# Nitrogen Use Efficiency in Spinach *(Spinacia oleracea* L.*)*

MSc Thesis Plant Breeding PBR80436

Supervisors:
J.R. Chan Navarrete, MSc
Dr. Gerard van der Linden
Dr. Edith Lammerts van Bueren

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Wageningen UR
Huzen, Mayra
891109380070

# Table of Contents

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# Abstract

Introduction: Nitrogen availability is in many environments the limiting factor for crop growth. Spinach especially has inefficient nitrogen use efficiency: uptake and utilization of nitrogen. High addition of nitrogen has negative effects on the environment but also causes risks of increasing the amount of nitrate in the harvestable product. This study aims to gain insight in traits and processes that are involved in nitrogen use efficiency and elucidate which traits contributing to nitrogen use efficiency are influenced by environment factors and assess the genetic variation for these traits.

Method: Two types of experiments were done: a hydroponic experiment and two field trials. In the hydroponic experiment eight genotypes were used to analyse differences within these genotypes at low nitrogen availability with two different application methods (Ingestad and depletion). In the field trials 24 genotypes were used to analyse differences between genotypes at different nitrogen levels (No application, 100 kg/ha and 150 kg/ha). In addition, the correlations between experiments were analysed.

Results: Plants at low nitrogen levels showed more stress under depletion conditions than under Ingestad conditions. To cope with low levels of nitrogen plants invest in root:shoot ratio, specific leaf area, root dry weight and longer roots. The field trials showed the same behavior in traits under low nitrogen as in the hydroponics experiment. Root characteristics were more difficult to study, because only part of the roots could be harvested. No significant differences were found between nitrogen levels and genotypes in the field trial for root traits.

Conclusions: Evaluation in hydroponics is best to do under Ingestad conditions since plants do not die in this condition as they do under depletion conditions. Traits that are essential for nitrogen use efficiency are root shoot ratio, specific leaf area, dry matter percentage and chlorophyll content. Increasing root shoot ratio and decreasing specific leaf area under low nitrogen conditions are adaptation mechanisms. This gives the plant the opportunity to have more root area per unit of shoot to take up nutrients in deprived conditions and to have thicker leaves and thus a stronger structural defence instead of growing too fast. Shoot dry weight was least influenced by environmental effects, while root characteristics were most influenced by environmental effects. Genotypes 16, 23, 33 and 41 are least influenced by the environment.

# 1. Introduction

## 1.1 Background

In many environments, nitrogen availability is the limiting factor for growth (Berendse and Aerts, 1987). Therefore, growth of agricultural food production is associated with increases in the use of nitrogen fertilizers. In general, plants only use around 50% of nitrogen input, which signifies a loss in money and energy (Elia *et al.*, 1998). At the same time, the increase in fertilizers has a consequential negative impact on the soil biodiversity and functioning of non-agricultural neighbouring ecosystems. However, an increase in food production is necessary to supply sufficient food for world consumption. Since the world population is still increasing, it is necessary to develop new cropping strategies and select or breed genotypes that can grow under sustainable, low nitrogen conditions (Cassman *et al.,* 2002). This study is part of a project that aims at developing breeding strategies for nitrogen use efficiency (NUE) in spinach.

## 1.2 Spinach

Spinach (*Spinacia oleracea L.)* is an annual crop of the Amaranthaceae family and has a leaf rosette that is consumed as a crop at its vegetative stage. Spinach is mainly a dioecious species, which can be found as monoecious in some cases, it is diploid (2x=2n=12), and is mainly wind-pollinated. The crop has a short growth cycle, three to five weeks for baby leaf production up to eight weeks for the industry market. The leaf blades and part of the petioles are harvested. The harvest usually takes place at the end of the vegetative phase, just before the plants start bolting. The fresh weight of the leaf rosette is very important, because it determines crop yield. The size of the leaf area per plant is determined by the rate and duration of leaf appearance, rate and duration of leaf expansion, mature leaf area and rate of leaf senescence (Biemond, 1995). Spinach has high nutritional value and is rich in vitamin C and antioxidants, which is necessary for vitamin A production (Cao *et al.,* 1998).

## 1.3 Nitrogen

There are two main forms of nitrogen sources in the soil: nitrate ($NO_3^-$) and ammonium ($NH_4^+$). Plants can store unassimilated nitrate for a short period, which is necessary for maintenance of growth in periods of nitrate shortage (unless it is in the vacuole where it can be store longer) (Pilbeam, 2011). Applied nitrogen not taken up by the plant is vulnerable to leaching and denitrification, which has a negative effect on the environment (Cassman *et al*., 2002), such as water pollution, air pollution and indirectly the loss of biodiversity (through eutrophication and acidification) (Erisman *et al*., 2008). An example of water pollution is eutrophication of freshwater which is the proliferation of algae that reach densities so high that it reduces the light available for rooted water plants (Hirel *et al.*, 2007).

## 1.4 Nitrogen in spinach and NUE

Spinach is not very efficient in both the nitrogen uptake and utilization and requires considerable amounts of nitrogen for growth and establishment of the dark green colour (Biemond *et al*., 1996; Smolders *et al*., 1993). The combination of high nitrate input and low nitrate reduction by spinach leads to high levels of nitrate in the

marketable product (Biemond *et al.*, 1996). For instance, if spinach is grown in a greenhouse, the plants can accumulate substantial amounts of nitrate in the leaves, because mineralization of soil organic matter gives a surplus of nitrate to the plant. This is due to the greenhouse effect in which solar radiation is present that warms the greenhouse. This amount is often higher than the levels permitted by EU law (Hirel *et al*., 2011). The European Union has regulations for nitrate content of leaf vegetables. The maximum permitted level of nitrate in freshly harvested spinach must be under 3000 mg $NO_3^-$/kg when harvested from 1 November to 31 March and must be under 2500 mg $NO_3^-$/kg when harvested between 1 April and 31 October . Deep-frozen spinach has a maximum permitted level for nitrate of 2000 mg $NO_3^-$/kg (Briggs, 2011). NUE can be divided in nitrogen uptake efficiency and nitrogen utilization efficiency. N uptake is the ability of the plant to absorb nitrogen from the soil and N utilization is the ability to produce yield (leaf) as a consequence of the nitrogen absorption (Hirel *et al*., 2011). For spinach to keep a satisfactory yield at low nitrogen conditions, a high NUE is necessary. Depending on whether plants are produced for biomass or grain yield, the definition of NUE differs (Masclaux-Daubresse *et al*., 2010). One definition for spinach is: the maximum economic yield produced per unit of nitrogen supplied to the plant (Fageria and Baligar, 2005). In general, NUE is a complex trait and according to Biemond *et al.*, (1996) NUE can be affected by differences in the amount of nitrogen applied, the timing of the nitrogen addition and the soil type in which the nitrogen is used. These three differences will be further explained below. Different varieties within a species can be influenced differently by the same environment (genotype x environment). Therefore different varieties can be used in different environments (Baresel *et al*., 2008).

## Amount of nitrogen used, timing of nitrogen application and soil type further explained

The amount of nitrogen fertiliser can affect plant growth, however it does not affect all traits of the plant. For instance, in spinach, nitrogen application had a positive effect on yield dry matter but had no effect on partitioning of dry matter to the leaf blades, petioles and stem independently (Biemond *et al*., 1996). Both amount and timing of the addition of nitrogen can have an effect on the NUE under hydroponics. For instance, the model of Ingestad, in which a steady state of nitrogen supply is used to study NUE, enables growth of plants with suboptimal levels of nitrogen fertilisation. This model shows that deficiency symptoms disappear when the internal concentration is stable, independent of level (Ingestad, 1982). Deficiency symptoms that show early on are not desirable in spinach production for consumption. Nutrition/growth relationships are shown to be different from the ones observed under varied external concentration. Relative growth rate under varied external concentration is lower than under a stable external concentration (Ingestad, 1982). NUE is dependent on both soil type and soil biodiversity. The growth response of spinach plants (relative growth rate and nitrogen content) on three different soil types (loamy sand, silt loam low mineral-N and silt loam high mineral-N), shows considerable differences in patterns of nitrogen uptake between soil types. High mineral-N silt loam had the highest nitrogen uptake, followed by loamy sand (Smolders *et al*., 1993). There are also some indications that soil fauna has a positive effect, but this may be an indirect effect (Brussaard *et al.*, 2007). Management

activities like tillage, seeding, weed and pest management, irrigation and harvesting are also known to affect the NUE in cereal crops like wheat, rice and maize (Cassman *et al.,* 2002).

## 1.5 The effect of different traits on NUE of plants

NUE is determined by different plant traits and genetic variation for the traits need to be identified in order to improve NUE. Many studies have already explored NUE in potato, maize, wheat and rice (Cassman *et al.,* 2002; Baresel *et al.,* 2008*)*. Growth and net - photosynthesis have already been shown to be negatively affected by a shortage in nitrogen supply, resulting in a lower level of free sugars in the roots (Buysse and Merckx, 1995). Growth is the trait that determines yield and it depends on the available amount of nitrogen in the plant (Agren 1985). In wheat, N uptake contributed more to N efficiency than N translocation efficiency from the vegetative parts to the grains. N translocation is part of nitrogen utilization (Baresel *et al*., 2008*).*

For instance, in potato, leaf area and nitrogen concentration per unit leaf area, are traits that change in the plant to adapt to nitrogen limitation. Adaptation of the size of the leaves is necessary to maintain photosynthetic capacity; there is more photosynthetic capacity with larger leaves (Vos *et al*., 2004). For maize leaf nitrogen content, photosynthetic capacity and radiation use efficiency are traits that are more sensitive to limitations in nitrogen than leaf area expansion and light interception, which suggests different strategies between species (Vos *et al*., 2004). Also, the importance of the root system, in taking up nitrogen under limiting conditions, has been shown in maize, rice, wheat and barley in several studies (Guingo *et al*., 1998; Kamara *et al*., 2003; Gallais and Coque, 2005 as cited in Hirel *et al*., 2007). Components of root morphology mostly affected by N application are length, number of apices and frequency of branching and root architecture.

## 1.6 Project Goal

Improving NUE in field grown spinach is desirable to improve crop yield, to reduce the cost of production and to maintain environmental quality (Campbell *et al.*, 1995; Fageria and Baligar, 2005; Grant *et al.*, 2002). Various (complementary) approaches can be used to improve NUE such as conventional breeding, molecular genetics and alternative farming techniques (Hirel *et al*., 2011). For this research the focus is on breeding for better NUE, since breeding for NUE has already proven to be promising in other crops such as barley (Bingham *et al*., 2012). Improving NUE through breeding is primary to improve the efficiency of crop production (Berendse and Aerts, 1987). Knowledge about processes and traits that are mostly determining NUE and their genetic variation available among such traits for effective selection and breeding of NUE are important. The hydroponic system is being explored as a method to determine the traits related to NUE, so also a selection method can be developed.

## 1.7 Research Objective

This study aims to discover the processes and traits that are highly affected by different nitrogen conditions and the correlation of each process or trait with NUE under hydroponics and field conditions. This will contribute to identify breeding targets and provide information about new breeding strategies for low input varieties of spinach.

## 1.8 Research Questions

- How do the different methods of nitrogen application (Ingestad and depletion) in the hydroponics influence plant performance?

Which traits are essential for nitrogen use efficiency and which are not?

- How do the environmental effects influence plant performance? What traits are different between hydroponics and the field trials?

- How are the hydroponics experiments correlated with the field experiments regarding traits, nitrogen levels and nitrogen application methods?
- Do cultivars differ in ranking in the different experiments?
- What are advantages and disadvantages of using hydroponics and field trials as an experimental method for nitrogen use efficiency in spinach?

# 2. Materials and Methods

For this paper two experiments were performed: I) a hydroponic experiment that had four separate units, each one with their own re-circulating system, two systems had depletion conditions and two had Ingestad conditions (Ingestad, 1982); and II) two field experiments at different locations, with three different nitrogen levels.

## 2.1 Hydroponics

### 2.1.1 Plant Material

In the hydroponics experiment eight cultivars were used (F1 hybrids). These lines were selected because of their difference in growth under low nitrogen. For each condition six containers with each 24 plants were sown, three replicates for each genotype per container (figure 1). In table 13 of appendix 1 all names and characteristics of the genotypes are provided. The experimental design of the hydroponics experiment is provided in appendix 2.

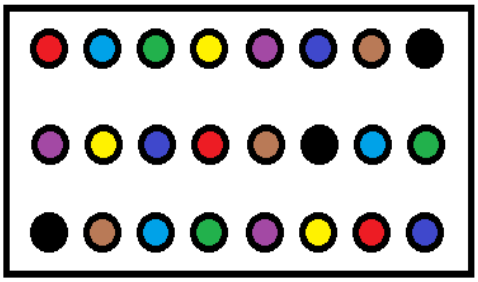

**Figure 1. Hydroponics container, each colour represents a genotype.**

### 2.1.2 Nitrogen Conditions

In the hydroponics experiment there were two conditions, low nitrogen and high nitrogen. The two conditions were based on the relative growth rate (RGR) established in previous experiments by Chan (2011). Low nitrogen had a RGR of 0.10 and high nitrogen had a RGR of 0.18. The nitrogen application in hydroponics was evaluated at both depletion conditions and Ingestad conditions (Ingestad, 1982). Depletion conditions meaning a nitrogen addition at once at the start of the experiment and Ingestad meaning the stable (daily) addition of nitrogen throughout the experiment. Both experiments contain the same amount of N, but applied from the beginning of the experiment or on daily dose. Appendix 3 shows the nitrogen additions for the hydroponics experiment. To control the border effects a light reflecting fence was put around the plants at the moment they started overlapping.

### 2.1.3 Planting and Harvesting

For the hydroponics experiment the seeds were sown in rock wool in the first week of January and were grown for two weeks before transplanting them in the hydroponic system. They were rinsed with tap water every two days. After transplanting, the plants grew in the hydroponics containers for four weeks. Two harvests were conducted (two and four weeks after transplanting). For each harvest three containers per treatment were harvested.

## 2.2 Field Trial

Two field trials were conducted at the breeding companies, one at Pop Vriend, Andijk and one at Rijk Zwaan, Fijnaart. The Pop Vriend trial was located in an open field and in clay soil. Rijk Zwaan had clay soil too and also has an open field, but a more enclosed field (still two open sides though).

### 2.2.1 Plant Material

In the field experiments 24 genotypes were used. In total 216 plots were sown: 24 genotypes x 3 replicates x 3 N-levels. These genotypes were all commercial F1 hybrid cultivars. In table 12 of appendix 1 all names and characteristics of the genotypes are provided. The experimental design of both the field trials are provided in appendix 4 and 5 respectively.

### 2.2.2 N-Levels

The genotypes in the field experiment were grown under three conditions (0 kg N/ha applied, an application for a final of 100kg N/ha and a for 150 kg N/ha). This setup was executed at two sites both with clay soil. Soil samples were taken beforehand to calculate the amount of nitrogen already present in the soil (done and only seen by the companies). With this knowledge nitrogen could be added to reach 100 or 150 kg/ha in total depending on the condition. Besides this, more soil samples were taken during the growing season, which will give information about the leaching or denitrification of nitrogen. Fertilizers used were 50% KAS and 50% ENTEC.

### 2.2.3 Planting and Harvesting

The seeds for the field experiment were sown on March 28$^{th}$ at Rijk Zwaan and on April 5$^{th}$ at Pop Vriend and were grown for seven weeks depending on the time of bolting. Harvests of the shoots will be done for all genotypes, but harvests for the roots were only done for four selected genotypes that were also used in the hydroponics experiment (parental lines in table 13, appendix 1). Two root harvests were performed at the same time of the of the first and last shoot harvest. Shoot harvesting was done at five time points evenly distributed over the growing period, starting after the second set of true leaves appeared until bolting. For Pop Vriend a sixth harvest was done to measure fresh weight and dry matter percentage, for Rijk Zwaan this was done together with the fifth harvest.

## 2.3 Trait Assessment

In both the hydroponics as well as in the field experiment several parameters were measured and calculated. Below all traits that were considered are explained.

### 2.3.1 Measured Traits

**Fresh and dry weight (g):** Fresh weight (FW) was measured for the root (RFW) and shoot (SDW) twice for the hydroponics (at week 2 and 4). After measuring the fresh weight the plant parts were dried (overnight) in an oven with a temperature ranging from 30-70°C, depending on the size of the plant parts. After drying the plant parts were weighed again for measuring the dry weight (DW). For the field trial the fresh weight was measured only once for the shoot at the end harvest. Fresh weight in the field was measured with a HALDRUP machine for the Pop Vriend trial. At Rijk Zwaan 10 plants were weighed to measure fresh weight. Dry weight was measured with every shoot harvest. This was done by harvesting 10 plants for each plot, which were placed in calibrated porated bags. The shoots were cleaned and counted and the average weight per plant was calculated. These plants were dried and weight the same way as with the hydroponics.

**Leaf number (sets of leaves)**: Leaf number (LN) was measured both in the hydroponics experiment only. This trait was measured from the start of the experiment up till the last

harvest every three or four days in the hydroponics experiment. Leaf number is measured per set of leaves.

**Leaf area ($cm^2$)**: Leaf area (LA) was measured by the Licor Leaf Area Scanner (LI-3100C). The leaves were put in the scanner separately by breaking them from the stem. Leaf area was measured during the two harvests for the hydroponics experiment, not for the field trial.

**Chlorophyll content ($\mu g/cm^2$)**: Chlorophyll content (CC) was measured with a SPAD meter (502 Minolta). The SPAD meter measures how much light is absorbed at a specific wavelength by the sample. The instrument measures transmission of red light at 650 nm and infrared light at 940 nm. Chlorophyll absorbs light with a wavelength of 650 nm and does not absorb light with a wavelength of 940 nm (Hoel and Solhaug, 1998). The measurements are expressed in SPAD units. Chlorophyll content was measured only when the plants were large enough for the measurement not to be destructive. The measurement is done both in the hydroponics experiment (first and second leaf) as in the field trial. For the field trials the measurements were done three times total and once a week. For RZ the measurements started 39 days after planting for the first leave set and 46 days after planting for the second leaf set. For PV they started 39 days after planting for both the first and second leaf set.

**Root characteristics**: Due to time constraints only the roots from the inner row of plants from each container (24 plants per treatment) were measured in the hydroponics at week 2 and 4. For the field trial roots were harvested at the beginning and the end of the growing period for root analysis. The samples that were taken reached to 20 cm deep in the soil below the plants for the first harvest and to 30cm deep in the soil below the plants for the second harvest and had a diameter of 5 cm. They were analysed per category of 10cm. Root characteristics were measured by scanning the roots and analysing them by a software program: WinRHIZO Pro 2005. This program measured the average root diameter (ARD) in millimetres, total root length (RL) in meters and area of the roots (RSA) square centimetres in the scanned images. The images were scanned at 400dpi and with grey levels. For the field trial only root length density could be measured, since only part of the roots were harvested.

**Soil Coverage (percentage of green pixels per area)**: Soil coverage (SC) was only measured in the field trial, because the genotypes were sown in plots and every genotype can be evaluated in a specific area. With each time point a photograph is taken from above (at the same place every time). A frame was used to keep the camera at the same distance and angle every time. This photograph was processed by MATLAB using DIPLib (a script developed by Delft TU) (Luengo & Hendriks *et al.,* 2005) and gave the soil coverage by counting pixels within a specified area. Soil coverage can be used to calculate photosynthetic exposure through time.

**Stomatal Conductance ($mmol/m^2/s$)**: Stomatal conductance (SC) was measured with a porometer (Model SC-1 by Decagon Devices). The porometer measures the rate $CO_2$ (carbon dioxide) entering or water vapour exiting through the stomata of a leaf. Plants need this gas exchange for cellular respiration. $CO_2$ is necessary for photosynthesis and water vapour is necessary for transpiration (Tricker *et al*., 2005). Due to a lack of time the stomatal conductance of only one container per condition is measured for the hydroponics. Stomatal conductance is not measured in the field trial due to lack of time.

### 2.3.2 Calculated Traits

**Root : shoot ratio:** The root:shoot ratio (RS) is based on the dry weight of both shoot and root and gives information about the biomass partitioning. The calculation is done according to this formula: Root:Shoot Ratio = Dry Weight Root / Dry Weight Shoot.

**% Dry Matter:** The ratio between dry and fresh weight is calculated for the hydroponics experiment. The calculation is done according to this formula: % Dry Weight (DM%) = Total Dry Weight / Total Fresh Weight * 100%.

**Specific Leaf Area ($cm^2/g$):** Specific leaf area (SLA) is the ratio between leaf area and the dry weight of the shoot. The specific leaf area gives information about the distribution of biomass in the leaf (how thick the leaf blade is). The calculation is done according to this formula: Specific Leaf Area = Leaf Area / Dry Weight Shoot.

