# Genetics of Nitrate Accumulation in Lettuce

## Genetica van Nitraatophoping in Sla

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# Genetics of Nitrate Accumulation in Lettuce

## Proefschrift

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in de landbouw- en milieuwetenschappen
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## Abstract

Reinink, K., 1993. Genetics of nitrate accumulation in lettuce. Thesis, Agricultural University Wageningen.

This study evaluated the prospects of breeding for low nitrate content in lettuce (*Lactuca sativa* L.). A lettuce collection was screened and accessions with low nitrate content were identified. These were used to study the genetics of nitrate accumulation. Nitrate accumulation inherited quantitatively, in a mainly additive fashion with only minor effects of dominance. No important maternal effects were detected. Estimates of the additive genotypic variance and the environmental variance were used to evaluate the prospects of introgression of the low-nitrate trait in modern lettuce cultivars and of a further reduction of the nitrate level by combining genes from two low-nitrate accessions. Nitrate content in lettuce showed important genotype × environment interactions. Genotypes responded differentially to changing environmental conditions related to the daylength or light-intensity at harvest. In a detailed study of two low-nitrate accessions this interaction was shown to be related to differences in the rate change of dry matter content under changing daylengths.



CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Reinink, K.

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## Stellingen

1. Door veredeling kan het nitraatgehalte van sla aanzienlijk verlaagd worden. De genetische variatie is echter onvoldoende om bij het huidige teeltregime in de winterteelt te kunnen voldoen aan een nitraatnorm van 2500 ppm.
   Dit proefschrift

2. Het ontmoedigen van de consumptie van verse bladgroenten in de winter is niet in het belang van de consument.

3. Het gebruik van de term 'avirulentiegen' is misleidend en moet worden afgeraden.
   Shaner, G., E.L. Stromberg, G.H. Lacy, K.R. Barker & T.P. Pirone, 1992. Nomenclature and concepts of pathogenicity and virulence. Annu. Rev. Phytopathol. 30: 47-66.

4. Crute en Davis veronderstellen ten onrechte dat de waardplantspecificiteit van *formae speciales* van *Bremia lactucae* goed correleert met de taxonomische afstand tussen plantensoorten.
   Crute, I.R. & A.A. Davis, 1977. Specificity of *Bremia lactucae* from *Lactuca sativa*. Trans. Br. mycol. Soc. 69: 405-410.
   Lebeda, A. & I.W. Boukema, 1991. Further investigations of the specificity of interactions between wild *Lactuca* spp. and *Bremia lactucae* isolates from *Lactuca serriola*. J. Phytopathology 133: 57-64

5. Programma's voor de introgressie van resistentie tegen *Bremia lactucae* uit *Lactuca saligna* in cultuursla zijn mislukt doordat men gefixeerd was op dominante overgevoeligheidsresistentie.
   Netzer, D., G. Globerson & J. Sacks, 1976. *Lactuca saligna* L., a new source of resistance to downy mildew (*Bremia lactucae* Reg.). HortScience 11: 612-613.
   Crute, I.R. & J.M. Norwood, 1979. Downy mildew of lettuce; Resistance studies. Rep. natn. Res. Stn for 1978, Wellesbourne, Warwick, p. 77.
   Crute, I.R., P.L. Gordon & J.M. Norwood, 1984. Downy mildew of lettuce; Novel sources of seedling resistance. Rep. natn. Res. Stn for 1983, Wellesbourne, Warwick, pp. 77-78.

6. De indeling van waardplantresistentie tegen insekten in antixenosis (effecten op gedrag) en antibiosis (effecten op fysiologie) suggereert ten onrechte dat dit elkaar uitsluitende fenomenen zijn.

7. Zonder gebruik te maken van moleculaire merkers valt het aantal genen dat betrokken is bij een kwantitatief overervende eigenschap niet betrouwbaar te schatten.
   Mayo, O. & A.M. Hopkins, 1985. Problems in estimating the minimum number of genes contributing to quantitative variation. Biom. J. 27: 181-187
   Mulitze, D.K. & R.J. Baker, 1985. Evaluation of biometrical methods for estimating the number of genes, 1. Effect of sample size. Theor. Appl. Genet. 69: 553-558.
   Mulitze, D.K. & R.J. Baker, 1985. Evaluation of biometrical methods for estimating the number of genes, 2. Effect of type I and type II statistical errors Theor. Appl. Genet. 69: 559-566.

8. Gezond verstand vormt een intellectuele handicap van de eerste orde.
   Verhoeven, C., 1992. Alleen maar kijken. Essays over de mens als toeschouwer. Ambo, Baarn, pp. 22-37.

9. Wetenschap zegt noodzakelijkerwijs meer dan ze weet.

10. Als een olifant hoger kan springen dan een muis, dan mag van schaalvergroting van onderzoeksinstellingen een sterke efficiëntieverhoging verwacht worden.

## Woord van dank

Het onderzoek dat tot dit proefschrift heeft geleid vormde een onderdeel van het reguliere onderzoek van het voormalige Instituut voor de Veredeling van Tuinbouwgewassen (IVT) en van het latere DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO). Dit betekent dat behalve de promovendus een groot aantal collega's aan dit werk hebben bijgedragen. Een aantal van hen wil ik bij naam bedanken. Remmelt Groenwold heeft ongetwijfeld de grootste bijdrage in dit nitraat-onderzoek geleverd. Zijn zeer nauwgezette hulp bij de opzet, uitvoering en verslag-legging van de proeven heeft dit onderzoek tot een succes gemaakt. Dat in al de jaren dat het onderzoek heeft geduurd vrijwel geen enkele proef mislukt is, is in hoge mate aan Remmelt te danken. Ook Marcel van Nes heeft drie jaar als assistent bij dit onderzoek geholpen. Recht van de schoolbanken begon Marcel aan dit onderzoek. Als een volleerd assistent-onderzoeker, die zowel in de kas als in het lab zelfstandig kan werken, begon hij drie jaar later aan zijn volgende baan. Veel heb ik ook te danken aan Alex van Silfhout. Hij verzorgde de planten en was verantwoordelijk voor de zaadteelt en het dorsen van het slazaad. De tienduizenden nitraatbepalingen die voor dit onderzoek nodig waren, werden op een voortreffelijke manier door Mevr. Hannie Hogendijk uitgevoerd. De "nitraatploeg" was de groep van medewerkers die de sla hebben geoogst en vermalen. Dit was een nat karweitje dat meestal 's winters bij lage temperatuur moest worden uitgevoerd. Mijn dank hiervoor aan Sjef Brom, Ab Wessels, Peter Saat, Jaap van den Berg, Nettert van der Linde, Bart van Kesteren en Paulien van de Poel.

Na het doen van proeven komt onvermijdelijk het publiceren. Vaak is dit niet het meest eenvoudige deel van het onderzoek. Maar ook hierbij waren er volop mensen die een helpende hand hebben gestoken. Allereerst natuurlijk de promotor, Professor Parlevliet, en de copromotor, Piet Stam, die het schrijfwerk op afstand hebben begeleid. De statistici Hans Jansen en Fred van Eewijk hebben belangrijke bijdragen geleverd wanneer de weerbarstige werkelijkheid zich weer eens moeizaam in statistische modellen liet persen. Voor het becommentariëren van concept-artikelen wil ik Minne Nieuwhof en Pim Lindhout bedanken. Pim's afkeer van wijdlopige formuleringen heeft uiteindelijk de drukkosten van dit proefschrift aardig gedrukt. Ook Greet Blom-Zandstra, die als plantenfysioloog op het DLO-Centrum voor Agrobiologisch Onderzoek (CABO-DLO) aan nitraatophoping in sla werkte, wil ik bedanken voor de gezamenlijke discussies over het onderzoek en het becommentariëren van concept-artikelen.

De niet onaanzienlijke kosten van dit onderzoek werden voor het overgrote deel door het Ministerie van Landbouw, Natuurbeheer en Visserij betaald. De assistentie door

Marcel van Nes voor een periode van drie jaar werd betaald door het Produktschap voor Groenten en Fruit. Arnoud Bootsma, die als gewetensbezwaarde voor een periode van 18 maanden in dit onderzoek deelnam, werd betaald door de Nederlandse Vereniging voor het Tuinzaadbedrijfsleven. Verder wil ik de directeur van het vroeger IVT en het huidige CPRO-DLO, Nic Hogenboom, bedanken voor de mogelijkheden die geboden werden om dit stuk onderzoek te bewerken tot een proefschrift.

Als laatste wil ik mijn echtgenote, Ina van Trijp, noemen. Zonder haar was dit proefschrift wellicht veel eerder af geweest. Het zou dan echter ook een stuk minder leuk zijn geweest.

## Contents

# 1 General introduction

*For about 15 years there has been an increasing public concern about the high human nitrate intake caused by high nitrate levels in food (especially vegetables) and drinking water. In agricultural and biological research, this concern has stimulated the study of various aspects of nitrate accumulation in plants. This thesis is the result of breeding research into nitrate accumulation in lettuce.*

*In the first section of this introduction (1.1) general information is presented on health risks that are possibly associated with high nitrate consumption. Further, the main sources of human nitrate consumption are discussed and the importance of vegetables in this respect. The legal regulations are outlined that have been imposed to reduce the nitrate level in marketed vegetables. The next section (1.2) provides information on the production of leafy vegetables in the Netherlands and other EC countries, clarifies why lettuce was chosen for breeding research into nitrate accumulation and gives background information on lettuce breeding. The last section of this introduction (1.3) lists the research questions that were dealt with in this thesis and presents the general approach to these questions.*

## 1.1 Nitrate accumulation and human health

*Health effects*

High nitrate contents in food products are considered to be a potential risk to the health of the consumer. Nitrate is not toxic in quantities normally present in food and drinking water and is neither carcinogenic nor mutagenic (Speijers et al., 1989). The presumed harmful effects of high nitrate consumption are due to the effects of nitrate-derived nitrite and the products of reactions of nitrite with organic compounds. Nitrite can be formed from nitrate by bacterial reduction both before and after consumption. Formation of nitrite before consumption can be minimized by careful storing and preparation of food. Ingested nitrate is quickly absorbed in the upper part of the digestive tract, from where it is partly transported to the salivary gland and excreted again in the oral cavity. In the oral cavity bacterial reduction to nitrite occurs. The fraction of ingested nitrate which is endogenously reduced to nitrite depends on the amount of nitrate consumed. For healthy adults it is estimated to be maximally 5% at high nitrate consumption (Gezondheidsraad, 1990). No reduction of nitrate occurs in the stomach of healthy adults, because the low pH of the stomach prevents bacterial growth. At relatively high stomach pH, which is found in infants and patients suffering from stomach disorders, reduction of nitrate can also occur in the stomach and a larger proportion of the ingested nitrate can then be reduced to nitrite (Van Duijvenbooden and Matthijsen, 1989).

The health effects of nitrite are both

direct and indirect. Direct effects are due to the oxidation of hemoglobin (Hb) by nitrite to methemoglobin (metHb). Nitrite oxidizes the ferrous iron ($Fe^{2+}$) of the heme group to the ferric state ($Fe^{3+}$), which cannot bind oxygen (Fan et al., 1987). High levels of metHb result in reduced oxygen transport capacity of the blood, which can cause tissue hypoxia. Normally about 1% of adult Hb circulates as metHb. Levels of metHb above 10% lead to clinically detectable symptoms, called methemoglobinemia or cyanosis. MetHb levels above 60% can be lethal.

The relevance of the intake of nitrate via food and drinking water for the occurrence of methemoglobinemia is under discussion. High intake of nitrate via food and water is not a necessary condition for the development of methemoglobinemia, as substantial amounts of nitrite can also be formed endogenously following intestinal infections (Hegesh and Shiloah, 1982). The Dutch Health Council concluded that methemoglobinemia is a multifactorial disease, in which bacterial infections play an important role. Although the development of the disease is not dependent on exogenous nitrate, high uptake of nitrate via food and water could be an additional risk factor (Gezondheidsraad, 1990).

Indirect health effects of nitrite could be caused by the endogenous formation of N-nitroso compounds from nitrite and a large range of so-called nitrosatable organic compounds (Mirvish, 1975; 1983). N-nitroso compounds are characterized by the presence of a nitrosyl (N-NO) group. These compounds can be formed under acidic conditions in the stomach. Groenen et al. (1984) have shown that after consumption of fish in combination with nitrate-rich leafy vegetables, endogenous formation of volatile N-nitrosamines can occur. Many N-nitroso compounds have been found to be carcinogenic in animal tests (Bogovski and Bogovski, 1981) and it is highly probable that most of these compounds are also carcinogenic for man (Speijer et al., 1989). Because these compounds are formed in the stomach, the primary potential health effect is an increased occurrence of gastric cancer, but carcinogenic effects on other organs cannot be excluded.

The relevance of nitrate intake for the endogenous nitrosation reaction is not clear. The results of epidemiological studies on the relationship between high nitrate intake and the frequency of occurrence of gastric cancer are conflicting. By searching the epidemiological literature Speijers et al. (1989) concluded that no clear relationship was found between nitrate intake and gastric cancer. Forman (1987) stated that nitrate intake is not a rate-limiting factor in the endogenous synthesis of N-nitroso compounds and that even low to moderate exposures to nitrate might be sufficient for nitrosation, given other favourable conditions.

The conflicting results of epidemiological studies could result from the fact that nitrate exposure is considered in isolation from other factors involved in the nitrosation process, such as the dietary provision of nitrosatable substrates, the physiological state of the stomach and modifiers of the nitrosation reaction. Some compounds (e.g. vitamins C and E) have a strong inhibitory effect on the nitrosation reaction (Hartman,

1983; Mirvish, 1983; Oshima and Bartsch, 1981; Weisburger, 1986). Leafy vegetables are an important source of nitrate, but they also contain vitamins C and E (Mirvish, 1983) and, in general, the consumption of leafy vegetables correlates negatively with the incidence of gastric cancer (Haensel and Correa, 1975; Tannenbaum and Correa, 1985).

*Human nitrate intake*

In plants, nitrate is mainly accumulated in the roots, stems and leaves. Reproductive organs (fruits, seeds) in general have a very low nitrate content. Nitrate accumulation by plants is strongly stimulated by low light intensities and thus is most important for crops grown in late autumn, winter and early spring. There are large differences between plant species in nitrate accumulation. Wright and Davidson (1964) listed Amaranthaceae, Chenopodiaceae, Cruciferae, Compositae, Gramineae and Solanaceae as plant families with a potential for accumulation of high levels of nitrate. Other reviews of nitrate content in plant species are given by Corré and Breimer (1979), Maynard et al. (1976) and Venter (1978). In Europe, leafy vegetables (lettuce, spinach, endive) and root crops (beetroot, radish) are important sources of human nitrate intake.

The acceptable daily intake (ADI) is defined as the maximum amount of a compound that can be consumed daily without a risk. The Joint FAO/WHO Expert Committee on Food Additives has indicated an ADI for nitrate of 220 mg for a person of 60 kg (JECFA, 1974). The present ADI-value for nitrate is under discussion because it does not take account of the endogenous conversion of nitrate to nitrite and the subsequent synthesis of N-nitroso compounds (Groenen et al., 1984; Van Went-De Vries, 1988). The ADI for nitrite is 8 mg for a person of 60 kg (JECFA, 1976).

Except for young infants, the main source of human nitrate intake is the consumption of vegetables. In the Netherlands the average daily intake of nitrate was estimated at 143 mg, of which 120 mg is derived from vegetables (Van Duijvenbooden and Matthijsen, 1989). The consumption of a meal with nitrate-rich vegetables can easily result in the consumption of two to three times the ADI (Kamsteeg and Butijn, 1981). Drinking water is a second important source of nitrate and is increasingly important because of the rising nitrate pollution of drinking water due to the excessive use of fertilizers in agriculture.

*Maximum admissible limits*

In several European countries public health authorities have taken measures to prevent further increases of nitrate consumption and, where possible, to reduce the nitrate intake. Official maxima have been imposed on nitrate levels of drinking water and some vegetables. According to the guidelines on the quality of drinking water of the European Community, the admissible nitrate concentration is limited to 50 $mg \cdot l^{-1}$. In the Netherlands maximally admissible nitrate contents for lettuce, spinach and endive were first imposed in 1982 (Staatscourant, 1982). For lettuce the maximum nitrate content was 5 g per kg fresh weight for the winter period (November 1 - March 31) and 4 $g \cdot kg^{-1}$ for the summer period (April 1 - October 31). These were lenient levels

***Table 1.1.*** **Per capita consumption of leafy vegetables in the Netherlands in 1990 (PGF, 1992)**

| Vegetable | Latin name | Annual consumption (kg) | Nitrate accumulator |
|---|---|---|---|
| Lettuce | *Lactuca sativa* | 3.22 | yes |
| Chicory | *Cichorium intybus* | 3.31 | no |
| Endive | *Cichorium endiviae* | 2.56 | yes |
| Spinach (fresh) | *Spinacia oleracea* | 0.81 | yes |
| Leaf celery | *Apium graveolens* | 0.20 | yes |
| Purslane | *Portulaca oleracea* | 0.12 | yes |

***Table 1.2.*** **Production data for the most important nitrate accumulating leafy vegetables in the Netherlands in 1990 (PGF, 1992)**

| | Open field | | Glasshouse | | Total | |
|---|---|---|---|---|---|---|
| | kg $\cdot 10^6$ | hfl $\cdot 10^6$ | kg $\cdot 10^6$ | hfl $\cdot 10^6$ | kg $\cdot 10^6$ | hfl $\cdot 10^6$ |
| Lettuce | 45 | 50 | 58 | 152 | 104 | 205 |
| Endive | 26 | 17 | 14 | 25 | 40 | 41 |
| Spinach | 58 | 15 | 2 | 3 | 60 | 18 |

because at that time no low-nitrate cultivars were available and there was little practical experience of cultural measures influencing the nitrate content. Later, the maxima have gradually been lowered. The maximum level for the winter period was lowered to 4.5 $g \cdot kg^{-1}$ in 1985 and will be further reduced to 3.5 $g \cdot kg^{-1}$ in 1995 (Van der Wees, 1991). The summer maximum was lowered to 3.5 $g \cdot kg^{-1}$ in 1985, to 3 $g \cdot kg^{-1}$ in 1988 and to 2.5 $g \cdot kg^{-1}$ in 1992. In the Netherlands the ultimate aim is to reduce the maximum nitrate contents in vegetables year-round to 2.5 $g \cdot kg^{-1}$ (Staatscourant, 1992). In other European countries maximum levels for nitrate content in vegetables have also been imposed or are in preparation (Schwemmer, 1990; Winkhoff, 1992).

## 1.2 Lettuce

*Why research on nitrate accumulation in lettuce?*

Lettuce is an important vegetable crop in the Netherlands. With a production value of 205 million hfl in 1990, lettuce ranked fourth after tomato, cucumber and sweet pepper (PGF, 1992). For the following reasons research on reducing the nitrate content in vegetables has mainly been focussed on lettuce:

1. Lettuce is a leafy vegetable, which contributes largely to human nitrate intake.
2. In the Netherlands the per capita consumption of lettuce is highest of all nitrate accumulating leafy vegetables (Table 1.1).
3. Lettuce has the highest production value of the leafy vegetables produced in the Netherlands (Table 1.2).
4. In the Netherlands a substantial part of the lettuce is produced in glasshouses in the period from October 1 to April 1, when nitrate accumulation is highest (Table 1.3).
5. In other EC countries lettuce is also by far the most important leafy vegetable produced (Table 1.4) and several of these countries also have problems with nitrate accumulation in vegetables.

*Breeding of lettuce*

The lettuce crop has several features which are relevant to the research presented here. In comparison to other leafy vegetables, lettuce breeding is intensive and at an advanced level. This fact increases the chance that the results from breeding research are quickly incorporated into commercial breeding programs. In Europe, Dutch breeding firms dominate the market. This is illustrated in Table 1.5. Outside Europe, intensive breeding of lettuce is carried out in the USA (Ryder, 1986). The breeding efforts lead to the introduction of a wide range of new cultivars each year, differing in type (butterhead, crisphead, cos, latin and cutting lettuce), colour (a wide range of green and red colours), adaptation to specific cultural and environmental conditions and resistances to biotic and abiotic stress factors. Table 1.6 shows the number of new lettuce cultivars registered in the Netherlands in the period 1980-1991.

Lettuce is in the family *Asteraceae* (*Compositae*). It is an autogamous species. The inflorescence is a panicle with many flower heads. Each flower head has about 15 florets. The five stamens of each floret are fused to form a tube, through which the style passes at the beginning of flowering. The pistil is pollinated inside the tube, resulting in strict self-pollination. Per plant, several

***Table 1.3.*** **Percentage of the annual production of three nitrate-rich leafy vegetables auctioned between October 1 and April 1. Mean values for the Netherlands over the period 1988-1991 (Source: PGF)**

| | Percentage based on: | |
|---|---|---|
| | Physical [1] units | Value |
| Lettuce | 49 | 60 |
| Endive | 30 | 47 |
| Spinach | 20 | 36 |

[1]Kilograms for endive and spinach, heads for lettuce

***Table 1.4.*** **Production of the most important leafy vegetables in the European Community in 1988 in $10^6$ kg (no substantial production in Denmark and Luxembourg, Eurostat, 1990)**

| Crop | Country[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | D | GR | E | F | IRL | I | NL | P | UK |
| Lettuce | 77 | 84 | 57 | 599 | 343 | 6 | 418 | 127 | 30 | 239 |
| Endive | 5 | 10 | 38 | 75 | 177 | · | 263 | 43 | · | · |
| Spinach | 21 | 39 | 44 | 52 | 80 | · | 91 | 47 | · | · |
| Chicory | 88 | · | · | 3 | 206 | · | 211 | 72 | · | · |

[1]B: Belgium; D: Germany; GR: Greece; E: Spain; F: France; IRL: Ireland; I: Italy; NL: the Netherlands; P: Portugal; UK: United Kingdom.
·: no data available

***Table 1.5.*** **Total number of lettuce cultivars registered in countries of the European Community in the period 1980-1990 (EC cultivar list)**

| Year | Country[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Total EC[2] | D | DK | E | F | I | NL | UK |
| 1980 | 401 | 27 | 12 | · | 132 | 71 | 220 | 67 |
| 1982 | 441 | 25 | 8 | · | 112 | 72 | 256 | 57 |
| 1984 | 482 | 27 | 7 | · | 116 | 71 | 297 | 54 |
| 1987 | 572 | 25 | 7 | 80 | 127 | 75 | 383 | 52 |
| 1988 | 646 | 41 | 4 | 81 | 137 | 74 | 426 | 59 |
| 1990 | 776 | 40 | 4 | 91 | 147 | 83 | 548 | 57 |

[1]B: Belgium; D: Germany; GR: Greece; E: Spain; F: France; IRL: Ireland; I: Italy; NL: the Netherlands; P: Portugal; UK: United Kingdom.
[2]As a cultivar can be registered in several countries, the total is smaller than the sum of cultivars per country.
·: no data available

***Table 1.6.*** **Number of new lettuce cultivars registered in the Netherlands in the period 1980-1991 (NAKG)**

| Year | 1980 | 1981 | 1982 | 1983 | 1984 | 1985 | 1986 | 1987 | 1988 | 1989 | 1990 | 1991 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 22 | 18 | 36 | 51 | – | 49 | 51 | 62 | 55 | 70 | 80 | 84 |

thousand seeds can be obtained. Pedigree selection is the dominant breeding method in lettuce. Cultured lettuce is an annual, but when grown at high temperatures, high light intensities and long daylength in glasshouses, three or more generations can be achieved per year. In breeding programs this procedure can be used to speed up the production of the early generations, in which the lines are only tested for features with a high heritability (resistances). Selected lines are tested in later generations under normal growing conditions for traits with a lower heritability.

Large collections of lettuce cultivars and uncultivated *Lactuca* species are available in genebanks (Boukema et al., 1990) and much is known about the genetics of the crop (Landry et al., 1987; Robinson et al., 1983).

## 1.3 The scope of this thesis

For large parts of the year (autumn, winter and early spring) the ultimate goal for the maximum admissible nitrate content in lettuce of 2.5 g per kg fresh weight cannot be achieved with the existing cultivars and the current cropping systems. When lettuce is grown on nutrient solution, a substantial reduction of nitrate content can be achieved by manipulating the concentrations of the salts in the solution (Van der Boon et al., 1990). However, this cropping system is not used in practice and the possibilities to manipulate the nitrate content of plants grown under winter conditions in soil are much smaller (Roorda van Eysinga and Van der Meijs, 1985). Therefore, cultivars with a genetically reduced capacity to accumulate nitrate would be of great value. It is likely that when the final admissible levels of 2.5 $g \cdot kg^{-1}$ are imposed, without such cultivars lettuce production will not be possible in the Netherlands for large periods of the year.

The research presented in this thesis investigated the prospects of lowering the nitrate content of lettuce by breeding and supports lettuce breeders in their work to produce cultivars with low nitrate content. Primary aims of this study were to gain knowledge about: a) the variation between lettuce accessions for nitrate content, b) the genetics of nitrate accumulation and c) the selection method to be applied in breeding for low nitrate content. Although knowledge of the physiology of nitrate accumulation can also be very relevant to plant breeding programs, this was not included as a main topic in this thesis because extensive physiological research on nitrate accumulation was carried out by others (Behr and Wiebe, 1988; 1992; Blom-Zandstra, 1990; Steingröver, 1986).

The research began with an evaluation of the variation shown by accessions of lettuce and related *Lactuca* species for nitrate accumulation (Chapter 2). Because it was known that modern lettuce cultivars do not show very large differences in nitrate content, a germplasm collection was screened to find accessions with very low nitrate levels. Such low-nitrate accessions would be very useful to practical plant breeding as donors of the low nitrate trait and could also be used to study the genetics of nitrate accumulation. A number of accessions representing a wide range of *Lactuca* germplasm were tested in two experi-

ments, one with plants grown in soil, the other with plants grown on nutrient solution. Accessions with extremely low nitrate content in both experiments were chosen for ensuing studies on the genetics of nitrate accumulation (Chapters 3 and 4) and on aspects of genotype × environment (G×E) interactions (Chapters 5 and 6).

Chapters 3 and 4 describe a study of the genetics of nitrate accumulation in lettuce. Detailed information on the genetics of this trait enables breeders to develop optimal breeding programs. This knowledge can also be used to predict the potential results of selection. The genetic study aimed to find out whether nitrate accumulation has a qualitative or a quantitative inheritance, what the heritability of the trait is, whether there are differences between reciprocal crosses, whether there are important effects of dominance involved and whether the level of nitrate in lettuce can be further reduced by combining genes for low nitrate from different low-nitrate accessions. Two types of crosses were studied: crosses between accessions widely differing in nitrate content and crosses of which both parents had low nitrate content. Chapter 3 describes a quantitative analysis of the means of reciprocal $F_1$ and $F_2$ generations and first backcrosses to both parents ($BC_1^1$ and $BC_1^2$). Chapter 4 describes a quantitative analysis of the components of variation in $F_2$ and $F_3$ generations. Additive genotypic variances, estimated from $F_3$ variance components and from the covariance between $F_2$ plants and corresponding $F_3$ lines, were used to predict the level of nitrate that could potentially be realized in inbred lines by selection for low nitrate content.

Chapter 5 describes a study of G×E interactions for nitrate accumulation in lettuce. Variation in environment was created by growing lettuce accessions in different periods of the year. The occurrence of G×E interactions would mean that the order of tested accessions for nitrate content is not the same when grown in different periods of the year. Knowledge of the occurrence and size of such interactions are important for breeders because G×E interactions may impose severe restrictions on the selection process and on the use of selected cultivars. Important effects of G×E interaction could mean that the cultivation of a certain low-nitrate genotype is restricted to limited periods of the year, i.e. that period in which the cultivar has low nitrate content. Furthermore, when G×E effects are important, selection for low nitrate should be carried out under growing conditions comparable to the conditions in the period of the year for which cultivars are bred.

Chapter 6 describes a further study of G×E interactions for nitrate content. This study elaborated the inheritance and characteristics associated with G×E interaction. This was done by crossing two low-nitrate accessions with a different pattern of change of nitrate content when harvested in successive periods of the year. The parents of the cross and 25 random $F_3$ lines were grown in four successive experiments. The pattern of change of nitrate content of the $F_3$ lines in the successive harvests was related to that of their parents and to the pattern of change of dry matter content.

## References


Behr, U. & H.-J. Wiebe, 1988. Beziehungen zwischen dem Gehalt an Nitrat und anderen Osmotica des Zellsaftes bei Kopfsalatsorten (*Lactuca sativa* L.). Gartenbauwissenschaft 53: 206-210.

Behr, U. & H.-J. Wiebe, 1992. Relation between photosynthesis and nitrate content of lettuce cultivars. Scientia Horticulturae 49: 175-179.

Blom-Zandstra, M., 1990. Some physiological aspects of nitrate accumulation in lettuce (*Lactuca sativa* L.). PhD-thesis, State University Utrecht, The Netherlands, 81 p.

Bogovski, P. & S. Bogovski, 1981. Animal species in which N-nitroso compounds induce cancer. Int. J. Cancer 27: 471-474.

Boon, J. van der, J.W. Steenhuizen & E.G. Steingröver, 1990. Growth and nitrate concentration of lettuce as affected by total nitrogen and chloride concentration, $NH_4/NO_3$ ratio and temperature of the recirculating nutrient solution. J. Hort. Sci. 65: 309-321.

Boukema, I.W., Th. Hazekamp & Th. J.L. van Hintum, 1990. CGN Collection Reviews: The CGN Lettuce Collection. December 1990. Centre for Genetic Resources, The Netherlands (CGN), Wageningen, The Netherlands, 27 p.

Corré, W.J. & T. Breimer, 1979. Nitrate and nitrite in vegetables. Literature survey. Centre for agricultural Publishing and Documentation, PUDOC, Wageningen, The Netherlands, no. 39, 85 p.

Duijvenbooden, W. van, & A.J.C.M. Matthijsen, 1989. Integrated Criteria Document Nitrate. Report No. 758473012, Nat. Inst. of Public Health and Environmental Protection, Bilthoven, The Netherlands, 196 p.

Eurostat, 1990. Agriculture - Statistical Yearbook 1990. Eurostat, Luxembourg, Theme 5: Agriculture, forestry and fisheries, ISBN 92-826-2596-6, 261 p.

Fan, A.M., C.C. Wilhite & S.A. Book, 1987. Evaluation of the nitrate drinking water standard with reference to infant methemoglobinemia and potential reproductive toxicity. Regulatory toxicology and pharmacology 7: 135-148.

Forman, D., 1987. Dietary exposure to N-nitroso compounds and the risk of human cancer. Cancer Surveys 6: 719-738.

Gezondheidsraad, 1990. Nitraat, toetsing van een ontwerp-basisdocument. Gezondheidsraad, Den Haag, Report 1990/07, 83 p.

Groenen, P. J., M.W. Cock-Bethbeder, E.J. van der Beek, W. van Dokkum, L.A. Prins, J.A. Westra & E. Bussink, 1984. Vorming van nitrosaminen in het lichaam van de mens na het eten van nitraatrijke groenten. CIVO-TNO report no. 84.108/020311. CIVO-TNO, Zeist, The Netherlands, 71 p.

Haensel, W. & P. Correa, 1975. Developments in the epidemiology of stomach cancer over the past decade. Cancer Res. 35: 3452-3459.

Hartmann, P.E., 1983. Review: Putative mutagens and carcinogens in foods. I Nitrate/nitrite ingestion and gastric cancer mortality. Environ. Mutagen. 5: 111-121.

Hegesh, E. and J. Shiloah, 1982. Blood nitrates and infantile methaemoglobinemia. Clinica Chimina Acta 125: 107-115.

JECFA, 1974. Toxicological evaluations of certain food additives with a review of general principles and of specifications. Joint FAO/WHO Expert Committee on Food Additives, 17th Report, FAO Nutrition Meeting Report Series 53, Rome, 53 pp.

JECFA, 1976. Toxicological evaluations of certain food additives. Joint FAO/WHO Expert Committee on Food Additives, 20th Report, FAO Nutrition Meeting Report Series 62, Rome, 56 pp.

Kamsteeg, J. & C.A.A. Butijn, 1981. De invloed van het bewaren, wassen en bereiden op het vitamine c- en nitraatgehalte van spinazie. Vakblad voor Huishoudkunde 2: 98-107.

Landry, B.S., R.V. Kesseli, B. Farrara & R.W. Michelmore, 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics 116: 331-337.

Maynard, D.N., A.V. Barker, P.L. Minotti & N.H. Peck, 1976. Nitrate accumulation in vegetables. Advan. Agron. 28: 71-118.

Mirvish, S. S., 1975. Formation of N-Nitroso compounds: chemistry, kinetics, and in vivo

occurrence. Toxicology and applied pharmacology 21: 325-351.

Mirvish, S. S., 1983. The etiology of gastric cancer, intragastric nitrosamide formation and other theories. J. natn. Cancer Inst. 71: 630-647.

Oshima, H. & H. Bartsch, 1981. Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in the urine. Cancer Res. 41: 3658-3662.

PGF, 1992. Jaarverslag 1991, Produktschap voor Groenten en Fruit, P.O. Box 90403, 2509 LK Den Haag, The Netherlands, 200 p.

Robinson, R.W., J.D. McCreight & E.J. Ryder, 1983. The genes of lettuce and closely related species. Plant Breeding Reviews 1: 267-293.

Roorda van Eysinga, J.P.N.L. & M.Q. Van der Meijs, 1985. Effect of nitrogen nutrition and global radiation on yield and nitrate content of lettuce grown under glass. Comm. in Soil Sci. Plant Anal. 16: 1293-1300.

Ryder, E.J., 1986. Lettuce Breeding. In: M.J. Bassett (ed.) Breeding vegetable crops. AVI Publishing Co. Inc. Connecticut, USA: p. 433-473.

Schwemmer, E., 1990. Nitrat in Gemüse. Gemüse 3: 173-175.

Speijers, G.J.A., G.F. van Went, M.E. van Apeldoorn, G.K. Montizaan, J.A. Janus, J.H. Canton, C.A.M. van Gestel, C.A. van der Heijden, E. Heijna-Merkus, A.G.A.C. Knaap, R. Luttik & D. de Zwart, 1989. Appendix to Report No. 758473012, Integrated Criteria Document Nitrate; Effects. Nat. Inst. of Public Health and Environmental Protection, Bilthoven, The Netherlands, 198 p.

Staatscourant, 1982. Vaststelling maximaal toelaatbaar gehalte nitraat in bladgroenten. Nederlandse Staatscourant, 15 September.

Staatscourant, 1992. Wijziging regeling nitraatgehalte groenten (Warenwet). Staatscourant, 7 mei, no. 87, p. 35.

Steingröver,. E., 1986. Nitrate accumulation in spinach. PhD-thesis State University Groningen, The Netherlands, 84 p.

Tannenbaum, S. R. & P. Correa, 1985. Nitrate and gastric cancer risks. Nature 317: 6675-676.

Venter, F., 1978. Untersuchungen über den Nitratgehalt in Gemüse. Stickstoff 12: 31-38.

Went-De Vries, G. F. van, 1988. Betekenis van toxicologische effekten van nitraat, nitriet en nitrosamines in relatie tot de normstelling voor nitraat. Report LAC, nr.88-1, Ministry of Agriculture and Fishery, p. 39-49.

