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Mgr. Tereza Mašková

Decisions in seed reproduction in plants

Rozhodující fáze v semenném rozmnožování rostlin

Disertační práce

Školitel: prof. RNDr. Tomáš Herben, CSc.

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Prohlášení autora / Author's declaration:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tuto práci ani její podstatnou část jsem nepředložila k získání jiného nebo stejného akademického titulu.

I hereby declare that I made this thesis independently, using only the mentioned references. I did not submit this thesis nor its part for any other degree or diploma.

V Praze dne 17. 7. 2019

Tereza Mašková



Contents

Abstract	13
Abstrakt	15
1 Introduction	17
1.1 Seed nutrient stoichiometry	20
1.2 Root:shoot biomass partitioning	21
1.3 Root system development	22
1.4 My findings	23
1.5 Conclusions	25
References	25
2 Maternal support in plants: CNP stoichiometry in seeds varies to match expected nutrient limitation by seedlings	33
Abstract	33
2.1 Introduction	34
2.2 Materials and Methods	35
2.3 Results	37
2.4 Discussion	43
References	45
3 Root:shoot ratio in developing seedlings: how seedlings change their allocation in response to seed mass and ambient nutrient supply	51
Abstract	51
3.1 Introduction	52
3.2 Materials and Methods	53
3.3 Results	56
3.4 Discussion	58
References	61
4 The roles of interspecific variability in seed mass and soil resource availability in root system development	67
Abstract	67
4.1 Introduction	68
4.2 Materials and Methods	70
4.3 Results	75
4.4 Discussion	76
References	80

5	The effect of rhizoboxes on plant growth and root:shoot biomass partitioning	95
	Abstract	95
5.1	Introduction	96
5.2	Materials and Methods	96
5.3	Results	98
5.4	Discussion	99
	References	101

Author contribution statement:

This thesis consists of general introduction, two published papers and two manuscripts. All papers are co-authored. My contributions to particular papers are as follows:

- (i) **Mašková T**, Herben T. Maternal support in plants: CNP stoichiometry in seeds varies to match expected nutrient limitation by seedlings.

TM and TH designed the study, TM performed the species selection, measurement of seed mass and managed stoichiometry data collection, TM and TH analyzed and interpreted results, TM wrote the text with contributions of TH. Both authors approved the final version of the manuscript.

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TM designed and performed the experiment, AK analyzed the data, TM and AK interpreted results, TM wrote the text with contributions of AK. Both authors approved the final version of the manuscript.

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Abstract

Seed reproduction is a key part of the life cycle of the most plant species. It allows for the dispersal of species in space and time and, thus, significantly affects dynamics of plant populations and communities. Seed formation, germination and seedling establishment are subjected to selection pressures from the environment and lead to optimization of maternal investments (maternal care), manifested by the number of seeds and amount and composition of nutrients stored in individual seeds. The thesis aims to answer two questions: (i) whether maternal investments in terms of seed mass and seed nutrient stoichiometry is optimized according to the environmental conditions in which seedling development is expected and (ii) how nutrient availability, considering nutrients both stored in the seed by the mother plant and those available in the substrate, affects seedling growth and development.

To answer the first question, we focused on interspecific comparisons of seed nutrient stoichiometry linking it with data on seed mass and species niche along gradients of nutrient availability. We used a phylogenetically informed comparative approach to explore the ratio between phosphorus, nitrogen and nonstructural carbon in seeds from 510 wild herbaceous species. We analyzed seed nutrient stoichiometry with particular emphasis on the context of environmental conditions in which the species typically occur and on their seed mass. Despite strong phylogenetic constraints on seed nutrient stoichiometry, we showed that proportions of stored nitrogen and nonstructural carbon are also optimized according to environments in which the species occur and thus are expected to germinate. The result was similar for both carbon and nitrogen; i.e., plants preferentially store nutrients that are expected to be limiting in the environment of the future seedling. Further, we showed that seed phosphorus content is connected with seed mass. This results suggest that seed nutrient stoichiometry is under selection pressures of both seed mass and environmental conditions.

To answer the second question, we evaluated the impact of available nutrients, a pool stored in substrate versus a pool stored in a seed, on seedling root:shoot biomass partitioning and root system development. The pools of nutrients are not interchangeable as they affect seedling growth and development via different mechanisms. Better resource provisions from the seed allow the seedling to be more flexible and to more effectively use nutrients available in the environment. Growing seedlings in classical pots and rhizoboxes resulted in a final methodological study that allows us to validate results of our experiments.

In this thesis, we demonstrated that interplay between amount and ratio of nutrients stored in a seed, nutrients available in soil and fine-tuned biomass allocation in early ontogeny are crucial for successful seed reproduction and occurs

both on evolutionary and ecological time scales. Our results underline the importance of maternal care for seedling establishment and consequently for population and community dynamics.

Key words: Biomass allocation · Nutrient stoichiometry · Root system development · Seeds · Seedlings

Abstrakt

Generativní rozmnožování je klíčovou částí životního cyklu většiny rostlin. Umožňuje šíření rostlinného druhu v prostoru i čase, a tím podstatně ovlivňuje dynamiku rostlinných společenstev i dlouhodobé přežívání druhů. Tvorba semen, jejich klíčení, následný vývoj a přežívání semenáčů jsou podrobeny selekčním tlakům prostředí, což vede k optimalizování mateřské péče, která se u rostlin projevuje množstvím vyprodukovaných semen a množstvím živin uložených v jednotlivých semenech. Cílem práce je zodpovězení dvou otázek: (i) zda je mateřská péče, ve smyslu velikosti jednotlivých semen a živinové stechiometrie semen, optimalizována dle podmínek prostředí, ve kterých je předpokládáno klíčení semen a (ii) jak dostupnost živin, jednak uložených do semen mateřskou rostlinou, jednak dostupných ze substrátu, ovlivňují růst a vývoj semenáče.

Pro zodpovězení první otázky jsme se zaměřili na mezidruhové srovnání živinové stechiometrie semen, kterou jsme spojili s daty o hmotnosti semen a živinovými nároky druhů. Použili jsme fylogeneticky informovaný srovnávací přístup ke zkoumání stechiometrie fosforu, dusíku a nestrukturálního uhlíku v semenech 510 bylinných druhů rostlin. Analyzovali jsme stechiometrii živin s důrazem na kontext podmínek prostředí, ve kterých se druhy typicky vyskytují a také jejich hmotnost semen. Navzdory tomu, že se živinová stechiometrie semen ukázala silně ovlivněná evoluční historií druhů, ukázali jsme, že proporce dusíku a nestrukturálního uhlíku v semenech je zároveň optimalizována pro prostředí, ve kterém daný druh roste. U obou jmenovaných prvků platí, že je přednostně ukládán ten, který je očekáván v prostředí jako limitující. Dále jsme ukázali, že obsah fosforu v semenech je spojen s jejich hmotností. Tyto výsledky naznačují, že živinová stechiometrie semen je pod selekčním tlakem jak velikosti semen tak i podmínek prostředí.

K zodpovězení druhé otázky jsme zhodnotili dopad dostupnosti živin, ze semen versus z prostředí, na alokaci biomasy u semenáčů a vývoj jejich kořenového systému. V obou případech se ukazuje, že živiny dostupné v semeni a v substrátu jsou nezaměnitelné v tom smyslu, že ovlivňují růst a vývoj semenáče jiným způsobem. Shodně v obou případech také vychází, že lepší zásobení semenáčů živinami ze semen jim umožňuje flexibilněji a efektivněji využívat živiny dostupné v substrátu. Pěstování semenáčů v rhizoboxech a v klasických květináčích pak vyústilo v poslední metodologickou studii, která potvrzuje interpretaci výsledků předchozí studie.

V předkládané práci ukazujeme, že souhra mezi množstvím a stechiometrií živin uložených v semenech, živin dostupných v substrátu a jemné ladění v alokaci biomasy v raných stádiích ontogeneze jsou nezbytné pro úspěšné generativní rozmnožování a probíhají jak na ekologické, tak i na evoluční časové

škále. Naše výsledky zdůrazňují důležitost mateřské péče pro úspěšné přežívání semenáčů, a tím pro celkovou dynamiku rostlinných populací a společenstev.

Klíčová slova: Alokace biomasy · Semena · Semenáčky · Vývoj kořenového systému · Živinná stechiometrie

Chapter 1

Introduction

Plants are modular (Rees and Crawley 1989) and highly phenotypically plastic (Bradshaw 1965; Schlichting 1986) organisms. These attributes are crucial to sessile organisms. Plants have no other options to obtain everything they need to live and cope with all the pitfalls that threaten their existence than to plan precisely and adapt their growth to the environmental conditions in which they grow. Fast escape from unfavourable conditions or hiding and waiting until the dangerous conditions go away is generally difficult for plants that are not dormant by seeds or bulbs and tolerance of local conditions or avoidance of certain conditions are key options for successful survival.

Plants can adapt their growth to various environmental factors, such as light (Kurashige and Agrawal 2005; Barišić et al. 2006), water availability (North and Nobel 2002; Weigelt et al. 2005; Espinoza et al. 2013), nutrient amount or heterogeneity in the soil (Johnson and Biondini 2001; Hodge 2003), the presence of other individuals (including the same or different species; Gersani et al. 1998; Gruntman and Novoplanski 2004), or soil obstacles (Massa and Girloy 2003; Falik et al. 2005). Successful survival of plants in one developmental stage is hence dependent on decisions made during the previous stage that are based on the ability to estimate the future conditions of the environment, and that permit the development of appropriate structures and form.

While such decisions are made at every life stage, the most tricky decisions are probably those associated with the transition from one generation to another by generative reproduction because these two generations can be distant in time or space. Consequently, the environmental conditions experienced by the next generation are uncertain. First important decision-making steps in generative reproduction are made by the mother plant during seed production, particularly optimization of seed nutrient stoichiometry and seed mass. Further, after germination, seedlings must decide how to invest stored resources, both in terms of root:shoot biomass allocation and root system development or stem development itself Fig. 1.1.

Both these decision-making steps can be made on an evolutionary or ecological time scale. Maternal investment in seeds in terms of seed size and seed nutrient stoichiometry is primarily decided on an evolutionary time scale. In contrast, the manifestation of maternal care (in terms of the absolute amount of stored nutrients) in seedling establishment and successful survival takes place both on evolutionary and ecological time scales as the evolutionarily determined maternal

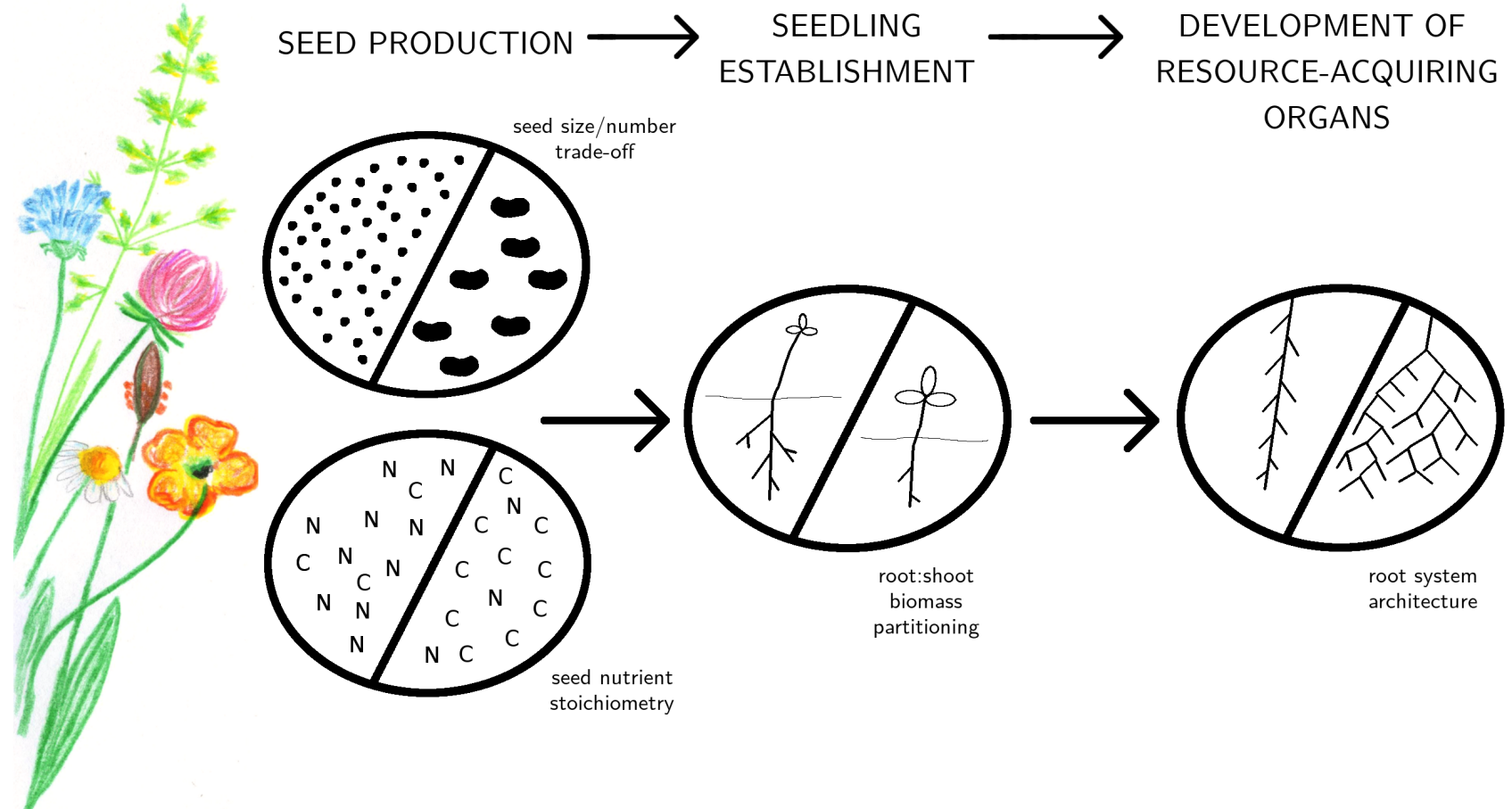


Fig. 1.1: Important decision-making steps during plant generative reproduction. Seed production is shaped during the evolutionary time, as revealed by interspecific comparisons; seedling establishment and resource-acquiring organ development occurs on an ecological time scale as an individual response to current environmental conditions, but interacting with maternal decisions (seed size and stoichiometry) in evolutionary time.

provision interacts with nutrient availability in the environment which varies over an ecological scale. From the methodological point of view, the former is typically revealed using phylogenetically informed interspecific comparisons, whereas the latter are best studied by examining differences between individuals in response to a range of environmental conditions and constructing reaction norms.

The recruitment and survival of seedlings is an important part of the life cycle of plants, and understanding the processes that lead to successful seedling survival is one of the key insights necessary to understand community dynamics and composition (Grubb 1977; Howard and Goldberg 2001; Henry et al. 2004). Still little is known about the key determinants of the seedling survival, such as phenotypic plasticity of seedling development (Fenner 1987; Pohlman et al. 2005; Xue et al. 2019), Understanding of these processes in seedlings is lagging behind knowledge on adult plants, where numerous studies demonstrate how they are phenotypically plastic in many aspects of their growth, ranging from root system development (Bell and Sultan 1999; Kembel and Cahill 2005) and biomass allocation patterns (Aerts et al. 1991; Poorter et al. 2012) to seed production in terms of the seed production timing and seed size (Meyer and Root 1993; Clauss and Aarssen 1994; Parciak 2002). It is not certain whether these findings are generalizable to seedlings. In the similar vein, we know very little about the evolutionary history of seed nutrient stoichiometry and the selection pressures that alter this stoichiometry (Kerkhoff et al. 2006), as well as the direct impact of maternal support (in terms of absolute amount of stored nutrients) on seedling growth.

Seedlings differ from adult plants due to the simple fact that they are dependent on nutrients stored in the seeds from which they germinated. These nutrients are special because they are species specific. Species identity includes specific information about amount of nutrients in the seeds available to the seedling. On the other hand, nutrients in the environment are more or less unpredictable and this unpredictability increases with the dispersal distance. Even seedlings that germinate in the closest vicinity to the mother plant cannot be certain about soil nutrient availability due to resource heterogeneity on a microspatial scale, and this uncertainty strongly increases with dispersal distance. A second significant advantage of seed reserves is that they are not subject to direct competition. These private resources are used by seedlings for development into self-sufficient individuals that are able to successfully survive and grow after exhaustion of these nutrients (Hanley et al. 2004). Seed reserves have primarily structure building function (i.e., development of resource-acquiring organs, including roots and shoots of seedlings) and maintenance function (e.g., supporting respiration especially in dense shade; Löwe and Dillenburg 2011).

Individual plant species differ remarkably in seed mass; for example, the smallest seeds of orchids weigh only $0.31 \mu\text{g}$ (Arditti and Ghani 2001), whereas seeds of *Lodoicea maldivica* can weigh as much as 18 kg (Edwards et al. 2015). On the other hand, intraspecific variation of seed mass is lower by several orders of magnitude (Harper et al. 1970). Moreover, mother plants grown in nutrient poor conditions prefer to reduce the number of seeds than their size (Vega et al. 2001). There may be considerable variability in seed mass between populations. Variability caused by the environmental conditions in which mother plant has grown has been documented for some species (Dorne 1981; Wellstein et al. 2013).

Further, there is developmentally based variability in seed mass. In many species, seed mass differs according to position in inflorescence, e.g., proximal versus distal branches of panicle of grasses (Simmons and Crookston 1979; Muchow 1990) or marginal versus central flowers of anthodium of Asteraceae (Forsyth and Brown 1982). Moreover, this seed mass heteromorphism can further be shaped by environmental conditions (Venable and Levin 1985; McGinley 1989). However, in spite of all these sources of intraspecific variation in seed mass, interspecific variability in seed mass is exceedingly larger than intraspecific variation. Consequently it is reasonable to design experiments that concentrate only on the interspecific variation. Also, intraspecific variability in seed mass is rather phylogenetically conservative (Hodgson and Mackey 1986; Westoby 1998) and most of the variability in seed mass can be attributed to family and genera (Peat and Fitter 1994).

While the seed mass of species is strongly determined by species phylogeny (Lord et al. 1995; Moles et al. 2005; Sims 2012), it still can be optimized according to life form or habitat type (Mazer 1989). The basic optimization mechanism involves seed size-number trade-off: the choice between producing many small seeds and relying on at least a couple of them to develop into adult plants, or producing only a few large seeds with good seedling provisions. Species with large seed mass have advantages during the seedling phase, and their improved survival has been shown many times, especially in extreme conditions, e.g., poor light or water availability (Grime and Jeffrey 1965; Leishman and Westoby 1994; Urbieta et al. 2008). On the other hand, species with smaller seed mass may have an advantage in early successional environments because they produce more seeds per unit canopy area per year (Jakobsson and Eriksson 2000) and they are likely to have longer dormancy (Rubio de Casas et al. 2017) and faster germination (Moles and Westoby 2004). In addition, chances of seed dispersal largely increase with decreasing seed mass, although dispersal syndrome and seed release height modify significantly this relationship (Moles et al. 2007; Muller-Landau et al. 2008; Thomson et al. 2011). Seedling survival of small-seeded species is also less dependent on seed reserves (Milberg and Lamont 1997). Furthermore, large seeds cannot be produced by small plants due to seed weight, indicating the physical limitations of seed size variability (Thompson and Rabinowitz 1989). Consequently, the multitude of processes involved in the seed-size number trade-off precludes simple solution of this trade-off (Smith and Fretwell 1974).

1.1 Seed nutrient stoichiometry

The absolute amount of nutrition provision by the mother plant (approximated by seed mass) is an important trait for explaining many ecological processes, but this is not the only way how plant maternal care is manifested. It is obvious that species with similar seed masses but entirely different proportions of basic nutrients (i.e., structural and nonstructural carbon, nitrogen, phosphorus) provide completely different starting conditions to their seedlings. Huge interspecific differences in seed nutrient composition are known for individual crop species (Kamel et al. 1982; Hoover and Sosulski 1991; Evers et al. 1999; El-Adawy and Taha 2001; Stevenson et al. 2007); nevertheless, to the best of my knowledge, the comparative data for wild species are generally lacking. Earle and Jones (1962),

Jones and Earle (1966) and Barclay and Earle (1974) studied the interspecific variability of the oil and protein content of many species to identify new potential plants for cultivation, but without asking broader ecological questions. The only ecologically conceived study by Bu et al. (2018) showed that relationships between basic seed macronutrient stoichiometry and phylogeny, life-history traits and altitude, as a proxy of environmental conditions, were important for the life cycle of plants.

The most variable component of seed mass is likely to be the amount of structural carbon. It does not play any role in seedling nutrient provision, but has a number of other functions. Testae with different thicknesses or different structures on the seed surface protect the embryo and are involved in seed dormancy (Debeaujon et al. 2000) or dispersal (van der Pijl 1982). Structural carbon is not used for seedling nutrition after germination although the abovementioned processes represent important selection pressures on seed mass. From the methodological point of view, it is important to note that while it contributes (often significantly) to seed element stoichiometry, it is not available for seedling growth and potentially confounds ecological interpretations of simple stoichiometric ratios.

Seedling establishment requires nutrients that can easily be mobilized during germination. These include non-structural carbon (stored in seeds as amylopectin, fructans or lipids; energy storage), nitrogen (stored in seeds as proteins; mechanical or catalytic functions) and phosphorus (stored in seeds mainly as phytate; important for nucleic acids and ATP). Immediately after germination, the seedling completely relies on these stored nutrients. Gradually, as it grows and develops, its root system and the above-ground part develop access to necessary nutrients from the environment, namely, nitrogen (in the form of ions NH_4^+ or NO_3^-) and phosphorus (in the form of the anion $H_2PO_4^-$) from the soil by roots and carbon (in the form of CO_2) through the leaves. Furthermore, individual nutrients are depleted from the seeds after different periods (Fenner 1986; Hanley and Fenner 1997). Thus, plant adaptation to certain type of environment might involve the ability to store nutrients that are lacking in the environment in seeds to provide the seedling a competitive advantage. Therefore, I hypothesize that plants from environments that differ in their nutrient availability will exhibit differences in seed nutrient stoichiometry. Namely, species adapted to shaded conditions, in which carbon limitation is stronger than limitation of soil-borne resources, will preferentially incorporate more nonstructural carbon into their seeds, and vice versa. Similarly, under unfertile conditions there will be pressure to store proportionally more nitrogen and phosphorus in the seed because above-ground competition in such an environment is weak and carbon availability is not limiting.

1.2 Root:shoot biomass partitioning

Seedling development refers to the first growth and development of its roots. The root system is an indispensable organ from the beginning of life of a new individual because it anchors the plant into the ground and is also necessary for water recovery. After some time (which can vary greatly between species; Moles and Westoby 2004), the aboveground part of the seedling starts to grow. Precise timing of the development and speed of growth of the aboveground part

is a crucial mechanism for coping with competition for light in the environment (Seiwa and Kikuzawa 1991; Galen and Stanton 1999; Moles and Westoby 2004).

The competition is strongly linked to the amount of nutrients in the environment (Weiner 1990; Wilberts et al. 2014); however, below- and aboveground competition differs in terms of symmetry. Belowground competition is mostly symmetric, meaning that a larger root system results in a proportional increase in nutrients received. In contrast, aboveground competition is typically highly asymmetric; i.e., the individual with the largest shoot captures the most light regardless of the size of the neighbours (Tilman, 1988; Weiner, 1990). Survival in this asymmetric aboveground competition is often a matter of life and death; therefore, it is a driving force of root:shoot biomass partitioning, especially in nutrient-rich environments (Keddy et al. 1997). The newly establishing seedling must carefully consider how it invests reserves stored in the seed. Seedling root:shoot biomass partitioning is one of the most important decisions in the early phase of ontogeny.

