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Genotype and Environment effects on nitrate accumulation in a diversity set of lettuce accessions at commercial maturity: the influence of nitrate uptake and assimilation, osmotic interactions, and shoot weight and development

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ABSTRACT

BACKGROUND. The causes of the natural variation in nitrate accumulation and associated traits are studied using a diverse population of 48 mature lettuce accessions grown hydroponically in winter and summer seasons. Information on the effects of genotype (G), environment (E) and their interactions will inform future selection strategies for the production of low-nitrate varieties more suited to meeting EU requirements for harvested produce.

RESULTS. The effects of G, E and G x E interactions were all significant, with nitrate concentrations lower but covering a wider range in summer. Concentrations of nitrate-N were positively correlated with those of water and total-N, and negatively with assimilated-C in the shoot in both seasons, with all relationships partitioned according to morphotype and/or seasonal type. Corresponding relationships between nitrate-N and assimilated-N, or with shoot fresh or dry weight were generally weak or inconsistent. Nitrate concentrations at an early growth stage were strongly related to those at maturity in the winter, but not in summer when light levels were less variable.

CONCLUSIONS. The effects of genotype and environment on nitrate accumulation in lettuce are strongly influenced by morphotype, with most G x E interactions between accessions within the same morphotype predominantly of the non-crossover type. All low nitrate-accumulating genotypes have increased concentrations of organic solutes (concentration regulation) and reduced water (volume regulation) to help stabilise osmotic potential within the shoots. Variability in nitrate accumulation arises more from differences in uptake than in efficiency of its chemical reduction. Genotypic differences in nitrate accumulation can be masked by changes in head morphology during maturation, provided they are not confounded by substantial changes in intercepted light. Recent selection strategies do not appear to have produced lower nitrate-accumulating cultivars.

Keywords: genotype; environment; interactions; nitrate; water; assimilated-C; assimilated-N; osmotic; shoot weight; lettuce; *Lactuca sativa*; *Lactuca serriola*; varieties; maturity; screening; diversity set

INTRODUCTION

Nitrate is an important plant nutrient which is taken up by the roots and accumulates naturally in vegetative tissues as a result of the combined effects of nitrogen (N) supply, aerial environment (E) and genotype (G).[1-3] Plant nitrate concentrations tend to rise when its rate of uptake exceeds that of its chemical reduction,[4,5] particularly when environmental stresses, such as low light intensity or short day length, restrict the energy for photosynthesis and nitrate assimilation.[6,7] Any nitrate that accumulates in plant tissues is not only used as a temporary store of N,[8,9] but also acts as a replacement osmoticum for other plant solutes,[10-13] helping maintain turgor and drive leaf expansion.[14-16]

Most annual crops accumulate some nitrate, but many leafy (salad) vegetables maintain higher concentrations in their tissues than other types of crop.[1,2,17] Salad crops form an important part of many western diets,[18] and fears that their consumption could result in excessive nitrate intake has led to the introduction of maximum permissible limits on nitrate concentrations in the shoots of lettuce and spinach in Europe.[19,20] Draft EU legislation has also been published to extend this legislation to other crops,[21] despite recent evidence that nitrate is unlikely to be a significant hazard to human health, and may even be beneficial.[17,22-24] This legislation is of particular concern to protected lettuce growers in northern latitudes, because poor light quality in their glasshouses can cause substantial increases in nitrate concentration in marketed produce, especially in wintertime. Opportunities for controlling nitrate accumulation by restricting N supplies at this time of year are limited.[6,7]

Nitrate accumulation is also subject to considerable genetic variability within and between different species of lettuce, including cultivated types and their wild relatives.[11,25-28] This suggests that a breeding approach could be adopted to produce new low-nitrate varieties, particularly as the inheritance of this trait not only appears to be relatively simple, but is also controlled by dominant genes.[29-32] As protected lettuce is now grown throughout the year, the identification of suitable breeding material must consider the effects of seasonal variations in light, which have the potential to cause significant interactions between genotype (G) and environment (E).[33] However, preliminary work with a limited selection of *Lactuca sativa* accessions representing different morphotypes suggested that the ranking of nitrate concentration was relatively insensitive to growing season.[28] This implies that any G x E interactions in nitrate accumulation by lettuce are predominantly of the non-crossover type.[34] If these results are confirmed using a wider range of accessions, it would simplify the production of low-nitrate breeding material, as selection trials would not need to be replicated in different seasons.