Weisberger, J.H., 1986. Role of fat, fiber, nitrate, and food additives in carcinogenesis: a critical evaluation and recommendations. Nutrition and Cancer 8: 47-62.

Wees, A. van der, 1991. Overheid scherpt nitraatnormen aan. Weekblad Groenten en Fruit/Glasgroenten 17, 26 April, p 37.

Winkhoff, J., 1992. Kein nationaler Alleingang - Nitrathöchstmenge für Kopfsalat! Gemüse 28: 273.

Wright, M.J. & K. L. Davidson, 1964. Nitrate accumulation in crops and nitrate poisoning in animals. Advan. Agron. 16: 197-247.

# 2 Genotypic Differences in Nitrate Content in *Lactuca sativa* L. and Related Species and Correlation with Dry Matter Content

*135 Accessions of cultivated lettuce* (Lactuca sativa *L.) and 21 accessions of wild* Lactuca *species were grown in winter in two experiments and the nitrate content was measured. In the first experiment the plants were grown on nutrient film, in the second in large pots filled with peat soil. Within each of the five plant types of cultivated lettuce that were distinguished, accessions were found with a low nitrate content. In butterhead lettuce, nitrate content was negatively correlated with dry matter content and positively with plant fresh weight. Five butterhead accessions, one with extremely high nitrate content and four with low nitrate contents, were selected for further research.*

## 2.1 Introduction

A high nitrate content is an undesirable property in leafy vegetables, because a high intake of nitrate can be dangerous to human health (Chapter 1). Nitrate accumulation is greatly increased by low light intensities (Blom-Zandstra and Lampe, 1985), causing problems in winter grown crops. Since 1980 more than 1000 lettuce accessions were screened in several trials for nitrate accumulation (Eenink et al., 1984). Large differences in nitrate accumulation were discovered between accessions. However, in most of these trials the coefficient of variation for nitrate content was high (above 25%) and selected accessions behaved rather variably in later trials. The use of small pots (800 g potting soil), which can easily be depleted locally of nitrate or water, could be an important source of error (Groenwold, 1985).

The primary aim of this study was to identify accessions which accumulate only low levels of nitrate when grown under low light conditions. Such accessions would be of great value in practical breeding programs and in research on the genetics and physiology of nitrate accumulation. To obtain more reliable results than in previous research, attention was paid to improve the uniformity of growing conditions. A second aim was to study the relationship between nitrate content and some other plant characteristics, i.e. dry matter content and fresh weight. This was done to confirm results of Eenink (1984), who reported that nitrate content was negatively correlated with both dry matter content and with plant fresh weight.

## 2.2 Materials and methods

*Accessions*

Based on their low nitrate contents in previous screening experiments, 151 accessions were selected for further research. Three modern butterhead cultivars (Panvit, Pascal and Pinto), with high nitrate accumulation, and two accessions which were known to accumulate only moderate levels of nitrate (CGN4567 and CGN9331) were added as controls. The group of accessions tested included both

cultivated and wild material. Of the cultivated accessions, 61 were of the butterhead type, 29 of the cos type, 19 of the crisp type, 19 of the cutting (loose leaved) type and 7 of the latin type. The group of wild *Lactuca* accessions consisted of 18 accessions of *L. serriola*, two of *L. saligna* and one of *L. virosa*. The lettuce collection tested in this research is presently maintained by the genebank of the Centre for Genetic Resources (CGN), the Netherlands.

*Growth conditions*

The 156 accessions were grown in two experiments in a glasshouse. In experiment 1 the plants were grown on recirculating nutrient film. To verify the results of experiment 1 and to compare the results obtained from plants grown on nutrient film with those from plants grown in soil, in a second experiment the plants were grown in large pots containing 4.5 l of peat soil. Experiment 1 was sown on October 29, 1984 and harvested on February 11-15, 1985. Experiment 2 was sown on October 31, 1984 and harvested on February 4-7, 1985. The experiments were grown according to a randomized block design, with five replicates and one plant per plot in experiment 1, and four replicates with two plants per plot (in 2 pots) in experiment 2.

Plants were grown under natural daylight and at minimum day and night temperatures of 11°C and 3°C respectively. The glasshouse was ventilated when temperature exceeded 18°C during the day or 7°C at night. Relative humidity ranged between 60 and 90% and the global radiation varied from 39 to 308 J. $cm^{-2} \cdot d^{-1}$. In experiment 1, five per cent of the nitrogen was given as ammonia up to five days before the start of harvest. To have the ammonium in the potting soil, used in experiment 2, converted to nitrate, this soil was stored in a glasshouse for three months before it was used in the experiment.

Plants were harvested at a young stage because previous research (Eenink et al., 1984) had shown a good relationship between the nitrate content of young plants and that of plants in later stages of growth.

*Harvest and analysis*

Replicates were harvested on consecutive days. In experiment 1 only the leaves were harvested; in experiment 2 the entire shoot was harvested. The harvested plant material was weighed, dried for 20 hours at 70°C and dry matter weight was determined for each plant. The dried material was pulverized and the nitrate content was analyzed in an aqueous extract of the samples, using a Skalar autoanalyzer. Nitrate content was calculated per kg fresh matter. Analysis of variance was done for nitrate content, fresh weight and dry matter content, both for the total group of accessions and for each plant type separately. Correlation coefficients were calculated using mean values for accessions per experiment.

To test for genotype × experiment interactions for nitrate content, the results of experiments 1 and 2 were log-transformed to equalize the error variances in the two experiments. The mean values for the accessions in the two experiments obtained in that way were included in an analysis of variance. Individual interaction terms of accessions

have been Bonferroni-tested against zero.

## 2.3 Results

*Fresh weight*
Mean fresh weight per plant was only slightly different between the plant types within cultivated lettuce and averaged 35 g in experiment 1 and 14 g in experiment 2. Wild *Lactuca* accessions produced less fresh matter: the mean fresh weight per plant was 23 g in experiment 1 and only 9 g in experiment 2.

*Nitrate content*
In both experiments large and highly significant genotype effects on nitrate content were found within all cultivated lettuce types and between the accessions of the wild species. Table 2.1 shows the observed range, the mean value, the variance ratio for effect of genotypes and the coefficient of variation per plant type per experiment. Compared with former experiments, the coefficients of variation for nitrate content in these two experiments were satisfactorily low. The accessions that were used had been selected for their low nitrate content in previous experiments. The high nitrate values of some of the accessions in these two experiments demonstrates the insufficient discrimination of these previous experiments. The experiment with plants grown on nutrient film had about the same coefficient of variation than the experiment with plants grown in large pots with peat soil. Mean nitrate content showed little differences between the cultivated plant types. Wild *Lactuca* accessions had a higher mean nitrate content in experiment 1. Within all plant types a wide range of nitrate contents was found. The lowest values occurred in the butterhead type, the highest among the wild *Lactuca* species. The range of nitrate contents was largest within the group of wild *Lactuca* species. Within cultivated lettuce, the butterhead type had the largest range for nitrate content. Table 2.2 gives the nitrate content in fresh matter and the ranking number of the control cultivars and of four other butterhead accessions, which were chosen for further research because of their low nitrate contents. For the other cultivated plant types and for the wild *Lactuca* accessions, the accession with the lowest mean nitrate content calculated over both experiments is listed. Table 2.2 shows that several lettuce accessions had a lower mean nitrate content over both experiments than the low-nitrate controls which were included in the experiments.

*Interaction between accessions and experiments for nitrate content*
A significant genotype × experiment interaction was found. However, at a significance level of $p=0.01$, the test of Bonferroni revealed that only 10 out of 156 accessions showed a significant interaction. Of the accessions listed in Table 2.2, the butterhead type CGN4892 and the crisp type CGN4518 showed significant interactions: the nitrate content of CGN4892 was relatively high in experiment 1 and low in experiment 2, while CGN4518 showed the opposite behaviour.

***Table 2.1.*** **Observed range and mean nitrate content (g per kg fresh matter), variance ratio (VR) for genotype effect and the coefficient of variation (CV) per plant type per experiment. Degrees of freedom for variance ratios can be deduced from 'Materials and methods'**

| Plant type | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | range ($g \cdot kg^{-1}$) | mean ($g \cdot kg^{-1}$) | VR | CV (%) | range ($g \cdot kg^{-1}$) | mean ($g \cdot kg^{-1}$) | VR | CV (%) |
| Butterhead | 1.3-3.7 | 2.5 | $29.8^{***}$ | 9 | 1.7-4.2 | 3.2 | $16.5^{***}$ | 9 |
| Cos | 1.6-3.0 | 2.4 | $15.1^{***}$ | 9 | 2.3-4.2 | 3.3 | $6.7^{***}$ | 10 |
| Crisp | 1.5-3.2 | 2.5 | $20.3^{***}$ | 8 | 2.5-4.1 | 3.2 | $5.5^{***}$ | 11 |
| Latin | 1.9-3.0 | 2.4 | $18.4^{***}$ | 9 | 2.7-4.1 | 3.4 | $28.7^{***}$ | 6 |
| Cutting | 1.9-3.0 | 2.4 | $2.7^{**}$ | 13 | 2.5-3.7 | 3.2 | $4.5^{***}$ | 8 |
| Wild species | 1.6-5.1 | 2.8 | $33.7^{***}$ | 11 | 2.6-6.4 | 3.3 | $14.9^{***}$ | 14 |
| Total | 1.3-5.1 | 2.5 | $22.7^{***}$ | 10 | 1.7-6.4 | 3.3 | $11.8^{***}$ | 10 |

*** : significant at p=0.001; ** : significant at p=0.01

*Dry matter content*

Large and highly significant genotype effects on dry matter content were found in both experiments. Table 2.3 shows the mean values and range of dry matter content per plant type and experiment. Wild *Lactuca* accessions had the highest mean value and the largest range of dry matter content in both experiments. Variation for dry matter content was very small for crisp lettuce in both experiments. The coefficient of variation for dry matter content was 6% in both experiments.

*Relationship between nitrate content and dry matter content*

The coefficients of correlation between nitrate content and dry matter content for the plant types in experiments 1 and 2 are shown in Table 2.4. A high and negative correlation was found between nitrate content and dry matter content in butterhead lettuce. For the other cultivated types this correlation was much lower. This could partly be caused by the smaller ranges of nitrate contents and dry matter contents in these other plant types, compared to the butterhead types. Fig. 2.1 shows the relationship between dry matter content and nitrate content for the butterhead cultivars in the two experiments.

*Relationship between nitrate content and plant fresh weight*

If low nitrate accumulators are high dry matter accumulators, as found for the butterhead cultivars, a positive correlation between nitrate content and fresh weight can be expected if the accessions have about the same net photosynthesis. Table 2.5 gives the coefficients of correlation between nitrate content and plant fresh weight and between dry matter

***Table 2.2.*** **Nitrate content in fresh matter ($g \cdot kg^{-1}$) of the control cultivars and of four butterhead accessions with low nitrate content, which were selected for further research. For the other plant types, the accession with the lowest mean nitrate content over the two experiments is listed. Ranking numbers are given in parentheses, ranging from 1 (low) to 156 (high)**

| Plant type | CGN [1] code | Accession name | Nitrate content ($g \cdot kg^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | | | Experiment 1 | | Experiment 2 | |
| Butterhead | 9277 | Panvit [2,4] | 3.5 | (153) | 4.4 | (155) |
| | 5133 | Pascal [2] | 3.5 | (152) | 3.8 | (137) |
| | 11439 | Pinto [2] | 3.2 | (142) | 3.6 | (115) |
| | 4567 | d'Hiver de Tremont [3] | 2.6 | (85) | 3.0 | (43) |
| | 9331 | Grosse Brune Têtue [3] | 1.9 | (18) | 2.6 | (13) |
| | 5233 | Reichenauer Winter [4] | 1.3 | (1) | 1.7 | (1) |
| | 4892 | Winter Butterkopf [4] | 1.9 | (16) | 1.9 | (2) |
| | 4944 | Trocadero Light 76 [4] | 1.9 | (22) | 2.8 | (29) |
| | 5811 | *L.sativa capitata* [4] | 1.8 | (10) | 1.9 | (3) |
| Cos | 4766 | Kahou | 1.8 | (12) | 2.3 | (6) |
| Crisp | 4518 | Batavia Rouge Grenobloise | 1.4 | (3) | 2.5 | (8) |
| Latin | 4840 | Verte d'Hiver | 2.1 | (34) | 2.7 | (17) |
| Cutting | 4919 | Tidig Gul | 2.1 | (31) | 2.5 | (10) |
| Wild species | 5009 | *Lactuca serriola* | 1.8 | (11) | 2.5 | (9) |

[1] Centre for Genetic Resources, P.O. Box 16, 6700 AA Wageningen, the Netherlands.
[2] Controls with high nitrate content
[3] Controls with low nitrate content
[4] Butterhead accessions selected for further research

content and plant fresh weight for the butterhead cultivars in experiments 1 and 2. The relationship between nitrate content and plant fresh weight is shown in Fig. 2.2. Nitrate content and fresh weight were positively correlated and fresh weight was negatively correlated with dry matter content. Butterhead cultivars with extremely low nitrate content (and high dry matter content) tended to produce less fresh matter per plant than accessions with a higher nitrate content. High nitrate accumulators show a wide variation in fresh weight per plant.

## 2.4 Discussion

Within each of the cultivated plant types of lettuce, accessions with low nitrate content were identified. This offer good prospects for breeding for low nitrate content, as the desired characteristic can be found in accessions of the same plant type, thus avoiding backcrosses to reobtain the desired plant type. The large range of nitrate contents, especially in the butterhead type, offers a good starting point for further research on the genetics and physiology of nitrate accumulation.

***Table 2.3.*** **Mean values and range of dry matter content (%) per plant type per experiment**

| Plant type | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | mean | range | mean | range |
| Butterhead | 6.8 | 5.4- 9.1 | 7.2 | 5.8- 9.7 |
| Cos | 7.2 | 6.2- 8.6 | 6.7 | 5.8- 9.1 |
| Crisp | 6.9 | 6.3- 7.3 | 6.8 | 6.3- 7.4 |
| Latin | 7.0 | 6.2- 8.0 | 7.2 | 6.1- 8.4 |
| Cutting | 7.0 | 6.3- 8.6 | 7.1 | 6.2- 8.6 |
| Wild species | 8.7 | 7.3-12.5 | 9.4 | 7.6-12.5 |
| Total | 7.2 | 5.4-12.5 | 7.4 | 5.8-12.5 |
| *Coefficient of variation* | *6%* | | *6%* | |

***Table 2.4.*** **Coefficients of correlation between nitrate content in fresh matter and dry matter content, per plant type per experiment**

| Plant type | Expt 1 | Expt 2 |
|---|---|---|
| Butterhead | -0.83*** | -0.85*** |
| Cos | 0.00 | -0.33 |
| Crisp | -0.54* | 0.04 |
| Latin | -0.01 | -0.39 |
| Cutting | -0.38 | -0.54 |
| Wild species | -0.43* | -0.46* |

*** : significant at p=0.001
* : significant at p=0.05

***Table 2.5.*** **Coefficients of correlation between nitrate content in fresh matter and plant fresh weight and between dry matter content and plant fresh weight for the butterhead types per experiment**

| Correlation coefficient | Expt 1 | Expt 2 |
|---|---|---|
| Nitrate content -plant fresh weight | 0.50*** | 0.47*** |
| Dry matter content -plant fresh weight | -0.55*** | -0.45*** |

*** : significant at p=0.001

A high and negative correlation was found between nitrate content in fresh matter and dry matter content in butterhead accessions. Butterhead accessions with very high dry matter content also had a low plant fresh weight. The question arises whether a causal relationship exists between dry matter accumulation and nitrate content. Also in spinach a mutant is known which is characterized by a combination of an extremely low nitrate content and a very high dry matter content (Handke and Junge, 1984). The fresh matter produc-

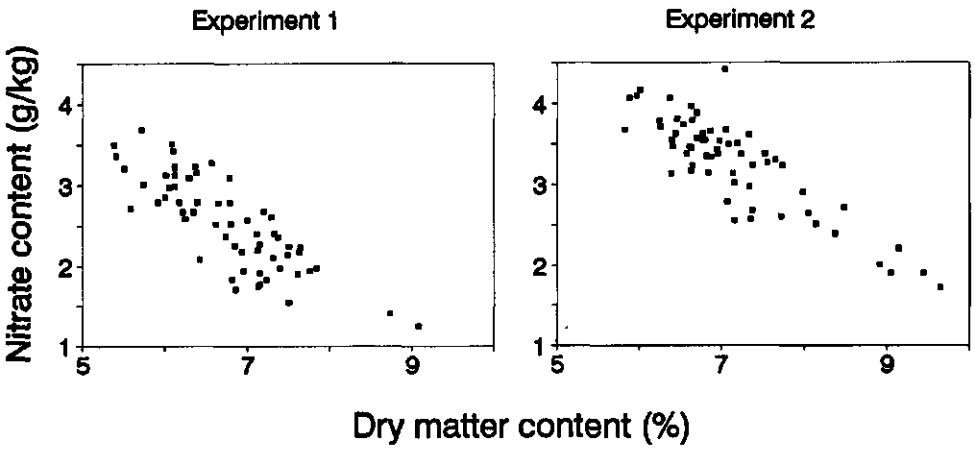


***Fig. 2.1.*** **Relationship between nitrate content ($g \cdot kg^{-1}$) and dry matter content (%) for the butterhead types in experiments 1 and 2.**

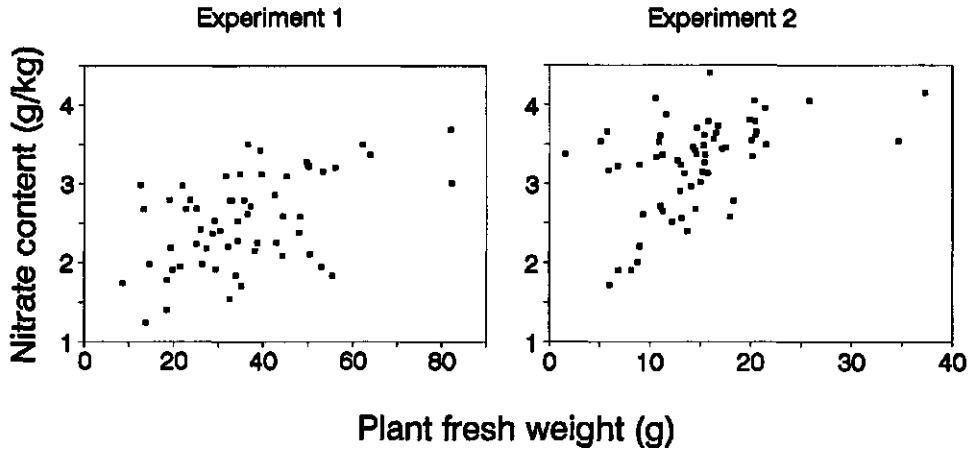


***Fig. 2.2.*** **Relationship between nitrate content ($g \cdot kg^{-1}$) and plant fresh weight at harvest (g) in experiments 1 and 2.**

tion of this mutant was also significantly lower than that of control cultivars. However, if there is a causal relationship between nitrate and dry matter content in lettuce, this relationship can explain only a part of the genotypic differences. The correlation between both traits was high within the group of butterhead accessions, but only slightly significant or absent within the other plant types.

Blom-Zandstra and Lampe (1985) showed that nitrate may serve as an

osmoticum to compensate for a shortage of carbohydrates at low light intensities. The negative relationship between nitrate and dry matter content found in butterhead lettuce, could be explained if accessions with a high dry matter content also are characterized by a relatively high content of carbohydrates in their vacuole. A higher content of carbohydrates in the vacuole would reduce the need for nitrate for maintaining osmotic pressure.

The positive correlation between plant fresh weight and nitrate content does not support the earlier observations of Eenink (1984), but is in accordance with the results of Handke and Junge (1984) with the spinach mutant. The latter authors state that at present a low content of nitrate is more important than a high fresh matter production. For lettuce grown in glasshouses in winter, where a high fresh matter production has always been one of the most important breeding characteristics, this statement cannot be endorsed, if the winter production of lettuce is to remain profitable. The variation in the tested accessions, however, seems large enough to enable selection of fast growing accessions with low nitrate content.

## References


Blom-Zandstra, M. & J.E.M. Lampe, 1985. The role of nitrate in the osmoregulation of lettuce (*Lactuca sativa* L.) grown at different light intensities. Journ. Exp. Bot. 36, 1043-1052.

Eenink, A.H., 1984. Vorderingen in het veredelingsonderzoek gericht op reductie van nitraatgehaltes in kropsla. Bedrijfsontwikkeling 15, 254-256.

Eenink, A.H., M. Blom-Zandstra, P.C.H. Holleman, P. Aarts & R. Groenwold, 1984. Research on the reduction of nitrate content in lettuce via breeding. Proc. Eucarpia Meeting Leafy Vegetables, Versailles, 28Febr.- 2 March 1984, 100-109.

Groenwold, R., 1985. Nitraat: voetangels en klemmen voor het onderzoek. Tuinderij, 4 juli, 32-33.

Handke, S. & H. Junge, 1984. Green matter yield and nitrate content in a dioecious spinach genotype with a long vegetative phase. Proc. Eucarpia Meeting Leafy Vegetables, Versailles, 28 Febr.- 2 March 1984, 110-114.

# 3 Genetics of Nitrate Content in Lettuce, 1: Analysis of Generation Means

*The genetics of nitrate content in butterhead lettuce (*Lactuca sativa *L.) was studied using the mean values of five parental genotypes and several generations obtained from crosses between them. One high and four low nitrate parents were chosen. A diallel analysis showed additive genetic effects to be the major source of variation in generation means. Estimates of additive genetic effects differed significantly between experiments, indicating genotype × experiment interactions. Effects of dominance were relatively small. The size and direction of dominance varied between experiments. Reciprocal differences were of limited size and also varied between experiments. The inheritance of nitrate content in lettuce fitted the additive-dominance genetic model.*

## 3.1 Introduction

Screening of the lettuce collection of the Centre for Genetic Resources (CGN, Wageningen, the Netherlands), revealed large genotype differences in nitrate content of the shoot (Chapter 2). In cultivated lettuce the largest differences were found within the butterhead genotypes. Previous research on 16 $F_2$s and three $F_3$s of crosses between lettuce genotypes showed a quantitative inheritance of nitrate content (Reinink and Groenwold, 1987). However, these crosses were made before the screening of the lettuce collection was completed, the crosses were made between genotypes of different plant types and no $F_1$ or backcross populations, and only three $F_3$ populations were included. Therefore a more comprehensive study of the genetics of nitrate accumulation in lettuce was considered necessary. Because breeding for low nitrate content in the Netherlands is mainly relevant for the butterhead type, as this is the main type produced in glasshouses during the winter period, butterhead accessions were selected for a detailed study of the genetics of nitrate accumulation. The screening results described in Chapter 2 revealed larger genotypic differences for nitrate content within the butterhead type than available to Reinink and Groenwold (1987).

The aim of this research was to study the inheritance of nitrate content in crosses between butterhead genotypes that differed strongly in nitrate content and in crosses with two low-nitrate parents. The first type of cross represents the situation for most practical breeding programs. Modern glasshouse cultivars, which are high-nitrate genotypes, are crossed with genebank accessions, chosen for their low nitrate content, but otherwise lacking many traits required in modern cultivars. The results of this study should show how difficult it is to introduce the low-nitrate trait into modern butterhead cultivars. The second type of cross, between low-nitrate butterhead accessions, was studied to find whether a further reduction of the nitrate content is possible by accumulating genes for low nitrate content from

*Table 3.1.* **Genebank code, accession name, country of origin and mean nitrate content (g per kg fresh weight) in two previous screenings experiments (Chapter 2) of the parental butterhead genotypes**

| Parent | CGN[1] code | Accession name | Country | Mean nitrate in previous experiments ($g \cdot kg^{-1}$) |
|---|---|---|---|---|
| $G_1$ | 9277 | Panvit | Netherlands | 4.0 |
| $G_2$ | 5233 | Reichenauer Winter | Switzerland | 1.5 |
| $G_3$ | 4892 | Winterbutterkopf | Germany | 1.9 |
| $G_4$ | 4944 | Trocadero Light 76 | Italy | 2.4 |
| $G_5$ | 5811 | *L. sativa capitata* | Romania | 1.9 |

[1]CGN: Centre for Genetic Resources, P.O.Box 16, 6700 AA Wageningen, the Netherlands.

several low-nitrate parents.

This chapter describes the results of three experiments designed to test whether the relatively simple additive-dominance (AD) model is adequate in the study of the genetics of nitrate content in butterhead lettuce. In one experiment a diallel set of crosses was grown to evaluate additive, dominance and reciprocal effects. In two other experiments the applicability of the AD model was tested using parental, $F_1$, $F_2$ and backcross (BC) generations of crosses between one high-nitrate genotype and four low-nitrate genotypes.

## 3.2 Materials and methods

*Genotypes and experimental design*

Table 3.1 presents data about the lettuce genotypes selected as parents in this study. The selection was based on the screening results presented in Chapter 2. One genotype ($G_1$), representative of modern butterhead cultivars grown in glasshouses in winter, was chosen for its extremely high nitrate content. The other four were low-nitrate genotypes ($G_2$, $G_3$, $G_4$ and $G_5$). These genotypes are not adapted to cultivation in glasshouses in winter.

In experiment 1, a diallel set of crosses were grown, containing the parental genotypes and the $F_1$s resulting from all possible crosses between the parents, including reciprocals. The experiment utilized 60 plants of each parent and 30 plants of each $F_1$. The plants were grown in a randomized block design with 30 replicates. Each replicate contained one plant of each $F_1$ and two plants of each parent.

In experiment 2, generations were tested which resulted from crosses between the high-nitrate genotype $G_1$ and three low-nitrate genotypes: $G_2$, $G_3$ and $G_4$. The experiment utilized 30 plants of each parent and $F_1$, 50 plants of the $F_2$ and 100 plants of each BC from crosses of the $F_1$ on both parents ($BC_1^1$ and $BC_1^2$). The cross between $G_1xG_5$ could not be evaluated in experiment 2 because not all generations were available at that time. In experiment 2 plants were grown in a randomized block design with ten replicates. Each replicate contained three plants of each parent and $F_1$, five plants

of each $F_2$ and ten plants of each BC. Plants were completely randomized within replicates.

Experiment 3 was carried out to confirm the results of the previous two experiments. Generations were tested which resulted from crosses between the high-nitrate parent and the four low-nitrate parents: $G_1xG_2$, $G_1xG_3$, $G_1xG_4$ and $G_1xG_5$. The experiment utilized 20 plants of each parent and $F_1$ generation and 40 plants of the $F_2$ and BC generations. Plants were grown in a randomized block design with four replicates. Each replicate contained five plants of each parent and $F_1$ and ten plants of each $F_2$ and BC. Plants were completely randomized within replicates.

All plants of the $F_1$ and BC generations were carefully checked morphologically at several ages and plants resulting from self-fertilization were excluded from the analysis.

*Growth conditions*

All plants were grown in the same glasshouse. Seeds were sown in trays and the plants were transplanted to 80 $cm^3$ slippots filled with peat-based compost, which were then placed in gullies with a recirculating nutrient solution (NFT-system). Plants were grown under natural daylight conditions at minimum day and night temperatures of 12°C and 7°C, respectively. The glasshouse was ventilated if the temperature exceeded 15°C during the day or 9°C at night. The nutrient solution was continually monitored for pH and electroconductivity (EC) and a full chemical analysis of the solution was carried out every two weeks. The mean nitrate concentration of the nutrient solution was 14 $mmol \cdot l^{-1}$, with extreme values ranging from 12 to 15 $mmol \cdot l^{-1}$. Because ammonia is known to reduce the nitrate content of plants grown on a nutrient solution (Van der Boon et al., 1990), the concentration of ammonia in the nutrient solution was kept below 0.1 $mmol \cdot l^{-1}$. The pH was maintained at 6.0 and the EC at 2.0 $ms \cdot cm^{-1}$.

In experiment 1 seeds were sown on October 8, 1987, plants transplanted on November 6, 1987, and harvested from January 18 to 20, 1988. In experiment 2 seeds were sown on September 21, 1987, plants transplanted on October 15, 1987, and harvested from November 30 to December 4, 1987. In experiment 3 seeds were sown on November 10, 1989, plants transplanted on December 27, 1989, and harvested from February 26 to March 1, 1990.

*Harvest and chemical analysis*

Harvesting was carried out between 08.30 h and 11.00 h on several successive mornings. Plants were cut from the roots, weighed, ground in a blender and the sap pressed through cheesecloth into tubes, which were then closed and stored at -18°C prior to further analysis. The roots of all plants were checked and those with brown roots were excluded from the analysis. After thawing and dilution, the nitrate concentration of the sap was measured using an autoanalyser (Skalar, Breda, the Netherlands).

*Statistical analyses*

Means and variances for nitrate concentration were calculated for each population after correction for the effects of blocks. Significance of differences between means were tested with a two

***Table 3.2.*** **Matrix of coefficients used to estimate genetic effects from generation means (Mather and Jinks, 1982)**

| Genetic effect [a] | Generation [b] | | | | | |
|---|---|---|---|---|---|---|
| | $P_1$ | $P_2$ | F1 | $F_2$ | $BC_1^1$ | $BC_1^2$ |
| m | 1 | 1 | 1 | 1 | 1 | 1 |
| a | 1 | −1 | 0 | 0 | 0.5 | −0.5 |
| d | 0 | 0 | 1 | 0.5 | 0.5 | 0.5 |

[a] m: midparent value; a: additive genetic effect; d: dominance effect

[b] $P_1$ is the high-nitrate parent, $P_2$ the low-nitrate parent, $BC_1^1$ is the $F_1(P_1x(F_1(P_1xP_2)))$ and $BC_1^2$ is the $F_1(P_2x(F_1(P_1xP_2)))$

***Table 3.3.*** **Diallel table with mean nitrate concentration ($NO_3^-$ ; $g \cdot l^{-1}$) and residual variance of individual plant measurements ($s_e^2$ ; $g^2 \cdot l^{-2}$) of parents and $F_1$s (after correction for effects of replicates) of a diallel set of crosses between five butterhead genotypes. The number of harvested plants per population (*N*) is given in brackets. For details about parental genotypes see Table 3.1**

| Female parent | | Male parent | | | | |
|---|---|---|---|---|---|---|
| | | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $G_5$ |
| $G_1$ | $NO_3^-$ | **4.87** | **3.80** | **4.03** | **3.81** | **4.18** |
| | $s_e^2$ | 0.0561 | 0.0530 | 0.0652 | 0.0508 | 0.0395 |
| | *N* | *(60)* | *(27)* | *(27)* | *(30)* | *(30)* |
| $G_2$ | $NO_3^-$ | **3.83** | **2.58** | **2.90** | **2.78** | **2.98** |
| | $s_e^2$ | 0.0241 | 0.0542 | 0.0349 | 0.0271 | 0.0369 |
| | *N* | *(30)* | *(59)* | *(21)* | *(29)* | *(30)* |
| $G_3$ | $NO_3^-$ | **3.93** | **2.86** | **3.22** | **2.94** | **3.20** |
| | $s_e^2$ | 0.0462 | 0.0437 | 0.0286 | 0.0419 | 0.0324 |
| | *N* | *(30)* | *(29)* | *(60)* | *(30)* | *(30)* |
| $G_4$ | $NO_3^-$ | **3.68** | **2.75** | **2.82** | **3.23** | **2.99** |
| | $s_e^2$ | 0.0388 | 0.0406 | 0.0817 | 0.0533 | 0.0214 |
| | *N* | *(29)* | *(30)* | *(8)* | *(57)* | *(15)* |
| $G_5$ | $NO_3^-$ | **3.99** | **2.92** | **3.23** | **3.08** | **3.39** |
| | $s_e^2$ | 0.0459 | 0.0187 | 0.0554 | 0.0334 | 0.0310 |
| | *N* | *(29)* | *(30)* | *(28)* | *(29)* | *(60)* |

sided t-test. Differences between populations in residual variance were analyzed using Bartlett's test for homogeneity of variances (Snedecor and Cochran, 1980). In experiment 1 the significance of additive and dominance effects and of reci-

procal differences between $F_1$s were analyzed using the fixed model described by Hayman (1954). In this model the total variation in the diallel table is subdivided into four major components: ***a*** measures the variation between the mean effects of each parental line whether used as male or female parent; ***b*** measures the variation due to dominance; ***c*** measures the variation due to the average maternal effects of each parent and ***d*** measures the variation in the reciprocal differences that cannot be ascribed to ***c***. The measure of variation due to dominance (***b***), is subdivided into three components: $b_1$ measures the mean dominance of all $F_1$s; $b_2$ measures the variation due to differences in mean dominance between the progeny of each parent and $b_3$ measures the variation due to dominance not ascribable to $b_1$ or $b_2$.

In experiments 2 and 3 the joint scaling test (Mather and Jinks, 1982) was used to test the applicability of the AD model. Means for nitrate concentration were analyzed for each set of generations (P's, $F_1$, $F_2$, $BC_1$s) using weighted regression according to the model:

$$Y = m + k_1 a + k_2 d,$$

where Y is the generation mean, m is the midparent value, a is the additive genetic effect, d is the dominance effect and $k_1$ and $k_2$ are coefficients specific for each generation (Table 3.2). The weight used in the regression was the number of analyzed plants per generation divided by the estimated residual variance of the generation. The significance of each estimated effect was tested with a t-test and the adequacy of the model was tested with a Chi-square test (Mather and Jinks, 1982). The proportion of variance explained by the AD model was judged using the adjusted squared correlation coefficients:

$$R^2_{adj} = 1 - (MS_{residual} / MS_{total}).$$

### 3.3 Results

*Experiment 1*

The mean nitrate concentration of the plant sap, residual variance and number of harvested plants per generation in experiment 1 are given in Table 3.3. Just as in the previous screening experiments (Table 3.1), $G_1$ and $G_2$ had the highest and lowest nitrate content of the parental genotypes, respectively. Bartlett's test showed significant differences between the estimates of the residual variances of the parents and $F_1$s included in experiment 1 ($0.025<p<0.05$). Two parents, $G_3$ and $G_5$, had low estimates for residual variance, while the other three had significantly higher estimates. The estimates of the residual variance for the $F_1$s ranged from 0.0187 to 0.0817 $g^2 \cdot l^{-2}$. However, the highest estimate was based on only seven degrees of freedom, owing to a large number of self-fertilized plants in the cross $G_4 x G_3$. The second highest estimate was 0.0652 $g^2 \cdot l^{-2}$, based on 26 degrees of freedom. No relationship could be found between the $s^2_e$ values of parents and their $F_1$s, indicating a non-heritable cause of the observed differences in residual variance. By comparing the residual variances of those populations which were grown in more than one experiment, it was found that significant differences in residual

variance were not reproducible. Also in another research (Chapter 5), in which the parental genotypes were tested in 18 experiments, no indications were obtained that genotypes $G_1$ to $G_5$ differed systematically in residual variance. Therefore, further calculations were made using the pooled residual variance. The average $s_e^2$ value over all $F_1$s was 0.042 $g^2 \cdot l^{-2}$ and the average over all parental genotypes was 0.045 $g^2 \cdot l^{-2}$. This means that the heterozygous $F_1$s are not significantly more stable for nitrate content than the homozygous parental genotypes. No significant relationship was found between the level of nitrate content and the residual variance (r=0.24), indicating that the differences in residual variance are not an effect of scale. The mean coefficient of variation over all parental genotypes and $F_1$s was 6.2 %.

