Improving the fertigation of soilless urban vertical agriculture through the combination of struvite and rhizobia inoculation in *Phaseolus vulgaris*

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1. Abstract

Soilless crop production is a viable way to promote vertical agriculture in urban areas, but relies intensively on the use of mineral fertilizer. Thus, the benefits of fresher, local food and of avoided transportation and packaging associated to reduced food imports could be counteracted by an increase in nutrient-rich wastewater, contributing to freshwater and marine eutrophication. The present study aimed to explore the use of mineral fertilizer substitutes in soilless agriculture. *Phaseolus vulgaris* (common bean) was fertilized with the combination of slow-releasing fertilizer struvite (a source of N, P, and Mg) that is a by-product of wastewater treatment plants and inoculated with Rhizobium (a N_2-fixing soil bacteria). The experiment included three bean production lines: A) 2g/plant of struvite and rhizobium inoculation, B) 5g/plant of struvite and rhizobium inoculation, both irrigated with a Mg, P and N- free nutrient solution, and C) control treatment irrigated with a full nutrient solution and no inoculation. Plant growth, development, yield and nutrient content were determined at 35, 62 and 84 days after transplanting, as well as the biological N_2 fixation using the ^{15}N natural abundance method. Treatments A and B resulted in lower total yields per plant than the control C (59.35± 26.4g^a $plant^{-1}$ for A, 74.2±23.0g^a $plant^{-1}$ for B and 147.71± 45.3g^b $plant^{-1}$ for C). For A and B, nodulation and N_2 fixation capacity seamed to increase with the initially available struvite, but overtime reached deficient levels of Mg and close to deficient levels of P which could explain the lower yields. Nevertheless, we conclude that the combination of struvite and the N_2-fixing bacteria

covered N needs of the plant throughout the growth cycle. However, further studies are needed to determine optimal struvite quantities for vertical agriculture systems that can meet P and Mg requirements throughout the lifetime of the plant.

2. Introduction

From 1950 to 2018, the population living in urban areas has grown more than four-fold to an estimated 4.2 billion. This unprecedented rise of population has greatly increased global food demand, creating great pressure on natural resources (United Nations, 2019). In response, new ways to efficiently produce vegetables while minimizing the use of land are being explored (Sanyé-Mengual *et al.*, 2015, 2018). One of these initiatives is vertical farming with the use of soilless production systems with growing media or substrates (Sonneveld and Voogt, 2009) to reduce transportation and packaging of foodstuffs to cities (Sanyé *et al.*, 2012). However, vertical agriculture relies intensively on the use of mineral fertilizer which results in nitrates and phosphate discharged in wastewater, contributing to freshwater and marine eutrophication (Anton *et al.*, 2005; Gopalakrishnan *et al.*, 2015; Sanjuan-Delmás *et al.*, 2018).

This extensive use of mineral fertilizers not only affect the environment when used, but can also signify a great cost of production and extraction like in the case of nitrogen fertilizers due to the Haber-Bosh process and phosphorous due to phosphate rock extraction. The wide use of these nutrients has made vertical farming rely entirely on them, making this agricultural practice unsustainable in the long run. The high energy cost of synthetic nitrogen production and the ever depleting sources of phosphate rock, added to the environmental cost of their disposal and emission to water and air, make the search for alternatives a necessity to further implement these technologies in a sustainable way.

Many strategies have been described in the past years for the implementation of organic fertilization in vertical farming, embracing a circular economy framework to reduce new resource input into the cities. Some examples include fertilization based on gray water and urine (Ikeda and Tan, 1998; Karak and Bhattacharyya, 2011) and the use of bio-fertilizers like Rhizobium in the cultivation of legumes (Kontopoulou *et al.*, 2015; Savvas *et al.*, 2018). While these strategies may reduce the direct input of inorganic fertilizer, their use often result in lower crop yields and require in some cases more infrastructure of the irrigation system.

The present study aimed to add to this growing pool of knowledge on vertical urban agriculture by exploring the use of mineral fertilizer substitutes while optimizing crop yield and minimizing infrastructure requirements. In this study we analyzed the growth, development and production of common bean (*Phaseolus vulgaris*) fertilized with the combination of the slow releasing

fertilizer struvite, a Phosphate-rich waste of wastewater treatment plants, and the soil bacteria Rhizobium which is able to fix nitrogen from the atmosphere. The combination of these alternative fertilizers can be implemented easily in terms of cost and space, and promotes the recycling of nutrients within the city.

Struvite ($MgNH_4PO_4.6H_2O$), a crystalline by-product of wastewater treatment plants, formed by spontaneous or induced precipitation, which usually contains high concentrations of N and P (Rahman *et al.*, 2014), regarded as a viable slow-releasing fertilizer due to its high content of P, Mg and N, which average 12.5%, 9.9% and 5.7%, respectively (Ahmed *et al.*, 2018) and is suitable for plant growth (Degryse *et al.*, 2017; Ahmed *et al.*, 2018). Due to struvite's high nutrient concentrations, there are many ongoing efforts to optimize induced precipitation to make wastewater a valuable resource for providing a P alternative to the use of the depleting phosphate rock (Massey, M.S., Davis, J.G., Sheffield, R.E., Ippolito, 2007; Cordell, Drangert and White, 2009; Talboys *et al.*, 2016; Degryse *et al.*, 2017).

A further positive aspect of struvite as an agricultural fertilizer substitute is its slow solubility in granular form (Talboys *et al.*, 2016) and under alkaline and neutral pH conditions of the soil (Bhuiyan, Mavinic and Beckie, 2007). Thus, the risk of nutrient leaching and water eutrophication is rather small under these conditions when struvite is compared to common readily soluble fertilizers (Ahmed *et al.*, 2018). Furthermore, the removal of approximately a 30%-40% of the N and P from the wastewater to produce this substance can prevent eutrophication in urban water cycles (González Ponce, López-de-Sá and Plaza, 2009; Antonini *et al.*, 2012). Its granular form also makes struvite easily manageable and could be applied in larger scale productions mixing it with the soil or applying it to the substrate in hydroponic production systems. The use of struvite has already been tested in agriculture as a substitute for phosphate from other sources, showing promising results with low or even no yield losses reported (González Ponce, López-de-Sá and Plaza, 2009; Cabeza *et al.*, 2011; Liu *et al.*, 2011; Ackerman *et al.*, 2013; Degryse *et al.*, 2017; Ahmed *et al.*, 2018)

Although struvite already contains N available to the plant, legumes are highly N demanding (McKey, 1994). Therefore, average N contents in struvite would not be sufficient for soilless crops to reach commercial yields, needing a second source of N to do so. This could be obtained from Rhizobium, which is capable of forming an endosymbiotic interaction with leguminous plants entering root cells and forming nodules. These nodules enable atmospheric N_2 fixation and the formation of ammonia (NH_3). The plant benefits from the bacteria generating these compounds while the bacteria can profit from photosynthesis derived compounds (Long, 1989).

This symbiosis on the other hand may entail a major requirement of nutrients from the plant, like phosphorous, to satisfy the needs of the bacteria and a successful nodulation (Olivera *et al.*, 2004). The possible N_2 fixation depends on a successful rhizobia root colonization, which is influenced by diverse factors, like the above mentioned phosphorous fertilization, salinity, drought and an initial N availability (Ntatsi *et al.*, 2018; Savvas *et al.*, 2018).

Rhizobium as a second source of N was chosen due to the lower inputs needed to achieve nitrogen intake by the plant (Gopalakrishnan *et al.*, 2015). When using the N_2 fixing bacteria Rhizobium in hydroponic cultivation, Kontopoulou et al. (2017) described the need to apply an initial N fertilization until the nodulation in the root medium is given, to further encourage nodulation and therefore N fixation, plant growth and production. Even though previous studies reported a lower production capacity in N_2 fixing plants compared to common beans with N fertilization (Olivera *et al.*, 2004; Kontopoulou *et al.*, 2017), the combination of the two N sources (Struvite and N_2-fixing bacteria) was made to analyze the possibility to overcome such lower yields (Savvas *et al.*, 2018).

