Development of a breeding strategy for nitrogen use efficiency in spinach (*Spinacia oleracea* L.)

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Abstract

Spinach (*Spinacia oleracea* L.) is one of the most consumed leafy vegetables worldwide and it is considered to be highly nutritious. Spinach is a short-cycle leafy crop that has a high demand for nitrogen in order to rapidly come to a harvestable product that has the required dark green colour within a reasonable harvest window. In commercial production of spinach the recovery of N is poor, which may result in environmental pollution. To increase sustainability of both organic and conventional spinach cultivation there is a need to reduce the dependency on high levels of nitrogen. Growers therefore urgently need cultivars with a satisfactory yield under reduced N input conditions. Nitrogen use efficiency (NUE), defined as the ability to produce high biomass per unit N applied, is low in spinach. The present study aims to evaluate spinach genotypes for selectable traits under varying N supply and provide tools and knowledge to facilitate the development of varieties with good yield, quality and stability under low N input. To minimise environmental variation affecting the identification of traits related to NUE a screening method was developed using a hydroponics system. The genetic diversity for NUE related traits was first studied with 24 commercial cultivars under contrasting levels of N supply based on the Ingestad model with a steady-state N application. This demonstrated that the hydroponics screening strategy as a pre-screening tool enabled reliable detection of heritable variation among cultivars for NUE-related traits under optimal as well as suboptimal N input. Shoot dry weight and leaf area were preferred selectable traits for the detection of heritable differences contributing to NUE in spinach. The effect of N application strategy was examined in seven cultivars grown under hydroponics conditions with low and high N levels supplied either as a single bulk N application resembling N fertilization in field cultivation, or a steady-state N application according to Ingestad. The latter application strategy provided more stable and reproducible conditions for determination of genetic differences in NUE under low N conditions for a short-cycle leafy vegetable crop. Several tools for molecular genetic evaluation of NUE in spinach were provided as well, including a SNP marker set for marker-assisted breeding, a genetic mapping population with a corresponding genetic map, and the identification of two major QTL regions contributing to growth under low N conditions. With these tools, an efficient strategy for breeding for NUE efficiency in spinach would include screening under controlled conditions at high and low N using leaf area, biomass and root to shoot ratio as selectable traits, and QTL identification of genetic factors that can be targeted and combined using marker-assisted selection. An in depth

genotype by environment interaction analysis using six field trials showed that environmental factors like temperature, soil, and management strongly influence nitrogen availability in the soil in a short cycle crop like spinach. This severely complicates selection and breeding for NUE of spinach under field conditions, and emphasizes the importance of performing trials under better controllable conditions for genetic dissection of NUE and discovery of genetic factors contributing to NUE. It also underscores the importance of validating these findings in various field trials. Multi-environment field trials with different levels of N fertilization will then allow selection of cultivars that combine stable performance under various low input growing conditions with high yields under more favorable conditions.

Keywords: *Spinacia oleracea* • breeding • nitrogen use efficiency • screening method • Ingestad method • N application regimes • G×E • genetic map • SNP markers • QTLs for NUE

"The greatest service which can be rendered to any country is to add a useful plant to its culture"

-Thomas Jefferson, 1800

To Marcela and Elena, obviously

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Chapter 1

General Introduction

Chapter 1. General Introduction

Spinach origin and wild relatives

The origin and domestication of *Spinacia oleracea* are still largely unknown (Andersen and Torp 2011). Spinach was first mentioned in China around 600 A.D. where it was referred as the herb of Persia (Hallavant and Ruas 2014). Early archaeobotanical evidence demonstrates that spinach was introduced into Spain by the Moors, where it was cultivated since at least the 11th century and from where it later was introduced into France and the rest of Europe (Hallavant and Ruas 2014). There is documentation that spinach was used as a common garden vegetable in Germany (1280), England and France (1500) (Ryder 1979, Nonnicke 1989, Decoteau 2000, Swiader and Ware 2002). In North America, spinach was brought by early colonists and by 1806 three varieties were recognized. The first savoy variety was introduced in 1828 (Nonnicke 1989, Decoteau 2000).

S. oleracea is a wind-pollinated, bisexual and diploid species (*2n*=12) of the Amaranthaceae family (ex-Chenopodiaceae subfamily Chenopodioideae), which also includes crop species like sugar beet, chard and quinoa. The wild ancestor of spinach is not exactly known, but currently the general assumption is that one of the two wild species *S. tetrandra* Stev. and *S. turkestanica* Iljin is the probable progenitor of spinach. Those two wild species related to spinach are a valuable source of genes conferring resistance to common diseases to cultivated spinach (Andersen and Torp 2011).

Main breeding goals

The breeding of spinach is mainly focused on resistance to diseases and pests. Spinach suffers from at least 35 pests and diseases but less than one third of them may cause significant crop losses (Koike et al. 2011). Resistance to downy mildew (*Peronospora farinosa* f.sp. *spinaciae*) is the primary emphasis in breeding (Morelock and Correll 2008). The fast rate of development of new races of downy mildew, currently already 15 official races are known (Plantum 2014), requires frequent introduction of new resistance genes. Currently, an important goal of the spinach breeding companies is to find genes that confer durable resistance to all the downy mildew races. Another focus of spinach breeding is yield

improvement. The parental lines of modern hybrid varieties are selected for unisexual flowering to facilitate hybrid seed production. The main gains in spinach yield have largely been attributed to the development of hybrid cultivars (Morelock and Correll 2008). Research indicates that heterosis between inbred lines depends on their genetic distance. Mukhanova et al. (1979) determined that spinach F1 hybrids yielded 30 to 40% more than the parental lines. By 2006, 85-90% of the spinach production was coming from hybrids (Morelock and Correll 2008).

Spinach is mainly a dioecious species but monoecious plants do occur. There is a high interest in the use of strict male lines with a high degree of homozygosity. Monoecious plants can be self-pollinated, which enables the efficient production of such lines. Yamamoto et al. (2014) determined that the genes for dioecism (Y) and monoecism (M) were closely linked. Therefore, those genes are also a target in breeding.

Although spinach is considered as one of the most nutritious leafy vegetables, the growing interest in human nutrition is a new impetus to breed for optimal profiles of vitamins, minerals and antioxidants (Morelock and Correll 2008). Spinach is rich in important constituents of human diets such as magnesium, potassium, calcium and iron (Zhang et al. 1989; Mills and Jones 1996; USDA 2002). It is also a good source of antioxidants and one of the vegetables with the highest values for oxygen radical absorbance capacity (ORAC) (Prior 2003). Likewise, spinach ranks highest for β-carotene and second behind kale (*Brassica oleracea* L. var. acephala D.C) for lutein (Holden et al. 1999, Lefsrud et al. 2007). Lutein is a phytochemical effective in the prevention of age-related macular degeneration and preliminary studies have shown a considerable variation in fresh leaves lutein content of spinach cultivars (Murphy 2001). An unfavourable health-related trait of spinach is its high content of oxalic acid (Kitchen et al. 1964) that can lead to the formation of kidney stones upon digestion of spinach (Wang et al. 2015) and the high nitrate content which may produce methaemoglobinaemia in young infants (Santamaria 2006). The reduction of oxalates and enhancement of the production of beneficial health-related compounds thus are important breeding targets in spinach.

Spinach breeding is mainly carried out by private breeding companies, which occasionally work together to solve common problems. An example is the International Working Group on *Peronospora farinosa* (IWGP). The IWGP is an organization set up by the Dutch seed association Plantum including Naktuinbouw (Netherlands Inspection Service for Horticulture) and the spinach breeding companies: Pop Vriend Seeds, Monsanto, Rijk Zwaan, Nunhems

(Bayer Crop Science), Takii, Sakata, Bejo Zaden, Enza Zaden, Syngenta, and Advanseed. The IWGP is supported by the University of Arkansas and the University of California Cooperative Extension in the United States (US). IWGP monitors the development of new races of *P. farinose* on spinach by testing potential field isolates on a differential set of cultivars that cover the full range of available resistances. IWGP is the responsible to denominate new races of *P. farinosa* (Plantum 2014, Naktuinbouw 2015). This organisation supports the breeding the companies for resistant spinach cultivars.

Spinach cultivation and seed production

Spinach is one of the most consumed leafy vegetables worldwide. It has been considered highly nutritious for generations and is mostly consumed fresh (Lucier and Plummer 2003). As a versatile food, it also is an ingredient of many cooked dishes. Therefore, its production can be divided to generate commodities for both fresh (bunched or bagged) and processed (sterilized and pelleted or frozen) use.

Spinach production has shown a stable increase in recent years worldwide. From 2010 to 2012 there was an increase of approx. 1.5 million tonnes (see Table 1). The major producer of spinach in the world is China with a production of approx. 19.5 million tons in 2012. Far behind China, the second largest producer in 2012 was the United States of America with 0.3 million tonnes.

The total area of spinach in the Netherlands is currently approx. 2000 ha of which some 390 ha is cultivated organically and 1720 ha conventionally (CBS 2015). In 2010, frozen spinach covered approx. 38.4% of the total Dutch spinach market with a turn-over of 27.8 million euros, while fresh and sterilized spinach had a respective market share of 55.5% and 6.0% with turn-over of 40.2 and 4.4 million euros. In the last few years, the total spinach market in the Netherlands increased to approx. 80 million euros due to the increase of baby leaf production (personal communication, H. Verwegen 2015). The Netherlands is market leader for the export of both conventional and organic frozen spinach products.

Table 1.1. General data on area and production of spinach from 2010-2012 based on the data of FAOSTAT (2015).

	Harvested area (× 1000 ha)			Production (× 1000 tonnes)		
Year	**2010**	**2011**	**2012**	**2010**	**2011**	**2012**
China	655.1	678.5	750.5	18,129.5	18,782.9	19,513.0
Netherlands	1.8	1.8	1.8	29.5	34.0	29.0
USA	17.5	17.3	17.8	397.6	409.4	354.0
Europe	31.5	34.5	31.4	523.6	590.8	547.1
World	840.6	867.3	938.3	20,235.9	20,980.9	2,1662.6

Spinach produces a rosette during the vegetative growth phase and is usually produced in cool seasons. The spinach leaves can be rounded to pointed and may range from flat to fully savoy (crinkled). Spinach is an annual crop with a short time to harvest: three to five weeks for babyleaf production and five to eight weeks for leaf spinach for the fresh and frozen-industry market (Prior 2003). Processing spinach is allowed to grow for longer periods of time than fresh spinach (48 to 90 days after planting (DAP)), resulting in higher yields of a product with larger and thicker leaves. Baby leaf spinach can be harvested 21 to 40 DAP, depending on the season. Baby leaves have lower yield but are sold at a higher price and they are sold fresh in bags and also in mixed salads (Koike et al. 2011, Simko et al. 2014).

The planting densities have increased over the years to increase yield; for baby leaf production the sowing density is between 8.6 - 9.9 million seeds per hectare (Koike et al. 2011).

Spinach can be cultivated successfully in many climates. However, for seed production spinach favours areas with long days and cool maritime weather conditions. Nowadays, Denmark is the main seed production area in the world. It has perfect conditions because of its geographical location that allows seeds to be produced during long summer days with a mild coastal climate. In 2012 it was estimated that the Danish spinach seed production covered 75% of the global market (Deleuran 2012). The seed originally had spikes but currently the seed is smooth, which is easier to handle and can be planted accurately (Morelock and Correll 2008). A large percentage of the spinach seed production activities in Denmark occurs for seed companies from the Netherlands. The Netherlands is world leader in spinach breeding and serves not only a major part of the European seed market (95%), but

also serves to a large extent the markets in the US (95%) and Oceania (80%). In recent years the demand for spinach seed has more than doubled due to the increased cultivation of baby leaf spinach requiring a relatively high sowing density (Baas 2006).

Organic spinach production

Consumer demand for organic products is increasing, reflected in the significant market growth of 11.5% in the US, the world's largest organic market (Willer and Lernoud 2015). Organic agriculture refrains from chemical-synthetic inputs such as fungicides, pesticides and mineral fertilisers (Kristiansen et al. 2006). Organic management is based on organically derived inputs such as compost and animal manure and aim at stimulating long-term biological self-regulatory processes to achieve resilience for stable crop production. However, organic growers have fewer means to control the growing conditions in the short-term when weather or soil conditions are less favourable. This requires cultivars with stable performance under variable growing conditions. Currently, organic growers depend largely on cultivars bred for the conventional management system based on high-external input (Lammerts van Bueren et al. 2002). Organic spinach growers in the Netherlands have indicated that their produce deteriorates more rapidly than conventionally grown crops due to limited availability of nitrogen during the growing period. As a consequence the harvest window is much shorter than for cultivation under high input conditions (Lammerts van Bueren and Ter Berg 2009). The spinach value chain partners have identified adaptation to low input of nitrogen in order to maintain quality and a reasonable harvest window as the most essential crop characteristic for spinach (Lammerts van Bueren and Ter Berg 2009).

Nitrogen: the most essential nutrient in agriculture

The global increase in production area of vegetables such as spinach is accompanied by an increase in nitrogen (N) consumption. N is essential for agricultural production and it is the macronutrient that most frequently limits plant growth in an agricultural cropping system and therefore the most consumed macronutrient (Table 2). For every 20-50 g of N taken up from the soil, a non-legume plant is able to produce approximately 1 kg of dry biomass (Robertson and Vitousek 2009).

Table 1.2. Forecast for the global demand of the three main fertilizers for 2011 to 2015 (in thousand tonnes) (FAO 2015).

Year	**2014**	**2015**	**2016**	**2017**	**2018**
Nitrogen (N)	113,147	115,100	116,514	117,953	119,418
Phosphate (P_2O_5)	42,706	43,803	44,740	45,718	46,648
Potash (K_2O)	31,042	31,829	32,628	33,519	34,456
Total ($K+P_2O_5+K_2O$)	186,895	190,732	193,882	197,190	200,522

The atmospheric dry air consists of 79% N_2 and represents the largest global N resource. In the 1920s and 1930s, Haber and Bosch discovered a way to use it for the synthesis of ammonia (NH_3), a fertilizer widely used in crop production systems. The Haber-Bosch reaction has drastically changed the global use of N fertilizers. However, the process requires high amounts of energy. The United Nations Environment Programme for the Industry and the Environment (1998) estimated that 873 m^3 of natural gas is needed for the production one metric ton of N fertilizer synthesized through the Haber-Bosch process. Other primary N fertilizers used for crop production, i.e. ammonium nitrate, urea and calcium ammonium nitrate are chemical derivatives of ammonia (UNIDO and IFD 1998).

The discovery of the Haber-Bosch process and its broad commercialization resulted in a substantial and still increasing use of nitrogen in agriculture (Robertson and Vitousek 2009). Nowadays, the fossil energy-based N resources used annually for crop production exceed the amount of N fixed each year by all natural processes together (Galloway et al. 2008). Currently, our society is seen as a nitrogen-based economy (Robertson and Vitousek 2009). The Haber-Bosch process represented an essential breakthrough for modern agriculture. It is considered that half of the current world population is able to survive due to this NH_3 synthesis process (Erisman et al. 2008).

N metabolism is influenced and modulated by multiple factors such as leaf growth, leaf photosynthesis, storage and translocation of N reserves; all these factors are of foremost importance to sustain the growth of the plants (Hirel et al. 2007).

Nitrogen: from the soil to the harvest

Nitrate is the principal nitrogen source for wild and cultivated crop species. After nitrate uptake by specific transporters through the root cell membrane (Orsel et al. 2002), reduction of nitrate occurs predominantly in the shoot cytoplasm in two steps by i) nitrate reductase (NR), which reduces nitrate to nitrite and ii) nitrite reductase (NiR), which reduces nitrite to ammonium. Root-specific transporters (Glass et al. 2002) also allow a direct absorption of available ammonium (Loqué and von Wirèn 2004). The ammonium uptake across the root cell membrane is highly regulated to avoid a toxic accumulation within the plant (Loqué and von Wirèn, 2004). Ammonia is then transferred to glutamine by glutamine synthetase (GS). The reaction catalysed by GS is considered to be the major N assimilation route facilitating the incorporation of inorganic nitrogen into organic molecules in conjunction with Ferredoxin-dependent glutamate synthase (Fd-GOGAT). Fd-GOGAT recycles glutamate and incorporates carbon skeletons into the cycle. Glutamine and glutamate are used as amino group donors for other amino acids used for protein synthesis and as constituents of DNA and RNA synthesis (Hirel and Lea 2001).

Over-fertilization: an environmental problem and a risk for humans

The use of N has increased food production at a faster rate than the world's population growth (Robertson and Vitousek 2009). N fertilization as such has a clear positive effect on agricultural systems. As a consequence annual N applications have increased ten-fold from 1950 to 2008; a development often creating situations with an unbalanced N supply (over-fertilization), which may adversely affect non-agricultural environments (Robertson and Vitousek 2009). Inappropriate management of N fertilization is accompanied by elevated environmental costs. A great part of the N added to the soil does not reach its ultimate and main target: produce sufficient plant biomass for the human diet (Lasaletta et al. 2014). Excessive use of nitrogen in agriculture has resulted in environmental problems such as water eutrophication and increased greenhouse gas production (Erisman et al. 2008). The leaching of soil N from fertilizers can pollute freshwater and marine ecosystems causing excessive growth of algae (Hirel et al. 2007). Nitrates from commercial fertilizers may in this way contaminate water resources used for human consumption in both the developed and developing world (Wolfe and Patz 2002). In N-poor ecosystems extra N is expected to cause a reduction of species diversity, since species differentially respond to N (Robertson and

Vitousek 2009). Species responsive to N may out-compete less responsive ones. In all, N is the major fertilization cost for production but at the same time the pollution costs due to over-fertilization are very high. In the EU these were estimated to be between €70 billion to €320 billion per year (Sutton et al. 2011).

The Declaration of the World Summit on Food Security (FAO 2009) calls for an average annual increase in food production of 44 million metric tonnes to feed approximately 9 billion people by 2050 (Tester and Langridge 2010). Accordingly, N fertilizer application is expected to increase by approximately threefold in the next 40 years (Good et al. 2004). One solution to reduce dependency on high levels of N is creation of crops able to grow well under low N input conditions (Xu et al. 2012).

Improving nitrogen use efficiency

Nitrogen use efficiency (NUE) can be determined in different ways, as agronomic efficiency (yield per unit of N applied) or as physiological efficiency (total biomass per unit of N uptake) (Dresbøll and Thorup-Kristensen 2014). NUE can be broken down into two components: uptake efficiency (NUpE) and utilisation efficiency (NUtE). NUpE is the capacity of the root system to uptake N from the soil (which usually is considered as the percentage of available N acquired by plant) and NUtE is the fraction of the N acquired that is converted in total plant biomass (Xu et al. 2012). The most widely applied measure for NUE is to evaluate the yield per unit of added N.

Dresbøll and Thorup-Kristensen (2014) emphasized that NUE can be analysed not only as a characteristic of a plant but also a characteristic of a specific crop and of a cropping system. Much research in the past has focused on improving NUE through agronomic management (Swain et al. 2014). Used strategies include methods i) to adjust the crop rotation system, ii) to provide dedicated decision support tools to farmers, iii) to improve the N fertilization management of cropping systems, and iv) to improve irrigation. For decades, farmers focused on general improvement of the soil fertility to improve response to N.

In addition, crop breeding strategies are now being explored to adapt plants to low input conditions (Lemaire and Millard 1999). Breeding for improved NUE should be an essential part of an integrative approach to reduce high demands of N for crops.

Improving NUE of a crop through breeding has only recently gained more interest and has proven to be difficult, as NUE is an inherently complex plant trait. It comprises many components including N uptake, translocation, assimilation, and remobilization. Each is under control of genetic factors which may be expressed differentially under low or high input conditions. Factors determining maximal NUE differ under high and low N conditions (Gallais and Hirel 2004, Hirel et al. 2007, Chardon et al. 2010, Xu et al. 2012).

Since most breeding programs select plants under optimal N conditions and there is a need to reduce the amount of applied nitrogen in agriculture it is likely that there is scope to improve NUE under low input growing conditions. Low N efficiency of annual cropping systems has several causes, such as a short growing cycle, the inability to remove N from soil efficiently and the habit of most growers to use more N than strictly required to minimize economic risks (Robertson and Vitousek 2009). NUE has been reported to decrease when N input increases (Zebarth et al. 2004), therefore the optimal cultivar requires both a good response to N and to be high yielding under low N conditions (e.g. Ospina et al. 2014).

Problems of spinach cultivation under reduced nitrogen conditions

Spinach has a high demand for nitrogen in order to rapidly come to a harvestable product with a dark green colour as required by the market (Smolders et al. 1993, Stagnari et al. 2007). The nitrogen use efficiency of spinach is low; studies by Neeteson and Carton (1999) determined that from the recommended fertilization (215 – 290 kg N ha^{-1}) 160-220 kg N ha^{-1} was not recovered in the harvested product. In the past the subject of several agronomic and physiological studies was the relation between nitrogen fertilization and (1) crop growth to optimize the production of spinach (e.g. Smolders and Merckx 1992, Biemond 1995, Biemond et al. 1996), and (2) seed yield to optimize seed production (Deleuran et al. 2005). No breeding studies have been reported on selection of spinach genotypes for NUE or on the development of cultivars that can deliver a good and commercially attractive yield under low nitrogen fertilization conditions (e.g. 100 kg N ha^{-1}). From organic spinach production it is known that the yield stability of the current cultivars under low input conditions is too low. In 2008, 50% of the organic spinach fields could not be harvested (Lammerts van Bueren and Ter Berg, 2009). A set of 21 spinach accessions had been evaluated for adaptation to organic, low input growing conditions; none responded well to low nitrogen availability (Serpolay et al. 2011).

Plant breeders consider the adaptation to low nitrogen availability also as crucial for the realization of a sustainable conventional production. Regulatory requirements in agriculture underline the need for NUE cultivars. Since 1991 there is in the European Union the Nitrates Directive (Directive 91/676/EEC) known as NiD in effect to reduce water pollution caused or induced by nitrate and phosphorus from agricultural sources. NiD intends to reduce the environmental impact of fertilizer and manure, and legally restricts the annual farm application to 170 kg ha^{-1} of nitrogen, or in case of derogation to inputs up to 250 kg N ha^{-1} (Oenema 2004, van Grinsven et al. 2012).

As the increase of nitrogen levels in fertilisation may lead to an increase of nitrate concentration in leafy vegetables such a spinach, and can lead to health problems, the European Union also took measures to regulate the content of nitrate of leafy vegetables, and as spinach may have a high nitrate content, the EU limits it for fresh spinach harvested from 1 November to 31 March 2005 to maximally 3000 mg NO_3^- kg^{-1}, from 1 April to 31 October to 2500 mg NO_3^- kg^{-1} and for deep-frozen or frozen spinach to 2000 mg NO_3^- kg^{-1} (Santamaria 2006).

It is expected that in the near future nitrogen will be the limiting factor for spinach production both in conventional and organic agriculture, which stresses the need for selection for NUE as an essential part of future spinach breeding.

Nitrogen use efficiency is a complex trait

There are no reports on breeding for NUE in spinach. Most studies on improving NUE are done with long cycle and seed producing crops such as oilseed rape, maize, rice and wheat (see e.g. Hirel et al. 2007). Such crops differ substantially in their physiology with respect to N metabolism e.g. due to translocation of nitrogen from leaves to the reproductive parts in the plant compared to a short cycle, leafy vegetable such as spinach. Multiple researches showed that NUE is a complex trait with quantitative inheritance, and that it is important to dissect NUE into underlying component traits (Gallais and Hirel 2004, Hirel et al. 2007, Chardon et al. 2010, Xu et al. 2012). For spinach, plant traits influenced by nitrogen and therefore relevant to be included in this research are leaf chlorophyll content, leaf area and shoot biomass (Biemond 1995). Kerbiriou et al. (2013) indicated that also the genetic variation among lettuce cultivars in root development under water and nitrogen stress conditions influences differentially the shoot growth, but below-ground traits are not easy to assess for

practical breeders. Breeders are especially interested to identify easily assessable traits that are related to NUE (Lammerts van Bueren et al. 2014).

In a number of studies with low N levels leaf nitrogen content has shown to be highly correlated with chlorophyll content (Lefsrud et al. 2007). Chlorophyll content is an example of a trait that can be easily assessed with a hand-held chlorophyll meter (SPAD meter). In the case of maize, measurements during the vegetative growth stage with this device gave reliable estimates of the leaf N content (Hirel et al. 2007). Plants grown under growth-limiting N levels were stunted, showed characteristic leaf chlorosis and had reduced contents of chlorophyll a and chlorophyll b pigments. Additionally, chlorophyll measurements can be indicative of breaking down of chlorophyll (senescence) and remobilization of N and other nutrients upon leaf ageing (Smart 1994). Nitrogen has also shown to affect spinach leaf area expansion and final leaf size (Biemond 1995). It is thought that N influences the cell divisions and the time of division of meristematic zone; in all the more N the more mitotic cells (Dreccer 2006).

In the case of shoot biomass, Lefsrud et al. (2007) found in a spinach study with two varieties small differences in response to N. Several agronomic studies were performed to improve the response to nitrogen fertilization for spinach. The effects of the N concentration of different N sources (nitrate and ammonium) and the effect on the partitioning of the dry matter were studied by Smolders and Merckx (1992) and Smolders et al. (1993). Biemond et al. (1996) investigated the relation between dry matter and N accumulation in spinach leaf blades, petioles and stems and how the nitrate concentration decreases with leaf age, and another study examined the higher affinity for nitrate instead of ammonium (Elia et al. 1998). N use efficiency based on the root uptake of field grown vegetable crops was studied by Neeteson and Carton (1999) and the effects of how and when fertilizers were applied by Deleuran et al. (2005). These studies were carried out with a limited number of cultivars and have not resulted in well-defined criteria for large-scale screening of spinach germplasm for NUE.

Screening methodologies for NUE

The efficiency of N use is a heritable multi-faceted characteristic controlled by a complex of highly interrelated plant characteristics which are also influenced by environmental factors (Xu et al. 2012). Many studies on genetic variation for NUE in various crops reported large interactions between genotype and N stress and other environmental conditions (Cabrera-

Bosquet et al. 2007, Anbessa et al. 2009, Beatty et al. 2010, Wei et al. 2012, Chen et al. 2013). The investigations of Bouchet et al. (2014), using an oilseed rape mapping population are a good example of such studies in which they determined genomic regions (Quantitative Trait Loci (QTLs)) associated with yield and assessed their stability under contrasting nitrogen nutrition regimes. They found many QTLs related to yield and yield components that were stable across N conditions within trial years but not across trial years. Kerbiriou (2014) found in her study on the contribution of genetic variation of lettuce root traits to (the variation in) resource capture and field performance that the mechanisms regulating resource capture use efficiency showed a lot interdependency between genotype and environment which masked the direct genotypic effects.

Thus, genetic and breeding studies to uncover heritable genotypic differences in plant growth related to NUE are therefore challenged to develop effective, reliable and relatively high throughput testing methods, both for the development of marker-aided selection tools and for phenotypic selection. To be able to better control the environmental factors this study aimed at the development of a controlled procedure for NUE screening and trait discovery. The basis is a good control of the N availability, whereas all other conditions should be optimal for growth. van Loo et al. (1992) developed a screening procedure using hydroponics to improve plant growth in perennial ryegrass. Spinach is quite suitable for testing under such conditions because of its small plant size and a short life cycle. Smolders and Merckx (1992) evaluated a single spinach cultivar on hydroponics and showed that the relative growth rate (RGR) of plants depends of the nutrition treatment and decreases during development in all treatments. The use of a hydroponics system has the advantage that different N application scenarios can be tested, such as a single application at the start of the experiment, split applications or various growth-dependent N applications based on the model presented by Ingestad (1982). N application using the latter strategy is proportional to the daily growth as expected at a certain level of N limitation. The advantage is that with this strategy plants experience a more or less equal strain due to N over the whole growing period.

With a reliable screening method of spinach cultivars for growth under different N input conditions, traits contributing to NUE can be identified, and genotypes contrasting for NUE selected. These can for instance be used as parents to generate a dedicated mapping population segregating for genes controlling NUE, and to unravel the genetic control of genotypic differences in NUE by mapping genomic regions controlling traits that contribute to NUE. This not only requires adequate phenotyping but also extensive molecular genetic

characterization of such a population. For spinach, little genetic information is available. There is only one genetic linkage map of spinach publicly available (Khattak et al 2006), which was used to map genes involved in sex expression, followed by other studies to fine-map the sex locus (Onodera et al. 2011, Yamamoto et al. 2014).

Although screening methods under controlled conditions can be very useful in uncovering traits contributing to a complex trait like NUE and genes linked to such related traits, these results should always be validated in practice under a broad range of field conditions (Gutierrez 2012).

Scope of research

The present study aims to develop breeding tools and identify spinach genotypes that will facilitate the development of varieties that perform well in terms of yield, quality and stability under low input of nitrogen. This will help to solve problems that hamper the development sustainable conventional as well as organic spinach production. To meet the objectives of the present thesis, research was proposed to answer the following research questions:

1. How can spinach genotypes be efficiently evaluated for NUE to optimize growth under low N input conditions?
2. What is the magnitude of genetic variation for traits affecting NUE in spinach?
3. What is the genetic basis of the variation in NUE-related traits in spinach?
4. What is the impact of genetic variation in NUE-related traits under field conditions and rates of N application?

Outline of this thesis

The present thesis consists of six chapters that include this introduction (Chapter 1). The initial investigations were focused on the development of an adequate screening procedure for growth at low N using hydroponics with controlled application of N (Chapter 2). This resulted in selection criteria for improving growth at low N supply. The screening procedure was subsequently used to evaluate the genetic diversity for NUE among a set of spinach cultivars (Chapter 3). The genetic diversity assessment was the basis for selection of two parents contrasting in growth in response to N. These were used to generate a dedicated mapping

population (F2) for genetic analysis of NUE and NUE-related traits as is described in Chapter 4. To this end a large set of polymorphic gene-based SNP markers were generated and used for characterizing the mapping population and to create a molecular map. In parallel F2 plants from the mapping population were selfed to get F2:3 lines which subsequently were evaluated on hydroponics. The molecular data and map were finally used to analyse the genetic variation among F2:3 lines to identify QTLs for NUE-related phenotypic traits. Chapter 5 comprises a GxE study with cultivars that were also used in the hydroponics study described in Chapter 3. The basis of this study was a multi-environment trial with differences in N fertilization, management and sowing time in which the progression of shoot growth and soil coverage was recorded. Prior to the GxE analyses a non-parametric curve fitting procedure was used to achieve data better enabling a cross comparison of cultivar performance over environments and time. On top of these analyses, trait-specific stability analyses were performed as well as and factorial regression analyses to get insight on the impact of the environment on the cultivar performance. Synthesis of the main outcomes of the research presented in the preceding chapters finally is given in Chapter 6 with emphasis on their implementation in the context of a breeding company, societal implications and on potential follow up research.

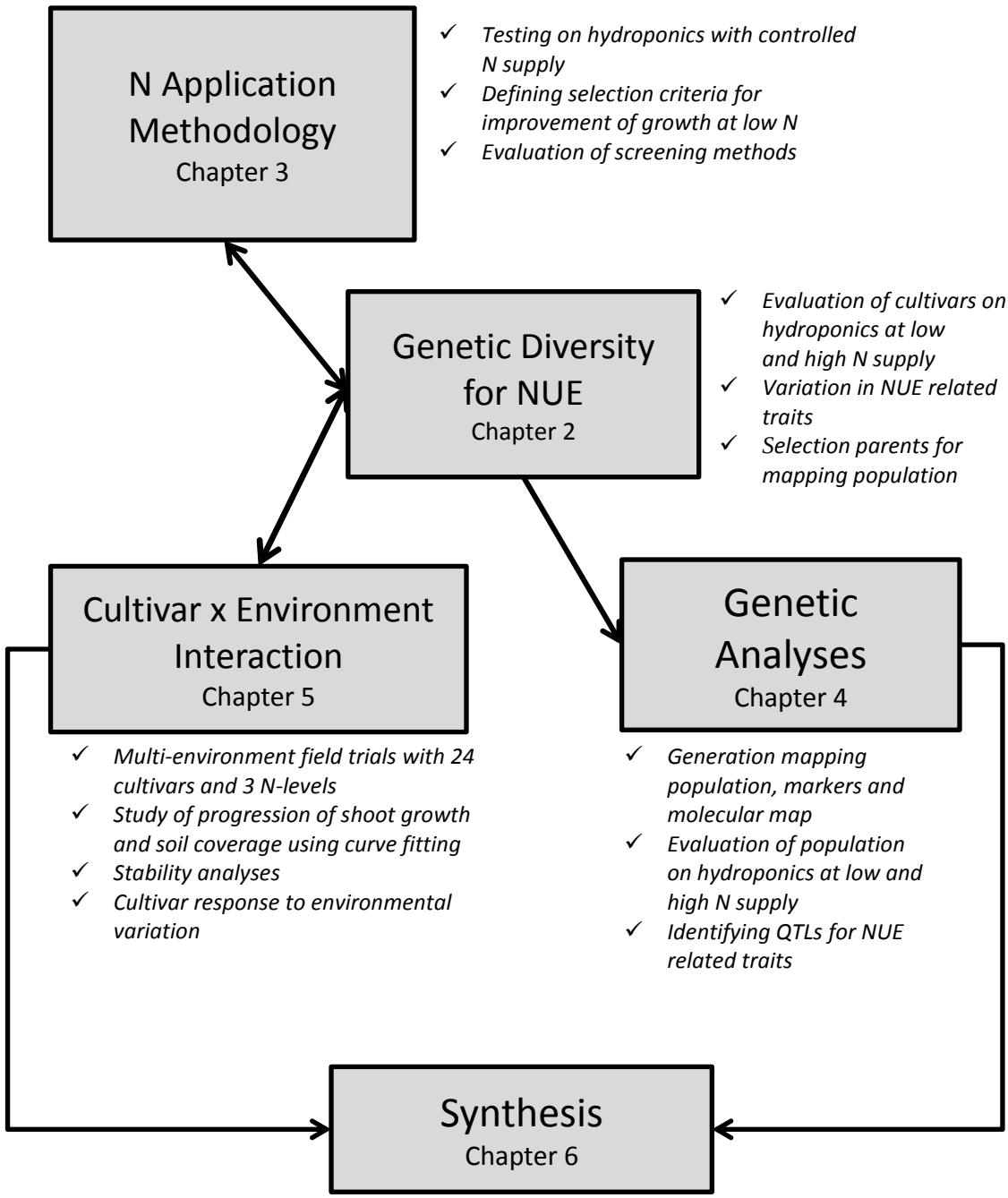

Figure 1.1. Methodological framework of this thesis

Chapter 2

Genetic diversity of nitrogen use efficiency in spinach (*Spinacia oleracea* L.) cultivars using the Ingestad model on hydroponics

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Abstract

Spinach is a leafy vegetable that requires a high N fertilization to have a satisfactory yield and quality, in part because it has poor nitrogen use efficiency (NUE). Therefore, there is a need to breed for cultivars with an excellent NUE. To this end the genetic diversity for NUE-related traits was studied in a diverse set of commercial cultivars. This set was evaluated in a hydroponic system using the Ingestad model; the system was set at a relative growth rate of 0.14 and 0.18 g g^{-1} day^{-1} (low and high N, respectively). Experiments were performed at low and high plant density. Traits monitored for single plants included fresh and dry weight, leaf area, specific leaf area, dry weight ratio between root and shoot, and chlorophyll content. The high density experiment showed more genotypic variation for the observed traits than the low density one. Biomass production was considerably lower at low than at high N. Path analysis revealed that leaf area had the highest direct effect on NUE, while specific leaf area was an important trait determining variation in NUE at low N. Slow and fast growing genotypes were shown to use different strategies to utilize N, and these strategies are expressed differently at high and low N availability. This indicates that improving spinach for NUE is feasible using the analysed genotypes as source material, and different strategies can be targeted for adaptation of spinach cultivars to low N conditions.

Keywords: Spinach • Hydroponics • Ingestad model • NUE • Nitrogen use efficiency

2.1. Introduction

Spinach (*Spinacia oleracea* L.) is a popular vegetable crop with a gradual increase in production. In 2011, the global area under cultivation was 0.88 million ha and in the preceding decade the global production increased by 128 % (FAOSTAT 2013). The growth in spinach consumption may be associated with its excellent nutritional value. This leafy vegetable is considered to be an important source of nutrients such as vitamins C and A, carotenoids, flavonoids, folic acid, calcium and magnesium (Koh et al. 2012). Europe represents only 3.72 % of the worldwide spinach production (FAOSTAT 2013).

As many leafy vegetable species, spinach requires a high amount of nitrogen (N) for optimal growth, which is often realized by N over fertilization (Barker et al. 1971, Cantliffe 1973,

Stagnari et al. 2007). N fertilization not only influences yield but also leaf appearance, expansion and senescence and thus product quality (Biemond 1995). In commercial production of spinach the recovery of N is poor, which may result in elevated N concentrations in the soil (Biemond et al. 1996). This may have a negative impact on the environment; N not used for growth can cause release of the greenhouse gas nitrous oxide (N_2O) that may contribute to global warming even more than carbon dioxide. In addition, excessive nitrate (NO_3^-) may cause eutrophication of aquatic ecosystems and pollution of soil and surface waters (Wolfe and Patz 2002).

Spinach takes up NO_3^- from the soil efficiently but is known to be relatively inefficient in nitrate reduction (Stagnari et al. 2007, Koh et al. 2012). This implies that fertilizer that is mainly consisting of NO_3^- cannot be utilized immediately (Nunes-Nesi et al. 2010). Slow reduction can even result in high NO_3^- levels in spinach leaves, which upon ingestion can be harmful to humans (Santamaria 2006). Spinach growers in part circumvent this adverse health effect of slow NO_3^- reduction by using fertiliser with a relatively high NH_4^+ proportion (Wang et al. 2009a). However, the uptake of NH_4^+ is less efficient.

In general, N has a favourable effect on spinach yield. Under low-input and often also under organic growing conditions, yields are low due to poor adaptation to nutrient limitation. Crops grown under such conditions tend to senesce more rapidly than those under high input conditions. Strict EU regulations on N fertilisation however (European Commission 2010) force spinach growers to reduce N fertilization. The N input for a sustainable production of spinach should be approximately 100 kg N ha^{-1}. Growers therefore need to realize a yield under these conditions that is economically viable through the use of cultivars adapted to reduced input conditions. Therefore, genetic improvement of nitrogen use efficiency (NUE) is a challenge of utmost importance for spinach breeders (Fageria and Baligar 2005).

