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FACTORS AFFECTING NITRATE VARIABILITY IN LETTUCE

CLAIRE GREENWAY

A thesis submitted in partial fulfilment of the requirement of The Nottingham Trent University for the degree of Master of Philosophy

The research programme was carried out in collaboration with the following partners;

Horticulture Research International
Horticultural Development Council
Hydro Agri UK Ltd
NV Produce Marketing
Seven Oaks Salads
Snaith Salad Growers
Tesco Stores Ltd

November 2001

DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the authors individual contribution. Tha author has attended approriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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(Candidate)

Signed.....
(Director of studies)

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ABSTRACT

Investigations were carried out in order to determine plant nitrate variability within lettuce and in addition to determine a suitable technique for rapid, on-field nitrate testing of lettuce. Hydroponic and soil studies were conducted at Horticulture Research International (HRI), Stockbridge House, Selby, North Yorkshire, in order to investigate the factors associated with nitrate variability in lettuce. Three hydroponic trials were conducted during an 18 month period of research, which included a study to determine inherent nitrate variability in lettuce, a study to determine the influence of different nutrient rates on plant nitrate concentration and a study to determine nitrate variability among lettuce cultivars. In addition to determining plant nitrate concentration, fresh weights of the lettuce were also assessed in all of the hydroponic studies. The influence of varying light intensity and soil nitrogen concentrations on plant nitrate concentration and head weight of lettuce was investigated during two soil studies. Soil trials were conducted during a winter and summer growing period in order to compare the influence of each treatment on nitrate variability and head weight within a winter and summer lettuce crop. A third soil trial involved raising eight lettuce varieties, which consisted of both butterhead and continental types, in order to determine cultivar variability in nitrate content and head weight.

An initial hydroponic investigation illustrated the relatively controlled growing conditions and resulting crop uniformity that are achievable within an NFT system. The level of nitrate variability within the hydroponic lettuce crop was limited and was not found to be significant between individual heads. Furthermore, the head weights of the hydroponic lettuce were generally consistent. In the second hydroponic trial there was significant nitrate variability between lettuce raised at different nutrient rates. An approximate increase of 25% plant nitrate concentration was observed in hydroponic lettuce when the nutrient supply was increased from 100ppm to 150ppm, and a further 20% increase was observed when the nutrient supply was increased from 150ppm to 200ppm nitrate-nitrogen. The head weight of lettuce was also significantly influenced by an increase in the nutrient rate from 100ppm to 150ppm, and from 100ppm to 200ppm. There was however, no significant increase in the mean head weight of lettuce when the nutrient solution was increased from 150ppm to 200ppm nitrate. Significant nitrate variability was found among the three lettuce cultivars during the third hydroponic trial, with the continental variety Miami exhibiting the highest nitrate concentrations and lowest head weight of the cultivars grown. The results from the two soil trials showed reduced nitrate variability within the winter crop compared to a significant level of nitrate variability within the summer lettuce crop, which was attributed to the differences in soil nitrogen concentration and light intensity. The results of the soil variety trial were in agreement with previous investigations, and showed significant nitrate variability between continental and butterhead lettuce varieties, with the highest nitrate concentrations observed in continental types. In addition, the head weights of continental varieties grown in soil were significantly lower compared to the head weights of butterhead lettuce. A negative correlation between head weight and nitrate concentration was observed in the butterhead cultivars, but a similar relationship was not observed within the continental varieties.

The Nitrachek meter was found to be a reliable and accurate method for use as a rapid nitrate testing technique despite the observance, during the later part of the investigations, of some degree of underestimation in the nitrate readings obtained with this method. The Horiba Cardy meter showed promise for use as a rapid nitrate testing method, with recommendations for further investigations in order to establish the repeatability of this technique. The Ion

Selective Electrode, Orion model, once again proved to be the most reliable method for determining sap nitrate concentrations from lettuce samples.

The identification of a reliable extraction method was deemed to be an essential component in the determination of a rapid nitrate testing technique. A standard, established technique for the extraction of nitrate from vegetables was not found to be in use amongst UK or European analytical laboratories. Indeed, the range of extraction methods currently used throughout the UK alone was found to be extensive. A great deal of variability was observed amongst the nitrate values obtained with different methods of nitrate extraction using lettuce samples that were obtained from the same crop. In particular, it was found that hot water nitrate extraction could significantly influence the nitrate concentration of extracts from lettuce used for analysis. Therefore, during the final stages of the investigation a series of small scale studies were conducted to determine the effect of nitrate extraction procedure on the mean nitrate concentration of extracts from lettuce. It was concluded that further studies would be necessary in order to validate a suitable nitrate extraction procedure for use along side the chosen technique for nitrate determination.

ACKNOWLEDGEMENTS

I would like to begin by thanking Dr W.R Carlile for his continued support throughout the research project. In particular, I would like to convey my appreciation to Bill for his commitment during the painstaking process of drafting the thesis and to acknowledge all the journeys over to Penkridge at weekends that could have been spent doing other things. Thanks Bill.

I would also like to thank staff at Nottingham Trent University, in particular Debbie Baker, for driving up and down to North Yorkshire several times to sample lettuce.

Thanks go to staff at HRI, Stockbridge House, for their invaluable technical expertise and the assistance provided during the setting up of the lettuce trials and throughout the research project.

Finally, I would like to express my deepest gratitude to family and friends who have had to put up with my tales of woe as completion of this thesis went on, and on, and on ...

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Chapter 1

Introduction

1.1 Importance of Nitrogen Nutrition in Plant Systems

Higher plants are autotrophic organisms that can synthesise molecular compounds from inorganic nutrients (Campbell, 1993). The assimilation of nutrients obtained from the local environment involves complex biochemical reactions, an example of which are the processes involved in nitrogen assimilation. Nitrogen is a key element in plant cells. It is located in the bases of nucleosides, nucleotides and amino acids, which are components of nucleic acids and proteins. The essential nature of nitrogen becomes clear when it is considered that only carbon, oxygen and hydrogen are more abundant in plant cells (Pitman and Cram, 1976). Nitrogen appears in its reduced form as proteins and nucleic acids, which provide the means for codification, storage and translation of genetic material. Nitrogen in this form accounts for 1.5 to 5 % of the dry weight of plants and of that amount 80 to 90 % is protein (Novoa and Loomis, 1981). High quality plant production as a means of supplying protein-rich food is therefore highly dependent on the availability of nitrogen. The nitrogen content of plants is also important to seed viability and germination. Understandably the importance of nitrogen has been recognised by the agricultural industry for many years. In agricultural and horticultural plant production, nitrogen is supplied in the form of fertiliser applications to ensure maximum crop yield and quality. To highlight the importance of nitrogen in plant growth, development and metabolism, it is necessary to consider the role of nitrogen in the biosphere and its availability to plants.

1.2 Nitrogen Availability on Earth

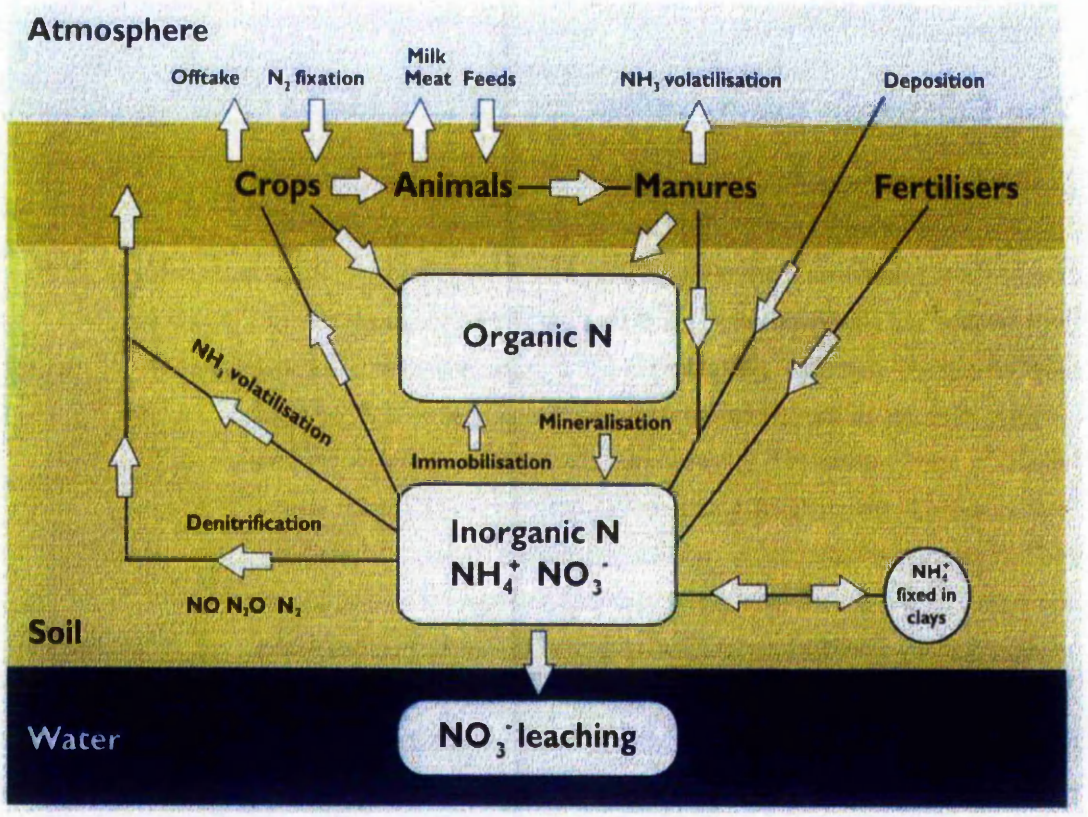
Nitrogen is one of the most common elements on Earth, accounting for 78% by volume of the total atmosphere (Taiz and Zeiger, 1991). The total amount of nitrogen in soils can vary from between 3000 to 6000 kg N/ha in arable soils, to up to 9000 kg N/ha in soils under grass (Anon, 1988). However only a fraction of all nitrogen is biologically available to plants (Crawford, 1995). For example, of the Earth's yearly input of nitrogen, which is estimated at 150-300 million tonnes, only 0.0025 % is available for biological use. This is because nitrogen most commonly exists as an inert dinitrogen (N_2) molecule with an exceptionally stable triple covalent bond. Nitrogen in this unreactive form is inaccessible to many biological systems. Since it is essential that plants are able to utilise nitrogen to form proteins, nucleic acids and other nitrogenous compounds, a more reactive form of nitrogen must be synthesised. This is accomplished when nitrogen is converted to the more reactive forms of ammonia (NH_4^+) and nitrate (NO_3^-).

1.3 Sources of Plant Nitrogen

Soil acts as a major reservoir for nitrogen (Jarvis, 1996). The total amount of nitrogen in soils is high. As previously stated, arable soils in the UK commonly contain between 3000 and 6000 kg N/ha, and soils under grass can contain up to 9000 kg N/ha. The major sources of nitrogen are derived from commercial fertilisers, animal manure, wet and dry deposition from the atmosphere and through natural or industrial processes of nitrogen fixation (Anon, 1999a). In horticultural and agricultural systems the most important source of nitrogen is from nitrogen fertiliser applications. However, biological nitrogen fixation provides the

majority of soil nitrogen worldwide that can be used by plants. Nitrogen is lost from ecosystems through leaching and surface run-off, gaseous transfers of nitrogen and nitrous oxides during the processes involved in nitrification and denitrification, and by ammonia volatilisation. The recycling of nitrogen, in its numerous forms within an ecosystem, is known as the Nitrogen Cycle (Figure 1.1).

Figure 1.1; Simplified diagram of nitrogen cycle (Anon, 1999a)

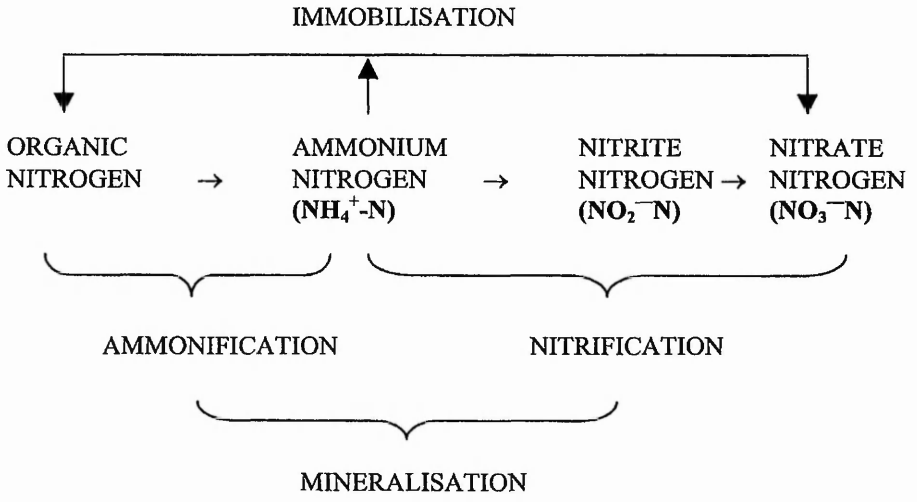


1.3.1 Mineralisation/Immobilisation

In the soil, organic matter undergoes decomposition and mineralisation in which nitrogen in its organic form is converted to inorganic compounds (Figure 1.1). The two most commonly occurring inorganic forms of nitrogen in the soil are ammonium (NH_4^+) and nitrate (NO_3^-). Transformation of organic nitrogen into NH_4^+ and NO_3^- is carried out by heterotrophic soil microorganisms (Haynes and Goh, 1978). The process of mineralisation, which release nutrients from organic nitrogen compounds into more accessible inorganic nitrogen compounds, is important in terms of plant production. This is because plants can only utilise inorganic forms of nitrogen in the production of amino acids and proteins.

Mineralisation is closely related to the process of immobilisation. Immobilisation occurs when inorganic nitrogen compounds are assimilated into organic nitrogen constituents of the cells of soil microbes. Degradation of the microbial tissues, which occurs as the soil microbial population turns over, releases the bound nitrogen and contributes once more to the pool of nitrogen in the soil (Jenkinson, 1990). This continuous transfer of nitrogen by the processes of mineralisation and immobilisation is extremely important to plants since it contributes to the supply of available soil nitrogen. The amount of mineral nitrogen in the soil is therefore largely dependent on the processes of mineralisation and immobilisation.

Figure 1.2 Diagram representing the major processes involved in the transformation of nitrogen in soils (Haynes and Goh, 1978).



1.3.2 Nitrification

Nitrification is the process in which the reduced, inorganic nitrogen compound ammonium is oxidised to nitrate (Figure 1.2). This is a key step in the soil nitrogen cycle. Ammonium is relatively immobile and transformation to nitrate, which is highly mobile, is beneficial to plants as the latter is more easily extracted from the root environment. Nitrification is therefore significant to plant productivity. It provides a more accessible source of nitrogen that can be used in the production of amino acids and proteins. The oxidisation process

involved in nitrification is carried out by two groups of autotrophic bacteria *Nitrosomonas* (1) and *Nitrobacter (2)* illustrated in the following reactions;

Figure 1.3 Steps in the nitrification process;

(1) $NH_4^+ + 1½\ O_2 \rightarrow NO_2^- + 2H^+ + H_2$ (*Nitrosomonas*)

(2) $NO_2^- + ½\ O_2 \rightarrow NO_3^-$ (*Nitrobacter*)

Nitrification rate is influenced by a variety of soil factors, which include air and water content, soil texture and structure, temperature, pH, level of substrate (NH_4^+) and the size of the microorganism population within the soil. Nitrification occurs most rapidly at optimum conditions, when temperatures are above 7-10°C and when the soil is well aerated and moist. Nitrification rate is also influenced by the supply of ammonium, which in agricultural and horticultural systems is derived primarily from soil applied ammoniacal fertilisers. These and other factors, such as diffusional constraints, can govern nitrification rates. In some soils, the rate of net mineralisation may exceed nitrification rates and this can lead to NH_4^+ accumulation at certain times of the year (Jarvis and Barraclough, 1991).

1.3.3 Nitrogen Fixation

Industrial Nitrogen Fixation:

Fixation of nitrogen derived from the atmosphere can occur by natural or industrial processes. One of the most important industrial fixation processes to be used has been the Haber process (Gallon and Chaplin, 1987). The reactions involved in this process occur under elevated temperatures of 200°C and pressure of 200 atmospheres. Molecular nitrogen combines with hydrogen to form ammonia, a more reactive form of nitrogen, which can be utilised by plants as can nitrate formed by the oxidation of ammonia. The agricultural industry has been the major beneficiary of the Haber process, with an increase in crop production and particularly cereal crop production. Worldwide industrial nitrogen fixation amounts to approximately 50 million tonnes annually (Taiz and Zeiger, 1991). Despite the obvious importance of industrial nitrogen fixation as a means of producing plant available nitrogen, it is the natural process that accounts for a much greater proportion of nitrogen fixation. The naturally occurring processes that contribute to the total amount of fixed nitrogen include lightning (through electrical discharge in the atmosphere), ultra violet irradiation and the internal combustion engine. The quantities of nitrogen fixed in this way are usually very small, in temperate climates they account for only a few kg NO_3-N ha^{-1} yr^{-1}. Under tropical conditions, the amount fixed by natural processes such as lightning is higher, but rarely exceeds 10 kg NO_3^- N ha^{-1} (Mengel and Kirkby, 1987). By far the most important naturally occurring process to contribute to nitrogen fixation is known as biological nitrogen fixation (Tinker cited in Hewitt and Cutting, 1979).

Biological Nitrogen Fixation:

Biological nitrogen fixation accounts for almost the same amount of nitrogen fixed annually through commercial processes, such as the Haber process (Green, Stout & Taylor, 1990). Free-living, symbiotic bacteria and some cyanobacteria carry out biological nitrogen fixation. Symbiotic bacteria include *Rhizobium* and *Actinomyces* species, and their host plants include clover species (*Trifolium*), angiosperms and legume species (*Pisum and Vicia*). The nitrogen

fixing capabilities of these kind of symbiotic bacteria can be considerable. Daly in Mengel and Kirkby, (1987), reported approximately 150 kg N ha^{-1} fixation by the symbiotic bacteria *Actinomyces albi* under favourable conditions living in symbiosis with alder (*Alnus rugosa*). Free-living bacteria such as the aerobic, anaerobic and facultative groups, include *Azotobacter, Clostridium* and *Klebsiella*. The quantity of nitrogen fixed by these organisms is generally low, and in the range of 5 to 10 kg N ha^{-1} (Mengel and Kirkby, 1987). Cyanobacteria that are capable of fixing atmospheric nitrogen include the genus *Anabacteria*. The symbiotic and free-living organisms possess the nitrogenase enzyme that catalyses the reduction of the unreactive nitrogen molecule to form ammonia and ammonium (Figure 1.4). This enzyme is a complex of two component proteins, which are both extremely oxygen sensitive. The intensive process of biological nitrogen fixation requires a great deal of energy. Sixteen moles of ATP are needed to produce one mole of dinitrogen (Dixon, 1986). The majority of nitrogen that is biologically fixed occurs through the symbiotic relationships between microorganisms and plants.

Figure 1.4 The conversion of atmospheric nitrogen into ammonia;

$$N_2 + 16ATP + 8e^- + 10H^+ \xrightarrow{\textit{nitrogenase}} NH_4^+ + 16ADP + 16Pi + H_2O$$

Many microorganisms responsible for biological nitrogen fixation exist in the soil. The microbial associations with plants that include peas, beans, clover, lupin, soybean and peanut are characterised by nodules, which are located on the root of legumes. The nodule is the site of infection and also the site of nitrogen fixation. These organisms are known as symbiotic rhizobia. There are also some non-leguminous plants, principally trees and shrubs, which have root nodules infected with symbiotic nitrogen fixing bacteria of the class Actinomycetes. Other plants, primarily lichens, liverworts and pteridophytes have symbiotic associations with blue-green algae. The enormous benefit for plants in all these symbiotic associations is that a supply of nitrogen, in the form of ammonia, is provided close to the plant root where it is needed. Atmospheric nitrogen fixed in this way is taken up through the root system of the plant where it is assimilated into organic nitrogen containing compounds. Agriculturally speaking, biological nitrogen fixation is of immense importance as a source of soil nitrogen, since demands for nitrogen in crop production worldwide cannot be met by nitrogen fertiliser application alone. When there is no additional supply of nitrogen, in the form of fertiliser application, the plants' only source is from biologically fixed nitrogen.

1.3.4 Nitrate and Ammonium Metabolism by Plants

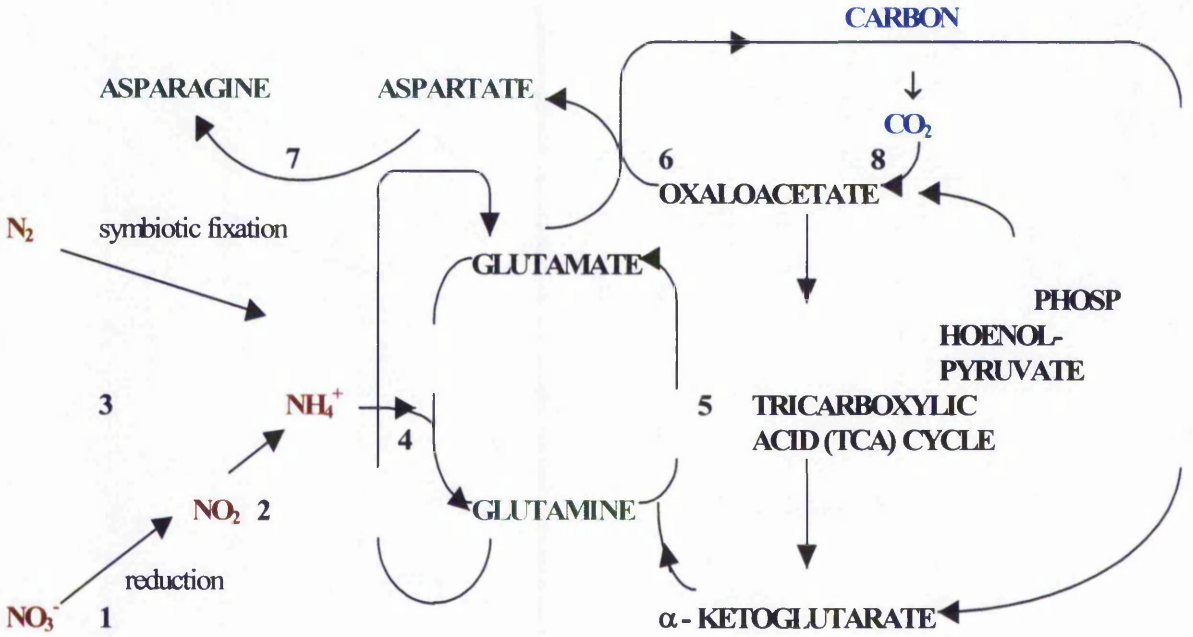
As previously stated, the two major forms of nitrogen taken up by plants are nitrate (NO_3^-) and ammonium (NH_4^+) ions. Most plants prefer the "safer", nitrate form of nitrogen compared with ammonium form. This may be because plants are more capable of extracting nitrate from the root environment than ammonium. Nitrate is also thought to be the preferred form of nitrogen due to the toxicity of ammonium and ammonia to the plant. Ammonium cannot be accumulated to any degree without damaging the plant. Nitrate on the other hand is not toxic to the plant and can accumulate at relatively high concentrations without detrimental effect to the plant (Gallon & Chaplin, 1987). Plants may also prefer nitrate-nitrogen because it is the most abundant form of nitrogen in the soil environment to which they have become adapted. Although both forms of nitrogen can be utilised by plants, crop

and vegetable species are most frequently supplied with the ammonium form, which is derived from ammoniacal fertilisers (Novoa & Loomis, 1981). Ammonia can be oxidised to nitrate during nitrification when conditions are suitable. In situations when ammonia is not oxidised to nitrate, a large proportion can be exported into the host cells where it is assimilated and used for plant growth processes (Mengel & Kirkby, 1987).

1.3.5 Ammonium Assimilation

The three enzymes which are of greatest importance in the assimilation of ammonia and oxidised nitrate are glutamate dehydrogenase, glutamine synthetase and glutamate synthase (Haynes & Goh, 1978). Glutamate dehydrogenase is located in the mitochondria of root cells, and acts as a catalyst in the assimilation of ammonia. Due to the relatively low NH_4^+ availability *in vivo*, it is unlikely that glutamate dehydrogenase has a significant role to play in ammonia assimilation. It is widely accepted that assimilation of ammonia takes place via the glutamine synthetase and glutamate synthase (GOGAT) cycles (figure 1.5). Glutamine synthetase is located in the chloroplasts and cytoplasm of plant-cells, and catalyses the formation of glutamine from glutamate. Glutamate synthase, which is also located in chloroplasts, catalyses the reductive transfer of an amide amino group from glutamine to 2-oxoglutarate to form two molecules of glutamate.

Figure 1.5 General scheme of nitrogen assimilation in higher plants showing enzymes involved. Glutamine, asparagine and aspartate are primary amino acids transported to other cells and plant organs. Photosynthate is used via the TCA cycle to generate carbon skeletons for amino acid biosynthesis. Carbon for amino acid synthesis can also be derived from non-photosynthetic CO_2 fixation of respired and/or atmospheric CO_2 (Dennis and Turpin, 1990).



Enzymes:

1: Nitrate Reductase (NR)
2: Nitrite Reductase (NiR)
3: Nitrogenase
4: Glutamine Synthetase (GS)
5: Glutamate Synthase (GOGAT)
6: Asparate Aminotransferase (AAT)
7: Asparagine Synthetase (AS)
8: Phosphoenolpyruvate Carboxlase (PEPC)

1.3.6 Nitrate Assimilation

In most soils, and particularly in horticultural/agricultural soils, the major form of inorganic nitrogen is nitrate. Nitrate is formed after the nitrification process of ammonium, derived primarily from mineralisation of nitrogen fertilisers and existing soil organic matter. Mineralisation and nitrification are important steps in the production of nitrate-nitrogen and contribute to the global rate of nitrate assimilation.

When the plant is supplied with nitrogen in the form of nitrate, a considerable amount of energy is required for its assimilation. This is because nitrate must be converted into ammonia before the nitrogen can be utilised for amino acid and protein production. Nitrate assimilation occurs during the two-stage process of nitrate reduction, and is dependent on the inducible enzyme nitrate reductase (Step1 Figure 1.6) as well as nitrite reductase (Step 2 Figure 1.6). Nitrate reductase is the most important factor in nitrate-nitrogen assimilation since it is the first, rate limiting enzyme between nitrate and amino acid production (Campbell, 1985). The location of nitrate assimilation varies from plant species to species and can occur in the plant root or leaves (Pate, 1980). For example, sub-tropical plant species predominately reduce nitrate in the shoot, whereas temperate plant species reduce nitrate mostly in the root. The site of reduction is also affected by the availability of soil nitrate. For example, when nitrate is low, higher proportions are reduced in the root once the storage pools of nitrate in the plant have been exhausted. In contrast, nitrate reduction occurs in leaves when soil fertiliser levels are high, and the root capacity for reducing nitrate is insufficient to cope with the supply of nitrate. The latter situation often leads to high nitrate levels in leafy vegetables, which are examined in chapter two.

Figure 1.6 The process of nitrate reduction;

Step 1) $NO_3^- - NAD(P)H + H^+ \rightarrow NO_2^- + NAD^+ + H_2O$ (*nitrate reductase*)

Step 2) $NO_2^- + 6e^- + 8\,H^+ \rightarrow NH_4^+ - 2\,H_2O$ (*nitrite reductase*)

The conversion of nitrate to nitrite is mediated by the enzyme nitrate reductase (Step1). This enzyme is thought to be a key compound in the regulation of nitrate assimilation and reduction processes in higher plants (Blom-Zandstra and Lampe, 1983). Nitrate reductase can be induced and inactivated by a complex regulatory system based either on synthesis control, repression or both. There are many factors thought to affect nitrate reductase synthesis and subsequent nitrate assimilation. It is believed that more than one enzyme is involved in the regulation of nitrate reductase. The availability of carbon skeletons and protein precursors may also have a marked influence on nitrate reductase. The exact nature of nitrate reductase regulation has been difficult to define, and is further complicated by a possible end product inhibition by amino acids, as well as the complications arising from nitrate partitioning. It is clear however that the substrate nitrate may act as a regulator in the synthesis of nitrate reductase.

Studies suggest that there is a close relationship between nitrate metabolism and photosynthesis. Many of the reactions involved in nitrogen metabolism are photosynthesis dependent in that they rely on the derivatives of photosynthetic processes such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NAD(P)H) (Wallsgrove cited in Dennis and Turpin, 1990). The process of nitrogen assimilation in the shoot is influenced by light and associated with oxygen evolution (Miflin, 1980). The enzyme involved in this initial process, nitrate reductase, and can be found only in the cytosol of roots and leaves. Light can influence nitrate metabolism through its effect on the supply of photosynthate, from which the synthesis of the reductant enzyme, nitrate reductase, is generated. Nitrite reduction, the second step in the nitrate assimilation pathway, has also been demonstrated to be closely linked to photosynthesis. At low light intensities the rate of photosynthesis in the chloroplast is limited, as are the products of photosynthesis namely

ATP, NAD(P)H, and reduced ferredoxin. In addition, the photosynthetic-dependent processes of nitrate reduction are also limited. Thus under conditions of low light intensity, typically found during winter periods, nitrate accumulation has been observed in the leaves of higher plants. A close correlation has been found between activity of the reductant nitrate reductase, photosynthetic rates and nitrate concentration in plant cells (Minotti and Jackson, 1970). Together with findings from several similar studies, the evidence suggests that the steps in nitrate assimilation are linked to photosynthetic processes (Behr and Wiebe, 1992).

The conversion of nitrate to nitrite by nitrate reductase rarely leads to nitrite accumulation in plant tissue. This is because the enzyme nitrite reductase, which readily converts nitrite to ammonia, exists at much higher levels of activity than nitrate reductase. It is the complex processes involved in ammonia and nitrate assimilation at the cellular level that regulate the quantities of inorganic nitrate-nitrogen found in plant tissue. Novoa and Loomis, (1981), pointed out that nitrate reductase activity is regulated by the flow of nitrates from the soil into plant tissues rather than by the presence of accumulated nitrates in the tissue. Although it is certain that the activity of nitrate reductase does exert an effect on the assimilation of nitrate in plant tissue, there are many other factors that can also influence nitrate uptake and/or assimilation. The activity of nitrate reductase is clearly a function of a complex set of inter-related factors.

1.4 Nitrogen Fertilisers in Agriculture and Horticulture

Since the 1950's there has been an increased demand on food production world-wide, largely due to rising populations and consumer needs (Avery, 1997). Advances in science and technology have more than tripled world-crop yields between 1960 and 1992 (Hanson, 1997). In developed countries, the agricultural industry has been able to meet these requirements mainly through plant breeding allied to an increased use of fertilisers and agrochemicals together with improved farming practises. Nitrogen fertiliser usage in the UK has increased from 60,000 tonnes/year in the mid-nineteen thirties to 1,580,000 tonnes/year by the-mid nineteen eighties (Anon, 1988). Nitrogen fertilisers in particular now represent approximately sixty percent of all directly applied fertiliser nutrients. Experts predict that European farmers will be supplied with approximately 9 million tonnes of nitrogen fertiliser by 2010 (Anon, 2001).

In the UK, fertilisers have played a major role in achieving high yields of cereal and vegetable crops. Over the past thirty years in Britain, yields of wheat have increased roughly in proportion to increases in the levels of nitrogen fertiliser application (Greenwood, 1990). There is evidence of a strong relationship between average yield of grain and nitrogen fertiliser application rates (Greenwood, 1989). There seems little doubt that without the growth in commercial fertiliser use, the expansion in food production as seen in the latter part of this century would not have been possible.

Fertilisers in the UK have always been readily available at a relatively low cost to growers, and although the government has offered guidance to farmers concerning fertiliser practise since 1905 there have been no official recommendations until recently (Anon, 1995a). Present day recommendations of fertiliser rates are based on factors known to affect the nutrient requirement of a particular crop. The factors that must be considered when making a prediction of fertiliser application rates include;

- plant species, expected yield and quality of the crop
- past cropping history, rainfall and soil texture
- the amount of mineral nitrogen in the soil at the start of the growing season
- the rate of decomposition of soil organic matter
- the level of available nitrogen lost before plant uptake

The recommendations that are made by the Department for Environment, Food & Rural Affairs, (DEFRA, formerly the Ministry of Agriculture Food and Fisheries (MAFF)), are useful but by no means exact. This is because large variations in any or all of the previously mentioned factors can exist between individual fields or glasshouses and from growing season to season. It has been difficult therefore to forecast the precise fertiliser level for crops in different growing conditions. Growers have tended to use maximum levels of nitrogen fertiliser to ensure that nitrogen is not a limiting factor in plant growth and that high yields are obtained (Maynard *et al.*, 1976). Quantities of nitrogen are invariably lost from agricultural systems. Nitrogen losses from the soil occur through the processes of leaching and ammonia volatilisation, or accumulate in crop tissues as nitrate (Powlson, 1997). Loss of nitrogen from agriculture and horticulture presents environmental and health threats to humans and livestock (Walters cited in Hewitt and Cutting, 1979). The environmental and health issues have created awareness within the industry for a need to develop strategies to maximise fertiliser use and efficiency.

1.5 Effect of Nitrate on Environment, Drinking Water and Foodstuffs

Approximately 1.6 million tonnes of nitrogen are applied annually as fertiliser. Various factors, including rainfall, irrigation, soil and crop type, method and form of fertiliser can affect the efficiency of nitrogen fertiliser uptake. Nitrogen recovery rates can vary according to the extent of these factors. It is possible to achieve high recovery rates if nitrogen fertiliser application is kept to within optimum rates. For instance, a crop of winter wheat in the UK fertilised at the standard rate of 190 kg/ha recovered over 97% nitrogen and left only 1-5 kg of nitrogen as nitrate in the soil (Addiscott and Powlson, 1989). However, recovery rates rarely reach optimum values, and for industrialised countries less than 50% of commercially applied nitrogen enters the crop (Tinker, 1979; Greenwood, 1990). Of the amount taken up by the crop, some of the nitrogen is returned to the soil in crop residues at harvest. The remaining nitrogen in the soil that is not taken up by the crop is not only an economic loss to the farmer but is also an environmental threat through the process of nitrate leaching.

The process of nitrate leaching into aquifers and rivers has been a major environmental issue in recent years. This led to the 1980 EC Directive on the Quality of Water Intended for Human Consumption setting guidelines for nitrate concentration in drinking water. The EU guide of 50mg/l nitrate in drinking water was reviewed in April 1997 by the World Health Organisation. On the basis of the latest scientific evidence it was concluded that the level of 50mg/l nitrate in drinking water should be maintained and since then the established maximum limit for nitrate in surface water used for abstraction for drinking has been set at this level. In addition to this directive, DEFRA policy aims to limit losses of nitrate and other forms of nitrogen pollution from agriculture by promoting practises that reduce such losses.

Leaching and Eutrophication

Nitrate leaching from agricultural land leads to increased eutrophication of waterways, an increase in the nitrate content of drinking water and foodstuffs, and can contribute to the release of "Greenhouse" gases for example methane and nitrous oxides (Anon, 1999a). Nitrate leaching is a significant contributor to eutrophication in marine and inland waters. Eutrophication occurs when an increase of nutrients such as nitrate encourages excessive algal growth. The ecological balance is disturbed as large increases in vegetative growth of algae leads to de-oxygenation, and this can lead to reductions in plant and animal species. Leaching rates are particularly high on sandy soils, and the drainage water from these soils plays a large part in regeneration of groundwater supplies. The amount of water supplied by groundwater systems varies from region to region across Britain, but is estimated at approximately 30% (Anon, 1988). It is mineral nitrogen, formed in the soil during autumn, which contributes most to losses of nitrogen by leaching.

Nitrates in Food and Water Supply

Another aspect of nitrogen pollution that is constantly under investigation is contamination of drinking water and foodstuffs. The nitrate content of foodstuffs and drinking water has been of interest due to its possible toxic effect on human health (Greenwood, 1990). Nitrate occurs in foodstuffs through its use as a preservative or naturally through the metabolic processes of plants (Liu and Shelp, 1995). The importance of nitrate in plant metabolism means that our primary intake, approximately two-thirds of the total dietary intake of nitrate, comes from vegetables (Richardson and Hardgrave, 1992). High nitrate levels, particularly in leafy vegetables such as lettuce and spinach and in drinking water, are primarily derived from an increased use of nitrogen fertilisers (McCall and Willumsen, 1998). For instance high nitrate concentration in drinking water can result from excessive nitrogen fertiliser applications which lead to nitrate leaching (Anon, 1995b). Nitrate accumulation in leafy vegetables is also influenced, among other things, by high rates of nitrogen fertiliser application (Ysart, Clifford and Harrison, 1999). Measures to limit dietary intake of nitrate have focused on vegetable crops, which are known to accumulate high levels of nitrate in their tissues. Vegetables and salad crops such as spinach, lettuce and endive are included in many of these investigations since they are known to make a significant contribution to dietary nitrate intake. It has been generally regarded as good practice to incorporate low nitrate-accumulating cultivars, and adopt fertility and management practises aimed at reducing nitrate concentrations.

1.6 The Risks to Human Health

Public concern over the health effects of nitrate have originated from observations that cows have died as a result of digesting nitrate rich herbage (Van Diest, 1986). Acute cases of nitrate or nitrite toxicity result in methaemoglobinaemia, and when the proportion of methaemoglobin is 70% of the total haemoglobin, the result may be fatal. Similar reported cases of methaemoglobinaemia in human beings are confined to infants under 6 months of age, although adults who are anaemic or who have genetically high methaemoglobin levels in their blood may also be liable to nitrite poisoning (Maynard *et al.*, 1976). Infantile methaemoglobinaemia is very rare in the UK. There have been fourteen cases attributable to nitrate in drinking water in the past thirty-five years (Anon, 1988). The last reported death from methaemoglobinaemia was in 1950, and the last reported and confirmed case was in 1972. Infantile methaemoglobinaemia is usually easy to diagnose and the condition may be alleviated by consumption of acceptable levels of nitrate.

Another possible adverse effect of dietary nitrate and nitrite is in the formation of potentially hazardous nitrosamine compounds. Studies have shown nitrosamine compounds to be carcinogenic (Magee and Barnes, 1967 cited in Wolff and Wasserman, 1972, Bruning-Fann and Kaneene, 1993). Nitrates and nitrites are ingested with food or water. In a healthy human these compounds are quickly absorbed from the stomach and upper gastrointestinal tract and are eventually excreted in urine. Under these conditions, nitrosamine formation is not favourable. Nitrosamine formation seems to occur when acidity in the gastrointestinal tract decreases and produces conditions that are favourable for nitrate-reducing bacteria. However, the evidence of nitrate as a role in gastric cancer is slight, and studies in Britain have found no correlation between dietary intake of nitrate and cancer (Beresford, 1985). Indeed in recent years some authors have disputed claims that nitrate can cause stomach cancer (Duncan *et al*, 1995). Despite the uncertainties, there is circumstantial evidence that the processing of food can result in the formation of N-nitroso compounds and may cause human cancer (Magee, 1987).

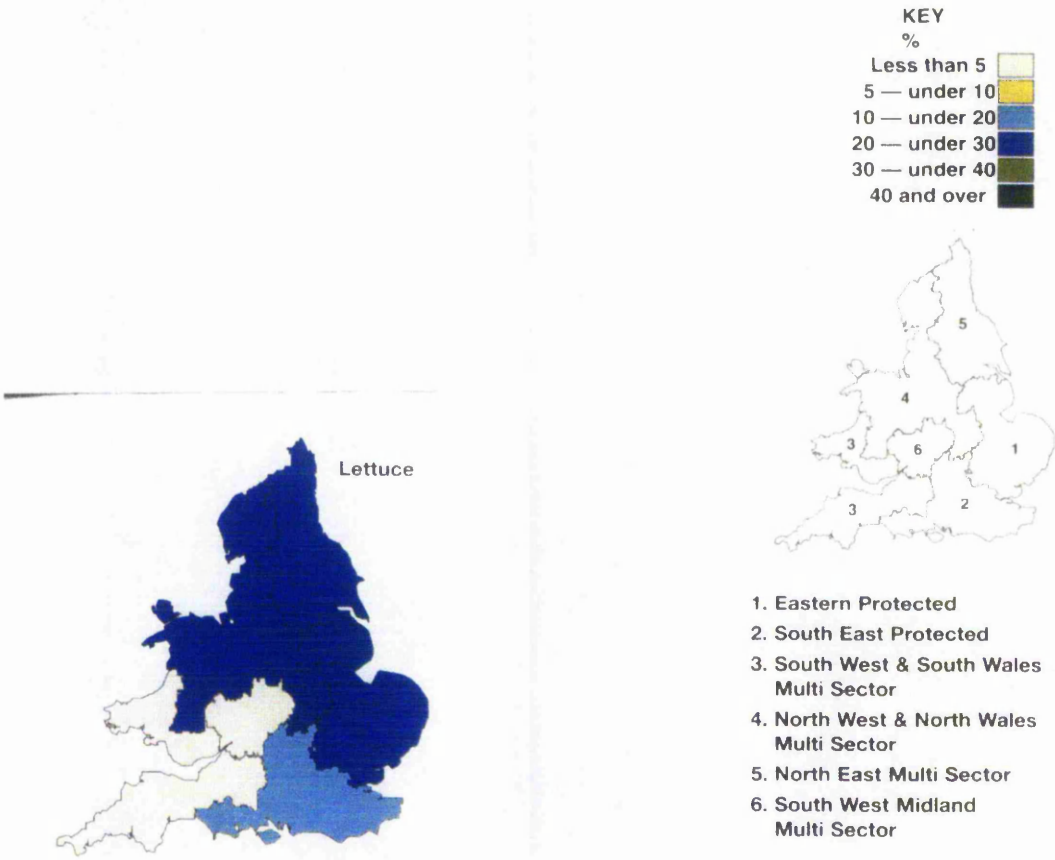
The consumption of green, leafy vegetables, such as lettuce and spinach, is generally considered to be beneficial to health. Indeed, the actual health benefits of a diet rich in vegetables may far outweigh the possible health risks posed by high nitrate levels in the vegetables. Benjamin at St Bartholomew's Hospital, London claims that nitrate in salad crops works as a disinfectant by killing bacterial pathogens (Anon, 1998d). However, there is good evidence to suggest a risk of N-nitroso formation during storage and food production especially of vegetables, which are naturally high in nitrate. In order to limit the possible risk of methaemoglobinaemia and stomach cancer resulting from dietary intake of nitrate, the EC Scientific Committee for Food set Acceptable Daily Intake of 3.65mg per kg body weight for the nitrate ion and 0.06mg per kg for the nitrite ion (Anon, 1998h). The implications of this regulation are far-reaching, particularly for the vegetable producing industries in the UK and Europe. Since the majority of dietary nitrate is ingested in the form of vegetables, it is the horticultural industry that has been the focus of recent legislation aimed at reducing excessive nitrate levels in certain vegetables.

1.7 Lettuce production in the UK

In the UK horticultural industry, much of the protected salad crops such as cucumber, tomatoes and lettuce are grown under glass (Anon, 1998b). Approximately four hundred hectares of glasshouses are currently devoted to lettuce production throughout the year (Figure 1.7). However, the area of crop grown has been in steady decline over recent years and has decreased by approximately 10 percent annually (Anon, 1998b). The value of glasshouse lettuce production is approximately £40 million per annum. The soil types used in lettuce production vary across the UK from free draining sands to organic peats. Lettuce production is favoured by soils in good physical condition, rich in organic matter and pH neutral (Anon, 1999c). Lettuce demand for nutrients is low in comparison to other vegetable crops, with average total nitrogen uptake for a medium yield being in the range 50-100 kg per ha. Fertiliser levels of 100 kg per ha nitrogen in outdoor conditions, with increases in nitrogen doses during winter cropping periods, are recommended to growers (Anon, 1999a). It is advised that one third of the nitrogen dose is given before planting, followed by one third a month after transplanting and the remaining third to be given before harvest. However, most growers in the UK apply nitrogen fertiliser in the form of a single base dressing (Lee, 1999). In the third and fourth weeks before harvesting, the crop takes up seventy percent of the total nutrients received.

The extended growing season in protected systems such as glasshouse environments makes it possible to produce lettuce all year round. Growing systems under glass create different conditions compared to an outdoor soil environment. The differences include a reduced variability in humidity level, limited light levels throughout the year and generally higher temperatures under glass compared to outdoor growing environments. Environmental conditions under glass vary throughout the year, with low light levels and temperature during winter conditions and increased light levels and temperature during summer months. It would be beneficial to investigate all aspects of the interactions between radiation levels, temperature, soil available nitrogen and nitrate accumulation in glasshouse lettuce. This might enable growers to manipulate growth conditions in the glasshouse environment to provide soil nitrogen concentrations, light intensity and temperature in a combination that is optimum for high yield but also results in low nitrate concentrations in the lettuce crop.

Figure 1.7 Protected lettuce production in UK (Anon, 1998b)



Discussions between lettuce growers, the National Farmers Union (NFU) and the Ministry of Agriculture, Fisheries and Food (DEFRA) have created a revised version of the Code Of Good Practise for the production of horticultural crops including lettuce, which considers new findings from research. Measures to limit nitrate levels in lettuce have been put forward as part of the lettuce protocol of the Assured Produce scheme. The protocol contains recommendations that, if followed by growers, are intended to reduce nitrate levels in lettuce. The following statements represent growing practises that are encouraged in order to reduce nitrate levels in a lettuce crop (Anon, 1998a).

The code indicates that nitrate accumulation occurs most rapidly under conditions that result in slow growth during winter. The influence of low light levels, as experienced during winter periods in particular, have led to the recommendation that crops are grown under optimum light-transmitting glasshouse conditions. In order to achieve optimum light-transmitting conditions, it is suggested that glass is cleaned regularly and shaded areas within the glasshouse are avoided. To determine soil nitrogen content, and thus calculate the amount of nitrogen fertiliser application required, soil sampling and analysis for mineralised nitrogen should be undertaken before each crop is planted. However, it is important to avoid sampling before soil sterilisation has taken place as this process releases bound nitrogen and increases concentrations in the soil. Following soil nitrogen analysis an estimation of the amount of nitrogen fertiliser that needs to be applied in order to achieve 100mg/l at planting can be made. This calculation is based on an amount of nitrogen sufficient to ensure the lettuce crop reaches harvest. For example, an application of $10g/m^2$ ammonium nitrate fertiliser (34.5% N) incorporated into 20cm of soil raises nitrogen level by 13mg/l. Base dressing required to raise N level to 65mg/l can therefore be determined relatively easily from these calculations. If starting NO_3-N level exceeds 120mg/l, it may be necessary to flood the soil to reduce the nitrate level.

The type of nitrogen fertiliser used can influence the final amount of nitrogen available to the crop. For instance, the amount of nitrogen released in the soil from slow release and organic nitrogen fertilisers may be unpredictable. This is due to nitrogen release being dependent on certain soil conditions, in particular temperature. Base dressing of fertilisers has been found to be an adequate method on most soils and is recommended in the Code of Good Practise. However, on sandy soils there is a greater risk of leaching, and it may then be necessary to increase nitrogen fertiliser rates or apply additional fertiliser dressings throughout the crop growing season. Soil conductivity can influence nutrient uptake by plants, and should be adjusted with potassium sulphate applications. Although there are no official recommendations concerning particular lettuce cultivars that should be grown, the Code of Good Practise states that lettuce cultivars should exhibit low or less variable nitrate concentrations. This advice is of considerable relevance in view of the current nitrate limits set by EC Regulation 194/97. An additional suggestion made to growers is to trim the outer leaves of lettuce at harvest in order to lower head nitrate concentrations. It is advised that the period of time the lettuce crop is stored prior to sale is limited in order to avoid possible conversion of nitrate to nitrite. A final recommendation that can be found in the Code of Good Practise concerns growing the lettuce crop under nutrient film technique rather than in soil. This advice is based on the belief that soil-less growing systems allow improved control of nutrient application, availability and therefore the eventual nitrate content of plants. However, almost all the lettuce raised under glass in the UK is grown in soil.

As stated earlier, the problem of nitrate leaching into water-courses together with the accumulation in drinking water and foodstuffs, (Section 1.5) has long been recognised by the

agricultural and horticultural industry. The problem is not confined to the UK and is common to all intensively farmed areas throughout Europe. In closed growing systems, the most widely used methods for lettuce production in the UK, the practise of flooding the soil before planting is common and can lead to nitrate leaching into drains. Consequently, there are currently guidelines coming into force to reduce the leaching of nitrates from flooding of soil in closed systems (Lee, 1999). As stated previously, at present approximately two thirds of dietary nitrate intake is in the form of vegetables and this has led to an abundance of research that has been primarily concerned with nitrates in leafy vegetables such as spinach and lettuce (Richardson, 1907 cited in Maynard *et al.*, 1976; Barker *et al*, 1971; Siciliano *et al*, 1975; Maynard *et al.*, 1976; Hayashi and Chino, 1985; Liu and Shelp, 1995; Forlani *et al.*, 1997).

In recognition of the high nitrate content of leafy vegetables such as lettuce, the European Community has approved legislation as part of its programme on agricultural contaminants in food. The proposal relating to nitrate levels in lettuce is European Commission Regulation No. 194/97, which came into force February 1997. This regulation specifies the maximum level of nitrate in lettuce, *Lactuca sativa,* and spinach, *Spinacia oleracea.* Nitrate limits for lettuce are set at 4500ppm (mg/kg) fresh weight for crops harvested from November to April inclusive, and 3500ppm (mg/kg) fresh weight for crops harvested from May to October inclusive. Since February 1997 the regulation has been amended, in April 1999, by EC Regulation 864/99. Member states may use a temporary derogation from the maximum nitrate levels but must demonstrate that thy follow the Code of Good Practise in order to minimise nitrate levels in lettuce or spinach. UK lettuce growers are currently operating under this and the UK Code has been assembled to include the most effective ways in which to minimise nitrate content. UK growers must also participate in the UK Monitoring Programme for Nitrate in Lettuce and Spinach, which began in May 1996. The implications of the Code of Good Practise and monitoring programme on lettuce production in the UK are discussed in the following section.

1.8 Nitrates in Leafy Vegetables and Lettuce

Estimates for the amount of nitrate consumed by the average person in Britain have been made which suggest that lettuce account for approximately 20% of the total intake of nitrate (Greenwood and Hunt, 1986). Glasshouse lettuce in particular makes a significant contribution to dietary nitrate intake and protected glasshouse systems are the predominant growing conditions for UK grown lettuce. Therefore, many studies have involved investigations into nitrate accumulation in protected lettuce crops.

Nitrate levels in lettuce can be affected by environmental factors including irradiation level, temperature, the water and air content of soil, soil pH, and plant root structure (Richardson and Hardgrave, 1992), cultural practises such as the level and method of nitrogen fertiliser application (Greenwood and Hunt, 1986), and genetic factors (Reinink, Groenwold and Bootsma, 1987). Van der Boon, Steenhuizen and Steingrober, (1990) showed that nitrate content of hydroponically raised lettuce was affected by light intensity, temperature during growth, total nitrogen application, ratio of ammonium/nitrate and chloride concentration in the nutrient solution. These factors are often interrelated and each or all of them may affect the processes of nitrate absorption, assimilation and translocation. For example, Hoff and Wilcox (1970), (cited in Maynard *et al.*, 1976), showed a temperature / light interaction using tomato (*Lycopersicon esculentum* Mill.). In their study, temperature exerted its greatest effect on tomatoes at high nitrogen rates and low light intensity. Light exerted its greatest effect at high temperature and high nitrogen rate. The processes of nitrate absorption, assimilation and

translocation can be modified by external factors in such a way that nitrate in plant tissues is always in a dynamic state. It is often difficult, if not impossible, to determine the effects of one particular factor such as nitrogen fertiliser rate on crop nitrate concentration. This is because additional environmental variables that occur in the soil can be so pronounced as to completely mask any effect of fertiliser rate on crop nitrate concentration. In recent years, an increasing proportion of research has been undertaken in controlled-environment conditions using methods such as nutrient film technique (NFT). Investigations using NFT reduce additional environmental variables known to influence plant nitrate content and enable the effects of a specific factor on nitrate concentrations, such as light or nitrogen nutrition, to be determined

The distribution of nitrate within the lettuce plant can vary. It has long been recognised that leaf blades have a lower nitrate content than stems and petioles (Scaife *et al.*, 1986). The younger leaves of lettuce are known to contain less nitrate compared to the older leaves, and it has been recognised for some time that changes in nitrate uptake occur with plant age (Swiader and Freiji, 1996). For example, in one study the average nitrate concentration in the outer leaves of lettuce was found to be 346 ppm NO_3-N, fresh weight compared to an average nitrate concentration of 132 ppm NO_3-N fresh weight for inner lettuce leaves (Minotti cited in Maynard *et al.*, 1976). In another study, the nitrate content of outer leaves was found to be three and a half to four times higher than inner leaves of lettuce (Aggelides *et al.*, 1999). Simple cultural practises that aim to reduce nitrate content of lettuce have utilised this knowledge by removing outer leaves at harvest.

1.8.1 Seasonal Variations

Climatic variables, occurring from season to season or even over a matter of hours, have been shown to have an appreciable effect on nitrate concentrations (Van Eysinga, 1984a; Ysart *et al.*, 1999). Figure 1.8 shows diurnal fluctuations in the percentage of nitrate-nitrogen of beet plants over a fifty-two hour period. Monthly results of nitrate analyses of UK grown protected lettuce show a clear trend of increasing nitrate levels during winter months compared with lettuce grown in summer months (Anon, 1999a). For example mean nitrate levels were 1831 and 2027 mg/kg in July and August respectively, compared to mean values of 3499 and 3930 mg/kg in December and January. Month to month variations in radiation levels have been shown to induce three-fold differences in nitrate content of lettuce (Figure 1.9, Van Eysinga, 1984a).