Table 3.4 presents the results of the analysis of variance for a diallel table as proposed by Hayman (1954). Per replicate, only one randomly chosen parental plant was included in this analysis to obtain an equal number of plants per cell of the diallel table. The mean parental effect, ***a***, is highly significant, indicating large additive genetic variation between the parents. The significance of the variation due to dominance, ***b*** indicates the presence of dominance effect. The high significance of $b_1$, which measures the mean dominance of all $F_1$s, indicates that the dominance deviations are predominantly in one direction. Table 3.3 shows that dominance is predominantly directed towards low nitrate content. The significance of $b_2$, which measures variation due to differences in mean dominance between the progeny of each parent, indicates that the five parental genotypes vary for the mean dominance deviation of their $F_1$s. The mean dominance deviations for $G_1$ to $G_5$ are -0.076, -0.006, -0.124, -0.266 and -0.095 $g \cdot l^{-1}$, respectively. This shows that the low-nitrate genotypes $G_2$ and $G_4$ differ strongly for mean dominance deviation, indicating that they vary in number or size of effect of dominant alleles. Although $b_3$, the part of the dominance deviation that cannot be ascribed to $b_1$ or $b_2$ and which is unique to each $F_1$ (Mather and Jinks, 1982), is significant, its size is only small compared to the other parameters, indicating only limited effects of specific combining ability.

The parameter which measures the average maternal effect of the parental genotypes, ***c***, is highly significant. The mean maternal effect for $G_1$ to $G_5$ are 0.098, 0.037, -0.013, -0.092 and -0.030 $g \cdot l^{-1}$, respectively. This shows that the significance of ***c*** was mainly determined by the maternal effects of $G_1$ and $G_4$. The occurrence of reciprocal differences means that the ***a*** item must be tested against the ***c*** mean square (Mather and Jinks, 1982). Reciprocal differences, not ascribable to ***c*** (***d***) are not significant, indicating that all maternal effects can be adequately described by the average maternal effect per parent. A separate t-test of all corresponding pairs of $F_1$s showed only significant reciprocal differences for $G_1 \times G_4$ (0.13 $g \cdot l^{-1}$, $p<0.05$) and $G_1 \times G_5$ (0.19 $g \cdot l^{-1}$, $p<0.001$). In both cases the $F_1$ with the high-nitrate genotype $G_1$ as female parent had the highest nitrate content.

***Table 3.4.*** **Analysis of variance of the diallel table for nitrate concentration ($g \cdot l^{-1}$) obtained in experiment 1. (SS: sum of squares; %SS: percentage of total sum of squares; df: degrees of freedom; MS: mean square; VR: variance ratio)**

| Parameters [1] | SS | %SS | df | MS | VR | Probability |
|---|---|---|---|---|---|---|
| ***a*** | 220.52 | 83.8 | 4 | 55.131 | 226.6 | <0.001 |
| ***b*** | 6.59 | 2.5 | 10 | 0.659 | 15.2 | <0.001 |
| $b_1$ | 1.77 | 0.7 | 1 | 1.772 | 40.8 | <0.001 |
| $b_2$ | 4.17 | 1.6 | 4 | 1.043 | 24.0 | <0.001 |
| $b_3$ | 0.65 | 0.2 | 5 | 0.130 | 3.0 | 0.01-0.05 |
| ***c*** | 0.98 | 0.4 | 4 | 0.245 | 6.0 | <0.001 |
| ***d*** | 0.42 | 0.2 | 6 | 0.071 | 1.6 | >0.1 |
| $\Sigma(a,b,c,d)$ | 228.52 | 86.8 | 24 | | | |
| Replicates | 7.05 | 2.7 | 29 | 0.243 | 5.6 | <0.001 |
| Residual | 27.70 | 10.5 | 637 [2] | 0.043 | | |

[1] ***a***: variation between the mean effects of each parental line; ***b***: variation due to dominance; $b_1$: mean dominance over all $F_1$'s; $b_2$: variation due to differences in mean dominance between the progeny of each parent; $b_3$: variation due to dominance not ascribable to $b_1$ or $b_2$; ***c***: variation due to the average maternal effects of each parent; ***d***: variation in the reciprocal differences that cannot be ascribed to ***c***.
[2] 59 values missing

***Table 3.5.*** **Mean nitrate concentration ($NO_3^-$ ; $g \cdot l^{-1}$) and residual variance of individual plant measurements ($s_e^2$ ; $g^2 \cdot l^{-2}$) of parents, $F_1$, $F_2$, $BC_1^1$ and $BC_1^2$ generations of crosses between four butterhead genotypes tested in experiment 2. The number of harvested plants per generation (*N*) is given in brackets. For details about parental genotypes see Table 3.1**

| Cross ($P_1$×P2) | | Generation [a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $F_1$ | $F_2$ | $BC_1^1$ | $BC_1^2$ |
| $G_1$×$G_2$ | $NO_3^-$ | **4.89** | **2.87** | **4.17** | **4.03** | **4.64** | **3.57** |
| | $s_e^2$ | 0.0682 | 0.0461 | 0.0364 | 0.0899 | 0.0550 | 0.0842 |
| | *N* | *(30)* | *(30)* | *(30)* | *(50)* | *(98)* | *(98)* |
| $G_1$×$G_3$ | $NO_3^-$ | **4.89** | **3.33** | **4.28** | **4.21** | **4.64** | **3.85** |
| | $s_e^2$ | 0.0682 | 0.0399 | 0.0548 | 0.0785 | 0.0658 | 0.0552 |
| | *N* | *(30)* | *(30)* | *(27)* | *(50)* | *(100)* | *(96)* |
| $G_1$×$G_4$ | $NO_3^-$ | **4.89** | **3.11** | **3.90** | **3.93** | **4.44** | **3.49** |
| | $s_e^2$ | 0.0682 | 0.0532 | 0.0306 | 0.1327 | 0.0691 | 0.0664 |
| | *N* | *(30)* | *(30)* | *(30)* | *(50)* | *(100)* | *(99)* |

[a] $BC_1^1$ is the $F_1(P_1x(F_1(P_1xP_2)))$; $BC_1^2$ is the $F_1(P_2x(F_1(P_1xP_2)))$

*Experiments 2 and 3*

Table 3.5 gives the mean nitrate concentration and residual variances for each generation in experiment 2. Once again $G_1$ and $G_2$ were the most extreme parents. In this experiment, unlike experiment 1, $G_4$ had a significantly lower nitrate concentration than $G_3$. Table 3.6 gives the mean nitrate concentrations and residual variances obtained in experiment 3. In this experiment $G_4$, not $G_2$, had the lowest nitrate concentration. Of the four reciprocal $F_1$s included in experiment 3, only the $F_1$s of $G_1 \times G_4$ were significantly different from each other ($F_1(G_1 \times G_4) - F_1(G_4 \times G_1) = -0.26$ g· $l^{-1}$, $P<0.001$). In contrast to the results of experiment 1, no reciprocal difference between the $F_1$s of $G_1 \times G_5$ were found in experiment 3. Although the cross $G_1 \times G_4$ showed a significant reciprocal effect in both experiments 1 and 3, the direction of the effect was opposite in the two experiments.

Table 3.7 presents the estimates from weighted regression of the genetic effects according to the AD model. For the three crosses tested both in experiments 2 and 3, the analysis was carried out per experiment and on the pooled values of both experiments. Although for $G_1 \times G_2$ in both experiments and for $G_1 \times G_4$ in experiment 3, the data deviated significantly from values predicted by the AD model, all the pooled means were consistent with it. Testing the AD model on other scales (logarithmic, square root and square-transformation) did not improve the fit. The values of the adjusted squared correlation coefficient ($R^2_{adj}$) show that even in those cases where the data did not fit the AD

***Table 3.6.*** **Mean nitrate concentration ($NO_3^-$ ; g· $l^{-1}$) and residual variance of individual plant measurements ($s_e^2$ ; $g^2 \cdot l^{-2}$) of parents, $F_1$, $F_2$, $BC_1^1$ and $BC_1^2$ generations of crosses between five butterhead genotypes tested in experiment 3. The number of harvested plants per generation (*N*) is given in brackets. For details about parental genotypes see Table 3.1**

| Cross ($P_1 \times P_2$) | | Generation [a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $F_1$ | $F_1'$ | $F_2$ | $BC_1^1$ | $BC_1^2$ |
| $G_1 \times G_2$ | $NO_3^-$ | **4.81** | **2.94** | **3.94** | **4.08** | **3.83** | **4.34** | **3.34** |
| | $s_e^2$ | 0.0362 | 0.0288 | 0.0435 | 0.0584 | 0.0810 | 0.0638 | 0.0691 |
| | *N* | *(20)* | *(20)* | *(16)* | *(19)* | *(40)* | *(39)* | *(39)* |
| $G_1 \times G_3$ | $NO_3^-$ | **4.81** | **3.62** | **4.35** | **4.26** | **4.17** | **4.42** | **3.97** |
| | $s_e^2$ | 0.0362 | 0.0778 | 0.0991 | 0.0399 | 0.0783 | 0.1033 | 0.0686 |
| | *N* | *(20)* | *(20)* | *(12)* | *(20)* | *(40)* | *(40)* | *(40)* |
| $G_1 \times G_4$ | $NO_3^-$ | **4.81** | **2.74** | **3.42** | **3.68** | **3.69** | **3.96** | **3.18** |
| | $s_e^2$ | 0.0362 | 0.0775 | 0.0477 | 0.0224 | 0.1185 | 0.1171 | 0.0765 |
| | *N* | *(20)* | *(15)* | *(20)* | *(19)* | *(40)* | *(40)* | *(37)* |
| $G_1 \times G_5$ | $NO_3^-$ | **4.81** | **3.59** | **4.27** | **4.36** | **4.24** | **4.63** | **4.00** |
| | $s_e^2$ | 0.0362 | 0.1442 | 0.0622 | 0.0335 | 0.0998 | 0.0819 | 0.0884 |
| | *N* | *(20)* | *(20)* | *(20)* | *(18)* | *(39)* | *(40)* | *(39)* |

[a] $F_1'$ is the $F_1(P_2 \times P_1)$; $BC_1^1$ is the $F_1(P_1 \times (F_1(P_1 \times P_2)))$; $BC_1^2$ is the $F_1(P_2 \times (F_1(P_1 \times P_2)))$

***Table 3.7.*** **Estimated genetic effects with their standard deviations (in italics) for nitrate concentration ($g \cdot l^{-1}$) in four crosses. The adjusted squared correlation coefficient ($R^2_{adj}$) indicates the proportion of variance explained by the additive-dominance (AD) model. (m: midparent value; a: additive genetic effect; d: dominance genetic effect; df: degrees of freedom of $\chi^2$-test for goodness of fit of the AD model)**

| Cross | Expt | Genetic effect | | | Test of adequacy of AD-model | | | |
|---|---|---|---|---|---|---|---|---|
| | | m [a] | a [a] | d | $\chi^2$ | df | Probability | $R^2_{adj}$ |
| $G_1 \times G_2$ | 2 | 3.92 | 1.04 | 0.29*** [b] | 11.1 | 3 | < 0.005 | 0.991 |
| | | *0.026* | *0.023* | *0.046* | | | | |
| | 3 | 3.84 | 0.94 | 0.10* | 13.8 | 4 | < 0.005 | 0.985 |
| | | *0.026* | *0.026* | *0.047* | | | | |
| | mean | 3.87 | 0.99 | 0.20*** | 5.2 | 3 | 0.1-0.25 | 0.997 |
| | | *0.019* | *0.018* | *0.034* | | | | |
| $G_1 \times G_3$ | 2 | 4.13 | 0.79 | 0.20*** | 2.6 | 3 | 0.25-0.5 | 0.997 |
| | | *0.026* | *0.023* | *0.050* | | | | |
| | 3 | 4.20 | 0.57 | 0.04 | 7.8 | 4 | 0.1 | 0.965 |
| | | *0.032* | *0.032* | *0.055* | | | | |
| | mean | 4.16 | 0.66 | 0.12** | 3.9 | 3 | 0.25-0.5 | 0.995 |
| | | *0.021* | *0.029* | *0.038* | | | | |
| $G_1 \times G_4$ | 2 | 4.00 | 0.91 | -0.10* | 1.8 | 3 | 0.5-0.75 | 0.998 |
| | | *0.026* | *0.024* | *0.045* | | | | |
| | 3 | 3.76 | 0.99 | -0.19*** | 36.9 | 4 | < 0.005 | 0.947 |
| | | *0.034* | *0.034* | *0.048* | | | | |
| | mean | 3.87 | 0.93 | -0.16*** | 8.9 | 3 | 0.025-0.05 | 0.994 |
| | | *0.021* | *0.020* | *0.035* | | | | |
| $G_1 \times G_5$ | 3 | 4.22 | 0.61 | 0.12* | 3.8 | 4 | 0.25-0.5 | 0.981 |
| | | *0.037* | *0.037* | *0.055* | | | | |

[a] All m- and a-estimates were significantly different from zero ($p<0.001$).
[b] *, ***: significant at $p=0.05$ and $p=0.001$, respectively, according to a two-sided t-test.

model, the percentage of variance explained was very high.

As could be expected from the choice of greatly differing parents, in all crosses the estimates of the additive genetic effect (a) were highly significant. In contrast to the results of experiment 1, in experiments 2 and 3 dominance was predominantly directed towards high nitrate content. Significant estimates of the dominance effect (d) were positive for crosses $G_1 \times G_2$, $G_1 \times G_3$ and $G_1 \times G_5$ and negative only in $G_1 \times G_4$. These d-estimates for individual crosses were on average 0.1 to 0.2 $g \cdot l^{-1}$ higher than those estimated in experiment 1.

Figure 3.1 shows the observed generation means for nitrate concentration and the expected values according to the best fitting AD model for crosses in experiments 2 and 3. Also from Fig. 3.1 it is clear that even in those cases where the data did not fit the AD model, deviation from the expected values was relatively small. The largest deviation observed was 5.0% for the $BC_1^1$ of $G_1 \times G_4$ in experiment 3.

## 3.4 Discussion

Until now very little is known about the genetics of nitrate accumulation in vegetable crops. Known to the author are only two papers on this topic (Subramanya et al., 1980; Reinink and Groenwold, 1987), both on research in lettuce. The results of the above mentioned papers were conflicting (see Reinink and Groenwold, 1987), which could be due to the choice of the genotypes or the environmental conditions. A better understanding of the genetics of nitrate accumulation is needed to support practical plant breeding programs and to obtain information on the size of the reduction of nitrate content which could be reached by plant breeding.

Lettuce is strictly autogamous and the parental genotypes used in this study can be considered to be completely homozygous. As the parents were selected for their extreme values for nitrate content, the group of parental genotypes was considered as a non-random, fixed population and the diallel analysis was restricted to the detection of additive, dominance and reciprocal effects within this group of parental genotypes. No further analyses of genetic variance components were made because these would require assumptions (Wright, 1985) which are unrealistic (parents in linkage equilibrium) or which were proved to be wrong (absence of reciprocal effects).

The diallel analysis of experiment 1 showed that additive effects are of major importance to explain the variation between generation means (Table 3.4). Effects of dominance were also shown, but were of relatively small size. Low nitrate content was partially dominant in experiment 1. Reciprocal differences, indicating cytoplasmic or other maternal effects were also shown by the diallel analysis, but were of very limited size. The results on dominance conflict with those from Subramanya et al. (1980), who found complete dominance of low nitrate content in five lettuce crosses, but are consistent with results on $F_2$ generations obtained by Reinink and Groenwold (1987).

In other studies (Chapter 5) it was shown that the parental genotypes used in this study display genotype × environment interaction when tested under different environmental conditions. In terms of the genetic parameters used in this study this means that the additive genetic effect (a) is not constant from one experiment to another. In the experiments presented in this paper several traits are shown to vary between experiments. In experiment 1, dominance was mainly directed towards low nitrate content, which agrees with the results of Reinink and Groenwold (1987). However, estimated dominance effects in experiments 2 and 3 (Table 3.7) were predominantly directed towards high

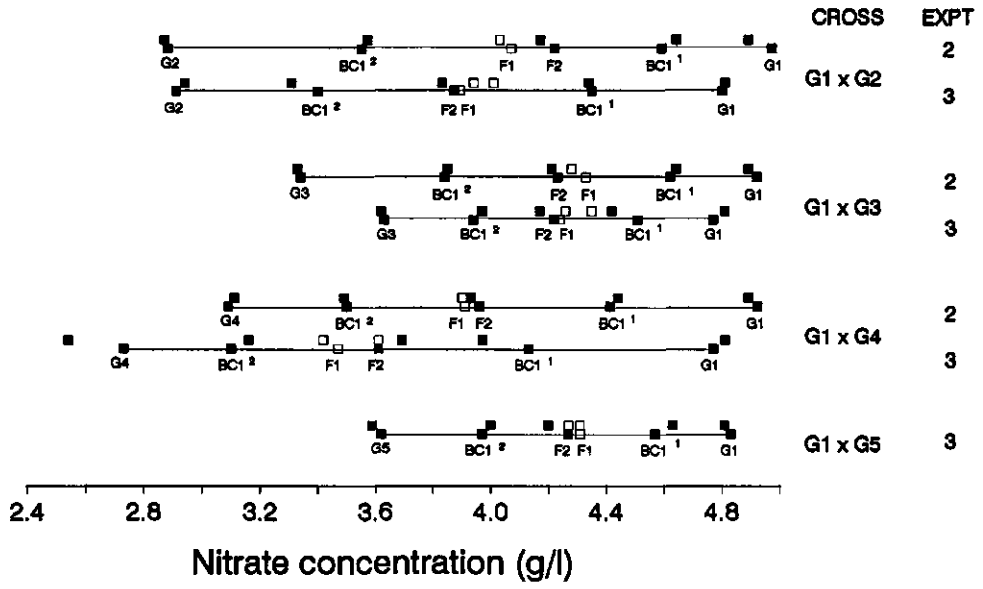


***Fig. 3.1.*** **Generation means for nitrate concentration ($g \cdot l^{-1}$) of the crosses tested in experiments (EXPT) 2 and 3. Squares above the lines present the observed means and squares on the lines give the expected means according to the best fitting additive-dominance model. Generation names are given below the lines.**

nitrate content. Only the crosses with $G_4$ as one of the parents showed partial dominance of low nitrate concentration in all experiments. In experiment 1, $G_4$ was shown to have by far the largest (negative) dominance deviation.

The additive genetic effect was also found to vary between experiments. Table 3.7 shows that the a-estimates of $G_1 \times G_2$ and $G_1 \times G_3$ obtained from experiments 2 and 3 differ significantly. For $G_1 \times G_4$ the a-estimates obtained from these two experiments were not significantly different, in agreement with other results (Chapter 5), in which $G_1$ and $G_4$ displayed a similar reaction to changing environmental conditions.

Maternal effects also differed between experiments. The diallel analysis of experiment 1 showed relatively small but significant maternal effects. When used as female parent, the high-nitrate genotype $G_1$ caused an increase in nitrate content and the low nitrate parent $G_4$ a decrease. However, the reciprocal differences shown in experiment 1 for $G_1 \times G_4$ and $G_1 \times G_5$ were not found in experiment 3, in which no difference between the reciprocal $F_1$s of $G_1 \times G_5$ were observed, and the reciprocal difference between the $F_1$s of $G_1 \times G_4$ was opposite to the first experiment. This shows that, although some maternal effects for nitrate content were found, these effects were of little importance and not reproducible.

The estimates of residual variance also showed conflicting results when comparing different experiments. In experiment 1 Bartlett's test showed significant differences in residual variance within the group of parental genotypes and within the group of $F_1$s. These differences were not related to the mean value of nitrate content and therefore could not be removed by changing scales. No relationship could be detected between the mean residual variance of parents and their offspring, indicating a non-genetic cause of the differences. The differences in residual variance found in experiment 1, were not confirmed by experiments 2 and 3.

Experiments 2 and 3 tested the applicability of the AD model, in which environmental effects are additive and genes are independent in action (no non-allelic interaction) and distribution (no linkage). Only crosses between parents greatly differing in nitrate content were used to test the AD model, because effects rendering this model inadequate (non-allelic interaction, linkage, maternal effects) were expected to be detected most efficiently in these crosses. Furthermore, in breeding programs aimed at the incorporation of low nitrate content into modern cultivars, the breeder will generally begin by crossing adapted lettuce genotypes, generally having high nitrate contents with unadapted genotypes with low nitrate contents. This situation is best represented by the type of crosses tested in experiments 2 and 3.

The restrictive AD model did not fit the data for all crosses in both experiments. Departures from the AD model can be caused by non-allelic interaction, possibly in combination with linkage (Mather and Jinks, 1982). However, in all cases the percentage of variance explained by the AD model was extremely high (95-99%). In experiment 3, which included reciprocal $F_1$s the rejection of the AD model for $G_1 \times G_4$ can partly be explained by the reciprocal difference observed for this cross. The varying results of $G_1 \times G_2$ and $G_1 \times G_4$ in experiments 2 and 3 show that the deviations from the AD model do not display a similar trend in both experiments. For this reason, and also because the AD model explained virtually all variation in generation means, no effort was made to test more complicated models, e.g. including two-locus interactions.

Thus, it can be concluded that the inheritance of nitrate content in lettuce can be satisfactorily described by the relatively simple AD genetic model. Therefore, predictions can be made about the potential of crosses for the selection of low nitrate lines in later generations, based on estimates of means and additive genetic variances, obtained in early generations (Jinks and Pooni, 1976). Estimates of the additive genetic variance, needed to make these predictions for the crosses used in this study, will be presented in the next chapter.

## References


Hayman, B. I., 1954. The analysis of variance of diallel tables. Biometrics 10: 235-244.

Jinks, J. L. & H. S. Pooni, 1976. Predicting the properties of recombinant inbred lines derived by single seed descent. Heredity 36: 253-266.

Mather, K. & J. L. Jinks, 1982. Biometrical Genetics.-3rd ed. Chapman and Hall, London, 396 pp.

Reinink, K. & R. Groenwold, 1987. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica 36: 733-744.

Snedecor, G.W. & W.G. Cochran, 1980. Statistical methods.-7th ed. The Iowa State University Press, Ames, Iowa, USA, 507 pp.

Subramanya, R., G. Vest, & S. Honma, 1980. Inheritance of nitrate accumulation in lettuce. HortScience 15: 525-526.

Van der Boon, J., J. W. Steenhuizen & E. G. Steingröver, 1990. Growth and nitrate concentration of lettuce as affected by total nitrogen and chloride concentration, $NH_4/NO_3$ ratio and temperature of the recirculating nutrient solution. J. Hort. Sci. 65: 309-321.

Wright, A. J., 1985. Diallel designs, analyses, and reference populations. Heredity 54: 307-311.

# 4 Genetics of Nitrate Content in Lettuce, 2: Components of Variance

*Components of variance for nitrate content were estimated in $F_2$ and $F_3$ generations of ten crosses. Additive genotypic variances (A) were estimated from $F_3$ variance components and from the covariance between $F_2$ plants and corresponding $F_3$ lines. Estimates of wide sense heritability of the $F_2$ from crosses between a high-nitrate genotype and four low nitrate genotypes ranged from 0.44 to 0.74 and the estimates for $\sqrt{A}$ ranged from 0.25 to 0.40 $g \cdot l^{-1}$. Estimated wide sense heritabilities of $F_2$s from six crosses involving two low nitrate parents ranged from 0.15 to 0.52. The parents of four of the low nitrate crosses showed relatively large effects of genotype × environment (G×E) interaction in successive experiments: the nitrate content of the parents reacted differently to environmental changes between experiments. Estimates of $\sqrt{A}$ for crosses between low nitrate genotypes without large effects of G×E interaction ranged from 0 to 0.19 $g \cdot l^{-1}$. The estimated probability of selecting transgressive low nitrate lines in the progeny of a cross between a high and a low nitrate genotype was low (P=0.002 - 0.039), indicating that large populations should be evaluated to combine the positive traits of modern high-nitrate cultivars with low nitrate content from genotypes not adapted to modern cropping practices. In the progenies from crosses between two low nitrate genotypes without important G×E effects, the estimates of the probability of obtaining transgressive low-nitrate lines were low (P=0.04 - 0.06). With the growth conditions used in this study, the probability of selecting lines with a nitrate content compatible under all winter conditions with the proposed future maximum permissible level of 2.5 g nitrate per kg fresh matter is low. Therefore the solution of this problem should be found in a combination of low-nitrate cultivars and cultural measures that reduce the nitrate content of the crop.*

## 4.1 Introduction

In previous research (Chapter 3) the nitrate contents of parental, $F_1$, $F_2$ and backcross populations of crosses between five lettuce genotypes of the butterhead type were described. Additive genetic effects constituted the major source of variation in generation means and only small effects of dominance were found. Both additive and dominance effects showed genotype × experiment interactions. Differences between reciprocal crosses were limited. It was concluded that the inheritance of nitrate content in lettuce could be described by a relatively simple additive-dominance genetic model.

This paper further elaborates the genetics of nitrate content in lettuce, using segregating $F_2$ and $F_3$ generations obtained from two types of crosses. In the first type the two parents differed strongly in nitrate content. These crosses are representative of breeding programs for the introgression of a low nitrate content from unadapted genotypes into modern cultivars. In the second type of

*Table 4.1.* **Genebank number (CGN code), cultivar name, country of origin and nitrate level of five lettuce genotypes of the butterhead type**

| Parent | CGN [1] code | Name | Country of origin | Nitrate [2] level |
|---|---|---|---|---|
| $G_1$ | 9277 | Panvit | Netherlands | high |
| $G_2$ | 5233 | Reichenauer Winter | Switzerland | low |
| $G_3$ | 4892 | Winterbutterkopf | Germany | low |
| $G_4$ | 4944 | Trocadero Light 76 | Italy | low |
| $G_5$ | 5811 | *Lactuca sativa capitata* | Romania | low |

[1]CGN: Centre for Genetic Resources, P.O. Box 16, 6700 AA, Wageningen, the Netherlands.
[2]see Chapter 2.

cross both parents had a low nitrate content. Based on the results the optimal breeding strategy can be developed and the potential results of breeding programs with these lines can be estimated.

## 4.2 Material and methods

*Experiments*

Five lettuce genotypes of the butterhead type were used as parents (Table 4.1). These genotypes were the same as used in previous studies (Chapter 3). Genotype $G_1$ (cv. Panvit) was chosen for its high nitrate content and genotypes $G_2$ to $G_5$ for their low nitrate contents. Crosses between these genotypes were made and $F_2$ and $F_3$ seed produced. In seven experiments 14 $F_2$ populations were studied. These crosses represented all combinations of two parents and four reciprocal combinations. The experiments included 200 plants of each $F_2$ and 40 plants of the corresponding parental genotypes. In experiments in which $G_1$ was not one of the parents, 20 plants of this high-nitrate genotype were included as an internal standard (Table 4.2).

The experimental design for the $F_3$ populations is shown in Table 4.3. All $F_3$ populations, except $F_3(G_1 \times G_5)$ were produced from the tested $F_2$ plants. No reciprocal $F_3$ populations were included, which reduced the number of $F_3$ populations to ten. Each $F_3$ line was represented by two plants. Each of the $F_3$ experiments included one quarter of the available $F_3$ lines of nine parental combinations. The $F_3(G_1 \times G_5)$ was tested one year later in a similar set of four experiments.

The parental genotypes and the segregating populations were distributed evenly over the replicates. In the experiments with $F_3$ material, the two plants per line were grown in the same replicate. Plants were completely randomized within replicates.

*Growth conditions*

Seeds were sown in trays and the plants were transplanted to 80 $cm^3$ perforated pots filled with peat soil, which were placed in gullies with a recirculating nutrient solution (NFT-system). Plants

***Table 4.2.*** **Experimental design to determine the nitrate content in $F_2$ populations**

| | Experiment number | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Sowing date | 25.09.86 | 29.09.86 | 8.10.86 | 13.10.86 | 6.10.86 | 3.11.86 | 13.10.88 |
| Harvest dates | 17-18.12.86 | 22-23.12.86 | 19-22.1.87 | 2,4-6.2.87 | 9,11-13.2.87 | 24-27.2.87 | 31.1-3.2.89 |
| Replicates | 4 | 4 | 4 | 4 | 4 | 5 | 4 |
| | Number of harvested plants | | | | | | |
| $G_1$ | 40 | 39 | 20 | 20 | 20 | 20 | 36 |
| $G_2$ | 40 | – | – | 39 | 40 | – | 28 |
| $G_3$ | – | 40 | 40 | 38 | – | 40 | 27 |
| $G_4$ | – | – | – | 40 | – | 40 | 19 |
| $G_5$ | – | – | 40 | – | 38 | 40 | 37 |
| $F_2(G_1 \times G_2)$ | 198 | – | – | – | – | – | – |
| $F_2(G_2 \times G_1)$ | 198 | – | – | – | – | – | – |
| $F_2(G_1 \times G_3)$ | – | 198 | – | – | – | – | – |
| $F_2(G_3 \times G_1)$ | – | 199 | – | – | – | – | – |
| $F_2(G_1 \times G_4)$ | – | – | – | – | – | 198 | – |
| $F_2(G_1 \times G_5)$ | – | – | – | – | – | – | 178 |
| $F_2(G_2 \times G_3)$ | – | – | – | 199 | – | – | – |
| $F_2(G_2 \times G_4)$ | – | – | – | 199 | – | – | – |
| $F_2(G_2 \times G_5)$ | – | – | – | – | 197 | – | – |
| $F_2(G_5 \times G_2)$ | – | – | – | – | 198 | – | – |
| $F_2(G_4 \times G_3)$ | – | – | – | – | – | 200 | – |
| $F_2(G_3 \times G_5)$ | – | – | 199 | – | – | – | – |
| $F_2(G_5 \times G_3)$ | – | – | 199 | – | – | – | – |
| $F_2(G_4 \times G_5)$ | – | – | – | – | – | 200 | – |

were grown under natural daylight conditions at minimum day and night temperatures of 12 °C and 7 °C respectively. The glasshouse was ventilated when the temperature exceeded 15 °C during the day or 9 °C at night. The nutrient solution was continually monitored for pH and electroconductivity (EC). The mean nitrate concentration of the nutrient solution was 13.5 mmol· $l^{-1}$, with extreme values ranging from 10 to 17 mmol· $l^{-1}$. The concentration of ammonia was maintained below 0.1 mmol· $l^{-1}$, the pH at 6.0 and the EC at 2.0 mS·$cm^{-1}$.

*Harvesting and chemical analysis*

The replicates of experiments were harvested on successive mornings between 08.30 h and 11.00 h. The leaves of the $F_2$ plants (except for $G_1 \times G_5$), were harvested leaving only the smallest

***Table 4.3.*** **Experimental design to determine the nitrate content in $F_3$ populations**

| | Experiment number | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Sowing | 14.10.87 | 23.10.87 | 26.10.87 | 9.11.87 | 13.10.88 | 25.10.88 | 16.11.88 | 1.12.88 |
| Harvest | 25-28.1.88 | 15-18.2.88 | 22-25.2.88 | 14-17.3.88 | 31.1-3.2-89 | 14-16.2.89 | 6-8.3.89 | 20-23.3.89 |
| Replicates | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 3 |
| | Number of harvested plants | | | | | | | |
| $G_1$ | 30 | 30 | 29 | 30 | 36 | 28 | 28 | 23 |
| $G_2$ | 30 | 30 | 30 | 30 | 28 | 30 | 25 | 27 |
| $G_3$ | 30 | 30 | 29 | 30 | 27 | 30 | 25 | 20 |
| $G_4$ | 29 | 30 | 30 | 30 | 19 | 27 | 25 | 21 |
| $G_5$ | 30 | 30 | 30 | 30 | 37 | 26 | 27 | 25 |
| $F_3(G_1 \times G_2)$ | 87 | 85 | 86 | 83 | - | - | - | - |
| $F_3(G_1 \times G_3)$ | 88 | 86 | 86 | 85 | - | - | - | - |
| $F_3(G_1 \times G_4)$ | 85 | 88 | 84 | 85 | - | - | - | - |
| $F_3(G_1 \times G_5)$ [1] | - | - | - | - | 85 | 94 | 89 | 85 |
| $F_3(G_2 \times G_3)$ | 86 | 88 | 85 | 84 | - | - | - | - |
| $F_3(G_2 \times G_4)$ | 85 | 86 | 88 | 83 | - | - | - | - |
| $F_3(G_2 \times G_5)$ | 58 | 58 | 58 | 53 | - | - | - | - |
| $F_3(G_4 \times G_3)$ | 86 | 84 | 88 | 85 | - | - | - | - |
| $F_3(G_3 \times G_5)$ | 86 | 83 | 85 | 85 | - | - | - | - |
| $F_3(G_4 \times G_5)$ | 83 | 84 | 83 | 85 | - | - | - | - |

[1]Because seed of the $F_3(G_1 \times G_5)$ was not available, this cross could not be included in the first set of $F_3$ experiments.

young leaves for regrowth of the plant for seed production. Of the $F_3$ plants and the $F_2(G_1 \times G_5)$ plants the entire shoot was harvested. The harvested material was weighed and ground in a blender. The sap was pressed through cheesecloth and collected in tubes, which were stored at -18°C until further analysis. The roots of all plants were checked and those with brown roots were excluded from analysis. After thawing and diluting, the nitrate concentration of the sap was measured using an autoanalyser (Skalar, Breda, the Netherlands).

*Statistical analysis of $F_2$ populations*

Means and phenotypic variances for nitrate concentration were calculated for each parent genotype and population after correction for effects of replicates. Significances of differences between means were tested with a two sided t-test, modified for unequal group size or unequal variances of homozygous lines and segregating populations (Snedecor and Cochran, 1980). For each

experiment, the residual variance ($\sigma_e^2$) was estimated as the weighted mean of the variances of all parent genotypes included in the experiment. The genotypic variance of the $F_2$ populations ($\sigma_{gF2}^2$) was estimated by subtracting the estimate of the residual variance from the calculated phenotypic variance of the $F_2$ population: $\hat{\sigma}_{gF2}^2 = \hat{\sigma}_{pF2}^2 - \hat{\sigma}_e^2$. A 95% confidence interval for $\hat{\sigma}_{gF_2}^2$ was calculated following the procedure described by Tai (1989).

*Statistical analysis of $F_3$ populations*

Means and phenotypic variances of parental genotypes and $F_3$ populations were calculated. For each population, the importance of genotype × environment (G×E) interactions for nitrate content in the series of four experiments conducted was evaluated by means of the data for both parents of the cross. The effects of experiments, genotypes and G×E interactions were fitted for each combination of two parents and the proportion of variance explained by each factor was judged by their contribution to the adjusted squared correlation coefficients:

$$R_{adj}^2 = 1 - (MS_{residual} / MS_{total}).$$

The importance of G×E interactions relative to the sum of variance components containing genetic effects (genotype and G×E effects) was expressed as:

$$\text{Ratio} = R_{adj\ (ge)}^2 / R_{adj\ (g+ge)}^2,$$

where $R_{adj\ (ge)}^2$ is the adjusted squared correlation coefficient attributed to G×E interaction and $R_{adj\ (g+ge)}^2$ is the adjusted squared correlation coefficient attributed to both genotype and G×E effects. For $F_3$s from parents not displaying important G×E interaction, additive genotypic variances and confidence intervals were estimated from the pooled results of the four experiments. For $F_3$s from parents showing important G×E interactions, genotypic variances and confidence intervals were estimated separately for each of the four successive experiments.