Availability of nutrients for the seedling is essentially a combination of available soil resources and resources stored in the seeds by the mother plant. It is likely that nutrients from these two different pools will differ in their impact on seedling development because nutrients stored in the seed are special given that they are not subject to competition and are also quite predictable for establishing the growth of new individuals in unpredictable and highly competitive conditions. Therefore, I hypothesize that each of these two pools of nutrients will have different effects on seedling root:shoot biomass partitioning during the first phase of their ontogeny. Moreover, I hypothesize that seedlings of small-seeded species will be more sensitive to soil nutrient availability because their seeds do not provide sufficient nutrients for successful development, and the requirements for establishing a functional root system as quickly as possible are stronger in these plants compared with large-seeded species.

1.3 Root system development

When the seed germinates, the radicle first breaks through the testa, and root system development is the first goal of new plants. The radicle changes to an elongated main root, and lateral roots arise soon thereafter. Although, the basic architecture and growing pattern of root systems of each species are genetically determined (Fitter et al. 1991), the structures are strongly shaped by actual environmental conditions. The newly established root can make the current root system denser or can extend it into a larger volume of soil. A dense root net with many lateral roots of higher order is advantageous in the environments with higher nutrient availability. A dense root system leads to increasing benefits from the area closest to the plant. However, if the surrounding area lacks nutrients, the plant does not gain an appropriate amount of resources by making other lateral roots, and it is likely more advantageous to send roots further. This choice is risky because the plant has no information on nutrient availability in distant soil regions, where resource availability can be even lower (He et al. 2003) or competition may be stronger (Gersani et al. 1998; McNickle and Cahill 2009). Plants must precisely balance the cost and possible benefits of generating new

roots, i.e., whether the new root will be able to gain more resources from the environment compared with the cost of root generation. (O'Brien et al. 2007).

In adult plants, nutrient availability in soil directly affects root system morphology, such as length of main and lateral roots and number of lateral roots or root thickness (Day et al. 2003; Hill et al. 2006; Giles et al. 2017). Generally, soil nutrient deficiency leads to reduced root system branching and faster growth of individual roots (Forde and Lorenzo 2001; Hill et al. 2006), which is mainly interpreted as an effort to identify nutrient richer patches. However, changes in root system morphology also alter the ability of the root system to utilize soil volume and transport nutrients (Fitter 1985; Bouda et al. 2018). However, the method used to generate new root is not only driven by quantity and distribution of soil resources but also water availability; therefore, not all morphological characteristics of the root system are necessarily affected only by nutrient availability. Moreover, as water availability is predictable, plants send their roots to a depth where water is usually more accessible (Lynch 1995; Hund et al. 2009).

The plant root system is modified to achieve the highest nutrient and water acquisition efficiency. Adult plants from nutrient-rich environments exhibit a higher rate of root phenotypic plasticity (Robinson and Rorison 1983; Fransen et al. 1999); however, species from nutrient-poor environments can make better use of short-term increases in nutrient availability (Campbell and Grime 1989). In contrast to adult stages, nutrients from the environment are likely to exhibit a reduced direct impact on growth and development (in terms of the amount of the nutrients directly used for growth) during the first phase of seedling ontogeny due to the incompletely developed root system of seedlings and the necessity to develop a skeleton of a structurally reliable root system. It is not clear how nutrients from the soil and seed interact with each other and how these two nutrient pools differently impact the establishment of the seedling root system. Similar to root:shoot biomass partitioning issues, I also hypothesize that nutrients from the seeds and from the soil will have different impacts on root system morphology and development. I also assume that seedlings of small-seeded species and their root system will be more sensitive to nutrient availability in the environment.

1.4 My findings

In this thesis, I focus on the main decision-making steps in plant generative reproduction cycle, namely maternal provision of seeds and usage of these resources during seedling establishment. Coping with all environmental limitations and the establishment of a self-sufficient individual under the constraints of all environmental limitations are the main goals of each germinating seed. This process includes building the root system and shoot part such that they will effectively perform all their functions after seed reserves are exhausted and lead to success in competition with other individuals. In this study, I examine effects both of the nutrients available in the soil and those stored in the seed. The question remains whether seedlings of large-seeded species have the option (because of higher nutrient reserves from the mother plant) to adapt their root:shoot biomass partitioning more flexibly and optimize a root system for future needs, whereas seedlings of small-seeded species focus on short-term goals, i.e., finding at least some resources.

To understand the role of maternal provisions (in terms of total amount of nutrients measured as seed mass and their stoichiometry) in seedling development in the first phase of ontogeny, we performed chemical analysis of seed nutrients reserves and several experiments in controlled laboratory conditions. I present their results in this thesis.

In the second chapter, we measured seed nutrient stoichiometry (namely, proportion of seed nitrogen, phosphorus and nonstructural carbon) in 500+ wild herbaceous species. We investigated the relationship between individual nutrients stored in seeds with the seed mass and environmental conditions in which species typically occur. We found that amounts of macronutrients in seeds are not accidental; while they are largely determined by the evolutionary history of species, their proportions also reflect seed size and environmental conditions in ways that are different for each measured element. Phosphorus storage was affected mainly by seed mass; small-seeded species stored proportionally higher amounts of phosphorus in their seeds, likely due to the poor accessibility of soil phosphorus in the environment. In contrast, proportions of nitrogen and nonstructural carbon in seeds are driven by environmental conditions in which species typically occur. We found similar patterns for both elements; specifically, species preferentially store the nutrient that is expected to be lacking in the environment.

In the third chapter, we asked questions about how the seedling use resources stored in seeds by the mother plant for its growth. Therefore, we observed seedling root:shoot biomass partitioning according to nutrients available in the soil and seed mass of species. We showed that seed mass is an important determinant for biomass allocation patterns during seedling ontogeny. Large-seeded species change their root:shoot ratio faster over time, and increasing nutrients in the soil affected these species more in terms of faster development of shoot parts. This ability could be a key advantage in asymmetric aboveground competition and thus could constitute a selective factor for optimum seed mass in highly productive environments.

In the fourth chapter, we observed the role of soil nutrient availability and species-specific seed mass on seedling root system development. The main purpose of that study was to show how the development of the seedling root system is affected by available nutrients both in soil and seed. We wanted to know whether, and how, these nutrients interact with each other. We used rhizoboxes to precisely record the root system architecture. We found that both sources of nutrients are important for seedling root system development in the first phase of ontogeny, but each source affected different sets of root system parameters. Whereas nutrients available in the soil primarily affected root system branching and root size, seed mass had greater effects on root system shape. Importantly, I would also like to point out that seeds contain not only soil-borne resources, but also carbon, both structural and nonstructural. As we show in the second chapter, seed nutrient stoichiometry is phylogenetically constrained; consequently we are using pairwise comparisons of species within one family to correct for phylogenetic effects of seed size and nutrient stoichiometry.

The fifth chapter presents the methodology and shows the limitations of the use of rhizoboxes for root system visualization in the experiments. We showed that rhizoboxes could affect seedling behaviour; root:shoot biomass partitioning slightly differed between plants growing in the rhizoboxes and regular pots. On

the other hand, we found no differences between total biomass of plants growing in different type of pots. These findings suggest that while individual plants detect space constraints of the rhizobox, their root systems developed in a similar fashion in the rhizoboxes and regular pots. Therefore, we believe that rhizoboxes are a good tool for observing behaviour, albeit not for evaluating absolute plant growth. We thus conclude that our conclusions from the experiment in which we used the rhizoboxes are valid.

1.5 Conclusions

Understanding patterns of maternal care is one of the key approaches to understand how organisms perceive their environment on an evolutionary time scale. Fine-tuning seed mass and seed nutrient stoichiometry according to the expected environment where the offspring develop is driven via the evolutionary optimization of the maternal care. In this thesis, we showed that despite the strong phylogenetic constraints of seed size and seed nutrient provision, maternal care in plants reflects the expected environmental conditions and is optimized for good seedling provision and, subsequently, for increasing the success of seedling establishment. Moreover, on an ecological time scale, the amount of nutrient provision from the mother plant plays an important role during seedling establishment. We also showed that higher amounts of nutrients stored in the seeds allow seedlings to adapt their growth and development according to environmental conditions more flexibly, underlining the importance of maternal care for subsequent seedling development.

This thesis provides a few answers to the questions that were initially posed. However, with each of these answers, more questions arise, and I am increasingly curious about all of the possibilities regarding the support that mother plants provide for their offspring, the limits of maternal support and how maternal support is constrained at different levels. Regardless of the answers to these questions, at the moment the seed leaves the plant, the cards are dealt, and the new exciting game of life begins.

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Chapter 2

Maternal support in plants: CNP stoichiometry in seeds varies to match expected nutrient limitation by seedlings

Tereza Mašková and Tomáš Herben

Abstract

Maternal care is one of the key fitness parameters in all organisms. In plants, maternal care has a form of provision of nutrients to seedlings by seeds. Although seed mass (the overall amount of nutrients) is the most studied proxy for this maternal provision, this proxy completely neglects the seed nutrient stoichiometry, i.e., the proportions of light-dependent resources (carbon) and soil-dependent resources (nitrogen and phosphorus), which can strongly affect seedling survival. We tested the hypothesis that seed nutrient stoichiometry is fine-tuned to the expected conditions of seed germination, namely, that plants from light-limited conditions will have proportionally more carbon than plants that are not light-limited and vice versa. We used a phylogenetically informed comparative approach to examine proportions of nitrogen, phosphorus and nonstructural carbon in 510 species and the relationships of these elements to the environment in which the seedlings typically germinate. We showed that, in spite of the strong phylogenetic signal in nutrient stoichiometry, proportions of nitrogen and nonstructural carbon change according to our hypothesis, i.e., seeds are primarily provided with nutrients expected to be limiting in a given environment. This enables seedlings to develop independent resource-acquiring organs quickly. Furthermore, we demonstrated that seed nutrient stoichiometry depends on seed mass; namely, the proportion of phosphorus is high in small seeds, and the proportion of unusable structural carbon is high in large seeds. All these results suggest that seed nutrient stoichiometry is under selection driven by both seed mass and the environment in which seedlings are likely to germinate.

Key words: C:N:P ratio · Evolutionary history · Nitrogen · Nonstructural carbon · Nutrient stoichiometry · Phosphorus · Seed size

2.1 Introduction

Maternal care is one of the key components of fitness in all organisms. In plants, the key component of maternal care is the provision of resources in the seed to offspring (Roach and Wulff 1987). As an emerging seedling cannot absorb the resources necessary for life in its early ontogeny from the environment, it is dependent on the resources stored in the seed (White and Veneklaas 2012). The development of initial resource-acquiring organs (fine roots and leaves) is thus entirely determined by the maternal provision of resources that the seedling draws from the seed, both in terms of the overall amount of nutrients and in their proportions. Once these organs are developed, seedling growth is autocatalytic, but the initial offset determined by the maternal investment can determine the fate of the seedling, particularly under conditions of asymmetric competition, which tends to amplify initial size differences (Schwinning and Weiner 1998). It is therefore important to endow the seedling with resources best suited for the environment where the seedling is likely to develop.

The amount of these resources is primarily determined by the mass of the seed. The seedlings of large-seeded species have an advantage in early phases of growth (Milberg and Lamont 1997; Weiner et al. 2010; Lönnberg and Eriksson 2013), but the cost of this advantage is the decreased number of seeds produced by parental individuals (Harper et al. 1970; Jakobsson and Eriksson 2000; Bruun and Brink 2008), limited dispersal potential of such seeds (Venable and Brown 1988; Hammond and Brown 1995; Westoby et al. 1996) and higher risk of predation (Reader 1993; Maron et al. 2012). Biogeographical and ecological patterns of seed mass convincingly show that evolution of this trait has been strongly shaped by selection on either seed mass or seed number (Moles and Westoby 2004; Gallagher and Leishman 2012; Kavanagh and Burns 2014).

However, seed mass is not the only component of maternal care in plants, although it is its most studied component. A variable part of the overall seed mass is built from structural tissues that serve additional functions such as embryo protection, dormancy or dispersal. Consequently, this carbon cannot be mobilized for seedling growth at emergence and thus does not meaningfully contribute to the maternal provision of usable carbon to seedlings. Seed mass thus does not constitute the best proxy of the nutrient (carbon) provided to seedlings, as dispersal-related structures (appendages, etc.) are highly variable among individual plant species and thus blur the relationship between seed mass and the maternal provision of carbon (Hintze et al. 2013).

Second, individual nutrients, namely, nonstructural carbon, nitrogen and phosphorus, need not be stored in the same proportions. Interspecifically, seeds are arguably the most variable part of the plant body in terms of their chemical composition (Kerkhoff et al. 2006; Bu et al. 2018). The contents of major nutrient components, such as nonstructural carbon (oils, polysaccharides), nitrogen and phosphorus, in seeds exhibit enormous variations across species (Barclay and Earle 1974; Kerkhoff et al. 2006; Bu et al. 2018), which are comparable to the variation in seed size. This means that the maternal provision of individual nutrients to seedlings is highly variable. The good provision of seeds with nutrients that are limiting may strongly contribute to seedling survival (Milberg and Lamont 1997; Vaughton and Ramsey 2001). It is known that C:N:P stoichiometry

affects seedling development (Milberg and Lamont 1997; Vaughton and Ramsey 2001), and it is likely that the existing high variation in the seed C:N:P stoichiometry should be interpreted in this respect, although we know little about the true determinants of it.

We therefore hypothesize that plant species from different environments have differing seed nutrient stoichiometry. We assume that seeds will have a relatively low content of nonstructural carbon in nutrient-poor and light-rich conditions where light is not limiting. Under such conditions, belowground nutrients are in short supply, and competition for these nutrients will be stronger. We therefore predict that plants preferentially store nitrogen and phosphorus in their seeds at the expense of nonstructural carbon. In contrast, plants from carbon-limited environments (i.e., shaded habitats) will proportionately incorporate more non-structural carbon into their seed.

We work with two additional hypotheses. First, we assume that seed size places a constraint on the overall amount of nutrient storage. As all plants have to provision their seedlings with a certain amount of nutrients, we hypothesize that small-seeded species must use the whole seed for nutrient storage only and thus have limited options to develop the seed coat or other structures on the seed surface used for seed protection, dormancy or dispersal. In contrast, large-seeded plants can afford the luxury of extra structural carbon for structures used for dispersal or protection. We thus hypothesize that the sum of all stored non-structural nutrients will make up a larger proportion of the overall seed mass in seeds that are small than in seeds that are large. Second, we assume that proper investment in seed reproduction is much more important in annual plants, which reproduce regularly by seeds and have no other possibilities for escape (e.g., clonality or postponing reproduction to the next season), than in perennial plants. In contrast, long-lived perennials persist as adults and may be able to wait for a rare event that would be favorable for seedling establishment. We therefore hypothesize that the maternal investments and, hence, the environmentally driven variation in seed nutrient stoichiometry will be stronger in annual plants than in perennial plants.

We examine these hypotheses using a large set of herbaceous species across the Angiosperm phylogenetic tree that were selected to cover major lineages and ecological groups. We measured the contents of nonstructural carbon, nitrogen and phosphorus in their seeds and determined the correlations of these nutrients with the parameters of the habitats where these species typically germinate and with the seed mass and lifespan of these species.

2.2 Materials and Methods

2.2.1 Species selection

The species were selected from Central European Eu-Dicot and Monocot flora to cover a wide range of seed masses, ecological conditions and lineages of the phylogenetic tree. Aquatic and semiterrestrial species were excluded because their seeds' nutrient stoichiometry is likely to be optimized in response to different

important factors than the factors that affect terrestrial species, e.g., the availability of oxygen in water. Additionally, parasitic and hemi-parasitic species were excluded because they gain nutrients through their hosts and finding the host, not self sustenance, is the primary goal of their seedlings. Using these criteria, we selected 510 species from 51 families covering a phylogenetically diverse group of species. We acquired all seeds from a local commercial supplier (Planta Naturalis; www.plantanaturalis.com), which grew all the species in one place, so the impacts of different environmental conditions (e.g., temperature, precipitation or altitude) were excluded.

2.2.2 Measurements

We counted 100 seeds per species, allowed them to dry in the air and weighed them to estimate species seed mass (Kleyer et al. 2008). Then, we analyzed the contents of nitrogen, phosphorus and nonstructural carbon (further referred to as nutrients) of the seeds. Nonstructural carbon was measured as the sum of fructans, starch and oils corrected to carbon content (we estimated the C weighted fraction in each measured compound as 44.45% C for fructans and starch and 77.2% C for oils (Rasor and Duncan 2014; Orsavova et al. 2015)). The nitrogen content was measured by flow injection analysis after Klejdahl mineralization. The phosphorus content was measured by flow injection analysis after perchloric acid mineralization. The contents of starch and fructans were measured by the enzymatic procedure Megazyme (McCleary et al. 1994). The limit of detection for N and P was 0.02 g/l, while the limit of detection for starch and fructans was 5 g/l. To determine the oil contents of seeds, whole seeds were dried at 105°C for 46 hours, and then the oil content was measured by Soxhlet (ISO 2009). The measured starch content was zero for thirteen species. We replaced this zero by the number 0.00025 (half of the detection limit reported by lab) for statistical analysis. The contents of nitrogen, phosphorus, fructans and starch were measured by the Analytical Laboratory of the Institute of Botany ASCR in Třeboň, and the content of oils was measured by the Department of Chemistry of the Czech University of Life Sciences in Prague.

As a proxy of species niches along major environmental gradients, we used Ellenberg indicator values for nutrients, light and pH (Ellenberg 1992) and indicator values of disturbance severity (Herben et al. 2016). We excluded species that were reported by Ellenberg et al. (1992) as indifferent to the given factor. Data about the Ellenberg indicator values for nutrients, light, and pH were available for 433, 461, and 390 species, respectively. We further used disturbance severity indicator values (Herben et al. 2016) to approximate the disturbance niche of species, i.e., the degree of soil disturbance preceding seedling establishment. Data about disturbance severity were available for 389 species.

Data about annual/perennial lifespan were taken from the Clonal Plant Database (CLOPLA) (Klimešová et al. 2017). Such data were available for 506 species. We used a phylogenetic tree from the Daphne database (Durka and Michalski 2012) as a source of information on the phylogenetic relationships of species. Phylogenetic data were available for 483 species.

2.2.3 Data analysis

We calculated a correlation matrix between proportions of stored nutrients (C, fructans, starch, oils, N, P). We examined the structure of these correlations using principal components analysis (PCA) in the `vegan` package, version 2.5-3 (Oksanen et al. 2018), in R version 3.4.4 (R Core Team 2018).

We determined the phylogenetic signal for each stored nutrient (nitrogen, phosphorus, fructans, starch, oil and nonstructural carbon). We used the `pgls` function from the package `caper` in R (Orme et al. 2013), fitted Pagel’s λ maximum likelihood (1999) and calculated its upper and lower confidence limits.

We examined relationships between environmental proxy variables (Ellenberg indicator values for pH, nutrients and light and indicator values of disturbance severity) and the proportions of stored nutrients (nitrogen, phosphorus and nonstructural carbon) using both nonphylogenetic and phylogenetic linear models. In both cases, we used a stepwise model selection approach, starting with models with all environmental predictors, seed mass, annual lifespan and interactions of the latter with all environmental predictors. We simplified the models using backward selection and the Akaike information criterion to find the best model. In the phylogenetic analyses, we first fitted the initial model using restricted maximum likelihood, determined the most likely value of Pagel’s λ , and used this value to simplify the same model fitted using unrestricted maximum likelihood. The final model was then refitted with REML to report values of individual regression coefficients.

We also used standard major axis regression on log-transformed data using `lmodel2` to determine the shape of the relationship between seed nutrient stoichiometry and seed mass. We performed separate analyses for each observed stored nutrient (nitrogen, phosphorus, starch, fructans, oils and nonstructural carbon). Furthermore, we ran one analysis for all observed nutrients together (calculated as the sum of the absolute values of nitrogen, phosphorus and nonstructural carbon) called storage size in the seed. All stored nutrients, seed mass and storage size values were absolute values (i.e., mg per seed). Stored nutrients, storage size and seed mass values were all logarithmically transformed. In this regression, a slope significantly different from 1 indicates deviation from linearity in the relationship between the stored nutrient and seed mass.

2.3 Results

The stored nutrient contents ranged from 0.12% to 1.46% for phosphorus, from 0.82% to 9.87% for nitrogen, from 0% to 82.22% for starch, from 0.4% to 6.67% for fructans, from 0.7% to 58.75% for oils and from 2.08% to 60.68% for nonstructural carbon (calculated as the sum of starch, fructans and oils corrected for different carbon contents). Single observed nutrients were correlated with each other (see Table 2.1 for details). The strongest relationship was between the content of nitrogen and phosphorus (Pearson $R = 0.62$) and between starch and oils ($R = -0.4$). Correlations between individual stored nutrients can also be seen in the PCA plot (Fig. 2.1). The first axis explained 38.6% of the variability and represented primarily variation in phosphorus and nitrogen. The second axis

explained 30.6% of the variability and represented the form of carbon storage (fructans and starch versus oils).

Table 2.1: Correlation matrix of nutrients stored in seeds. The percentage of stored nutrients was used. Coefficient between total nonstructural carbon and its single components (starch, fructans and oils) is not shown because nonstructural carbon was calculated from these three components.

	Phosphorus	Nitrogen	Starch	Fructans	Oils	Total nonstructural carbon
Phosphorus	-	0.626	0.001	0.216	0.385	0.385
Nitrogen		-	0.114	0.366	0.143	0.253
Starch			-	-0.017	-0.395	-
Fructans				-	-0.183	-

All measured seed parameters showed a strong phylogenetic signal, although its magnitude varied. The content of nonstructural carbon (Pagel's $\lambda = 0.927$), particularly each of its components (Pagel's λ for starch, fructans and oils were 0.887, 0.906 and 0.934, respectively), was highly determined by phylogeny. In particular, polysaccharides were found primarily in Fabaceae, Malvaceae, Amaranthaceae, and Caryophyllaceae, whereas oils prevailed in many other families (Fig. 2.2). In contrast, the contents of nitrogen (Pagel's $\lambda = 0.688$) and phosphorus (Pagel's $\lambda = 0.827$) showed weaker phylogenetic signals (see Table 2.2).

Table 2.2: Phylogenetic signal in nutrients stored in the seed. The percentage of stored nutrients was used. Total nonstructural carbon was calculated from carbon content in starch, fructans and oils.

Stored nutrient (%)	Pagel's λ	95% CI
Phosphorus	0.827	0.718–0.896
Nitrogen	0.688	0.524–0.809
Starch	0.887	0.821–0.932
Fructans	0.906	0.857–0.941
Oils	0.934	0.884–0.965
Total nonstructural carbon	0.927	0.870–0.961

The Ellenberg indicator values covered almost the entire environmental gradient (with values ranging from 2 to 9 for pH, from 2 to 9 for light and from 1 to 9 for nutrients, all from the possible range of 1-9). The index of disturbance severity ranged from 0.089 to 1 (from the possible range 0-1).