Figure 1.8 Diurnal fluctuations in NO_3-N of beet plants over 52 hour period (Minotti and Stankey cited in Maynard *et al.*, 1976)

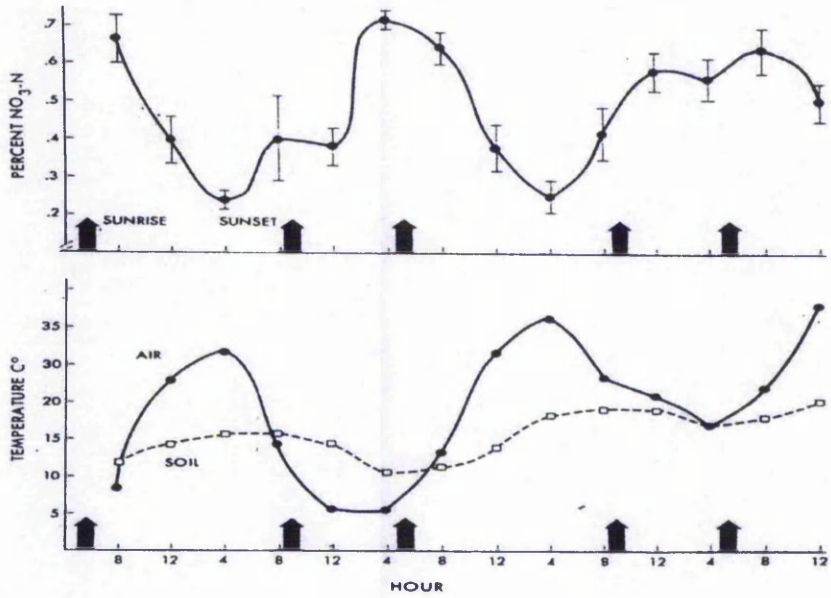
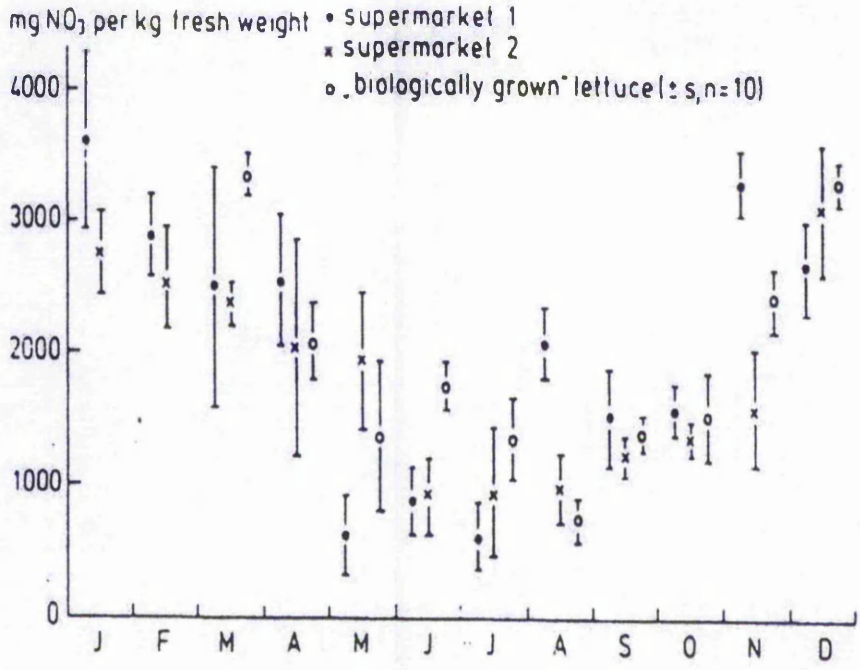


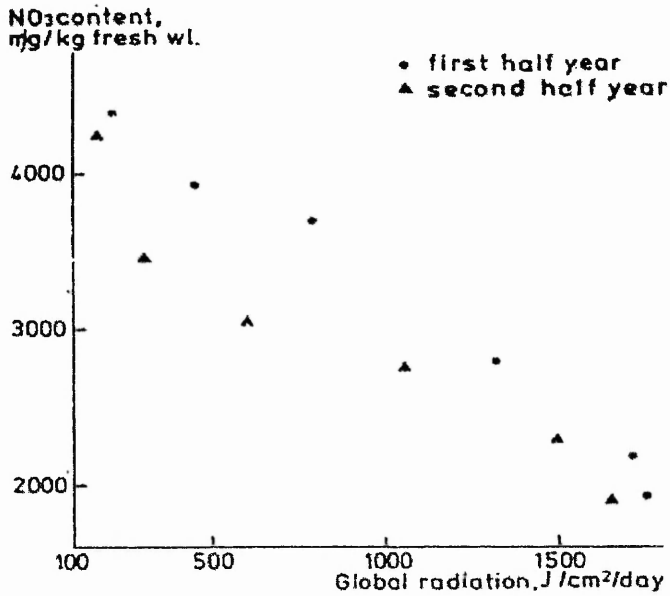
Figure 1.9 Seasonal pattern of nitrate concentration of lettuce in Switzerland (Van Eysinga, 1984a)



1.8.2 Light

The major factor influencing nitrate concentrations within plants has been shown to be light (Lorenz and Weir, 1974; Knight and Mitchell, 1983; Ysart *et al.*, 1999). Several experiments have illustrated a clear relationship between irradiation and nitrate accumulation in vegetables (Schonbeck *et al.*, 1991; Santamaria *et al.,* 1997). It has been established that nitrogen metabolism and the associated assimilation of nitrate has complex interactions with light (Jones and Sheard cited in Hewitt and Cutting, 1979). Many studies have established low light level as one of the most important factors contributing to nitrate accumulation in lettuce (Van Diest, cited in Lambers *et al.,* 1986). The relationship between light levels and the nitrate content of vegetables results in diurnal fluctuations in nitrate content throughout the year (Steingrover, 1986). Nitrate content of glasshouse lettuce strongly depends on global radiation (Van Eysinga, 1984 and 1987). Low radiation levels in winter are typically associated with high nitrate content in lettuce crops and a contrasting situation occurs in summer months (Figure 1.9). When radiation levels are high the nitrate content of the lettuce crop is low (Schonbeck *et al.*, 1991). Van der Boon *et al.* (1990) found that the nitrate content of lettuce at harvest was negatively correlated with light intensity. Suggestions of a linear relationship, with an r^2 value of -0.94, between mean daily solar irradiance and nitrate-nitrogen, (NO_3^-N), in glasshouse lettuce have been made (Figure 1.10, Van Eysinga, 1984b). Steingrover *et al.*, (1986), have suggested that nitrate translocation from the storage pool in the vacuole to the metabolic pool in the cytoplasm is a light mediated step.

Figure 1.10 Relationship between average nitrate content of lettuce and global radiation in The Netherlands over 12 month period (Van Eysinga, 1984b)



These light effects may be mediated through the activity of nitrate reductase, which is influenced by a variety of factors including light levels. Increases in irradiation have been shown to increase nitrate reductase activity (Lillo, 1983). In photosynthetic tissue variations in nitrate reductase activity generally occur on a diurnal basis, with peak rates during light periods and lowest rates at the end of a dark period. In contrast, during periods of low irradiation, nitrate reductase activity was reduced (Mengel and Kirkby, 1987). These fluctuations in nitrate reductase activity or turnover may help to explain the high levels of nitrate in winter lettuce compared to relatively low nitrate concentrations in summer grown lettuce.

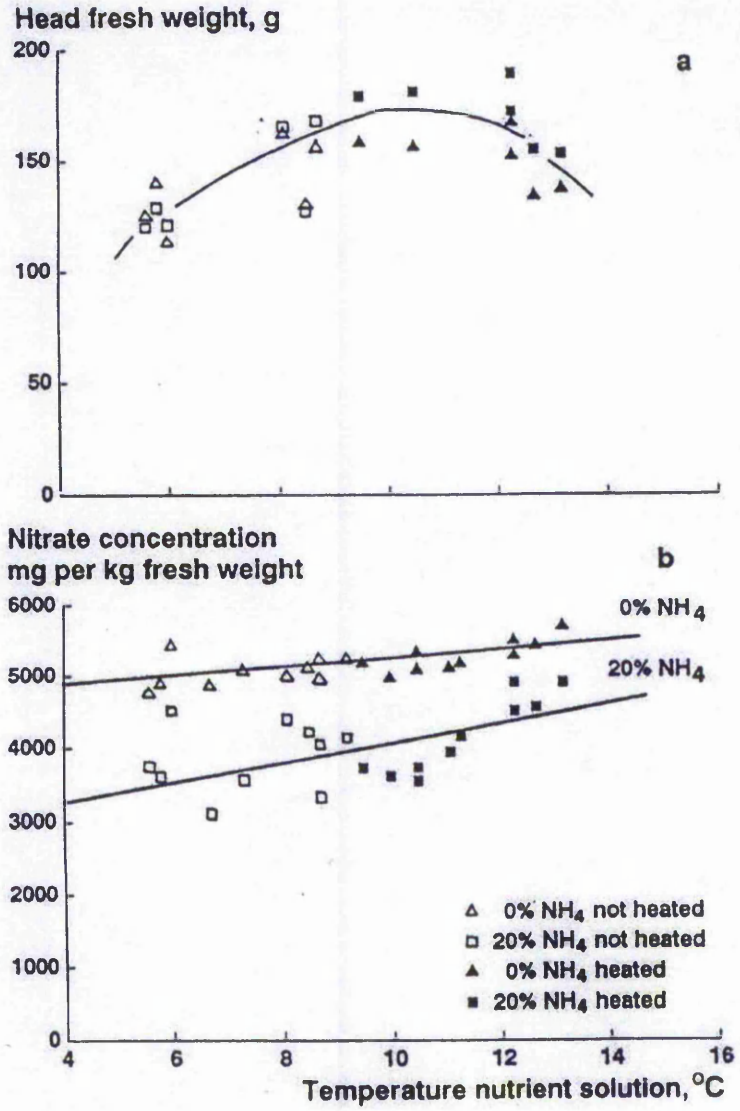
A photoperiod influence on nitrate levels in vegetables has also been shown to exist, although the particular response of a plant to variations in light duration is largely dependent on crop species (Van Diest, 1986). It has been reported that shortened photoperiods significantly increase nitrate levels in leaves and roots of some plant species (Cantliffe, 1972). Conversely, extended photoperiods have been shown to reduce nitrate levels.

1.8.3 Temperature

High temperatures have been shown to increase the nitrate content in a range of plants (Cantliffe, 1972) and in a study by Richardson *et al.* (1992), the head weight of glasshouse lettuce increased slightly when the temperature increased from 7°C day/4°C night to 10°C day/7°C night. When light is not a limiting factor, high temperature can also contribute to the increased rate of photosynthesis (Hall and Rao, 1994). High temperature is an associated factor when nitrate accumulates in plants grown under drought conditions (Viets and Hageman, 1971). A reduction or inactivation in the enzyme responsible for nitrate reduction has been shown at high temperatures. Reduction or inactivation of this enzyme is partly due

to reduced stomatal conductance and subsequent decrease in photosynthesis at high temperatures. A study by Van der Boon *et al.*, (1990) showed there was a clear relationship between nitrate concentration of lettuce and average nutrient solution temperature (Figure 1.11). An increased nitrate accumulation in the lettuce plants was found at the higher temperature of the nutrient solution. This finding is further supported by a study which found that the absorption of nitrate by lettuce was influenced to a greater extent by a rise in air and root temperature than by an increase in ammonium concentration (Frota and Tucker, 1972). In the study by Van der Boon *et al.*, (1990), winter lettuce treated with 0% NH_4 showed higher concentrations of nitrate mg per kg fresh weight compared to lettuce treated with 20% NH_4. This finding can be explained by the preference of plants to utilise NH_4 instead of NO_3^- when it is supplied in the nutrient feed.

Figure 1.11 Effect of temperature of the nutrient solution in winter on nitrate concentration of lettuce (Van der Boon *et al.*, 1990).



Temperature has been shown to exert a greater effect on plant nitrate concentrations at higher soil-nitrogen levels (Schonbeck *et al*,. 1991). The interaction between temperature and soil-nitrogen was illustrated in a study which showed that at low or no nitrogen fertiliser application, nitrate did not accumulate until temperature rose to 25°C (Onwueme *et al.*, 1971). The effect of temperature on soil nitrate availability will also influence nitrate uptake and subsequent accumulation in the plant. It has been shown that high temperatures, which increase the rate of mineralisation and nitrate content in the soil, can lead to increased levels of nitrate accumulation in the plant (Viets and Hageman, 1971). Low soil temperatures can limit nitrification, root growth, tissue permeability and active absorption of nitrate from the soil. This can result in a lowering of tissue nitrate concentrations in plants that are grown at lower temperatures (Van Diest cited in Lambers *et al.*, 1986). It has also been suggested that heat stress, during particularly high or low temperature conditions, can reduce nitrate reductase activity and this can contribute to nitrate accumulation (Onwueme *et al.*, 1971).

1.8.4 Soil Nitrogen

The amount of nitrogen in the soil is critical to the success of a lettuce crop for the reasons previously discussed in this chapter that relate to nitrogen metabolism by plants. Indeed, the increased use of nitrogen fertiliser in crop production and the resulting increases in yield are testament to the influence of this nutrient on plant growth. Nitrogen fertilisers provide by far the most significant source of soil nitrogen to a lettuce crop (Greenwood, 1990). Sorenson *et al.*, (1994), illustrated that high rates of nitrogen fertiliser can lead to high nitrate concentrations in the crop. The amount of soil nitrogen that is available to a crop, primarily derived from commercial fertilisers, can be influenced by solar radiation. For instance at low light intensities the level of soil nitrogen has little effect on crop nitrate content. However in summer months the effect of soil nitrogen is more pronounced (Van Eysinga and Van der Meys, 1985). Under low light levels, Van Eysinga *et al.*, (1985), found that applied nitrogen did not significantly increase the nitrate content in plant tissue. However, under levels of high irradiation, plant nitrate content increased markedly. The nitrate content of lettuce fertilised with 5kg ammonium nitrate limestone (ANL) was significantly and positively correlated with global irradiation, with an r^2 value of 0.89. The interactive effect of the level of available soil nitrogen and irradiation can account for 60% of the annual variation in nitrate content (Van Eysinga, 1987). This interaction can have many implications for minimising nitrogen fertiliser application in winter lettuce and deserves special consideration. It seems that during periods of low illumination nitrate uptake and assimilation processes do not occur as rapidly as in periods of high illumination. This suggests that the level of nitrogen fertiliser applied to the crop during winter months should be reduced in order to minimise nitrate-nitrogen losses through leaching. The level of nitrogen fertiliser applied to a lettuce crop in summer periods can be higher, since the plant is able to assimilate the nitrate more efficiently, thereby avoiding the excessive accumulation of nitrate in the soil and subsequent losses through leaching.

1.8.5 Agronomic practises affecting soil structure/composition

Agronomic practises are known to affect mineralisation processes in the soil and can therefore influence the availability of soil nitrogen. For instance, it is important that soil cultivation is avoided in wet conditions, when the risk of creating pans is high. If soil structure is compacted, drainage is impeded and this can result in poor crop growth. It is therefore important that the soil is cultivated under suitable conditions.

The air content of soil can affect nitrification, which occurs primarily in moist, well drained and aerated soils. The nitrification rate will contribute to the available nitrogen in soils in the form of nitrate. Conditions that do not favour nitrification include waterlogged and poorly aerated soils, together with low soil temperatures. Under these conditions, nutrients supplied within commercial fertilisers may be lost through processes that include leaching and denitrification. Irrigation and drainage practises can also influence soil nitrate concentrations since soil moisture affects the movement of nutrients, including nitrate, through the soil. Dry periods reduce nitrate uptake in lettuce crops since the mobility of nitrate is severely restricted in dry soils Azcon *et al.*, (1996). It is therefore important that the lettuce crop, which is a shallow rooting crop, is adequately irrigated throughout the growing season to ensure sufficient supply of nitrogen and to prolong nitrate uptake.

The properties of a soil differ according to climate, microbial population, topography and parent material, all of which interact at different periods of time. The soil is further influenced by human intervention, including agronomic practises, some of which have already been mentioned. Cultivation of the soil environment can create additional sources of nitrate variation arising from nitrogen residues remaining in the soil from grazing animal populations and previous fertiliser application. It is known that soil pH influences nitrate uptake. Nitrate uptake is inhibited in soils with a low pH and also by the presence of ammonium ions in the soil (Scaife *et al.*, 1986; Santamaria *et al.*, 1997). Lettuce plants are not tolerant of acidic soil conditions and are most suited to neutral soils. In short, it is important to optimise soil conditions through recommended agronomic practises in order to ensure a sufficient supply of soil nitrogen is available throughout the growing period.

1.8.6 Nitrogen Nutrition

The source of nitrogen, rate, time and method of nitrogen application are all known to influence nitrate content of lettuce (Greenwood and Hunt, 1986). It is widely recognised that one of the most important factors affecting crop nitrate concentration in agricultural systems is the amount of soil nitrogen in the form of nitrogen fertilisers (Maynard, 1976, Lorenz and Wier, 1974). For all crops, more than 85% of added nitrogen fertiliser is applied at times of active growth and uptake. The nitrate concentration in the nutrient solution surrounding plant roots is known to exert a profound effect on nitrate uptake of the plant (Wheeler *et al.*, 1998). It has been suggested that when all other environmental parameters are equal, excessive nitrogen fertiliser application is the primary factor influencing nitrate accumulation in plants (Van Diest, 1986). Data from studies by Greenwood and Hunt (1986) support the assertion that foliage crops, including lettuce, contain high concentrations of nitrate when grown with high rates of nitrogen fertilisers. They showed that a lettuce crop supplied with nitrogen fertiliser, at a rate of 100 kg/ha^{-1}, contained 3393 $\mu g/g^{-1}$ compared with lettuce supplied with maximum nitrogen fertiliser, at a rate of 392 kg/ha^{-1}, which contained 4776 $\mu g/g^{-1}$. McCall and Willumsen (1998), showed that reducing NO_3^-N availability from 260 to 200 kg N ha^{-1} significantly reduced the nitrate concentration of fresh lettuce. They showed that a further reduction of NO_3^-N to 120 kg N ha^{-1} significantly reduced both the nitrate content and fresh weight of the lettuce.

Greenwood and Hunt, (1986), estimated from study data and a survey of household food consumption, that the average British person consumes approximately 60 mg NO_3^-N week^{-1} in field vegetables. They calculated that if nitrogen fertiliser were withheld, consumption would approximately halve to 30 mg NO_3^-N week^{-1} and if excess fertiliser were applied, consumption would approximately double to 120 mg NO_3^-N week^{-1}. The average nitrate

uptake of arable crops, when supplied with an adequate amount of soil nitrogen, is in the range of 200-300kg/ha (De Willigen, cited in Lambers *et al.*, 1986). In situations of over-fertilisation, plants are unable to utilise and convert the inorganic nitrogen into proteins or amino acids. Nitrate may therefore accumulate at high concentrations in plants and / or leach into waterways.

The form of nitrogen in the fertiliser used to supply a lettuce crop affects individual plant nitrate concentrations (Gunes *et al.*, 1994; Scaife *et al.*, 1986). Previous studies have investigated the use of different forms of nitrogen fertiliser, varying rates of nitrogen fertiliser and different methods of fertiliser application on lettuce. Different forms of nitrogen fertiliser include urea-based, compound fertilisers that contain varying rates of nitrate and ammonium, fertilisers that include a nitrification inhibitor, organic nitrogen fertilisers and slow-release fertilisers. The most common "straight" nitrogen fertilisers, of which ammonium nitrate is the most well-known, are given below (Mengel and Kirkby, 1987).

		% Nitrogen
Ammonium sulphate	$(NH_4)_2SO_4$	21
Ammonium chloride	NH_4Cl	26
Ammonium nitrate	NH_4NO_3	35
Nitrochalk	$NH_4NO_3 + CaCO_3$	21
Ammonium nitrate sulphate	$NH_4NO_3.(NH_4)_2SO_4$	26
Potassium nitrate	KNO_3	14
Urea	$CO(NH_2)_2$	46
Calcium cyanamide	$CaCN_2$	21
Anhydrous ammonia	NH_3	82

It has been shown that nitrate based fertilisers can increase the nitrate content of plants to a greater extent than urea and ammonium based fertilisers (McCall and Willumsen, 1998 and Van Diest cited in Lambers, Neeteson and Stulen, 1986). A study by Myczkowski *et al.*, (1986) used four types of nutrient feed containing different levels of nitrate and ammonium. The lowest level of nitrate in tissue was shown by lettuce supplied with $68\%NO_3^-/39\%NH_4^+$ and $60\%\ NO_3^-/31\%\ NH_4^+$. The highest nitrate concentrations were observed in plants supplied with $100\%\ NO_3^-$. Substituting 40% of the available NO_3^--N with NH_4-N has been shown to significantly reduce the nitrate content of fresh lettuce (McCall *et al.*, 1998). The interaction between NH_4^+ and NO_3^- is complicated. The presence of NH_4^+ may inhibit or have little effect on net NO_3^- uptake. In a study by Santamaria *et al.*, (1997), it was assumed that NH_4^+ may have stimulated nitrate reductase activity, thereby reducing the accumulation of nitrate in plant tissue. They showed that supplying nitrogen in its mixed form, containing NH_4^+ and NO_3^-, enabled nitrate concentrations in endive leaves to linearly decrease during the light period. However, if nitrogen was supplied in the NO_3^- form only and/or it was supplied in excess, the leaf nitrate concentration increased even during the light period.

Methods of fertiliser application in crop production include base dressings, liquid application and foliar sprays. Protected crops, which are grown under glass, can be supplied with fertilisers simultaneously in the irrigation system. This process of fertilisation is termed fertigation. The most common method of nitrogen fertiliser application is as a broadcast dressing, either as a single or split dressing application, which is incorporated into the soil

before planting (Lee, 1999). Deficiencies in nitrogen at the seedling stage of vegetables have been noted in some circumstances, despite an adequate supply of nitrogen fertiliser before planting (Costigan cited in Greenwood, 1990). This situation may arise in a lettuce crop following distribution of fertiliser granules within the soil. At early stages of plant growth the seedling root is not long enough to intercept the zones in which nitrate concentration has been increased by means of diffusion from the fertiliser granules. To overcome this difficulty, a small amount of fertiliser may be injected directly beneath the seedling. This method has been shown to produce higher crop yields and 60 percent increased recovery of nitrogen in vegetable crops when compared to "broadcast" NPK fertilisers (Greenwood *et al.*, 1989). Fertiliser application as a foliar spray has been shown to increase the percentage of nitrogen and yield of vegetable crops, but it is not certain that foliar application can improve overall nitrogen use in a lettuce crop (Penny *et al.*, 1976). In addition, an experiment by Barker, Peck and MacDonald (1971), showed that nitrate levels in lettuce could be reduced if nitrogen fertiliser was applied as a side-dressing rather than broadcast at equivalent rates before planting.

It has been suggested that the presence of ammonium ions together with a nitrification inhibitor in the soil can inhibit nitrate uptake, thereby reducing nitrate accumulation in the crop (Richardson and Hardgrave, 1992). Indeed, nitrification inhibitors used alongside fertiliser application are one method growers have used to control nitrate accumulation in vegetables. Inhibitors, such as dicyandiamide (DCD) block the oxidation of NH_3^- to NO_2^- by bacteria of the species *Nitrosomonas, Nitrosocystus* and *Nitrosospira*. Nitrification inhibitors reduce major NO_3^- losses and extend the period over which ammonium-based fertilisers can be applied (Bhuija and Walker in Mengel *et al.*, 1987). Nitrification inhibitors are usually applied in autumn alongside NH_4-N fertilisers. The most effective commercial nitrogen fertiliser found to lower nitrate content of glasshouse lettuce was found to contain a nitrification inhibitor (Richardson and Hardgrave, 1992). Scaife, Saraiva-Ferreira and Turner, (1986), found that ammonium sulphate and a nitrification inhibitor gave good growth and very low sap nitrate levels. Beresniewiecz *et al.*, (1988) showed that the use of a nitrification inhibitor, Triamide Phosphoryl (TAP), as a nitrogen/phosphorus fertiliser controlled nitrate levels in lettuce, spinach and tomato plants. The study showed that TAP was as good a source of nitrogen for plants as ammonium nitrate. Plants grown with TAP contained, at harvest, ten times less nitrate than plants grown with ammonium nitrate.

Slow-release fertilisers may be composed of natural organic matter, synthetic or formulations of compounds. They are either materials with a low solubility, or are conventional fertiliser materials with coatings on the granules. As their name suggests, this type of fertiliser releases nutrients over a longer time period to coincide with plant nutrient requirement throughout the growing period. The surface coatings on individual fertiliser granules are responsible for delaying nutrient availability. The use of slow-release fertilisers has been found to reduce the nitrate concentration in a lettuce crop in the first cropping season. However after the second cropping season the nitrate content of the crop is not significantly different to a crop supplied with standard nitrogen fertiliser (Lee, 1999). The use of slow-release fertiliser is more expensive compared to the price of commercial fertiliser.

Timing of fertiliser application is crucial if the crop is to efficiently utilise the nitrogen. There is some evidence that crops do not respond to fertiliser application levels to the same extent during winter months compared to summer months. As noted previously, this may be due to the influence of light on nitrate uptake. To prevent residues of nitrogen fertiliser remaining in

the soil following application to a winter crop, it is therefore important to reduce fertiliser application rates during winter months compared to summer months.

1.8.7 Interactions between light, temperature and soil nitrogen and the effect on plant nitrate accumulation

A light, temperature and soil nitrogen interaction on plant nitrate accumulation has been noted (Hoff and Wilcox cited in Maynard, 1976). It seems that temperature exerts its greatest effect on nitrate accumulation when there is low light and high nitrogen. Conversely, light has its greatest effect on nitrate accumulation when temperature is high and soil nitrogen is high. Understanding the relationship between light level, temperature and availability of soil nitrogen is extremely important to lettuce growers. The combined effects of irradiance, light duration and temperature are such that to achieve low nitrate concentrations in lettuce growers are recommended to harvest lettuce on cool days with a long photoperiod and high levels of irradiation (Van Diest cited in Hewitt and Cutting, 1986). During periods of high irradiation, typically throughout summer months, demand for soil nitrogen increases with greater photosynthetic activity (Knight and Mitchell, 1983). However, the increased nitrate uptake does not usually result in excessive nitrate accumulation in the crop since the process of nitrate assimilation into organic compounds is also increased by light.

The availability of soil nitrogen is closely connected to soil temperature (Jarvis, *et al.*, 1996). It is the process of mineralisation that releases nitrogen in the soil, and net mineralisation generally increases and becomes less variable at higher temperatures. Therefore at higher temperatures and irradiation levels, an increase in soil nitrogen availability may coincide with the increased nitrogen demand of the plant. Such an interaction between factors has been demonstrated in a study by Van Eysinga *et al.*, (1985). Under high light levels, the nitrate concentration of the lettuce was low, approximately 1000 mg/kg NO_3^- fresh weight. The nitrate content of the lettuce increased notably with increasing nitrogen fertiliser application to approximately 2500 mg/kg NO_3^- fresh weight. Under low light levels, the nitrate content of the lettuce was high, at approximately 3000-3500 mg/kg NO_3^- fresh weight. The increase in lettuce nitrate concentration with increasing nitrogen fertiliser application was less marked at the low light level, at approximately 500ppm NO_3^-.

1.8.8 Variation in nitrate assimilation by different cultivars

Different nitrate concentrations have been shown to exist in cultivars of lettuce (Reinink, Groenwold and Bootsma, 1987). Schroder and Bero, (2001), have noted significant differences concerning the uptake of nitrogen between lettuce varieties raised in a hydroponic system. A wide variation in nitrate concentration has been noted by van Diest, cited in Lambers *et al.*, (1986), between the genotypes "Atlas", "Du Bonjard", "Grosse brune" and "Valmaine". The nitrate concentrations ranged from 4265, 4130, 3190 to 2050 mg.kg fresh weight respectively. Significant differences between nitrate content of butterhead compared with continental varieties have been reported (Maynard, 1976). The general trend is for butterhead varieties to have lower nitrate levels compared to continental varieties. Differences in nitrate levels between butterhead and continental varieties may be due to morphology, (Behr and Wiebe, 1992) but are more likely to be due to genetic differences leading to reduced nitrate uptake (Blom-Zandstra and Eenink, 1986).

Plant characteristics thought to be responsible for nitrate uptake and accumulation have been studied, which include enzyme activity, photosynthesis, transpiration rates, root capacities and leaf cell characteristics. However, the variation in nitrate concentration that is attributable to differences in nitrate assimilation and head morphology, (Plates 4, 5, and 6, chapter two) appear to play a minor role (Behr and Wiebe, 1992). An inverse correlation has been found between nitrate content and the concentration of other osmotica in a study using 19 lettuce cultivars (Behr and Wiebe, 1992). In their study, Behr and Wiebe showed that with a low nitrate concentration contained higher levels of malate, chloride, fructose and glucose. The results of these previous studies suggest that the differences in nitrate concentration between cultivars may result from different photosynthetic rates between lettuce types through which nitrate, acting as an osmoticum, is replaced by sugars.

Both uptake and assimilation systems are thought to be genetically determined (Ferraro-Mery *et al.*, 1997). However the strong genotype × environment (GE) interactions that have been observed in several lettuce varieties suggests that the differences may be strongly related to their photosynthetic capacity (Blom-Zandstra and Eenink, 1986; Reinink, 1991). Light intensity is of particular importance in the GE interactions, which can be related to daily annual variation in nitrate levels (Reinink, 1991). Recent work has confirmed a dual role for nitrate in plant cells. In addition to being a source of nitrogen for the synthesis of several organic compounds, nitrate also serves to function as an osmoticum (Behr and Wiebe, 1988). When nitrate is accumulated in the cell vacuole it maintains turgor pressure (Steingrover, 1986). Reinink, (1993), illustrated a significant genotypic variation for osmolarity in lettuce and endive. They suggested that the differences in osmolarity could result in similar variations in nitrate accumulation. Large scale screening of lettuce plants for nitrate accumulation at the seedling stage has been proven to give good correlation with nitrate accumulation in adult plants. Practises to lower nitrate levels in lettuce could involve the use of similar varietal screening, together with cultural methods known to reduce nitrate levels (Schonbeck *et al.*, 1991).

1.9 Nitrate Analysis

1.9.1 Predictive models for determining crop nitrogen requirements

The present strategy of optimising nitrogen fertiliser use and reducing nitrate levels in UK lettuce has made it necessary to determine the nitrate status of the crop before any additional fertiliser applications are applied (Anon, 1995a). Conditions under glass, the growing environment for the majority of UK grown lettuce, are vastly different to outdoor growing conditions. Predictive models have been quite successful at determining crop nutrient requirements in an outdoor growing environment. These computer based recommendation systems are based on data from scientific investigations, which aim to provide effective means of managing nitrogen fertiliser application. There have been several models developed that deal with fertiliser management of arable, horticultural and grassland crops. They include WELL-N, FERTIPLAN, SUNDIAL-FRS, NFERT and MANNER computer systems.

WELL-N was developed at Horticulture Research International, Wellesbourne, for use on horticultural and some arable crops (Burns, 1999). It is a complex and dynamic computer system that uses various cultural details to calculate nitrogen fertiliser requirements. The model is designed to use actual weather data to predict ongoing changes in top dressing or irrigation requirements during the growing period. However, any assumption based on

outdoor growing conditions cannot be directly applied to a protected environment. Differences in soil conditions exist between field and glasshouse environments. The root development of crops varies in glasshouse conditions and this affects the predicted growth patterns as well as the way they respond to nitrogen fertiliser application. Finally, there are relatively lower light levels and higher temperatures in protected environments. It is therefore necessary to modify the existing WELL-N model, taking into account the previous assumptions that were based on a field environment.

Experiments carried out as part of DEFRA funded projects are providing data relating to plant to plant variability in nitrate levels for protected lettuce crops (Burns, 1991). This information is being used to develop an equivalent nitrogen fertiliser management system for protected crops using the existing WELL-N model. A recommendation system of this kind would provide advice for both base dressing and supplementary nitrogen fertiliser applications by top dressing or liquid feeds. It is envisaged that this predictive system will provide accurate nitrogen fertiliser recommendations for protected lettuce growers (Anon, 1998d).

1.9.2 Methods of nitrate analysis

The EC Regulation 194/97 has deemed it necessary to determine the nitrate content of the lettuce crop before it is harvested. In addition to providing information on the nitrate level of a crop prior to harvest, nitrate analysis also allows the farmer to correct nutrient deficiencies at various stages in the growing period. Crop nutrient analysis therefore gives the grower better control of crop production. Lettuce is sampled at the nursery four times per year on a seasonal basis according to the sampling procedure outlined in the Assured Produce Scheme protocol (Anon, 1998a). The choice of analytical laboratory is made by the grower, and should be "a competent laboratory accredited by UKAS or a participant of FAPAS (Food Analysis Performance Assessment Scheme) proficiency test scheme, which uses a validated method of analysis" (Anon, 2000b).

Laboratories taking part in the FAPAS proficiency test are required to use a validated method of nitrate analysis. These laboratories are required to achieve satisfactory performance in appropriate quality control checks undertaken by FAPAS. The process involves distribution of duplicated samples of known nitrate concentration to different laboratories where nitrate analysis is carried out. The results are related to known or actual nitrate concentrations of the distributed sample. The results are represented as "z-scores", which indicate the degree of variation about the true nitrate value of each sample. Z-scores between +2 and –2 indicate satisfactory determination of nitrate and the laboratory is judged to be competent for nitrate analysis.

It is imperative that the method of analysis used to determine nitrate content is accurate and reliable. Current laboratory techniques for nitrate-nitrogen determination in lettuce and other vegetables include using anion-exchange high performance liquid chromatography (Hunt and Seymour, 1985; Schuster and Lee, 1987), ion chromatography (Swiader and Freiji, 1996), potentiometric procedures (Anon, 1990) and nitrate selective electrodes (Bedwell *et al.*, 1995).

1.9.3 Rapid testing methods for nitrate analysis

Laboratory methods of analysis used in the UK usually give high levels of accuracy, but are often time-consuming and expensive. If plant analysis is to help towards corrective methods during the growing period, the time taken to complete the analytical procedure is an important factor to consider. Rapid testing methods for nitrate analysis were first developed in the UK during the 1940's (Burns, 1999). Since 1976, "Merko-quant" test strips, which were primarily developed by BDH Chemicals Ltd. for nitrate determination in water, have been shown to provide a convenient, reliable sensitive method of nitrate analysis in a range of vegetables including lettuce (Scaife and Bray, 1977; Prasad and Spiers, 1982). Merkoquant test-strips determine nitrate through a colorimetric reaction. This occurs when a solution reacts with the aromatic amine and N-(1 napthyl) ethylene diamine, which is impregnated on two squares of filter paper attached to the thin plastic test-strip (Scaife and Bray, 1977). Investigations to determine the reliability of Merkoquant test-strips have involved comparison with existing methods of nitrate analysis (Bischoff *et al.*, 1996). In this study, the results obtained by nitrate analysis of water samples using the test strips were compared with results from two analytical laboratory methods, namely high performance liquid chromatography and colorimetric analysis using a colorimetric ion analyser. There was a good correlation between the test strip results and the results obtained by high performance liquid chromatography and colorimetric ion analysis.

The recent introduction of a portable, battery operated nitrate-selective electrode (Horiba "Cardy" meter) offers the possibility of quick, quantitative analysis of soil solution and undiluted petiole sap of vegetables including lettuce (Hartz *et al.*, 1993). Although the instrument has not been validated for use in the UK, several studies have set out to evaluate its use as an on-farm tool for monitoring plant nitrate content. The Horiba "Cardy" meter results were highly correlated with those obtained by laboratory analysis of dry petiole tissue (Hartz *et al.*, 1993).

It has been mentioned that laboratories in the UK may differ in the technique they use for analysis of nitrate. In addition to the variations in the method of nitrate analysis used by a particular laboratory, the procedure used to extract nitrate and the form in which the plant material is analysed can differ. The sample portion taken for analysis can comprise complete or trimmed lettuce heads, or representative samples taken from the lettuce which are pooled (Anon, 1998a). Extraction of nitrate can be carried out on fresh (Official Methods of Analysis, 1990), oven dried (Cataldo, Harron, Schrader and Youngs, 1975), frozen (Beljaars, van Dijk and van der Horst, 1994) or freeze dried plant tissue (Anon, 1999b). The use of water, at various dilution ratios, is commonly included in extraction procedures and this can be either hot or cold. In some instances, extraction may involve the use of clarifying agents such as activated carbon (Hunt and Seymour, 1985), or proteinating agents such as Carrez solutions I (30% $ZnSO_4$) and II (15% $K_4Fe(CN)_6$) (Blanco *et al.*, 1995). Nitrate may be extracted with a hydraulic press or in a mechanical blender, and the use of a boiling water bath has been noted (Anon, 1998a,g). The extract is usually analysed immediately but can be stored without significantly affecting nitrate levels, usually at -20°C, for up to sixty days (Khakural and Alva, 1996).

The abundance of techniques available for nitrate analysis of plant tissue, and more importantly the absence of a standardised method for nitrate extraction, can result in marked differences in the nitrate values obtained between laboratories (Bedwell *et al.*, 1995; Lee, 1999).

Aims of the Investigation

The aims of the current study involved two broad, but inter-linked areas of research.

Firstly, factors influencing plant to plant nitrate variability in soil and hydroponically raised glasshouse lettuce were studied. The investigation involved evaluation of plant nitrate variability as influenced by individual factors including light intensity, nitrogen nutrition rate and planting density, as well as the interactive effect of these factors, in both a winter and summer lettuce crop. Additional trials were carried out to determine variation in nitrate assimilation by different cultivars including butterhead and continental lettuce. The lettuce trials were conducted under glass in both soil and hydroponic systems.

The second area of the study, conducted concurrently with the first area of study, was to determine a rapid, nitrate testing technique for on-farm use with lettuce crops. A thorough evaluation of existing techniques for nitrate determination of plant extracts was investigated with an aim of establishing a suitable technique for monitoring nitrate concentrations in lettuce crops in the nursery.

Chapter 2

2.0 Investigations into factors affecting nitrate variability in lettuce in hydroponic growing systems

2.1 Introduction

Hydroponic growing systems, such as Nutrient Film Technique (NFT), provide a means of growing crops in soil-less culture in which the nutrient supply is more effectively controlled than in a soil-based system. The yield rate of crops grown in controlled environments, including hydroponic systems, are often higher compared to the yield rate of field grown crops (Knight and Mitchell, 1983). Chapman and Carter (cited in Knight and Mitchell, 1983) showed that it is possible to produce a marketable leaf lettuce in 22 days in a controlled environment, compared to a 55 day growth period when producing lettuce of similar quality in the field environment. Nutrient film technique (NFT) ensures the crop is supplied with nutrients throughout the growing period via a recirculating system. The crop nutrient requirement can be easily determined by monitoring crop nutrient uptake, and the composition of the nutrient solution can be adjusted to ensure the nutrient supply matches the demands of the crop. Hydroponic systems reduce the possibility of excessive fertiliser use in crop production, and if the system uses a recirculating nutrient supply, environmental pollution is also reduced. In addition to improvements in water and fertiliser use efficiency, soil-less culture systems may also give better crop production in terms of quantity and quality (Heinen and Harmanny 1992).

Maintaining a healthy crop is of utmost importance in any growing system, and a hydroponic growing system is no exception. In general, hydroponic systems do not present any major problems with pest or disease outbreak. The following recommendations are suggested for maintaining a healthy growing environment (Wells, 1995):

1. Ensure the crop grows well at all times by providing sufficient light, nutrients and other environmental requirements.
2. In the occurrence of disease, empty solution tanks and clean with 2% bleach solution. The germination area, including benches and solution tanks should also be disinfected.
3. The equipment should be washed between each use.
4. Avoid transporting foreign plant material into the glasshouse, as it may contain pests and pathogens that may infect the crop.
5. Keep the solution tanks shaded to prevent the growth of algae. Although the algae will not harm the crop directly, the presence of algae may weaken the crop to potential disease.

The introduction of EC regulation 194/97 stipulates that lettuce growers within the UK, operating under a derogation, adopt growing procedures that aim to reduce nitrate concentrations in lettuce. Reduction of nitrogen supply during the growing period, as a method for ultimately reducing nitrate content of harvestable crops, has been previously attempted in hydroponics (Van Diest, 1986). Although the investigations of Van Diest, (1986), were successful in producing crops with low nitrate content, the essential requirements of high crop yield and quality were not achieved. The ability to tailor nutrient supply to crop nutrient demand as well as reducing tissue nitrate content is highly desirable to

growers. It is possible that hydroponic systems could help to lower nitrate concentrations in lettuce by reducing the nutrient supply during the growing period (Andersen and Nielsen, 1992). It has even been alleged by Santamaria, (1997), that using the ammonium form of nitrogen in soil-less culture could offer the chance to produce nitrate-free endive heads (*Cichorium endivia* L.var. *crispum* Hegi). Hydroponic growing systems therefore appear to offer a means of reducing nitrate concentrations in UK grown lettuce.

Increased fertiliser use efficiency, as seen in NFT systems, has potential benefits both economically and environmentally. Developments towards more efficient energy conversion systems, crop modelling procedures, computer technology and new cultivars, have led to an increase in the number of horticultural crop species grown in controlled-environment systems. The benefits of using NFT systems for lettuce production has resulted in increased numbers of hydroponically produced lettuce in many countries (Economakis, 1991). Despite the obvious advantages of raising crops hydroponically, most protected salad crops in the UK which include lettuce, are grown in soil-based, protected systems. The task of monitoring and operating a modern hydroponic system. requires specialised equipment and well-trained staff. This may be a contributing factor preventing UK growers changing to soil-less culture. In addition, there is always a risk of mechanical breakdown or interruptions in the electrical supply, both of which pose a serious threat to the success of the crop. However, probably the most important factor deterring growers from adopting a soil-less system must be the long-standing tradition of growing lettuce in soil, together with the economic viability of setting-up such a system.

2.2 Inherent variation in nitrate concentration of hydroponically raised lettuce

2.2.1 Introduction

Many investigations have used hydroponic systems to study factors affecting nitrate metabolism and accumulation in lettuce plants (Myckowski, Rozek, Sady and Wojtaszek, 1986; Carrasco and Burrage, 1992; Gunes, Post *et al.*, Kirkby and Aktas, 1994; Urrestarazu *et al.*, 1998). Hydroponic systems provide a controlled environment in which factors known to influence nitrate uptake in soil-based systems, such as nutrient availability at the root zone, can be reduced or eliminated. The factors known to affect the nitrate concentration of crops, such as nutrient concentration and irradiation levels, can therefore be investigated more easily since plant nitrate variability resulting from other external factors has been limited.

Hydroponic systems were used in a series of investigations to determine plant nitrate variability in a controlled growing environment. The aim of the preliminary study, using hydroponically raised lettuce, was to determine inherent nitrate variability in lettuce. Nutrient film technique provides a closed, relatively constant system in which factors known to influence plant nitrate concentration are limited. The reduced plant nitrate variability would enable inherent variability within lettuce to be investigated. In the second investigation, plant nitrate variability in a hydroponic system was studied in relation to three nutrient treatment concentrations. The third hydroponic investigation involved plant nitrate variability between three lettuce cultivars.

2.2.2 Materials and Methods

A summer crop (cv Flandria) was sown into 4 × 4cm rockwool blocks and transplanted at the 5th leaf stage into hydroponic growing channels on 13 August 1998. The crop was grown using nutrient film technique (NFT) and fed with a standard hydroponic recipe containing 190ppm nitrate-nitrogen (Appendix section, page 1). Five 3m × 0.2m NFT channels were used, each containing one row of twelve plants. The channels were laid side-by-side to give a plant spacing of 25cm × 22cm (plate 2). Changes in the nitrate concentration of the nutrient solution were estimated by analysis of the solution, which was sampled at twice weekly intervals throughout the growth period. The pH, EC and flow rate of the nutrient solution were set at approximately 6.0, 2.2 mS cm^{-1}, and 5.5 l/min respectively and recorded throughout the growing period. pH, EC and flow rate were not adjusted during the growing period. Ten heads were harvested from each channel on 2 September 1998. The untrimmed heads were frozen prior to determination of sap nitrate content. The method used to extract nitrate from the lettuce tissue involved blending whole, untrimmed lettuce heads in a Philips HR 2835 blender with approximately equal volume to weight ratio of cold de-ionised water. The nitrate content of the lettuce was determined using the analytical techniques of the Ion Selective Electrode, Nitrachek meter and Ion Chromatograph (Chapter four). The nitrate results presented in this chapter were obtained following analysis with the ion selective electrode (nitrate probe).

Plate 1: Small-scale lettuce production in NFT at HRI, Stockbridge House.

2.2.3 Results

Environmental data for the growing period of 13 August to 2 September 1998 together with results of pH, EC and flow rate are included in the appendix, pages 2 to 4. Sap nitrate concentration of individual lettuce within one channel are shown in figure 2.1. The mean sap nitrate concentration and mean fresh weights of lettuce in each channel are shown in figures 2.2 and 2.3 respectively. Vertical bars represent the standard error of the means. The nitrate concentration of the nutrient medium within the five channels is shown in figure 2.4.

Figure 2.1 Sap nitrate concentration of individual lettuce heads, cv. Flandria, raised within channel number four of a five channel hydroponic system, at HRI Stockbridge House.

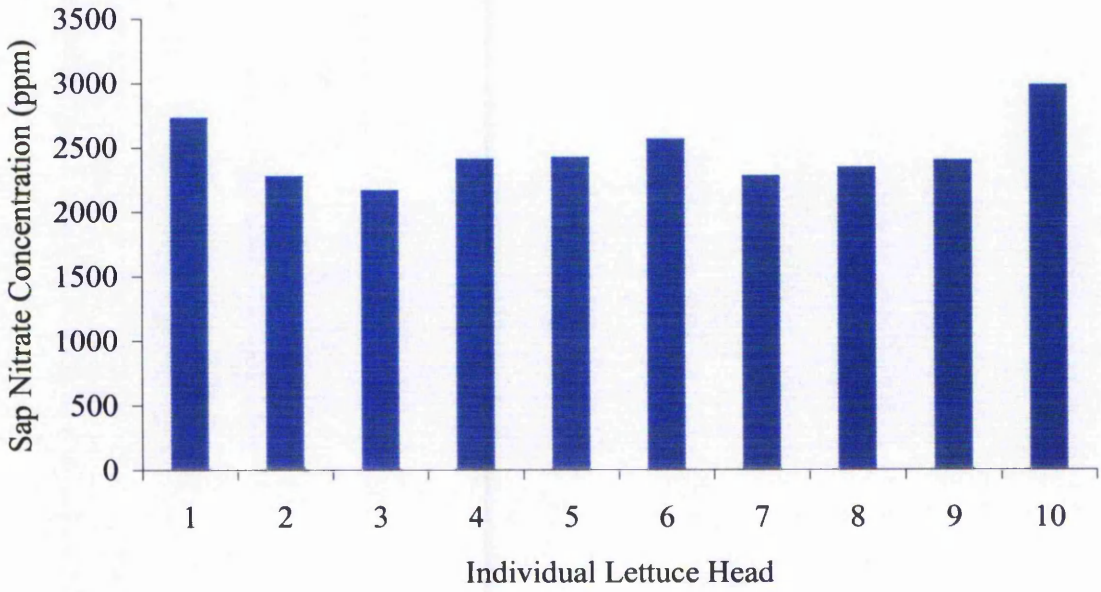


Figure 2.2 Mean sap nitrate concentration (ppm) of lettuce, cv. Flandria, raised in five NFT channels supplied with one nutrient treatment, at HRI Stockbridge House.

LSD = 479.69 at p = 0.001
LSD = 366.14 at p = 0.01
LSD = 274.06 at p = 0.05, at n = 10 throughout.

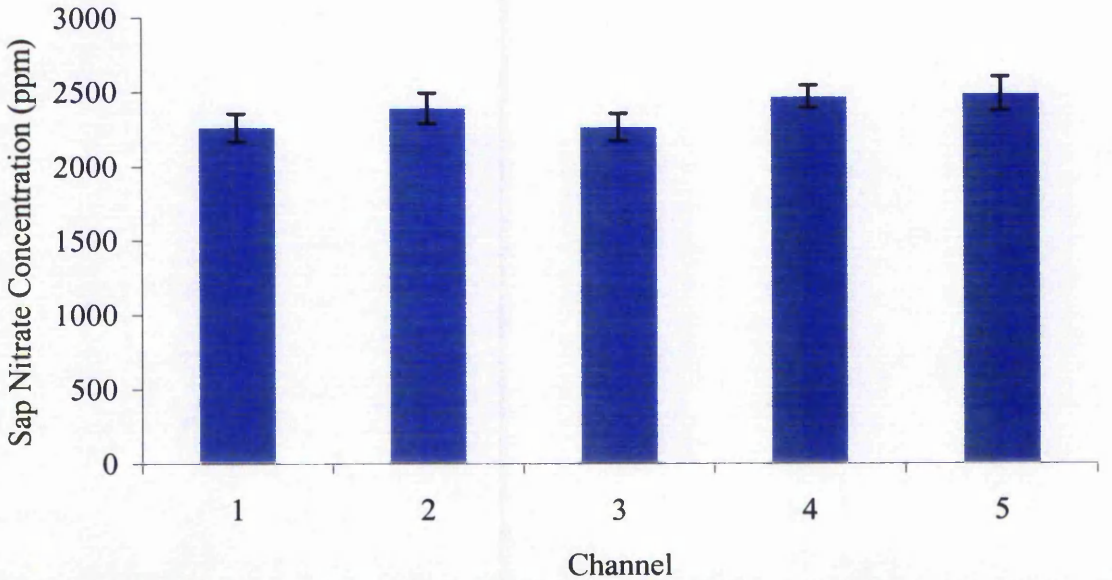


Figure 2.3 Mean fresh weight of hydroponically raised lettuce, cv. Flandria, at HRI Stockbridge House.

LSD = 39.95 at p = 0.001
LSD = 30.49 at p = 0.01
LSD = 22.83 at p = 0.05, at n = 10

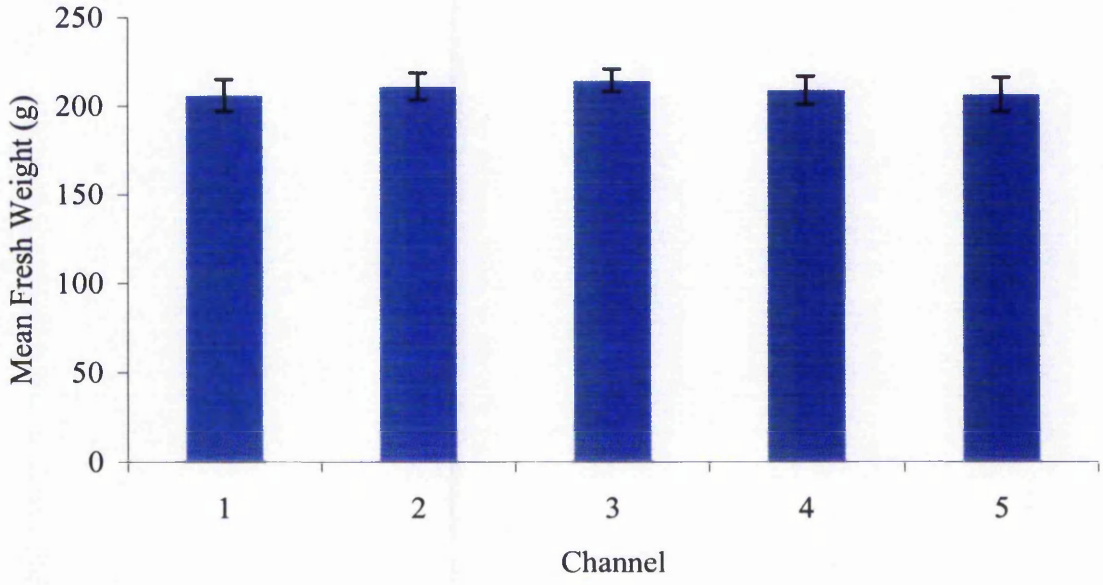


Figure 2.4 Nitrate-nitrogen concentration (ppm) of nutrient medium supplied to lettuce cv. Flandria during the growing period 17 August to 1 September, 1998.

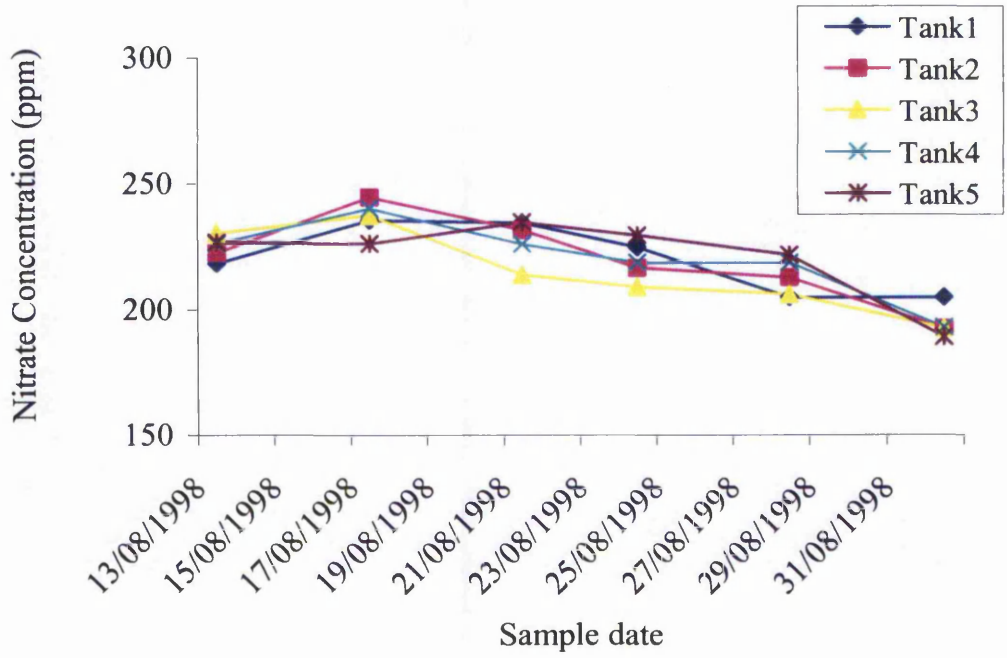


Figure 2.1 shows the variation in nitrate concentrations obtained between individual lettuce replicates within channel number four following analysis by the ion selective electrode (ISE). The nitrate concentration of individual lettuce within the channel ranged from 2172 ppm to 2991 ppm.

