The effect of nitrogen starvation on PSI and PSII activity in pea (*Pisum sativum*)

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Degree project in Biology 10p
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Acknowledgements

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Summary

This investigation addresses how photosynthetic efficiency is affected when pea (Pisum sativum) plants are restricted to a sole nitrogen source (i.e. ammonium or nitrate). The pea plants were watered with different nutrient solutions without NO$_3^-$ or NH$_4^+$ for different time-periods in order to assay for nitrogen content. The soluble ammonium and nitrate content was measured throughout the entire growth period. No major differences were observed in nitrogen content during the starvation period up to 25 days. For technical reasons, cultivation of plants could not be extended beyond this time. The chloroplasts and thylakoids were isolated after 25 days and assayed for chlorophyll contents and photosynthetic activity.

The outcome of these tests indicates a small but unambiguous decrease in the photosynthesis activity for all treatments, relative the control.
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1 Introduction
For a long period of time the effect of nitrogen starvation on plant growth and morphology as well as metabolism has attracted scientist’s attention [1] [2]. Many studies have focused on the effects on plants depending on the nitrogen compounds (NO$_3^-$ or NH$_4^+$) administered. But still, fairly little is known about the consequences of nitrogen starvation on the photosynthetic system of plants, the chloroplasts (see figure 1). The aim of this project has been to investigate the status of the chloroplast after nitrogen deficiency by analyzing the amount of chlorophyll per chloroplast as well as the effect on PSI and PSII activity.

![Figure 1. An illustration of a chloroplast containing thylakoids (N. M. State University 2001).](image)

1.1 An overview of nitrogen
Nitrogen is a mineral which, in addition to carbon, oxygen and hydrogen, constitute the major parts of the plant body. Approximately 1.5 % of a plants dry matter is nitrogen [3]. Nitrogen is an important part of the structure in proteins, amino acids, nucleic acids, hormones, chlorophylls and other organic molecules. The plant absorbs nitrogen as nitrate (NO$_3^-$) and ammonium (NH$_4^+$) [3].

The atmosphere is the largest source of nitrogen since the air contains approximately 78% nitrogen gas (N$_2$). N$_2$ can be available to plants through discharge (thunderstorms) and by nitrogen fixing bacteria in the soil. Microorganisms decompose nitrogen-containing compounds in soil organic matter to produce the inorganic nitrogen ions NH$_4^+$ and NO$_3^-$ (i.e. mineralization): they initially convert organic nitrogen to NH$_4^+$ and subsequently oxidize the NH$_4^+$ to NO$_2^-$ and then NO$_3^-$ (i.e., nitrification) [4] as seen figure 2.
Many plant families include species that form symbiotic relationships with nitrogen fixing bacteria, which in turn give roots built-in source of fixed nitrogen for assimilation into organic compounds [5].

According to Salisbury & Ross [3], large seeds sometimes contain enough of the essential elements to grow into mature plants, which is why it might take some time before any signs of nutrient deficiency can be observed.

Nitrogen has high mobility and the deficiency will therefore be noted as a yellowing of the older foliage. As the deficiency continues, the new growth will ultimately suffer similar yellowing and appear weak and spindly [6].

Nitrogen is the only essential element that can be absorbed as both a cation (NH$_4^+$) and an anion (NO$_3^-$). Plants absorb NH$_4^+$ much faster than NO$_3^-$, and the form of nitrogen can therefore have a significant effect on the uptake of other nutrient by competitive inhibition [1].

Cations (e.g. NH$_4^+$) are driven into the cell by the membrane potential and the accumulation of anions, (e.g. NO$_3^-$) in the cell is coupled by the transport of the inward diffusion of H$^+$ through a co-transporter [5].

If the plant needs to increase the inward diffusion of cations, in this case NH$_4^+$, the plant can exchange ammonium with hydrogen ions, which in the long run will cause lower pH in the soil.

### 1.2 Background – Nitrate vs. Ammonium

Bloom [4] explains the use of nitrogen in the plant as follows: “Plant acquisition of NH$_4^+$ and NO$_3^-$ from the soil requires three basic steps: absorption of the ion from the soil solution into the plant, translocation of the ion within the plant, and assimilation of the ion into an organic form such as an amino acid. The energy expended for these purposes
differs greatly between $NH_4^+$ and $NO_3^-$ and can comprise a significant, if not predominant, fraction of the total energy that a plant consumes.”