Linear models between the proportion of stored nutrients and the proxy of species environmental niche, seed mass and annual lifespan showed significant relationships between stored nutrients and the environment just for nitrogen and nonstructural carbon. The amount of nonstructural carbon was positively correlated with the Ellenberg indicator value for nitrogen ($p < 0.001$; Fig. 2.3a) and with the annual lifespan of species ($p < 0.01$). These two relationships were marginally significant in the phylogenetically corrected model ($p=0.06$ and $p=0.07$). The amount of stored nitrogen was negatively correlated with the index of disturbance severity ($p < 0.001$) and marginally positively correlated with the

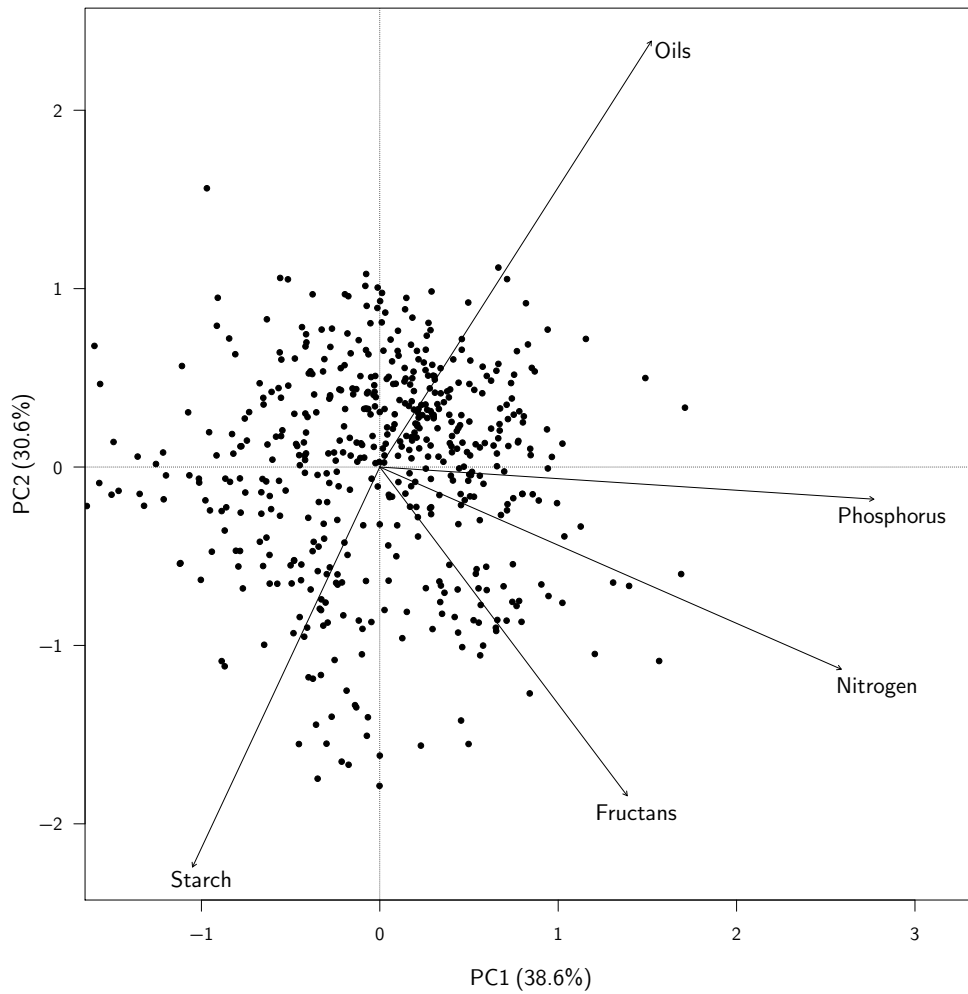


Fig. 2.1: Principal component analysis between proportions of basic macronutrients stored in seeds of 510 central European herbaceous species (dots). The first and second ordination axes account for ca. 38.6% and 30.6% of overall variation in the data. Starch, Fructans and Oils indicated carbon contained in these compounds.

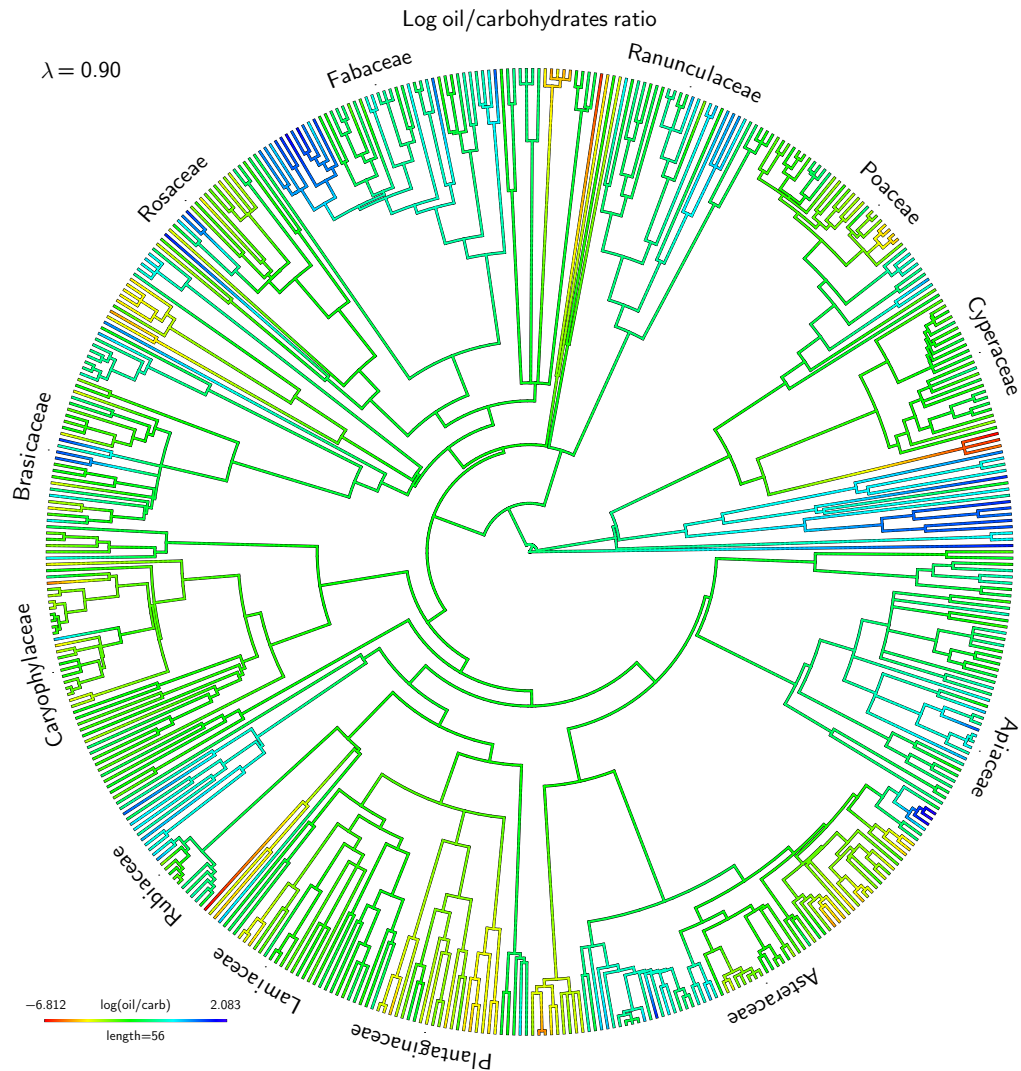


Fig. 2.2: Phylogenetic pattern in storing nonstructural carbon as oils or carbohydrates. Red colour indicate species with relative high storage of carbohydrates in their seeds, blue colour indicate species with relative high storage of oil in their seeds.

Ellenberg indicator value for light ($p = 0.059$, Table 2.3). The negative relationship between stored nitrogen and the index of disturbance severity was also significant in the phylogenetically corrected model ($p < 0.01$; Fig. 2.3b). There was no relationship between the amount of stored phosphorus and the proxy of species environmental niche or annual lifespan in either model, but both models showed a strong negative relationship between the amount of stored phosphorus and the seed mass of species ($p < 0.001$).

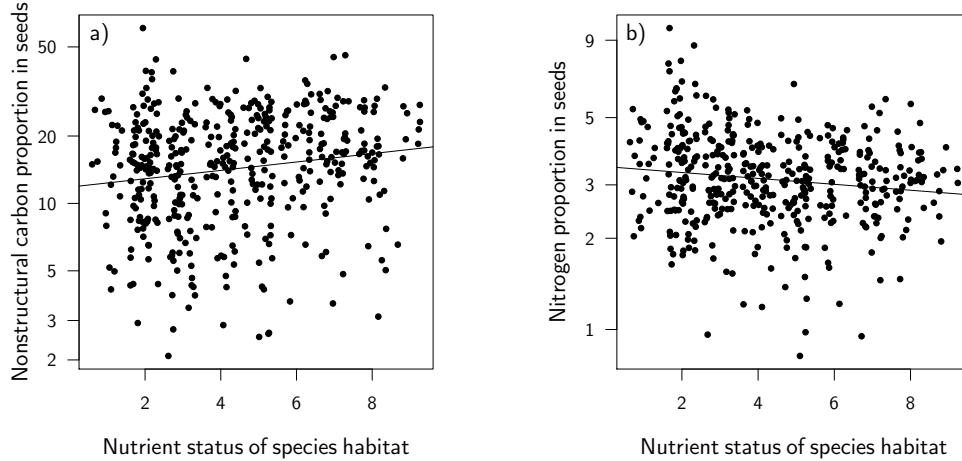


Fig. 2.3: Relationship between nutrient status of species habitat (approximated by Ellenberg indicator value for nutrients) and proportion of nonstructural carbon ($R^2 = 0.022$) (a) and proportion of nitrogen ($R^2 = 0.016$) (b) in seeds.

The seed masses of species ranged from 0.011 mg to 80.3 mg. There were linear relationships between stored nitrogen and oils and seed mass ($R^2 = 0.95$ for nitrogen and 0.74 for oils) (see Table 2.4). However, there was a significant deviation from linearity in the relationship between phosphorus content and seed mass (95% confidence interval did not cover unity, $R^2 = 0.95$). The phosphorus content was higher for small-seeded species than for species with larger seeds (Table 2.4; Fig. 2.4).

As mentioned above, there was a linear relationship between stored oil and seed mass, but the other nonstructural carbon components and the total content all nonstructural carbon were in a nonlinear relationship with seed mass (95% confidence intervals were higher than 1 for starch and fructans, $R^2 = 0.24$ for starch and $R^2 = 0.83$ for fructans (95% confidence intervals were lower than 1 for nonstructural carbon; $R^2 = 0.87$; Table 2.4). Furthermore, there was also a nonlinear relationship between storage size and seed mass (95% confidence intervals were lower than 1; $R^2 = 0.92$; Table 2.4), which means that species with small seeds use more of the seed biomass for nutrient storage than species with large seeds.

Table 2.3: Relationship between proportions of individual nutrients stored in seeds and species niche along environmental gradients, seed mass and annual lifespan, analyzed both by nonphylogenetic and phylogenetic models. Seed mass was log-transformed. Stored nutrients and proxies of environmental gradients were scaled to zero mean. — indicates effects excluded by the stepwise model selection approach. . indicates significant relationships. Nutrients – Ellenberg indicator value for nutrients, Light – Ellenberg indicator value for light, pH – Ellenberg indicator value for pH.

	Phosphorus		Nitrogen		Total nonstructural carbon	
	lm	pgls	lm	pgls	lm	pgls
Adjusted R^2	0.05**	—	0.04**	—	0.09***	—
Estimated λ	—	0.62	—	0.66	—	0.90
Nutrients	—	0.05	—	-0.01	0.18**	0.11 .
Light	—	0.05	0.11 .	0.05	—	-0.03
pH	—	-0.01	—	0.01	—	-0.01
Disturbance severity	—	0.03	-0.19 **	-0.17 *	0.14	0.09
Seed mass	-0.13	-0.19 ***	—	-0.14 ***	-0.15 ***	-0.05
Annual lifespan	—	-0.25	—	-0.10	-0.52 *	-0.30 .

Table 2.4: Relationship between nutrients stored in the seeds and seed mass (model II regression). Absolute amount of stored nutrients was used, and all variables were log-transformed. Nonstructural carbon was calculated from starch, fructans and oils. Storage size was calculated as the sum of phosphorus, nitrogen and nonstructural carbon. R^2 was calculated from ordinary least squares regressions. Regressions with significant nonlinearity are indicated in bold.

Stored nutrient	Model II slope	95% CI	R^2
Phosphorus	0.688	0.524–0.809	0.954
Nitrogen	0.827	0.718–0.896	0.954
Starch	0.887	0.821–0.932	0.230
Fructans	0.906	0.857–0.941	0.826
Oils	0.934	0.884–0.965	0.740
Total nonstructural carbon	0.927	0.870–0.961	0.874
Storage size	0.955	0.932–0.976	0.921

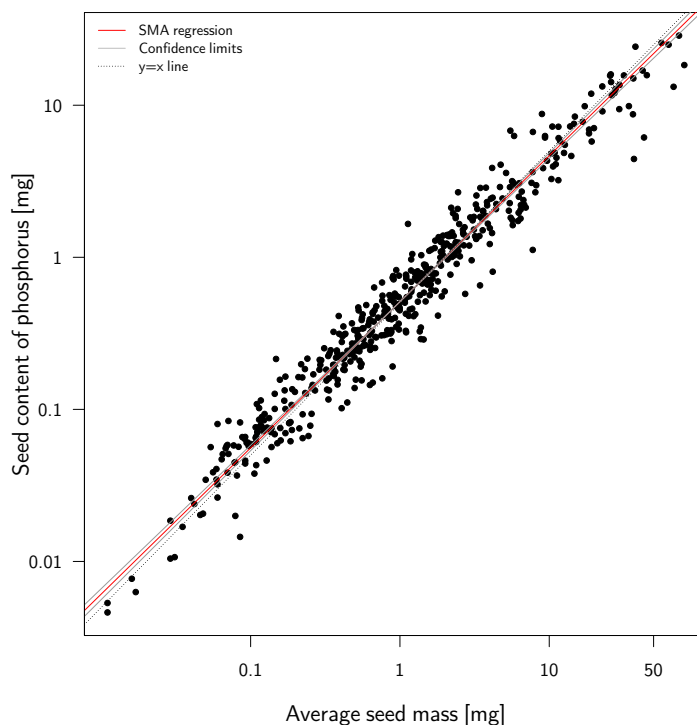


Fig. 2.4: Relationship between phosphorus stored in the seeds and seed mass (model II regression). An absolute amount of stored phosphorus was used, and both variables were log-transformed. Slope of SMA regression = 0.961, $R^2 = 0.954$, — SMA regression, — confidence limits, ··· $y = x$ line.

2.4 Discussion

Our data strongly support the notion that interspecific variability in seed nutrient stoichiometry is high and is highly conserved in evolution. This is particularly the case for nonstructural carbon, whereas nitrogen and phosphorus are much less phylogenetically conserved. The form in which carbon is stored is also highly conserved, often at the family level (Levin 1974). This shows that the overall high seed-trait conservatism known for seed mass (Moles et al. 2005b) and dispersal syndromes (Buoro and Carlson 2014) also holds true for nutrient stoichiometry, particularly the stoichiometry of nonstructural carbon and different types of stored carbon.

Despite this great influence of evolutionary history on seed nutrient stoichiometry, the contents of basic macronutrients in seeds showed a clear tendency to be optimized for the environmental conditions in which the species typically occurs and therefore where seeds are likely to germinate. In particular, the proportions of nitrogen and nonstructural carbon were affected by the species niche along environmental gradients. Consistently for all observed nutrients, plants tended to preferentially store the nutrient that was expected to be lacking in the typical species environment. Specifically, seeds of species from nutrient-rich environments typically had a lower proportion of nitrogen and a higher proportion of nonstructural carbon than seeds of species from nutrient-poor environments. Importantly, the relationship between nitrogen and nutrient turnover (approximated by the in-

dex of disturbance severity) in the environment remained strong and significant in the phylogenetic analysis, indicating that this relationship is due to recent evolution/selection. These findings suggest that the type of competition is the main driver of nutrient storage in seeds (as was shown previously for the coevolution of seed mass and light requirements for germination (Milberg et al. 2000)). Species from environments with strong above-ground competition, where there is pressure to have fast shoot development, stored more nonstructural carbon, and species from environments with strong below-ground competition preferentially stored nitrogen in their seeds. We therefore conclude that seed nutrient composition should be associated with the need to cope with competition at the seedling stage.

In an earlier study from the Tibetan Plateau, the seed nitrogen content was found to be related to altitude (Bu et al. 2018). While the authors interpreted this trend as a result of the need for faster development in the shorter growing seasons of higher altitudes, we believe that the growing season length is not necessarily the only explanation for the relationship. The relationship between the nitrogen proportion in seeds and soil nutrient availability is likely to explain the relationship between seed nitrogen content and altitude because nitrogen in the soil may be less available at higher altitudes due to decreased decomposition (Soethe et al. 2008; Thebault et al. 2014; but see Wilson 1993) and carbon limitation, since competition for light is less severe in high than in low altitudes (Coomes and Allen 2007).

We also partly confirmed our hypothesis that seed mass affects seed nutrient stoichiometry. This is specifically true for phosphorus, which is stored in higher proportions in small-seeded species than in large-seeded species. Additionally, there was a tendency for the proportion of phosphorus to be balanced with respect to the proportions of nitrogen and nonstructural carbon. Plants probably modulate their phosphorus content because of the generally low phosphorus availability in the environment (Richardson et al. 2009). It was previously shown that nitrogen reserves in the seeds are exhausted earlier than phosphorus reserves (Slot et al. 2013). It seems likely that plants are optimizing the proportion of phosphorus in their seeds to cover the first phase of their ontogeny without a fully developed root system. This trend was evident across all phylogeny lineages. Therefore, we suppose that the ability to store a high proportion of phosphorus is common in all species, and the degree to which this tendency will manifest is dependent on the seed mass of the species, which could be connected with other plant traits (Moles and Westoby 2006), environmental factors (Salisbury 1974; Moles et al. 2005a), dispersal strategies (Westoby et al. 1996) or granivore pressure (Chen and Valone 2017). In contrast to this finding, intraspecific variation in the relationship between seed mass and the proportion of phosphorus has been shown to be positive (Vaughton and Ramsey 2001; Obeso 2012), suggesting that different constraints and tradeoffs operate at interspecific and intraspecific scales.

We also confirmed strongly phylogenetical constraints in the oil-starch partitioning in seeds, which supports our general knowledge based primarily on crop species. The biochemical pathways for the production starch and oils are not independent; they influence each other (Martin and Smith 1995), although the exact nature of their relationships is still not known (Lin et al. 2006). The metabolic production of oils requires more energy than the synthesis of starch

(Johnson and Alric 2013); therefore, the energy density of oils is higher than that of starch. This implies that oils should be preferably stored by small-seeded species, but our data do not support this implication, as the content of oils did not show any relationship to seed size, in contrast to polysaccharides.

Our results show that seed nutrient stoichiometry is the seed trait of similar importance as seed mass and hence deserves much closer attention. Both of these traits are influenced by different selection pressures and are important at different stages in the plant life cycle. While seed mass is important for dispersal and seed survival before germination and during germination, nutrient stoichiometry is primarily important for optimizing seedling development. In contrast to seed mass, nutrient stoichiometry is unlikely to play an important role in seed dispersal, which is largely determined by seed mass and structural carbon components. However, nutrient stoichiometry is also likely (together with seed mass) to affect seed herbivory, as animal herbivores are known to select their food based on its nutrient contents (Greig-Smith and Wilson 1985; Díaz 1996; Ríos et al. 2012).

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Chapter 3

Root:shoot ratio in developing seedlings: how seedlings change their allocation in response to seed mass and ambient nutrient supply

Tereza Mašková and Tomáš Herben

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Abstract

Root:shoot (R:S) biomass partitioning is one of the keys to the plants' ability to compensate for limiting resources in the environment and thus to survive and succeed in competition. In adult plants, it can vary in response to many factors, such as nutrient availability in the soil or reserves in the roots from the previous season. The question remains whether, at the interspecific level, reserves in seeds can affect seedlings' R:S ratio in a similar way. Proper allocation to resource-acquiring organs is enormously important for seedlings and is likely to determine their survival and further success. Therefore, we investigated the effect of seed mass on seedling R:S biomass partitioning and its interaction with nutrient supply in the substrate. We measured seedling biomass partitioning under two different nutrient treatments after two, four, six and twelve weeks for seventeen species differing in seed mass and covering. We used phylogenetically informed analysis to determine the independent influence of seed mass on seedling biomass partitioning. We found consistently lower R:S ratios in seedlings with higher seed mass. Expectedly, R:S was also lower with higher substrate nutrient supply, but substrate nutrient supply had a bigger effect on R:S ratio for species with higher seed mass. These findings point to the importance of seed reserves for the usage of soil resources. Generally, R:S ratio decreased over time and, similarly to the effect of substrate nutrients, R:S ratio decreased faster for large-seeded species. We show that the seed mass determines the allocation patterns into new resource-acquiring organs during seedling development. Large-seeded species are more flexible in soil nutrient use. It is likely that faster development

of shoots provides large-seeded species with the key advantage in asymmetric above-ground competition, and that this could constitute one of the selective factors for optimum seed mass.

Key words: Biomass partitioning · Interspecific comparison · Nutrient availability · R:S ratio · Seed mass · Seedling development

3.1 Introduction

Root:shoot (R:S) biomass partitioning is one of the mechanisms by which plants cope with limitations imposed by growth-constraining resources in the environment (Bloom, 1985; Bonifas and Lindquist, 2006) and may ultimately influence the rate of plant growth (Poorter, 1989). Thus, plants distribute higher proportions of biomass into leaves and stems in nutrient-rich environments where above-ground competition for light is strong, whereas in nutrient-poor environments, where below-ground competition prevails, they allocate a higher proportion to roots (Tilman, 1985). The relationship between type of competition (above- versus below- ground) and biomass partitioning is not linear, because above-ground competition is mostly highly asymmetric (Tilman, 1988; Weiner, 1990), in contrast to symmetric or just weakly asymmetric below-ground competition (Cahill Jr and Casper, 2000; Raynaud and Leadley, 2005). Biomass partitioning in adult plants develops in response to many factors and may show strong lags in these responses (Kobe et al., 2010; McCarthy and Enquist, 2007).

Allocation plasticity is also immensely important for seedlings as the seedling phase is the most vulnerable stage in the generative reproduction cycle for most of plant species and a swift and well-tuned allocation response can have a direct impact on their survival (Lloret et al. 1999). Seedlings are not able to obtain all their necessary resources from the environment (Deleens et al. 1984; White and Veneklaas, 2012; Nadeem et al. 2013), resources stored in the seeds are hence driving force of their early growth (Modi and Asanzi 2008; Liu et al. 2010). Importantly, reserves stored in the seed, i.e. nonstructural carbon, nitrogen and phosphorus are fully available to the developing seedling, and are highly predictable compared with unpredictable availability of soil nutrients and light.

The ultimate success of a seedling depends on the development of its own resource-acquiring organs (leaves and roots), but also how well they can respond to the ambient environment, using the predictable maternal resources of all nutrients in the seed (determined primarily by seed mass). Indeed, the proportions of maternally-provided and acquired resources change during seedling development. Whereas seedlings invariably develop roots first (to obtain water), their relative investment into leaves versus roots as carbon- and nutrient-acquiring organs, respectively, can also change over time (Gedroc et al., 1996; McConnaughay and Coleman, 1999).

Despite different composition, reserves stored in seeds probably can play the same role for seedlings as storage organs do for perennials: allowing the plastic redistribution of the resource, thus supporting optimal biomass allocation in changing conditions (Mironchenko and Kozłowski, 2014). Surprisingly, we know only a little about the role of seed mass in seedling biomass partitioning. Seedling biomass partitioning has been shown to change in time (Nadeem et al. 2013) and response to environmental conditions (Parker et al. 2006), but there are no com-

parative data how it changes among species. Huge interspecific variation of seed mass, and hence amount of stored resources can be used to determine how are these mechanisms, described on one species, working at interspecific level.

The main purpose of this study, therefore, is to distinguish how different nutrient sources (reserves stored in the seeds and nutrients available in the substrate) affect seedling development, particularly biomass allocation, and how it interacts with resource supply from the soil. We hypothesize that (i) each of these two pools of nutrients will have different effects on seedling development, based on the assumption that preferential development of root systems in nutrient-poor environments occurs to compensate for lack of below-ground nutrients or to search for nutrient-rich patches. Further, we specifically hypothesize that: (ii) large seed mass provides enough resources for development and therefore seedlings from larger seeds will be less responsive to substrate nutrient supply during their early ontogeny (first 12 weeks); and (iii) expect that the effect of seed mass on biomass partitioning will decrease with time, whereas the effect of substrate nutrients will increase, with this switch occurring earlier in small-seeded species.

