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"Improved split-root system for the study of systemic sulfur allocation and incorporation into bacteroid proteins"

verfasst von / submitted by

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Abstract

The symbiotic interaction between legumes and rhizobium bacteria involves the nutrient exchange between both partners and enables the plants to grow on nitrogen deficient soils.

This metabolic exchange is located in the newly formed root organs, the nodules, where plants supply symbionts with sugar, sulfur and micronutrients while the bacteria fix atmospheric nitrogen (N) to build amino acids. Sulfate is the form of sulfur, crucial for the bacterial nitrogenase enzyme biosynthesis, responsible for N_2-fixation, and has been shown to be the form that directly transported towards the symbiont.

The question remains whether sulfate can also be systemically transported to the rhizobia via shoots or via incorporation into sulfur-containing amino acids.

For this, I developed a robust split-root system and found that sulfate can most likely only be directly (locally) transported to the bacteroids; there were no indications for an alternative systemic transport mechanism.

The development of the split-root system resulted in a very detailed protocol and allowed the study of systemic nutrient transport in the model legume, *Lotus japonicus.* For this, I used a split-root system, with one root being inoculated and kept without sulfate and the other not being inoculated but receiving ^{34}S-labeled sulfate through a nutrient solution. While previous experiments could demonstrate that sulfate is incorporated into rhizobium nitrogenase, I could not find any clear indications for a sulfur incorporation even after nine days. No significantly difference was detected in the relative isotope abundance when applying sulfate indirectly through split-roots.

My results indicate that sulfate is the sole form of sulfur that can be transported to the microsymbionts. It further suggests that sulfate is not transported from shoots to roots but may be transported in other forms, which are not able to enter the symbiosome.

The split-roots system, developed during this study, is more robust than previous protocols and may be easily adapted to other research questions such as to determine the exact sulfate concentration needed for symbiotic N-fixation and nodule formation as well as for further research on autoregulation of nodulation for instance.

Deutsch

Die symbiotische Wechselwirkung zwischen Hülsenfrüchten und Rhizobium-Bakterien beinhaltet den Nährstoffaustausch zwischen den beiden Partnern und ermöglicht den Pflanzen, auf stickstoffarmen Böden zu wachsen.

Dieser Stoffwechselaustausch findet in den neu gebildeten Wurzelorganen, den Knöllchen, statt, in denen Pflanzen Symbionten mit Zucker, Schwefel und Mikronährstoffen versorgen, während die Bakterien atmosphärischen Stickstoff (N) binden, um Aminosäuren zu bilden. Sulfat ist die Schwefelform, die für die Biosynthese des bakteriellen enzyms, Nitrogenase (für die N-Fixierung), entscheidend ist. Es wurde gezeigt, dass Sulfate direkt zum Symbionten transportiert wird.

Es bleibt die Frage, ob das Sulfat auch systemisch über den Sproß oder indirekt durch Einbau in schwefelhaltige Aminosäuren zu den Rhizobien transportiert werden kann.

Dafür habe ich ein robustes „Trennwurzelsystem“ (Split-Root-System) entwickelt und festgestellt, dass Sulfat höchstwahrscheinlich nur direkt und nicht über den Sproß, zu den Bakteroiden transportiert werden kann; Es gab keine Hinweise auf einen alternativen, systemischen Transportmechanismus.

Die Entwicklung des Split-Root-Systems führte zu einem sehr detaillierten Protokoll und ermöglichte die Untersuchung des systemischen Nährstofftransports in der Modellhülsenfrucht *Lotus japonicus*. Dafür verwendete ich dieses Split-Root-System, wobei eine Wurzel symbiont inoculiert und ohne Sulfat und die andere nicht symbiotisch angezogen wurde, aber ^{34}S-markiertes Sulfat über eine Nährlösung erhielt. Während frühere Experimente nachweisen konnten, dass markiertes Sulfat nach fünf Tagen zu 10% in die Nitrogenase eingebaut wird, konnte ich nun auch nach neun Tagen keine klaren Hinweise für einen Schwefeleinbau finden. Es wurde bei indirekter Sulfatapplikation über das Split-Root-System kein signifikanter Anstieg in der relativen Isotopenhäufigkeit festgestellt.

Meine Ergebnisse zeigen, dass Sulfat die einzige Form von Schwefel ist, die zu den Mikrosymbionten transportiert werden kann. Es kann ferner vermutet werde, dass Sulfat nicht vom Sproß zu den Wurzeln transportiert wird, aber eventuel in anderen Formen, die jedoch nicht in der Lage sind, in den Symbiosom einzudringen.

Das in dieser Studie entwickelte Split-Roots-System ist robuster als frühere Protokolle und kann leicht an andere Forschungsfragen angepasst werden, um z.B. die genaue Sulfatkonzentration zu bestimmen, die für die symbiotische N-Fixierung benötigt wird sowie für die weitere Forschung an der Autoregulierung der Knöllchenbildung, für die dieses System ursprünglich konzipiert wurde.

Contents

1. Introduction

1.1. *Lotus japonicus* and *Mesorhizobium loti*

Legumes (family Fabaceae) are very important considering their role in agriculture – the symbiotic N-fixation (SNF) results in importing 40 million tons of nitrogen into agricultural systems each year (Herridge, Peoples, and Boddey 2008). Legumes are used in cross rotations because they provide excess nitrogen to surrounding nonlegume crops. (Udvardi and Poole 2013). This process is also valuable because it is at no charge and has no negative impact on the environment. *Lotus japonicus* is a wild legume that can be found all over the world and thus became an important model legume for research on symbiosis with nitrogen fixing bacteria. The full genome structure has been published in 2008 (Sato et al. 2008). Another model legume is *Medicago truncatula* which is nodulated by *Synrhizobioum meliloti* and develops indeterminate nodules. In contrast to the model legume *Medicago truncatula* (López et al. 2008) *Lotus japonicus* forms determinate nodules in a specific symbiotic interaction with the *Rhizobium*, *Mesorhizobium loti* (formerly known as *Rhizobium loti*). This strain was first isolated from a root nodule on *Lotus corniculatus* (Jarvis, Pankhurst, and Patel 1982) and is ever since used for studies on *Lotus spp.* symbiosis (Stougaard 2001). Having different types of nodules (determinate and indeterminate) makes *Medicago* and *Lotus* good model legumes. Several *Lotus japonicus* mutants (LORE1 (Madsen et al. 2005)) and *Mesorhizobium* mutants strains exist (LPS, CGS (D'Antuono et al. 2005), R7A-nodZ, nolL (Rodpothong et al. 2009), NifA-RpoN (Sullivan, Brown, and Ronson 2013)).