In order to determine how effective the two alternative fertilizers are in providing N to the plant, the ^{15}N natural abundance method was employed to define the source of N throughout the experiment (Shearer and Kohl, 1989). While plants with N acquired from symbiotic atmospheric N_2 fixation show lower richness of the ^{15}N isotope, corresponding to the atmospheric abundance (0.3663%), plant tissues subjected to other sources of N can reveal greater amounts of the ^{15}N isotope, depending on the N fertilizer applied (Robinson, 2001).

The main objective of this study was to determine if a struvite and rhizobium combination is a plausible substitute of mineral fertilizers in an effort to reduce emissions of N and P to the environment in urban vertical agriculture. Additionally, we made observations of plant development, growth and nutritional state and identified the main N source of the crops by using the ^{15}N natural abundance method.

3. Materials and Methods

3.1 Experimental site, materials and growth conditions

This experiment was conducted in the Rooftop Greenhouse Laboratory (RTG-Lab) of the Environmental Science and technology building (ICTA-UAB) located in the Universitat Autònoma de Barcelona Campus (42º29'24" E, 45º94'36" N) (Sanjuan-Delmás *et al.*, 2018). The bean variety used for this experiment was the *Phaseolus v. Pongo* that had been previously germinated in a commercial greenhouse 10 days before transplanting in the RTG-Lab. The

production system was soilless with perlite substrate in 40L bags and the use of fertigation through a 2 L/h drip irrigation system.

The bean seeds where treated with a commercial product (Nadicom GmbH©) containing a mixture of *Rhizobium phaseoli* and *Rhizobium giardinii* strains for the inoculation before planting. These plants were again exposed to the same bacteria strain (5ml per plant) 5 days later, once transplanted in the RTG-Lab. The inoculation procedure was an exposure of the plant seeds with the liquid commercial product before planting and after the addition of the liquid commercial mix to the plant seedling. Once the plants were thus inoculated, they were irrigated with an Mg, P and N free solution (Table 1b in the supplementary information) increasing the application of K_2SO_4 to adjust to the K requirements. The control plants on the other hand were irrigated with a full nutrient solution. These nutrient concentrations were maintained throughout the entire experiment. The crops were irrigated 4 times a day for 3 minutes, a total amount of 400ml per day per plant.

The inoculated plants were treated with two different struvite amounts placed inside the perlite bag around the root area and surface, varying the concentration of P and N available to the plant from struvite: A) with 2g (1.02 m*mol* of P; 0.46 m*mol* of N) of granulate struvite per plant and B) with 5g (2.57 m*mol* of P; 1.15 m*mol* of N) of granulate struvite per plant. The amount of struvite most optimal for growth had been determined in a previous experiment conducted in the same i-RTG, in which 2.57 m*mol* P was deemed sufficient for common bean fertilization to reach an equivalent commercial production as mineral-fertilized beans. To ensure no loss of struvite due to runoff each plant was planted inside an extra 1L bag, containing perlite and the corresponding amount of struvite, with small holes to allow water drainage.

Each treatment was disposed in four randomly arranged rows with 16 plants each (4 perlite bags with 4 plants per bag planted in a frame of 0.125m^2) making a total of 64 plants per treatment (A; B; and Control), 192 plant in total (Figure 1b of the supplementary information). Due to the irrigation system and leachate recovery system randomization could only be made for entire lines of 4 bags.

Plants were germinated and transplanted in duplicate and thinned to one plant 21 days after transplanting (DAT).

The conditions in the greenhouse were monitored with T107 sensing devices (Campbell Scientific) along the cropping area measuring temperature, relative humidity and radiation, during the entire experiment (see Table 2b of the supplementary information). To assure proper

irrigation plant drainage volume, as well as pH and electric conductivity of the leachate were recorded every day for each irrigation line.

The phenological stages of the bean plants were determined each week. This information was assessed to identify the plant growth, development and productivity in time, creating a clear view of the plant cycle, growth and production peaks that allow a correct comparison of the plant development between treatments and control. This was performed by counting leaves, flower buttons and open flowers. The production of ripened bean pods was also counted and weighed for each harvest. These measurements were performed for each of the eight plants in the two middle bags of each row (see figure 1b) starting 14 days after transplanting (DAT). To ensure uniform counting, leaves under 5 cm length were not take into account, as well as only fully formed flower buttons with a white coloration and fully open flowers were counted. For the bean pods a minimum length of 11 cm was considered for harvesting while bean pod below this measurement were left for the next harvest. The average number and bean pod weight per treatment was then calculated for each week. At the same time and on a weekly basis a chlorophyll content measurement was performed (with an SPAD CCM-200 plus; Opti-Sciences, Inc.) on the same 8 plants in the center of each row (see figure 1b of the supplementary information).

3.2 Description of plant sampling methods

To determine the changes in the plant development as well as the nutritional state and ^{15}N, samples were taken during the three different stages of the crop. The first sampling took place 35 DAT, right before bean pod production started; the second sample 62 DAT, during the productive phase of the plants and the last sampling took place 84 DAT, at the end of the productive stage, which marked the last day of the experiment.

The samples consisted of 8 randomly chosen plants per treatment (excluding the 8 central plants of each row which were kept for the phenological analysis). Each plant was washed with deionized water, dried off the excess water and separated onto four main organs: leaves, shoots, roots and nodules. These where then weighted separately to determine their fresh weight (FW). All organs were put separately in envelopes and left to dry in an oven at 65º C until a stabilized dry weight (DW) was obtained, approximately after 7-8 days. Means of the obtained values were calculated for each treatment, each organ and time. The nodules were counted prior to their drying to determine the mean nodulation of each plant. In addition, fruit samples from each treatment were taken at three different times (49, 62 and 77 DAT) closely matching the three plant harvests.

Moreover, the 25% of the total sampled leaves for each plant were separated to determine their area before the drying process. To do so, these fresh leaves were scanned with a reference pixel obtaining the leaf area using the Image J software (Rueden *et al.*, 2017). This leaf area was further extrapolated to the 100% of leaf biomass of the plant. The Leaf Area Index (LAI) was then calculated dividing the total leaf area by the plantation frame of our crop (0.125m^2).

3.3 Nitrogen isotopic ($\delta^{15}N$) analysis

The goal of inoculating treatments A and B with Rhizobium was for the plant to indirectly fix N_2 from the air and meet its N needs this way. In order to determine how much of the N assimilated by the plant came from the atmosphere, we used the natural abundance method (Shearer and Kohl, 1989) to identify the origin of N obtained by the plant, which in our case should be either struvite or atmospheric N. While treatment A and B were actively inoculated with Rhizobium strains and fertilized with struvite containing N, the control treatment was fertilized through a standard N fertilization administered through the irrigation. Further nitrogen sources where discarded due to the laboratory conditions and the production on inert perlite.

The analysis was performed with an elemental analyzer- isotopic ratio mass spectrometer (EA-IRMS; Thermo Fisher Scientific). The devices used were a Flash EA 1112 analyzer and a Delta V Advantage spectrometer, coupled using a Conflo III interface. The plant and struvite samples were weighed in tin capsules and introduced in the EA-IRMS system to obtain the $\delta^{15}N$ values calculated with the following equation (Eq 1) (Robinson, 2001):

$$Eq\ 1: \qquad \delta^{15}N = \frac{Sample\ atom\ \%^{15}N - 0.3663}{0.3663} \times 1000$$

Equation 1: The $\delta^{15}N$ is our natural tracer for our N sources, the sample atom %15N is the previously obtained value of our plant sample, and the value 0.3663 is a standard value that represents the percentage of ^{15}N in the atmosphere.

