1) use various amounts of fertilizer, (2) be performed using soil samples from several distances from the place of fertilizer application, (3) cover a period of time similar to the extent of the study (including a measurement before the fertilization), (4) follow a pairwise arrangement with one fertilized and one control patch in a pair (within-pair distance should be large enough to avoid contamination but significantly lower than between-pair distance), (5) determine the concentration of several ions (NH^{4+}, NO^{3-}, PO_4^{3-}) and (6) take place at the same site in conditions as similar as possible to those in the study array.

Nutrients are only one among several potential predictors of FR placement in the field. Šmilauerová (2001) observed that root abundance can be affected by the functional group composition of neighbouring species. Also, in the experiment (Šmilauerová, 2001) several blocks varied in root biomass but not in the combined biomass of roots and rhizomes. In my experiment, fine underground biomass was not divided into roots and rhizomes and therefore differences in root : rhizome biomass ratio might have been overlooked.

4.2 Response of *Rumex acetosa* to artificial patch

Model plants did not respond to the elevated level of nutrient concentration in a neighbouring patch by increase in total root biomass (MR). The species, *Rumex acetosa,* had previously been reported to have increased its total root biomass under fertilizer treatment (Kołodziejek, 2019), a result not corroborrated by the findings of current study. *Rumex acetosa* was also able to forage for nutrients in a greenhouse experiment (Keser et al., 2015). In my experiment, I was unable to track any directional root growth stimulated by the fertilizer patch. This result could have been caused by the factors mentioned earlier (see chapter 4.1 Overall response to artificial patch) or inadequate separation of roots from different quarters. Cutting the whole-plot monolith into quarters prior to washing out the soil was arguably advantageous as it was unlikely to cause any shift of the roots between quarters. Unfortunately, it is possible that some root samples contained part of the epigeogeneous rhizome, and therefore were disproportionately heavier. Furthermore, the roots of model plants were not divided into any subcategories by size. That turned out to be a drawback, as the data on root biomass sorted by root diameter could have helped compensate for imprecise cutting. Incorporating additional measurements, such as root length and topology, might also be helpful in disentangling the causes of root system spatial distribution.

One possible reason for the observed lack of reaction is that the model plants were unable to reach the enriched patch by the time the nutrient levels dropped down to background soil level. In contrast, visual inspection showed that at least 2–3 individuals were able to reach the patch and proliferate inside it. However, those observations might have been an exception and many plants might not have been able to grow into the enriched patch.

From the considerably greater death rate of plants in treatment plots compared to control plots it seems that the intensity of competition increased in proximity to the enriched patch (as reported by McNickle et al., 2016). Interestingly, the proportion of plants in the treatment group that had reached flowering stage by the end of the experiment was about four times higher than in control plots. This could be explained by the plants' accidental detection of the nutrient-rich patch and subsequent increase in nutrient uptake by means of increased root growth and/or physiological uptake capacity. Another possibility is that the addition of nutrients merely strengthened the role of aboveground competition (DeMalach et al., 2016; Lamb et al., 2009) and thus promoted shoot growth and, consequently, flowering of model plants.

Many studies describing the morphological responses are quite short in comparison with this experiment (Caldwell et al., 1996; Campbell & Grime, 1989; Hodge et al., 1999; Mommer et al., 2012) and only a few consider the long-term consequences (Fransen et al., 2001; Li et al., 2016). This study aimed to observe the reaction to a long-term localized source of nutrients. However, the results of the available phosphorus content analysis suggest that the patch was depleted prior to the re-application of fertilizer. It is also possible that the plants did react after the first and/or second application of fertilizer, yet their root system later grew based on different stimuli than nutrient concentrations (after those dropped down to the background soil level).