1.2. **Symbiosis and nutrient exchange**

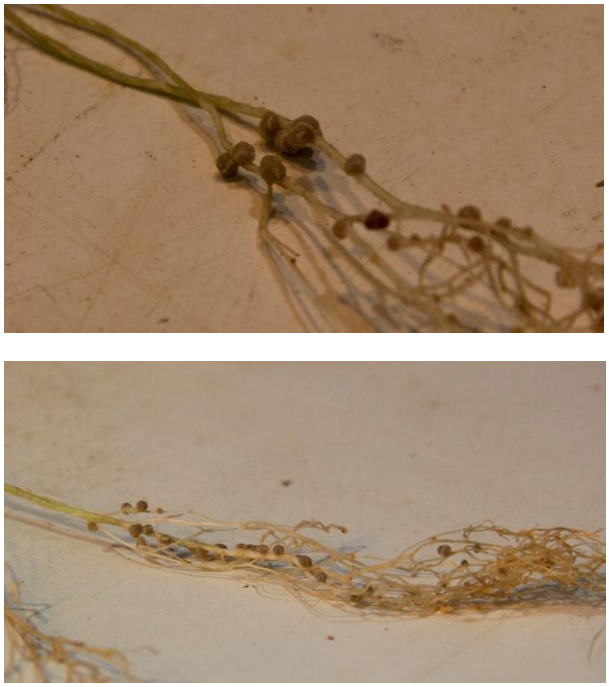


Figure 1 Root nodules.

In 1886 H. Hellriegel discovered the symbiotic interaction between legumes and rhizobia. He described the ability of the rhizobia to provide the plant with elementary fixed nitrogen from the atmosphere. To date, it is well established that the plants in return, provide the microsymbionts with carbon compounds fixed through photosynthesis (Udvardi and Poole 2013). SNF takes place in newly formed plant organs, the root nodules, form after inoculation of the root by rhizobia (Fig.1). After infection, bacteria differentiate into bacteroids enclosed by the plant-derived symbiosome membrane. This new organelle is called symbiosome. In *Lotus japonicus*, symbiosome usually contains several bacteroids. (Oldroyd et al. 2011) The symbiosis between legumes and rhizobia is valuable because it allows plants to grow on N-poor soils, which would not be possible without SNF. It is an important input of N for plants, considering the effect that high amounts of N have on eutrophication in agriculture (Vitousek et al. 1997).

The role of sulfur in SNF

Sulfur is one of the essential nutrients for plant growth and functioning (Takahashi et al. 2011). It is a limiting nutrient, right next to nitrogen. It is usually taken up as sulfate from the soil by specific sulfate transporters (Takahashi et al. 2000). It has been shown that SNF is dependent on sulfur while it is sensitive to nitrate (Zhao, Wood, and Mcgrath 1999). Legumes, grown under sulfate deficiency were hampered in SNF. However, it was not clear if this effect was an indirect effect due to a general sulfur starvation of the plant or directly linked to N-fixation process.

A key enzyme that enables SNF is the bacterial nitrogenase which is rich in sulfur. Nitrogenase is composed of two components, a homodimer - iron (Fe) protein (dinitrogenase reductase) which contains a single [4Fe-4S] cluster between the subunits and a heterotetramer - molybdenum-iron (MoFe) protein (dinitrogenase) with a [8Fe-7S] cluster between subunits. The Fe protein is encoded by NifH gene, while MoFe is encoded with NifD. (Yang, Danyal, and Seefeldt 2011). They both have sulfur in their structure which indicates why sulfur is so important for SNF and makes them good marker enzymes when analyzing the role of sulfur.

Wienkoop and Saalbach in 2003. showed for the first time the transport of sulfur from the plant to the nodules. They identified a highly expressed sulfate transporter in the symbiosome membrane which revealed a direct link of sulfate for the symbiosis. The transporter was identified as a symbiotic sulfate transporter 1 (SST1). The *sst1* mutants showed a decrease of Fe protein and leghemoglobin contents and a reduction of N_2 fixation rates at around 90% (Krusell 2005). However, it was still unclear if SST1 regulated sulfate uptake towards the bacteria and why a regulated sulfate uptake was necessary for the functioning of the symbiosis.

Sulfur protein incorporation studies using stable isotope metabolic labeling

Peptides containing a stable isotope have the same characteristics as unlabeled peptides concerning chromatography. The only difference is their mass. In metabolic labeling, the labeled isotope is introduced to an organism trough the growth medium. (Schulze and Usadel 2010). Stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O and ^{34}S) are used for labeling in MS analysis because they are easily distinguished from the most abundant isotopes that occur in nature (^{1}H, ^{12}C, ^{14}N, ^{16}O and ^{32}S) (Freund and Hegeman 2017). ^{15}N labeling was first used to study protein phosphorylation in bacteria (Oda et al. 1999). In plants, ^{15}N was used to address the assimilation in extraradical mycelium in mycorrhizal roots (Jin et al. 2005), for quantification of nitrogen transfer between mycorrhizal plants (He et al. 2009), quantitative proteomic analysis in Arabidopsis thaliana (Nelson et al. 2007) etc. Stable isotope labeling in planta (SILIP) is a method developed for efficient labeling of soil-grown plants (Schaff et al. 2008).

SILIP, using ^{34}S was used for the first time and applied in order to determine the transport rate of sulfur through the symbiosome membrane and the importance of sulfur for nitrogenase biosynthesis. It was shown that sulfate is transported at a high rate across the symbiotic membrane and accumulated in the bacteroids (Schneider et al. 2019). It remained open, if the SST1 is the only way for sulfur to cross the symbiosome membrane or if other forms than sulfate (e.g. s-containing amino acids) may be able to pass through. Furthermore, it remained unclear if sulfate was also systemically transported through the plant in order to reach and maintain symbiosis.

1.3. Split-root system

The split-root system plays a major role in unraveling systemic and local regulation mechanisms. Two separate root strands can be treated differently while they are linked via the same shoot system (Larrainzar et al. 2014). This allows to determine if there is a response in the untreated part of the split-root system, meaning that long-distance transport mechanisms, which involve the plant canopy, are active (implying systemic regulation). In contrast, when the response can only be seen in the treated part of the system, then this is a local response. So far, split-root systems were used to research autoregulation of nodulation (AON). These studies showed inhibition of nodulation on one side of the split-root system when the other side was previously inoculated and SNF fully functional. This was found in soybean (Kosslak and Bohlool 1984), subterranean clover (Sargent et al. 1987), alfa-alfa (Caetano-Anolles, Lagares, and Bauer 1990), common bean (George, Robert, and Bohlool 1992), common vetch (van Brussel et al. 2002), pea (Li, Kinkema, and Gresshoff 2009), model legumes *Lotus japonicus* (Suzuki et al. 2008) and *Medicago truncatula* (Jeudy et al. 2010). Split-root systems are also used for determining the effect of biotic and abiotic stresses on symbiotic N-fixation. Mostly researched abiotic factor is drought which has been shown to inhibit SNF (Arrese-Igor et al. 2011). Another factor that as well inhibits SNF and leads to nodule senescence is nitrogen supply. Systemic regulation of SNF is shown after longer exposure period of nitrate (Daimon and Yoshioka 2001). Split-root system is a great tool for understanding systemic vs local regulation in the plant, and all time and concentration critical processes that are crucial for well development of the nodules and their function. The split-root system in combination with SILIP enables nutrient allocation studies and gain insights into systemic transport regulations.