Most genotype studies of nitrate accumulation in lettuce have been made at a relatively early stage of growth, in the belief that such effects are consistent throughout the lifetime of a plant.[25,35] However, studies on other species have raised doubts about this assumption because reproductive organs (such as flowers and fruits or grains) which develop later in growth have lower nitrate concentrations than leaves, roots, stems and petioles.[1,2] Furthermore, changes in the proportion of vascular tissue as a plant ages are also likely to affect its net nitrate concentration.[36] A recent study with lettuce found that morphological changes in the plant (particularly after the start of heart formation) affected net nitrate accumulation; this was attributed to a reduction in the transfer of nitrate through the xylem to the newer leaves in the shoot.[28] The associated increases in the proportion of younger leaves (with lower nitrate) to that of older ones (higher in nitrate) often results in a net reduction in shoot nitrate concentration over time, with these changes likely to mask many of the earlier differences between genotypes.[26,28,37-40] Thus in order to provide a more accurate reflection of the potential contributions of lettuce to dietary nitrate intake, varieties should be screened using plants that are commercially mature.

This paper describes a study in which a set of 48 lettuce accessions (representing the diversity within the genepool) are used to characterise the effects of genotype, environment and their interactions on the accumulation of nitrate in their shoots at commercial maturity in two experiments carried out in contrasting seasons (winter and summer). The influence of shoot weight and development, and of other relevant plant properties associated with nitrate uptake and reduction, and with osmotic processes within the shoot are also investigated. The diversity set chosen for study included a group of accessions (subset A) consisting mostly of older (*L. sativa*) cultivars which had previously been screened for nitrate at an early stage of growth,[28] together with another group of predominantly modern cultivars (subset B) used specifically for either winter or summer glasshouse production. In addition to allowing a comparison of newer and older genetics, comparing data for subset A with those for corresponding accessions in the previous study[28] will provide a greater understanding of the extent to which genotypic effects on nitrate accumulation (and any environmental effects between seasons) are influenced by developmental stage for a range of different lettuce morphotypes. This new study will also examine whether there are any inherent differences in nitrate accumulation between winter (*ie* short day) and summer (*ie* day neutral) lettuce types. The inclusion of three Lactuca serriola accessions allows a comparison between undomesticated and domesticated germplasm.

EXPERIMENTAL

Plant material and associated maturity assessment

A diversity set consisting of 48 different lettuce accessions including 45 lettuce (*L. sativa* L.) cultivars, and three *L. serriola* L. lines, was sourced from the Warwick Genetic Resources Unit (GRU), from seed stocks produced in-house at Warwick, or from commercial seed suppliers (see Table 1). The lettuce cultivars were chosen to represent five major lettuce morphotypes: crisphead, butterhead, cos (romaine), leaf (bunching or cutting), and stem (stalk or asparagus) lettuce.[18] The selected types varied in head density and morphology (including firm and loose hearting, and non-hearting types), in leaf form (length, shape, texture and size) and in colour (light to dark green, red and blush or tinted leaves).

Accessions 1 to 24 (designated as diversity subset A), consisting of older cultivars and two *L. serriola* accessions, had been used in a previous screen in which the plants were sampled at a relatively early stage in growth.[28] Accessions 25 to 48 (diversity subset B) included more modern cultivars (mostly butterhead types) intended for glasshouse production. All accessions were categorised according to seasonal type (*ie* between short day and day neutral lettuce types used predominantly for commercial production in winter and summer respectively) to allow comparisons of their performance at contrasting times of year.