According to Salisbury & Ross [3], crop plants and many native species absorb most nitrogen as $NO_3^-$ because $NH_4^+$ is so readily oxidized to $NO_3^-$ by nitrifying bacteria. Thus, climax communities of conifers and grasses absorb most nitrogen as $NH_4^+$ because nitrification is inhibited either by low soil pH or by tannins and phenolic compounds. Also Woolfolk & Friend [2] agrees with Salisbury & Ross [3] and point out that general acid-tolerant species, such as evergreen conifers, grow best in $NH_4^+$-dominated solutions, whereas species adapted to more basic soils, such as deciduous hardwoods, grow best in $NO_3^-$-dominated solutions [2].

When the nitrate is absorbed by the roots, a plant enzyme can reduce the nitrate back to ammonium, and then other enzymes can incorporate the $NH_4^+$ into amino acids and other organic compounds [5].

Bloom [4] points out that plant species vary greatly in their response to $NH_4^+$ or $NO_3^-$. This can be illustrated by looking at the extremes, such as cranberry that cannot tolerate $NO_3^-$ as a nitrogen source, whereas radish cannot tolerate $NH_4^+$ as its sole source. Nonetheless, the majority of species meet their nutritional requirements for nitrogen by using both $NH_4^+$ and $NO_3^-$. 

Wang & Below [7] also emphasize that the form of nitrogen available to the plant is an important environmental variable that affect plant growth. Root proliferation and overall plant growth are usually greater with a mixture of $NH_4^+$ and $NO_3^-$ than with either form alone.

Moreover, $NO_3^-$ as a mobile anion is more susceptible to leaching than $NH_4^+$. For these reasons, the concentration gradient of $NO_3^-$ through the soil profile is more variable than that of $NH_4^+$, and $NH_4^+$ is probably a more reliable source of nitrogen for plants [4].

Muhlestein et al. [1] agrees with earlier studies but also point out that several short term studies indicate that the detrimental effects of high levels of $NH_4^+$ can be ameliorated if root-zone pH is controlled between pH 5 and 6. Muhlestein et al. [1] claim that high ammonium increases seed protein nitrogen which should enhance flour quality and improve the nutrition level.

Thus, how is the plant affected depending on their nitrogen source? A well known fact is that one of the most important processes in the world is taking place in the thylakoid membrane in the chloroplasts (see figure 1), i.e. the light driven reactions of photosynthesis, seen in figure 3. Both P680 and P700, in the thylakoid membrane, are chlorophyll molecules with proteins attached that play important roles in both photosystem I and II (PSI & PSII), and as mentioned earlier, nitrogen is a very important part of proteins and chlorophylls among other. So the conclusion is that the nitrogen source will affect the photosynthetic efficiency in the plants.
1.3 Purpose of this project

The aim of this project has been to investigate the effect of nitrogen starvation and different nitrogen sources on physiological parameters such as chlorophyll content and photosynthetic activity. This has been achieved by growing pea plants in various sets of nutrient solution, with different nitrogen sources. After the starvation period, in this case 25 days, chloroplasts and thylakoids have been isolated and assayed for chlorophyll content and photosynthetic activity. The photosynthetic activity was measured as oxygen consumption in PSI and oxygen evolution in PSII.
2 Materials and methods

2.1 Growth conditions

Pea seeds (*Pisum sativum*) cv. Kelvedon wonder were soaked in Milli-q water for approximately 18 hours and then planted in vermiculite. All peas were divided into four separately contained sets where each set was watered with different nutrient solutions. One set was used as control, given a complete set of nutrients, whereas the remaining three were given nutrition solutions according to table 1:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment:</th>
<th>Given nutrient solution:</th>
<th>[N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Complete set of nutrients 1:1 of both NH₄⁺ and NO₃⁻</td>
<td>71.4 mM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-NH₄⁺</td>
<td>Complete set of nutrients except ammonium</td>
<td>71.4 mM</td>
</tr>
<tr>
<td>2</td>
<td>-NO₃⁻</td>
<td>Complete set of nutrients except nitrate</td>
<td>71.4 mM</td>
</tr>
<tr>
<td>3</td>
<td>-N</td>
<td>Complete set of nutrients excluding NH₄⁺ and NO₃⁻</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Illustration of the different pea sets.