$\delta^{15}N$ values provide an indication of the source of N in the plant tissue. Values close to 0 denotes that the plant's N source is mainly from atmospheric N_2 fixation, while higher values can be interpreted as indicating mixed sources or dominated by N from struvite. The $\delta^{15}N$ value obtained for struvite used in the experiments was 7.1‰. To determine the relative contributions from the two sources considered we use Eq 2, which yields an estimate of the percentage of N derived from N_2- fixation (%Ndfa) (Shearer and Kohl, 1993; Unkovich *et al.*, 2002; Arndt *et al.*, 2004)

$$Eq\ 2: \qquad \%Ndfa = \frac{\delta^{15}N\ Source\ 2 - \ \delta^{15}N\ Sink}{\delta^{15}N\ Source\ 2 - 'B'value} \times 100$$

Equation 2: %Ndfa (Nitrogen derived from N_2 fixation from the atmosphere), δ15N Source 2 (‰) corresponds to the δ15N value of struvite, δ15N Sink (‰) corresponds to the δ15N value from the sample, 'B' value corresponds to the δ15N of N_2 fixation taking in account possible fractionation.

The 'B' value is the isotopic fractionation observed in N_2-fixing *Phaseolus vulgaris, which* was set to -1.16‰ corresponding to the lowest $\delta^{15}N$ value obtained (Shearer and Kohl, 1989; Peoples, Boddey and Herridge, 2002; Kermah *et al.*, 2018) and similar to the values found by Kontopoulou et al. (2017) in common bean fertilized without N and inoculated with Rhizobium.

The Biologically fixed Nitrogen (BNF) was further calculated with the obtained %Ndfa value as well as the obtained value of Nitrogen content in the plant. To extrapolate to Kg/ha the theoretical plant density of 8 plants/m^2 was used.

Finally the Nitrogen use efficiency (NUE) for all treatments was estimated. The methodology followed to do these calculations was given by Weih, 2014 who provides a tool to successfully calculate the NUE. To do this the provided information was:

-The N content at the initial stage of the plant in g/m^2 (previous to the main production stage at 35 DAT)

-The N content at the main productive stage in g/m^2 (chosen at 84 DAT)

-The N content in the harvested yield in g/m^2

-The yield biomass g/m^2

-The added N to the soil in g/m^2 (in this case Perlite)

3.4 Plant nutritional analysis:

Dried and grinded plant organs were weighed (up to 0.25g) and digested using a Single Reaction Chamber microwave (Milestone Ultrawave) with concentrated HNO_3. The digested samples were then diluted with HNO_3 1% (v/v) and analyzed with Optical Spectrometry (ICP-OES) (Perkin-Elmer, Optima 4300DV). All samples were weighted, digested and analyzed in duplicate.

3.5 Statistical analysis

All statistical analysis in this experiment were performed with the R studio software. Significance in our values was tested with one-way ANOVA $p<0.05$. Kruskal-Wallis test was further use to assess the statistical significance of treatments when the ANOVA was significant.

4. Results

4.1 Phenology, biomass and yield

The weekly recording of the phenologic growth of the bean plants showed differences between all treatments (Figure 1). In this figure, we can see the evolution throughout the crop development of the biomass production as well as the production of flowers and finally the production of bean pods. The control plants (Treatment C) showed a greater biomass growth as well as a faster development transitioning from flower buttons to open flowers and bean pod production. Although the performance of the treatments was similar at the earlier stages of growth, once the production stage started, greater differences were perceived. It was during the 40 DAT and 50 DAT that the treatments A and B start to perform worse in leaf production as well as in the formation and opening of flower buttons in comparison to the control plants (C). Between 60 DAT and 70 DAT a second production peak can be seen for the control treatment as well as a fast generation of flower buttons, while the treatments A and B show a declining pattern in bean pod production.

<Figure 1>

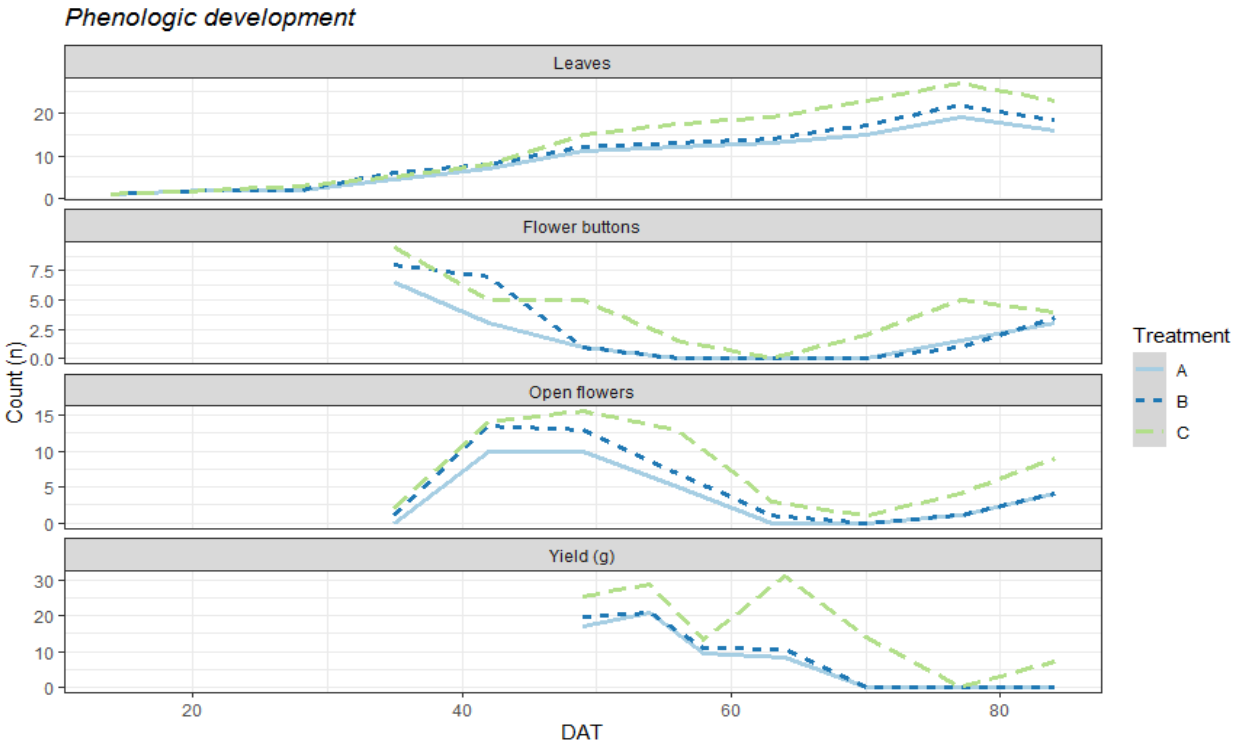


Table 1 shows the changes in the results of the plant measurement made on the sampled plants at three different development stages. While the first period of plant sampling, 35 DAT, shows almost no significant differences between the treatments (only in the case of nodule fresh and dry weight), further samplings at 62 and 84 DAT showed greater differences between treatments. At this point the leaf and shoot fresh weights were greater in the control treatment as well as the measured leaf area index. The only value without significant difference between

treatments throughout the experiment was the root fresh weight. A persistent significant difference throughout the three samplings between the treatments A and B and the control treatment C was the fresh and dry weight of the nodules which reached a maximum of 1.22 g, 1 g and 0.42 g for treatment A, B and C, respectively. On the other hand, treatment B (with higher amounts of struvite) also showed higher amounts of nodules as well as greater weights than the other two treatments during the second and third sample periods, even though no significant difference was seen.