As the different accessions varied in their rate of plant development, all replicates of each accession were sampled on one of three dates, when they were judged to have reached commercial maturity. A total of 16 accessions were sampled on each date in both experiments, with the final group comprising the weakly hearting and non-hearting lines. However, as the development rates also varied between winter and summer, individual accessions were not necessarily sampled in the same group in both experiments.

Plant raising and culture

Lettuce seeds were germinated in the dark at 20^{o}C on moist paper tissues in petri dishes in an incubator. After 36 to 48 hours, about 24 seeds (all with radicles of between 1 to 2 mm in length) of each accession were transferred individually to separate 40 mm rockwool cubes (BHGS Ltd, Evesham, UK), which were kept moist with tapwater in the glasshouse. After emergence, the resulting seedlings were selected for uniformity at the first true leaf stage and transferred in their cubes to four duplicate recirculating Nutrient Film Technology (NFT) systems (two each in separate glasshouse compartments) for the start of the experiments.

Each NFT system comprised eight individual flat-bottomed gullies (5.2 m in length) arranged on a 1 in 60 gradient along a north–south axis and spaced at 220 mm centres on a separate glasshouse bench. The gullies were fitted with segmented lids in which circular 45 mm holes had been cut to accommodate the rockwool cubes at transplanting. The holes were located at 320 mm intervals along the lids, allowing 14 plants to be grown in each gully (including a guard plant at either end). The position of the first hole in adjacent gullies alternated between 240 and 480 mm from one end, producing a staggered plant arrangement with a diagonal spacing of about 270 mm between successive gullies, and an overall density of 14.2 plants m^{-2} on each bench. All holes left unoccupied after plant sampling were covered to exclude light. A complete nutrient solution containing nitrate (at 8 mol m^{-3}) as the sole N source and an adequate concentration of all other nutrients[28] was continuously pumped from linked storage tanks (with a total capacity of 800 L) to the top end of the eight gullies; this then flowed down the gradient and drained back into the original tanks. This solution was used at half-strength during the first 5 days following transplanting, and at full strength thereafter. Concentrations of nutrients were checked at weekly intervals by laboratory analysis, and solutions supplemented or replaced when nitrate concentrations had fallen by about 30%. All solutions were adjusted to pH 6.0 using dilute H_2SO_4 at 1-2 day intervals.

Glasshouse environment

Automatic venting, fans and heating were used to maintain glasshouse day/night temperatures close to 25/10 ^{o}C (± 3 ^{o}C) in summer, and 18/8 ^{o}C (± 2 ^{o}C) in winter. Supplementary lighting was used for all but the last 2 weeks of growth in the winter to prevent the hypocotyls of the plants from becoming elongated. Light intensity above the crop canopy was measured at 5 minute intervals using solarimeters (Delta-T Devices, Cambridge, UK) positioned on an east-west axis at opposite ends of the gullies and recorded automatically using Squirrel 1201 data loggers (Grant Instruments, Cambridge, UK). The data for both experiments is shown in Figure 1.

Details of the screening experiments

The 48 lettuce accessions (Table 1) were screened for nitrate accumulation using the four NFT systems under both winter and summer conditions. Individual seedlings of each accession were laid out in a Latinised row-column alpha design with two replicates each in a single block at opposite ends of the gullies in each NFT system, giving four replicates per glasshouse compartment and eight replicates in total. Seedlings for the winter screen were transferred to the NFT systems on 27 October 2004 and grown on until maturity. The resulting plants were sampled destructively in three equal groups (each of 16 accessions) on one of three dates: 4 January 2005 (for earliest hearting accessions), 7 January (for slower hearting accessions), and 11 January (for weakly hearting and non-hearting accessions). Seedlings for the summer screen were transplanted on 25 May 2005, with corresponding sampling dates of 29 June, 1 July and 4 July 2005 respectively, depending on maturity. Accessions sampled in the summer experiment were generally heavier than those in the winter.