**Relative Growth Rate:** Relative growth rate (RGR) is also based on the dry weight, in this case the total dry weight. The calculation is done according to this formula: RGR = [Ln(SDWt2 / SDWt1)]/ t2-t1. SDWt1 and SDWt2 refer to the cultivar means for SDW at beginning and end of the time interval (t1 and t2).

Statistical analysis: for all statistical analysis the program Genstat 15th Edition SP1 was used. First the normal distribution was tested and for the hydroponics the data was normally distributed, but variances were not equal, thus a REML (restricted maximum likelihood) was used, since those assumptions are not needed for a REML. The data of the field trial was normally distributed so there an ANOVA (analysis of variance) was performed to look at significant differences between genotypes. For both Pop Vriend and Rijk Zwaan the experiment was divided in blocks and plots, therefore the experiment was analysed as a split-plot design.

Also correlations were calculated between all traits within the hydroponics and within the field trial. A rank summation index (RSI) was used to establish a correlation between the hydroponics and field trials. For the RSI the traits with the highest correlation with NUE were used (Previous experiment Chan Navarrete). The same traits were used for this experiment, because then the results can be compared to the previous experiments. The traits with the highest correlation were SDW, LA for the hydroponics and SC for the field trial, CC and RGR. Of those traits CC and RGR were correlating the least, so they were given a value of 10% each, and SDW and either LA or SC got a value of 40%.

Thermal time is calculated for both field trials to get a better comparison between the field trials. This was done by using the temperature to count the heat sum. Thermal time is a summation of the cumulative mean temperature of each day. It is calculated in units of degree-days (°C d). The formula used is thermal time = $\sum$ Ta + Ta (t-1) + Ta (t-2) etcetera, where Ta is the average temperature and t is time in days.

# 3. Results

The results are divided in three parts: results of the hydroponics experiment, the results of the field trials and the correlation between the hydroponics and field trials. As mentioned in the materials and methods, a REML was used for the hydroponics and an ANOVA was used for the field trials. The nitrogen effect, genotype effect and nitrogen x genotype interaction were analysed. Appendix 7 and 8 show the result of the REML of Ingestad and depletion from harvest 2. The outcome of the ANOVA analysis from the two field trials is represented in appendix 15 and 16.

## 3.1 Hydroponics

### 3.1.1 Germination and Growth

The germination for each genotype is given in figure 2. The germination % was determined from sowing until planting in the hydroponics. Especially the germination percentage of genotype 7 and 41 were low, therefore genotype 7 and 41 were represented with around half the replications of the other genotypes for all treatments. Genotype 41 also showed a decrease in germination after the second measurement meaning those plants died.

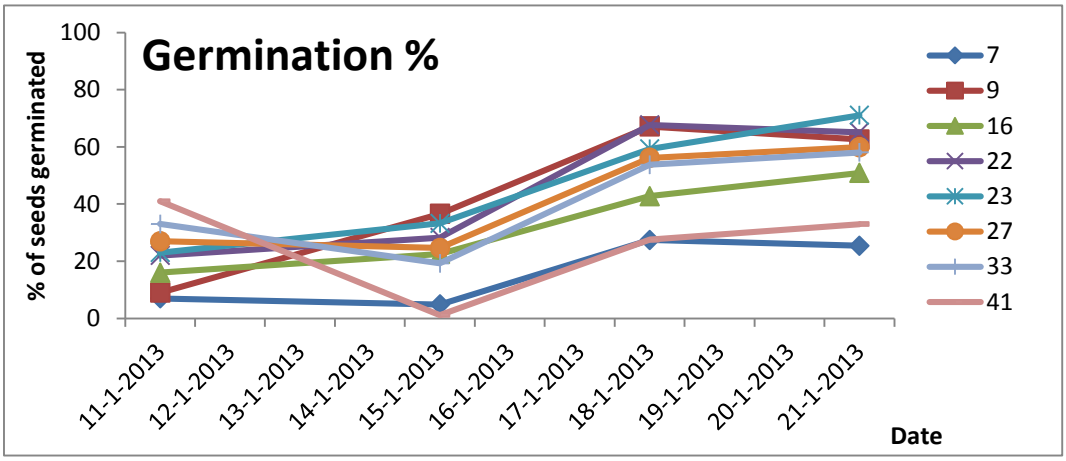


**Figure 2. Percentage of germination through time measured from sowing until planting (four measuring time point) of the hydroponics experiment.**

Shoot Dry Weight (SDW) and Leaf Area (LA) are both higher under high nitrogen levels than under low nitrogen levels (figure 3). However, this is only the case after four weeks of growing. SDW and LA seem to be higher under depletion conditions than under Ingestad conditions. Specific Leaf Area (SLA) shows a decrease after four weeks of growing compared to the SLA after two weeks of growing, but only under low nitrogen. This decrease is present under both Ingestad and depletion conditions but under depletion there is a larger decrease than under Ingestad. Root Dry Weight (RDW) and Root Length (RL) increase through time, without a difference between the two application methods or nitrogen levels. Dry Matter Percentage (DM%) shows a decrease after four weeks of growing for all conditions compared to DM% after two weeks of growing, except under depletion conditions under low nitrogen. In this treatment the plants show an increase in DM%. The Root:Shoot Ratio (RS) is higher under low nitrogen than under high nitrogen conditions. This difference is already visible after two weeks of growing. Under high nitrogen RS decreases through time (lower after four weeks of

growing than after two weeks of growing). The RS is higher under Ingestad conditions than under depletion conditions for both nitrogen levels. This means there is more investment in the roots under Ingestad conditions.

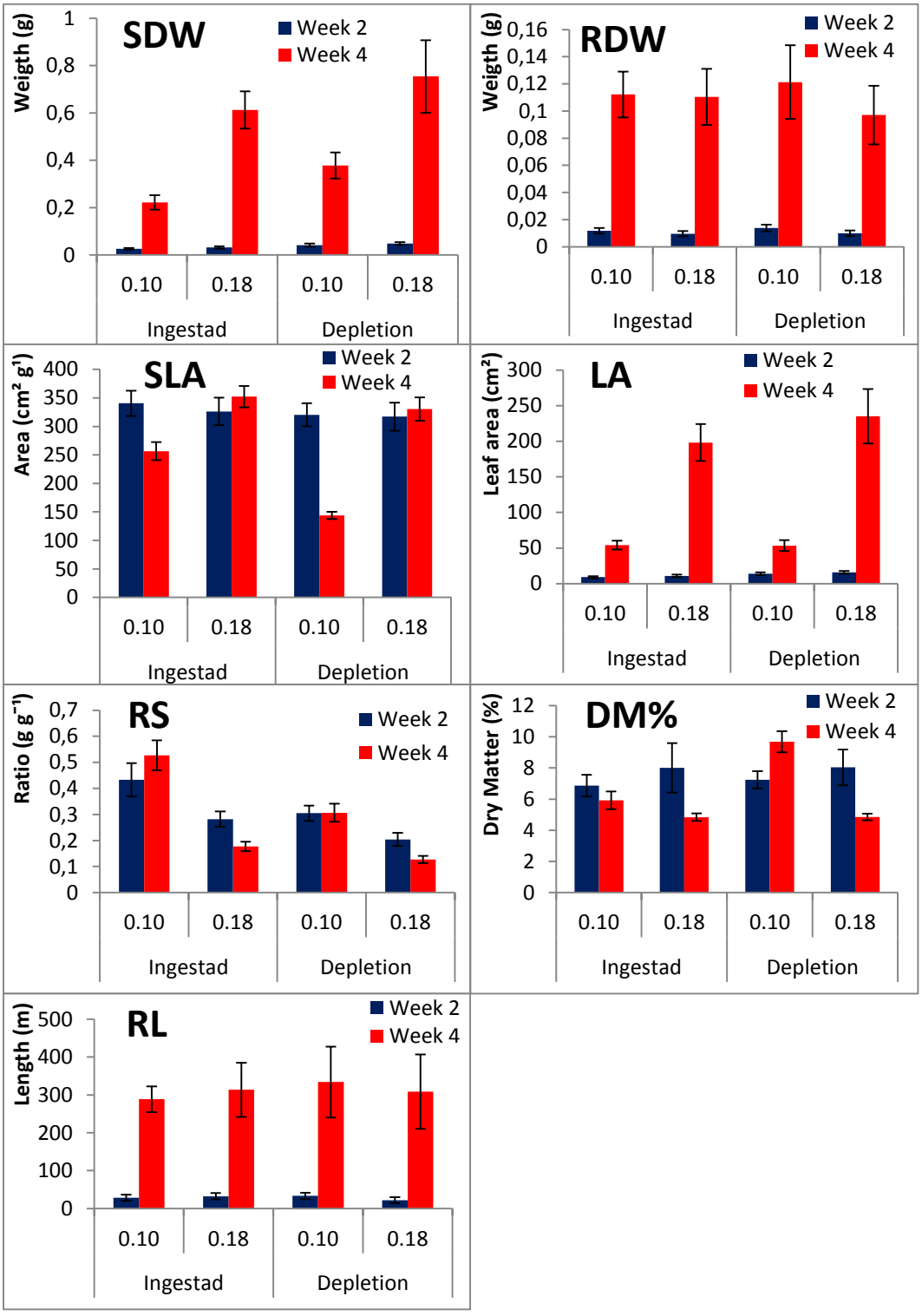


**Figure 3. Mean of all genotypes in the hydroponics experiment, for two and four week after planting, for Shoot Dry Weight (SDW), Root Dry Weight (RDW), Specific Leaf Area (SLA), Leaf Area (LA), Root:Shoot Ratio (RS), Dry Matter Percentage (DM%) and Root Length (RL). Error bars represent standard error at p=0.05.**

The relative growth rates are given in table 1. The relative growth rate (RGR) was calculated between the first and second harvest (15 days apart). Average RGR for the low nitrogen condition was 0.137, which is higher than the intended 0.10, while the average RGR for the high nitrogen condition was around the intended 0.18. Still, the difference in RGR between low and high nitrogen additions is large enough to have clear effects. Plants at the low nitrogen level had the same RGR under Ingestad and depletion but the health status was clearly different. Plants under depletion conditions showed more stress than plants under Ingestad conditions (like yellowing and dying of part of the leaves).

**Table 1. Nitrogen conditions with their measured relative growth rate.**

| Condition | Nitrogen | RGR |
|---|---|---|
| **Ingestad** | 0.10 | 0.137 |
| **Ingestad** | 0.18 | 0.182 |
| **Depletion** | 0.10 | 0.137 |
| **Depletion** | 0.18 | 0.170 |

For most traits there was no interaction between the first and second harvest with respect to the response of the genotypes to the different nitrogen levels and to the different additions of nitrogen. However, the differences between responses to the nitrogen levels and to the additions of nitrogen were more distinct for the second harvest (the harvest after week 4). Therefore only the data of the second harvest will be shown in the next part of the results. Graphs of harvest 1 are given in appendix 6.

### 3.1.2 Genotype and Analysis

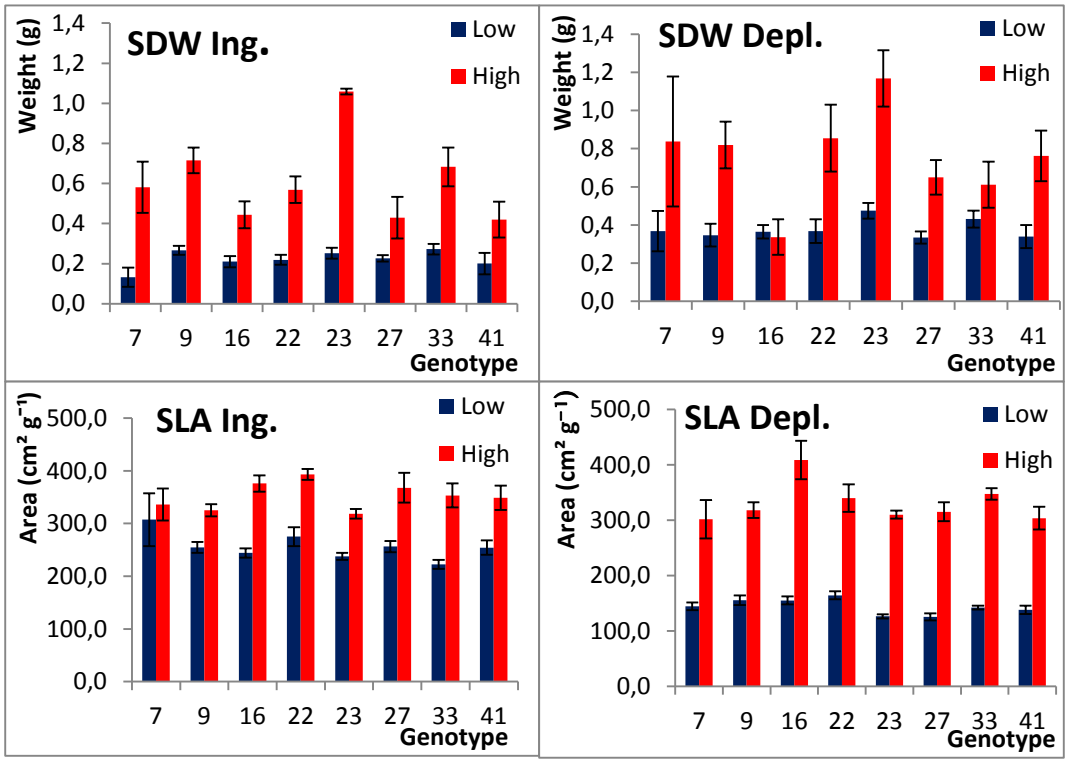


**Figure 4. Mean of Shoot Dry Weight (SDW) and Specific Leaf Area (SLA) for both Ingestad (Ing.) and Depletion (Depl.) of the hydroponics experiment for four weeks after planting. Error bars represent the standard error at p=0.05.**

In Figure 4 the shoot dry weight (SDW) and specific leaf area (SLA) are shown. Except for SLA Ingestad for genotype 7 and SDW depletion for genotype 16, both SDW and SLA were always higher under high nitrogen conditions. So, low nitrogen conditions affect the growth and SLA negatively. For SLA this difference is most clear under depletion conditions. However, the effect of nitrogen level on SDW is the same under Ingestad and depletion conditions for most genotypes, except for genotype 16 and 33 that performed better under Ingestad conditions. The difference in SDW under high and low nitrogen was largest for genotype 23 (this genotype was especially performing well under high nitrogen conditions). For SLA the difference was largest for genotype 16, most clear under depletion conditions (253 $cm^2\ g^{-1}$).

Figure 5 shows the Root Dry Weight (RDW), Root Length (RL) and Root:Shoot Ratio (RS). The REML analysis showed that there was no nitrogen effect for RDW and RL, but there was a genotype effect for both RDW and RL and a genotype x nitrogen effect for RDW under Ingestad conditions. Genotype 23 had heavier and longer roots than the other genotypes under high nitrogen conditions. Genotype 33 and 41 invested more in roots under low nitrogen conditions (RDW and RL) than under high nitrogen conditions. There are no significant differences between the rest of the genotypes for this trait.

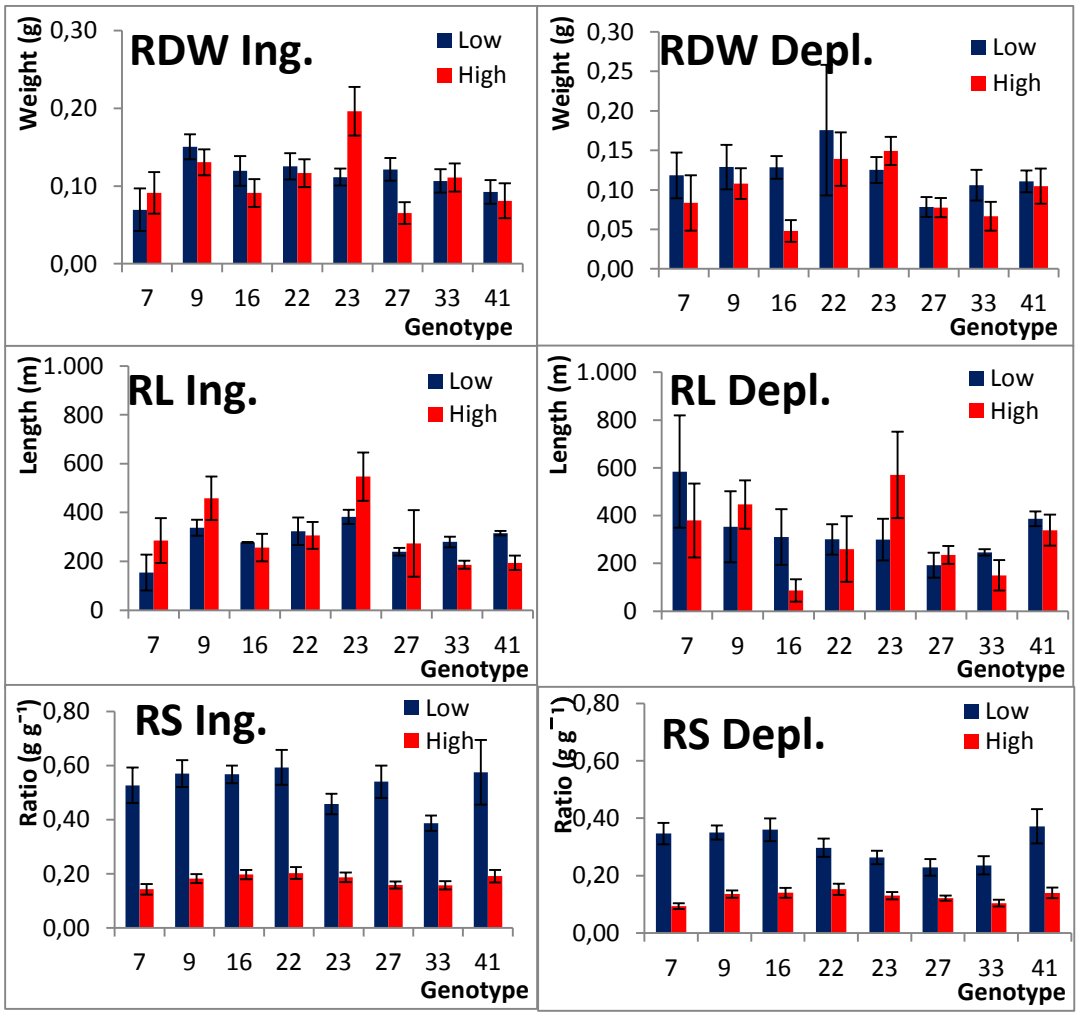


**Figure 5. Means of Root Dry Weight (RDW), Root Length (RL) and Root:Shoot Ratio (RS) of 8 cultivars grown in a hydroponic system with two levels of nitrogen(low and high) according the Ingestad (Ing.) and Depletion (Depl.) method for four weeks after planting. Error bars represent standard error at p=0.05.**

RS was highest under low nitrogen conditions for all genotypes (also seen after two weeks for almost all genotypes). So, all genotypes invested more in their roots when they were under low nitrogen conditions. This result is consistent under both Ingestad and depletion conditions. However, there was less difference between low and high nitrogen for genotype 27 and 22 under depletion conditions than under Ingestad conditions. RS was higher for low nitrogen under Ingestad conditions than for low nitrogen under depletion conditions.

The dry matter percentage is given in figure 6. DM% was higher under low nitrogen conditions than under high nitrogen conditions for all genotypes, except genotype 41 under Ingestad conditions. The difference between low and high nitrogen was larger under depletion conditions. The difference between low and high nitrogen was larger for genotype 33 than for other genotypes, this was the case for both Ingestad and depletion conditions. The genotype with the least difference between low and high nitrogen was genotype 41.

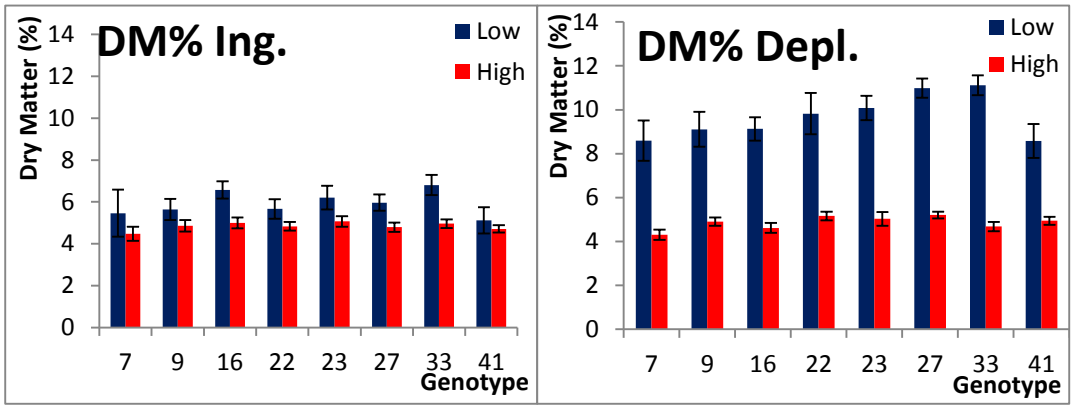


**Figure 6. Mean of Dry Matter % (DW%) for both Ingestad (Ing.) and Depletion (Depl.) of the hydroponics fot four weeks after planting. Error bars represent standard error at p=0.05.**

Figure 7 shows the mean of all genotypes for the chlorophyll content. This graph shows the trend of chlorophyll through time for the first two leaf sets. In appendix 9 the chlorophyll content for every genotype separate is shown. The REML analysis showed that there was no significant genotype x nitrogen interaction in chlorophyll content. However, the decrease in CC under depletion conditions was larger than under Ingestad conditions. Under low nitrogen the decrease in CC at depletion conditions was at least 4 SPAD units more than the decrease in CC at Ingestad conditions. For high nitrogen (1$^{st}$ leaf set only) the decrease at depletion was at least 3 SPAD units more than the decrease at Ingestad.