*Estimation of the additive component of the genotypic variance in $F_3$ populations*

To be able to predict the probability of obtaining lettuce lines with transgression for low nitrate content from a specific cross, the mean and the variance of all random inbred ($F_\infty$) lines must be estimated (Jinks and Pooni, 1976). The genotypic variance of the $F_3$ is a linear combination of two components: the additive genotypic variance (A) and the dominance genotypic variance (D). Assuming that nitrate content is caused by independently acting genes, the absence of linkage, stochastic variation and G×E interactions, the genotypic variance of the $F_\infty$ equals A, which can be estimated in several ways in an $F_3$ population (Mather and Jinks, 1982). The dominance component of the genotypic variance (D) diminishes in advanced generations of an inbreeding program due to increasing homozygosity. Unbiased estimates of A and D (designated $\hat{A}_1$ and $\hat{D}_1$) can be made in the $F_3$ population from the between line and within line variances (Mather and Jinks, 1982). Van Ooijen (1989) has shown that in most situations $\hat{D}_1$ has a very limited practical value because it has a very large mean square error and is highly and negatively

correlated with $\hat{A}_1$. Furthermore, Van Ooijen has shown that A can usually be estimated with a smaller mean square error using a biased estimator ($\hat{A}_2$), proposed by Jinks and Pooni (1980):

$$\hat{A}_2 = 2 \cdot \hat{\sigma}^2_{gbF_3},$$

in which $\sigma^2_{gbF_3}$ is the genotypic between $F_3$ line variance. Although $\hat{A}_2$ has a bias of $\frac{1}{8} \cdot D$, it outperforms the unbiased estimator $\hat{A}_1$ in almost all cases of practical interest (Van Ooijen, 1989).

In the present study two other estimators of the additive genotypic variance, designated $\hat{A}_3$ and $\hat{A}_4$, are used. $\hat{A}_3$ is calculated as:

$$\hat{A}_3 = 4/3 \cdot (\hat{\sigma}^2_{pF_3} - \hat{\sigma}^2_e),$$

in which $\sigma^2_{pF_3}$ is the overall phenotypic variance of the $F_3$. It can be shown (see appendix) that although the bias of $\hat{A}_3$ is twice as large as that of $\hat{A}_2$ (i.e. $\frac{1}{4} \cdot D$), in the experimental set up used in this study $\hat{A}_3$ outperforms both $\hat{A}_1$ and $\hat{A}_2$. Confidence intervals for $\hat{A}_3$ were calculated according to Tai (1989).

A fourth estimator of A ($\hat{A}_4$) used in this study is based on the covariance between the values for $F_2$ plants and the means of corresponding $F_3$ lines:

$$\hat{A}_4 = 2 \cdot \sigma (F_2, \overline{F}_3).$$

The bias of $\hat{A}_4$ is of the same size than that of $\hat{A}_3$, i.e. $\frac{1}{4} \cdot D$ (Mather and Jinks, 1982):

$$E(\hat{A}_4) = 2 \cdot E(\sigma (F_2, \overline{F}_3)) = A + \frac{1}{4} \cdot D$$

Because $\hat{A}_4$ is calculated using $F_3$ line means from one experiment and $F_2$ data from another, it can be expected to be less influenced by G×E interactions than $\hat{A}_3$ (Casler, 1982). A disadvantage of $\hat{A}_4$ is that because it is based on a covariance, no method is known to calculate mean square errors and confidence limits.

In combination with the parental mean value, the estimate of A can be used to predict the proportion of random inbred lines in advanced generations ($F_\infty$) exceeding a certain threshold value (Jinks and Pooni, 1976). This prediction assumes a normal frequency distribution of nitrate content in the $F_\infty$ population and the absence of stochastic variation, epistasis, linkage and G×E interactions. The threshold value used can be equal to the mean of one of the parents, in which case the frequency of transgressive segregants is predicted. For nitrate content in lettuce another interesting threshold value is the maximum permissible level which in the near future will be imposed by legal measures. Therefore, for each cross, not only the fraction of $F_\infty$ lines showing transgression towards low nitrate contents was predicted, but also the fraction of lines with a nitrate content below the future maximum level under mid-winter conditions.

## 4.3 Results

*$F_2$ populations*

The nitrate concentration of the $F_2$ populations and parents are given in Table 4.4. The nitrate concentration of $G_1$, which was included in all experiments, ranged from 4.50 to 5.08 $g \cdot l^{-1}$. This relatively small range indicates that the

***Table 4.4.*** **Mean nitrate concentrations ($g \cdot l^{-1}$) in $F_2$ experiments of genotype $G_1$, the parents $P_1$ and $P_2$ ($P_1$ is identical to $G_1$ in some crosses), the midparent ($\bar{P}$) and the $F_2$ of 14 crosses**

| Cross $P_1 \times P_2$ | Experiment No | Type [1] | Nitrate concentration ($g \cdot l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $G_1$ | $P_1$ | $P_2$ | $\bar{P}$ | $F_2(P_1 \times P_2)$ | $F_2(P_2 \times P_1)$ |
| $G_1 \times G_2$ | 1 | h×l | 5.03 | 5.03 | 3.03 | 4.03 | 4.13 * | 4.08 |
| $G_1 \times G_3$ | 2 | h×l | 4.96 | 4.96 | 3.44 | 4.20 | 4.21 | 4.22 |
| $G_1 \times G_4$ | 6 | h×l | 4.76 | 4.76 | 2.47 | 3.62 | 3.52 * | - |
| $G_1 \times G_5$ | 7 | h×l | 5.08 | 5.08 | 3.73 | 4.40 | 4.49 | - |
| $G_2 \times G_3$ | 4 | l×l | 4.90 | 2.63 | 3.18 | 2.91 | 3.06 * | - |
| $G_2 \times G_4$ | 4 | l×l | 4.90 | 2.63 | 2.93 | 2.78 | 2.86 * | - |
| $G_2 \times G_5$ | 5 | l×l | 4.50 | 2.36 | 3.14 | 2.75 | 2.93 * | 2.88 * |
| $G_3 \times G_4$ | 6 | l×l | 4.76 | 3.41 | 2.47 | 2.94 | - | 2.81 * |
| $G_3 \times G_5$ | 3 | l×l | 4.91 | 3.20 | 3.16 | 3.18 | 3.11 * | 3.15 |
| $G_4 \times G_5$ | 6 | l×l | 4.76 | 2.47 | 3.34 | 2.91 | 2.84 * | - |

[1] h×l: cross between a high- and a low-nitrate genotype; l×l: cross between two low-nitrate genotypes.
* : $F_2$ mean significantly deviating from midparent value at P=0.05.

***Table 4.5.*** **Estimates of residual ($\sigma^2_e$, $g^2 \cdot l^{-2}$) and genotypic variances ($\sigma^2_{gF_2}$, in $g^2 \cdot l^{-2}$, with 95% confidence interval) for nitrate concentration in $F_2$ experiments**

| Cross ($P_1 \times P_2$) | Experiment No | Type [1] | Variances for nitrate concentration | | | | |
|---|---|---|---|---|---|---|---|
| | | | $\hat{\sigma}^2_e$ | $\hat{\sigma}^2_{gF_2(P_1 \times P_2)}$ | $\hat{\sigma}^2_{gF_2(P_2 \times P_1)}$ | $\hat{\bar{\sigma}}^2_{gF_2}$ | |
| | | | | | | mean | 95% conf. interval |
| $G_1 \times G_2$ | 1 | h×l | 0.060 | 0.070 | 0.054 | 0.062 | 0.029 - 0.090 |
| $G_1 \times G_3$ | 2 | h×l | 0.056 | 0.040 | 0.039 | 0.039 | 0.011 - 0.063 |
| $G_1 \times G_4$ | 6 | h×l | 0.037 | 0.101 | - | 0.101 | 0.072 - 0.134 |
| $G_1 \times G_5$ | 7 | h×l | 0.107 | 0.142 | - | 0.142 | 0.083 - 0.206 |
| $G_2 \times G_3$ | 4 | l×l | 0.052 | 0.009 | - | 0.009 | -0.009 - 0.024 |
| $G_2 \times G_4$ | 4 | l×l | 0.052 | 0.038 | - | 0.038 | 0.016 - 0.061 |
| $G_2 \times G_5$ | 5 | l×l | 0.031 | 0.046 | 0.053 | 0.050 | 0.032 - 0.066 |
| $G_3 \times G_4$ | 6 | l×l | 0.037 | - | 0.047 | 0.047 | 0.027 - 0.067 |
| $G_3 \times G_5$ | 3 | l×l | 0.045 | 0.027 | 0.034 | 0.030 | 0.010 - 0.048 |
| $G_4 \times G_5$ | 6 | l×l | 0.037 | 0.054 | - | 0.054 | 0.033 - 0.076 |

[1] h×l: cross between a high- and a low-nitrate genotype; l×l: cross between two low-nitrate genotypes.
- : Not determined.

experiments with $F_2$ populations were carried out under similar environmental conditions. Nitrate means of reciprocal $F_2$s did not differ significantly from each other. Deviations of $F_2$ means from the midparent value were small. However, because of the high number of degrees of freedom, the deviation from the midparent value was still significant at P=0.05 for nine $F_2$s. Of the genotypes selected for low nitrate, $G_3$ and $G_5$ had considerable higher nitrate concentrations than $G_2$ and $G_4$.

The estimates of variance components in the $F_2$ experiments are given in Table 4.5. Estimates of residual error ($\hat{\sigma}_e^2$) ranged from 0.031 to 0.107 $g^2 \cdot l^{-2}$. There were no significant differences in genotypic variance between reciprocal $F_2$s. The estimates for genotypic variances in h×l (high-nitrate parent × low-nitrate parent) crosses were all significantly different from zero and ranged from 0.039 to 0.142 $g^2 \cdot l^{-2}$, with the lowest estimate for $F_2(G_1 \times G_3)$ and the highest for $F_2(G_1 \times G_5)$. In the l×l (two low-nitrate parents) crosses the estimates of genotypic variance ranged from 0.009 to 0.054 $g^2 \cdot l^{-2}$. All estimates of genotypic variance in $F_2$s of l×l crosses, except for $F_2(G_2 \times G_3)$, deviated significantly from zero. In all $F_2$s a unimodal frequency distribution for nitrate concentration was found, indicating a quantitative inheritance without detectable major genes. This is illustrated in Figure 4.1 for an $F_2$ of the h×l type ($G_1 \times G_4$) and an $F_2$ of the l×l type ($G_4 \times G_5$). If an average value for $\sigma_e^2$ of 0.05 $g^2 \cdot l^{-2}$ is taken as an estimate of the residual variance in future experiments, estimates of the wide sense heritability in the $F_2$ generation may range from 0.44 ($G_1 \times G_3$) to 0.74 ($G_1 \times G_5$) for the h×l crosses and from 0.15 ($G_2 \times G_3$) to 0.52 ($G_4 \times G_5$) for the l×l crosses.

*$F_3$ populations*

In contrast to the experimental set up for the $F_2$ plants, in which most populations were tested in separate experiments, the $F_3$ experiments were designed to evaluate all populations and parents simultaneously. The large number of plants meant that four repeats of the experiment were necessary to be able to test a sufficient number of $F_3$ lines per combination of parents. Figure 4.2 presents the means for nitrate concentration per experiment for each parent combination and corresponding $F_3$. The parents show G×E interactions: the nitrate content of the parent genotypes reacted differently to changes in environmental conditions between experiments. The ratio of adjusted squared correlation coefficients (Ratio = $R^2_{adj\ (ge)}$ / $R^2_{adj\ (g+ge)}$ ) is given in Figure 4.2. All combinations of parents, except $G_1 \times G_3$ and $G_2 \times G_3$ showed a significant interaction at P=0.01. However, the importance of the interactions expressed by the ratio varied between crosses. The largest ratio was found for the combination of $G_2 \times G_4$ (Ratio = 0.89). The ranking of $G_2$ and $G_4$ for nitrate content reversed in the course of the four successive experiments. Other parent combinations with G×E interactions amounting to a fraction of more than 10% of the summed effects of genotype and G×E interactions, were $G_4 \times G_3$, $G_3 \times G_5$ and $G_4 \times G_5$. In most cases the $F_3$ means were close to the midparent value. This means that compared to both parents the $F_3$ lines have an intermediate response to environmental changes.

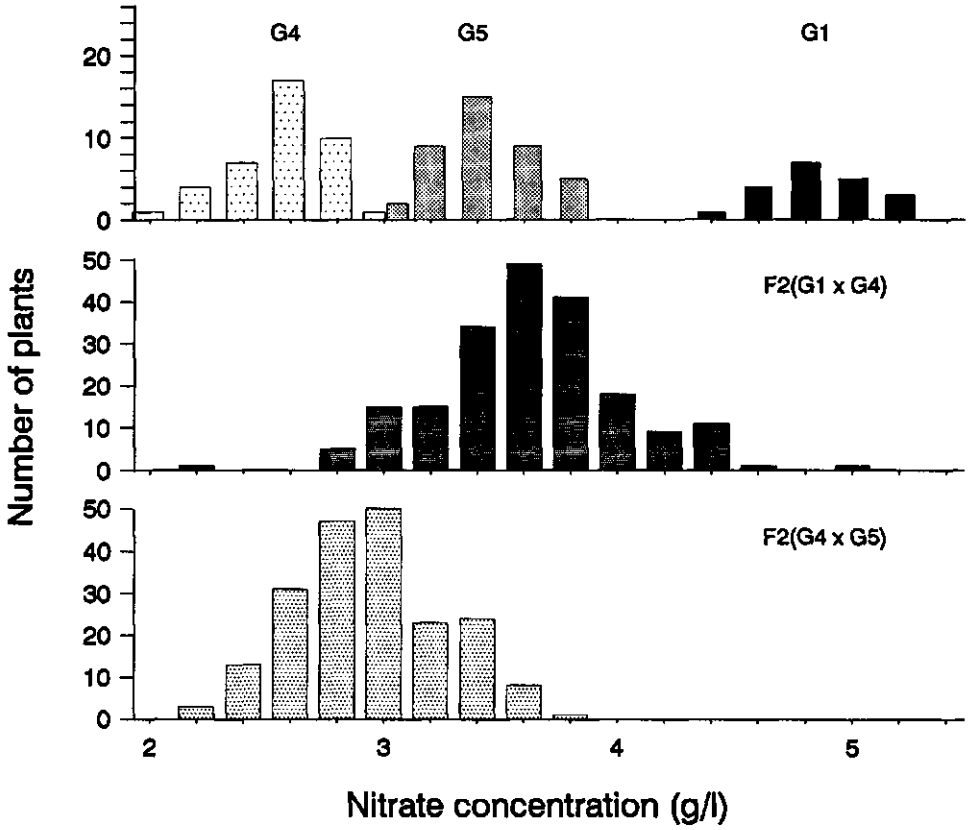


***Fig. 4.1.*** **Frequency distributions of nitrate concentration for parental genotypes and $F_2$s of a cross between a high- and a low-nitrate genotype ($G_1 \times G_4$) and a cross between two low-nitrate genotypes ($G_4 \times G_5$).**

Estimates of the additive genotypic variance were made per experiment when the parents of a cross displayed relatively large G×E interactions ($G_2 \times G_4$, $G_4 \times G_3$, $G_3 \times G_5$ and $G_4 \times G_5$) and were based on the pooled results of the four successive experiments when the parents of a cross displayed relatively small G×E interactions ($G_1 \times G_2$, $G_1 \times G_3$, $G_1 \times G_4$, $G_1 \times G_5$, $G_2 \times G_3$ and $G_2 \times G_5$). Table 4.6 presents the estimates of the additive genotypic standard deviation ($\sqrt{\hat{A}_3}$ and $\sqrt{\hat{A}_4}$) for the crosses with relatively small effects of G×E interactions. The estimates of the genotypic standard deviation based on the genotypic variance of the $F_3$ ($\sqrt{\hat{A}_3}$) and based on the covariance between $F_2$ plants and corresponding $F_3$ lines ($\sqrt{\hat{A}_4}$) were similar, except for $G_2 \times G_3$. For this cross a

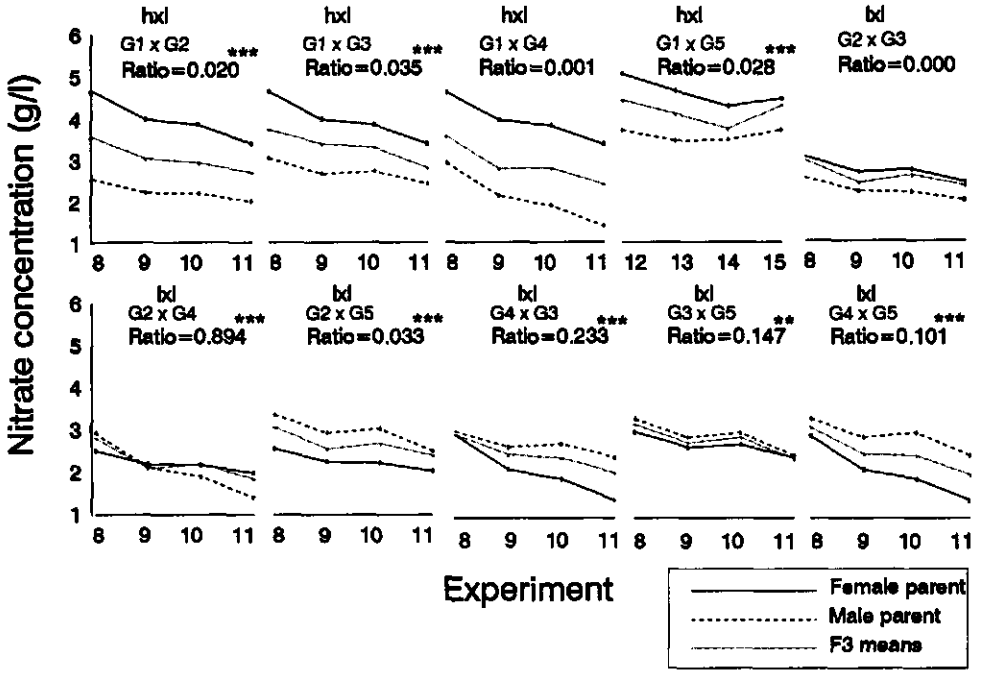


***Fig. 4.2.*** **Nitrate concentration of parents and $F_3$ populations of ten crosses in four successive experiments. The type of cross, the significances of genotype × experiment (G×E) effects for the parent combination and the ratio of the proportion of the adjusted squared correlation coefficient attributable to G×E effects to the combined effects of genotype and G×E effects (Ratio) are indicated in the figure (**: P < 0.01; *** : P < 0.001; h×l: a cross between a high-nitrate genotype and a low-nitrate genotype; l×l: a cross between two low-nitrate genotypes).**

negative estimate of $\hat{A}_3$ was obtained, resulting in a zero estimate for $\sqrt{\hat{A}_3}$. The $\sqrt{\hat{A}_4}$ estimate for this cross was 0.16 g·$l^{-1}$. Because $G_2$ consistently had a lower nitrate content than $G_3$ in the four $F_3$ experiments, some genotypic variance should be present and the $\sqrt{\hat{A}_3}$ estimate for $G_2 \times G_3$ must be an underestimation of $\sqrt{A}$. The largest estimates of $\sqrt{A}$ were found for $G_1 \times G4$ ($\hat{A}_3$=0.40 g·$l^{-1}$; $\sqrt{\hat{A}_4}$ = 0.37 g·$l^{-1}$).

The mean difference between the parents for nitrate concentration and the estimated genotypic standard deviation were used to predict the probability of obtaining transgressive $F_\infty$ lines with a nitrate content less than the lowest parent of the cross (Table 4.6). For the two crosses with the most extreme differences in nitrate content between parents ($G_1 \times G_2$ and $G_1 \times G_4$) the probability of obtaining transgressive low-nitrate

***Table 4.6.*** **Overall difference between parents in nitrate concentration ($P_1$-$P_2$ in $g \cdot l^{-1}$) and estimates for the additive genotypic standard deviation ($\sqrt{\hat{A}_3}$ (with 95% confidence interval) and $\sqrt{\hat{A}_4}$, $g \cdot l^{-1}$) and the probability of transgression towards low nitrate concentrations in advanced generations ($P_{low}$) for six crosses with parents not displaying important genotype x experiment interactions**

| Cross ($P_1 \times P_2$) | $P_1-P_2$ | $\sqrt{\hat{A}_3}$ | | $\sqrt{\hat{A}_4}$ | $P_{low}$ [1] |
|---|---|---|---|---|---|
| | | mean | 95% conf. int. | | |
| $G_1 \times G_2$ | 1.73 | 0.30 | 0.25-0.35 | 0.29 | 0.002 |
| $G_1 \times G_3$ | 1.23 | 0.25 | 0.20-0.30 | 0.26 | 0.007 |
| $G_1 \times G_4$ | 1.87 | 0.40 | 0.35-0.45 | 0.37 | 0.010 |
| $G_1 \times G_5$ | 1.02 | 0.29 | 0.23-0.34 | - | 0.039 |
| $G_2 \times G_3$ | -0.49 | 0 | 0.00-0.09 | 0.16 | 0.063 |
| $G_2 \times G_5$ | -0.72 | 0.19 | 0.12-0.24 | 0.19 | 0.037 |

[1]Estimates of $P_{low}$ based on $\hat{A}_3$, except for $G_2 \times G_3$.

lines was low: P=0.002 and P=0.010, respectively. Higher probabilities for transgression were obtained in the less extreme cross $G_1 \times G_5$ (P=0.039) and the two crosses between two low-nitrate genotypes $G_2 \times G_3$ (P=0.063) and $G_2 \times G_5$ (P=0.037).

The estimates for $\sqrt{\hat{A}_3}$ and $\sqrt{\hat{A}_4}$ obtained per experiment for the crosses with parents showing important G×E interactions are given in Table 4.7. The behaviour of cross $G_2 \times G_4$, with the largest relative effects of G×E interactions, is particularly interesting. The order of both parents for nitrate content reversed during the course of the four experiments. The absolute difference between both parents was largest in the first (experiment 8) and the last (experiment 11) experiment of the series and smaller in the middle two experiments (experiments 9 and 10). This is reflected in the $\sqrt{\hat{A}_3}$ estimates, which are largest for experiments 8 and 11 and smaller for experiments 9 and 10. In contrast, the $\sqrt{\hat{A}_4}$ estimates decrease from experiment 8 to 11. This can be explained because the conditions under which the $F_2$ was grown most resembles the conditions of experiment 8: the parental difference $G_2$-$G_4$ was -0.3 $g \cdot l^{-1}$ in the $F_2$ experiment (Table 4.4). When G×E interactions occur, $\sqrt{\hat{A}_4}$ will tend to be higher when the environments in which the $F_2$ and $F_3$ were grown are more alike. The $\sqrt{\hat{A}_3}$ and $\sqrt{\hat{A}_4}$ estimates for the other three crosses in Table 4.7 ($G_4 \times G_3$, $G_3 \times G_5$ and $G_4 \times G_5$) show that a considerable genotypic variation is still present in these crosses between low-nitrate genotypes. This indicates that these genotypes differ for genes for low nitrate content. The occurrence of G×E interactions means that for these crosses no general predictions of the probability of transgression towards low nitrate contents can be made.

*Table 4.7.* **Difference between parents for nitrate concentration ($P_1$-$P_2$ in $g \cdot l^{-1}$), estimates for the additive genotypic standard deviation ($\sqrt{\hat{A}_3}$ (with 95% confidence interval) and $\sqrt{\hat{A}_4}$, $g \cdot l^{-1}$) for four crosses in four experiments. The parental genotypes of these crosses displayed significant genotype × experiment interactions**

| Cross ($P_1 \times P_2$) | Experiment | $P_1$-$P_2$ | $\sqrt{\hat{A}_3}$ | | $\sqrt{\hat{A}_4}$ |
|---|---|---|---|---|---|
| | | | mean | 95% conf. int. | |
| $G_2 \times G_4$ | 8 | -0.42 | 0.39 | 0.30-0.50 | 0.36 |
| | 9 | 0.07 | 0.18 | 0.00-0.31 | 0.28 |
| | 10 | 0.29 | 0.13 | 0.00-0.24 | 0.22 |
| | 11 | 0.59 | 0.28 | 0.16-0.39 | 0.21 |
| $G_4 \times G_3$ | 8 | -0.09 | 0.30 | 0.20-0.40 | 0.26 |
| | 9 | -0.56 | 0.13 | 0.00-0.27 | 0.25 |
| | 10 | -0.83 | 0.27 | 0.17-0.37 | 0.24 |
| | 11 | -1.04 | 0.43 | 0.32-0.54 | 0.27 |
| $G_3 \times G_5$ | 8 | -0.30 | 0.17 | 0.00-0.27 | 0.25 |
| | 9 | -0.24 | 0.28 | 0.14-0.40 | 0.28 |
| | 10 | -0.28 | 0.28 | 0.18-0.38 | 0.25 |
| | 11 | -0.04 | 0.28 | 0.15-0.39 | 0.28 |
| $G_4 \times G_5$ | 8 | -0.34 | 0.33 | 0.24-0.44 | 0.24 |
| | 9 | -0.77 | 0.35 | 0.22-0.46 | 0.33 |
| | 10 | -1.11 | 0.43 | 0.33-0.54 | 0.43 |
| | 11 | -1.07 | 0.29 | 0.16-0.40 | 0.17 |

## 4.4 Discussion

*Transgression towards low nitrate content*

Two questions were raised in this study on the genetics of nitrate content in lettuce. The first question concerns the probability of obtaining low-nitrate segregants from crosses between modern high-nitrate lettuce cultivars and genotypes with a low nitrate content. Of the ten crosses studied, four were of the high × low (h×l) nitrate type. In the $F_2$ and $F_3$ generations of these crosses the nitrate content inherited as a quantitative trait with heritabilities of intermediate size. Estimates of the additive genotypic variance A from the genotypic variance of the $F_3$ generation and from the covariance between $F_2$ plants and corresponding $F_3$ lines were in close agreement. The estimates for the probability of obtaining segregants with the same or lower nitrate content than the low-nitrate parent ($P_{low}$) ranged from 0.002 for $G_1 \times G_2$ to 0.039 for $G_1 \times G_5$. These esti-

mates still may be too high because the efficiency of selection can be decreased if the assumptions (normal distribution, no linkage, epistasis, stochastic variation or G×E interaction) do not hold. One of these that will almost certainly not hold is the absence of G×E interactions. The parents of three of the four h×l crosses showed statistically significant (although relatively small) G×E interactions for nitrate content. As a consequence of the low values for $P_{low}$ large numbers of $F_2$ derived lines will have to be grown to keep the nitrate level of the low-nitrate parent in a breeding program. The practical consequences are considerable, because each line has to be tested by a large number of plants to measure the genotypic value of the lines accurately. In most cases several backcrosses with high-nitrate genotypes have to be made to combine all the required traits of a modern lettuce cultivar (adaptation to specific growth conditions, resistances etc.) with low nitrate content. Each backcross with a genotype with high nitrate content will further reduce the probability of obtaining lines with the same or lower nitrate content than the low-nitrate parent. Consequently, to be successful a breeding program aimed at developing low-nitrate cultivars in lettuce must involve large numbers of plants.

The second question raised in this study concerns the possibility to obtain lines with extremely low nitrate content from crosses between two low-nitrate genotypes (l×l crosses). Six l×l crosses were made and the progenies analyzed. In the $F_2$ generation considerable genotypic variation was present in most of the l×l crosses, which was reflected in the estimated wide sense heritabilities ranging from 0.15 to 0.52. These results seemed to indicate that a substantial reduction of nitrate content below the level of the low-nitrate parents used in this study could be possible. The results obtained from the $F_3$ populations, however, did not support this optimism. For two l×l crosses that did not show important G×E interactions ($G_2 \times G_3$ and $G_2 \times G_5$), notwithstanding the relatively small differences between parents for nitrate content, the probabilities of obtaining transgressive low-nitrate segregants were low (Table 4.6). Because the other four l×l crosses showed important G×E interactions no valid prediction of the fraction of transgressive low-nitrate lines in future selection experiments could be made.

Potentially a higher frequency of low-nitrate lines could obtained by combining the genes for low nitrate from more than two genotypes. Pooni and Jinks (1985) presented formulas to predict means and genotypic variances of inbreds derived from three-way and double crosses using parental means and estimated variances of single crosses. Using these formulas, it can be shown that no three-way or double cross involving the parental genotypes used in this study, yields a higher probability of obtaining low-nitrate lines in the $F_\infty$ than the single cross $G_2 \times G_4$. This means that three-way or double crosses are not effective in obtaining a higher frequency of low-nitrate lines. Lower nitrate contents could be obtained, however, by crossing selected $F_\infty$ lines with low nitrate from different parent combinations or by exploring other genotypes to broaden the available genotypic variation for low nitrate.

*Genotype × environment interaction*

The occurrence of G×E interaction was a complicating factor in this study. In the four successive $F_3$ experiments several patterns of behaviour of the nitrate content in the parents of a cross were observed (Fig. 4.2). The parents of crosses $G_2 \times G_3$ and $G_2 \times G_5$ showed parallel lines for nitrate content over the four experiments, indicating the absence of G×E interactions. The G×E interactions for combinations of parental genotypes were shown as diverging lines in the four successive experiments ($G_4 \times G_3$ and $G_4 \times G_5$), converging lines ($G_3 \times G_5$) and even an inversion of the ranking of parents for nitrate content for $G_2 \times G_4$. This inversion was also found in the second series of experiments (experiments 12-15) and in other studies (Chapter 5) and is reproducible when these two genotypes are repeatedly harvested during the first months of the year. This reproducibility opens possibilities for research on the physiological background of this G×E interaction (Chapter 6).

To obtain an estimate of the genotypic variance unbiased by G×E interaction, Casler (1982) proposed to estimate A from the covariance between $F_2$ plants and $F_3$ line means, with the two generations grown in separate environments. However, this estimate is not really free from effects of G×E interaction, because the $F_2$ and $F_3$ experiments may be similar with respect to factors causing the interaction. Algebraically this can be shown as follows. Because of randomization of the experiments in which the parental genotypes and the $F_3$ offspring are grown, environmental effects are uncorrelated with genotypic and interaction effects and the parent-offspring phenotypic covariance is

$$\sigma_{p_{PO}} = \sigma_{g_{PO}} + \sigma_{ge_{PO}}$$

where $\sigma_{g_{PO}}$ is the covariance between genotypic effects of parents and offspring and $\sigma_{ge_{PO}}$ is the covariance between genotype × environment interaction effects of parents and offspring. When parents and offspring are grown in separate experiments with randomly assigned environments, $\sigma_{ge_{PO}}$ will have a zero expectation. However, in the case of two experiments, one for the $F_2$ and one for the $F_3$ population, similarity between the $F_2$ and $F_3$ environments will result in a positive value of $\sigma_{ge_{PO}}$ and inflated estimates of A. This effect was found in the present results. Taking the parental difference in the $F_2$ and $F_3$ experiments as a measure of the similarity between experiments with respect to environmental factors relevant for G×E interaction, the highest estimates of A based on the parent-offspring covariance ($\hat{A}_4$) were obtained when the $F_3$ was tested in an experiment showing about the same parental difference as the experiment in which the $F_2$ was tested (Tables 4.4 and 4.7).

*Future permissible nitrate levels*

The fear of the effects of high nitrate intake on public health has led to the introduction of maximum permissible values for the nitrate content in vegetables in several European countries (Chapter 1). In the Netherlands, the aim is to impose ultimately a maximum nitrate content for lettuce of 2.5 $g \cdot kg^{-1}$ fresh matter. The maximum limit is based on

a measurement of nitrate via dry matter and recalculation of the nitrate content in fresh matter. In our study the nitrate content was measured directly in sap pressed from the harvested shoot. This measuring procedure is quicker and because less steps have to be taken before nitrate is measured, there is less opportunity for making errors. Previous research (Reinink and Groenwold, 1986) has shown that both measurements are highly correlated, although the contents measured in pressed sap were about 10 % higher than measured via dry matter.

A relevant question is whether the genotypic variation for nitrate content allows the selection of lines with a nitrate content in winter below the future maximum. In some of the experiments described in this paper all parental genotypes had nitrate contents above 2.5 $g \cdot l^{-1}$. The highest nitrate contents in the $F_3$ experiments were obtained in experiment 12, with contents for $G_1$ to $G_5$ of 5.1, 2.9, 3.3, 3.2 and 3.7 $g \cdot l^{-1}$, respectively. With these parental means and using the estimates A in Table 4.6, in all of the crosses without important G×E interactions the probability of finding lines with a nitrate content below 2.5 $g \cdot l^{-1}$ was negligible ($P<0.0001$). For the crosses with relatively large effects of G×E interactions, the probability of finding $F_\infty$ lines with levels below 2.5 $g \cdot l^{-1}$ were estimated using the $\sqrt{\hat{A}_3}$ estimates of experiment 8, which had the highest nitrate contents of the experiments in Table 4.7. The estimates were 0.07 for $G_2 \times G_4$, 0.005 for $G_4 \times G_3$, <0.0001 for $G_3 \times G_5$ and 0.002 for $G_4 \times G_5$. These estimates have been made neglecting the effects of G×E interaction on the selection result and the 10% overestimation of nitrate content because of the measurement in pressed sap.

However, the situation sketched above is more or less a worst case scenario. Most of the winter harvests had nitrate contents considerably lower than experiment 12 and under those conditions the probability of finding lines with a nitrate content compatible with the maximum limit will be much larger. Furthermore, the experimental conditions in this study (plants grown on nutrient solution with high nitrate and very low ammonia concentration) cause high nitrate contents. A combination of cultivars with a genetically reduced capacity for nitrate accumulation and cultural measures could lead to acceptable nitrate contents for most of the lettuce crops grown in winter. Van der Boon et al. (1990) described how the nitrate content of a lettuce crop grown on nutrient solution can be reduced by manipulating the concentrations and ratios of the nitrate, ammonia and chloride ions in the nutrient solution. Growing low-nitrate cultivars on nutrient solution could reduce the nitrate content of winter grown lettuce strongly. The possibilities to manipulate the nitrate content of plants grown under winter conditions in soil are smaller than when grown in nutrient solution (Roorda van Eysinga and Van der Meijs, 1985). However, also for lettuce growing in soil research aimed at reducing the nitrate content by cultural measures is continuing (van Amersfoort and Boersma, 1991; Muller and Schirmer, 1990; van Oeveren, 1991; Boersma, 1991; Mol, 1992) and a combination of low-nitrate cultivars and cultural measures is likely to lead to acceptable nitrate levels.