To distinguish between the impacts of these two pools of nutrients on seedling development, we cultivated seedlings of 17 species — covering a wide range of seed mass — in two different nutrient regimes and measured their biomass allocation during the first 12 weeks of their ontogeny. We examined differences in root and shoot allocation and their changes over time in relationship to differences in seed size and nutrient supply in the substrate. Both at the design and analysis stages, we took into account phylogenetic relationships, because seed mass is strongly phylogenetically conservative trait (Westoby et al. 1996) and its effect is likely to be confounded by many other differences when compared naïvely over large phylogenetical distances.

3.2 Materials and Methods

3.2.1 Species selection

We selected seventeen common central European eudicot species from nine families, with seed mass ranging over three orders of magnitude (see Table 3.1), spread regularly over the phylogenetic tree. In all cases but one, for which it was not possible, two species per family were selected, with their seed mass differing as much as possible. Species known to need special treatment to germinate were excluded from the candidate list. All seeds were acquired from a commercial supplier (Planta Naturalis, www.plantanaturalis.com). We determined species-specific seed masses by weighing 100 air-dried seeds per species (Kleyer et al., 2008). We used this species-specific seed masses as an approximation of the all resources available to the seedlings. We are aware that this is a crude approximation due to possible differences in seed coat or attached structures (such as spines or pappus) between individual species. However, we do not think this is a serious problem in our study given large differences between seed masses of individual species. Further, none of the species we used had attached structures such as spines, except for two species from the Asteraceae family, where both species had a small pappus.

Table 3.1: Species used in the experiment and their seed masses.

Species	Avg. per-seed mass (mg)	Family
<i>Inula britannica</i>	0.071	Asteraceae
<i>Lychnis viscaria</i>	0.081	Caryophyllaceae
<i>Sisymbrium officinale</i>	0.096	Brassicaceae
<i>Campanula glomerata</i>	0.122	Campanulaceae
<i>Campanula trachelium</i>	0.171	Campanulaceae
<i>Dianthus deltoides</i>	0.181	Caryophyllaceae
<i>Nigella arvensis</i>	0.901	Ranunculaceae
<i>Lotus corniculatus</i>	1.104	Fabaceae
<i>Ranunculus acris</i>	1.146	Ranunculaceae
<i>Plantago lanceolata</i>	1.265	Plantaginaceae
<i>Filipendula vulgaris</i>	1.600	Rosaceae
<i>Lithospermum arvense</i>	1.616	Boraginaceae
<i>Lepidium campestre</i>	2.495	Brassicaceae
<i>Centaurea cyanus</i>	3.447	Asteraceae
<i>Anchusa officinalis</i>	4.421	Boraginaceae
<i>Lathyrus vernus</i>	14.642	Fabaceae
<i>Agrimonia eupatoris</i>	18.485	Rosaceae

3.2.2 Plant cultivation

We used two nutrient treatments — pure deionized water and a universal fertilizer solution (Wuxal Super; manufactured by AGLUKON Specialdünger GmbH & Co.KG, Düsseldorf; N:P:K = 8:8:6; see Table 3.2 for details) diluted in water to 0.1% concentration. In the experiment, each of these nutrient treatments was used for one half of the seeds and seedlings. The fertilizer concentration used was in the lower half of the range recommended by the fertilizer manufacturer. As substrate, we used expanded perlite (expanded amorphous volcanic glass). We chose perlite because it leaches practically no nutrients, enabling us to fully control the amount of available nutrients by means of our watering and fertilization treatments.

Seeds in both nutrient-level treatments were germinated individually (to preclude neighbor effects) in Petri dishes on filter paper moistened with 3 ml of the respective fertilizer solution. Deionized water was added throughout germination whenever the filter paper seemed to be almost dry. The Petri dishes were kept in a growth chamber (Adaptis A 1000 with TC kit, Conviron, Canada; light intensity $225 \frac{\mu\text{mol}}{\text{cm}^2\text{s}}$ at a distance of 12.5 cm from the light source) under the following diurnal temperature regime: 20°C for 12 hours during the day and 10°C for 12 hours during the night. The relative air humidity was set to 50% during the day and 70% during the night. Each seed was transferred into its own individual experimental pot (size $7 \times 7 \times 8$ cm) on the day the radicle emerged through the testa to filter out the effect of that different species differ in their germination lag time. Cultivation of the plants took place in the same growth chamber as that used for germination and with the same temperature, humidity and light settings.

Initially, we aimed to have six replicates per species for each of the two nutrient levels and for each of the four harvesting intervals – after two, four, six and twelve

Table 3.2: Nutrient content of the fertilizer (Wuxal Super) in the concentrate supplied by the manufacturer. It was diluted further to reach the highest administered concentration in the experiment (0.1% *v/v*).

%w/w		g/l	
1.935	NH_2	amide nitrogen	24
3.71	NH_3	ammoniac	46
2.258	NO_3^-	nitrate	28
8	P_2O_5	phosphate	99.2
6	K_2O	potassium	74.4
0.887	S	sulphur	11
0.01	B	boron	0.124
0.004	Cu	copper	0.049
0.02	Fe	iron	0.248
0.012	Mn	manganese	0.148
0.004	Zn	zinc	0.049
0.001	Mo	molybdenum	0.012

weeks of cultivation. This would have yielded 48 pots per species and 768 pots in total. As not all plants survived transplantations, the real number of replicates per species per nutrient level per harvesting interval ranged from 4 to 6, with the final number of experimental pots 756.

We divided each harvested plant in to their above- and below-ground parts at the boundary between epicotyl and hypocotyl, let them dry at 65°C for two days and weighed them to assess their R:S biomass ratios. We did not measure the seed remains at the surface of substrate not connected with seedling itself. We believe that it was composed mainly of structural carbon, which cannot be used by the seedling.

3.2.3 Data analysis

We used two linear mixed-effect models (LME) with species as a random effect to examine changes in total biomass (sum of root and shoot biomass) and budget of different nutrient sources in total biomass (total biomass:seed mass ratio). Fixed effects included time, nutrient supply and seed mass. We handled phylogenetic relationships of species using phylogenetic eigenvectors (Diniz-Filho et al. 2008). We used principal coordinate analysis (PCoA; function `dudi.pco` from the `ade4` package version 1.7.4., Dray and Dufour 2007) to extract the first eight eigenvectors from the matrix of phylogenetic distances from the *Daphne* phylogeny (Durka and Michalski, 2012). These eigenvectors capture 95% of total phylogenetic variation and were used as additional fixed effects in the model. Total biomass, total biomass:seed mass ratio and seed mass were log-transformed, and the whole model was fitted by maximizing log-likelihood. We used the `nlme` package version 3.1-127 (Pinheiro et al., 2016) in R version 3.2.5 (R Development Core Team, 2016). R^2 was estimated using Nakagawa and Schielzeth's R^2 GLMM (Johnson, 2014) as implemented in the `rsquared` function from R package `piecewiseSEM` (Lefcheck, 2016).

To determine allometry in R:S biomass partitioning, we used the standard major axis regression, employing lmodel2 package version 1.7.2 (Legendre, 2014) in R version 3.2.5 (R Development Core Team, 2016) for logarithmically transformed root and shoot biomass.

To analyze the effects of nutrient supply and seed mass on development of R:S ratio of seedlings over the course of the experiment, we used a linear mixed-effect model (LME) with species as a random effect. Fixed effects included time, nutrient supply, seed mass and their second and third-order interactions. We dealt with the phylogenetic relationships of species using the same phylogenetic eigenvectors as in the analysis of total biomass. R:S ratio and seed mass were log-transformed and the whole model was fitted by maximizing log-likelihood the same way as in the analysis of total biomass.

When visualizing the analyzed data, we first grouped the species by families to take their phylogenetic relationships into account, and classified species as relatively small- or large-seeded within each family. *Plantago lanceolata*, the single species from the family Plantaginaceae, was treated as a large-seeded species, because its seed mass is among the largest in this family.

3.3 Results

Total biomass of seedlings increased, unsurprisingly, significantly with nutrient supply ($p < 0.001$, explained variability = 28.9%, see Fig. 3.1a), seed mass ($p = 0.003$, explained variability = 5.3%, see Fig. 3.1b) and time ($p < 0.001$, explained variability = 39.5%, see Fig. 3.1c). Impact of internal seed mass reserves on total biomass (calculated as total biomass:seed mass ratio) decreased significantly with nutrient supply ($p < 0.001$, explained variability = 30.1%) and time ($p < 0.001$, explained variability = 41.2%) and increased with seed mass ($p < 0.001$, explained variability = 5.7%)

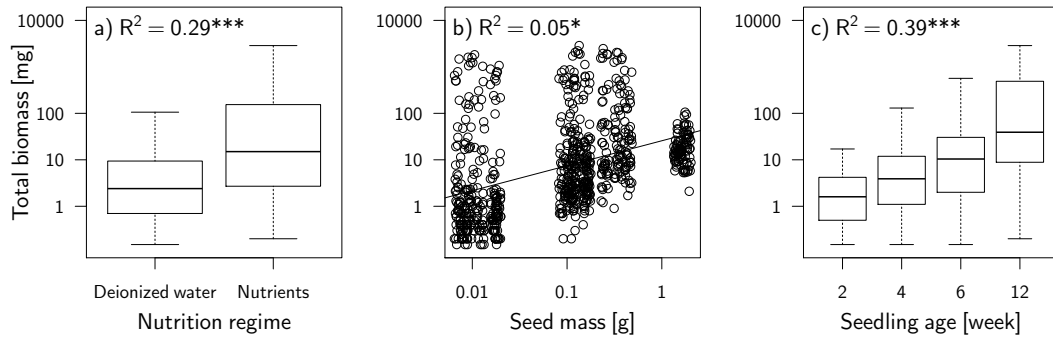


Fig. 3.1: Responses of total biomass to nutrient supply (a), seed mass of species (b) and age of seedling (c). Total biomass was log-transformed.

We found only a weak signal of allometry in root and shoot biomass (95% confidence interval of slope ranged from 1.02 to 1.08; Supplementary Fig. 3.1). For this reason we did not consider allometric relationships between the roots and shoots in the further analyses.

Geometric mean of the R:S ratio varied in deionized water treatment from 0.04 (for *Lathyrus vernus* after two weeks of cultivation) to 2.94 (for *Plantago*

lanceolata after 12 weeks of cultivation) and in nutrition supply treatment from 0.06 (for *Lathyrus vernus* after two weeks of cultivation) to 0.98 (for *Inula britannica* after 12 weeks of cultivation). After two weeks of growing, all individual plants in the experiment had developed green cotyledons leaves which allowed them photosynthetic activity.

Seedling R:S biomass partitioning (across plants harvested at all time intervals) was affected both by seed mass and the nutrients available in the substrate. Relative allocation of biomass to roots decreased with amount of available nutrients in the substrate ($p < 0.001$; explained variability = 39.7%; mean R:S ratio decreased from 1.18 in deionized water to 0.37 in the nutrient treatment; Fig. 3.2a). Biomass allocation to roots decreased with seed mass of the species ($p < 0.001$; explained variability = 5.7%; mean R:S ratio decreased from 1.27 for the smallest-seeded species (*Inula britannica*) to 0.57 for the largest-seeded species (*Agrimonia eupatoria*; Fig. 3.2b).

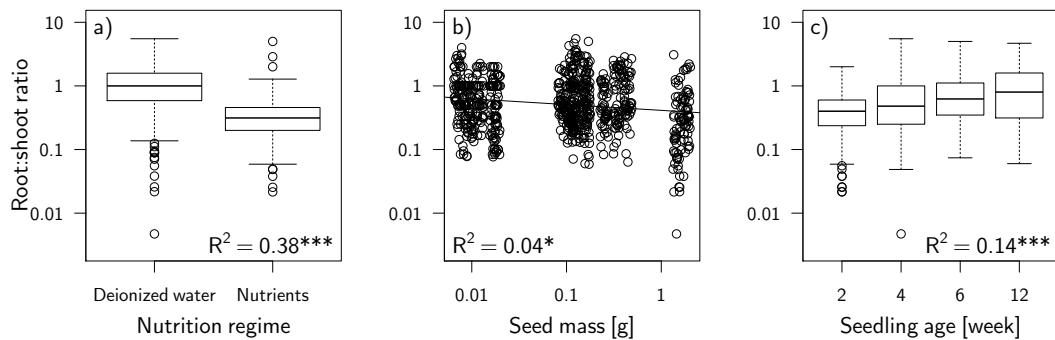


Fig. 3.2: Responses of R:S ratio to nutrient supply (a), seed mass of species (b) and age of seedling (c). R:S ratio was log-transformed.

Importantly, the effect of increased nutrient supply on biomass partitioning differed among species. An interaction among nutrient supply and seed mass of species showed that large-seeded species changed their biomass allocation with changing substrate nutrient supply more than small-seeded species: namely, they allocated relatively more into their shoots ($p < 0.001$; $R^2 = 1.3\%$; Fig. 3.3).

Generally, across treatments and species, the proportion of relative biomass allocation to roots increased over time ($p < 0.001$; $R^2 = 13.1\%$; mean R:S ratio increased from 0.5 in the second week to 1.04 in the twelfth week) but in a more detailed view, the time dynamics were changing in relation to both nutrition in the substrate and seed mass. All interactions with time were significant ($p < 0.001$ both for interaction with nutrient supply and seed mass; $R^2 = 4.3\%$ and 4.7% for interaction with nutrient supply and seed mass respectively; Table 3.3). The increase in R:S ratio was faster for large-seeded species and for the treatment without added nutrients.

The third-order interaction among seedling age, nutrient supply and seed mass was significant, but the explained variation was very low ($p = 0.003$, explained variability = 0.4%). The impact of seed mass on biomass allocation in relationship to nutrient supply lasted longer for large-seeded species (Supplementary Fig. 3.2).

3.4 Discussion

We found differences between large- and small- seeded species in their biomass partitioning, especially in the way how they respond to nutrient supply accros the time. In a striking contrast to our hypothesis, the large-seeded species were more sensitive to increased nutrient supply. The developmental trajectory over time during the observed initial period of seedling ontogeny also differed between large- and small- seeded species, and it interacted with nutrient supply. Although these interactions had less impact to seedling biomass partitioning than the main effects, we focus on them because the fact that seedlings grow larger over time and in response to higher nutrient supply is well known and is not subject of this work.

We show, for the first time at the interspecific level, that biomass partitioning is affected not only by soil nutrient supply, but also by the reserves stored in the seeds (seed mass). Our results are generally in agreement with previous studies that documented the relevance of soil nutrient availability for biomass partitioning during different developmental stages of seedlings (Gedroc et al., 1996) and adult plants (Glimskär and Ericsson, 1999; Cambui et al., 2011), but it seems that seed size plays an additional important role for the dynamics of seedling development. Increased amounts of reserves in the seed increased the above-ground biomass allocation of seedlings. Interestingly, both nutrients sources that we examined, viz. nutrients available in the substrate and reserves stored in the seeds — affected the biomass allocation in a similar way (more available nutrients in whichever pool led to higher investment in shoot biomass; Fig. 3.4), although they constitute

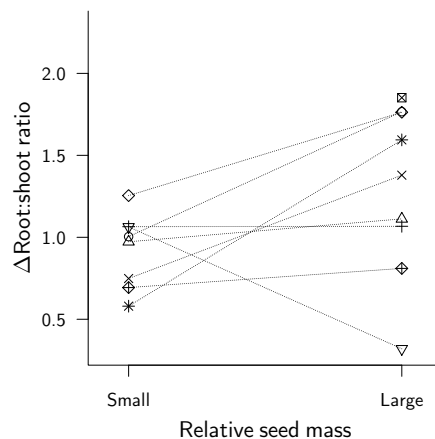


Fig. 3.3: Effects of two independent sources of nutrients (reserves stored in the seeds and nutrients available in the substrate) on seedling development measured as allocation of biomass. Δ R:S ratio was calculated as $\log(\text{R:S ratio in nutrient supply}) / (\text{R:S ratio in deionized water})$ and it shows difference between R:S ratio in deionized water and R:S ratio in nutrient supply for a given species. Lower values of this difference indicate stronger shift of the R:S ratio in response to increased nutrient supply in the substrate. Higher amount of nutrients available in the substrate translated into higher investment in above-ground biomass. This trend was stronger for large-seeded species (with the exception of *Fabaceae*).

Table 3.3: Relationship between seedling biomass allocation (measured as R:S ratio) over time, substrate nutrient supply, seed mass and their interaction (linear mixed-effect model, species used as random effect, model phylogenetically constrained). R:S ratio was log-transformed. Time—age of seedling; nutrient—substrate nutrient supply.

Fixed effect	Coefficient	<i>p</i> value	R^2
Time	0.06	<0.001	0.131
Nutrients	-0.25	<0.001	0.397
Seed mass	-1.29	<0.001	0.057
Nutrient*seed mass	0.24	<0.001	0.013
Time*nutrient	-0.03	<0.001	0.043
Time*seed mass	0.13	<0.001	0.047
Time*nutrient*seed mass	-0.02	0.003	0.004

pools with very different dynamics and ecological and evolutionary predictability. Using interspecific comparisons of species differing in their seed mass, we showed that seedling development was not affected only by reserves stored in the seed or nutrients available in the substrate per se, but also by the interaction of these two nutrient sources. This pattern was not an effect of size, because biomass partitioning was measured as the log-transformed R:S ratio which is essentially size-independent due to almost complete absence of allometric effects. Contrary to our initial hypothesis, large-seeded species were more sensitive to increase in substrate nutrient supply than small-seeded species after phylogenetic correction.

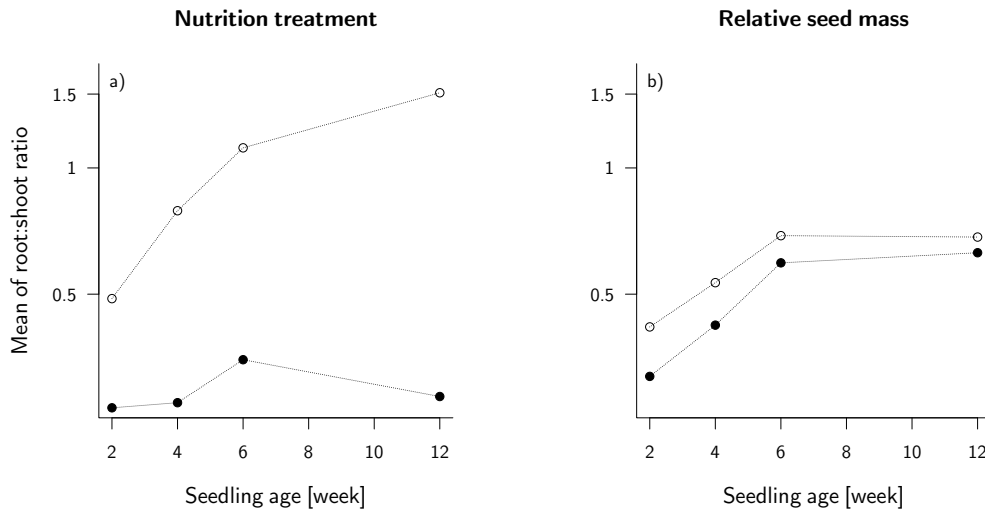


Fig. 3.4: Time courses of seedling biomass allocation. Biomass allocation differed between nutrition regimes and was dependent on relative seed mass.

Based on this result, we propose that greater seed size is an adaptation for fast development of shoots, a trait that may be highly beneficial in nutrient-rich and productive environments. In such environments, fast-growing shoots may be an important adaptation for success in asymmetric above-ground competition for light (Morris and Myerscough, 1991; DeMalach et al., 2016), which is supported

by the fact that species of productive environments tend to have larger seeds (Herben et al. 2017).

In our experiment, we used only successfully recruited seedlings and measured their biomass partitioning after given times. Thus we have information on the impact of nutrient supply on seedling condition, because seedling establishment is often a key part of generative life cycle of plants (Karban and Thaler, 1999; Jurena and Archer, 2003). Large-seeded species are generally considered to have an advantage during the seedling phase over small-seeded species, especially in various stressful conditions (reviewed in (Leishman et al., 2000; Westoby et al., 2002)). Nevertheless, most of the previous studies assessed the survival rate of recruited seedlings as a pass-fail process, counting how many seedlings were surviving after a given time and not examining viability or competitive ability of seedling or saplings later. In contrast to this, we measured seedling R:S ratio as an approximation of competitive ability (which seems to be a good approximation for seedlings which are always much smaller than surrounding adult individuals), although we do not know the direct impact of R:S ratio on fitness in the field.

The changes in biomass partitioning over time differ for each of the nutrient treatments and also for species with different seed masses. Moreover, each of these nutrient pools affected the temporal dynamic of seedling biomass partitioning in different ways (see Fig. 3.4 for comparison of the time dynamics of R:S development for two different pools of available nutrients). Faster increase in the R:S ratio in the treatment without added nutrients was probably caused by relatively rapid and large development of roots searching for nutrient richer patches — this is in agreement with optimal biomass partitioning theory (Bloom, 1985). While such a phenomenon has been reported a number of times (e.g., Walters and Reich, 2000; Shipley and Meziane, 2002; Portsmouth and Niinemets, 2007), there are also contrasting reports showing deviations from this theory’s predictions in extreme conditions (Canham et al., 1999; Espeleta and Donovan, 2002; Dijkstra and Cheng, 2007).

Our data showed a pattern that conforms with optimal biomass partitioning theory not only in terms of the observed bigger changes in R:S ratio in the nutrient-poor treatment, but also lower mean of R:S ratio during ontogenetic development for large-seeded species. Compared to the small-seeded species, these species probably store more than the minimum necessary reserves in their seeds and thus can dynamically and quickly respond to the environmental conditions into which their seedling emerge. This could be a mechanism that increases probability of seedling survival for large-seeded species and thus compensates the number of seeds. We assume that this mechanism can be working across the whole gradient of soil fertility under field conditions because we used fairly extreme conditions (pure deionized water and high concentration of nutrient supply) in the experiment. However, the question remains how much is this pattern masked by variation of other environmental factors, such as light, competition with neighbors or composition of microbiota in the soil.

Finally, we would like to draw an analogy between reserves in the seed and reserves stored in roots and rhizomes in adult plants. We hypothesize that seed reserves play a similar role for a seedling as carbohydrate reserves in roots at the beginning of the growing season for an adult plant. The resources stored in the roots during the growing phase are essential at the beginning of the next season

just as seed reserves are at the beginning of seedling development (Chapin et al., 1990; Loescher et al., 1990). It remains to be seen whether the effect of nutrients stored in roots/rhizomes has similar effects on dynamics of aboveground plant parts vs. nutrient-acquiring parts of their belowground structure.

Our study showed that substrate nutrient supply for seedling development is important, but it strongly differs in a nontrivial manner among species. We showed that seedling biomass partitioning during their early ontogeny strongly depends on the resources stored in the seeds (seed mass), and that these resources determine the magnitude of the response to substrate nutrients. Large-seeded species were more swift in their R:S response to increased substrate nutrients. We also showed that large-seeded species could have an advantage during the seedling development due to stored resources that are available and ready to use. This also means that the advantage of seed size is mainly in the larger pool of carbon (which is limiting in such environments) and much less in the larger pool of soil-borne resources, such as nitrogen or phosphorus. Thanks to these stored resources, large-seeded species are more flexible, can effectively and quickly use nutrients available in the soil and develop shoots faster – which will provide them the key advantage in asymmetric above-ground competition. This could constitute a selective factor for optimum seed mass, namely under highly productive conditions.