Figure 2.2 illustrates mean nitrate concentration of ten lettuce replicates raised in each of the five NFT channels. The mean nitrate concentrations of lettuce raised in the five channels ranged from 2238 to 2484 ppm. Statistical analysis of variance using the mean nitrate concentrations of the lettuce showed no significant variation between concentrations of nitrate within lettuce raised in the five channels ($p=0.001$; LSD± 479.69).

Figure 2.3 shows the mean fresh weights of the lettuce within each channel. The mean head weights ranged from 206 to 214 g. Statistical analysis of variance using the mean head weights of the lettuce showed no significant differences between hydroponic channels at harvest. The crop appeared to be uniform at harvest, in terms of both head weight and quality. At harvest there were no visible signs of insect infestation, foliar damage or tipburn (plate 5).

Figure 2.4 shows that the nitrate concentration of the nutrient solution remained at approximately 230 ppm throughout the growing period until the final week when the nitrate concentration dropped to below 210 ppm. The environmental data, given in the appendix, shows mean daily humidity, temperature and weekly values for irradiation throughout the growing period of the crop. Temperature showed a gradual decline from 21.8 to 14.2 °C and weekly irradiation from 95.65 to 52.81 MJ/m^2. Relative humidity increased during this period from 56.9 to 86.4%.

2.2.4 Discussion

The pH, EC, flow rate and nitrate concentration values obtained from the nutrient media within the five channels were similar throughout the growing period. This suggests that the system was efficient in supplying equal concentrations of nutrients to the lettuce within each channel. This is reflected in the general uniformity and overall quality of the crop. The nitrate concentration in the nutrient medium of all five tanks decreased during the final week of the growing period. It is likely that nitrate uptake of the crop increased during the last week of growth and this was reflected in the reduced nitrate concentration of the nutrient medium.

There were no significant differences in mean nitrate concentration of the lettuce between the five channels ($p=0.001$). As expected from the overall uniformity of the crop at harvest, there was no significant variation in head weights ($p=0.001$). Figure 2.1 shows the nitrate concentrations within an individual NFT channel ranged from 2172ppm to 2991ppm. A large degree of variation in nitrate concentration is not unusual for a lettuce crop. Plant nitrate variability in soil grown lettuce has been the subject of many investigations and is considered in chapter 3. Plant nitrate variability in hydroponic lettuce has been studied to a lesser extensively compared to plant nitrate variability in soil-grown lettuce. However, it is generally assumed that a controlled growing system reduces the level of plant nitrate variability within the lettuce. Previous studies have shown that the range of nitrate concentrations of lettuce raised in the hydroponic system showed much less variation when compared with the nitrate concentrations of lettuce raised in soil (Forlani *et al.*, 1997).

The nitrate concentrations from the hydroponic lettuce sampled in this study do not show significant variation between channels. Although there may be some random fluctuations in the behaviour of the tank systems, the results do not suggest that individual NFT channels influence the variation in nitrate concentration of the lettuce. It is likely that the range of nitrate concentrations obtained from lettuce within a channel, and the variation in mean nitrate concentrations between channels are largely due to inherent differences in uptake by individual lettuce heads.

2.3 Variation in nitrate concentration of hydroponic lettuce supplied with different nutrient treatments

2.3.1 Introduction

Glasshouse studies, using nutrient film technique (NFT), have been undertaken with an aim to reducing nitrate accumulation in lettuce. Van der Boon *et al.*, (1990), carried out a five-year series of experiments in which lettuce (*Lactuca sativa capitat* L.) was grown in recirculating nutrient solution. In these experiments both ammonium/nitrate ratios and total nitrogen concentrations in the nutrient feed were varied in order to reduce the nitrate content of the lettuce heads. The nitrate content of the lettuce was influenced by total nitrogen concentration and ammonium/nitrate ratio in the nutrient solution. Reducing the nitrate supply in the nutrient solution, and replacing it with ammonium led to decreased nitrate concentration of the lettuce. Reducing the nitrate supply by lowering the nitrate concentration in the nutrient medium, without replacement with ammonium, led to marked decreases in the nitrate content of spring lettuce. Temporarily omitting nitrogen from the medium solution, although reducing the nitrate concentration of lettuce, also reduced yield and quality to such an extent that the treatment was abandoned. In a similar study by Blom-Zandstra and Lampe (1983), a decrease in nitrate concentration of lettuce was obtained with little or no depression in yield using a nutrient solution without nitrogen. In a separate study by Andersen and Nielsen (1992), lettuce (var. Capitata) was grown in soil-less culture with a reduced supply of nutrients in proportion to the daily uptake of nutrients in a crop with free access to nutrients. The results from this study showed that it is possible to reduce the nitrate concentration in lettuce plants by 30-40% whilst simultaneously maintaining a high yield and quality.

In the second study from the series of investigations into plant nitrate variability using NFT, nutrient solutions varying in nitrate concentration were supplied to a lettuce crop. The aim of the study was to investigate the variation in nitrate concentration of hydroponic lettuce supplied with nutrient feeds containing three rates of nitrate-nitrogen.

2.3.2 Materials and Methods

A winter lettuce crop (cv Rachel) was transplanted in a hydroponic system at the 5th leaf stage on 21 September 1998. The crop was fed by nutrient film technique using three nutrient medium solutions containing 100ppm, 150ppm and 200ppm nitrate-nitrogen. The highest nutrient rate, 200ppm nitrate-nitrogen, was representative of a hydroponic lettuce feed used in studies at HRI, Stockbridge House (appendix). The three nutrient treatments were replicated in three channels to give a total of nine channels. Each channel contained twelve lettuce plants. The channels were laid side-by-side to give an approximate plant spacing of 25cm × 22cm. Flow rate, pH, and EC were set at approximately 5.5l/min, 6.0 and 2.2 mS cm $^{-1}$. Flow

rate, pH, and EC were regularly monitored, together with samples of the nutrient solution in order to establish plant nitrate uptake during growth. Ten heads were harvested from each channel on 28 October 1998. The untrimmed heads were frozen prior to analysis for nitrate concentration. The nitrate content of the lettuce was extracted as described in chapter 4 and analysed using the lab-based ion selective electrode (nitrate probe).

2.3.3 Results

Environmental data are given in the pages 2 to 6 of the appendix, together with pH, EC and flow rate measurements for the growing period 21 September to 28 October, 1998. Figure 2.5 shows mean nitrate concentration of hydroponically raised lettuce supplied with three nitrate-nutrient treatments. Figure 2.6 shows mean fresh head weight of hydroponically raised lettuce supplied with three nitrate-nutrient treatments. Vertical bars represent the standard error of the means. The mean nitrate concentration within the tanks used to supply lettuce with three nutrient treatments are presented in figure 2.7.

Figure 2.5 Mean nitrate concentration (ppm) of hydroponically raised lettuce, cv. Rachel, at harvest supplied with three rates of nitrate-nutrient treatments. Investigation conducted at HRI Stockbridge House from 21 September to 28 October, 1998.

LSD = 196.6 at p = 0.001
LSD = 151.3 at p = 0.01
LSD = 114.1 at p = 0.05, at n = 30

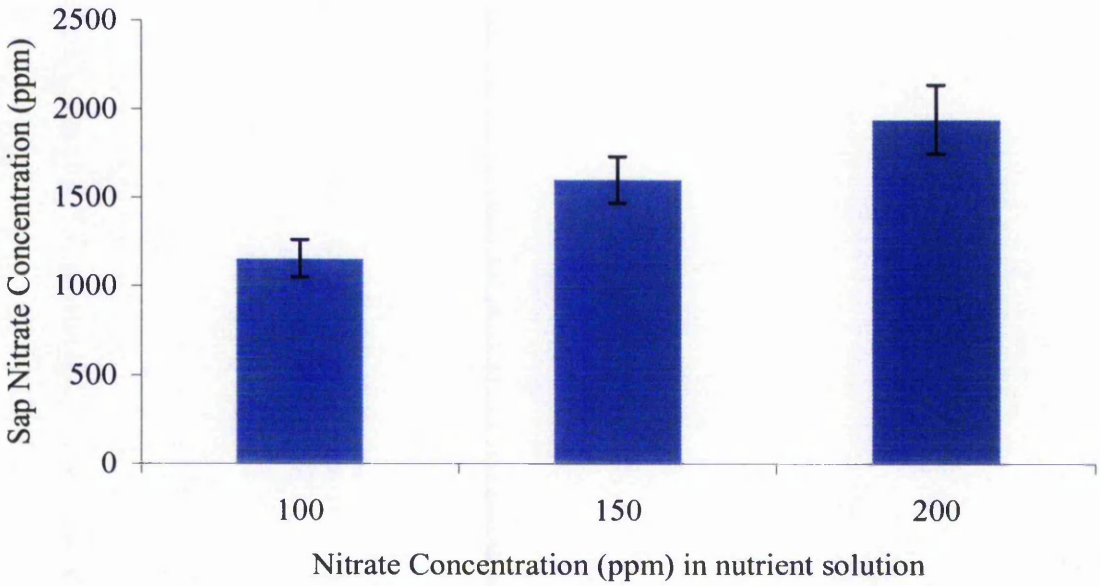


Figure 2.6 Mean fresh head weight of hydroponic lettuce, cv. Rachel, supplied with three rates of nitrate-nutrient solution. Investigation conducted at HRI Stockbridge House from 21 September to 28 October, 1998.

LSD = 26.04 at p = 0.001
LSD = 20.08 at p = 0.01
LSD = 15.13 at p = 0.05, at n = 30

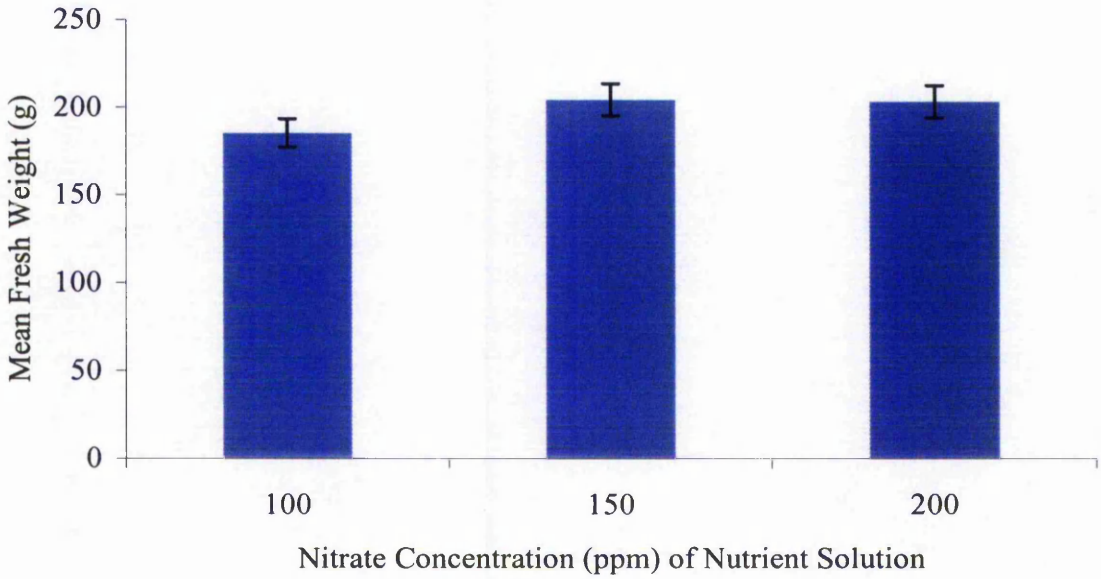
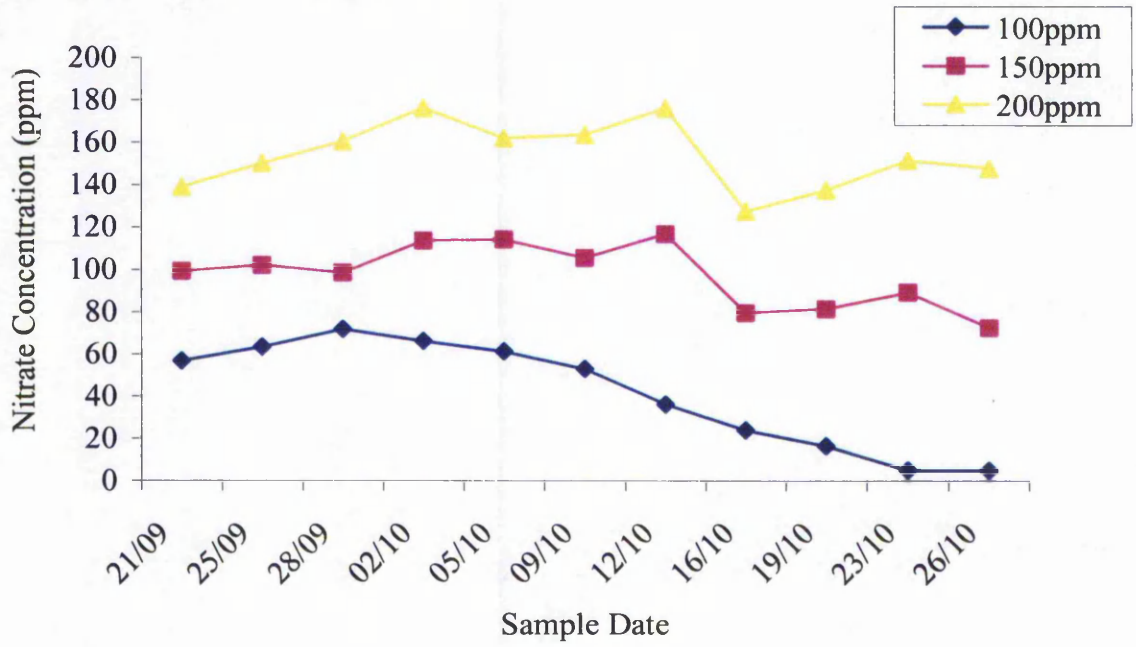


Figure 2.7 Mean nitrate concentration of nitrate-nutrient used to supply lettuce cv. Rachel within an hydroponic system. This investigation was undertaken at HRI Stockbridge House from 21 September to 28 October, 1998.



In the environmental data (given in page 6 of the appendix) daily mean temperature ranged from 11.8 °C to 17.9 °C, relative humidity ranged from 62.9 % to 86.9 % and weekly irradiation levels were within the range 28.03 and 43.85 MJ/m^2. The pH of the nutrient medium within the tanks ranged from 4.08 to 7.04, flow rate was set between 5 – 5.5 l/min and EC values decreased from 2.06 to 1.34 during the growth period. A complete set of data relating to these results can be found in the appendix section. Nitrate uptake in lettuce supplied with 100ppm nitrate-nitrogen was approximately 6.35 mg/day/plant over a ten-day growth period from 16 October to 26 October (figure 2.7). Average daily uptake rate of plants supplied with 150ppm nitrate-nitrogen during the ten-day period was approximately 7.13 mg/day/plant. The uptake rate of lettuce supplied nutrient solution containing 200ppm nitrate was approximately 4.8 mg/day/plant during the same growing period.

Statistical analysis of variance was carried out on the mean head weights of the lettuce raised in the three treatments. Significantly lower mean head weights were obtained by lettuce supplied with 100ppm nitrate-nitrogen compared to the mean head weight of plants supplied with 150ppm and 200ppm nitrate-nitrogen ($p=0.05$). Mean fresh head weight of lettuce supplied with nutrient at a concentration rate of 100ppm nitrate-nitrogen was 185.2 g, compared to 204.3 g and 203.6 g in treatments supplied with 150ppm nitrate-nitrogen and 200ppm nitrate-nitrogen respectively. Approximately 10% increase in head weight was observed when the nitrate concentration of the nutrient solution was increased from 100ppm to either 150ppm or 200ppm (figure 2.6). Mean head weight of lettuce supplied with 150ppm nutrient-nitrate was not significantly different to the mean head weight of lettuce supplied with 200ppm nutrient-nitrate.

The mean nitrate concentration of plants supplied with the lowest nutrient rate, 100ppm, was 1194.5 ppm compared to 1600.3ppm and 1985.2 ppm in treatments supplied with 150ppm

and 200ppm nitrate-nitrogen respectively (figure 2.5). Statistical analysis of variance was carried out on the mean nitrate concentrations of lettuce raised under the three nutrient treatments. A highly significant difference ($p=0.001$) in mean nitrate concentration was observed between lettuce supplied with 100ppm nutrient-nitrate compared to lettuce supplied with 150ppm nutrient-nitrate ($p=0.001$). Nitrate concentration in lettuce supplied with 200ppm nutrient nitrate was also significantly higher ($p=0.001$) than lettuce supplied with both 100ppm and 150ppm nutrient-nitrate solutions.

2.3.4 Discussion

Unfortunately, the results from this investigation may have been influenced by the presence of bird faeces on the leaves of the lettuce crop. The level of contamination and distribution of faeces throughout the crop was impossible to assess and no doubt varied between individual lettuce plants. It is feasible to assume that ammonia based substances within the faeces may have affected the accuracy of the nitrate results obtained. Despite this, the investigation did show a significant reduction ($p=0.001$) in crop nitrate concentration, together with mean head weight, at the lowest nutrient feed.

Increases in the nutrient medium pH observed throughout the growing period of the second hydroponic trial, as shown in the appendix, may be attributed to secretion of hydroxyl ions during uptake and reduction of nitrate and other anions from the root. Nitrate uptake increased steadily throughout the growing period, figure 2.7, and this, together with possible nitrate reduction and the associated secretion of OH^-, would lead to the increases in pH found. Changes in the anion to cation ratio of the nutrient solution are illustrated by the EC values shown in the appendix. EC decreased throughout the growing period from 2.06 $mScm^{-1}$ to 1.34 $mScm^{-1}$ (Pages 4 to 5, Appendix). The decrease in EC values reflects the depletion of nutrients from the medium, principally in the form of nitrate-nitrogen, as plant nitrate uptake rate increased (figure 2.7). The nitrate concentration in the nutrient medium gradually decreased throughout the growing period, a finding further supported by the EC and pH results.

There were noticeable differences in the concentrations of nitrate within the nutrient solutions throughout the growing period (figure 2.7). The removal of nitrate-nitrogen from nutrient media was highest in plants supplied with 100ppm and 150ppm nutrient solution. The removal of nitrate-nitrogen from nutrient solution was lowest in treatments containing 200ppm nitrate-nitrogen, and was recorded at 4.8 mg/day/plant during a ten-day growing period. These results may be due to an increased demand for plant nitrogen where nitrate-nitrogen supply is a limiting factor. The demand for nitrogen by the lettuce plants treated with the highest concentration of nitrate in nutrient solution may be reduced since nitrate-nitrogen is not a limiting factor. Under these conditions, plant nitrate uptake rate may be lower since the nitrate concentration of the nutrient solution is sufficiently high to maintain optimum plant growth. In previous studies by Cardenas-Navarro *et al.*, (1999), it has been suggested that there is a negative correlation between uptake rate and endogenous nitrate concentrations.

Approximately 20% reduction in lettuce nitrate concentration was obtained when the nutrient treatment was reduced from 200ppm to 150ppm nitrate-nitrogen. Reducing the nutrient medium concentration from 150ppm to 100ppm led to a further 25% decrease in tissue nitrate concentration (figure 2.5). These results are in agreement with a previous hydroponic study,

which was concerned with producing lettuce of low nitrate content by reducing the nitrogen supply (Andersen and Nielsen, 1992). In the study by Andersen and Nielsen, (1992), lettuce, was grown hydroponically and supplied with four nutrient treatments. Lettuce supplied with treatment A had free access to nutrients in a maintenance solution. In contrast, lettuce supplied with treatments B, C and D had reduced nutrient rates at 90%, 80% and 65% of the maintenance solution. The findings of this investigation illustrated that nitrate in the tissue of lettuce plants on a reduced supply of nutrients was significantly lower than that of plants supplied with the maintenance solution.

Reducing the nitrate-nitrogen supply of lettuce had a lesser effect on the mean head weight of lettuce then on tissue nitrate concentration (figure 2.6). The mean head weight of the lettuce was not significantly reduced when the nitrogen supply was decreased from 200ppm to 150ppm. However, plants supplied with 100ppm nitrate-nitrogen, the lowest nutrient rate, experienced a significant reduction in mean head weight ($p=0.01$). The intermediate nutrient rate produced lettuce of similar mean head weight to those supplied with the highest nutrient rate. The results of this study indicate that the intermediate nutrient rate may be sufficient to produce a crop of similar yield to a crop supplied with the highest nitrate-nitrogen rate. Reducing the nutrient rate from 200ppm to 150ppm, while not significantly affecting yield, did significantly reduce tissue nitrate concentration ($p=0.001$).

The results from this study show that it was possible to significantly reduce nitrate levels in hydroponic lettuce without significantly affecting yield by decreasing the nitrogen supply from 200ppm to 150ppm nitrate-nitrogen. In a study by Cardenas-Navarro *et al.*, (1999), the influence of environmental factors on nitrate accumulation in two lettuce cultivars was examined. They found a significant and concurrent reduction of both plant nitrate and water contents when a limited nitrogen regime was applied to plants. The results from the study by Cardenas-Navarro *et al.*, (1999), were interpreted as an effect of homeostatis for endogenous nitrate concentration. The results presented in this chapter are also in agreement with a previous study by McCall and Willumsen, (1998), involving lettuce grown in soil. In this study by McCall and Willumsen, (1998), reducing NO_3^-N availability from 260 to 200 kg N ha^{-1} significantly reduced nitrate content while fresh weight was unaffected. Further reducing NO_3^-N availability to 120 kg N ha^{-1} significantly reduced both nitrate content and fresh weight. Using the results from the present study in an attempt to achieve efficient management of nitrogen fertiliser use in a hydroponic system, it could be suggested that 150 ppm nitrate-nitrogen concentration is an acceptable rate to achieve high yield and low nitrate levels in hydroponic lettuce.

2.4 Variation in nitrate concentration of different lettuce cultivars raised hydroponically

2.4.1 Introduction

In soil, varying nitrate concentrations may occur in different lettuce types grown under the same conditions (Maynard *et al.*, 1976). These distinct genetic variations which affect nitrate concentration are especially important due to the wide range of lettuce varieties currently grown throughout the UK and Europe. In a study by Minotti (unpublished, cited in Maynard, 1976), the highest nitrate concentrations were found in crisphead varieties. Minotti found differences as great as fivefold when he screened fourteen cultivars under various field situations in soil. In a study by Reinink *et al.* (1987), nitrate content was measured in 135

genotypes of soil and hydroponically grown cultivated lettuce (*Lactuca sativa* L,) which included 61 butterhead types, 29 cos types, 19 crisp types, 19 cuttings types and 7 latin types. In addition, 21 genotypes of wild *Lactuca* species were included in the experiment by Reinink *et al.*, (1987). The modern butterhead cultivar, "Panvit", showed the highest mean nitrate concentrations (g/kg) in fresh matter of both hydroponically raised lettuce and soil grown lettuce. Hydroponically raised lettuce cv."Panvit" contained 3.5g/kg, (i.e. 3500ppm), nitrate and soil grown lettuce cv."Panvit" contained 4.1 g/kg, (4100ppm), nitrate compared to a known low nitrate accumulating butterhead cultivar, "Reichenauer", which contained 1.3g/kg, (1300ppm), nitrate and 1.7g/kg, (1700ppm), nitrate when raised in a hydroponic and soil environment, respectively. In an additional soil study by Schonbeck *et al.* (1991), the nitrate concentration in selected lettuce and endive cultivars was investigated. In the preliminary tests by Schonbeck *et al.*, (1991), four lettuce varieties were found to contain 25-50% less NO_3N compared to the greenhouse butterhead varieties "Diamante" and "Salina". The lettuce cultivars selected on the basis of their low nitrate concentrations for further evaluation were " Green Ice" (intermediate between leaf and iceberg), "Red Salad" (red leaf), "Patty" (butterhead), and Winter Destiny" (romaine).

Among the methods used to reduce nitrate concentrations in lettuce, one of the most potentially attractive methods is to breed low nitrate accumulating varieties. Previous research has shown large genotypic variation for nitrate concentration in soil grown lettuce (Reinink, 1992). The genotypic variation in nitrate content of lettuce, has been suggested to be quantitatively inherited. In a study by Reinink, (1992), the inheritance and characteristics associated with genotype × environment interaction for nitrate content were investigated for two lettuce genotypes. Both cultivars were outdoor butterhead types, previously selected for their low nitrate content under low light conditions. The study by Reinink, (1992) showed a close relationship between low nitrate content and poor growth under low light conditions. The occurrence of genotype × environment interactions can create difficulties in predicting the outcome of breeding programmes aimed at reducing nitrate content in lettuce. Breeding for genotypes that exhibit typically low nitrate concentrations, may re-enforce or weaken plant characteristics that are associated with low nitrate accumulators. For example, butterhead cultivars with extremely low nitrate content generally produce less fresh matter per plant than cultivars with a high nitrate content (Reinink *et al.*, 1987). However, from the genotypes already studied, there may be a large enough variation in nitrate concentration to allow a selection programme aimed at developing cultivars that are low nitrate accumulators, as well as being fast growing.

An important genotype × environment interaction has been established in previous studies by Reinink, (1991). In these studies, genotype × environment interactions were found and they were related to the large annual variation in light intensity. It was shown in a study by Schonbeck, Rivera, O'Brien, Ebinger and DeGregorio, (1991), that variety selection together with the adoption of specific cultural practises, can significantly reduce nitrate concentration in winter glasshouse lettuce. In the study by Schonbeck *et al.*, (1991), transferring hydroponic lettuce to a nutrient solution containing reduced NO_3 – N did not affect tissue nitrate or yield. However, lifting soil-grown lettuce and setting the roots in water 40 hours before harvest resulted in a 19% decrease in NO_3 – N. The possibility of incorporating a similar regime into the current growing procedure for lettuce in the UK may be limited. Despite this, the study by Schonbeck *et al.*, (1991), has illustrated that with suitable procedures such as a controlled growing environment and cultural selection, nitrate concentrations in lettuce can be successfully reduced.

Previous soil studies have investigated the genotypic variation in nitrate content of butterhead, romaine and iceberg lettuce types (Schonbeck *et al.*, 1991). Evidence from previous research suggests that differences in nitrate accumulation between cultivars may caused by the function of nitrate ion as an osmoticum, accumulated in the cell vacuole to maintain turgor pressure (Blom-Zandstra & Lampe, 1983; Steingrover, 1986). The theory implies a positive correlation can be expected between mean nitrate concentration and plant sap osmolarity in lettuce cultivars (Reinink, van Nes & Groenwold, 1994). Although a significant genotypic variation has been found for both osmolarity and nitrate content, no correlation has been found between both traits (Reinink, 1993). The non-specific osmotic nature of nitrate results in the ability of nitrate to be replaced by organic solutes and other inorganic anions. It has been suggested that variation in nitrate concentration between lettuce cultivars is caused by cultivar specific accumulation of sugars, which could replace nitrate as an osmoticum (Behr & Wiebe, 1992). Research conducted to investigate the role of nitrate in plant metabolism and photosynthetic activity support this claim, since lettuce cultivars with the highest sugar content also have significantly higher photosynthetic activity. There is a close correlation between sugars and nitrate, with an r^2 value of 0.99, and photosynthetic rate and nitrate content, with a r^2 value of 0.79. The results from the studies by Behr and Wiebe, (1992), suggest that nitrate, acting as an osmoticum, is replaced by the sugars which are produced during increased rates of photosynthesis.

Hydroponic systems have also been used to determine genotypic variation in lettuce, but the inclusion of additional continental lettuce types in these studies has not been found. In the third study into plant nitrate variability using hydroponic growing systems, variation in nitrate content of both butterhead and continental lettuce cultivars was examined. Nitrate variability within the butterhead cultivar "Rachel" and plant nitrate variability within the continental cultivars "Miami" and "Krizabri" was examined in an NFT system.

2.4.2 Materials and Methods

Three varieties of lettuce "Miami" (lollo bionda), "Krizabri" (oak leaf) and "Rachel" (butterhead) were raised in a hydroponic system at HRI, Stockbridge House between 6 April and 31 April 1999. The lettuce varieties were supplied with a standard lettuce feed containing approximately 190ppm nitrate-nitrogen (Page 1, appendix). The varieties were replicated in three channels and each channel contained twelve plants. Changes in the nitrate concentration of the nutrient solution were estimated by analysis of solution sampled at twice weekly intervals throughout the growth period. The pH, EC and flow rate of the nutrient solution were maintained between 5-6.5, 1.63-2.28 mS cm^{-1} and at approximately 5.5 l/min (Pages 7 to 8, appendix). Ten heads from each channel were harvested on 31 April 1999. The untrimmed heads were frozen prior to analysis for nitrate content as described in chapter 5. The nitrate content of the lettuce heads was determined using three analytical methods detailed as in chapter 4. For the purposes of this chapter, the nitrate results obtained by analysis using the nitrate probe are presented.

Plate 2 Continental lettuce, "Miami", a Lollo Bionda cultivar.

Plate 3 Continental lettuce, "Krizabri", an Oak Leaf cultivar.

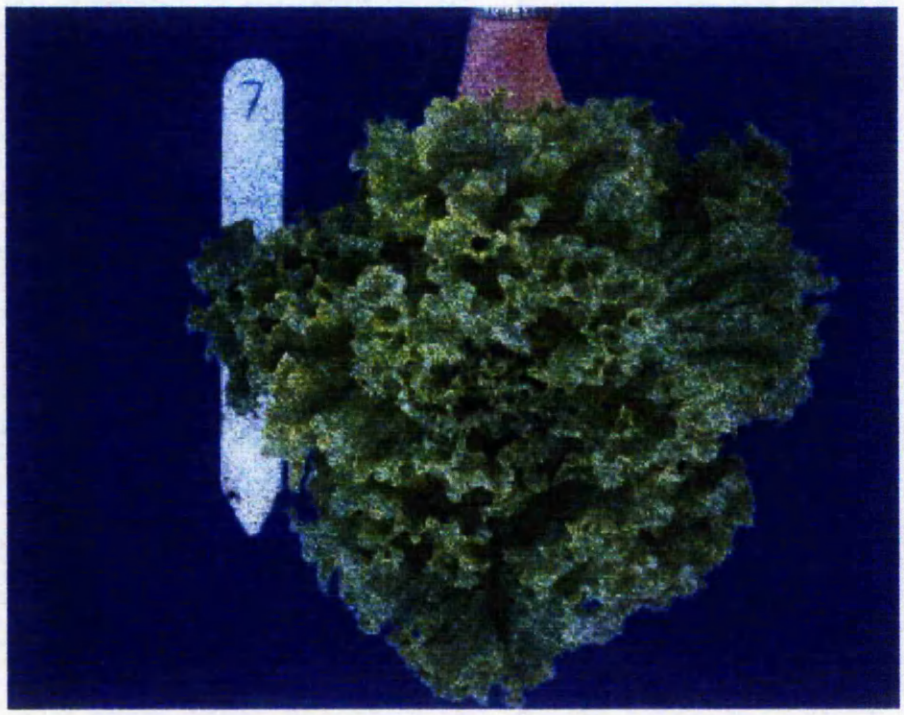


Plate 4 Butterhead lettuce, "Rachel", raised in NFT trial, HRI Stockbridge House.

Plate 5 Evidence of tipburn/stunted growth in NFT Lettuce Cultivar trial, at HRI Stockbridge House.

2.4.3 Results

The environmental results obtained for the growing period from 6 April to 31 April 1999 are given in the appendix, pages 7 to 8, together with pH, EC and flow rate of the nutrient solution supplied to the hydroponic lettuce. Figures 2.8 and 2.9 show mean nitrate concentration and mean fresh head weight, respectively, of lettuce culitvars raised in nutrient film technique. Vertical bars represent the standard error of the means. Nitrate concentrations of the nutrient solution during the period 16 to 30 April 1999 are given in figure 2.10.

During the growing period a pump failure resulted in temporary interruption of the nutrient supply to the plants. The cultivar most affected by the reduced supply of nutrients was Rachel. Plants showing signs of stunted development were omitted from the sampling procedure. The number of plants sampled at harvest from the cultivar "Rachel" was therefore reduced to twenty heads. In addition, there was evidence of tipburn on some plants (plate 5). The plants most badly affected by tipburn were omitted from the sampling procedure.

pH, flow rate and EC were set at approximately 6.0, 5-5.5 l/min and 2.2 respectively. The nitrate concentration of the nutrient medium was set to approximately 190ppm nitrate-nitrogen. The final nitrate concentration in the tanks was within the range of 180ppm to 200ppm (figure 2.10). The environmental data, appendix page 8, relating to the period 16 to 30 April 1999, shows mean daily temperature ranged from 9.8 °C to 19.8 °C, mean humidity values ranged from 53.9% to 97.5% and weekly irradiation values between 76.26 and 120.10 MJ/m^2.

Figure 2.8 Mean nitrate concentration of hydroponically raised lettuce cultivars Miami, Krizabri and Rachel at harvest. Experiment conducted at HRI Stockbridge House. Results attained following statistical unbalanced analysis of variance.
LSD = 235.57 at p = 0.001, n = 30, except Rachel (n=19)
LSD = 181.58 at p = 0.01
LSD = 136.73 at p = 0.05

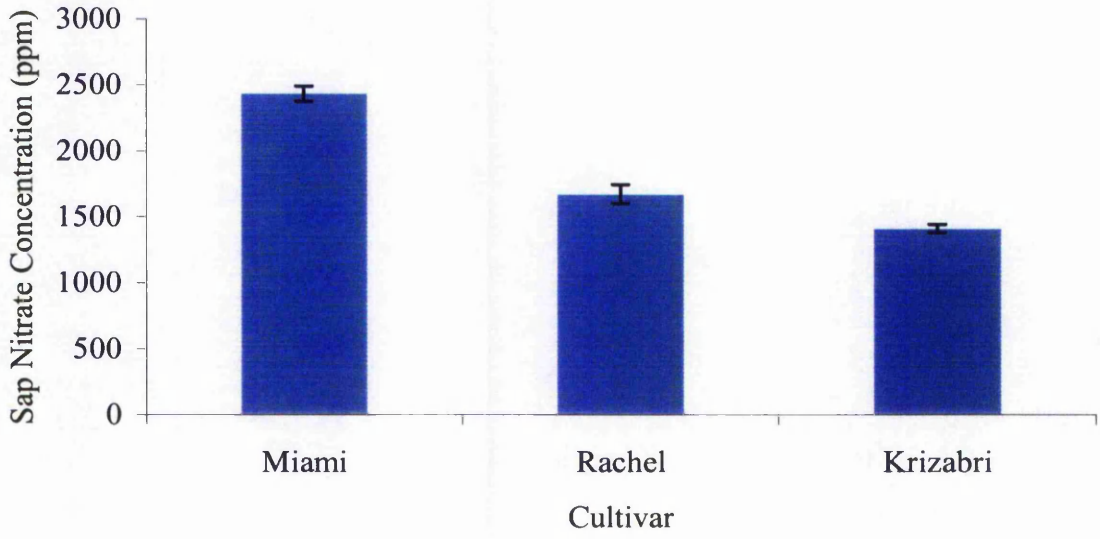


Figure 2.9 Mean head fresh weights of lettuce cultivars Miami, Rachel and Krizabri, raised in a NFT system at HRI Stockbridge House.
LSD = 61.09, p = 0.001, n = 30, except Rachel (n=19)
LSD = 46.08, p = 0.01
LSD = 34.21, p = 0.05

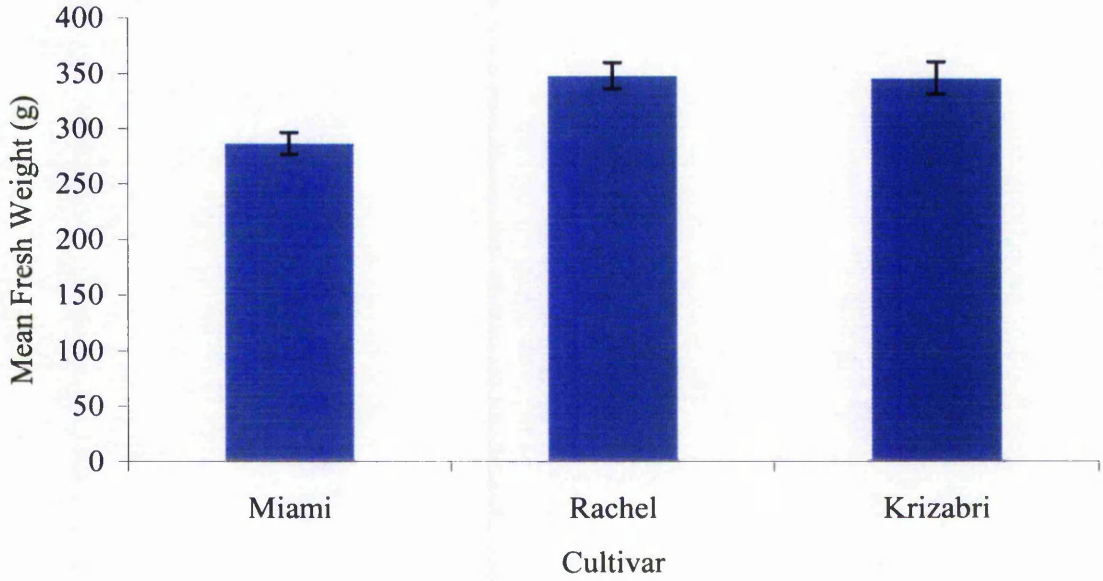
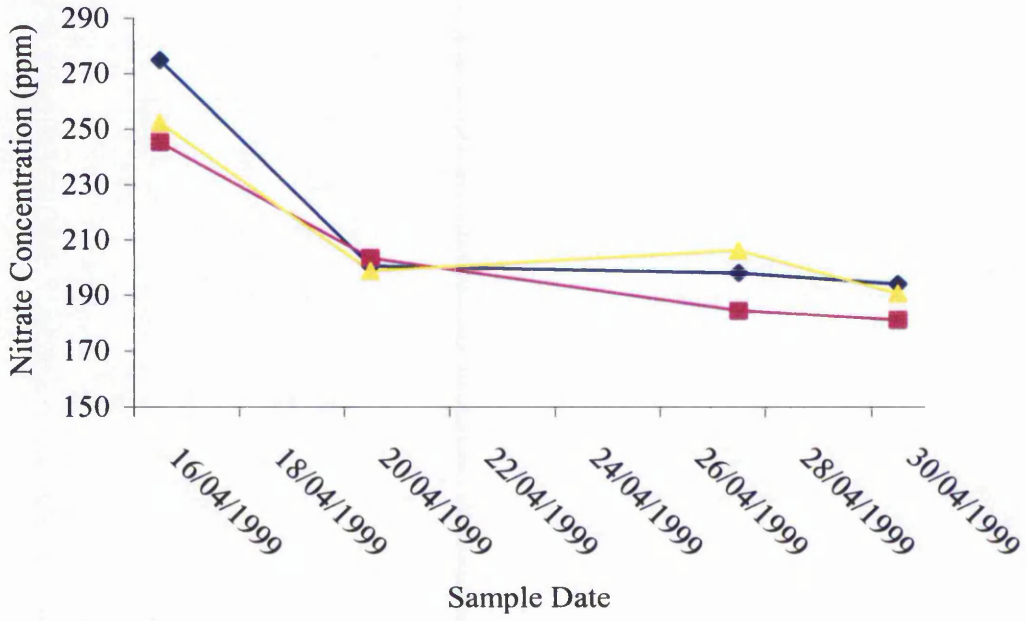


Figure 2.10 Mean nitrate concentration of nutrient solution used to supply lettuce cultivars Miami, Krizabri and Rachel in a NFT system at HRI Stockbridge House, during growing period 16 April to 30April 1999.



Statistical analysis of variance showed that there was a highly significant difference in nitrate concentration between the three lettuce cultivars used in the study ($p=0.001$, figure 2.8). The mean sap nitrate concentration of the continental variety "Miami", at 2437ppm, was significantly higher than both the butterhead variety "Rachel" and the continental variety "Krizabri" ($p=0.001$). The lowest mean nitrate concentration was observed in "Krizabri", at 1409ppm. "Rachel" had an intermediate mean nitrate concentration of 1668ppm. The butterhead variety, "Rachel", had significantly higher tissue nitrate concentration compared to continental variety, "Krizabri", and significantly lower tissue nitrate concentration compared to continental variety "Miami" ($p=0.001$).

Statistical analysis also illustrated a significant variation in mean head fresh weight between the three lettuce cultivars ($p=0.001$, figure 2.9). The lowest mean head weight was obtained in the continental variety "Miami". The continental cultivar "Krizabri" and the butterhead variety "Rachel" produced similar head weights. The mean head weight obtained by the continental variety "Miami", at 286.3g, was significantly lower than the mean head weight of both the butterhead variety "Rachel", at 348 g, and the continental variety "Krizabri", at 345.6 g. The mean fresh weight of the cultivar "Miami" at harvest was approximately 20% lower compared to the mean fresh weights of Rachel and Krizabri.

2.4.4 Discussion

Results from previous studies have suggested that differences in nitrate concentration in lettuce may be directly related to nitrate uptake or assimilation rates (Behr and Wiebe, 1992). It is difficult to determine nitrate uptake rates from the data obtained from analysis of the nutrient media in this investigation since during the growing period, several leaks occurred within the recirculating system. This led to a decrease in volume of the nutrient solution in all tanks, and may account for the noticeable increase in nitrate concentration of the nutrient solution from the start of the growing period until 16 April (data not included). An increase in tank volume, on 23 April, indicates the addition of nutrient solution to the tanks (appendix pages 7 to 8). During this period, the nitrate concentration in the nutrient media decreased noticeably. The decrease in nitrate concentration of the nutrient solution can be attributed to addition of nutrient media of a lower nitrate concentration than the existing tank solution. The values obtained for nitrate concentration of the nutrient solution from the period 6 April to 16 April and on 23 April are therefore deemed unreliable for use in determination of nitrate uptake rates and have not been included in this chapter. However, from the limited data available nitrate uptake appeared to be greatest in the lettuce cultivar "Miami", while the cultivars "Krizabri" and "Rachel" had apparently lower nitrate uptake during the same growth period (figure 2.10). The nitrate uptake results agree with the soil studies of Behr and Wiebe, (1992), whose findings suggested that differences in nitrate content of lettuce varieties may be due in part to cultivar differences in nitrate uptake.

A wide variation in the nitrate concentration of different lettuce cultivars have been noted during previous soil studies (Reinink *et al.*, 1987). In an additional study by Reinink and Eenink, (1988), genotypic differences in nitrate accumulation in the shoots and roots of lettuce were observed. In previously unpublished studies at HRI Stockbridge House, differences in mean nitrate concentration were found between butterhead and continental lettuce varieties raised in soil. In the studies conducted at HRI, Stockbridge House, lettuce varieties were grown under the same cultural and nutritional conditions. In general, the continental types exhibited higher nitrate concentrations than butterhead varieties. The differences in nitrate concentration appeared to be inversely related to the final yield of the lettuce cultivars. From these experiments it has been suggested that part of the genetic variation may be accounted for by the different amounts of nitrogen required for biomass production (Burns, 1999). In the studies conducted at HRI Stockbridge House, the butterhead cultivars "Flandria", "Vagus" and "Benjamin" had lower mean plant nitrate concentrations than the continental cultivars "Loretta". "Brigida", "Pinokkio" and "Charita". The cultivar with the highest mean plant nitrate concentration, at 5007 ppm, was recorded in the continental variety "Loretta". It should be noted that the nitrate concentration results obtained from the studies conducted at HRI Stockbridge House are, in general, considerably higher than those presented in this chapter. This may be explained by the use of different extraction methods throughout the HRI Stockbridge House studies compared to the methods employed in the current study, and is discussed later in the thesis.

The results from the hydroponic variety trial show that the continental variety "Miami" contained significantly higher mean nitrate concentrations than the butterhead "Rachel" and the continental cultivar "Krizabri" ($p=0.001$). Mean head weight was significantly lower in "Miami" compared to "Rachel" and "Krizabri". "Krizabri" resulted in significantly lower mean nitrate concentration and higher mean head weight compared to "Miami" ($p=0.01$). This result is in agreement with two previous soil variety trials carried out at HRI Stockbridge House, showing significant variation in mean nitrate concentrations and head

weights in different lettuce cultivars. Significantly larger head weights were observed within "Krizabri" and the four butterhead varieties, "Tziganne", "Scott", "Wendel" and "Gaby", in the study at HRI Stockbridge House when compared to the lower head weight and higher nitrate concentration of "Miami" (Anon, 1998f).

Differences in mean nitrate concentration observed between the lettuce cultivars (figure 2.8) may be an indirect effect of head morphology (Lee, 1999). Cultivar variation existed in the structure of leaves forming the lettuce head, particularly between butterhead and continental varieties (Plate 6). Leaf orientation and shape may determine the area of leaf that is exposed to irradiation and subsequent assimilation of nitrate within the leaf. The arrangement of leaves about the lettuce head may also determine the amount of shading a leaf is subjected to, and the quantity of light that the lower leaves receive. The reduction in light levels resulting from a shading effect could lead to nitrate accumulation. A quantitative study of leaf shape, orientation and number was not undertaken as part of this study. However, the shape and orientation of leaves in the continental varieties "Miami" and "Krizabri" were visibly different to the butterhead "Rachel" (Plates 2, 3 and 4, respectively). Variations in leaf form and arrangement between the continental varieties "Miami" and "Krizabri" also existed (Plate 6). The differences in head morphology between continental and butterhead varieties and associated shading effects may partly explain the significantly higher mean nitrate concentrations observed in "Miami". It is possible that the differences in leaf shape and orientation created increased levels of shading during the growing period of "Miami" compared to the butterhead variety "Rachel". However, no direct relationship has previously been established between the morphology of lettuce heads and uptake or assimilation of nitrate (Behr and Wiebe, 1992).

The continental variety "Krizabri", although morphologically different to the butterhead "Rachel", did not exhibit significantly different mean nitrate concentrations. It was assumed in this study that since all three cultivars were harvested simultaneously, the physiological maturity of the different lettuce cultivars was similar. However, there may be differences in the growth pattern of individual lettuce cultivars. It is possible that part of the variations in nitrate content between the cultivars were due to differences in growth patterns and physiological maturity of the lettuce. For example, plant maturity in different lettuce cultivars at harvest may influence the number of leaves per head (Blom-Zandstra and Eenink, 1986). Cultivar differences in the number of leaves per head would influence photosynthesis and nitrate assimilation, as well as fresh weight. The effect of leaf number on the process of nitrate assimilation could subsequently influence nitrate concentrations in the whole lettuce. In addition, nitrate concentrations in lettuce are known to be affected by leaf age, with older leaves typically containing higher concentrations of nitrate than younger, less mature leaves (Poulsen, Sorensen and Johansen, 1994). It is possible that significantly lower nitrate concentrations in "Krizabri" compared to "Miami" were due to a combination of physiological differences existing at the time of harvest. It is possible that "Krizabri" was less mature at harvest and contained fewer mature leaves per head compared to "Miami". A high proportion of immature leaves per head in "Krizabri" could explain the significantly lower mean nitrate concentrations in this continental cultivar. Although there is no evidence of an earlier maturity date in the continental variety "Miami" compared to the variety "Krizabri", the relative maturity of each cultivar at harvest should be considered in a discussion of mean head nitrate concentrations.

The results suggest that cultivar differences in nitrate concentration between "Miami", "Krizabri" and "Rachel" may be attributed to a combination of morphological differences,

physiological variation resulting from relative maturity, and cultivar variation in nitrate uptake rate. A tentative suggestion, with reference to previous investigations, can be made relating the observed variation in plant nitrate concentration to cultivar differences in osmolarity. However, such a statement is speculative and requires further investigation. From the results of the study presented in this chapter it would seem that the continental variety "Krizabri" is the most attractive cultivar in terms of mean head eight and mean nitrate concentration, if a low nitrate concentration is the most important factor. "Krizabri" exhibited significantly lower mean nitrate concentration than both "Miami" and "Rachel", together with a high mean head weight.

Chapter 3

3.0 Factors affecting plant to plant nitrate variability within a protected, soil-raised lettuce crop.

3.1 Introduction

As stated previously in chapter one, nitrate levels within protected lettuce crops are subject to wide variations despite being raised in similar soil and environmental conditions (Mengel and Kirkby, 1987). It is important to examine all of the factors known to influence nitrate concentration in lettuce, in order to minimise variation in nitrate concentrations and ensure crop uniformity is achieved. Since both the level of nitrogen fertiliser and light intensity have been established as factors influencing nitrate concentration in glasshouse lettuce, they were incorporated as variables in the study to determine plant to plant variability in protected lettuce. The variability within a winter lettuce crop and summer lettuce crop raised under different light regimes and nitrate-nitrogen rates was examined. Although similar studies have been conducted concerning the influence of light and soil nitrogen levels on glasshouse lettuce, the purpose of this study was to confirm these findings by carrying out glasshouse investigations specifically under UK conditions. In addition to studying the effect of the interaction between soil nitrogen and light level on lettuce nitrate concentration, the influence of planting density was also investigated. A summary of the plant density results are given in the appendix page 30, and are referred to in the general discussion (chapter 6).

3.2 Materials and Methods

3.2.1 Soil investigation using winter lettuce

A winter lettuce crop (cv Flandria) was transplanted into sandy-loam soil, and grown between the period November 1997 to March 1998 at HRI, Stockbridge House. The trial was designed to provide varying growth conditions under glass with a range of light, soil nitrogen and planting regimes. The trial was replicated twice, with each trial consisting of three fully randomised blocks. Light conditions were simulated to achieve three light regimes at approximately 0% estimated light reduction, 10% and 20% estimated light reduction. The reduced light conditions were achieved using multiple layered insect netting, supported 30cm above the crop, until approximate conditions of "10%" and "20%" light reduction were achieved. The "0%" light reduction condition was achieved without the use of insect meshing and represented ambient light conditions within the glasshouse. Ambient light levels within the glasshouse were recorded using a solarimeter and shown in figure 3.3. The three soil nitrate-nitrogen regimes were achieved by incorporating varying rates of ammonium nitrate fertiliser into the soil at the pre-planting stage. The soil was flooded beforehand in order to remove nitrate. The three soil conditions were set at 50 mg/l, 100 mg/l and 150 mg/l nitrate-nitrogen. Planting density was also varied to give three planting treatments at 16 plants/m^2, 25 plants/m^2 and 33 plants/m^2.

The three variables; light, soil nitrate and planting density, were represented as treatments consisting of all possible combinations. The "0%" light reduction treatments were supplied with three ammonium nitrate fertiliser rates to give soil nitrate concentrations at 50ppm, 100ppm and 150ppm nitrate-nitrogen. The three treatments were given the abbreviations S1L1P2, S2L1P2, and S3L1P2 to represent treatments of 50ppm soil nitrate-nitrogen with

"0%" light reduction, 100ppm soil nitrate-nitrogen with "0%" light reduction and 150ppm soil nitrate-nitrogen with "0%" light reduction, all at a planting density of 25 plants/m^2. The "10%" light reduction treatments were supplied with ammonium nitrate fertiliser at rates that gave soil nitrate concentrations at 50ppm, 100ppm and 150ppm nitrate-nitrogen. The three treatments were given the abbreviations S1L2P2, S2L2P2, and S3L2P2 to represent the treatments of 50ppm soil nitrate-nitrogen with "10%" light reduction, 100ppm soil nitrate-nitrogen with "10%" light reduction and 150ppm soil nitrate-nitrogen with "10%" light reduction. The "20%" light reduction treatments were supplied with three ammonium nitrate fertiliser rates to give soil conditions of 50ppm, 100ppm and 150ppm nitrate-nitrogen. The three treatments were given the abbreviations S1L3P2, S2L3P2 and S3L3P2, which represented the treatment at 50ppm soil nitrate-nitrogen with approximately "20%" light reduction, 100ppm soil nitrate-nitrogen with approximately "20%" light reduction and 150ppm soil nitrate-nitrogen with approximately "20%" light reduction.

Lettuce was sampled at a planting density of 25 plants/m^2 from replicated plots of each of the nine treatments on 16 March 1998. In total, ten lettuce heads were sampled from each treatment from plots exposed to different light and soil nitrogen levels. In addition to the ten lettuce heads sampled from the nine treatments at a planting density of 25 plants/m^2, ten heads were sampled from three treatments at a higher planting density. The three additional treatments contained lettuce at a planting density of 33 plants/m^2 under "0%" light reduction conditions. The three treatments were given ammonium nitrate fertiliser to provide soil nitrate-nitrogen conditions at 50ppm, 100ppm and 150ppm. The treatments were given the abbreviations S1L1P3, S2L1P3 and S3L1P3 to represent 50ppm soil nitrate-nitrogen with "0%" light reduction and a planting density of 33 plants/m^2, 100ppm soil nitrate-nitrogen with "0%" light reduction and a planting density of 33 plants/m^2 and 150ppm soil nitrate-nitrogen with "0%" light reduction and a planting density of 33 plants/m^2.

A total of 120 lettuce heads were sampled from the winter lettuce trial. The lettuce were weighed and individually bagged, and stored at -20°C prior to nitrate determination as outlined in chapter two and as detailed in chapters four and five.

Summary list of nine treatments sampled during the winter investigation at a planting density of 25 plants/m^2

- estimated 0% light reduction and 50 ppm soil nitrate-nitrogen (S1L1).
- estimated 0% light reduction and 100 ppm soil nitrate-nitrogen (S2L1).
- estimated 0% light reduction and 150 ppm soil nitrate-nitrogen (S3L1).
- estimated 10% light reduction and 50 ppm soil nitrate-nitrogen (S1L2).
- estimated 10% light reduction and 100 ppm soil nitrate-nitrogen (S2L2).
- estimated 10% light reduction and 150 ppm soil nitrate-nitrogen (S3L2).
- estimated 20% light reduction and 50 ppm soil nitrate-nitrogen (S1L3).
- estimated 20% light reduction and 100 ppm soil nitrate-nitrogen (S2L3).
- estimated 20% light reduction and 150 ppm soil nitrate-nitrogen (S3L3).