This development was observed by Fransen & De Kroon (2001) in a 2-year study with two grass species, *Holcus lanatus* and *Nardus stricta*. At the beginning, *Holcus* was able to proliferate its roots into the nutrient-rich patch in a high nutrient concentration treatment. Contrastingly, *Nardus* did not place its roots preferentially into the enriched part of soil under any treatment. During the first season of the experiment, the patch was depleted and the initial foraging response of *Holcus*, which was also mirrored in aboveground biomass gain, was no longer an advantage. Rather, after the nutrient levels within and outside the patch evened out, *Holcus* no longer benefited from the proliferation and at the end of the experiment its shoot biomass was smaller than under homogeneous conditions. Therefore, it is possible that the outcome of my study was affected by its duration. In conclusion, shorter timespan of a study and/or more stable nutrient input could allow for the detection of a morphological response.

Both the distance from a patch and its size are likely to play an important role in the occurrence and magnitude of a response (Wijesinghe et al., 2001; Wijesinghe & Hutchings, 1999, 1997). Campbell et al. (1991) postulated the existence of a trade-off between the scale on which a species can forage and the precision of its foraging response. Several studies have found support for this theory (Grime & Mackey, 2002; Wijesinghe et al., 2001), while other results were not in agreement with it (Einsmann et al., 1999; Farley & Fitter, 1999b; metaanalysis by Kembel & Cahill Jr., 2005). Later on, Grime (2007) argued that the critics of the scale-precision trade-off hypothesis had misinterpreted the original scope, which was restricted to mowed grasslands. Kembel et al. (2008) responded to his article with an analysis of a broad dataset which brought no support for the trade-off hypothesis.

Hutchings et al. (2003) conducted an experiment with pots divided into four quadrants, where soil in each two opposite quadrants was of the same quality, either nutrient-poor or nutrient rich. They found that the performance of species with smaller root systems was significantly limited when the initial position of the plant was in the nutrient-poor rather than nutrient-rich quadrant. Irrespective of the starting quadrant nutrient status, species with large root systems tended to allocate a similar proportion of roots to rich quadrants. On the other hand, species with small root systems placed most of their roots in the quadrant where they began to grow. Also, Martínková et al. (2018) encountered somewhat similar differences in response to nutrient heterogeneity arising from root system size. In the context of my study, the model species' root system falls into the small size category. Hutchings et al. (2003) hypothesized that in the presence of competition, the disadvantage of plants with small root systems in heterogeneous soil will increase, resulting in stronger growth suppression or death. My data provide support for this prediction as the death rate in treatment plots was significantly higher than in control plots.

The magnitude of a response to soil nutrient heterogeneity can depend on root symbiosis with mycorrhizal fungi (Cui & Caldwell, 1996; Šmilauerová, 2001; Wijesinghe et al., 2001): in the presence of mycorrhiza, smaller amount of roots is usually produced. The model species, *Rumex acetosa*, was earlier reported not to form mycorrhizal associations (Grime et al., 1987; Harley & Harley, 1987). More recently, mycorrhizal structures have been observed in this species: vesicles in 4.0 % (± 4.0; Pawlowska et al., 1997) and 14.4 % (± 1.2; Veresoglou et al., 2011) of the roots; and arbuscules in 18.2 % (± 1.0; Veresoglou et al., 2011)

of the roots. Eriksen et al. (2002) reported vesicles in 3, arbuscules in 2 and internal hyphae in 8 out of 15 plants collected in three grasslands in Norway. Even though the species is apparently capable of forming mycorrhizal associations, the evidence acquired suggests it occurs only rarely. Therefore it seems reasonable to believe that the model plants in my experiment did not obtain any significant proportion of nutrients by means of mycorrhiza.

The nature of a response to uneven distribution of nutrients in soil depends also on clonality (Slade & Hutchings, 1987; Weiser et al., 2016). However, it is important to distinguish between different types of clonality. For example, species with large lateral spread capability can react to heterogeneous conditions by changing the length of spacers (de Kroon & Hutchings, 1995; Slade & Hutchings, 1987). On the other hand, some rhizomatous species possess a highly limited ability of vegetative spread, and therefore are likely to interact with underground environment in a similar way to non-clonal perennial species (Šmilauerová & Šmilauer, 2007; Weiser et al., 2016). To conclude, although the study was conducted using a clonal species, the results presented might be applicable to both non-clonal and clonal perennials, provided they do not spread laterally over distances larger than a few centimetres.