See appendix A for complete recipes on the different nutrient solutions.

All plants were cultivated in a growth chamber at 21.7 ±1.5 °C until they developed floral organs (i.e. blooming), but individual plants of each treatment were also harvested at day 15, 18, 21, 24 and 25. The light/dark cycle was 16 h/8 h and during the light period the intensity was kept constant of 50 µmol·s⁻¹. The first days, until the plants were approximately 2 cm, they were watered with only Milli-Q water. After that the plants were watered as indicated in table 1 and appendix A, approximately every third day.

At day 6, 11 and 16 the growth were measured and when the plants started to generate flowers, at day 31, the remaining plants were harvested and the flower-frequency was registered.

2.2 Ammonium and nitrate measurements

Plants from the different treatments were harvested at day 15, 18, 21, 24 and 25 in order to determine the amount of ammonium and nitrate as an effect of the treatment. Approximately 0.8 g of plant material was grinded with 8 ml of distilled water for each treatment. The mixture was then transferred to four Eppendorf tubes and centrifuged at 18 000 × g for 10 minutes. The nitrate and ammonium content in the supernatant was then measured using the FIA star 5000 analyzer (Foss TECTATOR).

2.3 Chloroplast and thylakoid preparation

The final harvest was made after 25 days, and the chloroplasts and thylakoids were isolated according to Dahlin and Cline [8]. Chlorophyll concentrations were measured in 80 % acetone according to Lichtenthaler & Wellburn [9].
2.4 Oxygen measurements

Oxygen measurements were performed separately in PSI and PSII (see figure 3) using the Oxygraph V 2.32 equipment, including both software and hardware (Hansatech Instr. Ltd.). A cooling/heating device Ecoline RE 104 (Lauda) was also connected to the oxygen chamber. Circulating water held a constant temperature of 20 °C around the oxygen-chamber. The light source used was a Pradovit P300 slide-show projector that was kept 50 cm from the oxygen chamber, resulting in a light intensity of 360 µmol²s⁻¹.

To assay the photosynthetic efficiency in the both PSI and PSII, two experiments were made on each harvest; the first experiment was performed on PSII where the oxygen evolution was measured, whereas the second experiment was based on PSI where oxygen consumption was registered. The Oxygraph software was calibrated for 20 °C and 101.3 kPa (normal air pressure). 2.5 ml of reaction medium (containing 50 mM sorbitol, 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA and 1 mM HEPES, pH 7.6) was used as a base before adding the predefined amount of thylakoid suspension to reach a total quantity of 100 µg chlorophyll for each measurement mixture. The chamber was then sealed with a lid and all reagents were added with a Hamilton syringe.

In the PSII reactions, 50 µl 10 mM phenyl-p-quinone (PPQ) was used as an electron-acceptor. For PSI, 10 µl 1.0 M methyl-viologen (MV) was first applied, acting as an acceptor. When the mixture was stable, 50 µl 10 mM diuron, 3-(3.4-dichlorophenyl)-1.1-dimethyleurea (DCMU) was added to prevent the reduction of PQ and disconnect water from the electron transport chain. Thereafter 50 µl 10 mM 2.6-dichlorophenolindophenol (DCPIP) was used as an electron donor before adding 50 µl 1.0 M Na-ascorbate in order to re-reduce it. See detailed experimental scheme (figure 4).

2.5 Chlorophyll determination

Purified and intact chloroplasts were used for chlorophyll determination according to Dahlin & Cline [8].

![Figure 4. Experimental scheme. An illustration over the chemical substances (in bold) used when testing the effect on PSII and PSI in the thylakoid membrane as indicated within the parenthesis.](image-url)
3 Results

3.1 Plant morphology
Measurements of the plants during the observation period only indicate minor differences in size between the different samples (see table 2). The result can be roughly interpreted as that the different samples are similar in size, at least until day 16.

<table>
<thead>
<tr>
<th></th>
<th>Growth [cm]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 6</td>
</tr>
<tr>
<td>Control</td>
<td>1.7</td>
</tr>
<tr>
<td>-NH₄⁺</td>
<td>1.7</td>
</tr>
<tr>
<td>-NO₃⁻</td>
<td>1.7</td>
</tr>
<tr>
<td>-N</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 2. Growth of the pea plants after the different treatments.