1.4. **Objectives of the thesis**

Legumes play an important role in agriculture and it is very important to understand how they will react to changes of abiotic factors due to global warming and new agricultural techniques. Understanding the importance of symbiosis between legumes and rhizobia is crucial for more successful agriculture in the constantly growing human population. So far it is well known that plants are not able to take up atmospheric nitrogen, so one solution to this problem was to use fertilizers but this leaves another problem – eutrophication. Therefore, to engineer plant-Rhizobium interaction and SNF to other plants would enable a drastic reduction in needs of N-fertilization. However, besides fixed carbon, another essential element for SNF functioning is sulfur (Schneider et al. 2019). It is taken up by the plant and transported to the bacteroids in high amounts via the symbiosome membrane specific SST1. In contrast to nitrogen poor soils, the limitation of sulfate inhibits SNF. Sulfur is needed in high amounts, but it is not clear if it can be transported systemically through the plant in order for optimal provision to the bacteroids. Split-root system of *Lotus japonicus* was developed for a study of autoregulation of nodulation by Suzuki et al. in 2008. I adjusted their protocol and applied ^{34}S-metabolic labelling to study sulfur allocation regarding the role of sulfate in SNF. Is sulfate systemically transported to the root system and can its incorporation into bacteroid proteins be traced?

Specific goals were:

1. To define, how long it takes until sulfur incorporation into nitrogenase is detectable under normal conditions,
2. to improve the existing split-root protocol for robustness and reproducibility and
3. transform it into a new protocol,
4. to answer the question, whether ^{34}S is incorporated into the nitrogenase, when only applied to the non nodulated side of the root system.

2. Materials and methods

2.1. Lotus japonicus germination and root cut

Lotus japonicus seeds were treated with sulfuric acid, on a shaker for 20 minutes, then washed with distilled water. After washing, bleach was used to treat the seeds for 20 minutes again in the shaker. After treating with commercial bleach, seeds were washed with sterile water and left overnight in the water with gentle agitation. This preparation was used to weaken the seed coat and to start germination. After the overnight step in the sterile water, seeds were transported to plant agar plates (B&D nutrient medium (Broughton and Dilworth 1971) solidified with 0.8% plant agar) and incubated in the dark for 3 days at room temperature. If germination was successful, plates were transferred to light conditions and left to grow in the plates for 5 days. After this, plants were ready for the “root cut” (Fig. 2). To achieve adventitious root formation, cutting of the hypocotyl of the seedlings is necessary. Plants are then planted in pots with vermiculite and watered with B & D medium containing 0.5 mM KNO_3 solution for 25 days. When adventitious roots were formed, only the plants which had roots with equal size (at least 4 cm) were selected and transported to the split root system (Fig. 2).

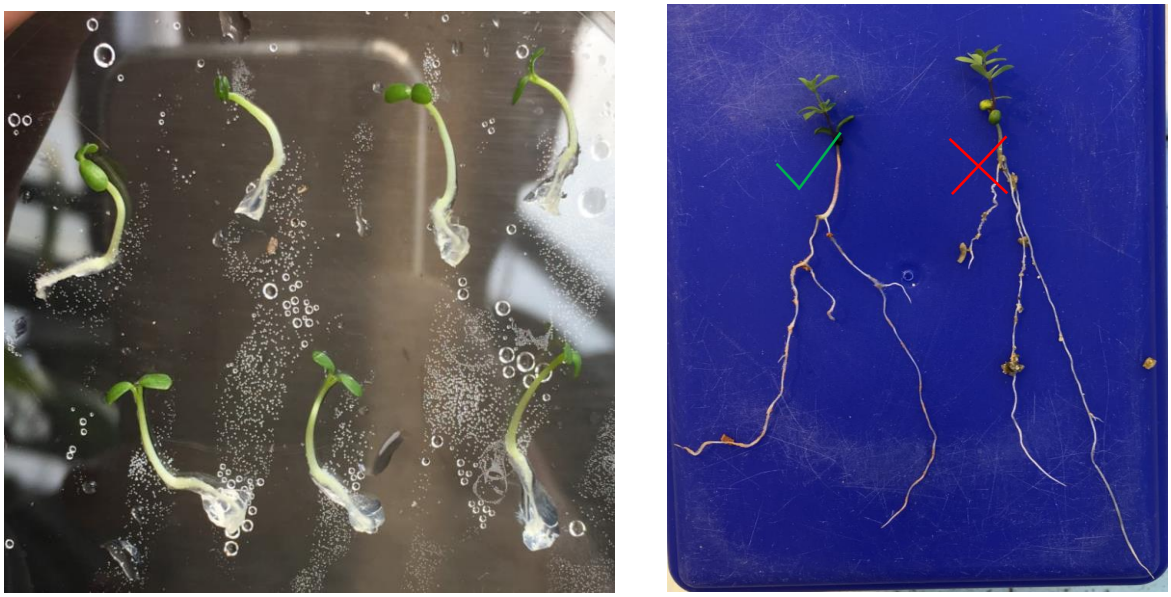


Figure 2 Left – seedlings ready for root-cut. Right – example of same root length and a plant where one root took over.

2.2. **Preliminary control analysis of a time course of ^{34}S incorporation**

Plants were grown normally (no split-root system) and fully inoculated as described before and according to (Schneider et al. 2019). Plant nodules were harvested at time point 0, 24, 48 and 72 hours of ^{34}S-labelling.