## 4.5 Appendix

*Comparison of estimators of the additive component of genotypic variance (A) in an $F_3$ population*

The estimator of the additive genetic variance used in this study was:

$$\hat{A}_3 = 4/3 \cdot (\hat{\sigma}^2_{pF_3} - \hat{\sigma}^2_e),$$

in which $\sigma^2_{pF_3}$ is the phenotypic variance of the $F_3$ population and $\sigma^2_e$ the residual variance. $\hat{A}_3$ will be compared to two other estimators, using the mean square error (MSE) as a measure of the performance of the estimator (Van Ooijen, 1989). The first estimator is unbiased:

$$\hat{A}_1 = 4/3 \cdot (2 \cdot \hat{\sigma}^2_{gbF_3} - \hat{\overline{\sigma}}^2_{gwf_3}),$$

in which $\sigma^2_{gbF_3}$ is the genotypic between $F_3$ line variance and $\overline{\sigma}^2_{gwF_3}$ is the mean genotypic within $F_3$ line variance. The second estimator is biased, but was shown to have a smaller MSE than $\hat{A}_1$ in almost every situation (Van Ooijen, 1989):

$$\hat{A}_2 = 2 \cdot \hat{\sigma}^2_{gbF_3}.$$

Table 4.8 was used for the analysis of components of variance in the $F_3$ generation. The bias and MSE's of the three estimators are summarized in Table 4.9. In the experiments presented in this chapter two plants per line were used. For comparison of the MSE's of the estimators, values of 43 were taken for $df_b$ and $df_w$, 86 for $df_t$ and 130 for $df_i$ when A was estimated for each experiment separately. When A was estimated from the pooled results of the four successive experiments, a value of 172 was taken for $df_b$ and $df_w$, 345 for $df_t$ and 520 for $df_i$. To compare the performance of the estimators two situations were calculated, both for estimates based on single experiments and estimates based on pooled experiments. In the "realistic" case, values for parameters A, D and $\sigma^2_e$ were chosen in accordance with past experience. In this realistic case the value of D is small compared to A, because previous research has shown a predominantly additive inheritance of nitrate concentration in lettuce (Chapter 3). In the "unfavourable" case relatively large values for D and $\sigma^2_e$ were taken, resulting in an relatively large MSE of $\hat{A}_3$ compared to the MSE's of $\hat{A}_1$ and $\hat{A}_2$. For the "realistic" case the following estimates of A, D, and $\sigma^2_e$ were taken: A=0.08 $g^2 \cdot l^{-2}$ ; D=0.016 $g^2 \cdot l^{-2}$ and $\sigma^2_e$ = 0.05 $g^2 \cdot l^{-2}$, giving values for *E*(MSB), *E*(MSW), *E*(MST) and *E*(MSI) of 0.154, 0.072, 0.113 and 0.05 $g^2 \cdot l^{-2}$, respectively. For the "unfavourable" case the following values were taken: A=0.08 $g^2 \cdot l^{-2}$, D=0.08 $g^2 \cdot l^{-2}$ and $\sigma^2_e$ = 0.08 $g^2 \cdot l^{-2}$, giving values for *E*(MSB), *E*(MSW), *E*(MST) and *E*(MSI) of 0.2, 0.11, 0.155 and 0.08 $g^2 \cdot l^{-2}$, respectively. The ratios of MSE($\hat{A}_3$) to MSE($\hat{A}_1$) and MSE($\hat{A}_3$) to MSE($\hat{A}_2$) are given in Table 4.10. In all presented situations the unbiased estimator $\hat{A}_1$ has the largest MSE. $\hat{A}_3$ and $\hat{A}_2$ have about the same performance when the genotypic variance has an important dominance component D ("unfavourable" case) and the estimate of A is based on the pooled results of four experiments. In all other situations the MSE($\hat{A}_3$) is much smaller than the MSE($\hat{A}_2$), indicating a better performance of estimator $\hat{A}_3$.

***Table 4.8.* Analysis of variance of the $F_3$ population**

| Mean square | Name | Degrees of [1] freedom | Expectation for the mean square |
|---|---|---|---|
| MSB | between lines | $df_b = l-1$ | $\sigma_e^2 + \bar{\sigma}^2_{gwF_3} + n \cdot \sigma^2_{gbF_3}$ |
| MSW | within lines | $df_w = l \cdot (n-1)$ | $\sigma_e^2 + \bar{\sigma}^2_{gwF_3}$ |
| MST | total | $df_t = (l \cdot n)-1$ | $\sigma_e^2 + \sigma^2_{gF_3}$ |
| MSI | within parents | $df_i = p \cdot (i_p-1)$ | $\sigma_e^2$ |

[1] l : number of $F_3$ lines; n : number of plants per line; p : number of homozygous parental lines included in the experiment; $i_p$ : number of plants of parental line p; $\sigma^2_{gF_3}$ : genotypic variance of the $F_3$; $\sigma^2_{gbF_3}$ : genotypic between $F_3$ line variance; $\bar{\sigma}^2_{gwF_3}$ : mean genotypic within $F_3$ line variance; $\sigma_e^2$ : residual variance.

***Table 4.9.* Bias and mean square error (MSE) of three estimators of the additive genotypic variance in an $F_3$ population using two plants per $F_3$ line (n=2)**

| Estimator | Bias | MSE [1] |
|---|---|---|
| $\hat{A}_1$ | 0 | $\text{var}(\hat{A}_1) = \frac{16}{9} \cdot \frac{2 \cdot E^2(MSB)}{df_b} + \frac{64}{9} \cdot \frac{2 \cdot E^2(MSW)}{df_w} + \frac{16}{9} \cdot \frac{2 \cdot E^2(MSI)}{dfi}$ |
| $\hat{A}_2$ | $\frac{1}{8} \cdot D$ | $\text{var}(\hat{A}_2) + \frac{D^2}{64} = \frac{2 \cdot E^2(MSB)}{df_b} + \frac{2 \cdot E^2(MSW)}{df_w} + \frac{D^2}{64}$ |
| $\hat{A}_3$ | $\frac{1}{4} \cdot D$ | $\text{var}(\hat{A}_3) + \frac{D^2}{16} = \frac{16}{9} \cdot \frac{2 \cdot E^2(MST)}{df_t} + \frac{16}{9} \cdot \frac{2 \cdot E^2(MSI)}{df_i} + \frac{D^2}{16}$ |

[1] MSE for $\hat{A}_1$ and $\hat{A}_2$ taken from Van Ooijen (1989) with n=2.

***Table 4.10.* Ratio of mean square errors (MSE) for three estimators of the additive genetic variance A ($\hat{A}_1$, $\hat{A}_2$ and $\hat{A}_3$), for a realistic case and for a situation unfavourable for the biased estimators $\hat{A}_2$ and $\hat{A}_3$, both when A is estimated per single experiment and when the estimate of A is based on the pooled results of four successive experiments**

| Situation | $\frac{MSE(\hat{A}_3)}{MSE(\hat{A}_1)}$ | $\frac{MSE(\hat{A}_3)}{MSE(\hat{A}_2)}$ |
|---|---|---|
| Realistic, single experiment | 0.17 | 0.45 |
| Realistic, pooled experiments | 0.18 | 0.48 |
| Unfavourable, single experiment | 0.21 | 0.62 |
| Unfavourable, pooled experiments | 0.37 | 0.98 |

## References


Amersfoort, R. van & J. H. Boersma, 1991. Bemesting van sla moet anders. Groenten en Fruit/Glasgroenten 37: 22-23.

Boersma, J. H., 1991. Gericht bemesten helpt nitraat verlagen. Groenten en Fruit/Glasgroenten 43: 12-13.

Boon, J. van der, J.W. Steenhuizen & E.G. Steingröver, 1990. Growth and nitrate concentration of lettuce as affected by total nitrogen and chloride concentration, $NH_4/NO_3$ ratio and temperature of the recirculating nutrient solution. J. Hort. Sci. 65: 309-321.

Casler, M. D., 1982. Genotype x environment interaction bias to parent-offspring regression heritability estimates. Crop Science 22: 540-542.

Jinks, J.L. & H.S. Pooni, 1976. Predicting the properties of recombinant inbred lines derived by single seed descend. Heredity 36: 253-266.

Jinks, J.L. & H.S. Pooni, 1980. Comparing predictions of mean performances and environmental sensitivity of recombinant inbred lines based upon $F_3$ and triple test cross families. Heredity 45: 305-312.

Mather, K. & J. L. Jinks, 1982. Biometrical Genetics.-3rd ed. Chapman and Hall, London, 396 p.

Mol., C., 1992. Achtergrond - meststoffen. Langzaam vrijkomen geeft minder nitraat. Groenten + Fruit/Glasgroenten 49, (4 dec. 1992): 10-11.

Müller, H. & H. Schirmer, 1990. Ein Beitrag zum Einsatz des Nitrifikationshemmstoffs Nitrapyrin (N-Serve) zur Verminderung des Nitratgehaltes im Gemüse. Agribiol. Res. 43: 74-81.

Oeveren, L. van, 1991. Telers verzamelen gegevens voor verder onderzoek. Groenten en Fruit/Vollegrondsgroenten 28: 10-11.

Ooijen, J.W. van, 1989. Estimation of additive genotypic variance with the $F_3$ of autogamous crops. Heredity 63: 73-81.

Pooni, H.S. & J.L. Jinks, 19985. Predicting the properties of first cycle inbreds and second cycle hybrids extractable from two, three and four parent crosses. Heredity 54: 397-411.

Reinink, K. & R. Groenwold, 1986. Vergelijking van de bepaling van het nitraatgehalte in sla in drogestof en in perssap. Zaadbelangen 40: 174-176.

Reinink, K. & R. Groenwold, 1987. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica 36: 733-744.

Roorda van Eysinga, J.P.N.L. & M.Q. Van der Meijs, 1985. Effect of nitrogen nutrition and global radiation on yield and nitrate content of lettuce grown under glass. Comm. in Soil Sci. Plant Anal. 16: 1293-1300.

Snedecor, G.W. & W.G. Cochran, 1980. Statistical methods.-7th ed. The low State University Press, Ames, Iowa, USA, 507 pp.

Tai, G.C.C, 1989. A new procedure to construct confidence intervals for genotypic variance and expected response to selection. Genome 32: 307-308.

# 5 Genotype × Environment Interaction for Nitrate Content in Lettuce

*Previous results indicated the occurrence of genotype × environment (G×E) interactions for nitrate content in lettuce. Because of the important influence of light intensity on nitrate accumulation two types of interactions may be expected: interactions related to daily variation and those related to annual variation. In the present study both types were investigated using eight butterhead accessions which were repeatedly harvested. No daily variation in nitrate concentration and no corresponding G×E interactions were found, irrespective of the level of global radiation. In contrast, a large annual variation and important corresponding G×E interactions were found. Joint regression analysis on environmental means and on physical factors related to light intensity showed a differential response of genotypes to changing environmental conditions. Multiple joint regression on daylength and change in daylength accounted for two-thirds of the interaction variance. However, deviations from regression were still significant indicating non-linearity of the relationship or the existence of other environmental factors contributing to G×E interaction.*

*A negative relationship was found between genotype means for nitrate and the concentration of organic solutes (organic acids and monosaccharides). Important effects of G×E interaction were also detected for these organic solutes. This is in accordance with the hypothesis that nitrate is accumulated to maintain osmotic pressure and that in low-nitrate genotypes nitrate is replaced by organic solutes. Significant genotype differences in osmolarity of the expressed sap were also found. However, no correlation was found between osmolarity and nitrate accumulation.*

## 5.1 Introduction

In lettuce a large genotypic variation for nitrate accumulation in winter-grown lettuce was found (Chapter 2). Later experiments (Reinink and Groenwold, 1988; Reinink and Blom-Zandstra, 1989) showed genotype × environment (G×E) interactions.

Light intensity strongly influences the nitrate content of leafy vegetables (Blom-Zandstra and Lampe, 1985; Blom-Zandstra et al., 1988; Roorda van Eysinga and van der Meijs, 1985; Steingröver et al., 1986a). Under low light intensities nitrate is accumulated in the vacuoles to maintain osmotic pressure (Blom-Zandstra and Lampe, 1985; Steingröver et al., 1986a). Experiments with varying light intensities have shown a negative relationship between the concentration of nitrate and the concentration of organic solutes (Behr and Wiebe, 1988; Blom-Zandstra and Lampe, 1985; Blom-Zandstra et al., 1988). The highest nitrate contents occur in crops harvested in late autumn, winter and early spring.

Because of the strong influence of light intensity on nitrate content, this

G×E interaction could be caused by the differential reaction of genotypes to various levels of light intensity. The most important rhythms of light intensity are the daily and annual cycles. This chapter presents the results of a study of the influence of both cycles on the nitrate content in lettuce and the importance of G×E interaction. G×E interaction in the daily cycle could be of practical value if genotypes were to be found with a large fall in nitrate content during the day. When harvested in the afternoon, these genotypes could be marketed with a low nitrate content. Results obtained by Danek-Jezik (1986) suggested that such genotypes actually exist. For breeding purposes it is important to know the size of the G×E interaction in the annual cycle. If this interaction is only small in comparison to the main effect of genotypes, selection can be made throughout the whole year. If, on the other hand, the annual G×E interaction is important, selection should be made under conditions similar to the environment for which the low-nitrate cultivars are bred, i.e. winter conditions.

In two of the experiments additional measurements were made of the osmolarity of the expressed sap and the concentrations of monosaccharides and organic acids. These measurements were related to the genotypic differences in nitrate content.

## 5.2 Materials and methods

Eight lettuce genotypes of the butterhead type (Table 5.1) were grown in 18 experiments. From previous experiments (Chapter 2; Blom-Zandstra et al., 1988) the genotypes were known to exhibit large differences for nitrate content.

Annual variation in nitrate content was investigated by repeatedly harvesting the eight genotypes throughout the year. Each harvest consisted of an experiment with 64 plants in a randomized block design with eight replicates and with each plant being an experimental unit. A total of 18 harvests was realized over a period of 15 months. Details about harvest dates, plant age at harvest, number of days of growth on nutrient solution, plant weight, global radiation, daylength and daily change in daylength at harvest are given in Table 5.2.

The daily variation of the nitrate content was investigated in four seasons. In experiments 3, 5, 8 and 13 (Table 5.2), in addition to the normal harvest time at 08.30 h, two extra harvests were done at 11.30 h and at 15.30 h. Per harvest time eight plants of each genotype were harvested.

All the plants were grown in the same glasshouse. Sowing was done in trays and the plants were transplanted to 80 $cm^3$ slippots filled with peat soil, which were then placed in gullies with a recirculating nutrient solution (NFT-system). Plants were grown under natural daylight conditions at minimum day and night temperatures of 12°C and 7°C respectively. The glasshouse was ventilated if the temperature exceeded 15°C during the day or 9°C at night. The nutrient solution was continually monitored for pH and electroconductivity (EC) and every two weeks a full chemical analysis of the

***Table 5.1.*** **Details of the lettuce accessions used to study G×E interactions for nitrate concentration**

| Accession | CGN [1] code | Name | Country of origin | Mean nitrate content in screening experiments ($g \cdot kg^{-1}$) [2] |
|---|---|---|---|---|
| a | 9277 | Panvit | Netherlands | 4.0 |
| b | 9273 | Deci-Minor | Netherlands | - |
| c | 11439 | Pinto | Netherlands | 3.4 |
| d | 9331 | Grosse Brune Tête | France | 2.2 |
| e | 5233 | Reichenauer Winter | Switzerland | 1.5 |
| f | 4892 | Winterbutterkopf | Germany | 1.9 |
| g | 4944 | Trocadero Light 76 | Italy | 2.4 |
| h | 5811 | *L. sativa capitata* | Romania | 1.9 |

[1]CGN: Centre for Genetic Resources, P.O.Box 16, 6700 AA Wageningen, the Netherlands.
[2] see Chapter 2.

solution was done. The mean nitrate concentration of the nutrient solution was 14 $mmol \cdot l^{-1}$, with extreme values ranging from 10.4 to 16.3 $mmol \cdot l^{-1}$, and the concentration of ammonia kept below 0.1 $mmol \cdot l^{-1}$. The pH was maintained at 6.0 and the EC at 2.1 $ms \cdot cm^{-1}$ in harvests 1-11 and at 1.5 $ms \cdot cm^{-1}$ in harvests 12-18. Global radiation was recorded at the automatic meteorological station "Haarweg", 2.5 km from the experimental site.

Plants were harvested while relatively young. The plant fresh weight aimed for at harvest was between 50 and 100 g. However, in some experiments, the realized mean plant weight at harvest was outside this range (Table 5.2). Harvesting was done between 08.30 h and 09.30 h. In the four experiments on daily variation, a second harvest was done between 11.30 h and 12.30 h and a third between 15.30 h and 16.30 h. Plants were cut from the roots, weighed, ground in a blender and the sap pressed through cheesecloth in tubes, which were then closed and stored at -18°C prior to further analysis. The roots of all plants were checked and those with brown roots excluded from the analysis. After thawing and dilution, the nitrate concentration of the sap was measured using an autoanalyser (Skalar, Breda, the Netherlands).

*Additional analyses*

In experiment 6 (plants grown at low light intensities, see Table 5.2) and experiment 12 (plants grown at high light intensities) additional measurements were made of the osmolarity of the expressed sap and the concentrations of monosaccharides and organic acids. The osmolarity ($mosmol \cdot kg^{-1}$) of the plant sap was measured cryoscopically with an osmometer (Gonotec, Berlin,

*Table 5.2.* **Harvest date, plant age at harvest (number of days after sowing), number of days of growth on nutrient solution (NS), mean plant fresh weight at harvest (FW), mean global radiation over a period of ten days before harvest ($GR_{10}$), daylength at harvest (DL) and change in daylength at harvest (ΔDL) of 18 experiments**

| Expt | Harvest date yy mm dd | Plant age d | Period on NS d | FW g | $GR_{10}$ $KJ \cdot cm^{-2} \cdot d^{-1}$ | DL h | ΔDL $s \cdot d^{-1}$ |
|---|---|---|---|---|---|---|---|
| 1 | 87 4 8 | 62 | 28 | 82 | 1.05 | 13.2 | 240 |
| 2 | 87 5 6 | 48 | 26 | 157 | 1.50 | 14.9 | 210 |
| 3 | 87 7 3 | 32 | 11 | 28 | 1.60 | 16.4 | - 70 |
| 4 | 87 9 10 | 36 | 13 | 46 | 1.12 | 12.7 | -250 |
| 5 | 87 10 7 | 40 | 23 | 70 | 0.97 | 10.9 | -240 |
| 6 | 87 11 5 | 57 | 34 | 94 | 0.37 | 9.0 | -210 |
| 7 | 87 11 25 | 64 | 40 | 47 | 0.16 | 8.1 | -140 |
| 8 | 88 1 6 | 92 | 56 | 41 | 0.10 | 7.7 | 90 |
| 9 | 88 2 19 | 116 | 65 | 55 | 0.48 | 9.9 | 230 |
| 10 | 88 3 18 | 123 | 64 | 112 | 0.59 | 11.7 | 250 |
| 11 | 88 3 30 | 85 | 54 | 118 | 0.62 | 12.5 | 250 |
| 12 | 88 4 26 | 78 | 35 | 184 | 1.73 | 14.3 | 230 |
| 13 | 88 5 10 | 53 | 35 | 134 | 1.70 | 15.2 | 200 |
| 14 | 88 5 18 | 41 | 21 | 105 | 2.08 | 15.6 | 170 |
| 15 | 88 6 3 | 43 | 21 | 71 | 1.45 | 16.2 | 110 |
| 16 | 88 6 14 | 47 | 19 | 80 | 1.42 | 16.4 | 50 |
| 17 | 88 6 20 | 46 | 17 | 75 | 1.83 | 16.5 | 10 |
| 18 | 88 6 30 | 50 | 17 | 55 | 1.41 | 16.4 | - 50 |

Germany). An estimate of the total organic anion content was obtained by measuring the carboxylate level (c-a estimate). The cell sap was added to cellulose powder and gently ashed at 500°C to convert all carboxylates and nitrates into oxides of the metal cations. Addition of excess standard acid and titration to pH 5 gave ash alkalinity. To obtain (c-a), expressed in eq $\cdot$ $dm^{-3}$, the value was corrected for the nitrate concentration of the plant sap. This (c-a) was used as an estimate of the total organic anion concentration, according to Dijkshoorn (1973) and Blom-Zandstra and Lampe (1985).

Monosaccharides (glucose and fructose) were determined according to Nelson (1944). Saccharose was not determined because previous research (Blom-Zandstra and Lampe, 1985; Behr and Wiebe, 1988) has shown no accumulation of saccharose in leaf vacuoles of lettuce.

*Statistical analyses*

The nitrate concentration was first analyzed separately for each experiment

and then across experiments. Within experiments, a two-way model with the main effects of blocks and genotypes was used. Differences between experiments in residual variance were analyzed using Bartlett's test for homogeneity of variances (Snedecor and Cochran, 1980). Experimental means were then regressed on several environmental variables using weighted regression with the reciprocal of the residual variance of the experimental mean as weight.

An overall weighted analysis including all experiments was done on genotype means per experiment following the model:

$$y_{ij} = \mu + g_i + e_j + (ge)_{ij} + \bar{\epsilon}_{ij}$$

where $y_{ij}$ is the observed mean value of genotype i (i= a,...,h) in environment j (j=1...18), $\mu$ represents the overall mean, $g_i$ the effect of the i-th genotype, $e_j$ the effect of the j-th environment, $(ge)_{ij}$ the effect of interaction between the i-th genotype and the j-th environment and $\bar{\epsilon}_{ij}$ is the mean random error of the i-th genotype in the j-th environment. In this analysis, the weights used were the reciprocals of the residual variances of genotype means per experiment. Homogeneity of residual variances of the genotypes was tested using Bartlett's test after fitting the above model.

The observed G×E interaction was further analyzed by a joint weighted regression analysis on environmental mean values, according to Eberhart and Russell (1966). In this analysis, the experimental mean is assumed to be an integrated measure of the experimental conditions to which the genotypes show specific responses. The regression model is:

$$(ge)_{ij} = \beta_i \cdot e_j + \delta_{ij}$$

where $\beta_i$ is the linear regression coefficient for genotype i and $\delta_{ij}$ is a deviation.

In a second analysis of the G×E interaction, instead of a biological quantification of the environment, joint weighted regressions (simple and multiple) were done on measured (global radiation) and calculated (daylength and change in daylength) environmental variables (Table 5.2). The proportion of explained variance by regressions was judged using the adjusted squared correlation coefficients:

$$R^2_{adj} = 1 - (MS_{residual} / MS_{total}).$$

G×E interaction was partitioned into components assignable to individual genotypes, according to Wricke (1962) and Shukla (1972). For each genotype, the stability variance prior to ($\hat{\sigma}_i^2$) and after using covariates ($\hat{s}_i^2$) was estimated (Shukla, 1972). These estimates are equivalent to the interaction mean square per genotype, $\hat{\sigma}_i^2$ represents an unbiased estimate of the variance of $((ge)_{ij} + \bar{\epsilon}_{ij})$, $\hat{s}_i^2$ represents an unbiased estimate of the variance of $(\delta_{ij} + \bar{\epsilon}_{ij})$ (Becker and Léon, 1988). The distributions of $\hat{\sigma}_i^2$ and $\hat{s}_i^2$ in weighted analysis and under the hypothesis of zero interaction variance have approximate F distributions with (m-p-1) and (>120) degrees of freedom, where m is the number of experiments and p is the number of covariates in the regression (Shu-

kla, 1972).

The results of the experiments on daily variation of nitrate concentration were analyzed separately for each experiment according to a split plot design with harvest times considered as plots and genotypes as subplots.

Analyses of variance, involving main effects of genotypes and experiments and interaction effects were carried out for the measurements of the osmolarity of the expressed sap and the concentrations of organic acids and monosaccharides.

## 5.3 Results

Genotype means for nitrate concentration in 18 experiments and marginal means are presented in Table 5.3. The means per experiment ranged from 2.09 $g \cdot l^{-1}$ in experiment 4 to 3.79 $g \cdot l^{-1}$ in experiment 7. Marginal means for genotypes ranged from 1.77 $g \cdot l^{-1}$ for genotype g to 3.46 $g \cdot l^{-1}$ for genotype a.

The standard deviations for experimental means were obtained after fitting the effects of blocks and genotypes. Analysis of the corresponding residual variances using Bartlett's test showed significant heterogeneity of variances across the experiments ($p<0.001$). Indications of a negative relationship between mean nitrate concentration and residual variance were obtained (Fig. 5.1 A). The higher residual variance of experiments with low nitrate concentrations coincided with high positive within-genotype (non-genetic) correlations between nitrate concentration and fresh weight (Fig. 5.1 B). Standard deviations for genotype means were obtained after fitting the effects of blocks, experiments and G×E interaction. The corresponding residual variances of individual genotype means were not significantly different from each other (Bartlett's test: $0.75< p <0.90$).

Weighted regression of experimental means on mean global radiation had the highest correlation when the mean global radiation was calculated over a period of ten days before harvest ($GR_{10}$, Table 5.2). However, this regression explained only a rather small proportion of the differences between experiments: $R^2_{adj}=0.43$. A larger proportion was explained using regression on daylength at harvest (DL, Table 5.2): $R^2_{adj}=0.60$. Figure 5.2 presents the relationship between experimental means for nitrate concentration and $GR_{10}$ (Fig. 5.2 A) and DL (Fig. 5.2 B). The relatively low proportion of explained variance in both regressions indicates important influences of environmental factors other than daylength or light intensity, or non-linearity of the relationship.

Mean fresh weight per experiment ranged from 28 to 184 g per plant (Table 5.2). The addition of fresh weight as an independent variable to the regression of the experimental means for nitrate concentration on $GR_{10}$ or DL increases the $R^2_{adj}$-value by 0.05 and 0.03, respectively. This increase is not significant ($0.25<p<0.10$), indicating that within the range observed in these experiments the fresh weight has no significant influence on the nitrate concentration. This is in accordance with previous results of

***Table 5.3.*** **Mean nitrate concentration ($g \cdot l^{-1}$) of eight genotypes in 18 experiments and marginal means with standard deviations of marginal means. For calculation of $s_e$ see text**

| Experiment | Genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | h | mean ± $s_e \cdot 100$ |
| 1 | 3.11 | 2.84 | 2.63 | 1.99 | 2.20 | 2.41 | 1.25 | 2.38 | 2.34 ± 2.7 |
| 2 | 3.38 | 3.22 | 2.85 | 2.82 | 3.00 | 2.95 | 2.18 | 3.20 | 2.96 ± 2.2 |
| 3 | 3.07 | 2.33 | 2.51 | 2.12 | 2.69 | 2.60 | 1.03 | 2.36 | 2.32 ± 3.1 |
| 4 | 3.20 | 2.66 | 2.23 | 1.64 | 2.19 | 2.17 | 1.06 | 1.60 | 2.09 ± 5.1 |
| 5 | 3.92 | 3.37 | 3.03 | 2.65 | 2.94 | 2.93 | 2.01 | 2.94 | 2.96 ± 2.1 |
| 6 | 4.15 | 3.97 | 3.44 | 2.81 | 2.87 | 3.23 | 2.34 | 3.29 | 3.26 ± 2.8 |
| 7 | 4.85 | 4.51 | 4.01 | 3.50 | 3.14 | 3.62 | 3.08 | 3.61 | 3.79 ± 1.5 |
| 8 | 4.55 | 4.20 | 3.43 | 2.94 | 2.62 | 3.05 | 2.82 | 3.07 | 3.34 ± 2.9 |
| 9 | 3.72 | 3.51 | 3.34 | 2.43 | 2.18 | 2.53 | 1.92 | 2.83 | 2.81 ± 2.0 |
| 10 | 3.58 | 3.30 | 3.29 | 2.39 | 2.16 | 2.68 | 1.74 | 2.73 | 2.73 ± 2.5 |
| 11 | 3.31 | 3.13 | 2.96 | 2.28 | 1.80 | 2.15 | 1.37 | 2.18 | 2.40 ± 4.5 |
| 12 | 3.44 | 3.33 | 3.25 | 2.56 | 2.84 | 3.04 | 1.93 | 3.06 | 2.93 ± 2.0 |
| 13 | 3.20 | 3.05 | 2.95 | 2.70 | 2.61 | 2.90 | 1.91 | 3.14 | 2.81 ± 3.8 |
| 14 | 2.89 | 2.30 | 2.30 | 2.24 | 1.93 | 2.41 | 1.46 | 2.27 | 2.23 ± 4.5 |
| 15 | 2.70 | 2.43 | 2.17 | 2.00 | 2.19 | 2.39 | 1.27 | 2.14 | 2.18 ± 4.6 |
| 16 | 3.14 | 2.71 | 2.43 | 2.26 | 2.41 | 2.44 | 1.54 | 2.46 | 2.42 ± 4.6 |
| 17 | 2.75 | 2.47 | 2.23 | 2.13 | 2.33 | 2.19 | 1.29 | 2.62 | 2.25 ± 3.7 |
| 18 | 3.27 | 2.38 | 2.56 | 2.17 | 2.55 | 2.39 | 1.62 | 2.81 | 2.47 ± 3.6 |
| Mean ± $s_e \cdot 100$ | 3.46 ± 1.9 | 3.10 ± 1.9 | 2.87 ± 2.0 | 2.42 ± 1.9 | 2.48 ± 2.0 | 2.67 ± 2.0 | 1.77 ± 1.9 | 2.70 ± 2.1 | 2.68 |

Reinink and Eenink (1988).

Table 5.4 presents the results of an overall weighted analysis of genotype means per experiment for nitrate concentration. As expected both genotypes and experiments have large main effects. The G×E interaction adds up to eight per cent of the total sum of squares and the value of the mean square for G×E interaction is 9.1. Under the hypothesis of absence of G×E interaction, due to weighting, the expectation for the G×E interaction mean square would be 1. Thus, the G×E interaction is significant ($p<0.001$).

A significant G×E interaction means that the eight genotypes do not show the same reaction to changing conditions from one experiment to another. Joint weighted regression on environmental means shows that thirty per cent of the total sum of squares for G×E interaction could be explained by variation for the linear regressions

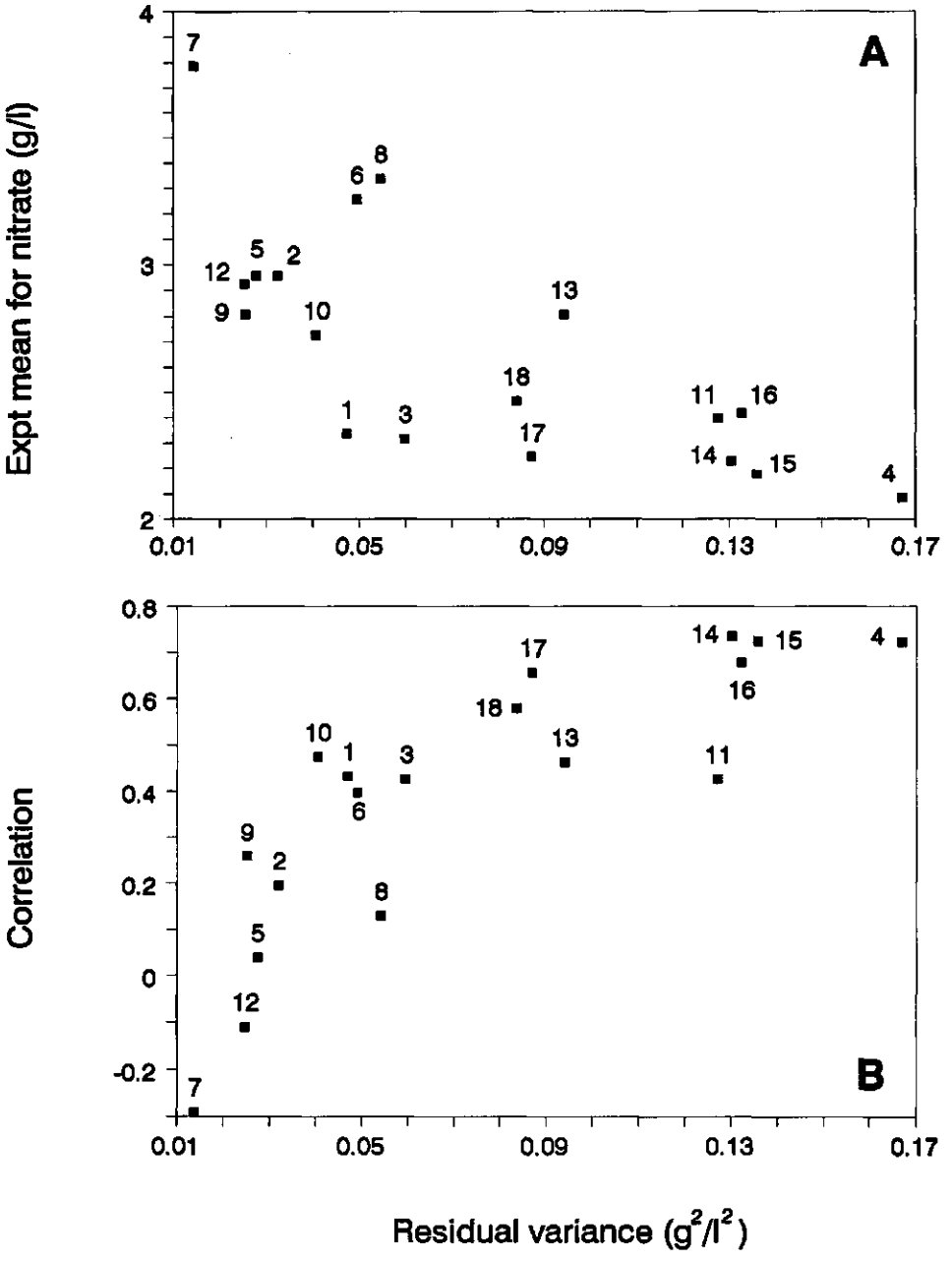


*Fig. 5.1.* **Relationship between experimental mean for nitrate concentration ($g \cdot l^{-1}$) and the residual variance per experimental unit for nitrate concentration ($g^2 \cdot l^{-2}$) (A, $R^2_{adj}$=0.50) and between the within-genotype correlation between nitrate concentration and fresh weight and the residual variance for nitrate concentration (B, $R^2_{adj}$=0.63) in 18 experiments.**

***Table 5.4.*** **Weighted analysis of variance of the nitrate concentration of eight genotypes in 18 experiments and joint weighted regression of the G×E interaction terms on the experimental mean**

| | Df | SS | %SS | MS |
|---|---|---|---|---|
| Experiments | 17 | 6160 | 47 | 362.3*** |
| Genotypes | 7 | 5909 | 45 | 844.1*** |
| G×E interaction | 119 | 1083 | 8 | 9.1*** |
| Regressions | 7 | 323 | | 46.0*** |
| Deviations | 112 | 760 | | 6.8*** |
| Total | 143 | 13151 | 100 | |

***: significant at p=0.001

(Table 5.4). The variance ratio of the regressions, using the deviations mean square as error, is 6.8. This means that the explained proportion is highly significant, although $R^2_{adj}$ is only 0.26.