On day 31, when all plants started to generate flowers, a slight difference in the number of flowers could be observed as the control had 6.0 % plants that had generated flowers whereas 18.6 % of the pea plants generated flowers in sample 1 (-NH₄⁺) (see table 3).

<table>
<thead>
<tr>
<th>Frequent of Flower (day 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>flower/no flower</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>-NH₄⁺</td>
</tr>
<tr>
<td>-NO₃⁻</td>
</tr>
<tr>
<td>-N</td>
</tr>
</tbody>
</table>

Table 3. Effect of nitrogen deficiency on the production of flower. The table shows that there were for example a total of 68 plants in the control and that 4 had flowers on day 31.

3.2 Nitrate and ammonium content
The soluble content of nitrate and ammonium was measured at day 15, 18, 21, 24 and 25 in order to find when it is possible to measure the effects of the nitrogen starvation. This would in turn indicate the most suitable time for when to harvest and make photosynthetic measurements (i.e. PSI and PSII activity). The result of the first measurements of both nitrate and ammonium at day 15 was similar for all treatments. Measurements at day 18 and 21 indicated that the plant utilizes ammonium and stores nitrate. The differences between the treatments became more substantial at day 24 and 25 (see figure 5).
Figure 5. Bars showing the nitrate (A) and ammonium (B) content in all treatments on day 24 and 25 when the differences between the treatments became more obvious. $2 \geq n \geq 4$, where $n$ represents the number of samples that the result is based on. For example, the control at day 25 had a mean value of 10 µg ammonium per g fresh weight and the standard error was from 7-13 µg/g fresh weight.
3.3 Chlorophyll content
The total chlorophyll content of isolated chloroplast is showed in pg Chl/chloroplast. Table 4 illustrates that all samples are affected by the treatment. The Chl/chloroplast content is between 71-87 %, relative to the control.

<table>
<thead>
<tr>
<th>Chl/Chloroplast [pg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>-NH₄⁺</td>
</tr>
<tr>
<td>-NO₃⁻</td>
</tr>
<tr>
<td>-N</td>
</tr>
</tbody>
</table>

Table 4. Showing the amount of Chl/chloroplast.

3.4 Tests on PSII and PSI
The result is showed in activity (nmol oxygen/mg chlorophyll/min) in figure 6 and 7. In the statistical process, the highest and lowest values in each treatment were discarded.

3.4.1 Photosystem II activity

![Figure 6](image)

Figure 6. The mean value \( [5 \geq n \geq 8] \) and standard error of the oxygen evolution in PSII after the different treatments of pea plants, were \( n \) represents the number of samples that the result is based on.

All treatments resulted in lower photosynthetic PSII activity (approximately 20 %) as compared to the control. But on the other hand, PSII compared among the different treatments, exhibited fairly similar activities.
3.4.2 Photosystem I activity

Figure 7. The mean value [7 ≥ n ≥ 10] and standard error of the oxygen consumption in PSI after the different treatments of pea plants, were n represents the number of samples that the result is based on.

Similar to the PSII activity, the activity of PSI was 15-20 % lower for all treatments when compared to the control. However, the data within each treatment exhibited fairly large deviations from the mean values.
4 Discussion

4.1 Plant morphology

The growth measurements indicated no results according to nitrogen scarcity, probably since it requires a very small amount of nitrogen for a plant to grow mature, along with the fact that the pea seed already contains adequate nitrogen reserves [10][3]. On day 31, when the plants started to generate floral organs, the differences between the different cultivation treatments became more substantial; the \(-\text{NH}_4^+\) and \(-\text{NO}_3^-\)-treatments had a remarkably higher occurrence of flowers, this could be related to stress caused by the treatment. Stress is believed to increase the accumulation of ethylene gas which is the hormone that, among other things, also has an impact on the senescence (e.g., the production of floral organs) c.f [6]. The stress can be related to either \(\text{NH}_4^+\) or \(\text{NO}_3^-\) deficiency. It is also possible that the nitrogen uptake indirectly affects the pH in the growth medium. Absorption of either \(\text{NH}_4^+\) or \(\text{NO}_3^-\) will decrease/increase the pH level and further inhibit absorption of nitrogen and other nutrients [3] [4] [11]. Of course, this assumption demands additional tests, such as pH measurements of the growth medium.