Summary list of three treatments sampled during the winter investigation at a planting density of 33 plants/m^2

- estimated 0% light reduction and 50 ppm soil nitrate-nitrogen (S1L1).
- estimated 0% light reduction and 100 ppm soil nitrate-nitrogen (S2L1).

- estimated 0% light reduction and 150 ppm soil nitrate-nitrogen (S3L1).

3.2.2 Soil investigation using summer lettuce.

The second investigation into plant variability in a protected lettuce crop involved a summer lettuce crop (cv Rachel), that was transplanted and grown in sandy-loam soil from June to July 1998. The summer crop was harvested on 28 July 1998.

The summer trial was identical in format to the winter trial. In the summer trial the light regimes, soil nitrate-nitrogen concentrations and planting densities mirrored those of the winter trial. As in the winter investigation, ten lettuce heads were harvested from the nine treatments which are summarised below. Lettuce was harvested from the summer trial on 28 July, 1998. Each untrimmed head was weighed, bagged and stored individually at -20°C. Nitrate analysis was carried out on whole lettuce heads following the procedures that are outlined in chapter two and detailed in chapters 4 and 5.

Details of the nine treatments sampled during the summer investigation follow the same criteria as the winter treatments at a planting density of 25 plants/m^2 shown on page 54.

3.3 Results

3.3.1 Soil investigation using winter lettuce

A summary of the environmental data relating to the winter crop growing period showing weekly mean values relating to temperature, humidity and irradiation levels for the period November 1997 to March 1998 is given in the appendix, page 9. Mean nitrate concentrations and mean fresh head weight of winter lettuce for this growing period are presented in figures 3.1 and 3.2 respectively. Vertical bars represent the standard error of the means. The nitrate results presented in figure 3.1 were obtained following nitrate analysis using a nitrate probe. Irradiation levels within the glasshouse throughout the winter growing period are given in figure 3.3.

Figure 3.1 Mean nitrate concentration of winter lettuce crop cv. Flandria, raised in soil under glasshouse conditions at HRI Stockbridge House during November to March 1998, following nitrate probe analysis.
LSD = 426.56, (p=0.001) LSD = 337.41, (p=0.01) LSD = 254, (p=0.05), at n=10

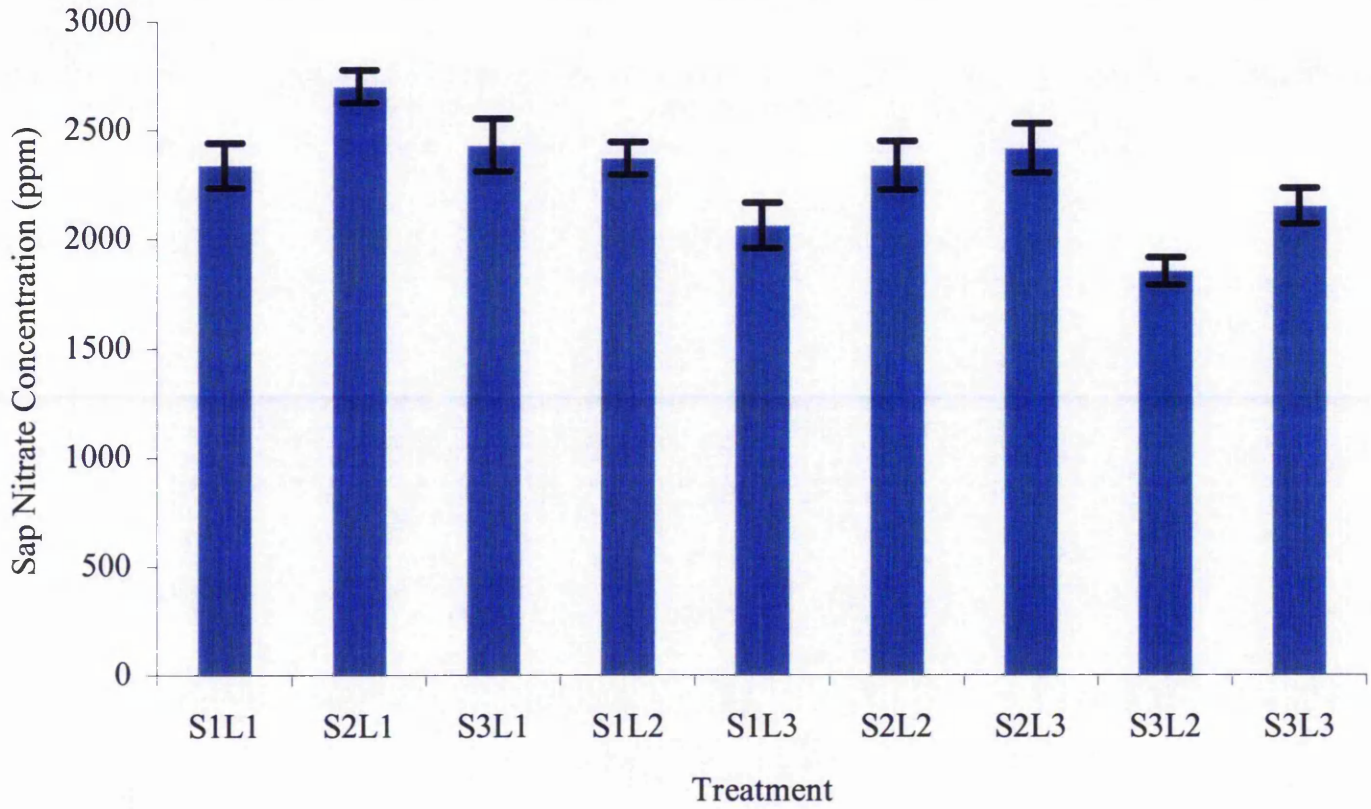


Figure 3.2 Mean fresh weights of winter lettuce cv Flandria, raised in soil under glasshouse conditions during November to March 1999, at HRI Stockbridge House.

LSD = 10.06 (p=0.5)
LSD = 7.81 (p=0.6) , n=10

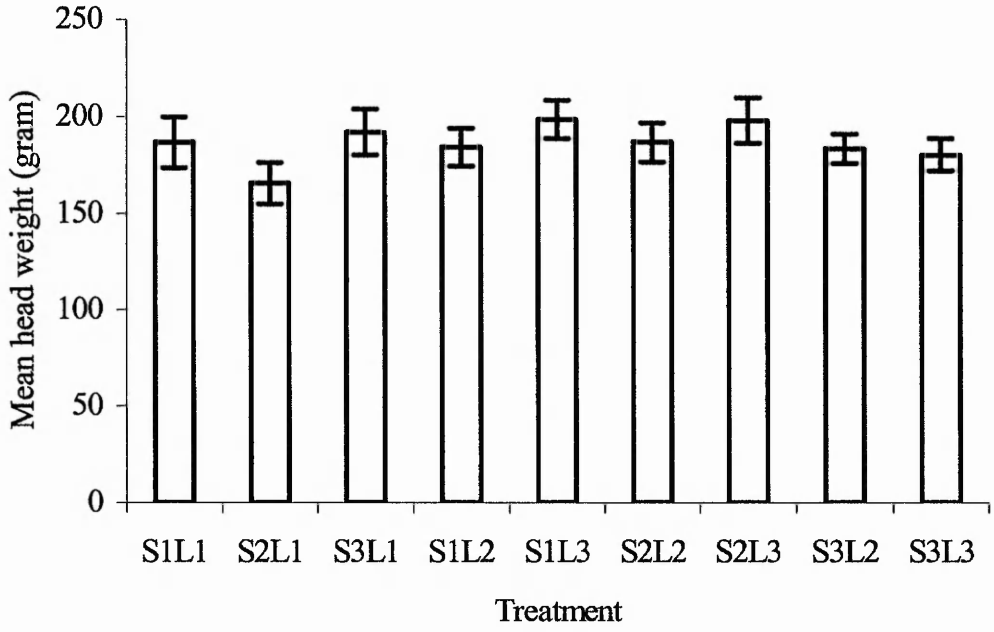


Figure 3.3 Irradiation levels throughout growing period of winter lettuce crop cv Flandria, from November to March 1998, under glasshouse conditions at HRI Stockbridge House.

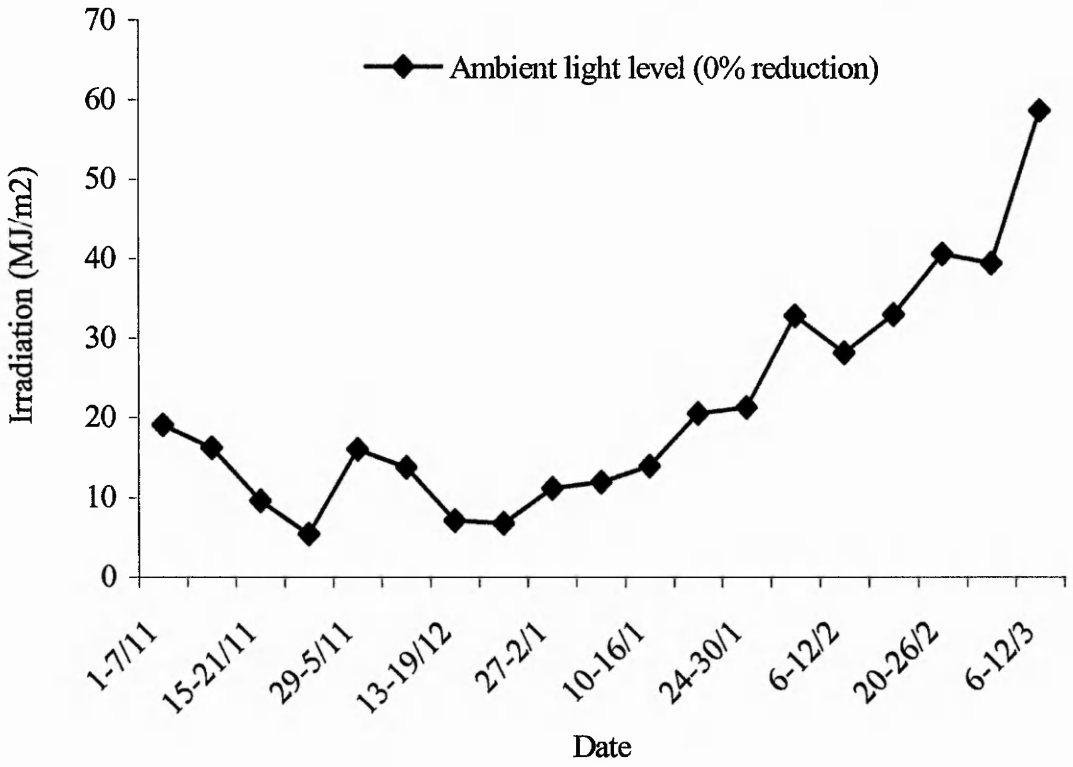


Table 3.1

Summary table of mean nitrate concentration (ppm) of winter lettuce cv Flandria, as shown in figure 3.1.

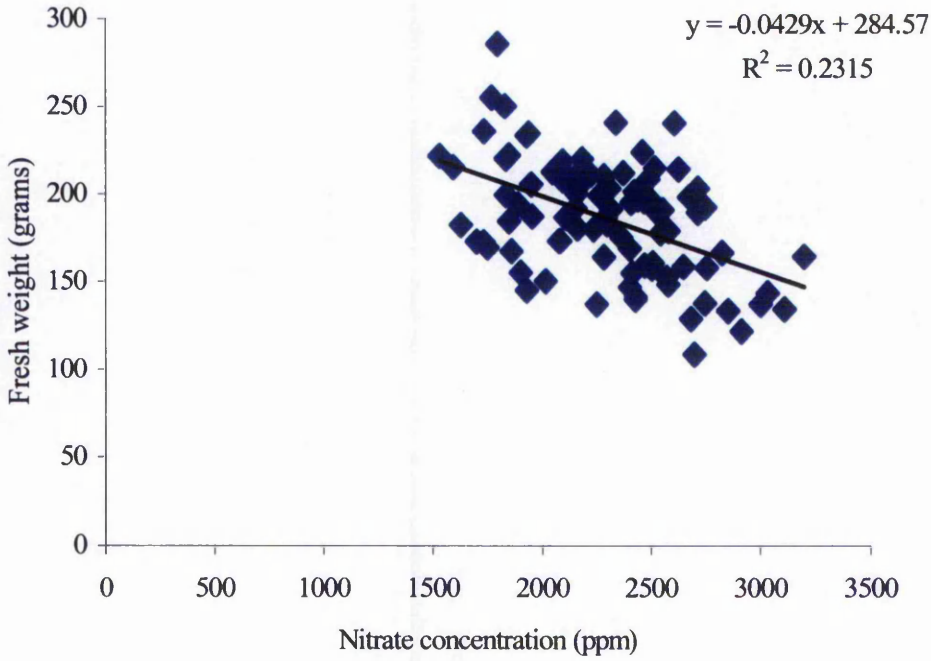
	L1 "0%" light reduction	*L2* "10%" light reduction	*L3* "20%" light reduction
S1 50ppm Nitrate Nitrogen	**2339.40 ppm (2dp)**	**2370.02 ppm (2dp)**	**2060.10 ppm (2dp)**
S2 100ppm Nitrate Nitrogen	**2702.27 ppm (2dp)**	**2336.80 ppm (2dp)**	**2415.93 ppm (2dp)**
S3 150ppm Nitrate Nitrogen	**2430.66 ppm (2dp)**	**1852.41 ppm (2dp)**	**2150.31 ppm (2dp)**

The winter lettuce trial at harvest showed little variability in head quality or size. In general, the crop appeared to have a high level of uniformity. There were no signs of disease that may have affected the development, quality, or indirectly influence the nitrate concentration of the lettuce.

Mean nitrate concentrations ranged from 1852 ppm to 2702 ppm nitrate in winter lettuce cv Flandria (figure.3.1). The highest mean nitrate concentration, at 2702ppm, was obtained from lettuce treated with 100ppm soil nitrate-nitrogen and "0%" light reduction. The mean nitrate results were subjected to statistical analysis of variance, table 7 in appendix, which illustrated a significant difference between nitrate concentration of lettuce sampled from S2L1 treatment and nitrate concentration of lettuce sampled from each of the remaining eight treatments (p=0.05). The lowest mean nitrate concentration, at 1852ppm, was observed in lettuce treated with 150ppm soil nitrate-nitrogen and with a "10%" reduction in natural light levels (treatment S3L2). Lettuce sampled from treatment S3L2 was significantly lower in mean nitrate concentration compared to lettuce sampled from all the other treatments (p=0.05), except in lettuce supplied with 50ppm soil nitrate-nitrogen and "10%" light reduction. Significantly higher nitrate concentrations were found in lettuce supplied with 150ppm soil nitrate and "0%" light reduction compared to lettuce supplied with equal soil nitrate-nitrogen treatment and 10% reduced light (p=0.001) and 20% reduced light (p=0.05, figure 3.4). Significant differences were observed between nitrate concentrations of lettuce raised at "0%" reduced irradiation and lettuce raised at "20%" reduced irradiation within soil nitrate-nitrogen treatments at 50ppm, 100ppm, and 150ppm (p=0.05).

Mean fresh weight of lettuce sampled from the nine treatments ranged from 165.3 gram to 198.4 gram (figure 3.2). The highest mean fresh weight occurred in lettuce treated with 50ppm soil nitrate-nitrogen and a "20%" reduction in natural light levels (S1L3 treatment). The lowest mean fresh weight was found to be in lettuce treated with 100ppm soil nitrate and "0%" light reduction (treatment S2L1). Statistical analysis of variance using the fresh weight of lettuce showed no significant differences between the lettuce sampled from the nine treatments. No correlation was found between mean fresh weight and mean nitrate concentration of lettuce sampled from the nine treatments (figure 3.4). Results from regression analysis are given in the appendix, page 26.

Figure 3.4 Relationship between nitrate concentration and fresh weights of winter lettuce raised under nine treatment conditions at varying light levels and soil nitrogen concentrations.



3.3.2 Soil investigation using summer lettuce

Environmental data, including daily mean temperature, humidity and weekly irradiation levels, are shown in the appendix, page 10. Mean nitrate concentrations and mean fresh head weight of summer lettuce are presented in figures 3.5, and 3.6 respectively. Vertical bars represent the standard error of the means. The nitrate values presented in figure 3.5 were obtained using nitrate probe analysis. Estimated irradiation levels within the three light treatments throughout summer growing period are given in figure 3.7. Relationships between nitrate concentration and fresh weight of summer lettuce are shown in figures 3.8, 3.9 and 3.10.

Figure 3.5 Mean nitrate concentration of summer lettuce crop cv. Rachel, raised under glasshouse conditions at HRI Stockbridge House during period 1 July to 28 July, 1998. Analysis undertaken with ISE meter (nitrate probe analysis).

LSD = 701 (p=0.001)
LSD = 538.2 (p=0.01)
LSD = 403.9 (p=0.05) at n=9

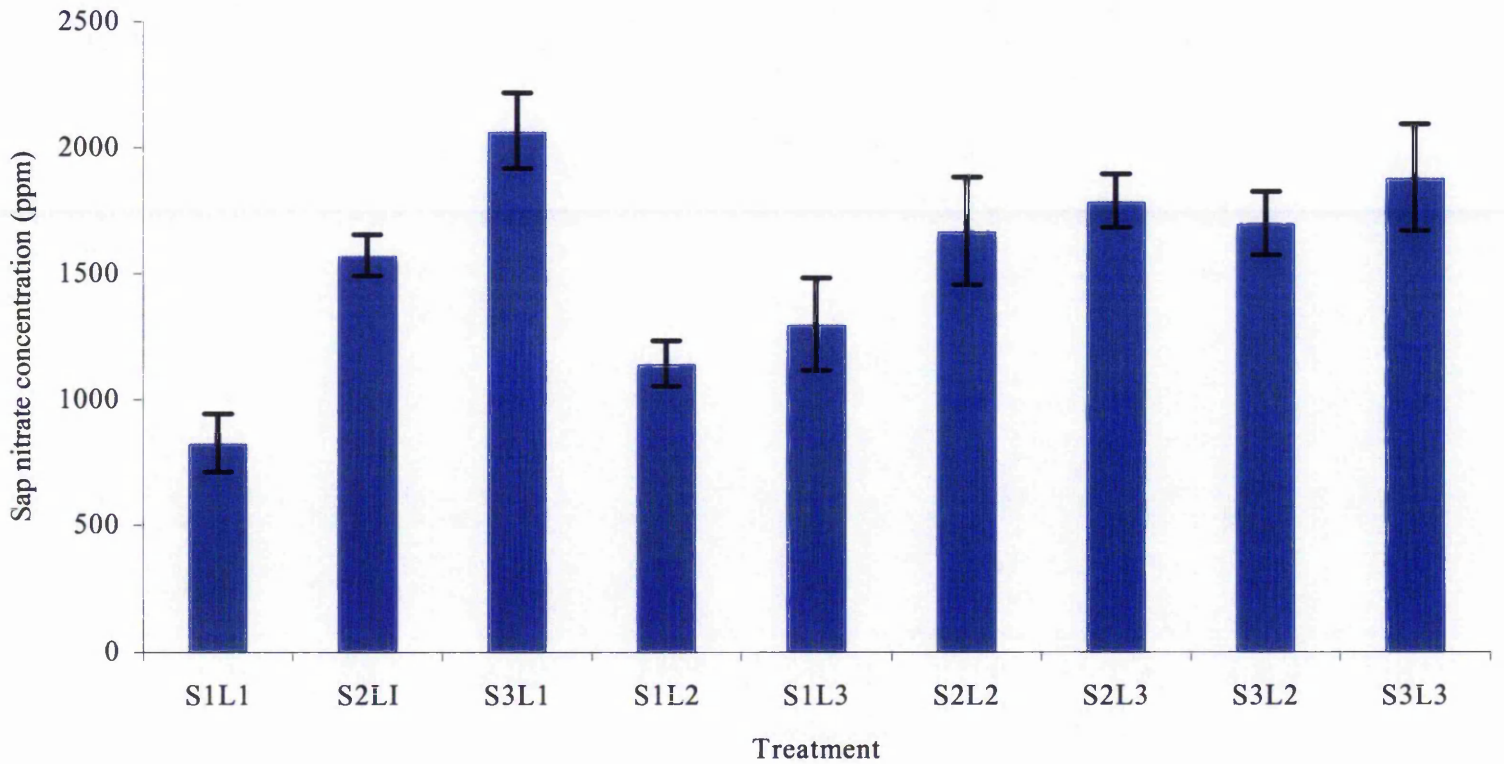


Figure 3.6 Mean fresh weights of summer lettuce cv. Rachel, grown under glasshouse conditions at HRI Stockbridge House (1 July to 28 July, 1998).

LSD = 64.47 (p=0.001
LSD = 49.69 (p=0.01)
LSD = 37.41 (p=0.05) at n=9

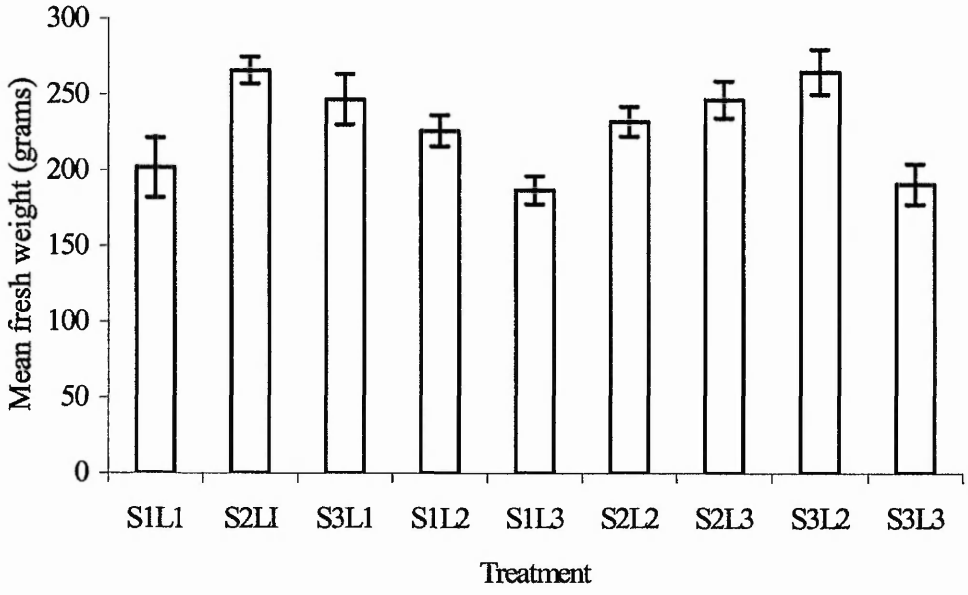


Figure 3.7 Estimated weekly Irradiation levels under glass at HRI Stockbridge House during growing period of summer lettuce crop cv. Rachel from 29 June to 28 July, 1998.

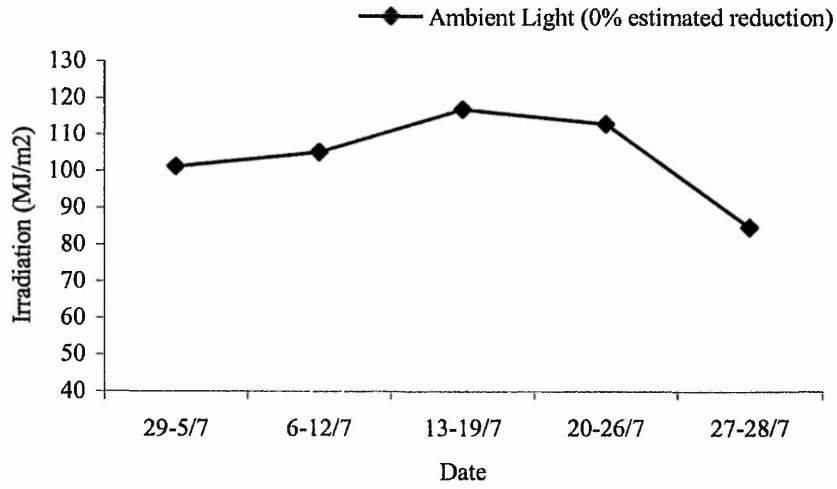


Table 3.2

Summary table of mean nitrate concentration (ppm) of summer lettuce cv Rachel, as shown in figure 3.5.

	L1 "0%" light reduction	*L2* "10%" light reduction	*L3* "20%" light reduction
S1 50ppm Nitrate Nitrogen	**825.36ppm (2s.f)**	**1142.30ppm (2s.f)**	**1297.97ppm (2s.f)**
S2 100ppm Nitrate Nitrogen	**1572.60ppm (2s.f)**	**1667.66ppm (2s.f)**	**1787.15ppm (2s.f)**
S3 150ppm Nitrate Nitrogen	**2065.85ppm (2s.f)**	**1698.48ppm (2s.f)**	**1878.35ppm (2s.f)**

In terms of appearance, the summer lettuce trial appeared was uniform. Mean nitrate concentrations ranged from 825ppm to 2066ppm in the summer lettuce crop (figure 3.5). The lowest mean nitrate concentration, at 825ppm, was obtained in lettuce grown in 50ppm soil nitrate-nitrogen and "100%" natural light levels (treatment S1L1). Statistical analysis of variance, table 8 in appendix illustrated a significant difference between the mean nitrate concentrations of lettuce from treatment S1L1 and the mean nitrate concentrations of lettuce within each treatment ($p=0.05$), with the exception of treatment S1L2. A significant difference existed between the mean nitrate concentration of lettuce from treatment S1L1 and the mean nitrate concentration of lettuce treated with 50ppm soil nitrate-nitrogen and a "20%" reduced light level (treatment S1L3, $p=0.05$).

The highest mean nitrate concentration, at 2066ppm, was obtained in lettuce treated with 150ppm soil nitrate-nitrogen and "0%" light reduction (treatment S3L1). The nitrate concentration of lettuce sampled from treatment S3L1 was significantly higher compared to the mean nitrate concentrations of lettuce sampled from treatments S1L1 and S2L1 ($p<0.000$). There was a significant increase in the nitrate concentration of lettuce when the nitrate-nitrogen treatment was increased from 50ppm to 100ppm within each of the three light treatments ($p=0.05$). There was a general trend of increasing nitrate concentration within lettuce raised at both 50ppm and 100ppm soil nitrogen treatments when the light levels were reduced from "0%" reduction to "10%", and then to "20%". However, the differences between the treatments were not significant.

Mean fresh weight of summer lettuce ranged from 186.6 grams to 265.6 grams (figure 3.6). The lowest mean fresh weight, at 186.6 grams, was observed in lettuce grown in 50ppm soil nitrate-nitrogen and a "20%" light reduction (S1L3 treatment). Statistical analysis of mean fresh weight showed a highly significant variation between lettuce from the S1L3 treatment and lettuce from treatments S2L1 and S3L2 ($p=0.001$) with lower head weight in lettuce from treatment S1L3 compared to lettuce from treatments S2L1 and S3L2. A significantly lower mean fresh weight existed in lettuce sampled from S1L3 treatment compared to the mean fresh weight of lettuce from treatment S2L3 and S3L1 ($p=0.01$) and also S1L2 and S2L2 ($p=0.05$). The highest mean fresh weight, at 265.6 gram, was in lettuce treated with 100ppm soil nitrate-nitrogen and "100%" natural light levels (treatment S2L1). A highly significant difference existed between the mean fresh

weight of lettuce from S2L1 treatment and the mean fresh weight of lettuce from S1L1, S1L3 and S3L3 treatments ($p=0.001$). A significant variation was shown between the mean fresh weights of lettuce sampled from treatment S2L1 and the mean fresh weight of lettuce from treatment S1L2 ($p=0.05$). No correlation was found between mean nitrate concentration and mean fresh weight of the summer lettuce sampled from the nine treatments (figures 3.8, 3.9 and 3.10). Results from regression analysis are shown in the appendix, pages 27, 28 and 29.

Figure 3.8 Relationship between nitrate concentration and fresh weight of summer lettuce cv. Rachel grown at 50ppm soil nitrate at HRI Stockbridge House from 1 July to 28 July, 1998.

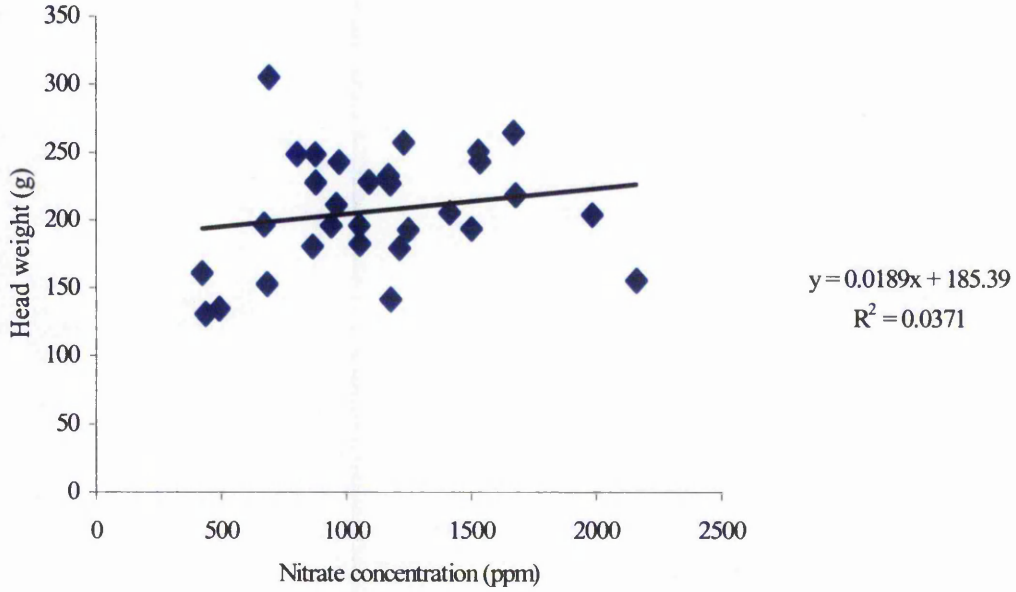


Figure 3.9 Relationship between nitrate concentration and fresh weight of summer lettuce cv. Rachel grown at 100ppm soil nitrate at HRI Stockbridge House from 1 July to 28 July, 1998.

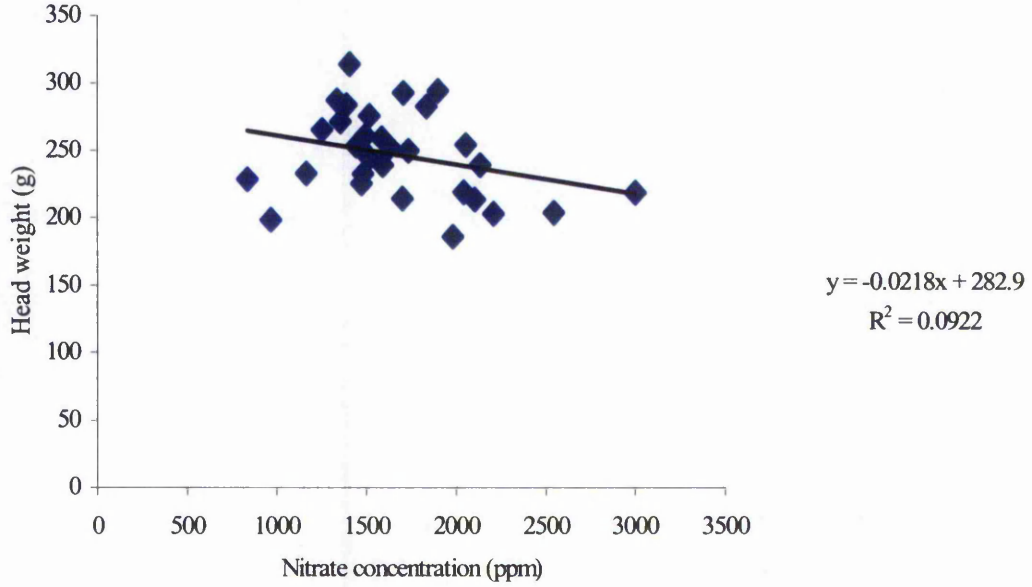
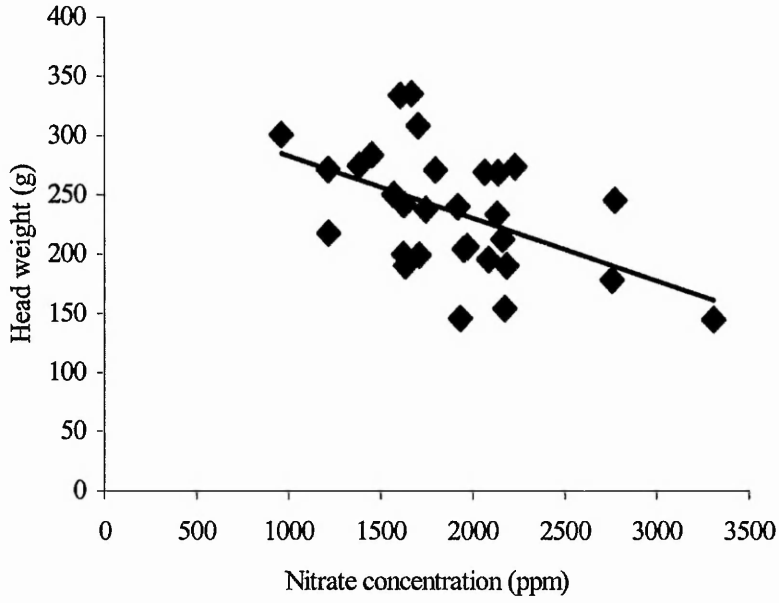


Figure 3.10 Relationship between nitrate concentration and fresh weight of summer lettuce cv. Rachel grown at 150ppm soil nitrate at HRI Stockbridge House from 1 July to 28 July, 1998.



3.4 Discussion

3.4.1 Nitrate variability in summer and winter lettuce.

The winter and summer lettuce trials were designed in order to create similar growing conditions within each of the nine treatments. However, the possibility that environmental factors such as soil moisture, soil organic matter content and more likely, increased temperatures throughout the summer growing period, contributed to the variation in lettuce nitrate concentration makes it difficult to draw direct comparisons between lettuce from the winter and summer trial. For this reason a comparison of summer and winter results following statistical analysis of variance using the mean nitrate concentrations and mean fresh weights of summer and winter lettuce have not been included in this report. Nevertheless, it is possible to determine the nitrate concentrations and head weights of lettuce, and to investigate the effect of a range of soil nitrate concentrations and light regimes on these two parameters within a winter and summer crop separately.

Lettuce growth during winter periods is considerably slower compared to growth during summer months, and this can be largely attributed to lower winter temperature (Anon, 1999c). Slow development of lettuce during winter growing periods would influence nutrient uptake and nitrate concentrations in the plant (Campbell, 1993). Delayed crop development, together with reduced mineralisation rates affecting soil available nitrogen during the winter period and low light levels, may explain the absence of a significant influence of increased nitrate supply on the nitrate concentrations in winter lettuce.

Despite the possibility that additional factors other than seasonal variation may contribute to the differences in nitrate concentration, a clear difference between nitrate concentration in winter and summer lettuce was shown. The mean nitrate concentration of winter lettuce within the nine treatments was generally higher and less variable compared to the mean nitrate concentrations of lettuce from the summer crop. These findings are in agreement with previous studies which have shown that seasonal variation in nitrate concentrations occurs in glasshouse lettuce (Tempereli cited in Van Eysinga, 1984a). The seasonal variation in nitrate concentration can be attributed to the different light intensities experienced during winter and summer months. The winter irradiation levels during the lettuce growing period, are clearly lower than those experienced during the summer growing period (figures 3.3 and 3.7 respectively). The maximum irradiation level during the winter growing period did not exceed 70MJ/m^2, whereas throughout the summer growing period irradiation levels did not fall below 80 MJ/m^2. In addition, warmer temperatures throughout the summer growing period would result in faster growth of the summer lettuce crop.

Light has long been established as a major factor influencing nitrate concentration in lettuce. A relationship was shown between average nitrate content of lettuce and global radiation in The Netherlands during a 12 month period (Van Eysinga, 1984b). From this study it was concluded that the nitrate content of lettuce is affected by the intensity of global radiation, and that the nitrate concentration of lettuce is therefore dependent on the growing period within the year. Additionally, in a report by Ysart, Clifford and Harrison,

(1999), nitrate levels in lettuce were recorded as higher during winter months (October – March), with a mean nitrate concentration of 3124 mg/kg, compared to summer months (April – September), when a mean nitrate concentration at 2382 mg/kg. It can therefore be concluded from previous investigations and those from the present study, that the increased light levels experienced during the summer trial contributed to the lower mean nitrate levels in lettuce.

The higher nitrate concentrations shown in winter lettuce may also be attributed to the function of the nitrate ion acting as an osmoticum (Blom-Zandstra and Lampe, 1983). It has been established that under conditions of low light, typical of winter growing periods, nitrate concentration in lettuce are high due to a low availability of alternative organic osmotica. Under high light periods a proportion of nitrate, acting as an osmoticum, can be replaced by sugars which are produced during increased rates of photosynthesis. High correlations have been shown between plant nitrate concentration and the rate of photosynthesis (Behr and Wiebe, 1992). It is possible that the high nitrate concentrations in winter lettuce were due to a reduced rate of photosynthesis and corresponding lack of replacement sugars with which to replace nitrate in the plant tissue.

3.4.2 Nitrate variability within the winter lettuce crop.

Nitrate variation in lettuce may have been subject to a treatment × plot interaction, which may have masked, to some extent, the effect of varying light regimes and soil nitrate concentrations. Lettuce heads were sampled from replicated plots of the same treatment across the trial. Soil nitrate-nitrogen concentrations were assumed to be identical in replicated treatments. However, soil conditions may have varied from across the trial and this could have influenced the amount of available soil nitrate-nitrogen in replicated treatments. Variability in soil structure between replicated plots could also have occurred in the investigation and this may have further influenced the availability of soil nitrogen. An interaction between the treatment conditions and additional environmental factors, specific to the plot location, may have contributed to the variability in nitrate concentration of the lettuce, although a statistical analysis of the plot effect has not been undertaken in these studies. This must be taken into consideration in a discussion of this investigation. It would be useful to repeat the investigation, incorporating additional techniques such as soil sampling , in order to reduce the occurrence of a plot × treatment interaction.

In these studies, increasing the soil nitrate concentration from 50ppm to 100ppm, and then to 150ppm did not significantly increase the nitrate concentration in winter lettuce. A reduction of light level from "100%" irradiation to "10%" and then to "20%" reduced irradiation did not lead to significant increases in nitrate concentration of lettuce at any of the three soil nitrate levels (figure 3.3). The combined effect of high soil nitrate concentration (150ppm), together with reduced light level ("20%" reduced irradiation) did not produce significantly higher nitrate concentrations in winter glasshouse lettuce. Despite this, there were significant differences between nitrate concentration of lettuce raised under varying light and nitrate-nitrogen regimes. Furthermore, there was significant variation between some treatments that may be attributed to the combined interaction of light and soil nitrogen treatments (table 7 in appendix).

The results are in agreement with previous studies concerned with the effect of nitrogen application rate on nitrate concentration in lettuce. Investigations by Van Eysinga, (1987), showed that the nitrogen content of the soil had little effect on the nitrate content of lettuce at low light levels. During the winter period, when light levels are low, nitrate concentrations are high in lettuce even at low soil nitrogen levels and do not appreciably rise with increasing soil nitrogen availability. Low temperature is known to reduce the amount of plant-available soil nitrogen. In a study by De Willigen cited in Lambers *et al.*, (1986), the rate of mineralisation and nitrification increased from 0.08 to 0.22 mg/kg/day and from 3.9 to 8.3 mg/kg/day respectively, following an increase of 10°C in temperature. Applying the findings of De Willigen cited in Lambers *et al.*, (1986), to the study presented in this chapter, it could be suggested that the low soil temperatures during the winter period reduced the rate of mineralisation and nitrification, thereby limiting soil-available nitrogen. Therefore, application of increased rates of nitrogen fertiliser may not have increased soil available nitrogen or resulted in a significant increase in the nitrate content of the lettuce.

3.4.3 Nitrate variability within a summer lettuce crop.

Similarly to the winter lettuce results there was a possibility that a significant plot interaction may have masked the influence of different light regimes and soil nitrate concentrations on lettuce nitrate concentration.

There was significant variation in mean nitrate concentrations within the summer lettuce (figure 3.5). In contrast to the results obtained from the winter lettuce, a clear trend was noticeable between nitrate concentrations and the nine treatments. The general trend was of increasing nitrate concentrations at high rates of soil nitrate concentration and reduced light levels. Increasing soil nitrate concentration from 50ppm to 100ppm significantly increased nitrate concentrations of summer lettuce ($p=0.05$). The influence of high soil nitrate rates on sap nitrate concentration was most marked at the higher light levels such as "0%" and "20%"light reduction. The results from the present study, although not statistically significant, point towards the findings of Van Eysinga and van der Meijs, (1985), who suggested that an interaction between soil nitrate and light conditions may influence the nitrate concentration of lettuce. Their investigations found that under high light conditions, when the nitrate content of the crop is generally low, increasing the nitrogen application rate led to considerable increases in tissue nitrate concentration. In the studies by Van Eysinga and van der Meijs, (1985), the nitrate concentration of lettuce grown under high light conditions was found to be approximately 1000mg/NO_3/kg (1000ppm), and increased to 2500mg/NO_3/kg (2500ppm) with increasing nitrogen application rates. In the present study, the lowest mean nitrate concentrations were obtained in lettuce raised under "0%" light reduction and with the lowest soil nitrate rate (S1L1). Previous literature has indicated that low light levels and high soil nitrate-nitrogen availability leads to high nitrate levels in glasshouse lettuce (de Willigen, cited in Lambers *et al.*, 1986). However, among the highest mean nitrate concentrations obtained from lettuce in this study were those raised under "100%" irradiance with the highest rate of soil nitrate (S3L1). This may be attributable to the interactive effect of high soil nitrate concentration and high irradiation. The "100%" irradiation conditions

would accelerate plant development, to a greater extent than the "10%" or "20%" reduced light treatments, and increase nitrate nutrient uptake from the soil thereby significantly increasing the nitrate content of the lettuce.

Temperature may have been an additional factor that influenced nitrate concentrations in summer lettuce. The average daily temperature during the summer period was approximately 10°C higher compared to temperatures during between the winter period (Pages 9 to 10, appendix). Such increases in temperature would lead to increased mineralisation and nitrification in the soil, and this may have raised the amount of plant available soil nitrogen. Therefore, increased summer temperatures would lead to a more noticeable effect of high soil nitrate application on the nitrate concentration of lettuce. This appeared to be the case in the summer lettuce trial, with significant differences in lettuce nitrate concentration between treatments that had varying soil nitrate concentration. As previously stated, increased temperature and light level can influence growth rate and nutrient demand in plants. Increased nutrient demand may lead to an increase in nutrient uptake, including soil nitrate. When nitrate assimilation into proteins and nucleic acids is low, during periods of slow growth, nitrate can accumulate within the plant. However, during periods of high light intensity nitrate accumulation is lower, and this has been attributed to an increased growth rate and the associated assimilation of nitrate into proteins and nucleic acids (Santamaria, Elia and Gonnella, 1997). The assimilation of nitrate into nitrogen containing compounds has been attributed to the enzyme nitrate reductase, whose activity rate has been shown to increase during periods of high light levels and faster growth. Although increased temperatures and high soil nitrate concentrations may both have contributed to an overall reduction in plant nitrate concentrations, the major factor determining nitrate concentrations in summer lettuce was probably the increased growth during the summer period and the rapid assimilation of nitrate into nitrogen containing compounds.

As previously mentioned, light levels have also been shown to influence the role of nitrate as an osmoticum, through the replacement of sugars generated by photosynthetic processes (Blom-Zandstra *et al.*, 1992). It is possible that the low nitrate concentrations in summer lettuce raised under 100% irradiance were in part due to a replacement of nitrate as an osmoticum by sugars. However, this cannot be confirmed without additional studies involving analysis of parameters such as plant osmolarity, photosynthesis, and nitrate uptake rates.

3.4.4 Variation in mean fresh weight in summer and winter lettuce

The mean fresh weights of the winter trial (figure 3.2) were generally lower and less variable compared to the fresh weights obtained from the summer trial (figure 3.6). A similar trend was observed in the mean nitrate concentrations of winter and summer lettuce. However, no correlation between nitrate concentration and fresh weight was established in either trial (figures 3.5 and 3.8 to 3.10). It seems that the fresh weight of summer lettuce was significantly influenced by the treatment conditions, ($p=0.001$). However this may have been potentially masked by plot effects. In contrast, the fresh weight of winter lettuce was not significantly affected by the treatments.

The generally higher fresh weights in summer lettuce compared to winter lettuce is further evidence of the well established and previously discussed interactive effect between temperature and irradiation, Knight and Mitchell, (1983).

As previously stated, increasing nitrogen fertiliser rate is known to enhance yield in lettuce (Knight and Mitchell, 1983). The results from the winter lettuce trial did not show any significant influence of soil nitrate rate on mean fresh weight. However, statistical analysis of variance revealed a significant difference in the mean head weights of lettuce within the nine treatments ($p=0.001$, figure 3.6).

Reduction of light availability did not significantly influence the winter lettuce yield (figure 3.2). Application of increased rates of nitrogen fertiliser does not necessarily increase productivity when additional factors are limiting to growth. For instance during winter the additional growth limiting factors would include low light level and temperature. Low temperatures, reduced light and shortened photoperiods would account for a general reduction in the rate of plant growth. A reduction in plant growth rate would lower nutrient plant nutrient requirements. It is possible that during slower growth in winter, a soil nitrate concentration at 50ppm was adequate to meet the nutrient requirements demanded for plant growth, and therefore increasing soil nitrogen concentration did not significantly influence lettuce head weight. In contrast, irradiation and length of photoperiod during the summer growing period were generally adequate to ensure light was not a limiting factor in plant growth.

3.5 Nitrate concentrations in different cultivars of lettuce raised in soil.

3.5.1 Introduction

The consumer has been educated in recent years by the vast array of salad leaves currently on offer. The wide varieties of lettuce available today differ in taste, texture and colour. In response to this trend, lettuce breeders are looking at new varieties of iceberg and continental types of lettuce (Anon, 1998e). In previous years the most popular variety was unquestionably Iceberg lettuce. In more recent times however, there has been a resurgence in older types of Cos lettuce, with estimates that Cos will eventually take up to 40% of the UK outdoor market (Joep van Balen cited in Anon, 1998e). Another type of lettuce that is expected to impact on the market of iceberg is Batavia. Cos and Batavia are successful commercially due primarily to their flavour. In addition, both varieties exhibit good shelf life in processing packs that are increasing in popularity. Further types are ideal for salad packs, with Lollo rossa exhibiting an attractive degree of redness in the head. Leaf lettuce such as oak leaf varieties, branch from a single stalk in a loose bunch rather than forming a tight head. The leaf colour ranges depending on variety giving red and green types, which offer resistance to a range of diseases. The leaves are suitable for the processing market, although they are more perishable than head lettuce.

There is an increasing necessity for lettuce breeders to adopt programmes aimed at creating new varieties suitable for this market and to select varieties on the basis of taste, texture and colour. However, factors other than taste and colour should be considered if breeders are to select a new and successful lettuce cultivar, and among these are included

resistance to pest and disease. It has been established in experiments conducted by MAFF (currently DEFRA), that nitrate levels in outdoor grown lettuce appear to vary according to variety (Anon, 1999b). Typically, continental varieties contain higher concentrations of nitrate compared to butterhead varieties (Lee, 1999) and this was also established in the hydroponic studies detailed in chapter two. Nitrate concentrations of different lettuce varieties, which were obtained from grower holdings throughout the UK, indicate that there are widely variable nitrate levels in continental and butterhead glasshouse lettuce. Nitrate concentrations in continental lettuce ranged from 3750 to 5180 mg/kg compared to butterhead lettuce, which ranged from 965 to 2230 mg/kg nitrate (Anon, 1999b). Although the Code of Good Practise does not specify particular lettuce varieties that can be grown, it is important that growers of protected and outdoor lettuce are aware of the nitrate levels that can be expected in a particular type of lettuce. With an aim towards growing new lettuce varieties that present means of achieving the EC maximum nitrate levels, growers should remain vigilant with the continental type lettuce presently available.

3.5.2 Materials and methods

The soil trial with different cultivars was raised in winter and sown on 9 November 1998 into 4cm × 4cm peat blocks, prior to transplanting on 22 December. The trial consisted of eight winter lettuce varieties, which are shown in table 3.3. The butterhead varieties were spaced at 20 × 25cm and the continental varieties were spaced at 25 × 25cm. The eight variety plots within each bay were randomised, and the bays were replicated three times in a Trojan Square design. The fertiliser regime involved Ammonium nitrate application to achieve 70ppm nitrogen in the top 0-20cm of the soil profile. Ten heads from each cultivar were harvested, at midday, on 31 March 1999. The heads were weighed and frozen prior to determination of nitrate concentration according to the procedures outlined in chapters 4 and 5.

Table 3.3 Winter glasshouse lettuce used in soil variety trial, Stockbridge House, December 1998- April 1999.

Variety (short-day)	Type
Yorvik	Curly
Tziganne	Butterhead
Scott	Butterhead
Wendel	Butterhead
Gabby	Butterhead
Miami	Lolla Bionda
Bercie	Batavia
Krizabri	Oakleaf

3.5.3 Results

Figures 3.11 and 3.12, respectively, show mean nitrate concentration and mean fresh weight of lettuce cultivars. Vertical bars represent standard errors of the mean. Weekly mean temperature °C, humidity % and irradiation values MJ/m^2 are shown in page 11 of the appendix.

Figure 3.11 Mean nitrate concentration of eight protected lettuce cultivars raised in soil growing system at HRI, Stockbridge House during growing period from December 1998 to April 1999. LSD = 468.6 (p=0.001), LSD = 366.2 (p=0.01), LSD = 272.7 (p=0.05), n = 10.

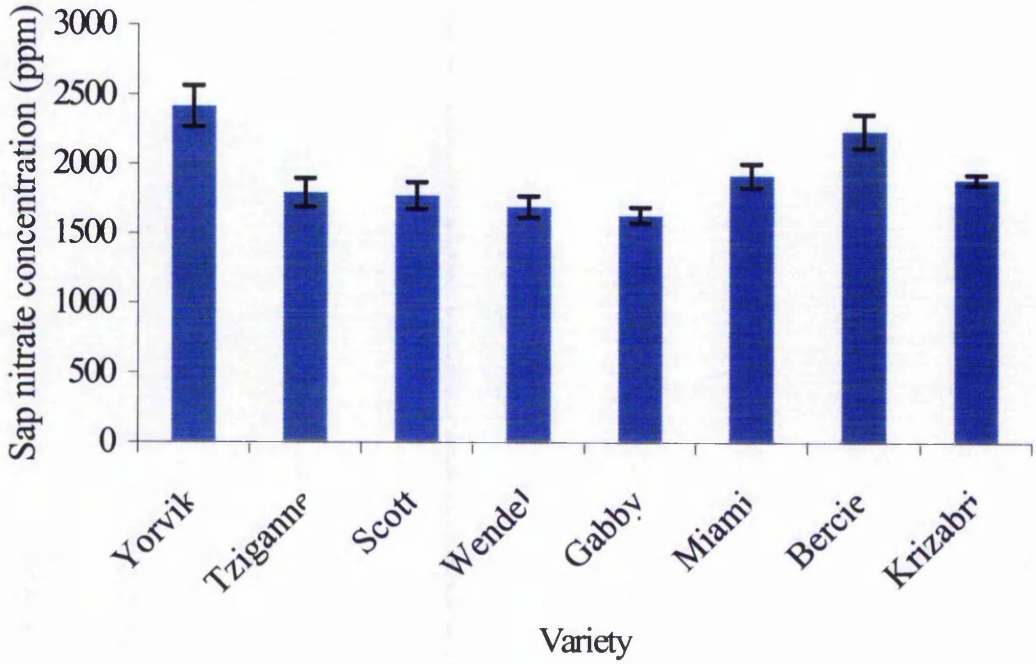
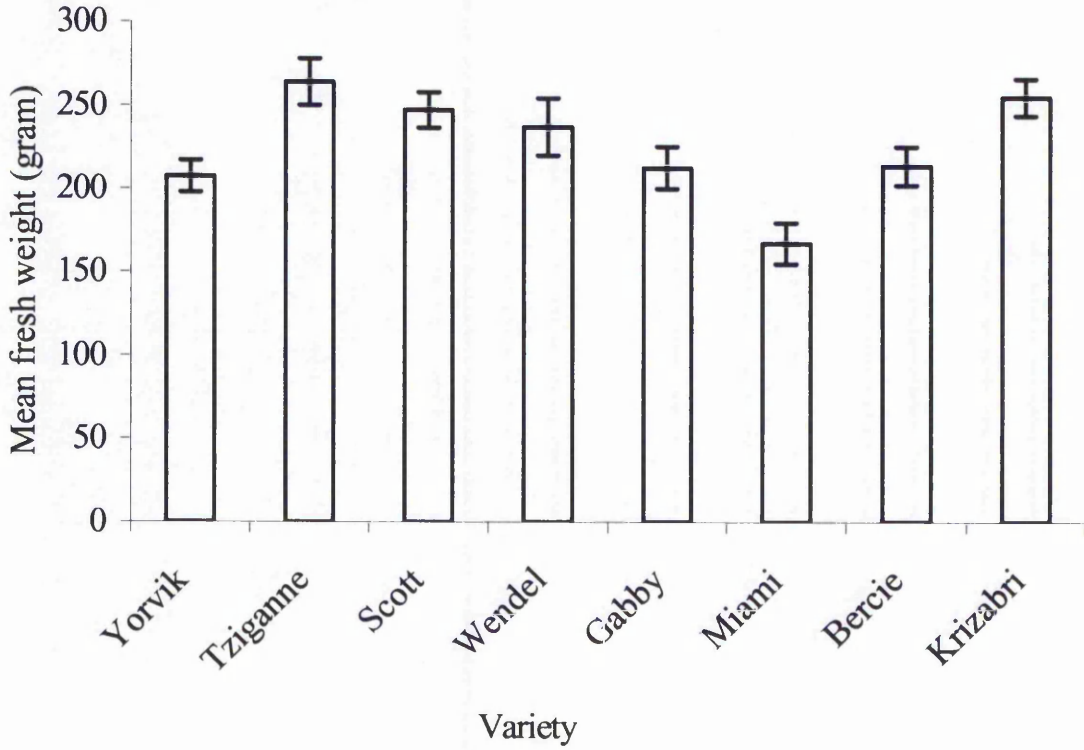


Figure 3.12 Mean fresh weights of protected lettuce cultivars raised in a soil growing system at HRI Stockbridge House during growing period from December 1998 to April 1999. LSD = 60.9 (p=0.001) LSD = 46.0 (p=0.01) LSD = 35.3 (p=0.05), n = 10.