Using the experimental mean as predictor variable, means quantifying the environment biologically. Instead, a joint regression can also be done on physical factors. Because of the well-known physiological relationship between nitrate content and light intensity, obvious candidates for predictor variables in this analysis are the global radiation during a certain period before harvest ($GR_n$), the daylength (DL) and the change in daylength (ΔDL). The change in daylength differentiates between experiments done in spring and autumn, which could otherwise have about the same daylength or amount of global radiance. Table 5.5 presents the results of simple and multiple joint weighted regression on these predictor variables. Again, in regression on global radiation the highest $R^2_{adj}$-value was obtained by regression on $GR_{10}$ ($R^2_{adj}$ = 0.57). An equally good fit was obtained by regression on DL ($R^2_{adj}$ =0.56). Simple regression on ΔDL had a zero value for $R^2_{adj}$. However, in combination with $GR_{10}$ or DL, $R^2_{adj}$ increased significantly by about 0.1 ($p<0.001$). For both multiple regressions the $R^2_{adj}$-value was 0.66 (Table 5.5). This indicates that apart from the total amount of radiation or daylength, the time of year, i.e. increasing or decreasing daylength, can also explain differential reactions between genotypes. The correlation between $GR_{10}$ and DL was high (r=0.91). Both correlations between $GR_{10}$ and ΔDL and between DL and ΔDL were low; respectively r=0.17 and r=0.21. The $R^2_{adj}$ values of regressions on measured experimental factors were much higher than after regression on experimental means. However, even in the regressions with the highest $R^2_{adj}$ value, the deviations mean square was still 3.1. This means that the unexplained part of the G×E interaction is still three times higher than the average within-experiment error and is still highly

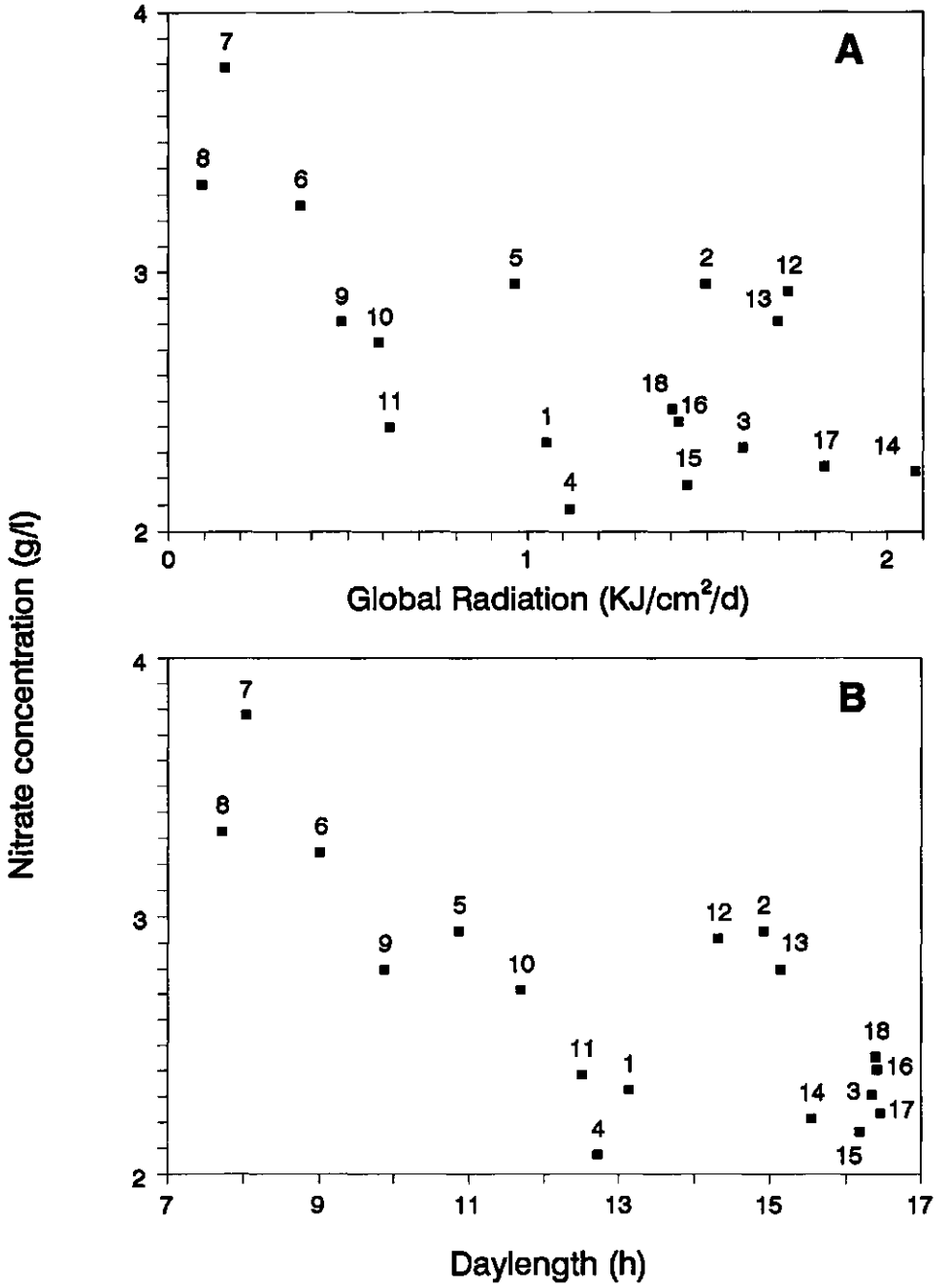


*Fig. 5.2.* **Relationship between experiment means for nitrate concentration and mean global radiation over a period of ten days before harvest (A, $R^2_{adj}$=0.43) and between experiment means and daylength at harvest (B, $R^2_{adj}$=0.60) in 18 experiments.**

*Table 5.5.* **Explained proportion of the G×E interaction mean square ($R^2_{adj} \cdot 100$) after joint weighted regression on physical factors and multiple regression on combinations of two factors. ($GR_n$: Mean global radiation during last n days before harvest; DL: daylength at harvest; ΔDL: change of daylength at harvest)**

| | $GR_1$ | $GR_2$ | $GR_4$ | $GR_6$ | $GR_8$ | $GR_{10}$ | $GR_{12}$ | $GR_{14}$ | DL |
|---|---|---|---|---|---|---|---|---|---|
| | 45 | 43 | 46 | 49 | 51 | 57 | 57 | 56 | 56 |
| DL 56 | 54 | 55 | 56 | 55 | 55 | 57 | 58 | 58 | -- |
| ΔDL 0 | 52 | 48 | 52 | 55 | 59 | 66 | 65 | 65 | 66 |

*Table 5.6.* **Partitioning of the G×E interaction sum of squares into components assignable to individual genotypes before and after joint regression on the experimental mean, on daylength (DL) and after joint multiple regression on DL and change of daylength (ΔDL)**

| | Before regression | | After regression on: Expt mean | | DL | | DL+ΔDL | |
|---|---|---|---|---|---|---|---|---|
| Genotype | $SS_{G\times E}$ | $\hat{\sigma}_i^2$ | $ß_i$ | $\hat{s}_i^2$ | $ß_i$ | $\hat{s}_i^2$ | $\hat{s}_i^2$ | $SS_{residual}$ |
| a | 171 | 11.9*** | 0.24 | 9.5*** | -0.065 | 3.4*** | 3.1*** | 41 |
| b | 188 | 13.2*** | 0.31 | 8.2*** | -0.073 | 2.3*** | 1.2 | 19 |
| c | 115 | 7.5*** | -0.01 | 8.5*** | -0.019 | 8.0*** | 5.3*** | 65 |
| d | 35 | 1.2 | -0.05 | 1.6 | 0.018 | 1.4 | 1.7 | 25 |
| e | 324 | 23.9*** | -0.41 | 15.3*** | 0.089 | 7.2*** | 3.3*** | 43 |
| f | 68 | 3.8*** | -0.15 | 3.1*** | 0.037 | 1.8* | 1.8* | 26 |
| g | 84 | 5.1*** | 0.22 | 2.8*** | -0.029 | 4.3*** | 4.7*** | 59 |
| h | 97 | 6.1*** | -0.16 | 5.4*** | 0.042 | 3.2*** | 3.5*** | 45 |
| Total | 1083 | 9.1*** | 0.0 | 6.8*** | 0.0 | 4.0*** | 3.1*** | 323 |

***: significant at p=0.001; *: significant at p=0.05

significant (p<0.001).

Table 5.6 presents the results of partitioning the G×E interaction sum of squares into components assignable to individual genotypes before and after joint regressions on experimental means, on DL and on DL + ΔDL. The $SS_{G\times E}$ values given in Table 5.6 are identical to Wricke's ecovalence ($W_i$) (Wricke, 1962). For ranking purposes, $W_i$ and $\hat{\sigma}_i^2$ are equivalent (Becker and Léon, 1988). The values of $SS_{G\times E}$ and $\hat{\sigma}_i^2$ show that the genotypes differ greatly in their contribution to the G×E interaction. The largest difference was found between genotypes d, with no

significant interaction and genotype e, which contributed strongly to the G×E interaction. It is interesting to note that both genotypes have about the same mean nitrate concentration across experiments (Table 5.3). All stability variances ($\hat{\sigma}_i^2$) were highly significant, except the one for genotype d. Joint regression on experimental means reduced the estimates of stability variance ($\hat{s}_i^2$), but all except the one for genotype d remained highly significant. The genotypes with the highest (a) and lowest (g) mean for nitrate concentration had about the same slope of the regression line ($\beta_i$). Genotype e was considerably less sensitive to environmental change than the other genotypes in this analysis.

For all genotypes except g the $\hat{s}_i^2$-value after regression on DL was lower than after regression on the experimental mean. Again genotype e was the least sensitive to changes in environmental conditions, in this case daylength. The additional improvement after multiple regression on DL+ΔDL is mainly due to a better fit for only three genotypes: b, c and e. Even so, the majority of the stability variances are highly significant, but compared with the $\hat{\sigma}_i^2$-values, multiple regression resulted in a considerable reduction of the stability variances, especially for those genotypes with large initial $SS_{G\times E}$-values (a, b and e).

Figure 5.3, which presents the regression lines of genotype means per experiment on experimental means (Fig. 5.3 A) and on daylength (Fig. 5.3 B), shows the differential reaction of genotypes to changing environmental conditions.

Table 5.7 presents the means and variance ratios for nitrate concentrations in four experiments with three harvest times per day. None of these experiments showed either a significant main effect of the harvest time or an interaction between genotypes and harvest time.

Table 5.8 gives the results of analyses of variance for osmolarity and concentrations of nitrate, organic acids and monosaccharides in experiments 6 and 12. For all traits significant effects of experiments and of genotypes were found. Significant effects of G×E interaction were found for the concentrations of nitrate, organic acids and monosaccharides, but not for osmolarity. Genotype means for osmolarity, organic acids and monosaccharides are presented in Table 5.9. The largest difference observed for osmolarity, between genotypes c and e, amounted to 35 mosmol·kg$^{-1}$. Correlations between nitrate concentration and osmolarity were not significant (r=−0.13 and −0.47 for experiments 6 and 12, respectively). Genotypes a and g, with the largest difference for nitrate content, did not differ significantly for osmolarity. Genotypic means for nitrate and organic acids were negatively correlated (r=−0.91$^{**}$ and −0.83$^{**}$ in experiments 6 and 12, respectively). The negative correlation between genotypic means for monosaccharides and nitrate was not statistically significant at the number of degrees of freedom in these experiments (r = −0.43 and −0.62 for experiments 6 and 12, respectively). Interestingly, genotype g, with the lowest nitrate content in both experiments had the highest

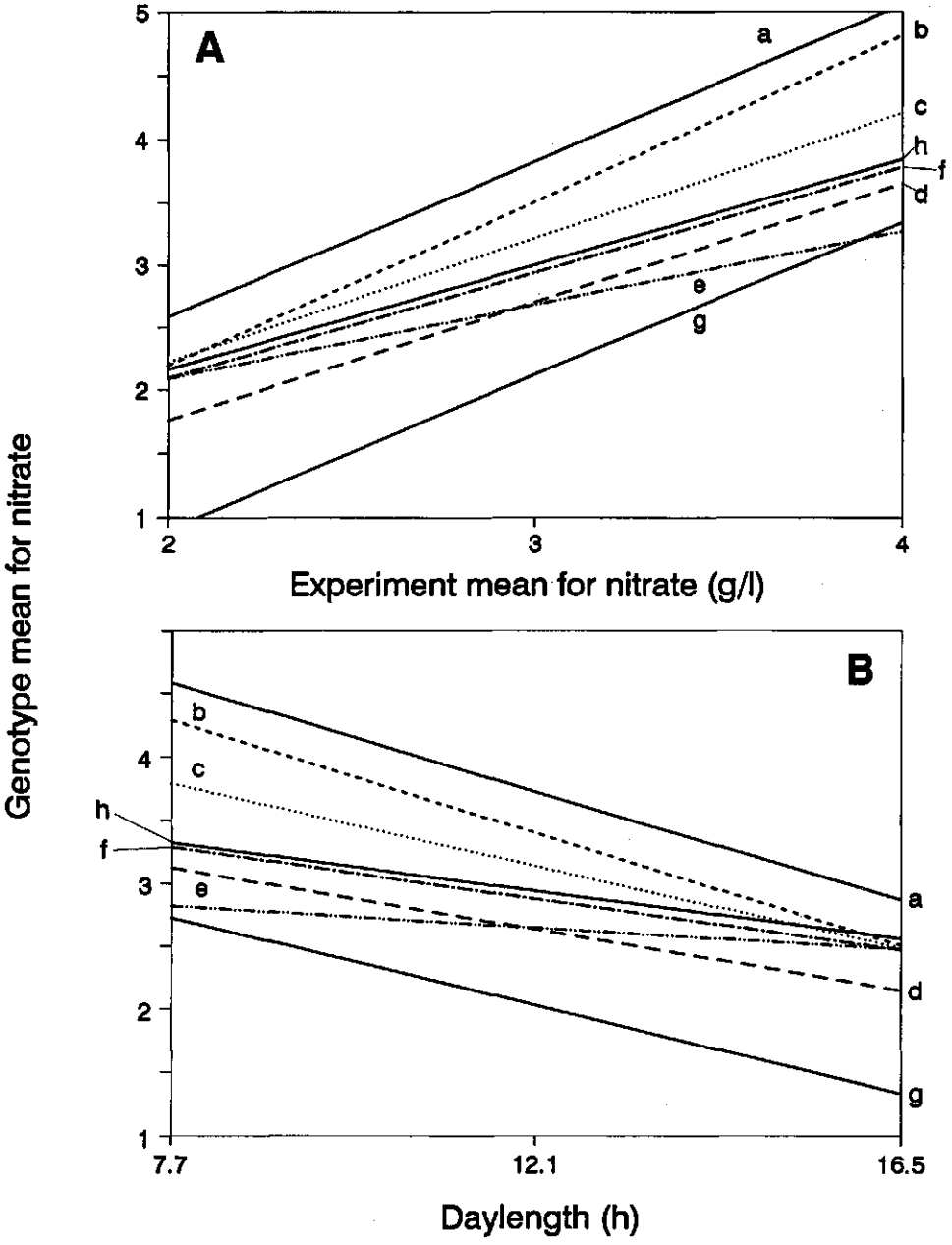


*Fig. 5.3.* **Regression lines obtained by joint weighted regression of genotype means per experiment on experiment means (A, $R^2_{adj}$=0.26) and on daylength at harvest (B, $R^2_{adj}$=0.56). Slopes of regression lines in A: $b_i = 1 + \beta_i$; in B: $b_i = -0.129 + \beta_i$ (for $\beta_i$'s see Table 5.7).**

*Table 5.7.* **Means and variance ratios for nitrate concentration ($g \cdot l^{-1}$) in four experiments with three harvest times and the corresponding mean global radiation over a period of ten days before harvest ($GR_{10}$ in $KJ \cdot cm^{-2} \cdot d^{-1}$). ($VR_t$: variance ratio for effects of harvest time; $VR_g$: for genotype effect; $VR_{gt}$: for interaction between genotypes and harvest time)**

| Experiment | Nitrate means | | | Variance ratios | | | $GR_{10}$ |
|---|---|---|---|---|---|---|---|
| | 8.30h | 11.30h | 15.30h | $VR_t$ | $VR_g$ | $VR_{gt}$ | |
| 3 | 2.32 | 2.35 | 2.30 | 0.2 | 65.8*** | 1.1 | 1.60 |
| 5 | 2.90 | 3.02 | 2.94 | 2.8 | 97.5*** | 0.9 | 0.97 |
| 8 | 3.34 | 3.42 | 3.37 | 1.1 | 263.5*** | 0.5 | 0.10 |
| 13 | 2.81 | 2.90 | 2.82 | 0.4 | 42.4*** | 1.2 | 1.70 |

*** : significant at p=0.001

*Table 5.8.* **Variance ratios for effects of experiments ($VR_e$), genotypes ($VR_g$) and genotype × experiment interaction ($VR_{ge}$) for osmolarity and concentrations of nitrate, organic acids and monosaccharides in experiments 6 and 12**

| Variance ratio | D.f. [1] | Trait | | | |
|---|---|---|---|---|---|
| | | Osmolarity | Nitrate | Organic acids | Monosaccharides |
| $VR_e$ | 1 | 41.4*** | 22.8*** | 701.1*** | 783.7*** |
| $VR_g$ | 7 | 9.2*** | 122.6*** | 14.2*** | 34.5*** |
| $VR_{ge}$ | 7 | 1.7 | 6.1*** | 3.6** | 6.8*** |

[1]Degrees of freedom
** : significant at p=0.01
*** : significant at p=0.001

concentration of monosaccharides in both experiments, while genotype e, also a low nitrate accumulator, accumulated less monosaccharides than the high-nitrate genotype a.

## 5.4 Discussion

In this study no evidence was found of a daily cycle of the nitrate concentration in lettuce. This result conflicts with results reported for spinach grown hydroponically in a growth chamber (Steingröver et al., 1986a). In spinach, a decrease was found during the day followed by an increase during the initial hours of the night. The latter increase could be circumvented by supplying light of low intensity during the night (Steingröver et al., 1986b). These authors suggested that this phenome-

***Table 5.9.*** **Mean osmolarity (mosmol· $kg^{-1}$) and concentrations of organic acids and monosaccharides (mmol· $l^{-1}$) in expressed sap of eight genotypes in two experiments (E6 and E12). For corresponding nitrate concentrations see Table 5.3**

| Genotype | Osmolarity | | | Organic acids | | | Monosaccharides | | |
|---|---|---|---|---|---|---|---|---|---|
| | E6 | E12 | Mean | E6 | E12 | Mean | E6 | E12 | Mean |
| a | 242 | 285 | 264 | 24.8 | 64.3 | 44.5 | 9.9 | 27.4 | 18.7 |
| b | 243 | 267 | 255 | 18.7 | 67.3 | 43.0 | 11.3 | 27.9 | 19.6 |
| c | 224 | 251 | 237 | 26.9 | 60.4 | 43.6 | 6.9 | 19.1 | 13.0 |
| d | 235 | 265 | 250 | 33.3 | 71.5 | 52.4 | 10.5 | 28.2 | 19.3 |
| e | 250 | 294 | 272 | 29.7 | 65.3 | 47.5 | 9.6 | 22.8 | 16.2 |
| f | 249 | 283 | 265 | 27.6 | 66.6 | 47.1 | 12.7 | 26.4 | 19.6 |
| g | 244 | 297 | 271 | 37.8 | 74.4 | 56.1 | 14.7 | 34.0 | 24.4 |
| h | 240 | 272 | 256 | 29.2 | 67.0 | 48.1 | 10.1 | 22.8 | 16.5 |
| LSD(0.05) | 18 | 18 | 11 | 5.3 | 5.3 | 3.4 | 2.4 | 2.4 | 1.6 |

non could be used in practice to reduce nitrate concentrations in glasshouse-grown spinach. Quinche (1982) tested the daily variation of nitrate concentration in several vegetable species grown in soil in a glasshouse. He found that on sunny days vegetables with erect leaves (parsley, corn-salad, spinach) showed a decrease in nitrate during the day. The amount of nitrate decrease depended on the level of global radiation. In contrast, no decrease during one day could be found in either lettuce or radish. However, these two crops were tested in only one experiment. Danek-Jezik (1986) measured the nitrate content during one day of six field-grown lettuce cultivars. For some cultivars, she found a decrease in nitrate content in the morning and an increase in the afternoon. Not all cultivars behaved in the same way, indicating G×E interactions. She recommended harvesting lettuce plants in the early afternoon to obtain minimum nitrate levels. In our results, the nitrate concentration in hydroponically grown lettuce did not change during one day, irrespective of the level of global radiation. This is in agreement with results obtained by Blom-Zandstra and Lampe (1985), who showed that the process of increase or decrease in nitrate concentration in hydroponically grown lettuce plants following a change in light intensity takes more than 14 days. For plant breeding purposes this means that hydroponically grown lettuce plants can be harvested throughout the day, without the danger of additional variation caused by a daily cycle of the nitrate concentration. The decisive factors resulting in some authors finding a daily cycle in nitrate concentration still need to be confirmed. Candidates for such factors are

plant species, plant weight at harvest and the growth medium.

As expected, an important annual variation in nitrate concentration was found. The experimental mean for nitrate concentration was correlated with the amount of global radiation and with daylength; two highly intercorrelated factors. Regression on global radiation gave the highest correlation when mean radiation values over a period of ten days before harvest were used. This again indicates that the reaction of the nitrate level in the lettuce plant to changing light intensity is not a matter of hours. The period of ten days is in agreement with results obtained by Roorda van Eysinga and Spaans (1985) with radish, who found the highest correlation between nitrate concentration and global radiation over a period of five to ten days before harvest.

The residual variance was not constant throughout the year. Although plants grown under high light conditions had a lower mean nitrate concentration, in general they had a considerably higher residual variance compared with those grown under low light conditions. Inflation of the residual variance was associated with a higher positive correlation between the residuals for nitrate concentration (after fitting a model with the effects of blocks and genotypes) and those for fresh weight. This means that after correcting for the effects of blocks, under high light intensities within genotypes the largest plants had the highest nitrate concentration. Under low residual variance such a non-genetic correlation was not obtained (Fig. 5.1 B).

In addition to significant genotype and environmental effects, the experiments on annual variation also demonstrated an important G×E interaction. Both by joint regression of genotype means per experiment on experimental means and by regression on physical factors, it was shown that genotypes respond differently to changing conditions. In studies on G×E interaction for yield, a positive correlation is often found between the regression slope and the varietal mean (Hardwick, 1981). For nitrate in lettuce no such relationship between sensitivity to environmental change and the mean level of nitrate accumulation was found. The most extreme genotypes, a and g, reacted very similarly in both regressions (Fig. 5.3). Genotype e showed the lowest sensitivity to environmental change. Genotype e was selected for its extremely low nitrate content under low light conditions (Chapter 2; see also Table 5.1), and is one of the lettuce genotypes used by commercial seed firms as parent in breeding programmes for low nitrate concentration. Reinink and Blom-Zandstra (1989) reported that under different growth conditions the relative nitrate concentration of genotype e was much higher.

The regression of genotype means for nitrate concentration on the prediction variates daylength and change in daylength explained the larger part of the G×E interaction. However, for five of the eight genotypes the stability variance according to Shukla (1972) remained highly significant, indicating non-linearity of the relationship or the existence of other physical factors to

which the genotypes react differently.

The joint regression analysis with the experimental mean used as a biological quantification of the environment has been criticized by various authors: regression slopes and deviations are not independent (Hardwick and Wood, 1972), the covariate is not independent from the data analyzed and therefore the model has only descriptive value and no predictive value (Lin et al., 1986), the technique constrains interactions into a linear form (Knight, 1970), the comparison of the regression component of interaction against deviations assumes that the deviations are homogeneous for the genotypes (Freeman, 1973) and the regression fit may be strongly influenced by outlying data points (Westcott, 1986). Although Westcott (1986) strongly disapproved of the joint regression analysis on experimental means, other authors (Hill, 1975; Becker and Léon, 1988) have stressed the general usefulness of the analysis to plant breeding research as long as its empirical nature and the biological and statistical limitations are kept in mind.

To obtain a better understanding of the causes of interaction a physical quantification of the environment is preferable to the use of the experimental mean. However, physical measurements are very seldom available (Westcott, 1986) and when available usually no single physical factor can discriminate effectively between environments (Hill, 1975). In those cases, the fit of the joint regression will be worse in comparison to regression on the experimental mean (for a recent example see Gorman et al., 1989). For nitrate, however, joint regressions on physical factors related to light intensity showed better results than regressions on the environmental mean. This reflects the strong influence of light intensity on nitrate concentration. The fact that daylength was somewhat more effective as a prediction variable than global radiation could be due to the fact that the global radiation was not measured inside the glasshouse but at a nearby meteorological station.

The joint regression analysis divides the G×E interaction into a predictable part corresponding to regressions and an unpredictable part corresponding to deviations. Although the regression on physical factors accounts for the larger part of the G×E interaction, the deviations part is still significant. This phenomenon is very common to this analysis (Hill, 1975). However, the linear model has a considerable predictive value for the genotypes concerned. Even after regression on the experimental mean, which accounts for less than one-third of the G×E interaction, almost the same classification of genotypes follows from the estimated regression lines than from regression on daylength (Fig. 5.3).

The occurrence of G×E interactions has important consequences for practical plant breeding. It means that selection should be done as far as possible under conditions similar to those for which cultivars are bred. For cultivars of lettuce with a low nitrate concentration this will primarily be for glasshouse production in late autumn, winter and early spring. However, also within this restricted period interactions could occur. An additional advantage

of selection under low light conditions is the smaller residual error of experiments conducted under such conditions.

When analysing the stability of genotypes under changing environmental conditions, two concepts of stability are used: the static and the dynamic concept (Becker and Léon, 1988). In the static concept stable genotypes have the same performance under all conditions, in the dynamic concept stable genotypes do not show non-additivity. According to the static concept, lettuce genotype e is the most stable one in the group of lettuce genotypes tested. According to the dynamic concept, genotype d is the most stable. The question is: which response pattern should be preferred by plant breeders? Theoretically, the most preferable low-nitrate genotype would be a stable one according to the static concept. This genotype would have the same (low) nitrate concentration under all conditions. However, considering the low-nitrate genotypes in this study, selected for their low nitrate concentration from a large collection (Chapter 2), this ideal genotype is not available and the most pragmatic choice would be genotype g, with a low nitrate concentration in winter, a strong decrease in nitrate concentration with increasing light intensity and low stability variance. Only under mid-winter conditions does genotype g sometimes exceed genotype e in nitrate concentration. Under all other conditions this genotype has the lowest nitrate concentration of all genotypes tested.

Knowledge about the response pattern of genotypes is important both for advanced breeding material and for genotypes used to initiate a breeding program for low nitrate concentration. Lettuce cultivars are always bred for a specific period of the year and for specific cultivation conditions. However, even in a restricted period, e.g. mid-winter, the amount of radiation can vary considerably depending on weather conditions. Therefore, also within this restricted period, effects of G×E interaction could be important. For genotypes used as parents in a breeding program for low nitrate content it is especially important to know the reaction type for nitrate because G×E interactions are partly inherited (Hill, 1975; Powell et al., 1986). Therefore, these parents should be tested for nitrate accumulation under all environmental conditions relevant to later selection.

Various recent papers reported a negative relationship between the concentrations of nitrate and organic solutes (sugars and organic acids) in expressed cell sap (Behr and Wiebe, 1988; Blom-Zandstra and Lampe, 1985; Ourry et al., 1989; Reinink and Blom-Zandstra, 1989; Steingröver et al., 1986b; Veen and Kleinendorst, 1985). This negative relationship is explained by the hypothesis that nitrate is accumulated for osmotic purposes in the cell vacuole. Thus, the large effect of low light conditions on nitrate accumulation can be explained by a substitution of organic solutes in the vacuoles by nitrate when the production of photosynthates is low. Also genotype differences for nitrate accumulation could be related to differences in contents of organic solutes.

Behr and Wiebe (1988) reported that the means of 19 lettuce cultivars for nitrate concentration were negatively correlated with the concentrations of organic acids and monosaccharides. These results were confirmed by the results in this paper: genotype means for nitrate were negatively correlated with genotype means for organic acids. Important G×E interactions were not only found for nitrate, but also for organic acids and monosaccharides.

When plants accumulate nitrate to maintain osmotic pressure, genotypes with a reduced osmotic value of the vacuole sap would need less solutes and thus less nitrate. No significant differences in osmolarity were detected by Behr and Wiebe (1988) between 19 lettuce cultivars. In contrast, the present results have shown significant and repeatable genotypic differences in osmolarity. The largest difference observed amounted to 35 mosmol·$kg^{-1}$. Such a difference in osmolarity of the cell sap could have important consequences for the total amount of solutes needed to maintain osmotic pressure. If a difference of 35 mosmol·$kg^{-1}$ was entirely realized by anorganic ions, with nitrate as the anion, this would be equivalent to a difference in nitrate concentration of 17.5 mmol·$l^{-1}$, or 1.09 g·$l^{-1}$. Thus, genotypic differences in osmolarity, as observed here, could lead to very substantial differences in solute requirement and thus in nitrate accumulation. However, for the eight genotypes tested here no correlation between nitrate content and osmolarity was observed, whereas a positive correlation would have been expected if differences in osmolarity would be important to explain genotypic differences in nitrate accumulation. The absence of such a relationship could mean that these genotypes did not display the whole range of nitrate content possible, and that a further reduction of nitrate level could be achieved by combining the low nitrate mechanism of genotype g with the low osmolarity of genotype c. However, it could also mean that for physiological reasons a combination of extremely low nitrate content with low osmolarity is not possible. An analysis of a segregating population for both traits is needed to elucidate this point.

## References


Becker, H.C. & J. Léon, 1988. Stability analysis in plant breeding. Plant Breeding 101, 1-23.

Behr U. & H. J. Wiebe, 1988. Beziehungen zwischen dem Gehalt an Nitrat und anderen Osmotica des Zellsaftes bei Kopfsalatsorten (*Lactuca sativa* L.). Gartenbauwissenschaft 53, 206-210.

Blom-Zandstra M. & J.E.M. Lampe, 1985. The role of nitrate in the osmoregulation of lettuce (*Lactuca sativa* L.) grown at different light intensities. Journ. Exp. Bot. 36, 1043-1052.

Blom-Zandstra M., J.E.M. Lampe & F.H.M. Ammerlaan, 1988. C and N utilization of two lettuce genotypes during growth under non-varying light conditions and after changing the light intensity. Physiol. Plant. 74, 147-153.

Danek-Jezik, K., 1986. Nitratvergleiche bei Treibsalatsorten in verschiedenen Entwicklungsstadien. Gartenbauwissenschaft 51, 190-192.

Dijkshoorn, W., 1973. Organic acids and their role in ion uptake. In: G.W. Butler and R.W. Bailey (eds), Chemistry and Biochemistry of Herbage, Vol. 2, pp. 163-188.

Academic Press, London. ISBN 0-12-148102.

Eberhart, S.A. & W.A. Russell, 1966. Stability parameters for comparing varieties. Crop Sci. 6, 36-40.

Freeman, G.H., 1973. Statistical methods for the analysis of genotype-environment interactions. Heredity 31, 339-354.

Gorman, D.P., M.S. Kang & M.R. Milam, 1989. Contribution of weather variables to genotype x environment interaction in grain sorghum. Plant Breeding 103, 299-303.

Hardwick, R.C., 1972. The analysis of GE interactions: what does it mean if varietal stability is linearly related to varietal performance. Euphytica 30, 217-221.

Hardwick, R.C. & J.T. Wood, 1972. Regression methods for studying genotype-environment interactions. Heredity 28, 209-222.

Hill, J., 1975. Genotype-environment interactions - a challenge for plant breeding. J. Agric. Sci. 85, 477-493.

Knight, R., 1970. The measurement and inter pretation of genotype-environment interactions. Euphytica 19, 225-235.

Lin, C.S., M.R. Binns & L.P. Lefkovitch, 1986. Stability analysis: Where do we stand? Crop Sci. 26, 894-900.

Ourry, A., B. Gonzalez & J. Boucaud, 1989. Osmoregulation and role of nitrate during regrowth after cutting of ryegrass (*Lolium perenne*). Physiologia plantarum 76: 177-182.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Powell, W., P.D.S. Caligari, M.S. Phillips & J.L. Jinks, 1986. The measurement and interpretation of genotype by environment interaction in spring barley (*Hordeum vulgare*). Heredity 56, 255-262.

Quinche, J.P., 1982. Fluctuations des teneurs en nitrates des légumes au cours de la journée. Revue suisse Vitic. Arboric. Hortic. 14, 85-87.

Reinink, K. & M. Blom-Zandstra, 1989. The relation between cell size, ploidy level and nitrate concentration in lettuce. Physiol. Plant. 76, 575-580.

Reinink, K. & A.H. Eenink, 1988. Genotypical differences in nitrate accumulation in shoots and roots of lettuce. Scientia Hortic. 37, 13-24.

Reinink, K. & R. Groenwold, 1988. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica, 36, 733-744.

Roorda van Eysinga, J.P.N.L. & M.Q. van der Meijs, 1985. Effect of nitrogen nutrition an global radiation on yield and nitrate content of lettuce grown under glass. Commun. in Soil Sci. Plant Anal. 16, 1293-1300.

Roorda van Eysinga, J.P.N.L. & L. Spaans, 1985. Nitrate and bromide contents of glasshouse radish as affected by soil content and global radiation. Commun. in Soil Sci. Plant Anal., 16, 1307-1318.

Shukla, G.K., 1972. Some statistical aspects of partitioning genotype-environmental components of variability. Heredity 29, 237-245.

Snedecor, G.W. & W.G. Cochran, 1980. Statistical methods. Seventh edition. The Iowa State University Press, Ames, Iowa, USA, 507 pp.

Steingröver, E., P. Ratering & J. Siesling, 1986a. Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity. Physiol. Plant. 66, 550-556.

Steingröver, E., J. Siesling & P. Ratering 1986b. Effect of one night with "low light" on uptake, reduction and storage of nitrate in spinach. Physiol. Plant. 66, 557-562.

Veen, B.W. & A. Kleinendorst, 1985. Nitrate accumulation and osmotic regulation in Italian ryegrass (*Lolium multiflorum* Lam.). J. Exp. Bot. 36, 211-218.

Westcott, B., 1986. Some methods of analysing genotype-environment interaction. Heredity 56, 243-253.

Wricke, G., 1962. Über eine Methode zur Erfassung der ökologischen Streubreite in Feldversuchen. J. Pflanzenzüchtg. 47, 92-96.

# 6 Relationship between Effects of Seasonal Change on Nitrate and Dry Matter Content in Lettuce

*Lettuce genotypes respond differently to changes in environment conditions between experiments with respect to the nitrate content in fresh matter. To study the inheritance and characteristics associated with genotype x environment (G×E) interaction for nitrate content, two lettuce genotypes known to show large G×E interaction, and 25 $F_3$ lines from a cross between them were studied in four successive experiments. G×E interactions were observed both for nitrate content and for dry matter content: with increasing daylength one parent showed nearly constant nitrate content and decreasing dry matter content, while with the other parent the reverse was true. Similarly, under increasing daylength, $F_3$ line means showed a high and negative correlation between the rate of change of nitrate and dry matter content, suggesting that these are pleiotropic effects. None of the $F_3$ lines had a transgressive low nitrate content in all experiments, but some $F_3$ lines had significantly lower nitrate relative to the lowest nitrate parent in some experiments, while they were not significantly different from the lowest nitrate parent in the other experiments. This suggests that a small further reduction of nitrate content is possible by recombination of genes for low nitrate content from both parents.*

## 6.1 Introduction

In several studies it was observed that the nitrate accumulation shown by lettuce genotypes reacted differently to changes in environment conditions (Chapters 2-5; Reinink and Blom-Zandstra, 1989). A large and repeatable interaction for nitrate accumulation associated with seasonal changes in environment was observed with two lettuce cultivars, 'Reichenauer Winter' (CGN5233) and 'Trocadero Light 76' (CGN4944). It was observed repeatedly that in the mid-winter period CGN5233 had the lowest nitrate content of all genotypes tested, while it had intermediate nitrate contents in periods with higher irradiance. In contrast, CGN4944 had a somewhat higher nitrate content than CGN5233 in mid-winter, but a much lower nitrate content in the remainder of the year (Chapters 3 and 5). To study the inheritance and characteristics associated with genotype x environment (G×E) interaction for nitrate content, CGN5233 and CGN4944 and 25 random $F_3$ lines from a cross between them were grown in four successive experiments.

