

Investigating Root Storage and Exudation in the *Brachypodium* Genus

by

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ABSTRACT

In plants, sources are the tissues where a specific nutrient is absorbed or fixed into a plant, and sinks are the tissues in the plant where the nutrients are dispersed after they are absorbed or fixed. In the case of carbon, leaves tend to be the source, as most photosynthesis occurs in the leaves, and one of the sinks for carbon are root tissues. Because carbon-based compounds are the main source of cellular energy in plants, understanding the source-sink dynamics of carbon in plants gives us an insight into which functions or processes the plant is investing in. Specifically, the two aspects of source sink dynamics we investigate in this study are root exudation and root storage. We look at the difference of exudation between annual and perennial species and compare the levels of carbon storage in roots that arise from shoot apical meristems or from root apical meristems. These two functions occur in response to surplus carbon content in a plant, which we artificially prompted by withholding nitrogen from our plants. We use multiple species from the *Brachypodium* genus, an annual, *B. distachyon*, and a perennial, *B. sylvaticum* for this study. We chose this genus because it has evolved annuality and perenniality multiple times, and it is a good model for several economically valuable species. Most conclusively, we found that there is a significantly higher levels of carbon storage in shoot-borne roots compared to seminal root tissues.

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Introduction:

Plant tissues are specialized to perform specific tasks that are essential to the overall function and maintenance of the organism (Esau, 1977). The accumulation of nutrients is an example of this phenomenon, with leaf tissues gathering the majority of carbon, and root tissues gathering the bulk of other nutrients like nitrogen, phosphorus, and water. The movement of these nutrients throughout the plant are a major part of source-sink dynamics. Source refers to the tissue where the nutrient is absorbed or fixed, and sink refers to the tissues where nutrients are transported after they are absorbed or fixed. In the case of carbon, leaves are the source, as most photosynthesis takes place in the leaves, and roots are one of the sinks, getting carbon from the leaves to elongate and expand the root system. Source-sink dynamics refer to the relationship between different tissues and how they supply one another with the proper materials to complete essential plant functions. Studies in source-sink dynamics help us to understand both the mechanisms and the fitness effects for patterns of nutrient movement. For our study, we want to answer the question, how do root type anatomy, life history strategy, and nutrient manipulation affect the movement of carbon from leaves to the roots and out into the rhizosphere?

When plants absorb or fix different nutrients, not all of them are put into use for growth, reproduction, or other plant functions right away (Chapin, Schulze, & Mooney, 1990). For example, when a plant has excess carbon, some of it is stored in different plant tissues until it is needed (Prescott et al., 2020). The roots contain a sizable portion of these storage tissues and their anatomy may give us a clue as to how storage is organized within the root tissues. Storage is not only an important mechanism in trees and other plants with longer life cycles. Storage in grasses helps provide insight into fitness benefits of the various phenological strategies employed by both annuals and perennials in the *Brachypodium* genus. This study will give a glimpse into the diversity of storage strategies within the genus.