4.2 Chlorophyll/chloroplast

The measurements of chlorophyll in chloroplasts indicate that plants of all treatments are affected by the given unique customized nutrient solution. The chlorophyll molecule contains nitrogen [6], and the accumulation of chlorophyll had decreased as a result from the nitrogen starvation. This decrease could have different origins in the different treatment as further discussed in chapter 4.4.

4.3 Nitrogen content

The results of this investigation only show the quantity of soluble nitrogen in the plants, and consequently the nitrate/ammonium stored in the plant. Hence, there is no indication of the nitrogen contents in for example pigments or macromolecules. The result of the measurements of both nitrate and ammonium from day 15 was similar for all treatments. Three days later, at day 18, the nitrate content in sample 1 (-\(\text{NH}_4^+\)) was almost twice the amount, whereas in the other treatments it had only increased slightly (data not shown). This indicate that the plants utilizes most of the given ammonium (e.g., for amino acids) while most of the given nitrate is stored without being metabolized [12].

The relatively large difference between day 24 and 25 (seen in figure 4), can perhaps be explained by the differences in the plant’s daily cycle as the different samples were collected at different times during that daily cycle.

The nitrate levels in the control increased with time. This can be interpreted as if the plant have a problem using the nitrate and instead store it in the vacuole, maybe partly for osmotic balance [6]. Ammonium works the other way around as it decreases with age in all the treatments, possibly because the younger plants had not developed the enzyme-system required in order to absorb ammonium, i.e. glutamine synthetase and glutamate synthase [6].
Sample 1 (-NH$_4^+$) has a relatively high level of ammonium that can be explained by the fact that nitrate can be reduced in a two-step process into ammonium [5].

Sample 2 (-NO$_3^-$) has, as expected, a low content of nitrate. It can not be excluded that some of the detected nitrate originates from the vermiculite used in the pots, this is also seen in sample 3 (-N). Furthermore the ammonium content is similar to the control, as expected.

Sample 3 (-N) indicate a surprisingly high level of both ammonium and nitrate. Possible reasons may be either contamination of the vermiculite, and/or that the pea is a typical large-seed plant [3] [10], and actually fairly insensitive for nitrogen starvation during the first 3-4 weeks of growth.

4.4 PSII & PSI activity

The measurements, as shown in figure 5 and 6, indicates a decrease in efficiency after nitrogen starvation. This phenomenon occurs both in the PSI and PSII tests. The same tendency is also observed in the chlorophyll/chloroplast content as discussed in chapter 4.2. The conclusion is therefore that treatment 3 suffers from nitrogen deficiency related symptoms and that treatment 1 and 2 suffer from unbalanced nutrient supply, but for different reasons as described below;

Sample 1 (-NH$_4^+$) gets all its nitrogen from nitrate, but nitrate is often absorbed so fast that there are rapid increases in the pH of the growth medium. An optimum would be below 6 [13], but since absorption of nitrate (and other anions) is accompanied by absorption of H$^+$ or excretion of OH$^-$, to maintain charge balances, this pH is not easy to preserve. At high pH values, iron and some other elements precipitate as hydroxides and are then unavailable to the roots. In this case the pH problem can be minimized by supplying part of the nitrogen as an ammonium salt since the absorption of NH$_4^+$ and other cations occurs simultaneously with absorption of OH$^-$ or transfer of H$^+$ from the root to the surrounding solution [3]. It is also possible that the dominance of anion causes an unbalance in the anion/cation ratio. Furthermore, the plant can immediately use the ammonium, e.g. for the synthesis of amino acids, whereas nitrate on the other hand has to undergo reduction, via the enzyme nitrate reductase and nitrite reductase [6], before it can be utilized. This reduction is an energy demanding process for the plants [4].

Sample 2 (-NO$_3^-$) on the other hand gets all its nitrogen from ammonium. Systems without adequate pH control that contain NH$_4^+$ as a sole nitrogen source quickly become acidic [4], and since the optimal pH for absorption ammonium is above 8 [13], the pH inhibit further NH$_4^+$ absorption. On the other hand, as mentioned earlier, if the root-zone pH is controlled within the interval 5 to 6, the detrimental effects of high levels of NH$_4^+$ can be minimized [1]. Sample 2, as well as sample 1, can suffer from an unbalance in the cation/anion ratio, due to the dominance of cation in the nutrient supply.