Plant sampling and analysis

All samplings started at about 0900 hours and were completed at around 1100 hours. The shoots were cut off at the base of the stem and the weight of fresh matter (FM) measured without any leaf trimming. Corresponding weights of dry matter (DM) were determined after oven drying at 80-90°C for between 24 and 48 hours. Concentrations of water in the shoots were calculated as g g^{-1} in DM from the difference. The dried material was milled to 1.0 mm and nitrate determined on water extracts using Flow Injection Analysis.[13] Average shoot nitrate concentrations were expressed directly as mg kg^{-1} in FM (to be consistent with the units used for current EU limits[20]), and as nitrate-N g kg^{-1} in DM (to facilitate comparisons with other elemental constituents in the shoots). Assimilated-C (carbon) and total-N were determined directly by IR analysis following combustion of the milled samples using a CN2000 Analyser (LECO Corporation, Michigan, USA) as g C kg^{-1} and g N kg^{-1} in DM respectively. Corresponding concentrations of assimilated-N (*ie* of organic forms of N) were calculated from the difference between those of total-N and nitrate-N. The concentration of total-N approximates to the total uptake of nitrate-N per unit plant weight, because the proportions of total-N in the roots are much smaller than in the shoots.

Statistical analysis

Statistical analyses were conducted in Genstat (Version 9.1, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK). A residual or restricted maximum likelihood analysis (REML; a generalised analysis of variance which can be applied to unbalanced data) was used to estimate standard errors of differences (SEDs) for each variate, and to assess the statistical significance of the genotype effects under both winter and summer conditions. These REML analyses were then repeated for both experiments by including the accumulated light levels over both 7 and 10 day periods immediately prior to the three sampling dates as covariates to determine whether any small differences in irradiance arising from partitioning the plant population into early, medium and late harvesting groups significantly affected the estimated genotype effects. Further REML analyses were used to compare the winter and summer experiments and determine the overall significance of environment (*ie* season) and genotype x environment interactions. A χ^2 test on the Wald statistic was used to assess statistical significance in each analysis. To equalise the variances over each dataset, a logarithmic transformation was applied to all fresh and dry weight data, and an angular transformation to the water concentration data (after conversion to percentages) prior to statistical analysis. No transformations were necessary for the nitrate concentrations (on either a DM or FM basis), nor for the total-N, assimilated -N or assimilated-C concentration data. Unless otherwise stated, statistical significance was determined at the 5% level.

A sequential linear regression approach (analysis of parallelism) was also set up in Genstat to investigate the interrelationships between the replicate means of selected variates for each accession, and examine whether these were modified by lettuce morphotype. This analysis fitted three separate sequential models to the data: the first comprising a single regression line for all morphotypes; the second, parallel regression lines with a different intercept for each morphotype; and the third, different regression lines with separate slopes and intercepts for each morphotype. The changes in residual deviance between these nested models were assessed using an accumulated analysis of variance to determine whether the second or third steps in this process significantly improved the overall fit to the data. All of the fits were also assessed by estimating the percent variance accounted for by the regression (R_{adj}^2 values), with significance levels determined using standard F tests. The same approach was also used to determine whether equivalent relationships for the butterhead cultivars (which represented the largest proportion of the whole diversity set) were affected by their seasonal type (*ie* by their normal season of production).

RESULTS AND DISCUSSION

Comparisons within seasons

Effect of genotype

REML analyses showed that nitrate concentrations varied significantly between accessions (genotypes) within each season ($P<0.001$) irrespective of whether they were expressed on a

DM or FM basis. Additional REML analyses on nitrate concentrations showed that these genotype effects were not significantly influenced by accumulated light levels during either a 10 or 7 day period immediately prior to each sampling date. This indicates that any differences in environmental conditions experienced by the groups of accessions sampled on the three different dates (depending on maturity) did not materially affect any conclusions in either experiment. Corresponding REML analyses for shoot fresh and dry weights, and the concentrations of water, assimilated-C, total-N and assimilated-N were also strongly affected by genotype in both experiments (all significant at $P<0.001$).