NUE is defined as the ability to produce high biomass per unit N applied (Gourley et al. 1994). NUE is a complex trait and is the result of two main components (Benincasa et al. 2011): uptake efficiency (NUpE), which relates to the capacity of plants to take up available N from the soil, and utilization efficiency (NUtE), referring to the plant's efficiency of utilising the N that is taken up to produce biomass (Hirel et al. 2007). Agronomic and physiological studies on N fertilization have been mainly focussed on optimizing spinach cultivation (e.g. Smolders and Merckx 1992, Biemond 1995, Biemond et al. 1996), even though spinach was for a while used as a model crop to study NO_3^- accumulation and its

regulation (Breimer 1982, Steingrover 1986). The impact of low N availability on growth and harvestable yields are not yet well understood. There also is little experimental knowledge on ways to develop NUE spinach cultivars adapted to low input conditions.

Biemond (1995) reported that sufficient N must be available at the start of the growth to realize optimal growth in spinach. Several authors have indicated that spinach can acquire nutrients better with improved root systems (Smolders and Merckx 1992, Hirel et al. 2007). Biemond et al. (1996) determined that with increased N availability the total green leaf area increased through a higher leaf expansion rate. This demonstrates the strong interdependence of N availability and crop growth.

The objective of the current study is to gain better insight in genetic diversity in NUE and (selectable) traits that affect NUtE and NUpE of spinach. However, selection for genotypic differences in NUE is not straightforward. Under low-input field conditions there is no good control over N availability which introduces environmental variation that will mask the genotypic variation. In addition, N fertilisation is normally applied prior to sowing, so seedlings start with an excessive amount of N but the crop may deplete the N in the soil at later growth stages. This implies that under these conditions fast growing cultivars tend to exhaust the N reserves in the soil faster than the slow growing cultivars. Fast growers therefore will suffer earlier from N stress, which may mask useful NUE properties.

To circumvent these problems and identify traits related to NUE that can be targets for breeding, a diverse set of cultivars was tested in this study on a hydroponics system under controlled greenhouse conditions. In this system, N is daily supplied over the whole growing period in dosages that are proportional to the plant growth as suggested by Ingestad (1982). The Ingestad approach was previously used successfully to identify traits for NUE in grasses (van Loo et al. 1992; Dolstra et al. 2007). The biggest advantage of this test procedure over field testing is that within each N treatment plants experience more or less the same internal N availability, or limitation. The tests were conducted at suboptimal and optimal conditions for growth (further referred to as low N and high N, respectively). Our results indicate that this approach allows identification of variation in NUE traits in cultivated spinach that may be targets for breeding more N use efficient spinach cultivars.

2.2. Materials and methods

Plant material and germination

A diverse set of 22 commercial spinach F1 hybrid cultivars was chosen varying in growth rate and other characteristics (Table 2.1). During data processing the cultivars could be classified as slow or fast growers on the basis of the relative growth rate (RGR) observed at high N. The slow growers had a RGR B 0.18 g g^{-1} day^{-1} and the fast growers a RGR 0.18 g g^{-1} day^{-1}.

To produce seedlings for testing on hydroponics, seeds were primed at 13˚C for 2 days and allowed to germinate on wet filter paper. After the cold treatment, the germinated seeds were transferred onto wet cubic rock wool blocks with a height of 5 cm and a top surface of 2 cm^2; the blocks were watered daily with tap water. After 2 weeks, cubic blocks with seedlings were planted into the hydroponics system for evaluation.

Table 2.1. Origin, market type and date of release of F1 hybrid cultivars of spinach used to assess genetic variation for traits associated with NUE.

Cultivar	Date of Release	Company
Grandi	4-6-2008	Enza Zaden
Corvette	29-12-2010	
Corvair	28-3-2011	
Ranchero	8-10-2012	
Thunderbolt	22-1-2013	
Chevelle	22-1-2013	
Hudson	21-10-2010	Pop Vriend
Piano	30-1-2013	
Cello	29-8-2011	
Celesta	28-3-2011	
PV 0293	Not released	
Palco	Not released	Nunhems
Novico	28-3-2011	
Andromeda	29-5-2012	
NUN00905SP	Not released	
NUN00915SP	Not released	
Crocodile	21-9-2006	Rijk Zwaan
Eagle	12-6-1999	
Rhino	15-12-2002	
Sparrow	28-3-2011	
Beaver	28-3-2011	
Marabu	20-6-2007	

Experimental setup and conditions

The hydroponics system in a greenhouse consisted of four units, each with a capacity of 500 L and 16 containers of 40 cm long, 30 cm wide and 20 cm high. All containers of a single unit were connected in parallel to a large container with 300 l nutrient solution. Every container had 24 plant positions (three rows with eight holes). Nutrient solution within a unit was circulated with an equal passage rate of nutrient solution through the containers. Each treatment consisted of two independent, replicated units.

A Hoagland nutrient solution without any source of N was used and N was added daily in a 3:1 ratio of KNO_3 and NH_4Cl, respectively. The application rate in each unit was calculated from the growth rate and based on the model of Ingestad (1982). The daily N application aimed to create an environment with a stable relative plant growth rate (RGR) of either 0.14 (low N) or 0.18 g g^{-1} day^{-1} (high N).

Two separate hydroponics experiments were carried out to study the performance of 24 genotypes (22 cultivars (G) plus two dummies) for NUE and NUE related traits under high and low N. The photoperiod in both experiments in the greenhouse was set at 12 h day/12 h night. The day/night air temperatures in the greenhouse compartment were set to be 20/16 ^{0}C and the relative humidity was set to 50-60%.

The experiments differed in plant density. The first experiment had a low density similar to 133 plants per m^2 (referred to as LD experiment) and it was performed from week 46 to 50 of 2011. Containers from two adjacent units differing in N level were treated as main plot and sets of containers as subplot. Each subplot consisted of six containers, each having four rows of four plants. The 24 genotypes were randomly assigned to one of 24 rows available per subplot. This setup was chosen to minimize light competition between plants, in particular between plants from different cultivars. The experiment had a randomized block design. The second experiment referred to as the high density (HD) experiment was equivalent to a plant density of 200 plants per m^2 and it was performed from week 9 to 13 of 2012. Containers from two adjacent units differing in N level were treated as main plot and individual containers as subplot. Seedlings of all genotypes under study were randomly assigned to the 24 plant positions available per container. The second experiment was designed as a split-plot. Both experiments were performed with two hydroponic units (replicates) per N treatment. 14 days after starting the N treatment, half of the containers of every hydroponic

unit were harvested and the remaining ones were harvested after 28 days. All the traits were evaluated at these time points, except chlorophyll content (CC).

Plant traits

Biomass

At harvest, the plants were patted dry with industrial paper tissue. The plants were divided in a root and shoot fraction and weighed separately to determine root fresh weight (RFW) and shoot fresh weight (SFW). The sum of both equals the total plant fresh weight (TFW).

The shoot and root fraction of each plant were dried for 2 days at 70ºC to get measures for plant SDW and RDW. TDW was calculated as TDW = SDW + RDW. The plant root-to-shoot ratio (R:S) was determined as R:S = RDW/SDW.

Leaf area (LA) (cm^2)

After harvest but before drying, the leaf area of plants was determined with a Licor Leaf Area Scanner (LI-3100C).

Specific leaf area (SLA) ($cm^2\ g^{-1}$)

It was calculated by the formula SLA = LA/SDW.

Chlorophyll content (CC) (SPAD units)

CC of leaves was measured 13, 21, and 27 days after transplanting of seedlings on hydroponics with SPAD 502 (Konica Minolta, Osaka, Japan). SPAD values were collected for the first and the second appearing pair of leaves of each plant.

Relative growth rate ($g\ g^{-1}\ day^{-1}$)

To determine the growth rate, RGR of each cultivar was calculated for the time interval (in days) between harvests by $RGR = [Ln\ (SDW_{t2}/SDW_{t1})]/t_2 - t_1$. SDW_{t1} and SDW_{t2} refer to the cultivar means for SDW at the beginning and end of the time interval (t1 and t2, 14 days between 2 weeks and 4 weeks after planting).

Nitrogen use efficiency (NUE) (g SDW g^{-1} N)

A Kjeldahl analysis was used to determine the N percentage of shoot dry matter. NUE was calculated as the SDW divided by the N content in the dry shoot. This trait was only assessed for samples of harvest two of the HD experiment.

Statistical analysis

The data was analysed with GenStat v15.0 for descriptive statistics, correlations and analyses of variance (ANOVA) for each trait. The latter took into consideration that the first experiment had a randomized block design and the second a split-plot design. The data was analysed for each harvest time separately. The number of 'main plots' per harvest was two in the LD experiment and eight in the HD experiment. Correlation analyses were performed using cultivar means for the traits assessed in both experiments at high and low N. As several traits showed a high correlation and they were all linked to plant growth, a path analysis (Dewey and Lu 1959) was performed using GenStat v15.0 to determine the contribution of each of NUE-related traits to NUE as determined in the HD experiment. A path analysis calculates standardized partial-regression coefficients which are measures of the direct influence of explanatory variables on the genotypic variation found for NUE. The analysis gives a picture of the direct and indirect causal relationships between the explanatory variables and the response variable NUE. The method requires a priori knowledge or experimental evidence on the causal relationships (Dewey and Lu 1959). Broad sense heritabilities were calculated as follows:

$$h^2m = \sigma_g^2 / (\sigma_g^2 + \sigma^e/n)$$

in which σ_g^2 corresponds to the cultivar variance and σ_e^2 to the experimental variance, and n refers to the number of replicates (4 and 9 for LD and HD respectively).

2.3. Results

General response to N at low and high plant density

Two hydroponic experiments with a low plant density (LD) and a high plant density (HD) design were performed with in both cases RGR set at 0.14 g g^{-1} - day^{-1} (low N) and 0.18 g g^{-1} day^{-1} (high N). After 2 weeks on hydroponics the SDW of plants grown at low and high N differed significantly at HD but not at LD (Figure 2.1). LA was not significantly different between treatments after 2 weeks in both experiments. For these traits the mean plant performance after 2 weeks was slightly better at LD compared to HD. High N obviously had a positive effect on SDW and LA after 2 weeks of growth on hydroponics. Compared to low N conditions, plants under high N initially invested more in LA as plants grown at high N had a lower root-to-shoot ratio (R:S) and a higher SLA (Figure 2.1). After 4 weeks on hydroponics the plants in both experiments showed on average a strong adverse effect of low N availability on SDW and LA. SDW was reduced at LD by 66% and at HD by 72%; the corresponding reductions of LA were 59 and 71% (Supplementary Table 2.A). The beneficial effects of N on shoot growth and leaf area development were associated with a lower average R:S and higher average SLA. Differences for these two morphological traits tended to be larger under LD.

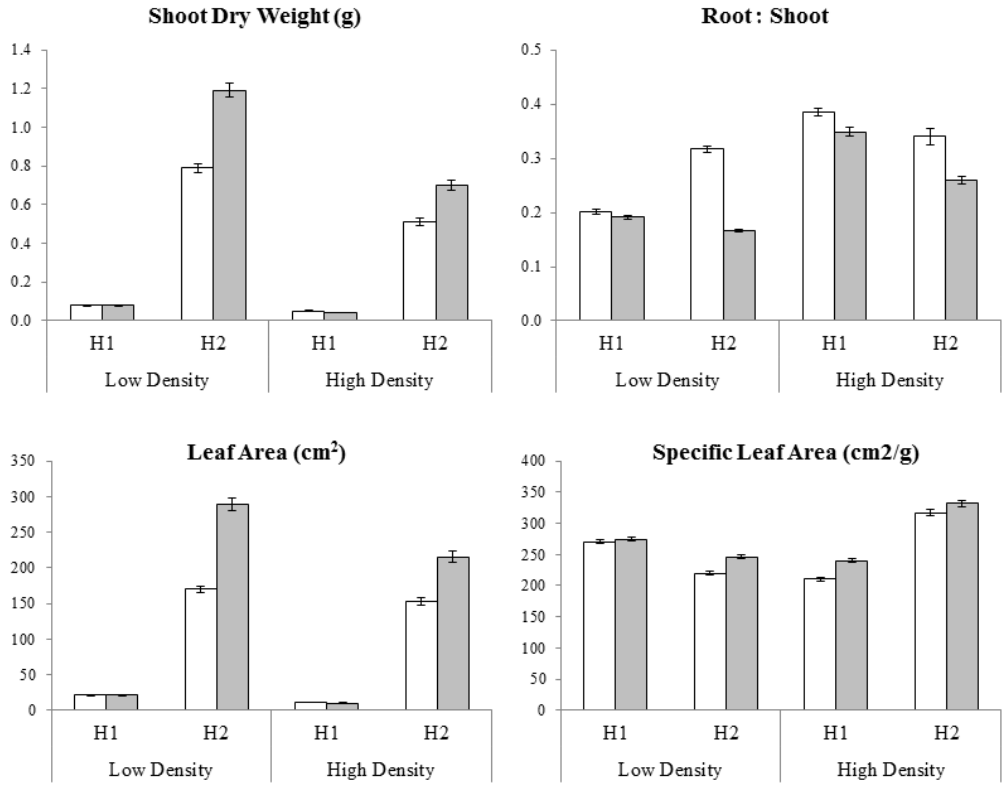

Figure 2.1. Trait means at low and high density for two different N treatments (relative growth rate 0.14 g g^{-1} day^{-1} in white and 0.18 g g^{-1} day^{-1} in grey) after 2 and 4 weeks on hydroponics (H1 and H2, respectively)

Genetic variation for NUE related traits

In the following sections the differences among cultivars in response to N will be discussed for the HD experiment only. This experiment is the more suitable and informative, as it has a design with a plant density that is equivalent to field conditions (~200 plants m^{-2}). The performance of the cultivars in the LD setup at low and high N is summarized in Table 2.2 and described in more detail in the Supplementary Tables 2.A and 2.B.

Table 2.2. Descriptive statistics for the traits evaluated at Harvest 2 (4 weeks after planting) in the high density (HD) experiment at Relative Growth Rate (RGR) set at 0.14 and 0.18. CC=chlorophyll content; LA=leaf area; SFW=shoot fresh weight; RFW=root fresh weight; TFW= total plant fresh weight; SDW=shoot dry weight; RDW=root dry weight; TDW=total plant dry weight; SLA=specific leaf area; R:S=root to shoot ratio; N%=nitrogen percentage of SDW; NUE=nitrogen use efficiency

Trait	RGR	Statistics					
		Mean	Min	Max	σ_g	σ_e	h^2_m
CC (SPAD)	0.14	29.45	23.95	34.19	2.72	2.69	0.89
	0.18	29.83	25.79	33.11	1.95	2.39	0.84
LA (cm^2)	0.14	153	81	198	23.0	59.8	0.54
	0.18	216	128	316	44.6	91.8	0.65
SFW (g)	0.14	7.70	3.61	13.06	1.68	3.15	0.69
	0.18	12.00	6.40	18.57	2.90	5.65	0.68
RFW (g)	0.14	6.01	2.85	9.12	1.17	3.12	0.53
	0.18	4.22	2.29	6.21	0.90	2.54	0.50
TFW (g)	0.14	13.71	6.46	22.18	2.77	6.12	0.62
	0.18	16.13	8.69	24.77	3.66	7.93	0.63
SDW (g)	0.14	0.51	0.32	0.81	0.08	0.24	0.44
	0.18	0.70	0.40	1.04	0.16	0.36	0.60
RDW (g)	0.14	0.17	0.10	0.24	0.03	0.08	0.44
	0.18	0.18	0.09	0.26	0.03	0.10	0.50
TDW (g)	0.14	0.69	0.41	1.05	0.10	0.31	0.46
	0.18	0.88	0.46	1.30	0.19	0.45	0.58
SLA (cm^2/g)	0.14	317	274	367	15.64	66.5	0.31
	0.18	332	301	404	17.41	65.0	0.36
R:S	0.14	0.34	0.28	0.40	0.02	0.07	0.38
	0.18	0.26	0.20	0.33	0.03	0.04	0.73
RGR (g g^{-1} day^{-1})	0.14	0.16	0.13	0.19	0.01	0.04	*
	0.18	0.19	0.16	0.26	0.02	0.04	*
N%	0.14	4.19	3.89	4.48	0.10	0.21	0.62
	0.18	5.15	4.94	5.39	0.10	0.28	0.50
NUE (g g^{-1} N)	0.14	13.24	9.00	19.58	1.68	5.58	0.42
	0.18	14.34	8.78	20.66	3.22	6.65	0.65

*: *statistic not available*

At the final harvest, N level generally had a significant main effect on all traits except for CC, RDW and NUE, while for none of the traits tested the G×E interaction was significant. The set of cultivars tested showed variation for all traits studied at Harvest 2, after 4 weeks of growth, with heritability estimates on a cultivar mean basis ranging from 0.31 to 0.89 (Table 2.2). The range in mean performance of the cultivars for the commercially relevant traits SFW, SDW and LA was large. Most of the cultivars responded to increased N with higher SDW and increased LA, except for cvs Crocodile and Celesta, which had lower LA and a

lower or similar SDW (Supplementary Tables 2.A, 2.B). Cv. Ranchero was highly responsive to the increase in N with a 70 % increase in LA and SDW (Supplementary Tables 2.A, 2.B). The lower N application rate resulted on average in only a slightly lower N % in SDW and a small, insignificant reduction of NUE. The genotypic variation for NUE, however, was fairly large at high as well as at low N. Some cultivars showed a large differential response to different N applications; the respective cultivar means for NUE at low and high N were for cv. Crocodile 13.07 and 9.76 for cv. Celesta 10.66 and 9.83.

Table 2.3. Matrices of correlation coefficients based on cultivar means for a selected set of traits as determined in the HD experiment at Harvest 2 (4 weeks after planting). CC=chlorophyll content; LA=leaf area; SDW=shoot dry weight; RDW=root dry weight; TDW=total plant dry weight; SLA=specific leaf area; R:S=root/shoot ration; N%=nitrogen percentage of SDW; NUE=nitrogen use efficiency

	CC	**LA**	**SDM**	**RDM**	**SLA**	**R:S**	**N%**	**NUE**
CC	-	-0.40	-0.35	-0.17	-0.15	0.47	0.33	-0.41
LA	-0.44	-	0.82	0.81	0.21	-0.01	0.21	0.70
SDM	-0.48	0.97	-	0.88	-0.31	-0.15	0.11	0.93
RDM	-0.50	0.90	0.95	-	-0.19	0.31	0.30	0.73
SLA	0.29	-0.55	-0.69	-0.71	-	0.20	0.33	-0.39
R:S	0.15	-0.56	-0.55	-0.27	0.30	-	0.59	-0.34
N%	0.51	-0.06	-0.08	-0.14	-0.08	-0.26	-	-0.11
NUE	-0.55	0.92	0.97	0.91	-0.62	-0.51	-0.15	-

The coefficients above the diagonal refer to the low N evaluation (RGR set at 0.14) and those below the diagonal to the high N evaluation (relative growth rate set at 0.18). Correlation coefficients with a value of $|r|>0.49$ are significantly different from 0 ($P = 0.01$).

Correlation and path analyses

Cultivar means obtained from evaluations at low and high N in the HD experiment (Supplementary Tables 2.A, 2.B) were used to generate two correlation matrices. Coefficients of correlation between a selected subset of these traits determined at low and high N are presented in Table 2.3. Excluded from the set were fresh matter traits as well as TDW, since these were highly correlated to SDW, RDW and LA. Traits like CC and N % did not show any significant correlation with other traits at both low and high N. It seems that there was a weak but insignificant correlation between these traits. The traits that may be affected by the allocation of assimilates, SLA and R:S, were not significantly correlated with other traits except for SLA with RDW at high N. The correlation matrices shown in Table 2.3 were used as basis for two separate path analyses to get a better insight in the causal relations among NUE related traits at low and high N, respectively. NUE was used as response variable in both analyses and the traits LA, RDW, CC and SLA as explanatory variables (Figure 2.2).

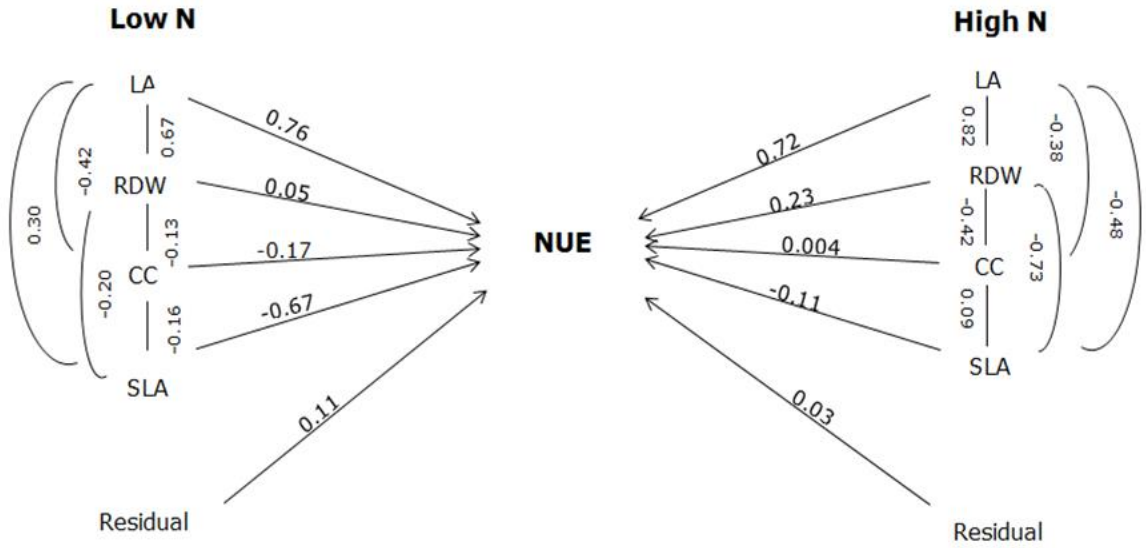

Figure 2.2. Path analysis of factors that influence NUE at low N (relative growth rate set at 0.14) and high N (relative growth rate set at 0.18) using the Ingestad model at high density (HD). The figures along the arrows refer to path coefficients and the others to correlation coefficients. LA=leaf area; RDW=root dry weight; CC=chlorophyll content; SLA=specific leaf area; NUE=nitrogen use efficiency.

In the path diagrams, connecting lines without arrows indicate mutual relationships between explanatory variables by correlation coefficients. Single-arrowed lines represent the direct

influence of explanatory variables on the response variable as indicated by path coefficients. The explanatory variables (LA, RDW, CC and SLA) explained almost all variation in NUE between cultivars at low and high N. LA at both N conditions had a major contribution to the variation in NUE; 58 and 52 % at low and high N, respectively. SLA had a strongly negative direct effect on NUE at low N and explained about 45 % of the variation in NUE between cultivars. At high N however the direct influence of SLA on NUE was small. RDW had a very high correlation with NUE at low as well as at high N, but hardly contributed directly to the variation for NUE at low or high N.

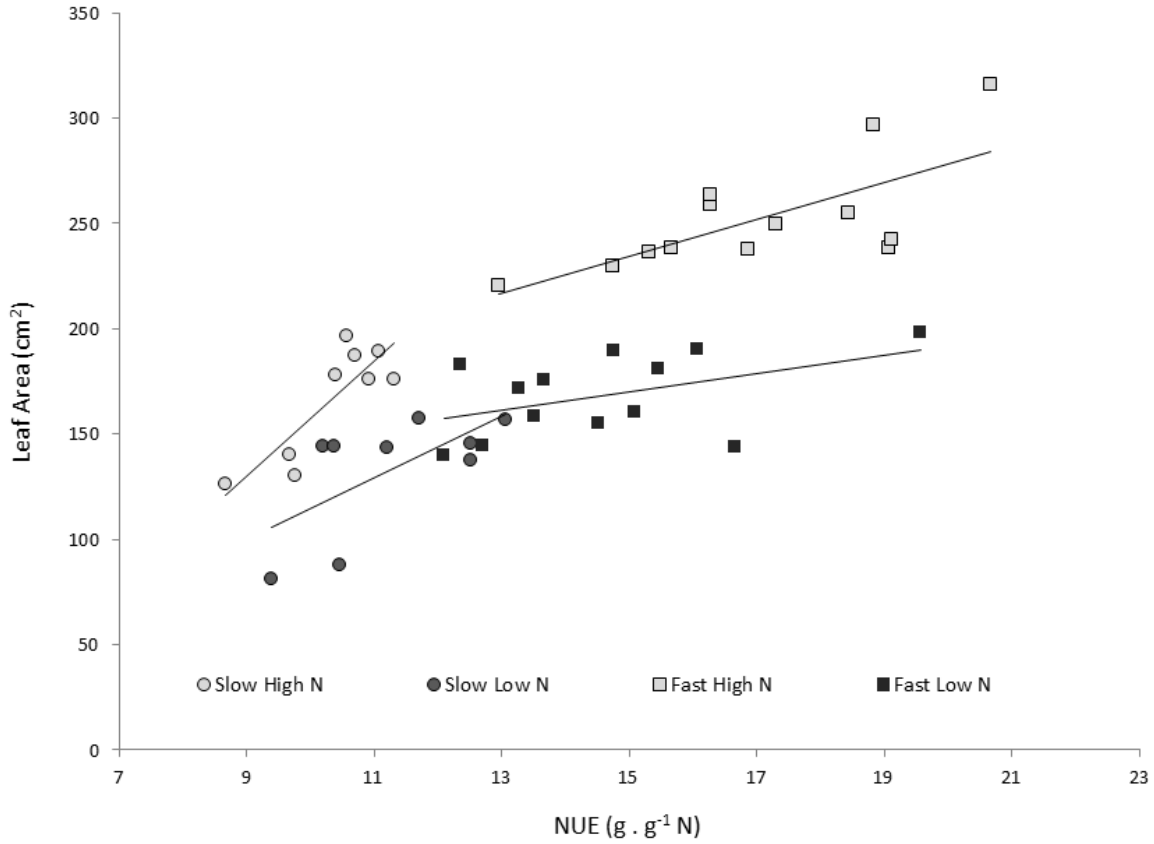

Figure 2.3. Relationship between leaf area (LA) and nitrogen use efficiency (NUE) for slow and fast growers at low and high N in high density (HD) experiment

The relationship between LA and NUE

Figure 2.3 shows the relationship between LA and NUE at low and high N. The cultivars were classified as slow or fast growers on the basis of the RGR observed at high N, with RGR $\leq$ 0.18 g g^{-1} day^{-1} for low growers and for fast growers RGR > 0.18 g g^{-1} day^{-1}. The slow

growers were found to have a relatively small LA and low NUE with a clearly positive relation between NUE and LA (Figure 2.3). The class of fast growers had high NUE and a much weaker positive relation between NUE and LA. Fast growing cultivars did not utilize the available N as efficiently for leaf growth as the slow growers. The variation observed in NUE of fast growing cultivars was not or only to a small extent due to differences in LA.

2.4. Discussion

The Ingestad approach to screen for NUE

Our study shows that the Ingestad approach in a hydroponics system is a useful tool to screen cultivars for variation in NUE and the traits related to NUE in spinach. Under field conditions, the genetic factors that contribute to NUE are difficult to assess because the measurements reflect the combined effects of a variable environment and the genotype (Glimskär and Ericsson 1999, Baresel et al. 2008, Xu et al. 2012). An often overlooked variable factor is the decreasing N availability during the growth cycle of the crop. With the Ingestad approach, the plants are supplied with amounts of N that matches either a suboptimal growth rate (constant stress) or an optimal growth rate, which enables to determine NUE in a reproducible way without disturbance due to luxurious N uptake. The plants are subjected to a constant stress level, allowing the plants to adapt and stay viable even under nutrient limitation (Ingestad and Agren 1995). Although this presents advantages in dissecting the genetic complexity of NUE and discovering traits contributing to NUE, the Ingestad approach in hydroponics does not represent field conditions. As the hydroponic system distributes N uniformly and in a controlled way over the plants, it is likely that not all genotypic differences relevant to N uptake from soil are expressed or can be discovered. Therefore the screening as done in this study mainly reveals variation in N utilization. Although the use of hydroponics may imply that not all results from this study can be readily extrapolated to field-grown spinach, the accurate and reproducible results on traits with relatively high heritabilities does enable the identification of traits that contribute to NUE. Traits such as LA, SLA and SDW as determined in our study may serve as promising selection criteria for breeding, and their relevance to spinach cultivation should be confirmed by field tests.

Implications of plant density

Substantial differences among the cultivars were observed at the two N levels in the high and low density experiments. The higher biomass production at LD could be caused by a better light interception. At HD, plants may need to compete more with each other for light once the plot canopy closes. Grindlay (1997) has shown that leaves that are deeper in the canopy and cannot intercept as much light as leaves higher in the canopy, photosynthesize less and utilize less N, resulting in reduced leaf growth. Similarly, the reduced light interception of the plants grown at HD may lead to reduced LA, compared to the LD grown plants. According to Grindlay (1997) RGR is reduced when crop growth is limited by the N supply. However, we observed that the average RGR in the period between Harvests 1 and 2 at were similar at both plant densities for low N and high N (0.17-0.16 g g^{-1} day^{-1} and 0.20-0.19 g g^{-1} day^{-1} respectively) (Supplementary Tables 2.A, 2.B). This implies that the differences in biomass were already established 14 days after starting the N treatment. In this period light interception does not yet play a role as the leaves of plants are not yet competing for light. The differences in biomass could therefore not only be due to plant competition for light. We cannot rule out differences in (outdoor) light conditions between the experiments to play a role as well, as the LD experiment was performed in winter and the HD experiment in spring. Another factor could be competition at the root level or root sensing (Bais et al. 2006) as at HD there was less rooting space per plant and more random inter-cultivar competition. The lower trait heritabilities under LD conditions may also point to an extra source of random variation in the LD setup, perhaps small container-to-container differences as a consequence of the limitation of the number of genotypes tested per container to four (Supplementary Table 2.A). Therefore, the HD setup is likely best suited for NUE trait selection in spinach.

Biomass partitioning

The R:S increased at low N in our experiments, which is in agreement with a change in biomass partitioning in favour of roots under N-limiting conditions as reported in many other studies. Smolders et al. (1993) produced a model for NUE in spinach in which the net N assimilation rate of the shoot is explained by the N content in the shoot dry weight for plants grown in pots and in hydroponics. The path analysis demonstrated that in addition to the shoot dry weight contribution reported by Smolders et al. (1993), LA contributes more than

other traits to NUE at both low and high N, and LA and SDW are highly correlated at low and high N conditions. When the Ingestad approach is used, all N given on a daily base will be utilized by the plant for growth and the luxurious N conditions are thus avoided. This allows the plant to adapt to limited N conditions by biomass partitioning to guarantee photosynthetic activity that is balanced with N availability, which is reflected in the changes in root to shoot ratio.

Biemond et al. (1996) concluded that there was little variation in growth pattern of spinach in response to N, but their findings were based on the use of a single cultivar (Trias of C.W. Pannevis BV). We found broad genetic variation in the tested set of 22 commercial cultivars for a number of traits, including LA, SDW and the partitioning of biomass in shoots and roots at optimal and limited N conditions.

Several studies report that NO_3^- acts as a signal that alters C metabolism in shoots and increases the R:S (Grindlay 1997, Scheible et al. 1997a, Hermans et al. 2006, Remans et al. 2006). NO_3^- produces a strong response in root growth, acting not only as nutrient but also as a signal for cell growth. Split root experiments indicated that the inhibition of root growth is triggered by the accumulation of nitrate in the shoot, not in the root (Scheible et al. 1997b). Hermans et al. (2006) suggest that at N stress, application of NO_3^- to the root system stimulates lateral root growth at that root site. The breakdown of photosynthesis proteins in older leaves during N stress and senescence contributes to the reallocation of N to young leaves, which may indirectly help to support a more fine root formation (Stitt and Krapp 1999). The increase in R:S, RL and SRL observed in our study under deficient N conditions agrees with an adaptation of C partitioning that improves the plant's ability to acquire mineral elements by optimizing root morphology towards lateral root proliferation (Grindlay 1997). However, under hydroponic conditions with the Ingestad model, the investment in roots is likely to have less impact on plant growth as N is readily available to the roots. The consequences to plant growth might therefore be different in field grown spinach, for which the R:S increase at low N is a well-documented response. In addition, soil-grown plants have a higher carbon cost per unit root weight than hydroponically grown plants (Evans and Poorter 2001).

The genetic response in hydroponics is a measurable genetic factor that may contribute to NUE in the field, and that can be used as a selectable trait. The impact of such a trait on crop production however should always be validated in field trials.

Carbon/nitrogen ratio (C/N)

The reduction of leaf area to reduce the photosynthetic area and the increased root length as measured in the current study may be responses to maintain a balanced carbon to nitrogen ratio (C/N). At low N, the increase in C/N and the accumulation of carbohydrates suppress photosynthesis by decreasing the amounts of photosynthetic components such as ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) (Noguchi and Terashima 2006). The decreased SLA therefore may be an indication of a coordinated reduction in photosynthesis capacity to adapt to limited N availability. Boese and Huner (1990) observed that at lower temperatures leaves thicken (reducing the SLA) which may help to avoid damage due to photo inhibition at high light intensities; conditions with low temperature and high light intensities often occur in spring and autumn, the common periods for cultivation of spinach in NW-Europe (Noguchi and Terashima 2006). The C/N balance is restored by investing in structural components that require less N, which is reflected in higher investment in the roots (Figure 2.1). Grindlay (1997) stated that when leaf expansion is limited under N shortage SLA can be reduced by half due to accumulation of dry matter in the form of starch and cell wall material.

We did not find a correlation between CC and NUE or N content. CC is a genotype dependent trait that is related to colour and the number of photosystem II reaction centres per leaf surface area. The relationship between CC and N content is well documented (Evans 1989; Lawlor et al. 1989), and CC correlates with photosynthesis rates. Liu et al. (2006) found a strong positive correlation between CC and the total N content in a pot experiment in spinach. Our results do not show a relation between CC and NUE, and we believe that this is based on the use of the Ingestad model in our research. The plants received an exponentially increasing amount of N every day. The plants could therefore acclimate to the low N availability, and maintain an adequate C/N balance with adapted leaf area and growth. In most other systems N is depleted, and the limiting amount of N is not enough to sustain the photosynthetic activity of the plant. This results in chlorophyll breakdown and hence a decrease in CC.

The relationship between NUE and LA: slow and fast growers' clusters

Several papers report that NUE is influenced by N availability. However, we observed under our hydroponic conditions on average no significant difference in NUE between high and low

N (Table 2.2). In contrast, the correlated biomass traits were highly affected by the N level. The overall N level effect for NUE was not significant because the genotypes tested presumably respond differently to N limitation and use different strategies to cope with it (Figure 2.3). We further investigated the relationship between LA and NUE, and observed that faster growing cultivars were more responsive to high N conditions and in general had under those conditions a relatively high NUE while slower growers had a higher NUE at low N conditions (Figure 2.3). This is in agreement with two strategies to cope with N limitation as proposed by Lambers (1987): (a) maximizing biomass accumulation (LA and/or SDW) and decreasing NUE; and b) maximizing NUE and decreasing the investment in biomass. The fast growing cultivar Ranchero followed the first strategy and was highly responsive to N, increasing LA from 189.6 to 315.5 cm^2, while the slow growing cultivar Crocodile used the second strategy, increasing NUE from 9.69 to 13.07 (g SDW g^{-1} N) from high to low N. The difference in biomass production between slow and fast growers was not directly associated with differences in biomass partitioning, as both groups increased the relative investment in root growth and augmented lateral root branching at low N. Even though the fast growers have a higher NUE under low and high N conditions than the slow growers, the latter tend to increase the NUE under low N conditions (on average to 9.93%), while the fast growers decrease the NUE at low N conditions (-11.89%). The differences in response reflect different strategies to cope with N limiting conditions. The fast growers in general have the higher NUE at both conditions, but lack the capacity to increase NUE under N limitation. The ability to adapt NUE under low N that was detected mainly in slow growers may be an interesting trait for improving spinach varieties for growth under low N conditions.

2.5. Perspectives

The results of the hydroponic experiments revealed that sufficient genetic diversity in NUE is available among commercial spinach genotypes to breed for cultivars with improved performance under N-limiting conditions. LA was positively correlated with NUE and identified as a major source of variation for NUE in spinach, while SLA affects it negatively. Fast growing cultivars were shown to have different strategies to cope with limited N availability than slow growing cultivars. The ability to increase NUE under low N conditions of slow growing cultivars in particular may be a trait that can be used in breeding for more

nitrogen efficient cultivars that will enable cultivation of spinach at reduced levels of N fertilization such as in organic agriculture.

This study developed a relatively easy methodology to determine variation in traits related to NUE in spinach and for genetic studies. To what extent genetic expression of the variation of these traits are correlated with the phenotypic performance under field conditions still needs to be tested.