<Table 1>

(1)	Leaf DW (g) per plant	Shoot DW (g) per plant	Roots DW (g) per plant	Nodules n per plant	Nodules DW per plant (g)	LAI
A	1.12^{a}±0.22	0.46^{a}±0.08	0.44^{a}±0.10	132.50^{a}±80.35	0.16^{b}±0.07	0.57^{a}±0.12
B	1.31^{a}±0.46	0.56^{a}±0.19	0.51^{a}±0.15	156.75^{a}±60.82	0.12^{b}±0.06	0.62^{a}±0.23
C	1.33^{a}±0.57	0.58^{a}±0.18	0.53^{a}±0.14	148.75^{a}±48.23	0.05^{a}±0.02	0.65^{a}±0.27

(2)	Leaf DW (g) per plant	Shoot DW (g) per plant	Roots DW (g) per plant	Nodules n per plant	Nodules DW per plant (g)	LAI
A	3.97^{a}±1.25	2.02^{a}±0.72	0.80^{a}±0.28	127.88^{a}±63.85	0.14^{b}±0.09	1.14^{a}±0.51
B	3.69^{a}±1.53	2.24^{a}±1.01	0.87^{a}±0.34	172.25^{a}±132.66	0.15^{b}±0.14	1.22^{a}±0.64
C	6.44^{a}±3.09	3.85^{a}±1.96	0.95^{a}±0.44	82.25^{a}±62.47	0.01^{a}±0.01	2.38^{b}±1.33

(3)	Leaf DW (g) per plant	Shoot DW (g) per plant	Roots DW (g) per plant	Nodules n per plant	Nodules DW per plant (g)	LAI
A	5.86^{a}±2.96	3.09^{a}±1.45	1.77^{a}±0.79	136.88^{b}±106.31	0.15^{b}±0.13	1.74^{a}±0.92
B	7.40^{a}±2.17	4.53^{a}±1.48	2.49$^{a\,b}$±0.57	186.25^{b}±48.79	0.24^{b}±0.11	1.80^{a}±0.67
C	11.11^{b}±1.51	6.91^{b}±1.42	3.35^{b}±0.88	39.13^{a}±24.76	0.02^{a}±0.02	3.72^{b}±0.87

When observing the SPAD measurements (Figure 2b supplementary information) no great differences in chlorophyll content were observed throughout the experiment.

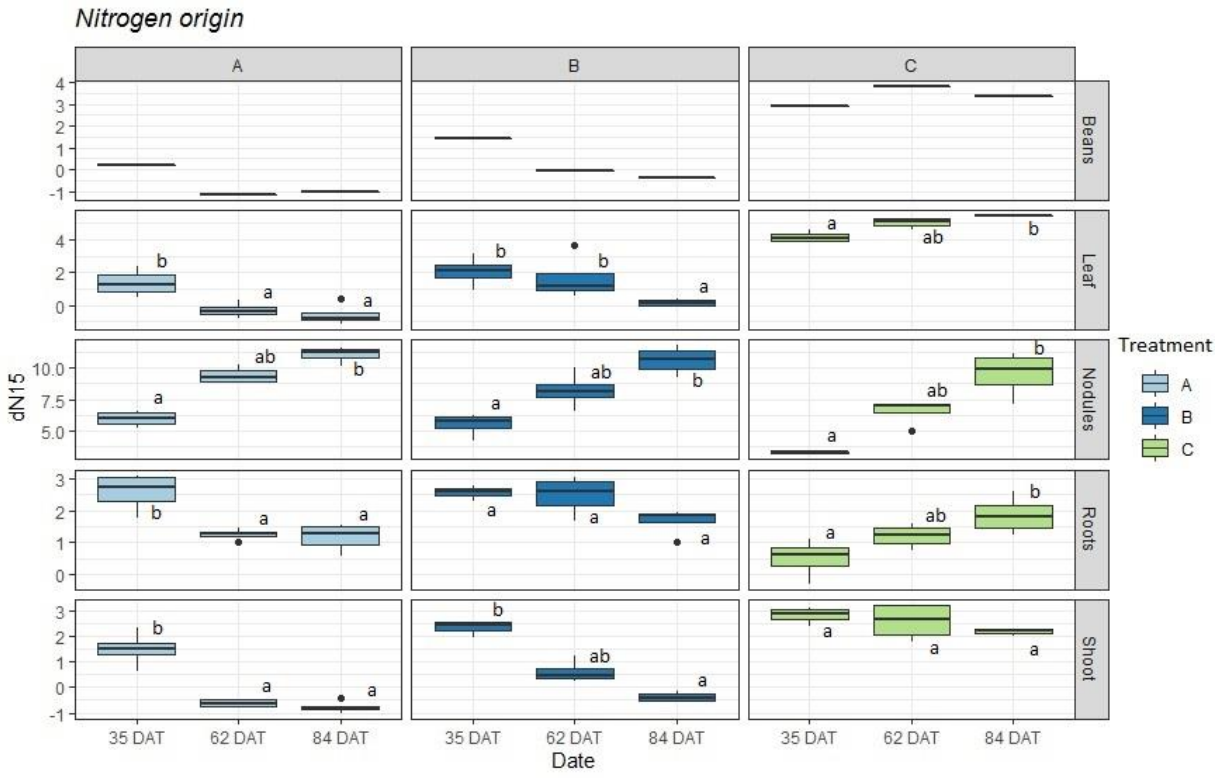
The final productions obtained in all three treatments where 1899.2g, 2375.6g and 4726.7 g of green bean pods for treatments A, B and C, respectively. Albeit the plants treated with struvite and rhizobium produced about half of the yield of the mineral fertilized plants, it is important to point out that they were healthy throughout the experiment. The average yields given per plant where 59.35± 26.4g^{a} plant^{-1} for A, 74.24±23.0g^{a} plant^{-1} for B and 147.71± 45.3g^{b} plant^{-1} for the control treatment C. These production differences can also be seen in figure 1 where obtained

yields are given in time, showing greater production peaks and a faster capacity to develop flower buttons and open flowers after each harvest.

4.2 d^{15}N, %Ndfa and Biologically fixed N

The results obtained for the $\delta^{15}N$ values of plant tissues and bean pots (Figure 2 and Figure 3b in the supplementary information) show a great variability in the enrichment of all organs except the nodules. While the treatment C shows a clear enrichment in time the pattern for the treatments A and B is the opposite. In the case of the nodules all three treatments experience a clear enrichment in time. The treatment B gives an intermediate $\delta^{15}N$ value between A and C, with a less abruptly decreasing $\delta^{15}N$ value compared to the tissues exposed to the treatment A. It was also interesting to observe that the major decrease in the $\delta^{15}N$ value for treatment A occured between day 35 and 62 after transplanting, remaining rather constant at 84 DAT. While in the case of the plants from treatment B the value at 62 DAT has not fallen as drastically, experiencing a more considerable change at 84 DAT.