2.3. **Establishment of split root system**

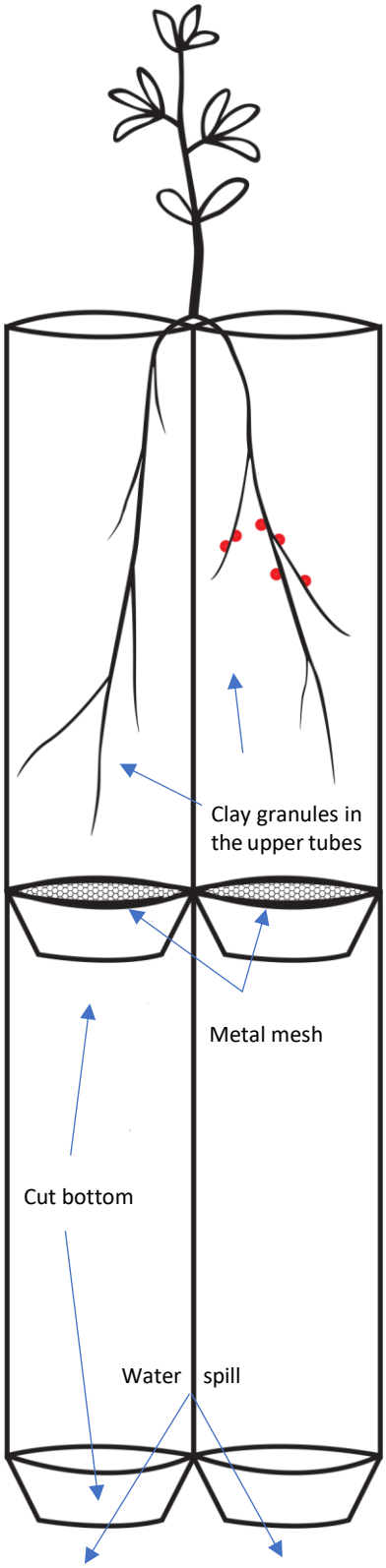


Figure 3 Split-root system setup

Falcon tubes were used instead of pots for the split root systems. Tips of the tubes were cut, to provide a free space for water to run through, and a mesh was placed on the bottom of each tube to keep the granules inside the tubes (Fig. 3). For cutting the tubes first soak them in hot water to soften the plastic.

For the first set I used one tube for each root. In the second set I used two tubes, one on top of the other, for each root (Fig. 4). I taped the tubes together. Half of the tubes were filled with sterilized clay granules soaked with B & D solution containing 1 mM KNO_3. When the set-up was ready, roots were separately placed gently in different tubes and the tubes were filled up to the top with a spoon. The plants were left to adjust for 1 day. One root was inoculated, and plants stayed in the split root system for 28 days.

Figure 4 Falcon tubes taped together for split-root system

2.4. Inoculation

Mesorhizobium loti was cultivated on yeast-mannitol-agar with congo red (YEMA, table 1.) medium in plates 25°C in the dark and, when enough colonies were grown, stored at 4°C. Rhizobia was later resuspended in YEM solution, incubated at 25°C for 3 days and immediately used for inoculation. Only one root in the system was inoculated (root A) while the other was not (root B).

Ingredients	**g/l**
Yeast extract	1
Mannitol	10
Dipotassium phosphate (K_2HPO_4)	0.5
Magnesium sulfate ($MGSO_4x7H_2O$)	0.2
Sodium chloride	0.1
Congo red	0.025
Agar	20
Total	31.825
Final pH (at 25°c)	6.8 (+/- 0.2)

Table 1. Yeast-Mannitol-Agar with congo red

Inoculation took place one day after setting up of the split root system and again 3 days after the first inoculation. Roots were inoculated with 2 ml of solution with rhizobia each time. Inoculation was checked after 3 weeks, and if there was not sufficient number of nodules developed, again after 5-7 days. The roots were watered with different concentrations of KNO_3. The side with nodules was watered with 0.5 mM KNO_3, and the

side without nodules was watered with 3 mM KNO_3 in one trial and then with 2 mM KNO_3 in the second trial.

Tubes were labeled accordingly to visually separate the side with an inoculated root from the side with the root that was not infected (Fig. 5).

Figure 5 Split root system with both strong roots. Tubes labeled according to inoculation and watering conditions.

2.5. ^{34}S - Sulfate metabolic labeling in *Lotus japonicus*

4 weeks after the first inoculation plants were checked for sufficient nodulation. If there were enough nodules, experiment could be started. Plants were watered only with water for 2 days to wash out any remaining nutrients and divided into two subsets - control plants and plants for the ^{34}S uptake (S-plants). Plants were watered with appropriate solutions every 3 days and the nodules were harvested on the 10th day. One control group was kept in the conditions that were leading up to the experiment, to check if the nodule senescence is connected to sulfate deficiency or the nodules are getting old just with time. Roots were watered differently both in control and in the S-plants. In control plants, ‘root A’ was watered with sulfate-free growing solution, while ‘root B’ was watered with the nutrient solution that contained non-labeled sulfate (Na_2SO_4). In S-plants, ‘root A’ was watered with sulfate-free growing solution while ‘root B’ was watered with growing solution which contained labeled sulfate ($Na_2{}^{34}SO_4$). (Fig. 6, 7) In the sulfate-free solution, all sulfates in the B&D medium were replaced with chlorides or phosphates.

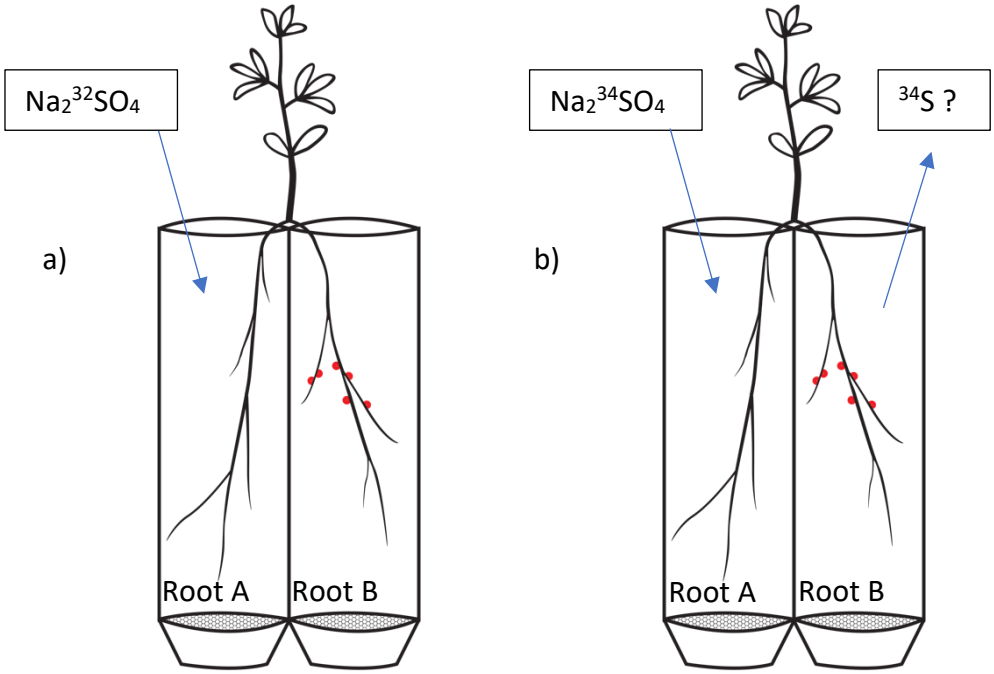


Figure 6 Two groups: a) control and b) treatment. a) Root A was watered with a solution containing 32S, the most abundant isotope in nature, and 2 mM KNO3, while b) Root A was watered with a solution containing 34S, heavy isotope, and 0.5 mM KNO3

Figure 7 Final look of the split-root system.