4.3 Root system morphology assessment

In this study, patterns of root biomass distribution were recorded as a measure of root foraging behaviour. However, assessing only the allocation of root biomass may not reflect important properties of the event. For instance, it might be the dynamics of root growth (Fransen & De Kroon, 2001; Gross et al., 1993) or changes in root system morphology that are more connected to the nutrient acquisition process (Hou et al., 2017). There are several possible approaches to the assessment of root system morphology: part of them are non-destructive techniques [such as minirhizotron tubes (Fransen & De Kroon, 2001; Padilla et al., 2013) or X-ray computed tomography (Pfeifer et al., 2015)] and others involve the destruction of plants. The latter typically include excavation of soil cores/monoliths, followed by separation of roots (Fitter, 1982; Herben et al., 2007) or freezing of samples (Caldwell et al., 1996). Unfrozen samples are usually handled using water and sieve, “dry excavation” (i.e. extraction of the root system without the use of water) is used only

rarely (Brisson & Reynolds, 1994; Pecháčková et al., 1999). Therefore, information on horizontal distribution of a root system is typically lost during the separation of roots from the soil.

Two techniques for studying root distribution under ground were tested out in my study: (1) hammering a nailboard into the ground, excavating a monolith and washing out the soil; and (2) excavation and cutting of the monolith into parts from which the soil is washed out. Even though the nailboard was not chosen in the end, I believe that it might be possible to use a similar tool with success. The advantage of the method lies in the conservation of information on horizontal root distribution. However, when the root densities are high, washing the soil out becomes rather problematic, as shown by the testing of the nailboard method. I propose that both of the methods might be applied together, gaining advantage from each of them. The resulting approach would consist of hammering not one, but several smaller nailboards into the ground, excavating the whole monolith and then cutting it apart into several small soil monoliths (each with a nailboard). Smaller-sized nailboards would probably make obtaining of the roots more effective and less laborious. This technique could allow for spatially informed assessment of root morphological and topological characteristics, combining the advantages of "dry" and "wet" methods for studying root systems.

4.4 EIV distribution

The EIV data showed marked autocorrelation up to distances from ca 6 to ca 10 metres. Mean quarter EIVs carried only limited amount of information due to small number of species with assigned EIVs and were therefore ineffective for tracking any potential within-plot spatial heterogeneity. The EIVs might perhaps be applicable on such a small scale in certain conditions (e.g. those described in Sádlo et al., 2011) but the accuracy of the estimates would probably benefit from the inclusion of additional predictors, such as species diversity.

While EIV-based estimates cannot precisely describe short-term soil heterogeneity at small spatial scales, they possess information on soil parameters' spatial variation on a large temporal scale. In a similarly sized field array, Jackson & Caldwell, (1993) found soil nutrient concentration data (ammonium, nitrate, phosphate and potassium) to be spatially autocorrelated on scales smaller than 1-3 metres, while the smallest distances (12.5 cm) still showed substantial variation. Furthermore, Janik (2008) assessed the spatial autocorrelation of soil moisture in two grassland arrays (4×4 metres and 10×10 metres) and found the *range*

value to be approximately 3 metres. The range value is clearly smaller when measured values are used as opposed to EIV data, probably also thanks to the finer spatial grain of the measurements. The greater *range* value in EIV-derived variograms is arguably a product of the large-scale nature of EIVs. The actual parameters may show dependence on a smaller spatial and temporal scale, while the vegetation provides us with long-term "averages" of these fluctuations.