Every genotype showed the same decrease for the chlorophyll content in time, although there were some differences in the amounts of CC. Especially for the 1$^{st}$ leaf set under low nitrogen conditions there was variability between genotypes. Genotype 7 and 33 were the same as the mean, while 27 and 41 were higher and genotype 22 and 23 were lower than the mean for both leaf sets at every time point. Genotype 9 was only lower for the 2$^{nd}$ leaf set under high nitrogen conditions at every time; the rest of the genotypes were the same as the mean. The same is true for genotype 16; this genotype was only higher for the 1$^{st}$ leaf set under high nitrogen conditions at every time point.

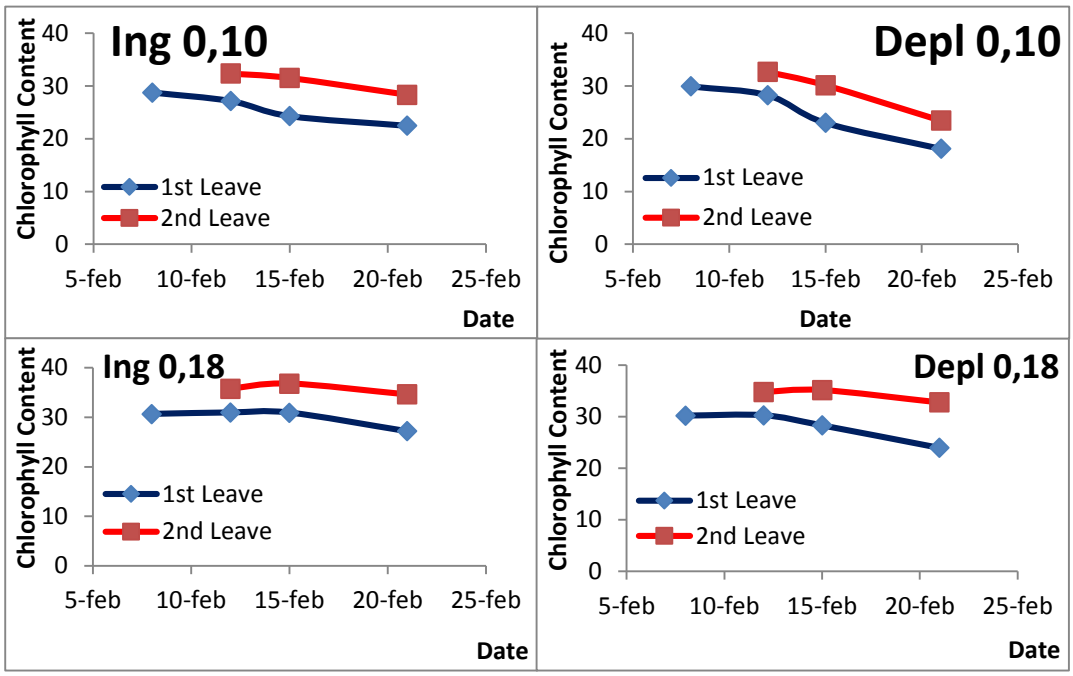


**Figure 7. Means of 8 genotypes for chlorophyll content for both Ingestad and depletion (Hydroponics) and both nitrogen levels. Measurements started 14 days after planting for the first leaf set and 18 days after planting for the second leaf set. Measurements were done every three to four days. CC is given SPAD units. Error bars represent standard error of the mean at p=0.05, but are too small to be visible for chlorophyll content.**

Additional graphs for hydroponics not shown in the results can be found in appendix 10 (figure 22). The figure shows the averages of all genotypes for Leaf Area (LA), the Leaf Number (LN) four weeks after planting, the Surface Area (RSA) of the roots and the Shoot Fresh Weight (SFW).

**Table 2. Correlations for hydroponics experiment of both Ingestad and Depletion of 1) Shoot Dry Weight, 2) Specific Leaf Area, 3) Leaf Area, 4) Root Dry Weight, 5) Root Length, 6) Surface Area Root, 7) Root:Shoot Ratio and 8) Dry Matter Percentage; and 9-15) Chlorophyll Content of the 1st and 2nd leaf set measured at 14, 18, 21 and 26 days after planting. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| **1** | SDW | - | | | | | | |
| **2** | SLA | 0.0208 | - | | | | | |
| **3** | LA | **0.9318** | 0.3337 | - | | | | |
| **4** | RDW | **0.6072** | **-0.4270** | **0.3963** | - | | | |
| **5** | RL | **0.7149** | -0.2770 | **0.5467** | **0.9156** | - | | |
| **6** | RSA | **0.7423** | -0.3277 | **0.5588** | **0.9627** | **0.9643** | - | |
| **7** | RS | **-0.4686** | **-0.4361** | **-0.5582** | **0.2921** | 0.0822 | 0.1203 | - |
| **8** | DM% | -0.0936 | **-0.7374** | **-0.3350** | 0.0060 | -0.0389 | -0.0379 | -0.0305 |
| | | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **9** | CC 1-8 | -0.053 | -0.0377 | -0.076 | -0.124 | -0.1675 | -0.1083 | -0.1583 |
| **10** | CC 1-12 | 0.197 | 0.2572 | 0.250 | -0.098 | -0.0873 | -0.0388 | **-0.4241** |
| **11** | CC 1-15 | 0.191 | 0.5436 | 0.361 | **-0.220** | -0.1105 | -0.1167 | **-0.4745** |
| **12** | CC 1-21 | 0.231 | 0.3353 | 0.328 | -0.007 | 0.0449 | 0.0408 | -0.2341 |
| **13** | CC 2-12 | 0.058 | 0.1713 | 0.110 | -0.126 | -0.1500 | -0.1206 | -0.3012 |
| **14** | CC 2-15 | 0.386 | 0.3822 | 0.475 | -0.017 | 0.0429 | 0.0707 | **-0.4711** |
| **15** | CC 2-21 | 0.405 | 0.5418 | 0.563 | -0.035 | 0.0246 | 0.0647 | **-0.4199** |

| | | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|
| **9** | CC 1-8 | 0.058 | - | | | | | |
| **10** | CC 1-12 | -0.124 | **0.710** | - | | | | |
| **11** | CC 1-15 | **-0.427** | **0.505** | **0.774** | - | | | |
| **12** | CC 1-21 | -0.301 | 0.181 | **0.516** | **0.546** | - | | |
| **13** | CC 2-12 | -0.143 | **0.515** | **0.608** | **0.571** | **0.3892** | - | |
| **14** | CC 2-15 | **-0.312** | **0.436** | **0.643** | **0.703** | **0.6044** | **0.6473** | - |
| **15** | CC 2-21 | **-0.560** | **0.324** | **0.548** | **0.666** | **0.4886** | **0.5709** | **0.8005** |

Table 2 is a correlation table for all traits mentioned in the results. The SDW was highly correlated with LA and also but to a lesser extent with RL and RSA. Also RDW was highly correlated with RL and RSA. RL and RSA were also highly correlated with each other. Fewer correlations were found for the CC. The bold number indicate the significance for $p<0.05$ calculated by Genstat. Correlations between SDW and RDW per genotype are given in appendix 11 (table 16). Correlations between SDW and RDW were not significant for genotype 9, 16 and 27.

## 3.2 Results Field Trials

### 3.2.1 Germination and Growth

Figure 8 shows the germination of both Pop Vriend (PV) and Rijk Zwaan (RZ) measured at two time points. Time points were different for each trial because of different sowing dates. The germination was slightly higher at Pop Vriend (60%) than it was at Rijk Zwaan (50%), but there were no significant differences between genotypes between the two companies.

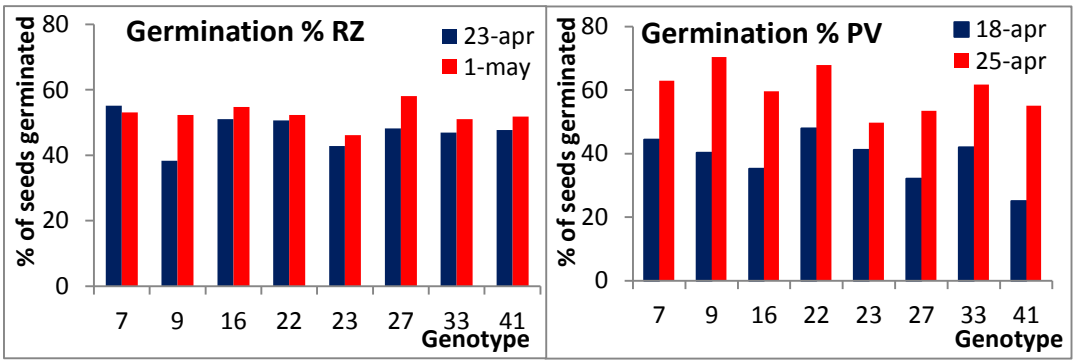


**Figure 8. Percentage of germination through time measured from sowing until planting. There were two measuring time point, for PV the first was 13 days after sowing (18-apr) and the second was 20 days after sowing (25-apr), and for Rijk Zwaan the first was 13 days after sowing (23-apr) and the second was 21 days after sowing (01-may).**

**Table 3. Results of the soil samples taken at Pop Vriend Seeds (one taken at the start of the trial and one at the end of the trial) together with the relative growth rate between harvest 1 and harvest 3 (RGR 1-3), between harvest 2 and harvest 4 (RGR 2-4) and between harvest 3 and 5 (RGR 3-5).**

| PV | Nitrogen Condition | $NO_3^-$ (mg/l) | $NH_4^+$(mg/l) | Available N (kg/ha) | RGR 1-3 | RGR 2-4 | RGR 3-5 |
|---|---|---|---|---|---|---|---|
| **Start** | NA | 6.07 | <0.5 | 36 | | | |
| | 100kg/ha | 17.67 | 1.10 | 112 | | | |
| | 150 kg/ha | 23.37 | 1.73 | 150 | | | |
| **End** | NA | 0.70 | <0.5 | 3 | 0.116 | 0.046 | 0.067 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 100kg/ha | 1.60 | <0.5 | 10 | 0.126 | 0.048 | 0.081 |
| | 150 kg/ha | 1.40 | <0.5 | 9 | 0.133 | 0.051 | 0.080 |

**Table 4. Results of the soil samples taken at Rijk Zwaan together with the relative growth rate, in the same way as with the table of PV.**

| RZ | Nitrogen Condition | $NO_3^-$ (mg/l) | $NH_4^+$(mg/l) | Available N (kg/ha) | RGR 1-3 | RGR 2-4 | RGR 3-5 |
|---|---|---|---|---|---|---|---|
| **Start** | NA | 24.5 | <0.5 | 147 | | | |
| | 100kg/ha | 43.4 | <0.5 | 264 | | | |
| | 150 kg/ha | 10.1 | 0.6 | 61 | | | |
| **End** | NA | 0.6 | <0.5 | 4 | 0.133 | 0.073 | 0.078 |
| | 100kg/ha | 1.5 | <0.5 | 9 | 0.147 | 0.081 | 0.078 |
| | 150 kg/ha | 2.3 | <0.5 | 14 | 0.149 | 0.093 | 0.078 |

Table 3 and 4 show the results of the soils samples taken at the start and end of respectively PV and RZ. The RGR in the table is based on solely the SDW, because there were not enough harvests for the roots to include RDW.

Nitrogen levels in the soil at PV were close to what was expected, low nitrogen levels for no application (NA) and almost exactly 100 and 150 kg/ha for the other two treatments, as they were intended to be. For RZ the nitrogen levels were not what they were intended. The NA level was expected to have the lowest amount of nitrogen available, which is not the case. There has probably been a mix-up with the soil samples and for the results the NA nitrogen level will be considered to be the lowest and 150 kg/ha the highest. Plants at PV showed less stress (larger and greener plants). The RGR was slightly higher at the beginning of the trial for RZ than for PV (table4), but was similar for both trials during the last measured period (3-5). The largest RGR was found in the beginning of the field trial (RGR 1-3). After this the RGR decreased and stayed stable for RZ but increased slightly for PV in the end.

### 3.2.2 ANOVA Analysis and Graphs

SDW of the average of all genotypes is shown for both field trials in figure 8 against thermal time. All graphs with thermal time on the X-axis have the unit degrees of Celsius-days (°C d). This graph is an exponential curve in this case, while a S-curve was expected (precious experiments of Chan Navarrete). The plants grew faster at RZ than at PV (also shown by RGR), but they also started to flower earlier than the plants at PV. Plants were sown eight days earlier at RZ than at PV. The SDW under NA was lower than the SDW under 100 kg/ha and 150 kg/ha. Appendix 11 shows the SDW for the eight genotypes used in the hydroponics. Especially performing well in NA was genotype 27 (high SDW). Under the highest nitrogen condition (150 kg/ha) genotype 16 had the lowest SDW.

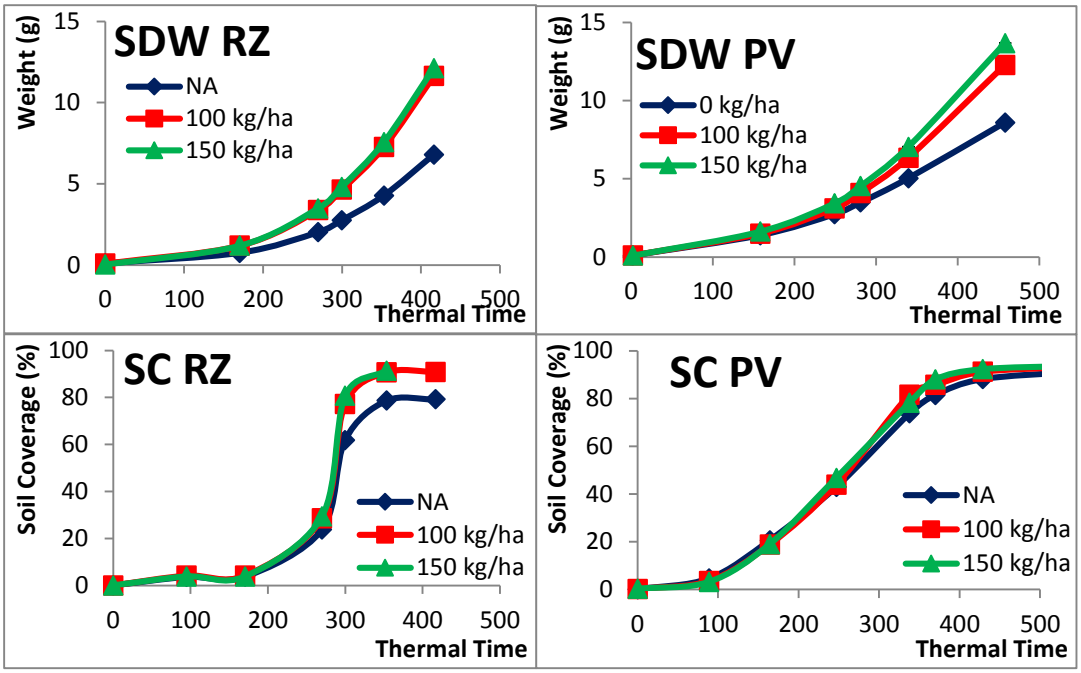


**Figure 9. Mean of Shoot Dry Weight (SDW) and Soil Coverage (SC) for both the Rijk Zwaan (RZ) and the Pop Vriend (PV) field trial. Error bars represent standard error at p=0.05, but are too small to be seen for SC.**

Development of soil coverage (SC) is for both RZ and PV a logistic curve (figure 9), even though the sowing date of PV was earlier, which could explain the differences in steepness of the curves. More time points were taken before exponential growth of SC at RZ than at PV. The delay in SC at RZ was compensated by a faster exponential growth at RZ than at PV. At RZ the end point for NA was lower than the 100 and 150 kg/ha, while at PV there was no difference between the three treatments for all harvests. The SC of the plants under the 100 and 150 kg/ha conditions were the same for both trials after 400-500 °C d.

SDW was clearly reduced at PV in the NA condition, while SC was the same for all conditions. For RZ both SDW and SC were affected. Appendix 13 and 14 show the graphs of RZ and PV per genotype for SC. These graphs show that all genotypes for PV showed the same pattern while for RZ the difference between NA and 100 and 150 kg/ha was smaller for genotype 7, 9 and 16 than for the other genotypes (appendix 13 and 14). Genotype 22 covered the soil the fastest of all genotypes at both at RZ and PV. At RZ genotype 7 also covered the soil quickly, while at PV both genotype 7 and genotype 9 covered the soil quickly. This difference between genotypes was not seen for SDW or for comparable traits (SDW, LA and SLA) in the hydroponics.

DM% was calculated for 10 plants during the last harvest of RZ, but at PV DM% was calculated for the whole plot for every genotype and was based on traits measured with a Haldrup machine. When looking at the mean of all genotypes the DM% (figure 10) for NA was the same for both trials, but DM% for NA and 100 kg/ha was higher at PV than at RZ. For most genotypes DM% is slightly higher at PV than at RZ. At both trials more nitrogen means lower DM%, but the standard errors are also larger at PV than at RZ. All genotypes had a higher DM% with less nitrogen, though one genotype had more

difference between treatments then the other. At PV two genotypes did not have any measurements (genotype 10 and 26), because for these genotypes no extra plots were sown for this measurement.