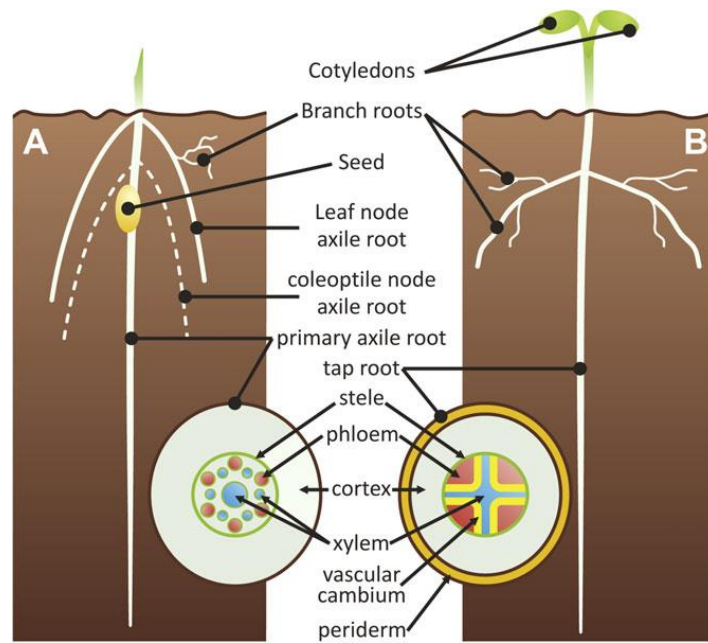


Fig. 1. Components of monocotyledon (A) and dicotyledon (B) root systems. Cross-section diagrams of the monocot primary axile root (A) and dicot tap root (B) illustrate the differences in vascular tissue arrangement. Reproduced from Journal of Experimental Botany 63, 3467–3474 (Chochois et al., 2012). Copyright © Oxford University Press, 2012.

In monocots, and more specifically grasses, the root system is split into two parts (Esau, 1977). The first is a seminal root, sometimes called a tap root, that emerges directly from the embryo (Rich & Watt, 2013). It has several smaller roots branching off of it, up to a third degree. The second part of the root system is comprised of adventitious roots that differentiate from the stem (Rich & Watt, 2013). These can include, and in *Brachypodium* do include, roots that differentiate from the coleoptile node and roots that differentiate from leaf nodes (Rich & Watt, 2013). Even though these roots emerge from the stem, they still are located below the surface of the soil. The primary seminal root tends to elongate straight downward, whereas these stem-borne nodal roots tend to branch more horizontally (Rich & Watt, 2013).

Several studies indicate functional differences between the nodal and seminal roots, including differences in timing of absorptive capacity and response to changing soil conditions (Rich & Watt, 2013). Like most monocot species, the seminal roots have a stele that consists of large central metaxylem vessel surrounded by smaller metaxylem (Chochois, Vogel, & Watt, 2012).

However, the nodal roots are not necessarily always constructed similarly. The basal region of nodal roots of some grasses, including the *Brachypodium* genus, have a stele with a pith of parenchyma cells instead of the large central metaxylem (Chochois, Voge, Rebetzke, & Watt, 2015). Within a single plant, parenchyma cells can perform several different functions in different tissue systems, but one of its most common functions is storage (Esau, 1977). In addition to the several differences in function between nodal and seminal roots, this anatomical difference suggests a potential storage disparity between the nodal and the seminal roots. Does this additional parenchyma found in the nodal roots increase its storage capacity compared to seminal roots?

In this study we will measure the soluble sugar concentration of root tissues as a proxy for total root carbon storage. We will assay soluble sugars because they are one of the major ways that plants store carbon, and have also been measured to study source-sink dynamics in previous studies (Martínez-Vilalta et al., 2016).

In addition to storage, root exudation is another important facet of source-sink dynamics. Root exudation refers to the transfer of nutrients from the inside of a living plant to the external environment through pores in root tissues (Canarini, Kaiser, Merchant, Richter, & Wanek, 2019). Similar to storage, root exudation also occurs in response to excess carbon (Prescott et al., 2020). Root exudation has been studied in several grasses and provides evidence to show that it is an integral part of the mutualistic relationship between grasses and the microorganisms that inhabit the surrounding rhizosphere (Hamilton, Frank, Hinchey, & Murray, 2008). Exudation is also elicited as a response to defoliation and herbivory, and in response to excess carbon (Hamilton et al., 2008; Shen, Yang, Xiao, & Zhou, 2020). Further, root exudation has been compared in annual and perennial species, but the studied species were not closely related, often comparing trees to herbaceous species (Phillips, ERLITZ, Bier, & Bernhardt, 2008). When comparing two species that are not closely related there are thousands of genetic, environmental, and functional differences that could potentially be responsible for different levels of exudation. Our study will observe two species, one annual and one perennial, in the same genus. Because they are genetically similar, it is far more likely that an observed difference in exudation would be caused by their different life history strategies.