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Supplementary data

Table 2.A. Descriptive statistics from calculated date of nitrogen use efficiency (NUE) related traits under a relative growth rate (RGR) of 0.14 and 0.18 g g^{-1} day^{-1} at Harvest 2 for the low density experiments. LA=leaf area; SDW=shoot dry weight; RDW=root dry weight; SLA=specific leaf area; R:S=root to shoot ratio

	LA (cm^2)		**SDW** (g)		**RDW** (g)		**SLA** (cm^2/g)		**R:S**		**RGR** (g g^{-1} day^{-1})	
Genotype	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**
Cultivar mean												
Grandi	139.7	174.2	0.60	0.64	0.20	0.14	232.4	241.4	0.34	0.22	0.15	0.19
Corvette	165.0	310.2	0.66	1.17	0.21	0.23	253.4	269.3	0.32	0.19	0.14	0.17
Corvair	160.7	310.7	0.66	1.23	0.24	0.21	244.8	252.1	0.36	0.17	0.15	0.18
Ranchero	219.0	375.1	0.99	1.49	0.31	0.25	224.1	254.8	0.33	0.17	0.15	0.21
Thunderbolt	194.3	333.3	0.86	1.44	0.27	0.22	224.8	232.4	0.31	0.15	0.22	0.21
Chevelle	185.2	182.2	0.93	0.81	0.25	0.13	208.5	225.1	0.26	0.16	0.17	0.17
Hudson	167.0	338.5	0.84	1.36	0.22	0.22	204.0	252.1	0.28	0.16	0.16	0.21
Piano	189.3	378.8	0.95	1.62	0.26	0.2	199.1	235.0	0.26	0.12	0.21	0.19
Cello	149.4	306.8	0.75	1.25	0.25	0.18	208.8	244.2	0.36	0.14	0.15	0.24
Celesta	163.0	166.2	0.71	0.71	0.18	0.11	231.6	250.4	0.26	0.16	0.17	0.16
PV 0293	147.0	274.7	0.79	1.17	0.25	0.20	186.4	234.9	0.32	0.17	0.16	0.19
Palco	218.5	370.5	0.93	1.44	0.32	0.23	232.4	258.7	0.33	0.16	0.16	0.19
Novico	147.1	226.5	0.68	1.05	0.23	0.18	230.3	211.0	0.33	0.15	0.15	0.21
Andromeda	272.4	508.8	1.25	2.08	0.38	0.28	220.3	245.5	0.34	0.13	0.21	0.21
NUN00905SP	255.4	367.2	1.18	1.58	0.42	0.27	218.9	232.8	0.36	0.17	0.20	0.21
NUN00915SP	204.6	299.3	0.91	1.15	0.28	0.16	227.6	261.5	0.30	0.14	0.17	0.19
Crocodile	119.3	264.4	0.61	1.17	0.16	0.18	201.4	229.5	0.25	0.16	0.15	0.21
Eagle	170.5	287.9	0.83	1.15	0.25	0.16	211.4	255.4	0.31	0.14	0.15	0.20
Rhino	179.8	233.0	0.84	1.06	0.29	0.22	210.5	217.5	0.34	0.22	0.18	0.20
Sparrow	152.1	342.3	0.83	1.42	0.20	0.16	189.4	242.3	0.25	0.11	0.17	0.22
Beaver	157.5	244.2	0.72	0.81	0.22	0.14	236.9	301.7	0.32	0.19	0.19	0.19
Marabu	66.4	133.4	0.32	0.6	0.11	0.12	188.3	240.4	0.28	0.19	0.14	0.15
General mean	168.4	286.9	0.78	1.18	0.24	0.19	220.05	246.24	0.32	0.17	0.17	0.20
SEM	55.99	79.60	0.27	0.30	0.08	0.05	15.98	25.45	0.07	0.02	*	*
***h^2*m**	0.26	0.56	0.24	0.64	0.41	0.38	0.81	0.39	0.02	0.65	*	*
F-probability (genotype)	0.24	0.03	0.26	0.01	0.11	0.13	<0.001	0.12	0.48	0.01	*	*
F-probability (N-level)	0.01		0.01		0.01		0.13		0.02		*	

*: *statistic not available*

Table 2.B. Descriptive statistics for calculated data of nitrogen use efficiency (NUE) related traits under a relative growth rate (RGR) of 0.14 and 0.18 g g^{-1} day^{-1} at Harvest 2 for the high density experiments. LA= leaf area; SDW= shoot dry weight; RDW= root dry weight; SLA= specific leaf area; R:S= root to shoot ratio

	LA (cm^2)		**SDW** (g)		**RDW** (g)		**SLA** (cm^2/g)		**R:S**		**RGR** (g g^{-1} day^{-1})		**NUE** (g g^{-1} N)	
Genotype	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**	**0.14**	**0.18**
Cultivar mean														
Grandi	143.7	174.2	0.41	0.50	0.16	0.14	367.1	369.5	0.38	0.27	0.15	0.18	11.19	11.32
Corvette	157.4	196.4	0.47	0.54	0.19	0.14	342.5	375.8	0.39	0.26	0.16	0.18	11.70	10.56
Corvair	171.1	220.4	0.55	0.67	0.22	0.17	315.2	347.6	0.40	0.26	0.16	0.21	13.27	12.96
Ranchero	190.1	315.5	0.61	1.04	0.21	0.26	323.9	310.5	0.34	0.24	0.15	0.21	16.08	20.66
Thunderbolt	144.3	238.1	0.5	0.91	0.17	0.23	293.3	300.6	0.33	0.25	0.17	0.22	12.70	19.07
Chevelle	124.5	176.3	0.48	0.55	0.13	0.14	280.5	333.4	0.30	0.25	0.16	0.17	12.51	10.92
Hudson	144.5	189.3	0.48	0.54	0.18	0.12	325.3	359.1	0.37	0.23	0.18	0.18	10.21	11.08
Piano	139.4	242.3	0.45	0.8	0.13	0.19	325.5	332.7	0.30	0.24	0.17	0.23	12.10	19.10
Cello	158.1	258.8	0.54	0.86	0.19	0.23	308.3	305.9	0.36	0.27	0.18	0.23	13.52	16.27
Celesta	144.4	130.1	0.41	0.4	0.14	0.09	351.2	404	0.34	0.29	0.17	0.19	10.37	9.77
PV 0293	137.8	187.6	0.45	0.56	0.15	0.15	305.6	354.1	0.35	0.28	0.16	0.18	12.51	10.69
Palco	175.5	236.3	0.53	0.78	0.18	0.2	350.4	307.9	0.35	0.26	0.17	0.19	13.68	15.32
Novico	143.3	238.5	0.65	0.73	0.21	0.19	276.6	341.3	0.34	0.27	0.15	0.21	16.67	15.67
Andromeda	180.4	259.2	0.58	0.84	0.18	0.20	338.9	330.3	0.32	0.24	0.16	0.21	15.47	16.28
NUN00905SP	182.9	237.6	0.52	0.84	0.18	0.22	359.2	317.5	0.34	0.27	0.17	0.22	12.36	16.87
NUN00915SP	198.1	255.0	0.81	0.88	0.24	0.24	274.2	302.8	0.31	0.26	0.19	0.19	19.58	18.43
Crocodile	156.7	140.4	0.54	0.48	0.18	0.13	299.3	311.5	0.33	0.27	0.16	0.18	13.07	9.67
Eagle	189.3	249.7	0.63	0.82	0.23	0.21	313.4	332.0	0.36	0.26	0.19	0.21	14.76	17.30
Rhino	81.2	144.9	0.32	0.52	0.10	0.17	304.6	302.7	0.34	0.33	0.19	0.18	9.39	10.39
Sparrow	155.2	229.5	0.53	0.78	0.15	0.16	315.3	316.5	0.29	0.20	0.16	0.21	14.52	14.75
Beaver	157.9	296.9	0.52	0.95	0.16	0.22	316.7	319.7	0.28	0.23	0.16	0.26	15.09	18.83
Marabu	88.6	127.6	0.32	0.40	0.12	0.12	286.2	329.0	0.35	0.32	0.13	0.18	10.45	8.67
General mean	152.9	215.7	0.51	0.70	0.17	0.18	316.9	332.0	0.34	0.26	0.16	0.19	13.24	14.34
SEM	28.8	44.3	0.11	0.17	0.04	0.05	35.8	33.8	0.03	0.02	*	*	1.86	2.22
***h*²m**	0.54	0.65	0.44	0.60	0.44	0.50	0.31	0.36	0.38	0.73	*	*	0.42	0.65
F-probability (genotype)	0.009	<0.001	0.023	<0.001	0.024	0.009	0.43	0.215	0.052	<0.001	*	*	0.034	<0.001
F-probability (N-level)	<0.001		<0.001		0.476		0.044		<0.001		*		0.088	

*: *statistic not available*

Chapter 3

Differential effects of nitrogen application strategies on growth and nitrogen response of spinach (*Spinacia oleracea* L.) cultivars

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To be submitted

Abstract

Nitrogen fertilizer application is fundamental for high production of agricultural crops. Over-fertilization however can lead to environmental pollution, which often occurs with spinach production. Optimal utilisation of applied nitrogen of the crop through integrated improvement of crop Nitrogen Use Efficiency (NUE) and optimizing management strategies are likely to have the highest impact on minimizing N used for spinach cultivation. We examined genetic differences among spinach cultivars grown under two different nitrogen application strategies: a bulk application that resembles field situations and a steady-state application, both at low and high nitrogen availability. Spinach plants were better able to adapt to low N conditions with steady-state N application than with bulk application. The present study demonstrated that the cultivars responded differently with respect to traits related to NUE depending on the N application method. The application strategy affected the timing and duration of the physiological stress. We can conclude that the steady-state provides stable and reproducible conditions that allow determination of genetic differences in NUE under low N conditions for a short-cycle leafy vegetable crop such as spinach.

Keywords: Spinach • Nitrogen use efficiency • Hydroponics • Application strategies • Ingestad model • Single bulk application

3.1. Introduction

Nitrogen (N) is the macronutrient that most frequently limits plant growth (Fageria and Baligar 2005). The almost two-fold increase in food production over the last 40 years has been largely attributed to the increased use of N fertilizer (Chimungu and Lynch 2014). It is estimated that less than 50 % of N fertilizer applied is taken up for crop production; the rest is lost from the rhizosphere through erosion, surface run-off, leaching and volatilization, causing

environmental pollution (Raun and Johnson 1999). Additionally, the production of inorganic N fertilizers is energy-consuming and fuel dependent, which leads to a large carbon footprint for these fertilizers and considerable price fluctuations because of dependency on the price of fossil fuel.

One of the enabling strategies to lower N use in crop production is genetic improvement of the crop's Nitrogen Use Efficiency (NUE). The definition of NUE applied in this study is the yield produced per unit of added N (Dresbøll and Thorup-Kristensen 2014). NUE is already an important target for breeding research in many crops such as wheat (Cabrera-Bosquet et al. 2007), barley (Anbessa et al. 2009, Beatty et al. 2010), rice (Wei et al. 2012, Chen et al. 2013), and oilseed rape (Koeslin-Findeklee et al. 2014).

NUE is intrinsically connected to the way the nitrogen is made available to the plants. The ratio of ammonium and nitrate influences not only nitrate uptake efficiency, but also uptake of other minerals including phosphate, thereby affecting the plant performance and thus, indirectly, NUE (Cassman et al. 2002). In addition, the rate and frequency at which nitrogen is supplied and available to the plant has a severe impact on plant growth and yield (Moll et al. 1982). Studies on genotypic differences of NUE and NUE-related related traits, in particular in short cycle crops, are often done using a single N application (Chardon et al. 2010, Koeslin-Findeklee et al. 2014, Kerbiriou et al. 2014). N is mostly applied just before sowing and the genotypic response for growth to the N application is assessed frequently during crop growth. However, applying nitrogen to the crop only at the start of the crop cycle may result in shortages at later stages due to run-off and leaching from the soil. Genotypes with high NUE when N is abundant, at early growth stages may suffer more at later stages when the high photosynthetic capacity can no longer be met by N uptake and transport, resulting in early senescence and N remobilisation from old leaves and internal stores. For a leafy vegetable like spinach, the imbalance between N and C and subsequent leaf senescence

can have severe consequences for the harvestable yield (Terashima and Evans 1988). In addition, selection of traits contributing to NUE is complicated by variable environments with respect to N availability (Xu et al. 2012). The effects of early vigour and high NUE at early stages of growth (under relatively high N availability) may be confounded by the effects of the traits that play a role at later growth stages when N demand is high but N availability is decreasing. For genetic studies and identification of genetic factors contributing to NUE and growth, dissecting NUE in contributing traits at different stages of development and at well-defined levels of stress at each stage will improve uncovering heritable variation and the chance of identifying contributing genetic factors.

Ingestad (1982) proposed conditions of steady state N stress for assessing NUE by applying daily limited amounts of N in quantities proportional to the plant growth. The ultimate aim of such an approach is to have plants with a limiting steady-state availability for N in the plant, which maximizes the chance of finding genetic differences for NUE (Ingestad 1982; Ingestad and McDonald 1989; McDonald 1990). The Ingestad model was successfully used to select for NUE in grasses (Van Loo et al. 1992, Dolstra et al. 2007).

In the current study we compared the response of spinach cultivars grown under steady state conditions according to Ingestad (1982) with plants grown under single application of N in a hydroponics system. A set of seven cultivars shown to have differential responses to N in a previous study using a similar hydroponics set-up with Ingestad conditions (see Chapter 2, Chan-Navarrete et al. 2014) was evaluated for a variety of traits that contribute to NUE under both N application strategies, and at two N levels (optimal and N-limiting).

Our results indicated that the involved spinach cultivars had diverse and differential responses to different N application methods in particular under N-limiting conditions, which has implications for genetic diversity assessment for NUE in spinach, and for the choice of selection environment and conditions in breeding programs.

3.2. Materials and Methods

Plant materials

Seven commercial spinach F1 hybrid cultivars were selected out of a set of 24 cultivars used in a previous study (see Chapter 2, Chan-Navarrete et al. 2014). The selected cultivars differ in NUE and traits contributing to NUE, and the set comprised slow as well as fast growers (Table 3.1).

The seeds of each cultivar were primed at 13^{o}C for two days on wet filter paper and the germinated seeds were transferred to wet cubic rock wool blocks with a height of 5 cm and a top surface of 2 cm^{2}; the blocks were watered daily with tap water. After two weeks, cubic blocks with seedlings were planted on the hydroponics system for evaluation.

Table 3.1. F1 hybrid spinach cultivars with their origin and their growth habit as used to assess genetic variation for traits associated with NUE.

Cultivar	Company	Growth habit
Chevelle	Enza Zaden	Slow
Cello	Pop Vriend	Fast
Novico	Nunhems (Bayer CropScience)	Fast
Andromeda	Nunhems (Bayer CropScience)	Fast
Crocodile	Rijk Zwaan	Slow
Sparrow	Rijk Zwaan	Fast
Marabu	Rijk Zwaan	Slow

Note: The growth habit classification is based on a hydroponics study on NUE (Chapter 2; Chan-Navarrete et al. 2014)

Experimental setup

The hydroponics system as described in detail in Chapter 2 (Chan-Navarrete et al. 2014) was used to study the differences in response to single bulk or steady-state N application at high N and low N availability in a temperature-controlled sunlit greenhouse compartment at UNIFARM (Wageningen, The Netherlands). The system comprised six units, each with a capacity of 500 l and 16 containers of 40 cm length, 30 cm width and 20 cm height. Only four units were used for the current experiment, one per N treatment (see Figure 3.1). All containers of a single unit were connected in parallel to a large container with 300 l nutrient solution. Every container had 24 plant positions (three rows with eight holes each). The nutrient solution within a unit was circulated with an equal passage rate through the containers.

The temperature of the nutrient solution was cooled to 12 ^{0}C by means of a cooling system present in the large containers connected each in the units of hydroponics system. The photoperiod in the experiment was 12 h day/ 12 h night. The day/night air temperatures in the greenhouse compartment were set to be 20/16 ^{0}C and the relative humidity was set to 50-60%. The experiment was performed from week 4 to 8 of 2013.

The basic nutrient medium used in this experiment was a Hoagland nutrient solution without N. The N application method based on the nutrition model of Ingestad (1982) consisted of daily N applications using a mixture of KNO_3 and NH_4Cl in a 3:1 ratio. This proportion of nitrate and ammonium was reported as optimal for the production of good quality spinach (Wang et al. 2009b). The daily application was aiming at a stable relative plant growth rate (RGR) of either 0.10 (Low N) or 0.18 g g^{-1} day^{-1} (High N). For the single N application conditions one single bulk N application was given immediately after the transfer of the seedlings to the hydroponics system. The amount of N given was equal to the total N amount

applied over to the corresponding Ingestad-based N treatment. The duration of the experiment was 28 days.

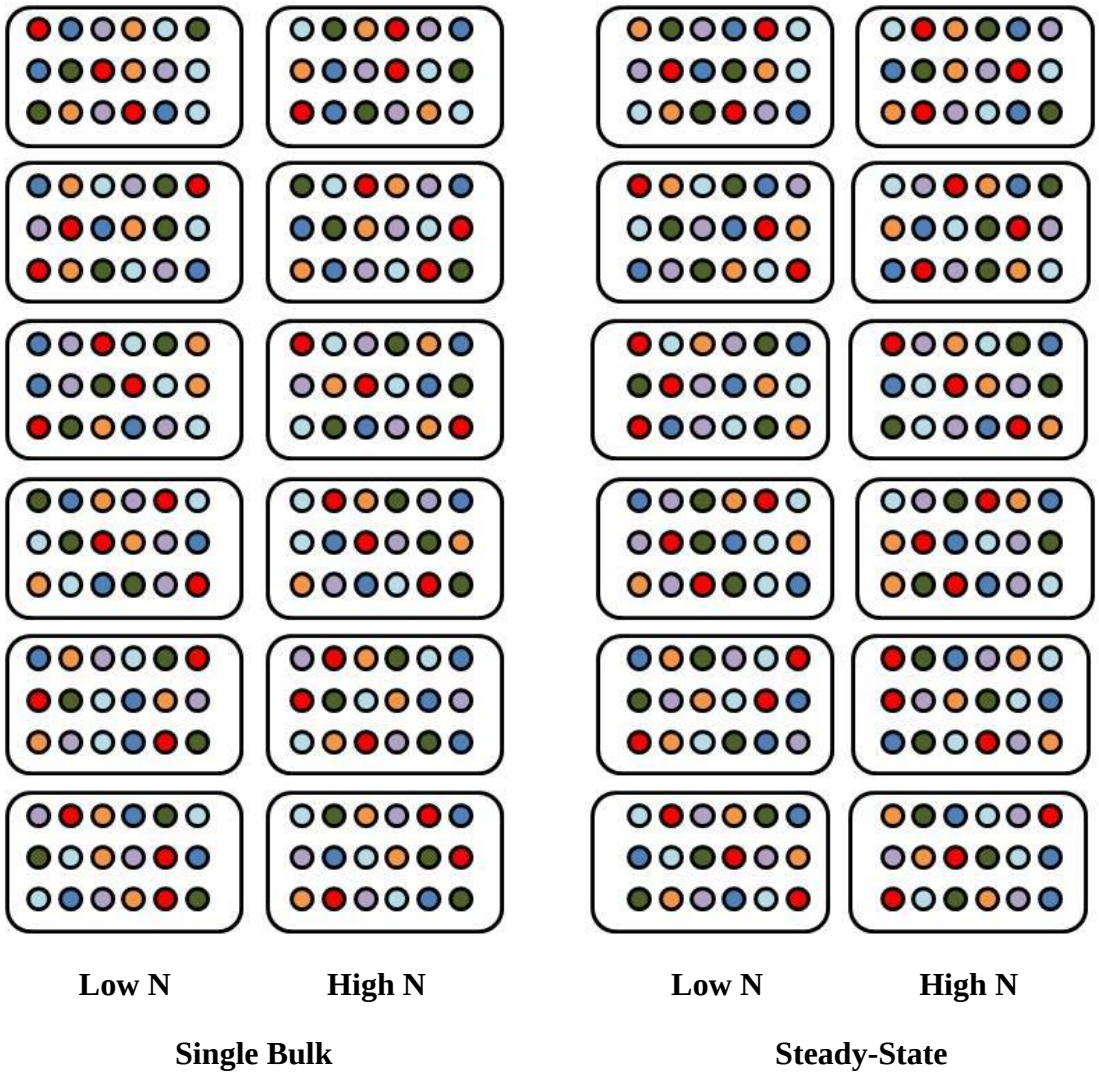

Figure 3.1. Experimental design with six containers per N application treatment. The rows of each container was randomly filled with single plants from each cultivar tested in the current study.

The experiment had a hierarchical design in which each combination of application method and N-level was assigned to a single hydroponics unit. A seedling of each cultivar was randomly assigned to one of the eight plant positions within each container row (three cultivar replicates per container). The open plant positions were filled with dummy plants. The experiment comprised of six containers per hydroponics unit. Plants from half of the

containers (three containers) of each hydroponics unit were harvested at Day 14 (Harvest 1) and the rest at Day 28 (Harvest 2) and used to determine multiple traits.

Evaluation of cultivars

At both Harvest 1 and Harvest 2 the plants were patted dry with industrial paper tissue and divided in a root and shoot fraction, which were weighed separately to determine the plant's **root fresh weight (RFW)** and **shoot fresh weight (SFW)**. The plant shoot fraction, including leaf blade and petioles, was used to determine the **leaf area (LA)** (cm^2), with a Licor Leaf Area Scanner (LI-3100C). The shoot and root fraction of each plant were dried for two days at 70˚C to get measures for plant **shoot dry weight (SDW)** and **root dry weight (RDW)** from which the **root-to-shoot ratio (R:S)** was calculated as R:S = Root Dry weight / Shoot Dry Weight. The **dry matter percentage of the shoot (DM%)** was calculated as DM% = (SDW/SFW) x 100%. The measurements of LA and SDW were used to calculated the **specific leaf area (SLA)** (cm^2 g^{-1}) as SLA = LA / SDW. Prior to harvest the **chlorophyll content (CC) (**SPAD readings**)** of the oldest pair of leaves of each plant was determined with a SPAD 502 chlorophyll meter (Konica Minolta, Osaka, Japan). Roots were preserved in a solution of 10% ethanol, and scanned and analysed with the software WinRhizo (Regent Instruments Inc., Ottawa, Canada) within two weeks after harvest. The root traits obtained were **average diameter of roots (ADR)** (mm), **total length of roots (TLR)** (m) and **surface area of roots (SAR)** (cm^2)**.** The **nitrate contents (NO_3^-)** (ppm) of Harvest 2 leaf samples were measured with a LAQUA twin nitrate meter (Spectrum Technologies Inc, Aurora, USA), using the supernatant from 0.10 g dry leaf material in 1 ml of H_2O.

Statistical analysis

The experiment was statistically analysed for the two harvests separately using GenStat v16.0 (VSN International Ltd). Descriptive statistics and analyses of variance (ANOVAs) were performed while taking into consideration the hierarchical design of the experiment. It had for each combination of application method and N-level a randomized block design with 9 replicates per cultivar. Cultivar means were calculated per harvest time for each combination of application method and N-level. NUE-related traits differentially affected by the N application method were included as dependent variates in a multiple regression analysis with SFW and SDW as response variate. These traits were chosen as they correspond best with the yield of a spinach crop and NUE. The multiple linear regression was performed to quantify the strength of the relationship between SFW or SDW to R:S, LA, DM%, SLA and RDW using a the *RSearch* procedure of GenStat to determine the best model, the estimate of each trait and the level of significance.

3.3. Results

Differences between N application methods

All the cultivars were affected by N level under both application strategies and at both harvests (Figure 3.2 and Table 3.2), with stronger effects at Harvest 2 (H2) (see also supplementary Figure 3.A, and Supplementary Table 3.A). At Low N, shoot dry weight (SDW) and leaf area (LA) were lower and SLA and R:S were higher than at High N at both harvests. DM% however was slightly lower at Low N compared to High N at H1, but higher at H2.

The application strategy significantly affected growth of the spinach cultivars. The direction of the trait value differences between High and Low N was similar under steady state and single N application, but for steady state-grown plants the effect of N was smaller than for single N application-grown plants.

At High N, single N application resulted in plants with higher SDW and SFW than the Ingestad N application at both harvests, but at Low N SFW was highest for steady state plants (Figure 3.2 and Supplementary Figure 3.A). A striking difference between plants grown under steady state and single N application was found for R:S, especially under Low N, with R:S considerably higher for steady state plants. Under Low N, steady-state-grown plants had considerably lower SDW while LA was relatively similar to single N application-grown plants, which accounted for a higher SLA for plants under steady state conditions. The high DM% for single N application plants at Low N compared to steady state plants and plants grown at High N at Harvest 2 indicated that at single N application conditions, the plants had difficulty maintaining leaf water status at later stages of vegetative development of the crop. This was supported by a stronger decrease in chlorophyll content (SPAD) (Table 3.2 and Supplement Figure 3.A) for low single N application plants, indicating that the metabolic status of the leaves of these plants was deteriorating faster than for plants grown under steady state conditions.

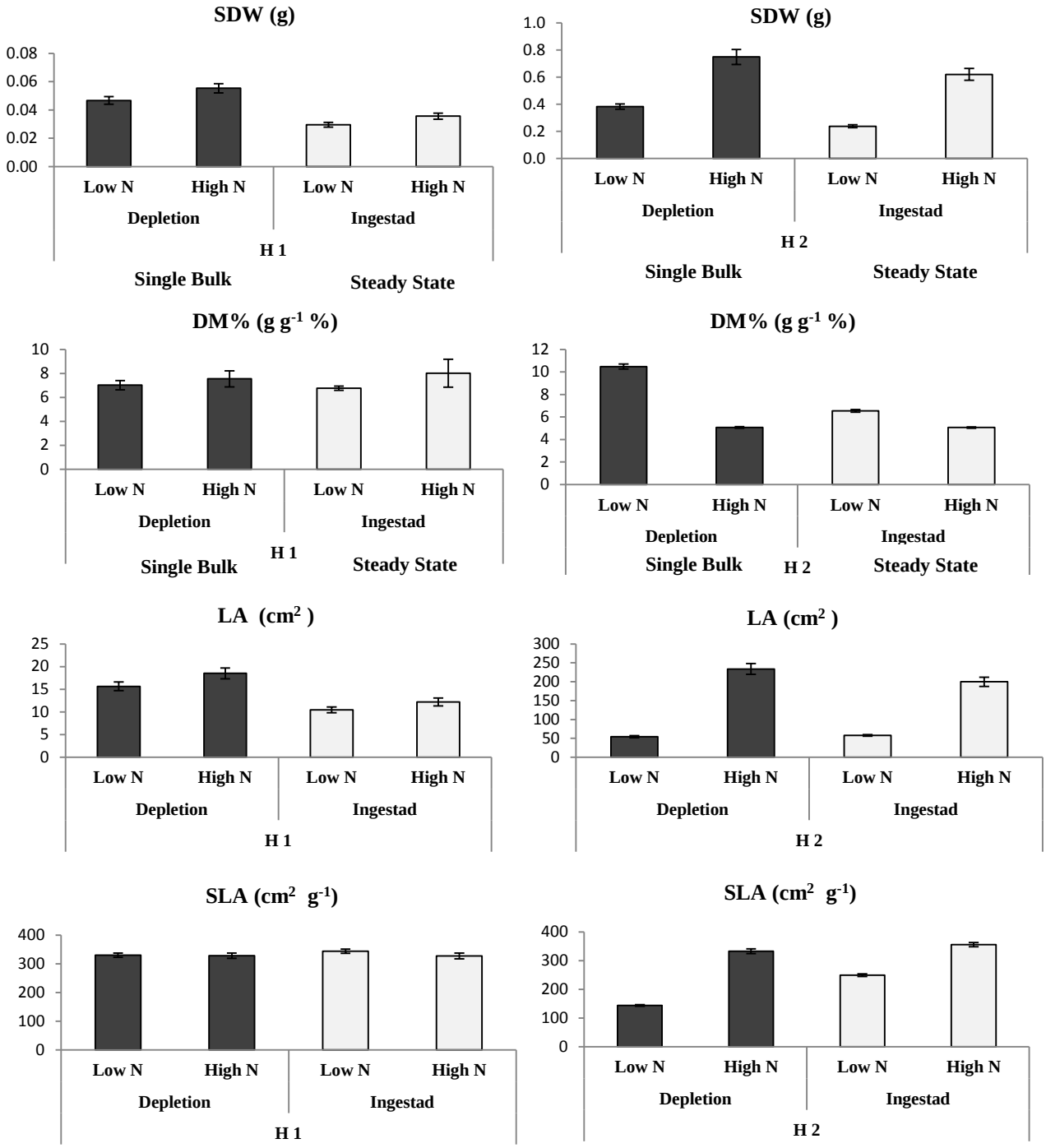

Figure 3.2. Average plant performance for four NUE-related traits assessed after 14 and 28 days on hydroponics (H1, H2) under either Single Bulk N application or Steady State conditions at Low N and High N. SDW= shoot dry weight; DM%= dry matter percentage of the shoot; LA= leaf area; SLA= specific leaf area. The error bars depict the standard error of means.

Table 3.2. Means and ranking (in brackets) for six growth traits determined for seven cultivars tested with two N application strategies: Single Bulk (SB) and Steady-State (SS), both at High and Low N. The data was collected at Harvest 2. SDW=shoot dry weight, R:S=Root to shoot ratio, LA=leaf area, SLA=specific leaf area, NO_3^{--}=free nitrate concentration, CC=chlorophyll content

Cultivar	SDW (g)				R:S (g g^{-1})			
	Low N		High N		Low N		High N	
	SB	SS	SB	SSS	SB	SS	SB	SS
Chevelle	0.35 (5)	0.27 (2)	0.82 (3)	0.72 (2)	0.35 (3)	0.57 (3)	0.14 (4)	0.18 (5)
Cello	0.36 (4)	0.21 (6)	0.34 (7)	0.44 (5)	0.36 (2)	0.57 (4)	0.14 (2)	0.20 (2)
Novico	0.37 (3)	0.22 (5)	0.86 (2)	0.57 (4)	0.30 (4)	0.59 (1)	0.15 (1)	0.20 (1)
Andromeda	0.48 (1)	0.25 (3)	1.17 (1)	1.06 (1)	0.26 (5)	0.46 (6)	0.13 (5)	0.19 (4)
Crocodile	0.33 (7)	0.23 (4)	0.65 (5)	0.43 (6)	0.23 (7)	0.54 (5)	0.12 (6)	0.16 (6)
Sparrow	0.43 (2)	0.27 (1)	0.61 (6)	0.68 (3)	0.24 (6)	0.39 (7)	0.10 (7)	0.16 (7)
Marabu	0.34 (6)	0.20 (7)	0.76 (4)	0.42 (7)	0.37 (1)	0.58 (2)	0.14 (3)	0.19 (3)
Mean	*0.38*	*0.23*	*0.75*	*0.62*	*0.30*	*0.52*	*0.13*	*0.18*
SEM	*0.02*	*0.01*	*0.05*	*0.04*	*0.01*	*0.02*	*0.01*	*0.01*

Cultivar	LA (cm^2)				SLA ($cm^2.g^{-1}$)			
	Low N		High N		Low N		High N	
	SB	SS	SB	SS	SB	SS	SB	SS
Chevelle	53.9 (5)	66.3 (1)	247.4 (3)	228.3 (2)	155.3 (2)	255 (3)	318.1 (4)	325.0 (6)
Cello	56.1 (4)	50.5 (6)	119.1 (7)	159.6 (5)	155.0 (3)	244 (5)	408.7 (1)	376.0 (2)
Novico	58.6 (3)	58.0 (4)	259.0 (2)	219.7 (3)	164.2 (1)	275 (1)	340.1 (3)	393.0 (1)
Andromeda	59.7 (2)	59.2 (3)	355.5 (1)	320.6 (1)	126.2 (6)	238 (6)	310.1 (6)	318.5 (7)
Crocodile	42.1 (7)	57.3 (5)	194.7 (6)	138.4 (6)	125.3 (7)	256 (2)	315.2 (5)	367.9 (3)
Sparrow	60.6 (1)	59.5 (2)	232.4 (4)	205.4 (4)	142.2 (4)	222 (7)	347.5 (2)	353.3 (4)
Marabu	45.4 (6)	48.4 (7)	212.9 (5)	132.8 (7)	138.0 (5)	254 (4)	303.7 (7)	348.8 (5)
Mean	*54*	*56.2*	*233.6*	*198.4*	*144.0*	*253.0*	*331.4*	*353.9*
SEM	*2.8*	*2.1*	*13.9*	*11.4*	*2.8*	*5.6*	*8.0*	*7.1*

Cultivar	NO_3^- (ppm)				CC (SPAD)			
	Low N		High N		Low N		High N	
	SB	SS	SB	SS	SB	SS	SB	SS
Chevelle	*	*	*	*	17.77 (4)	21.82 (6)	23.08 (5)	26.04 (5)
Cello	1600 (1)	1127 (4)	5067 (1)	5033 (2)	16.63 (5)	23.57 (3)	23.01 (6)	29.94 (1)
Novico	1243 (3)	1203 (1)	3633 (4)	3967 (5)	8.27 (7)	20.61 (7)	21.81 (7)	23.59 (7)
Andromeda	1180 (5)	1170 (3)	2967 (5)	3333 (6)	24.51 (1)	22.29 (5)	24.89 (3)	28.17 (4)
Crocodile	1300 (2)	1057 (5)	3833 (2)	4800 (3)	19.26 (3)	23.97 (2)	25.94 (1)	28.83 (3)
Sparrow	1073 (6)	937 (6)	3733 (3)	5100 (1)	20.96 (2)	23.34 (4)	23.39 (4)	25.71 (6)
Marabu	1230 (4)	1200 (2)	2900 (6)	4333 (4)	16.47 (6)	25.52 (1)	25.50 (2)	29.08 (2)
Mean	*1269*	*1105*	*3689*	*4428*	*18.04*	*22.31*	*24.1*	*27.14*
SEM	*307*	*215*	*1111*	*933*	*4.44*	*2.34*	*1.37*	*2.04*

Cultivar-specific responses

Significant differences between cultivars were found for all traits ($P < 0.05$) (summarized in Figure 3.2 and Table 3.2).

Ranking of cultivars for SFW (supplementary Table 3.A, in brackets) was similar under single N application and steady state conditions at High N, but there were striking differences for a few cultivars in particular at Low N. The highest SFW (2.92g) under low single N application was found for cv. Cello, but it was only ranked 6th under steady state conditions (2.73g). Cv. Chevelle on the other hand had the highest SFW under steady state conditions (3.74g), while it was average under single N application conditions (2.60g).

The differential response of the cultivars to N availability under both N application strategies was examined by ANOVAs performed separately for each combination of harvests (H1 or H2) and N-level (Low or High N) (Table 3.3). The analyses over application strategies showed for both harvests significant differences between cultivars ($P<0.05$) for all traits, except for DM% at High N (both harvests) and SDW under Low N conditions at H2. Significant Genotype by Application strategy (G×A) interaction ($P<0.05$) was only observed for a few traits at H1 at High N, and at H2 at Low N. The genotype-specific differences in response to the N application strategy were obviously more expressed after prolonged exposure to Low N, in particular for R:S, SLA, and DM%. At High N however, SFW, SDW, LA and R:S were differentially affected by N application strategy at an early stage (H1).

Cv. Cello was the least affected by Low N availability under single N application conditions, with 67% reduction in SFW and 53% in LA under Low N relative to High N availability, while most cultivars showed a reduction of more than 80% (SFW) and 73% (LA). At the same time, cv. Cello's root length (3-fold increase) and root surface area (RSA) were much higher under Low N compared to High N (Supplementary Table 3.B). In addition SFW, SDW

and LA at the first harvest were even reduced (25%, 20% and 27%) at High N compared to Low N under single N application. The internal (free) nitrate concentrations of cv. Cello under High single N application however was highest of all cultivars (Figure 3.3, Table 3.3), indicating that N uptake was not inhibited.

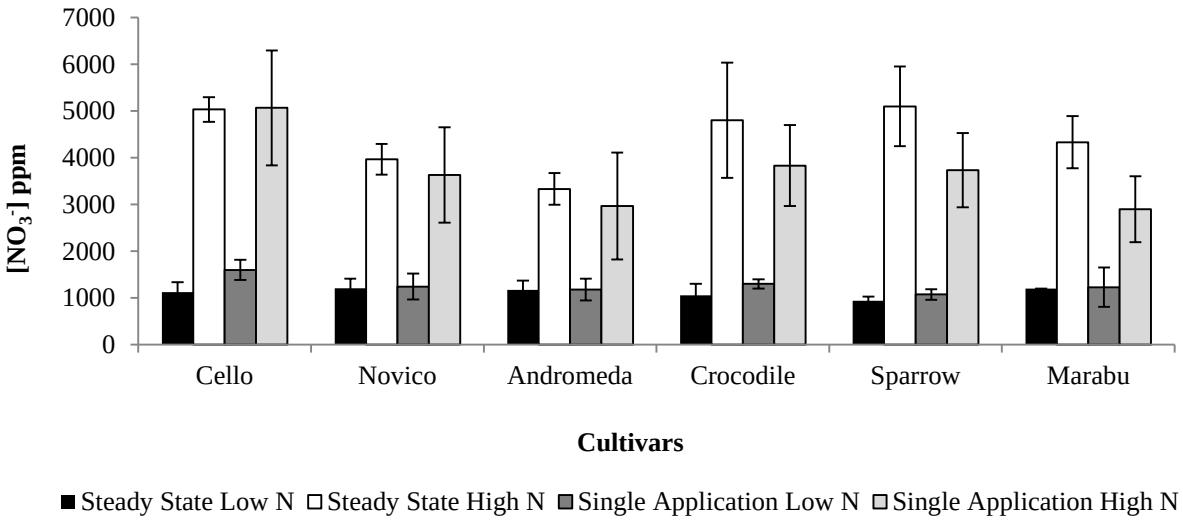

Figure 3.3. Average nitrate concentration of six cultivars under steady state and single N application conditions at Harvest 2. *Note:* cv. Chevelle was not included due to loss of the plant material at storage and cv. Marabu at Steady-State Low N had only one sample.

Cultivar Andromeda on the other hand was highly responsive to N. It had the highest SFW, SDW and LA under High N conditions, both under steady state and single N application conditions, and had the strongest increase in SFW, SDW and LA compared to Low N of all cultivars. Even at Low N, cv. Andromeda had relatively high SFW, SDW and LA compared to the other cultivars.

Under steady state conditions cv. Crocodile was the least affected by Low N compared to High N conditions, with the smallest SFW, SDW and LA reductions. This is at least partly due to limited responsiveness to higher levels of N. Cv. Crocodile had the highest absolute and relative decrease in root biomass at High N compared to Low N at steady state conditions

(42%; Supplementary Table 3.B), which may have limited its N uptake efficiency at High N. Cv. Sparrow was among the best performers under single Low N application conditions, with highest SDW and LA together with Andromeda. Sparrow however was the least responsive cultivar to a single High N application. In contrast to cv. Andromeda and the other cv. Sparrow invested under all application regimes relatively little in the roots, as can be deduced from the R:S cultivar ranking. Growth of cv. Sparrow under single High N conditions may therefore be limited by its relatively poorly developed root system.