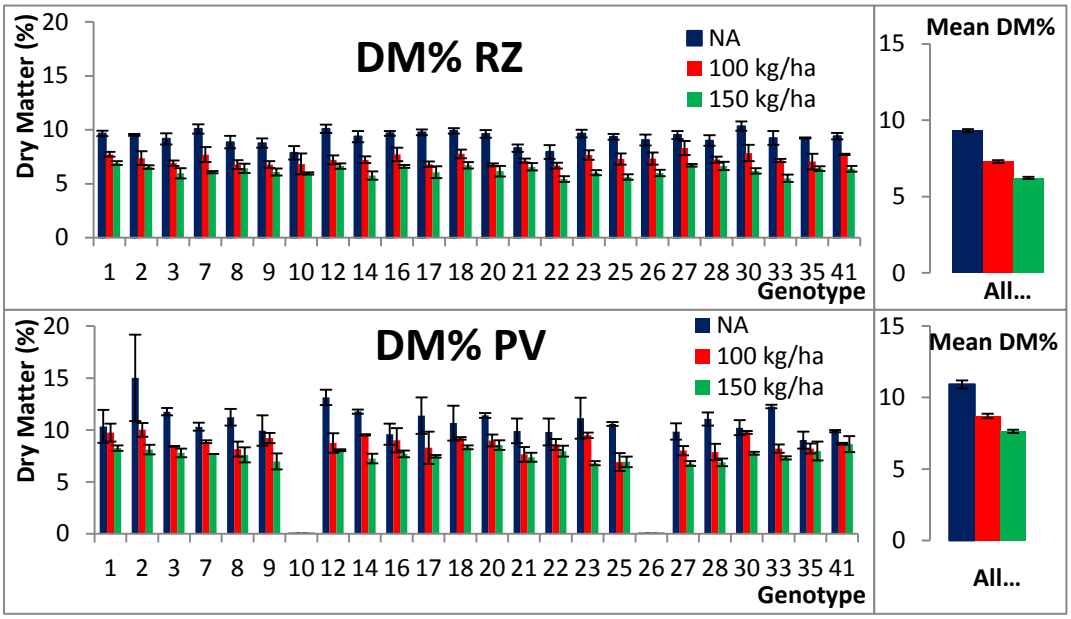


**Figure 10. This figure gives the Dry Matter % for each genotype separate for RZ and PV (on the left), and for the mean of all genotypes (on the right). Error bars represent standard error at p=0.05.**

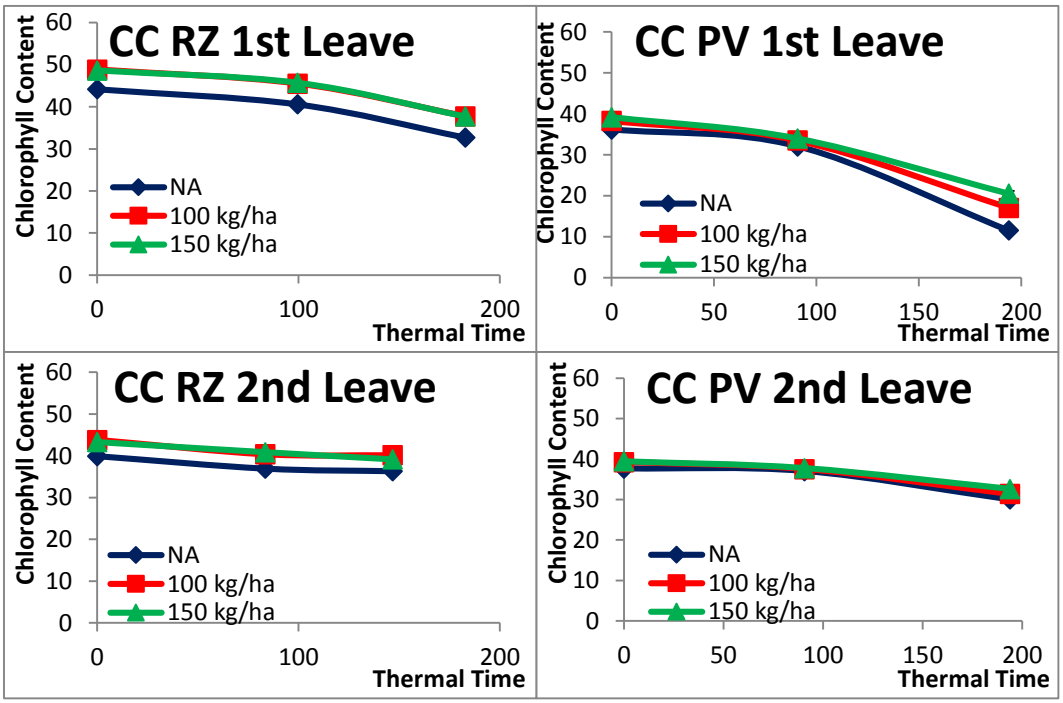


**Figure 11. Mean Chlorophyll Content (CC) of all genotypes of the first and second leaf set for Rijk Zwaan (RZ) and Pop Vriend (PV). At RZ measuring started 39 days after planting for the first leaf set and 46 days after planting for the second leaf set. Measuring at PV started 39 days after planting for both the first and second leaf set. Measuring was done once a week. CC is given SPAD units. Error bars represent standard error at p=0.05, but are too small.**

Chlorophyll content (CC) was measured for the first and second leaf set at three time points for each trial (figure 11). The CC of the first leaf set decreased through time in

both trials. However, this decrease was smaller at RZ than at PV. Also, the CC was lower at PV (± 40 to 20) than at RZ (± 45 to 35). For the second leaf set this trend was the same. In almost all cases the CC for NA were the lowest and no difference was indicated between 100 and 150 kg/ha, except for the 2nd leaf set at PV under NA where the CC was the same as for 100 kg/ha and 150 kg/ha.

Root characteristics as Root Dry Weight (RDW), Root Length Density (RLD), Average Root Diameter (ARD) and Root Surface Area (RSA) were only measured for four genotypes. For that reason also the Root:Shoot Ratio (RS) could be calculated for these four genotypes. RS is based on the RDW recovered from the top 30cm of soil. The RS for the first harvest showed no significant differences between nitrogen levels, genotypes and trial, so only the RS of the second root harvest (at the fifth shoot harvest) is shown here (figure 12). This was also the case for the other traits, therefore only the results of the second root harvest are shown.

For all genotypes RS seems higher if less N is available, though this was not significant for all genotypes and both trials (figure 12). The only significant difference between RZ and PV was for genotype 7 under NA conditions, though all values for RS look higher at PV compared to RZ. For genotype 7 there was no difference between treatments at PV, while RS was significantly higher for NA at RZ. Again there was no difference for RS between the 100 and 150 kg/ha conditions.

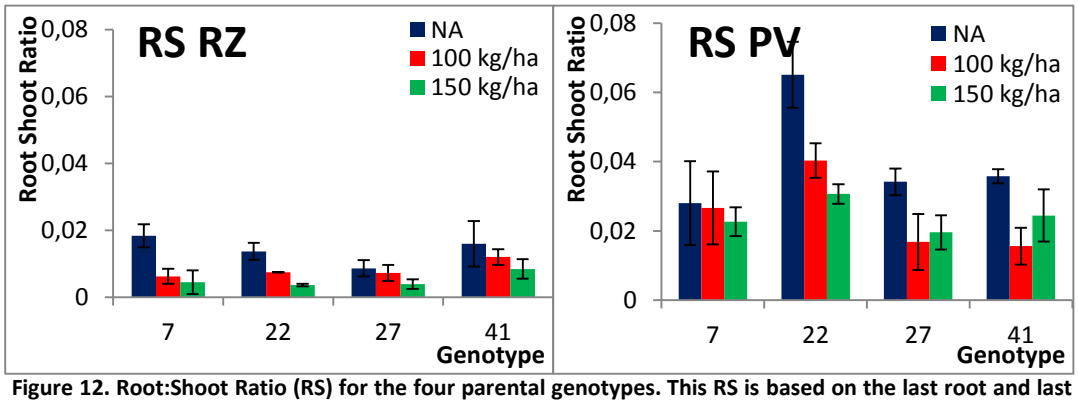


**Figure 12. Root:Shoot Ratio (RS) for the four parental genotypes. This RS is based on the last root and last shoot harvest (same date). Error bars represent standard error at p=0.05.**

Average Root Diameter is not shown here, because there were no significant differences between genotypes, nitrogen levels and field trials. RDW, RLD and RSA are shown (figure 13) since there was significance between genotypes, nitrogen levels and field trials. One difference was that genotype 41 had a higher RDW and RSA for 100 kg/ha than for the other nitrogen levels, but this was only the case at RZ. Genotype 7 and 22 had a higher RDW for NA than for the other nitrogen levels, but for both genotypes this was only the case at one company.

ANOVA tables for RZ and PV are given in appendix 15 and 16. These tables show significance for genotype and nitrogen effect for SDW, SC and CC. The genotype x nitrogen interaction was only significant for a few traits, namely SFW, DM% and CC at one time point for PV, and SDW Harvest 2 (H2), RLD H1, ARD H2 and SC from H1 and

onwards for RZ. Root characteristics were not significantly different between genotype and nitrogen levels for both RZ and PV, except for small genotype effects for ARD and RS. The difference between RZ and PV is that at RZ there was significance for genotype x nitrogen effect for SC which is not the case for PV.

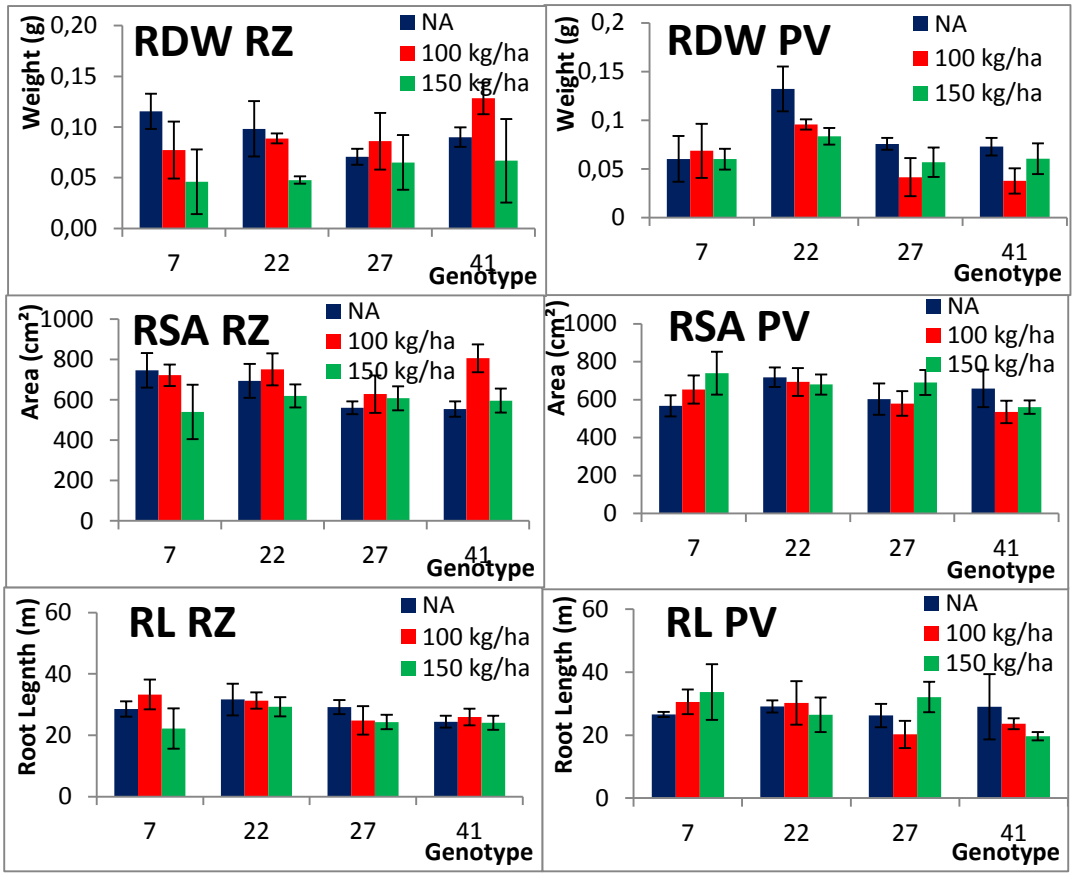


**Figure 13. Root Dry Weight (RDW), Root Length (RL) and Root Surface Area (RSA) for the four selected genotypes for both the RZ and PV trial. Error bars represent standard error at p=0.05.**

**Table 5. Correlations between Soil Coverage (SC) and Shoot Dry Weight (SDW) for every harvest (H1 = harvest 1; H2= harvest 2). Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| | RZ Soil Coverage | | | | | PV Soil Coverage | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **SDW** | **H1** | **H2** | **H3** | **H4** | **H5** | **H1** | **H2** | **H3** | **H4** | **H5** |
| **H1** | **0.487** | | | | | **0.520** | | | | |
| **H2** | | **0.674** | | | | | **0.406** | | | |
| **H3** | | | **0.656** | | | | | **0.499** | | |
| **H4** | | | | **0.702** | | | | | **0.483** | |
| **H5** | | | | | **0.514** | | | | | 0.096 |

Tables 5 and 6 show the correlations between SC and SDW for both field trials. Table 5 shows that SC and SDW for every harvest and both field trials are significantly correlated (except for H5 at PV). SDW and SC were positively correlated in all cases.

All root traits mentioned (RDW, RLD and RSA) are significantly correlated for both field trials within the harvests (tables 6 and 7). The bold numbers give significant correlations

at p<0.05. Average Root Diameter (ARD) showed no significant correlations with other root or plant traits and is therefore not shown.

**Table 6. Correlation between root characteristics of RZ. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| | RDW H1 | RDW H2 | RLD H1 | RLD H2 | RSA H1 | RSA H2 |
|---|---|---|---|---|---|---|
| **RDW H1** | - | | | | | |
| **RDW H2** | 0.1096 | - | | | | |
| **RLD H1** | **0.6168** | 0.0833 | - | | | |
| **RLD H2** | -0.0796 | **0.3919** | -0.0299 | - | | |
| **RSA H1** | **0.5626** | 0.2362 | **0.6748** | 0.1246 | - | |
| **RSA H2** | 0.1703 | **0.5881** | 0.1639 | **0.7840** | 0.2131 | - |

**Table 7. Correlation between root characteristics of PV. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| | RDW H1 | RDW H2 | RLD H1 | RLD H2 | RSA H1 | RSA H2 |
|---|---|---|---|---|---|---|
| **RDW H1** | - | | | | | |
| **RDW H2** | -0.0567 | - | | | | |
| **RLD H1** | **0.6019** | 0.0484 | - | | | |
| **RLD H2** | 0.0060 | 0.1369 | 0.1700 | - | | |
| **RSA H1** | **0.5241** | 0.1852 | **0.6756** | -0.0156 | - | |
| **RSA H2** | -0.0091 | **0.4307** | 0.1276 | **0.8017** | 0.0200 | - |

The correlations between the two field trials are given in table 8. This table shows that the SDW of every harvest is significantly correlated with the same harvest of the other field trial. There is also a significant correlation between the DM% at RZ and the DM% at PV. The root characteristics have no significant correlations between each other.

**Table 8. Correlation between the field trial at RZ and the field trial at PV both at 100 kg/ha. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| | SDW H1 | SDW H2 | SDW H3 | SDW H4 | SDW H5 | RDW H1 | RDW H2 |
|---|---|---|---|---|---|---|---|
| **SDW H1** | **0.3281** | | | | | | |
| **SDW H2** | | **0.4075** | | | | | |
| **SDW H3** | | | **0.6050** | | | | |
| **SDW H4** | | | | **0.5180** | | | |
| **SDW H5** | | | | | **0.4400** | | |
| **RDW H1** | | | | | | -0.0883 | |
| **RDW H2** | | | | | | | 0.1787 |
| | **RS H1** | **RS H2** | **RLD H1** | **RLD H2** | **RSA H1** | **RSA H2** | **DM%** |
| **RS H1** | -0.4515 | | | | | | |
| **RS H2** | | 0.2513 | | | | | |
| **RLD H1** | | | 0.1689 | | | | |
| **RLD H2** | | | | 0.3753 | | | |
| **RSA H1** | | | | | -0.0134 | | |
| **RSA H2** | | | | | | 0.2104 | |
| **DM%** | | | | | | | **0.4764** |

## 3.2 Correlations between Field and Hydroponics

Tables 9 and 10 show the correlation values between the depletion condition of the hydroponics experiment and RZ (table 9) and between the depletion condition of the hydroponics and PV (table 10) for every trait separately. The low nitrogen level of the hydroponics was compared to the NA of the field trial and the high nitrogen level of the hydroponics was compared to the 100 kg/ha of the field trial. The only significant correlation for RZ was the SDW from the hydroponics with the SDW of H5 of the field trial. For PV there was also a significant correlation for SDW, but here also for H1, H2 and H4 (besides H5). Another significant correlation for PV was the RDW of the hydroponics with the RDW of the second harvest. Other traits like RS, DM%, ARD, RLD and RSA showed no significant correlation for both field trials with the hydroponics.

**Table 9. Correlation between the depletion condition of the hydroponics experiment and the different harvests of Rijk Zwaan. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| RZ | Hydroponics traits | | | | | | |
|---|---|---|---|---|---|---|---|
| | RDW | SDW | RS | DM% | ARD | RLD | RSA |
| RDW H1 | -0.1548 | | | | | | |
| RDW H2 | -0.2040 | | | | | | |
| SDW H1 | | 0.4442 | | | | | |
| SDW H2 | | 0.4376 | | | | | |
| SDW H3 | | 0.4323 | | | | | |
| SDW H4 | | 0.2892 | | | | | |
| SDW H5 | | **0.5065** | | | | | |
| RS H1 | | | 0.1534 | | | | |
| RS H2 | | | 0.4157 | | | | |
| DM% | | | | 0.4764 | | | |
| ARD H1 | | | | | 0.3755 | | |
| ARD H2 | | | | | 0.2889 | | |
| RLD H1 | | | | | | 0.1574 | |
| RLD H2 | | | | | | -0.4111 | |
| RSA H1 | | | | | | | -0.2868 |
| RSA H2 | | | | | | | -0.1346 |

**Table 10. Correlation between the depletion condition of the hydroponics experiment and the different harvests of Pop Vriend. Values represent the direction of the correlation (minus being negative) and bold numbers are significant at p<0.05.**

| PV | Hydroponics traits | | | | | | |
|---|---|---|---|---|---|---|---|
| | RDW | SDW | RS | DM% | ARD | RLD | RSA |
| RDW H1 | 0.1206 | | | | | | |
| RDW H2 | **0.6977** | | | | | | |
| SDW H1 | | **0.5205** | | | | | |
| SDW H2 | | **0.5088** | | | | | |
| SDW H3 | | 0.3007 | | | | | |
| SDW H4 | | **0.4862** | | | | | |
| SDW H5 | | **0.3264** | | | | | |
| RS H1 | | | -0.1909 | | | | |
| RS H2 | | | 0.4298 | | | | |
| DM% | | | | -0.1144 | | | |
| ARD H1 | | | | | -0.2707 | | |

| | | | |
|---|---|---|---|
| **ARD H2** | 0.0191 | | |
| **RLD H1** | | 0.2114 | |
| **RLD H2** | | 0.0907 | |
| **RSA H1** | | | 0.2024 |
| **RSA H2** | | | -0.3266 |

To calculate the correlation of the performance of genotypes under field and hydroponic conditions a Rank Summation Index (RSI) was performed. For hydroponics the traits that were used are SDW (40%), LA (40%), RGR (10%), CC 1st leaf set (5%) and CC 2nd leaf set (5%). For the field trials the same traits were used, only SC was used instead of LA. The total RSI of all experiments separately and total are given in table 11. For hydroponics the low nitrogen condition was used, and for the field trials 100 kg/ha (this was done to compare to a previous experiment performed by Rafael Chan Navarette). A higher number (higher ranking) means that the genotype has a better performance for the specific trait.

**Table 11. Rank Summation Index (RSI) of both Ingestad and Depletion for hydroponics and both Rijk Zwaan and Pop Vriend for the field trial.**

| | Hydroponics | | | Field Trials | | |
|---|---|---|---|---|---|---|
| **Cultivar** | **Depletion** | **Ingestad** | **Total** | **RZ** | **PV** | **Total** |
| **7** | 15,00 | 11,25 | 13,13 | 58,13 | 66,25 | 62,19 |
| **9** | 35,63 | 70,63 | 53,13 | 6,88 | 58,75 | 32,81 |
| **16** | 55,63 | 24,38 | 40,00 | 43,75 | 32,50 | 38,13 |
| **22** | 64,38 | 46,88 | 55,63 | 63,75 | 35,63 | 49,69 |
| **23** | 82,50 | 61,25 | 71,88 | 63,75 | 53,75 | 58,75 |
| **27** | 23,13 | 47,50 | 35,31 | 35,63 | 48,75 | 42,19 |
| **33** | 59,38 | 75,00 | 67,19 | 63,13 | 39,38 | 51,25 |
| **41** | 17,50 | 13,13 | 15,31 | 15,00 | 15,00 | 15,00 |

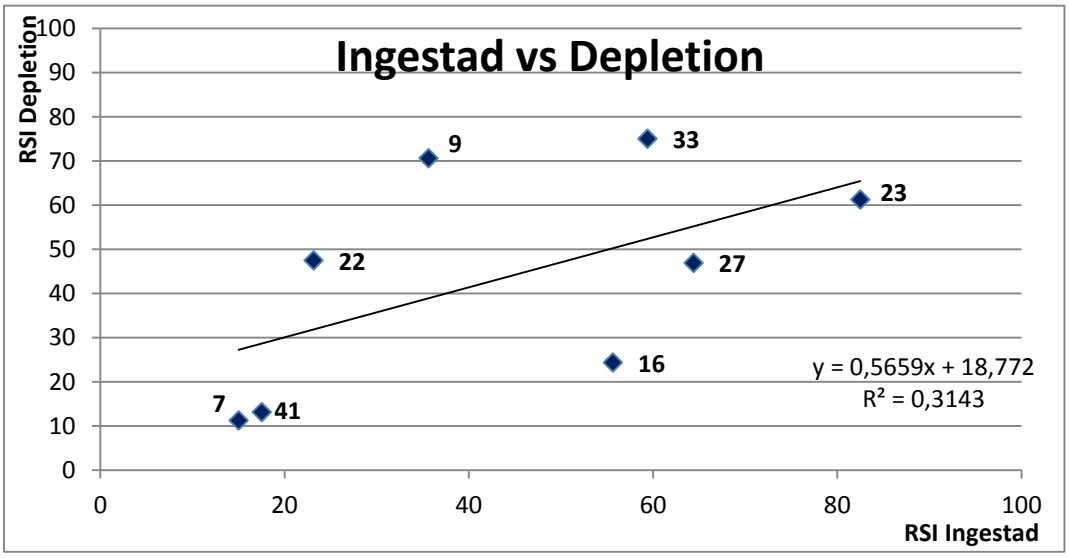


**Figure 13. Correlation graph for the correlation between Ingestad and depletion based on the RSI.**

As can be seen in table 9 there is a lot of variation between field and hydroponics, but also for some genotypes between the two hydroponics and between the two field trials. Although genotype 7 was rather stable within the hydroponics experiment and within

the field trial, this genotype had a large difference between the field and hydroponics. However, this was not the case in previous experiments where the performance was more similar between hydroponics and field. This could indicate that there was indeed a mix-up with genotype 7.