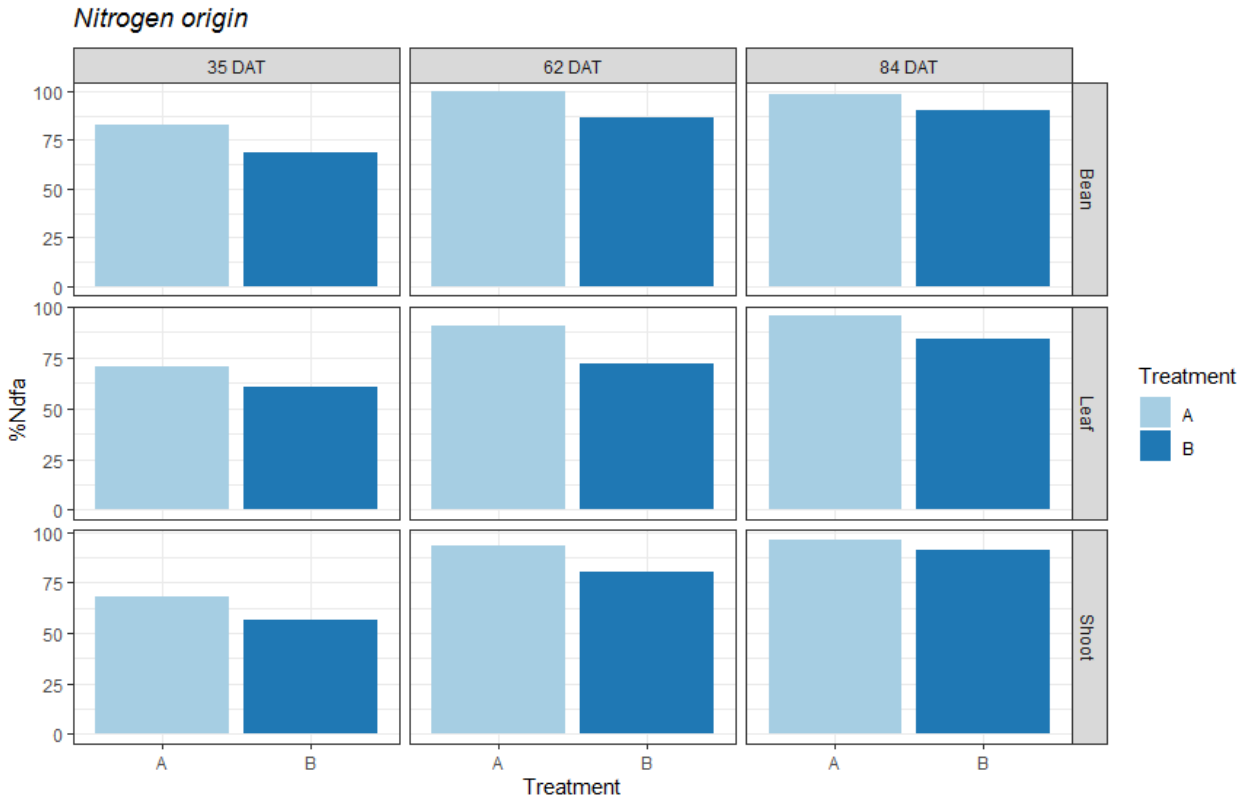
<Figure 2>



When calculating the percentage of atmospheric N fixed during our three sampling periods we obtained the values shown in figure 3. This figure shows the approximate percentage of N derived from atmospheric fixation in relation to the total N obtained by the plant.

As we can perceive from the figure the percentage of fixed N_2 in all three tissues are higher for plants with the treatment A with values of 65% to 80% at 35DAT, reaching 90% by the end of the experiment (84 DAT). On the other hand, the treatment B shows lower values throughout the experiment with initial values close to 50% to 60% (35 DAT) reaching final values of 80% at 84 DAT.

<Figure 3>



While plants with less struvite in the root medium (treatment A) increased the percentage of fixed N_2 more rapidly (from 70% (35 DAT) to 90% (62 DAT) in the leaves), the plants from treatment B take longer to rise this value (from 60% (35 DAT) to 71% (62 DAT) in leaves). This corresponds to the results for $\delta^{15}N$ values observed in figure 2.

Table 2 shows the results for the estimation of biological fixed nitrogen (BNF) expressed in kg/ha. These results show an extrapolation of the total N found in the plants for each treatment to the magnitude of kg/ha. The percentages of N from atmospheric origin obtained previously are further used to attain the kg/ha of biologically fixed nitrogen for each treatment as well as the N from struvite used by the plant.

<Table 2>

Date	Treatment	% Ndfa plant	Total N in plant kg/ha	Kg/ha Biologically fixed N	Kg/ha N from Struvite
35 DAT	A	68%	7.54±1.03[a]	5.38±1.04[b]	2.16
	B	60%	8.59±2.19[a]	5.33±1.38[b]	3.26
62 DAT	A	89%	24.67±4.96[a]	22.92±4.07[b]	1.75
	B	73%	24.62±6.20[a]	18.70±5.09[b]	5.92
84 DAT	A	90%	27.25±12.79[a]	25.40±12.96[b]	1.85
	B	82%	35.04±9.16[a]	29.21±7.79[b]	5.83

Here we can see that as the percentages of atmospheric derived N, and the total N found in the plant increase so does the kg/ha of Biologically fixed N. While the plants with the treatment A have higher values for the biologically fixed N during the first two sampling periods at 84 DAT an increase of the fixation percentage as well as total N in the plants from the treatment B rise their amount of biologically fixed N. On the other hand, the use of N from struvite only increases for the treatment B and stays constant for treatment A.

4.3 Nutrient content:

The nutrient contents in the above-ground plant organs are presented in figure 4. The observed concentration of nutrients in leaves for the three treatments were within sufficient levels except for less than optimal concentrations of Mg^{2+} at 62 DAT for treatment A and 84 DAT for treatments A and B. And close to deficient levels of K and P in both treatments A and B at 62 and 84 DAT according to Hochmuth *et al.*(2018). In the case of N, both in leaf and shoot tissues, no deficiency levels were found in any of the treatments as well as no significant difference between treatments. On the other hand, a clear decline of P and Mg^{2+} in time can be seen on treatments A and B in the leaves as well as P in the shoots. The control treatment (C) on the other hand remains stable.

<Figure 4>

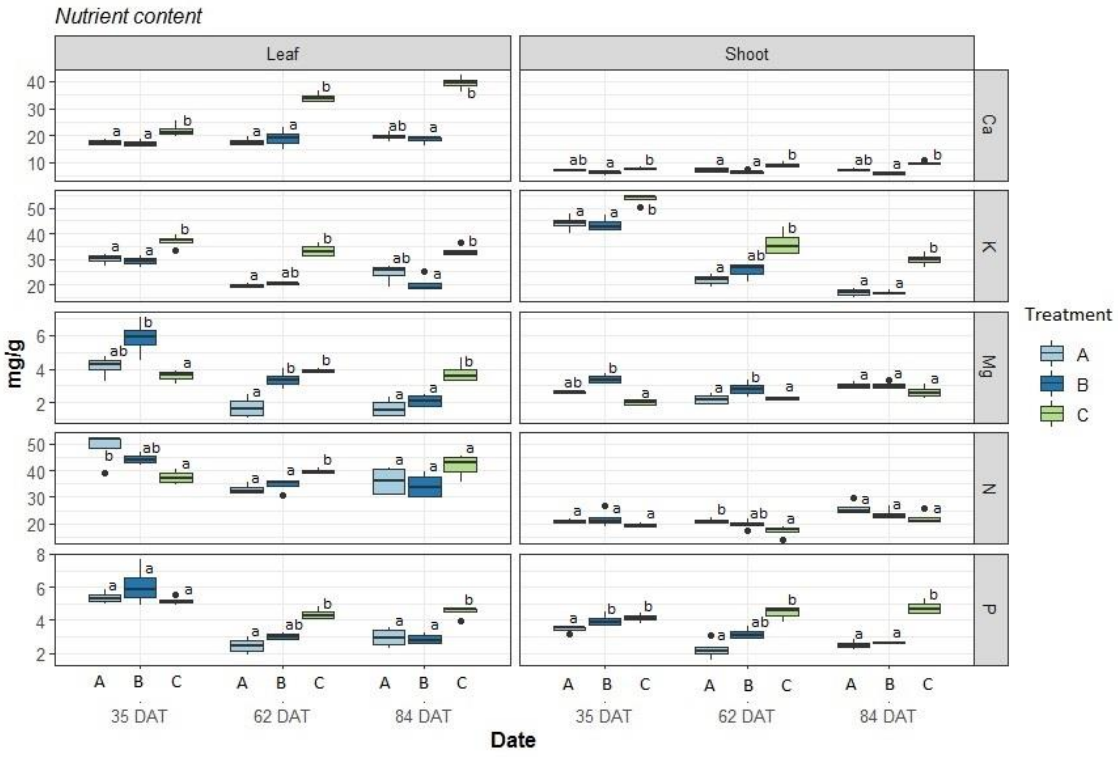


Figure 4b shown in the supplementary information also indicates the total nutrient content bound to the total biomass of the sampled plants. Here, we can observe that the treatment B with a higher amount of struvite shows results in between both other treatments. In the case of Mg at 35DAT in leaves treatment B shows levels as high as the control treatment, but while the latter stays constant in time both A and B are reduced. The same trend can be seen for P both in leaves and shoots. In the case of N we can see an increase in all treatments being faster for control C, while A and B increase in a similar fashion.