Nitrate concentrations in the untrimmed heads ranged from 11.3 to 31.2 g N kg^{-1} DM and from 2289 to 6422 mg kg^{-1} FM in winter, whereas in summer the concentrations varied between 7.5 and 30.5 g N kg^{-1} DM and between 2896 and 5488 mg kg^{-1} FM, see Table 2. Average nitrate concentrations in the DM of the *L. sativa* accessions were significantly larger in winter than summer (23.5 vs 21.4 g N kg^{-1} DM), but not when expressed on a FM basis (3920 vs 4287 mg kg^{-1} FM). Corresponding average concentrations for the *L. serriola* types were lower in summer irrespective of how they were expressed (9.3 vs 24.0 g N kg^{-1} DM and 4166 vs 5578 mg kg^{-1} FM). Nitrate concentrations for some *L. sativa* cultivars at the upper end of the range exceeded the proposed increased new EU limits for lettuce (4000 mg kg^{-1} FM in summer and 5000 mg kg^{-1} FM in winter[21]) especially in summer, although these limits will only apply to trimmed produce from which some of the older leaves with higher nitrate have been removed. However, these results are in line with other data which show that it can be more difficult to meet existing EU limits in summer than in winter.[41]

Figure 2 shows data for each of the accessions arranged in order of increasing nitrate-N concentration in the tissue DM for both the winter and summer experiments. The largest differences between successive accessions occurred at lower and higher concentrations, with smaller differences in intermediate parts of the range. Amongst the *L. sativa* accessions, average concentrations were higher for butterhead and leaf lettuce and lower for the crisphead and cos morphotypes in both seasons. There was also a difference in behaviour between the *L. serriola* and *L. sativa* accessions in the summer experiment, when the former exhibited the lowest nitrate-N concentrations on a DM basis, although the same was not evident under winter conditions. Corresponding data for nitrate concentrations in the FM of *L. sativa* accessions showed a broad similarity in the ranking of accessions to that in the DM for each experiment (data not shown), but with some differences in the middle part of the range (particularly in the winter), where quite small errors of estimation could have contributed to relatively large effects on their ranking. However, the behaviour of two of the *L. serriola* types (accessions 4 and 12) were somewhat inconsistent, with both exhibiting the highest concentrations in the FM under winter conditions, but not in summer. This seasonal difference in nitrate concentration is not unexpected because these wild relatives are examples of early colonising summer weed species which evolved in arid habitats under high-light and long-day conditions, and appear to be less able to control nitrate accumulation when grown hydroponically in winter. The same two *L. serriola* accessions also generated the highest concentrations in their FM when subset A of the population was sampled at an earlier stage of growth in a previous winter screen.[28]

A direct comparison between concentrations of nitrate in the FM with those of nitrate-N in the DM showed a strong correlation between the two (R_{adj}^2 = 50.1 and 59.5 % in winter and summer experiments respectively; both significant at $P<0.001$). However, analyses of parallelism revealed that both relationships were influenced by morphotype, with the data best described by a series of parallel lines (one for each morphotype) in each experiment, as shown in Figure 3. Allowing for morphotype in this way increased the R_{adj}^2 values to 73.2 and 81.1 % in winter and summer respectively (both significant at $P<0.001$), and revealed a slightly steeper slope for the relationships in summer, see Table 3. There was no further significant improvement in the fit by allowing the slopes of the regression lines for each morphotype to vary independently in either experiment. This partitioning implies that the relationships between the concentrations of nitrate and water in the shoot vary between morphotypes, so any changes in ranking arising from the conversion of nitrate concentrations from a DM to a FM basis (*eg* for the *L. serriola* accessions) were largely a consequence of differences in average shoot water concentration between the various types (see below). It also follows from this partitioning that any such changes in ranking between individuals would have been less had a population of just one morphotype been screened, much as is normally practiced by most commercial breeders. Because of the strong correlation between nitrate concentrations in the FM and the DM, subsequent concentration data are presented only on a DM basis, except where there are differences likely to affect the interpretation of the data.