Overall, the correlation based on the RSI between Ingestad and depletion is weak. Genotypes 9, 22 and 33 have a higher ranking under depletion conditions than under Ingestad conditions (figure 13). Genotype 16, 23 and 27 have a higher ranking for Ingestad than for depletion. Genotype 7 and 41 have the same ranking in both conditions as well as the lowest ranking (performance) of all genotypes. However, genotype 7 cannot be trusted as mentioned before.

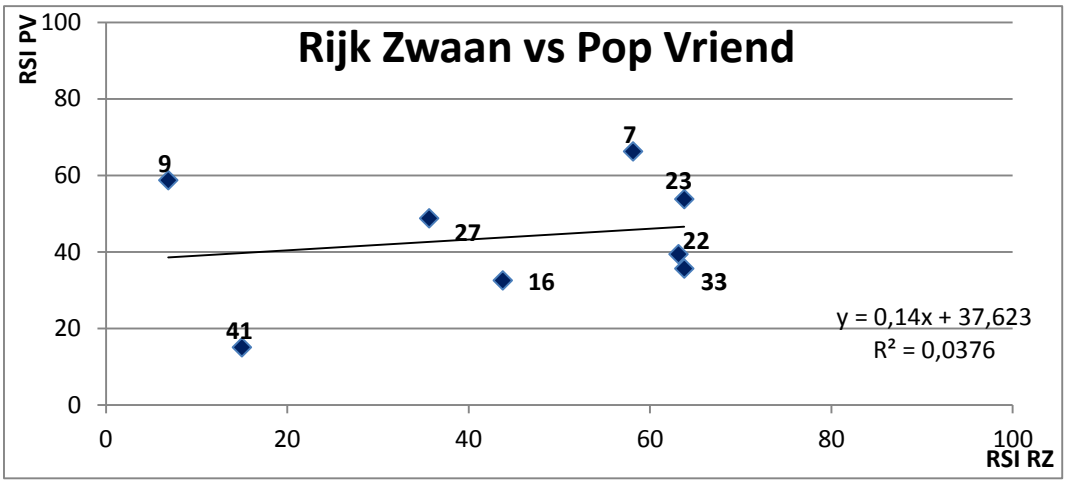


**Figure 14. Correlation graph based on the RSI for the correlation between Rijk Zwaan and Pop Vriend.**

Figure 14 shows the correlation matrix between the two field trials (Rijk Zwaan and Pop Vriend). The rankings of genotype 7, 27 and 41 were most similar between the two experiments. Genotype 9 had a higher ranking for the PV trial than for the RZ trial and had the most difference between the two field trials. The other genotypes (16, 22, 23 and 33) had a higher ranking for the RZ trial than for the PV trial.

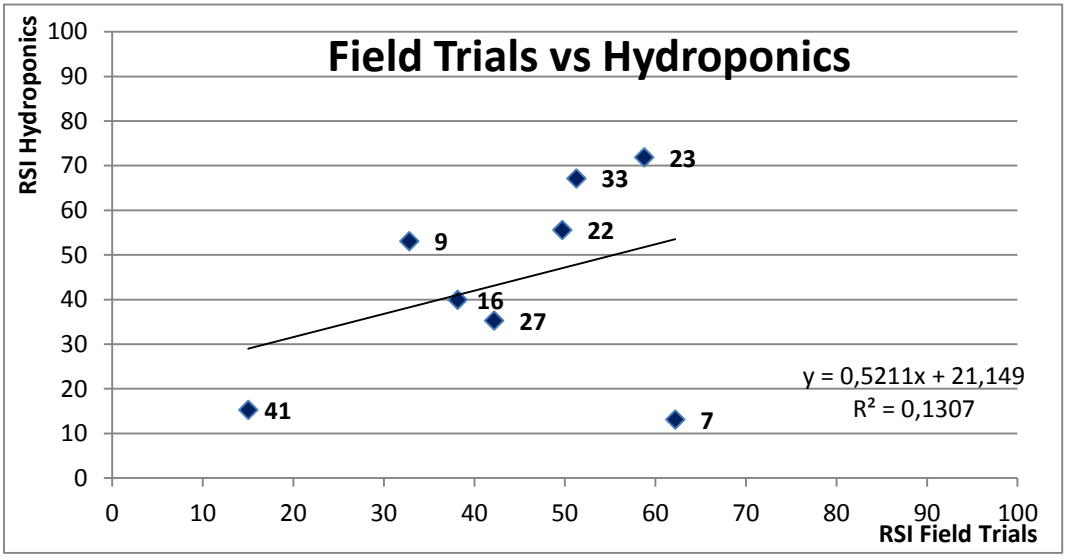


**Figure 15. Correlation graph based on the RSI for the correlation between the field trials (average of RZ and PV) and the hydroponics experiments (average of Ingestad and depletion).**

The correlation graph that describes the correlation between the field trials and the hydroponics is given in figure 15. Genotype 7 has the most difference in RSI between the field and hydroponics experiments. This is in agreement with the fact that genotype 7 was performing differently than normal in the hydroponics experiment and may have been mixed-up with another genotype.. Genotype 16 and 41 had the most similar RSI between the field trials and the hydroponics. Except for genotype 7, the ranking of the genotypes is very similar to the previous experiment in 2012 of Rafael Chan Navarrete (figure 16). The correlation is high for most genotypes, when taking out of account genotype 7. Excluding genotype 7 will give a $R^2$ of 0,7809.

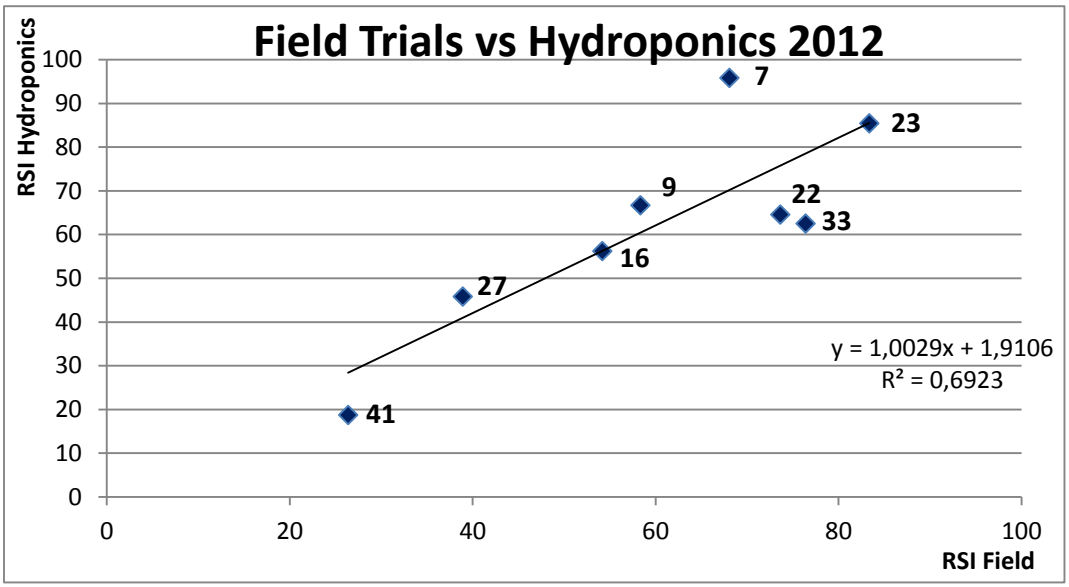


**Figure 16. Correlation graph based on the RSI for the correlation between the field trials and the hydroponics experiment of Rafael Chan Navarrete in 2012.**

# 4. Discussion

As the performance of genotype 7 in the hydroponics was not the same (smaller plants, all other traits are the same) as in previous hydroponic experiments (Personal communication Chan Navarrete, 2013), there are two possibilities. The first possibility is that there was a mix-up with another genotype and the second possibility is that the germination of that genotype was so bad that the plants have fallen behind. Due to lack of time a mix-up cannot be excluded, but also germination of genotype 7 was not good. Therefore the results of genotype 7 will only be used to look for differences between nitrogen conditions and nitrogen levels, and not for the differences between genotypes and correlations with field trials.

## 4.1 Hydroponics: Ingestad vs. Depletion

Plants under depletion conditions at low nitrogen show more stress (yellowing and dying) than plants under Ingestad conditions in low nitrogen. This could be explained by the fact that plants under Ingestad conditions have a longer time to adapt to a shortage of nitrogen, because they endure a low nitrogen level from the start of the experiment. The plants under depletion get all the nitrogen at the start and the shortage of nitrogen arises later on in the experiment, after the plants have taken up all the nitrogen. Therefore plants under depletion conditions do not have to adapt to low nitrogen levels in the beginning of the experiment, but only when the nitrogen is gone later on in the experiment. This could also explain why the largest differences between Ingestad and depletion only become visible after four weeks of growing. The stress in depletion was not yet present and plants under Ingestad conditions were already adapted to less nitrogen. After two weeks most stress is found under Ingestad conditions in low nitrogen, but after four weeks most stress is found under depletion conditions of low nitrogen. Stress is shown by yellowing and wilting. Although dying is not desirable for spinach production, differences between nitrogen levels and genotypes are more clear in depletion conditions, especially in low nitrogen where yellowing and dying of the plants occurred towards the end of the experiment. To answer the questions related to NUE, Ingestad would be a better method, since nitrogen stress on plants is desirable, but not to the degree of dying, since nothing can be measured in dead plants. Under Ingestad conditions the levels of stress are more controllable.

Under low nitrogen conditions, RS is higher under Ingestad conditions than under depletion conditions. RS is lowest under low nitrogen conditions (for both Ingestad and depletion). The difference in RS between low and high nitrogen is already visible after two weeks for most genotypes. Increasing RS seems an adaptation mechanism to low nitrogen. In a period of nitrogen shortage, which is the case more under Ingestad than under depletion conditions two weeks after planting, less investment in the shoots is seen. RS changes to increase uptake of nutrients such as nitrogen and to improve physiological efficiency by which plants, under short supply, utilize nutrients for growth (Marschner, 1995). A higher RS under Ingestad conditions is obtained by having less SDW, but the same RDW as under depletion. So, in this case it looks like investment in the roots goes as the cost of shoot investment. When the nutrient availability is relatively high (which is the case in depletion after two weeks), less nutrients are moved

to the roots, because the roots already have access to sufficient nutrients (Agren and Franklin, 2003). This is the case for depletion low nitrogen after two weeks and even more for both application methods at high nitrogen. The genotypes that invest relatively more in their roots under low nitrogen conditions invest mainly in RDW and RL, and the RSA also increases (either through longer roots or more lateral roots). These increases seem important for the uptake of nitrogen for the plant. This agrees with the research of Eissenstat (1992) that indicated that longer roots have higher chances to take up more nitrogen from the soil. Longer roots have more surface area to take up nutrients.

Also SLA is a trait that is higher for Ingestad (less SDW, same LA) than for depletion for all genotypes under low nitrogen levels. This difference only was clear at four weeks. A higher SLA means the leaves of the plants are thinner. In plants with thinner leaves more N is allocated to Rubisco, which is directly used for photosynthesis (Poorter and Evans, 1998). The allocation to Rubisco is necessary to grow faster, but gives a weaker structure of the leaves (less N in cell walls). In thicker leaves, more N is allocated to cell walls, which is useful for strong structural defenses (Feng *et al.*, 2009). There is probably a tradeoff for the plant to either grow faster or have strong structural defenses. In a period of stress (after four weeks most stress is found in depletion low nitrogen), the plants focus on the strong structural defense instead of allocating N to Rubisco for photosynthesis. Since already much is invested in existing leaf area and thus photosynthetic capacity, growth needs to be slowed down to also focus on the structural defenses. Plants under continuous stress (Ingestad low nitrogen) or no stress (high nitrogen conditions for both conditions) focus more on growing fast instead of structural defenses. To be able to do this it is necessary to maximize N usage for photosynthetic output.

It has been reported that, looking at different species, low chlorophyll content was found in thinner leaves (Poorter and Evans, 1998). At the beginning of the experiment the chlorophyll content under depletion condition was higher than under Ingestad conditions. However, after four weeks Ingestad had a higher chlorophyll content than depletion for most genotypes. The decrease in chlorophyll content in depletion in the last week could be due to chlorosis, because the nitrogen stress was too high. Chlorosis was seen in plants under depletion low nitrogen and not at all in the other conditions (not quantified, but scored yes or no). Although chlorosis of older leaves can lead to a loss of photosynthesis it can be necessary to remobilize N, that is derived from Rubisco degradation, to the younger leaves which is necessary for the growth of younger leaves and reproduction. This could mean that the chlorosis was due to remobilization of N (Feller *et al.*, 2008; Wingler *et al.,* 2006).

DM% at week four was higher for low nitrogen levels than for high nitrogen levels, but highest under depletion conditions. DM% has a negative correlation with SLA. In this experiment it is seen that when a plant has thinner leaves (higher SLA), it has less DM%. The differences in DM% are due to a difference in SFW, not SDW. With more nitrogen present there is more water in the plant (lower DM%), so there is a negative correlation between nitrogen availability and DM%, meaning there was less water content under

low nitrogen conditions and more water content in the plant under high nitrogen conditions. Under low nitrogen conditions this means the stomatal density is lower than under high nitrogen conditions, which has more transpiration as a result and therefore DM% increases (Hamid *et al.,* 1990; Lake and Woodward, 2008). The differences in RS were mainly due to changes in RDW and were already present after two weeks under Ingestad conditions. These traits increased as a consequence of the low nitrogen and are a possible adaptation necessary for nitrogen uptake efficiency.

## 4.2 Field Trials: Rijk Zwaan vs. Pop Vriend

The soil samples of Rijk Zwaan showed that the nitrogen levels were different than the intended three nitrogen levels. Looking at the graphs of the traits it looks like something did go wrong with the soil samples or the results of the soil samples. An indication for this is that the amount of 264 kg/ha is not possible, when the NA contained 61 kg/ha and at most 150 kg/ha was added, nitrogen levels should never reach more than 211 kg/ha. These observations suggest that the soil sample for 150 kg/ha was incorrect. However, the results (comparison with PV, SDW and SC for instance) showed that the order of the original nitrogen levels was right; the NA level contained the lowest amount of nitrogen of the three treatments and the 150 kg/ha level the highest. Mineralization could also have created an increase in N level of the soil, but would still not explain the difference at the N level of 150 kg/ha.

For PV there were no significant differences between the 100 kg/ha and 150 kg/ha conditions for all traits, which could indicate that 100 kg/ha is enough fertilizer for the plants to reach their maximum shoot weight, though previous experiments showed that there were differences between those two treatments (personal communication Chan Navarrete, 2013). The trial of RZ also showed no difference in SDW and SC for the two highest applications. Also here it looks like there is a maximum for the production of SDW and SC. This maximum for SDW and SC could be the result of a maximum for nitrogen uptake of the plant from the soil. It has been found that the maximum yield for Spinach is under 150 kg N/ha, but with 50 kg N/ha left in the soil. This would mean that the maximal uptake of N is around 100 kg/ha (Schenk *et al.,* 1991). In this case applications of nitrogen of 100 kg/ha or more resulted in maximum growth of the plants and no visible stress symptoms.

The DM% is highest under low nitrogen conditions and decreases when the nitrogen level increases at both RZ and PV. However, DM% is higher at PV than RZ for all nitrogen levels. A possibility is that the differences are due to environmental differences. The minimal, maximum and average temperature was the same for both trials (respectively 6,3°C; 18,3°C and 12,3°C). However, the relative humidity was higher at PV (81,6%) than at RZ (73,9%). An increase in air humidity creates a decrease in transpiration which results in an increased stomatal density necessary for compensation (Lake and Woodward, 2008). A higher stomatal density results again in more transpiration which will create a decrease in water content of the plant and an increase in dry matter percentage (Hamid *et al.,* 1990). The difference in DM% can also be a result of different soil types. Even though both companies have a clay soil, the exact composition of the

soil was not measured. Plants grown on clay soil have a higher amount of DM% than plants grown on sandy soils (Masoni *et al.,* 2007). This could be due to a lower relative bulk density in clay soil than in sand soil (Keller and Håkansson, 2010). A difference in bulk density of the soil between the companies could explain the difference of DM%, but so far there are no indications that this is the case.

The CC was lower at the end of the field trial at PV than at RZ, especially for the first leaf set. This is probably due to the fact that chlorophyll measurements were done later in the growing period at PV than at RZ as a results of dying of the youngest leaves at RZ. Explanations for this can be that they had water stress, since water was not draining properly at RZ. Normally stress would have a decrease of CC as a result, but it was not possible to confirm this by measuring the CC since the leaves were too fragile to measure. At RZ therefore the decrease of the CC as a consequence of nitrogen stress could not be measured at all. Drainage was better at PV, though the same amount of rain fell as at RZ, and therefore there was less water stress at PV. As a consequence the CC measurements could be taken for a longer time at PV, which resulted in the decrease of CC what probably was caused by the nitrogen stress.

There are less significant differences between nitrogen levels for root characteristics in the field trials than in the hydroponics experiment. An explanation for this could be that only a small soil sample was taken and only part of the root could be harvested, with the consequence that standard errors were high. Therefore there are no obvious differences within the two field trials. However, RS is higher at PV than at RZ. Since there are no significant differences between the field trials for RDW, the difference in RS is due to differences in SDW (keeping in mind than the root samples were incomplete). Although more thermal time passed at PV from the first to the last harvest, the SDW at PV was the same as the SDW at RZ. This was also the case for RDW, which would suggest less nitrogen stress at PV.

## 4.3 Correlation between Hydroponics and Field Trials

The SDW of both the plants at RZ and at PV were significantly correlated with the SDW of the plants of the hydroponics (table 9, 10). It looks like SDW is least influenced by environmental conditions. This correlation took into account the nitrogen levels and genotypic differences, which means that also these factors could have an influence on SDW but had the same influence on SDW in the field and the hydroponics. For the root characteristics there are no significant correlations between hydroponics and the two field trials. This could be due to less replications in the field and less precision of root harvesting in the field trials. And of course the root environments are completely different between hydroponics and the field trials. Plant performance between the experiments at the different nitrogen levels seems to correlate well. In both the hydroponics and the field trials SC/LA, SDW and CC decrease whenever nitrogen levels decrease, but DM% and RS increase with decreasing nitrogen levels.

The ranking summation index between the field trials and the hydroponics showed that the most similar ranking between the experiments is found for genotype 16, 23, 33 and

41. Thus, these genotypes are most stable between conditions and least influenced by the environment. This coincides with earlier results of Rafael Chan Navarrete. Genotype 7 showed the most difference in ranking between experiments. This is in line with the hypothesis of a mix-up in the hydroponics and the bad germination that was mentioned earlier. Both Ingestad and depletion have the same correlation to the field trials (low N in hydroponics compared to 100 kg/ha for the field trials). In the field the nitrogen application is done at the beginning of the trial, which is in line with depletion. But one of the fertilizers that was used (Entec) slowly releases the nitrogen through time, which is in line with Ingestad. The other fertilizer that was used was KAS, which immediately releases the nitrogen.

Correlations based on the RSI (SDW, RGR, CC and LA or SC) are high between hydroponics and the field trials for most genotypes, except genotype 7 (table 11 and figure 15). For one genotype there is a higher correlation than the other, which could indicate that one genotype is more influenced by the environment than the other. With hydroponics it is possible to apply nitrogen stress in a steady state (Ingestad) without senescence and luxurious N uptake (taking up more nitrogen than necessary for growth and storing it). The most important advantage of the hydroponics experiment is that the root environment and nutrient supplies can be completely controlled. Another advantage of hydroponics experiments is that harvesting shoots and especially roots is easier and faster and that these experiments can be done in the greenhouse which makes it possible to do year round. Also, studies can be performed without the problem of environmental influences. However, this could also be the drawback of the system. Plants are influenced by the environment (soil types, soil life, weather etcetera) and these influences are left out in the hydroponics experiment. Therefore, hydroponics is a good system for making selections for breeding and for studying different processes in plants, but in combination with field trials that will make it possible to incorporate environmental influences.

# 5. Conclusions

- Plants are in better health until the end of the experiment under Ingestad conditions, instead of dying in the end of the experiment as they do under depletion conditions. Therefore It is better to evaluate traits related to NUE under Ingestad conditions than under depletion conditions, since traits cannot be measured in dead plants.
- Traits that are essential for nitrogen use efficiency are RS and SLA. Increasing RS under low nitrogen conditions is an adaptation mechanism. This gives the plant the opportunity to have more root area per unit of shoot to take up nutrients in deprived conditions. SLA is lower under low nitrogen conditions, which means plants invest in thicker leaves and thus a stronger structural defence instead of thinner leaves due to growing fast under high nitrogen conditions. Both DM% and CC were negatively correlated with SLA. So, plants also have more chlorophyll content and less water content under low nitrogen conditions.
- SDW was least influenced by environmental effects (significant correlation between hydroponics and the field trials), while root characteristics were most influenced by environmental effects (not significant correlation). However, root environment was completely different between hydroponics and the field experiments.
- The results of the hydroponics are comparable to the results of the field trials for genotype 16, 23, 33 and 41. This means that these genotypes are least influenced by the environment. Genotype 7 had the most difference between hydroponics and the field trials, but this can be explained by a probable mix-up with the seeds.
- Advantage of a hydroponics experiment is that nutrient supplies and the root environment can be completely controlled. Harvesting shoots and especially roots is much easier and faster in a hydroponics experiment and these experiments are possible to do year round. The drawback of hydroponics is that the influence of the environment on the plants cannot be researched.