The general appearance of the lettuce within each variety was healthy and disease free. Weekly temperatures ranged from 6.6 °C to 13.4 °C during the growing period. Humidity levels were fairly consistent throughout the growth period, and were between 77.8% and 90.6%. Irradiation levels rose steadily from 8.91 MJ/m^2 at the beginning of the growing period to 79.45 MJ/m^2 at the time of harvest (Page 11, appendix).

All of the butterhead cultivars included in the trial, which included Tziganne, Scott, Wendel and Gabby, contained lower mean nitrate concentrations compared to the continental types Yorvik, Miami, Bercie and Krizabri (figure 3.11). Mean nitrate concentrations within the cultivars ranged from 1630 ppm to 2414 ppm. The cultivar that exhibited the highest mean nitrate concentration in lettuce sap was the continental variety Yorvik. The butterhead variety, Gabby, gave the lowest mean nitrate concentration. Statistical analysis of variance using the mean nitrate concentrations found a significant difference between the lettuce cultivars ($p=0.001$). Yorvik, a curly type continental variety, contained significantly higher mean nitrate levels compared to each of the butterhead varieties and additionally when compared to the continental varieties Miami and Krizabri. Gabby, a butterhead variety, exhibited significantly lower mean nitrate levels compared to the continental types Yorvik and Bercie.

Analysis of variance using mean fresh weight of lettuce illustrated a significant difference ($p=0.001$) between the eight lettuce cultivars, which ranged from 167 gram to 264 grams (figure 3.14). The lowest mean fresh weight was observed in the continental variety Miami, at 167 gram, and the uppermost mean fresh weight was observed in the butterhead variety Tziganne, at 264 gram. The mean fresh weight of Miami was significantly lower than the mean fresh weights of Tziganne, Krizabri, Scott and Wendel ($p=0.001$). Tziganne was found to have a significantly higher mean fresh weight compared to the lollo bionda variety, Miami. With the exception of the oak leaf variety, Krizabri, the continental varieties generally gave lower mean fresh weights compared to the butterhead varieties.

Regression analysis did not reveal a correlation between fresh weight and nitrate concentration of the butterhead lettuce cultivars Tziganne, Scott, Wendel and Gabby (Page 30, appendix). Further regression analysis between the nitrate concentration and fresh weights of the continental cultivars Yorvik, Miami, Bercie and Krizabri did not reveal a correlation (Page 31, appendix).

3.5.4 Discussion

The results are in agreement with previous soil studies using a range of lettuce varieties, conducted at HRI Stockbridge House. In general, the nitrate concentration results obtained indicate that the continental varieties Yorvik, Miami, Bercie and Krizabri, were higher than the butterhead varieties Tziganne, Scott, Wendel and Gabby. Previous studies at Stockbridge House using the same lettuce cultivars showed that the continental varieties Yorvik and Bercie contained significantly higher nitrate concentrations, compared to the butterhead varieties Tziganne, Scott, Wendel and Gaby (Anon, 1998f). Similar cultivar variation in nitrate concentration has been observed in a study by Eenink *et al.*, (1984), using four lettuce cultivars.

The eight cultivars clearly differed in terms of leaf form, arrangement, colour and texture of leaves. Although no quantitative leaf measurements were taken, it can be speculated that a physiological difference in leaf number and or size could affect processes indirectly related to nitrate uptake and assimilation. As previously discussed in chapter 2, the degree of shading may directly influence nitrate assimilation, and this could have varied between butterhead and continental cultivars. Plates 2, 3 and 4 are typical examples of butterhead and continental lettuce cultivars and illustrate the different head morphology. An additional explanation for the observed nitrate variation between

lettuce cultivars may be due to differences in root distribution. However, differences in root distribution and structure between plots and even individual lettuce may have occurred throughout all of the soil studies. The study did not seek to determine differences in root distribution between lettuce cultivars. It is therefore speculative to suggest that differences in root structure were present between lettuce cultivars, or that the root structure of different cultivars directly influenced nitrate levels in the lettuce via nitrate assimilation and translocation in the root.

With the exception of Krizabri, the general trend in mean fresh weight was of a higher fresh weight in the butterhead varieties compared to the fresh weights of the continental types. An inverse relationship between nitrate concentration and fresh weight of butterhead lettuce has been observed in a study by Reinink, Groenwold and Bootsma, (1987). A similar correlation was observed in both dry weight and fresh weight of glasshouse lettuce in a study by Eenink *et al.*, (1984). The results obtained in the butterhead varieties used in the present study do not support the previous findings of a negative correlation between fresh weight and nitrate concentration. Although a negative correlation was not found between mean nitrate concentrations and mean fresh weight in the butterhead varieties Tziganne, Scott, Wendel and Gabby, there did appear to be some trend between butterhead and continental cultivars. In general, the butterhead varieties contained lower nitrate concentrations and higher mean head weights compared to the continental varieties, which were typically low in head weight but higher in nitrate concentration (Figures 3.11 and 3.12). It could be that part of the genetic variations in nitrate concentration may be accounted for by differences in the amounts of nitrogen required for the production of biomass.

In terms of marketability, the butterhead variety Tziganne, which offers the lowest nitrate concentration and the highest yield is most appealing. Among the continental types, the cultivar producing the highest weight together with a low nitrate concentration is Krizabri.

Chapter 4

Development of a rapid nitrate testing technique.

4.1 Introduction

4.1.1 Nitrate monitoring in crop production.

Information on crop nitrogen requirement is particularly beneficial in the implementation of a nitrogen fertiliser strategy. Determination of the nitrate concentration in lettuce plants would ensure that where necessary, precise concentrations of supplementary nitrogen dressings or liquid nitrogen feed are applied to the crop to match its requirements. Regular plant nitrate analysis can also be used to reveal chronic nitrogen deficiencies or excesses, and distinguish the cause of visual deficiency symptoms that are easily confused with virus damage. As a result of plant nitrate analysis, it may be necessary to make corrective nutrient applications immediately, in order to prevent serious loss to the crop. Therefore certain characteristics, one of the most important being speed, are required for the ideal nitrate analysis method. In addition to these requirements, the method selected should be reliable and sensitive in order to provide an accurate guide to the nitrate content of the crop

As previously highlighted, Commission Regulation (EC) No. 194/97 (amended in April 1999 by EC Regulation No. 864/99) sets maximum levels for nitrates in lettuce at 4500ppm (fresh weight) for crops harvested from November to April inclusive and 3500ppm for crops harvested from May to October inclusive. UK lettuce growers operate under a derogation, currently due for review in November 2001, which exempts production for the domestic market provided the growers follow an industry Code of Good Practise. The aim of the code is to minimise nitrate concentration in lettuce through implementation of the cultural advice given to lettuce growers. The practical and financial benefits of implementing a Code of Good Practise are wide ranging and include improved crop uniformity, waste minimisation and maximisation of Class 1 lettuce production, reduction in nitrate leaching into water courses and improved economics of lettuce production due to a reduction in fertiliser costs. In short, the viability of the UK glasshouse lettuce industry will be improved if UK growers continue to follow the Code of Good Practise and attempt to achieve the standards imposed by EC Regulation 194/97.

The cultural advice consists of knowledge from research conducted throughout EU Member States, particularly The Netherlands. Within the advice is information regarding the analytical procedures, involved in nitrate monitoring and sampling, that should be followed. Sampling and analytical procedures are deemed to be essential elements of due diligence in the cultural advice given to UK growers. In accordance with the Code of Good Practise, UK growers must use a "competent" laboratory that follows a "validated" method of analysis. As previously noted (Chapter one Page 24) to qualify as "competent" the laboratory would be required to hold UKAS (United Kingdom Accreditation Service) accreditation and participation in FAPAS (Food Analysis Performance Assessment Scheme) or a similar proficiency testing scheme, ideally z-scoring between +2 and -2 in FAPAS nitrate rounds.

Nitrate testing techniques that are generally conducted under laboratory conditions are often laborious and expensive. The choice of laboratory largely determines the method used to

establish nitrate concentrations in the plant tissue sample, and also the level of accuracy (Bischoff, Hiar & Turco, 1996). At present, nitrate determination of lettuce samples is made immediately before harvest to provide an indication of the nitrate levels within the harvestable crop. Additional nitrate monitoring, demanded by customers or enforcement authorities, may also be requested. However, nitrate testing during the growing period for the purpose of nitrogen fertiliser management is not a requirement, and is left to the discretion of the individual grower. Although plant analysis cannot be used to determine basal fertiliser applications for the current crop, it may be possible to considerably reduce the recommended dressing rate following plant analysis where top-dressing of extra nutrients forms part of the growing plan. In the development of a sustainable management system for nitrogen fertiliser application in protected lettuce, the determination of the nitrate status of plants at different growth stages would be a useful requirement. To encourage lettuce growers to adopt such a strategy would involve the development of alternatives to the present nitrate testing techniques currently used to analyse nitrate concentrations in lettuce.

4.1.2 Methods used to determine plant nitrate concentration.

There are many methods for nitrate determination that include spectrophotometric techniques, liquid and gas chromatography and potentiometry using a nitrate selective electrode (Blanco, Martinez, Mangas, Dapena & Gutierrez, 1995). The two commonly used laboratory methods for nitrate determination in solution samples are Ion chromatography (IC) and rapid flow analysers (RFA) (Khakural & Alva, 1996). During the past decade, laboratories in The Netherlands have been studying leafy vegetables and their products for nitrate concentration. Many of the laboratories that carry out nitrate determination have used a fully automated continuous-flow technique (CF) for nitrate and nitrite determination (Beljaars, van Dijk & van der Horst, 1994). The CF technique is an automated version of an official procedure prescribed by the Dutch Food Act, (1984). Extracted nitrates are reduced to nitrite in the system by metallic cadmium, and then nitrite reacted with sulfanilamide and *N*-1 naphthylethylenediamine to form a reddish-purple azo dye. The dye is measured colorimetrically at 530nm. The CF method has been shown to offer many advantages to the earlier methods, and no significant differences have been found among Dutch and Belgian participants using CF and other analytical procedures (Beljaars *et al.*., 1994).

Chromatography can be defined as "the separation of components in a mixture by partitioning between the mobile phase (either a gas or liquid) and a stationary phase (either a liquid or a solid)" (Anon, 1989). There are three major disciplines of chromatography that can be identified in terms of the mobile phase, which are gas, liquid and super critical fluid. High Performance Liquid Chromatography (HPLC) was first introduced in the late 1960's (Anon, 1989). The use of HPLC for determination of nitrate-nitrogen in vegetables has been described in an investigation conducted by Hunt & Seymour, (1985). HPLC has been used for some time to determine the NO_3-N content in soil, but was found to be unsatisfactory for direct analysis in vegetable extracts. This was due to the interference caused by organic matter present in the vegetable extracts. The adapted method incorporated nitrate-free activated carbon to absorb the organic matter in the vegetable extracts. When the anion-exchange HPLC was used with an automated system, it was found to provide accurate, rapid and precise measurement of NO_3-N in vegetables. Indeed, it has been widely regarded that of all the current methods for nitrate analysis, the most sensitive are chromatography-based. When this method is suitably modified, it can detect NO_3 as low as 0.02 ± 0.005 μM in a nutrient medium containing additional anions (Swiader & Freiji, 1996).

Ion chromatography has been perhaps the most valued technique for the determination of nitrate concentration to date, due in part to the high level of accuracy and repeatability of the method. Since its introduction in the mid 1970's, Ion Chromatography has become a widely used analytical technique for the determination of all types of ions (Anon, 1989). In a study by Khakural & Alva, (1996), two methods of nitrate analysis were used to determine the nitrate concentration of water samples. The methods used were ion chromatography (IC) and rapid flow analyser (RFA). There was good agreement between the nitrate results obtained by the two methods.

Nitrate selective electrodes have been a popular method for determining nitrate uptake patterns in water culture systems such as NFT (Deane-Drummond & Glass, 1983). Ion selective electrodes (ISE) are suitable for measurement under NFT conditions and for use on plant, soil and water samples because they provide fast and direct measurements that are relatively cheap. However, nitrate selective electrodes detect single ions only. Nitrate ions in aqueous solution diffuse through a semi-permeable membrane in the sensing module of the electrode. The potential difference created between the nitrate electrode and the reference electrode is used to determine the nitrate ion concentration in the solution. The ISE meter estimates nitrate within the range 0.1 to 100ppm, and for nitrate determination of samples exceeding this range a suitable dilution is necessary.

Most of the conventional laboratory methods are time-consuming, expensive and complicated. The time taken to carry out the analytical procedure is one of the major disadvantages with these methods. Staff may be required to visit the crop site and obtain samples, which are then labelled, bagged and taken to the laboratory for nitrate determination. The entire procedure normally takes one week. The need for trained staff to carry out the procedure, together with the demand on resources such as laboratory equipment means that the cost of nitrate analysis is high. In addition, the cost of nitrate analysis is often made on a per sample basis. The expensive nature of nitrate analysis means that regular nitrate monitoring of the crop during the growing season is a rarity.

Sensitivity is generally dependent on the technique used to determine nitrate concentration and can therefore vary between individual laboratories. As previously mentioned, the code of good practise for lettuce growers in the UK suggests that nitrate analysis is undertaken by accredited laboratories who are able to show z-scoring between +2 and -2 in FAPAS nitrate rounds. The high cost, together with the additional inconveniences associated with the present methods of nitrate analysis illustrate their unsuitability for application in routine crop monitoring. A rapid nitrate testing technique for on-farm use, which would reduce the time factor and high cost associated with nitrate determination off-site would be very useful to lettuce growers. This would be particularly useful to UK growers operating under the EU derogation and following the sampling advise detailed in the Code of Good Practise.

4.1.3 Rapid nitrate testing techniques.

Although application for plant nitrate testing was not envisaged by the manufacturers of the test strips, there is potential for "Merckoquant" test strips in the development of a suitable rapid nitrate testing technique of plant sap. For some crops, the petiole or stem can be crushed in a sap press and the extracted sap can be collected and analysed for nitrate concentration. The test strips have been successfully used to assess nitrogen nutrition in some vegetable

crops and ornamental crops (Prasad & Spiers, 1982 and Scaife & Stevens, 1983). In these investigations, NO_3-N was determined by timing the development of colour on the test strip. Unfortunately, due to the subjective nature of this method of nitrate determination it has not been adopted commercially. "Merckoquant" test strips cannot determine nitrate concentrations in excess of 500ppm and for many leafy vegetable and salad crops, such as lettuce and spinach, plant nitrate levels generally surpass this limit. For application of "Merckoquant" test strips in the determination of lettuce nitrate concentrations, a suitable extraction and dilution method is required beforehand.

A rapid nitrate testing technique, that removes the need for sample dilution and extraction, has been developed by the Horiba company in Japan. The Horiba "Cardy" meter is a battery operated, portable nitrate-selective electrode, model C-141, that provides quick, quantitative analysis of soil solution and undiluted plant sap. The Cardy Nitrate Meter has a nitrate detection range of 0 to 9900 ppm. A high level of correlation was shown between the meter and conventional laboratory methods of nitrate analysis on dry petiole tissue and soil solutions by Hartz, Smith, LeStrange & Schulbach, (1993). The Cardy meter has been widely used in the United States, but has not been extensively used in Europe. Investigations using the meter suggest that it may be considered applicable for on-farm nitrate analysis of fresh petiole sap (Hartz *et al.*., 1993).

4.1.4 Development of a rapid nitrate testing technique for on-farm application, using glasshouse lettuce as a test crop.

The aim of the investigation was to determine a rapid nitrate testing technique, that could be used by lettuce growers on-site. A limited number of steps would be advantageous in the prescribed process to determine nitrate in the lettuce. A review of existing literature did not yield a uniformly accepted extraction procedure with which to obtain nitrate from fresh or frozen lettuce. The origin of many of the diverse nitrate extraction procedures currently used in commercial laboratories is unknown. Many of the nitrate extraction procedures were found to use fresh plant material, and involved lengthy reactions with reducing compounds and or periods of boiling (Bedwell *et al.*). For the purposes of the present investigations a procedure was used that involved cold water extraction. The modified procedure was based on an unpublished method for nitrate determination in fresh lettuce (Burns, 1999). This method was chosen for its simplicity and the rapidity with which the procedure could be completed. In addition, the method did not involve the use of hazardous extraction compounds and could be carried out in non-laboratory situations. It was anticipated that the method, when used alongside the rapid nitrate testing "Merckoquant" meter, could be incorporated in an on-farm nitrate testing procedure.

The process of freezing lettuce heads was a necessary step due to the large number of heads sampled at each harvest. The effect of freezing lettuce heads was expected to disrupt cell walls and membranes, which could be advantageous to the blending process and overall extraction procedure. The process of freezing lettuce samples has been stated as an aid to the homogenisation of the sample during blending and the possible release of stored vacuolar nitrate (West Yorkshire Analytical Services, personal communication 1998). Prolonged storage of non-acidified solution samples, at -20°C for up to eighty days, did not significantly affect nitrate concentration in a study by Khakural & Alva, (1996). In a separate study by Schuster & Lee, (1987), nitrate concentration in carrots was not altered during a ten week storage period at -18°C and nitrite formation did not occur. In chapter 5, the findings from a

small-scale study to determine the effect of freezing lettuce heads on nitrate concentration of lettuce sap samples are detailed. The t-test results from the preliminary studies, detailed in chapter 5, seem to suggest that there are no significant differences between the nitrate concentration obtained following extraction from frozen lettuce compared to the nitrate concentration following extraction from fresh lettuce (appendix).

4.2 Determination of a suitable rapid testing technique using hydroponic lettuce samples.

4.2.1 Materials and Methods

Ninety lettuce heads were sampled from the hydroponic lettuce trial outlined in chapter two. The trial involved raising butterhead lettuce, cv. Rachel, supplied with nutrient media at 100ppm, 150ppm and 200ppm nitrate-nitrogen. Each nutrient treatment was replicated in gullies containing twelve lettuce. Ten lettuce heads were sampled from each gully, and the heads were weighed and stored at –20°C prior to nitrate determination. For the purpose of determining a suitable rapid nitrate testing technique, sap was extracted from each lettuce head and analysed by four techniques for nitrate determination as outlined in section 4.2.3.

Extraction of nitrate from lettuce

In view of the large number of individual lettuce samples to be analysed individually, the lettuce was stored at -20°C in a freezer. Nitrate extraction was undertaken on partially frozen untrimmed heads and the extracts were analysed for nitrate immediately, or frozen until analysis was carried out. The method used for nitrate extraction involved blending whole, untrimmed partially frozen heads in a Philips HR 2835 blender. An approximately equal volume to weight ratio of de-ionised water was used to extract the highly soluble nitrate. Blending was carried out for approximately 30 seconds, or until a homogenous slurry was obtained. The slurry was passed through four layers of muslin cloth ($1mm^2$), and the filtrate was collected and stored at -20°C until analysis. Prior to nitrate determination, the filtrate was allowed to defrost at room temperature with intermittent shaking of samples in order to mix any sediments in solution. The filtrate was diluted with de-ionised water at 1:50ml. The nitrate concentration of the samples was determined by the following analytical techniques.

Analytical techniques for nitrate determination.

In total, four nitrate analytical methods were used in the development of a rapid-testing technique, which were;

1. Ion Selective Electrode (ISE) – Orion Research Inc. nitrate specific and reference electrodes (Plate 7).
2. Nitrachek meter – Merck Ltd (Plate 8).
3. Horiba Cardy meter, - Horiba Co. model C-141 (Plate 9)
4. Ion Chromatograph, - Dionex Corp. model DX 100

Nitrate analysis using the Ion Selective Electrode method.

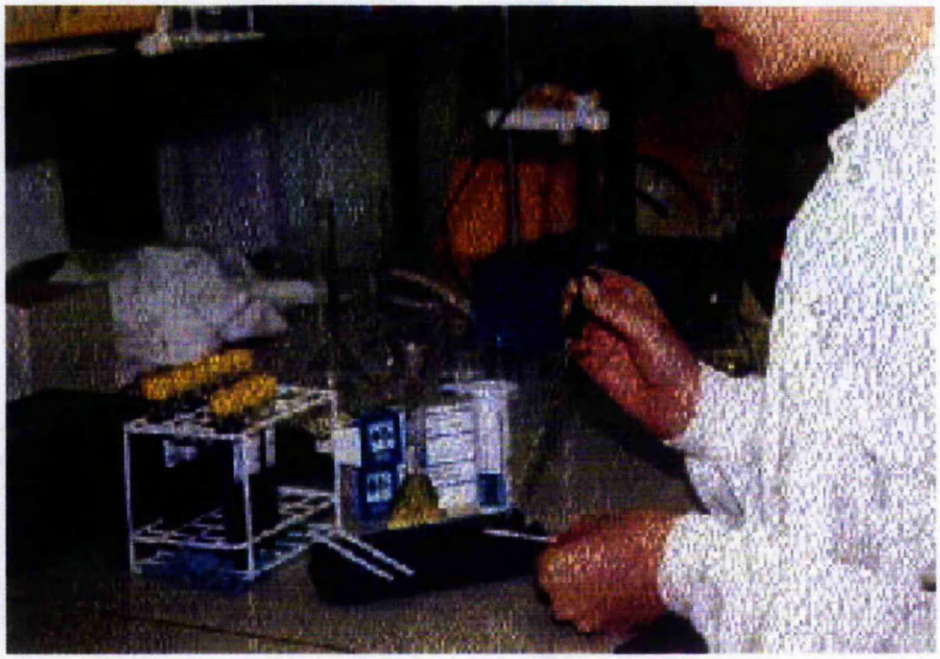
The ISE meter was calibrated using standard nitrate solutions from stock solution of 1000ppm nitrate, as detailed on page 1, appendix. The standard operating procedure of Turner & Carlile, (1982) was used to determine nitrate concentration. 100ppm and 10ppm nitrate standards were prepared from the 1000ppm stock nitrate solution in deionised water. 20ml of the 100ppm and 10ppm solutions were put into 50ml beakers and to each beaker, 0.4ml ionic strength adjuster (2M Ammonium sulphate) and a magnetic stirring bead were added. The sample was placed on a magnetic stirrer and the nitrate specific and reference electrodes were lowered into the solution to a depth of approximately 0.5cm. The reading was allowed to stabilise and the nitrate value was recorded. The electrodes were cleaned with deionised water between each sample measurement. After every ten samples the ISE meter was re-calibrated (if necessary) in order to maximise the accuracy of the nitrate values obtained. It was important that nitrate determination was carried out when the samples were at the same temperature, in order to minimise temperature changes in solution. The ISE meter is known to be sensitive to temperature fluctuation (Hartz *et al.*., 1993).

Plate 7: Nitrate analysis of diluted lettuce sap using the Ion Selective Electrode method.

Nitrate analysis using Nitrachek meter.

The nitrachek meter was calibrated by inserting a blank test strip and waiting for the digital read-out to show "cal". The sample solution was pipetted onto both squares of filter paper on the blank test strip. Colour development was complete after one minute and a digital read-out was given following insertion of the test-strip into the meter. Removal of the used test-strip and re-calibration using a blank test-strip ensured the Nitrachek meter was set for additional nitrate analysis.

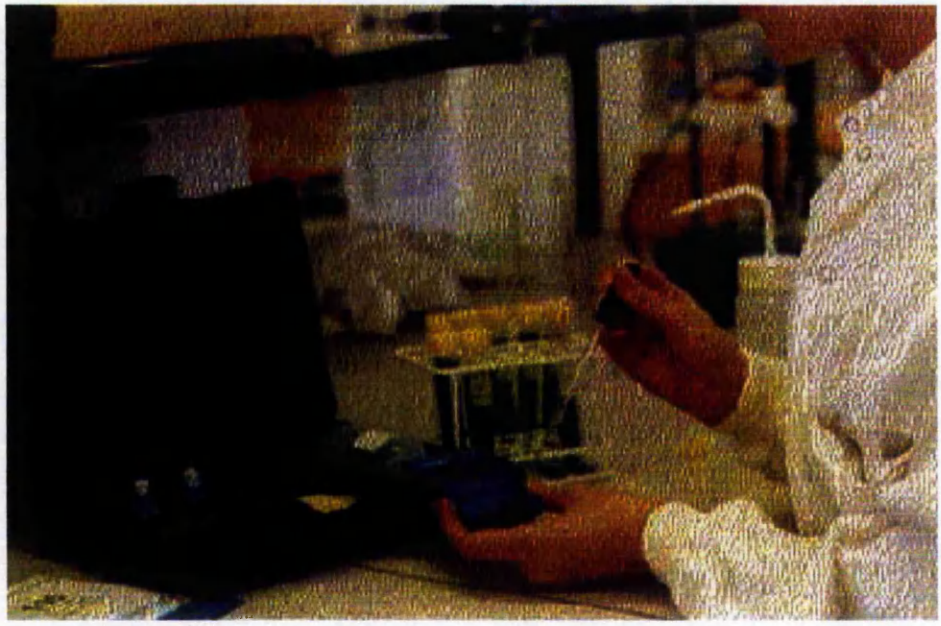
Plate 8: Nitrate analysis of diluted lettuce sap using the Nitrachek meter method



Nitrate analysis using Horiba Cardy meter.

The Cardy meter was calibrated prior to analysis of sample nitrate concentration using standard nitrate solutions at 150ppm and 2000ppm NO_3^-. A sheet of sampling paper was placed directly on the sensor pad and 3 to 5 drops of the 2000ppm standard solution were added to this. After the display had stabilised, the meter dial was adjusted until the desired value was displayed. The sensor was then rinsed with distilled water until a zero read-out was obtained. The procedure was repeated using the 150ppm standard solution. The meter was calibrated after every ten samples. A small volume of sample solution was pipetted onto the piece of sampling sheet that covered the sensor pad. The reading was allowed to stabilise for approximately 30 seconds. The digital reading provided the measured nitrate concentration of the sap.

Plate 9: Nitrate analysis of diluted lettuce sap using the Horiba Cardy meter method.



Nitrate analysis using Ion Chromatography

The Ion Chromatograph used to determine nitrate concentration in lettuce samples was a Dionex DX-100, two-column set system. The Dionex system was equipped with an AS14 IonPac separator column that separated and eluted anions, including nitrate. The column was supplied with a carbonate / bicarbonate eluent and supressor regenerant solution of dilute sulphuric acid. Details of the regenerant and eluent solutions are given in the appendix. The Dionex system was calibrated at the beginning of a series of samples, using linear regression on a range of nitrate standard solutions at 0ppm, 1ppm, 5ppm, 10ppm, 25ppm, and 50ppm. Linear regression analysis with zero intercept produced a calibration curve and equation from which the nitrate concentrations of the samples could be calculated. Approximately 0.5ml of sample solution was pipetted into auto analyser vials with a 0.45μm filter cap. The auto analyser was set-up with lettuce sample solutions, which were intermittently stacked with standard nitrate solutions in order to monitor the consistency of nitrate determination during long periods of analysis. The DX-100 Ion Chromatograph can detect a range of anions and their concentrations and the presence of nitrate was indicated as a peak with a retention time at approximately six to seven minutes. The nitrate concentration was determined from the equation;

peak area = m × concentration (ppm) *where* **m = x coefficient of calibration curve**

The nitrate concentration (ppm) of the sample was calculated by dividing peak area by x coefficient. The nitrate concentrations of the samples were displayed as a series of results, which were generated and stored on Dionex-100 computer software.

4.2.2 Results

The results presented in this section relate to the mean nitrate concentrations of hydroponic lettuce supplied with three nutrient treatment concentrations. The three treatment conditions were replicated in three gullies and each was supplied with nutrients from individual tanks. A detailed description of the experiment is given in chapter 2. Each sample of lettuce extract was analysed four times using the four chosen methods of nitrate determination. Analysis of lettuce extract by the Ion Selective Electrode, Nitrachek meter and Horiba Cardy meter was carried out concurrently. Analysis of lettuce extract by Ion Chromatography was performed in two, separate over-night runs. The results were subjected to two-way analysis of variance using Minitab v.12 statistical package, together with determination of least significant difference values. Regression analysis of the results was carried out to determine the relationship between the four methods of nitrate determination and is shown on pages 18 to 22, appendix. The relationship between all four methods of nitrate analysis is shown in figures 4.1.4 to 4.1.8. The results obtained from regression analysis are included in the appendix.

There is some variability between the nitrate concentration of lettuce within the replicated gullies of the same treatment (figures 4.1.1, 4.1.2 and 4.1.3). All four methods of nitrate analysis revealed an increase in the nitrate concentration of lettuce raised in gully number two and three compared to gully number one, in the nutrient treatment containing 100ppm and 150ppm nitrate-nitrogen. There was a highly significant difference in nitrate concentration of lettuce raised in replicated gullies supplied with the same treatment at 100ppm, 150ppm and 200ppm ($p=0.001$). In general, the variation between gullies was seen in each of the four chosen methods of nitrate determination. The Horiba Cardy meter did not appear to reflect the nitrate variability between gullies to the same extent as the Ion Selective Electrode meter, Ion Chromatograph and Nitrachek meter. This may indicate some limitation in the overall sensitivity of the Horiba Cardy meter.

Figures 4.1.1, 4.1.2 and 4.1.3 show the mean nitrate concentrations of hydroponic lettuce supplied with three nutrient treatments. Throughout the treatments, there were highly significant differences ($p=0.001$) between the mean nitrate concentrations obtained by the four methods of nitrate analysis. The greatest level of variability between the nitrate concentrations obtained by the four methods of analysis was within the 200ppm nitrate-nitrogen treatment (figure 4.1.3). However, the basic level of agreement between nitrate concentrations obtained by the four methods of analysis was high, particularly within the 100ppm and 150ppm nitrate-nitrogen treatments (figure 4.1.1 and 4.1.2). In general the nitrate concentrations obtained by the Ion Selective Electrode, Nitrachek meter and Ion Chromatograph were not significantly different (figures 4.1.1 and 4.1.2). However, the nitrate concentrations obtained by the Horiba Cardy meter were significantly higher compared to the mean nitrate concentrations obtained by the Ion Selective Electrode, Nitrachek meter and Ion Chromatograph ($p=0.001$). There was a general trend for lower nitrate readings obtained by the Ion Chromatograph when compared to other methods (figures 4.1.1 and 4.1.3). In a few cases the differences between nitrate concentrations obtained by the Ion Chromatography

method and the nitrate concentrations obtained by the Ion Selective Electrode, Nitrachek meter and Horiba Cardy meter were highly significant (p=0.001).

The least variable method for determining nitrate concentration was the Ion Selective Electrode technique (as seen in figures 4.1.1, 4.1.2 and 4.1.3). The Horiba Cardy meter and the Ion Chromatograph were the two most variable methods for determining nitrate concentrations in lettuce sap.

In general, the nitrate concentrations obtained by the ISE meter were not significantly different to the nitrate concentrations obtained by the Nitrachek meter. However, there was some variation between the nitrate concentrations obtained by the Nitrachek meter and the nitrate concentrations obtained by the Ion Selective Electrode within one replicate of a 200ppm nitrate-nitrogen treatment (figure 4.1.3).

Figure 4.1.1 Mean nitrate concentration of lettuce raised in NFT with 100ppm nitrate. Nitrate analysis using ISE (probe), Nitrachek (Nchek), Ion Chromatography (Dionex) and Horiba Cardy meter (Hcardy).

LSD = 179 (p=0.001); LSD = 140 (p=0.01) ; LSD = 106 (p=0.05) n=10

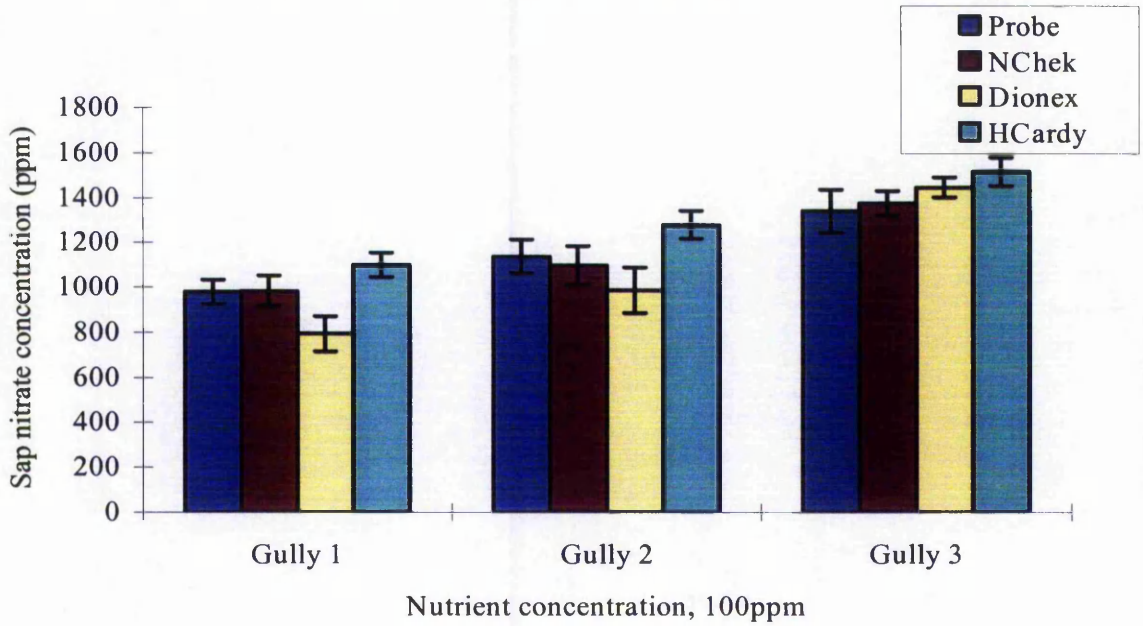


Figure 4.1.2 Mean nitrate concentration of lettuce raised in NFT with 150ppm nitrate nutrient rate. Nitrate analysis using ISE (probe), Nitrachek (Nchek), Ion Chromatography (Dionex) and Horiba Cardy meter (Hcardy).

LSD = 179 (p=0.001); LSD = 140 (p=0.01) ; LSD = 106 (p=0.05) n=10

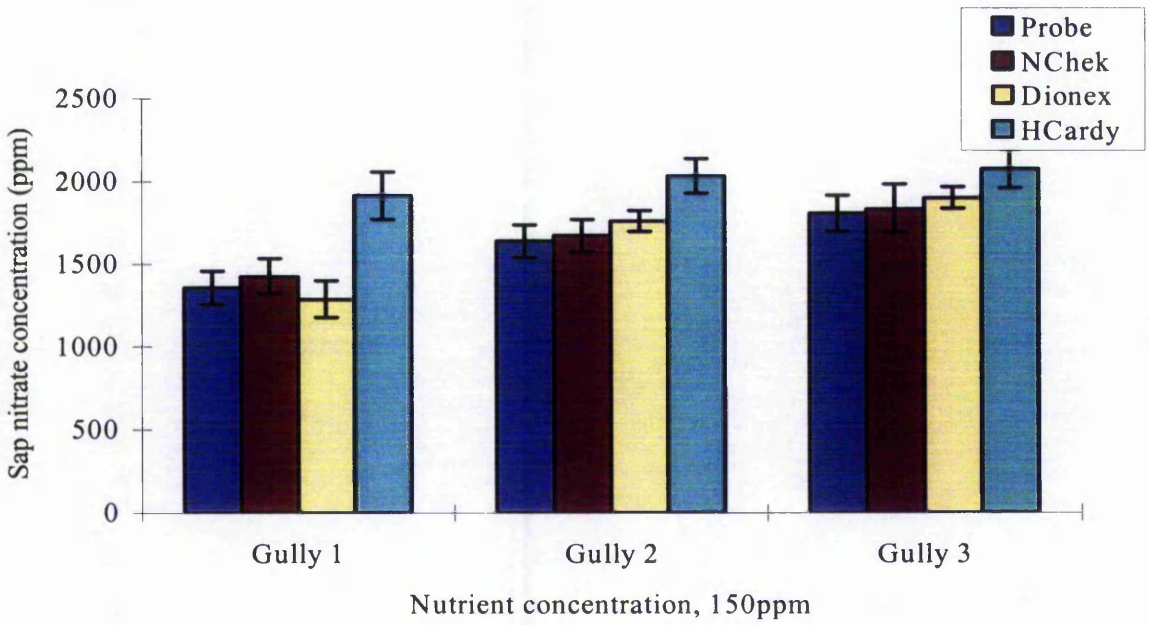
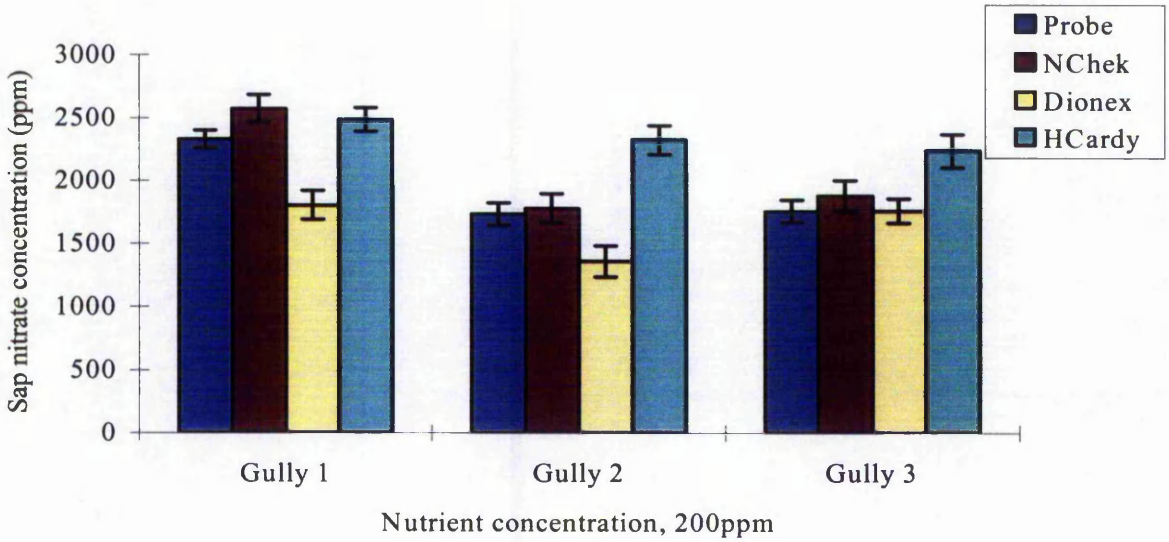


Figure 4.1.3 Mean nitrate concentration of lettuce raised in NFT with 200ppm nitrate nutrient rate. Nitrate analysis using ISE (probe), Nitrachek (Nchek), Ion Chromatography (Dionex) and Horiba Cardy meter (Hcardy).

LSD = 179 (p=0.001); LSD = 140 (p=0.01) ; LSD = 106 (p=0.05) n=10



Regression analysis of the nitrate results was used to establish the level of correlation between the four methods of nitrate analysis. The level with an r^2 value of correlation between the Nitrachek meter and the Ion Selective Electrode was particularly high, with a r^2 value of 0.896 (3sf) (figure 4.1.4). The high r^2 value reflects the results shown in figure 4.1.1 to 4.1.3, with limited variation between the nitrate concentrations obtained by these two methods of analysis (p=0.01). There was a good level of agreement between the nitrate readings obtained by the Ion Chromatograph method and the Nitrachek meter, r^2 = 0.679 (3sf) (figure 4.1.5). However, several outlying values, which are shown in figure 4.1.5, contributed to the observed significant differences between nitrate values obtained by the Nitrachek meter those obtained by the Ion Chromatograph (figure 4.1.3).

The lowest level of correlation was shown between the Nitrachek meter and the Horiba Cardy meter, with an r^2 value of 0.607 (3sf) (figure 4.1.6). The level of correlation, although reasonable, was indicative of the general trend for overestimation of nitrate values by the Horiba Cardy meter and the highly significant variation between it and each of the three methods of nitrate analysis (p=0.001, figures 4.1.1, 4.1.2 and 4.1.3). The significantly higher nitrate readings obtained by the Horiba Cardy meter are further reflected in figures 4.1.7 and 4.1.8. The relationship between the nitrate results obtained by the Horiba Cardy meter and the nitrate results obtained by the Ion Selective Electrode gave an unsurprisingly similar correlation to that of the Horiba Cardy meter and Nitrachek, with an r^2 value of 0.661 (3sf) (figure 4.1.7). In figures 4.1.1, 4.1.2 and 4.1.3, the Ion Chromatograph produced significantly lower nitrate readings compared to the higher nitrate estimates given by the Horiba Cardy meter (p=0.001). Interestingly, a good level of correlation was found between the nitrate results obtained by the Horiba Cardy meter and the nitrate results obtained by the Ion Chromatograph, with an r^2 value of 0.721 (3sf). Despite the high r^2 value, which indicated a

good level of agreement between the two methods of analysis, there was a general inconsistency between the nitrate readings obtained. However, while the difference between the nitrate readings obtained from the Horiba Cardy meter and Dionex was highly significant, the trends emerging from the two sets of results were found to be in agreement and this could explain the high r^2 value obtained.

In each instance, regression analysis reflected the, sometimes excellent, level of agreement between the four methods of nitrate determination. However, regression analysis did not fully reflect the highly significant differences between nitrate readings obtained by the Horiba Cardy meter and the three methods of nitrate analysis ($p=0.001$). As previously stated, the correlation between the Nitrachek method and the two standard laboratory techniques, the Ion Selective Electrode and Ion Chromatograph was generally good throughout.

Figure 4.1.4 Relationship between Nitrachek meter and Ion Selective Electrode, used to measure nitrate concentration of hydroponically raised lettuce.

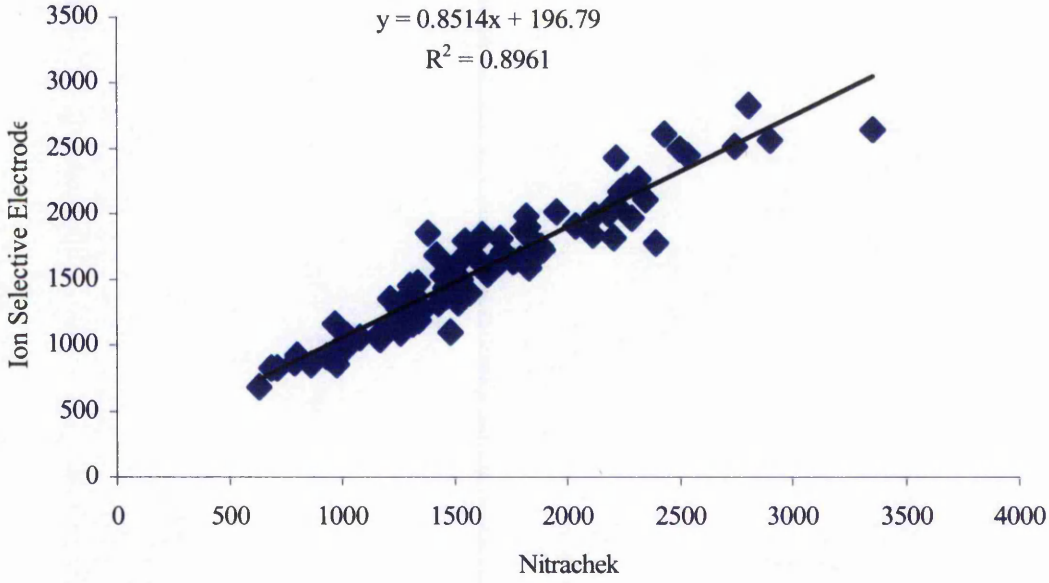


Figure 4.1.5 Relationship between Nitrachek meter and Ion Chromatograph – Dionex, used to measure nitrate concentration of hydroponically raised lettuce.

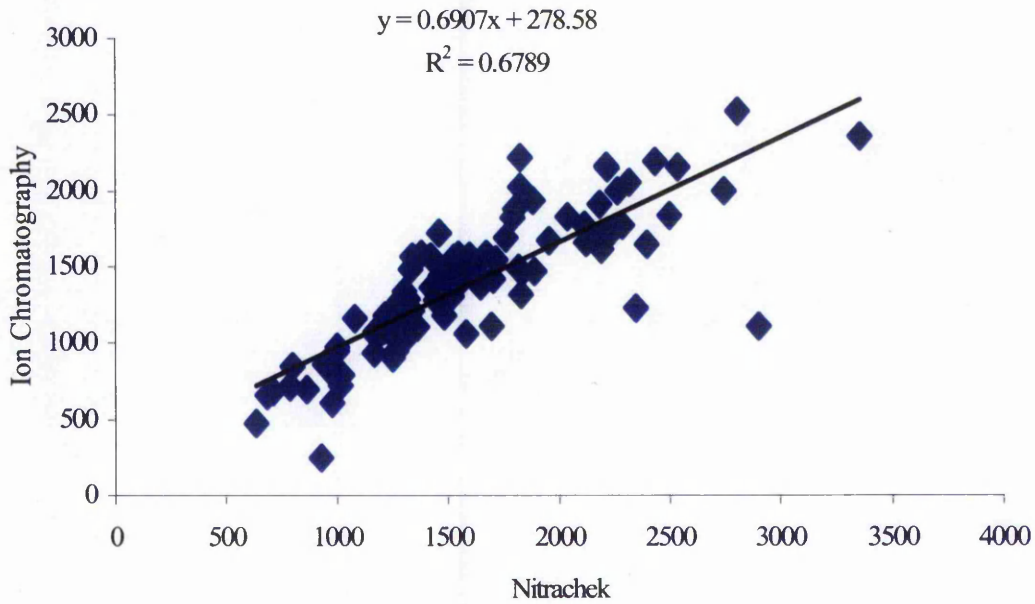


Figure 4.1.6 Relationship between Nitrachek meter and Horiba Cardy meter, used to measure nitrate concentration of hydroponically raised lettuce.

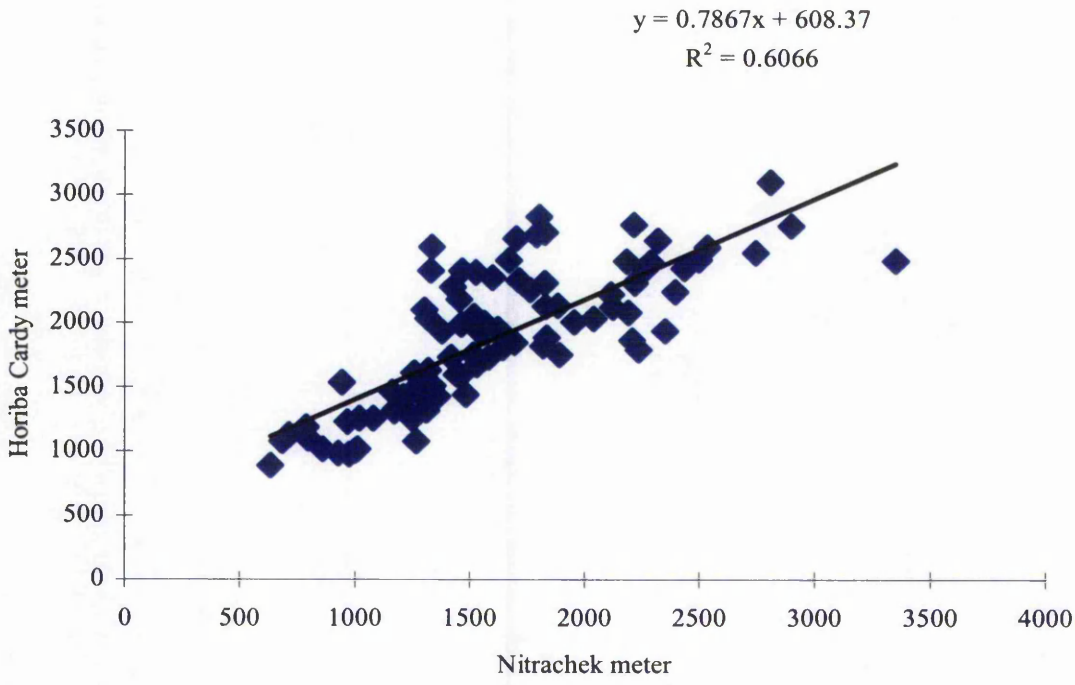


Figure 4.1.7 Relationship between Horiba Cardy meter and Ion Selective Electrode, used to measure nitrate concentration of hydroponically raised lettuce.

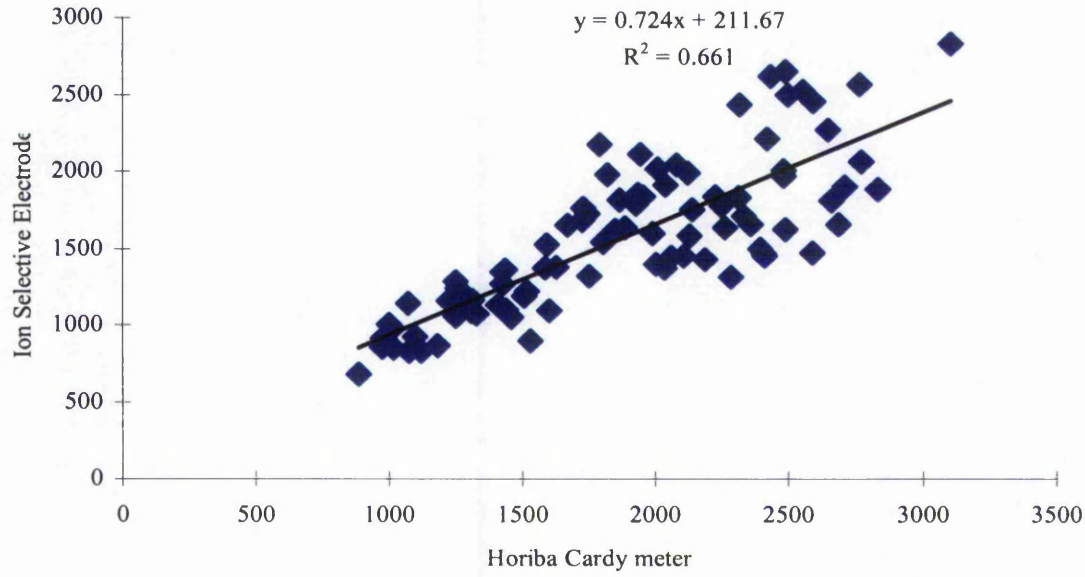
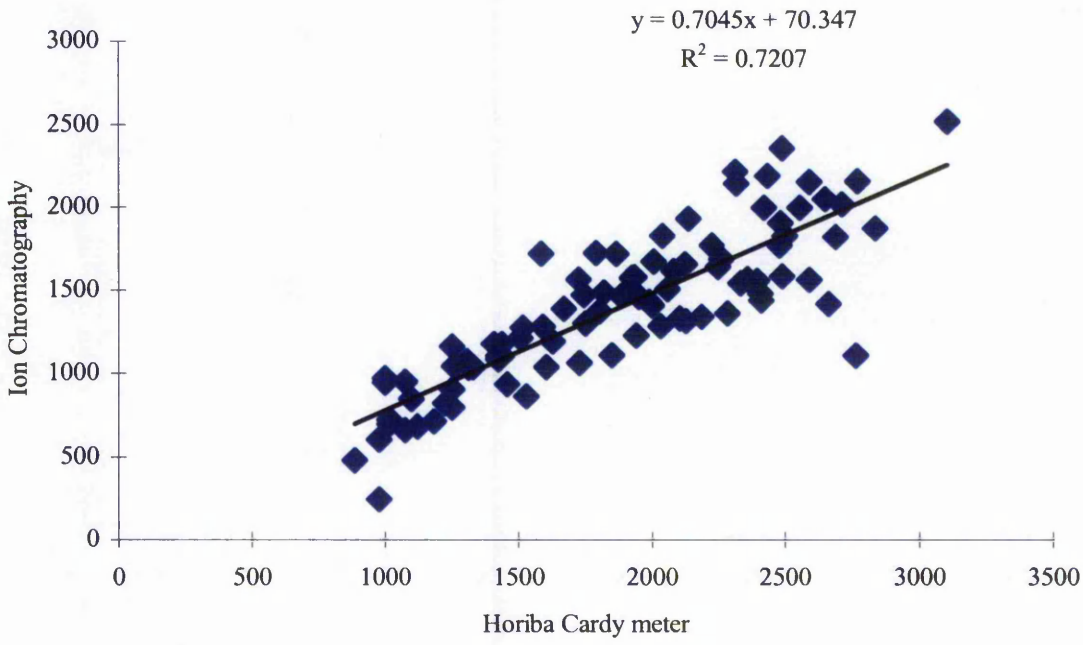


Figure 4.1.8 Relationship between Horiba Cardy meter and Ion Chromatograph – Dionex, used to measure nitrate concentration of hydroponically raised lettuce.



4.2.3 Discussion

As stated in the results section in this chapter, significant differences were revealed between the nitrate readings obtained by the rapid nitrate testing methods and the lab-based methods of nitrate analysis. There have been several reports concerning the performance and reliability of the Nitrachek meter for the purposes of rapid nitrate determination (Scaife & Bray, 1977). In a study by Prasad & Spiers, (1982), Merckoquant test strips were evaluated for use as an indicator of nitrogen nutrition in a range of popular pot plants. Prasad & Spiers, (1982), compared the Merckoquant test strip method for nitrate determination with the established method for determining total N concentration, the micro-Kjeldahl method. A linear relationship was found between the test strip method and the micro-Kjeldahl method for sap nitrate, with an r^2value of 0.94, and from this result it was decided that the test strips would provide an acceptable method for determining sap nitrate concentration (Prasad & Spiers, 1982).