To study these different topics, we will use multiple species in the *Brachypodium* genus, specifically the annual grass *B. distachyon* and the perennial grass *B. sylvaticum*. There are several different benefits to using the *Brachypodium* genus as a model system. Firstly, it is closely related to several staple crops, including wheat, rice, and barley, making our findings applicable to economically important species (Chochois et al., 2015). Further, the *Brachypodium* genus is the subject of multiple genomic studies, with several reference genomes for both our study species and a pangenome for *B. distachyon* (Gordon et al., 2017). It is also smaller in stature, making it easier to work with than other similar grass species. Finally, within the genus, there are several different annual and perennial species, that are not segregated into different clades (Vogel, 2016). This means annuality or perenniality has evolved independently multiple times within the genus. This allows us to investigate the differences like level of root exudation between annual and perennial plants fewer confounding factors related to the genetic gap between two species. In order to determine if nodal roots have a higher storage capacity than seminal roots, we will only be using *B. sylvaticum*, as having a perennial life history may be more conducive to increased storage.

Our experiment seeks to answer several questions relating to source-sink dynamics, using two processes that are both stimulated by excess carbon content. Do the nodal root tissues store more carbon than the seminal root tissues? Do perennial plants exude more carbon out of their roots than annual plants? Root storage and exudation of carbon both involve the mechanism of moving carbon, in the form of photosynthate, down the phloem from the leaves to the roots. This movement will give us an insight into the strategies employed by plants under different environmental conditions to give them the optimal fitness.

Materials and Methods:

Plant Growth

B. distachyon and *B. sylvaticum* seeds were placed in petri dishes lined with damp filter paper, covered with an additional sheet of damp filter paper, sealed with parafilm, and placed into a 5° C refrigerator for 4 weeks to synchronize germination. Seeds were then moved into 262 cm³ pots (D16H – 2x7” Heavyweight Deepot Cell) lined with mesh and filled with porous ceramic

topdressing profile (Greens Grade) saturated to field capacity with water. In addition, we also placed several seeds into 1642 cm³ pots (TP49 – 4x9.5” – “Short One” Mini-Treepot Usage), to check if smaller pots restrict the development of the root systems of our test plants. The pots were kept in a growth chamber (Biochambers FXC-9 growth chamber) with a 12-hour daytime, 12-hour nighttime cycle. Daytime temperature was set at 25° C, nighttime temperature was set at 20° C, and chamber humidity was set at 50%. Plants were kept under half strength light before tillering stage. Plants were watered three times a week, alternating between adding nutrients to the water or not. In addition, the racks of plants were fully randomized, and their position was rotated after each watering event. Before the tillering stage, all plants were given a control nutrient solution, (Dyna-Gro GROW™ 7-9-5). Plant germination date was recorded in order to properly assess the age of the plants in the experiment. Once tillering began, the plants were switched to a modified Hoagland nutrient solution (1500 μM K₂SO₄, 2000 μM MgSO₄, 4000 μM CaCl₂, 45 μM Fe-Na*EDTA, 4.6 μM H₃BO₃, 0.5 μM MnCl₂*4H₂O, 0.2 μM ZnSO₄*7H₂O, 0.8 μM Na₂MoO₄, 0.2 μM CuSO₄, 6000 μM NH₄NO₃, 600 μM KH₂PO₄) for 1 week before splitting into separate experimental groups. At this point, the lights were switched to full strength and any individuals that had not germinated were removed from the experiment. Our experiment included 65 plants, 7 *B. distachyon*, all in the 262 cm³ pots, and 58 *B. sylvaticum*, with 5 in the 1642 cm³ pots, and 53 in the 262 cm³ pots. The three experimental groups only altered the nitrogen concentrations, leaving the other nutrients concentrations the same throughout the experimental groups. The three nitrogen concentrations were the control group with 6 mM, the decreased group with 1.2 mM, and the starved group with 600 μM. Watering pattern continued to be three times a week with every other watering event including nutrient solution addition. The nutrient manipulation occurred for three weeks, but 23 *B. sylvaticum* individuals, all in 262 cm³ pots, were harvested after two weeks, and the remaining 42 individuals were harvested after the three weeks of nutrient manipulation had concluded.