Inside the array, M values increased in the opposite direction than R and N values, however, the pattern was less clear for N values (Figure 17). There are several possible reasons for the observed patterns of EIV distributions, including a nearby forest edge (north of the array) and consequent differences in light availability or perhaps even microtopographic variation (Moeslund et al., 2013). However, the available data are too limited for any generalisation, such as a reciprocal relationship between soil moisture and soil reaction at small scales. Also, R EIVs might not be adequate for estimating small-scale distribution of soil pH, as even on larger scales their performance has been questioned (Schaffers & Sýkora, 2000). It is likely that extending the grid of sampling points would be useful for improving the precision of the estimates and perhaps shed light on soil parameters interactions at small to intermediate scales.

Although aboveground biomass and FR biomass were loosely associated, only aboveground biomass was efficient for predicting N values. This is in accordance with results of other researchers (Hill & Carey, 1997; Schaffers & Sýkora, 2000) who have found strong positive relationship between N values and productivity measured as aboveground yield. Contrastingly, M values were positively associated with FR biomass, but not with aboveground biomass. Increasing soil water availability had previously been shown to promote root growth (Van Vuuren et al., 1997). Recently, Moeslund et al. (2013) found that M EIVs are correlated with topographic wetness index data obtained using Light detection and ranging (LiDAR) technology. They concluded that topography is an important driver of soil moisture, and consequently, soil moisture affects the diversity of vegetation (see also Silvertown et al., 1999). In my experiment, the relationship between soil moisture and diversity was impossible to assess as no direct measurements of soil moisture were made (see Zeleny & Schaffers, 2012, for a discussion of the use of vegetation composition derived data). On the level of quarters, aboveground biomass increased with species diversity. This trend has

been commonly reported (Flombaum & Sala, 2008; Ruijven & Berendse, 2005), yet the causality behind it has been questioned by Grace et al. (2007), who argued that additional factors apart from diversity are responsible for productivity variation.

4.5 Foraging responses in the field

Natural grassland soils present a complex environment with high root densities, at least in the top soil layers (Herben et al., 2020). Several field experiments carried out in Bohemia show the ability of grassland species to increase root investment in patches of elevated nutrient availability (Šmilauerová, 2001; Šmilauerová & Šmilauer, 2006, 2002). These results might, however, be confounded by the removal of plants (including roots and rhizomes) from the experimental patches. Studies by Caldwell et al. (1991, 1996) showing interactions between roots of different species managed to create almost natural conditions, yet these conditions were quite different from those in my study array. Firstly, only two species were growing in their experimental patches, a shrub and a grass. Secondly, the study was conducted on a site in the mountains in Utah, USA, in a former shrub steppe, again differing from the context of my study.

In order to better understand the causes of a foraging response (or its absence) in the field, several species differing in their foraging abilities (when grown alone) should be used in an experiment, preferably with treatments of several nutrient levels and patch distances to the plant. However, studies of such complexity would present a difficult task, for example with respect to distinguishing the roots of focal plants from their neighbours (Hodge, 2004). Nevertheless, I believe they might be valuable in connecting the indices supporting the adaptive value of root foraging (Hodge et al., 1999; Keser et al., 2015; Robinson et al., 1999) or those questioning it (de Kroon et al., 2009; Dong et al., 2002; Fransen & De Kroon, 2001; Hutchings & de Kroon, 1994; James et al., 2010; van Vuuren et al., 1996) with the context of a natural environment. With such a perspective, it might be possible to investigate whether plant nutrient acquisition and/or competitive strength is increased by foraging abilities (James et al., 2009; Keser et al., 2014) or determined rather by growth rate (Aanderud et al., 2003; DeMalach et al., 2016) or other factors. Furthermore, organic sources of nutrients are only rarely used in studies of root foraging, despite the fact that the naturally occurring patches consist of organic material (Fransen et al., 1998;

Hodge et al., 1999; Tibbett, 2000). Hence, a robust assessment of plant root plastic responses in the field may benefit from the incorporation of realistic nutrient sources and perhaps also soil microbial communities (Hodge et al., 1999).