The results presented in this chapter were from a study that incorporated the Nitrachek meter and the Horiba Cardy meter for evaluation as techniques for rapid nitrate determination. Previous work, by Hartz, Smith, LeStrange & Schulbach, (1993), has been carried out using the Horiba Cardy meter together with nitrate test strips to determine the nitrate concentration in broccoli (*Brassica oleracea* L.). The nitrate-nitrogen concentration of fresh petiole sap in broccoli was determined using the portable Horiba Cardy meter, and was highly correlated with nitrate-nitrogen concentration in dry petiole tissue of broccoli (Hartz *et al..*, 1993). Hartz *et al..*, (1993) found a similar relationship between the nitrate-nitrogen concentration of fresh petiole sap determined with the Horiba Cardy meter and nitrate-nitrogen concentration in dry petiole tissue of lettuce determined with a conventional laboratory technique, with an r^2 value of 0.77. Hartz *et al..*, (1993), concluded that the Horiba Cardy meter showed promise as an on-farm tool for nitrate monitoring in crops, but suggested that the reliability of the Horiba Cardy meter could be improved if used in conjunction with periodic testing by conventional laboratory methods

The results presented in this chapter were obtained from analysis of hydroponically grown lettuce, stored at -20°C, by the four chosen methods for nitrate determination. A comparison between the two rapid nitrate methods and the two established laboratory methods suggested that the most reliable and accurate method for rapid nitrate determination was in fact the Nitrachek meter. The Nitrachek meter performed comparably to the established Ion Selective Electrode method. Variability between the nitrate values obtained by the Ion Selective Electrode and the Nitrachek meter, although not highly significant, can be partly accounted for by the higher level of accuracy provided by the Ion Selective Electrode compared with the Nitrachek method. The Ion Selective Electrode method gave nitrate readings correct to one decimal place, whereas the Nitrachek meter gave nitrate readings as whole numbers. When a multiplication factor was applied to the initial nitrate readings, in order to give the total nitrate concentration of each lettuce, the small discrepancies between the two methods was magnified. This would explain the unavoidable variation between the two methods of nitrate analysis. The excellent repeatability and accuracy of the Nitrachek meter may be attributed to its limited operating procedure, which reduced the possibility of user error. In a study by Bischoff, Hiar & Turco, (1996), nitrate test strips were evaluated using water samples and the individual user error involved in operating the Nitrachek meter was investigated. Bischoff *et al..*, (1996) found a good agreement between the "readers" results and the analytical methodologies used. Bischoff *et al..*, (1996) concluded that nitrate test strips could provide a

reliable and quick test for nitrate contamination in well water, when used by non-technical persons.

The Horiba Cardy meter, when used to determine the nitrate concentration of lettuce sap, gave more variable and significantly higher nitrate values compared to the other analytical methods ($p=0.001$). The Horiba Cardy meter tended to over estimate nitrate concentrations in lettuce sap, compared to the other methods. Although the Horiba Cardy meter is known to be highly temperature sensitive (Hartz *et al..*, (1993), temperature fluctuations within the laboratory at the time of analysis would have been small and would not have significantly influenced the nitrate readings. The most likely cause of over estimation by the Horiba Cardy meter was due to the error introduced when converting the displayed nitrate value on the meter to actual nitrate concentrations. The Horiba Cardy meter has a 2-digit display range up to 99ppm. The nitrate detection range is increased automatically to 990ppm nitrate by multiplication of the displayed value by 10, and the range is further increased to 9900ppm by multiplication of the displayed value by 100. Therefore, estimated nitrate values at the higher end of the nitrate concentration range are subject to ± 100ppm error.

The laboratory based Ion Selective Electrode method for nitrate determination was found to be the least variable of all the four methods investigated. This has been found to be the case in numerous studies. In an investigation by Heinen and Harmanny, (1992), Ion-Selective Electrodes were evaluated in an automated NFT system and the readings obtained were found to be highly replicable. Indeed, the "Orion" Ion Selective Electrode has been found to be consistently efficient for use in laboratories and is highly recommended as a tool for determining nitrate concentrations in soil and water samples (Carlile, personal communication). As a method for rapid nitrate analysis, the Ion Selective Electrode method is feasible since it does not require gases for nitrate detection and it could be used as a portable instrument. However, the Ion Selective Electrode does require frequent calibration using standard nitrate solutions and the incorporation of a magnetic stirrer adds to the difficulty of transporting the equipment. For these reasons, the Ion Selective Electrode is predominantly used in laboratory situations. The performance of the Ion Selective Electrode was superior to the laboratory based Ion Chromatograph method, which tended to under estimate nitrate concentrations in lettuce sap.

There are some notable disadvantages in the use of ion selective electrodes, which include variation between individual electrodes, their sensitivity dependence on temperature, a decrease in sensitivity with time and the ability to detect only a single ion (Bailey, Haggett, Hunter, Albery & Svanberg, 1988). However, with regular calibration and maintaining of the ion selective electrode probes, many of these problems can be easily overcome. Furthermore, ion selective electrodes provide a much quicker method of nitrate detection compared to the ion chromatography or rapid flow analysis methods when nitrate is the sole ion of interest.

There are several possible explanations for the often significantly lower nitrate concentrations obtained by the Ion Chromatograph ($p=0.001$). Analysis of lettuce samples by the Ion Chromatography method was not carried out at the same time as nitrate analysis by the other three methods. Lettuce samples that were stored at -20°C for up to three days prior to analysis by the Ion Chromatograph. Although it has been shown that the nitrate concentration of plant extracts does not significantly change during short periods of storage, sediment can form within the solution and this is often difficult to dissolve back into solution. 10μL of sample is drawn through a 0.45μm filter cap before injection into the detection column of the Ion Chromatograph. Therefore, any nitrate that has effectively come out of solution and formed

part of the sediment during storage, may be removed during the filtration process and will not be detected by the Ion Chromatograph. It has also been suggested that nitrate adheres to the vial wall, and this may reduce the nitrate concentration of the solution (Davies, personal communication).

However the main factor influencing the nitrate readings obtained by the Ion Chromatograph can be attributed to the holding time prior to analysis. The holding time was dependent on the number of samples that needed to be analysed and ranged from eight to twelve hour periods. It has been suggested that extended sample holding time, up to 24 hours, is only acceptable if the sample is acidified at pH = 2.0 (Khakural and Alva, 1996). The extended holding periods prior to the analysis of samples could have resulted in chemical reduction of nitrate to nitrite. Evidence for the chemical reduction of nitrate to nitrite was provided by the Ion Chromatograph and further confirmed following analysis of the samples using the Nitrachek meter. Following analysis of some samples, the presence of nitrite was detected and indicated by a violet dye on the Nitrachek meter and represented in the print-out of peak area from the Ion Chromatograph results. Chemical reduction of nitrate to nitrite, together with the possibility of microbial activity, may have influenced the nitrate concentration in sample. Some evidence of this was apparent in samples analysed for nitrate following a holding time of over 12 hours. The samples analysed in the final stages of the twelve hour runs often contained appreciable amounts of nitrite, and the nitrate readings obtained were lower when compared to the nitrate values obtained by the other methods.

Despite the limitations to the accuracy of nitrate determination using the Horiba Cardy meter, there was one obvious advantage in its use compared to that of the Nitrachek meter. The Nitrachek meter, like the two standard laboratory methods of nitrate analysis, required sample dilution and filtration prior to nitrate determination. These additional steps in the overall process of nitrate determination may have introduced experimental error and influenced the nitrate readings. In view of the application of the two proposed rapid nitrate testing methods for use on-farm, the least complicated and time consuming Horiba Cardy method would hold sway over the more detailed procedure required for operation of the Nitrachek meter. However, it would be unwise to sacrifice the superior accuracy of the Nitrachek meter in favour of a simpler analytical procedure alone. The results point towards more extensive investigations using Horiba Cardy, and suggestions for these are explored in the general discussion.

4.3 The limitations of the Nitrachek method for nitrate determination of lettuce sap.

4.3.1 Introduction

The Nitrachek meter was used as a method for determining nitrate throughout the studies to investigate nitrate variability in lettuce. In general the level of accuracy and repeatability from the Nitrachek meter was excellent and correlated well with the two laboratory based methods of nitrate determination. The method had been previously shown to be suitable for development as a rapid testing technique. The results have supported previous studies by Scaife and Stevens, (1983), who showed a high level of accuracy and repeatability with the Nitrachek meter when compared to established laboratory techniques. However, some discrepancies were noticed in the accuracy and repeatability of the Nitrachek meter during a study to determine the nitrate concentration of lettuce cultivars raised in soil. Deficiencies in the Nitrachek meter need to be identified and reduced prior to any recommendations for its application as a rapid testing technique. The results presented in this chapter, together with the results from a short series of investigations show some of the disadvantages of the Nitrachek meter. The trial used in this study consisted of eight lettuce cultivars consisting of butterhead and continental types raised as part of a variety screening trial. Details of the experiment and the procedure used to determine nitrate concentration in the soil grown lettuce cultivars are described in chapter 3, section 3.5. In addition to using lettuce samples from the soil variety trial, the comparisons of the Nitrachek meter, Ion Selective Electrode and Ion Chromatograph were also made using a range of standard nitrate solutions.

The aim of the study was to identify the least variable and most variable method of nitrate analysis. The Horiba Cardy meter was not included in this set of investigations. It was anticipated that the laboratory based Ion Selective Electrode method, which had shown consistency and accuracy in nitrate determination throughout the previous studies, would prove to be the least variable of the methods. Previous studies using the Ion Chromatograph had highlighted significant inaccuracies in nitrate determination. The discrepancies were found to be due to incomplete optimisation of the anion column and Dionex equipment. Following repair and replacement of the anion column the accuracy and repeatability of the Ion Chromatogram greatly improved. It was also noted that storage, at - 20 °C for extended time periods prior to nitrate determination using the Ion Chromatograph, may have affected nitrate concentrations in the extracted lettuce sap and consequently influenced the nitrate values obtained. Following thorough re-checking and optimisation of the Ion Chromatograph and Ion-Selective Electrode methods, they were deemed reliable for nitrate determination. It was suspected that the Nitrachek meter could be responsible for the significant differences recently noted between the methods. If the assumption proved to be correct, and the Nitrachek meter was under-estimating nitrate values, an appropriate correction factor would be applicable for use with the Nitrachek meter to improve its reliability in nitrate determination.

4.3.2 Materials and Methods

The methods of nitrate extraction and analysis used in the initial part of the investigation to determine the limitations of the Nitrachek method are detailed earlier in this chapter. The initial investigation involved nitrate analysis of winter lettuce cultivars raised in soil during the winter growing periods 22 December, 1998 to 31 March, 1999, at HRI Stockbridge

House. Nitrate analysis of the lettuce was undertaken using Ion Chromatography, Ion Selective Electrodes and the Nitrachek method of nitrate analysis.

In the second investigation, the accuracy of the Nitrachek method itself was investigated by determining the percentage nitrate recovery from standard nitrate solutions using test strips from different batches.

In the third study, Ion Chromatography, Ion Selective Electrodes and the Nitrachek methods were used to determine the percentage nitrate recovery from standard nitrate solutions. In this study, standard nitrate solutions at 5ppm, 10ppm, 25ppm and 50ppm were made from a stock nitrate solution (Page 1, appendix). Nitrate concentration was measured in the standard nitrate solutions using each of the three methods of nitrate analysis. The percentage recovery was then calculated.

4.3.3 Results

The mean nitrate concentrations of the eight lettuce cultivars, determined by the three analytical techniques, are shown in figure 4.1.9. Regression analysis of the nitrate values obtained by each method was carried out, in order to establish the level of correlation between each of the three analytical methods. The results from regression analysis of the nitrate values obtained by the three methods are shown on pages 23 to 25, appendix. The relationship between the Nitrachek meter and the Ion Chromatography method (Dionex) is shown in figure 4.2.0. The relationship between the Nitrachek meter and the Ion Selective Electrode method is shown in figure 4.2.1. The relationship between the Ion Chromatography method and Ion Selective Electrode method is shown in figure 4.2.2.

Figure 4.1.9 show significant differences in the nitrate values obtained by all three methods of nitrate analysis ($p=0.001$). There was a trend of significantly ($p=0.001$) higher nitrate values following analysis by the Ion Selective Electrode in the cultivars Yorvik, Tziganne and Scott. The nitrate values obtained by the Ion Chromatograph were significantly ($p=0.001$) lower than those obtained by the Ion Selective Electrode. However, the Nitrachek meter revealed significantly lower nitrate values in all of the lettuce cultivars analysed ($p=0.001$).

There was a weak correlation between the Nitrachek meter and the Ion Chromatograph, with an r^2 value of 0.48 (figure 4.2.0). However, the level of correlation between the Nitrachek meter and the Ion Chromatograph was not as high as the correlation previously shown, with an r^2 value of 0.68 (figure 4.1.5). The nitrate values obtained with the Ion Chromatograph method are clustered around a higher nitrate concentration range compared to the nitrate values obtained with the Nitrachek meter (figure 4.1.9). A similar relationship was found between the Nitrachek meter and the Ion Selective Electrode, with an r^2 value 0.53, with a level of correlation that reflected the highly significant differences between the nitrate values obtained by the two methods ($p=0.001$). Figure 4.2.1 shows numerous outlying nitrate values that can be associated with the significantly lower nitrate readings obtained by the Nitrachek meter compared to the nitrate values obtained with the Ion Selective Electrode. A higher level of correlation was found between the two laboratory based methods, Ion Selective Electrode and Ion Chromatograph, with an r^2 value of 0.65 (figure 4.2.2). Although there were some outlying values surrounding the regression between the Ion Selective Electrode and the Ion Chromatograph values, in general the data was tightly grouped and gave a reasonably good correlation.

The percentage nitrate recovery from standard nitrate solutions was previously established during preliminary studies using the Nitrachek meter. A reduction in the accuracy and repeatability of the Nitrachek meter is evident by comparison of the previously established percentage nitrate recovery values with the current percentage nitrate recoveries (batch 1 and 2, respectively, figure 4.2.3). In figure 4.2.4, the percentage nitrate recoveries of the chosen laboratory methods, the Ion Selective Electrode (probe) and Ion Chromatograph (dionex), are shown together with the percentage nitrate recovery values obtained with the Nitrachek meter. The percentage nitrate recovery results, shown in figure 4.2.4, indicate the high percentage recovery of nitrate from standard solutions by both the Ion Selective Electrode and Ion Chromatograph methods. In general, the percentage nitrate recovery obtained with the Ion Selective Electrode and Ion Chromatography methods are close to 100%. However, it is clear that the percentage nitrate recovered by the Nitrachek meter was considerably less compared to the other methods of analysis, at approximately 80%, and this was most marked in the solutions containing higher concentrations of nitrate. Interestingly, the Nitrachek meter showed considerably higher percentage nitrate recovery from standard solutions containing 5ppm nitrate compared to the other two methods (figure 4.2.4).

Figure 4.1.9 Mean nitrate concentrations of lettuce cultivars raised in soil during 22 December, 1998 to 31 March, 1999 at HRI Stockbridge House. Nitrate analysis carried out by Ion Selective Electrode (probe), Ion Chromatography (Dionex) and Nitrachek method.

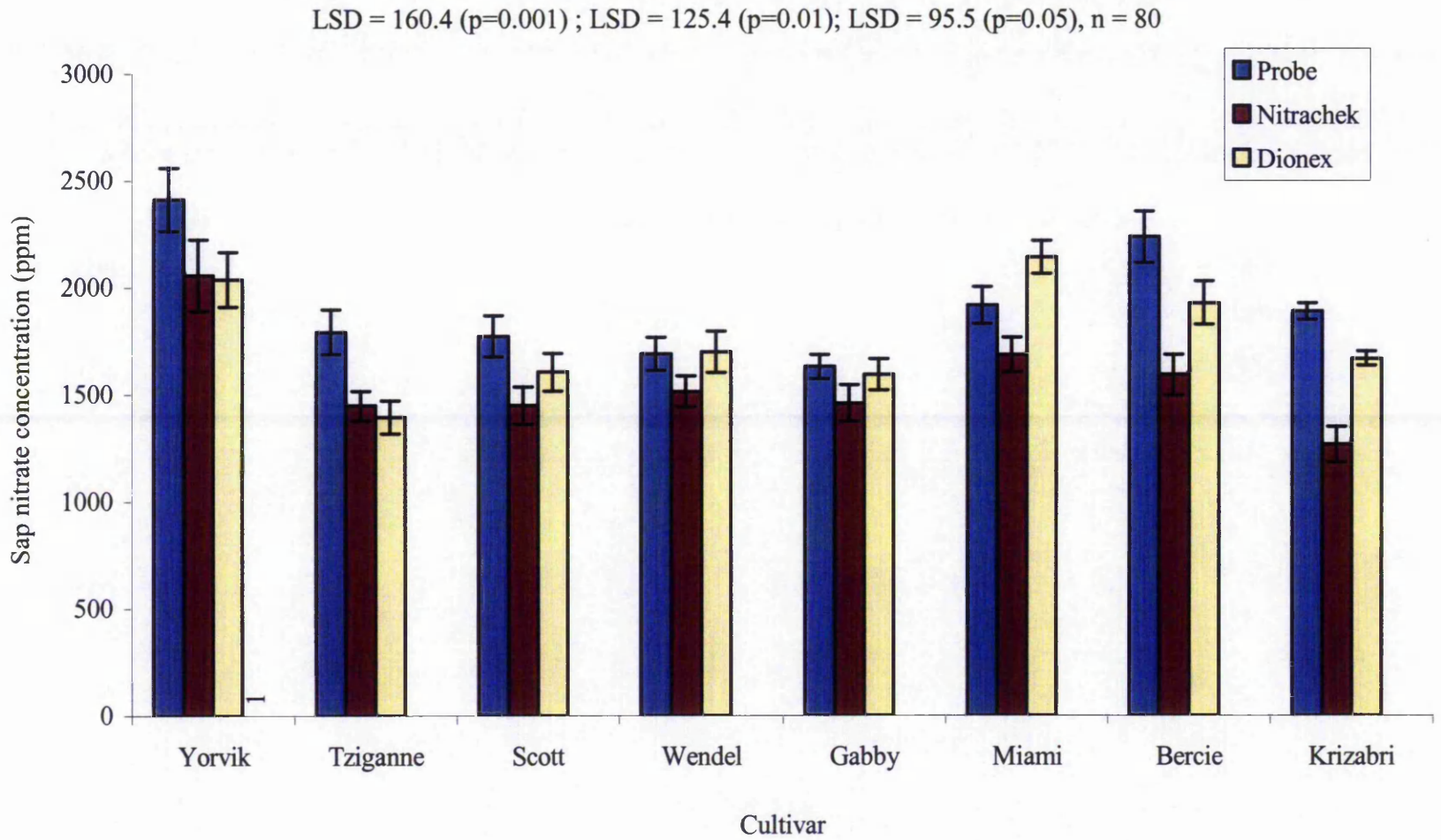


Figure 4.2.0 Relationship between the Nitrachek meter and Ion Chromatograph method following analysis of sap extracts from soil raised lettuce cultivars.

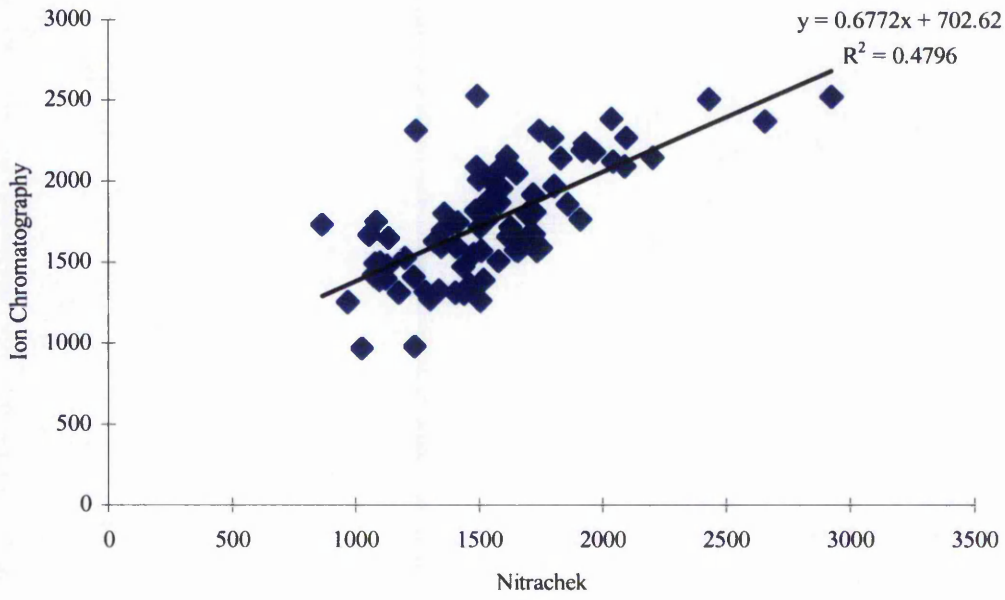


Figure 4.2.1 Relationship between the Nitrachek meter and Ion Selective Electrode methods following analysis of sap extracts from soil raised lettuce cultivars.

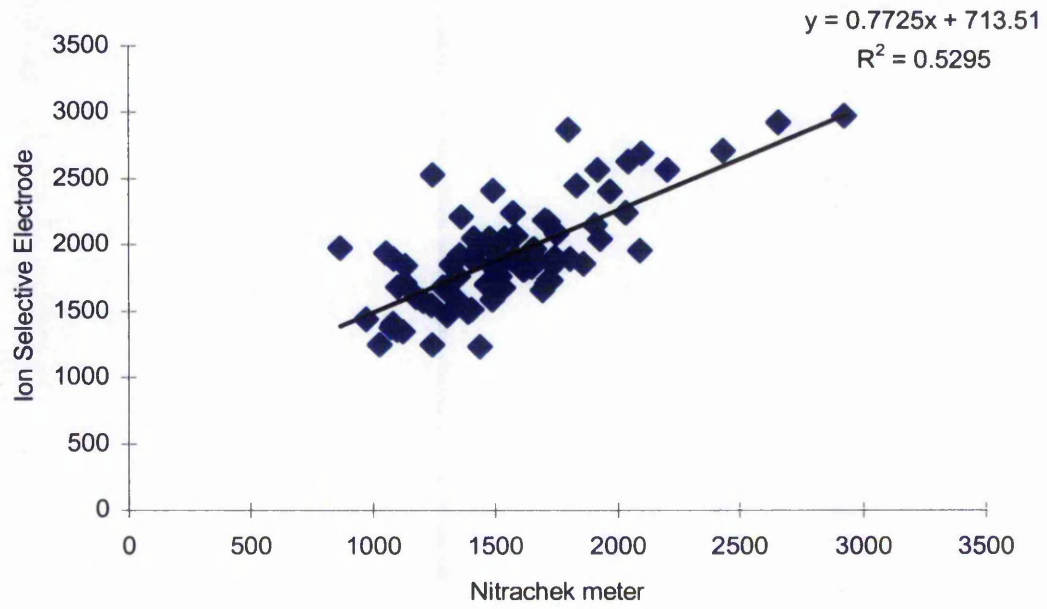


Figure 4.2.2 Relationship between the Ion Selective Electrode and Ion Chromatograph following analysis of sap extracts from soil raised lettuce cultivars.

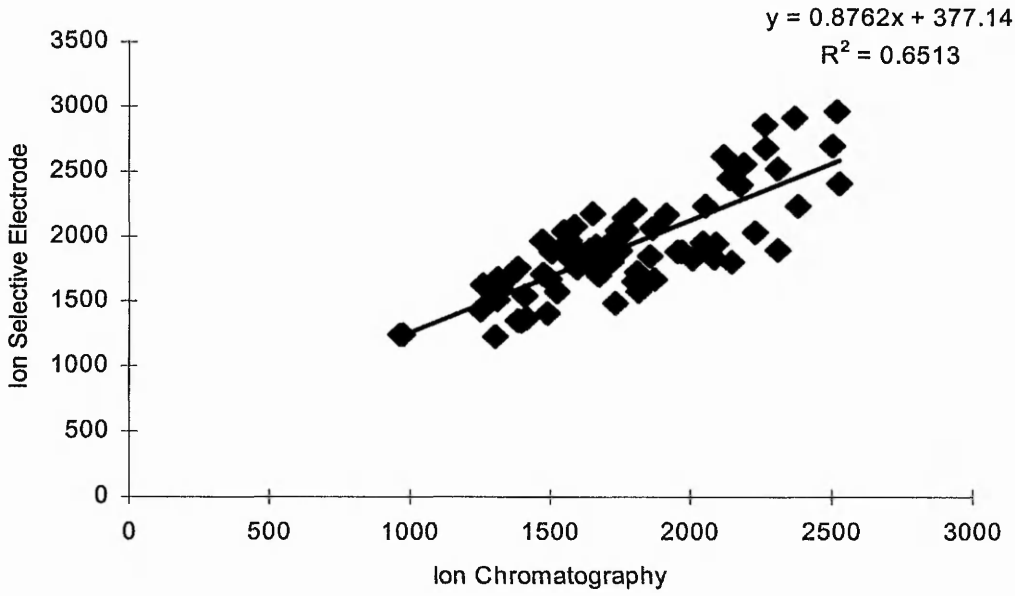


Figure 4.2.3 Measurement of nitrate in standard solutions using test strips from a variety of batches. Test strips from batch 1 were previously found to give accurate readings. Test strips from batch 2 were experiencing inaccurate readings.

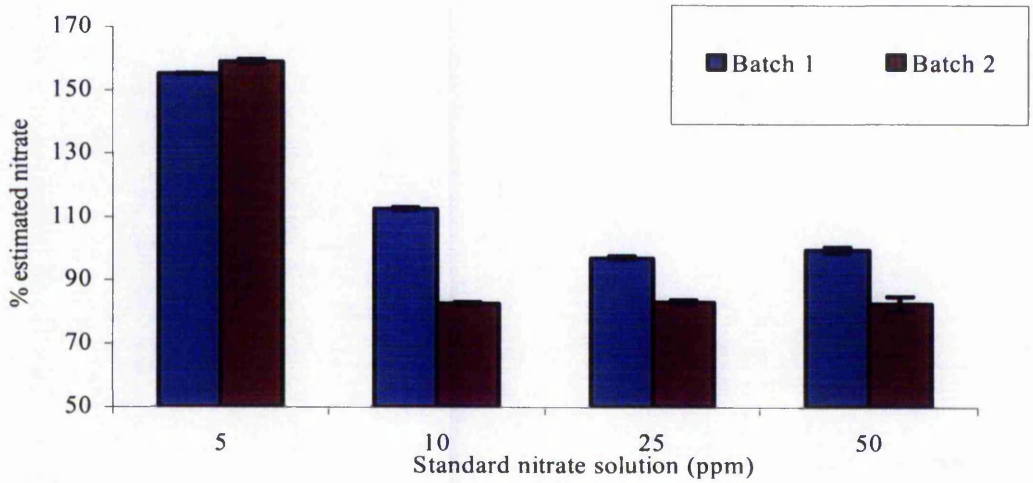
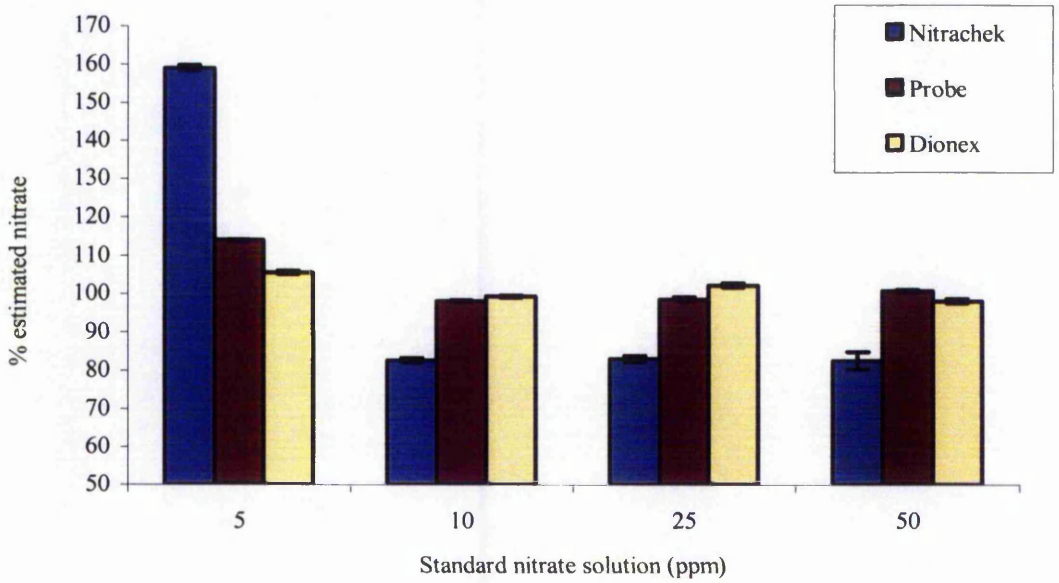


Figure 4.2.4 Measurement of nitrate in standard solutions using three methods of nitrate analysis. Nitrachek meter, Ion Selective Electrode (probe) and Ion Chromatograph (dionex). Test strips from batches 1 were used in this investigation.



4.3.4 Discussion

Statistical analysis using the nitrate results showed that there were significant differences between the three methods of nitrate analysis, with highly significant differences between the nitrate values obtained by the Nitrachek meter and those obtained with the Ion Selective Electrode ($p=0.001$). There were also significant differences between the nitrate values obtained by the Nitrachek meter and those obtained by the Ion Chromatograph ($p=0.01$). The results suggest that there may be limitations in the accuracy of the methods used to determine nitrate concentration. It is reasonable to suggest that the incorporation of a calibration step into the analytical procedure would increase the accuracy and reliability of the nitrate testing method. In both of the standard laboratory techniques, the Ion Selective Electrode and Ion Chromatograph, a calibration step was carried out prior to analysis of samples using standard nitrate solutions. During the analysis of lettuce sap by the two standard laboratory techniques, the calibration procedure was repeated to ensure maximum accuracy was maintained. On the basis of the repeated calibration procedure it was assumed that the Ion Selective Electrode and Ion Chromatography methods were satisfactorily detecting nitrate from lettuce samples. Calibration of the Nitrachek meter, using standard nitrate solutions, was not a requirement of the analytical procedure and was not therefore included during the analysis of lettuce sap. It was assumed that the pre-calibration step, as instructed in the Nitrachek user guide and involving insertion of a blank test strip prior to sample analysis, was sufficient to maintain the level of accuracy previously observed with the Nitrachek meter.

Previous analysis of lettuce samples with the Nitrachek meter showed excellent correlation between this method and the Ion Selective Electrode method, as previously detailed in this chapter. However, the results that are presented in this section do not show such a good correlation between the two methods. This may be attributed to a reduced level of accuracy and reliability in the rapid Nitrachek meter. The absence of a calibration step contributes to the application of the Nitrachek meter as a rapid, nitrate testing technique. The percentage nitrate recovery results, presented in figures 4.2.3 and 4.2.4, confirmed the suspicion that the Nitrachek meter was underestimating nitrate concentrations in lettuce sap. The results suggest that the Nitrachek meter is responsible for the variability between the nitrate values obtained by the three methods in this second study. Figure 4.2.4 indicated consistent percentage nitrate recovery, close to 100%, from standard nitrate solutions with both the Ion Chromatograph and Ion Selective Electrode methods. The percentage recovery of nitrate from standard nitrate solutions using the Nitrachek meter was considerably lower from these standard solutions, except in the 5ppm standard solution which showed considerable overestimation of nitrate.

It has been observed that the test strips may contribute to the inaccurate nitrate values obtained by the Nitrachek meter. Variability existed between the nitrate values obtained using test strips from different batches (figure 4.2.2). The batch to batch consistency of the test strips is a matter for consideration when considering the Nitrachek meter as an on-farm tool for nitrate determination. Although quality control measures should reduce the possibility of variation amongst batches of test strips, there is clearly a need for additional testing of individual batches prior to their use for nitrate analysis.

The results appear to imply that the test strips are a major factor contributing to the level of accuracy in nitrate determination using the Nitrachek meter. The design and construction of the Nitrachek meter means it is difficult to calibrate or repair parts of the equipment, unlike the Ion Chromatograph and Ion Selective Electrode methods. These methods allow replacement of nitrate detection components such as the probe head or anion detection column. However, it is possible to improve the accuracy of the Nitrachek meter by conducting calibration prior to analysis of samples using a range of standard nitrate solutions. Determination of a relevant correction factor together with its application would improve the reliability of the nitrate readings obtained by the Nitrachek meter.

Chapter 5

Factors affecting the nitrate concentration of lettuce in samples used for analysis.

5.1 Introduction

In May 1996, the UK Monitoring Programme for Nitrate in Lettuce and Spinach was undertaken in order to obtain information on the nitrate concentrations of UK crops (Anon, 1997b). The programme has been continued throughout 1997, 1998 and 1999 and the results of the surveillance programme have been detailed in Joint Food Safety and Standards Group Food Surveillance Information Sheet (Anon, 1999b). The survey was also intended to help in the negotiations on a review of the European Commission Regulation 194/97. The results of the 1998 and 1999 survey have been used to estimate dietary exposure for consumers of lettuce and spinach, and to assess the risk to health. Although the determination of crop nitrate concentration throughout the growing period is not a requirement for UK lettuce growers, there has been increased interest in recent years for a suitable method for to enable growers to determine the nitrogen status of lettuce plants. It is anticipated that growers and producers will need a suitable extraction and analytical technique to conduct their own tests for nitrate.

The Code of Good Agricultural Practise cited in the Assured Produce Protocol – Protected Lettuce, (Anon, 1998a), provides advice for protected lettuce growers concerning sampling procedures when harvesting lettuce for nitrate analysis. Sampling and analytical procedures are essential elements of due diligence. Sampling should be undertaken four times a year, on a seasonal basis. The prevailing sampling conditions are important since they can lead to unexpected nitrate concentrations. For instance, additional sampling should be carried out following a period of prolonged dull weather, as the nitrate concentration in the crop would be unusually high. The storage procedures are also important and there should be optimal conditions in order to minimise changes in the crop that may affect the nitrate concentration.

Growers are advised to send the samples to competent analytical laboratories that use validated methods of nitrate analysis (Anon, 1998a). The cultural advice in the Code of Good Practise stresses that the laboratory used must be accredited by UKAS and participate in FAPAS or a similar proficiency testing scheme. However, the European Commission has not adopted a prescribed method of nitrate analysis. The absence of a prescribed method for nitrate analysis has led to the adoption of a variety of extraction methods and analytical techniques by commercial laboratories in the UK. Furthermore, lettuce growers have reported wide variability in nitrate values in crop samples from one harvest, which were sent to different laboratories for analysis (Berrevoets, personal communication). Variation in the nitrate concentration of lettuce determined by different analytical laboratories may contribute to the natural nitrate variability that is common within a lettuce crop. Therefore, some of the differences in nitrate concentration within a lettuce crop may be attributed to the handling procedures and extraction methodologies adopted by the individual laboratory.

The variability in nitrate readings obtained by different laboratories probably reflects the diversity of extraction procedures used by individual analytical laboratories. It seems likely that the nitrate extraction technique is the main factor determining the sap nitrate concentration reported by individual laboratories. The majority of nitrate analysis previously published in analytical journals has involved extraction from dried plant material (Hunt and Seymour, 1985). For instance, dried plant material was used in a method adopted from a

study by Bremner and Keeney cited in Usher and Telling (1975), which involved nitrate determination by steam distillation. This extraction method, which is currently cited in the Assured Produce Protocol for Protected Lettuce, (Anon, 1998a), requires lettuce that had been dried in a forced-draft oven, maintained at 100 ± 2°C for eight hours. An example of the use of dried plant material in the potentiometric determination of nitrate is cited in the AOAC Official Methods of Analysis, (Anon, 1990). In this method, the sample was prepared in a forced air oven at 60°C until a constant weight was obtained.

Although the use of dried plant material is an established method for the determination of nitrate content (Cataldo *et al.*, 1975; Anon, 1990) it requires specialised drying apparatus and can be a time consuming procedure. In addition, calculations regarding nitrate concentration can only be made on a dry weight basis and can not be directly related to the nitrate content of fresh material. The use of freeze dried plant material while common to some laboratory technique prior to nitrate analysis (Cataldo *et al.*, 1975) would not be suitable in the development of an extraction procedure for on-farm testing of nitrate. The safety concerns surrounding the uncontrolled use of liquid nitrogen in a field environment would be considerable. However, sap nitrate from fresh material is a measure of the current nitrogen status of the crop, although it may not necessarily be closely related to dry weight or measurements determined from dry material (Ulrich and Hills cited in Prasad and Spiers, 1982). Some authors consider that nitrate analysis can be improved by analysing plant sap rather than dry plant tissue, and by expressing the results as sap concentrations rather than in amounts of nutrient relative to the dry matter of the present (Sciafe and Bray, 1977).

Among the methods for nitrate extraction using fresh plant material, there exists a wide degree of variability. For example, in a study by Hartz, Smith, LeStrange and Schulbach, (1993), the midribs of the youngest wrapper lettuce leaves were analysed directly without dilution or filtration. The nitrate content of the sap from the wrapper leaves of fresh lettuce was highly correlated with conventional laboratory analysis that used dry plant material. This study showed that lettuce sap extracted from fresh tissue can offer a reliable alternative method to previous nitrate extraction methods using dry plant material.

Although the method described in a study by Hartz *et al.*, (1993), does not involve the extraction in an aqueous solution, many extraction procedures do require blending with water followed by further dilution prior to nitrate analysis. Aqueous solutions are used in the extraction of nitrate from plant tissues and meat tissue due to the high solubility of nitrate in water (Usher and Telling, 1975). For example, laboratories in The Netherlands currently employ two methods for analysis of nitrate in lettuce – a "wet" method and a "dry" method. There is a good correlation between the two methods (de Kreij, personal communication). The wet method involves homogenisation of 50 gram of a chopped lettuce sample in a mechanical blender with 200 ml water for 30 seconds. The nitrate concentration of the lettuce is determined after filtration and reduction with a cadmium column and auto-analyser. The extraction technique applied has been found to be subject to further variation, and can involve either cold or hot water extraction, with or without the addition of an alkaline buffer, and periods ranging from 5 minutes to 2 hours have been noted (Usher and Telling, 1975). The inclusion of an extraction buffer in sample preparation has also been noted in a study by Beljaars, van Dijk and van der Horst, (1994). An extraction buffer is used to prevent the conversion of nitrate to nitrite. However, the incorporation of a buffer is generally only important if the determination of nitrite is also required.

In extraction procedures that use water to extract soluble nitrate, the volume of water used is an important consideration. In a protocol for the determination of nitrate in fresh vegetables and water, devised by Swiss researchers and taken from an original by Kunsch, Scharer and Temperli, (1990), a method for nitrate analysis in leafy vegetables has been outlined. The procedure involves taking opposite quarters from eight lettuce heads and macerating the whole sample in a food processor without the addition of water. To 40 grams of lettuce homogenate, 360 ml water are added and then blended for 30 seconds. In this procedure, no filtration is required and nitrate concentration is determined using Ion Selective Electrodes. The adapted method of Kunsch *et al.*, (1990), uses a combined sample of edible portions of individual lettuce heads. This method can be used to provide an estimate of the overall nitrate concentration of a lettuce crop. However, it may be necessary to determine the nitrate variability within a lettuce crop, and in this case nitrate analysis of individual lettuce heads are required.

There are clearly a wide variety of procedures that can be involved in sample preparation and nitrate determination. For instance, it is common for the sample to be chopped or homogenised in a blender, and water can be added at the blending step or the sample can be frozen to aid homogenisation (West Yorkshire Analysts, personal communication). It is common for European laboratories to use cold water extraction procedures (de Kreij, 1998), although the use of hot water extraction in UK laboratories appears to be popular (Lee A, personal communication). In addition to the use of hot water in the extraction process, it has also been noted that a boiling water bath can be incorporated. Extraction procedures that make use of boiling water baths are usually time-consuming and often complicated. For use alongside a rapid nitrate testing technique, it would be beneficial to develop a reliable, quick extraction procedure. In the search for an appropriate extraction procedure, the existing methods that involve complicated equipment and lengthy periods of boiling may appear unsuitable.

The relative complexity of extraction procedures for nitrate analysis is variable. The extraction procedures used by analytical laboratories in the UK are particularly complicated and time consuming. However, the procedures that are most commonly used in European countries such as The Netherlands (Korperl-Arkesteijn and van Elderen, 1994) and Switzerland (Kunsch, Scharer and Temperli, 1990), appear to be less complicated and on the whole a great deal simpler. In view of the large differences between laboratories in the UK in particular, it is clearly appropriate to question the reliability of nitrate results obtained from the laboratories. In order to determine a reliable nitrate extraction procedure, extraction methods that are currently used in analytical laboratories throughout the UK and Europe were chosen and included in the investigation. Initial studies involved small-scale investigations of the individual components of extraction procedures and their influence on nitrate concentrations in lettuce samples. For example, the use of hot water and cold water in nitrate extraction from lettuce samples was undertaken, and the efficiency of nitrate extraction was compared. It was not possible to investigate all the factors of nitrate extraction and, for the purposes of this preliminary study, only those factors thought to have a significant effect on the nitrate concentration of lettuce samples were investigated. Furthermore, time-constraints meant it was not possible to use large sample sizes in the small-scale investigations and for many of the studies a minimum of three heads were used. For the purposes of the small-scale studies, which are reported below, butterhead lettuce was used and was sampled from retail outlets. In addition to the small-scale investigations, the variability between four of the existing extraction procedures was studied in order to establish their effect on the sap nitrate

concentration of lettuce samples. In this study, butterhead lettuce was sampled from an existing glasshouse trial at HRI, Stockbridge House.

5.2 Small-scale methodology studies

As previously mentioned, a literature review of existing nitrate extraction procedures practised throughout the UK and Europe has shown a highly variable range of techniques. The following series of investigations were designed to incorporate all of the major features of nitrate extraction procedures, and to ascertain the influence of these features on the nitrate concentration of the lettuce sample.

5.2.1 The nitrate concentration in sap sample of lettuce following extraction procedures using different lengths of time during the blending process.

Introduction

The extraction technique described in a study by Blanco.D *et al.*, (1995) suggests homogenising lettuce in a Polytron mixer for five minutes. In contrast, it has been suggested that mixing lettuce tissue for approximately thirty seconds in a blender is sufficient time to thoroughly macerate the plant tissue and obtain complete nitrate extraction (Kreij, Personal Communication; Burns, Personal Communication). In a separate study by Hunt and Seymour, (1985), the plant material was shaken in aqueous solution for thirty minutes. The following investigation compared the effect on nitrate concentration from lettuce extracts of one minute and ten minute blending periods. It should be noted that low sample sizes were used throughout the small scale methodology studies and therefore the findings may not be conclusive.

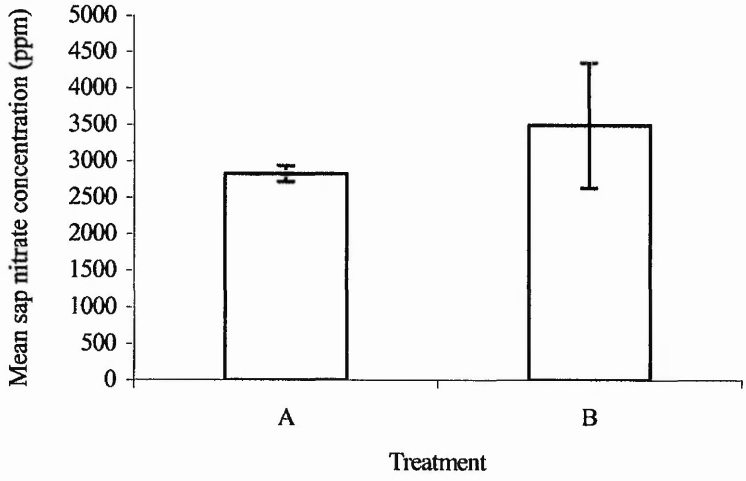
Materials and methods

Butterhead lettuce were randomly selected from a retail outlet and weighed to ensure uniformity in size. The lettuce was halved, re-weighed and labelled "A" and "B". Each half of lettuce labelled "A" was extracted in an equal volume of cold, de-ionised water to weight of lettuce and blended for approximately 1 minute prior to filtration using muslin cloth. Lettuce labelled "B" was extracted in an equal volume of cold, de-ionised water to weight of lettuce and blended for approximately ten minutes prior to filtration using muslin cloth. Sap extracts from both treatments, "A" and "B", were then analysed for nitrate concentration using the nitrate probe, as detailed in chapter 4 section 4.23.

Results

The mean nitrate concentrations of lettuce sap from treatment "A" and treatment "B" are shown in figure 5.1. Lettuce extracted by blending in water for one minute resulted in a sap nitrate concentration of 2825 ppm nitrate compared to a mean nitrate concentration of 3488 ppm following blending for ten minutes. Despite the clear difference between sap nitrate concentration, t-test analysis did not reveal this variation to be significant ($t = 1.88$, $p=0.1$). It is clear from figure 5.1 that there was greater variability in sap nitrate concentration of lettuce within treatment "B" compared to the variability in sap nitrate concentration within treatment "A".

Figure 5.1 Mean nitrate concentration of summer lettuce sampled from a retail outlet. Nitrate analysis conducted using the nitrate probe. Treatment "A" involved blending for one minute, Treatment "B" involved blending for ten minutes.



Discussion

The results clearly show that the duration of blending influenced the nitrate concentration of the sap extract. The greater variability in nitrate concentrations from treatment "B" may have been partly due to one or more anomalous values taken from the sample or due to the low number of replicates used in the investigation. However, treatment "B" extracts were taken from the same lettuce as treatment "A" extracts and it could be assumed that the nitrate concentration of each half would be similar. Furthermore, anomalies between lettuce samples would be reflected in the nitrate values obtained in both treatments.

From the results in figure 5.1 it could be tentatively suggested that the higher incidence of nitrate variability within treatment "B" compared with treatment "A" was due to the increased blending period in treatment "B", and that increasing the blending period from one to ten minutes improves the efficiency of nitrate extraction from lettuce.

5.2.2 The nitrate concentration in sap samples of lettuce extracted for different time periods.

Introduction

The prolonged storage of samples at –20°C for periods of fifty to eighty days prior to nitrate determination has been shown to have little effect on nitrate concentration (Khakural and Alva, 1996). However, the possible effects of nitrate reduction and/or leaching on the nitrate concentration of extracts stored at room temperature prior to analysis have received little attention.

Materials and methods

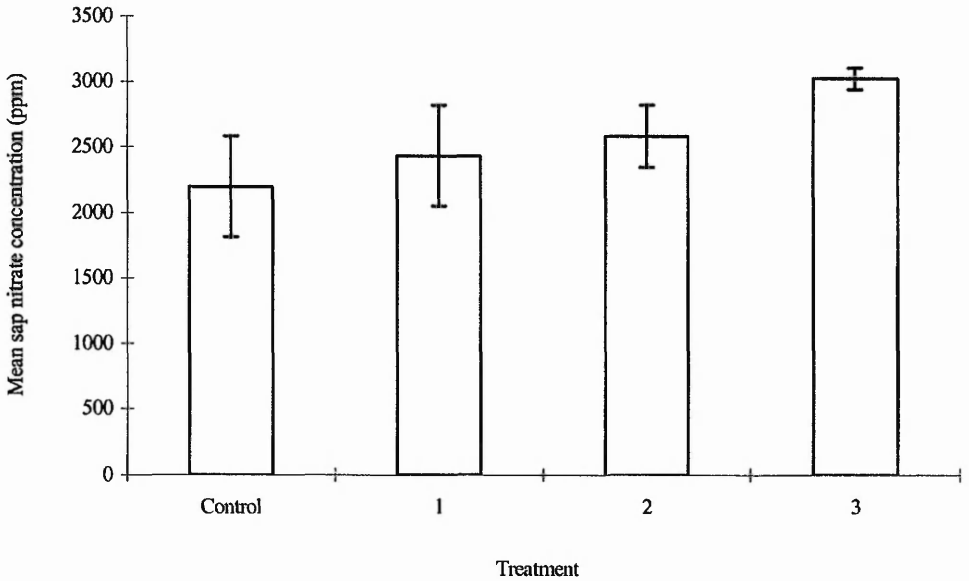
Three butterhead lettuce were randomly selected from a batch at a local retail outlet and weighed to ensure uniformity in size and quality. Sap from was extracted in approximately equal volume of cold, de-ionised water to weight of each lettuce and blended for approximately 1 minute. The slurry was then filtered through four layers of muslin cloth and the extract was collected in a glass beaker. Analysis was conducted with the nitrate probe according to the analytical procedure detailed in chapter 4 section 4.23. A sample of the lettuce sap was analysed immediately, at time zero. Samples of the sap were then analysed at one hour intervals to establish three treatments. In treatment 1, lettuce sap was analysed after one hour, in treatment 2 the sap was analysed after two hours, and in treatment 3 lettuce sap was analysed after three hours. The mean nitrate concentrations of the lettuce sap following each treatment are presented in figure 5.2.

Results

In figure 5.2 it is clear that there was a trend of increasing nitrate concentration in lettuce sap following one hour, two hours and three hours intervals extraction time. Treatment three, with an extended extraction period of three hours, resulted in the highest sap nitrate concentration at 3022ppm nitrate. Following one-way analysis of variance for nitrate concentration following the four standing time treatments, the nitrate concentration in lettuce sap from treatment three was found to be significantly higher compared to the nitrate concentration of lettuce sap from treatment one ($p=0.01$). Although the nitrate concentration increased in lettuce sap from 2193 ppm to 2431 ppm after one hour, and from 2431ppm to 2582ppm after two hours, the difference in sap nitrate concentration between the extraction times was not found to be significant ($p=0.05$).

Figure 5.2 The mean nitrate concentration of summer lettuce sampled from a retail outlet following a time course at one hour intervals. Control treatment at time zero, treatment one after a one hour interval, treatment two after a two hour interval and treatment three after a three hour interval. Nitrate concentrations were determined with the nitrate probe.

LSD = 1672, (p=0.001); LSD = 1078, (p=0.01), LSD = 765, (p=0.05), n=3



Discussion

The nitrate variability within the treatments diminished as the time period for extraction increased. This suggests that the process of nitrate leaching into solution was more effective with increased extraction time and resulted in an overall improvement in nitrate extraction. These results point towards additional investigations to determine the minimum extraction period to obtain optimum nitrate extraction.

5.2.3 Comparing the nitrate concentration of lettuce sap using different volumes of water in the nitrate extraction procedure

Introduction

As previously mentioned in this chapter, a range of nitrate extraction techniques exist each using a variety of fresh plant material : water extraction ratios. A review of current literature and unpublished extraction protocols has revealed extractant dilution ratios ranging from 1:1 fresh plant tissue : water, (Burns, Personal Communication), to 0.1:50 freeze-dried plant tissue : water, (Hunt and Seymour, 1985). In the following study, dilution ratios of 1:2 (treatment A) and 1:1 (treatment B) were used to determine the effect of dilution ratio on the nitrate extraction procedure.

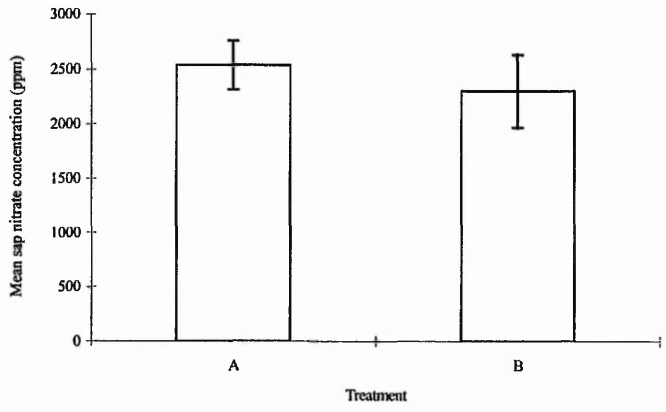
Materials and methods

Three butterhead lettuce were randomly selected from a retail outlet and each head was weighed to ensure uniformity in size. The untrimmed heads were cut in half and re-weighed, with each half being labelled "A" or "B. Lettuce labelled "A" was extracted in approximately twice the volume of water to weight of lettuce, and lettuce sap was extracted according to the procedure detailed in chapter 4. Lettuce labelled "B" was extracted in approximately equal volume of water to weight of lettuce, and lettuce sap was extracted using the procedure detailed in chapter 4 section 4.22. Lettuce sap from both treatments was analysed immediately using the nitrate probe method of analysis, as detailed in chapter 4 section 4.23.

Results

The mean nitrate concentration of lettuce sap from treatment "A" and the mean nitrate concentration of lettuce sap from treatment "B" is shown in figure 5.3. The mean nitrate concentration of lettuce extracted in an equal volume of water to weight was 2300ppm nitrate, compared to the mean nitrate concentration of lettuce extracted in twice the volume of water to weight which was 2536 ppm nitrate. Although the nitrate concentration of sap from treatment A was higher compared to the nitrate concentration of sap from treatment B, t-test analysis of the sap nitrate concentrations for the two treatments did not reveal a significant difference ($t = 1.45$, $p = 0.01$).

Figure 5.3 Mean nitrate concentration of summer lettuce sampled from a retail outlet and extracted in twice volume of water to weight of lettuce, treatment "A", and equal volume of water to weight of lettuce, treatment "B". Nitrate concentration determined using the nitrate probe.



Discussion

The nitrate concentration of extracts from treatment A and B were not significantly different. While it is not possible to draw firm conclusions from the results, due to the limited number of replicates and dilution ratios used in the small-scale investigation, it could be suggested that improved nitrate extraction occurred at the increased dilution ratio as seen in treatment A. However, since nitrate is extremely soluble in water and no significant difference in nitrate concentrations between treatment A and B was observed, it could be suggested that optimal nitrate extraction may be achievable at lower dilution ratios, for instance the 1:1 dilution ratio in treatment B.

5.2.4 The sap nitrate concentration of fresh lettuce compared to the sap nitrate concentration of frozen lettuce following nitrate extraction and analysis.