Measuring of Soluble Sugar Content

Individuals to be tested for soluble sugar content were removed from Profile, and had the excess Profile washed off. Then the plant was separated into shoot tissue, nodal root tissue, and seminal root tissue. The shoot tissue was placed into an envelope and then into a drying oven at 70° C for a minimum of 48 hours. The nodal root tissues were split in half, with each root cut into distal

and proximal segments and placed into separate 15 mL falcon tubes. The seminal root tissue was also placed into a 15 mL falcon tube, and then all three tubes were flash frozen in liquid nitrogen and placed into a -70° C freezer for storage. The frozen root tissue was then freeze dried. The dried shoot and root tissues were weighed to determine shoot mass, root to shoot ratio, mass of nodal root tissues and mass of seminal root tissues. After being freeze dried and weighed, the root tissues were placed into a 2 mL tubes with ball bearings and put into a FastPrep-24 Classic Bench-Top Bead Beating Lysis System to be ground into a powder. The root tissue was then put through a protocol to determine soluble sugar content as a percentage of dry weight. We followed the phenol-sulfuric acid protocol for total soluble sugar content measurement as outlined by Landhäusser (2018). However, that protocol has mostly been used to measure sugar concentrations in tree tissues and is therefore more calibrated toward the concentrations typical of woody tissue. Blumstein and Hopkins (2021) used the same protocol and measured the non-structural carbohydrate concentration of woody tissues to be between 15 to 30 mg/g. Kafi, Stewart, and Borland (2003), measured the non-structural carbohydrate content of root tissue in wheat (*Triticum aestivum*) was calculated as within a similar range in response to differing salinity conditions. Wheat is closely related to the *Brachypodium* genus, and the two plants have similar anatomy, so it is a reasonable proxy for our individuals (Chochois et al., 2015). Therefore, it will not be necessary to dilute the root tissue, as it will likely have a soluble sugar concentration that will be in the range for which the protocol is calibrated.

Measuring of Root Exudates

Individuals to be tested for levels of root exudation will be removed from their Profile, and have the excess rinsed away with deionized water. Once cleaned, the plants were placed into Erlenmeyer flasks filled with either 560 or 280 mL of solution depending on plant size, with parafilm at the opening to separate the aboveground tissue and belowground tissue. Half of the plants were put in deionized water, and half were placed into their assigned fertilizer solution. The flasks were covered in aluminum foil in order to keep light off of the root tissues and the solution. After being in the flasks for one week in the growth chamber, the plants were removed, and the root and shoot tissues were separated. Root tissues were frozen in liquid nitrogen and placed into a -70° C freezer, then freeze dried. Shoots were placed into a drying oven. Then the tissues were weighed to determine root to shoot ratio as well as other plant mass measurements.

The solution remaining in the flasks were run through the same phenol-sulfuric acid protocol to determine the amount of carbon compounds exuded by the plant roots (Landhäusser et al., 2018).

Data Analytics

Data was analyzed using Rstudio version 3.6.3 and using the *tidyverse* package (Wickham et al., 2019), the *rstatix* package (Kassambara, 2021), and the *gridExtra* package (Auguie, 2017). In order to test linear relationships between two quantitative variables, and pairwise t-tests were used to test relationships between categorical variables and quantitative variables. Level of significance was set 0.05, and pairwise t-tests that tested more than two groups were subjected to the Bonferroni correction.

Results:

Soluble Sugar Content

Soluble sugar content was measured as the percentage of dry weight for our three types of root tissue and tested with a pairwise t-test with a Bonferroni correction for 82 samples. Sugar content in distal nodal roots ($p = 7.87 \times 10^{-6}$) and proximal nodal roots ($p = 0.0207$) were both found to be significantly higher than in seminal roots. Distal nodal roots had a significantly higher sugar content than proximal nodal roots before correction but were not significant after correction ($p = 0.0935$). Another pairwise t-test with a Bonferroni correction showed that different nutrient levels had no significant or observable effects on soluble sugar content ($p = 0.638, 0.356, 1$).