Sample 3 (-N) almost certainly suffers from nitrogen deficiency. As mentioned earlier there are several large protein-complexes attached with both PSI and PSII, for example LHCI and LHCII (see figure. 3). LHCII contain some of the dominating thylakoid membrane proteins e.g. LHCP of PSII, which is the light harvesting chlorophyll a and b binding protein. [14]. These large complexes are likely to be affected when there is a
nitrogen shortage. It would be suitable to run gel-separation tests on the proteins of isolated thylakoids to be sure which molecules that are affected.

Conclusively no lethal effect on the pea plants is to be seen after 25 days of nitrogen starvation, from a sole nitrogen source (i.e. NO$_3^-$ or NH$_4^+$). Furthermore, the chloroplasts are relatively unaffected by the treatment with a slight decrease in chlorophyll content and photosynthetic activity.

The same decrease in efficiency in the plants is shown after 25 days regardless of they have had access to a single nitrogen source, or no nitrogen at all. It would be interesting to perform the same experiments in older plants, e.g. 40 days of growth period, to see when the starvation effects differ from the toxic effects seen in single nitrogen-source cultivation.
References


Figures

Figure 1: New Mexico state University (2001)

Figure 2: Physicalgeography.net (2006)

5 Appendix A

Nutrient solution recipes

Stock solution A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Molarity</th>
<th>Molecular Weight</th>
<th>Concentration in l water</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.2 M</td>
<td>136.09 g/mol</td>
<td>27.2 g/l water</td>
<td></td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.2 M</td>
<td>174.27 g/mol</td>
<td>34.9 g/l water</td>
<td></td>
</tr>
</tbody>
</table>

Stock solution B

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Molarity</th>
<th>Molecular Weight</th>
<th>Concentration in l water</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄ × 7H₂O</td>
<td>0.3 M</td>
<td>246.48 g/mol</td>
<td>73.9 g/l water</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 M</td>
<td>58.44 g/mol</td>
<td>5.8 g/l water</td>
<td></td>
</tr>
</tbody>
</table>

Stock solution C

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Molarity</th>
<th>Molecular Weight</th>
<th>Concentration in l water</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ × 2H₂O</td>
<td>1 M</td>
<td>147.02 g/mol</td>
<td>147.2 g/l water</td>
<td></td>
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</tbody>
</table>

Micro nutrients

<table>
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<th>Molarity</th>
<th>Molecular Weight</th>
<th>Concentration in l water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III)-EDTA-Na</td>
<td>0.05 M</td>
<td>367.05 g/mol</td>
<td>18.4 g/l water</td>
<td></td>
</tr>
<tr>
<td>MnCl₂ × 4 H₂O</td>
<td>0.007 M</td>
<td>197.91 g/mol</td>
<td>1.4 g/l water</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.0007 M</td>
<td>136.28 g/mol</td>
<td>0.1 g/l water</td>
<td></td>
</tr>
<tr>
<td>CuSO₄ × 5 H₂O</td>
<td>0.0008 M</td>
<td>249.68 g/mol</td>
<td>0.2 g/l water</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.002 M</td>
<td>61.83 g/mol</td>
<td>0.1 g/l water</td>
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<tr>
<td>NaMoO₄ × 2 H₂O</td>
<td>0.0008 M</td>
<td>241.95 g/mol</td>
<td>0.2 g/l water</td>
<td></td>
</tr>
</tbody>
</table>

Add 4 ml from A, B, C and micro nutrients for 4 l of Milli-Q water. Ensure that the pH is within the interval 5.9 to 6.1.

Nitrogen additive

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Molarity</th>
<th>Molecular Weight</th>
<th>Concentration in l water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂ × 4 H₂O</td>
<td>1 g N/l water</td>
<td>236.15 g/mol</td>
<td>8.43 g/l</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1 g N/l water</td>
<td>132.1 g/mol</td>
<td>4.71 g/l</td>
<td></td>
</tr>
</tbody>
</table>

Add one of the nitrogen additives (1 ml for 1 l Milli-Q water). Add 0.5 ml of each nitrogen additive for control solution.

*Note: Water always refers to Milli-Q water*