# 6. Recommendations

- Future experiments in hydroponics and the field should be done using more than four or eight genotypes to look for correlations. If so, hydroponics could be a nice system for selection in breeding and studying of plant processes, but always in combination with field trials that make it possible to incorporate environmental influences like soil type, weather etcetera.
- Nitrogen levels in the hydroponics were not measured for this experiment. For the next hydroponic experiment it would be useful to measure the nitrogen content to have more information about nitrogen use efficiency.
- This study showed that the hydroponics system can be a good system for plant research. Especially for root characteristics, because these traits are difficult to study in the field. However, to see if there is a significant correlation for root characteristics between hydroponics and the field, more root samples should be taken in the field for the next experiment.
- In organic agriculture nitrogen availability is less than in conventional agriculture and therefore the model of Ingestad (steady state instead of at once) would be in

theory the best way to apply nitrogen to the field, because less stress was found in plants grown in those conditions. In this way less input of fertilizer is necessary and this will reduce costs for farmers, though this method of applying nitrogen will increase mechanical labor. Future research can focus on new application methods for nitrogen for the field or fertilizers that have an even slower release of nitrogen than Entec, which can then be used to decrease both nitrogen fertilization as well as the mechanical labor.

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# Appendix 1: Genotypes

**Table 12. The 24 Genotypes used in the field experiment at Pop Vriend and Rijk Zwaan, 2013.**

| Cultivar | Origin | Cultivar Name |
|---|---|---|
| 1 | Enza Zaden | Grandi |
| 2 | Enza Zaden | Corvette |
| 3 | Enza Zaden | Corvair |
| 7 | Enza Zaden | Ranchero |
| 8 | Enza Zaden | Thunderbolt |
| 9 | Enza Zaden | Chebelle |
| 10 | Enza Zaden | Charger |
| 12 | Pop Vriend | Hudson |
| 14 | Pop Vriend | PV 9208 |
| 16 | Pop Vriend | PV 9273/Cello |
| 17 | Pop Vriend | PV 9274/Celesta |
| 18 | Pop Vriend | PC 0293 |
| 20 | Pop Vriend | PV 0294 |
| 21 | Nunhems | Palco |
| 22 | Nunhems | Novico |
| 23 | Nunhems | Andromeda |
| 25 | Nunhems | NUN 00905 SP |
| 26 | Nunhems | NUN 00915 SP |
| 27 | Rijk Zwaan | Crocodile |
| 28 | Rijk Zwaan | Eagle |
| 30 | Rijk Zwaan | Rhino |
| 33 | Rijk Zwaan | Sparrow |
| 35 | Rijk Zwaan | Beaver |
| 41 | Rijk Zwaan | Marabu |

**Table 13. Genotypes used in hydroponics experiment with their characteristics.**

| Cultivar | Criteria for selection |
|---|---|
| 7 | Strong parental line under low nitrogen |
| 9 | Small leaf area but moderate biomass production |
| 16 | High root production but low shoot biomass |
| 22 | Strong parental line under low nitrogen |
| 23 | High root production and high shoot biomass |
| 27 | Weak parental line under low nitrogen |
| 33 | High performance at field conditions |
| 41 | Weak parental line under low nitrogen, shows stability |

# Appendix 2: Experimental Design Hydroponics Experiment

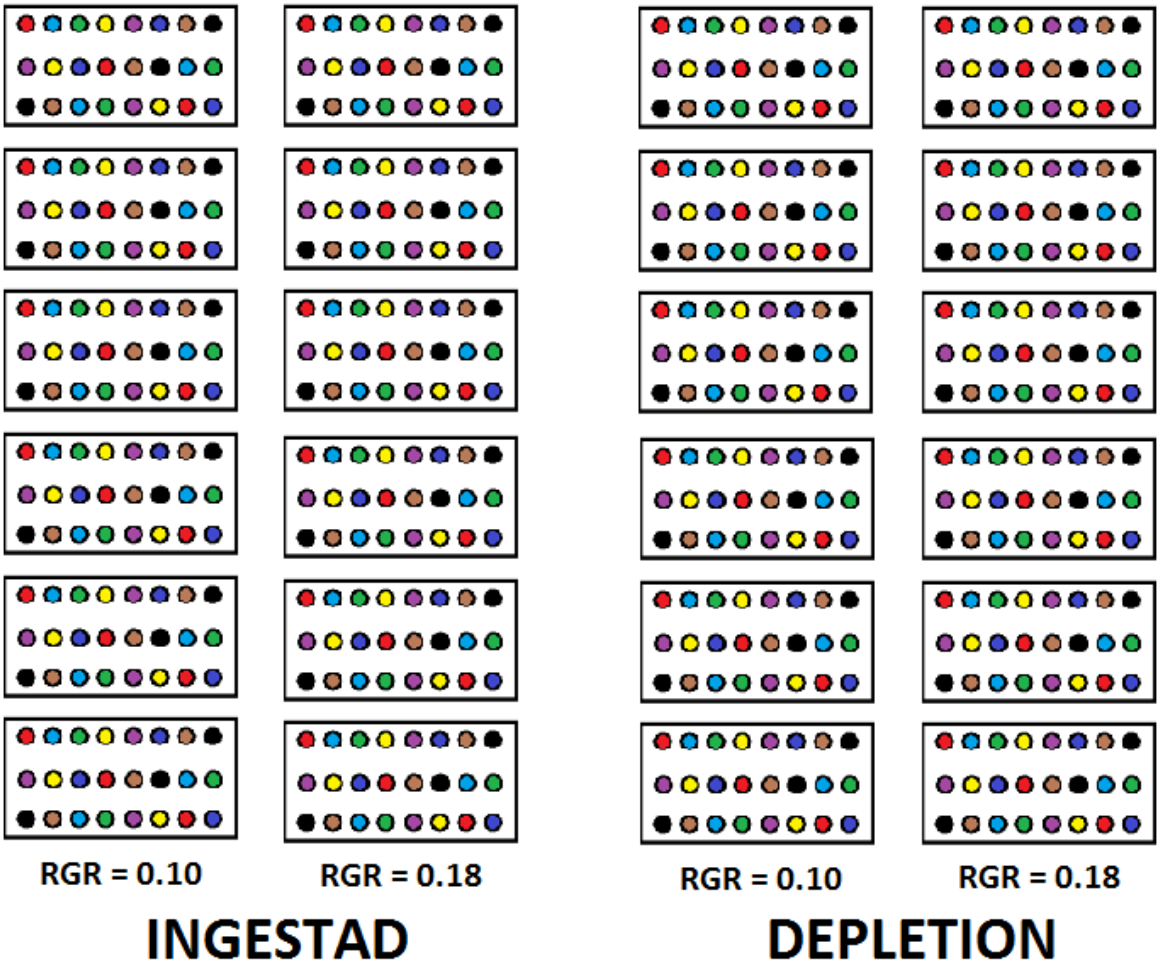



**Figure 17. Experimental design with six containers for each condition. Each container is filled with genotypes according to figure 1.**

# Appendix 3: Nitrogen Additions Hydroponics

**Table 14. Nitrogen added under Ingestad conditions in the hydroponics experiment.**

| mg | RGR=0.10 | 3 | 1 | RGR=0.18 | 3 | 1 |
|---|---|---|---|---|---|---|
| **192x** | **T** | **KNO3** | **NH4Cl** | **T** | **KNO3** | **NH4Cl** |
| **25-jan** | 1 | 58,37 | 10,30 | 1 | 109,46 | 19,31 |
| **26-jan** | 2 | 64,51 | 11,38 | 2 | 131,05 | 23,11 |
| **27-jan** | 3 | 71,30 | 12,57 | 3 | 156,90 | 27,67 |
| **28-jan** | 4 | 78,80 | 13,90 | 4 | 187,84 | 33,13 |
| **29-jan** | 5 | 87,08 | 15,36 | 5 | 224,88 | 39,66 |
| **30-jan** | 6 | 96,24 | 16,97 | 6 | 269,23 | 47,48 |
| **31-jan** | 7 | 106,36 | 18,76 | 7 | 322,33 | 56,85 |
| **1-feb** | 8 | 117,55 | 20,73 | 8 | 385,90 | 68,06 |
| **2-feb** | 9 | 129,91 | 22,91 | 9 | 462,01 | 81,48 |
| **3-feb** | 10 | 143,58 | 25,32 | 10 | 553,12 | 97,55 |
| **4-feb** | 11 | 158,68 | 27,98 | 11 | 662,21 | 116,79 |
| **5-feb** | 12 | 175,36 | 30,93 | 12 | 792,81 | 139,82 |
| **6-feb** | 13 | 121,13 | 21,36 | 13 | 593,23 | 104,62 |
| **7-feb** | 14 | 133,87 | 23,61 | 14 | 710,22 | 125,26 |
| **8-feb** | 15 | 147,95 | 26,09 | 15 | 850,29 | 149,96 |
| **9-feb** | 16 | 163,51 | 28,84 | 16 | 1017,98 | 179,54 |
| **10-feb** | 17 | 180,70 | 31,87 | 17 | 1218,74 | 214,94 |
| **11-feb** | 18 | 199,71 | 35,22 | 18 | 1459,10 | 257,33 |
| **12-feb** | 19 | 220,71 | 38,93 | 19 | 1746,86 | 308,09 |
| **13-feb** | 20 | 243,92 | 43,02 | 20 | 2091,37 | 368,85 |
| **14-feb** | 21 | 269,58 | 47,54 | 21 | 2503,82 | 441,59 |
| **15-feb** | 22 | 297,93 | 52,54 | 22 | 2997,62 | 528,68 |
| **16-feb** | 23 | 329,26 | 58,07 | 23 | 3588,80 | 632,94 |
| **17-feb** | 24 | 363,89 | 64,18 | 24 | 4296,58 | 757,77 |
| **18-feb** | 25 | 402,16 | 70,93 | 25 | 5143,94 | 907,21 |
| **19-feb** | 26 | 444,46 | 78,39 | 26 | 6158,41 | 1086,13 |
| **20-feb** | 27 | 491,20 | 86,63 | 27 | 7372,96 | 1300,34 |

**Table 15. Nitrogen added under depletion conditions in the hydroponics experiment.**

| mg | Depletion | 0,10 | | Depletion | 0,18 | |
|---|---|---|---|---|---|---|
| **192x** | **T** | **KNO4** | **NH4Cl** | **T** | **KNO4** | **NH4Cl** |
| **25-jan** | 1 | 5297,72 | 934,34 | 1 | 46007,65 | 8114,17 |
| **26-jan** | 2 | | | 2 | | |

# Appendix 4: Experimental Design Field Trial Pop Vriend

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **No application** | --> | 17 | 1 | 9 | 35 | 16 | 18 | 30 | 22 | 23 | 41 | 21 | |
| | | 7 | **21** | **3** | **23** | **16** | **18** | **8** | **2** | **23** | **9** | 14 | <-- |
| | --> | 16 | **22** | **14** | **28** | **23** | **10** | **12** | **12** | **8** | **17** | 30 | |
| | | 23 | **18** | **1** | **26** | **2** | **26** | **14** | **25** | **27** | **22** | 12 | <-- |
| | --> | 22 | **30** | **25** | **16** | **22** | **7** | **20** | **28** | **10** | **16** | 33 | |
| | | 27 | 33 | 8 | 12 | 3 | 28 | 25 | 7 | 2 | 14 | 20 | <-- |
| | | | | | | | | | | | | | |
| | --> | 9 | 8 | 1 | 17 | 23 | 7 | 2 | 16 | 41 | 30 | 28 | |
| | | 8 | **8** | **33** | **41** | **41** | **28** | **35** | **26** | **7** | **14** | 41 | <-- |
| | --> | 2 | **12** | **17** | **10** | **25** | **27** | **1** | **21** | **18** | **30** | 27 | |
| | | 21 | **20** | **35** | **9** | **17** | **30** | **21** | **20** | **35** | **33** | 9 | <-- |
| | --> | 35 | **27** | **7** | **2** | **9** | **3** | **33** | **1** | **3** | **41** | 18 | |
| | | 21 | 12 | 3 | 18 | 14 | 22 | 33 | 35 | 20 | 25 | 27 | <-- |
| | | | | | | | | | | | | | |
| **100 kg/ha** | --> | 3 | 33 | 22 | 27 | 21 | 12 | 17 | 25 | 8 | 28 | 41 | |
| | | 23 | **14** | **12** | **3** | **14** | **1** | **23** | **21** | **9** | **10** | 18 | <-- |
| | --> | 35 | **21** | **7** | **20** | **9** | **2** | **10** | **18** | **41** | **2** | 33 | |
| | | 8 | **27** | **35** | **22** | **35** | **28** | **22** | **28** | **3** | **27** | 16 | <-- |
| | --> | 21 | **10** | **23** | **41** | **21** | **8** | **27** | **14** | **1** | **33** | 9 | |
| | | 9 | 18 | 20 | 23 | 16 | 35 | 2 | 14 | 1 | 7 | 30 | <-- |
| | | | | | | | | | | | | | |
| | --> | 25 | 27 | 20 | 18 | 28 | 2 | 35 | 1 | 16 | 41 | 23 | |
| | | 27 | **18** | **26** | **2** | **17** | **18** | **41** | **30** | **16** | **25** | 2 | <-- |
| | --> | 7 | **17** | **8** | **9** | **26** | **25** | **16** | **35** | **22** | **12** | 22 | |
| | | 12 | **1** | **16** | **25** | **30** | **7** | **20** | **23** | **17** | **20** | 14 | <-- |
| | --> | 41 | **28** | **33** | **30** | **33** | **12** | **3** | **26** | **8** | **7** | 30 | |
| | | 22 | 7 | 9 | 33 | 14 | 30 | 17 | 8 | 21 | 3 | 12 | <-- |
| | | | | | | | | | | | | | |
| **150 kg/ha** | --> | 22 | 23 | 14 | 8 | 21 | 27 | 17 | 28 | 9 | 16 | 25 | |
| | | 2 | **41** | **3** | **23** | **30** | **21** | **35** | **18** | **21** | **14** | 14 | <-- |
| | --> | 12 | **33** | **26** | **9** | **33** | **14** | **17** | **30** | **27** | **28** | 16 | |
| | | 23 | **20** | **16** | **35** | **23** | **18** | **25** | **23** | **10** | **41** | 22 | <-- |
| | --> | 9 | **12** | **27** | **25** | **12** | **10** | **8** | **33** | **2** | **3** | 8 | |
| | | 33 | 18 | 30 | 2 | 7 | 41 | 1 | 3 | 35 | 12 | 20 | <-- |
| | | | | | | | | | | | | | |
| | --> | 30 | 22 | 41 | 25 | 33 | 9 | 12 | 7 | 16 | 1 | 14 | |
| | | 27 | **14** | **2** | **7** | **26** | **22** | **27** | **20** | **9** | **8** | 7 | <-- |
| | --> | 41 | **18** | **8** | **17** | **9** | **20** | **7** | **35** | **1** | **22** | 21 | |
| | | 30 | **30** | **22** | **10** | **2** | **3** | **41** | **26** | **12** | **17** | 18 | <-- |
| | --> | 35 | **28** | **21** | **1** | **16** | **28** | **1** | **7** | **16** | **25** | 33 | |
| | | 2 | 23 | 20 | 28 | 3 | 18 | 27 | 17 | 21 | 8 | 35 | <-- |

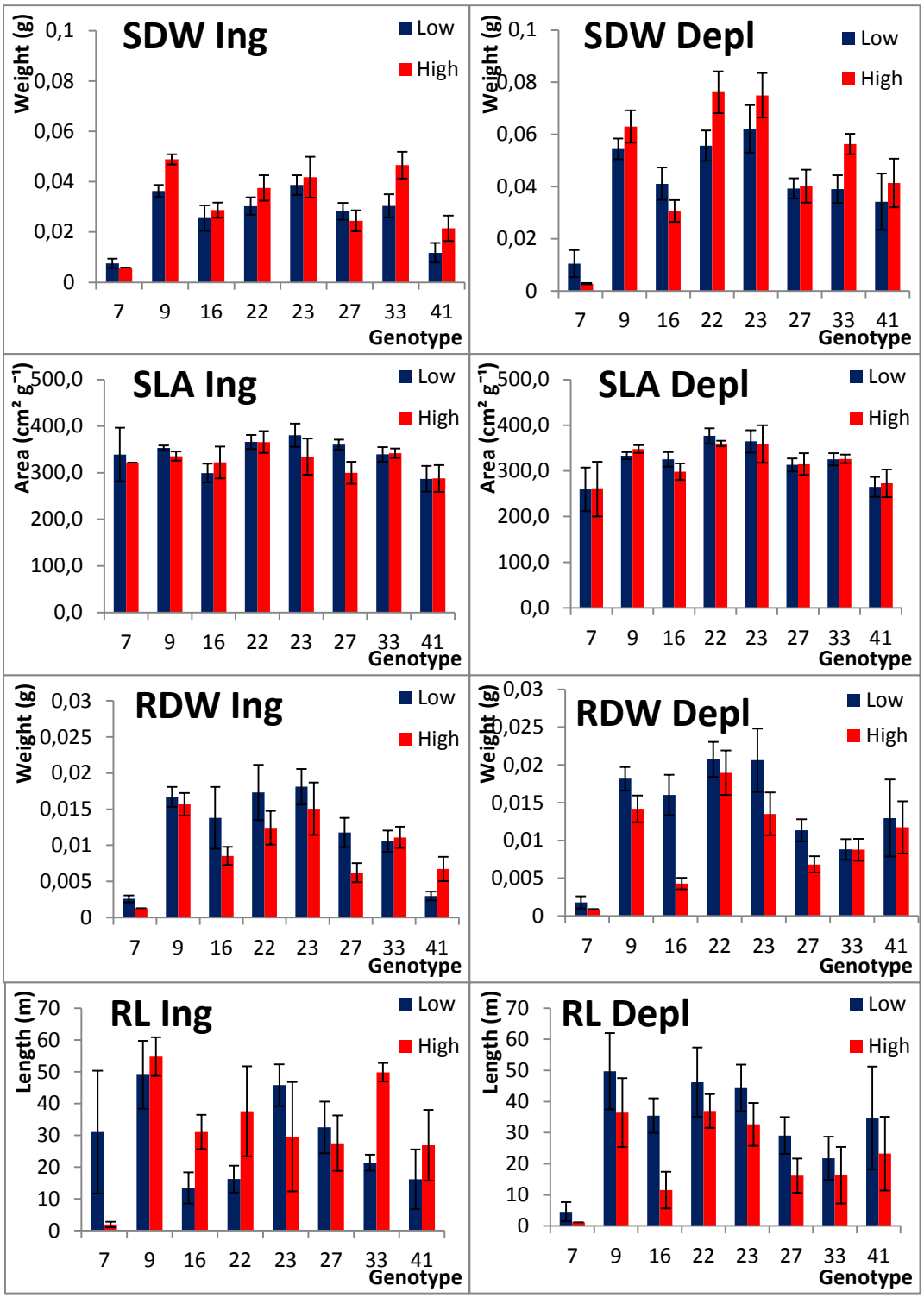
**Figure 18. Experimental design of the field trial at Pop Vriend.**