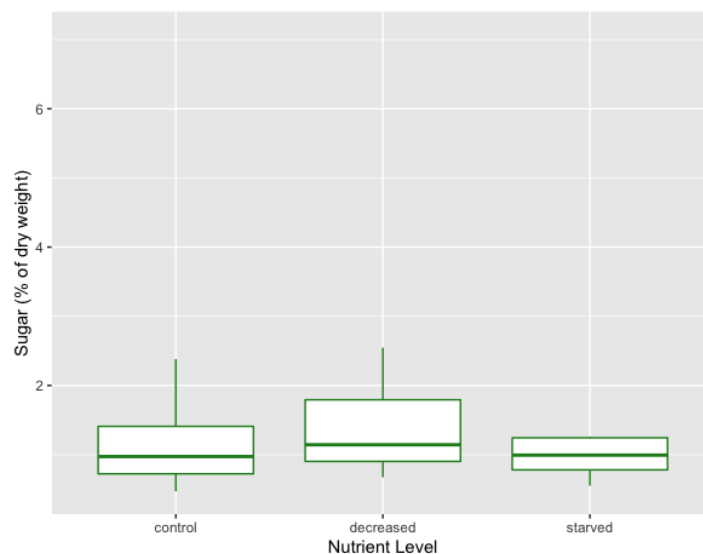


Fig. 2. The effect of applying fertilizers with different nutrient concentrations on the percent mass of dried root tissue that is composed of soluble sugars.

We then performed pairwise t-tests with the Bonferroni correction on groups separated by their soil nitrogen concentrations. In the group with control levels of soil nitrogen (6 mM, 41 individuals), sugar content in both distal nodal roots ($p = 6.39 \times 10^{-5}$) and proximal nodal roots ($p = 0.0123$) was significantly higher than in seminal roots, but there was no significant difference between sugar content in the two types of nodal roots ($p = 0.584$). In the group with decreased levels of soil nitrogen (1.2 mM, 21 individuals), there was a significantly higher soluble sugar content in distal nodal roots than in seminal roots ($p = 0.0012$), but sugar content in proximal nodal roots compared to in seminal roots was not significantly higher after Bonferroni correction ($p = 0.0667$). Again, there was no significant difference between the soluble sugar content of the two types of nodal roots ($p = 0.375$). In the group with starved levels of soil nitrogen (0.6 mM, 20 individuals), there were no significant relationships after correction ($p = 0.392, 0.0633, 1$). In all of the subsets, the mean and median of sugar content was lowest in seminal roots, then in proximal nodal roots, and highest in distal nodal roots (Figure 2).