Table 3.3. F-probabilities for Genotype and Genotype by Application strategy interaction (G×A) obtained from ANOVAs for six NUE-related traits measured at either Low or High N in two successive harvests. SFW=shoot fresh weight, SDW=shoot dry weight, R:S= root to shoot ratio, DM=dry matter percentage; LA=leaf area; SLA=specific leaf area

N Level	Trait	Harvest 1		Harvest 2	
		Genotype	GxA	Genotype	GxA
Low N	SFW	<.001	0.308	0.041	0.150
	SDW	<.001	0.513	0.088	0.591
	R:S	0.003	0.899	<.001	0.036
	DM%	<.001	0.319	<.001	0.053
	LA	<.001	0.370	0.025	0.280
	SLA	<.001	0.272	<.001	0.005
High N	SFW	<.001	0.025	<.001	0.610
	SDW	<.001	0.007	<.001	0.450
	R:S	0.006	0.001	<.001	0.505
	DM%	0.053	0.999	0.375	0.097
	LA	<.001	0.009	<.001	0.687
	SLA	0.006	0.566	<.001	0.322

Relationship between traits

The dependence of the plant-to-plant variation of SFW and SDW on the variation observed for the growth-related traits R:S, LA, DM% and SLA was analysed using a multiple regression approach. This was done for each screening condition separately in order to get a better insight in how N application strategy influenced the way these traits affected plant

growth. To facilitate comparison between screening conditions we normalised the response variates SFW and SDW (nSFW and nSDW in Table 3.4) as well as the dependent traits. The best multiple linear regression model chosen for the individual screening conditions explained nearly all variation for both SDW and SFW (R^2_{adj} > 95.6%) (Table 3.4). The best model for nSFW included all the dependent traits, with strongest roles for nLA and nSLA. For nSDW, the contributions of LA and SLA were even stronger for the steady-state/High N and both single N application conditions. Under all conditions the variation in nLA positively affected nSFW and nSDW, while nSLA had a smaller, consistently negative impact on both traits. The dependent trait nDM% had a moderate, significantly negative influence on nSFW under all screening conditions, whereas nR:S was just significant for the single bulk application at Low N only. However, the relative importance of the four different plant characteristics determining the normalized variation in SFW did not differ much from condition to condition.

Table 3.4. Multiple linear regression parameters calculated for four screening conditions describing the plant-to-plant variation for the response variates shoot fresh weight (SFW) and shoot dry weight (SDW) based on the best subset of four plant traits. All traits were normalised (n) prior to the regression analyses. Traits not included in the best subset are indicated by not applicable (NA). R:S=root/shoot ratio; LA=leaf area; DM%=dry matter percentage of the shoot; SLA=specific leaf area

Screening condition	**Regression coefficient**					R^2_{adj}
	Constant	**nR:S**	**nLA**	**nDM%**	**nSLA**	
*Response variate: n**SFW***						
Single Bulk/Low N	0.002^{***}	0.042^{ns}	0.977^{***}	-0.101^{**}	-0.110^{**}	95.6
Steady State/Low N	0.000^{***}	0.038^{ns}	0.905^{***}	-0.107^{**}	-0.204^{***}	97.1
Single Bulk/High N	0.036^{***}	-0.043^{*}	0.881^{***}	-0.183^{***}	-0.224^{***}	98.4
Steady State/High N	-0.062^{***}	-0.032^{ns}	0.900^{***}	-0.202^{***}	-0.245^{***}	97.9
*Response variate: n**SDW***						
Single Bulk/Low N	0.012^{***}	NA	0.958^{***}	NA	-0.308^{***}	97.7
Steady State/Low N	0.000^{***}	-0.101^{**}	0.816^{***}	0.063^{ns}	-0.215^{***}	95.6
Single Bulk/High N	0.027^{***}	NA	0.821^{***}	NA	-0.201^{***}	96.8
Steady State/High N	-0.059^{***}	NA	0.861^{***}	NA	-0.188^{***}	97.3

^{ns}: not significant; *; significant at $P<0.05$; **: significant $P<0.01$;***:$P<0.001$

3.4. Discussion

Improving growth of crops under low N input conditions is an important challenge for breeders. Complex traits like NUE are difficult to breed for, both due to the genetic complexity of NUE and the interaction with environmental factors (Xu et al. 2012). Here we demonstrated that traits contributing to NUE in spinach under optimal, but in particular under N limiting conditions were differentially expressed depending on the way N is made available to the plant, in a genotype-dependent manner.

N availability and the timing of N application were shown to have various morphological and physiological implications for plant growth that are particularly strong for growth of spinach under low N input conditions. Nevertheless there were significant differences at High N among cultivars in the way they respond to the N application method as well. Cultivar Andromeda was highly responsive to high N levels, had the highest SFW, SDW and LA under High N growth conditions, and was among the best performers at Low N as well (Table 3.2), while other cultivars did not respond to high N application with increased growth, but even with a reduction in growth parameters like SDW, SFW and LA at Harvest 1 (cv. Cello) and thus a strongly decreased NUE under High N. Several studies point to a positive relationship between amount of available N in the root environment and growth in leafy vegetables like lettuce (Liu et al. 2014); even though at very high concentrations the response to N was decreasing. The growth response of cv. Cello in our study however pointed to an inhibitory effect of high N levels in the root environment on its growth, or at least an inability to utilize the available high levels of nitrogen for growth. This inability to respond to high N availability may have resided in inadequate uptake of nitrogen or inefficient utilisation of N for growth after uptake. However, measurements of leaf free nitrate concentrations at the second harvest showed that cv. Cello had the highest internal nitrate concentrations of all tested cultivars (up to 6000ppm) under high single N application (Table 3.2 and Figure 3.3),

in line with increased nitrate content with increasing N fertilizer application found in spinach (Lee 1970), lettuce (Liu et al. 2014) and other crops (Gunes et al. 1995; Pavlou et al. 2006). The lack of responsiveness of cv. Cello to N may therefore have resided in inefficient use of N that is taken up, in particular at high N availability.

For cultivars Crocodile and Sparrow, the limited responsiveness to N may be associated with the decrease in root length and root biomass under steady state (cv. Crocodile) or single application (cv. Sparrow) high N conditions. However, the influence of a more proliferated root system for resource capture under non N-limiting conditions was likely to be minimal in soil (Kerbiriou et al. 2014), and is likely to have contributed even less under hydroponics conditions, in which N was circulated and brought to the roots, suggesting that the limited root biomass under High N would not have been an important factor for the limited N responsiveness of cv. Crocodile and cv. Sparrow.

Our results thus indicated that varying not only the N level, but also the N application strategy allows for selection of traits contributing to NUE and growth under limited N availability that would be lowly or not expressed at all. Single N application at the beginning of the growth cycle favour selection of traits that relate to delayed senescence and efficient N remobilisation, and possibly growth under luxurious N, while the steady state application according to Ingestad may enable selection for adaptation of root traits and the root to shoot ratio to low N, along with optimising the C/N balance. Plants grown under High and Low steady state application conditions showed no obvious N deficiency symptoms, as suggested by Ingestad (1982). This study however also confirmed the finding of Biemond (1995) that at the onset of growth sufficient N must be available to enable optimal growth. Given the difficulties of defining N deficiency stress under field conditions (Xu et al. 2012), optimal selection strategies for successful identification of genetic factors contributing to growth under low N conditions as described in this study and in Chapter 2, and combining these with

field test for validation may be the most effective approach towards NUE improvement of crops like spinach (Loudet et al. 2003, Hirel et al. 2007, Xu et al. 2012).

A trait that showed strong variation depending on the N application strategy was SLA, which was increased under steady state low N conditions, and even more strongly increased in plants grown under single N application conditions (Figure 3.2). Multiple studies have shown that plants can change the investment of N in photosynthetic components, re-allocate N within the leaf (Evans 1989, Evans 1993, Hikosaka and Terashima 1996, Hikosaka et al. 1998, Niinemets et al. 1998), or adapt SLA (Evans 1993, Hirose and Werger 1987, Sims et al. 1994) in response to environmental changes. Plants balance the demand for limiting resources amongst others by reducing ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) levels under conditions of reduced N supply (Quick et al. 1992), while under high N conditions Rubisco can act as a sink for assimilates, which would be reflected in higher LA with higher N availability, and higher SLA. De Pinheiro Henriques and Marcelis (2000), however, showed that the decrease in SLA under low but steady state N supply in another leafy vegetable, lettuce, could be largely attributed to a decrease in DM%. This was confirmed in our study in which the decrease in SLA under steady state low N conditions was not accompanied by a similar decrease in leaf thickness (which can be expressed as LA/SFW), but could be explained by a decrease in DM% as well. Reduced water content, and reduced cell elongation may therefore underlie the reduction in SLA in response to low N availability. In addition to dry matter partitioning and nitrogen partitioning, leaf free nitrate concentration was shown to be closely related to plant N concentration and N supply in lettuce grown under steady-state conditions (De Pinheiro Henriques and Marcelis 2000) which is consistent with data of Ingestad and McDonald (1989), Boot et al. (1992) and Van der Werf et al. (1993). The low free nitrate concentrations found in the leaves of the spinach plants grown under N-limiting conditions indicated a similar relationship in spinach.

Free nitrate is stored in the vacuole, and can serve as an osmoticum (Blom-Zandstra and Lampe 1985, Cardenas-Navarro 1999). The low free nitrate concentrations measured under low N conditions in our study would increase the osmotic potential of the plant leaf cells, and may have been at least partly responsible for the increase in DM%. In lettuce a similar increase in DM% was attributed to nitrate, acting either as an osmoticum, a cell wall relaxation agent or as a promotor of root hydraulic conductance (De Pinheiro Henriques and Marcelis, 2000). Furthermore, the lower concentration of nitrate in the vacuole may be compensated by an increase in vacuolar content of soluble carbohydrates, serving as osmolytes, possibly at the cost of growth (Blom-Zandstra 1989). The latter hypothesis was not supported by our data, as the variation in free nitrate among the tested spinach cultivars was not significantly correlated to SDW. Indeed, the cultivar (Cello) with the highest nitrate contents at high N availability was the lowest-yielding, as discussed above.

SLA and DM% were even more affected under low single N application conditions than under steady state N-limiting conditions. Schöttler and Tóth (2014) indicated that in response to a sudden sink limitation of photosynthesis, growth is restricted due to slowdown of the Calvin-Benson cycle and even light-induced damage to the photosynthetic electron transport system. The single application of N may have likely caused a similar sink limitation of photosynthesis after several weeks in our experiment, possibly inducing damage and chlorophyll breakdown. The much stronger loss of water (higher DM%) under these conditions at Low N may be an indication for N limitation-induced senescence, which was further supported by the stronger decrease in chlorophyll content of first and second leaves in plants growing under these conditions (Table 3.2).

3.5. Implications for spinach breeding and cultivation

Selection for a trait like NUE is challenging under field conditions. Environmental factors can impact NUE in many ways, and N availability is very difficult to control (Loudet et al. 2003, Hirel et al. 2007, Xu et al. 2012). Selection under more controlled conditions takes away several of these constraints, but as shown by others and in this paper, the applied conditions and stress interact with the genotypic response of the plants (Hirel et al. 2001; Loudet et al. 2003; Chapter 2, Chan-Navarrete et al. 2014). Choices on the level of stress to apply, the timing of the stress in relation to the plant phenology, and the application strategy do not only impact the magnitude, but sometimes even the direction of the response. And shown in this study, there was a strong differential genotypic response to the applied conditions. Selection and genetic dissection of traits linked to photosynthetic adaptation, like R:S, SLA and DM% can be studied under steady state conditions, but are likely to be confounded by senescence effects at later stage of vegetative growth in single N application strategies. The single High N application conditions may resemble field conditions, but the high N levels may induce an inhibitory response, as we have seen in our trials with the cultivar Cello. When aiming at unravelling traits contributing to NUE in spinach, it is therefore worthwhile to screen for contributing traits under different N application strategies.

The most stable trait for assessing NUE under different application strategies and N level was LA (Table 3.4). For selection of the best performing genotype, assessing LA at different stages of the growth cycle is likely to be reliable and new phenotyping and sensor technologies now allow more accurate phenotyping of LA in the field as well.

Acknowledgements

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Supplementary data

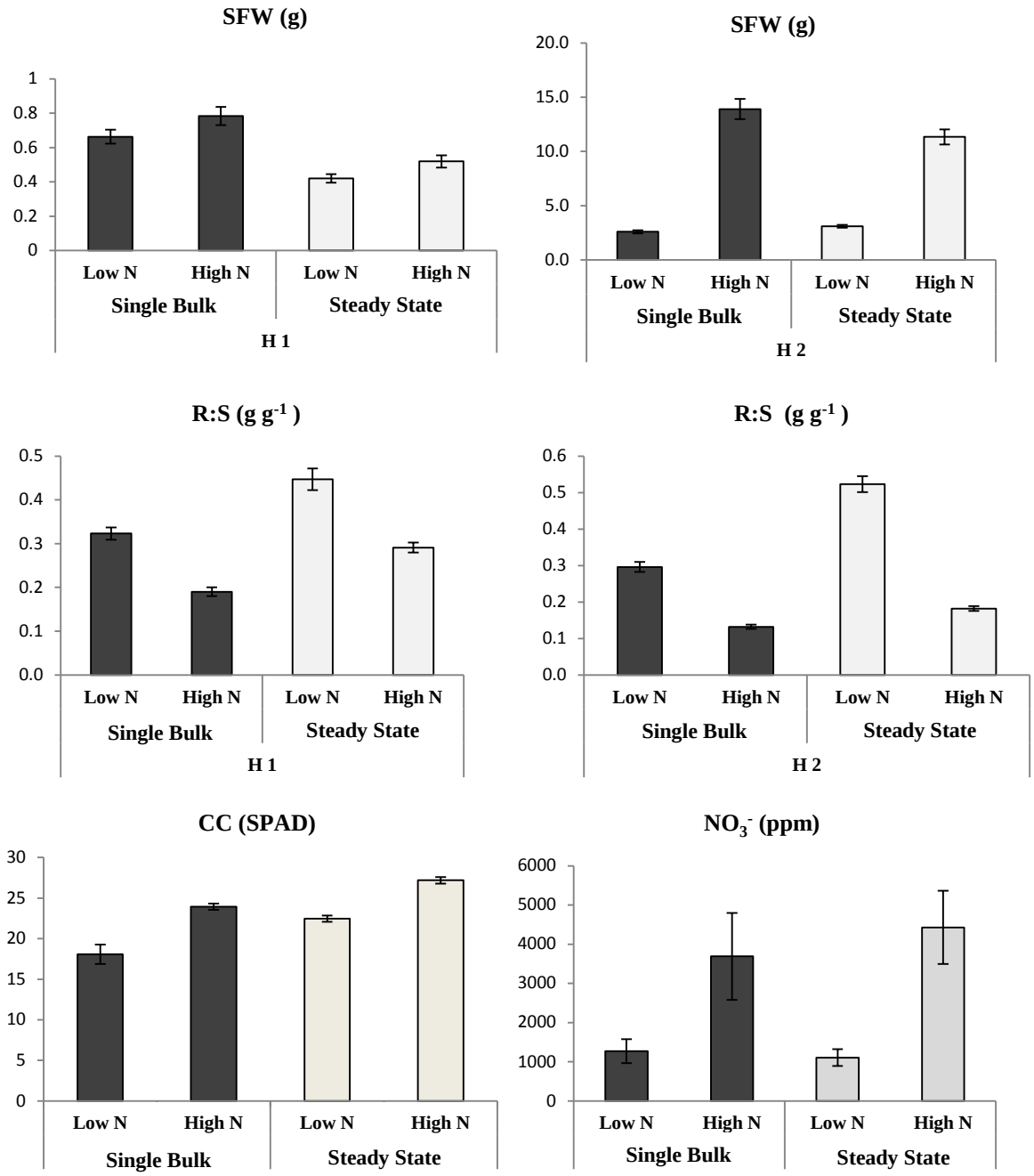

Figure 3.A. Average plant performance for four NUE-related traits assessed after 14 and 28 days on hydroponics (H1, H2) under either Single Bulk or Steady-State N application at Low N and High N. The error bars depict the standard error of means. SFW=shoot fresh weight, R:S= root to shoot ratio, CC=chlorophyll content

Table 3.A. Means and ranking (in brackets) for two growth traits (shoot fresh weight (SFW), dry matter percentage (DM%)) for seven cultivars grown with either the Single Bulk N application strategy (SB) or Steady-State strategy (SS), both at High and Low N. The data was collected at Harvest 2.

Cultivar	SFW (g)				DM% (g g^{-1} %)			
	Low N		High N		Low N		High N	
	SB	SS	SB	SS	SB	SS	SB	SS
Chevelle	2.60 (4)	3.74 (1)	15.05 (2)	13.24 (2)	9.73 (3)	6.15 (2)	5.04 (3)	5.02 (4)
Cello	2.92 (1)	2.73 (6)	6.81 (7)	7.83 (7)	9.61 (2)	6.82 (6)	4.72 (1)	5.18 (6)
Novico	2.55 (5)	3.14 (4)	14.85 (4)	10.69 (4)	10.34 (4)	6.17 (3)	5.29 (6)	5.00 (3)
Andromeda	2.83 (2)	3.30 (3)	21.86 (1)	18.87 (1)	10.60 (5)	6.75 (5)	5.18 (5)	5.24 (7)
Crocodile	2.16 (7)	3.13 (5)	11.58 (6)	7.90 (5)	11.58 (6)	6.45 (4)	5.35 (7)	4.95 (2)
Sparrow	2.82 (3)	3.36 (2)	12.23 (5)	12.59 (3)	11.66 (7)	7.27 (7)	4.78 (2)	5.10 (5)
Marabu	2.25 (6)	2.70 (7)	14.06 (3)	7.90 (5)	9.46 (1)	6.05 (1)	5.11 (4)	4.92 (1)
Mean	*2.59*	*3.16*	*13.78*	*11.29*	*10.39*	*6.52*	*5.06*	*5.04*
SEM	*0.11*	*0.14*	*1.72*	*1.53*	*0.21*	*0.11*	*0.07*	*0.06*

Table 3.B. Means and ranking (in brackets) for root dry weight (RDW), average diameter of roots (ADR), surface area of roots (SAR), and total length of roots (TLR) for seven cultivars grown with two N application strategies: Single Bulk (SB) and Steady-State (SS), both at High and Low N. The data was collected at Harvest 2.

	RDW (g)				ADR (mm)			
Cultivar	**Low N**		**High N**		**Low N**		**High N**	
	SB	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**
Chevelle	0.129 (3)	0.151 (1)	0.108 (3)	0.131 (2)	0.342 (5)	0.369 (2)	0.307 (6)	0.318 (7)
Cello	0.130 (2)	0.121 (4)	0.048 (7)	0.091 (5)	0.372 (3)	0.324 (7)	0.320 (5)	0.331 (5)
Novico	0.175 (1)	0.126 (2)	0.139 (2)	0.117 (3)	0.364 (4)	0.328 (5)	0.304 (7)	0.343 (3)
Andromeda	0.125 (4)	0.112 (5)	0.149 (1)	0.196 (1)	0.372 (1)	0.332 (4)	0.338 (3)	0.342 (4)
Crocodile	0.081 (7)	0.122 (3)	0.078 (5)	0.065 (7)	0.339 (6)	0.344 (3)	0.364 (1)	0.318 (6)
Sparrow	0.106 (6)	0.107 (6)	0.067 (6)	0.111 (4)	0.323 (7)	0.325 (6)	0.331 (4)	0.354 (2)
Marabu	0.111 (5)	0.094 (7)	0.104 (4)	0.081 (6)	0.372 (2)	0.376 (1)	0.356 (2)	0.380 (1)
Mean	0.122	0.119	0.099	0.113	0.355	0.343	0.331	0.341
SEM	0.010	0.006	0.013	0.015	0.007	0.008	0.008	0.008

	SAR (cm^2)				TLR (m)			
Cultivar	**Low N**		**High N**		**Low N**		**High N**	
	SB	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**
Chevelle	28.82 (3)	31.18 (2)	34.79 (2)	35.26 (2)	352.9 (2)	337.7 (2)	447.0 (2)	458.0 (2)
Cello	31.28 (2)	25.05 (5)	10.01 (7)	21.84 (5)	323.1 (3)	290.7 (5)	111.6 (7)	256.6 (5)
Novico	26.84 (5)	27.20 (4)	22.89 (5)	27.61 (3)	300.7 (4)	323.5 (3)	260.0 (4)	306.4 (3)
Andromeda	28.56 (4)	32.54 (1)	50.62 (1)	44.15 (1)	299.4 (5)	382.3 (1)	570.1 (1)	546.7 (1)
Crocodile	16.96 (7)	22.75 (7)	24.02 (4)	24.29 (4)	192.4 (7)	239.9 (7)	234.8 (5)	273.8 (4)
Sparrow	22.29 (6)	23.61 (6)	13.57 (6)	18.76 (7)	245.6 (6)	279.7 (6)	150.0 (6)	186.3 (7)
Marabu	36.44 (1)	30.70 (3)	32.83 (3)	19.63 (6)	386.5 (1)	315.0 (4)	338.9 (3)	194.2 (6)
Mean	27.31	27.58	26.96	27.36	300.10	309.80	301.80	317.40
SEM	2.19	1.38	4.84	3.25	22.75	15.91	57.12	47.47

Chapter 4

Genetic map construction and QTL analysis of nitrogen use efficiency in spinach (*Spinacia oleracea* L.)

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Abstract

Cultivation of spinach requires high amounts of nitrogen (N), which puts a strain on the environment. A sustainable solution to this problem is to breed for crops with higher N use efficiency (NUE). The aim of this study was to provide tools for molecular breeding and to elucidate the genetic variation of factors contributing to NUE in spinach. A cross was made between two F1 hybrid cultivars contrasting in NUE. Several F1 progeny were self-pollinated and based on evaluation of the F2 generation, a mapping F2 population (335 individuals) of a single F1 was selected. SNP markers for the genetic map were discovered by RNA sequencing of the two parent cultivars, and 283 SNP markers were used to produce a genetic map comprising of six linkage groups (P01-P06), ranging in size from 46 to 116 cM. NUE related traits were determined for a set of F2:3 families grown under low and high N conditions in a hydroponics system under an Ingestad N-addition model. Interval mapping analysis detected 39 trait-specific QTLs, with several QTLs accumulating on P01 and P02 of the linkage map. The QTLs and in particular the P01 and P02 regions provide potential targets for the improvement of NUE in spinach.

Keywords: *Spinacia oleracea* • Nitrogen Use Efficiency (NUE) • Quantitative Trait Locus (QTL) • Genetic map

4.1. Introduction

Nitrogen is the nutrient that most frequently limits plant growth (Fageria and Baligar 2005). In most current crop production systems plants rely on mineral fertilizers to meet their N demand. The high levels of N fertilization are often associated with environmental problems such as eutrophication of soil and surface water and emission of greenhouse gases (Lawlor et al. 2001). The environmental pollution together with human health issues has resulted in strict regulation of nitrogen fertilization in Europe (European Commission 2010). This legislation has a large impact on the cultivation of vegetable crops, in particular of spinach. Mitigation of the negative effects of lowering N input on productivity of spinach therefore is a major challenge. This holds true in particular for production systems that require low external input, such as organic agriculture, in which N inputs would preferably be reduced from 150 kg ha^{-1}

to approx. 100 kg N ha^{-1} or less (Fageria and Baligar 2005). A long term sustainable strategy contributing to cultivating spinach with less nitrogen is the genetic improvement of its nitrogen use efficiency (NUE), resulting in cultivars that can realize an economically acceptable yield even under low input conditions.

Nitrogen efficiency is a complex trait that is the resultant of two component traits: nitrogen uptake efficiency and nitrogen utilisation efficiency (Hirel et al. 2007). Insight in the physiological processes governing NUE under low N conditions and the genetic basis for NUE is essential for efficient breeding for this complex trait. A number of studies were conducted on N use of spinach, but these were mainly focused on maximizing spinach yield to get close to the yield potential of the cultivars under study (e.g. Smolders and Merckx 1992, Biemond 1995, Biemond et al. 1996), and on NO_3^- accumulation in relation to consumption quality (Breimer 1982, Steingröver 1986). These studies revealed that sufficient N must be available at the start of growth to realize optimal growth in spinach (Biemond 1995) and that spinach can acquire nutrients better with improved root systems (Smolders and Merckx 1992, Hirel et al. 2007). Biemond et al. (1996) found that with increased N availability the total green leaf area increased through a higher leaf expansion rate, emphasizing the strong dependence of spinach growth and yield on N availability.

To assess the potential of breeding cultivars with improved NUE it is necessary to (i) determine the genetic variation present in spinach germplasm for traits that govern NUE and (ii) develop a methodological setup that enables accurate quantification of and selection for the physiological and growth parameters that contribute to improved NUE (Baligar et al. 2001). Chapter 2 (Chan-Navarrete et al. 2014) of the present thesis evaluated traits related to growth and photosynthesis and their correlation with NUE in a hydroponics system. They reported that leaf area as well as Specific Leaf Area (SLA) were strong determinants of variation for NUE in spinach.

Insight in the genetics of a complex trait such as NUE can be gained by Quantitative Trait Locus (QTL) analysis of a dedicated segregating population. Spinach is a dioecious species with separate male and female plants and occasional monoecious plants (Correll et al. 2011). Spinach is diploid with $2n = 12$ chromosomes and has a genome size of 989Mb (Arumuganathan and Earle 1991). The genome sequence is not available yet, but the spinach mitochondrial and chloroplast genomes were sequenced (327 kb and 150 kb, respectively) (Stern and Palmer 1986, Schmitz-Linneweber et al. 2001). QTL analysis of a segregating

population requires a genetic linkage map with sufficient molecular markers distributed over the genome. For spinach only one genetic linkage map with a limited number of Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLPs) markers was published until now (Khattak et al. 2006). It was used to analyse genetic variation related to sex expression. Onodera et al. (2011) used the same molecular marker data to map genes for dioecism and monoecism in spinach.

The aim of the current study is to provide tools for molecular breeding for NUE in spinach, and elucidate the genetic factors determining the variation in NUE and traits contributing to NUE. NUE is a quantitative plant trait with a polygenic inheritance, which is influenced by N availability (Hirel et al. 2001). The latter is difficult to control under field conditions (Fageria and Baligar 2005) and this will complicate genetic analysis of NUE variation. Hence, a hydroponics system providing controlled evaluation conditions thus reducing the environmental effects as described by in Chapter 2 (Chan-Navarrete et al. 2014) of the present thesis.

A dedicated F2:3 mapping population derived from a cross between heterozygous parents was evaluated on a hydroponics system the parents were shown to differ highly for NUE under low N conditions (see Chapter 2; Chan-Navarrete et al. 2014). A genetic linkage map was constructed using a selected set of gene-based Single Nucleotide Polymorphisms (SNP) markers to enable a QTL analysis of the NUE evaluation data of the F2:3 lines. The analysis revealed several regions in the spinach genome with clustered QTLs that contribute to improve NUE, providing potential targets for molecular breeding for NUE in spinach.

4.2. Materials and methods

Plant material and mapping population

The mapping population consisted of a random set of F2:3 lines derived from a single F_1-plant that resulted from a cross between the hybrid spinach cultivars Ranchero (Enza Zaden) and Marabu (Rijk Zwaan). The parents were selected because they differed strongly in NUE (see Chapter 2; Chan-Navarrete et al. 2014). Ranchero is a cultivar with an upright growth habit and many dark oval-shaped leaves, suitable for spring, autumn and winter cultivation. Marabu is a cultivar with thick, dark green leaves suitable for cultivation in spring and early

autumn. Cv. Ranchero had high NUE, while NUE of cv. Marabu was relatively low under low N conditions (see Chapter 2; Chan-Navarrete et al. 2014).

In total 320 F2 plants were grown and self-pollinated to generate F2:3 lines. In parallel leaf material of each plant was sampled for DNA analysis and molecular genotyping with Single Nucleotide Polymorphisms (SNPs). The F2:3 lines were grown on hydroponics for phenotypic evaluation of NUE.

Discovery of gene-based markers

Leaf material of 10 young plants of Ranchero and Marabu, grown under optimal N conditions, was bulked. From both bulks, total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN). The RNA was quantified using Qubit (Invitrogen) and checked on a 1% agarose gel. After library preparation, the samples were sequenced on a HiSeq2000 sequencer (Illumina, Varshney et al. 2009), obtaining 2×100 bp paired-end reads. For Marabu, the overlapping ends of the paired-end reads were first merged with *FLASH* (http://ccb.jhu.edu/software/FLASH/, Magoč and Salzberg 2011), resulting in 72% merged read-pairs, with an average merged length of 140 bases. After merging, the merged and non-merged paired-end reads were quality-trimmed using *PRINSEQ* (http://prinseq.sourceforge.net/index.html, Schmieder and Edwards 2011). Bases with a PHRED Q-value lower than Q20 were trimmed, poly-A trailing bases longer than 20 bp were removed, and remaining sequences shorter than 50bp were discarded. Low complexity regions were filtered with the 'dust' option, and duplicate reads were removed. To remove chloroplast reads, the sequences were mapped against the chloroplast genome of spinach (AJ400848) using *Bowtie2* (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml, (Langmead and Salzberg 2012), and mapped reads were excluded from further analysis. The remaining reads were extracted using *SAMtools* (http://samtools.sourceforge.net/, Li et al. 2009) and used for a *de novo* transcriptome assembly using *Trinity* (http://trinityrnaseq.sourceforge.net/, Grabherr et al. 2011). The Trinity assembly was performed with minimal k-mer coverage of 2, generating 80,483 transcripts from 45900 components, with an N50 of 2004 bp. A component could be related to a 'unigene' and a component can have multiple transcript types (isoforms). To avoid redundancy, only one transcript of each component was used for marker development. The abundance of each isoform was determined with *RSEM* (RNA-Seq by Expectation-Maximization, included in the

trinity distribution), and for each component, the most abundant transcript was kept in the final transcript fasta sequence file. Finally, a set of 45900 transcripts with an N50 of 1491 was used as transcriptome reference sequence. The RNAseq reads from both genotypes were mapped to the reference transcriptome sequence using *Bowtie2*. On the resulting SAM file, SNPs were called using *QualitySNPng* (http://www.bioinformatics.nl/QualitySNPng/, Nijveen et al. 2013).

The screening resulted in discovery of 12477 candidate SNPs with a non-polymorphic flanking region of 50 bp on both sides of the SNP. SNPs polymorphic between the parents, but homozygous in Marabu or Ranchero were selected. This reduced the number of SNPs to 7704, present in 506 unique transcripts. Of these SNPs, 419 were used to genotype the F_2 plants.

Genotyping

The sequence information of 419 SNPs was used to develop KASPar assays for genotyping 335 F2 plants of the mapping population. The Competitive Allele Specific PCR (KASPar) platform (http://www.kbioscience.co.uk) is a PCR-based novel homogeneous fluorescent SNP genotyping system. The array included seven technical DNA replicates of each of the parents. The DNA was extracted with a modified C-TAB (Steward and Via 1993).

Construction of linkage map

High quality and informative molecular markers (no segregation distortion and less than 15% missing values) were used to generate a linkage map using software package JoinMap 4.1 (van Ooijen 2006) with the maximum-likelihood option for ordering markers within linkage groups. The Haldane mapping function was used to calculate the final maps (Haldane 1931). Possible segregation distortion was determined by testing the actual against the expected segregation ratio of 1:2:1, using the $\chi 2$ test of goodness of fit with two degrees of freedom.

Experimental setup

A randomly selected set of 96 F2:3 lines was evaluated for NUE using a hydroponics system in a temperature-controlled sunlit greenhouse compartment at UNIFARM (Wageningen, the Netherlands) as described in detail by Chapter 2 (Chan-Navarrete et al. 2014). The system consisted of six units, each having 16 containers with 24 plant positions. N application could be separately controlled for each of the six units. The daily application of N aimed at a stable relative plant growth rate (RGR) of either 0.10 or 0.18 $g \cdot g^{-1} \cdot day^{-1}$ based on the Ingestad (1982) model to acquire a steady-state N-nutrition level. We further refer to the 0.10 and 0.18 $g \cdot g^{-1} \cdot day^{-1}$ N rate as low N and high N, respectively.

Evaluation of F2:3 lines

The hydroponics experiment to evaluate the F2:3 mapping population for NUE and related traits was executed over a period of 35 days. The measurements were done at the end of the trial, except for non-destructive chlorophyll content measurements. Every measurement was done on individual plants except for NUE measurements, which were based on bulked samples of 4 plants (in total 3 samples per line for both N treatments).

The plants were patted dry at harvest with industrial paper tissue and divided in a root and shoot fraction. The shoot fraction was weighed immediately to determine **shoot fresh weight (SFW)**. The shoot and root fraction of each plant were dried for two days at 70˚C to get measures for plant **shoot dry weight (SDW)** and **root dry weight (RDW)**. The plant **root-to shoot ratio (R:S)** was determined as $R:S = RDW \cdot SDW^{-1}$. Other traits evaluated at harvest time were **Dry Matter Percentage (DM%)** ($g \cdot g^{-1} \cdot 100$), which is the percentage of the dry mass from the fresh weight of the shoot; **Leaf Area (LA)** (cm^2) determined with a Licor Leaf Area Scanner (LI-3100C) directly after harvest; **Specific Leaf Area (SLA)** ($cm^2 \cdot g^{-1}$) calculated as SLA = LA / SDW; **Leaf Number (LN)** scored the day before harvest time; **Stem length (SL)** (cm) measured with a metric ruler; **Flowering (Fl)**, scored as presence (1) or absence (0) of flower structures at harvest time. **Chlorophyll Content (CC)** (SPAD units) was measured with a SPAD 502 meter (Konica Minolta, Osaka, Japan) 21 (CCi) and 28 (CCh) days after transplanting of seedlings on hydroponics. SPAD values were collected on the first appearing pair of leaves of each plant, 192 one measurement per plant. **Nitrogen Use Efficiency (NUE)** (g SDW g^{-1} N) was calculated as the SDW divided by the N content in

SDW (determined with a Kjeldahl analysis). The latter measurements were performed pooled samples of 4 plants per line-treatment combination, resulting in 3 pooled samples per combination.

Statistical analysis

Descriptive statistics were applied to estimate the overall effects of the two N treatments and to quantify the variation present in the mapping population. The relationship between traits was studied with a correlation analysis. Analyses of variance (ANOVAs) were carried out for each N treatment separately using GenStat 16th; each having a randomized block design. Each block consisted of four adjacent containers within one of the units of the hydroponics system. In all, the experiment contained 24 blocks, i.e. six (units) x four (blocks/unit). To each of the 96 plant positions available within a block a single plant was randomly assigned from each of the 96 F2:3 lines to be evaluated. Broad sense heritabilities (h^2_m) were calculated for means over replicates of F2:3 lines (n=12), the genotypic variance (σ_g^2) and the experimental variance (σ_e^2) with the following formula:

$$h^2_m = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2/n)$$

For the analysis between N treatments the experimental design was evaluated as a split-plot design. The six hydroponics units were grouped in three subsets of two adjacent units. The two N-treatment levels were than assigned to one of the two units within a subset.

QTL analysis

QTL analysis of the phenotypic data was performed with the mapping software available in GenStat 16th Edition (VSN International 2013). Separate single trait association analyses (also known as linkage disequilibrium mapping) were performed separately for low and high N using the F2:3 line means for each phenotypic trait, the F2 linkage map and the marker data of the F2 progenitors of the F2:3 lines. A genome-wide permutation test according to Li and Ji (2005) was performed to calculate the threshold for QTLs. Only QTLs with significance level > 95% were taken into consideration. The linkage maps with QTLs were displayed with MapChart (Voorrips 2002).

4.3. Results

Molecular marker discovery

SNP discovery was done by RNA sequencing of spinach cvs Marabu and Ranchero. The cv. Marabu sequence was used as a reference to detect SNPs. The total number of cv. Marabu reads was 23755238, which after processing (see Material and Methods) resulted in 45872 unique transcripts. The cv. Ranchero sequence reads were mapped against this final transcript set and with the use of QualitySNPng (Nijveen et al. 2013) 27499 SNPs were identified. Of these, only 1351 SNPs were polymorphic between but homozygous within cvs Ranchero and Marabu, while 11781 SNPs were heterozygous in one parent only and 14317 SNPs were heterozygous in both parents.

The SNPs that were homozygous in both parents but polymorphic between parents were preferred for genetic map construction, as these were all expected to segregate in the F2 population. After filtering for insufficient flanking sequence information for assay development, a set of 704 SNPs in 506 unique transcripts was available for genotyping.

Genotyping

Genotyping of the mapping population was performed with KASPar assays using a selection of 419 SNPs from the set of 506 candidate SNP markers. Of these, 136 markers were not informative because of a lack of signal (59), a high percentage of missing values (19), distorted segregation (17) or simply lack of segregation (41). The 283 informative markers showed a 1:2:1 segregation ratio and were used to generate a genetic linkage map. To this end the marker data of 320 out of 335 genotyped F2 plants were used; data of 15 genotypes were omitted because they had a high number of markers with missing values.

Linkage map

The genetic linkage map was constructed using JoinMap 4.1 (van Ooijen 2006) with 283 informative markers and 320 genotypes. All the markers were incorporated in a map with a total size of 433.6 cM, divided over six linkage groups (Figure 4.1). This is in accordance with the basic number of spinach chromosomes. The linkage groups were coded P01 - P06 (P

for putative) as no chromosomal anchors were available to link the linkage groups to chromosomes. The linkage groups varied in size from 46.7 – 116.2 cM. Some linkage groups contained relatively large gaps (20 cM -46.51 to 66.08 cM- in P02 and 35 cM -24.17 to 59.19 cM- in P05). Substantial clustering of markers was observed as well.

Phenotypic evaluation of the mapping population

A random set of 94 F2:3 lines from the mapping population was evaluated for NUE-related traits on hydroponics at low N and high N. The results are summarized in Table 4.1 and visualized for selected traits in Figure 4.2. The N treatment affected plant growth considerably. In general, Shoot Fresh Weight (SFW), Shoot Dry Weight (SDW), Leaf Area (LA) and NUE were reduced at low N compared to high N conditions (Table 4.1), and Dry Matter percentage (DM%) and Root to Shoot ratio (R:S) were increased. N levels had no effect on Root Dry Weight (RDW), and Flowering (Fl) was increased at low N conditions. Chlorophyll Content (CC) was slightly but significantly negatively affected. Plants grown at high N conditions were bigger than plants grown at low N and had larger leaves with a relatively dark green colour.

The F2:3 line means for all traits determined at low and high N showed a continuous and in most cases normal distribution, as exemplified in Figure 4.2. Analyses of variance of the traits at low as well as at high N demonstrated highly significant F2:3 line-to-line variation ($p < 0.001$). There were large differences between the most extreme lines for all traits (Table 4.1). The heritability estimates (h^2_m) for all traits were intermediate to high, but varied considerably from trait to trait (Table 4.1). The heritability estimates of each trait at low and high N were mostly similar. The heritability estimates for R:S, SLA and DM% were relatively low as these traits represent ratios of measured traits.