Introduction

As previously mentioned, it has been suggested that the process of freezing lettuce samples may aid homogenisation (West Yorkshire Analysists, Personal Communication). The following investigation was conducted to determine the effect of freezing lettuce samples for twenty-four hours at –20 °C on the sap nitrate concentration of butterhead lettuce.

Materials and methods

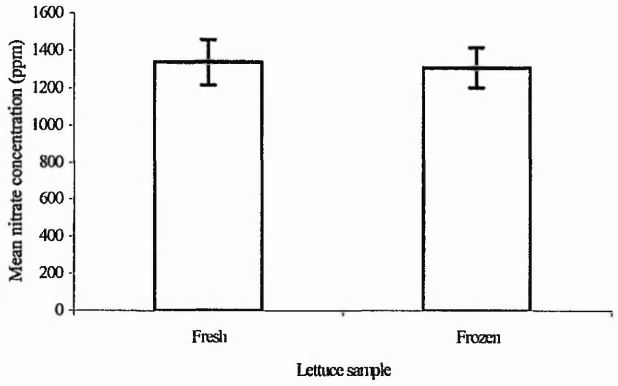
Five butterhead lettuce were sampled from a retail outlet and selected on the basis of uniformity in size, weight and quality. The untrimmed heads were cut in half and weighed to ensure each half was approximately equal in size. Each half of the lettuce head was bagged and labelled "A" or "B" respectively. The lettuce samples labelled "A" were stored over night in cold storage at 4°C, and the lettuce samples labelled "B" were stored over night at –20 °C in order to freeze the tissue. After 24 hours, all the samples were removed from storage and sap nitrate extraction was carried out on each sample individually according to the method detailed in chapter 4 section 4.22. The nitrate concentration of the lettuce sap was

determined using the nitrate probe, following the method described in chapter 4 section 4.23. The mean nitrate concentrations determined from the fresh and frozen lettuce sap are shown in figure 5.4.

Results

T-test analysis of the sap nitrate concentration of lettuce from the two treatments showed no significant variation between the mean nitrate concentration of fresh lettuce sap compared to the mean nitrate concentration of frozen lettuce sap ($t = 0.56$, $p = 0.01$). This result has been found to be in agreement with similar investigations that were conducted at a West Cranleigh nursery. In this study, a frozen extraction method was compared to an extraction method using fresh lettuce. The initial results from this study showed no significant variation in sap nitrate concentration between these two methods of analysis (Page 33, appendix).

Figure 5.4 The mean nitrate concentration of fresh lettuce extract, sampled from a summer lettuce crop, following storage at 4 °C and frozen lettuce sap stored at – 20 °C. The nitrate extraction procedure detailed in Chapter Four was followed and nitrate concentrations were determined using the nitrate probe.



Discussion

The results suggest that freezing lettuce samples does not improve the efficiency of nitrate extraction. Indeed, the nitrate concentration values were not significantly different. The results suggest that freezing lettuce at –20 °C prior to extraction does not lead to nitrate reduction and is therefore not detrimental to the nitrate extraction procedure.

5.2.5 The nitrate concentration of lettuce subjected to hot water nitrate extraction compared to the nitrate concentration of lettuce sap subjected to a cold water extraction procedure.

Introduction

The most significant difference between the variety of nitrate extraction techniques used throughout UK and European laboratories is in the use of hot water versus cold water as an extractant. It is widely regarding in The Netherlands that cold water extraction is the most reliable and convenient method for nitrate extraction from leafy vegetables and lettuce (Kreij,

Personal Communication). However, many UK laboratories use hot water extraction techniques (Usher and Telling, 1975; Lee, Personal Communication). The wide variances in nitrate values obtained from laboratories within the UK suggests that the main factor influencing nitrate concentration in extracts is in the use of hot water versus cold water in the extraction procedure. The following investigation involved both cold and hot water extraction techniques.

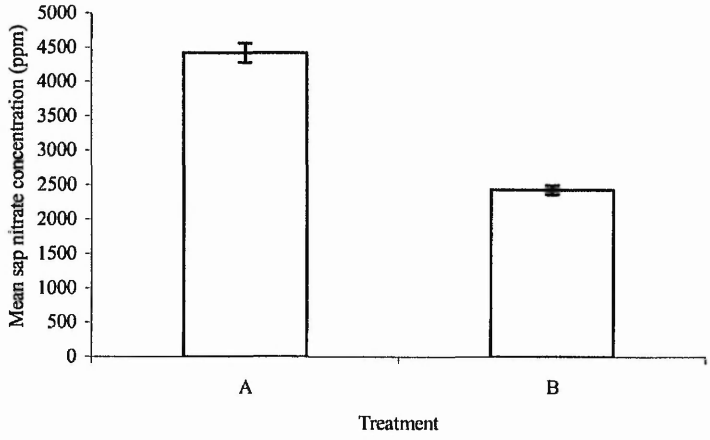
Materials and methods

Ten butterhead lettuce were sampled from a winter glasshouse lettuce trial and weighed to ensure uniformity of size and quality. The method chosen in this study was adapted from an unpublished procedure used by members of the MAFF (currently DEFRA)-LINK consortium (Palmer, Personal Communication). Five lettuces were labelled "A" and the remaining five lettuces labelled "B". From each sample of chopped lettuce labelled "A" 50 gram was taken and added to 500ml hot deionised water. The beaker and contents were placed in a water bath, set to approximately 60°C, and left for one hour with frequent stirring of the mixture. After one hour, the slurry was filtered through Whatman No. 40 paper and allowed to cool until nitrate determination was carried out. Chopped lettuce from the sample labelled "B" was extracted in an approximately equal volume of cold, deionised water using a blender. The slurry was filtered through muslin cloth. The extracted sap from treatment "A" and "B" was analysed for nitrate concentration using the nitrate probe, as described in chapter 4.

Results

The mean nitrate concentrations of lettuce sap from treatments "A" and "B" are shown in figure 5.5. T-test analysis of the sap nitrate concentration of lettuce from the two treatments revealed a clear difference in sap nitrate concentration following the use of hot water treatment compared to the cold water treatment ($t = 31.24$, $p = 0.01$). Lettuce sap, extracted by the cold water method, resulted in a mean nitrate concentration of 2384 ppm compared to lettuce sap extracted by the hot water method, which resulted in a mean nitrate concentration of 4419 ppm.

Figure 5.5 Mean nitrate concentration of winter lettuce extracts. Treatment "A", following extraction in hot, deionised water using a 60°C water bath and treatment "B", following extraction in cold deionised water. Nitrate concentrations were determined using the nitrate



Discussion

The results clearly show that the hot water extraction technique greatly increases the efficiency of nitrate extraction from lettuce samples. The solubility of nitrate would have been greatly improved at the increased temperature and extended extraction period.

5.3 A comparison of three extraction procedures currently used in UK laboratories

5.3.1 Introduction

As previously stated, a wide variety of nitrate extraction techniques are currently in use within UK and European laboratories. In this investigation, three nitrate extraction methods were studied. Two procedures used in this study were based on information provided by D A Lee, at HRI Stockbridge House. Method 1 was based on an unpublished procedure, (Collins, personal communication), and method 2 was based on an unpublished procedure provided by the consortium partner "Tesco", (Palmer, personal communication). The third technique, method 3, was initially selected from the nitrate variability studies and was adapted from an unpublished nitrate extraction procedure previously used at HRI, Wellesbourne (Burns, 1999).

5.3.2 Materials and Methods

Butterhead lettuce were used in a series of investigations to determine the variability between nitrate extraction procedures. In the first investigation, forty butterhead lettuce were sampled

from an existing glasshouse trial at HRI Stockbridge House. The lettuces were randomly selected from the trial and the fresh head weights were recorded to ensure that a uniform sample was attained. The untrimmed lettuce heads were weighed, bagged and cold stored overnight. Three nitrate extraction procedures were investigated using a sample size of ten lettuces for each method. The extracted sap from individual lettuce heads was stored at -20°C until nitrate determination was carried out. Two different methods of nitrate analysis were used for the determination of nitrate from lettuce sap. The nitrate determination techniques were the Ion Selective Electrode (nitrate probe) and the Nitrachek meter. The methods used to determine sap nitrate are described in detail in chapter 4. The three nitrate extraction procedures were carried out following the methods described below.

Method 1:

On receipt of the sample of ten lettuces, each head was cut in half and the base was removed. The lettuce was chopped and macerated using a blender to form a slurry. It was suggested in the protocol that blending could be aided at this stage by freezing, however during this investigation only fresh plant material was used. A 10g aliquot of slurry was taken from the sample. Nitrate was extracted from the slurry in 100ml recently boiled deionised water, and filtered through Whatman No. 40 paper. The extracted sap was analysed for nitrate content immediately using the nitrate probe and Nitrachek meter methods.

Method 2:

On receipt of the lettuce sample, individual untrimmed lettuce heads were chopped. A representative 50g sample was taken and placed in 500ml of water, and this was placed on a boiling water bath for 1 hour with frequent stirring. The slurry was allowed to cool to room temperature, and filtered through Whatman No. 40 paper, and analysed for nitrate immediately or stored at –20 °C until nitrate analysis.

Method 3:

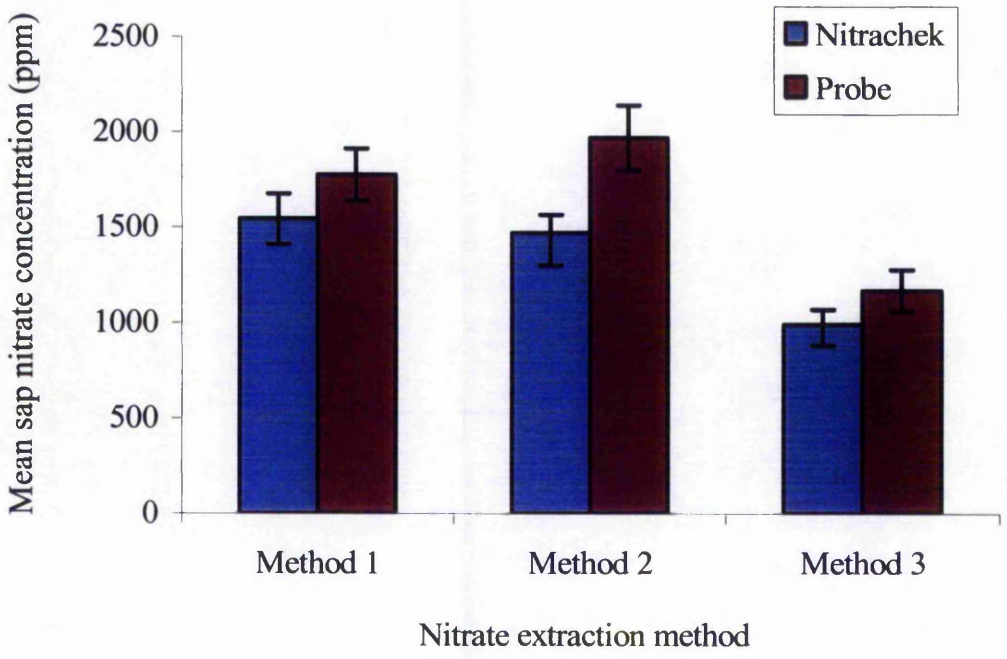
On receipt of the sample, the lettuces were stored at -20°C for 24 hours. The frozen, untrimmed lettuce heads were chopped and blended in approximately equal volume to weight ratio of cold, deionised water. The lettuce were blended for approximately two minutes, or until a homogenous slurry was obtained. The slurry was then filtered through four layers of muslin and analysed for nitrate immediately or stored at –20 °C until nitrate analysis.

5.3.3 Results

Results of the mean nitrate concentrations of lettuce sap, obtained by three different methods of nitrate extraction, are shown in figure 5.6. The nitrate concentration of lettuce sap was determined using the Nitrachek meter and the nitrate probe.

Figure 5.6 The mean nitrate concentration of summer butterhead lettuce extracts obtained by three different methods of nitrate extraction. Nitrate concentrations were determined with the Nitrachek meter and nitrate probe, and following three methods of nitrate extraction.

LSD = 881.8, (p=0.001); LSD = 680.0, (p=0.01); LSD = 511.6, (p=0.05)



The mean nitrate concentrations obtained following nitrate extraction using the three chosen methods and analysis with both the Nitrachek meter and nitrate probe techniques were clearly variable. Mean nitrate concentrations ranged from 991 ppm to 1545 ppm in butterhead lettuce following analysis with the Nitrachek meter, and from 1166 ppm to 1970 ppm following analysis with the nitrate probe (figure 5.1). Two-way analysis of variance was conducted on the mean nitrate concentrations of lettuce obtained by the three extraction methods using the nitrate probe and Nitrachek meter. Highly significant differences existed between the nitrate concentrations obtained by method two and the other two methods of nitrate extraction (p=0.001). Significantly lower mean nitrate concentrations were obtained following nitrate extraction method three compared to methods one (p=0.05), method two (p=0.01). Nitrate extraction method one did not produce significantly different mean nitrate concentrations compared to nitrate extraction method two. The mean nitrate concentrations obtained using extraction method one were 1545 ppm and 1775 ppm, following nitrate determination with the Nitrachek meter and nitrate probe respectively. The mean nitrate concentrations obtained using extraction method two corresponded well to the mean nitrate concentrations resulting from extraction method one, with mean nitrate concentrations at 1470 ppm and 1970 ppm, following nitrate determination with the Nitrachek meter and nitrate probe respectively.

5.3.4 Discussion

The results from the initial small-scale study indicate that the methodology of sap extraction from lettuce can significantly affect sap nitrate concentrations. For example, the sap nitrate concentration of lettuce subjected to hot water extraction was noticeably higher compared to the sap nitrate concentration of lettuce following cold water extraction (figure 5.5). Although not significantly different, the volume of water used to extract sap and the weight of each sample was shown to influence the sap nitrate concentrations obtained (figure 5.3). The efficiency of nitrate extraction may therefore be improved when higher volumes of water are used in the extraction procedure. The small-scale studies also showed improved efficiency of nitrate extraction as a result of increased and vigorous blending of the sample (figures 5.1 and 5.2). This may be due to the process of macerating the lettuce tissue and the subsequent release of nitrate during the blending process. Additionally, a lengthened extraction time would lead to increased leaching of nitrate into the solution leading to higher sap nitrate concentrations. The results from the small-scale study point towards further investigations incorporating all the factors known to improve the efficiency of nitrate extraction.

The investigation to determine the effect of three different extraction methods on the nitrate content of sap samples showed significant variation in the sap nitrate concentrations between the three methods (figure 5.6). The sap nitrate variation between the three extraction methods can be partly attributed to the different volumes and temperatures of water used to extract nitrate as specified in each method. The efficiency of nitrate extraction may have been influenced by the use of a high volume of water. Method three required the lowest volume to weight ratio, with 1 ml water to 1 gram frozen lettuce, and resulted in the lowest sap nitrate concentrations which may be attributed to a reduced efficiency of extraction. The results obtained in methods one and two, showing similar sap nitrate concentrations, were expected since equal volume to weight ratios were used. It seems likely that there is an optimal volume of water to weight of sample needed to ensure an efficient extraction of nitrate is achieved (Kunsch *et al.*, 1990). Further studies should be aimed at extracting small amounts of lettuce in large volumes of water.

Clearly, there was a significant increase in the efficiency of nitrate extraction following hot water extraction (as seen in method two, figure5.6, and shown in figure 5.5). However, the increase in sap nitrate concentration of lettuce extracted in hot water, may be partly attributed to the high volume of water to low weight of lettuce used. The results presented in this chapter support previous studies conducted by fellow consortium members and suggest that the higher the temperature the more nitrate is extracted (Lee A, personal communication).

The three extraction methods differed primarily in the volume of water used to extract nitrate, the weight of lettuce used and the water temperature. An additional difference between the methods of extraction concerned maceration of lettuce tissue, as in methods one and three, compared to chopping of the tissue, as in method two. It could be suggested that maceration would encourage optimal nitrate extraction due to disintegration of cellular tissue and release of stored nitrate during the blending process (Korperl-Arkesteijn *et al.*, 1994). Chopping alone, as in method two, may be an insufficient technique with which to achieve the necessary breakdown of cellular tissue to release all of the stored nitrate. Improving the efficiency of nitrate extraction by increasing the blending period, may also give more realistic nitrate results than an extraction procedure that required a relatively short period of blending.

Method two was selected due to the one hour time period needed for the extraction of nitrate. In comparison to method three, which required the shortest time period for extraction, at approximately two minutes. The results from this study show that a longer extraction period does not significantly influence the efficiency of nitrate extraction. This finding was in agreement with results from the previous small-scale studies, which showed that there was no increase in sap nitrate concentration with increased blending and extraction time from zero to one and then to two hours (Figure 5.2). While increasing extraction times in excess of two hours or more may improve the efficiency of nitrate extraction, as shown in figure 5.2, consideration should be given to the possibility of nitrate reduction that could occur and lead to inaccurate nitrate readings (Beljaars *et al.*, 1994).

As part of the small-scale studies, extracts from frozen lettuce were compared to extracts from fresh lettuce, with no significant differences being observed between the sap nitrate concentrations (Figure 5.4). In spite of these results, it has been suggested that freezing aids the homogenisation process thus, increasing the release of cellular nitrate and increasing the efficiency of nitrate extraction (West Yorkshire Analysts, personal communication). However, the low sap nitrate concentration in method three may does not support this finding (Figure 5.6). The nitrate results obtained from lettuce sap following extraction method three may be attributed to a combined effect of low water volume together with short blending time in the extraction procedure. Improvement in the efficiency of nitrate extraction by freezing the tissue sample may occur only when additional factors, also known to improve the efficiency of nitrate extraction, are included in the extraction technique. In method three, these additional factors were not present.

The combined effect of a high volume of water together with hot water during sap extraction, as seen in the small-scale study, significantly improves the efficiency of nitrate extraction. From the results, it could be suggested that hot water extraction together with vigorous blending or boiling of lettuce in a high volume of water for long periods of time would increase the efficiency of nitrate extraction. In contrast, the extraction of lettuce in a low volume of cold water, blended for a limited period only may result in incomplete extraction of nitrate, as seen in method three. However, the extraction procedure required for use alongside a rapid nitrate testing technique, should not be time consuming or complicated. Furthermore, there is relevance in developing an extraction technique that is consistent with the extraction processes that naturally occur within the human gut. It is unlikely that the concentrations of nitrate obtained in these studies would be obtained during digestion of lettuce and within the gut, since temperature conditions do not exceed 37°C (body temperature).

To this end, the use of extreme conditions in the extraction procedure, such as boiling for one hour in large volumes of water, may not prove to be suitable. Therefore, while it is important to obtain accurate estimates of the nitrate concentration in a lettuce sample, it is equally important in the development of a rapid nitrate testing technique that the chosen method can be replicated in field situations and on grower holdings.

Further work should be aimed at maximising the efficiency of nitrate extraction from samples of lettuce. These investigations would involve a comparison of extraction procedures that include a combination of factors known to increase the efficiency of nitrate extraction. An optimum extraction procedure might utilise a low fresh weight of lettuce, with a high volume of hot water together with vigorous blending of the slurry.

Chapter 6

6.0 Discussion

The preliminary focus of the work undertaken at the Nottingham Trent University, which contributed to the MAFF-LINK project, was to investigate factors affecting nitrate variability in lettuce. The factors studied were light level, soil nitrate concentration, plant density and lettuce cultivar using soil raised and hydroponically raised lettuce. However, as work proceeded the methods for extraction and analysis of nitrate came increasingly under scrutiny, and it became clear that no standard procedure for nitrate extraction and analysis existed among the Public Analysis laboratories in the UK. In order to clarify the situation regarding nitrate analysis and to develop a rapid nitrate testing technique suitable for on-farm nitrate determination using glasshouse lettuce as a model crop, analytical procedures were investigated. Since it was assumed at the start of the work that a standard protocol for the extraction of nitrate from lettuce would be in use in UK laboratories, this area of research was conducted in the later part of the project through a series of small-scale studies.

Although EC Regulation 194/97 states that "*...nitrate analysis should be carried out by competent analytical laboratories...*" the Commission has not specified a specific method of analysis. In spite of the absence of a specified procedure for nitrate analysis, the techniques employed for nitrate analysis throughout UK and European laboratories are generally accurate and reliable. It is the extraction methods used, prior to the determination of nitrate, that appear to produce significant variability, and are therefore in serious doubt. In order to ensure consistency among analysts, the Public Analyst laboratories which currently take part in the UK Monitoring Programme for Nitrate in Lettuce and Spinach must achieve satisfactory performances in the nitrate rounds of the Food Analysis Performance Assessment Scheme (FAPAS). FAPAS is a proficiency test run by the Central Science Laboratories (CSL) Food Science Laboratories and is designed to determine competency and credibility in various areas of analytical work. However in a recent MAFF report (Food Surveillance, 1997), it was noted that only two Public Analysts participating in FAPAS had gained accreditation in the area of nitrate analysis (Anon, 1997b).

While the importance of selecting the correct analytical technique was recognised as a significant factor in the development of rapid testing methods, the relative importance of extraction procedures on nitrate determination was not anticipated. Furthermore, a thorough study of the existing extraction techniques was not carried out at the start of the project due, in part, to time constraints and also limited access to the protocols used by the analytical laboratories. It is important to emphasise the distinction between identifying a rapid technique for nitrate analysis and establishing an accurate method for nitrate extraction from lettuce sap. Although determination of an appropriate extraction procedure was not an initial aim of the research investigation, the importance of investigating these issues are clear and have therefore been deemed appropriate and necessary for inclusion in the discussion. However, due to the late inclusion of these studies, only small-scale investigations were carried out.

On the basis of the limited availability of extraction protocols at the start of the research investigation, an extraction technique was used based on a method previously used for nitrate extraction at HRI, Wellesbourne (Burns, 1999). This extraction procedure was followed throughout the investigations, which are reported in chapters two and three, and involved the

extraction of sap from lettuce using roughly equal volume of cold, de-ionised water to weight of lettuce. The method was later found to be similar to the extraction procedure currently used by laboratories in The Netherlands (de Kreij, 1998) and in Switzerland (Kunsch *et al.*, 1990).

Significant variability in nitrate values between analytical laboratories have been previously reported by growers, and were also found between the commercial laboratories involved in the MAFF-LINK project. The primary reason for these differences has been attributed to the nitrate extraction technique used by the analytical laboratories (Lee, unpublished 1998). An additional investigation was co-ordinated by members of the MAFF-LINK project to compare several of the nitrate extraction techniques and determine their influence on sap nitrate concentration. Subsequent to this investigation, a series of separate small-scale experiments were undertaken as part of this study, in order to compare the efficiency of nitrate extraction from lettuce using a range of methods.

The findings of the unpublished MAFF-LINK study co-ordinated by Lee, (1999), found large differences in mean nitrate content of glasshouse lettuce between individual laboratories. Lee, (1999), concluded that the choice of laboratory could have a much larger influence on the nitrate content of the lettuce crop compared to the agronomic practises detailed in the Code of Good Practise. From these results it was hypothesised that the variations in nitrate concentration were due to the different sampling techniques, and more importantly, to the different sap extraction procedures used by the laboratories. Additional factors, including the volume of water and length of time taken for extraction, were also considered in the small-scale investigation. However, in view of the low number of replicates used in the small-scale studies, the results may be inadequate to use as a guide for developing an efficient nitrate extraction procedure. Indeed, the principal outcomes of both the MAFF-LINK investigation and small-scale studies are that further work is essential in order to establish a reliable and repeatable method, which achieves maximum efficiency of extraction.

Hot water extraction, using a high volume of water to low weight of sample, may be the most efficient method for extracting nitrate from lettuce and this has been referred to in chapter 5. It may be possible to quantify relative efficiencies of extraction methods, for instance those that involve cold water extraction or extraction using low volumes of water. The use of "correction factors", as seen with the nitrate meters, would ensure that the nitrate values obtained by alternative, and possibly less efficient, extraction methods were comparable to those achieved by following a "standard" extraction procedure. The inclusion of "correction factors" would improve the overall accuracy of nitrate determination regardless of the extraction procedure followed by an individual analytical laboratory. In this way, the influence of specific procedural steps within an extraction procedure on nitrate extraction efficiency, for example by using cold water rather than hot water, could be removed.

The results indicated that there were factors in the extraction procedure, in addition to the use of hot water, that affected the nitrate concentration in lettuce sap. For example the condition of the lettuce sample used during the extraction procedure varied between extraction methods. Two of the chosen methods required fresh lettuce, that was neither frozen nor freeze-dried, to be chopped prior to extraction in hot water. In contrast, a third method was carried out on frozen, chopped lettuce. The practise of freeze drying lettuce material, as noted in some extraction procedures, may have been incorporated into extraction methods in order to reduce the degradation of the sample during transportation or storage (Hunt and Seymour, 1985). Although it is clearly important to preserve the sample to ensure an accurate nitrate

result, freeze-drying is an expensive procedure and there are additional safety considerations when using liquid nitrogen. With these points in mind, it would be highly inappropriate to recommend an extraction procedure that involved the freeze-drying of lettuce samples on growers holdings.

A standard nitrate extraction procedure may be achieved by an adaptation to the hot water extraction method detailed in chapter five. The scientific basis behind hot water extraction and the incorporation of water baths in nitrate extraction procedures is unclear although there could be an assumption that higher temperatures aid leaching of substances into solution and increase nitrate solubility. The use of hot water, and particularly the vigorous activity of boiling, would disrupt cellular membranes of organelles such as vacuoles where much of the nitrate is contained. Since sap nitrate concentrations are appreciably higher following hot water extraction compared to cold water extraction, a thorough investigation of the differences between these types of extraction procedures is long overdue.

Similar discrepancies in extraction techniques have been noted by researchers involved in studies using peat-free growing media (Dickinson, 1995). In these instances, the extent of variation regarding extraction procedures has been the focus of discussion and has resulted in attempts at international standardisation (Schumilewski & Gunther, 1988; Gabriels, van Keirsbulck & Verdonck, 1991; Sonneveld & De Kriej, 1995; Baumgarten, 2000). By highlighting the variation that exists between the methods of nitrate extraction from lettuce, attention may be given to the determination of a standard procedure for use with a variety of leafy vegetables and salads. The establishment of a standard nitrate extraction procedure could be carried out by European Union member states, in much the same way that previous certification for methods of soil analysis have been achieved through the Comité Européan de Normalisation (CEN). It is important that when setting standard protocols for nitrate determination, all the countries that contribute to lettuce production within the European Union are involved. However, it is essential for Northern European countries to be involved in all future research concerning nitrate levels in lettuce, since these countries in particular are sensitive to the findings and implications of research. It is certainly the case that some of the most important issues emerging from these studies have been the wide variety of extraction techniques used not only within UK laboratories, but also in laboratories of different countries within European member states. Until there is a firmly established extraction procedure in place for nitrate determination, it will continue to be difficult to directly relate the results of these studies with those of other workers.

It was ascertained during the small-scale methodology studies that the extraction technique based on the cold water extraction method by Burns, (1999), and used extensively in continental Europe, may not be the most efficient method with which to obtain sap nitrate. Despite this, and for the purposes of consistency and uniformity of results, this method of extraction was followed throughout the entire investigation. *It should therefore be noted that the nitrate concentrations reported throughout this thesis are subject to a degree of underestimation when compared with results obtained by other methods of extraction and analysis.* While the general trends should not be discounted, they might not give a complete measure of lettuce nitrate content in comparison to the figures produced by those UK laboratories employing hot water as an extractant.

The results from the investigations presented in chapter four, for the most part, confirm the findings of previous studies by Bischoff, Hiar & Turco, (1996), that suggest that the Nitrachek meter offers a suitable technique for rapid nitrate determination in vegetable crops

such as lettuce. However, as previously mentioned in chapter four, the user of the Nitrachek meter may experience certain limitations concerning the accuracy of nitrate readings obtained. It was established during the rapid development studies, that the test strips used alongside the Nitrachek meter were not always reliable and some batches were reported to underestimate nitrate values. It is possible to calibrate the Nitrachek meter using standard nitrate solutions and to incorporate a correction factor into the final calculation of nitrate concentrations. However, this procedure may increase user error and should ideally be avoided if the Nitrachek meter is to be used by growers. This issue is a matter for quality control and requires further investigation by the manufacturers of the Nitrachek test strips. Despite this, the Nitrachek meter generally proved to be a reliable method for determination of sap nitrate concentration in lettuce, and compared extremely well against laboratory methods of nitrate analysis (Section 4.2, Figures 4.14, 4.15 and 4.16). Indeed, with the exception of results from the series of small-scale studies presented in Chapter 4, the level of correlation between the Nitrachek meter and the nitrate probe was particularly good, with an r^2 value of 0.9 being observed.

A possible advancement for the Nitrachek meter could involve the development of an increased nitrate concentration detection range. At present, the Nitrachek meter detects nitrate within the range of 0 to 500ppm, but this does not extend as high as the nitrate concentration in most leafy vegetables including lettuce. Therefore when using the Nitrachek meter on a lettuce sample, the extracted sap must be diluted before the nitrate concentration can be determined. To limit the possibility of user error occurring as a result of incorrect sap dilution, it would be useful for manufacturers of the Nitrachek meter to increase the range of nitrate detection. This refinement would remove the need for dilution of the plant sap and would be a huge advantage to the grower.

The laboratory based Ion Selective Electrode method of nitrate analysis showed continued reliability and consistency throughout the series of investigations within the current study. The high correlation between the nitrate readings obtained by Ion Selective Electrode and the other three methods, particularly the Nitrachek meter, confirm findings of previous studies and suggest that as a laboratory based method for nitrate analysis, the Ion Selective Electrode is hard to fault (Heinen at al, 1992). Indeed it may be suggested from the current studies that the nitrate readings obtained by Ion Selective Electrode analysis provide a "yard stick" against which the nitrate results obtained by the other three methods of analysis could be measured.

The Horiba Cardy meter showed considerable promise as a method for rapid nitrate determination, despite showing significantly higher nitrate readings compared to the other three methods of nitrate determination. The ideal use of the Horiba Cardy meter should involve nitrate determination of sap taken directly from the petiole (Khosla, personal communication, 1997). However this was not the method followed for nitrate determination in this study, which involved nitrate analysis of extracted sap from the whole lettuce head. The nitrate readings obtained with the Horiba Cardy meter may be improved by adopting the official mode of nitrate analysis. It would seem advisable therefore to test the accuracy and reliability of the Horiba Cardy meter on a greater number of replicates, possibly using the petiole sap of fresh lettuce. Future studies would involve nitrate determination of sap obtained from several petioles per lettuce head, since it is known that the nitrate concentration within a lettuce varies depending on the age and location of leaf and petiole (Viets and Hageman, 1971). It would be useful to establish a correlation between the

measurements obtained from whole head nitrate analysis and petiole sap analysis in order to validate both methods.

The use of Ion Chromatography in the detection of anions is an accurate and established method (Swiader and Freiji, 1996). However, during the initial stages of the investigations, the reliability of nitrate readings and repeatability of the Ion Chromatograph was found to be unsatisfactory, with significantly lower nitrate values obtained. The use of the Ion Chromatograph for detection of one anion, namely nitrate, is both time-consuming and expensive. Indeed, the principal purpose of Dionex is for multi-ion analysis of large samples, which requires constant running rather than setting up the equipment for short periods of analysis. The lengthy procedural systems involved with the running of the Dionex, together with the high turnover of reagents and disposable vials significantly reduces the appeal of this technique. Ion Chromatography is therefore not an ideal choice for rapid detection of a single anion, namely nitrate, from lettuce sap. In comparison, the laboratory-based Ion Selective Electrode technique does not require high levels of maintenance, is suited to analysis of single anions, is not expensive to run and has proved to be extremely reliable. Despite this, Ion Chromatography may be suitable for continuous detection of a range of anions from samples under laboratory conditions.

Notwithstanding the possible discrepancies due to sub-optimal extraction methods, some significant trends arising from the studies concerning factors affecting nitrate variability in lettuce were evident. This section of research was divided into a series of hydroponic studies and soil studies. All of the trials were conducted at HRI, Stockbridge House. Hydroponic growing systems were utilised in order to achieve a controlled, soil-less growing environment for lettuce. It was hoped that hydroponic growing systems would reduce the crop nitrate variability usually associated with additional environmental factors in a soil environment, and this would therefore enable the influence of individual factors on the nitrate content of lettuce, such as cultivar variation and nutrient feed concentration, to be determined. As anticipated, the preliminary hydroponic investigation, which involved raising butterhead lettuce fed with a standard lettuce feed, showed nitrate variability within a hydroponic lettuce crop to be low (Section 2.2, Figure 2.1 and 2.2) when compared to the later soil studies (Section 3.2, Figure 3.1 and 3.5). The initial hydroponic investigation showed that it is possible to reduce the nitrate variability within a lettuce crop by controlling the growing environment.

The next hydroponic investigation involved raising a lettuce crop supplied with three nutrient levels each providing nitrate-nitrogen at 100ppm, 150ppm and 200ppm. Despite the faecal contamination of the second hydroponic study by birds (Section 2.3) the investigation did show a significant reduction in crop nitrate concentration and mean head weight at the lowest nutrient feed (Section 2.3, Figure 2.5 and 2.6). The eventual nitrate concentration of lettuce supplied with 150ppm nitrate was not significantly different to the eventual nitrate concentration of lettuce supplied with 200ppm nitrate. However, the nitrate concentration and fresh weights of lettuce from both of these treatments was higher than lettuce supplied with the lower concentration nutrient feed. In similar hydroponic studies involving treatments of different nutrient concentration, it has been shown that the nitrate concentration of lettuce could be significantly reduced if the supply of nitrogen was limited or stopped prior to the harvest date (Alt & Strower cited in Urrestarazu, Postigo, Salas, Sanchez & Carrasco, 1998). The accumulation of nitrate in cell vacuoles is thought to be the fundamental cause of high nitrate levels in leafy vegetables such as lettuce. In a study by Urrestarazu *et al.*, (1998), it was shown that substitution of nitrate in the nutrient solution with chloride ions could

significantly reduce nitrate concentration in lettuce tissue. Similar nutrient regimes to those employed in the hydroponic study at HRI, Stockbridge House and those conducted by previous researchers. For example using a nutrient feed containing the minimum concentration of nitrate nitrogen for optimum growth, could reduce nitrate concentrations in lettuce. It has been established in previous investigations that nitrate uptake in the later stages of growth is largely superfluous to nitrogen demand and thus accumulates in the vacuoles of the plant cells, (Granstedt & Huffaker, 1982). The results of these studies together with results obtained from hydroponic investigations conducted at HRI, Stockbridge House, indicate that a reduction in the nitrogen supply during the final growth stage would indeed minimise nitrate accumulation without significantly affecting fresh weight.

While previous studies have shown that it is possible to control nutrient supply in a precise manner within a hydroponic system, similar control over the level of nutrients reaching plants within a soil-based growing system cannot be achieved to the same extent. The soil-growing environment can vary dramatically depending on characteristics such as organic matter content, pH, soil moisture and soil type, which in turn influence soil nitrogen availability and nitrate uptake patterns (Jarvis *et al.*, 1996). The benefits of using the results from hydroponic studies in the management of nitrogen fertiliser application and nitrate levels in soil-raised, glasshouse lettuce may be limited due to the differences between soil and hydroponic systems. However, some areas of nitrate research, that include studying the influence of temperature on plant nitrate concentrations, could benefit from the inclusion of hydroponic systems rather than the traditional soil-based growing systems in their investigations. Indeed, despite a great deal of nitrate research, the effect of temperature on plant nitrate concentration is still far from clear. This is due, in part, to the inability to distinguish between temperature effects on nitrate uptake and reduction from the effects of temperature on soil nitrogen dynamics and plant metabolism. By adopting a soil-less growing system in a future study of this kind, the results obtained could possibly relate more closely to the effect of temperature on nitrate uptake and reduction, without the additional influence of soil nitrogen dynamics.

The use of different lettuce cultivars in the third hydroponic trial highlighted the differences in nitrate accumulation between "continental" and "butterhead" lettuce, with highly significant differences being observed (Section 2.4, Figure 2.8). The results of these studies were in agreement with previous soil studies conducted at HRI Stockbridge House using a range of butterhead and continental cultivars (Lee, 1999). The general trends emerging from the current and previous investigations has been of higher nitrate concentrations in continental lettuce varieties compared to butterhead lettuce varieties. Nutrient supply failure and high humidity levels during the course of the current study resulted in tipburn and stunting of some plants and it would therefore be advisable to repeat the hydroponic variety trial with improved humidity levels while ensuring a constant supply of nutrient feed. The outcome of such a duplicate study would, hopefully, provide results confirming those from the initial hydroponic cultivar trial.

Nitrate variability between different lettuce cultivars has not been extensively studied in either soil or soil-less growing environments, although it is generally assumed that some of the nitrate variability is due to morphological variation between cultivars. There is some evidence that differences in nitrate concentration between lettuce cultivars is determined by the rate of nitrate uptake, which may be fixed through osmotic potential regulation in the root cells (Steingrover, 1986). It is believed that the genetic variation for osmolarity might determine plant nitrate concentration via regulation of nitrate uptake. It is not possible to attribute the observed cultivar differences in nitrate concentration in this study to cultivar

differences in osmolarity without determining plant sap osmolarity. However, the nitrate uptake results from this study, although estimated from limited hydroponic data, do suggest that the cultivar variation in nitrate concentration may be related to nitrate uptake rate. To establish a connection between nitrate variation between cultivars, additional parameters such as plant nitrate uptake rate and osmolarity should be investigated. An additional study should include the determination of osmotic potential, nitrate uptake rates throughout the growing period, nitrate assimilation rates through determination of nitrate reductase activity, and photosynthetic rate.

Although the results from the hydroponic investigations do provide important information related to nitrate variability within a lettuce crop, there are some important considerations in the use of soil-less growing systems. It has been observed in a previous investigation by Alt, (1980), that the buffering capacity within a hydroponic system is extremely limited. This means that factors influencing the composition of the nutrient solution, such as nutrient and water uptake can affect the nutrient medium to a greater extent than in a soil-based growing system. Determination of the anion-cation balance within the medium can provide a guide to nutrient depletion relative to the volume of water. As well as regular monitoring of pH, flow rate and nitrate content of the nutrient solution, EC determination in hydroponic systems can ensure that the composition of the nutrient medium is maintained for optimum crop growth. Furthermore, since hydroponics can provide ideal conditions for controlled investigations concerning crop nutrition, much consideration needs to be given to hydroponics as a practical alternative to the current method of lettuce production.

The inclusion of soil-based, protected trials in this research project was an obvious necessity since they are the major growing medium for lettuce in the UK. Growers have long been aware of the nitrate variability within a soil grown lettuce crop, and a great deal of research has focused on ways to minimise this variability through management of fertiliser application. There was a deliberate link between the nature of the soil investigations and the hydroponic studies. For example, the hydroponic study to determine nitrate variability among lettuce cultivars involved some of the same varieties used in a more extensive soil variety trial. The ability to relate the findings of hydroponic research to lettuce production in soil is heavily dependent on such close connections between investigations using soil and soil-less growing systems. Indeed, the findings from this type of hydroponic research may be extrapolated to soil situations and given a practical application. For this reason, although they are different growing systems, an association between soil and soiless growing media is acknowledged in these investigations.

As previously mentioned, extensive research has been conducted on factors affecting the nitrate content of leafy vegetables such as lettuce by among others McCall and Willumsen, (1998), Knight and Mitchell, (1983). However, few researchers have chosen to compare nitrate variability for both winter and summer lettuce raised in the same environment, and subjected to identical soil nitrogen concentration and irradiation regimes. For example, although a study by Ysart, Clifford and Harrison, (1999), showed that the nitrate concentration in protected lettuce was influenced by season, the lettuce was sampled from locations throughout the UK and therefore the effect of geographical variation and climate change on nitrate variability cannot be discounted. The inclusion of year-round soil studies, as detailed in the methodology section of chapter three, was a considered and practical approach to the aims and design of the investigations. By conducting soil trials during both a summer and consecutive winter season, in the same glasshouse environment, a realistic and valid representation of nitrate variability within a glasshouse lettuce crop was obtained

throughout the UK growing season. The results, particularly those obtained from the soil studies in Chapter 3, have provided the UK lettuce industry, who currently grow lettuce all year round, with a unique and particularly relevant insight into nitrate variability within glasshouse lettuce. Furthermore, throughout the soil investigations, the planting and growing practises currently recommended to growers were followed in order to replicate, as far as possible, actual growing conditions for UK glasshouse lettuce.

Previous values for nitrate variability within a lettuce crop have been largely based on the results obtained from pooled samples of lettuce. For instance the analytical method detailed in the EC Monitoring Programme document, V1/4800/96, recommends that ten lettuce heads are taken to form an individual sample to be analysed (Anon, 1997b). The methodology followed in the studies at The Nottingham Trent University, involved nitrate analysis of individual lettuce heads since the analysis of pooled lettuce samples does not ensure that the inherent nitrate variability within a lettuce crop is fully represented. In addition, no fewer than ten heads were sampled from individual treatments during both the soil and hydroponic trials. This was primarily to improve the accuracy of the nitrate results obtained and also to reduce the frequency of anomalous values, which are known to occur following the analysis of samples with a low number of replicates.

The results of the initial soil studies were in agreement with findings reported previously by Van Eysinga, (1984a), and showed low nitrate variability within the winter lettuce crop compared to a high level of nitrate variability within the summer lettuce crop. Growers have noted for some time that a lettuce crop has a better response to nitrogen fertiliser application during the summer season compared to the winter growing period. This can be attributed to improved soil nitrogen availability during summer months and an increase in nitrate uptake in summer lettuce. The greater availability of cellular nitrate in summer lettuce plants increases the frequency of plant nitrate x environment interactions, and this can influence nitrate concentrations within individual lettuce plants to a greater extent than in winter lettuce. Furthermore, the variability in soil nitrogen availability and environmental factors such as light, temperature and humidity within a lettuce trial, particularly under glass, can add to the nitrate variability within the crop (Van Eysinga and Van der Meijs, 1985). The increased nitrate variability within the summer lettuce crop, as shown in the results of the initial soil studies, is most likely due to rapid growth together with interactions between available soil nitrogen, and seasonal influences of temperature and light level variation on the processes of nitrogen metabolism.

The difference between nitrate levels in summer and winter glasshouse lettuce is a common occurrence. Mean nitrate levels within a summer lettuce crop are usually markedly lower compared to mean nitrate levels in a winter crop, and this was indeed observed in the results from summer and winter lettuce trials raised at Stockbridge House. The results were likely to be due to increased nitrate assimilation and plant activity in summer crops, (Stepowska & Kowalezyk, 2001), or may simply have been related to the shorter summer growing period that gave individual plants less time to absorb nitrate from the soil. These results are in agreement with a recent UK study (Ysart *et al.*, 1999), which showed a mean nitrate concentration of 2382 mg/kg in summer lettuce compared to 3124 mg/kg nitrate in winter lettuce. Ysart *et al.*, (1999), concluded from their results that there was a significant inverse relationship between sunlight/daylight hours and nitrate concentration in glasshouse lettuce. The increased irradiation levels and length of photoperiod, when viewed alongside the mean nitrate concentration results from the summer trial, suggest that a similar inverse relationship between sunlight/daylight hours and nitrate concentration may have indeed have occurred.

An additional factor associated with seasonal variation in nitrate concentration of lettuce is photoperiod. The length of photoperiod is known to significantly influence nitrate concentrations in protected lettuce. An increase of 1 hour sunshine per day led to approximately 7.5% decrease in the nitrate concentration of lettuce (Ysart *et al.*, 1999). Data relating to daylight hours during the lettuce trials were not available for the present study, and so the influence of photoperiod on the nitrate concentrations in lettuce could not be determined. Of course, photoperiods were shorter during the winter growing period in the winter soil study compared to those experienced during the summer growing period of the summer soil study. It can therefore be assumed that any increase in photoperiod during the summer trial may have further contributed to the reduction of nitrate concentration in the lettuce.

The increase in nitrate utilisation, nitrate assimilation and general plant production via the production of proteins and nucleic acids can also be seen in the mean fresh weights of summer lettuce, which are generally greater than the mean fresh weights within the winter lettuce crop. Furthermore, the nitrate concentrations and fresh weights of the winter crop did not show significant variation between treatments containing different levels of nitrogen fertiliser. Recommended fertiliser levels, cited in the Code of Good Practise, suggest applying sufficient nitrogen to achieve a soil level of 100 mg/l at planting (Anon, 1998c). However, the results from the soil study suggest that increasing the base fertiliser dressing, for the purposes of improving crop yield in winter, from 50ppm to 100ppm and particularly to 150ppm, may be unnecessary. It may therefore be advisable to apply nitrogen fertiliser at a much lower rate than is currently recommended, and to follow basal fertiliser applications with additional applications during the growing period as and when the crop requires it. The application of low nitrogen fertiliser rates is also advisable in order to minimise pollution due to nitrate leaching into water courses.

Methods for reducing nitrate concentrations in glasshouse lettuce, such as the use of alternative nitrogen fertilisers, have been previously detailed in chapters one and three. In a separate series of investigations incorporated within the MAFF-LINK programme of research, fertiliser type and the effect of timing and method of application on crop weight and nitrate concentration of glasshouse lettuce were evaluated. The major findings, which are not presented in this thesis, suggest that nitrate residues in lettuce are highest when grown with calcium nitrate fertiliser. The results of these and additional studies by Beresniewiecz *et al.*, (1988), indicate that alternative compounds that restrict nitrate accumulation in plants may also be suitable for use as nitrogen fertilisers.

While there are many growing practises that can be adopted in order to markedly reduce nitrate variability within the crop, one of the most important and unfortunately unpredictable factors to influence crop nitrate concentration is light. As previously mentioned, light levels in the UK are commonly low in winter and highly variable from region to region. Emerging scientific evidence, (Knight *et al.*, 1983), which has been supported by the results of the initial soil investigations, suggests that increasing light levels may reduce nitrate levels in lettuce, while maintaining good fresh weights. While there are recommendations for maximising light levels under glasshouse conditions, which include siting of glasshouse to obtain maximum light throughout each day, ensuring glass is clean and shading of the crop is limited, it is almost impossible to significantly increase the level or duration of irradiation received by a crop without exposing the plants to artificial light. The cost implications of installing and supplying artificial light throughout the winter growing period as a method for

reducing nitrate concentrations in lettuce are huge and renders it unworkable. However the findings from previous studies and those presented in chapter three, provide evidence of an interactive influence between soil nitrogen and light level on the nitrate concentration in summer lettuce, and this has led to alternative suggestions for minimising crop nitrate concentration. It seems that the most effective way to minimise nitrate concentrations in glasshouse lettuce within the UK, at the present time, is to confine the use of maximum fertiliser applications to periods of high irradiation, and to use minimum nitrogen fertiliser application during periods of low illumination.

Evidence of the problems faced by growers in achieving and maintaining consistent soil nitrogen concentrations across a lettuce trial is shown in a significant influence of plot position on nitrate concentration in both the summer and winter soil trials. The influence of plot position on mean nitrate concentration, represented as a significant block effect in the summer trial may be attributed to the variation in soil nitrogen concentration across the trial (Chapter 3, Figure 3.6). Similar discrepancies in soil nitrogen concentration between plots within the same trial leads to variability in nitrate uptake, assimilation and the subsequent concentration of nitrate within the crop, and this is often found to be the case within UK glasshouse lettuce. Current measures to manage soil nitrogen concentration and regulate the levels of nitrogen supplied to a lettuce crop involve soil nitrogen analysis at least twice throughout the growing period. Indeed, full soil analysis is recommended for each lettuce crop where lettuce follows a different crop (Anon, 1998a). However, in practical terms the economic cost and time needed to carry out a full soil analysis prior to individual lettuce crops may not always prove realistic. Therefore, while UK growers generally adhere to cultural advice, there may be occasions when these recommendations are not followed. Under circumstances when either time constraints or economic factors determine the frequency of soil nitrogen analysis, or when soil nitrogen supply within a trial is believed to be inconsistent, the availability of a nitrate testing kit for plant analysis may be beneficial in regulating nitrogen availability throughout the growing season.

It has already been mentioned that the outer leaves of lettuce are naturally high in nitrates compared to the inner leaves of lettuce. It is therefore beneficial to produce a high yielding lettuce crop, since trimming the outer leaves reduces the nitrate content of individual lettuce while maintaining satisfactory head weights. In order to achieve high yielding lettuce, recommendations are given to growers that include increasing plant spacing to a maximum of 25 plants per square metre. These recommendations are especially important when growing winter lettuce, as it is the winter crop that commonly exceeds the derogated maximum nitrate level. Increased plant spacing in winter and the subsequent production of large heads followed by trimming of outer leaves, offers a means of achieving a satisfactory mean nitrate concentration within a winter crop that falls within EC levels. Some of the work undertaken as part of the winter soil study involved investigating the effect of plant spacing on mean head weight and nitrate concentration (Page 32, appendix). The results from these investigations suggest that plant spacing influences both the mean head weights and mean nitrate concentration of winter lettuce. The mean head weight of lettuce was highest in plants that were grown at the lower planting density, 25 plants/metre, compared to the higher planting density of 33 plants/metre at all three soil nitrogen concentrations rates. Lettuce planted at a density of 25 plants/metre and supplied with the lowest soil nitrogen rate, at 50ppm nitrate-nitrogen, achieved greater head weights compared to lettuce planted at 33 plants/metre and supplied with soil nitrogen at a rate of 150ppm nitrate-nitrogen. The results suggest that in winter lettuce a density of 25 plants/metre is necessary to achieve maximum head growth and that plant density, rather than nitrate availability, is the limiting factor to

achieving high head weight. On the basis of these findings alone it could be suggested that a plant spacing of 25 plants/metre minimise the need for increased nitrogen fertiliser application above 50 ppm nitrate-nitrogen in order to achieve optimum crop yield. Furthermore, while a plant spacing of 25 plants/metre does not necessarily help to reduce plant nitrate concentrations, the apparent increase in head weight associated with lower planting densities allows for trimming of outer leaves and indirectly reduces the final plant nitrate concentration. The results provide further credence to the suggestion that high yields can be achieved with minimum fertiliser application rates when additional methods, in this case cultural, are implemented.

In summary, there are several methods a UK lettuce grower can adopt that will help to reduce nitrate concentrations within the crop. These include positioning of the glasshouse to ensure maximum utilisation of light levels throughout the growing season, applying low levels of nitrogen fertiliser during the winter growing period and increasing plant spacing to maximise crop yield and allow for trimming of outer leaves, which are naturally high nitrate. Nitrate levels in lettuce can also be minimised by harvesting in full sunlight conditions, (Schroder and Bero, 2001), and by removing the nitrogen supply for several days before harvest in hydroponic systems, (Santamaria *et al.*, 2001).

In recent years, trials have been conducted that involve growing new lettuce varieties that may offer means of achieving the proposed nitrate levels (Eenink *et al.*, 1984). The unpublished results from previous soil studies, conducted at HRI Stockbridge House, (Lee, 1999), showed significant nitrate variability between different continental and butterhead lettuce cultivars. These earlier findings are supported by the results of the soil variety trial reported in Section 3.5, Figure 3.11, which show that many continental varieties accumulate appreciably more nitrate compared to butterhead varieties.

It certainly seems that the demand for butterhead lettuce is decreasing in favour of prepared salads, which contain the more "exotic" or continental types of lettuce. Indeed, the current preference of retailers and the public in the UK is undeniably towards niche markets that may include leaves from continental varieties. Figures obtained from MAFF (now DEFRA) indicate that the total value of the glasshouse lettuce market in the UK in 1999 was approximately £16.5 million (Anon, 2000a). Despite this, 20% of the continental lettuce varieties that make up packs of prepared salads are imported from growers in France (Anon, 1998e). The advantages of including continental varieties within UK grower trials are obvious and would benefit the declining market for UK produced lettuce. In the long-term this might enable UK growers to compete more efficiently with the success of growers in countries such as France and Spain, where warmer climatic conditions and higher light intensities, especially in winter, are more favourable to crop growth and low nitrate concentrations. However, in order to encourage UK growers, the expected crop yield of recommended continental cultivars should be high and the anticipated nitrate content should fall within the EC limits. This is especially important for continental crops grown during the winter season. In the long term, breeding varieties that have lower nitrate levels may be more acceptable to growers than adopting different cultural methods. At the present time however, there are no official recommendations regarding alternative varieties that may supply low nitrate concentrations in the lettuce crop. The possibility that nitrate levels in continental lettuce might regularly exceed maximum EC nitrate levels may diminish the appeal of growing these varieties in the UK in winter.

Additional work should be concentrated on establishing a standard procedure for nitrate extraction and analysis for use throughout European analytical laboratories. The recording of plant nitrate uptake rates raised in soil-less growing systems would enable detailed information relating to plant nitrogen requirements at all stages of the growing period to be gathered. This information could then be used to improve the accuracy of nitrogen fertiliser application in soil-based growing systems as well as improve the timing of supplementary fertiliser applications. In this way, the nitrogen demands of the developing crop would be met while the risk of applying excessive amounts of fertiliser would be reduced. The applications for the results of hydroponic investigations in soil-based growing systems can only be fully realised once the complexities of the soil environment are more clearly understood. Characteristics that are specific to the soil environment have been shown to exert a significant influence on soil available nitrogen and root development. The hydroponic growing environment is vastly different to the soil growing system, and this must be taken into consideration when determining the benefit of hydroponic studies. The increase in consumption of different lettuce varieties in pre-packed salads necessitates further research into ways to minimise nitrate levels in these novel cultivars. However, there is still clearly a requirement for establishing a winter lettuce variety that gives high yield, is resistant to pathogens and disease, while maintaining low nitrate concentrations. Indeed, it is becoming increasingly important for UK growers to produce a high yielding, lower nitrate accumulating lettuce variety considering the constraints within European in the form of nitrate limits.