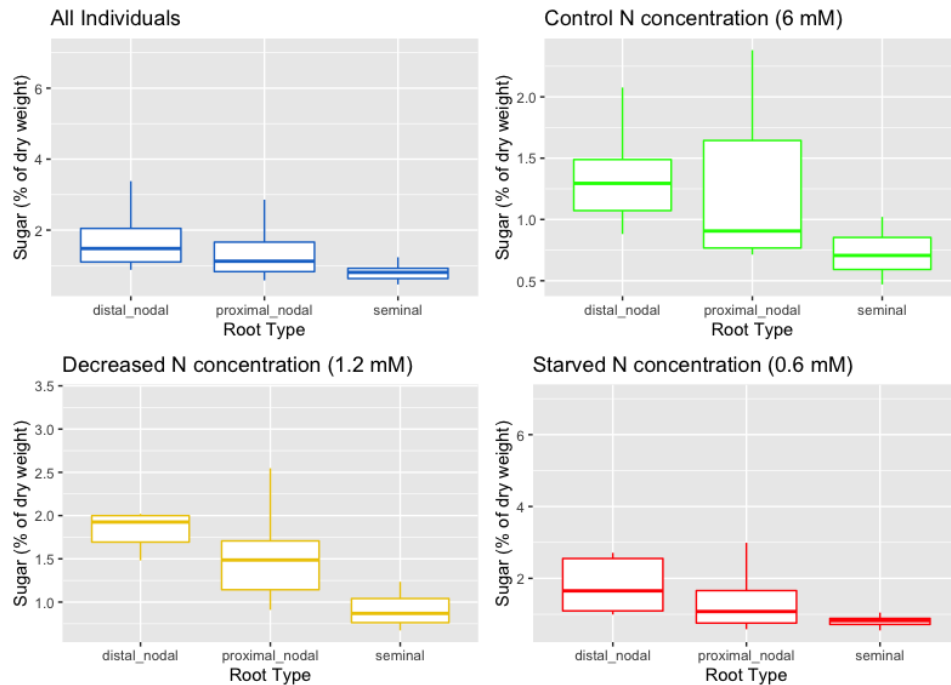


Fig. 3. Effect of root type on tissue sugar content, as a percentage of total dry weight of tissue. Blue is all individuals, green is individuals fertilized with control nutrient solution, yellow is individuals fertilized with decreased nutrient solution, and red is individuals fertilized with starved nutrient solution.

Root Exudates

After running the solutions remaining in the exudate flasks through the sugar assay protocol, and controlled for concentration of flask exudate solution, we found that there was no significant effect of species on the levels of exudation ($p = 0.664$). However, we did see an increased mean and median in *B. sylvaticum* compared to *B. distachyon*, and generally observe a slight increase of exudate level from the perennial compared to the annual.

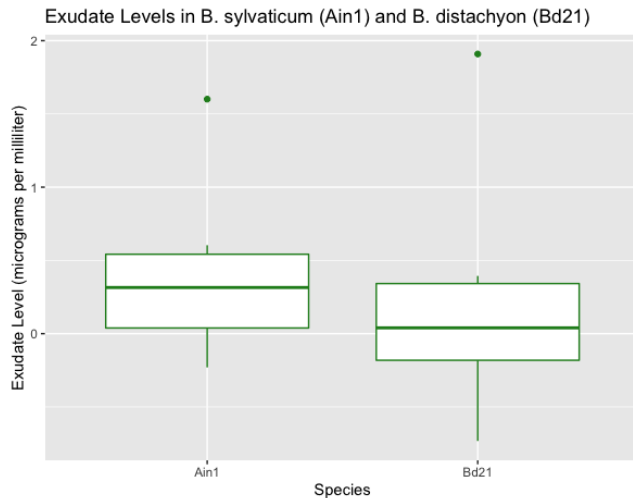


Fig. 4. Comparison of the exudate levels of the annual species *B. distachyon* and the perennial species *B. sylvaticum*. Exudate levels are measured as micrograms of soluble sugars per milliliter of solution.

Biomass Measurements

In addition to the measurements of soluble sugars, we also took measurements for dry mass of shoot tissues and root tissues, splitting their root tissues into seminal roots, proximal nodal roots and distal nodal roots for the individuals in the storage assay. This information, paired with the demographic information on pot size, nutrient levels, and plant age provided some patterns. Plant age had a significant linear relationship with both total plant biomass ($p = 1.203 \times 10^{-13}$, slope = 58.669) and root mass ($p = 2.994 \times 10^{-10}$, slope = 17.144), so all other biomass and total root mass tests were controlled for age by using average root mass added per day and average biomass added per day. So, when we investigate nutrient level's effect on total biomass, controlled for age, we see that there are no significant differences between control, decreased and starved groups ($p = 1, 0.0512, 0.547$), but there was an observed pattern of decreasing total biomass from control to decreased to starved. There was also no significant effect and no observable pattern of nutrient level on total root mass, controlled for age ($p = 1, 1, 0.874$). Similarly, there was no significant effect and no observable pattern of nutrient level on nodal root mass, controlled for age ($p = 0.55, 1, 0.524$). When we look at root to shoot ratio, there is an effect of the change in nutrient level, with individuals fertilized with starved nutrient solution having a significantly

higher root to shoot ratio than the individuals fertilized with control nutrient solution ($p = 0.00563$)