Supplementary

Supplementary data are available at Dryad: <https://datadryad.org/handle/10255/dryad.179154> and consist of the following. Supplementary Fig. 3.1: Relationship between root and shoot biomass. Supplementary Fig. 3.2: Time courses of seedling biomass allocation.

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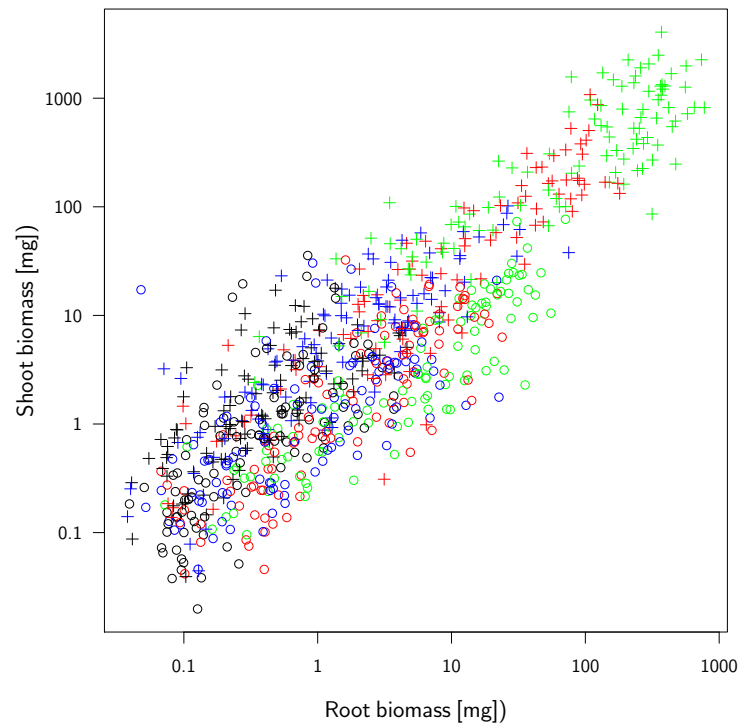
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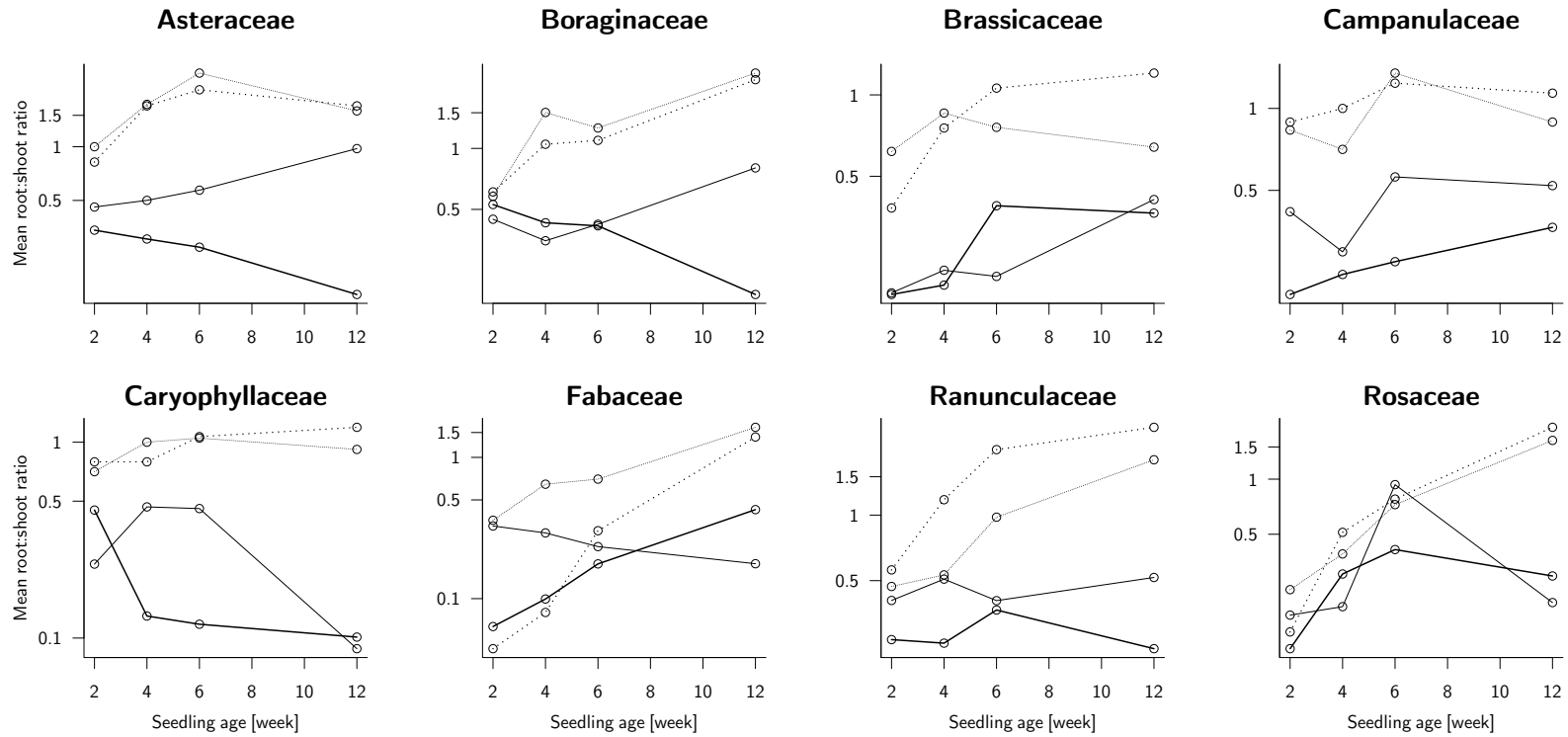
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Supplementary Fig. 3.1: Relationship between root and shoot biomass. 95% confidence interval of slope ranged from 1.02 to 1.08.



Supplementary Fig. 3.2: Time courses of seedling biomass allocation. Dotted line – deionized water; solid line – nutrient supply; bold line – large-seeded species in the family. Biomass allocation differed among nutrition regimes. The shift in biomass allocation over time due to nutrient supply differed among species according to their seed mass after phylogenetic correction. Display includes only families with pair of species available.

Chapter 4

The roles of interspecific variability in seed mass and soil resource availability in root system development

Tereza Mašková and Martin Weiser

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Abstract

Because plant roots serve mainly as organs for the uptake of water and nutrients, we aimed to test whether the development of seedling roots is influenced by the two principal nutrient sources—the substrate and the pool stored in the seed itself. Using seven Fabaceae species that differ in seed mass, we observed their early root system development under four levels of nutrient availability. Transparent-wall rhizoboxes allowed us to track root development and to score root system structure (length and number of roots), size (depth and width of root system) and shape (relative depth and width of root system). Seedling root system development depended on both the amount of nutrients contained in the substrate and on the seed mass of the species. Compared to an average seedling, effects of these two nutrient pools were (i) opposite and (ii) did not fully overlap. Small-seeded species developed wider root systems that branched earlier than large-seeded species. Increased availability of nutrients in the substrate led to proliferation of lateral roots, without any substantial impacts on the shape of root system or beginning of branching. The source of the nutrients affected the way they were used throughout early root system development, leading to different structures and dynamics. This may be one of the mechanistic links connecting seed mass and the realized niche of the species.

Key words: Root system development · Seed mass · Image analysis · Seedlings · Fabaceae · Rhizobox

4.1 Introduction

Vast differences among plant species exist in terms of their root system morphology (Fitter 1987; Kutschera et al. 1997) and plasticity (Gersani et al. 2001; Grime and Mackey 2002; Kembel and Cahill 2005), despite the fact that most of terrestrial plants have similar needs in terms of root function: nutrients and water acquisition and transport, and anchoring. Moreover, root system diversity and plasticity appears to be higher than what could be expected from the extended list of the root system functions (e.g. storage, overwintering, clonal propagation). This suggests that both the function and the local environment shape the root systems together. The developmental stage is likely to play a role as well, because plant growth responds to the environment (Bradshaw 1965; Ahn and Schmid 2017), and resource pools available to an individual plant change throughout its development.

For adult plants, the division between species-specific and environmentally plastic root system traits has already been documented (e.g., Lynch 1995). Among the species-specific ones, the root system architecture, i.e., the number of lateral roots and their positions, determines the exploited soil volume, but also affects root system performance (Fitter 1987; Bouda et al. 2018). On the other hand, the highly plastic morphological response of adult plant root systems to the soil environment is well documented (Audus 1975; Jackson and Barlow 1981; Vartapetian and Jackson 1997; Day et al. 2003). When compared to controls, nutrient deficiency generally produces faster-growing taproots and diminishes root branching in dicots, although further morphological responses are highly species specific (Forde and Lorenzo 2001; Hill et al. 2006). Soil nutrient availability seems to be the key driver of root system architecture and morphological plasticity, both at the evolutionary and ecological scale.

At the seedling stage, however, while part of the nutrients used are extracted from the soil environment, the plant also possesses nutrients that come from the seed itself. Thus, root system development may be affected by both nutrient pools. Compared to nutrients extracted from the soil, seed-stored nutrients are not subjected to competitive exploitation by neighbors, making this pool particularly reliable. Depletion of this pool is driven solely by the seedling itself, allowing more effective management of its use over time. On the other hand, the pool of nutrients in seed tissues from which the plant germinated (e.g., the cotyledons in legumes, endosperm in grasses) is necessarily limited (Leishman et al. 2000; Henery and Westoby 2001).

The limited amount of nutrients stored in the seed is likely to be used on growth of resource-harvesting body parts (adult plants, which could spend available resources for e.g. making storage organs or for flowering), so the seedling reaches self-sustainability in resource acquisition before exhausting this pool. While continuous acquisition of the nutrients from the environment allows for adjusting the (root system) development in response to the environmental gradients changing over time or space, the capacity for this response is limited while using the limited seed-stored pool.

The seed-stored nutrient pool differs among species (Kerkhoff et al. 2006), and thus the developmental constraints outlined above are manifested to different degrees in different species. Interspecific variation in the amount of nutrients

stored is enormous, exceeding corresponding intraspecific variation by several orders of magnitude (Harper et al. 1970; Ellison 2001). This contributes to differences in starting conditions among species and represents species-specific solutions to various tradeoffs in seed mass (Westoby et al. 1996; Coomes and Grubb 2003). Unsurprisingly, seedlings of species with small seeds are more resource-limited than those of species with larger seeds (Milberg and Lamont 1997; Leishman et al. 2000). Thus, particularly in species with small seeds, seed-size limitations may therefore lead to a tradeoff between the future need for a well-developed root system against the immediate need for belowground resources, as each of these goals is likely to be attained by a different root system morphology. For instance, thick roots with a large nutrient transport capability function as a backbone of the future root system, while thinner, but longer roots are able to exploit a larger soil volume immediately (Fitter 1985; Bouda et al. 2018). Finding at least some resources may be an important short-term goal, whereas efficiency of the whole root system may be more important over the long run (Fitter 1986).

Both the basic architecture of the root system and the seed mass are determined at the species level (Westoby 1998; Fitter et al. 1991; Moles et al. 2006), and there is evidence linking each of the traits to the environment of the species. For example, root system architecture at the species level reflects the water regime (Schenk and Jackson 2002), nutrient availability (Craine 2006) and even disturbance dynamics (Silver and Vogt 1993; Kiley and Schneider 2005).

The trade-off between seed size and number favors small but numerous seeds in highly heterogeneous or unpredictable environments, whereas fewer and larger seeds benefit reproduction in stable, competitive environments (Harper et al. 1970), but little is known about the direct relationship between seed size and the "hard" traits responsible for seedling recruitment and survival (Gross et al. 1992; Leishman and Westoby 1994; Hallett et al. 2011), root system architecture being among them. The attention paid to the seedling root system plasticity in response to different environmental factors is quite limited (Nicotra et al. 2002; Larson and Funk 2016) and there is only an indirect evidence of seedling response to the soil environment (Šmilauerová and Šmilauer 2007).

Here we investigated the roles that environmental nutrient richness and the amount of stored nutrients play in seedling root system development. We followed seedling root system development of seven species from the Fabaceae family (*Lotus corniculatus*, *Trifolium pratense*, *Trifolium medium*, *Securigera varia*, *Cytisus nigricans*, *Lathyrus pratensis*, *Lathyrus vernus*), grown in four different levels of nutrients over a period of four weeks. During the experiment, we tracked changes in root system architecture, size and shape. Because environmental and seed resource pools are not predictable to the same extent, we hypothesized that the amounts of resources in these two pools may affect seedling root system development in different ways. Because seedlings of small-seeded species possess smaller amounts of internal resources, we hypothesized that these seedlings are more sensitive to nutrient levels in the environment.

4.2 Materials and Methods

4.2.1 Plants selection and cultivation

We used seven species from the Fabaceae family, selected to represent a substantial range of seed masses (Table 4.1). We chose all the species from the same family in order to minimize differences among their life histories that are likely to occur with increasing phylogenetic distance, e.g., type of dormancy, type and origin of the storage tissue, etc. All seeds were bought from a commercial provider (Planta naturalis, Markvartice u Sobotky; www.plantanaturalis.com). These were natural ecotypes originally taken from the wild. This approach partly eliminates possible differences in maternal effects, since all the maternal plants were cultivated in conditions chosen to enable plants to produce the maximum number of high-quality seeds. All seeds had matured in the same season at the same place. Following the standard method (Kleyer et al. 2008), we counted 100 seeds per species, let them air-dry and weighed them to gain a species-specific average seed mass.

Table 4.1: Seed mass of species used and number of individuals per nutrition treatment in the experiment.

Species	Avg. per-seed	Number of individuals per nutrition treatment			
		0.0%	0.025%	0.05%	0.1%
<i>Lotus corniculatus</i>	1.104	12	11	12	11
<i>Trifolium pratense</i>	1.487	11	11	9	11
<i>Trifolium medium</i>	1.948	6	8	9	7
<i>Securigera varia</i>	3.511	11	6	11	8
<i>Cytisus nigricans</i>	5.718	9	9	8	9
<i>Lathyrus pratensis</i>	9.371	11	9	9	11
<i>Lathyrus vernus</i>	14.642	11	12	12	10

We used four fertilizer concentrations to test the effect of environmental nutrient levels. Throughout the experiment, basic macronutrients were added in the form of a universal fertilizer solution (Wuxal Super; manufactured by AGLUKON Spezialdünger GmbH & Co.KG, Düsseldorf [<https://wuxal.com/57-super.html>]; N:P:K = 8:8:6; see Table 3.2 for details), which was diluted in water to four different volumetric concentrations: 0.025%, 0.05%, 0.1% and 0.0% (pure deionized water). The highest administered concentration was in the lower half of the range recommended for hydroponics for the adult plants by the fertilizer manufacturer. We chose the upper limit according to our previous experience with the system, concentrations above this limit had frequently led to overfertilization. Supplementary Fig. 4.1 show the total biomass of seedling at the end of our experiment. Seeds of each species were divided into four equal groups; since each species has a different germination rate, the number of seeds used per species per treatment varied to try to give a constant number of seedlings for each group. Each of the groups were subjected to one of the four fertilizer treatments, and following germination, the seedlings were subjected to the same fertilizer concentrations as their respective seeds.

Seeds in each nutrient-level treatment were germinated individually in closed Petri dishes (diameter = 3.5cm) on filter paper moistened with 3ml of the respective fertilizer solution. Deionized water was added throughout the germination process whenever the filter paper seemed to be almost dry. The Petri dishes were kept in a growth chamber (Adaptis A 1000 with TC kit, Conviron, Canada; light intensity $225 \frac{\mu\text{mol}}{\text{cm}^2\text{s}}$ at a distance of 12.5cm from the light source) under the following diurnal temperature regime: 20°C for 12 h during the day and 10°C for 12 h during the night. Each seed was transferred into its own individual experimental container on the day the radicle emerged through the seed testa.

The experimental containers (rhizoboxes; inner dimensions: $19.5 \times 15 \times 1 \text{ cm} = h \times w \times d$) consisted of PVC boards sealed with silicone putty, with one of the larger sides transparent. Even the fastest-growing species (*Securigera varia* and *Trifolium pratense*) did not reach the bottom or side walls until the end of experiment in the fourth week. During the experiment, the transparent front side was covered with a non-transparent panel so that the roots developed in darkness; the cover was removed for observations. The containers were sloped at an angle of 40°, with the transparent (but covered) side facing downwards. This caused the roots to grow on the inner surface of the transparent panels.

We used expanded perlite (expanded amorphous volcanic glass) as a substrate matrix for the cultivation. It provides good aeration and leaches practically no nutrients, so we were able to fully control the amount of available nutrients by fertilization. Each container was filled with perlite and 200 ml of one of the fertilizer solutions (i.e., 0.025%, 0.05%, 0.1% or 0.0%), with each germinated seed transferred into a container having the same fertilizer concentration as that used for the seed during germination. The construction of pots did not allow water to run through. After filling the container with perlite and 200 ml of one of the fertilizer solutions, the water level reached 3/4 of the container height and gradually decreased over time, but the upper layers were wet all the time, soaking up the solution from the bottom. Therefore, the root systems were always in the wet part of the rhizobox, but were not flooded. Deionized water was added during the experiment if the water level in the container got down to ca. 1 cm above the bottom, in which case 50 ml of deionized water was added; typically this occurred after three weeks. This threshold corresponded to the point when the topmost layer was perceived as being dry when observed.

Cultivation of the plants took place in the same growth chamber as that used for germination, with the same temperature and light settings. Relative air humidity was set to 50% during the day and 70% during the night. The plants were cultivated for four weeks after germination. During this time, the plants were not limited by the volume of the rhizoboxes. In most of the individuals, the cotyledons were still present at the end of the experiment and all of the plants had at least some true leaves. Initially, we aimed to have at least 10 seedling replicates per species per nutrient level, which would have led to 40 containers per species and 280 containers in total, but due to technical reasons, we did not achieve the full set. Instead, the actual number of replicates per species per nutrient level ranged from 6 to 12 (Table 4.1). Root system development was checked weekly for four weeks, totaling 1096 observations.

Each week, we traced the root system (i.e., main root and lateral roots) of each seedling on the front side of the container with a permanent marker. We used

different colors of markers for each week to make the weekly growth increments of the roots recognizable in the final picture. During the experiment, only a negligible amount of tertiary root growth was observed (most of it for *Trifolium pratense*), in terms of both count and length. We therefore excluded tertiary roots from further analyses. After four weeks of growth (yielding four root tracings for each plant), we scanned the color picture of each root system from the front side of the container using an office table-top document scanner.

4.2.2 Measurements

Raw scanned images were not suitable for direct skeletonization—the process produced artifacts, because we did not draw each of the lines with constant press and speed, so the marker color and thickness of the line varied and could not be distinguished automatically. Therefore, we redrew them in GIMP (version 2.6.12 ("GIMP - The GNU Image Manipulation Program,"n.d.)) using the scanned originals as templates, and then skeletonized the redrawn images. For skeletonization, we used the built-in algorithm in ImageJ (version 1.45; Schneider et al. 2012). From each of the skeletonized images, we extracted parameters of root system structure, size and shape.

Structure The structure of the root system was characterized in terms of the length of the main root, total length of lateral roots, number of lateral roots, and mean length of lateral roots at the end of each week. The length of the roots was measured as the number of square pixels of corresponding color in the skeletonized image minus one, to measure the length of the line rather than the number of pixels (1 cm corresponded to 118 pixels in a straight line). Two pixels with a common side were counted as one; two pixels with a common vertex only were counted as $\sqrt{2}$ pixel. This gave the real distance between the center of the first and the center of the last square pixels. Pixels and lengths were counted using a Python script (available at <https://github.com/prvak/roots-analyzer/> and Supplementary Listing 1). With only a few exceptions, root systems branched to the second-order roots only (main root and non-branched lateral roots), so all were classified as herringbone-type (i.e. no dichotomous-type branching was observed), and thus the number of lateral roots and their length becoming the principal parameter.

Size and shape To describe both the size and shape of the whole root system, we marked its topmost (denoted A), leftmost (C), rightmost (D) and bottommost (B) points at the end of each week (see Fig. 4.1), yielding 16 (four per week) points per scanned image, and noted the coordinates of these points. The size parameters indicate the area occupied by the root system, and were expressed in terms of three parameters: the depth of the root system was defined as the vertical distance between points A and B, width of the root system as the horizontal distance between points C and D, and the area of the root system as the area of the ACBD tetragon for each of the weekly measurements.

We selected shape parameters to be size-independent. The root system shape was estimated using the coordinates of the ACBD vertices. Using these points, first, we calculated the position of the ACBD centroid — the vertical distance between point A and the point of intersection of the ACBD tetragon diagonals. The position of the ACBD centroid refers to the depth at which the majority of



Fig. 4.1: Image of the root system with sixteen marked points used for calculating size and shape parameters of the root system at the ends of each of the four weeks. Point A is the topmost, point B is the bottommost, point C is the leftmost, and point D is the rightmost. The numbers correspond to the measurement week. Each color represents a particular unique combination of root order (primary vs lateral) and its week length gain.

the lateral roots are to be found. However, we used its size-independent variant for the analysis — the relative depth of the root system, which was defined as the vertical position of the ABCD centroid divided by the root system depth. Further, we used a parameter named "relative width of the root system", which was defined as the width:depth ratio. We calculated these parameters for each of the four weeks. All measured data are available from Dryad Digital Repository: <https://doi.org/10.5061/dryad.gv33qc2>.

4.2.3 Data analysis

Because we observed ten parameters per each root system for each time, the nature of these data was multivariate and hierarchical. Namely, the data about root system shape would be difficult to interpret in a non-multivariate way, since the tetragon shape cannot be appropriately described by a single parameter. Therefore, we analyzed them using redundancy analysis (RDA), a multivariate analogue to multiple regression, using the *vegan* package version 2.3–1 (Oksanen et al. 2015) in R version 3.1.1 (R Core Team 2014).

We checked the linearity of the measured parameters in time, since root system growth in time was expected to be the main driving force of change. Accordingly, since the root system area and number of lateral roots was non-linear, we used the logarithmic and cubic root transformations, respectively, for further analysis of these parameters. Next, each response variable was scaled to zero mean and unit variance.

In each of the analyses, we used the same set of predictors (constraining variables): seed mass, nutrient level, time (the number of weeks since the beginning of the experiment) and their interactions. Marginal effects (all effects higher in the hierarchy being partialled out) of predictors and their interactions were reported in terms of adjusted R^2 (Peres-Neto et al. 2006), using the *RsquareAdj* routine of the *vegan* package. Because of the adjustment, the R^2 of the examined effects and covariables and residual variance do not have to sum up to 1, and the values could be even negative. In cases where an effect of seed mass (continuous predictor) was to be partialled out, we used species identity (as a nominal-scale data) instead, conservatively partialling out also the possible non-linear effects of seed mass. The hierarchical nature of the data makes estimating overall R^2 per model difficult, since "explained variance" occurs both at the level of individuals and at the level of individual observations; thus, we calculated the R^2 values both with and without partialling out the identity of the individuals. Also, the R^2 can be compared to the unconstrained variant of the model, i.e., to the sum of the same number of leading eigenvalues as there were predictors in the constrained model, with the same set of covariables in both cases, and we used this approach in describing marginal effects of the individual predictors (for details see Listing 2).

Statistical significance of the predictor effects was tested by permutation tests (function *anova* in the *vegan* package, 999 permutations). Permutation structure followed the model structure, so that all effects in the model hierarchy higher than the tested one were used as covariables. The permutation was done at the lowest available level; if involved, time was shuffled instead of freely permuted; if seed mass was to be involved as a covariable, species identity was used instead

the continuous seed mass. The tests were based on permutating residuals after fitting covariables (so-called reduced model of permutation).