4.6 Consequences of soil heterogeneity for communities

The current view of field soil nutrient heterogeneity in temperate grasslands entails small-scale spatial variability created by short-lived peaks of nutrient concentrations (Březina et al., 2019; Herben et al., 2018; Herben & Novoplansky, 2010; Lamb et al., 2004). In such a dynamically changing environment, plasticity in root physiology is likely advantageous (Cui & Caldwell, 1997; van Vuuren et al., 1996). However, long-term nutrient enrichment is not necessarily unrealistic, either as a result of human (Šmilauerová, 2001) or animal impact (Keenan et al., 2018). Long-term nutrient-rich patches might pose one explanation of root morphological plasticity: morphological changes are much slower than physiological modifications and sometimes begin so late that a short-term patch is already mostly depleted (van Vuuren et al., 1996). This explanation would imply that root proliferation increases the aboveground biomass or fitness of an individual, yet this pattern is not always observed (Cahill & Casper, 1999; James et al., 2010; Kembel & Cahill Jr., 2005).

It is likely that soil heterogeneity affects the intensity of competition (McNickle et al., 2016). The importance of soil heterogeneity effect might fade with increasing spatial scale: in some studies, plant individuals were notably influenced by it, while on population or community levels little variation was observed (Casper & Cahill, 1996; Tilman & Pacala, 1993). The diversity of a community has been linked to uneven soil nutrient distribution (Fitter, 1982). However, Stevens & Carson (2002) concluded that overall resource availability, rather than heterogeneity, affects species richness of a stand. Interestingly, both heterogeneity and simple abundance of nutrients have been hypothesized to increase the asymmetry of competition (DeMalach et al., 2016; Rajaniemi & Reynolds, 2004). The traits important for competitive success were shown to differ based on the total asymmetry of competition (DeMalach et al., 2016). The theory predicts that in soils of low nutrient availability, symmetric competition will prevail and favour slow-growing and long-lasting organs. Contrastingly, in fertile soils, competition will be more asymmetric and reward fast-growing individuals (DeMalach et al., 2016; Hou et al., 2017). Unlike the symmetry of aboveground competition, that of underground competition is still subject to debate (see e.g. Rasmussen et al., 2019). Recent studies suggest that on a small temporal scale,

heterogeneity might cause a shift towards asymmetry in underground competition (Rajaniemi & Reynolds, 2004), while in the long run it becomes symmetric (Fransen & De Kroon, 2001; Herben et al., 2018). Therefore, in nutrient-rich environments, aboveground performance differences might prove to be more important than belowground competition.

The evidence accumulated by recent research suggests that the role of root morphological plasticity in the field might be inferior. This underlines the importance of a provoking question asked by Robinson (1996): "Why do plants bother?" If the ability to proliferate roots in nutrient-rich patches does not ensure higher yield and if foraging scale or speed rather than precision seems to be rewarded, than why do plants do it? One possibility is that plastic changes of morphology might be beneficial when competition takes place in nutrient-poor soils, for example in the early stages of succession (Robinson et al., 1999). However, the answer may well be species-specific and depend on additional factors (Herben & Novoplansky, 2010), suggesting that broader as well as more detailed research is still needed (Hodge, 2006).

5 Conclusion

This experiment aimed to test the effect of a long-term artificial nutrient-rich patch on the distribution of model plant roots and neighbouring plants underground biomass. The treatment did not affect fine underground biomass in the proximity of the patch. Also, neither preferential allocation of roots toward the enriched patch nor an increase in total root biomass of model plants was recorded in treatment plots. Several possibilities for these results are discussed, including insufficient nutrient availability in the patch, too large a distance between the model plant and the patch or a response in traits not measured in this experiment. Either thanks to a plastic root response or increased aboveground competition, greater proportion of model plants flowered in treatment plots than in control plots. This pattern was accompanied by an elevated death rate in the treatment plots, suggesting that the fertilizer patch treatment increased overall competition intensity.