## 6.2 Materials and methods

Two lettuce cultivars were chosen based on the repeatable G×E interactions they had shown in previous research. The cultivars were: 'Reichenauer Winter' (CGN5233), which originated from Switzerland and 'Trocadero Light 76' (CGN-4944), which originated from Italy. Both cultivars are outdoor butterhead types previously selected for their low nitrate content under low light conditions

(Chapter 2). They were crossed and $F_3$ seed was produced.

Four successive experiments (experiments 1-4) were conducted in the winter of 1988-1989. In each experiment 32 (experiment 1) or 30 (experiments 2-4) plants of both parents and 12 plants of 25 randomly chosen $F_3$ lines of the cross CGN5233 × CGN4944 were included. Seeds were sown in trays with peat. For experiments 1 to 4 this was done on 13 Oct., 25 Oct., 16 Nov. and 1 Dec. of 1988, respectively. Ten days after sowing the plants were transplanted to 80 $cm^3$ perforated pots filled with peat. Five weeks after sowing the potted plants were placed in a glasshouse in gullies with a recirculating nutrient solution (NFT-system). The mean nitrate concentration of the nutrient solution was 12.4 $mmol \cdot l^{-1}$, with extremes ranging from 10.4 to 15.2 $mmol \cdot l^{-1}$. The concentration of ammonia was maintained below 0.1 $mmol \cdot l^{-1}$, the pH at 6.0 and the EC at 2.0 $mS \cdot cm^{-1}$. The plants were grown under natural daylight conditions at minimum day and night temperatures of 12°C and 7°C respectively. The glasshouse was ventilated if the temperature exceeded 15°C during the day or 9°C at night.

The experiments comprised four (experiment 1) or three (experiments 2-4) replicates, which were harvested on successive days in the periods 31 Jan.-3 Feb. (experiment 1), 14-16 Feb. (experiment 2), 6-8 March (experiment 3) and 20-23 March, 1989 (experiment 4). Plants were harvested between 08.30 h and 11.00 h. The roots of all plants were checked and those with brown roots (in most cases caused by a species of *Pythium*) were excluded from analysis. The percentage of $F_3$ plants excluded from analysis in experiments 1 to 4 was 10, 2, 5 and 6, respectively. Shoots were cut from the roots and individual plants were weighed, dried for 20 hours at 70°C and weighed again to determine the dry weight of the plant. The dried matter was ground and the nitrate concentration was analyzed in an aqueous extract with an autoanalyser (Skalar, Breda, the Netherlands). The nitrate content was expressed in g per kg fresh weight.

Means and variances were calculated for the parent cultivars and the $F_3$ population after correction for effects of experiments and replicates. 95% Confidence intervals of means were calculated from the observed residual variance and the number of tested plants, using the t-distribution. Linear regressions of parent and $F_3$ line means were performed for nitrate content and dry matter content on daylength at harvest, calculated according to Van Keulen et al. (1982). Correlations between characteristics were calculated using the $F_3$ line means, excluding the parent means.

## 6.3 Results

The mean nitrate contents of the parent cultivars and the $F_3$ in the four successive experiments ranged from 2.03 to 2.84 $g \cdot kg^{-1}$ (Table 6.1). The G×E interaction for nitrate content is shown by the reversed order of the two parents in experiments 1-2 and experiments 3-4. The calculated daylength at harvest for experiments 1 to 4 were 8.85, 9.64, 11.02 and 12.05 h, respectively. The nitrate content of CGN5233 showed no tendency to decrease with increasing

*Table 6.1.* **Means and 95 % confidence intervals (between brackets) for nitrate content ($NO_3$; g per kg fresh matter), dry matter content (DMC; %) and fresh weight (FW; g) of the two parental cultivars and the $F_3$ population in four experiments, and within-experiment correlations for $F_3$ line means between $NO_3$, DMC and FW**

| Trait | Experiment | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| **Nitrate content** | | | | |
| CGN5233 | 2.59 (*2.45-2.73*) | 2.14 (*2.05-2.23*) | 2.50 (*2.40-2.60*) | 2.52 (*2.38-2.67*) |
| CGN4944 | 2.84 (*2.56-3.12*) | 2.62 (*2.46-2.78*) | 2.18 (*2.07-2.29*) | 2.03 (*1.84-2.22*) |
| $F_3$ | 2.61 (*2.55-2.67*) | 2.36 (*2.32-2.40*) | 2.43 (*2.39-2.46*) | 2.37 (*2.32-2.42*) |
| **Dry matter content** | | | | |
| CGN5233 | 7.05 (*6.90-7.20*) | 6.98 (*6.84-7.12*) | 5.93 (*5.79-6.08*) | 6.06 (*5.86-6.25*) |
| CGN4944 | 5.84 (*5.60-6.09*) | 5.97 (*5.79-6.15*) | 5.69 (*5.57-5.81*) | 5.93 (*5.78-6.07*) |
| $F_3$ | 6.51 (*6.45-6.56*) | 6.43 (*6.39-6.46*) | 5.68 (*5.65-5.72*) | 5.73 (*5.69-5.78*) |
| **Fresh weight** | | | | |
| CGN5233 | 39.0 (*36.8-41.2*) | 38.8 (*37.0-40.5*) | 63.9 (*59.4-68.4*) | 108.9(*104.6-113.2*) |
| CGN4944 | 108.3 (*97.5-119.1*) | 101.2 (*93.5-108.9*) | 122.8 (*114.1-131.5*) | 212.9(*202.8-223.0*) |
| $F_3$ | 70.8 (*68.6-73.0*) | 81.4 (*79.5-83.3*) | 98.0 (*95.9-100.1*) | 153.3(*149.4-157.2*) |
| **Correlations** | | | | |
| $r_{F_3(NO_3;DMC)}$ | -0.26 | -0.30 | -0.15 | -0.41* |
| $r_{F_3(NO_3;FW)}$ | -0.17 | -0.19 | 0.07 | -0.11 |
| $r_{F_3(DMC;FW)}$ | -0.45* | -0.44* | -0.58** | -0.44* |

* : significant at P=0.05; ** : significant at P=0.01.

daylength. CGN4944 showed a continuous decrease in nitrate content in the successive experiments, amounting to a reduction of nitrate content of 29% when experiments 1 and 4 are compared. In all experiments the mean of the $F_3$ population for nitrate content was between those of both parents, but closest to the mean of CGN5233.

The parent and $F_3$ means for dry matter content ranged from 5.69 to 7.05% (Table 6.1). The dry matter content of CGN5233 was about 7% in the first two experiments and decreased to about 6% in experiments 3 and 4. In contrast, CGN4944 had about constant dry matter content of 5.9 % in all experiments. The mean dry matter content of the $F_3$ was between that of both parents in experiments 1 and 2, equal to the dry matter content of CGN4944 in experiment 3 and lower than the dry matter content of CGN4944 in experiment 4.

In the first two experiments CGN-5233 had a low fresh weight of 36% (experiment 1) and 38% (experiment 2) relative to CGN4944 (Table 6.1). In the last two experiments CGN5233 had higher fresh weight, resulting in relative values of 52% (experiment 3) and 51% (experiment 4) compared to the fresh weight of CGN4944. This increase in fresh weight of CGN5233 relative to CGN4944 is more than expected from the decrease in dry matter content alone. Again, the mean of the $F_3$ population was between that of both parents.

*Within-experiment correlations* for $F_3$ line means showed a significant negative correlation between nitrate and dry matter content only in experiment 4, no significant correlations between nitrate content and fresh weight and a significant negative correlation between dry matter content and fresh weight in all experiments.

Comparison of the two parent cultivars shows that CGN5233 lowered its dry matter content and maintained its nitrate content with daylengths at harvest increasing from about 9 to 12 h in experiments 1 to 4, while CGN4944 maintained its dry matter content and lowered its nitrate content. Another striking difference between both cultivars is that in comparison to CGN4944, CGN-5233 had a poor growth under low light conditions. To study whether this association in CGN5233 of poor growth with stable nitrate content and decreasing dry matter content with increasing daylength is causal or only coincidental, the means of individual $F_3$ lines were analyzed. In the case of a coincidental association an independent segregation of the traits is expected in the $F_3$ lines. As a measure for the dependence of nitrate content and dry matter content on daylength, for both parents and for each $F_3$ line regression coefficients ($b_i$) from linear regression of nitrate content ($b_{i(NO3;DL)}$ in $g \cdot kg^{-1} \cdot h^{-1}$) and dry matter content ($b_{i(DMC;DL)}$ in $\% \cdot h^{-1}$) on daylength at harvest were calculated. These regressions measured differences in response of the $F_3$ lines to increasing daylength in successive experiments. Previous research (Chapter 5) has shown that the calculated daylength at harvest has a high explanatory value for the nitrate content in cultivars of lettuce. As was already apparent from Table 6.1, the $b_{i(NO3;DL)}$ estimate was nearly zero for CGN5233 and strongly negative for CGN4944 (Fig. 6.1). For the $b_{i(DMC;DL)}$ estimates the situation was the reverse: a negative value for CGN5233 and a nearly zero estimate for CGN4944 (Fig. 6.1). Although for both regressions the $b_i$'s of most $F_3$ lines were between those of both parents, the majority of $F_3$ lines resembled CGN5233 more than CGN-4944 for both traits (Fig. 6.1). Some lines even had a stronger increase in nitrate and a stronger decrease in dry matter content with increasing daylength (especially line 9). There is a strong negative correlation ($r=-0.81$, $P<0.01$) between both $b_i$'s (Fig. 6.1), indicating that reduction of dry matter content and of nitrate content with increasing daylength are inversely related and do not segregate independently in the $F_3$ lines.

There was no significant correlation between $b_{i(NO3;DL)}$ and the mean fresh weight of the $F_3$ lines in the four experiments ($r=-0.08$, Fig. 6.2). Lines which were similar to CGN5233 with respect to the changes in nitrate and dry matter content under increasing daylength,

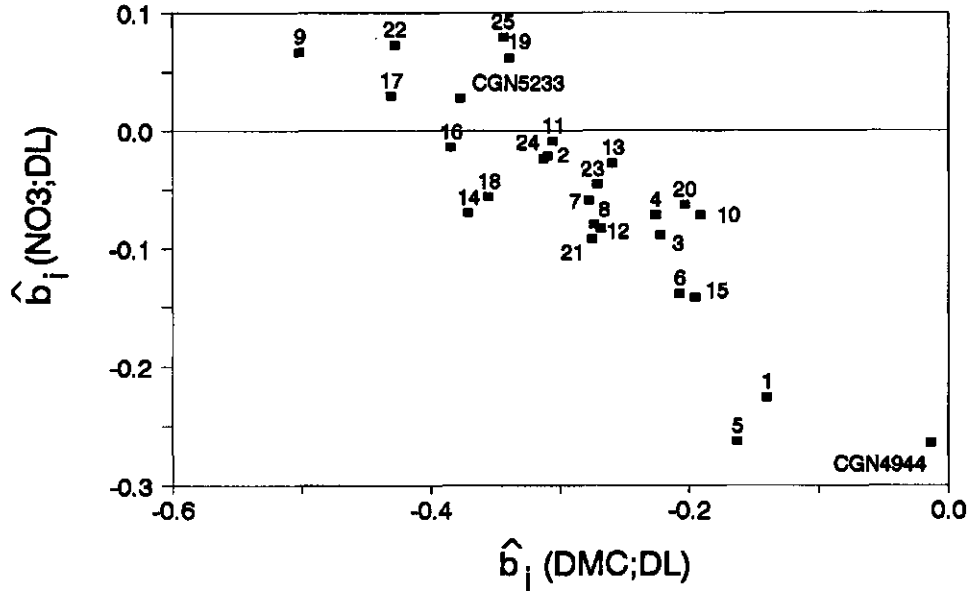


*Fig. 6.1.* **The relationship between regression coefficients from linear regressions of parent and $F_3$ line means for nitrate and dry matter content on daylength at harvest ($b_{i(NO3;DL)}$ ($g \cdot kg^{-1} \cdot h^{-1}$) and $b_{i(DMC;DL)}$, ($\% \cdot h^{-1}$), respectively).**

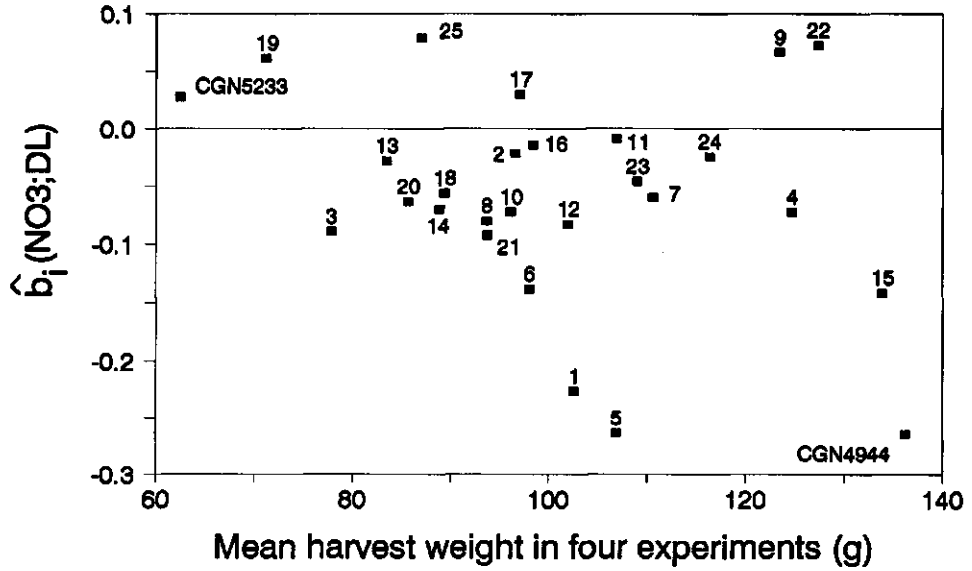


*Fig. 6.2.* **The relationship between regression coefficients from linear regressions of parent and $F_3$ line means for nitrate content on daylength at harvest ($b_{i(NO3;DL)}$, $g \cdot kg^{-1} \cdot h^{-1}$) and line means for fresh weight at harvest.**

were found both in the lower ranges (lines 19 and 25) and in the higher ranges of fresh weight (lines 9 and 22).

## 6.4 Discussion

One of the striking features observed when screening a large lettuce collection for nitrate content, was a strong negative correlation between nitrate and dry matter content in butterhead cultivars, whereas in other lettuce types such a correlation was either low or absent (Chapter 2). In the experiments presented in this chapter a significant within-experiment correlation between nitrate and dry matter content was only observed in experiment 4 ($r=-0.41$; Table 6.1). Probably, the correlations between nitrate and dry matter content were not significant (experiments 1 to 3) or only low (experiment 4) in the $F_3$ population tested here, because the ranges of nitrate and dry matter content were very restricted compared to the screening described in Chapter 2.

The relationship between dry matter content and nitrate content can be understood if a high dry matter content is associated with high contents of organic solutes in the vacuole. Plants growing under low light conditions accumulate high amounts of nitrate to compensate for a shortage of organic solutes (in lettuce mainly malate, fructose and glucose) in the cell vacuole, thus maintaining the osmotic pressure of the cell (Blom-Zandstra and Lampe, 1985; Steingröver et al., 1986; Behr and Wiebe, 1988). Reinink and Blom-Zandstra (1989) found evidence for a positive correlation between the dry matter content of lettuce cultivars and the concentration of organic solutes.

G×E interactions for nitrate content in lettuce have been reported before (Chapter 2-5; Reinink and Blom-Zandstra, 1989). The present study has shown a close relationship between the G×E interaction for nitrate content shown by the cultivars CGN5233 and CGN4944 and a G×E interaction for dry matter content. Under mid-winter conditions CGN5233 has a high dry matter content and accumulates relatively little nitrate. This could be explained if the high dry matter content in CGN5233 is associated with a high vacuolar concentration of organic solutes. Within the range of the experiments described here, CGN5233 reacted to increasing daylength by a decrease in dry matter content. Assuming a positive relationship between dry matter content and the vacuolar concentration of organic solutes, the decrease in dry matter content with increasing daylength in CGN5233 would lead to reduced contents of organic solutes and an increased need for nitrate. Thus the, negative effect of increasing daylength on the nitrate content could be much smaller in CGN5233 than what would be expected with constant levels of dry matter content under increasing daylength. This effect is shown as G×E interaction when CGN5233 is compared to CGN4944, a genotype that does not show a substantial change in dry matter content within the range of daylengths tested. The high and negative correlation (Fig. 6.1) between the rates of change of dry matter content and of nitrate with increasing daylength in a group of random $F_3$ lines shows that both characteristics cosegregate, which makes it likely

that these are pleiotropic effects.

Previous research (Chapter 2) has shown a large genotypic variation for nitrate content in lettuce, which is inherited quantitatively (Chapter 3; Reinink and Groenwold, 1987). However, if low nitrate content is associated with poor growth under low light conditions, as was found for CGN5233, at least part of the genotypic variation for nitrate content is not usable for practical purposes. Handke and Junge (1984) found a similar association of low nitrate content with low fresh matter production and high dry matter content in a mutant of spinach. The causal relationships between fresh matter production and dry matter content are not clear. On the one hand, a high dry matter content can be expected to result in a low fresh weight production, due to the higher energy input per unit fresh weight. On the other hand, accumulation of high contents of dry matter could also be a reaction of the plant to unfavourable growth conditions, thus being the result, rather than the cause of poor growth. However, the results of the $F_3$ lines have shown that a reaction to increasing daylength similar to that of CGN5233 (decrease of dry matter content and stable nitrate content) can be found both in lines with low or high fresh matter production. Furthermore, no significant within-experiment correlation was found between nitrate content and fresh weight of $F_3$ lines. This indicates that the relationship between poor growth and low nitrate in CGN5233 is not causal and the genotypic variation for nitrate content available for practical breeding is not only restricted to genotypes with the characteristics of CGN4944.

A next question of practical interest is whether the mechanisms leading to low nitrate content in CGN5233 and in CGN4944 can be combined to obtain a further reduction in nitrate content. Since the physiology of the mechanisms in both low nitrate genotypes is largely unknown, no prediction can be made whether the different mechanisms will re-enforce or weaken each others action when combined in one genotype. However, both from previous research (Chapter 4) and from the present results it can be concluded that a recombination of genes leading to a further reduction of nitrate content is possible: although none of the $F_3$ lines had a nitrate content lower than both parents in all experiments, some lines had lower nitrate content than the lowest nitrate parent in some experiments and were not significantly different from the lowest nitrate parent in the other experiments.

G×E interactions for nitrate content create additional difficulties for breeding programmes aiming to reduce nitrate content. The occurrence of G×E interaction means that selection results are only fully repeatable under identical environmental conditions. In most cases G×E interaction will lower the response to selection. Therefore, when important G×E interactions occur, it is important to only select in that period of the year in which the cultivars resulting from the breeding programme will be grown. Furthermore, it is important to characterize the parents of breeding programs with respect to G×E interaction to know whether large effects of interaction can be expected in segregating populations resulting from those parents. The obser-

vation of different mechanisms influencing the nitrate content may also lead to a further reduction of nitrate content by the combination of different mechanisms in one genotype.

## References


Behr, U., and Wiebe, H.J., 1988. Beziehungen zwischen dem Gehalt an Nitrat und anderen Osmotica des Zellsaftes bei Kopfsalatsorten (*Lactuca sativa* L.). Gartenbauwissenschaft, 53: 206-210.

Blom-Zandstra, M. and Eenink A.H., 1986. Nitrate content and reduction in different genotypes of lettuce. J. Amer. Soc. Hort. Sci., 111: 908-911.

Blom-Zandstra, M., Koot, H.T.M., Vanhattum, J. and Borstlap A.C., 1991. Interactions of uptake of malate and nitrate into isolated vacuoles from lettuce leaves. Planta, 183: 10-16.

Blom-Zandstra, M. and Lampe J.E.M., 1985. The role of nitrate in the osmoregulation of lettuce (*Lactuca sativa* L.) grown at different light intensities. Journ. Exp. Bot., 36: 1043-1052.

Blom-Zandstra, M., Lampe, J.E.M. and Ammerlaan, F.H.M., 1988. C and N utilization of two lettuce genotypes during growth under non-varying light conditions and after changing the light intensity. Physiol. Plant., 74: 147-153.

Handke, S. and Junge, H., 1984. Green matter yield and nitrate content in a dioecious spinach genotype with a long vegetative phase. Proc. Eucarpia Meeting Leafy Vegetables, Versailles, 28 Febr.- 2 March 1984: 110-114.

Keulen, H. van, Penning de Vries, F.W.T. and Drees, E.M., 1982. A summary model for crop growth. In: F.W.T. Penning de Vries and H.H. van Laar (Eds.) Simulation of plant growth and crop production. Pudoc - I11 - (Simulation monographs), Wageningen, The Netherlands: 87-97.

Reinink, K. and Blom-Zandstra, M., 1989. The relation between cell size, ploidy level and nitrate concentration in lettuce. Physiol. Plant., 76: 575-580.

Reinink, K. and Eenink, A.H., 1988. Genotypical differences in nitrate accumulation in shoots and roots of lettuce. Scientia Horticulturae 37: 13-24.

Reinink, K. and Groenwold, R., 1987. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica, 36: 733-744.

Steingröver, E., Ratering, P. and Siesling, J., 1986. Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity. Physiol. Plant., 66: 550-556.

# 7 General Discussion

*Public concern about health effects of high nitrate consumption has led to a large amount of biological and agricultural research into nitrate accumulation in vegetables. This thesis resulted from a study into the genetics of nitrate accumulation in lettuce and the potentials to reduce the nitrate content of lettuce by breeding. The main questions addressed in the present research were:*

1. *What is the genotypic variation for nitrate content in cultivated lettuce or in exploitable related Lactuca species?*
2. *What is the inheritance of differences in nitrate content between lettuce genotypes?*
3. *Is it possible to select lettuce cultivars with an acceptable low level of nitrate?*
4. *What is the most efficient way to select for low nitrate content in lettuce and which factors influence the selection efficiency?*

*The first part (7.1) of this general discussion deals with these questions in view of the results presented in Chapters 2 to 6. The second part (7.2) discusses recent insights concerning physiological mechanisms involved in nitrate accumulation and the possible physiological mechanisms causing the observed genotypic variation for nitrate content in lettuce.*

## 7.1 Perspectives of reducing the nitrate content of lettuce by breeding

### 7.1.1 Genotypic variation

Several studies have shown that even among modern lettuce cultivars differences can be observed for nitrate accumulation (Cools et al., 1980; Schaer and Habben, 1986; Behr and Wiebe, 1988). These differences in nitrate content can be exploited by growers and plant breeders. However, greater differences in nitrate accumulation were found when screening a much wider range of genetic material (Chapter 2). Extremely high nitrate contents were found within the group of wild *Lactuca* species. Accessions with a low nitrate content were found in all cultivated lettuce types (butterhead, cos, crisphead, latin, cutting), but especially in the group of butterhead cultivars.

The lowest nitrate contents were found in obsolete cultivars that are not adapted to modern cropping practices. These accessions can be used to breed for nitrate levels below those found in modern cultivars. The low level of nitrate found in these accessions would mean a valuable contribution to the efforts aimed at reducing the human nitrate consumption: in a series of 18 experiments CGN4944, the accession with lowest nitrate, had on average a nitrate content of 1.7 $g \cdot l^{-1}$ below that of the modern cultivar Panvit (Chapter 3). This equals an average reduction of about 50%.

The identification of these low-nitrate accessions had a considerable impact on

practical breeding programs aimed at incorporating the low nitrate trait in modern cultivars. Information on the low-nitrate accessions was given to breeding companies in 1985 and several programs were started with these accessions. However, for an efficient selection of low-nitrate genotypes, additional knowledge was required on the inheritance of low nitrate content and the stability of the low-nitrate trait under a wider range of environmental conditions. Another important question was to what extent the low nitrate trait can be transferred into modern cultivars.

### 7.1.2 Genetics of nitrate accumulation in lettuce

*The genetic model*

In a genetic study the first question to be answered is whether the trait under study is governed by one or a few genes with distinctively detectable effects (qualitative inheritance), or by a number of genes of which the individual effects can not be measured distinctively (quantitative inheritance). Genes with a qualitative inheritance are easy to handle in breeding programs. Previous experiments had shown a continuous variation in nitrate content among cultivars. Consequently, a qualitative inheritance was not expected for nitrate accumulation in lettuce. A qualitative model for the inheritance of nitrate content in lettuce was proposed by Subramanya et al (1980), with low nitrate being governed either by alleles at two loci with dominant complementary epistasis, or by one single dominant allele. The present study, using a much wider range of crosses and generations, has shown that a qualitative model was inadequate to explain the inheritance of nitrate accumulation in lettuce (Chapter 4). A quantitative analysis, as worked out for autogamous species by Mather and Jinks (1982), was more appropriate.

In Chapter 4 it was shown that nitrate content inherits mainly additively. The effects of dominance were relatively small, although sometimes statistically still highly significant. Consequently, generations will have a mean value for nitrate content close to the mean of their parents. No important maternal effects were observed: although there were some differences between reciprocal crosses, these were of limited size and varied between experiments. In conclusion, the inheritance of nitrate content in lettuce can satisfactorily be described by an additive-dominance genetic model (Mather and Jinks, 1982), excluding the influence of other factors such as linkage, epistasis and maternal effects.

*Frequency of low-nitrate segregants and accumulation of genes for low nitrate*

Subsequently, in a series of crosses the contribution of genetic and non-genetic sources to the variation for nitrate content were estimated in $F_2$ and $F_3$ generations (Chapter 5). The results of this study were used to answer two practical questions:

1. What is the probability of obtaining low-nitrate segregants from crosses between modern high-nitrate cultivars and low-nitrate accessions?
2. Can a further reduction in nitrate content be achieved by combining genes from different low-nitrate accessions?

To predict the probability as described in 1. for a specific cross, the mean and variance of the population of random inbred ($F_\infty$) lines that can be derived from that cross, should be estimated (Jinks and Pooni, 1976). These estimates can be made in various ways from data from parental, $F_2$ and $F_3$ generations, assuming independently acting genes and the absence of linkage, stochastic variation and G×E interactions. Under the further assumption of a normal frequency distribution for nitrate content in the $F_\infty$ population, the proportion of random inbred lines in advanced generations with a nitrate content below a specified threshold was estimated. Two threshold values were evaluated: a) the mean of the lowest nitrate parents to predict the frequency of transgressive segregants and b) a fixed nitrate level of 2.5 $g \cdot l^{-1}$. The latter threshold was used to evaluate whether lines can be selected that are compatible with the future maximum nitrate level.

In crosses between a high- and a low-nitrate genotype the estimated probability of selecting transgressive low nitrate lines was low (P=0.002 - 0.039). Because not only the nitrate content, but also many other agronomically important traits will segregate in these crosses, large populations should be evaluated to combine the positive traits of modern high-nitrate cultivars with low nitrate content from accessions not adapted to modern cropping practices. Thus, a breeding program for low nitrate content will be relatively expensive.

In most $F_2$ generations from crosses between two low-nitrate genotypes a considerable genotypic variation was found, as reflected in estimated wide sense heritabilities ranging from 0.15 to 0.52. These results indicated that a further reduction of nitrate content below the level of the low-nitrate parents used in this study is possible. However, the results obtained from the $F_3$ populations did not sustain this optimism. Notwithstanding the relatively small differences between the parents for nitrate content, estimates of the proportion of lines with a lower nitrate content than the parent with lowest nitrate were rather low (P = 0.04-0.06). The possibility of obtaining transgressive lines may be further reduced if the assumptions made (a normal distribution of nitrate content in an inbred population and the absence of linkage, epistasis, stochastic variation and G×E interaction) do not hold. Therefore it does not seem likely that combining genes for low nitrate content from different accessions will lead to a substantial further reduction of the nitrate content.

### 7.1.3 Genotype × environment interactions

*Occurrence of G×E interactions*

Significant genotype × environment (G×E) interactions were found in the initial screening (Chapter 2) and in other studies (Reinink and Groenwold, 1988; Reinink and Blom-Zandstra, 1989). In Chapters 3 and 6 this aspect of nitrate accumulation was studied in more detail.

The occurrence of G×E interactions means that the ranking of a set of genotypes depends on the particular environmental conditions of the experiment in which they are tested. Such interactions are revealed statistically by a significant

non-additivity of the data when two or more genotypes are grown in contrasting environments. In breeding programs the occurrence of G×E interactions is a complicating, but frequently met phenomenon. For nitrate content important effects of G×E interaction could mean that accessions selected for low nitrate content in one experiment may only have a mediocre performance in another test. Obviously this will hamper both the selection of accessions to be used as parents in breeding programs and the selection of superior genotypes from crosses. In fact, if important G×E interactions occur, there may not be a superior genotype at all. One genotype may be the best in one particular environment, while others perform better in other environments.

G×E interactions can be considered to result from differential reactions of accessions to environmental factors. Because it is often unknown to which factors the accessions reacted differentially, the biological interpretation of statistically significant interactions is frequently lacking. In the experiments described in this thesis, with plants grown in a glasshouse on nutrient film, many environmental factors potentially related to G×E interactions were kept constant. However, the amount of light, was not controlled as plants were grown under natural light conditions. A strong influence of the amount of light on nitrate accumulation was shown by several authors (Blom-Zandstra and Lampe, 1985; Blom-Zandstra et al., 1988; Roorda van Eysinga and Van der Meijs, 1985; Steingröver et al., 1986; Veen and Kleinendorst, 1985). Therefore, differential reactions of lettuce genotypes to different levels of light might be important to explain the G×E interactions observed.

The present research studied the effects of two natural rhythms of light intensity, the daily and the annual cycles, on the nitrate content of a set of lettuce accessions. G×E interaction for nitrate content in the daily cycle could be of practical value if genotypes were to be found with a large fall in nitrate content during the day. When harvested in the afternoon, these genotypes could be marketed with low nitrate. Results obtained by Danek-Jezik (1986) suggested that such genotypes actually exist. However, in our studies no daily variation in nitrate concentration and no corresponding G×E interactions were observed, irrespective of the level of global radiation (Chapter 3). For selection experiments this means that lettuce plants can be harvested throughout the day, without the danger of additional variation caused by a daily cycle of the nitrate concentration.

Because lettuce is grown and harvested throughout the whole year in the Netherlands, it was also important to know whether there are important G×E interactions for nitrate content with the annual cycle of light intensity. If this interaction were small in comparison to the main effect of genotypes, selections can be made throughout the whole year. If, on the other hand, the annual G×E interaction were important, breeders should select only under conditions similar to those for which the low nitrate cultivars are intended. This would mean that selection can only be done in restricted periods of the year.

A large seasonal variation and im-

portant corresponding G×E interactions were found. This interaction involved the reversal of rank of some genotypes (Fig. 3.3). However, the most extreme genotypes, CGN4944 and cv. Panvit reacted very similarly to environmental change and consequently the total range of nitrate content remained about constant in all experiments. Several of the other genotypes showed different patterns of change for nitrate content with changing environmental conditions. No correlation was observed between the sensitivity to environmental change and the mean level of nitrate accumulation, as frequently found for yield (Hardwick, 1972).

The occurrence of G×E interactions mean that it is important to have knowledge about the nitrate content, relative to standard cultivars, of accessions to be used in breeding programs in a wide range of environmental circumstances. With that information parents can be chosen that show similar changes in nitrate content under varying circumstances, thus not showing important G×E interactions. An example of such a low-nitrate accession is CGN4944, which had the lowest nitrate content of the accessions tested in almost every period of the year (Chapter 3), and did not show G×E interactions when compared with the modern high-nitrate cultivar Panvit.

If parents are used which show important G×E interactions, selection should be restricted to the period of the year for which the cultivars are bred. Different breeding programs are then necessary to select cultivars intended to be grown in different periods of the year.

*Exploitation of G×E interactions*

A differential reaction of low-nitrate accessions to environmental changes could mean that further progress in reducing the nitrate levels is possible. G×E interactions indicate physiological differences between the genotypes tested. Combining these different mechanisms in one genotype by crossing and selecting, if physiologically feasible, might lead to a superior performance for nitrate content. CGN4944 had the lowest nitrate content of the accessions tested, in almost every period of the year (Chapter 3). Only under mid-winter conditions did the accession, CGN5233, have a lower nitrate content than CGN-4944. This interaction was repeatable: also in the genetic studies (Chapter 5) in two different years CGN5233 had the lowest nitrate content of all parental genotypes in mid-winter, while in periods with higher irradiance CGN4944 had lowest nitrate content. The low nitrate content of CGN5233 in mid-winter was associated with a low fresh matter production and a high dry matter content (Chapters 2 and 5).

A causal relationship between poor growth and low nitrate content under mid-winter conditions might prohibit the production of breeding lines combining an acceptable production with low nitrate content. To gain more insight into this association, a further study was carried out in which CGN5233, CGN4944 and 25 random $F_3$ progeny lines were grown in four successive experiments, harvested in February and March. G×E interactions were observed for both nitrate content and dry matter content (Chapter 6). When the two experiments harvested in March were

compared to those harvested in February, the nitrate content of CGN5233 was nearly constant and the dry matter content decreased, while for CGN4944 the opposite was observed. Similarly, the $F_3$ line means showed a high and negative correlation between the rate of change of nitrate and of dry matter content with increasing daylength, suggesting that these are pleiotropic effects. A high dry matter content may be associated with high contents of organic solutes in the vacuole, thus reducing the need for nitrate as an osmoticum (see 7.2).

Some of the $F_3$ lines combined a reaction to increasing daylength similar to CGN5233 (decrease of dry matter content and stable nitrate content) with a high fresh matter production. Furthermore, no correlation was found between nitrate content and fresh weight of $F_3$ lines within an experiment. This indicates that the relationship between poor growth and low nitrate content in CGN-5233 is not causal. Thus, the physiological mechanism causing low nitrate content in CGN5233 can be employed for practical purposes.

A further reduction in nitrate content could be possible by combining the mechanisms leading to low nitrate content in CGN5233 and CGN4944. However, since the physiology of the mechanisms in both low-nitrate genotypes is largely unknown, no prediction can be made as to how the different mechanisms will interact when combined in one genotype. Although only a limited number of 25 $F_3$ lines were tested, the results presented in Chapter 6 indicate that a substantial reduction in nitrate content below that of both parents cannot be expected from such a combination. None of the $F_3$ lines had a nitrate content below both parents in all experiments. However, some lines had a significantly lower nitrate content than the lowest parent in some experiments and were not different from the lowest parent in the other experiments. Thus, averaged over experiments, a small improvement is possible.