Relationship between NUE-related traits

To get insight in the relationships between NUE and NUE-related traits two separate correlation analyses were carried out: one with line means from low N and one with line means from high N. The results are summarized in Figure 4.3.

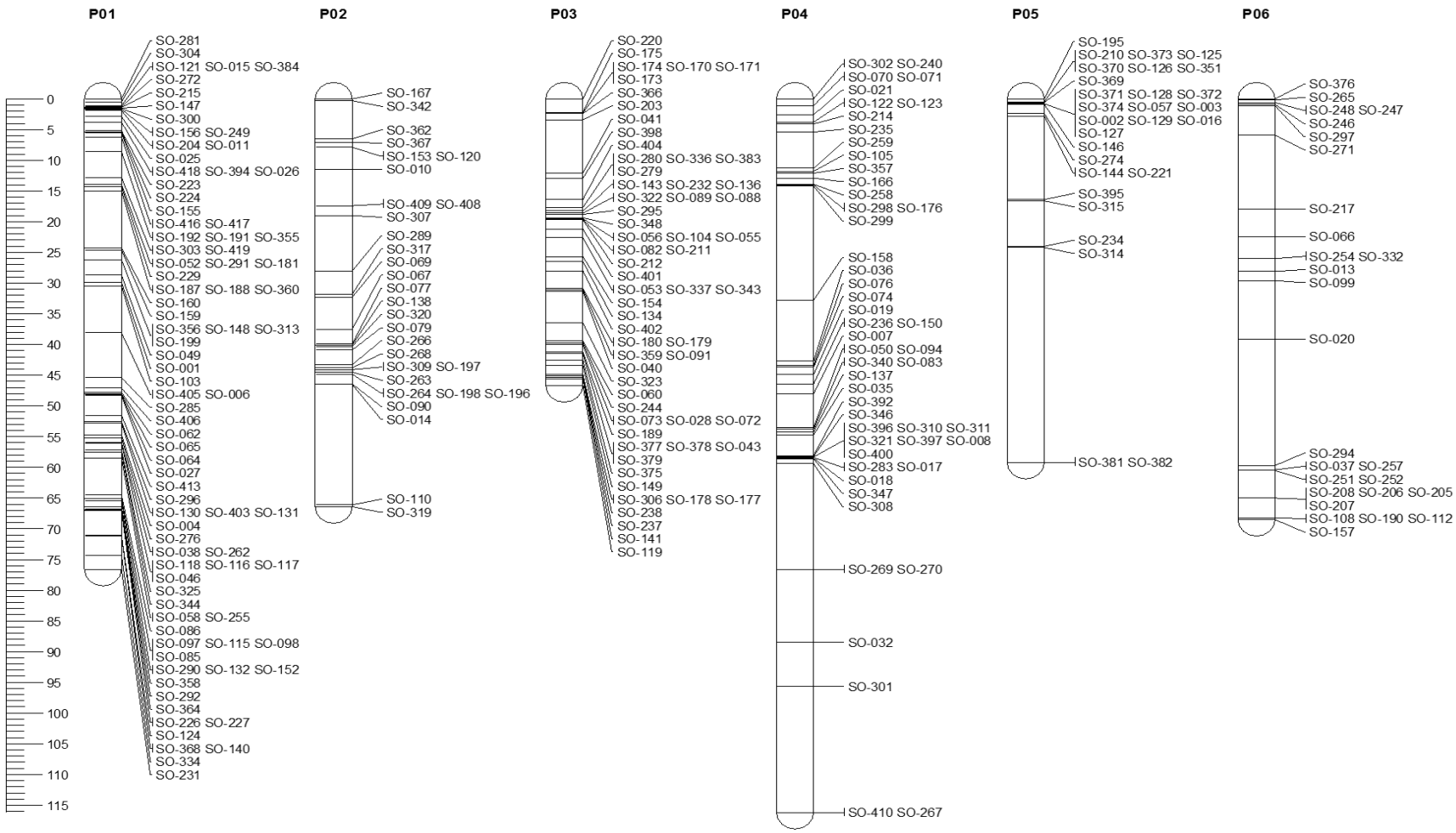

Figure 4.1. Genetic linkage map of spinach with six putative linkage groups (P01-P06). Locus names are indicated at the right-hand side of each group with their map position (cM) at the left-hand side.

The values on the diagonal represent the trait-specific coefficients of correlation between trait values of plants grown at low and high N. The correlation between N treatments for NUE (r = 0.42) was moderate, reflecting the significant line × N interaction for this trait (Table 4.1). At both N treatments NUE was highly correlated with SDW and not surprisingly also with SFW, LA and RDW (0.65 - 0.74 for low N and 0.78 - 0.89 for high N). The most interesting traits with a moderate negative correlation with NUE were the physiological traits SLA and R:S. Fl and SL are highly correlated but no obvious relation with any of the other traits including NUE was found. Remarkably, chlorophyll content at 28 days (CCh) showed no significant correlation to NUE at low N while at high N it was significantly correlated with NUE as well as with SFW, SDW, RDW and LA.

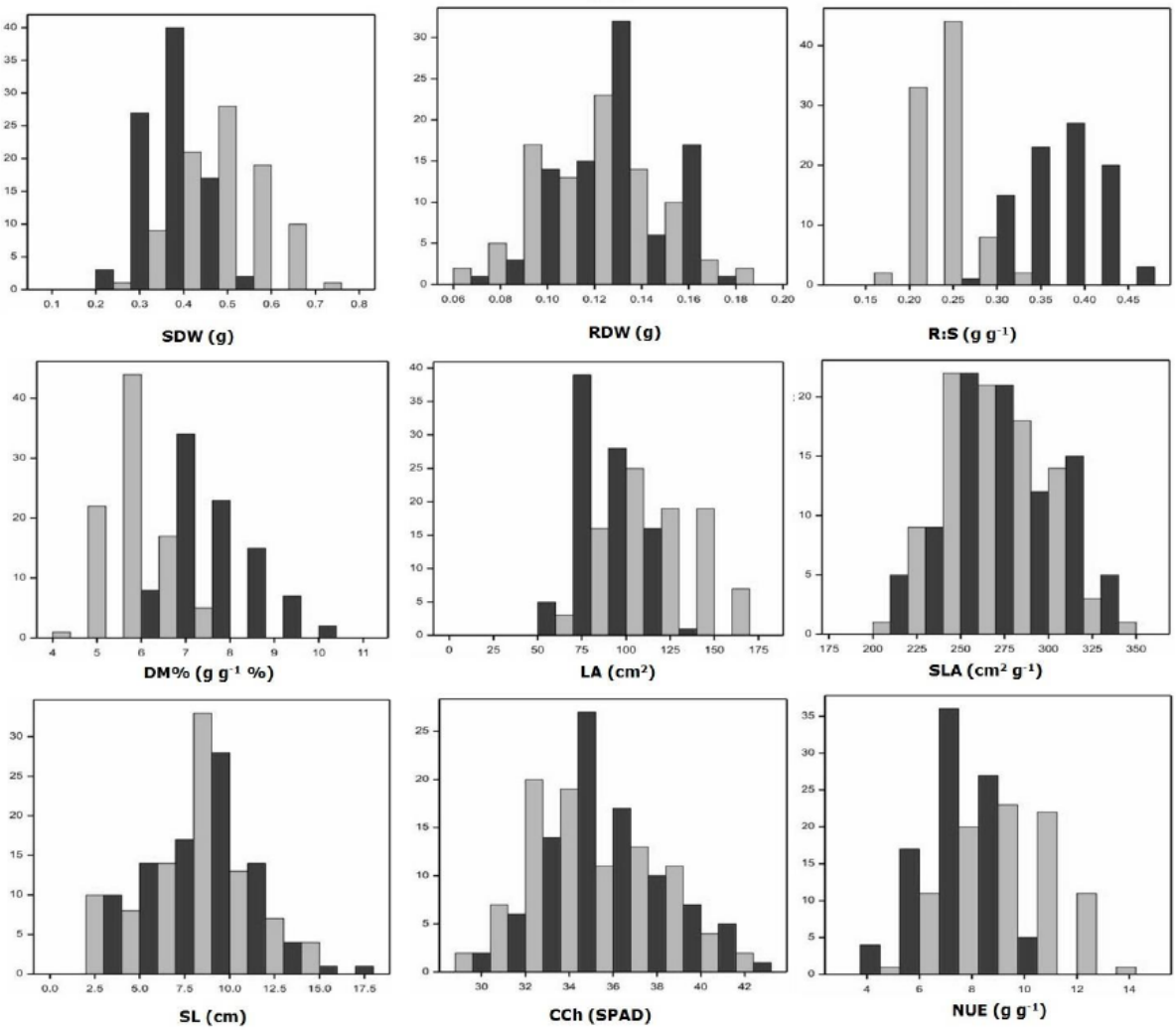

Figure 4.2. Distribution of trait means of F2:3 lines from the cvs Ranchero × Marabu population for nitrogen use efficiency (NUE)-related phenotypic traits at low N (black) and high N (grey). SDW=shoot dry weight; RDW=root dry weight; R:S=root to shoot ratio; DM%=dry matter percentage of the shoot; LA=leaf area; SLA=specific leaf area; SL=stem length; CCh=chlorophyll content at 28 days

QTL mapping for NUE-related traits

QTL analysis was performed to discover chromosomal regions that contribute to the variation observed within the mapping population grown under high N and low N conditions. Separate QTL analyses were performed with GenStat 16th (VSN International 2013) using the F2:3 line mean values from the phenotypic evaluation at low and high N. The genome-wide LOD score threshold was calculated to be 2.97. The QTLs for the NUE-related traits are summarized in Table 4.2 and are graphically represented in Figure 4.4. Nineteen and 20 QTLs for high and low N, respectively, were detected. The alleles from the male parent cv. Marabu had a favourable effect for a relatively large number of QTLs. The QTLs for which cv. Ranchero contributed the favourable allele were HN_R:S_2, HN_SLA_1, LN_NUE_1, LN_R:S_1, LN_SFW_2 and all the QTLs related to CC (Table 4.2). The cv. Marubu allele of the QTL for LA at P01 (3.8 cM) showed a differential response to N level with a relatively strong favourable effect at high N (9.11) and unfavourable effect at low N (3.79).

A number of QTLs co-localized at the top of P01 and at the bottom of P02. The QTLs related to Fl co-localized with multiple other QTLs at P01 and P02. Flowering is a developmental process that impacts N metabolism and therefore may be affecting several other traits. To investigate to what extent the QTLs were independent of flowering; Fl was used as a covariate in QTL mapping using the software MapQTL 6.0 (van Ooijen 2009). The QTLs were confirmed, indicating that these QTLs are not only dependent of Fl.

Biomass QTLs: Two QTLs were determined for SDW, one at low N and one at high N. Both of them were detected in the multiple-QTL region on P01, with a peak at 3.8cM and 15.1% explained variation for low N and 13.9% for high N. Under N limiting conditions, two QTLs were found, at P01 and P05 that explained 29.8% of the variation. For LN_SFW_1 the favourable allele is donated by cv, Marabu, but for LN_SFW_2 by cv. Ranchero. At high N, a single QTL was identified for this trait, explaining 18.7% of the variation. For high N conditions no QTL was found for DM%, but for low N conditions a QTL was found on P05 that explained 14.3% of the phenotypic variation. Two QTLs were detected for R:S for high N and one QTL for low N with an explained variation of 33.0% and 14.0%, respectively. Three QTLs were determined for LN at both low N and high N. At high N all three QTLs were found in P01, but at low N there were two QTLs at P01 and one at P02.

Table 4.1. Variation statistics for 13 nitrogen use efficiency (NUE)-related traits of F2:3 lines at Low N and High N. The statistics comprise population means, standard errors for line means (SEM), ranges between the most extreme line means and heritability estimates for line means (h^2_m). SFW=shoot fresh weight; DM%=dry matter percentage of the shoot; LA=leaf area; LNh=leaf number at 28 days; CCi=chlorophyll content at 21 days; CCh=chlorophyll content at 28 days; Fl=flowering time; SDW=shoot dry weight; R:S=root to shoot ratio; SLA=specific leaf area; SL=stem length; RDW=root dry weight

Trait	**Population mean**		**Range**		**SEM**		h^2_m	
	Low N	**High N**	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**
SFW (g)	**5.05**	**9.00**	2.39 - 7.86	4.94 - 13.56	0.06	0.14	0.72	0.72
DM% ($g \cdot g^{-1}$ %)	**7.44**	**6.03**	5.79 - 10.34	4.68 - 7.61	0.07	0.05	0.54	0.54
LA (cm^2)	**82.63**	**123.50**	44.41 - 132.05	64.44 - 177.94	1.01	1.90	0.73	0.70
LNh	8.35	8.49	6.03 - 11.83	6.33 - 11.83	0.06	0.05	0.90	0.87
CCi (SPAD)	41.50	41.85	36.47 - 47.27	37.43 - 47.13	0.14	0.15	0.73	0.71
CCh (SPAD)**	35.28	35.36	29.42 - 42.05	29.83 - 43.13	0.15	0.18	0.73	0.80
Fl**	**0.55**	**0.48**	0.00 - 1.00	0.00 - 1.00	0.01	0.01	0.87	0.84
SDW (g)	**0.35**	**0.52**	0.17 - 0.50	0.33 - 0.72	0.00	0.01	0.57	0.62
R:S ($g \cdot g^{-1}$)	**0.37**	**0.25**	0.17 - 0.50	0.33 - 0.72	0.00	0.00	0.35	0.21
SLA ($cm^2 \cdot g^{-1}$)	271.00	273.60	203.7 - 331.5	215.30 - 342.60	2.68	2.35	0.40	0.40
SL (cm)	7.92	8.60	2.46 - 16.48	2.58 - 15.63	0.15	0.14	0.84	0.89
RDW (g)	0.12	0.12	0.06 - 0.17	0.07 - 0.18	0.00	0.00	0.61	0.69
NUE ($g \cdot g^{-1}$)*	**7.09**	**9.88**	3.56 - 10.06	6.03 - 14.93	0.09	0.15	0.58	0.64

Population means depicted in bold differed significantly (p<0.05); **: Trait showing significant Line × N interaction (p<0.05)

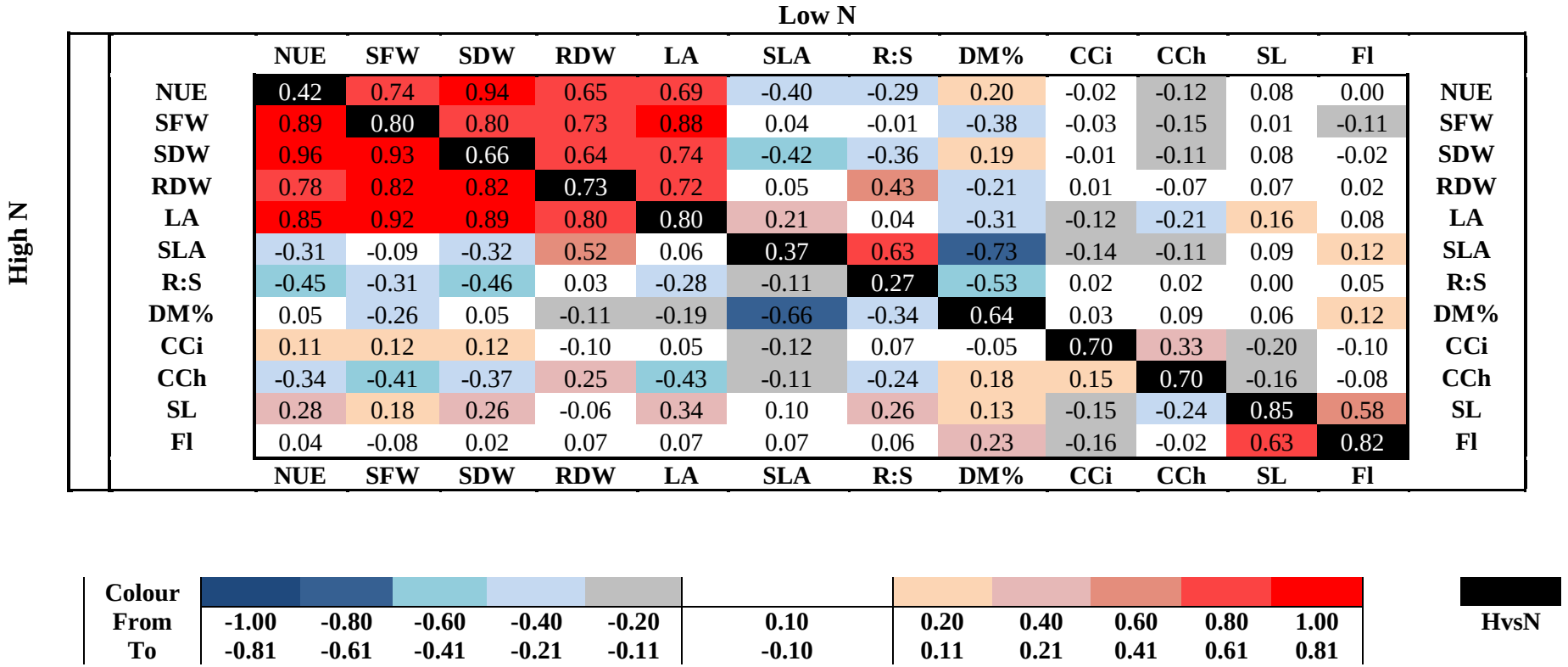

Figure 4.3. Correlations between the phenotypic line means for twelve nitrogen use efficiency (NUE)-related traits determined at Low and High N. The coefficients above the diagonal refer to traits determined at low N and the ones below the diagonal to the corresponding traits from the high N treatment. The values on the diagonal (black cells) represent coefficients of correlation between line means determined at low and high N. The colour scale (depicted below the correlation table) indicates the correlation strength. Correlation coefficients with $|r| > 0.20$ were significant for a $p<0.001$. SFW=shoot fresh weight; SDW=shoot dry weight; RDW=root dry weight; LA=leaf area; SLA=specific leaf area; R:S=root to shoot ratio; DM%=dry matter percentage of the shoot; CCi=chlorophyll content at 21 days; CCh=chlorophyll content at 28 days; SL=stem length; Fl=flowering time.

Table 4.2. Summary table on characteristics of QTLs determining genotypic variation for NUE-related traits observed in the cvs Ranchero × Marabu F2:3 population tested at high and low N.

N Treatment	QTL	Linkage Group	Peak	Position	LOD	R^2 %	Additive	Dominance
	HN_CCi_1	P02	66.1	43.3 - 66.4	6.23	25.0	-1.77	-0.63
	HN_CCi_2	P04	58.1	54.2 - 59.4	4.01	17.7	-2.37	-0.46
	HN_CCi_3	P06	17.9	17.9	4.44	19.2	-1.48	-0.10
	HN_CCh_1	P01	3.8	0.0 - 8.6	13.48	41.9	-2.37	-1.72
	HN_CCh_2	P03	26.4	12.1 - 31.1	4.07	17.9	1.66	-0.86
	HN_Fl_1	P01	2.9	0.00 - 6.2	5.33	22.2	-0.20	0.13
	HN_Fl_2	P01	30.5	30.5	3.06	14.1	-0.17	0.07
	HN_Fl_3	P02	46.5	43.3 - 46.5	11.04	37.1	0.28	0.09
High N	HN_LA_1	P01	3.8	1.1 - 3.8	3.74	16.7	13.89	9.12
	HN_LA_2	P02	66.4	66.1 - 66.4	3.88	17.2	16.88	8.56
	HN_LN_1	P01	5.5	0.0 - 8.6	4.79	20.4	-0.67	0.15
	HN_LN_2	P01	30.5	29.9 - 30.5	3.32	15.1	-0.64	0.09
	HN_LN_3	P02	46.5	40.2 - 66.4	6.11	24.6	0.78	0.40
	HN_R:S_1	P04	53.5	53.8 - 54.7	3.46	15.6	0.00	0.02
	HN_R:S_2	P06	39.1	29.6 - 39.1	3.93	17.4	0.01	-0.02
	HN_SDW_1	P01	3.8	2.9 - 3.8	3.01	13.9	0.05	0.03
	HN_SFW_1	P01	2.9	0.0 - 6.2	4.29	18.7	1.14	0.56
	HN_SLA_1	P02	66.1	66.1 - 66.4	5.56	22.9	19.62	-4.23
	HN_SL_1	P02	46.5	43.3 - 46.5	13.20	41.4	2.99	1.41
	LN_CCi_1	P01	6.2	0.0 - 8.6	4.07	17.9	-1.17	-0.81
	LN_CCi_2	P02	66.4	46.4 - 66.4	5.62	23.1	-1.65	-0.08
	LN_CCi_3	P04	58.1	53.5 - 59.4	4.50	19.4	-2.93	-1.09
	LN_CCi_4	P06	17.9	17.9	3.88	17.2	-1.34	-0.34
	LN_CCh_1	P01	3.8	0.0 - 6.2	8.45	31.2	-1.78	-1.44
	LN_DM%_1	P05	2.4	2.4 - 2.9	3.11	14.3	-0.37	0.45
	LN_Fl_1	P01	0.5	0.0 - 6.2	9.70	34.2	-0.26	0.06
	LN_Fl_2	P01	24.3	24.3 - 30.5	3.53	15.9	-0.18	0.11
	LN_Fl_3	P02	46.5	43.3 - 46.5	11.06	37.2	0.29	0.04
Low N	LN_LA_1	P01	3.8	3.8	3.13	14.3	8.93	3.79
	LN_LN_1	P01	0.0	0.0 - 6.2	8.10	30.2	-0.97	0.27
	LN_LN_2	P01	30.5	24.3 - 43.8	5.38	22.4	-0.92	0.10
	LN_LN_3	P02	46.5	43.3 - 66.4	3.77	16.8	0.77	0.25
	LN_NUE_1	P01	3.8	0.0 - 6.2	3.82	17.0	0.79	-0.10
	LN_R:S_1	P06	29.6	29.6	3.03	14.0	0.01	-0.02
	LN_SDW_1	P01	3.8	1.1 - 3.8	3.33	15.1	0.03	0.00
	LN_SFW_1	P01	3.8	3.8	3.01	13.9	0.57	0.09
	LN_SFW_2	P05	0.6	0.0 - 16.4	3.54	15.9	0.52	-0.41
	LN_SL_1	P01	0.0	0.0 - 6.2	3.83	17.0	-1.61	0.99
	LN_SL_2	P02	46.5	43.3 - 46.5	10.86	36.8	2.66	0.86

SFW=shoot fresh weight; DM%=shoot dry matter %; LA=leaf area; LNh=leaf number day 28; CCi=chlorophyll content day 21; CCh =at day 28; Fl=flowering time; SDW=shoot dry weight; R:S=root/shoot ratio; SLA=specific leaf area; SL=stem length; RDW=root dry weight

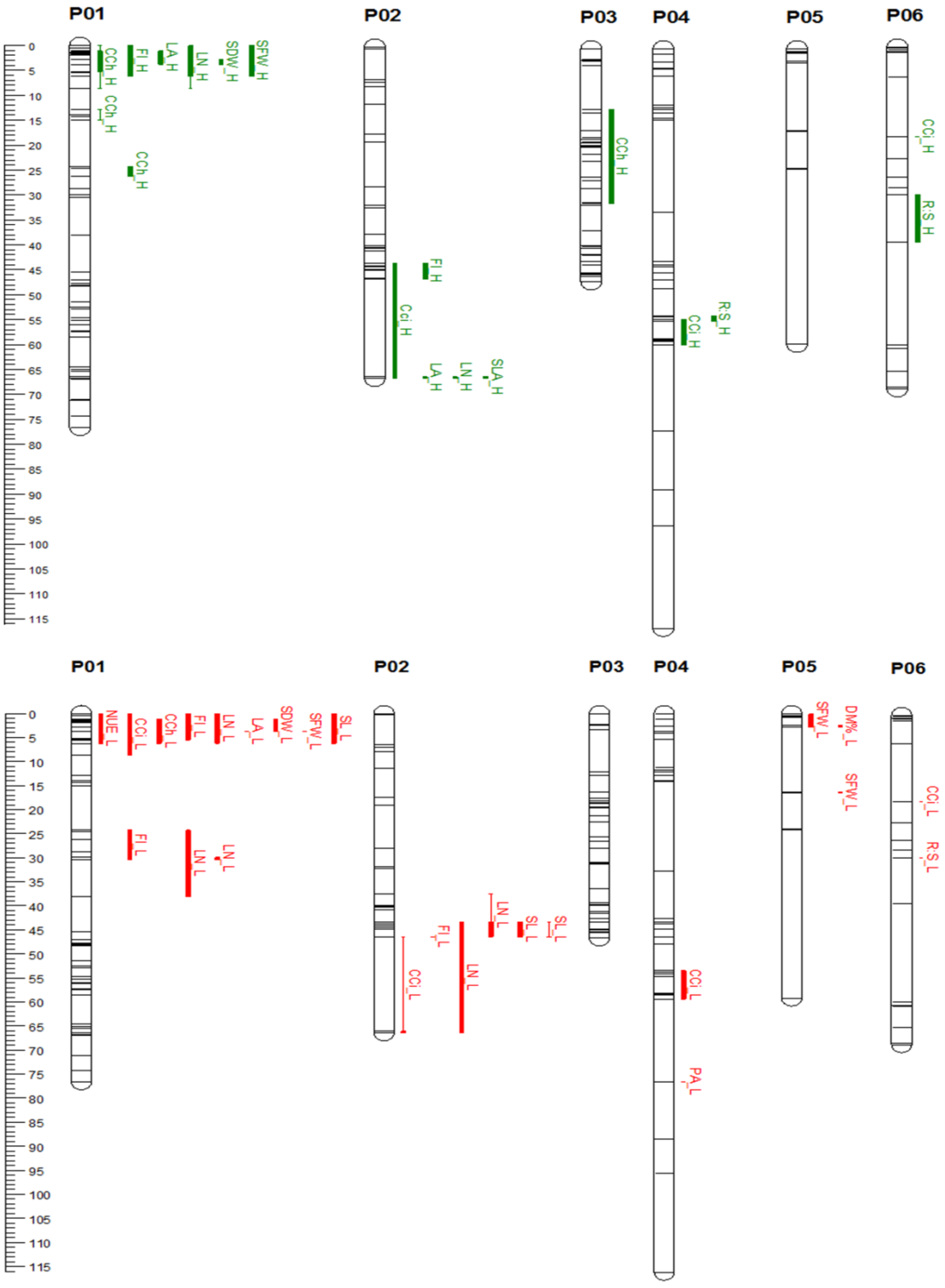

Figure 4.4. The location of QTLs for nitrogen use efficiency (NUE)-related traits detected in the high N and low N experiment is shown on separate genetic linkage maps by green and red vertical bars, respectively. Vertical bars represent the LOD-1 and the whiskers the LOD-2 support interval.

Leaf QTLs: for LA at high N conditions, the two QTLs explained 16.7% and 17.2% of the phenotypic variation and in low N conditions a single QTL that explained 14.3% of the variation was detected. A QTL for SLA was found only at high N conditions with a LOD value of 5.56. The explained variation is 22.9% and high values were driven by the Ranchero allele. The chlorophyll content measurements were performed at an intermediate time point (CCi) and at harvest (CCh). Multiple QTLs were observed for CCi that explained most of the phenotypic variation. For CCh, two QTLs were identified with in total 59.8% phenotypic variation for high N explained, and a unique QTL for low N was found that explained 31.2% of the variation. For all the QTLs found for CC, the dominant alleles were coming from Ranchero. Three QTLs were detected for LN at high N of which HN_LN_3 (in P02) explained 24.6% of the phenotypic variation. The QTLs found at low N co-localized with the ones detected at high N. LN_LN_1 on P01 explained under this condition most of the phenotypic variation (30.2%).

Flowering: Three QTLs were found at low N (two at P01 and one at P02) for Fl and also at high N, and these together explained most of the variation between lines. HN_Fl_3 and LN_Fl_1 co-localized with strong QTLs for SL at high and low N, respectively, in line with the high correlation between these traits.

NUE: A QTL controlling NUE was found at P01 at low N conditions. This QTL explained 17.0% of the phenotypic variation with a LOD value of 3.82. This QTL co-localized with multiple QTLs for SDW, LA, SFW, CCh, CCi, Fl, LN and SL (Figure 4.4). At high N conditions no QTL was detected.

4.4. Discussion

Cultivation of spinach, like many other leafy vegetables, requires high amounts of nitrogen. This puts a strain on the environment, consumes large amounts of fossil energy for the synthesis of synthetic N, and limits the opportunities for spinach as an organically produced crop. This study provides a first step towards a sustainable solution to this problem by providing molecular tools for breeders through identification of genetic factors governing NUE. Improved NUE is beneficial under high N conditions as it helps limiting the amount of nitrogen that leaches into the environment, but is at least equally important at low N availability as it enables the plant to produce more biomass and yield when N availability is

limiting. We created a genetic linkage map and several QTL regions were identified that can be breeding targets for improvement of NUE in spinach.

QTL analysis of complex traits (such as NUE) is often hampered by a low heritability of the stress-related traits detected under stress conditions (Lafitte et al. 2004). It is likely that often at least in part this is due to the lack of control over the stress conditions. Our results showed substantial variation under low N for most of the traits, and the heritability was high. This may be partly due to the experimental setup, which involved a hydroponics system and N addition rates according to the Ingestad model. The plants were thus exposed to a highly controlled steady state N stress in a controlled root environment, reducing the environmental variation and improving detection of heritable selection traits under low N availability. This set-up is particularly useful for a genetic study aiming at the traits contributing to N utilisation efficiency.

The genetic map presented in this study was based on SNP markers identified in actively transcribed genes. The map, composed of close to 283 markers in expressed genes divided over 6 linkage groups (matching the basic chromosome number in spinach), presents a significant improvement over the currently available published map by Khattak et al. (2006) with 110 markers and 7 linkage groups. Moreover, the Khattak map contained a considerable number of AFLP markers, which are not easily usable as allelic bridges between different molecular maps. The available sequence information of the SNP markers of the here reported map will enable integration with other maps and physical mapping using the spinach genome sequence, which is expected to become available soon. The current linkage map still contains several gaps and clusters of markers. This may be related to the fact that the markers originate from coding regions of the genome, which are mostly present in the euchromatin. Marker clustering within linkage maps is not restricted to gene-based markers, and not uncommon for random DNA-based markers as well (Qi et al. 1998; Haanstra et al. 1999; Vuylsteke et al. 1999; Young et al. 1999; Jeuken et al. 2001). Brugmans et al. (2002) suggested that the ideal distribution of markers to find functional or causal genes for a trait of interest is not necessarily regular spacing across the whole genome, but rather a concentration of markers in the coding regions of the genome, suggesting that the map presented here is well-suited for QTL analysis.

Gaps and clusters may also result from a lack of homology between the genomes of the parents, reducing recombination frequency. The pedigree of the female parent of the mapping

population map includes germplasm from the wild species *S. turkestanika* (M. van Diemen, personal communication 2013). However, this was also the case for the parents of the Khattak mapping population, but the markers on this map are not clustered as much as the markers on our map. Another explanation for the clustering and presence of gaps could be linked to the size and structure of the spinach chromosomes. A cytogenetic study of Ramanna (1976) showed that four of the six spinach chromosomes had a low short-to-long arm ratio, which is normally associated with a high number of rod bivalents at the late prophase I or metaphase I of meiosis (for a review, see Jones, 1987). Rod bivalents are due to the absence of chiasmata between the chromatids of the short arms of two homologous chromosomes and are therefore indicative for a low degree of recombination (Sanchez-Moran et al. 2002). Since spinach chromosomes are quite small, the overall number of chiasmata that occur within single chromosome pairs may be low, which may at least partly account for the clustering and gaps in our map. It is important to note that this may severely complicate breeding for traits for which the genes are located on the short arms of the spinach chromosomes.

Several phenotypic traits that were measured in this study may be determined by the same biological processes or even a single gene, which is exemplified by the accumulation of QTLs at two locations, i.e. on linkage groups P01 and P02 (Figure 4). The QTL effects may be strongly influenced by differences in flowering time of the plants; the response to abiotic stress is influenced by maturity of the plants (Vargas et al. 2006, Reynolds and Tuberosa 2008). Flowering influences N use efficiency because the fruits and flowers are strong N sinks (Schieving et al. 1992). Genotypic differences in flowering time may therefore directly affect traits related to plant growth and N use efficiency. Flowering differences were evaluated by measuring presence/absence of flowers (Fl), leaf number (LN) and indirectly, stem length (SL). The six QTLs for Fl co-localized with QTLs for biomass, suggesting that the genetic variation for biomass may be partly determined by flowering traits. However, separate QTL analyses with flowering as a covariate resulted in the same QTLs for biomass traits with similar LOD scores, indicating that the biomass trait QTLs on linkage groups P01 and P02 were to a larger extent determined by other factors than flowering.

The detected flowering QTLs together explained a large part of the total variation both under high and low N conditions, indicating that the majority of the genomic regions controlling flowering in this population were identified. Two other traits directly associated with flowering (stem length and leaf number) showed QTL co-localization with the flowering QTLs, emphasizing the significance of these QTL regions. Together with studies of Khattak

et al. (2006), Onodera et al. (2011) and Yamamoto et al. (2014) related to sex expression, these results present a basis for understanding the genetic control of flower development in spinach and similar species.

Nitrogen nutrition plays a crucial role in determining plant photosynthetic capacity in both natural and agricultural environments (Abrol et al. 1999). Because the photosynthetic apparatus utilizes a large part of the available N in the plant, N availability is a key external factor for photosynthetic capacity and plant growth. Photosynthetic capacity is dependent on leaf area and on chlorophyll content of the leaf. A number of studies have demonstrated the correlation of chlorophyll content with N availability. In maize, Crafts-Brandmer and Poneleit (1992) found a correlation between Rubisco content, chlorophyll content and photosynthetic activity, and Hageman and Lambert (1988) linked photosynthetic activity to leaf N concentration. SPAD readings were suggested to give good estimations of NO_3^- N concentrations in spinach, assisting in the evaluation of N availability in fields and assessment of optimal harvest time (Liu et al. 2006). In cereals, leaf chlorophyll content was decreased under low N conditions (Muchow and Davis 1988, Sinclair and Vadez 2002), and in spinach under suboptimal N conditions a considerable reduction of chlorophyll content (CC) was observed (Evans and Terashima 1987). In most cases, the decrease in CC under low N availability is directly related to stress-induced senescence. In our study CC was not affected by N level, seemingly contradicting results from others. However, our experiments used the N addition model of Ingestad (1982), and N is provided to the plants in an exponential and progressive manner according to a specific relative growth rate (0.10 and 0.18). At the low N addition rate, the plants are able to adapt to the low N availability (Gutshick 1999). The plants do grow slower, but these are likely able to balance photosynthetic capacity and growth to the available N, and the leaves hardly senesce (see Chapter 2; Chan-Navarrete et al. 2014). The CC measurements at harvest therefore may be indicative for physiological aging of the measured leaves, which was affected by low N availability. This is in agreement with the co-localization of CC QTLs at high and low N. The HN_CCh_1 and LN_CCh_1 QTLs localized in the QTL hotspot at P01, linking CC to biomass production both under low and high N conditions. The cv. Ranchero allele linked to delayed senescence (higher chlorophyll content at end harvest) located on P01 may be an interesting quality target for spinach breeding.

Nitrogen use efficiency was highly correlated with SFW, SDW, RDW and LA under both low and high N conditions. Under low N conditions, leaf area is typically limited, balancing it with the limited N availability (Evans and Terashima 1987, Evans 1989). The genotypes that

can retain a relatively high LA while balancing nitrogen and carbon generally would be expected to have higher NUE, which is exemplified by the positive correlation between LA and NUE (Figure 4.3) and the co-localization of QTLs for LA, NUE and biomass traits under high N conditions on P01 and P02, and under low N conditions at P1.

R:S was negatively correlated with NUE under high N conditions and DM% did not show any correlation. The lower (negative) correlation between R:S and NUE at low N was likely caused by prioritizing an investment in roots over shoots (the NUE measurements are based on leaf N content). The R:S QTLs HN_RS_2 and LN_RS_1 co-located on P06 at 29.6 cM, indicating that this QTL at last partly controls partitioning of assimilates independent of N availability. Under low N conditions, DM% increased and a low correlation with NUE was detected, as was also observed by Elia et al. (1998). In addition, we identified QTLs for SLA and LA at P02 under high N conditions, but these QTLs were not found under low N conditions. This points to genotypic differences in how assimilate usage is adapted at low N, and may reflect variation in changing from producing N-rich proteins and maximizing leaf surface area under high N to production and accumulation of carbohydrates (Blaby et al. 2013), production of low-N containing structural components, or production of osmolytes to adapt osmotic balance of the cells to cope with a reduction of the water content. The here observed reduction in LA and prioritization of investment in roots at low N conditions may reflect a response aimed at maintaining the balance between carbon and nitrogen. This is in line with the fact that plants possess an intricate regulatory mechanism that coordinates N metabolism with C metabolism (Nunes-Nesi et al. 2010). Nitrogen availability is directly linked to photosynthetic capacity because the proteins of the Calvin cycle and thylakoids represent the majority of leaf nitrogen (Evans 1989). Limited N availability increases the C/N ratio, with less N available for photosynthetic proteins, and the resulting accumulation of carbohydrates feeds back negatively on photosynthesis (Noguchi and Terashima 2006). The C/N balance can be further restored by a reduction of LA, decreasing photosynthetic capacity, and an increase in the thickness of the leaf, represented by a reduction of SLA (Kant et al. 2011). Indeed we find that SLA is inversely correlated with NUE (-0.40 and -0.31 under low and high N conditions, respectively), indicating that in the plant material evaluated, the restoration of the C/N balance as described above positively affects NUE. We also found that leaf area was highly positively correlated with NUE (0.69 at low N and 0.85 at high N). Moreover, under high N conditions, SLA and RDW are correlated (0.52), while under low N conditions, this correlation disappears. This may imply that with ample N available,

investment in roots is accompanied by large and thin leaves, matching increased N uptake capacity with higher photosynthetic surface. Under low N, SLA and RDW are no longer correlated: this may imply that plants grown under low N conditions tend to prioritize roots over shoots (R:S is increased), but this investment in roots is under these conditions not associated with higher SLA. The investment in roots does not compensate for the lower levels of N in the root environment, N-uptake is decreased, and therefore C/N needs to be adapted as well. The strong positive correlation of LA with NUE under low N indicates that there is substantial variation for selecting NUE related traits in the spinach population under study to optimize NUE under N-limiting conditions.