# Appendix 5: Experimental Design Field Trial Rijk Zwaan

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 150 kg/ha | --> | 41 | 7 | 2 | 22 | 14 | 9 | 25 | 35 | 23 | 28 | 17 | |
| | | 2 | **16** | **9** | **41** | **27** | **1** | **16** | **26** | **3** | **27** | 7 | <-- |
| | --> | 8 | **25** | **23** | **26** | **23** | **20** | **12** | **35** | **12** | **8** | 18 | |
| | | 12 | **3** | **22** | **20** | **22** | **14** | **2** | **9** | **1** | **20** | 30 | <-- |
| | --> | 23 | **7** | **17** | **14** | **18** | **3** | **17** | **25** | **33** | **10** | 41 | |
| | | 22 | **12** | **33** | **2** | **35** | **30** | **10** | **22** | **41** | **7** | 27 | <-- |
| | --> | 9 | **35** | **8** | **30** | **33** | **21** | **7** | **28** | **14** | **17** | 33 | |
| | | 14 | **18** | **28** | **10** | **26** | **9** | **28** | **16** | **18** | **23** | 21 | <-- |
| | --> | 16 | **1** | **27** | **21** | **25** | **8** | **41** | **2** | **21** | **30** | 35 | |
| | | 33 | 8 | 27 | 20 | 18 | 30 | 1 | 12 | 21 | 16 | 3 | <-- |
| No application | --> | 35 | 33 | 1 | 9 | 16 | 23 | 20 | 14 | 8 | 41 | 2 | |
| | | 33 | **25** | **41** | **21** | **8** | **10** | **28** | **28** | **18** | **35** | 9 | <-- |
| | --> | 27 | **20** | **27** | **1** | **7** | **20** | **3** | **33** | **1** | **21** | 8 | |
| | | 2 | **35** | **7** | **22** | **18** | **23** | **21** | **16** | **26** | **10** | 30 | <-- |
| | --> | 16 | **14** | **3** | **28** | **12** | **22** | **27** | **9** | **20** | **12** | 35 | |
| | | | | | | | | | | | | | |
| | | 7 | **10** | **23** | **17** | **41** | **17** | **35** | **23** | **3** | **41** | 14 | <-- |
| | --> | 22 | **2** | **26** | **16** | **1** | **33** | **26** | **17** | **27** | **8** | 18 | |
| | | 21 | **33** | **12** | **18** | **30** | **2** | **14** | **14** | **25** | **7** | 12 | <-- |
| | --> | 23 | **9** | **8** | **30** | **16** | **9** | **25** | **2** | **30** | **22** | 41 | |
| | | 25 | 7 | 21 | 27 | 3 | 18 | 28 | 22 | 30 | 12 | 17 | <-- |
| 100 kg/ha | --> | 18 | 20 | 41 | 35 | 28 | 8 | 3 | 22 | 27 | 1 | 7 | |
| | | 9 | **21** | **20** | **14** | **1** | **28** | **9** | **8** | **9** | **17** | 21 | <-- |
| | --> | 41 | **17** | **18** | **12** | **14** | **17** | **2** | **35** | **33** | **18** | 18 | |
| | | 30 | **22** | **26** | **35** | **21** | **20** | **35** | **7** | **20** | **1** | 14 | <-- |
| | --> | 7 | **7** | **28** | **23** | **22** | **10** | **23** | **41** | **23** | **2** | 33 | |
| | | 12 | **9** | **8** | **33** | **8** | **33** | **41** | **27** | **22** | **26** | 22 | <-- |
| | --> | 2 | **16** | **30** | **25** | **12** | **16** | **26** | **25** | **12** | **21** | 35 | |
| | | 23 | **1** | **41** | **2** | **7** | **30** | **18** | **3** | **10** | **30** | 16 | <-- |
| | --> | 8 | **27** | **3** | **10** | **27** | **25** | **3** | **14** | **28** | **16** | 27 | |
| | | 16 | 25 | 9 | 14 | 17 | 33 | 21 | 12 | 2 | 30 | 23 | <-- |

**Figure 19. Experimental design of the field trial at Rijk Zwaan.**

## Appendix 6: Harvest 1 Hydroponics (Graphs)



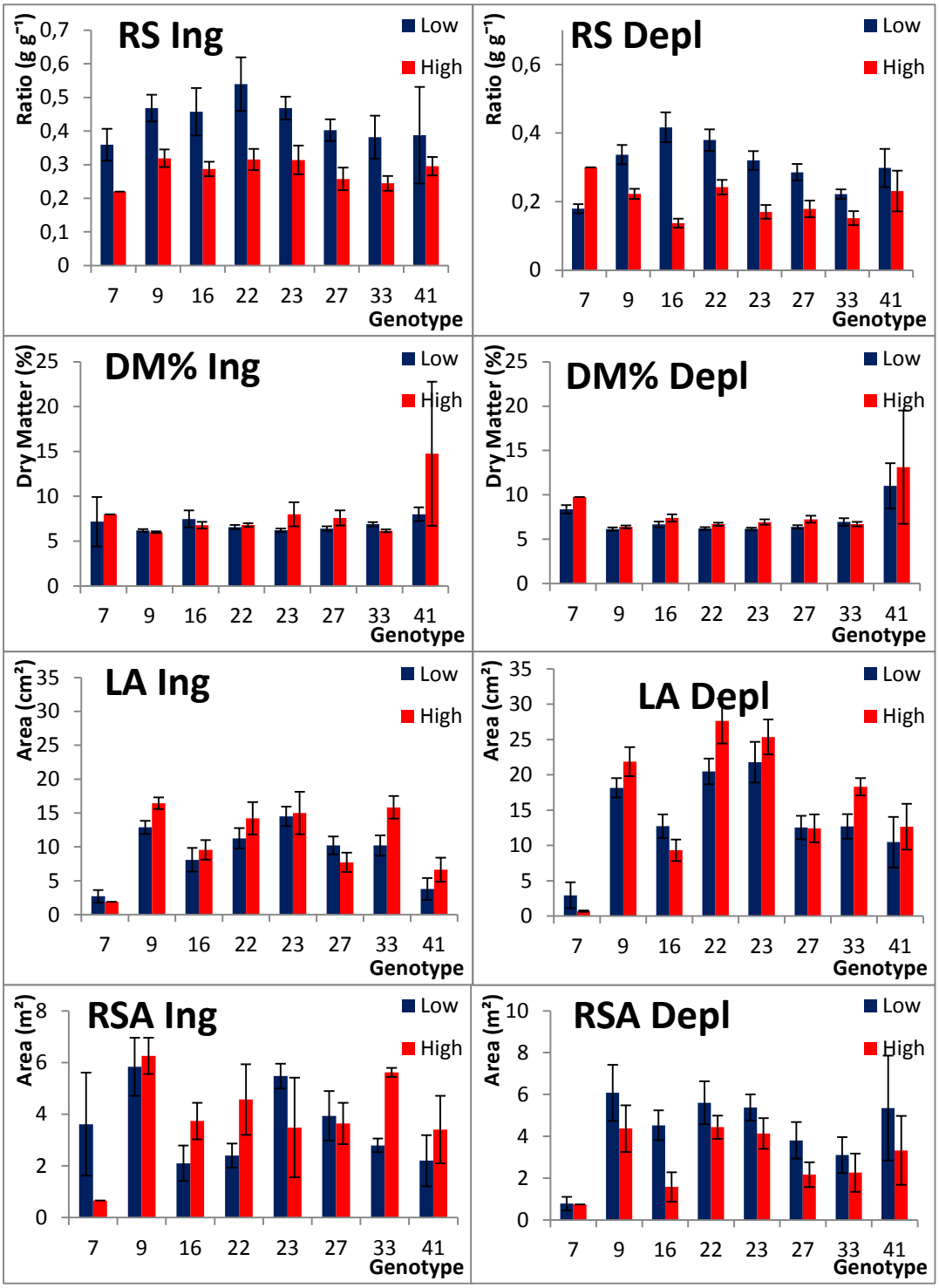



**Figure 20. Graphs of harvest 1 for both Ingestad and depletion conditions for Shoot Dry Weight (SDW), Specific Leaf Area (SLA), Root Dry Weight (RDW), Root Length (RL), Root:shoot Ratio (RS), Dry Matter Percentage (DM%), Leaf Area (LA) and Surface Area of the root (RSA). Error bars represent standard error.**

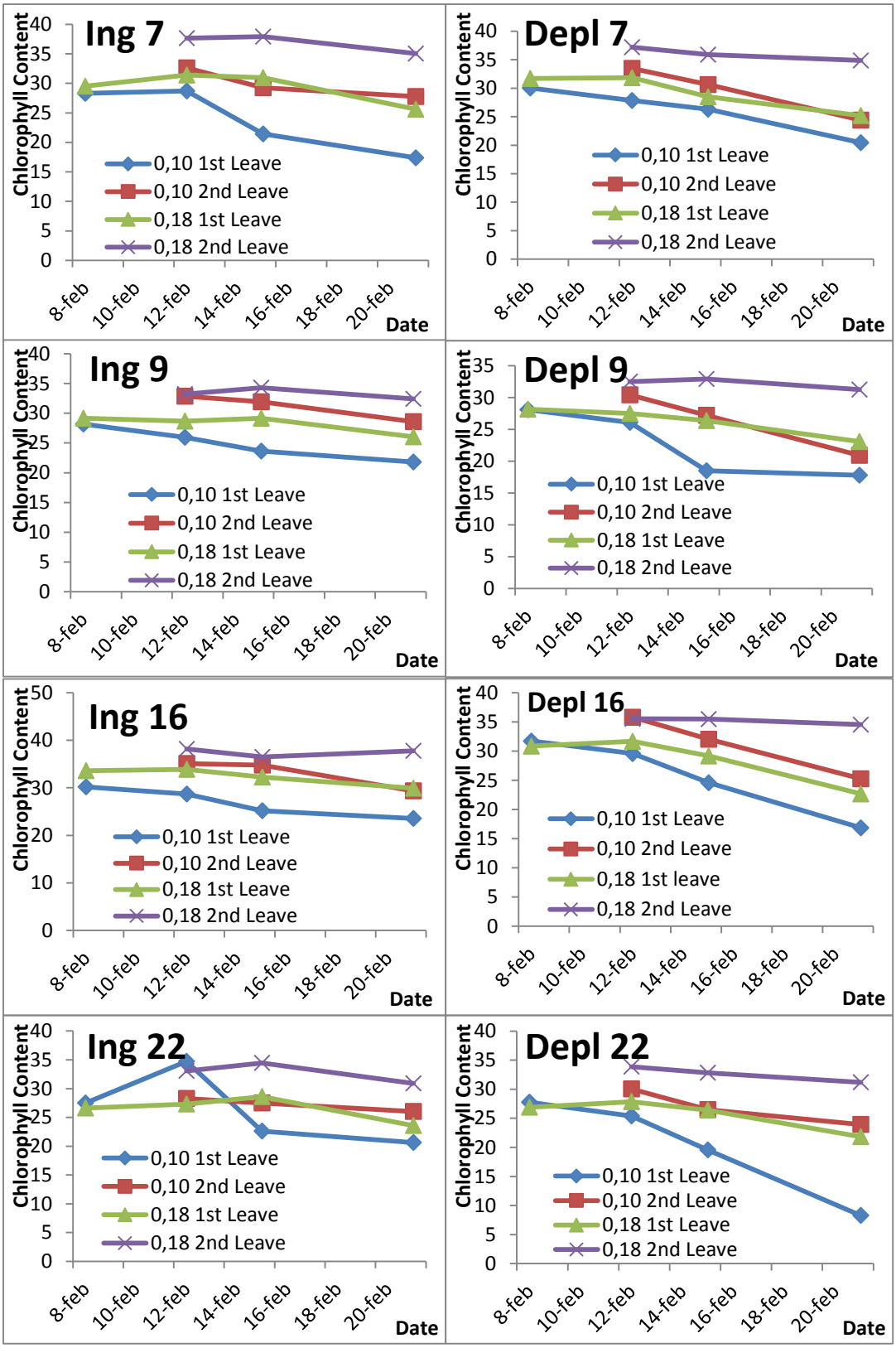
# Appendix 7: REML Analysis Hydroponics Ingestad

| | | DAS | Leaf | Genotype | Nitrogen | Genotype x Nitrogen |
|---|---|---|---|---|---|---|
| **Dry Weight** | **Total** | | | <0,001 | <0,001 | 0,001 |
| | **Shoot** | | | <0,001 | <0,001 | 0,002 |
| | **Root** | | | <0,001 | 0,860 | 0,001 |
| **Fresh Weight** | **Total** | | | <0,001 | <0,001 | <0,001 |
| | **Shoot** | | | <0,001 | <0,001 | <0,001 |
| | **Root** | | | <0,001 | 0,001 | 0,002 |
| | | | | | | |
| **Roots** | **Average Diameter** | | | 0,357 | 0,507 | 0,410 |
| | **Total Root Length** | | | 0,010 | 0,278 | 0,699 |
| | **Surface Area** | | | 0,026 | 0,655 | 0,677 |
| | | | | | | |
| | **Leaf Area** | | | <0,001 | <0,001 | <0,001 |
| | **SLA** | | | 0,012 | <0,001 | 0,131 |
| | **Root Shoot Ratio** | | | <0,001 | <0,001 | <0,001 |
| | **Dry Matter %** | | | <0,001 | 0,034 | 0,045 |
| | **Stomatal Conductance** | | | 0,205 | 0,968 | 0,508 |
| | | | | | | |
| | **Leaf Number** | 4 | | 0,016 | 0,742 | 0,994 |
| | | 8 | | <0,001 | 0,234 | 0,063 |
| | | 11 | | <0,001 | 0,345 | 0,359 |
| | | 15 | | <0,001 | 0,099 | 0,055 |
| | | 18 | | <0,001 | 0,002 | 0,184 |
| | | 21 | | <0,001 | <0,001 | 0,868 |
| | | 25 | | <0,001 | 0,031 | 0,202 |
| | | | | | | |
| | **Chlorofyll Content** | 14 | 1 | <0,001 | <0,001 | 0,003 |
| | | 18 | 1 | <0,001 | <0,001 | 0,470 |
| | | 18 | 2 | <0,001 | <0,001 | 0,145 |
| | | 21 | 1 | <0,001 | <0,001 | 0,230 |
| | | 21 | 2 | 0,002 | <0,001 | 0,405 |
| | | 27 | 1 | <0,001 | <0,001 | 0,043 |
| | | 27 | 2 | <0,001 | <0,001 | 0,021 |

# Appendix 8: REML Analysis Hydroponics Depletion

| | | DAS | Leaf | Genotype | Nitrogen | Genotype x Nitrogen |
|---|---|---|---|---|---|---|
| **Dry Weight** | **Total** | | | 0,015 | <0,001 | 0,105 |
| | **Shoot** | | | 0,009 | <0,001 | 0,053 |
| | **Root** | | | 0,116 | 0,237 | 0,840 |
| **Fresh Weight** | **Total** | | | 0,004 | <0,001 | 0,015 |
| | **Shoot** | | | 0,002 | <0,001 | 0,004 |
| | **Root** | | | 0,003 | 0,202 | 0,525 |
| | | | | | | |
| **Roots** | **Average Diameter** | | | 0,224 | 0,293 | 0,124 |
| | **Total Root Length** | | | 0,063 | 0,632 | 0,476 |
| | **Surface Area** | | | 0,041 | 0,762 | 0,394 |
| | | | | | | |
| | **Leaf Area** | | | <0,001 | <0,001 | 0,003 |
| | **SLA** | | | <0,001 | <0,001 | 0,075 |
| | **Root Shoot Ratio** | | | <0,001 | <0,001 | <0,001 |
| | **Dry Matter %** | | | 0,003 | <0,001 | 0,010 |
| | **Stomatal Conductance** | | | 0,897 | 0,570 | 0,564 |
| | | | | | | |
| | **Leaf Number** | 4 | | 0,011 | 0,228 | 0,718 |
| | | 8 | | <0,001 | 0,356 | 0,195 |
| | | 11 | | <0,001 | 0,070 | 0,984 |
| | | 15 | | <0,001 | 0,133 | 0,381 |
| | | 18 | | <0,001 | 0,792 | 0,386 |
| | | 21 | | <0,001 | 0,012 | 0,860 |
| | | 25 | | <0,001 | 0,056 | 0,687 |
| | | | | | | |
| | **Chlorofyll Content** | 14 | 1 | <0,001 | 0,510 | 0,687 |
| | | 18 | 1 | <0,001 | <0,001 | 0,275 |
| | | 18 | 2 | <0,001 | <0,001 | 0,316 |
| | | 21 | 1 | <0,001 | <0,001 | 0,210 |
| | | 21 | 2 | <0,001 | <0,001 | 0,881 |
| | | 27 | 1 | 0,003 | <0,001 | 0,133 |
| | | 27 | 2 | 0,011 | <0,001 | 0,964 |

## Appendix 9: Chlorophyll Content All Genotypes



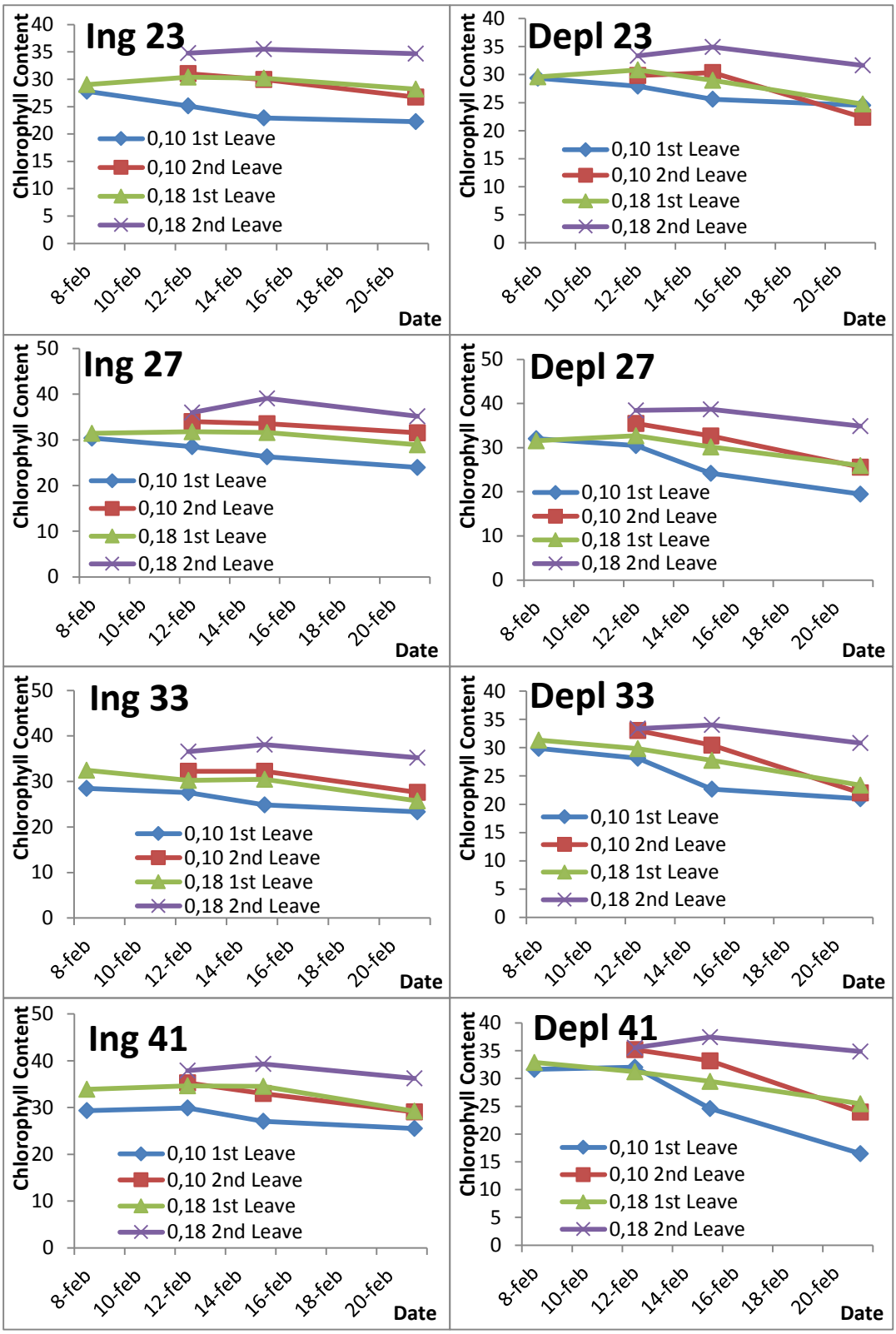



**Figure 21. Chlorophyll content per genotype for both Ingestad and depletion conditions.**

# Appendix 10: Additional Graphs Hydroponics

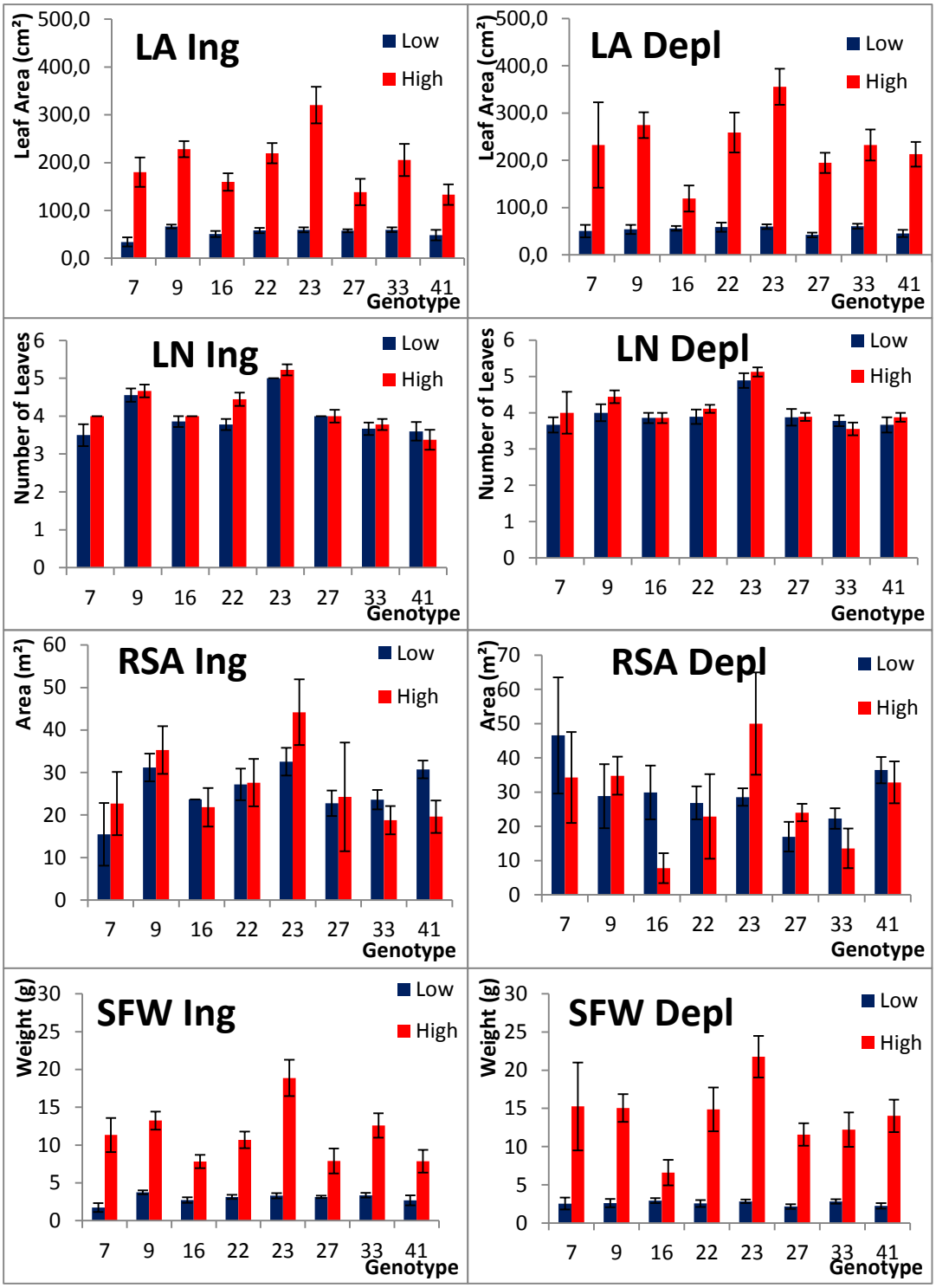



**Figure 22. Leaf Area (LA), Leaf Number (LN), Surface area (RSA) and Shoot Fresh Weight (SFW) of the roots per genotype for both Ingestad and depletion conditions of the hydroponics experiment four weeks after planting.**