6.1 Concluding remarks and future work.

In the UK and Europe there has been a great deal of expenditure and human resources spent on developing ways to minimise nitrate variability and reduce nitrate levels in lettuce in order to comply with the EC Regulation 194/97. Indeed, plant nitrate metabolism and the associated factors that affect nitrate levels in plants such as lettuce have been the subject of extensive study by scientific researchers worldwide (Lee, 1970; Lorenz & Weir, 1974; Walters & Walker cited in Hewitt and Cutting, 1979; Van Eysinga, 1987; Greenwood, 1990; Forlani *et al.*, 1997; Powlson, 1997; Anon, 1999a,c). The initial concern regarding nitrate levels in foodstuffs was sparked by the potential threat to human health from nitrate toxicity, methaemoglobinaemia and nitrosamine formation. However, actual case evidence of nitrate toxicity is rare and its association with cancer causing agents such as nitrosamine is the subject of some dispute.

Furthermore, the focus on lettuce and other leafy vegetables such as spinach as a source of dietary nitrate is not wholly tenable. While it is true to say that leafy, green vegetables do contain appreciably higher levels of nitrate compared to other vegetables, the relatively low quantities of these foodstuffs consumed by the public pose a minuscule threat to human health. There are numerous other sources of dietary nitrate and it could be suggested that cured meat, many of which contains the preservative sodium nitrate, might lead to a more serious risk of nitrate toxicity than consumption of lettuce. Furthermore, there have been reported benefits of consumption of dietary nitrate. One of the reported benefits of nitrate has been in the role it plays in the body's defence against pathogenic bacteria within the gut (Carlile, 1999). There is clearly room for additional research concerning the role of nitrates in crop production, which could well be linked to the future of the lettuce industry itself.

Despite a greater understanding in the scientific community concerning the factors associated with high nitrate levels in lettuce, the practical implications of these findings have yet to be fully realised within the lettuce industry. The main drive behind researching nitrate issues in the UK has come from lettuce growers themselves who could be penalised under EU law for the high nitrate concentrations found in lettuce crops and consequently are desirous of ways to reduce the nitrate levels. It is therefore interesting to note that while much of the funding for this type of research has been from the lettuce industry, via a lettuce grower levy, the financial health of the industry has been variable in recent years. Indeed, several producers in the UK have gone out of business or have adapted to the current climate by growing alternative crops. The sales of UK produced butterhead lettuce have been less successful in recent years and have been replaced in popularity by continental lettuce varieties in prepared salads. The sale of processed salad leaves in prepared salads has seen a year on year growth, currently set at 30%, and expected to continue at this level for the next few years (Anon, 1998e). However, these types of continental lettuce are not commonly grown in the UK and instead are imported from abroad. The competition now faced by the UK lettuce grower from South European countries, concerning the emergence of unusual lettuce varieties onto the market, makes the future of growing predominantly butterhead varieties in winter questionable. While UK growers may move towards continental lettuce production, consideration must be given to the growing conditions in which continental varieties thrive. Continental lettuce varieties were originally bred in much warmer climates than those generally experienced throughout the UK. Therefore, to achieve optimal continental lettuce production in the UK the current glasshouse facilities and cultural practises would require some adjustment. In addition to this practical consideration there is an additional issue of

high nitrate levels in continental cultivars. As previously mentioned, nitrate concentrations in continental lettuce cultivars are significantly higher compared to the more commonly grown butterhead cultivars.

The introduction of European Union derogation 194/97, which sets maximum nitrate concentrations at 3500ppm and 4500ppm in summer and winter lettuce respectively, has had major implications for North European lettuce producing countries. In order to ensure that the maximum nitrate levels are not exceeded within the lettuce crop, sampling and nitrate testing is carried out before harvest. Regular monitoring of plant nitrate concentration during the growing season is also recommended, as a means of determining nitrogen requirement of the crop during the growing season thereby maximising nitrogen fertiliser use efficiency while minimising nitrate accumulation. However, intermediate nitrate analysis is not a requirement of UK lettuce growers. Furthermore, the absence of a standardised protocol for nitrate extraction within the UK and Europe, as previously mentioned in chapter five, has been identified as the main reason for the high degree of variability between nitrate levels obtained. The level of variation between nitrate results obtained from different analytical laboratories is often significant and the variable nitrate extraction methods used means that the results obtained are not wholly representative of the true nitrate status of the crop. The unpublished findings of a study conducted by Lee, (1999) and those of the small-scale methodology studies presented in Chapter five, provide examples of at least five different extraction methods currently used by UK laboratories during routine nitrate analysis of lettuce samples. This current situation makes a mockery of the current nitrate limits listed under EC 194/97. In order for nitrate analysis to help towards the minimisation of nitrate accumulation in lettuce, there must firstly be standardisation of nitrate extraction procedures throughout Europe. Only then can the results obtained from analysis of lettuce samples claim to truly represent the nitrate status of the crop and the enforcement of nitrate limits through EC regulation 194/97 successfully enforced.

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APPENDIX:

STANDARD NUTRIENT FEED (Stockbridge House, Nick Hall)

Tank A	**200L**
Calcium Nitrate	12.6kg
Potassium Nitrate	3.5kg
Nitram	0.5kg
Iron (powder)	0.4kg
Nitric Acid (60%)	200mls

Tank B

Mono Potassium Phosphate	4.0kg
Magnesium Sulphate	7.4kg
Potassium Nitrate	6.0kg
Nitric Acid (60%)	200mls

Trace Elements (Tank B)

Manganese Sulphate	50g
Zinc Sulphate	35g
Borax	70g
Copper Sulphate	16g
Sodium Molybdate	2.5g

STANDARD NITRATE SOLUTION

Stock concentration: 1000ppm

1.63 gram KNO_3 / litre deionised water.

0.815 gram KNO_3 / 500ml deionised water

ANION REGEN SOLUTION

0.7 ml / litre cH_2SO_4

ELUENT SOLUTION

(0.5M) sodium carbonate + (0.5M) sodium bicarbonate

10.8 ml Na_2CO_3 solution + 1.2 ml $NaHCO_3$ solution / litre deionised water.

APPENDIX

pH, EC, Tank Depth and Flow Rate

Hydroponic Trial #1: (17/8/98 – 2/9/98), conducted at HRI Stockbridge House

TANK 1

DATE	pH	EC ($mScm^{-1}$)	Depth (metre)	Flow (litre/min)
13/08/98	5.93	2.20	55.00	5.40
17/08/98	6.04	2.32	54.00	5.40
21/08/98	5.68	2.21	47.50	5.40
24/08/98	5.64	2.21	42.50	5.40
28/08/98	6.21	2.10	36.00	5.40
01/09/98	6.37	1.99	28.00	5.40

TANK 2

DATE	pH	EC ($mScm^{-1}$)	Depth (metre)	Flow (litre/min)
13/08/98	5.91	2.10	55.00	5.50
17/08/98	6.08	2.22	52.50	5.50
21/08/98	5.55	2.22	49.00	5.50
24/08/98	5.49	2.21	53.50	5.50
28/08/98	6.20	2.12	38.00	5.50
01/09/98	6.55	1.99	32.00	5.50

TANK 3

DATE	pH	EC ($mScm^{-1}$)	Depth (metre)	Flow (litre/min)
13/08/98	5.97	2.20	55.00	5.40
17/08/98	6.03	2.20	52.50	5.40
21/08/98	5.41	2.20	48.00	5.40
24/08/98	5.40	2.19	46.00	5.40
28/08/98	6.18	2.10	41.00	5.40
01/09/98	6.58	1.96	35.00	5.40

TANK 4

DATE	pH	EC ($mScm^{-1}$)	Depth (metre)	Flow (litre/min)
13/08/98	5.95	2.20	55.00	5.00
17/08/98	5.96	2.22	55.00	5.00
21/08/98	5.31	2.21	49.00	5.00
24/08/98	5.43	2.19	43.00	5.00
28/08/98	6.20	2.11	40.00	5.00
01/09/98	6.69	1.96	34.00	5.00

TANK 5

DATE	pH	EC ($mScm^{-1}$)	Depth (metre)	Flow (litre/min)
13/08/98	6.09	2.20	55.00	5.10
17/08/98	5.43	2.27	55.00	5.10
21/08/98	5.24	2.21	49.00	5.10
24/08/98	5.45	2.21	42.50	5.10
28/08/98	6.25	2.14	38.00	5.10

01/09/98	**6.77**	**1.98**	**30.00**	**5.10**

Daily mean values for glasshouse temperature, humidity and weekly irradiation levels

Hydroponic Trial #1: (17/8/98 – 2/9/98), conducted at HRI Stockbridge House

Date	Temperature°C	Humidity %	Weekly Irradiation (MJ/m^2)
17/8	21.8	56.9	95.65
18/8	20.9	53.3	95.65
19/8	21.3	57.5	95.65
20/8	18.3	64.1	95.65
21/8	19.6	62.7	95.65
22/8	18.3	60.3	95.65
23/8	16.5	75.4	95.65
24/8	18.2	60	85.52
25/8	18	74.4	85.52
26/8	14.2	86.4	85.52
27/8	*	*	85.52
28/8	*	*	85.52
29/8	*	*	85.52
30/8	*	*	85.52
31/8	*	*	52.81

pH, EC, Tank Depth and Flow Rate

Hydroponic Trial #2 (21/9/98-26/10/98), conducted at HRI Stockbridge House

TANK1 (150ppm)				*TANK 2* (150ppm)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
21/9/98	4.08	1.82	50	21/9/98	4.58	1.97	51
25/9/98	5.78	1.73	49	25/9/98	5.69	1.92	50
28/9/98	5.88	1.70	47	28/9/98	5.45	1.90	48
2/10/98	5.74	1.69	46	2/10/98	5.53	1.87	46
5/10/98	5.85	1.69	45	5/10/98	5.75	1.87	45
9/10/98	6.03	1.61	42	9/10/98	6.04	1.79	42
12/10/98	6.21	1.69	39	12/10/98	6.22	1.75	40
16/10/98	6.45	1.47	35	16/10/98	6.48	1.66	36
19/10/98	6.48	1.39	33	19/10/98	6.56	1.59	34
23/10/98	6.61	1.49	29	23/10/98	6.72	1.58	31
26/10/98	6.63	1.39	27	26/10/98	6.74	1.48	27

TANK 3 (200ppm)				*TANK 4* (100ppm)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
21/9/98	4.79	2.00	51	21/9/98	4.99	1.79	51
25/9/98	5.60	1.98	50	25/9/98	5.60	1.78	49
28/9/98	5.34	1.91	48	28/9/98	5.38	1.75	49
2/10/98	5.32	1.97	47	2/10/98	5.55	1.75	47
5/10/98	5.45	1.97	45	5/10/98	5.80	1.72	45

9/10/98	5.83	1.89	43	9/10/98	6.19	1.64	44
12/10/98	6.07	1.84	41	12/10/98	6.44	1.58	41
16/10/98	6.37	1.77	36	16/10/98	6.65	1.57	42
19/10/98	6.48	1.70	35	19/10/98	6.72	1.45	35
23/10/98	6.70	1.65	32	23/10/98	6.88	1.35	31
26/10/98	6.73	1.51	31	26/10/98	6.86	1.24	30

TANK 5 (200ppm)				*TANK 6* (200ppm)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
21/9/98	5.65	2.04	47	21/9/98	5.18	2.06	50
25/9/98	5.51	2.05	49	25/9/98	5.57	2.06	49
28/9/98	5.26	2.04	46	28/9/98	5.30	2.05	47
2/10/98	5.22	2.05	45	2/10/98	5.19	2.05	45
5/10/98	5.27	2.04	45	5/10/98	5.15	2.05	45
9/10/98	5.65	1.97	43	9/10/98	5.62	1.99	43
12/10/98	6.03	1.93	40	12/10/98	6.01	1.92	41
16/10/98	6.40	1.82	35	16/10/98	6.40	1.83	36
19/10/98	6.50	1.79	33	19/10/98	6.54	1.80	35
23/10/98	6.72	1.63	30	23/10/98	6.78	1.71	31
26/10/98	6.81	1.56	27	26/10/98	6.82	1.61	27

TANK 7 (100ppm)				*TANK 8* (100ppm)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
21/9/98	5.23	1.86	50	21/9/98	5.16	1.88	50
25/9/98	5.63	1.84	49	25/9/98	5.89	1.86	49
28/9/98	5.31	1.82	47	28/9/98	5.66	1.84	47
2/10/98	5.44	1.80	46	2/10/98	5.75	1.83	46
5/10/98	5.60	1.78	45	5/10/98	5.90	1.83	45
9/10/98	6.07	1.72	43	9/10/98	6.33	1.78	43
12/10/98	6.31	1.65	41	12/10/98	6.57	1.72	41
16/10/98	6.70	1.55	36	16/10/98	6.88	1.64	36
19/10/98	6.82	1.48	35	19/10/98	6.93	1.56	35
23/10/98	6.99	1.38	32	23/10/98	7.01	1.37	31
26/10/98	6.96	1.35	30	26/10/98	7.04	1.34	30

TANK 9 (150ppm)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)
21/9/98	5.25	1.89	50
25/9/98	5.80	1.89	50
28/9/98	5.45	1.88	47
2/10/98	5.41	1.86	45
5/10/98	5.71	1.81	45
9/10/98	6.05	1.76	42
12/10/98	6.32	1.67	40
16/10/98	6.66	1.63	35
19/10/98	6.70	1.55	33

23/10/98	6.84	1.51	30
26/10/98	6.88	1.50	27

Daily mean values for glasshouse temperature, humidity and weekly irradiation levels

Hydroponic Trial #2: (21/9/98 – 28/10/98), conducted at HRI Stockbridge House

Date	Temperature°C	Humidity %	Weekly Irradiation (MJ/m^2)
21/9/98/	17	75.7	43.85
22/9/98	15.5	77.7	43.85
23/9/98	15.8	75.2	43.85
24/9/98	14.4	82.7	43.85
25/9/98	16.9	74.9	43.85
26/9/98	16.2	80.3	43.85
27/9/98	15.5	86.9	43.85
28/9/98	16.2	84.5	28.03
29/9/98	15.8	80	28.03
30/9/98	15	90.5	28.03
1/10/98	15.2	85.9	28.03
2/10/98	13.4	74.8	28.03
3/10/98	13	75	28.03
4/10/98	12.7	77.7	28.03
5/10/98	13.5	76.4	42.99
6/10/98	13.4	72.5	42.99
7/10/98	13.5	74.2	42.99
8/10/98	13.1	72.6	42.99
9/10/98	13.2	74.4	42.99
10/10/98	14.6	71.6	42.99
11/10/98	14.9	68	42.99
12/10/98	15	62.9	41.86
13/10/98	15.2	80.6	41.86
14/10/98	16	71.2	41.86
15/10/98	14.5	66.6	41.86
16/10/98	13.4	83.4	41.86
17/10/98	12.8	78.8	41.86
18/10/98	12.2	65.5	41.86
19/10/98	13.1	66.1	35.63
20/10/98	12.4	70	35.63
21/10/98	15.8	74	35.63
22/10/98	17.9	70.8	35.63
23/10/98	15.4	69.8	35.63
24/10/98	11.8	80.6	35.63
25/10/98	13.5	66.5	35.63
26/10/98	12.5	64.1	37.81
27/10/98	15.5	69.7	37.81
28/10/98	*	*	37.81

pH, EC, Tank Depth and Flow Rate

Hydroponic Trial #3 (6/4/99 – 30/4/99), conducted at HRI Stockbridge House.

TANK2	(MIAMI)			*TANK 2*	(MIAMI)		
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
6/4/99	5.81	2.28	40	6/4/99	5.64	2.23	40
13/4/99	5.42	2.52	33	13/4/99	5.50	2.39	33
16/4/99	5.98	2.34	25	16/4/99	6.25	2.20	20
20/4/99	6.65	2.38	25	20/4/99	6.95	2.22	20
23/4/99	6.21	2.12	52	23/4/99	6.30	1.97	52
27/4/99	6.43	2.03	46	27/4/99	6.63	1.86	46
30/4/99	6.80	2.19	37	30/4/99	6.86	1.90	39

TANK 3	(KRIZABRI)			*TANK 4*	(RACHEL)		
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
6/4/99	5.53	2.18	41	6/4/99	5.53	2.16	40
13/4/99	5.72	2.19	33	13/4/99	5.63	2.26	33
16/4/99	6.44	1.97	30	16/4/99	6.31	2.06	25
20/4/99	6.97	1.92	26	20/4/99	6.96	2.08	25
23/4/99	6.45	1.81	52	23/4/99	6.30	1.97	53
27/4/99	6.67	1.68	46	27/4/99	6.67	1.88	47
30/4/99	6.88	1.76	39	30/4/99	6.85	1.97	39

TANK 5	(KRIZABRI)			*TANK 6*	(KRIZABRI)	**Lettuce not**	**sampled**
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
6/4/99	5.36	2.16	39	6/4/99	5.30	2.17	35
13/4/99	5.84	2.23	30	13/4/99	5.58	2.35	33
16/4/99	6.63	2.04	20	16/4/99	5.30	2.19	30
20/4/99	7.01	1.95	22	20/4/99	5.22	2.38	26
23/4/99	6.50	1.84	52	23/4/99	5.94	2.13	53
27/4/99	6.79	1.65	44	27/4/99	6.25	2.01	47
30/4/99	6.95	1.77	35	30/4/99	6.51	2.09	41

TANK 7	(RACHEL)	**Lettuce not**	**sampled**	*TANK 8*	(RACHEL)	**Lettuce not**	**sampled**
Date	pH	EC ($mScm^{-1}$)	Depth (metre)	**Date**	pH	EC ($mScm^{-1}$)	Depth (metre)
6/4/99	5.00	2.15	37	6/4/99	5.02	52.10	50
13/4/99	5.26	2.32	35	13/4/99	5.43	2.16	35
16/4/99	4.81	2.18	30	16/4/99	4.84	1.98	30
20/4/99	4.83	2.36	29	20/4/99	5.40	1.95	29
23/4/99	5.66	2.08	54	23/4/99	5.61	1.87	53
27/4/99	6.06	2.01	49	27/4/99	6.15	1.79	46
30/4/99	6.32	2.11	45	30/4/99	6.47	1.82	37

Hydroponic Trial #3 (6/4/99 – 30/4/99), conducted at HRI Stockbridge House.

TANK 9 (MIAMI)			
Date	pH	EC ($mScm^{-1}$)	Depth (metre)
6/4/99	5.20	2.09	40
13/4/99	5.64	2.16	33
16/4/99	6.02	1.99	25
20/4/99	7.00	2.01	25
23/4/99	6.17	1.78	52
27/4/99	6.44	1.65	46
30/4/99	6.65	1.63	37

Daily mean values for glasshouse temperature, humidity and weekly irradiation levels

Hydroponic Trial #3: Daily mean glasshouse temperature °C, humidity % and weekly irradiation MJ/m^2 from the investigation to determine of plant nitrate variability in lettuce cultivars raised in a NFT system at HRI Stockbridge House during the growing period 6th – 31st April, 1999.

Date	Temperature°C	Humidity %	Weekly Irradiation MJ/m^2
6/4/99	19.8	53.9	86.09
7/4/99	15.6	58	86.09
8/4/99	16.2	62.5	86.09
9/4/99	18.2	74.5	86.09
10/4/99	15.4	75.1	86.09
11/4/99	14.6	75.7	86.09
12/4/99	14.7	79.1	78.89
13/4/99	11	80.1	78.89
14/4/99	13.1	93.2	78.89
15/4/99	11.3	90.5	78.89
16/4/99	10.3	86.4	78.89
17/4/99	11.4	88.1	78.89
18/4/99	12.3	93.9	78.89
19/4/99	11.7	95.9	76.26
20/4/99	9.8	87.9	76.26
21/4/99	13.9	88.8	76.26
22/4/99	16.4	90.8	76.26
23/4/99	13.8	97.5	76.26
24/4/99	15.2	93.9	76.26
25/4/99	15	90.9	76.26
26/4/99	13.4	86.7	120.10
27/4/99	17.2	87.1	120.10
28/4/99	17.1	88.9	120.10
29/4/99	15.6	88.1	120.10
30/4/99	14.7	94.5	120.10

Soil investigation using winter lettuce: Weekly means of temperature °C, humidity % and irradiation in glasshouse at Stockbridge House, during period from November 1997 to March 1998.

Date	Temperature °C	Humidity %	Irradiation MJ/m^2
1-7/11/97	9.6	87.8	19.04
8-14/11/97	11	83.2	16.17
15-21/11/97	9.8	85.6	9.53
22-28/11/97	10.6	87.5	5.40
29-5/12/97	9.6	90.1	15.99
6-12/12/97	7.0	91.2	13.72
13-19/12/97	8.9	91.4	7.12
20-26/12/97	7.4	85.9	6.73
27-2/1/98	7.7	86.3	11.10
3-9/1/98	8.2	91.4	11.94
10-16/1/98	8.2	88.7	13.92
17-23/1/98	5.2	86.4	20.48
24-30/1/98	5.2	87.6	21.28
31-5/2/98	6.3	87.8	32.78
6-12/2/98	12.6	85.3	28.13
13-19/2/98	9.0	78.8	32.94
20-26/2/98	9.1	78.4	40.55
27-5/3/98	8.8	79.4	39.43
6-12/3/98	9.5	83.3	58.68

Soil investigation using summer lettuce: Daily means of temperature°C, humidity % and irradiation. Values were recorded during the growth period of glasshouse summer lettuce cv.Rachel 1 July to 28 July, 1998 at HRI Stockbridge House.

Date	Temperature °C	Humidity %	Irradiationμmol $s^{-1}m^{-2}$
1/7	15.9	81.3	101.04
2/7	16.4	76.4	101.04
3/7	18.3	69.6	101.04
4/8	18.6	71.7	101.04
5/9	18.9	71.9	101.04
6/9	17.6	73.3	105.09
7/9	16.7	70.3	105.09
8/9	17	72.7	105.09
9/9	19.2	69.3	105.09
10/9	19	65.2	105.09
11/9	18.9	60	105.09
12/9	17.6	79.4	105.09
13/9	16.7	63	116.72
14/9	17.8	59.3	116.72
15/9	18.4	62.1	116.72
16/9	18.2	68.6	116.72
17/9	16	78.3	116.72
18/9	15.6	78	116.72
19/9	17.1	82	116.72
20/9	20.5	80.9	112.79
21/9	19.5	73.8	112.79
22/9	18.7	75.9	112.79
23/9	18	80.3	112.79
24/9	16.6	74.2	112.79
25/9	17	74.7	112.79
26/9	17.9	76.6	112.79
27/9	19.1	79.6	84.55
28/9	18.6	79.8	84.55

Winter soil variety trial: Weekly mean temperature °C, humidity % and irradiation values MJ/m^2 within glasshouse at HRI, Stockbridge House between 21 December 1998 to 4 April 1999.

Date	Temperature (°C)	Humidity (%)	Irradiation MJ/m^2
21-27/12/98	6.6	88.8	8.91
28-3/1/99	7.8	90.6	13.82
4-10/1/99	5.7	86.4	12.95
11-17/1/99	7.4	88	16.75
18-24/1/99	6.9	85.9	18.65
25-31/1/99	8	90.6	13.89
1-7/2/99	7.3	81.7	23.44
8-14/2/99	7.3	86.9	30.48
15-21/2/99	8.1	83.4	30.62
22-28/2/99	9.4	87.7	45.58
1-7/3/99	7.4	89.5	27.41
8-14/3/99	11	84.4	44.77
15-21/3/99	10.8	81.6	68.33
22-28/3/99	13.4	80.5	74.20
29-4/4/99	12.6	77.8	79.45

Analysis of variance of lettuce nitrate concentration in hydroponic growing media.

Table 1 One-way ANOVA of lettuce nitrate concentration in single treatment hydroponic growing media.

Source of Variation	DF	SS	MS	F	P
Tank Nr	4	522055	130514	1.41	0.245
Error	45	4159017	92423		
Total	49	4681072			

Table 2 One-way ANOVA of lettuce nitrate concentration in hydroponic growing media, supplied with three nitrogen nutrient treatments.

Source of Variation	DF	SS	MS	F	P
Treatment	2	9538659	4769329	32.26	0.000
Error	88	13011419	147857		
Total	90	22550078			

Table 3 One-way ANOVA of nitrate concentration of different lettuce cultivars, raised in a hydroponic growing system.

Source of Variation	DF	SS	MS	F	P
Treatment	2	16830991	8415496	119.32	0.000
Error	77	5430860	70531		
Total	79	2261851			

1A) Analysis of variance of lettuce fresh head weight in hydroponic growing media.

Table 4 One-way ANOVA of mean head weight of lettuce raised in a single treatment hydroponic growing media.

Source of Variation	DF	SS	MS	F	P
Treatment	4	619	155	0.24	0.913
Error	45	28823	641		
Total	49	29442			

Table 5 One-way ANOVA of mean head weight of lettuce raised in hydroponic growing media, supplied with three nitrogen nutrient treatments.

Source of Variation	DF	SS	MS	F	P
Treatment	2	7055	3527	4.08	0.020
Error	86	74297	864		
Total	88	81352			

Table 6 One-way ANOVA of mean head weight of different lettuce cultivars, raised in a hydroponic growing system.

Source of Variation	DF	SS	MS	F	P
Treatment	2	66315	33157	7.87	0.001
Error	75	315843	4211		
Total	77	382158			

2) Analysis of variance ANOVA of nitrate concentration of soil grown lettuce, raised under three light intensity and soil nitrogen treatment combinations.

Table 7 Two-way ANOVA of mean nitrate concentration of winter lettuce grown under treatment combinations of varying light intensity and soil nitrogen concentration, showing interaction between treatments.

Source of Variation	DF	SS	MS	F	P
Treatment "light"	2	1726430	863215	13.97	0.000
Treatment "nitrate"	2	1807260	903630	10.35	0.001
Interaction	4	1268278	317069	3.32	0.021
Error	36	3440106	95558		
Total	89	12197350			

Table 8 Two-way ANOVA of mean nitrate concentration of summer lettuce grown under treatment combinations of varying light intensity and soil nitrogen concentration, showing interaction between treatments.

Source of Variation	DF	SS	MS	F	P
Treatment "light"	2	458660	229330	0.89	0.430
Treatment "nitrate"	2	9132847	4566423	24.8	<0.0001
Interaction	4	1401063	350266	2.44	0.067
Error	32	4587751	143367		
Total	80	25527876			

2A) Analysis of variance of fresh head weights of soil grown lettuce.

Table 9 One-way ANOVA of fresh head weights of winter grown soil lettuce, raised under nine treatment conditions of varying light intensity and soil nitrogen concentration.

Source of Variation	DF	SS	MS	F	P
Treatment	8	8062	1008	0.92	0.508
Error	81	89116	1100		
Total	89	97178			

Table 10 One-way ANOVA of fresh head weights of summer grown soil lettuce, raised under nine treatment conditions of varying light intensity and soil nitrogen concentration.

Source of Variation	DF	SS	MS	F	P
Treatment	8	65142	8143	5.15	0.000
Error	72	113817	1581		
Total	80	178960			

3) Analysis of variance of mean nitrate concentration of different soil grown lettuce cultivars.

Table 11 One-way ANOVA of mean nitrate concentration of different lettuce cultivars, raised in soil.

Source of Variation	DF	SS	MS	F	P
Treatment	7	5197881	742554	7.96	0.000
Error	72	67185258	93313		
Total	79	11916409			

3A) Analysis of variance of fresh head weight of different soil grown lettuce cultivars.

Table 12 One-way ANOVA of fresh head weights of different soil grown lettuce cultivars

Source of Variation	DF	SS	MS	F	P
Treatment	7	69801	9972	6.37	0.000
Error	72	112695	1565		
Total	79	182495			

4) Analysis of variance of mean nitrate concentration of hydroponic lettuce showing the interaction between nutrient feed, "treatment", and method of nitrate determination (four analytical techniques detailed in chapter 4).

Table 13) Two-way ANOVA of mean nitrate concentration of hydroponic lettuce and method of nitrate analysis.

Source of Variation	DF	SS	MS	F	P
Treatment	2	50729814	25364907	191.03	0.000
Plot	3	10826914	3608971	27.18	0.000
Interaction	6	704421	117404	0.88	0.507
Error	348	46208434	132783		
Total	359	108469583			

5) Analysis of variance of mean nitrate concentration of lettuce sap showing the interaction between method of nitrate analysis and nitrate extraction technique.

Table 14) Two-way ANOVA of mean sap nitrate concentration of lettuce showing the interaction between method of extraction and technique for nitrate determination.

Source of Variation	DF	SS	MS	F	P
Treatment	1	1275167	1275167	8.62	0.005
Plot	2	5265187	2632594	17.79	0.000
Interaction	2	344024	172012	1.16	0.320
Error	54	7990177	147966		
Total	59	14874555			

APPENDIX:

Figure 1: Relationship between fresh weight and nitrate concentration in butterhead lettuce varieties raised in soil at HRI, Stockbridge House.

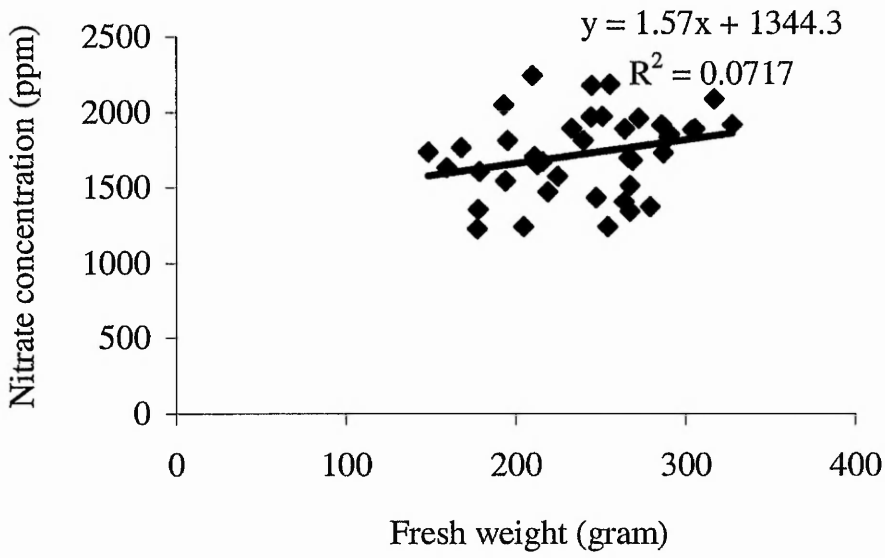


Figure 2: Relationship between fresh weight and nitrate concentration in continental lettuce varieties raised in soil at HRI, Stockbridge House.

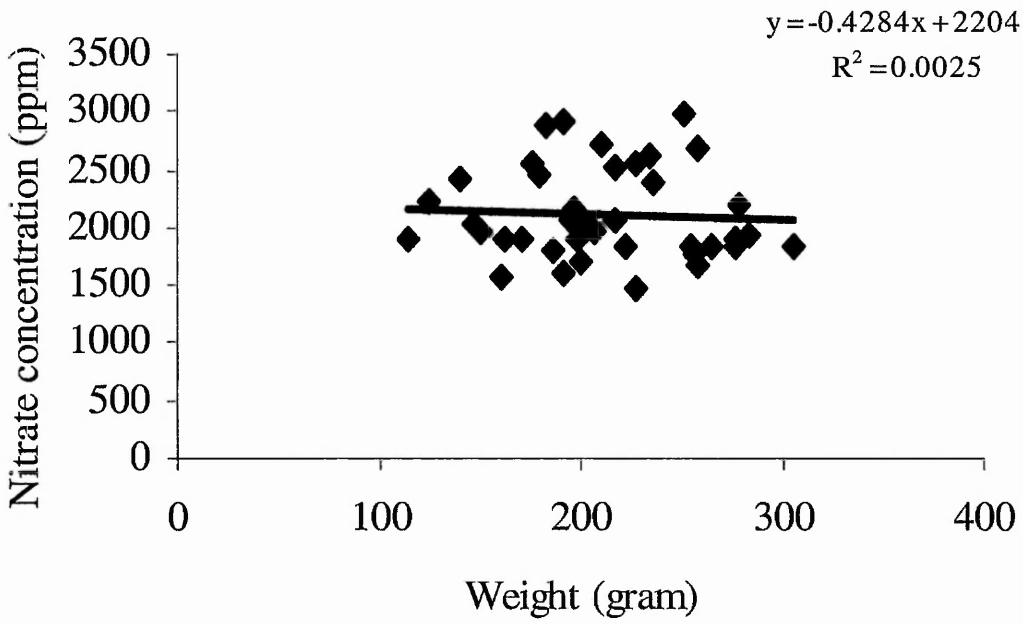


Figure 3 Relationship between fresh weight and nitrate concentration of winter lettuce raised in soil at HRI, Stockbridge House

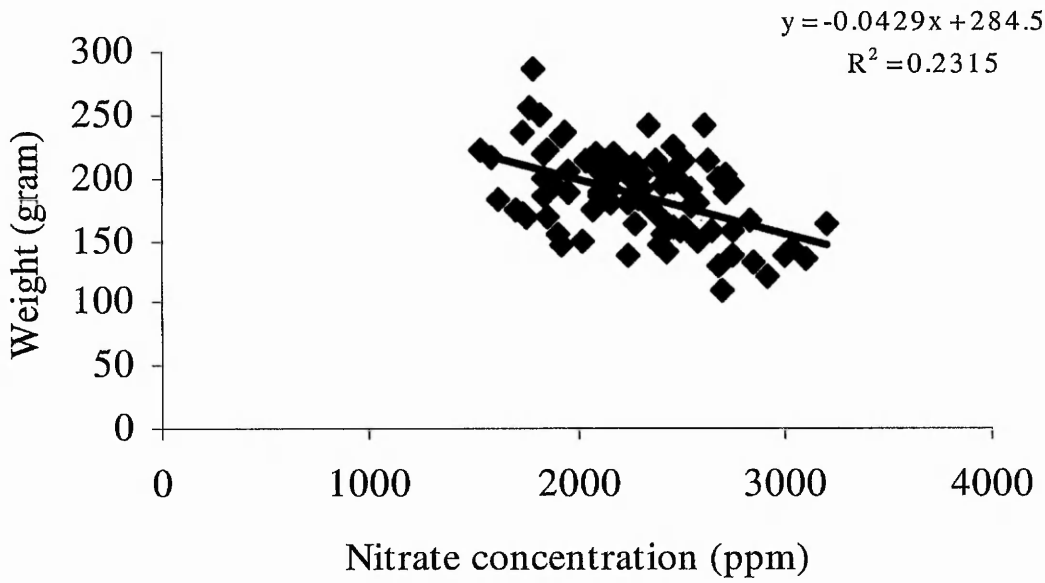
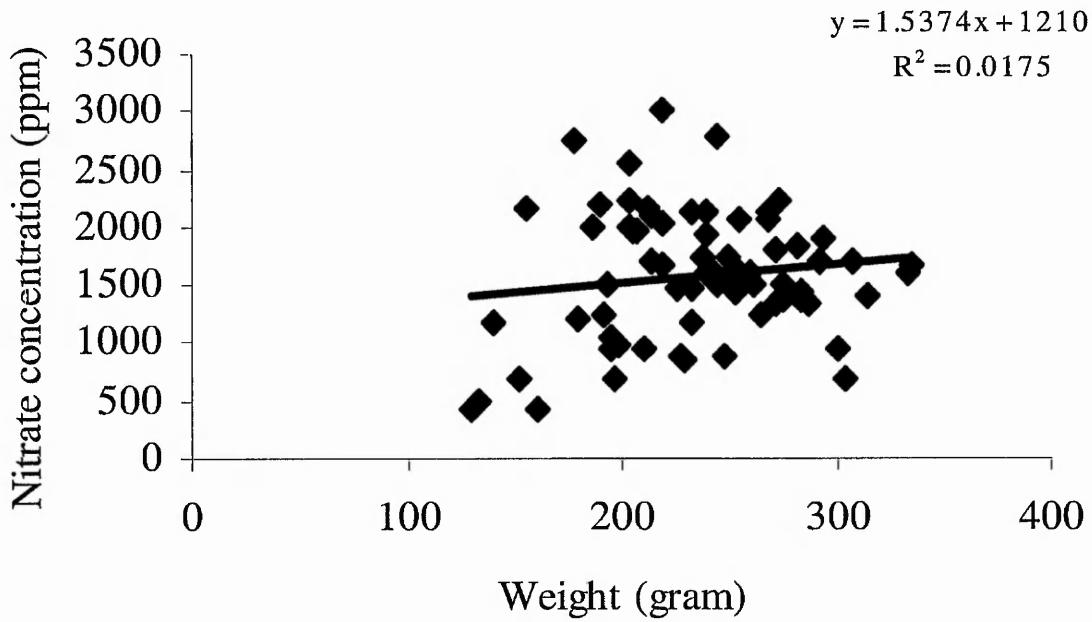


Figure 4 Relationship between fresh weight and nitrate concentration of summer lettuce raised in soil at HRI, Stockbridge House



Regression Statistics

1) Relationship between the Nitrachek metre and Ion Selective Electrode (Probe) methods of nitrate analysis.

Regression Statistics	
Multiple R	0.946627736
R Square	0.896104071
Adjusted R Square	0.894909865
Standard Error	159.3219309
Observations	89

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	19047162.76	19047162.76	750.3764069	1.50046E-44
Residual	87	2208362.556	25383.47766		
Total	88	21255525.32			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	196.7871603	52.97088172	3.71500632	0.000358373	91.50172089	302.0725998	91.50172089	302.0725998
X Variable 1	0.85143112	0.03108207	27.39299923	1.50046E-44	0.789652095	0.913210144	0.789652095	0.913210144

2) Relationship between Nitrachek and Ion Chromatography (Dionex) methods of nitrate analysis.

Multiple R	0.82432041
R Square	0.679504172
Adjusted R Square	0.675777477
Standard Error	262.0465939
Observations	88

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	12520602.77	12520602.77	182.3342265	5.82635E-23
Residual	86	5905483.895	68668.41738		
Total	87	18426086.67			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	277.4216665	87.14802229	3.183338637	0.002027246	104.1772727	450.6660603	104.1772727	450.6660603
1673.021555	0.690358775	0.051125875	13.50311914	5.82635E-23	0.588723983	0.791993567	0.588723983	0.791993567

3) Relationship between Nitrachek and Horiba Cardy methods of nitrate analysis

Regression Statistics	
Multiple R	0.77884506
R Square	0.60659958
Adjusted R Square	0.60207774
Standard Error	348.152268
Observations	89

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	16260165.02	16260165.2	134.148706	2.5804E-19
Residual	87	10545269.95	121209.995		
Total	88	26805434.97			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	608.370427	115.7526295	5.25578062	1.03857E-06	378.2993761	838.441479	378.2993761	838.4414794
X Variable 1	0.78667775	0.067920926	11.5822584	2.5804E-19	0.651677422	0.92167800	0.651677422	0.921678007

4) Relationship between Horiba Cardy and Ion Selective Electrode methods of nitrate analysis

Regression Statistics	
Multiple R	0.81302602
R Square	0.66101133
Adjusted R Square	0.65711493
Standard Error	287.785457
Observations	89

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	14050144.41	14050144.1	169.645797	3.81427E-22
Residual	87	7205380.909	82820.4702		
Total	88	21255525.32			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	211.670019	108.8124093	1.94527463	0.05497122	-4.606582805	427.946622	-4.606582805	427.9466223
X Variable 1	0.72398399	0.055584975	13.0248145	3.81427E-22	0.613502756	0.83446523	0.613502756	0.834465231

5) Relationship between Horiba Cardy and Ion Chromatography methods of nitrate analysis

Regression Statistics	
Multiple R	0.84894266
R Square	0.72070364
Adjusted R Square	0.717493337
Standard Error	243.4535835
Observations	89

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	13305862.6	13305862.6	224.4970774	8.01719E-26
Residual	87	5156459.315	59269.6473		
Total	88	18462321.92			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	70.34701737	92.05041525	0.764222705	0.446802324	-112.6132828	253.30731	-112.613	253.307317
X Variable 1	0.704547191	0.047022395	14.98322654	8.01719E-26	0.611085022	0.7980093	0.611085	0.79800936

6) Relationship between Nitrachek meter and IonChromatography using soil data.

Regression Statistics	
Multiple R	0.69253454
R Square	0.47960401
Adjusted RSquare	0.47293238
Standard Error	259.712124
Observations	80

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4848730.312	4848730.32	71.8858799	1.11013E-12
Residual	78	5261130.106	67450.3858		
Total	79	10109860.42			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	702.622087	127.8415259	5.49603951	4.72429E-07	448.1090986	957.135067	448.1090986	957.1350687
X Variable 1	0.67720162	0.079872301	8.47855406	1.11013E-12	0.51818805	0.83621514	0.51818805	0.836215194

7) Relationship between Nitrachek meter and Ion Selective Electrode using soil data.

Regression Statistics	
Multiple R	0.72765118
R Square	0.52947627
Adjusted R Square	0.52344381
Standard Error	268.112063
Observations	80

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6309460.342	6309460.32	87.7727111	2.08237E-14
Residual	78	5606957.997	71884.0768		
Total	79	11916418.34			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	713.514232	131.9763387	5.40637991	6.80464E-07	450.7694672	976.259001	450.7694672	976.2590011
X Variable 1	0.77250286	0.082455632	9.36870917	2.08237E-14	0.608346245	0.93665947	0.608346245	0.936659427

8) Relationship between Nitrachek meter and Horiba Cardy methods of nitrate analysis, using soil data.

Regression Statistics	
Multiple R	0.80703981
R Square	0.65131329
Adjusted R Square	0.64684296
Standard Error	230.803962
Observations	80

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7761321.617	7761321.67	145.696508	1.58389E-19
Residual	78	4155096.722	53270.4708		
Total	79	11916418.34			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.000%*	*Upper 95.000%*
Intercept	377.136222	130.2088644	2.89639434	0.00489607	117.9102272	636.362213	117.9102272	636.3622173
X Variable 1	0.87618383	0.072588979	12.0704808	1.58389E-19	0.731670294	1.02069742	0.731670294	1.020697472

9) Relationship between fresh weight and nitrate concentration of winter lettuce raised in soil at HRI, Stockbridge House.

SUMMARY OUTPUT winter soil

Regression Statistics	
Multiple R	0.481191827
R Square	0.231545574
Adjusted R Square	0.222813137
Standard Error	326.3628924
Observations	90

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2824246.419	2824246.419	26.51557442	1.57966E-06
Residual	88	9373120.904	106512.7375		
Total	89	12197367.32			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	3298.216109	197.7750708	16.67660183	5.55937E-29	2905.179412	3691.252806	2905.179412	3691.252806
X Variable 1	-5.391928094	1.047113049	-5.14932757	1.57966E-06	-7.47284685	-3.311009338	-7.47284685	-3.311009338

10) Relationship between fresh weight and nitrate concentration in summer lettuce raised in soil at 50ppm nitrate. HRI, Stockbridge House.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.192600448
R Square	0.037094933
Adjusted R Square	0.002705466
Standard Error	428.9174903
Observations	30

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	198443.3995	198443.3995	1.078671355	0.307881106
Residual	28	5151165.978	183970.2135		
Total	29	5349609.377			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	715.0876693	397.5743005	1.798626492	0.082867221	-99.30728169	1529.48262	-99.30728169	1529.48262
X Variable 1	1.959619815	1.886806003	1.038591043	0.307881106	-1.905331416	5.824571046	-1.905331416	5.824571046

11) Relationship between fresh weight and nitrate concentration in summer lettuce raised in soil at 100ppm nitrate. HRI, Stockbridge House.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.303623365
R Square	0.092187148
Adjusted R Square	0.05976526
Standard Error	31.36748525
Observations	30

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2797.638	2797.638	2.84336152	0.102865343
Residual	28	27549.74	983.9191		
Total	29	30347.37			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	282.9008366	22.47206	12.589	4.77036E-13	236.8688607	328.9328124	236.8688607	328.9328124
X Variable 1	-0.021809168	0.012934	-1.68623	0.102865343	-0.048302699	0.004684362	-0.048302699	0.004684362

12) Relationship between fresh weight and nitrate concentration in summer lettuce raised in soil at 150ppm nitrate. HRI, Stockbridge House.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.497755678
R Square	0.247760715
Adjusted R Square	0.220895026
Standard Error	45.40131201
Observations	30

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	19009.52538	19009.52538	9.222198529	0.005127432
Residual	28	57715.81571	2061.279133		
Total	29	76725.3411			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	335.3795264	33.81583676	9.917824265	1.15443E-10	266.1108471	404.6482057	266.1108471	404.6482057
X Variable 1	-0.052661206	0.017340977	-3.036807292	0.005127432	-0.088182628	-0.017139785	-0.088182628	-0.017139785

13) Relationship between fresh weight and nitrate concentration in butterhead lettuce varieties raised in soil at HRI, Stockbridge House.

SUMMARY OUTPUT

butterhead
soil

Regression Statistics	
Multiple R	0.267835345
R Square	0.071735772
Adjusted R Square	0.047307766
Standard Error	262.0864371
Observations	40

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	201714.3768	201714.3768	2.936620046	0.094739703
Residual	38	2610193.42	68689.30052		
Total	39	2811907.797			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	1344.346157	223.8479438	6.005622095	5.59254E-07	891.1896704	1797.502644	891.1896704	1797.502644
X Variable 1	1.569997528	0.916167938	1.713656922	0.094739703	-0.284687563	3.424682619	-0.284687563	3.424682619

14) Relationship between fresh weight and nitrate concentration in continental lettuce varieties raised in soil at HRI, Stockbridge House.

SUMMARY OUTPUT

continental
soil

Regression Statistics	
Multiple R	0.050427105
R Square	0.002542893
Adjusted R Square	-0.023705978
Standard Error	397.5413059
Observations	40

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15310.23891	15310.23891	0.096876279	0.757312063
Residual	38	6005485.415	158039.0899		
Total	39	6020795.654			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	2204.262255	296.7986024	7.426794592	6.55496E-09	1603.424876	2805.0996	1603.424876	2805.099635
X Variable 1	-0.428368713	1.376287039	-0.311249544	0.757312063	-3.214516261	2.3577788	-3.214516261	2.357778834

Figure 1: Mean nitrate concentrations of winter lettuce grown at different planting densities (25 and 33 plants/metre) and three soil nitrogen regimes. Trial conducted at HRI, Stockbridge House as part of winter soil trial detailed in chapter three.

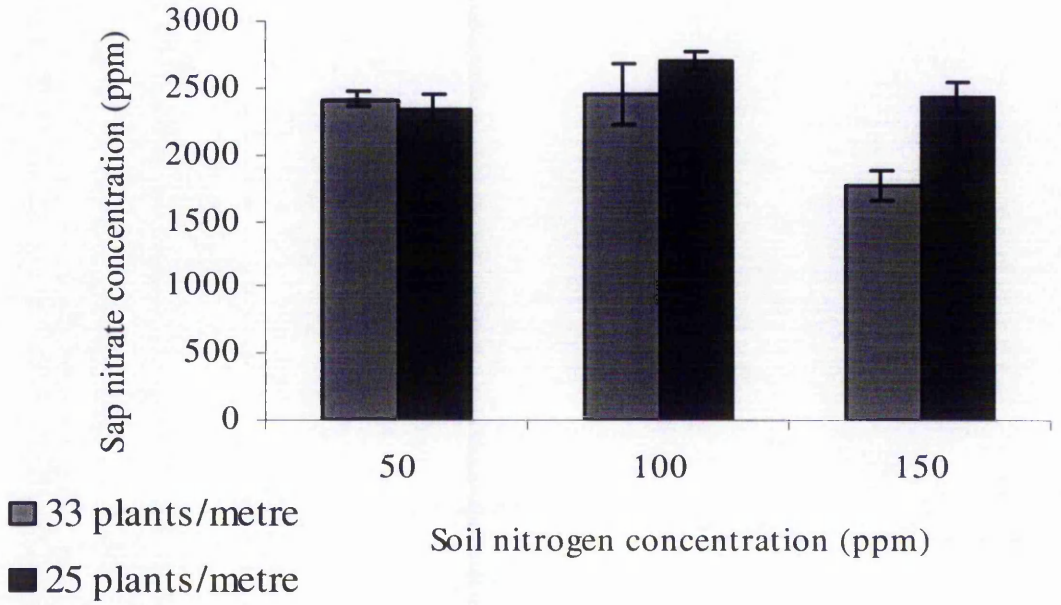


Figure 2: Mean fresh weight of winter lettuce grown at different planting densities (25 and 33 plants/metre) and three soil nitrogen regimes. Trial conducted at HRI, Stockbridge House as part of winter soil trial detailed in chapter three.

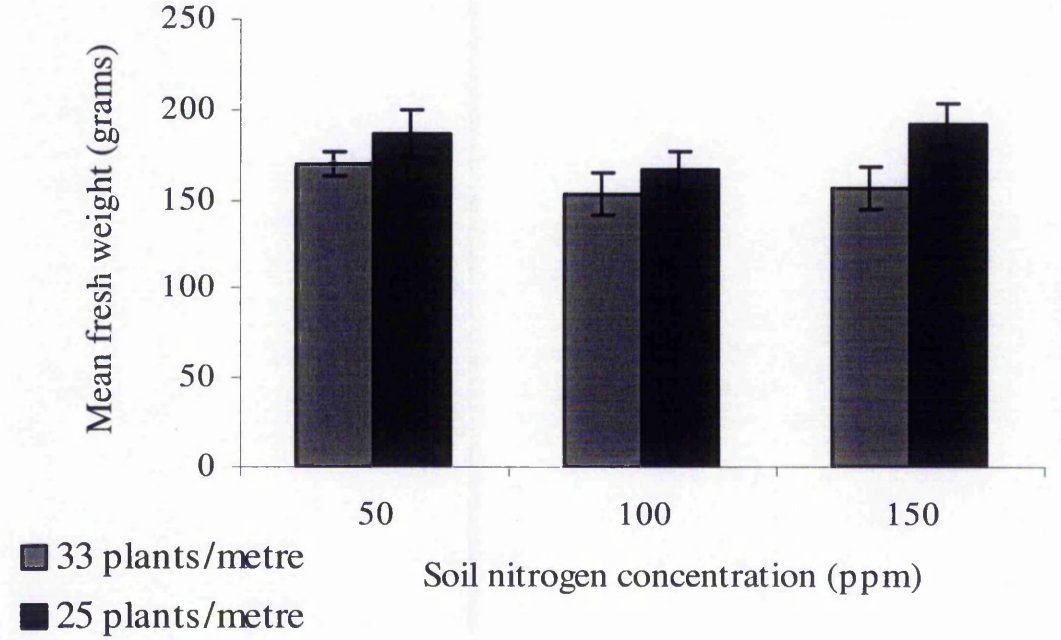
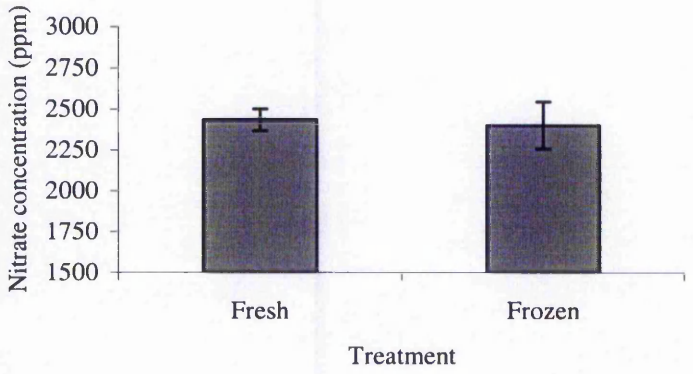


Figure 3 Mean nitrate concentration of lettuce sap following two different methods of nitrate extraction, conducted at West Cranleigh Nurseries, 1999.



Small-scale studies:

t – Test Paired Two Sample for Means

1) The difference between mean nitrate concentration of lettuce sap blended for 1 minute and the mean nitrate concentration of lettuce sap blended for 10 minutes.

The null hypothesis states that blending for 10 minutes does not significantly influence sap nitrate concentration.

.	Variable 1	Variable 2
Mean	2825	3488
Variance	12566.4	735477.8
Observations	3	3
Hypothesised mean	0	
df	2	
t stat (t)	-1.88	
$t_{(10\%)}$4 (T)	2.776	

T>t (-1.88<2.776). Therefore do not reject null hypothesis and conclude that there is no significant influence on the nitrate concentration of lettuce following an extended blending period of 10 minutes, (p=0.1).

2) The difference between the mean nitrate concentration of lettuce extracted in 150 ml water and the mean nitrate concentration of lettuce extracted in 300ml water.

The null hypothesis states that doubling the volume of water used during sap extraction, from 150ml to 300ml per lettuce head, does not influence the nitrate concentration of the extracted lettuce sap.

.	Variable 1	Variable 2
Mean	2300	2536
Variance	109826	49997
Observations	3	3
Hypothesised mean	0	
df	2	
t stat (t)	-1.45	
$t_{(10\%)}$4 (T)	2.776	

T>t (2.776>-1.45). Therefore do not reject null hypothesis and conclude that increasing the volume of water used during extraction from 150ml to 300ml does not significantly influence sap nitrate concentration in lettuce, (p=0.1).

3) The difference between mean nitrate concentration of sap extracted from fresh lettuce heads and mean nitrate concentration of sap extracted from frozen lettuce heads.

The null hypothesis states that using frozen instead of fresh lettuce heads in the extraction procedure does not influence nitrate concentration in the extracted sap.

.	Variable 1	Variable 2
Mean	1338	1309.5
Variance	14682.2	11378.5
Observations	5	5
Hypothesised mean	0	
df	4	
t stat (t)	0.56	
$t_{(10\%)}$4 (T)	2.306	

T>t, (2.306>0.56). Therefore do not reject the null hypothesis and conclude that using frozen lettuce material in the extraction procedure does not significantly influence sap nitrate concentrations, (p=0.1).

4) The difference between mean nitrate concentration of sap extracted from lettuce using cold, deionised water and mean nitrate concentration of sap extracted from lettuce using hot deionised water.

The null hypothesis states that using hot water in the extraction procedure compared to cold water does not influence sap nitrate concentration.

.	Variable 1	Variable 2
Mean	2434	4419.4
Variance	4480.96	19745.9
Observations	3	3
Hypothesised mean	0	
df	2	
t stat (t)	31.24	
$t_{(10\%)}$4 (T)	2.776	

T<t, (2.776<31.24). Therefore reject the null hypothesis and conclude that there is a significant influence on sap nitrate concentration following the use of hot water in the extraction procedure, (p=0.1).