4.5. Concluding remarks

The results presented in this study provide a first step towards molecular breeding for complex traits in spinach, in particular for nitrogen use efficiency and adaptation to growth under low input conditions. The identified QTLs may be targets for breeding programs aimed at improving NUE, both under high N and N limiting condition, thus providing tools to increase yields under low N input conditions, and decrease environmental strain at high N input. However, it should be taken into account that the approach chosen in this study, i.e. the use of a hydroponics system and N addition according to the Ingestad model, focused on QTL detection for traits related mostly to N utilization under highly controlled N stress conditions. The relevance of the detected QTLs under field conditions still needs to be proven.

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Chapter 5

Genotype by environment analysis of spinach cultivars in field trials with different nitrogen availability

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To be submitted

Abstract

Short cycle leafy vegetables like spinach are nitrogen demanding putting a lot of strain on the environment. We investigated the Genotype by Environment interaction (GEI) using a set of spinach cultivars that has shown to differ in nitrogen use efficiency under controlled conditions (steady state nitrogen availability in a hydroponics system). Twenty-four spinach cultivars were evaluated in six different locations in the Netherlands, each with three nitrogen (N) fertilization levels, in all at 18 environments. This evaluation comprised regular collection of shoot dry weight and soil coverage data of each cultivar. The data for both traits were plotted against temperature-normalized growth time (with a base temperature of 4^0C) and used successfully to fit smooth growth curves for calculating cultivar-specific characteristics describing the progression of soil coverage and shoot growth. For both traits, the progression over time was found to differ strongly from environment to environment. The differences between environments within location due to N fertilization, however, were relatively small but increasing with time. The lower rates of N fertilization reduced shoot growth by 0.6% to 31% compared to the higher rates of N The smooth curves were used to calculate the shoot dry yield at a standardized thermal time point (SDW_t90%) for each environment-cultivar combination. The variation for this trait as well as for other shoot growth parameters was highly influenced by the main factors of the genotype by environment study (N level and cultivar), but no significant cultivar by N level interaction was detected. An in-depth stability analysis using the Finlay-Wilkinson approach for three shoot-curve derived traits and a factorial regression analysis for a yield parameter was done to estimate the influence of environmental quality factors. This analysis showed that multiple factors from the environment were affecting the measured traits, thus complicating the interpretation of the N level effect on spinach growth, and emphasizing the importance of selection under as stable as possible environments in breeding programs for improved N use efficiency.

Keywords: Spinach • Genotype by Environment Interaction (GEI) • Smooth curves • Finlay-Wilkinson

5.1. Introduction

Food demand is increasing and the current agricultural cropping systems need to reduce fertilizer input while increasing yield. Nitrogen (N) is one of the most important nutrients to sustain plant yields. In modern agriculture, N is therefore applied in large quantities to compensate soil N shortages. However, more than half of the N added to crops is not utilised by the crops and exerts a negative effect on the environment (Lassaletta et al. 2014, Hodge et al. 2000). N not used for plant growth can be released as nitrous oxide (N_2O), a greenhouse gas that significantly contributes to global warming. In addition, excessive nitrate (NO_3^-) can pollute soil and surface waters causing eutrophication through leaching (Wolfe and Patz 2002). Lowering fertilizer input and breeding for cultivars with better nutrient use efficiency are the main challenges to mitigate these impacts on environment (Hirel et al. 2007).

Like most leafy vegetables, spinach requires a high amount of N fertilization for optimal growth and quality of the end product (Barker et al. 1971, Cantliffe 1973, Stagnari et al. 2007). N fertilization influences leaf appearance, leaf expansion, and high N levels are required for a harvestable product with the desired dark green leaves (Biemond 1995). However, the recovery of N is particularly poor at high N fertilization resulting in elevated N concentrations in the soil at commercial production (Biemond et al. 1996). Neeteson and Carton (1999) determined that 160-220 kg N ha^{-1} from the 215 – 290 kg N ha^{-1} recommended for spinach cultivation is not used for growth. Improved nitrogen use efficiency (NUE) at high N levels in spinach cultivars would reduce the amount of N left in the soil, and improved NUE at low N levels would allow lower N application rates while maintaining desired crop characteristics (Masclaux-Daubresse et al. 2010). NUE is therefore regarded as an important breeding goal for spinach (Fageria and Baligar 2005)

N use efficiency (NUE) from an agronomic perspective is defined as the amount of biomass produced (grain, fruit or forage) at a given amount of N applied (Good et al. 2004, Han et al. 2015). This definition includes soil and weather characteristics influencing the N available to the plant for uptake (Xu et al. 2012). The physiological definition of NUE focuses on the ability of the plant to take up and utilize the available N, and consists of two components: N uptake efficiency (NUpE), which is the ability to take up N from the soil efficiently and the N utilization efficiency (NUtE): the plant's efficiency to produce biomass with the N that is taken up (Hirel et al. 2007, Han et al. 2015). Between and within many crop species large differences in both NUpE and NUtE have been reported (e.g. Hirel et al. 2007). Considerable genetic variation was found for traits that contribute to NUE, including the total N uptake, N

translocation, and N assimilation among different varieties of the same species (Xu et al. 2012). Variation in NUE is reflected in traits that are sometimes relatively easily amenable to selection for breeding purposes such as chlorophyll content (Liu et al. 2006), fresh and dry weight production (Lefsrud et al. 2007), leaf area expansion and root:shoot ratio (Hirel and Lemaire 2006).

We previously studied the genetic diversity of spinach cultivars with respect to NUE and traits that contribute to NUE using a hydroponics system and a steady state N application rate based on expected growth (Ingestad 1982), see also Chan-Navarrete et al. (2014); chapter 2 of this thesis. These conditions favour the detection of genetic variation in NUtE. The variation based on root properties like scavenging for available nitrogen in the soil, and uptake N uptake differences are likely to have a much lower effect on leaf growth and yield under hydroponics than under field conditions. It also strongly reduces environmental variation that is hard to control under field conditions (soil properties, N leaching and availability, temperature differences). In combination with the steady state stress applied, these conditions reduce the complexity of NUE as a trait to select for, and thus better allows for detection of genetic factors driving spinach growth at different levels of N availability (Chan-Navarrete et al. 2015; Chapter 4).

However, spinach is a field crop. Field-grown crops are constantly subjected to changes in the soil and aerial environment during the crop cycle that leads to Genotype by Environment Interaction (GEI) (Crossa et al. 1999, Pilbeam 2010). Nitrogen capture and use efficiency are strongly affected by large GEI interactions (Li et al. 2015) and in other leafy crops, such as lettuce, their influence on crop performance and the genetic control of their expression can therefore be difficult to assess (Kerbiriou et al. 2014). Lettuce field trials showed strongly inconsistent cultivar effects across trials (both within and between years) affecting the expression of the various traits (Kerbiriou et al. 2016). GEI observed in multi-environment trials can have several causes, including differences in N availability. For instance, the best performing maize varieties at high N input were not necessarily the best ones when the N supply is lowered (Gallais et al. 2006). Insight in the effects of environmental factors on the genetic variation of NUE-related traits is therefore useful to breed for ideotypes suitable for specific field conditions, including low N-input (Barraclough et al. 2010).

This study aims to increase understanding of the genotype by environment interaction for NUE related traits in spinach. To this end, the set of cultivars that was shown to differ in NUE under controlled conditions with steady state N application rates (Chan-Navarrete et al. 2014;

Chapter 2) was evaluated in spinach field trials in 2012 and 2013. The trials were carried out in different regions of The Netherlands with different N fertilization regimes and included organically and conventionally managed trials. Shoot biomass and canopy development were monitored throughout the crop growth period and modelled using a non-parametric approach (Hurtado et al. 2012). The multi-environment dataset for curve-fitting-derived traits was used for GEI analysis as described by Malosetti et al. (2013). The results also allowed us to make a comparison of the NUE of the set of cultivars under field conditions and under controlled screening conditions in hydroponics with steady state N conditions.

5.2. Materials and Methods

Plant materials

A set of 24 spinach F1 hybrid cultivars, see Table 5.1, was evaluated in six field trials during spring and autumn of 2012-2013. The selected cultivars were generally slow-bolting types developed for spring cultivation, and represented a broad variation for growth and morphological characteristics. The selection of cultivars was composed based on information kindly supplied by the breeding companies Enza Zaden, Nunhems, Pop Vriend and Rijk Zwaan. The set of cultivars had shown to contain broad genetic variation for NUE under low N, controlled conditions in a hydroponics system (Chapter 2; Chan-Navarrete et al. 2014), see Table 5.1.

Field experiments

All the field trials included 24 cultivars in a split-plot design. Each of the trials contained three N levels (main plots) and three blocks per N level (subplots within main plot). In each block, the 24 cultivars were placed in randomized positions (three replicates per N level). The six field trials were conducted during the spring and autumn of 2012 and 2013 (Table 5.2). Four trials were managed conventionally with chemical weed control and application of mineral fertilizers (50% calcium ammonium nitrate (CAN) and 50% ENTEC). Two trials were managed organically using fertilizers acceptable for organic farming (Monterra pellets). Both CAN and ENTEC are mineral fertilizers based on NH_4NO_3, but ENTEC contains a nitrification inhibitor (DMPP: 3,4-dimethylpyrazole phosphate) that ensures the conversion from ammoniac into nitrate at a slow pace (ENTEC 2015). The combination of both mineral

fertilizers aims to provide stable N availability during the growth period. Monterra malt is a pelleted fertilizer for organic cultivation purposes based on organic material that allows homogenous fertilization over the plots (Memon fertilizers 2015); the formulation used was NPK 9-1-4 with 75% organic matter. Prior to fertilization and sowing, soil samples (30 cm deep) were taken to assess the total nitrogen content, and nitrogen application was adjusted to reach the three intended N levels (100, 150 and 200 kg N/ha). In three trials (Fijnaart1, Fijnaart2 and Andijk) the N levels at the start of the trial were about 50 kg N/ha lower than originally intended (see Table 5.2). The sowing density at each location was 300 seeds per m^2, with the seeds evenly distributed over ten rows per plot. The plots were approximately 7 m long and 1.5 m wide.

Table 5.1. Origin, year of release or registration and NUE of spinach F1 hybrid cultivars. NUE of cultivars was determined in a previous study on hydroponics under steady state low N conditions (Chan-Navarrete et al. 2014).

Cultivar	Year of Release/registration	NUE ($g\ g^{-1}$ N)
Grandi	2008	11.19
Corvette	2010	11.70
Corvair	2011	13.27
Ranchero	2012	16.08
Thunderbolt	2013	12.70
Chevelle	2013	12.51
Charger	2009	*
Hudson	2010	10.21
Piano	2013	12.10
Cello	2011	13.52
Celesta	2011	10.37
PV 0293	Not released	12.51
PV 0294	Not released	*
Palco	1999	13.68
Novico	2011	16.67
Andromeda	2012	15.47
NUN00905SP	Not released	12.36
NUN00915SP	Not released	19.58
Crocodile	2006	13.07
Eagle	1999	14.76
Rhino	2002	9.39
Sparrow	2011	14.52
Beaver	2011	15.09
Marabu	2007	10.45

*: NUE not determined

Table 5.2. Environmental and management conditions in 18 environments (Env) of the multi-environment field study in which the set of F1 spinach hybrids was evaluated. Indices shown were the mean temperature (MeanTemp), temperature and light sums (TempSum and LightSum respectively), the residual amount of nitrogen in soil at the end of each experiment (SoilN), and the difference in day length between start and end of experiments (DLD).

Env	Location	N-Level*	Soil	Manage-ment**	Time		Environmental Index				
					Sowing	Duration	TempSum	MeanTemp***	Light Sum	SoilN	DLD
					(Date)	*(d)*	*(^oCd)*	*(^oC)*	*(h)*	*(kg ha^{-1})*	*(h)*
1		L(100)								7.0	
2	Nunhem	M(150)	Sandy	C	23-3-2012	68	710.0	10.82	972.4	11.0	4.24
3		H(200)								23.0	
4		L(100)								68.7	
5	Lelystad	M(150)	Loamy	O	3-5-2012	43	571.9	13.69	661.6	90.0	1.74
6		H(200)								128.3	
7		L(49)								4.7	
8	Fijnaart1	M(100)	Clay	C	10-9-2012	57	755.6	11.65	524.5	4.3	-3.77
9		H(150)								6.0	
10		L(61)								4.0	
11	Fijnaart2	M(100)	Clay	C	23-4-2013	35	429.7	10.58	429.8	9.0	1.90
12		H(150)								14.0	
13		L(36)								3.0	
14	Andijk	M(100)	Clay	C	25-4-2013	43	559	10.23	491.9	10.0	2.34
15		H(150)								8.7	
16		L(100)								32.0	
17	Voorst	M(150)	Loamy	O	27-8-2013	42	650.3	14.83	456.3	35.0	-2.76
18		H(200)								36.0	

Note: *: L, M, or H for Low, Medium, or High N Level, respectively and in brackets the amount of available N (kg/ha) at start of experiment; **: C for Conventional and O for Organic; ***: mean over daily maximum temperatures

Environmental indices

Environmental indices describing the differences between the environments are given in Table 5.2. 'Duration' and TempSum reflected the duration of the growing period, expressed in days from sowing to final harvest and in thermal days, respectively. The set of indices further included meteorological data accumulated or averaged over the growing period, collected at a weather station of the Royal Netherlands Meterological Institute (KNMI 2014) in the vicinity of the locations of the field trials. MeanTemp was the mean maximum temperature per day and LightSum the accumulated hours of daylight (Table 5.2). SoilN referred to the residual N content in the top soil layer, calculated as the mean over soil samples (15 samples of 30 cm deep per N-block for each location) taken at the end of

growing period. DLD was the day length difference between beginning and end of the individual trials, with positive values for spring trials and negative values for autumn trials.

Crop parameters

Data of the crop parameters shoot dry weight (SDW) and soil coverage were collected on periodical intervals during crop growth (of every 3 or 4 days). The trials were ended at first signals of flowering (heading and bolting) (spring trials), because of disease invading the fields (Voorst, autumn trial), or physiological senescence (Fijnaart1, autumn trial). To be able to compare the final yields of the six trials we calculated the SDW at time point 90% (SDW_t90%) of the growing time (in ^{o}Cd) from sowing to harvest as for some trials the final harvest was not reliable due to above mentioned constraints.

Shoot Dry Weight

For shoot dry weight (SDW) measurements, shoots of ten randomly chosen plants from each plot (cut at the plant stem below the cotyledons) were rinsed with water to remove the soil residues, and dried at 70 ^{o}C for 48 hrs). SDW is the average SDW per plant of the 10 shoot samples. The first sampling was done when the first pair of true leaves started to expand and the second pair of true leaves appeared.

Soil Coverage

For soil coverage (SC), pictures were taken of an area within a frame of 60 cm by 60 cm with a wide-angle camera (26 mm lens), which was mounted 80 cm above the soil. The pictures were edited to 705 by 805 pixels, and analysed using MatLab (MathWorks R2011) and DIP*image*, a toolbox for scientific image processing from TUDelft. The percentage of coverage by the canopy (SC) was determined with a script developed by Gerie van der Heijden in MATLAB® version 7.8.0347 (R2009a), the MathWorks™ programme (see also Ospina et al. 2014).

Smooth curves: Modelling of time series

The collected datasets represent time series of measurements for SDW and SC for each cultivar in 18 environments, see Table 5.2. A smooth curve was fitted to the time series for SDW and SC to capture patterns in the data, similar to Hurtado et al. (2012) using a temperature-corrected timescale. This approach allows the SDW production and SC development over the growth cycle to be described in curve descriptors that can be compared between environments even if the curves are highly divergent. The replicate values per cultivar for each time point were averaged prior to curve fitting. The curves were fitted using accumulated thermal time (TempSum), which is the summation of daily mean temperature corrected with a base temperature of 4˚C [((Max. Temp + Min. Temp)/2) - base temperature] from sowing date, in degree-days. The base temperature used was according to the lettuce study of Kerbiriou et al. (2013). The fitted curves represent standardized time series consisting of 100 successive time sections. The time measure used for curve fitting was the temperature sum from sowing expressed in degree-days (˚Cd). We used a nonparametric modelling approach using P-splines as a flexible semiparametric description of the curves (Eilers and Marx 1996). P-splines are penalized B-splines resulting in smooth piecewise polynomial curves. For the implementation, we used the software environment R (R Core Team 2013). The R package mgcv (Wood 2006) includes the function gam with its option for P-splines.

Several curve characteristics derived from the fitted curves describe physiological processes. For the SDW curves, the inflection point of the curve indicates the time at which the shoot dry weight increase started to slow down and for SC, the time at which canopy development rate was the highest. The (empirical) slope at all time points was calculated directly from the fitted values of the curve. The mean slope (Mean) is a measure of the SDW production (or mean relative growth rate (RGR)) or canopy development rate of the cultivar over the observed growth period), and the maximum slope (Max) of SDW refers to the maximum change in SDW (or maximum RGR) of a particular genotype (Table 5.3). The area under the curve (AUC) of SDW values is a measure of biomass produced over the entire observation period of the crop cycle, while AUC for SC represents the total light interception capacity of the crop during the time of the trial. The SDW smooth curves for each cultivar-environment combination were used to estimate the SDW at 90% of the total growing period (referred to as SDW_t90%) as a measure of yield.

Table 5.3. Descriptors of the smooth curve characteristics determined for each shoot dry weight (SDW) and soil coverage (SC) curve.

Characteristic	Description
Mean	Average progression rate over all time points
Max	Maximum progression rate over all time points
AUC	Area under the curve
Ipoint	Inflection point of fitted curve

GEI analysis

The curve descriptors are expressed as single values per cultivars, as curves were fitted on the replicate averages of the measured cultivar trait values. The dataset for the GEI analysis therefore was a simple 2-way table of 24 cultivars by 18 environments. The raw dataset available for SDW was first inspected for outliers, and subjected to analysis of variance (ANOVA) between cultivars. The datasets were analysed with block structure using environment as blocking factor. A few outliers (with residuals larger than five times the overall residual error) were replaced by fitted values obtained from the ANOVA. For each trait, the interaction between the factors N level and Cultivar were analysed with ANOVA with the environments within location defined as block structure. All analyses were done with the statistical package GenStat 16 (VSN International 2013).

The GEI analysis over all environments was done as described by Malosetti et al. (2013) with GenStat 16 (VSN International 2013). This comprises (1) a stability analysis using a regression procedure developed by Finlay and Wilkinson (1963) and (2) a factorial regression analysis of the trait-specific sensitivity of cultivars to different environmental factors. The data for each trait in each environment were first centred by subtracting the trait mean. Finlay-Wilkinson analyses were done to get genotype-specific stability estimates (G_i and b_i) using the following model:

$$\mu_{ij} = G_i + b_i E_j + \varepsilon_{ij}$$

in which μ_{ij} represents the mean value (centred) of genotype i in environment j; E_j the mean value of all cultivars in environment j and ε_{ij} is the random error term.

SDW_t90% was also subjected to factorial regression as described by Malosetti et al. (2013) using the model:

$$\mu_{ij} = G_i + \sum_{k=1}^{k} b_{ik} Z_{jk} + \varepsilon_{ij}.$$

This model includes the environmental covariate Z_{jk} and results in estimates for the sensitivity to an environmental covariate of genotypes present in the model (b_{ik}). The environmental covariates were the environmental indices summarized in Table 5.2. These were centred prior to use.

To gain further insight in the influence of additional environmental factors on genotype performance a Finlay-Wilkinson regression analysis (FW) was first performed, followed by a factorial regression analysis. For the FW analysis, two SDW curve descriptors and a curve-derived trait were chosen that were influenced significantly by both Cultivar and N-level: AUC, Max (maximum progression rate) and SDW_t90%. FW is a linear regression analysis of cultivar performance on mean performance of all cultivars in an environment, resulting in estimates of mean trait performance, slope (b_i) and intercept (Gi) of cultivar-specific regression equations. The slope estimate reflects the sensitivity to environmental variation or environmental quality. Stable cultivars that are less affected by environmental factors have a low slope value. Cultivars with high as well as stable trait values combine a high mean trait value with a relatively low sensitivity (lower than 1). The cultivar-specific coefficient (G_i) reflects mean trait performance of cultivars relative to all cultivar means.

The cultivar-specific stability and sensitivity measures for shoot growth characteristics from the current study were finally compared with the performance data of the common cultivars upon steady-state low and high N supply as described in Chapter 2. This was done by means of a correlation analysis using the software package Genstat 16, including the procedure to calculate partial correlation coefficients, all with the ultimate aim to get a better insight in the relevance of screening for NUE.

5.3. Results

Time courses for development of canopy coverage and shoot growth

The progression of shoot dry weight (SDW) and canopy development (SC) in each environment were established to get an impression of the mean performance of the spinach cultivars in each of the environments. For this, the collected SDW and SC data for each cultivar were used to fit smooth curves as described in the material and methods section, with the time axis transformed from days to thermal days. The curve-derived values for the 100 successive time intervals for the cultivars were subsequently averaged for the comparison of N levels and environments, depicted in Figure 1 for SDW (Figure 1a for the spring trials, and

in Figure 1b for the autumn trials) and Fig 2 for SC (Figure 2a for the spring trials, Figure 2b for the autumn trials).

There was large variation between the different trials in the length of the trials (from sowing to harvest), both in normal days and in thermal days, with the spring trials generally being shorter than the autumn trials (Table 5.2). The soil coverage measurement data demonstrated that in all trials, the cultivars reached canopy closure at the time of the last harvest (Figure 2a and 2b). The shortest trial was the Fijnaart2 spring trial, which was sown relatively late in the season, and was terminated early because the plants started to bolt early. The Lelystad spring trial was sown even later, but germinated late, and lasted further into summer. This late germination, and the delay in progress of canopy development shortly after germination, can be linked to low temperatures that may have caused cold stress to the young plantlets in this particular trial (Supplementary Figure 5.A). All spring trials were terminated because of bolting. As bolting and flowering are promoted by long day conditions (Parlevliet 1968), plants in this trial did not show any bolting. The trial was terminated because of low temperatures and physiological senescence arresting growth. The organically managed Voorst trial was terminated prematurely because of downy mildew invading the field.

Within most locations N fertilization affected the development of shoot dry weight, with a significant effect of N availability ($p<0.001$) on SDW at later time points in four out of six trials, but not in the organically managed Lelystad and the Voorst trials (Figure 5.1). Different N availabilities for the plants in these trials were difficult to realize, as the release of N and mineralization is dependent on the weather and therefore difficult to control (Han et al. 2015). This is also reflected by the high amounts of nitrogen left in the soil at the end of these trials, especially in the spring Lelystad trial (Table 5.2).

As can be expected, the differences in shoot dry weight between N levels were generally most obvious at the later stages of the trials. The strongest reductions in SDW at low N relative to high N were observed for the Andijk spring and the Fijnaart2 (spring) trial, and the SDW differences were already significant ($P<0.001$) at a relatively early stage in the trial (Table 5.2, ANOVAs). The N levels in those trials were lower (36 and 61 kg/Ha for Low N at Andijk and Fijnaart2, respectively, and 100 and 150 for Medium and High N) than in the other trials, suggesting that the lower N levels in these trials were inducing a stronger and earlier reduction in SDW than in the other trials. SDW on average increased with increased N application rate, but differences between SDW under Medium and High N conditions were relatively small.

For all locations, the cultivar-dependent variation for almost all SDW curve parameters was significant except for the inflection point. The N-level dependent differences within locations were relatively small in comparison to the differences between the locations. Within each location, hardly any significant Cultivar × N level interactions were detected for SDW.

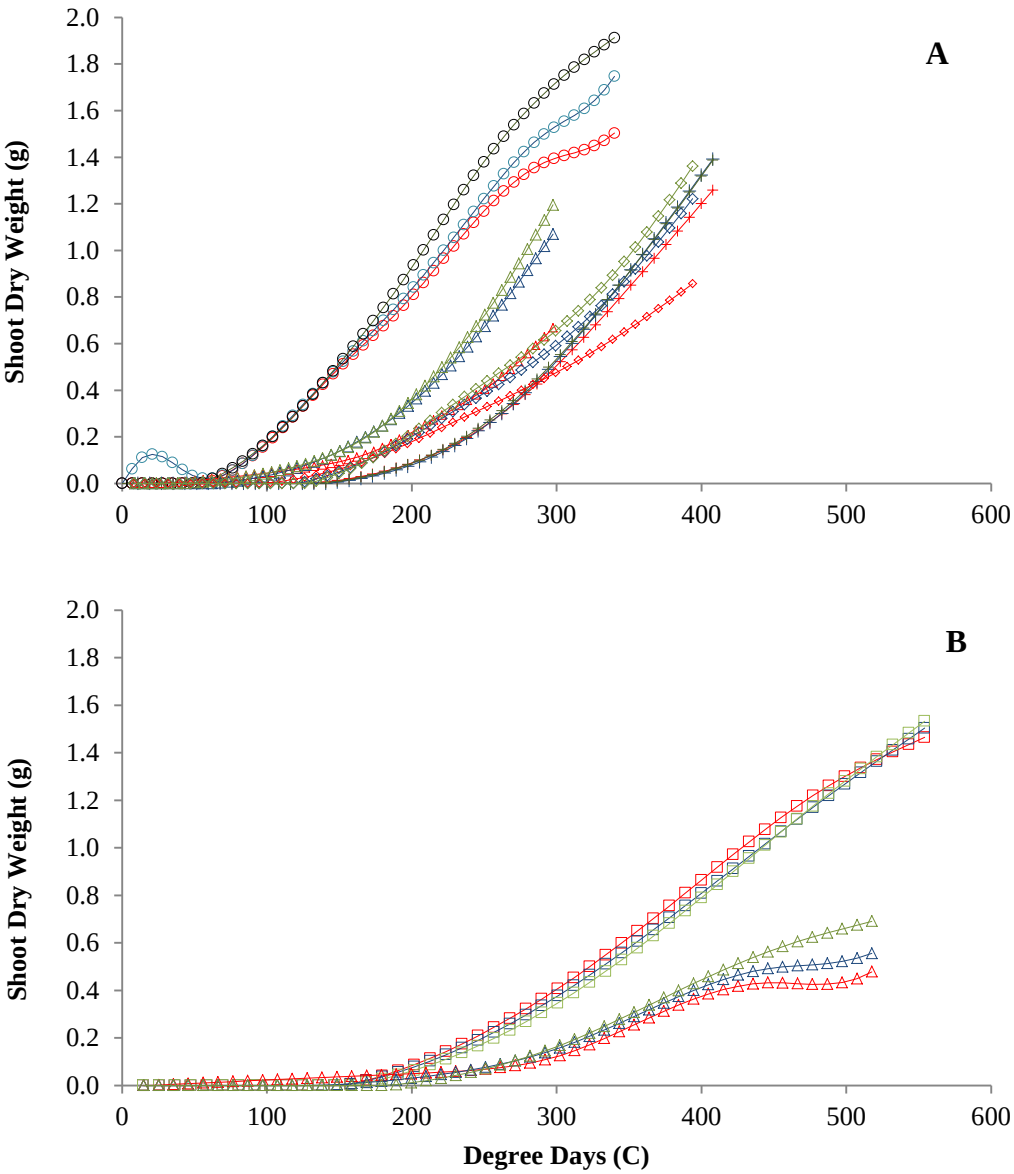

Figure 5.1. Smooth curves for shoot dry weight (SDW) averaged over 24 cultivars in 18 environments. Figure 5.1a presents the smooth curves for the spring trials, Figure 5.1b for the autumn trials. The N treatment levels L(ow), M(edium), and H(igh) are depicted in red, blue and green, respectively. Triangles (Δ): Fijnaart trials, diamonds (◊): Andijk trials, squares (□): Voorst trials, circles (○): Nunhem trials and dashes (+): Lelystad trials.

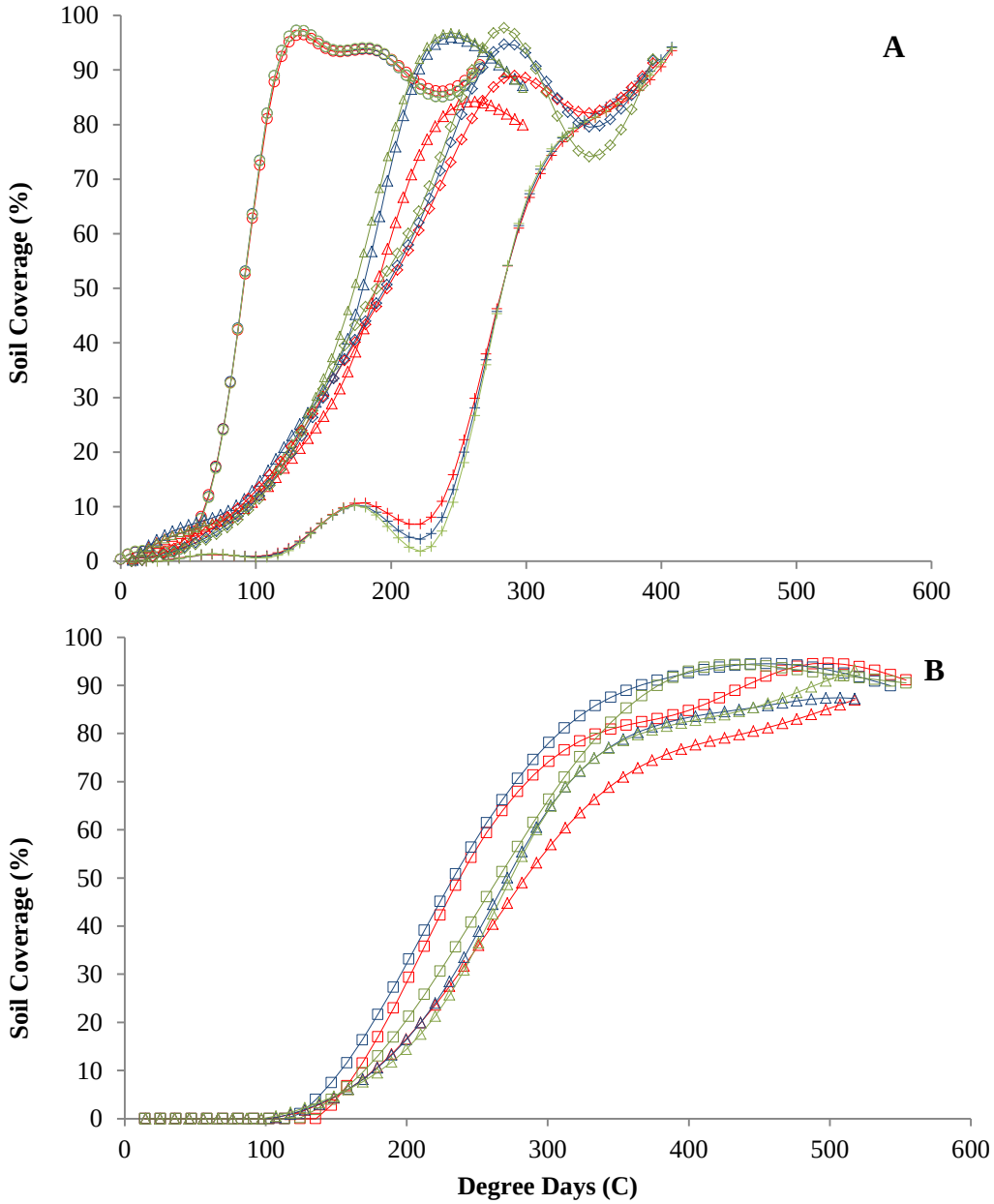

Figure 5.2. Soil coverage of the different field trials (18 environments by locations, N levels and seasons) over the mean of 24 genotypes and based on thermal time. Figure 5.2a presents the smooth curves for the spring trials, Figure 5.2b for the autumn trials The N treatment levels L(ow), M(edium), and H(igh) are depicted in red, blue and green, respectively. Triangles (Δ): Fijnaart trials, diamonds (◊): Andijk trials, squares (□): Voorst trials, circles (○): Nunhem trials and dashes (+): Lelystad trials

Effect of N level and environment on SDW curve characteristics.

The curve descriptors allow the comparison of performance of the cultivars in the strongly variable environments. Statistical analysis for the curve descriptors across all environments are summarized in Table 5.4. N-level as well as Cultivar were highly significant sources of variation for the area under the curve (AUC) and the progression rate characteristics Max and

Mean. There were no significant N-level × Cultivar interactions detected for the evaluated curve characteristics.

Table 5.4. Overall means, means for each N-Level, and Cultivar means for each shoot dry weight curve parameter as well as shoot dry weight at 90% of the growing time (SDW-t90%) obtained from the corresponding analyses of variance. The curve parameter values of the most extreme cultivars are shown (Max and Min). AUC=area under the curve; iPoint=inflection point of fitted curve

Factor	**Curve Parameter**					
	AUC	**iPoint**	**Min**	**Max**	**Mean**	**SDW_t90%**
			($x10^{-3}$)			*(g/plant)*
N-Level						
Low	142.1	354.3	-6.584	6.096	2.678	0.884
Medium	154.7	360.1	-4.777	7.471	3.288	1.025
High	161.8	358.7	-3.562	7.853	3.536	1.093
F-prob	**0.044**	0.727	0.334	0.079	**0.006**	**0.007**
Cultivar						
Min	135.3	335.0	-20.738	5.628	2.776	0.876
Max	167.8	384.0	-0.858	8.44	3.473	1.095
F-prob	**<0.001**	0.359	**0.003**	**0.009**	**<0.001**	**<0.001**
N-Level × Cultivar						
F-prob	0.966	0.700	**<0.001**	0.079	0.807	0.841
Mean	152.90	357.71	-4.974	7.14	3.17	1.00

For a proper GEI analysis, a trait must be selected that allows a good comparison of the performance of the cultivars. The trials in the different environments were highly diverse, as a result of environmental factors affecting growth as well as harvest time. We decided to focus on an agronomic trait that included the consequences of factors enforcing harvests, like bolting/flowering in trials, and diseases invading the trials. We used the SDW at 90% of the growing time (in ˚Cd) (SDW_t90%). SDW_t90% showed highly significant differences between N levels and between cultivars (Table 5.4). Low N level SDW_t90% was considerably lower than SDW_t90% of the M and H levels, while the latter two did not differ much. The magnitude of differences between cultivars was substantial and significant. No significant interaction between N level and Cultivar was detected. Our analyses indicated that other sources of environmental variation, such as management, weather and soil conditions compromised detection of G×N interactions when analysed over all environments. As a

comparison, a similar analysis was done using the SDW at the time soil coverage reached 90% of the maximum soil coverage, which would represent the SDW at similar stages of crop development in each environment. The results of the statistical analysis were comparable to the results using SDW_t90%, and the effects of N were relatively low as this measure would represent SDW at a relatively early stage of the crop growth at which N effects may not be strongly expressed in the phenotype. Therefore, we decided to continue with the latter trait for GEI analysis.

Table 5.5. Cultivar-specific stability measures *Gi* and *bi* from Finlay-Wilkinson regression analyses of the variation between cultivars in 18 environments and mean cultivar performance over environments for each parameter, for three parameters based on shoot dry weight (SDW): area under the curve (AUC), Mean Growth Rate (Mean) and SDW_t90%.

	AUC			**Mean**			**SDW_t90%**		
Cultivar	**Mean**	***Gi***	***bi***	**Mean**	***Gi***	***bi***	**Mean**	***Gi***	***bi***
Grandi	146.9	-5.996	1.078	3.160	-0.007	1.019	0.973	-0.028	1.071
Corvette	155.4	2.504	1.079	3.031	-0.136	0.862	0.991	-0.010	0.932
Corvair	135.3	-17.596	0.904	2.894	-0.273	0.826	0.912	-0.089	0.923
Ranchero	160.1	7.204	1.009	3.182	0.015	0.962	0.999	-0.002	0.892
Thunderbolt	167.8	14.904	1.118	3.378	0.211	0.989	1.080	0.079	1.043
Chevelle	167.5	14.604	1.251	3.399	0.232	1.232	1.095	0.094	1.312
Charger	157.1	4.204	1.055	3.376	0.209	0.931	1.032	0.031	0.932
Hudson	167.5	14.604	1.103	3.457	0.290	1.027	1.087	0.086	1.078
Piano	157.9	5.004	1.156	3.216	0.049	1.023	1.033	0.032	1.135
Cello	144.1	-8.796	0.907	3.028	-0.139	0.896	0.950	-0.051	0.861
Celesta	136.1	-16.796	0.883	2.776	-0.391	0.903	0.876	-0.125	0.867
PV0293	144.8	-8.096	0.943	3.150	-0.017	0.998	0.979	-0.022	0.990
PV0294	154.3	1.404	1.042	3.273	0.106	0.988	1.018	0.017	1.043
Palco	157.8	4.904	0.949	3.215	0.048	0.999	1.035	0.034	0.928
Novico	157.5	4.604	0.886	3.155	-0.012	1.096	0.986	-0.015	0.932
Andromeda	163.3	10.404	0.992	3.473	0.306	0.977	1.071	0.070	0.920
NUN0905sp	153.2	0.304	1.006	3.004	-0.163	1.014	0.993	-0.008	1.078
NUN0915sp	150.7	-2.196	1.011	3.036	-0.131	0.993	0.961	-0.040	0.994
Crocodile	152.9	0.004	1.067	3.388	0.221	1.076	1.052	0.051	1.077
Eagle	148.5	-4.396	0.903	2.970	-0.197	0.885	0.961	-0.040	0.918
Rhino	138.3	-14.596	0.736	2.847	-0.320	0.870	0.928	-0.073	0.784
Sparrow	161.6	8.704	1.030	3.365	0.198	1.111	1.060	0.059	1.097
Beaver	151.9	-0.996	1.000	3.213	0.046	1.254	1.004	0.003	1.156
Marabu	139.0	-13.896	0.893	3.028	-0.139	1.070	0.951	-0.050	1.035
F-prob		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001

Cultivar performance stability across environments

The results of the Finlay-Wilkinson analyses for AUC, Max and SDW_t90% are summarized in Table 5.5. The AUC represents a measure of biomass produced over the entire observation period of the crop cycle. The G_i-estimates and sensitivity estimates for AUC were highly correlated to SDW_t90% (r = 0.92, and r = 0.88, respectively), and the G_i-estimates for AUC and SDW_t90% were also significantly correlated to the sensitivity estimates (r = 0.83 and r = 0.79, respectively), indicating that stable cultivars in general tended to be the ones that produced less biomass. The cultivars that escaped this tendency showed relatively high and stable values for AUC across environments. These cultivars were Andromeda, Palco and Novico. Chevelle. The cultivars Piano, Hudson and Thunderbolt were the most responsive to environmental differences for both AUC and SDW_t90%. Good and stable performers for SDW_t90% were Andromeda, Palco, Charger and Ranchero.