Relationship with shoot water concentration

Table 2 summarises the main differences in water concentration between the accessions. In general, water concentrations were lower in *L. serriola* and stem lettuce, and higher in leaf and butterhead types in both seasons, although crisphead cultivars also had higher concentrations in winter. Average values for all lettuce types were greater in winter than summer, with the *L. serriola* accessions showing the largest seasonal difference due to very low summer values. Analyses of parallelism confirmed there were strong relationships between nitrate-N and water concentrations in the shoot DM, which differed between morphotypes. Figure 4 shows that the results were best described by a series of parallel lines (one for each morphotype) for all of the accessions in both winter (R_{adj}^2 = 44.4 %; P=0.001) and summer (R_{adj}^2 = 68.6 %; P<0.001), although the data for the butterhead cultivars were somewhat variable in both. Such dependence on water concentration originates partly from a process of volume regulation, in which the lettuce plants automatically adjust the proportions of their tissue water in tandem with changes in the concentration of all endogenous solutes (including nitrate) in order to help stabilise osmotic potential in their shoots.[13] The accumulation of alternative organic osmotica (including soluble carbohydrates, organic anions *etc*) in plants with lower nitrate concentrations also tends to increase their DM content,[10-13] and contributes to the reduction in the proportion of water present in their shoots. The parallel relationships within each experiment (Figure 4) show that there was essentially the same unit change in water concentration per unit change in nitrate concentration for all morphotypes, implying that the mediating effects of volume regulation were similar for each at the same time of year.

A statistical summary of the fit of the models to the data in Table 4 shows that the slope of the lines was lower under winter conditions than in summer, and reflects a larger unit change in water concentration relative to that of nitrate across the range of accessions in the winter. It also explains why the slopes of the relationships between nitrate concentrations in the FM against those in the DM were different under winter and summer conditions (see Table 3). This seasonal effect on slope is consistent with that for equivalent relationships for the same 22 *L. sativa* morphotypes in subset A when sampled at an earlier stage of growth,[28] although in the latter work, separate parallel relationships between nitrate concentration and water content were only observed under summer conditions, with all morphotypes following a single relationship in winter. Apart from these results, no other studies have been able to identify similar seasonal differences in the slope of such relationships.[42,43]

Relationship with assimilated-C concentration

Assimilated-C concentrations in shoot DM ranged from 310.7 to 387.8 g C kg^{-1} in winter and 345.0 to 429.6 g C kg^{-1} in summer (Table 2). The majority of this C forms part of the relatively stable structural material of the plants, with a smaller fraction representing the more labile non-structural solutes, which (amongst other functions) are used for balancing the osmotic potential of the plant sap when nitrate concentrations are low.[13] Comparisons of nitrate-N and assimilated-C concentrations in the shoot DM across all accessions revealed a significant negative correlation between the two in both winter and summer, which is consistent with the low-nitrate accessions using organic solutes instead of nitrate as part of an iso-osmotic control mechanism. Analysis of parallelism also showed significant partitioning in the relationships for both experiments, with the data best described by separate parallel lines, one for each morphotype (with R_{adj}^2 values of 82.6 and 68.4 % in winter and summer respectively; $P<0.001$), see Figure 5. These analyses also showed that the slopes of the lines were almost identical under winter and summer conditions, see Table 4.

The similarity of these slopes indicates that the unit change in nitrate-N concentration with that for the soluble fraction of assimilated-C was effectively the same across all accessions in both seasons, and implies that all morphotypes utilise similar proportions of organic osmotica for adjusting osmotic potential in winter and summer. Previous studies have shown that any changes in the ionic charge of nitrate are mostly balanced by organic anions, comprised largely of malate.[10] Assuming for our experiments, that malate in its doubly ionised form was the only organic anion involved, with the remaining soluble assimilated-C comprised entirely of simple carbohydrates, then a slope of 0.23 g of nitrate-N per g of assimilated-C for the relationships in Figure 5 would mean that assimilated-C accounted for approximately 76