# Appendix 11: Correlation between SDW and RDW

**Table 16. Correlation between RDW and SDW for every genotype separately. The bold numbers are significant for a p<0.05.**

| Genotype | Correlation | Significance |
|---|---|---|
| **7** | **0.6327** | **0.0048** |
| **9** | 0.2981 | 0.0774 |
| **16** | 0.4947 | 0.0054 |
| **22** | **0.3899** | **0.0187** |
| **23** | **00.7013** | **<0.001** |
| **27** | 0.2994 | 0.0806 |
| **33** | **0.5501** | **<0.001** |
| **41** | **0.6515** | **<0.001** |

# Appendix 12: Extra Graphs Shoot Dry Weight

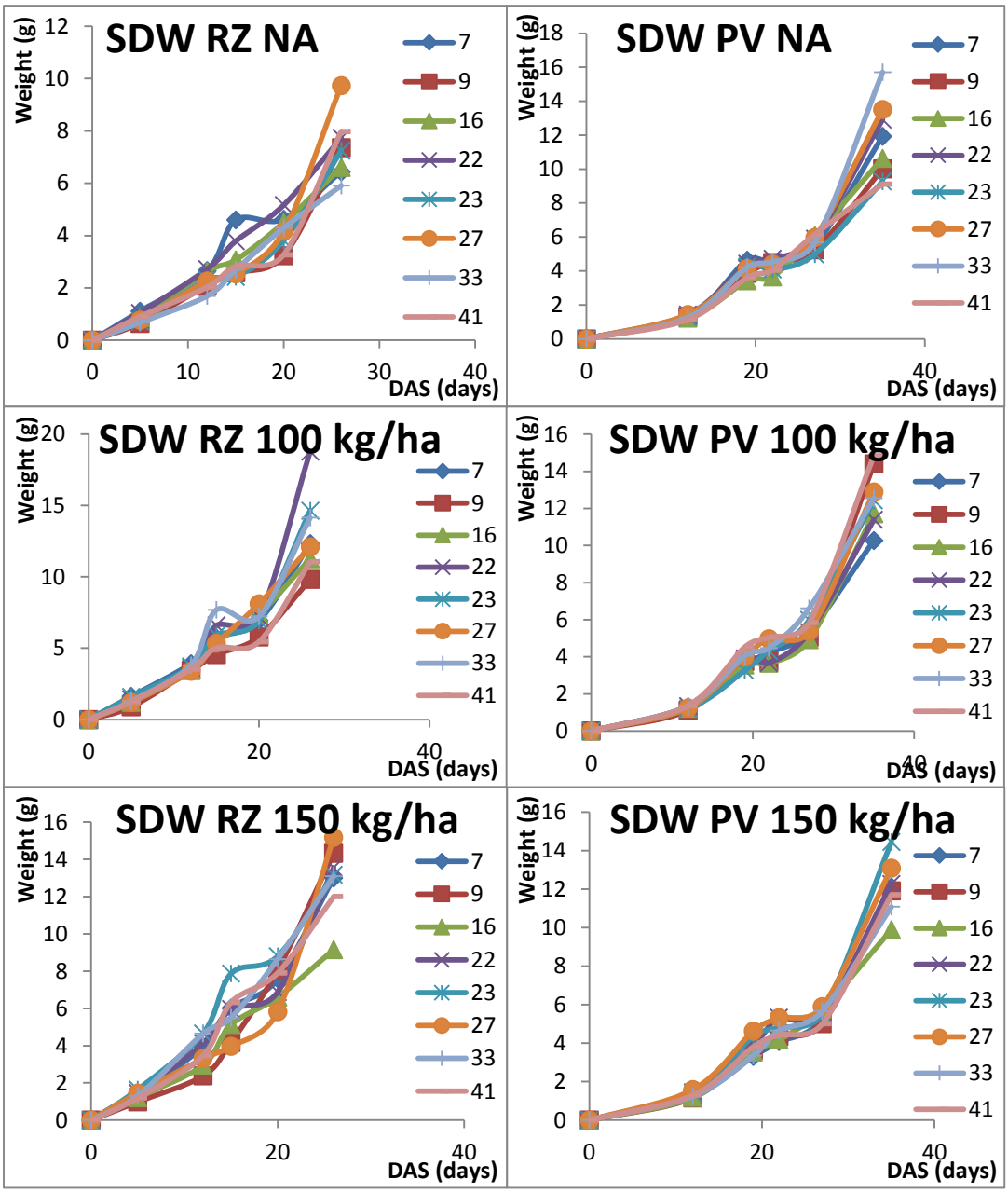



**Figure 23. Shoot Dry Weight (SDW) for eight genotypes at every N level of Rijk Zwaan (RZ) and Pop Vriend (PV).**

## Appendix 13: Extra Graphs Soil Coverage Rijk Zwaan

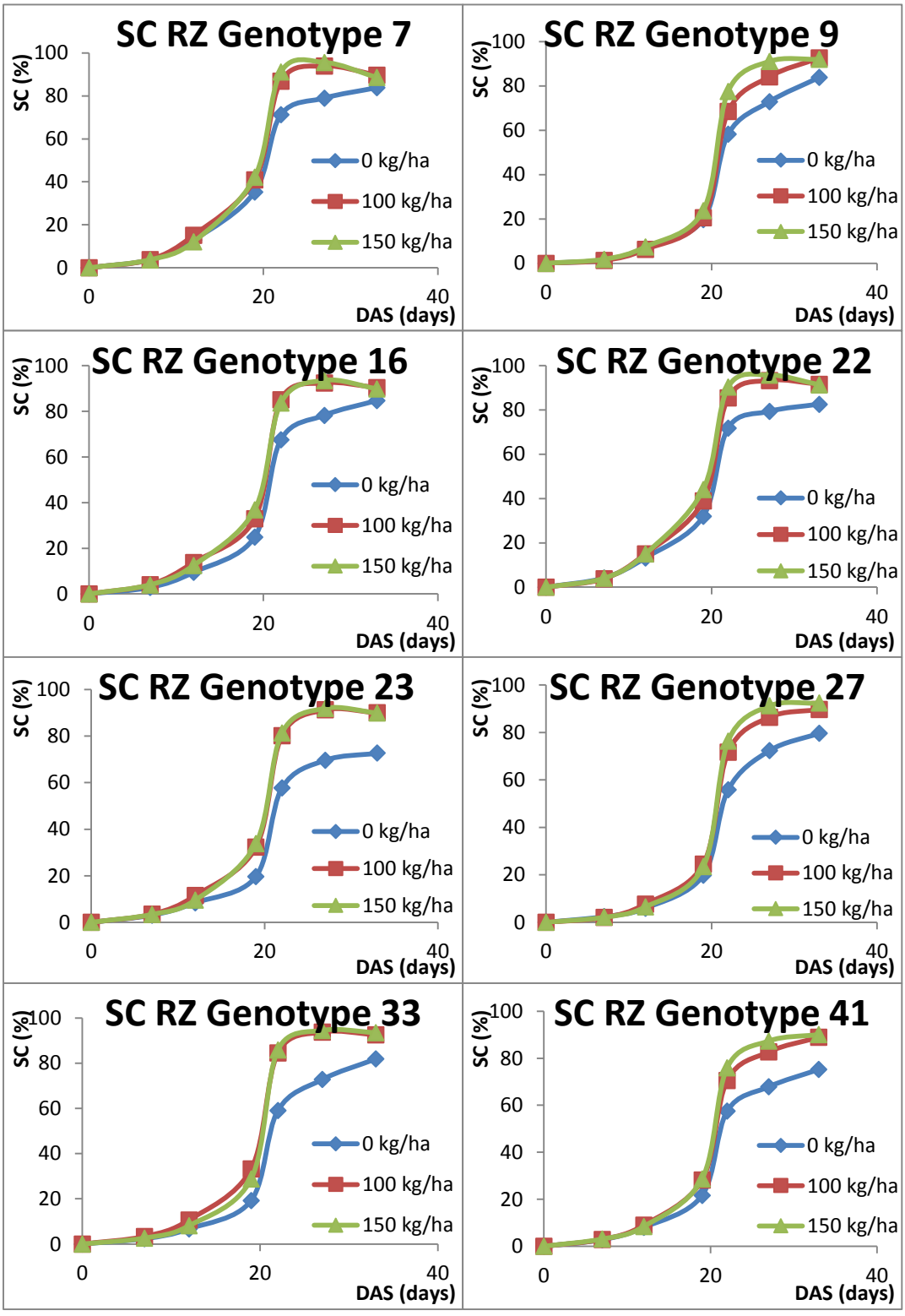



**Figure 24. Soil Coverage (SC) for every genotype at Rijk Zwaan (RZ).**

# Appendix 14: Extra Graphs Soil Coverage Pop Vriend

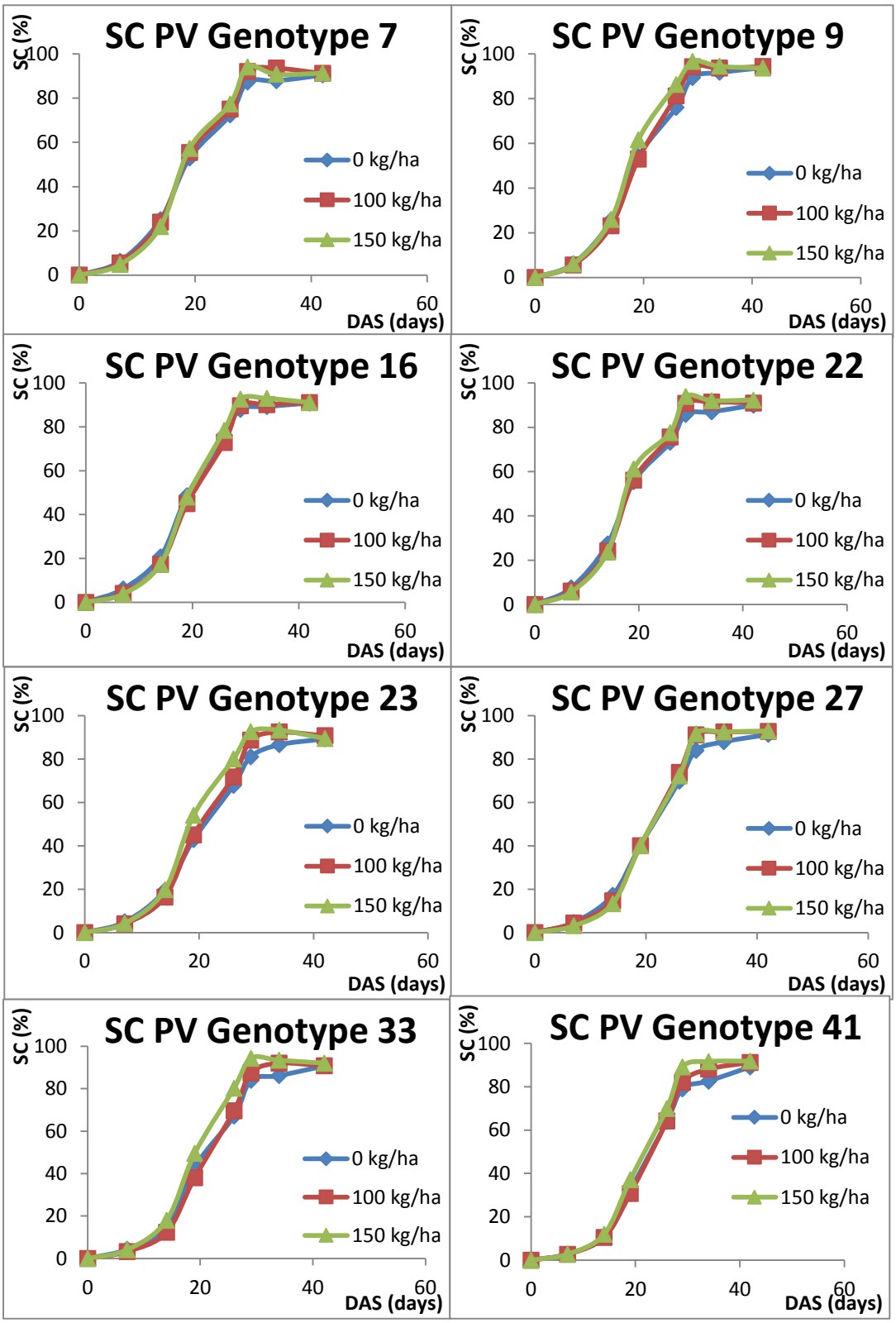



**Figure 25. Soil Coverage (SC) for every genotype at Pop Vriend (PV)**

# Appendix 15: ANOVA Table Rijk Zwaan

| | Harvest | Date | Leaf Set | Genotype | Nitrogen | Genotype x Nitrogen |
|---|---|---|---|---|---|---|
| **Shoot Dry Weight** | 1 | 06-05 | | <0,001 | 0,002 | 0,422 |
| | 2 | 13-05 | | <0,001 | 0,002 | 0,009 |
| | 3 | 16-05 | | <0,001 | <0,001 | 0,120 |
| | 4 | 21-05 | | 0,007 | 0,001 | 0,241 |
| | 5 | 27-05 | | 0,037 | <0,001 | 0,776 |
| **Shoot Fresh Weight** | 5 | 27-05 | | 0,007 | <0,001 | 0,347 |
| **Dry Matter %** | 5 | 27-05 | | <0,001 | <0,001 | 0,810 |
| **Root Dry Weight** | 1 | 06-05 | | 0,015 | 0,452 | 0,448 |
| | 2 | 27-05 | | 0,250 | 0,232 | 0,467 |
| **Root Length** | 1 | 06-05 | | 0,324 | 0,259 | 0,011 |
| | 2 | 27-05 | | 0,352 | 0,051 | 0,844 |
| **Surface Area Root** | 1 | 06-05 | | 0,695 | 0,636 | 0,891 |
| | 2 | 27-05 | | 0,570 | 0,038 | 0,513 |
| **Average Root Diameter** | 1 | 06-05 | | 0,049 | 0,498 | 0,729 |
| | 2 | 27-05 | | 0,311 | 0,037 | 0,036 |
| **Root:Shoot Ratio** | 1 | 06-05 | | 0,021 | 0,772 | 0,539 |
| | 2 | 27-05 | | 0,139 | 0,038 | 0,606 |
| **Soil Coverage** | | 23-04 | | <0,001 | 0,880 | 0,083 |
| | | 01-05 | | <0,001 | 0,189 | 0,226 |
| | | 06-05 | | 0,006 | <0,001 | 0,045 |
| | | 13-05 | | <0,001 | <0,001 | 0,005 |
| | | 16-05 | | <0,001 | <0,001 | 0,006 |
| | | 21-05 | | <0,001 | <0,001 | <0,001 |
| | | 27-05 | | <0,001 | <0,001 | 0,011 |
| **Chlorophyll Content** | | 06-05 | 1 | <0,001 | 0,012 | 0,896 |
| | | 13-05 | 1 | <0,001 | 0,003 | 0,209 |
| | | 21-05 | 1 | <0,001 | 0,018 | 0,249 |
| | | 13-05 | 2 | <0,001 | 0,006 | 0,067 |
| | | 21-05 | 2 | <0,001 | 0,003 | 0,357 |
| | | 27-05 | 2 | <0,001 | 0,004 | 0,401 |

# Appendix 16: ANOVA Table Pop Vriend

| | Harvest | Date | Leaf Set | Genotype | Nitrogen | Genotype x Nitrogen |
|---|---|---|---|---|---|---|
| **Shoot Dry Weight** | 1 | 07-05 | | <0,001 | 0,019 | 0,793 |
| | 2 | 14-05 | | <0,001 | <0,001 | 0,495 |
| | 3 | 17-05 | | <0,001 | 0,004 | 0,576 |
| | 4 | 22-05 | | <0,001 | 0,011 | 0,590 |
| | 5 | 30-05 | | <0,001 | 0,051 | 0,922 |
| **Shoot Fresh Weight** | 5 | 05-06 | | <0,001 | 0,465 | <0,001 |
| **Dry Matter %** | 5 | 05-06 | | 0,050 | 0,746 | 0,013 |
| **Root Dry Weight** | 1 | 07-05 | | 0,093 | 0,841 | 0,668 |
| | 2 | 30-05 | | 0,011 | 0,079 | 0,681 |
| **Root Length** | 1 | 07-05 | | 0,035 | 0,599 | 0,693 |
| | 2 | 30-05 | | 0,521 | 0,886 | 0,563 |
| **Surface Area Root** | 1 | 07-05 | | 0,061 | 0,340 | 0,368 |
| | 2 | 30-05 | | 0,402 | 0,247 | 0,687 |
| **Average Root Diameter** | 1 | 07-05 | | 0,024 | 0,441 | 0,396 |
| | 2 | 30-05 | | 0,738 | 0,929 | 0,876 |
| **Root:Shoot Ratio** | 1 | 07-05 | | 0,486 | 0,887 | 0,895 |
| | 2 | 30-05 | | 0,005 | 0,015 | 0,445 |
| **Soil Coverage** | | 25-04 | | <0,001 | 0,024 | 0,972 |
| | | 02-05 | | <0,001 | 0,480 | 0,883 |
| | | 07-05 | | <0,001 | 0,426 | 0,911 |
| | | 14-05 | | <0,001 | 0,003 | 0,806 |
| | | 17-05 | | <0,001 | <0,001 | 0,931 |
| | | 22-05 | | <0,001 | <0,001 | 0,074 |
| | | 30-05 | | <0,001 | 0,641 | 0,192 |
| **Chlorophyll Content** | | 14-05 | 1 | <0,001 | 0,017 | 0,051 |
| | | 22-05 | 1 | <0,001 | 0,009 | 0,160 |
| | | 29-05 | 1 | <0,001 | <0,001 | 0,144 |
| | | 14-05 | 2 | <0,001 | 0,060 | 0,301 |
| | | 22-05 | 2 | <0,001 | 0,329 | 0,493 |
| | | 29-05 | 2 | <0,001 | <0,001 | 0,025 |