Since the effect of the seed mass was to be estimated at the highest hierarchical level but the number of replicates per species was not constant thus making proper permutation unfeasible, for this particular estimation and the test we used species means, collapsing the data into seven points, which considerably constrained the permutation space. The means were calculated before data standardization. We estimated the amount of variance depicted by the ordination axes using the *anova* function.

Apart from examination of the full model, differences between the effects of seed mass and fertilizer amount were addressed by partitioning the variance into the parts that could be ascribed to the respective predictors and the interaction of the predictors with time. This allowed us to estimate the quantitative overlap between the effects of seed mass and fertilizer amount.

4.3 Results

Root system development was strongly determined by the predictors. The full model addressed approximately 31.1% of the full data variability ($p = 0.001$; approximately 33.56% after partialling out the effects at the level of individuals). With species identity instead of seed mass (assuming non-linear effects of seed mass or effects not correlated with seed mass), the model addressed approximately 49.1% of the full data variability. In the variant with all predictors as categorical variables (time, nutrients level, species identity), i.e. estimating the maximum variability addressable by the predictors (including their non-linear effects), approximately 55.1% of the overall variability of the data was addressed. This suggests that the species-specific seedling root system traits cannot be fully inferred from the seed mass.

We found no evidence (adj. $R^2 \sim 0$) for a three-way interaction, i.e., no evidence for the hypothesis that the development of root systems is shaped according to both seed mass and fertilizer concentration in a non-additive way. Also, we did not find any substantial evidence for the root system being consistently (with no dynamics) shaped by the non-additive effects of seed mass and fertilizer amount (adj. $R^2 \sim 0$). The support for non-additive effects of the fertilizer amount and growth in time was very weak (adj. $R^2 = 0.001$). Fertilizer level affected overall characteristics of the root systems (adj. $R^2 = 0.004$), but mostly in an additive way, i.e., the effects of fertilizer could be estimated regardless of the seed mass and (more or less) the age of the seedlings. On the other hand, seed mass affected the overall root system development: seedlings of different seed masses followed distinct growth trajectories (seed mass \times time; adj. $R^2 = 0.017$; see Table 4.2).

For the description of the traits and effects that follows, it should be noted that the effect of data centering and standardization manifests so that the overall mean, not zero, is the reference level. The first ordination axis was associated mainly with the root system development in time and covered 30.1% ($p = 0.001$) of the overall variability in data. However, the second ordination axis (1.2%; $p = 0.001$) shows that the effects of seed mass and fertilizer were, indeed, opposite of each other. Availability of the nutrients in the environment led to proliferation of lateral roots, both in terms of count and length. Also, seedlings in nutrient-

Table 4.2: Explanatory power and statistical significance of the individual predictors in the model of the whole set of root traits. The column "% of possible max. explained var." contains the predictor compared to the unconstrained axes of the similar models (the same set of covariables). Significance values are based on 999 permutations. Reported degrees of freedom do not fully reflect the permutation structure.

Predictor	Adjusted R^2	% of possible max. explained var.	df (model, residual)	pseudoF	p value
seed mass	0.075	30.8%	1, 5	1.483	0.264
fertilizer	0.004	2.9%	1, 1081	10.164	0.004
time	0.312	72.4%	1, 821	622.82	0.001
seed mass \times fertilizer	0.001	0.5%	1, 1086	2.038	0.304
seed mass \times time	0.017	8.2%	1, 1086	33.982	0.001
fertilizer \times time	0.001	0.5%	1, 1086	2.109	0.028
seed mass \times fertilizer \times time	0.000	0.5%	1, 813	1.058	0.241

rich environments produced relatively wider root systems. On the other hand, seedlings of the large-seeded species appeared to invest more into development of the main root, leading to deep-reaching and narrow root systems (Fig. 4.2; Supplementary Fig. 4.2).

In terms of associated variance in the data, the effects of seed mass and fertilizer amount substantially overlapped, but there was still a significant ($p = 0.001$) portion of variability that could be ascribed to the effects of seed mass but which was not covered by the effect of fertilizer amount (Fig. 4.3). Seedlings of the large-seeded species occupied the belowground space slower than their counterparts emerging from small seeds. The larger the seed, the later it developed lateral roots (Fig. 4.4).

4.4 Discussion

Our results show that both treatment parameters (the amount of available nutrients in the substrate and seed mass) play important roles in root system development in the early phase of plant ontogeny, but that their effects were, surprisingly, almost opposite to each other. Seed mass had a more profound effect on root system development than the amount of nutrients available in the environment of the developing plant. Nutrient availability in the substrate increased the amount of lateral roots and led to a relatively more shallow root systems, while seedlings of the large-seeded species tended to develop deeper and relatively less-branched root systems. This effect arose partly because of slower development of the lateral roots in the large-seeded species, an effect of seed mass that cannot be directly linked to nutrient availability in the environment.

In general, these results are in agreement with previous work that found that nutrient deficiency led to faster-growing taproots and diminished root branching (Forde and Lorenzo 2001; Hill et al. 2006). In addition to these earlier works, we were able to partly address some of the species-specific responses: seed mass might be the key trait. On the other hand, in treatments with the opposite effects

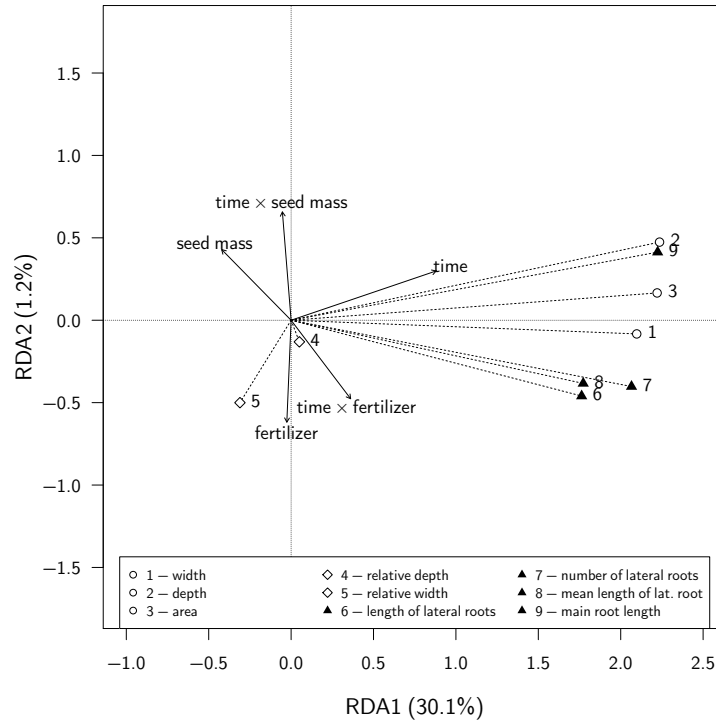


Fig. 4.2: Constrained ordination of the root system traits. The first and second ordination axes depict ca. 30.1% and 1.2%, respectively, of the overall data variability, each of them being statistically significant ($p = 0.001$). All the terms from Table 4.2 were used as constraining variables, but statistically non-significant ($p > 0.05$) interaction terms are not depicted. Symbols correspond to the groups of the root system traits: circles—size, diamonds—shape, triangles—structure. Individual traits are marked with numbers. Data are dominated by growth (change in time), the opposite effects of seed mass and fertilizer amount are visible along the second ordination axis.

of the seed mass and environmental nutrient availability, we found no evidence for a larger effect of environmentally available nutrients upon small-seeded species.

We found seedling root systems to branch more in the treatments with higher nutrient levels. This is also frequently the case for root systems of fully grown plants (Drew 1975; Robinson 1994; Hodge et al. 1999) although the response differs depending on nutrient stoichiometry and mobility in the substrate matrix (Craine and Dybzinski 2013). The proliferation rate of lateral roots, i.e., the number of active absorption zones in the nutrient patch, is the factor that underlies the absorption rate (Hodge et al. 1999; Robinson et al. 1999). Structural similarities across developmental stages, i.e., early seedlings and full-grown plants, probably reflects a common response to a common challenge (although some other, as yet undetected mechanisms may also play a role). We therefore argue that the proliferation of seedling lateral roots that we observed in nutrient-rich environments has the same cause and purpose as lateral root proliferation in developed plants. On the other hand, as we do not compare root systems of seedlings and full-grown plants directly, this statement is only qualitative, not

quantitative, i.e., root proliferation reflects environmental nutrient levels but the exact relationships may differ between seedlings and adults.

Whereas root system branching corresponded to the treatment environment, the development of the root system was strongly affected by seed mass. Compared to branching intensity, overall root system shape has been seldom studied (but see Gross et al. 1992), so any interpretation of our results for seedling root system shape by means of analogy with shape and function in adult plants must be more cautious. Nevertheless, one of the possible explanations invokes the trade-off between root systems that are effective at harvesting resources and those that are effective at transporting them (Fitter 1987). According to this scenario, larger-seeded species may simply start by building structures that will be fully used later in the life of the plant, whereas small-seeded species need to start with structures that are ready for use even though they will have to be reconstituted later (Larson et al. 2016).

The shape of the root system in adult plants is strongly related to the environment, namely water dynamics (Schenk and Jackson 2002). Therefore, another possible interpretation of the pattern we observed is that larger internal seed reserves allow seedlings to reach deeper into the soil, where the risk of drying out is lower. This could be an explanation of higher survival of seedlings of large-seeded species observed by Hallett et al. (2011). Because of small internal reserves, small seeds are forced to branch their roots as soon as possible to reach nutrients in the environment. Early and thus shallow branching of small-seeded seedlings puts their root system at a higher risk of drying out, as a higher portion of the root system is in the more dangerous shallow zone, even though deeper levels may eventually be reached (Stanton 1984).

Similarly, large-seeded species may be able to invest in roots that are thicker or have denser tissue (Wright and Westoby 1999), so that root systems and the plants may be less susceptible to stress and more tolerant of highly competitive environments (Semchenko et al. 2017). This allows us to speculate that the large-seeded species that dominate in the dense stands of late successional stages (Westoby 1998; Moles et al. 2006) are adapted to focus their competitive efforts in above-ground competition (Mašková and Herben 2018), being just able to tolerate belowground competition, while the small-seeded species do the

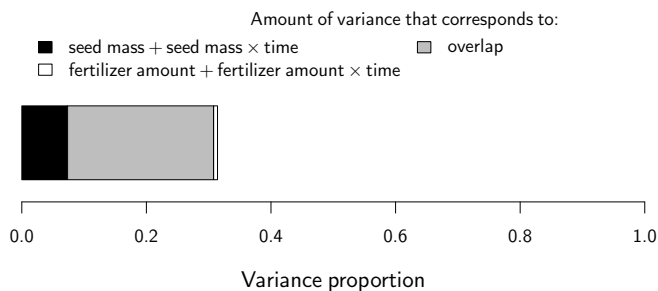


Fig. 4.3: Variability in data partitioned between the effects related to seed mass and fertilizer levels. These effects quantitatively overlap to a large extent, but there is a substantial and statistically significant ($p = 0.001$) portion of the variability that can be ascribed only to the effects related to the seed mass.

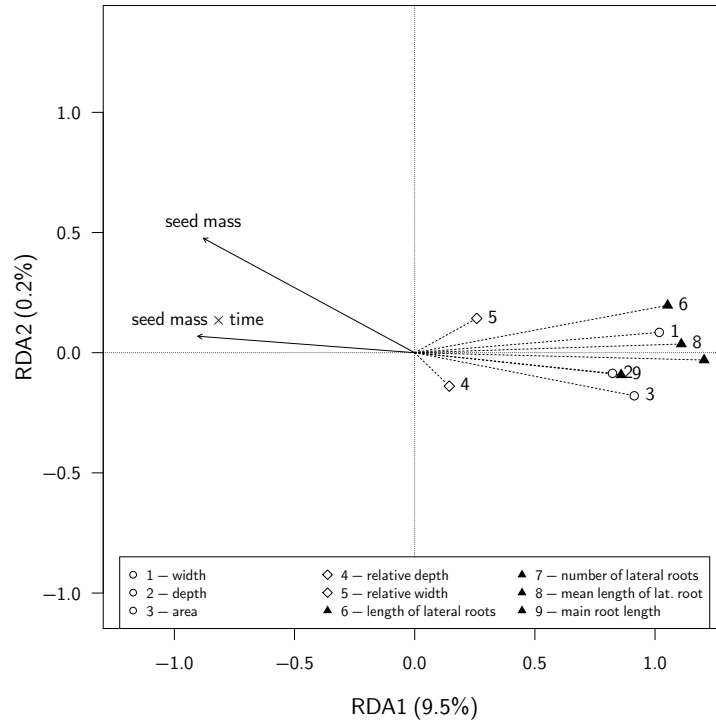


Fig. 4.4: Net effects of the seed mass. Constrained ordination of the root system traits after filtering out the portion of the data variability that could be ascribed to the effects of the fertilizer amount. Symbols correspond to the groups of the root system traits: circles—size, diamonds—shape, triangles—structure. Species with bigger seeds branch their root systems less and more slowly than small-seeded species.

opposite. Plants increase their amount of roots in belowground competitive situations (Gersani et al. 2001; Craine et al. 2005; Craine 2006), and the larger the root system extent, the higher the probability of being a successful, aggressive competitor belowground (Semchenko et al. 2017). The speculation about the seed mass–competition type relationship could be corroborated by estimating the allometry in seed stoichiometry: the proposed scenario would lead to large seeds being unproportionally rich in carbon and possibly nitrogen, which are the main building blocks of the aboveground structures, leaves and stems. In highly asymmetric competition for light, an early start could bring life-long profit (Schwinning and Weiner 1998; Olsen et al. 2005; Verdú and Traveset 2005), so ready-to-use nutrients stored in the large seeds are likely to enhance survival.

If the patterns we observed, especially the development of root system shape, prove to be general, the increased resistance to unexpected periods of drought as a feature of large seeds may be a mechanism that restricts the solutions to the tradeoffs in seed size and numbers in habitats where unexpected dry periods may occur (Amir and Cohen 1990; Wong and Ackerly 2005). In turn, both this causative relationship and the one linking seed mass and competitive ability could be extrapolated to address the broad applicability of the leaf-height-seed (LHS) scheme (Westoby 1998) for species differentiation across large scales, where seed mass is the key trait.

Both of the treatment parameters, seed mass and nutrient availability in the substrate, played important roles in the development of the root system in the seedling stage. However, these two nutrient pools were not interchangeable, and in terms of quantity of lateral roots, even had an opposite effect. Seed mass determined the relative shape of the root system, so that large-seeded species developed narrow and deep-reaching root systems, branching much later during the ontogeny than the small-seeded species. High availability of the nutrients in the environment led to increased length and number of lateral roots, with only a small impact upon the dynamics of this process. These differences between the roles of the nutrients pools may underlie the differences in the community assembly rules in various environments.

Supplementary

Supplementary data are available at Dryad: <https://datadryad.org/resource/doi:10.5061/dryad.gv33qc2> (Supplementary Fig. 4.1, Supplementary Fig. 4.1 and Listing 2) and <https://github.com/prvak/roots-analyzer/> (Listing 1) and consist of the following. Supplementary Fig. 4.1: total dry mass of individuals at the end of the experiment. Supplementary Fig. 4.1: measured parameters of root system during the time for individual species. Listing 1: Python code used for the image analysis. Listing 2: a sketch of the script used for data analyses.

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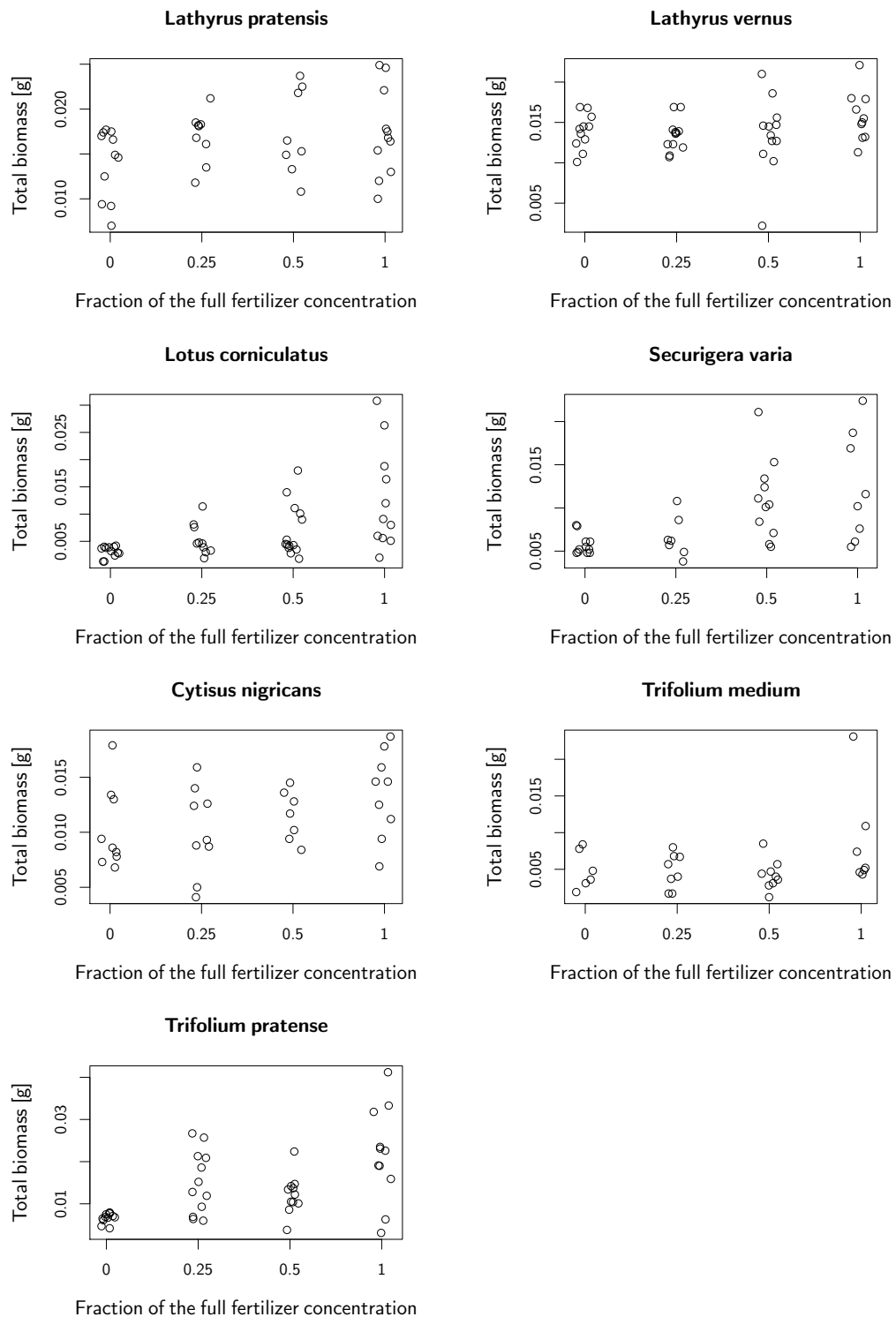
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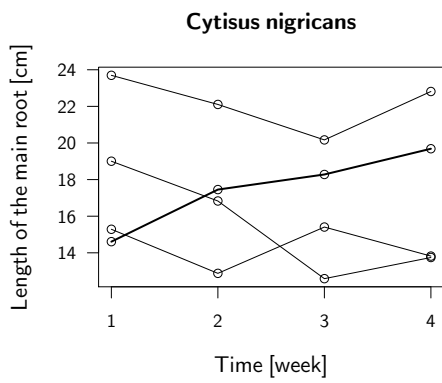
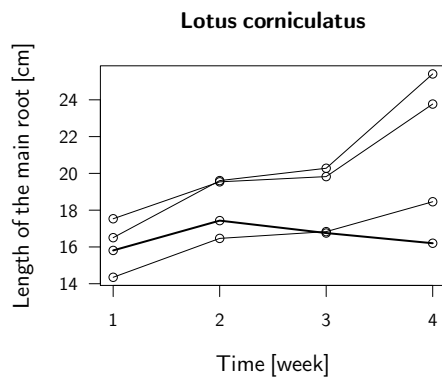
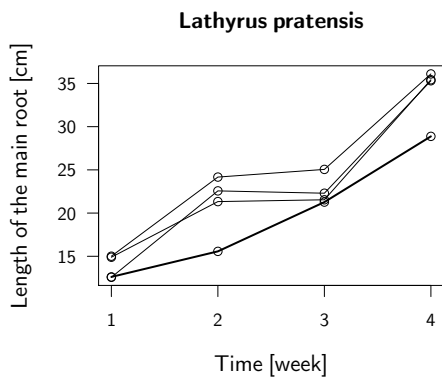
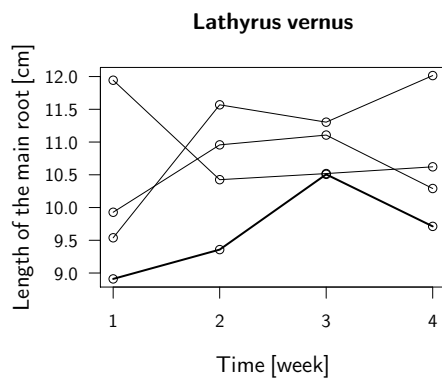
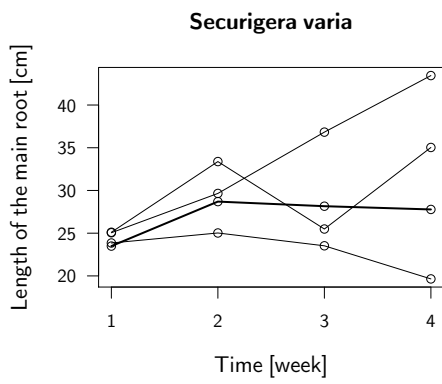
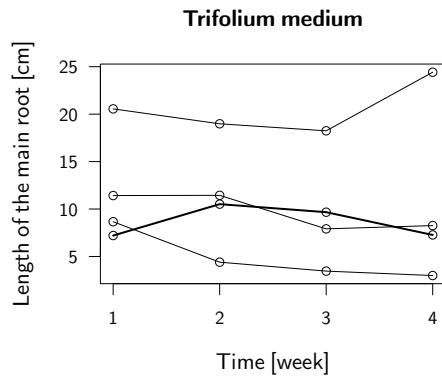
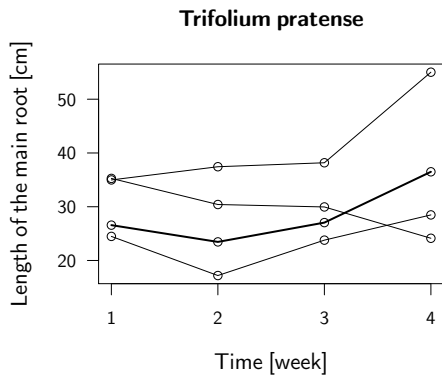
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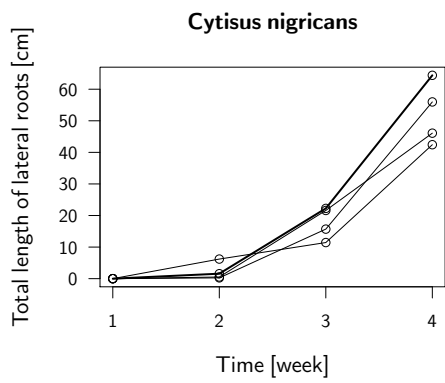
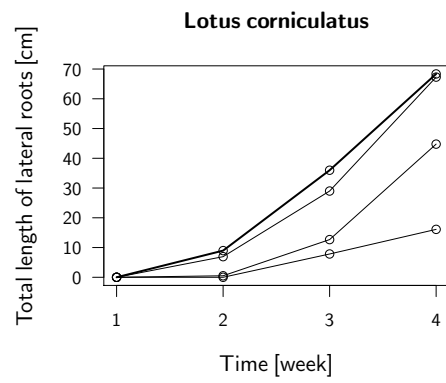
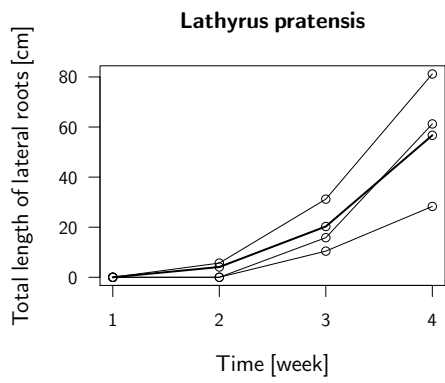
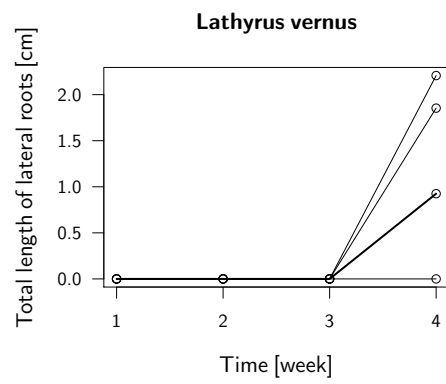
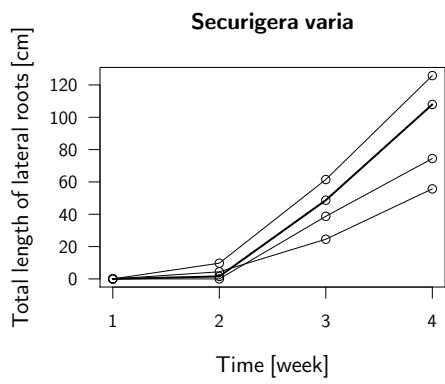
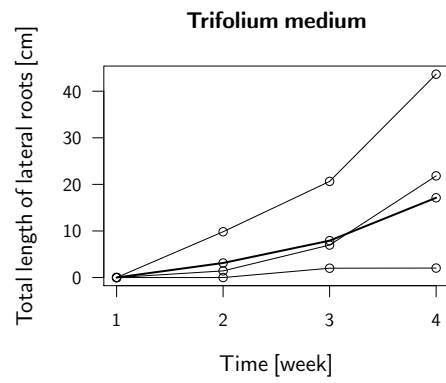
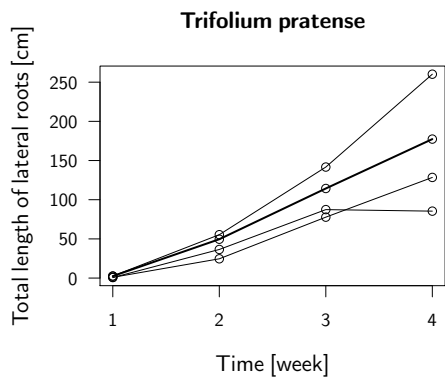


Supplementary Fig. 4.1: Total dry mass of individuals in the four administered nutrition supply levels, at the end of the experiment (after four weeks of growth).