% of any osmotic effects resulting from differences in nitrate concentration across the range of accessions. The assumptions used in this calculation are only approximate, but the result is broadly consistent with measurements on individual lettuce cultivars, where the proportion of organic acids plus soluble carbohydrates used to balance nitrate ranged from 60 or 68 % [12] to figures approaching 100 %.[44] Any deficit in this balance may be made good by changes to the concentrations of other inorganic ions within the shoot and by adjustments to its shoot water content.[13]

Relationships with total-N and assimilated-N concentrations

Nitrate accumulates in the shoots as a result of uptake by the roots, with its concentration modified by chemical reduction to organic forms of N (assimilated-N). As total-N in the shoot represents a far larger proportion of that in the root, its concentration in shoot DM provides a good approximation of total amount of N taken up per unit of plant dry weight. Table 2 shows that average total-N concentrations in shoot DM were greater in winter than in summer, although those for the wild relatives were consistently lower than those for the cultivated types in summer. Comparison of the shoot concentrations of nitrate-N and total-N revealed there was a strong positive correlation between the two for both experiments, with the data best described by a separate parallel line for each morphotype in each case, as shown in Figure 6. R_{adj}^2 values were 86.8 % in winter and 75.0 % in summer, both significant at $P<0.001$. The slopes of these relationships show that variations in shoot nitrate concentrations across all accessions accounted for 89.4 % of those for total-N in the winter, and 81.5 % in the summer, see Table 4. The large size of these slopes implies that the variations in shoot nitrate concentration were largely caused by differences in nitrate uptake.

This was confirmed by data for the corresponding relationships between the concentrations of nitrate-N and assimilated-N in the shoots for both experiments. Although average concentrations of assimilated-N in the lettuce shoots were about 60 % greater than those of nitrate-N (Table 2), simple statistical analyses of the pooled data for all accessions failed to show any correlation between the two in either experiment. However, analyses of parallelism revealed there was a separate relationship for each morphotype, with the data described by a series of parallel lines in both experiments; R_{adj}^2 values were 24.3 % in winter ($P=0.007$) and 52.1 % in summer ($P<0.001$). Statistical data for these analyses are summarised in Table 4. This shows that although the slopes of these relationships were negative, both were quite small compared with their standard errors, with only that for the summer experiment just significantly different from zero. This implies that the significance levels in these analyses arise largely from differences in average nitrate-N concentrations between morphotypes. The absence of any strong negative relationships here also implies that only a small part of the variations in nitrate-N concentration within each morphotype were caused by differences in the extent to which it was reduced in their shoots. This agrees with other smaller studies which found no substantial differences in nitrate reduction capacity between high and low nitrate accumulating cultivars within the same morphotype.[45,46] Taken together, our results indicate that nitrate uptake plays the dominant role in governing the variation in nitrate accumulation amongst lettuce accessions, much as suggested previously by Behr and Wiebe.[35]

Relationships with shoot weight at maturity

Previous studies have shown that nitrate concentrations in lettuce often tend to decline with increasing fresh weight as the plants approach maturity,[26,28,37-40] although when comparisons were made across a range of different butterhead cultivars harvested on the same date, accessions with larger fresh weights generally had higher nitrate concentrations in their tissues.[25] In the current experiments there was no significant correlation between nitrate-N concentration and fresh weight for the diversity set as a whole, even though all plants in each experiment were sampled within a week of one another. However, a more detailed investigation using analyses of parallelism revealed there were separate relationships for each morphotype, with the data best described by a series of parallel lines for each experiment, see Table 5. R_{adj}^2 values were 25.9 % for the winter experiment ($P=0.005$) and 41.3 % for the summer experiment ($P<0.001$); both were significant despite the variation between butterhead cultivars being quite large. Although the slope of the lines in the two experiments were positive, they were very small, with neither significantly different from zero. This implies that the significance of these analyses were due more to differences in mean nitrate-N concentrations between morphotypes than to any strong association with the fresh weights of their shoots. Clearly, there is only a weak relationship at best and, because of the large proportion of water in the shoots, it is possible that these apparent associations were the indirect result of the much stronger relationships between the concentrations of nitrate-N and water (see Table 4).