Finally the NUE obtained for all three treatments was 1.32 g/g; 0.55 g/g; 0.29 g/g for A, B and C respectively. The methodology of calculation taken in account was the N in the soil, while the fixed nitrogen was not taken in account, therefore the use efficiency can be very different for all three treatments.

5. Discussion

5.1 Plant growth and development

The results indicate that once the first production peak is reached, the control plants were highly capable to keep producing flower buttons, while the inoculated and struvite-fertilized plants took longer time. The relation between their development and the amount of struvite given to the plant seem to directly correlate. Generally, the biomass as well as bean pod production was higher in the control plants while the treatment B with a greater amount of struvite (5g) followed

behind. The treatment A with the lower amount of struvite (2g) was recorded to be the treatment with lowest growth and production rates. These findings coincide with those of previous literature (Nanjareddy *et al.*, 2014), where a lower availability of KNO_3 was directly linked to a reduction of leaf and flower formation. This reduction also seemed to be related to the availability of P and Mg in time due to struvite depletion, taking into account that the initial performance was similar in all three treatments.

When observing the SPAD measurements the chlorophyll content in all three treatments indicates that the N content in the leaves was not majorly affected by the treatments but rather the LAI. Lower P availability resulted in a reduction of LAI as well as the overall growth of the plant, which have been observed in treatments A and B. This difference was not as strong in the root weights (compared to the other plant organs), which has been reported to be less affected by P reduction in previous literature (Chaudhary and Fujita, 1998; Rao, Fredeen and Terry, 2008).

The lower nodule fresh and dry weight in the control treatment compared to the treatments A and B has been previously reported in other studies, where nodule fresh and dry weight were found to be considerably reduced when the inorganic NO_3^- fertilization was not restricted (Nanjareddy *et al.*, 2014; Kontopoulou *et al.*, 2017).

The increasing nodule number and weight of the B treatment (with greater struvite) throughout the experiment in comparison to the treatment A confirms Kontopoulou's (2017) findings, that initial low N fertilizations can restrict successful colonization. These differences, however, could also be given due to lower amounts of P in the treatment A compared to treatment B, since P is a limiting factor for a successful nodulation (Olivera *et al.*, 2004).

The lower bean productivities are similar to the study provided by Olivera et al. (2004), where bean production with lower P fertilization and Rhizobia inoculation turned out to be insufficient to reach production levels as high as conventionally fertilized beans. However, struvite fertilization seemed to increase the production of inoculated plants up to a 25% when the treatments for 2g and 5g per plant were compared (59.35± 26.4g $plant^{-1}$ in treatment A to 74.23±23.0g $plant^{-1}$ in treatment B).

5.2 The effect on atmospheric N fixation capacity

The above-ground organs showed a clear pattern throughout the 3 measurements in terms of N assimilation. The ^{15}N enrichment in the A and B treatments was lower than the C treatment, meaning that treatments A and B get most of their N from the atmosphere. This difference was further accentuated as time progressed, reflecting a greater dependence on N_2 fixation in the A

and B treatments. The difference between these two treatments (A and B) itself can be due to a greater availability of struvite in the root medium and therefore a greater availability of initial N and P for the treatment B opposed to the treatment A (Olivera *et al.*, 2004; Kontopoulou *et al.*, 2017).

The $\delta^{15}N$ reduction in treatments A and B through time corresponds to the availability of N provided by the struvite, assuming that it is reduced in time. This reduction can be seen when the concentrations of NO_3^- in the drained water are observed (see Table 3b of the supplementary information). While initially greater amounts of N are being detected in the leached water, by the end of the experiment very low concentrations are seen. Therefore, while the $\delta^{15}N$ value of the control treatment C remains constant in time (except in the nodules) the $\delta^{15}N$ values of the treatment A and B are progressively reduced in time corresponding to the available N provided by the struvite in the root medium.

This information indicates a change in the source of N from the plant that takes place during the time span of 35 to 62 DAT. We can therefore assume that the availability of struvite and therefore N in the root medium has been depleted mainly during that time forcing the plant to rely on atmospheric N_2 fixation. The results obtained for the %Ndfa also confirm that the fixation of N_2 increases in time in both treatments.

Nodules appear to be highly enriched with ^{15}N along all three harvests, especially in the treatment A and B. These results align with previous literature attributing the enrichment as a consequence of the export of ^{15}N depleted ureides and the import of ^{15}N enriched amino acids. Still these values do not have a great effect on the total plant enrichment if the nodule biomass is considered (Shearer *et al.*, 1986; Unkovich, 2013; Craine *et al.*, 2015).

The quantity of fixed nitrogen has not reached 40- 50 kg/ha which corresponds to low ranges expressed in previous literature (Farid and Navabi, 2015). While the treatment A, with less struvite has a higher value for BNF during the first two sampling times, the treatment B BNF increased by the end of the experiment. These findings are in agreement with those in the literature, where BNF was found to be restricted in the presence of plant available NO_3^-, as well as that the BNF value increased during mature stages of the plant with a sufficient NO_3^- fertilization during the plant early growth (Müller, Pereira and Martin, 1993; Hungria *et al.*, 2006; Kontopoulou *et al.*, 2017). However, no significant difference was observed between the treatments.

5.3 Plant health and Nitrogen assimilation

We conclude that all treatments had enough N since there were minimal differences in N concentrations in stem, leaves, bean pods, during plant growth and at the end of the experiment, as was also found by Kontopoulou et al., 2015. We attribute that the lower yields are rather caused by a reduced uptake of the cations K^+ and Mg^{2+} due to electrochemical imbalance generated with the reduced presence of NO^{3-} in the root medium. This idea is reinforced by the results obtained in the Figure 4b in the supplementary information, where N was gradually increased in all three treatments throughout the experiment, indicating that fixation on the case of treatment A and B was taking place. The values increased from less than 0.1 g of N at 35DAT up to 0.2g at 84DAT for both A and B treatments.

A slight increase of K by the end of the experiment in the plants with less struvite (treatment A) was most likely due to a lower availability of the cation Mg^{2+} facilitating the cation uptake (Marschner, 2002).

A declining N concentration in the leachates led us to believe that the reduction of P and Mg in the above-ground organs can also be related to the depleting struvite in the medium. This depletion occured faster in treatment A than B, which was related to the initial amounts of struvite given in each treatment (2g and 5g respectively). One key finding from this study is that for inoculated plants, greater amounts of P are needed to support the symbiosis and nodulation, as has also been observed by other authors (Olivera *et al.*, 2004; Ntatsi *et al.*, 2018; Savvas *et al.*, 2018). Whether the additional P required can be assimilated through more struvite in the substrate is something worth pursuing in future studies.

These findings lead to the idea that lack of N is not the limiting factor entirely responsible for the lower yields of A and B treatments but rather the progressive loss of P and Mg in the root medium as well as the reduced cation uptake. When looking at the NUE obtained for all treatments we can see that the plants with lower N input have a greater use efficiency. This difference is very clear in the treatment A with a three times higher efficiency compared to the treatment B. This difference can also be influenced by the atmospheric N fixation which is not given as “Soil” N in the calculation tool (Weih, 2014). Higher fixation capacity can therefore generate a higher NUE, which corresponds to our BNF results.