2.6. Protein extraction

Nodules were harvested and homogenized with ice-cold homogenization solution (HS1, table 2.) in Eppendorf tubes using small plastic pestles and then centrifuged at 4°C at 200 xg for 15 min. HS1 contains sucrose, an osmolyte which is used to preserve the bacteroid cells from breaking. Thus, in the supernatant only soluble proteins from the nodule plant fraction remain. The supernatant is taken and placed in a 2 ml safe-lock Eppendorf tube. Pellet is washed again with HS1, centrifuged for 5 minutes and the supernatant was added to the previously collected nodule plant fraction. Pellet was then resuspended in HS2 (table 1) using pipette tips, plastic pestles and sonication (3 times 30 seconds with 30 seconds break) for bacteroid cell disruption.

HS1	**HS2**
Hepes 50 mM (pH 7.5) (MW: 283.3 g/mol)	Hepes 50 mM
Sucrose 300 mM (MW: 342.3 g/mol)	DTT 10 mM
DTT 10 mM (MW: 154 g/mol)	PMSF 10 mM
PMSF 1 mM (MW: 174 g/mol)	

Table 2. Homogenization solutions – 1 and 2

The suspension was centrifuged at 4°C at 10.000 xg for 15 minutes. After collecting the supernatant in a 2 ml safe-lock Eppendorf tube, pellet was washed again with HS2 and centrifuged for 5 minutes. The remaining supernatant was added to the previous one. This supernatant contained nodule bacteroid proteins. Remaining pellet contained insolubilized membrane proteins and cell wall and was discarded. The supernatants (plant and bacteroid protein fractions) were precipitated overnight at -20°C in acetone/0.5% mercaptoethanol.

Urea-protein extraction protocol was used to further proceed with the extraction. Samples were centrifuged after the overnight precipitation for 15 minutes at 4000 x g, at 4°C and the supernatant was discarded. The pellet was washed once with ice-cold methanol and again centrifuged for 10 minutes at 21 x g, at 4°C. Pellets was left to dry, plant fraction pellets were frozen and bacteroid fraction pellets solved in Urea buffer. Protein extraction was proceeded for bacteroid fraction.

Bradford protein assay was used to determine the concentration of protein in the samples. BSA protein was used in following concentrations: 0.5, 1, 1.5, 2, 3 and 4 µl. The absorption of these concentrations was measured for three technical replicates each. Urea buffer was used as the blank. Samples were then solubilized in Milli-Q water, 2 µl of sample with 18 µl of water, and pipetted in the flat bottom well-plate. Each sample also head three replicates.

Reduction and alkylation of the proteins was done with 5 mM DTT and 10 mM IAA, respectively. Alkylation was stopped with 10 mM DTT. Trypsin buffer (table 3) was used to dilute the samples to a concentration of 2 M urea.

Trypsin buffer	**Stock**	**10 ml**
H_2O		8.85 ml
ACN (10%)	100%	1 ml
AmBic (50 mM)	1 m	0.5 ml
CaCl (2 mM)	200 mm	0.1 ml
DTT (5 mM)	Fresh	0.0077 g

Table 3. Trypsin buffer

1 µl of trypsin beads was added to each sample and left for slow mixing over night for protein digestion at 37°C. C18 stage tips were used for desalting of the digest: the samples were acidified 3% with formic acid (FA). C18 stage tips were washed with 100 µl MeOH, pipetting 10 times through each tip. MeOH was then washed twice with 100 µl 0.1% FA, each time by pipetting 10 times through the tip. Samples were centrifuged, separated from Trypsin and pipetted through prepared stage tips 10 times each. 0.1% FA was pipetted immediately after the pipetting of the sample for desalting. The contents left in the stage tips were washed twice with 100 µl MeOH into a low-bind eppi for elution. Samples were dried down in speed-vac and stored at -20°C.

Dried peptides were dissolved in 2% acetonitrile (ACN) with 0.1% FA. 0.5 µg of each sample was applied on reversed phase column (Acclaim PepMap, 3 µm, 100 Å, 75 µm × 50 cm; Thermo Fisher Scientific, Austria). LTQ-Orbitrap Elite was used for mass spectrometry measurements (Schneider et al. 2019).

2.7. $^{34}SO_4$ incorporation analysis

MaxQuant was used for protein identification. Schneider et al. (2019) presented a table with peptide sequences for Fe and MoFe proteins. I identified those specific sequences in MaxQuant msms file to obtain retention time values and mass to charge ratio which were used for the analysis. Xcalibur (LC-MS system software, version 2.2 SP1.48, Thermo Scientific) was used to extract peptide ion traces. The first isotopic peak of a peptide represents the monoisotopic, light peak (^{32}S) and the third isotopic peak is the putative, heavy peak (^{34}S). Relative isotope abundance, RIA, (Lyon et al. 2016) was calculated for each peptide (ratio of the heavy to the sum of heavy and light intensities). From each biological replicate the minimum RIA value for respective peptide was subtracted to correct the isotopic overlap.

2.8. Statistical analysis

Data was organized in two groups, control and treated. All RIA values were included in the statistical analysis.

For visual presentation, RIA values were presented as a percentage.

Data analysis was performed in RStudio (Version 1.2.1335). ANOVA was used to determine whether there is a significant difference between the control and the treated group. If ANOVA assumptions of normal distribution could not be reached, I performed Kruskal-Wallis for additional check. P-values of ANOVA were used for discussion.

3. Results and discussion

3.1. Time of ^{34}S-incorporation into nitrogenase subunits

In order to see whether and if so, after how many days sulfur is detectable to be incorporated into nitrogenase, nodules of *Lotus japonicus* roots were harvested at different time points after ^{34}S application. Three different Nif units were analyzed. Time course of ^{34}S-experiment showed a clear incorporation into nitrogenase subunits by the increase of the relative isotope abundance (RIA) already after 48 hours (Fig. 8). Here, RIA values were in average at 6% and increased to about 8% after 72 hours. This matches with results from Schneider et al. 2019, where RIA values reached 10% after 5 days of ^{34}S-labelling.