The *Gi*-estimates of the Max progression rate descriptor were positively correlated to *Gi* estimates of AUC and SDW_t90% (r = 0.64 and 0.67, respectively). The sensitivity estimates on the other hand were not significantly correlated. The cvs Andromeda, Charger, Thunderbolt and Ranchero combined low sensitivities with mean Max values, indicating that these cultivars grow relatively fast in the less favourable environments.

Influence of environmental conditions

A factorial regression analysis of the variation in SDW_t90% between the cultivars with environmental indices (see Table 5.2) was done to determine the contribution of the individual environmental factors to the variation in SDW production of the spinach cultivars. The results are summarized in Table 5.6. The regression coefficients indicated that SDW_t90% for most cultivars increased with MeanTemp and Duration, while the effect of LightSum on of cultivars varied considerably. The effect of DLD, which separates spring and autumn trials, was not significant; indicating that for SDW_t90% there is no significant difference between spring and autumn trials. All cultivars nevertheless appeared to have slightly higher SDW_t90% values in spring, with the exception of cv. Novico. Cv. Novico also was among the most responsive cultivars to LightSum.

SoilN was included in the set of environmental indices as an indicator of soil N depletion due to growth and possibly leaching due to rainfall. SoilN did not significantly contribute to the variation in SDW_t90%, even though for two of the trials (Voorst and Lelystad, the

organically managed trials) the SoilN values were significantly higher than for the other trials (Table 5.2). This indicated that in our trials, the N remaining in the soil after harvest was not a good indicator of N uptake by the crop.

Table 5.6. Cultivar-specific sensitivity measures (b_{ik}) from factorial regression analyses (Malosetti et al. 2013) of the variation between cultivars for shoot weight (SDW_t90%) based on the environmental variation for environmental indices. The sensitivity measures are coefficients of regression of the trait on the individual indices. Mean refers to the mean of the response variate, DLD refers to difference in day length between start and end of experiments

Cultivar	Mean	Environmental Index				
	(g/plant)	MeanTemp	Duration	SoilN	LightSum	DLD
			(x 10)	*(x 10)*	*(x 10)*	*(x 10)*
Grandi	0.973	0.224	-0.118	-0.070	0.017	0.531
Corvette	0.991	0.208	-0.196	-0.073	0.019	0.201
Corvair	0.912	0.169	-0.147	-0.062	0.018	0.404
Ranchero	0.999	0.181	-0.116	-0.067	0.016	0.357
Thunderbolt	1.079	0.196	-0.147	-0.059	0.018	0.504
Chevelle	1.095	0.244	-0.099	-0.067	0.019	0.588
Charger	1.032	0.204	-0.069	-0.059	0.011	0.555
Hudson	1.086	0.182	-0.109	-0.042	0.017	0.581
Piano	1.033	0.252	-0.139	-0.076	0.018	0.570
Cello	0.950	0.147	-0.140	-0.038	0.016	0.396
Celesta	0.876	0.149	-0.116	-0.050	0.016	0.353
PV0293	0.979	0.148	-0.156	-0.034	0.020	0.360
PV0294	1.018	0.195	-0.095	-0.047	0.015	0.525
Palco	1.035	0.168	-0.114	-0.052	0.014	0.486
Novico	0.986	0.123	-0.157	-0.046	0.021	0.385
Andromeda	1.071	0.146	-0.176	-0.031	0.019	0.296
NUN0905sp	0.993	0.151	-0.102	-0.052	0.020	0.526
NUN0915sp	0.961	0.162	-0.128	-0.054	0.020	0.377
Crocodile	1.052	0.195	-0.188	-0.077	0.021	0.482
Eagle	0.961	0.155	-0.128	-0.050	0.018	0.488
Rhino	0.928	0.094	-0.206	-0.029	0.021	0.166
Sparrow	1.060	0.179	-0.180	-0.063	0.023	0.434
Beaver	1.004	0.139	-0.097	-0.037	0.021	0.566
Marabu	0.951	0.159	-0.104	-0.056	0.017	0.551
F-prob	<0.001	<0.001	<0.001	0.931	<0.001	0.732

Cv. Rhino was low yielding and relatively poorly responsive to temperature as well as soilN. However, it was among the most responsive cultivars for LightSum. The most responsive to temperature increase were cvs Chevelle and Piano. Cv. Chevelle is a high yielding cultivar

performing particularly well under spring conditions. A longer duration of the trial was typically associated with a slightly lower SDW_t90%. Cv. Charger was the least sensitive to the duration of the trial.

5.4. Discussion

The main objective of this study was to get a better understanding of the impact of nitrogen availability and other environmental factors on the yield of field-grown spinach cultivars. To this end a set of cultivars shown to differ in NUE under controlled conditions (Chapter 2; Chan-Navarrete 2014) was evaluated in six field trials (over a period of two years and two different seasons) under a wide variety of environmental conditions with varying N availability. Two performance measures, *i.e.* soil coverage and plant shoot dry weight, were monitored throughout the growth period. The protocol used for soil coverage measurements was described in detail by Ospina et al. 2014 and has proven to result in good estimates of canopy development, and capacity of light interception. In the organic Lelystad trial the progression of soil coverage halted and even decreased during the exponential phase of SC. This was at least partly due to the environmental conditions, with low temperatures causing low mineralization of organic fertilizers, leaf damage and loss of canopy (Supplementary Figure 5.A) in agreement with the findings of Yadav (2010). In this trial there were also long periods without rainfall causing additional stress (Supplementary Figure 5.B)

Under field conditions, N availability is difficult to control and may change over the growth season. Monitoring growth characteristics throughout the growth cycle of the crop allows a more accurate description of the growth response of a crop to changing environmental conditions. The use of thermal time as a time measure facilitated the comparison of cultivar performance across environments. The curve parameters derived from the non-parametric smooth curves (as described by Hurtado et al. 2012) describing the progression for SDW and SC using thermal time proved to be useful descriptors of growth for which genotypic, developmental and environmental variation could be detected (Hurtado et al. 2012, Ospina et al. 2014, Ospina 2016).

The SDW curve parameters AUC, Mean growth rate and SDW_t90% formed the basis of the extensive GEI analysis done in this study. These traits were the most relevant parameters to do such analyses, but bolting and incidence of diseases affected the final harvest date and the duration of a trial, confounding the effects of other environmental factors on these traits. We

also considered the use of an alternative shoot yield parameter that is independent of harvest time and may better represent the effects of the environment (N, temperature, soil) on crop growth: SDW of each cultivar at the time of 90% soil coverage. This parameter however was not significantly affected by N levels (not shown). Because of the early closure of the canopy in the spinach plots, 90% soil coverage likely occurred at a stage at which the plants had hardly experienced limitation of N. The differences in SC and SDW due to N availability became more prominent later in the growth cycle of the crop. The impact of N availability on the progression of soil coverage therefore was small and only significant during the second half of the growing period, at which time the effect of N levels was also significant for all shoot growth characteristics. This further supports the notion that N is non-limiting at early growth stages, but that with increased plant size N requirement increases, while N availability becomes lower, resulting in N limitation of plant growth at later growth stages (Han et al. 2015). In a field trial, this would imply that fast growers deplete their N resources more quickly than slow growers, and show N deficiency symptoms faster. This complicates the interpretation of the differences in cultivar performance for NUE and NUE-related traits in (multi-environment) field studies, exemplified in Chapter 3 where two different N application strategies (steady state application of N and single bulk application) induced variable growth responses and favored different cultivars, both under low and high N availability, suggesting that different genetic factors may contribute to NUE under these two conditions.

No significant interaction between Cultivar and N-level was found for any soil coverage or shoot growth parameter (Table 5.4), whereas for most traits significant genotypic differences were present (Supplementary Tables 5.A and 5.B). A likely explanation for this is that N availability is difficult to control in the field because of unpredictable environmental variation influencing the availability of N to the plants such as leaching of N after heavy rains under conditions with mineral fertilizers and with additional mineralization of N from soil organic matter as can occurs under organic conditions. In addition, other environmental factors may mask genotypic differences in shoot growth in response to N. For instance, leaf expansion rate and leaf area are known to decrease under mild water deficits (Tardieu et al. 1999), interfering with the effect of N availability for this trait. Water availability is considered as the most important environmental factor that may affect NUE (Han et al. 2015). The variation for water availability between environments would then induce additional phenotypic variation that would confound the genetic variation in response to N levels. Accurate monitoring of additional environmental factors and frequent assessment of the plant traits can in part

circumvent the masking of genotypic differences. New phenotyping technologies as well as accessible and affordable monitoring tools for multiple environmental parameters is therefore essential for uncovering genotypic differences in NUE under field conditions (Araus et al. 2015, Hatfield 2015, Pratap et al. 2015).

Notwithstanding the challenges in the presented trials, monitoring of plant growth throughout the crop growth cycle, and curve fitting using thermal time enabled the comparison of the performance of 24 commercial cultivars known to differ in NUE (Chapter 2) in 18 environments and assessment of the influence of a set of environmental factors on the shoot growth traits AUC, Max and SDW_t90%. Finlay and Wilkinson regression analyses showed significant differences between cultivars in response to environmental quality that could partially be assigned to individual environmental factors (Tables 5.6 and 5.7). It is likely that the analyses did not include all relevant sources of variation since the set of environmental indices was not comprehensive. The FW analysis for SDW_t90% showed that the G×E interaction for this trait had a significant multifactorial basis, indicating not surprisingly that several factors were influencing growth of the plants simultaneously. This raised the question whether under field conditions spinach breeding for NUE can be effective. In many studies dedicated to mapping NUE and components of NUE in different crops the main challenge is the degree of phenotypic variation and the difficulty to acquire reliable data from field trials (Han et al. 2015). Progress can be made by multiyear and multilocation field trials, but this requires a dedicated effort. Another option would be to control the environmental factors that confound the effect of N availability. We have shown in Chapters 2, 3 and 4 that experiments in hydroponics (in which the N-availability was tightly controlled) allowed identification of traits contributing to NUE in spinach, such as leaf area and specific leaf area, and of genetic factors underlying these traits.

An important question that needs to be investigated is how the results and the performance of the cultivars in the current study relate to the response of the cultivars under controlled, hydroponic conditions with a steady-state supply of N (Chapter 2; Chan-Navarrete et al. 2014). To explore this question we compared cultivar-specific environmental sensitivity measures for shoot growth from this study (AUC, SDW_t90%) with several trait values for the cultivars measured on hydroponics in Chapter 2 in a correlation analysis (Supplementary Figure 5.C). The various FW-parameters determined in current field study generally showed low correlations with hydroponics traits (Supplementary Figure 5.C). Interestingly, the few stronger and significant correlations were mostly found with root to shoot ratio (R:S), both

under high and low N conditions. In fact, these were often higher than the correlations of R:S with hydroponics shoot yields. R:S under low N had strongest (negative) correlations with the stability measure *bi* of the FW analysis, while R:S under high N was strongly negatively correlated to the relative performance measure *Gi* and other yield measures in the field. Being able to invest in roots over shoots under low N conditions in particular may therefore be a trait that favors stability under variable field conditions, while partitioning nutrients to the roots especially under high N conditions but also under low N conditions appears to penalize yield. The hydroponics setup therefore may be a selection platform not only for improvement of nitrogen-use efficiency but also one enabling root trait phenotyping and selection contributing to yield in the field crop of spinach.

We also calculated partial correlation coefficients between nitrogen-use efficiency determined at low N and some sensitivity estimates for three environmental factors and the FW parameters for SDW_t90% determined in this study (Table 5.7). The latter was done to eliminate confounding effects due to correlation between the different parameters. The correlation found between each parameter and NUE_LN turned out to be low and insignificant. The absolute values for the corresponding partial correlation coefficients, however, were strikingly higher (Table 5.7). The results indicated that NUE_LN measured on hydroponics was a positive indicator of the overall shoot yield (SDW_*Gi*) probably as a consequence of a positive relation with major factors associated with growth (LightSum, Duration and MeanTemp). The negative partial correlation with SDW_*bi* on the other hand indicated that NUE_LN also was a relevant measure in relation to breeding for stable performance.

Table 5.7. Simple and partial coefficients of correlation between the genotype-specific estimates for NUE_LN and sensitivity parameters from the factorial regression analyses as well as the fresh weight (FW) parameters for shoot dry weight at 90% of the growing time (SDW_t90%). Partial correlation coefficients were calculated using all traits mentioned in this table.

Type of correlation	Sensitivity parameter (*bi*)			FW-parameter	
	MeanTemp	Duration	LightSum	*Gi*	*bi*
Simple (r)	-0.14^{ns}	0.03^{ns}	0.23^{ns}	0.11^{ns}	-0.03^{ns}
Partial (r_p)	0.49^{*}	0.63^{**}	0.64^{**}	0.54^{*}	-0.61^{**}

^{ns}: r not significantly different from nil; *, **: r_p significantly different from nil with $P<0.05$ and $P<0.01$, respectively

In conclusion, the current study has shown that low N fertilization had an adverse effect on mainly the later stages of spinach crop growth, after canopy closure in a densely grown crop. Our results showed significant differences between cultivars in crop biomass production and yield. The lack of significant interactions between cultivar and N-treatment for both shoot biomass and soil coverage was likely linked to the strong influence of environmental factors like temperature, soil, and management on nitrogen availability in the soil in a short cycle crop such as spinach. The GEI analyses showed substantial cultivar-specific differences for shoot growth traits, such as SDW_t90% in sensitivity to environmental quality in general and in sensitivity to specific environmental factors, in particular underscoring the importance of performing trials under better controllable conditions for genetic dissection of NUE and discovery of breeding traits (Hirel et al. 2007, Xu et al. 2012, Han et al. 2015). We therefore argue that the more efficient strategy for screening for NUE in spinach would be to select genetic diversity in germplasm (cultivars as well as inbred lines) under controlled conditions, identify selectable traits, followed by performance testing under field conditions of selected material and crosses of favorable genotypes.

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Supplementary Data

Table 5.A. Overall genotypic mean performance for growth curve characteristics (AUC: area under the curve, Ipoint: inflection point of fitted curve, Max: maximum progression rate) for shoot dry weight (SDW) across environments and the, shoot dry weight at 90% of the growing time (SDW_t90%)

Cultivar	AUC	Ipoint	Max *($x10^{-3}$)*	Mean *($x10^{-3}$)*	SDW_t90% *(g/plant)*
Grandi	234.7	585.4	5.59	1.98	1.00
Corvette	253.4	581.7	6.14	1.97	1.07
Corvair	224.9	582.2	4.65	1.86	0.98
Ranchero	261.0	551.4	5.44	2.02	1.05
Thunderbolt	278.0	551.1	5.70	2.18	1.17
Chevelle	262.9	582.5	5.30	2.14	1.12
Charger	262.6	592.8	5.92	2.15	1.13
Hudson	284.0	562.1	5.49	2.24	1.16
Piano	246.1	559.5	5.17	2.05	1.07
Cello	234.7	562.7	4.99	1.94	1.00
Celesta	227.0	586.9	4.81	1.78	0.91
PV0293	233.6	570.7	5.09	2.02	1.01
PV0294	248.0	584.3	5.68	2.08	1.03
Palco	263.4	561.1	5.15	2.10	1.12
Novico	270.9	536.2	5.51	1.99	1.11
Andromeda	261.1	566.9	6.42	2.23	1.10
NUN0905sp	255.7	550.0	4.65	1.94	1.05
NUN0915sp	256.7	571.2	5.37	1.96	1.03
Crocodile	253.5	551.4	5.99	2.18	1.11
Eagle	250.7	590.4	5.29	1.97	1.04
Rhino	227.5	562.7	4.54	1.84	0.95
Sparrow	276.3	609.7	6.38	2.29	1.15
Beaver	259.8	600.6	5.59	2.15	1.08
Marabu	224.2	556.2	4.55	1.89	0.97

Table 5.B. Overall genotypic performance for growth curve characteristics (AUC: area under the curve, Ipoint: inflection point of fitted curve, Max: maximum progression rate) for soil coverage across environments.

Cultivar	AUC	iPoint	Max *(x10^{-3})*	Mean *(x10^{-3})*
Grandi	26456	347.2	4.60	1.44
Corvette	26847	334.6	5.15	1.42
Corvair	25269	350.6	5.06	1.40
Ranchero	28162	315.4	5.76	1.44
Thunderbolt	28178	330.3	5.66	1.47
Chevelle	28864	326.6	5.01	1.47
Charger	28843	330.4	5.80	1.47
Hudson	29435	326.8	5.72	1.48
Piano	27483	351.0	4.70	1.44
Cello	28308	344.6	5.18	1.43
Celesta	27577	349.5	4.85	1.43
PV0293	26357	363.7	5.13	1.42
PV0294	27890	344.2	5.10	1.47
Palco	29286	339.5	5.99	1.49
Novico	31419	307.0	5.36	1.46
Andromeda	28438	328.1	5.05	1.44
NUN0905sp	28926	320.7	5.55	1.45
NUN0915sp	29175	323.4	5.63	1.45
Crocodile	27646	346.8	5.02	1.46
Eagle	28005	331.5	5.38	1.44
Rhino	29620	314.4	5.39	1.46
Sparrow	28608	339.7	5.28	1.46
Beaver	28222	324.9	5.64	1.45
Marabu	25763	357.8	5.25	1.43

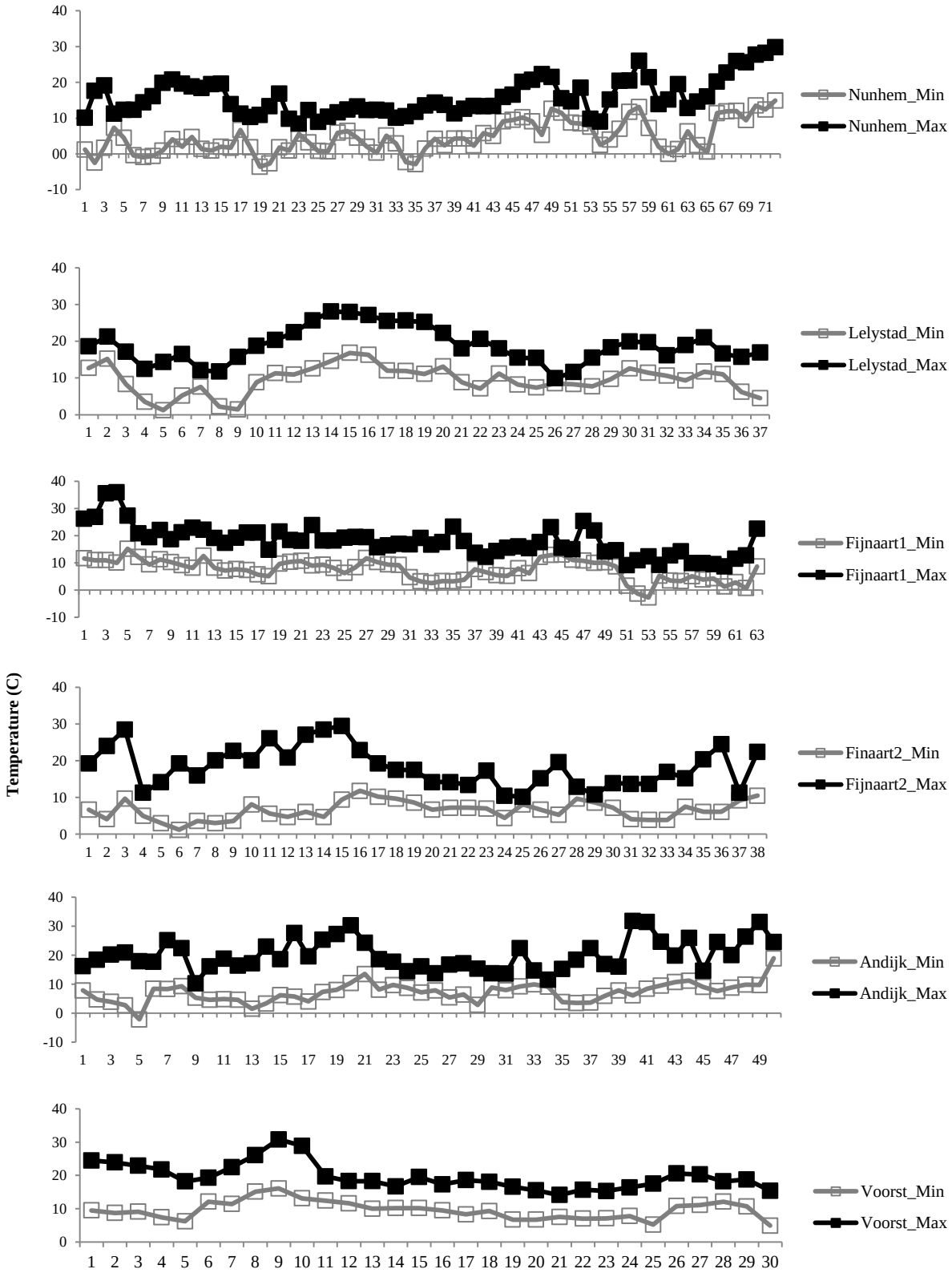


Figure 5.A. Daily maximum and minimum temperature trend over time in each location of the GEI study from sowing to the final harvest day.

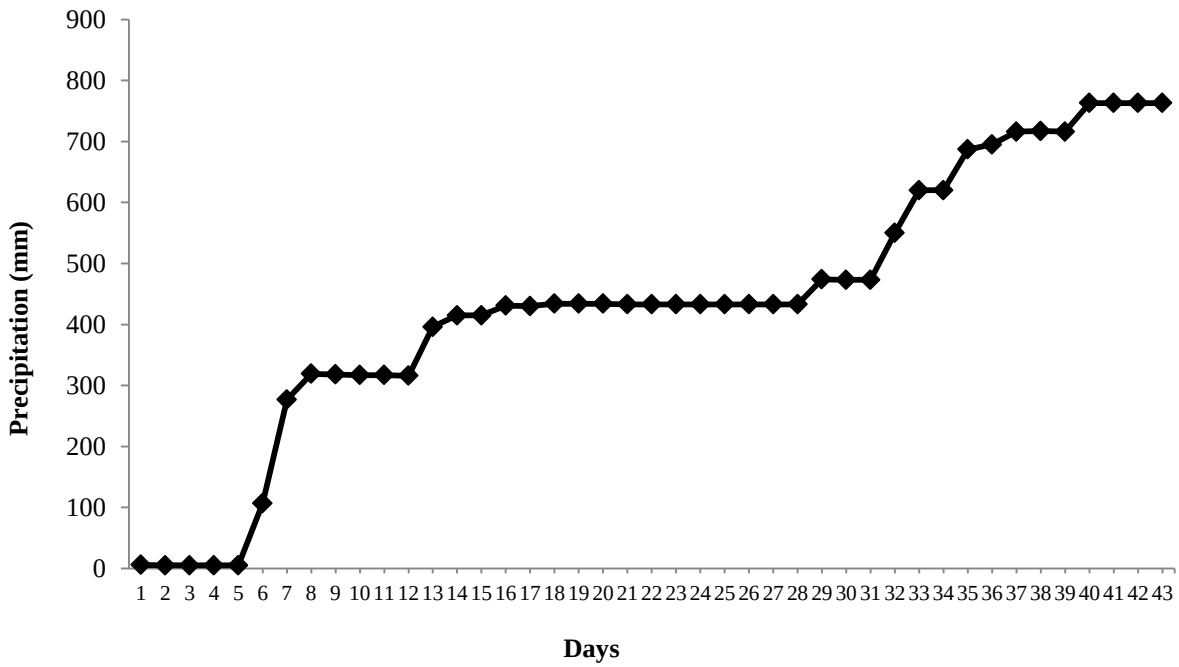


Figure 5.B. Accumulated precipitation in the Lelystad trial from sowing to the final harvest day.

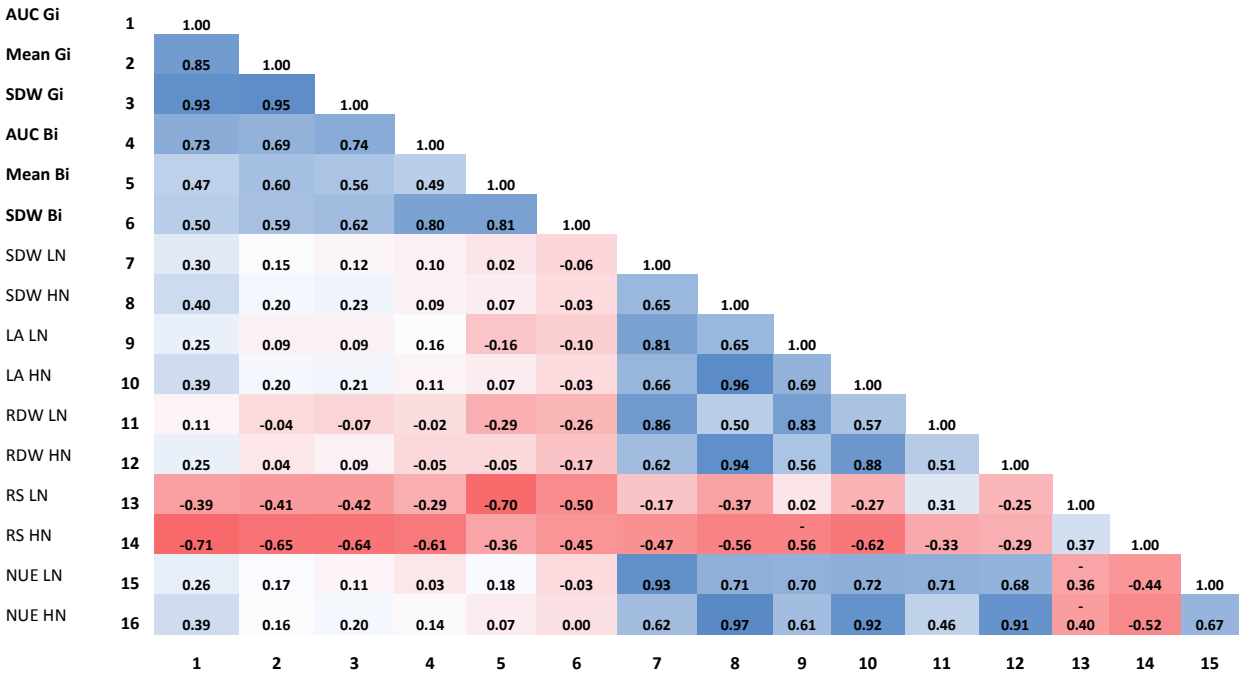


Figure 5.C. Coefficients of correlation between field trial parameters from Finlay-Wilkinson stability analyses (*Gi* as mean performance and *Bi* as stability) of the mean (average progression rate over all time points), AUC (area under de curve) and SDW_t90% (estimate the SDW at 90% of the total growing period) (in bold) and traits scored in tests under hydroponics conditions P((|r| > 0.42) = <0.05; df=20) (shoot dry weight (SDW), leaf area (LA), root dry weight (RDW), root to shoot ratio (R:S) and nitrogen use efficiency (NUE)) under low and high N conditions (LN and HN respectively). Negative correlations are highlighted from white to red being dark red the highest negative correlations. Positive correlations are highlighted from white to blue being the dark blue the highest positive correlations.

Chapter 6

General discussion

Chapter 6

General Discussion

Modern agriculture has greatly increased food availability and security but this was accompanied by a large increase of the use of fertilizers, often in excessive amounts. The major macronutrient supplied is nitrogen, the primary driver of plant growth and development. Over fertilization, however represents a major hazard for the environment. Excess of nitrogen fertilizer can lead to contamination of water resources and even contribute to global warming due to the production of NO_2, a potent greenhouse gas (Erisman et al. 2008). It is therefore essential to reduce the hazardous environmental effects of the N-application needed to support crop production. One sustainable method is the improvement of the nitrogen use efficiency (NUE) of crops.

NUE (defined in this thesis as the yield produced per amount of N applied) is inherently a complex trait that is difficult to breed for (reviewed in Han et al. 2015). Any environmental factor may influence the phenotypic variation as much as genotype. Several evaluation strategies can be applied to avoid masking genetic components of NUE by environmental factors. These include detailed phenotyping for component traits, minimizing environmental influence by controlling selection conditions, and combining data from multi-year multi-location trials. Breeding programs that aim at combining genetic factors (QTL) determining component traits for NUE that are related to yield may result in additional gains in yield under low N conditions (Hirel et al. 2007, Quarrie et al. 2005). A better understanding of the traits that determine NUE, how these traits and their contribution to NUE are affected by N levels, N management as well as other environmental conditions provide insight that will improve the success rate of breeding programs aiming at cultivars with improved NUE.

The focus of this thesis was to do so for spinach, a leafy vegetable crop. Spinach is a crop with a short life cycle that has a high demand for nitrogen to realize a good yield of leaves with a dark green colour as required by the market (Smolders et al. 1993, Stagnari et al. 2007). However, the efficiency of nitrogen use of the crop is low. In this thesis several aspects of breeding for NUE in the leafy vegetable spinach were further investigated in order to define parameters that would aid breeding for spinach cultivars that can grow better with less N fertilizer. The first step was to establish a screening method that would allow dissection of

NUE in component traits, and use it to evaluate genetic diversity for NUE in a set of spinach cultivars. The response of the plants to different N application strategies were investigated as well. The screening method was used for a genetic study to find genetic factors contributing to NUE in spinach, for which molecular and genetic tools were developed. As spinach is a field crop, we evaluated the same set of cultivars was analysed in field trials and compared their performance in the controlled screening environment. The results described in the previous chapters are summarized below.

6.1. Overview of results

The first research goal was to get insight into the availability of useful genetic variation for NUE within the genepool of cultivated spinach. Because of the known challenges of evaluating NUE under field conditions (Han et al. 2015) we investigated the possibility of using a system that would allow much better control of environmental conditions, and N availability. We focused on hydroponics using a wide set of commercial F1 hybrids that differed highly in growth under cultivation conditions commonly met in the Netherlands, according to spinach breeders. These cultivars were likely to vary for NUE as well because of the strong interdependency between growth and NUE while in spinach breeding improvement of vigour is an important selection criterion. A pilot study resulted in a hydroponics screening protocol that would be suitable for testing varieties as well as various types of families, based on growth-dependent N application as suggested by Ingestad (1982).

The testing procedure was used in comparative studies as described in Chapters 2, 3 and 4. Young seedlings of each entry were prepared from seeds and subsequently transplanted on a hydroponics system. Two N application regimes were applied; one aiming at a relative growth rate of 0.14 $g.g^{-1}$ and the other one at 0.18 $g.g^{-1}$ shoot dry matter (low and high N, respectively) per day. The experiments comprised plant monitoring for the following NUE-related traits: fresh and dry weight, leaf area, specific leaf area, dry weight ratio between root and shoot, and chlorophyll content. In the first study the cultivars were tested at low and high plant density (Chapter 2). This study showed the presence of heritable variation among cultivars for all NUE-related traits. Biomass production was considerably lower at low than at high N. It was decided to do all further testing at a high density since this experimental design showed a better discrimination of genotypic differences for the observed traits than the low

density planting design and is closer to the sowing density under field conditions. The NUE of the genotypes from the experiment described in Chapter 2 was determined using weight and N content of the shoot sample, and statistical analysis showed the presence of heritable variation between cultivars for this trait under low and high N availability. Path analysis revealed that under both conditions leaf area (LA) had the highest direct effect on NUE, while at the low N regime the direct effect of specific leaf area (SLA) also was important. Chapter 2 also shows that slow and fast growing genotypes differed in their strategy of utilizing N. The fast growers in general had higher NUE at both conditions, but lacked the capacity to increase NUE under N limitation. The ability to increase NUE under low N that was detected mainly among slow growers and may be an interesting trait for improving spinach varieties for growth under low N conditions.

In Chapter 3 the steady-state testing procedure based on the model of Ingestad was compared with a single bulk N application strategy, consisting of a single N dose given at the start of the experiment that resembled the cultivation conditions of spinach usually meets in the field. Both strategies were tested on the hydroponics system using two contrasting N levels and seven commercial hybrids, thus allowing us to gain insight in the genotype-specific effects of N application strategies on crop growth. The results of Chapter 3 showed that the application methods affected the genotypic performance for shoot growth differentially, which was likely associated with the timing and duration of the physiological stress. The Ingestad model further was shown to provide stable and reproducible conditions that allowed determination of genetic differences in NUE under low N conditions for a short-cycle leafy vegetable crop such as spinach.

On the basis of the results described in Chapter 2, two F1 hybrid cultivars contrasting in NUE (cvs Ranchero and Marabu) were selected as parents for a cross to produce a mapping population (Chapter 4). Several F_1 plants from this cross were self-pollinated. Their offspring was evaluated on hydroponics for segregation of NUE, and the most promising offspring of a single F1 plant was selected to establish a dedicated mapping population (F2) to enable genetic analyses of variation for traits affecting growth under conditions differing in N availability. The resulting population (335 individuals) was subsequently used to construct a SNP genetic linkage map (Chapter 4). The SNP markers (283) used for mapping represented polymorphisms in expressed genes discovered by sequencing mRNA from the two parent cultivars of the population. The final genetic map comprised six linkage groups (P01-P06), ranging in size from 46 to 116 cM.

The F2 plants from the mapping population were self-pollinated to generate so-called F2:3 families, of which 94 were subsequently evaluated on hydroponics (Chapter 4). The performance of the families was studied at high and low N conditions using the steady-state N application protocol based on the Ingestad model as developed in Chapter 2. The variation in family performance for each of the NUE related traits determined under both screening conditions was genetically dissected using the F2 genetic map and the marker data of the parental F2 genotypes of the tested families. Interval mapping analysis resulted in 39 trait-specific QTLs, with several QTLs accumulating on P01 and P02 of the linkage map (Chapter 4). The QTLs, in particular those in the P01 and P02 regions provide potential targets for the improvement of NUE in spinach.

In Chapter 5, the impact of Genotype by Environment interaction (GEI) was studied in a multi-environment study using the same set of 24 spinach cultivars that were evaluated under controlled conditions (steady-state nitrogen availability in a hydroponics system) in Chapter 2. The spinach cultivars were evaluated in 18 environments in the Netherlands, i.e. 6 different replicated trials with three nitrogen (N) fertilization levels (high, medium, low). The trials were conducted under both organic and conventional cultivation practices over a period of two years with testing in spring as well as in autumn. The progression of plant shoot dry weight and soil coverage was monitored periodically from sowing to the final harvest. To allow better comparison of cultivars under different environmental conditions shoot dry weight data and soil coverage were first plotted per environment against a temperature-normalized growth time with a threshold set at 4 ^{0}C. The resulting data were used to fit cultivar-specific smooth growth curves and various curve characteristics were calculated that formed the basis of Genotype by Environment Interaction analyses described in Chapter 5.

Genotype by N-level analyses for all shoot dry weight growth curve characteristics demonstrated significant differences among N-levels and substantial differences among cultivars. The low N fertilization treatment showed a considerable overall reduction of shoot growth compared to the medium and high N treatments. The analyses of variance however did not show significant cultivar by N-level interactions for the growth curve characteristics. The GEI study, on the other hand, showed significant cultivar by environment interactions which were substantiated by two cultivar-specific parameters from a Finlay-Wilkinson analysis and by various cultivar-specific parameters, showing their sensitivity to a set of environmental factors. Comparison of the NUE determined on hydroponics using a low steady-state application of N as described in Chapter 2 with the growth and Finlay-Wilkinson

parameters showed a positive (partial correlation) relationship with three environmental factors (mean temperature, light summation index and duration of the trial) and a negative relation with the stability estimate of the corresponding Finlay-Wilkinson analysis (Chapter 5). Our results indicated that several environmental factors were affecting shoot growth in spinach, thus complicating the interpretation of the N level effect on growth. It also emphasizes the importance of the fact that selection for improved NUE needs to be done under stable environmental conditions that are as much as possible non-limiting for other abiotic stresses than N availability.

6.2. Selection environment for NUE

The chosen screening environment under controlled conditions with the roots submerged in growing medium enabled a high level of control of both the above-ground and below-ground environment. In addition, two N application strategies were used: The steady-state N application used provides a steady state stress, but does affect the genotype-specific response of the spinach cultivars. The justification of using a system that enables control of environmental conditions, and soil nutrient conditions in particular is at least partly given in Chapter 5 of this thesis. The field conditions were very diverse and the evaluations had different N levels, seasons, soils and also management (conventional and organic), which gives an informative overview of the cultivar growth under various conditions relevant for spinach cultivation, but complicates genetic analysis for the NUE component. This section further compares screening conditions based on the comparison between: a) N level, b) application strategy, c) hydroponics vs field, d) organic vs conventional and e) seasons.

Hydroponics Low N and High N

An important question in selection for NUE and growth under N limitation, but also in selection for tolerance to other abiotic stresses, is whether selection should be done under stress or optimal conditions. This question also relates to the environment in which the selected genotypes will be cultivated (for instance: Is cultivation in practice done under conditions with continuous N-limitation or only incidentally?). It is obvious that for the first environment the genotypic performance under low N conditions is essential and a lot more

relevant than for the second environment. Several reports indicate that genotypes that do well under high N conditions maybe among the best performers under low N conditions, and vice versa. However the genetic basis of genotypic differences in response to low and high N may differ considerably (Moll et al.1982, Bertin and Gallais 2000; Gallais and Coque 2005).

In Chapter 2 a set of spinach cultivars was extensively tested under hydroponics conditions using a steady state N application strategy at both high and low N. We concluded that spinach cultivars differ in their strategies of coping with N limiting conditions: (a) some cultivars maximize biomass accumulation (LA and/or SDW) with a decrease in NUE and (b) other cultivars combine slow growth, i.e. a relatively low investment in biomass with an increased NUE. This finding is in line with the observations of Lambers (1987) who stated that fast growers in general have relatively high NUE under low high N, which limits the ability to cope with prolonged N limitation. Slow growers on the other hand invest relatively little in growth, which gives these the ability to adapt better to prolonged N limitation during later stages of the growing period. Through this adaptation mechanism the slow-growing type of genotypes might show a relatively high NUE at the end of the growing period under conditions with low N availability, and may have even realized a higher yield than fast growers. The genetic factors underlying this mechanism may be interesting for improving the crop yield of spinach cultivated under low N conditions.