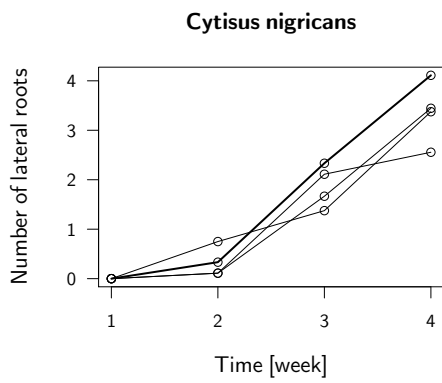
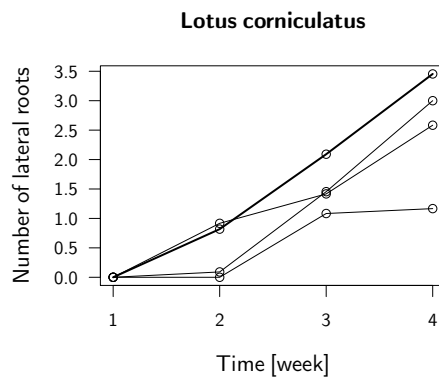
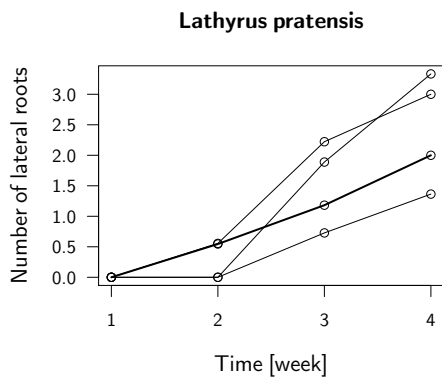
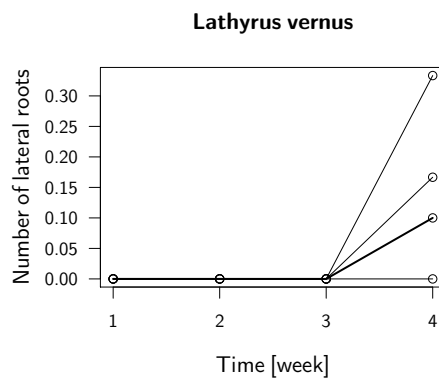
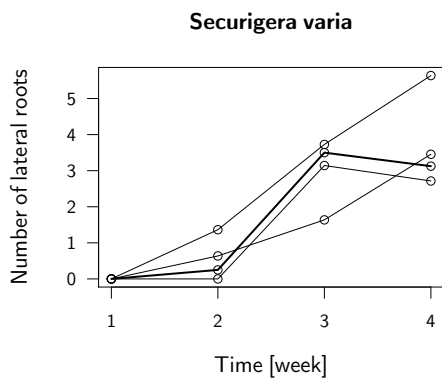
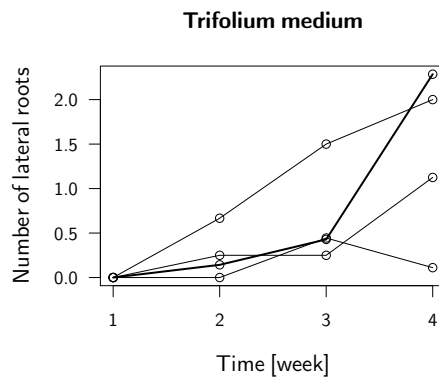
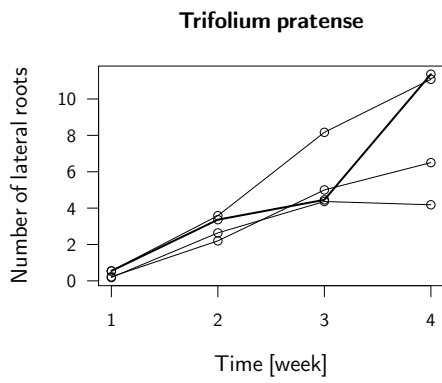
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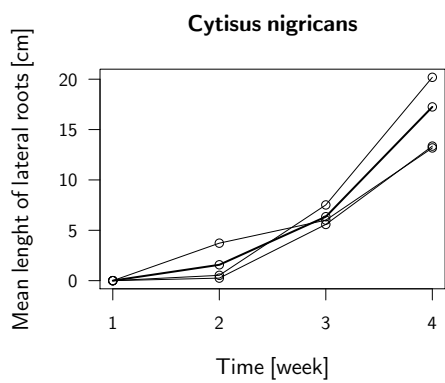
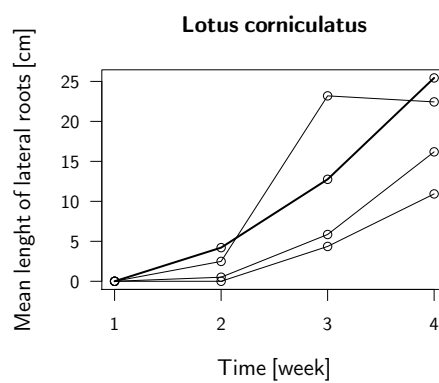
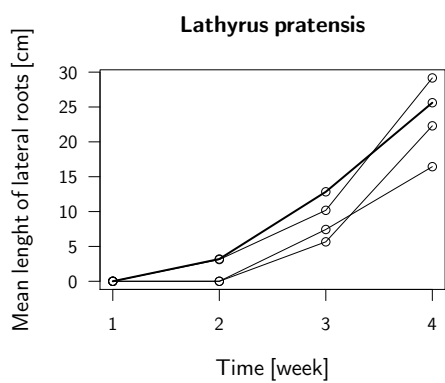
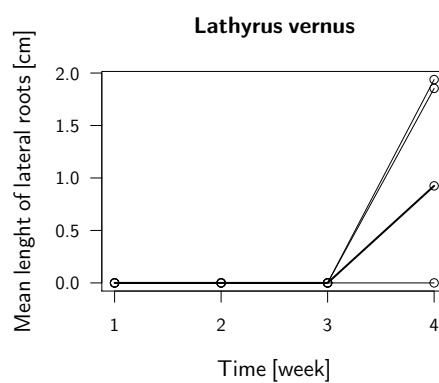
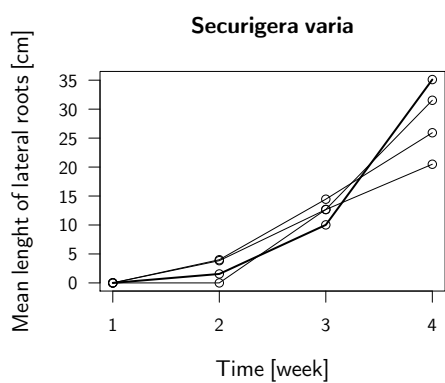
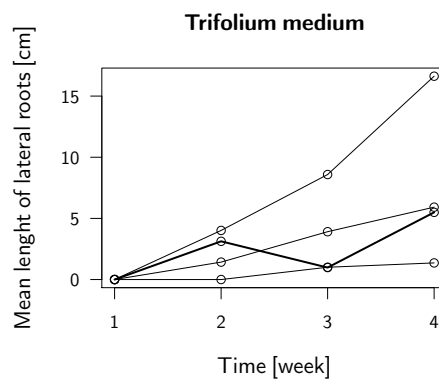
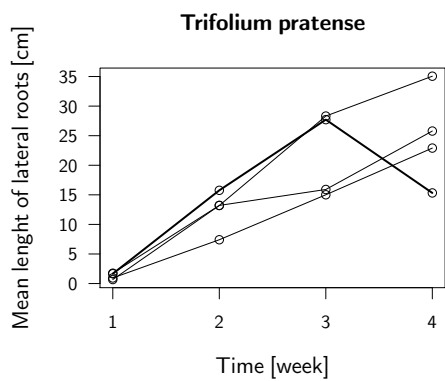
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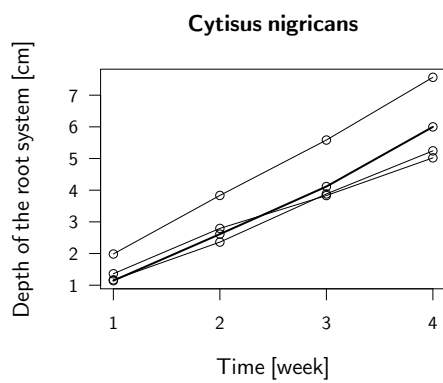
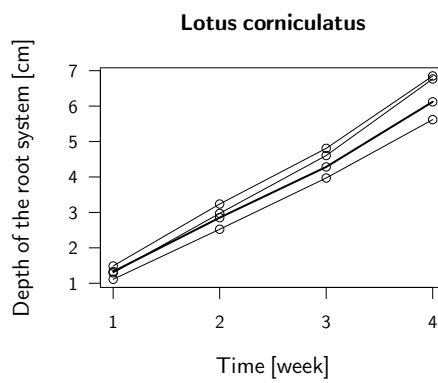
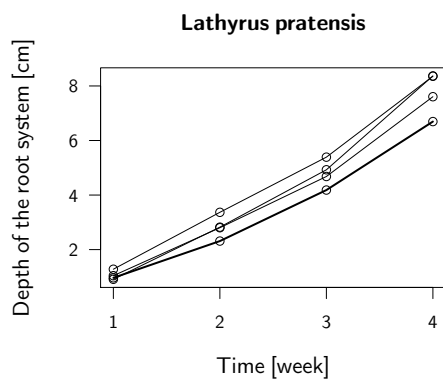
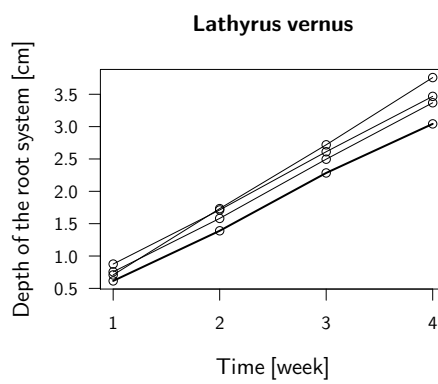
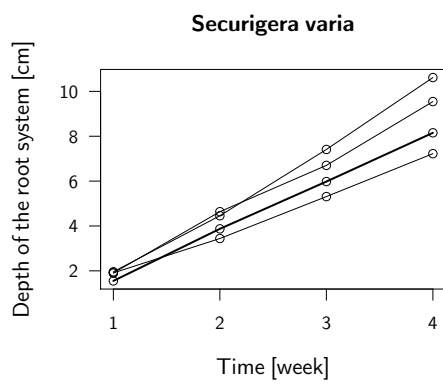
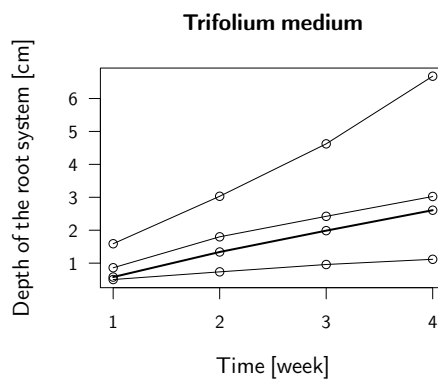
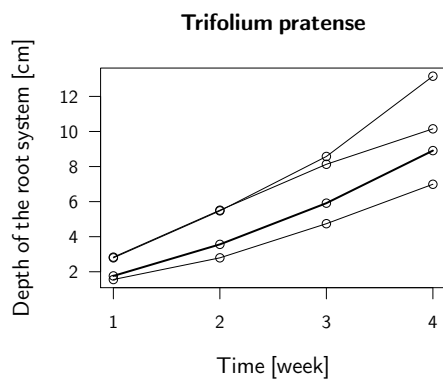
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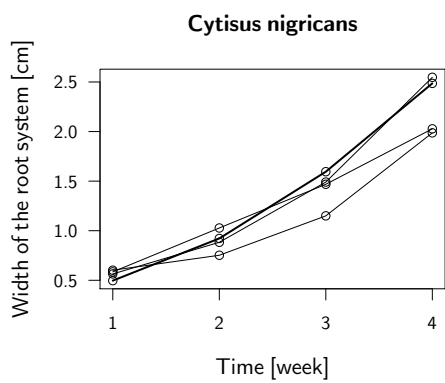
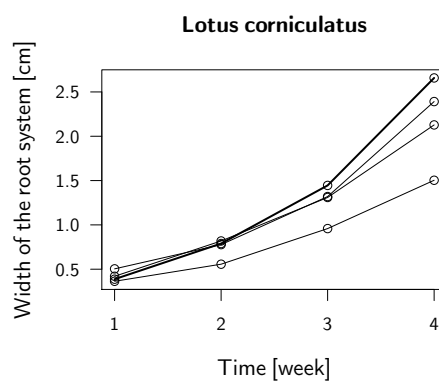
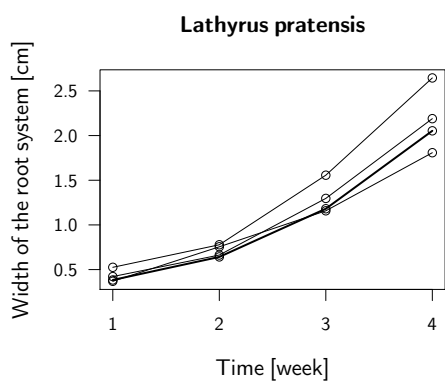
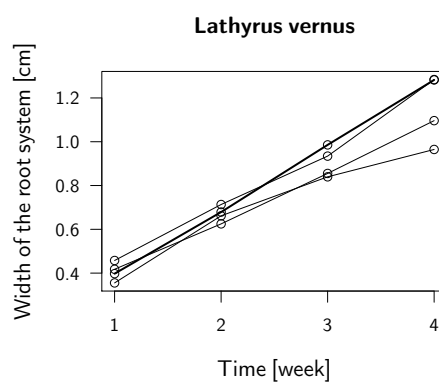
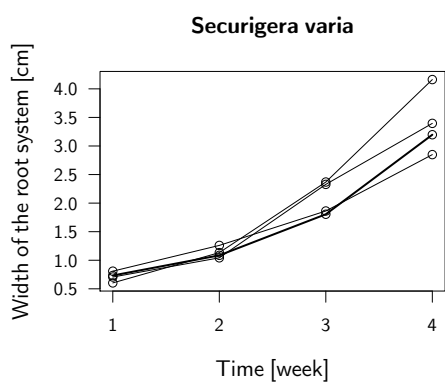
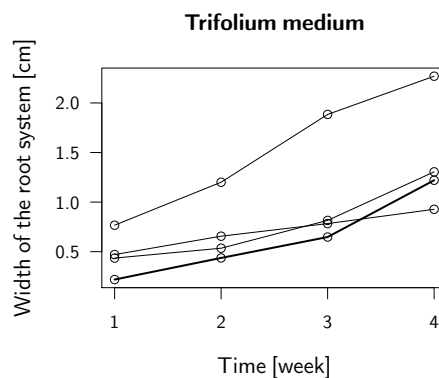
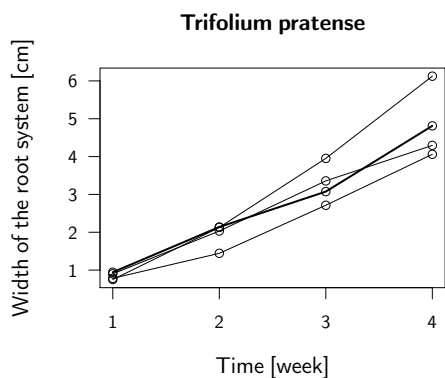
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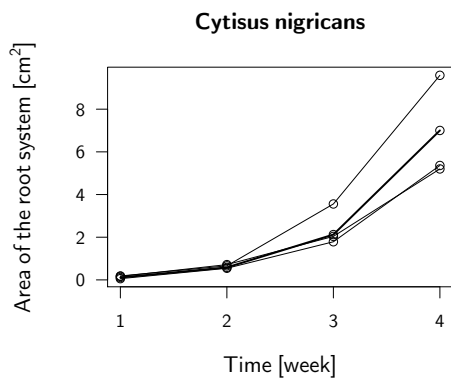
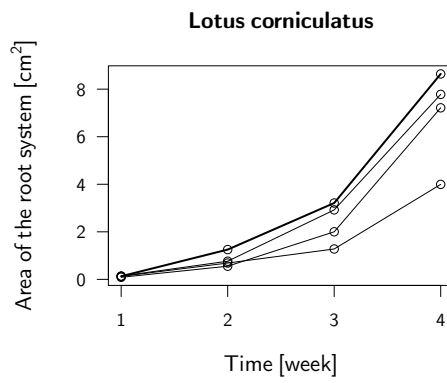
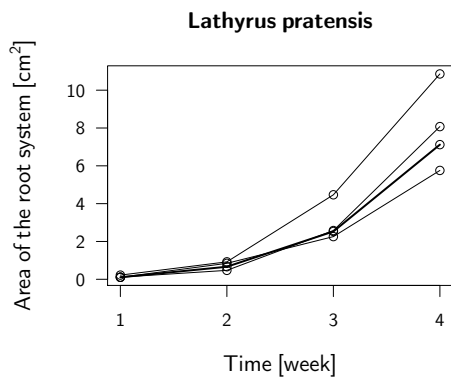
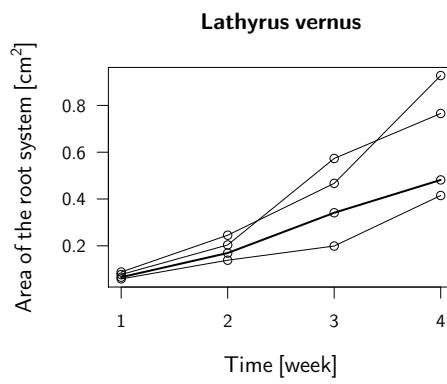
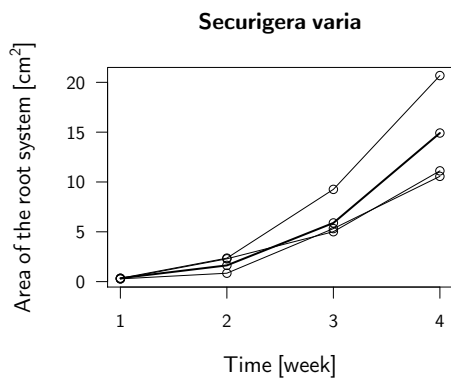
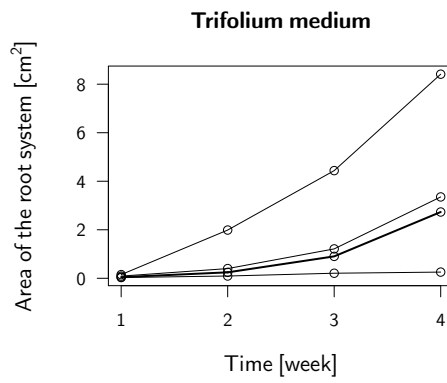
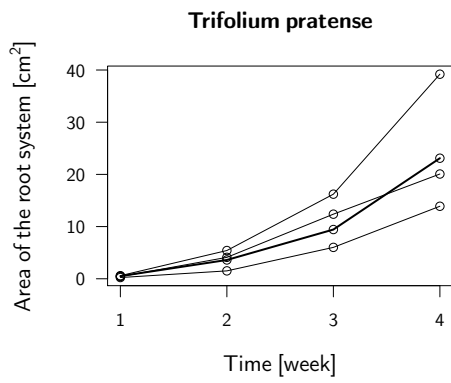
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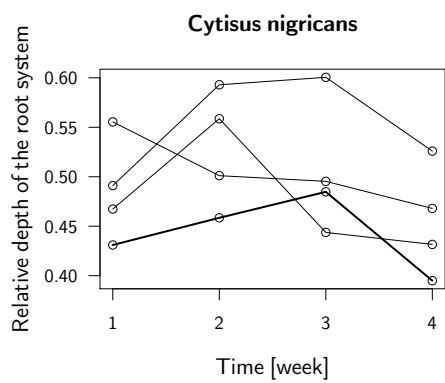
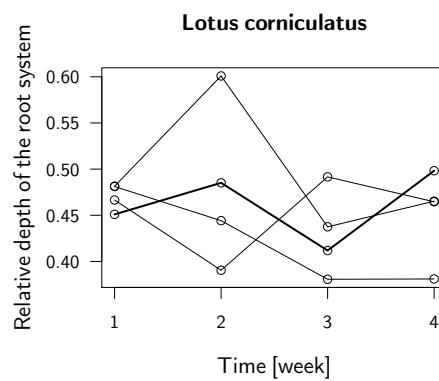
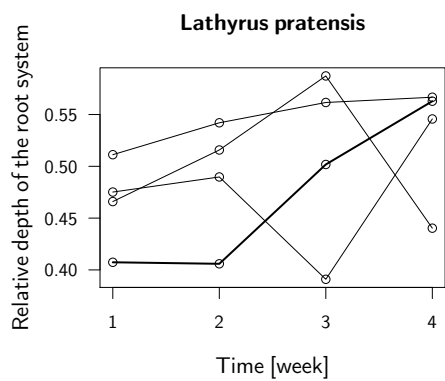
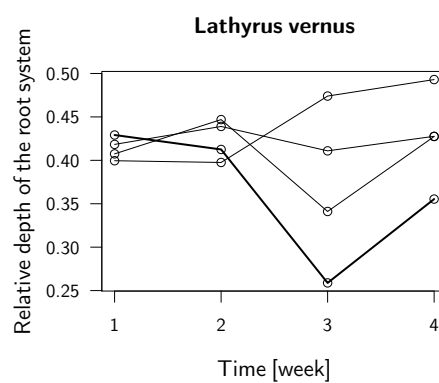
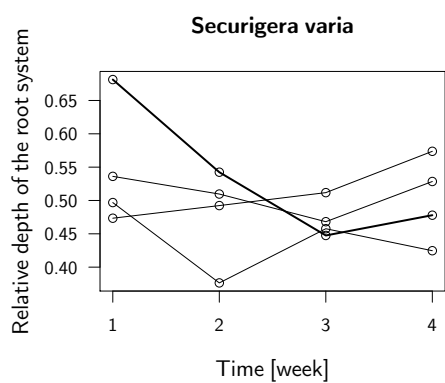
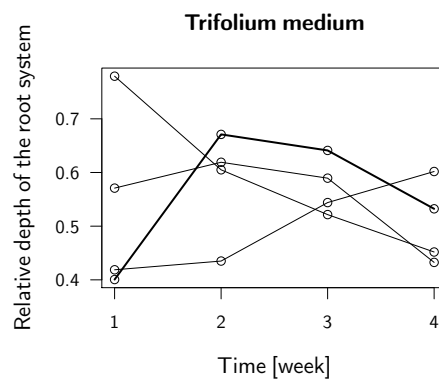
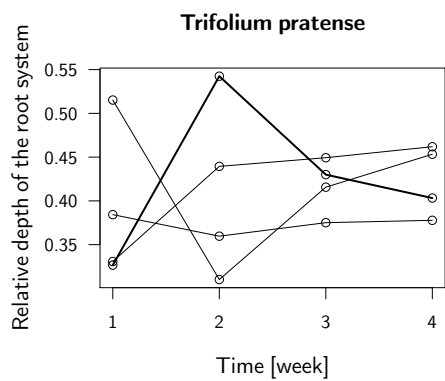