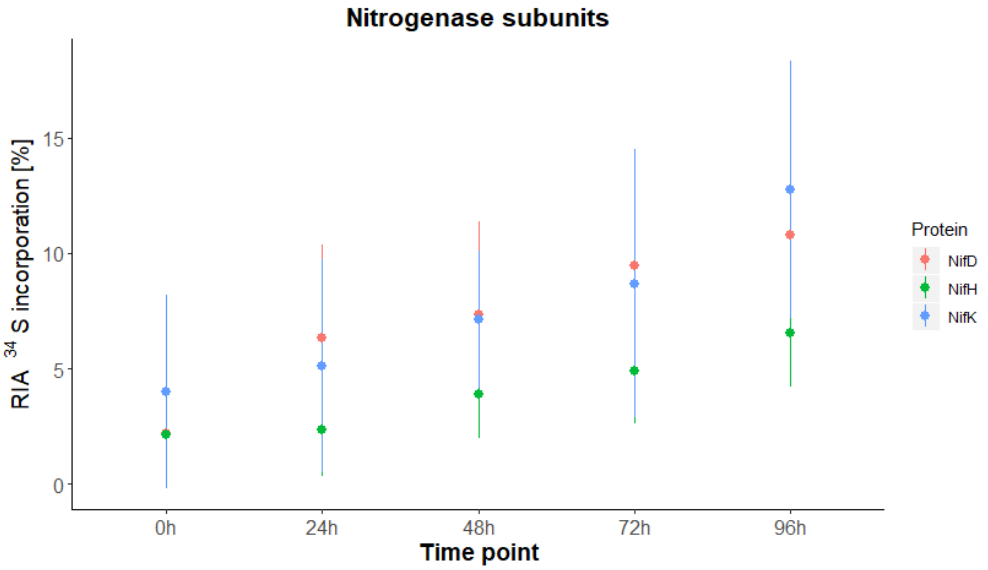


Figure 8 Relative isotope ratio of sulfur from three different nitrogenase subunits after 4 days of ^{34}S-labelling.

ANOVA - NIfD: p = 0.00958 after 48 h, NifH: p = 0.0103 after 72 h (p = 0.0564 after 48 h), NifK: p = 0.0168 after 48 h

In order to ensure detection of S-incorporation in our split-root system we increased labelling to 9 days.

3.2. Split-root system

During following the protocol by Suzuki et al. (2008), I was facing a lot of challenges. Thus, I decided to prepare a new protocol indicating some crucial issues, which I want to discuss here shortly. The new and detailed protocol is attached to the appendix.

With *Lotus japonicus* being a gentle plant, it is crucial to be very careful while handling it through the whole process. It is important to give it enough time to form nice seedlings. I found that if they don't have two nicely formed leaves, they won't be able to survive the root cut.

Another crucial step is to keep them in a humid environment, and well-watered the moment they are planted in a pot after the root-cut. If they are not watered immediately, they will dry out as it happened with my first set.

When they are successfully growing and forming new roots, time is of high importance for further actions. If they are transformed to a split-root system too early, they might not survive. Roots must be at least 4 cm long and strong for successful transfer. Around one third of all plants survived the root cut and formed two roots of equal size that were suitable for the split-root system. The stress of root-cut and replanting leaves the plants looking stressed and growing slower than they would normally grow. This is alright as long as they are green and progressing.

Once the plant is transferred to a split-root system and inoculated, it's important to keep it well watered and in humid conditions. Full functional nodulation took about 3 weeks, and it is important to stay on schedule with the picking of the nodules because they might be too young if checking earlier and too old if they are left for longer under the used growth conditions. Since not all the plants form strong roots that are equal in size, it is important to have at least three times more than one needs for later experiment and

always keep in mind that during the experiment some roots might break or die. This will not be obvious because the plant keeps growing and functioning with the other root. Because of this, it is important to really choose plants with both strong roots for planting in a split-root system.

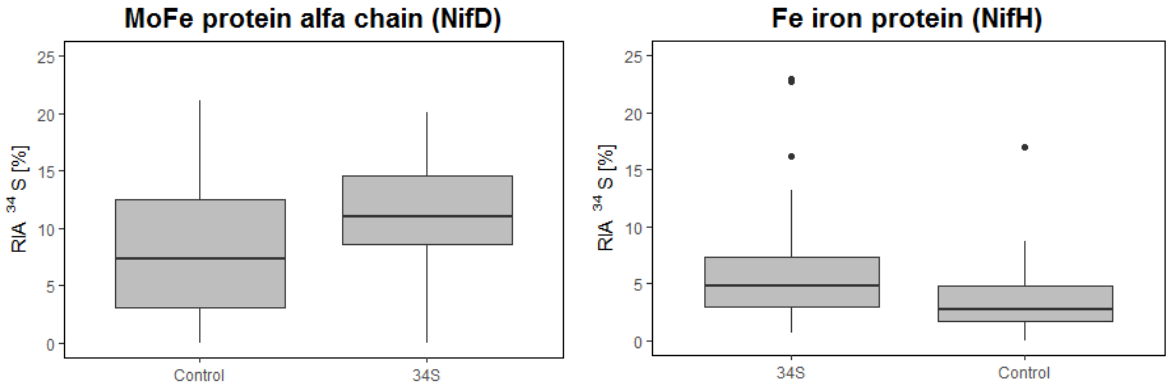
Another thing to be cautious about is the process of inoculation. Even one drop of the solution with rhizobia may lead to the formation of nodules on the site, which was not intended to form nodules.

Nitrogen supply through the watering solution needs consideration as well. If nitrogen is too low, nodule functioning might be reduced but also if nitrate concentration is too high (5-10 mM), nodule formation will be strongly hampered (Yassileva & Ignatov, 1996).

I observed that when the root without nodules were watered with 3 mM KNO_3, nodulation on the other side of the system was drastically slowed down. Number of nodules three weeks after inoculation was around 50% of the number of nodules observed in the experiment with 2 mM KNO_3. Therefore, I reduced the concentration of KNO_3 down to 2 mM, which lead to nice nodule formation on the other site but ensured overall good root/plant growth.

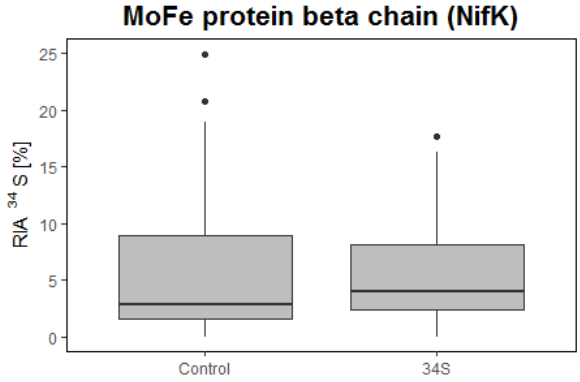
Mixing of the solutions (bacterial and nitrogen containing solutions), when washed through the system can be a significant problem if the roots outgrow the tubing of the system get in contact with the solution. Thus, the most important result of the first trial was the length of the roots after 4 weeks. They outgrew the length of the tubes leading to two similar roots strands with ^{34}S labelling contamination. When applying a double tube system as described in my protocol (enlarging the tubing system for root growth), contamination was successfully prevented.