### 7.1.4 Breeding for the future maximum

An important question is whether the level of nitrate content found in the accessions with low nitrate identified in this study allows the selection of breeding lines with a nitrate content below the future maximum of 2.5 g per kg fresh matter. In a few of the experiments described, all parental genotypes had nitrate contents above 2.5 $g \cdot l^{-1}$ (Chapter 5). When the parental means from the experiment with highest nitrate contents were used to make estimates of the chance of finding lines with a nitrate content below 2.5 $g \cdot l^{-1}$, only very low probabilities were obtained in crosses between a high- and a low-nitrate accession. When two low-nitrate accessions were crossed, the estimate of this probability was higher. However, these populations showed important effects of G×E interactions, and therefore no reliable prediction of the selection results can be made.

Notwithstanding these results, breeding for low nitrate content in lettuce can be effective. The estimated fractions of inbred lines below the future maximum were based on parental means from the experiment with highest nitrate content.

In that experiment all parents had nitrate contents above 2.5 g· $l^{-1}$. However, most of the experiments harvested in winter had nitrate levels considerably lower than this extreme experiment. Under those conditions a higher fraction of the breeding lines will have a nitrate content compatible with the maximum limit. Furthermore, the experimental conditions in this study (plants grown on nutrient solution with high nitrate and very low ammonium concentration) were chosen because they produce high nitrate contents in the crop. With another cropping system the levels of nitrate may be much lower. Therefore, a combination of cultivars with a nitrate content comparable to the low-nitrate accessions in this study and a cropping system designed to further reduce the nitrate content may very well lead to nitrate levels below the future permissible maximum in most, if not all, of the lettuce crops grown in winter.

## 7.2 Physiology of genotypic variation for nitrate content

*Nitrate as osmoticum*

The last part of this chapter discusses physiological mechanisms possibly involved in the large genotypic variation observed for nitrate content of lettuce. In the last decade physiological research has considerately improved our general understanding of nitrate accumulation. However, little is still known about the physiological mechanisms of genotypic differences in nitrate content. The results from different physiological studies into cultivar differences for nitrate accumulation (Blom-Zandstra et al., 1989; Behr and Wiebe, 1992) seem to indicate that several physiological processes are involved.

Recent work established that nitrate has at least two functions in the plant cell. Not only is nitrate a source of nitrogen for the synthesis of various organic compounds, it also has an important function as an osmoticum, accumulated in the cell vacuole to maintain turgor pressure (Behr and Wiebe, 1988; Blom-Zandstra and Lampe, 1985; Steingröver, 1986). The function of the nitrate ion as osmoticum is non-specific: nitrate can be replaced by organic solutes (carbohydrates, organic acids) and to a certain extent also by other inorganic anions (e.g. chloride). Thus, nitrate accumulates when organic solutes for storage in the vacuole are limited. This occurs at low-light conditions. The uptake of nitrate is determined by the demand for nitrogen in the shoot (Steingröver, 1986). Nitrate is not taken up in excess of what is needed to maintain osmotic pressure.

If nitrate is mainly accumulated for osmotic purposes, a few hypotheses can be proposed about physiological mechanisms affecting nitrate accumulation: genotypic differences in nitrate accumulation could result from differences in:

1. The total availability of organic solutes.
2. The osmotic potential of the leaf cell.
3. The preference for accumulation of nitrate relative to organic solutes.
4. The preference for accumulation of nitrate relative to other inorganic anions.

*Total availability of organic solutes*

In the cell vacuole the most important organic compounds used as solutes are

sugars (glucose and fructose) and organic acids. In lettuce the organic solute with highest concentration is malate (Blom-Zandstra and Lampe, 1985; Behr and Wiebe, 1988). The availability of these organic compounds depends on the photosynthetic activity of the plant and hence on the light intensity. An increase of the photosynthetic rate by increased atmospherical $CO_2$ levels and by lowered $O_2$ levels decreased the nitrate content of kohlrabi plants (Bentrup and Lenz, 1987). Behr and Wiebe (1992) observed genotypic variation for photosynthetic rate in lettuce. The cv. 'Bellona' had a higher rate of $CO_2$ assimilation and a lower nitrate content than cv. 'Panvit'.

Genotypic variation for photosynthetic activity (and hence for the availability of organic solutes) could be related to differences in growth habit. However, in lettuce no direct relationship was found between morphological characteristics and nitrate content (Chapter 2; Behr and Wiebe, 1992). This is also in agreement with the close relationship between the nitrate content in very young lettuce plants and in older plants (Eenink et al., 1984, Behr, 1988, Reinink and Eenink, 1988).

*Osmotic potential*

In leaf cells the relationship between water potential ($\psi_{cell}$), pressure potential (turgor pressure, ($\psi_P$) and osmotic potential ($\psi_\pi$) can be conveniently described as $\psi_{cell} = \psi_P + \psi_\pi$ (Barlow, 1983). In leaves $\psi_{cell}$ and $\psi_\pi$ are negative, while $\psi_P$ is positive. The negative osmotic potential is generated by the concentration of ions and other solutes in the vacuoles. The solute accumulation is essential for maintaining turgor and for water flow in the plant (Pitman, 1988). The water potential of the leaf must be lower than that around the roots to enable water flow from the growth medium to the leaves. When the plant is growing under water stress (e.g. a dry soil or a nutrient solution with high osmolarity), the concentration of solutes in the vacuoles of the leaves must be correspondingly high and the osmotic potential correspondingly low (Barlow, 1983). For Italian ryegrass Veen and Kleinendorst (1985) showed that a PEG mediated decrease in water potential of the nutrient medium resulted in an increase of organic solutes in the leaf cells at high light intensity and of the nitrate content at low light intensity.

Genotypic variation for the osmotic potential in leaf cells could lead to differences in solute requirement, and thus in nitrate accumulation. Differences in osmotic potential could be due to differences in total water potential in the leaf or due to differences in turgor pressure. Genotypic variation in total water potential could be due to differences in resistance to water flow between the roots and the shoot. Differences between genotypes in turgor pressure could be due to differences in cell wall elasticity.

Very little is known about genotypic variation for these parameters. In Chapter 3 genotypic variation for osmotic potential was discussed. The largest difference observed amounted to 35 mosmol· $kg^{-1}$. Such a difference between genotypes would mean significant differences in solute requirement and therefore could lead to important differences in nitrate accumulation. However, with the eight genotypes

tested in Chapter 3, no relationship was found between nitrate content and osmolarity. Therefore, more research is needed to investigate whether selection for a low osmolarity in the leaf can lead to reduced nitrate contents.

*Preference of accumulation of nitrate relative to organic solutes*

Genotypic differences in nitrate accumulation could also result from differences in how genotypes use their photosynthates. Genotypes that use a larger part of their photosynthates for growth will have less carbon-compounds available for non-structural osmotic purposes and will compensate this by accumulating more inorganic ions. Blom-Zandstra et al. (1988) showed that the differences between two lettuce cultivars in nitrate accumulation were probably not due to differences in the production of photosynthates, but to differences in the partition of carbohydrates over structural growth and osmotic purposes.

An interesting point is that when nitrate accumulation is caused by a high fraction of the photosynthates being used for structural purposes, a positive relationship should be found between nitrate content and growth. This agrees with the fact that most of the modern lettuce cultivars selected for high productivity in glasshouse production in winter have high nitrate contents. In contrast, when differences in nitrate accumulation are related to differences in total production of photosynthates, a negative relationship is expected between nitrate content and growth.

The G×E interactions found in this study when comparing nitrate and dry matter contents for CGN5233 and CGN4944 (Chapter 6) may also be related to differences in utilization of carbon-compounds. In mid-winter CGN5233 combines a high content of dry matter and extremely low nitrate content with a poor growth in comparison to CGN4944 (Chapter 6). Most likely the high accumulation of dry matter is associated with high contents of organic solutes in the vacuoles (Reinink and Blom-Zandstra, 1989). Under conditions with higher levels of irradiance, the growth of CGN5233 was much improved in comparison to CGN-4944, but its dry matter content was no longer extremely high and its nitrate content no longer extremely low. Thus, in CGN5233 the proportion of photosynthates utilized for non-structural and structural growth seems to be under environmental control.

*Preference of accumulation of nitrate relative to other inorganic anions*

The function of nitrate as an osmoticum is non-specific. To a certain extent it can be replaced by other solutes, such as chloride. Increasing the chloride content of the growth medium can result in a reduction of the nitrate content of the plants grown on that medium (Blom-Zandstra and Lampe, 1983; Burghardt and Ellering, 1988; Wehrman and Hähndel, 1985; Van der Boon et al., 1990). Thus, genotypes with a high capacity to accumulate chloride as osmoticum could have reduced nitrate contents. A negative correlation between the nitrate and chloride contents of lettuce cultivars was reported by Behr and Wiebe (1988). In our own research (unpublished results) a negative correlation was found between the nitrate and chloride contents of

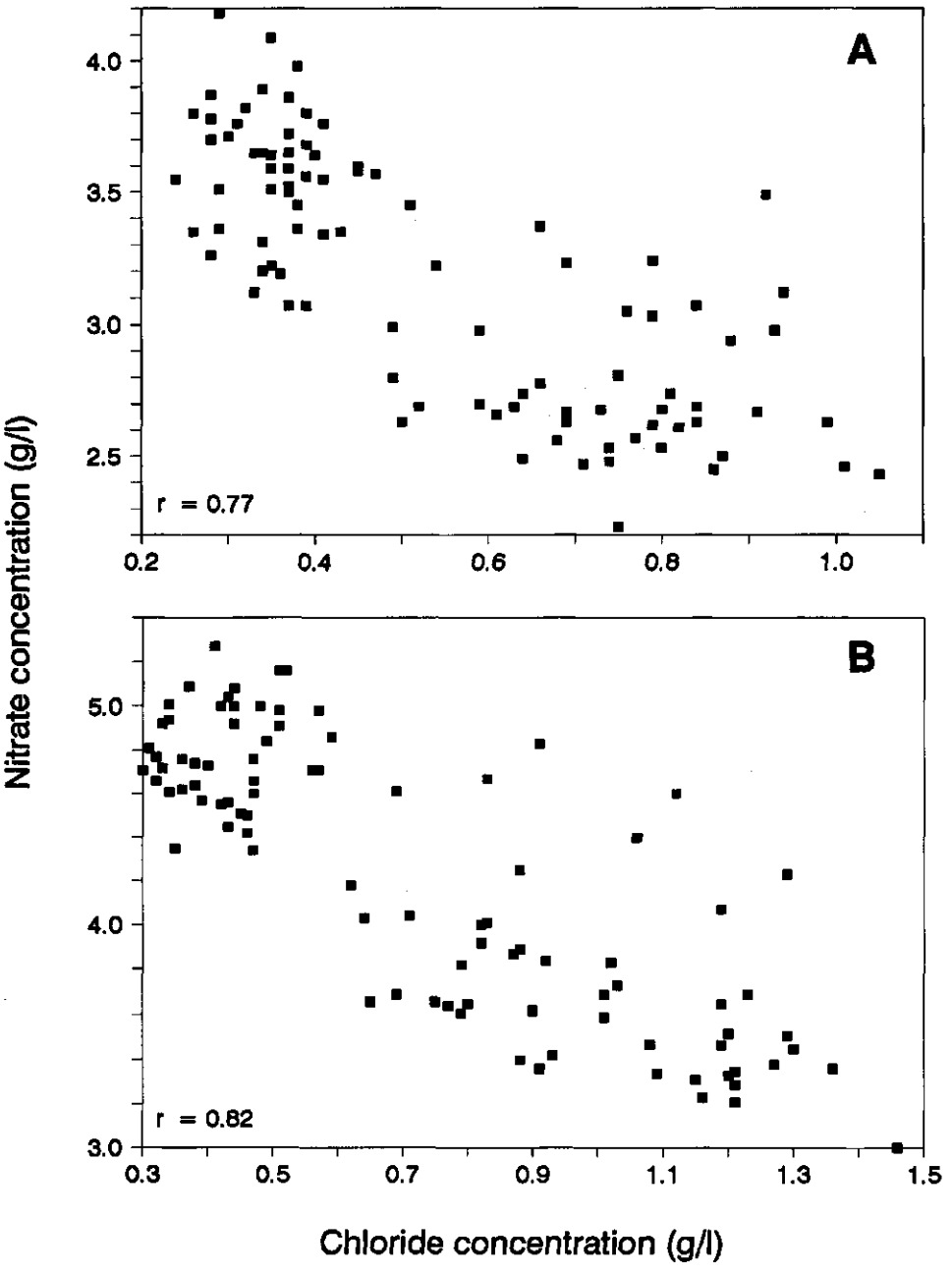


***Fig. 7.1.*** **Relationship between means of lettuce accessions for nitrate and chloride concentration in two experiments, harvested in November 1989 (A) and January 1990 (B).**

genotypes. This is shown in Fig. 7.1 for 95 lettuce genotypes harvested in November 1989 (Fig. 7A) and January 1990 (Fig. 7B). The experiments were carried out in the same way as those described in Chapters 3-6. Like the nitrate content, the chloride content of the lettuce genotypes also showed a significant genotype × experiment interaction. The low-nitrate accessions (CGN5233, CGN4892, CGN4944 and CGN5811) differed significantly for chloride content. This indicates that a further reduction of nitrate content could be achieved by combining genes from a high chloride accumulator with genes from a genotype with low nitrate but intermediate chloride content.

## References


Barlow, E.W.R., 1983. Water relations in the mature leaf. *In:* J.E. Dale & F.L. Milthorpe (Eds.), The Growth and Functioning of Leaves. Cambridge University Press, Cambridge, ISBN 0-521-23761-0: 315-345.

Behr, U., 1988. Sortenvergleich zum Gehalt an Nitrat und anderen qualitätsbestimmenden Inhaltstoffen in Kopfsalat (*Lactuca sativa* L. *var. capitata* L.) und Spinat (*Spinacia oleracea* L.). PhD-thesis, University Hannover, 149 pp.

Behr, U. & H.-J. Wiebe, 1988. Beziehungen zwischen dem Gehalt an Nitrat und anderen Osmotica des Zellsaftes bei Kopfsalatsorten (*Lactuca sativa* L.). Gartenbauwissenschaft 53: 206-210.

Behr, U. & H.-J. Wiebe, 1992. Relation between photosynthesis and nitrate content of lettuce cultivars. Scientia Horticulturae 49: 175-179.

Bentrup M. & F. Lenz, 1987. Einfluss von $CO_2$- und $O_2$-Behandlungen auf den Nitratgehalt von Kohlrabi. Gartenbauwissenschaft 52: 45-48.

Blom-Zandstra, M., 1990. Some physiological aspects of nitrate accumulation in lettuce (*Lactuca sativa* L.). PhD-thesis, State University Utrecht, 81 pp.

Blom-Zandstra, M. & J.E.M. Lampe, 1983. The effect of chloride and sulphate salts on the nitrate content in lettuce plants (*Lactuca sativa* L.). Journal of Plant Nutrition 6: 611-628.

Blom-Zandstra, M. & J.E.M. Lampe, 1985. The role of nitrate in the osmoregulation of lettuce (*Lactuca sativa* L.) grown at different light intensities. Journ. Exp. Bot. 36: 1043-1052.

Blom-Zandstra, M., J.E.M. Lampe & F.H.M. Ammerlaan, 1988. C and N utilization of two lettuce genotypes during growth under non-varying light conditions and after changing the light intensity. Physiologia Plantarum 74: 147-153.

Boon, J. van der, J.W. Steenhuizen & E.G. Steingröver, 1990. Growth and nitrate concentration of lettuce as affected by total nitrogen and chloride concentration, $NH_4/NO_3$ ratio and temperature of the recirculating nutrient solution. J. Hort. Sci. 65: 309-321.

Burghardt, H. & K. Ellering, 1988. Beeinflussung des Nitratgehaltes von Spinat durch unterschiedliche Kulturbedingungen. Gartenbauwissenschaft 53: 201-205.

Cools, M.H., M.Q. van der Meijs, J.P.N.L. Roorda van Eysinga & J.H. Stolk, 1980. Het nitraatgehalte van de huidige slarassen voor de teelt onder glas. Proefstation voor Tuinbouw onder Glas, P.O. Box 8, 2670 AA Naaldwijk, Report 79-45, 2pp.

Danek-Jezik, K., 1986. Nitratvergleiche bei Treibsalatsorten in verschiedenen Entwicklungsstadien. Gartenbauwissenschaft 51: 190-192.

Eenink, A.H., M. Blom-Zandstra, P.C.H. Holleman, P. Aarts & R. Groenwold, 1984. Research on the reduction of nitrate content in lettuce via breeding. Proc. Eucarpia Meeting Leafy Vegetables, Versailles, 28 Febr.- 2 March 1984: 100-109.

Jinks, J.L. & H.S. Pooni, 1976. Predicting the properties of recombinant inbred lines derived by single seed descend. Heredity 36: 253-266.

Hardwick, R.C., 1972. The analysis of GE inter-

actions: what does it mean if varietal stability is linearly related to varietal performance. Euphytica 30: 217-221.

Mather, K. & J. L. Jinks, 1982. Biometrical Genetics.-3rd ed. Chapman and Hall, London, 396 p.

Pitman, M.G., 1988. Whole plants. *In*: D.A. Baker & J.L. Hall (Eds.), Solute transport in plant cells and tissues. Longman Scientific and Technical, Essex, UK: 347-391.

Reinink, K. & M. Blom-Zandstra, 1989. The relation between cell size, ploidy level and nitrate concentration in lettuce. Physiologia Plantarum 76: 575-580.

Reinink, K. & A.H. Eenink, 1988. Genotypical differences in nitrate accumulation in shoots and roots of lettuce. Scientia Horticulturae 37: 13-24.

Reinink, K. & R. Groenwold, 1988. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica 36: 733-744.

Roorda van Eysinga, J.P.N.L. & M.Q. Van der Meijs, 1985. Effect of nitrogen nutrition and global radiation on yield and nitrate content of lettuce grown under glass. Comm. in Soil Sci. Plant Anal. 16: 1293-1300.

Schaer, T. & J. Habben, 1986. Nitratgehalt von Kopfsalatsorten unter Glass. Gemüse 3: 104-106.

Schwemmer, E., 1990. Nitrat im Gemüse. Gemüse 3: 172-175 & 5: 282-284.

Steingröver,. E., 1986. Nitrate accumulation in spinach. PhD-thesis State University Groningen, The Netherlands, 84 pp.

Steingröver, E., J. Siesling & P. Ratering 1986. Effect of one night with "low light" on uptake, reduction and storage of nitrate in spinach. Physiol. Plant. 66: 557-562.

Subramanya, R., G. Vest, & S. Honma, 1980. Inheritance of nitrate accumulation in lettuce. HortScience 15: 525-526.

Veen, B.W. & A. Kleinendorst, 1985. Nitrate accumulation and osmotic regulation in Italian ryegrass (*Lolium multiflorum* Lam.). J. Exp. Bot. 36: 211-218.

Wehrman, J. & R. Hähndel, 1985. Influence of chloride on yield and nitrate content of spinach. *In*: J.J. Neeteson & K. Dilz (Eds.), Assessment of Nitrogen Fertilizer Requirement, Inst. for Soil Fertility, Haren, The Netherlands: 95-99.

## Samenvatting

## Genetica van nitraatophoping in sla

*Het eten van veel nitraat is mogelijk ongezond. Groentetelers zoeken daarom naar mogelijkheden om het nitraatgehalte van hun gewassen te beperken. Veredeling kan hieraan een bijdrage leveren. Dit proefschrift beschrijft onderzoek naar nitraatophoping in sla. Nagegaan werd welke rasverschillen voor het gehalte aan nitraat bij sla voorkomen en wat de genetica van deze eigenschap is. Ook werd onderzoek gedaan naar seizoensinvloeden op de rangorde van rassen.*

In de laatste 15 jaar zijn het publiek en de beleidmakers in toenemende mate bezorgd geworden over de hoge nitraatgehalten die mensen consumeren via voedsel (vooral groenten) en drinkwater. Als gevolg daarvan werd op diverse plaatsen onderzoek gestart naar diverse aspecten van nitraatophoping. Dit proefschrift is het resultaat van een onderzoek naar de mogelijkheden om het nitraatgehalte in sla te verlagen door veredeling.

De keuze van het gewas sla was niet toevallig. Wat betreft productiewaarde is sla het vierde groentegewas in de Nederlandse tuinbouw (na tomaat, paprika en komkommer). Van de groep van bladgroenten, die in het algemeen nitraat-rijk zijn, wordt sla het meest gegeten. Daarbij komt nog dat een groot gedeelte van de slaproductie plaats vindt in kassen in de donkere periode van het jaar (late herfst, winter en het vroege voorjaar), wanneer de problemen met nitraatophoping het grootst zijn.

De overheid tracht overmatige nitraatconsumptie tegen te gaan door voor een aantal veel gegeten nitraat-rijke groenten (sla, andijvie, spinazie en rode biet) maximaal toelaatbare niveaus in te stellen. Om de telers tegemoet te komen waren deze normen aanvankelijk ongeveer gelijk aan wat in de praktijk gangbaar was. Vervolgens werden deze normen stapsgewijs verlaagd. Het plan is om uiteindelijk op een norm van 2,5 gram nitraat per kilogram vers gewicht uit te komen. Ook in het buitenland, waar het grootste gedeelte van de Nederlandse groenteproductie naar toe gaat, worden nitraatnormen gesteld of zijn ze in voorbereiding.

Voor sla geldt dat de telers in grote gedeelten van het jaar, vooral in de lichtarme periode, niet aan deze uiteindelijke norm kunnen voldoen met de gangbare teeltmethoden en rassen. Rassen die minder nitraat ophopen zijn daarom gewenst, omdat anders in de toekomst in bepaalde perioden van het jaar geen teelt van sla meer mogelijk zal zijn.

Het onderzoek van dit proefschrift was gericht op het verkrijgen van antwoorden op de volgende vragen:

1. Welke verschillen in nitraatgehalte kan gevonden worden in gecultiveerde herkomsten van sla of in voor de veredeling exploiteerbare verwante soorten van *Lactuca*?
2. Wat is de overerving van verschillen in nitraatgehalte tussen herkomsten van sla?

3. Is het mogelijk om slarassen te selecteren die kunnen voldoen aan de toekomstige maximum nitraatnorm?
4. Hoe kan in sla het efficiëntst op laag nitraatgehalte geselecteerd worden en welke factoren beïnvloeden de selectie-efficiëntie?

Het onderzoek begon met het doortoetsen van een grote sla-collectie op verschillen in nitraatgehalte. Er werden slaherkomsten geïdentificeerd die weinig nitraat ophopen, terwijl andere een zeer hoog nitraatgehalte hadden. Zeer hoge nitraatgehalten werden gevonden in wilde herkomsten. In alle typen van gecultiveerde sla (botersla, ijssla, bindsla, romaine-sla en snijsla) werden herkomsten gevonden met een laag nitraatgehalte. De laagste gehaltes werden aangetroffen in het boterslatype, in oude rassen, die niet voldoen aan moderne teelteisen. Deze rassen kunnen dus niet rechtstreeks in de teelt gebruikt worden, maar kunnen wel als kruisingsouder in veredelingsprogramma's benut worden, om zo een laag nitraatgehalte te combineren met de overige gewenste cultuureigenschappen. De geïdentificeerde laagnitraat herkomsten werden ter beschikking gesteld aan de Nederlandse veredelingsbedrijven die aan sla werken

Voor een studie van de overerving van het nitraatgehalte in sla werden vijf boterslarassen uitgekozen. Hiervan had het moderne glassla-ras Panvit een zeer hoog nitraatgehalte en de overige vier een laag nitraatgehalte. In diverse generaties verkregen uit kruisingen tussen deze vijf slarassen werd het nitraatgehalte bepaald. Het nitraatgehalte had een kwantitatieve overerving: in splitsende generaties werd een continue frequentieverdeling voor nitraat gevonden. De overerving van het nitraatgehalte werd grotendeels additief bepaald: de gemiddelde waarde van de generaties lag vrijwel steeds dicht bij het gemiddelde van beide ouders. Dit betekent dat eventuele effecten van dominantie slechts van geringe omvang waren, hoewel ze statistisch vaak nog wel zeer significant waren. Belangrijke moederlijke effecten waren eveneens afwezig. Het nitraatgehalte in sla kon worden verklaard met een simpel kwantitatief-genetisch model, dat alleen additieve en dominantie effecten veronderstelt en de overige genetische effecten, zoals reciproke verschillen en effecten van koppeling en epistasie, verwaarloost.

Het aandeel van genetische factoren in de totale variatie voor nitraatgehalte in splitsende generaties werd geschat in $F_2$ en $F_3$ populaties van de tien mogelijke combinaties van de vijf kruisingsouders. Uit deze schattingen bleek dat er in kruisingen tussen een hoog- en een laagnitraat ras slechts een geringe kans is om nakomelingen te vinden die het niveau van de laag-nitraat ouder evenaren. Omdat in deze kruisingen niet alleen het nitraatgehalte uitsplitst, maar ook veel andere belangrijke cultuur-eigenschappen, moeten grote populaties getest worden om de positieve eigenschappen van de moderne hoog-nitraat rassen te combineren met het lage nitraatgehalte van herkomsten die niet aangepast zijn aan de moderne teelteisen. Daarom zal veredelen voor laag nitraat relatief duur zijn.

De laag-nitraat rassen werden onderling gekruist om na te gaan of het mogelijk is om genen voor laag nitraat uit verschillende herkomsten bijeen te brengen om zo nog een verdere verlaging

van het nitraatgehalte te krijgen. De resultaten hiervan stemden echter niet optimistisch. Schattingen van het gedeelte van de nakomelingen met eenzelfde of lager nitraatgehalte dan de ouder met het laagste gehalte waren vrij laag (0.04-0.06). Bovendien traden er bij deze kruisingen relatief grote effecten van genotype × milieu (G×M) interactie op: de relatieve volgorde voor nitraatgehalte van de getoetste genotypen was afhankelijk van de proefomstandigheden. G×M-interacties reduceren de waarde die aan bovengenoemde schattingen gehecht moet worden. Het lijkt daarom niet waarschijnlijk dat het combineren van genen uit verschillende laag-nitraat herkomsten nog tot een substantiële verdere verlaging van het nitraatgehalte zal leiden.

In veredelingsprogramma's is het optreden van G×M-interacties een vervelend, maar veel voorkomend probleem. Het betekent dat een genotype die in het ene milieu geselecteerd wordt, in een volgende proef met andere milieuomstandigheden sterk kan tegenvallen. Om het optreden van G×M-interacties voor nitraatgehalte nader te bestuderen werd een aantal slarassen over een periode van anderhalf jaar herhaaldelijk geoogst. Omdat de planten onder natuurlijk licht in de kas werden geteeld varieerde de lichthoeveelheid, de milieufactor met de grootste invloed op het nitraatgehalte, sterk van proef tot proef. Zoals verwacht varieerde het nitraatgehalte sterk met de seizoenen. Ook werd een belangrijke G×M-interactie gevonden bij proeven geoogst in verschillende perioden van het jaar. De meest extreme rassen, CGN4944 met een laag nitraatgehalte en het hoog-nitraat ras Panvit reageerden echter vergelijkbaar op de seizoensveranderingen. Het maximale verschil in nitraatgehalte bleef daarom ongeveer constant gedurende het hele jaar. Het verschil in nitraatgehalte tussen beide herkomsten was gemiddeld over 18 proeven 1.7 $g \cdot l^{-1}$, wat betekent dat CGN4944 gemiddelde slechts half zo veel nitraat bevatte als Panvit.

Het optreden van G×M-interacties betekent dat het belangrijk is om van kruisingsouders in veredelingsprogramma's na te gaan hoe hun nitraatgehalte verandert onder veranderende milieuomstandigheden. Zo kunnen ouders gekozen worden die eenzelfde gedrag tonen, en dus onderling geen belangrijke interacties vertonen. Als rassen gebruikt worden die belangrijke effecten van G×M-interactie vertonen, moet geselecteerd worden onder de omstandigheden waarin de rassen die uit zo'n programma voortkomen later zullen worden geteeld.

Een verdere verlaging van het nitraatgehalte kan wellicht bereikt worden door laag-nitraat herkomsten, die duidelijke G×M-interacties voor het nitraatgehalte vertonen, en die dus fysiologisch van elkaar moeten verschillen, met elkaar te kruisen en in de nakomelingen te selecteren op recombinanten met een verlaagd gehalte. CGN4944 had het laagste nitraatgehalte van alle geteste herkomsten in vrijwel elke periode van het jaar. Alleen midden in de winter had een ander ras, CGN5233, een lager gehalte. Deze interactie werd in een aantal winterseizoenen waargenomen. Het extreem lage nitraatgehalte van CGN5233 midden in de winter ging gepaard met een laag versgewicht en een hoog drogestofgehalte. $F_3$-lijnen van

een kruising tussen deze twee laag-nitraat herkomsten werden herhaaldelijk geoogst in de periode van begin februari tot eind maart, met het doel de samenhang tussen veranderingen in nitraatgehalte, drogestofgehalte en versgewicht te onderzoeken. Zowel voor nitraatgehalte als drogestofgehalte traden G×M-interacties op. Het nitraatgehalte van CGN5233 was vrijwel constant en het drogestofgehalte nam beduidend af in de opeenvolgende oogsten. Bij CGN4944 nam juist het nitraatgehalte beduidend af, terwijl het drogestofgehalte vrijwel constant bleef in de opeenvolgende oogsten. In de $F_3$-lijnen werd een hoge en negatieve correlatie gevonden tussen de mate van verlaging van nitraatgehalte en van drogestofgehalte bij toenemende daglengte. Dit suggereert dat het hier gaat om pleiotrope (door dezelfde genen veroorzaakte) effecten. Er werden aanwijzingen gevonden dat een reactie zoals van CGN5233 (vrijwel constant nitraatgehalte en afnemende drogestofgehalte bij oogst onder toenemende daglengte) niet noodzakelijk gekoppeld is aan een slechte groei: ook $F_3$-lijnen met een relatief hoog versgewicht vertoonden zo'n reactie. Op grond van de nitraatgehalten van de $F_3$-lijnen in de opeenvolgende proeven wordt niet verwacht dat een combinatie van de mechanismen voor laag nitraat uit CGN5233 en CGN4944 tot een belangrijke verdere verlaging van het nitraatgehalte kan leiden.

Een belangrijke vraag is of met de in deze studie geïdentificeerde laag-nitraat herkomsten rassen kunnen worden geselecteerd die voldoen aan de toekomstige nitraatnorm van 2,5 g per kg vers gewicht. In een aantal proeven hadden de vier laag-nitraat rassen die in het genetisch onderzoek werden gebruikt allen een nitraatgehalte boven de toekomstige norm. De gemiddelden van de proef met de hoogste nitraatgehalten werden gebruikt om een schatting te maken van de kans om in een kruising tussen een hoog- en een laag-nitraat ras nakomelin-gen aan te treffen die onder die ongunstige omstandigheden aan de toekomstige norm kunnen voldoen. Deze fractie was verwaarloosbaar klein. Bij kruising tussen twee laag-nitraat genotypen was deze fractie groter. In deze populaties werden echter belangrijke effecten van G×M-interactie gevonden, waardoor geen betrouwbare voorspellingen gemaakt kunnen worden. Ondanks deze resultaten is veredeling van sla op een laag nitraatgehalte zeker zinvol. De voorspelling van de fractie nakomelingen die aan de toekomstige norm kan voldoen werd gedaan op grond van de proef met de hoogste nitraatgehalten. In de meeste andere proeven waren de nitraatgehalten van de kruisingsouders aanzienlijk lager. Onder die omstandigheden zal een grotere fractie van de nakomelingen een nitraatgehalte lager dan 2,5 $g \cdot kg^{-1}$ hebben. Bovendien waren de proefomstandigheden in deze studie, met planten op voedingsfilm met een hoge nitraatconcentratie en een lage ammoniumconcentratie, gunstig voor een hoge nitraatophoping. Wanneer in de praktische teelt laag-nitraat rassen gecombineerd worden met cultuurmaatregelen die nitraatophoping tegengaan, mag verwacht worden dat de meeste, zo niet alle teelten kunnen voldoen aan de nitraatnorm.

## Curriculum Vitae

Kees Reinink werd geboren op 21 september 1959 in het Drentse Odoornerveen. Hij leerde de landbouw al vroeg kennen aangezien zijn vader een akkerbouwbedrijf had, waarvan de inkomsten werden aangevuld met intensieve varkenshouderij. De scholing in Drente omvatte de lagere school te Schoonoord en het Atheneum van de Christelijke Scholengemeenschap te Emmen. De omgevingsfactoren zullen er niet geheel vreemd aan geweest zijn dat Kees in 1975 naar de Landbouwhogeschool in Wageningen ging. Hij haalde in 1976 zijn propaedeuse met lof, waarna hij begon aan een studie plantenveredeling. In 1978 haalde hij met lof het kandidaatsdiploma. De doctoraalstudie omvatte een 6-maandsvak plantenveredeling (een selectiemethodenproef in tarwe, begeleider Dr. Thomas Kramer), een 3-maandsvak genetica (een simulatie van selectiemethoden, begeleider Dr. Piet Stam), een 6-maandsvak plantenziektekunde, waarvan 3 maanden epidemiologie (verspreiding van valse meeldauw in spinazie, begeleider ir. Herman Frinking) en 3 maanden algemene plantenziektekunde en een 3-maandsvak wiskundige statistiek. Begin 1982 studeerde Kees met lof af. In november 1981 werd hij als onderzoeker aangesteld bij het Proefstation voor de Akkerbouw en de Groenteteelt in de Vollegrond (PAGV) te Lelystad. Hier was hij verantwoordelijk voor de introductie, de uitvoering en de experimentele verificatie van EPIPRE, een geautomatiseerd adviesmodel voor de bestrijding van ziekten en plagen in tarwe. In 1985 kreeg hij een baan bij het toenmalige Instituut voor de Veredeling van Tuinbouwgewassen (IVT). Hier werd hij belast met het veredelingsonderzoek aan sla. Sinds 1990 is hij leider van de sectie Resistentieonderzoek Groentegewassen van het Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO). Om zijn academische vorming te voltooien begon Kees in 1986 een deeltijdstudie wijsbegeerte aan de Rijksuniversiteit Utrecht. In 1988 haalde hij hiervan met lof de propaedeuse. In het doctoraal heeft hij zich gespecialiseerd op de geschiedenis van de moderne wijsbegeerte. Als afstudeerscriptie bestudeert hij onder begeleiding van Dr. Theo Verbeek de verhouding van subject en substantie bij Spinoza.

## Account

The Chapters 2 to 6 are revised versions of the following publications:

Chapter 2. Reinink, K., R. Groenwold & A. Bootsma, 1987. Genotypical differences in nitrate content in *Lactuca sativa* L. and related species and correlation with dry matter content. Euphytica 36: 11-18.

Chapter 3. Reinink, K., 1991. Genetics of nitrate content of lettuce, 1: Analysis of generation means. Euphytica 54: 83-92.

Chapter 4. Reinink, K., 1992. Genetics of nitrate content in lettuce, 2: Components of variance. Euphytica 60: 61-74.

Chapter 5. Reinink, K., 1991. Genotype × environment interaction for nitrate concentration in lettuce. Plant Breeding 107: 39-49.

Chapter 6. Reinink, K., 1993. Relationship between effects of seasonal change on nitrate and dry matter content in lettuce. Scientia Horticulturae 53: 35-44.