It seems that for model plants, the distance to the enriched patch was limiting in terms of nutrient acquisition. This might have important implications: the survival of species with smaller root systems could be affected more by the distance from nutrient enrichment than by their foraging abilities. The duration of the artificial patch was probably smaller than originally planned. Still, current study brings evidence that localized temporary elevation of nutrient concentration in natural grassland soil does not induce substantial shifts in belowground biomass. Recent studies suggest that the role of root morphological plasticity under natural conditions might be less important in habitats of intermediate to high nutrient levels. Further research under realistic conditions is needed to answer questions concerning soil heterogeneity, plant underground phenotypic plasticity and its adaptive value.

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Appendix 1

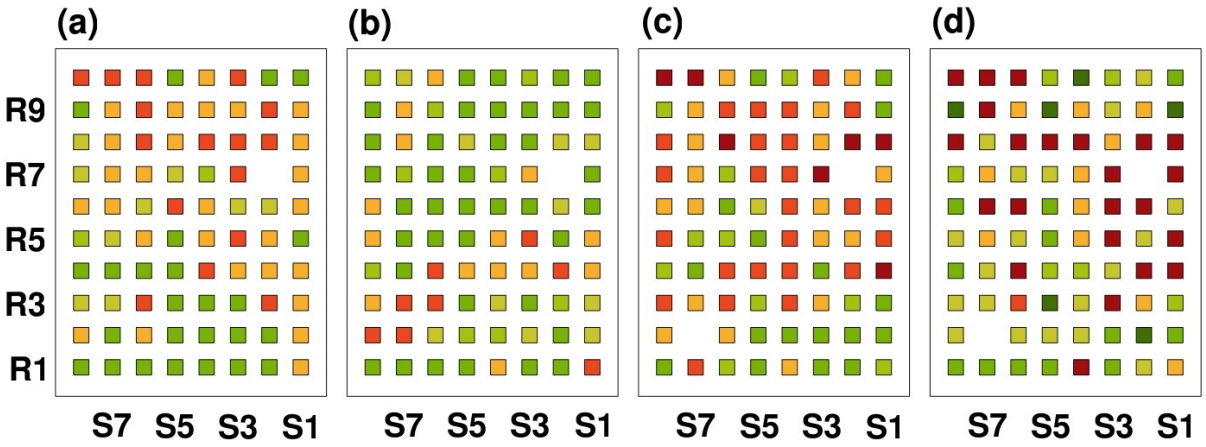

Figure S1: Whole-array survivorship development of model plants. Each graph shows model plants' observed vitality at a different time of evaluation: **(a)** – beginning of summer 2019, **(b)** – late summer 2019, **(c)** – autumn 2019, **(d)** – early summer 2020. Each point is colour coded by observed vitality: rank **0** – dead plant (■), rank **1** (■), rank **2** (■), rank **3** (■), rank **4** (■), rank **5** (■), rank **6** – flowering plant (■).

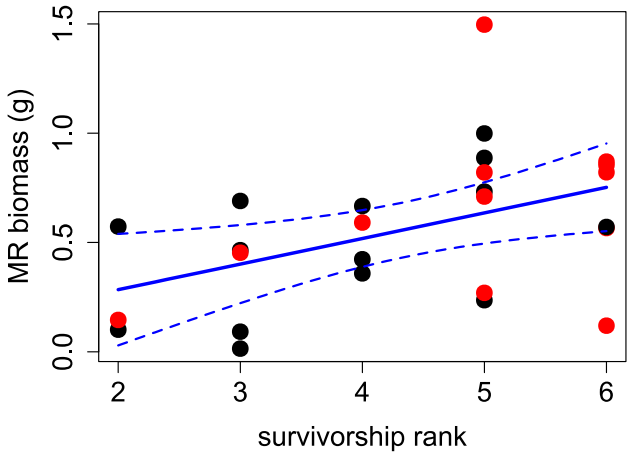

Figure S2: Total MR biomass across ranks of vitality. The black dots represent model plants from control plots, the red dots represent model plants from treatment plots. The horizontal axis shows survivorship ranks of model plants at the end of the field experiment. Only data of plants from excavated plots are shown. Model statistics: *Adj.* $R^2 = 0.181$, $F_{1,24} = 6.532$, $p = 0.017$, $n = 26$