3.3. Analysis of $^{34}SO_4$ incorporation into nitrogenase subunits of the split-root system



ANOVA, p-value = 0.0553

ANOVA, p-value = 0.11



ANOVA, p-value = 0.791

Figure 9 Relative isotope abundance of ^{34}S (RIA 32S [%]) for three nitrogenase subunits after 9 days of labeling, ANOVA n=10

Nine days after watering the non-nodulated root system with ^{34}S-labelling solution, and in order to determine if there was an incorporation of sulfur into the symbionts nitrogenase, different nitrogenase subunits were tested for an increase in its RIA. ANOVA was used to determine whether there was a difference between the treatments, indicative

for an ^{34}S-incorporation. Even after 9 days of application of ^{34}S-solution, there was no significant increase in RIA ($p < 0.05$) not in NifK nor in other subunits such as NifD and NifH. Hence, while local application of ^{34}S lead to a significant increase in RIA after 2 days, this could not be detected after 9 days of an indirect heavy sulfate nutrition. This result confirms that sulfate is the major source, transported through the symbiosme membrane to the bacteroids. Schneider et al. (2019) found that sulfate is transported via the SST1 with a high rate through the symbiosome membrane, crucial for nitrogenase biosynthesis. Interestingly, they detected a slight uptake of sulfur also in a *sst1* mutant plant. From this, they concluded that there might be a diffusion of sulfate or other sulfur containing compounds through the membrane.

Another possible might have been that sulfur is systemically transported in form of amino acids (cysteine and methionine) for protein synthesis. However, this cannot be confirmed from our results. The question also still remains, if and how sulfur is transported to the plant itself. In a next step, plant proteins should be extracted from the root and plant nodule fraction to check for labeled sulfur e.g. leghemoglobin.

The results show that within 9 days the plant did not transport labeled sulfur from one root to the other, nodulated roots. The experiment could be extended for another few days, but this might cause a problem with the nodule senescence. After nine days, some nodules were already getting old (green colour). One control group could be used to check how long the nodules can survive or whether new nodules are formed, when not supplied with sulfur for an extended period (which will be very unlikely). I would suggest checking for nodule formation earlier, e.g. 20 days after inoculation and if the number of nodules is sufficient, already starting the experiment and increasing the number of days in the experiment with few different time points (on day 10, 12, 14) and check if RIA values

are changing. This could answer if the plants need more time for systemic transport and incorporation of ^{34}S into bacteroids of the inoculated root.

Another interesting test would be to look for the minimum concentration of sulfate that is required for SNF. Now that we know that labeled sulfate is not incorporated into the proteins of the bacterial fraction in the nodule, we can use the slit-root system for instance to treat one site of a nodulated root with and one without sulfate. This would exclude a general sulfur deficiency response of the plant, in case nodulation cannot be established due to low sulfate on the nodulated site.

A closer look should in future also be made on several physiological parameters such as photosynthetic efficiency and nitrogenase activity to exclude that the plants suffer from stress and to determine whether S-deficiency in the bacteroids causes reduction of SNF and if so, whether the plant reacts e.g. with nodule sanctioning (AON). Therefore, a general protein abundance change analysis, comparing sulfur depleted with well-fed plant nodules, will be of additional information.

4. Conclusion

Taken together, *Lotus japonicus* is a plant that can be used in a split-root system without any problems regarding growth and strength of the plant, when following the tips of my new protocol.

I found that even though sulfate uptake and thus sulfur incorporation into bacteroid proteins seems rather quick under normal conditions and can be measured within 2 days of supply. However, it was not possible to detect it when applied indirectly through split-root system even after 9 days. Hence sulfate is the main source of sulfur transport through the symbiosome membrane and needs to be applied locally to the roots in order to maintain SNF. It also demonstrates that sulfur is not transported via other amino acid transport systems, as these could be systemic transport vessels for sulfur instead of sulfate. This observation is interesting as it also means that the split-root system can be used to determine the actual sulfate concentration needed for SNF or even nodule formation as well as for further tests on AON.

Hence, it is important to follow the new protocol to successfully and reproducibly perform split-root experiments. This system is robust and may serve for further labelling experiments to study local and systemic regulations.

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6. Appendix

How to split-root with *Lotus japonicus*

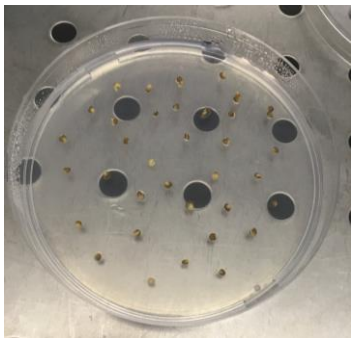
Germination

Material

1. Three falcon tubes
2. Sulfuric acid
3. Bleach
4. Agar plates
5. Sterile spoons
6. A box

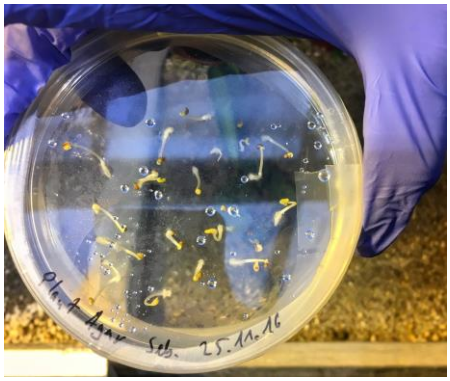
Method

1. Take twice as many seeds as you need to germinate.
2. Put them in a 50 ml falcon tube and add 5-10 ml of sulfuric acid.
3. Shake on shaker for 20 minutes.
4. Discard sulfuric acid and wash the seeds in the strainer with distilled water.
5. Transfer seeds in a new falcon tube and add 10 ml of bleach.
6. Shake on shaker for 20 minutes.
7. Discard bleach and wash seeds again with distilled water.
8. Put seeds in a falcon tube and add 20 ml of distilled water.
9. Shake on shaker over night.
10. Transfer seeds to agar (with B&D / check protocol) plates; (leave around 5 millimeters between seeds). Work in the laminar, use sterile spoons



11. Gently put agar plates in a box, close the box and wrap it in alufoil.

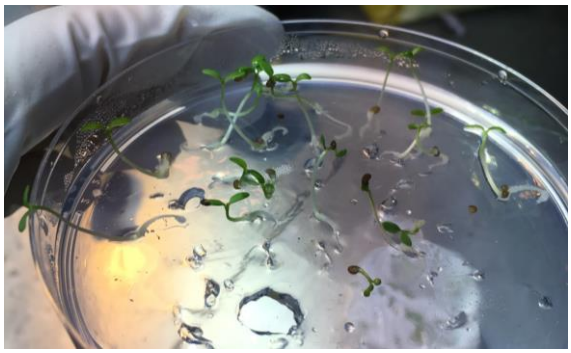
12. Leave seeds in the dark for 4-5 days.
13. Transfer plates to light in a greenhouse



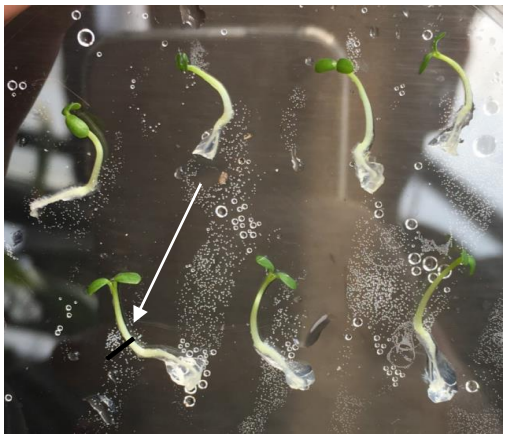
14. Check after 5 days. Plants should have formed small roots and at least two leaves.