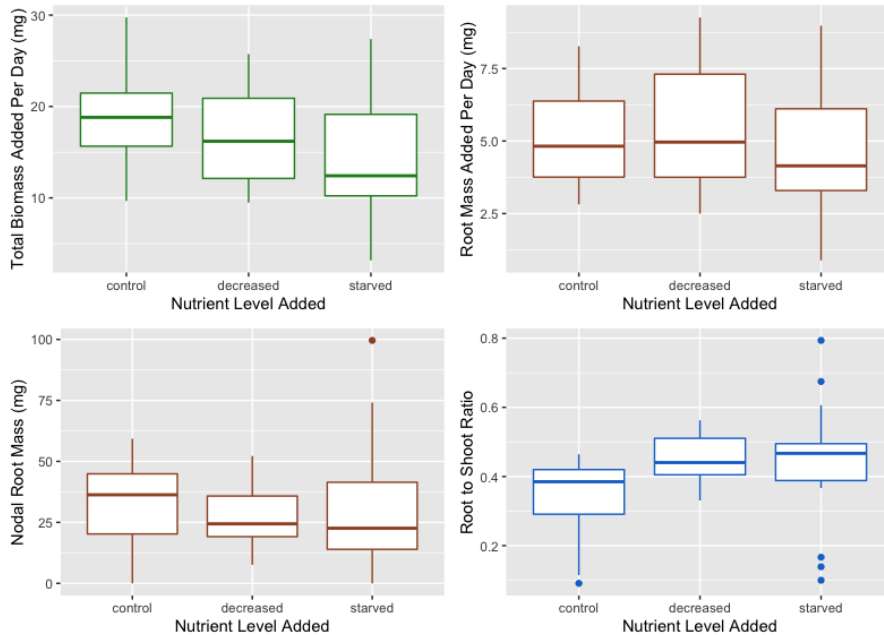


Fig. 5. The effect of altered nutrient levels on different plant tissue masses. From top left to bottom right: Total dried biomass of the plant divided by days since germination, Total dried root mass divided by days since germination, Dried nodal root tissue mass, Ratio of dried root mass to dried shoot mass.

In addition to nutrient level, pot size was another factor that could change the conditions of the plant development. The plants were either kept in smaller rounded 262 cm³ pots or in squared 1642 cm³ pots. When comparing the different biomass measurements with pot size, we found strongly significant results in total biomass ($p = 1.41 \cdot 10^{-13}$) and root biomass ($p = 1.09 \cdot 10^{-9}$), controlled for age, with both measures increasing on average by a factor of more than three. Root to shoot ratio did not show a significant difference between pot sizes ($p = 0.111$).

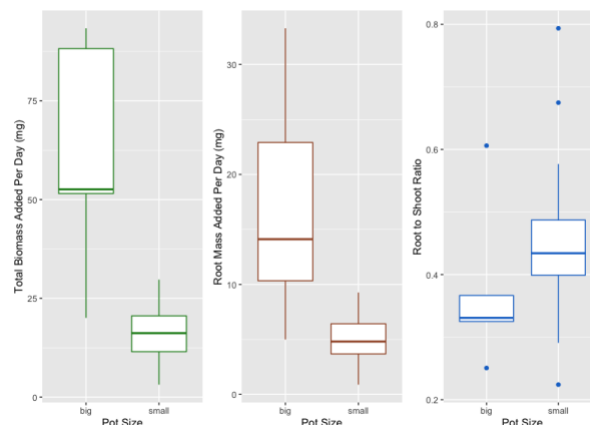


Fig. 6. The effect of pot size on different measures of biomass. From left to right: Total dried biomass divided by days since germination, Total dried root mass divided by days since germination, Total dried root mass divided by total dried shoot mass.

Discussion:

Source-Sink Dynamics

In this experiment we have used nutrient manipulation in an effort to increase excess carbon and prime carbon storage in the roots as well as increase root exudation of carbon compounds into the rhizosphere. Through this process we have learned several details about the source-sink dynamics of grasses in the *Brachypodium* genus. Let's begin with storage. Firstly, we see that the two main components of monocot root systems, seminal and nodal roots, have significantly different storage capabilities, with nodal roots having a higher percentage of their dry weight composed of soluble sugar when compared to seminal roots. This is possibly a mechanism of increased ground tissue cells, specifically parenchyma, in the pith of the roots of nodal root tissues, as opposed to the typical large central metaxylem. In addition to the difference between seminal and nodal roots, there was several instances of a difference in soluble sugar as a percentage of dry weight between the proximal and distal regions of nodal roots, but none were significant. This question requires further investigation, including anatomical research into where, along the longitudinal axis of the nodal roots, the center of the pith is composed parenchyma, and where it has a large central metaxylem. Then we can perhaps understand the difference in storage between the two portions of nodal roots. This discovery of yet another functional difference between nodal and seminal roots presents an opportunity to use them to our advantage. An interesting follow up experiment with this information would be to try to identify genes in the *Brachypodium* genus responsible for the differentiation between seminal and nodal roots. Then we could artificially select for those genes or artificially promote them more in an attempt to increase the storage capacity of the organism drastically. A large increase in storage potential could be used to improve reestablishment time for perennials or could be used to improve other grasses whose roots are harvested as crops, supporting development of perennial grain crops (Crain et al., 2020).

Next, our experiment with exudates did not yield a significant difference between the annual species *B. distachyon* and the perennial species *B. sylvaticum*. Multiple negative readings for soluble sugar concentration suggest that the solution was too diluted to detect level of exudation, despite the additional concentration that occurred from water absorption in the week after initial harvest of the plants. There was, however, still a slight pattern showing an increase in soluble

sugar content for *B. sylvaticum*, like our hypothesis predicted. In order to better test this hypothesis in the future, a similar experiment could be improved by concentrating the exudate solution by drying or boiling in order to increase the concentration to get an accurate reading. While we have no decisive results from this experiment, it does not necessarily mean that there is no effect of species on level of root exudation of soluble sugars or other non-structural carbohydrates. Further study is necessary in order to make a definitive conclusion.

Pot size

Larger size pots were initially included to ensure that the plants' root systems were not constricted by the 262 cm³ pots, as previous experiments with *B. distachyon* and *B. sylvaticum* showed no signs of being affected by that pot size. However, likely due to the increased length of growth time in this experiment compared to previous ones so that nutrient manipulation could begin after tillering, it became clear that the 262 cm³ pots were restricting plant growth. This occurred when controlling for the age of the plant, and under the same soil nutrient concentration and proportional watering levels. In future experiments, if growth is expected to last above 50 days under optimal growth conditions, larger pots, such as the 1624 cm³ pots will be necessary to more accurately reflect field conditions.

Additional Limitations of the Study

The main limitation of this study is the relative error associated with the protocol for the measurement of soluble sugars. Improved calculation of interference with multiple types of blanks, as well as a calculation of a standard curve as outlined in the Landhäusser paper (2018), would provide a decrease in error and perhaps elucidate more concrete relationships. In addition, assays that extract and measure from the same protocol could be run in order to get a complete picture of non-structural carbohydrates in the roots of the *Brachypodium* species. That way we could get a much larger picture of storage in these grasses, as starch is a major storage compound in plants (Chapin et al., 1990). Samples for this set of plants are still usable, so an improved dataset could be attained without a complete restart of the experiment.

Conclusions

The movement of carbon downward from photosynthetic tissues in the shoots down into the roots for storage, or out through the roots via exudation are incredibly important ecological processes for grasses (Prescott et al., 2020). They help to foster mutualistic relationships with soil microbes, improve reestablishment in future growing seasons, create stores for regrowth in case of herbivory, and more (Hamilton et al., 2008; Williams et al., 2022). In our experiment, we found out that the two main root types in the *Brachypodium* genus have different storage capabilities that can be tied to a small anatomical difference and learned how different environmental factors like pot size and nutrient concentration can affect the biomass of different portions of *Brachypodium* grasses. In addition to better understanding the movement of carbon throughout the plant, it gives us a more well-rounded idea of how the plants in the *Brachypodium* genus interact with their environment. We can use these insights in the model system and apply them to numerous grass crop species. This can include improving efforts to increase perennial crop abundance, decrease reliance on artificial fertilizers, and foster more mutualistic relationships with soil microorganisms. These improvements would be great steps in making our agricultural systems more sustainable.

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