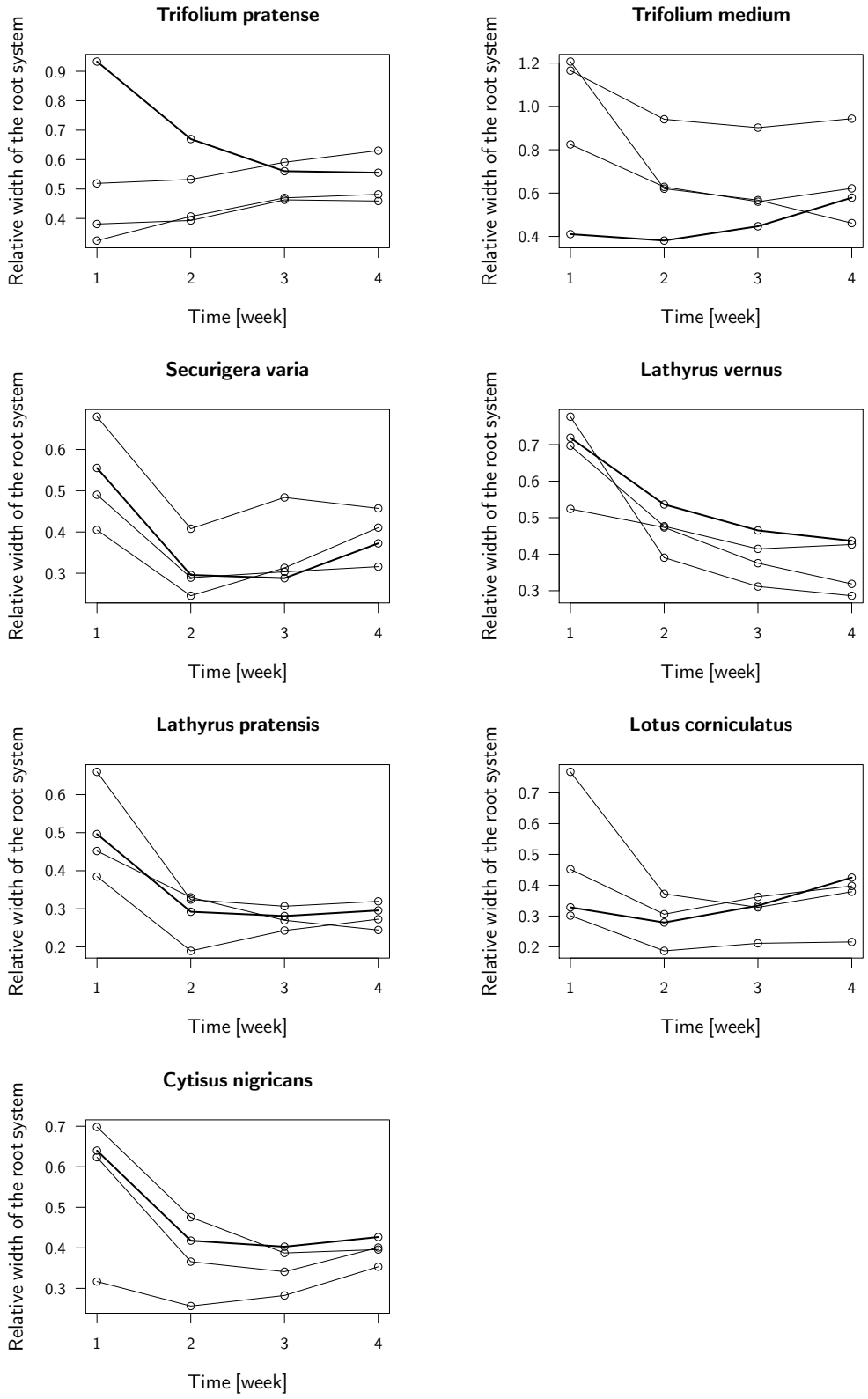
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G







Supplementary Fig. 4.2: All panels represent the raw data. The inference was based on their multivariate treatment (see the main text). Individual points depict means per trait, species, time and nutrition supply. Lines connect subsets with the same concentration of fertilizer and line thickness corresponds to nutrient supply. Panels: A = length of main root, B = total length of lateral roots, C = number of lateral roots, D = mean length of lateral roots, E = depth of the root system, F = width of the root system, G = area of the root system, H = relative depth of the root system, I = relative width of the root system.

Chapter 5

The effect of rhizoboxes on plant growth and root:shoot biomass partitioning

Tereza Mašková and Adam Klimeš

Abstract

Various types of flat rhizoboxes aid in root visualization and tracking in experiments where the focus is upon root system growth and development. While size of the pot is known to affect experiments, nothing is known about the impact of rhizoboxes – not only their volume, but also their shape might affect root and shoot growth. Therefore, we investigated how rhizoboxes change plant biomass and root:shoot biomass partitioning. We compared biomass and root:shoot ratio of plants growing in the usual ‘three-dimensional’, cuboid plant pots and flat ‘two-dimensional’ rhizoboxes about the same volume. We used two different nutritional treatments (deionized water and additional nutrients) for investigating whether the nutrient availability in the substrate changed the impact of rhizoboxes on plant growth. We used 15 species for the generalizability of our results across the phylogenetic tree. Compared to the usual pots, the plants invested relatively more in the roots when grown in the rhizoboxes, but this tendency was weak. This pattern was stable across nutrition treatments and across species. Further, we found no differences in total biomass of plants between pot type within nutrient treatments. With added nutrients, the plants had a higher biomass and lower root:shoot ratio compared to treatments without nutrient addition. Thus, species can be safely compared when grown in the rhizoboxes; rhizoboxes did not affect root system growth comparisons among species and nutrient levels. Also, they did not affect plant growth in terms of total biomass. However, they might affect root:shoot ratio of biomass partitioning – this should be taken into account when interpreting the results of rhizobox experiments.

Key words: Experimental container · Nutrient supply · Plant growth · Pot shape · Rhizobox · Root:shoot biomass partitioning

5.1 Introduction

Laboratory experiments are a routine tool in modern plant ecology for uncovering and understanding basic processes about plant behaviour, especially under different environmental settings or for multilevel experiments. For study of root system behaviour and plasticity, rhizoboxes are usually used because they allow continuous observation of uninterrupted root growth compared to plant cultivation by other means, e.g. pots or in the field, which only allow samples to be taken, hence disturbing or killing the individual plants under study.

Rhizoboxes can vary greatly in shape and size – from a petri dish (Gross et al. 1992) through a half-cylinder (Falik et al. 2005) to flat rectangular pots (Marschner and Römheld 1983; Schmidt 2018; Mašková and Weiser 2019). No matter the shape, during cultivation, rhizoboxes are inclined so that the root system is forced to grow along the flat front transparent wall. This pronouncedly deforms the space that the roots can occupy. Instead of three dimensions, the root system is forced to grow in essentially two. This reduction of dimensionality allows both full tracking of root system growth and also eases analysis of the root system than is the case in three-dimensional space.

It is silently assumed that this simplification into two dimensions does not fundamentally affect the behaviour of the plant root system – for example, if plants allocate more biomass into their root systems under some conditions than others, it would do so when the roots are grown either in three dimensions or a reduced two-dimensional space. On the other hand, it is known that the size of experimental pots can affect the results of an experiment (Poorter et al. 2012). It even turns out that the shape of regular pots (i.e., the ratio of their height and diameter; McConnaughay et al. 1993), the material the pots are made of (Bunt and Kulwiec 1970) or their colour (Markham et al. 2011) can slightly affect plant growth, mainly through their effect on soil and root temperature. But it is not known how the critical limitation of space to two dimensions in the rhizoboxes affects plant growth and behaviour. Plants in rhizoboxes may have a large fraction of their roots being ‘pot-bound’, with all kinds of secondary consequences, among others a changed root:shoot ratio (Herold and McNeil 1979).

The main goal of this study is to show whether total biomass and root:shoot biomass partitioning of plants growing in regular three-dimensional pots and flat “two-dimensional” pots of the same volume differ. Both total biomass and root:shoot biomass partitioning are closely related to soil nutrient availability, therefore we used two different levels of nutrient supply. We worked at an inter-specific level to generalize our results across the phylogenetic tree.

5.2 Materials and Methods

5.2.1 Plant cultivation

We grew plants in two differently shaped pots: regular pots – square ‘three-dimensional’ pots (upper size 7×7 cm, bottom size 5×5 cm, height 8 cm, the volume 290.7cm^3); and flat pots – rhizoboxes typically used for visualization of root systems (inner dimension $19.5 \times 15 \times 1$ cm h \times w \times d, the volume 292.5cm^3) consisting of PVC boards glued together with silicone sealant, with one of the

larger sides transparent. The transparent side was covered by a non-transparent panel during plant growth.

We chose perlite (expanded amorphous volcanic glass) as a substrate for cultivation of the plants. It provides good aeration and leaches practically no nutrients. We used two different nutrient treatments to assess whether the nutrient availability in the substrate changes the effect of the pot shape. We used universal fertilizer solution (Wuxal Super; manufactured by AGLUKON Spezialdünger GmbH & Co.KG, Düsseldorf; N:P:K = 8:8:6; see Table 3.2 for details) diluted in water to a 0.1% volumetric concentration, and pure deionized water. Half of the individuals in each type of pot were subjected to each nutrient treatment.

We used 15 common central European herbaceous species spread over the phylogenetic tree (Table 5.1). All seeds were acquired from a commercial supplier (Planta Naturalis, www.plantanaturalis.com).

Table 5.1: Plant species used and number of individuals per type of pot and nutrition treatment in the experiment.

Species	<i>Flat pots</i>		<i>Regular pots</i>	
	deionized water	nutrient supply	deionized water	nutrient supply
<i>Agrimonia eupatoria</i>	12	13	6	6
<i>Anchusa officinalis</i>	11	10	6	6
<i>Campanula glomerata</i>	11	8	6	5
<i>Campanula trachelium</i>	11	5	6	6
<i>Centaurea cyanus</i>	10	10	6	6
<i>Dianthus deltoides</i>	10	10	6	6
<i>Filipendula vulgaris</i>	9	10	6	6
<i>Inula britannica</i>	10	7	6	6
<i>Lathyrus vernus</i>	11	19	6	6
<i>Lepidium campestre</i>	11	11	6	6
<i>Lithospermum arvense</i>	10	10	6	6
<i>Lotus corniculatus</i>	12	11	6	5
<i>Lychnis viscaria</i>	10	9	6	6
<i>Nigella arvensis</i>	10	8	6	6
<i>Sisymbrium officinale</i>	10	10	6	6

All seeds were germinated individually in Petri dishes on filter paper moistened with 3 ml of the respective fertilizer treatment solution. Pure deionized water was added as needed so that the paper remained moist throughout. The Petri dishes were kept in a growth chamber (Adaptis A 1000 with TC kit, Conviron, Canada; light intensity $225 \frac{\mu\text{mol}}{\text{cm}^2\text{s}}$ at a distance of 12.5 cm from the light source) under the following diurnal temperature regime: 20°C for 12 hr during the day and 10°C for 12 hr during the night. On the day the radicle emerged through the seed testa, the seed was transferred into its own individual pot. Cultivation of the plants in pots took place in the same growth chamber used for germination and under the same temperature and light settings. The relative air humidity was set to 50% during the day and 70% during the night.

Initially, we aimed to have six replicates per species per nutrient treatment in the regular pots and ten replicates per species per nutrient treatment in the

flat pots. This would have led to 32 pots per species and 480 pots total. Due to technical reasons (not all plants survived transplantation), the actual number of pots per species per nutrient treatment ranged from 5 to 6 for regular pots and 5 to 13 for flat pots (see Table 5.1 for details).

We harvested plants after four weeks of growth, divided them into their root and shoot components at the boundary between epicotyl and hypocotyl, dried them at 65°C for 2 days and weighed them to assess their root and shoot biomass. We calculated total biomass (as sum of root and shoot biomass) and root:shoot ratio. We used total biomass and root:shoot ratio as an approximation of plant growth dynamics because it is known that other proxies of growth (e.g. SLA) are not correlated with pot size (Poorter et al. 2012). We used a value 0.005 for root:shoot ratio in cases where root:shoot ratio was 0 (in case that amount of root biomass was under the detection limit) for purpose of the logarithm.

5.2.2 Data analysis

To assess the effect of pot type on total biomass and root:shoot ratio, we ran phylogenetic hierarchical linear models in a Bayesian framework. Response (natural logarithm of total biomass (g) and of root:shoot ratio) was modelled for each species as:

$$Resp \sim Normal(\alpha + \beta_1 * P + \beta_2 * N + \beta_3 * P * N, \sigma)$$

where P is type of pot, N is nutrient level, σ is a standard deviation parameter and α , β are species-specific parameters. We modeled α , β as:

$$par \sim MultivariateNormal(\mu_{par}, phy * \lambda_{par} * \gamma_{par})$$

where *par* is parameter α , β_1 , β_2 or β_3 . μ_{par} is a mean parameter (specific for each α or β) and *phy* is a phylogenetic variance-covariance matrix, λ is a parameter which multiplies only off-diagonal elements of the variance-covariance matrix (thus Pagel's λ (1999)) and γ is a parameter (multiplies all elements of the matrix) specific for each α and β . The parameter λ was constrained to interval $[0, 1]$ and γ was constrained to be larger than 0.

For parameters we used uninformative Cauchy-distributed (with mean 0 and scale 5) priors, apart from parameters α and λ where we used uniform priors (on R for α and on interval $[0, 1]$ for λ). Models were evaluated using Hamiltonian Monte Carlo with a No-U-Turn sampler (Hoffman and Gelman 2014) with 4 chains, 4000 iterations each, 2000 of them as a warmup phase. Convergence was checked using \hat{R} statistics (\hat{R} was for all parameters within interval $[0.99, 1.01]$; Gelman and Rubin, 1992). Analyses were done in R (R Core Team 2016) using package rstan (version 2.17.3; Carpenter et al. 2017, Stan Development Team 2018). Phylogenetic information was taken from Durka and Michalski (2012).

5.3 Results

Total biomass of plants ranged from 0.1mg to 130mg and from 0.1mg to 59mg in the regular pots and flat pots, respectively. Plants under high nutrient treatment had 2.8 times more aboveground biomass (95% credible interval $[1.6820, 4.6450]$;

Fig. 5.1a). The total plant biomass was not affected by type of pots (plants in flat pots had 1.2 times more biomass; 95% credible interval [0.8338, 1.8340]) in both nutrition treatments (1.4 posterior mean of the interaction parameter; 95% credible interval [0.6703, 2.8205]; Table 5.2).

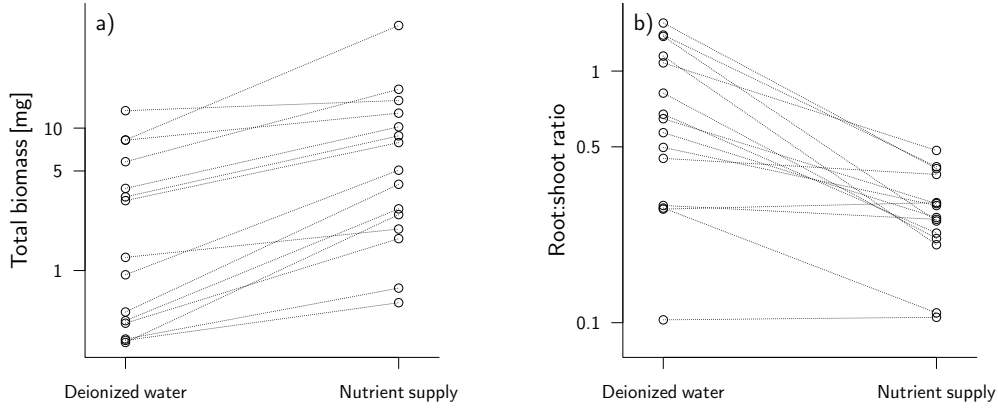


Fig. 5.1: Nutrient supply was the main driver of plant growth a) and behaviour b). Points indicate mean values for different species per nutrient supply, lines connect individual species. Root:shoot and total biomass were logarithmically transformed.

Table 5.2: Effect of pot type and nutrient treatment on logarithm of total biomass of plants. Values in brackets are on the (natural) logarithmic scale.

Effect	Parameter	Posterior mean	95%credible inreval
Type of pots	$\mu_{\beta 1}$	1.2310 (0.2078)	[0.8338,1.8340] (-0.1818,0.6065)
Nutrients	$\mu_{\beta 2}$	2.8075 (1.0323)	[1.6820,4.6450] (0.5200,1.5358)
Type of pots \times nutrients	$\mu_{\beta 2}$	1.3793 (0.3216)	[0.6703,2.8205] (-0.4000,1.0369)

The root:shoot ratio of plants ranged from 0 to 3 and from 0.003 to 4 in the regular pots and the flat pots, respectively. As expected, we found a lower root:shoot ratio in the higher nutrition treatment (0.5 times; 95% credible interval [0.2993, 0.8712]; Fig. 5.1b). Further, we found a marginal effect of type of pot on the root:shoot biomass partitioning of plants. Our results suggested a tendency to invest more into the below-ground biomass in the flat pots (a 1.4 times larger root:shoot ratio than in the regular pots; 95% credible interval [0.9928, 1.9429]; Fig. 5.2). This pattern did not differ between nutrition treatments (0.8 posterior mean of the interaction parameter; 95% credible interval [0.5207, 1.2638]; Table 5.3).

5.4 Discussion

Although the plant growth in rhizoboxes was comparable to the growth in regular pots, this study showed that the constraints of growth of seedling root system

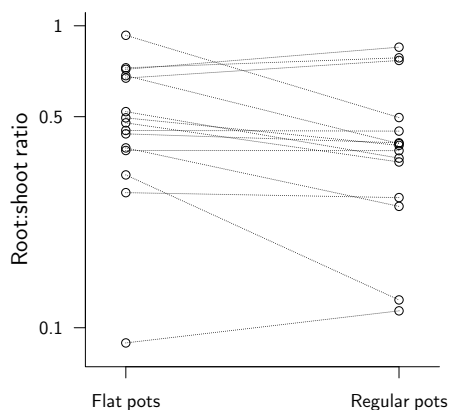


Fig. 5.2: Root:shoot ratio of plants growing in the flat pots and regular pots. Plants had a slight tendency to invest more into the below-ground biomass in the flat pots. Points indicate mean values for different species per nutrient supply, lines connect individual species. Root:shoot ratios were logarithmically transformed.

Table 5.3: Effect of pot type and nutrient treatment on logarithm of root:shoot ratio of plants. Values in brackets are on the (natural) logarithmic scale.

Effect	Parameter	Posterior mean	95%credible inreval
Type of pots	$\mu_{\beta 1}$	1.3824 (0.3238)	[0.9928,1.9429] (-0.0072,0.6642)
Nutrients	$\mu_{\beta 2}$	0.5092 (-0.6750)	[0.2993,0.8712] (-1.2063,-0.1379)
Type of pots \times nutrients	$\mu_{\beta 2}$	0.8081 (-0.2131)	[0.5207,1.2638] (-0.6525,0.2341)

in the two-dimensional space compared to the growth in three dimensional space could be stressful for plants and so possibly change their growth dynamics. As a result, plants growing in the flat pots partitioned their biomass slightly differently than the plants growing in the regular pots of the same volume. Although this effect was small, we think that it is necessary to consider it when interpreting the results of experiments with rhizoboxes. In the flat pots, plants have a tendency to invest higher proportion of biomass into their root system than plants in the regular pots, probably because they have to cope with obstacles in the form of pot side. Although the volume is the same for both type of pots, in the flat pots, substrate particles are distributed further away from the rooting point. Therefore, they are accessible only with more root growth and hence there are less substrate-bond resources available to the roots of the same total length, although the exact figures may vary according to the root intake capacity and mobility of the resources in the substrate matrix. Moreover, the pot surface between pot types differs substantially. Thus, the effects of the pot boundary (Herold and McNeil 1979; Ou 2014) or root self-inhibition (Falik et al. 2005) could have a higher importance in the flat pots than in the regular pots. Similar mechanisms play a role in terms of the effect of substrate texture (Semchenko et al. 2007).

On the other hand, we found almost no differences in total biomass of plants between the pot types. It seems that constraining space to two dimensions does

not mean a substantial difficulty for development of a root system capable of utilizing available nutrients. This supports the finding of Poorter et al. (2012), who showed that it was not pot size per se but rather plant mass per unit rooting volume that is relevant for plant growth. Nevertheless, it was shown that pot size affects plant growth (Keever et al. 1986; Poorter et al. 2012) and also could change the result of an experiment (Arp 1991), we therefore recommend carefully considering the pot volume as well for experiments with rhizoboxes.

The type of pot affected total plant biomass and root:shoot biomass partitioning much less than the nutrient supply. It has been shown many times before that the nutrient supply is an important driver of plant growth and root:shoot biomass allocation in pots (Gedroc et al. 1996) and also in the field (Delgado et al. 2011), and our results confirmed the same effect of nutrient availability on plant growth and development during their growth in rhizoboxes. In favour of the experiments based on rhizoboxes, the differences between usual pots and rhizoboxes were not amplified with an increased plant size induced by the fertilization. This suggests that the effects of rhizoboxes are not substantially allometric, hence the effects might be relatively easy to predict.

Rhizoboxes provide a great opportunity to study root system behaviour and dynamics. They are very useful for understanding the processes usually hidden below ground and allow for comparisons of root system growth among species under various conditions. However, comparisons within a species, or comparison of pot-based and rhizobox-based results might be affected by the possible differences in root:shoot biomass allocation.

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