Root-cut

1. Prepare enough pots - fill them with vermiculite and soak with water.
2. Take plants out of agar plates.



3. Cut hypocotyl just above the root.

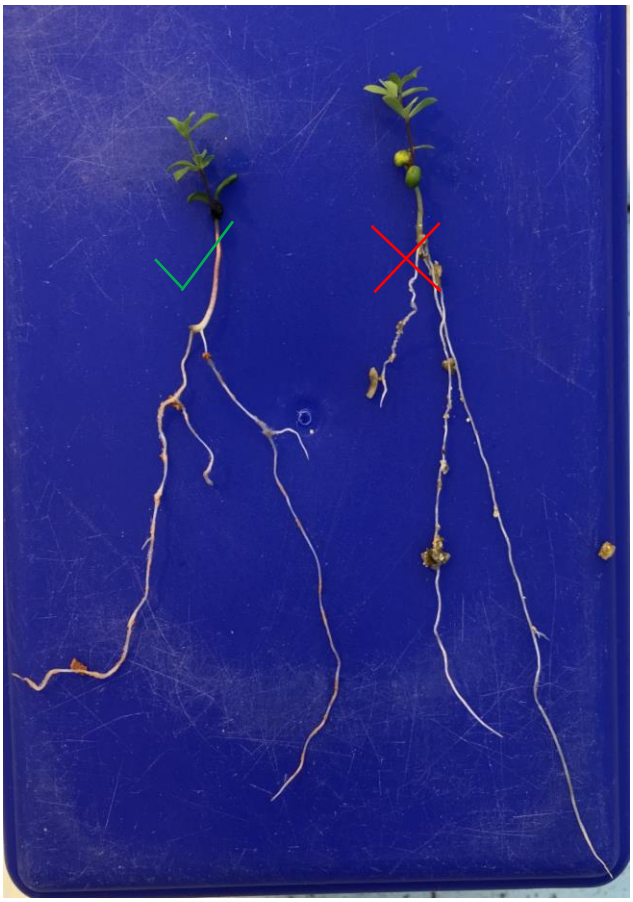


4. Immediately plant plants in vermiculite (5-6 plants per pot).
5. Spray all plants with water.
6. Cover to maintain humid conditions.

Developing the split-root system

1. Place pots in a shade for three days.
2. Move pots to light for 25 days.

3. Water every few days with 0.5 mM KNO_3 B&D solution.
4. After 25 days check roots: they should be at least 4 cm long.
5. Choose only plants with two roots with the same length.



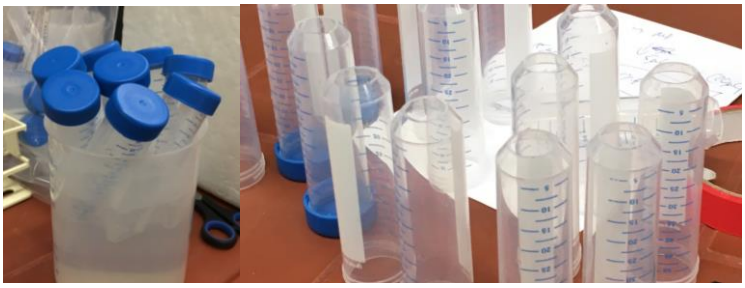
Preparation of the split-root system

Material

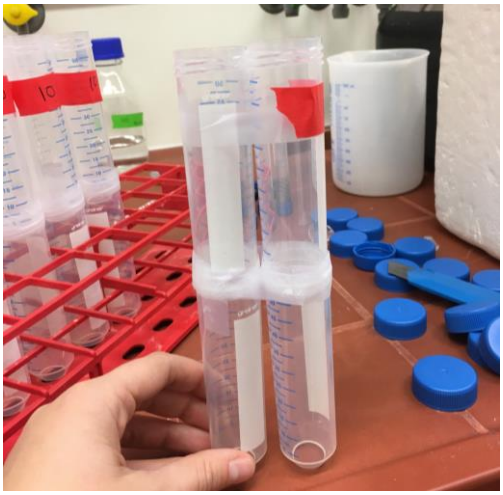
1. 4 falcon tubes (for one plant)
2. Tape
3. Mashes in the diameter of a falcon tube
4. Alufoil
5. Rack
6. Sterile clay granules soaked in 1 mM KNO_3 B&D solution
7.

Method:

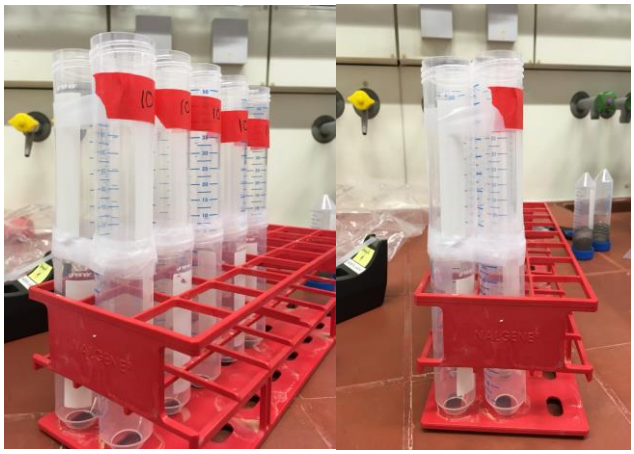
1. Cut bottoms of all tubes with a scalpel (soak tubes in hot water first to soften them)



2. Tape two tubes one on top of the other; repeat with another pair.
3. Tape two pairs of tubes together next to each other and label them according to root treatment.



4. Insert meshes on the bottom of the upper tubes.
5. Place tubes in racks.



6. Fill tubes halfway with clay granules with a spoon.

7. Insert gently one root to one tube and another root to the other tube. The stem of the plant should stay between two tubes.

8. Fill up tubes with clay granules to the top.

9. Water with water immediately after planting.
10. Wrap the whole rack with alufoil.

11. Water with 2 mM KNO_3 B&D solution if the root is not inoculated, and 0.5 mM KNO_3 B&D solution if it is inoculated.