The relative performance of the cultivars under high and low N conditions in hydroponics was compared using the cultivar rankings for yield. Figure 6.1 clearly shows the differential response in Shoot Dry Weight (SDW) of the cultivars observed in the hydroponics experiments discussed in Chapter 2. Our set of 24 cultivars included cultivars that were highly responsive to N, but relatively low yielding at low N conditions (cvs Beaver and NUN0905SP), cultivars that showed the opposite (cvs NUN0915SP, Eagle, Crocodile and Corvette), cultivars with low N response and low yields (cvs Rhino and Celesta), and cultivars that were among the best performers at both N levels (cvs Andromeda, Thunderbolt and Ranchero). A NUE breeding program in spinach may aim for a variety that is able to produce sufficient biomass under low N conditions but can fully exploit high N conditions for yield. Based on our hydroponics results, cvs Andromeda, Thunderbolt and Ranchero are the most interesting to be used under conditions varying in N availability. Although cv. Andromeda would be a good choice for cultivation under low N conditions based on its performance under high N, good performers under low N like NUN0915SP would be missed when selecting under high N only. Therefore, our results also indicate that to be able to fully

exploit the wide genetic variation of spinach cultivars for NUE, selection under both high and low conditions is necessary.

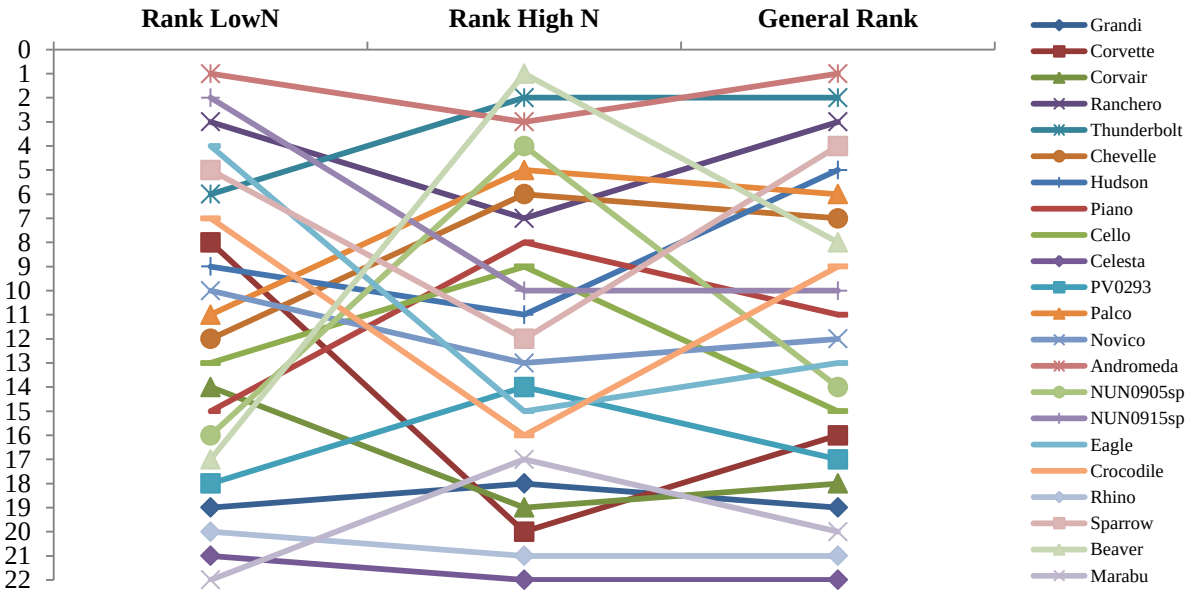


Figure 6.1. Ranking of the cultivars based on shoot dry weight (SDW) in hydroponics experiments (description see Chapter 2). The general rank is the average rank under both high and low N conditions.

Hydroponics versus Field

The results and conclusions discussed in the previous section are based on evaluations under controlled conditions. Controlled systems, like the hydroponics system we used, enable selection for genetic traits contributing to NUE without environmental variation masking the genetic variation, but may not always be able to predict which genotypes or cultivars will perform best under field conditions. There is an on-going debate on the relevance of testing plants under "artificial conditions" as the final aim is to improve the adaptation of the crops to field conditions. A number of reports indicate that these artificial systems are highly useful tools to gain insight in the traits contributing to NUE and other tolerance to abiotic stresses, and to find the genetic factors associated with those traits (Tuberosa et al. 2002, Long et al. 2013, Li et al. 2015). In fact, selection under hydroponics conditions are an important component in the success of two loci that have shown to be very useful in improving salt tolerance in rice cultivars (the Saltol locus (Bonilla et al. 2002) and the NAX1 locus (Munns et al. 2012). A QTL for NUE identified by evaluating barley on hydroponics corresponded to a QTL detected in field experiments (Hoffman et al. 2012), and the authors suggested that

screening of plants in early developmental stages grown in a hydroponics system could be a fast and cost effective method for early QTL detection.

In order to explore the applicability of hydroponics selection for genotypes and traits in spinach, we address the question: how comparable are the field and hydroponic conditions for spinach?

The hydroponic system has the advantage of allowing much tighter control of plant nutrition and the growing environment, and enables more extensive phenotyping. In the field, the environment may be responsible for much of the phenotypic variation, and N availability is partly dependent on the environmental conditions, which is exemplified by the GEI study in Chapter 5. Furthermore, phenotyping of in particular the root system in the field is difficult and laborious. In particular the root environment is quite different from the field situation, which may have consequences for root growth and architecture. We indeed observed that the spinach roots in hydroponics develop quite different from those in the field. In liquid media plants produce more lateral roots; root hairs are hardly formed, and there is, in contrast with field grown spinach, not an evident pivotal root. Heins and Schenk (1987) found that the root hair surface of spinach plants grown in the soil was ten times greater in comparison with spinach plants grown in a nutrient solution.

We used the shoot dry weight (SDW) data of the hydroponics experiments (Chapter 2) and the field data input of the Finlay-Wilkinson regression (Chapter 5) for our comparison. The correlation table in Figure 6.2 (also depicted in the supplementary data of Chapter 5) shows that the parameters for field conditions had low correlations to the different traits in hydroponics conditions: R^2 = 0.12 and 0.23 for SDW under low and high N respectively. The highest correlation observed (R^2=0.40) was between SDW HN and AUC *Gi* and this was a derived trait from SDW. Root Dry Weight (RDW) in culture on hydroponics is not significantly correlated with the field yield parameters, indicating that investment in roots as shown under hydroponics may not contribute to better growth under field conditions. It is therefore likely that the root variation and response in the field will be different from the hydroponics. With this in mind, the strong negative correlation between the R:S in hydroponics and the yield parameters, ranging for low N conditions from -0.29 to -0.70 and for high N from -0.36 to -0.71 is remarkable. This finding is in contrast to research in maize that determined that a higher R:S would increase the NUE (Yu et al. 2015). In maize the definition of NUE is based on the amount of grain produced by the amount of N provided,

while in spinach NUE is based on the vegetative biomass production by the amount of N provided. The different processes involved in producing seeds (for which remobilization of N from the shoot is an important factor) compared to leaves may be underlying this difference, and also exemplifies that for leafy vegetables, traits that contribute to high yield under N limiting conditions may be quite different from other seed producing crops like cereals. In addition, the difference in the relation between R:S and NUE between spinach and maize may be associated with the fact that spinach takes up NO_3^- from the soil efficiently but is known to be relatively inefficient in nitrate reduction (Stagnari et al. 2007, Koh et al. 2012).

The correlations between field parameters and hydroponics R:S were stronger at high N in hydroponics than at low N, except for the stability measurement for Mean and SDW_t90%, which were stronger for low N (-0.70 and -0.50 respectively). This may be an indication that under N limitation the plasticity of R:S biomass allocation and partitioning of nutrients and carbon is essential to be able to adapt to different environments (Gedroc et al. 1996, Tran et al. 2016).

A well-known phenomenon under decreasing nitrogen availability is an increase in R:S; in spinach grown in hydroponic conditions, Bottrill et al. (1970) found a 2.6 fold increase in R:S in plants grown under nitrogen deficiency, compared to control plants. However, the genetic differences for R:S has not been explored in depth until this research.

Of the tested cultivars, cvs Rhino and Marabu have higher R:S (0.33 and 0.32) under high N conditions, and hardly adapted R:S to low N conditions. These are among the lowest biomass producers in the cultures on hydroponics and cv. Rhino was also the most stable, but worst performer in the field trials. Cvs Ranchero, Andromeda and Palco were cultivars that increased R:S under low N compared to high N hydroponics conditions, and these were three of the four cultivars with high and stable SDW_t90% in the field experiments (the fourth cultivar was Charger, which was not included in the hydroponics trials due to problems with germination). This again points to a possibly important role for root plasticity for optimal production under varying N availability and environments. In addition, our results indicate that selection under hydroponics conditions for R:S and root plasticity may be relevant for breeding programs aimed resilient cultivars.

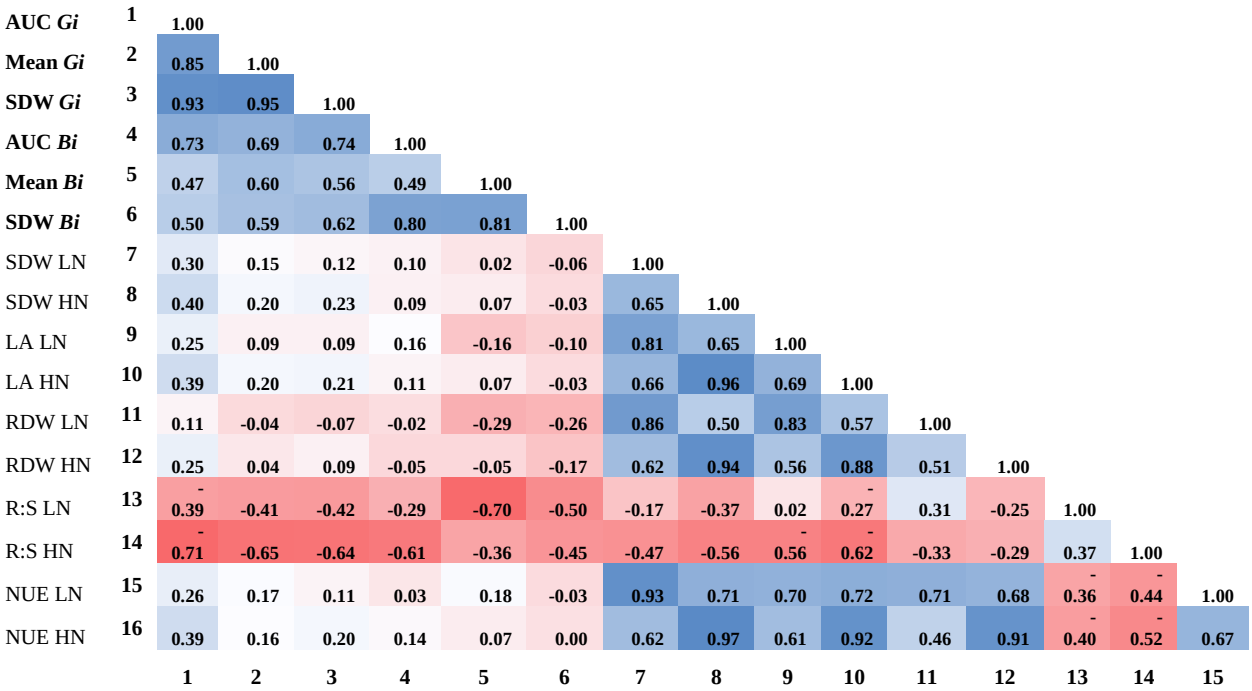


Figure 6.2. Coefficients of correlation between field trial parameters from Finlay-Wilkinson stability analyses (*Gi* as mean performance and *Bi* as stability) of the mean (average progression rate over all time points), AUC (area under de curve) and SDW_t90% (estimate the SDW at 90% of the total growing period) (in bold) and traits scored in tests under hydroponics conditions P((|r| > 0.42) = <0.05; df=20) (shoot dry weight (SDW), leaf area (LA), root dry weight (RDW), root to shoot ratio (R:S) and nitrogen use efficiency (NUE)) under low and high N conditions (LN and HN respectively). Negative correlations are highlighted from white to red being dark red the highest negative correlations. Positive correlations are highlighted from white to blue being the dark blue the highest positive correlations.

Ingestad versus single application

In the comparison between field and hydroponics conditions, it should be emphasized that we used steady state N conditions according to the Ingestad model for our evaluations described in Chapters 2 and 4. In field conditions, N availability is hardly ever stable. For a short cycle crop, a single application of N before sowing is normal practice, and may include the use of a nitrification inhibitor (ENTEC) as was applied in the conventional field trials discussed in Chapter 5. N will therefore be abundantly available at the start of the growth cycle, but as plants develop, their requirement for N will be higher, while N at the same time is used by the plants and therefore decreasing the concentration in the soil, despite the use of a nitrification inhibitor. When using organic fertilizer, N is released at a slower rate, which may match the plant's requirements better, but the mineralization process is strongly affected by weather conditions, and therefore difficult to control. Consequently, N availability for a field-grown

crop is often erratic and at best sub-optimal. In a study that evaluated processing spinach in winter conditions it was determined that a split application was more beneficial for yield and to match the N crop demand better than a single application using a nitrification inhibitor (as ENTEC) (Canali et al. 2011 and Canali et al. 2014).

With the study described in Chapter 3 we aimed to get further insight in the influence of N availability on the genetic variation for NUE in spinach by growing a set of seven hybrid cultivars under two different nitrogen application strategies: a single bulk application and a steady-state application, both at low and high nitrogen availability. Results showed that although yields were not higher, spinach plants were able to adapt their growth better to a low N steady-state application than to a single application, with less damage and leaf senescence. For a crop like spinach, leaf greenness is an important quality trait. Dark-green leaves are preferable, and (stress-induced) leaf senescence can already decrease the value of the crop harvest considerably. We have shown in Chapter 3 that plants grown under depletion conditions (that resemble the field conditions under current N management practices) start decreasing their internal NO_3^- stores at high N conditions relative to the steady state grown plants (Figure 6.3), along with slight reductions of chlorophyll content in leaves as an indication of initial stress (not shown). This might indicate that field grown spinach may benefit from a split N application strategy, or even from application of nutrients through fertigation, which may widen the harvest window and decrease the risk of yellowing of the leaves at the planned harvest especially under sub-optimal N conditions. It is important to realize that cultivars responded differently to the different application strategies (See Chapter 3, Table 2). Changes in N application management in cultivation may therefore influence the cultivar choice as well as the selection strategy when breeding for improved NUE, in particular choices on screening conditions.

Organic versus conventional field conditions

We calculated the correlations between conventional and organic trials described in Chapter 5, but the correlations were very low ($R^2 < 0.10$, data not shown)). There could be many reasons for this, including the high variation between any of the environments, even between conventional trials. However, there are some specific conditions for organic trials that may have added to the environmental variation. For instance, organic trials in general suffer more

from random environmental variation than conventional trials, which would complicate the detection of heritable variation.

With respect to nitrogen dynamics, it is known that the control of nitrogen availability in organic farming systems is not easy (Koopmans and Bokhorst 2002). Organic fertilisers are mostly characterised by slow release of nutrients. Nutrients are only available for the plants after mineralisation and the nitrogen is not immediately available for the plant compared to the water soluble nutrients in mineral fertilisers as applied in conventional management. The mineralisation process of organic fertilisers such as compost or animal manure is dependent on biological soil activity influenced by soil temperature and soil moisture. Under cold, early spring conditions mineralisation can be (too) low and delayed whereas later in the season with warmer soil conditions the mineralisation can go on too long causing sometimes too high levels of N availability, depending also on the additional release of nitrogen from soil organic matter and precrop residues (Koopmans and Bokhorst 2002).

6.3. Nitrate

Most plant species are able to take up both nitrate and ammonium but have a preference for one or the other. Spinach has a preference for nitrate above ammonium (Goh and Vityakon 1986, Elia et al. 1998, Lasa et al. 2001). A higher proportion of nitrate in comparison to ammonium results therefore in a higher yield, but also in an increase in nitrate content of the plants (Stagnari et al. 2007). Nitrate serves as the primary signal in regulating the nitrate reductase activity (Crawford 1995). The induction of the nitrate reductase after exposure to nitrate occurs within minutes and needs only very low concentrations (down to 10 µM) of nitrate (Crawford 1995).

We have shown in Chapter 3 that free nitrate concentrations vary between cultivars, and also between N application strategies in particular at the end of the growth cycle. To explore this in more detail, we have grown eight cultivars (Ranchero, Chevelle, Cello, Novico, Andromeda, Crocodile, Sparrow and Marabu) on a hydroponics system under single N application conditions (high N and low N), similar to what was described in Chapter 3. The plants were harvested 11, 21 and 28 days after transplanting to the system, and measurements included free nitrate concentrations ($[NO_3^-]$) in the leaves along with fresh and dry biomass and leaf area. Figure 6.3 shows that the free nitrate concentrations were high and stable over

time at high N conditions, while at low N, the already lowered nitrate concentration was further decreased at the second and third harvest to levels similar to the levels measured in Chapter 3 (Figure 3.2) both under steady state and single N application strategies. These levels are close to the lower limit for [NO_3^-] at low N of around 1200 ppm measured in the cultivars, which would be necessary to maintain the osmotic contribution of nitrate to maintain turgor (Cardenas-Navarro 1999). Interestingly, none of the growth-related traits (Shoot Dry Weight (SDW), Shoot Fresh Weight (SFW), Leaf Area (LA) and derived traits Dry Matter percentage (DM%) and Specific Leaf Area (SLA) were significantly decreased at Harvest 2. This indicates that the spinach cultivars depleted their internal nitrate reserve when N availability became limiting, thus maintaining growth. Depletion of the nitrate reserve already at Harvest 2 did have consequences for growth at Harvest 3, with a strong reduction in SDW. This observation may open up the possibility of predicting the optimal time of harvesting spinach in the field. Nitrate measurements are done as part of spinach cultivation, but mostly to assure that the harvested spinach does not contain nitrate levels that exceed the maximum allowed concentrations (Santamaria 2006). We propose that nitrate concentrations should be monitored at the end of the crop cycle, and when the nitrate concentration falls below a certain threshold the crop should be harvested within a few days as it is likely to experience N stress and to deteriorate shortly after the leaf free nitrate levels reach the minimum of approx. 1500 ppm.

Cardenas-Navarro et al. (1999) showed that in spinach, there appears to be a positive correlation between shoot water content and nitrate content in the shoot, which is likely related to the function of free nitrate as an osmolyte. Indeed we found a negative correlation between DM% (inversely related to water content) and ([NO_3^-] at Harvests 1 and 3, under low N, and at Harvest 3 under high N conditions.

Remarkably, this correlation was reversed at Harvest 2. De Pinheiro Henriques and Marcelis (2000), showed that the decrease in SLA under low but steady state N supply in another leafy vegetable, lettuce, could be largely attributed to a decrease in DM%, which was confirmed by our study described in Chapter 3. A similar strong (negative) correlation between DM% and SLA was found in the pilot study at Harvest 3 (-0.6), but this correlation was absent at Harvest 2. Apparently, other factors play a role during the dynamic process of remobilising free nitrate from the vacuolar store when N becomes limiting for growth. In addition to N availability in the root environment, light conditions appear to influence free nitrate accumulation as well. Blom-Zandstra et al. (1985) reported on the influence of light on the

accumulation of free nitrate, and proposed that under low light conditions, nitrate may serve as an osmoticum to compensate for the organic osmolytes. Light conditions also interact with nitrate assimilation, with a reduction of nitrate reductase activity with reduced light conditions (Riens and Heldt 1992). Kaiser et al. (2004) suggested that this reduction is regulated by photosynthesis. Although we cannot rule out that changes in light conditions may have affected free nitrate concentrations, the light conditions were controlled in this trial, and an important role for changing light conditions in on the nitrate measurements seems unlikely.

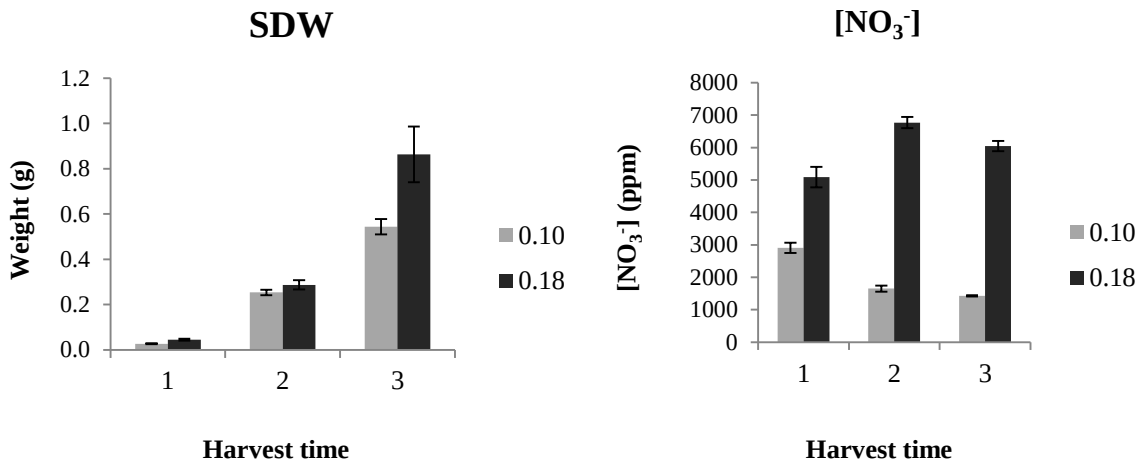


Figure 6.3. Over all means over the performance of eight genotypes at three harvest time points for Shoot Dry Weight (SDW), Shoot Nitrate Concentration ([NO3] determined at two different N levels: 0.10 (Low) and 0.18 (High). Error bars represent the standard error of the mean.

6.4. Tools for spinach breeding

An important aim of this thesis was to develop tools that would allow genetic analysis of NUE in spinach. For successful breeding, sources for genetic variation are essential. The evaluations for NUE as described in this thesis demonstrate that there is sufficient variation for NUE in cultivated spinach to make progress, but larger improvements may be possible if spinach wild relatives are included that are adapted to harsh, nutrient-poor environments. These genotypes may be less responsive to N, but may harbour traits that can contribute to improve NUE of cultivated spinach. The use of wild accessions and wild relatives of spinach in further breeding programs may lead to successful incorporation of favourable alleles associated with N use efficiency, but is also a major breeding strategy for disease resistance, for instance for the devastating spinach disease downy mildew (*Peronospora farinosa*).

Identification of novel sources of resistance to downy mildew should be complemented with studies of the genetics of resistance. For this, a high-density genetic map and mapping populations segregating for disease resistance are important (Correll et al. 2011).

For efficient introgression breeding of NUE-improving traits, molecular markers are an essential tool, for instance by helping to avoid effects due to linkage drag associated with the introgression from wild relatives. We developed molecular markers for breeding and showed their usefulness in a QTL analysis of NUE (Chapter 4). The molecular markers that were developed for the genetic analysis were discovered through RNA sequencing of the parents from the mapping population (Ranchero and Marabu). For our mapping analysis, we focused on a set of 419 markers that were homozygous in the parents, but polymorphic between the parents, of which 283 were used for the construction of the genetic map, as these were the markers that would be most informative in our specific mapping population. The SNP discovery approach however resulted in many more reliable gene-based SNPs (more than 27,000). This SNP dataset is therefore an important enabling tool for developing novel dedicated molecular breeding methods for spinach, not only for NUE but also for other complex traits and agronomically important traits.

The fact that for spinach the genetic map published by Chan-Navarrete et al. 2015 (Chapter 4) is only the second map publicly available illustrates how little molecular genetic information is available for spinach, and is at least partly due to the challenges of genetic analysis of complex traits of agronomical importance in spinach in particular. This thesis faces this challenge by providing the basic elements for molecular breeding in spinach. Another important development is the sequencing and assembly of the spinach genome, which may be available shortly (UC Davis 2016). The availability of an assembled spinach genome sequence will further facilitate marker development, QTL analysis and candidate gene discovery, as well as discovery of favourable alleles, and will together with the tools presented in this thesis pave the way for marker assisted selection (MAS) for traits like NUE to become an integrated part of spinach breeding programs (Correll et al. 2011).

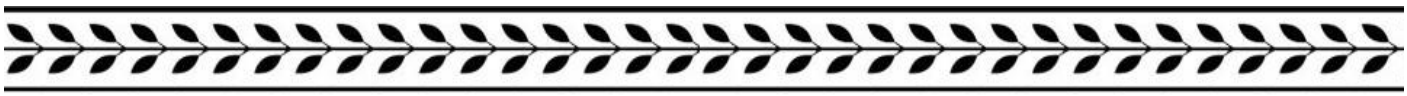
Essential prerequisites for MAS are genetic factors for selection, i.e. genes/QTLs with a clear beneficial effect of the traits under selection. The study described in Chapter 4 based on a dedicated F2:3 population identified several important and significant QTLs for NUE and traits contributing to NUE. The advantage of using such a population is that it enables progeny testing which is necessary for genetic studies on quantitative traits like NUE. However, the population is not ‘immortal’ like for instance populations of recombinant inbred

lines (RIL). A RIL population is difficult to realize for spinach because being cross-pollinating and dioecious, spinach may be prone to inbreeding depression. Indeed, continuously selfed spinach plants were shown to produce significantly less fruits than plants that were cross-pollinated (Miglia and Freeman 1996). Alternatively, the genetic constitution of the F2 plants may be conserved through intercrossing plants within F2:3 lines.

Further investments in vegetative propagation of spinach (like for instance *in vitro* culture) may be an important tool for crop improvement programs. Successful regeneration of spinach in *in vitro* culture however is dependent on genotypes and types of explants (Knoll et al. 1997, Zhang and Zeevaart 1999, Leguillon et al. 2003, Nguyen et al. 2013). An additional problem is that spinach plants regenerated *in vitro* are difficult to transplant to soil because of early bolting (Ishizaki et al. 2002). An *in vitro* culture method that is less genotype-dependent would help to maintain RIL populations for long term studies.

Molecular tools can only be fully exploited when they are combined with tools for phenotypic selection. The challenges that need to be met when selecting for NUE and growth under N-limiting conditions have been extensively discussed in this thesis (Chapter 5 and General Discussion), and it was demonstrated that controlled systems like the hydroponics system described and used in Chapters 2-4 can be an important tool for genetic dissection of the complex trait NUE in spinach. The hydroponics system was shown to be suitable for large-scale progeny testing for growth-related traits in spinach, such as shoot and root dry matter yield and leaf area. Spinach, in particular fits very well on the system since it is a vegetative crop with small plants and a short growing period. The system presents a uniform platform that can simulate outdoor growing conditions quite well while it enables a proper control of plant nutrition and other growing conditions. Other positive system features especially for breeding purposes are the possibility to design proper randomized tests based on single plants and the low seed requirement for progeny testing. As such, the hydroponics system is a promising (prescreening) tool to develop novel F1 hybrid cultivars for spinach. The system would be highly useful prior to any field evaluation for large-scale evaluation of inbred lines and test crosses with a few testers to get insight in the *per se* performance of the lines and their general as well as specific combining ability for traits relevant for breeding of spinach including low N-input conditions. The information collected in this way can be used for (1) selection of the most promising combinations of inbred lines for making experimental hybrids to be tested in the field and for (2) the start of a new breeding cycle.

References



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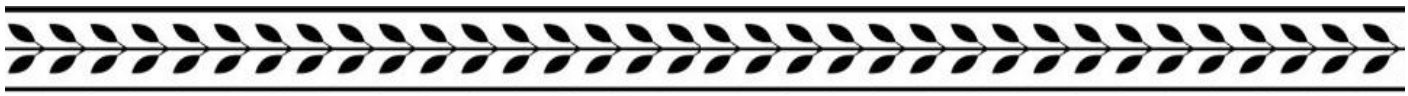
Summary



Summary

Spinach (*Spinacia oleracea* L.) is one of the most consumed leafy vegetables worldwide as it is considered to be highly nutritious. The high plant density required for the production of babyleaf spinach for fresh consumption (bagged), in particular together with the large and still increasing production area of spinach makes it an important crop for seed companies. Dutch breeding companies are market leaders in this crop. Spinach has a high demand for nitrogen in order to rapidly come to a harvestable product with a dark green colour as required by the market. In commercial production of spinach the recovery of N is poor, which may result in elevated soil N concentrations and N leaching to the groundwater causing environmental problems. To increase sustainability in both organic and conventional farming systems there is a need to reduce the amount of nitrogen fertilizer needed for commercial spinach cultivation. Therefore, genetic improvement of nitrogen use efficiency (NUE) of spinach crops is a breeding challenge of the utmost importance.

NUE is defined in this thesis as the amount of biomass produced by the crops per amount of nitrogen applied to the crop. It is a trait based on complex underlying mechanisms and different morphological and physiological plant characteristics all contributing to the efficient uptake and use of nitrogen. NUE is more and more addressed in research, but most studies on improving NUE of crops focused on arable grain crops such as oilseed rape, maize and wheat, for which nitrogen use related to yield is physiologically quite different from that in short-cycle leafy vegetables such as spinach. In contrast to the grain crops, information on NUE in spinach is still rather limited and tools for genetic studies are few.

This thesis therefore aimed at developing knowledge and tools for identifying spinach genotypes with selectable traits that improve yield, quality and stability under low input of nitrogen and to facilitate the development of cultivars for low N input conditions. The research was divided in four parts, presented in Chapters 2-5. First, the genetic diversity for NUE-related traits was studied using a diverse set of 24 commercial cultivars (Chapter 2). This set was evaluated under controlled conditions on a hydroponics system using two contrasting levels of N supply based on the Ingestad model with steady-state N application, thus minimizing the environmental variation. Plants were assessed individually for fresh and dry shoot weight, leaf area, specific leaf area, dry weight ratio between root and shoot, and chlorophyll content. This study demonstrated that the reduced shoot biomass production under low N versus high N conditions was most strongly affected by leaf area, while at low N

specific leaf area was an important determinant of variation in NUE as well. The hydroponics screening strategy as presented in this study enabled reliable detection of heritable variation among cultivars for NUE-related traits and NUE under optimal as well as suboptimal N input, with shoot dry weight and leaf area as preferred selectable traits for the detection of heritable differences in NUE in spinach.

The second study described in Chapter 3 was designed to get insight in the possible differential genotypic differences in response to N application strategy. Seven cultivars were grown under hydroponics conditions and N was supplied either as a single bulk N application resembling N fertilization in field cultivation, or a steady-state N application similar to the first study of this thesis, both at two contrasting N-levels (low and high). The application methods affected the genotypic performance for shoot growth differentially, which was likely associated with a difference in timing and severity of the stress perceived by the plants. Although shoot biomass production was not higher, spinach plants were able to adapt better under low N steady-state application than under single application, with less damage and leaf senescence. The Ingestad model thus provides stable and reproducible conditions that allow determination of genetic differences in NUE under low N conditions for a short-cycle leafy vegetable crop.

The third study presents tools for molecular breeding and their application in elucidating genetic variation of factors contributing to NUE in spinach (Chapter 4). To perform this genetic study an F2 mapping population was made from an F1 plant derived from a cross between two F1 hybrid cultivars contrasting in NUE. Single nucleotide polymorphisms (SNPs) in expressed genes were identified and subsequently used to produce a genetic map comprising six linkage groups (P01-P06), matching the haploid chromosome number of spinach. Ninety-four F2:3 families derived from the F2 mapping population were evaluated on hydroponics under steady-state N application at low and high N conditions. A QTL analysis resulted in 39 trait-specific QTLs, with two regions (linkage groups P01 and P02) accumulating a number of QTLs. QTLs for shoot biomass, chlorophyll content, leaf area at both high and low N, and for NUE at low N co-localized on P01. These QTLs are potential targets for the improvement of NUE in spinach.

The first three studies clearly show the applicability of a system that allows control of the environmental conditions, and the root environment in particular. The fourth study complements those studies with an evaluation of growth under varying levels of N availability in the field (Chapter 5). A set of 24 spinach cultivars was evaluated at six different locations

in the Netherlands in replicated field trials, each with three fertilization levels (low, medium, high). The set included 22 cultivars that were also evaluated in the hydroponics study of Chapter 2. The study comprised periodic assessment of two relevant traits, i.e. soil coverage and shoot dry weight, during the whole growing period to generate data for analyses of cultivar by environment interaction (GEI). The data for both traits were plotted against temperature-normalized growth time (with a base temperature of 4°C) and used to fit smooth growth curves for calculating cultivar-specific parameters that describe the development of soil coverage and shoot growth during the full growth cycle of the crop. The measured traits and derived parameters differed strongly from environment to environment. The differences due to N fertilization levels within each location were relatively small but increased over time, with reduced shoot growth at low N fertilization, indicating that low N fertilization had an adverse effect on mainly the later stages of spinach crop growth, i.e. after canopy closure in this densely grown crop. The variation for shoot dry weight as well other shoot growth parameters was highly significantly influenced by N fertilization and Cultivar. An in depth genotype by environment interaction analysis showed that the lack of significant interactions between cultivar and N-treatment for both shoot biomass and soil coverage was likely linked to the strong influence of environmental factors like temperature, soil, and management on nitrogen availability in the soil in a short cycle crop like spinach. This particularly emphasizes the importance of performing trials under better controllable conditions for genetic dissection of NUE and discovery of breeding traits, but also underscores the importance of testing these findings in various field trials.

In conclusion, the studies presented in this thesis resulted in knowledge and tools that can be implemented in efficient breeding strategies for the complex trait nitrogen use efficiency. These include a hydroponics system for evaluation of NUE in spinach that can be included as a prescreening tool for NUE. The studies further produced tools for molecular genetic evaluation of NUE, including a SNP marker set for marker-assisted breeding, a genetic mapping population with the corresponding genetic map, and the identification of two major QTL regions contributing to growth under low N conditions. With these tools, an efficient strategy for breeding for NUE efficiency in spinach would include screening under highly controlled conditions at high and low N using leaf area, biomass and root to shoot ratio as selectable traits, and QTL identification of genetic factors that can be targeted and combined using marker-assisted selection. The selections should then be validated in multi-environment field trials with different levels of N fertilization to be able to select cultivars that combine

stable performance under various low input growing conditions with high yields under more favorable conditions.

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Gracias Elena por motivarme a terminar la tesis. Ahora puedo prepararme para estrenar el título de papá. Cuando estés grande y leás ésto, espero que sepas que te esperaba con muchas ansias. Sin haberte alzado ya te amaba.

To the people reading which are struggling with work, thesis or anything else, just some piece of advice: happiness is a decision; then it is important to decide to be grateful. Therefore, no matter how busy or overwhelmed you are, keep in mind what Charles Chaplin said: "*We think too much and feel too little"*. Don't forget to feel, for example… today I feel simply joyful.

About the author

Jose Rafael Chan Navarrete was born on August 21st 1979 in San Jose, Costa Rica. He obtained a BSc in Biotechnology Engineering (Instituto Tecnologico de Costa Rica, 2002) and a first MSc. degree in Agronomical Science and Natural Resources with an emphasis in Biotechnology (Universidad de Costa Rica, 2007) in which the thesis was awarded with honors by a study of the "Genetic structure and mating type determination of *Pyricularia grisea* isolated from *Oryza glumaepatula* and *Oryza sativa* in the North Area of Costa Rica". He obtained a second MSc degree in Plant Sciences with an emphasis on Plant Breeding and Genetic Resources (Wageningen University, 2010) sponsored by a NUFFIC scholarship; his MSc thesis was titled "Phenotypic and genetic analysis of salinity tolerance in tomato introgression lines". The research presented in this PhD thesis is as a result of a joint project of Wageningen UR Plant Breeding with four breeding companies: Enza Zaden, Nunhems (Bayer CropScience), Pop Vriend Seeds and Rijk Zwaan under the Groene Veredeling program. He is currently working as a Biotech Breeder within the Leafy Vegetables department of Enza Zaden (Enkhuizen, NL) trying to make better leafy crops for the future generations.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- The development of a breeding strategy for nitrogen efficiency in spinach

Post-graduate courses (3.9 ECTS)

- Mixed model based on QTL mapping in GenStat; Biometris (2012)
- Root ecology (2012)
- BioNUT summer school; BioNUT (2013)

Laboratory training and working visits (1.2 ECTS)

- Visit to companies breeding for spinach; Enza Zaden/Vitalis, Nunhems, Pop Vriend and Rijk Zwaan (2010-2011)

Invited review of (unpublished) journal manuscript (1 ECTS)

- Journal of Food and Agricultural Sciences: a survey of nitrate concentrations in retail fresh leafy vegetables from daily markets of different locations (2011)

Competence strengthening / skills courses (2.1 ECTS)

- PhD Competence assessment; PE&RC (2011)
- Scientific writing; Wageningen in'to Languages (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC Day (2010)
- PE&RC Weekend (2010)
- PE&RC Day (2013)

Discussion groups / local seminars / other scientific meetings (5.6 ECTS)

- Varietal difference in potato root system and its implication to drought tolerance (2010)
- Projects meetings (2010-2014)
- Plant soil interactions (2011)
- Discussion meeting for root phenotyping with Copenhagen University department (2011)

- Literature discussion meetings (2011)
- Understanding and engineering salinity tolerance in crop plants; University of Amsterdam (2012)
- Global nutrient cycles and food security (2013)
- The efficiency of plant breeding (2013)
- Water saving in rice: combining genetic, physiological, agronomic and modelling approaches to combat drought stress in rice crops (2014)

International symposia, workshops and conferences (4.4 ECTS)

- EUCARPIA leafy vegetables (2011)
- EUCARPIA meeting on low-input and organic agriculture (2013)
- EUCARPIA leafy vegetables (2015)

Lecturing / supervision of practical's / tutorials (2.4 ECTS)

- Organic plant breeding (2011-2014)
- Design of plant breeding programs (2013)

Supervision of 8 MSc students (24 ECTS)

- Establishment of hydroponic conditions of spinach
- Hydroponics and gene expression of spinach
- Field trial evaluation and gene expression of spinach
- Field evaluation of NUE and comparison of N application methods
- Phenotypic evaluation of a mapping population+
- Nitrogen evaluation at organic and conventional field trials
- Genotype by environment interaction for Nitrogen Use Efficiency in Field -Grown Spinach (*Spinacia oleracea* L.)
- Physiology and genetic variation of nitrogen use efficiency in spinach (*Spinacia oleracea* L.)

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