For a production in a larger scale vertical farm a fertilization with Struvite and Rhizobium seems possible, especially with the greater quantity of struvite like in the treatment B, which shows a great compatibility with the soil bacteria as well as producing a larger yield than the crops fertilized with only 2g of struvite. Seeing the initial fixation capacity of the control treatment and the appearance of nodules during the first sampling stage the nodulation could be even given

with naturally occurring Rhizobium, which could make the fertilization process easier in soil-based agriculture. The limitation for larger scale production could be an exact application of the struvite in the root area. As seen in this study, there is a large difference in production between the application of 2g and 5g of struvite, a larger scale production in a vertical farm would mean a precise weighting of the struvite amounts per plant and a direct application to each rhizosphere of each plant. Like stated by Degryse, F. *et al.* (2017) the location of this slow releasing fertilizer can have a great impact on a successful nutrient delivery to the plant. Therefore, it could be highly time and resource consuming.

6. Conclusions

This work aimed to study the feasibility of using struvite and the inoculation of Rhizobium bacteria as alternative Mg, N and P fertilization methods for vertical agriculture systems. To this purpose, we quantified the nitrogen source, the production and the evolution of the phenological stages of *Phaseolus vulgaris* with Rhizobium inoculation and different quantities of struvite, and compared the results to a control treatment. Three main conclusions can be stated from this study.

First, both alternative fertilizer treatments supplied the necessary nutrients to fulfill the plant cycle in a soilless growing media. The lower yield compared to the control suggests the necessity to evaluate higher struvite quantities to fulfill plant requirement to achieve higher yields. Since previous experiments conducted with struvite suggested a successful performance with 5g/plant, its combination with the soil bacteria Rhizobium makes this quantity insufficient, due to the additional nutrition requirements of the bacteria itself. This can be seen by the great reduction in the yields of treatment A and B in comparison to the control.

Secondly, while the nodulation seemed not to be hindered by the nitrogen input through the struvite in the root medium, it didn't significantly improve it either, although the BNF seemed to increase in later stages for plants grown under the treatment with an initial greater quantity of struvite.

Thirdly, the limiting factor for struvite fertilized and rhizobium inoculated treatments didn't seem to be nitrogen, which maintained sufficient concentrations in the plant throughout the experiment, but rather potassium, due to a lower uptake capacity caused by an electrochemical imbalance generated with the reduced presence of NO^{3-} in the root medium as well as magnesium and phosphorus given the struvite depletion seen as a reduced concentration in the plant nutrient content over time.

An increase of the applied struvite might be a solution for a more sustained phosphorus and magnesium supply for vertical agriculture, but could also interfere in the nodulation capacity of the plant. In this sense, further studies should aim to determine optimal struvite quantities for hydroponic bean production in combination with Rhizobium inoculation.

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Tables:

Table 1: Results for the mean values per plant of fresh weight (FW) and dry weight (DW) of the different organs as well as the Leaf Area Index (LAI) m^2 $plant^{-1}$ of the three treatments (A= 2g Struvite + Rhizobium; B= 5g Struvite + Rhizobium; C = Control) in three different time periods. 35 DAT (1), 62 DAT (2) and 84 DAT (3).

(1)	*Leaf DW (g) per plant*	*Shoot DW (g) per plant*	*Roots DW (g) per plant*	*Nodules n per plant*	*Nodules DW per plant (g)*	*LAI*
A	1.12^{a}±0.22	0.46^{a}±0.08	0.44^{a}±0.10	132.50^{a}±80.35	0.16^{b}±0.07	0.57^{a}±0.12
B	1.31^{a}±0.46	0.56^{a}±0.19	0.51^{a}±0.15	156.75^{a}±60.82	0.12^{b}±0.06	0.62^{a}±0.23
C	1.33^{a}±0.57	0.58^{a}±0.18	0.53^{a}±0.14	148.75^{a}±48.23	0.05^{a}±0.02	0.65^{a}±0.27

(2)	*Leaf DW (g) per plant*	*Shoot DW (g) per plant*	*Roots DW (g) per plant*	*Nodules n per plant*	*Nodules DW per plant (g)*	*LAI*
A	3.97^{a}±1.25	2.02^{a}±0.72	0.80^{a}±0.28	127.88^{a}±63.85	0.14^{b}±0.09	1.14^{a}±0.51
B	3.69^{a}±1.53	2.24^{a}±1.01	0.87^{a}±0.34	172.25^{a}±132.66	0.15^{b}±0.14	1.22^{a}±0.64
C	6.44^{a}±3.09	3.85^{a}±1.96	0.95^{a}±0.44	82.25^{a}±62.47	0.01^{a}±0.01	2.38^{b}±1.33

(3)	*Leaf DW (g) per plant*	*Shoot DW (g) per plant*	*Roots DW (g) per plant*	*Nodules n per plant*	*Nodules DW per plant (g)*	*LAI*
A	5.86^{a}±2.96	3.09^{a}±1.45	1.77^{a}±0.79	136.88^{b}±106.31	0.15^{b}±0.13	1.74^{a}±0.92
B	7.40^{a}±2.17	4.53^{a}±1.48	2.49^{ab}±0.57	186.25^{b}±48.79	0.24^{b}±0.11	1.80^{a}±0.67
C	11.11^{b}±1.51	6.91^{b}±1.42	3.35^{b}±0.88	39.13^{a}±24.76	0.02^{a}±0.02	3.72^{b}±0.87

Table 2: Results for percentage of Nitrogen derived from atmospheric N_2 fixation (%Ndfa) in plant, Total amount of N in plant expressed in kg/ha (Leaves+Shoot+Root+Beans) and Biologically fixed N extressed in kg/ha. Results given for three treatments A) 2g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution B) 5g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution at three different time periods. 35 days after trans*planting, 62 days after* trans*planting and 84 days after* trans*planting.*

Date	*Treatment*	*% Ndfa plant^{-1}*	*Total N in plant kg/ha*	*Kg/ha Biologically fixed N*	*Kg/ha N from Struvite*
35 DAT	*A*	68%	7.54±1.03[a]	5.38±1.04[b]	2.16
	B	60%	8.59±2.19[a]	5.33±1.38[b]	3.26
62 DAT	*A*	89%	24.67±4.96[a]	22.92±4.07[b]	1.75
	B	73%	24.62±6.20[a]	18.70±5.09[b]	5.92
84 DAT	*A*	90%	27.25±12.79[a]	25.40±12.96[b]	1.85
	B	82%	35.04±9.16[a]	29.21±7.79[b]	5.83

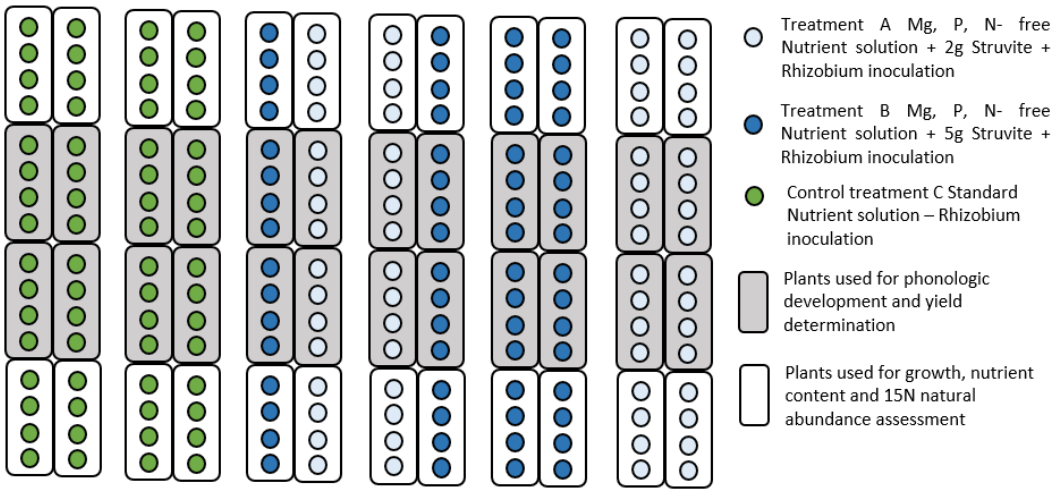
Figure Legends:

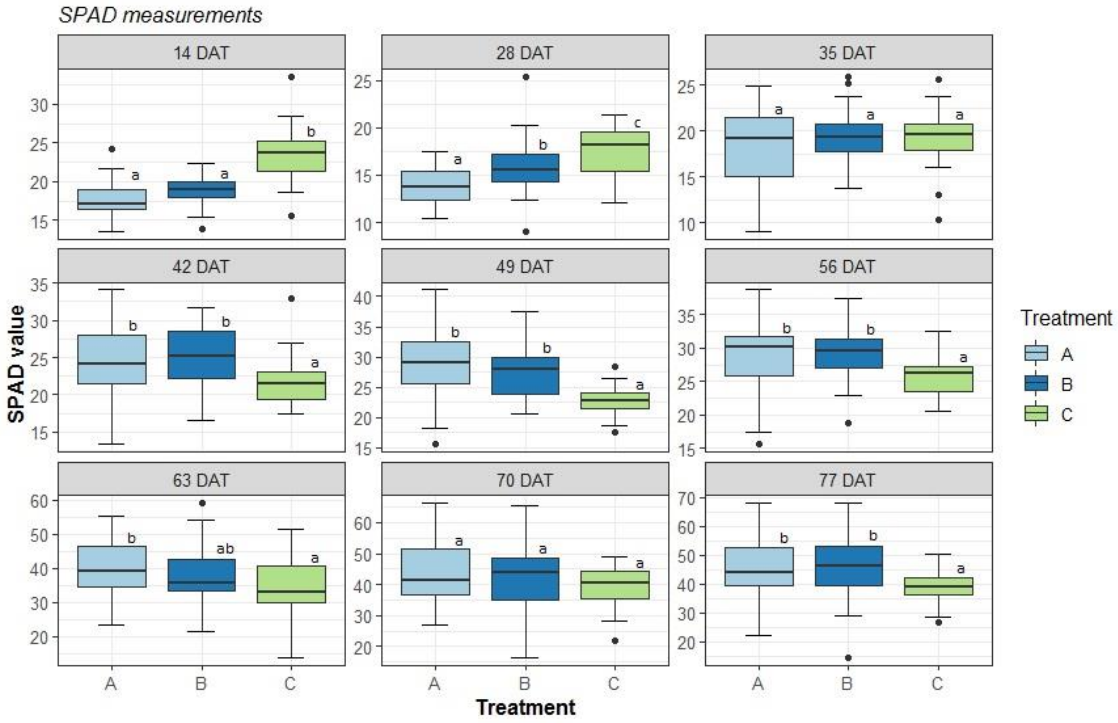
Figure 1: Graphic representation of the mean numerical count per plant for each organ (Leaves, flower buttons, open flowers) and yield in g/plant on a weekly basis, DAT representing the days after transplanting inside the iRTG. The colors represent the three treatments: (A) N.free solution with Rhizobium inoculation and 2g of struvite per plant. (B) N.free solution with Rhizobium inoculation and 2g of struvite per plant. (C) Complete nutrient solution without struvite and no inoculation treatment.

Figure 2: Boxplot representing the obtained $\delta^{15}N$ values for treatments A) 2g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution B) 5g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution and C) standard nutrient solution - Rhizobium inoculation. These observed values are given by plant organ in three different time periods. 35 days after trans*planting, 62 days after* trans*planting and 84 days after* trans*planting.*

Figure 3: Percentage of Nitrogen derived from atmospheric N2 fixation (%Ndfa) represented for treatments A) 2g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution B) 5g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution. These observed values are given for two plant organs (leaf; shoot) as well as the bean pods in three different time periods. 35 days after transplanting, 62 days after transplanting and 84 days after transplanting.

Figure 4: Nutrient concentration in Phaseolus vulgaris leaves and shoots, expressed in mg/g. Results given for three treatments A) 2g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution B) 5g of struvite + Rhizobium inoculation + P, Mg, N-free nutrient solution and C) standard nutrient solution - Rhizobium inoculation at three different time periods. 35 days after transplanting, 62 days after transplanting and 84 days after transplanting.


Treatment A Mg, P, N- free Nutrient solution + 2g Struvite + Rhizobium inoculation
Treatment B Mg, P, N- free Nutrient solution + 5g Struvite + Rhizobium inoculation
Control treatment C Standard Nutrient solution – Rhizobium inoculation
Plants used for phonologic development and yield determination
Plants used for growth, nutrient content and 15N natural abundance assessment


SPAD measurements
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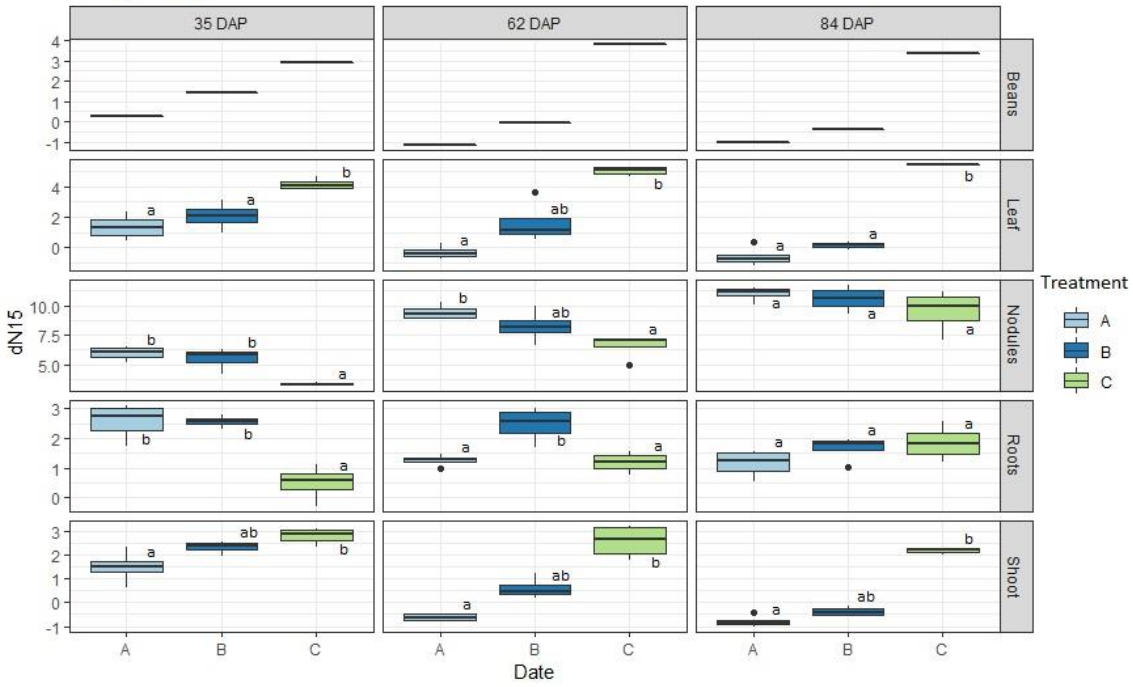
28 DAT
35 DAT
42 DAT
49 DAT
56 DAT
63 DAT
70 DAT
77 DAT
SPAD value
Treatment
A
B
C

Nitrogen origin



Nutrient content