This was tested by repeating the analyses of parallelism for the relationships between shoot nitrate-N concentration and its dry weight. These showed similar results to those for fresh weight, with the data once again best described by a series of parallel lines for each experiment; $R_{adj}^2 = 22.3$ % in the winter ($P=0.005$) and $R_{adj}^2 = 42.3$ % in summer ($P<0.001$). Table 5 shows that the slope of these lines was just positive in the winter experiment and just negative in the summer one; both were too small to be statistically significant. This demonstrates that there were no clear relationships between nitrate concentration and dry weight, and confirms that the above apparent weak association with fresh weight was largely an effect of differences in the proportion of water in their tissues.

Effect of plant development stage

Previous screening studies with lettuce have measured nitrate accumulation at a relatively early stage of growth, as it was generally believed that genotypic differences are independent of plant size.[25,35] However, doubts about this assumption have since been raised by Burns *et al*,[28] who found that changes in head morphology during heart formation could influence nitrate accumulation in some lettuce cultivars. To investigate this further, current nitrate-N concentration data for the 24 accessions in diversity subset A at maturity were compared with those for the same accessions sampled at an earlier stage of growth in the previous study of Burns *et al.*[28]

Previous conclusions about masking effects on genotypic differences in nitrate-N concentration from changes in head morphology appeared to be confirmed by an analysis of parallelism on data from the two summer experiments, which showed no significant relationship between the concentrations at the two stages of growth, either for the diversity set as a whole or for individual morphotypes. In contrast, however, corresponding results for the two winter experiments showed a strong positive relationship between nitrate-N concentrations at the later and earlier stages of growth, with no significant differences between morphotypes; $R_{adj}^2 = 77.0$ % (significant at $P<0.001$). The large slope of this line (1.54 ± 0.174 g g^{-1} of nitrate-N) was caused by relatively high concentrations of nitrate-N in the shoots at maturity, when light levels were between two and three times lower than at the earlier growth stage. Such large differences in accumulated light levels did not occur at the two growth stages in the summer experiments. Thus the current results would appear to confirm our previous conclusions that genotypic differences in nitrate accumulation can be masked by morphological changes in the shoot during maturation,[28] provided there are no substantial changes in intercepted light throughout this process.

Comparison between older and newer cultivars

Modern lettuce breeding strategies have focussed largely on the production of cosmetically acceptable cultivars with a natural resistance to downy mildew and other fungal diseases (Ward G, Snaith Salads, UK, pers. comm.). Selection for traits such as low nitrate accumulation have been given a lower priority, although recent screening methods have tended to use suboptimal N supplies which may increase the chances of cultivars with lower nitrate concentrations being selected.[47] REML analyses were therefore used to compare nitrate-N concentrations in subset A of the diversity set (which consisted primarily of older *L. sativa* cultivars) with those in subset B (largely comprising newer cultivars intended for modern glasshouse production) in order to see if there were any differences in nitrate accumulation between the two groups. The results showed that average nitrate-N concentrations in the DM were consistently greater for subset B than for subset A in both the winter (25.95 vs 21.05 g kg^{-1} DM) and summer (23.11 vs 18.20 g kg^{-1} DM) experiments respectively (both significant at $P<0.001$). However, these differences could have been confounded by the larger proportion of butterhead cultivars (which tend to accumulate more nitrate) among the accessions in subset B. A further comparison of the average concentrations of nitrate-N in the butterhead cultivars in the two experiments with those across all other morphotypes revealed average concentrations of 25.76 and 22.78 g kg^{-1} DM in winter and summer respectively for the butterhead cultivars and 21.22 and 18.51 g kg^{-1} DM respectively for all other accessions. These differences are very similar to those between the predominantly newer (subset B) and older (subset A) varieties above, and confirm that the latter were indeed strongly affected by the different proportions of the butterhead cultivars in each subset. Thus our results do not provide evidence that the selections and crosses used to produce the more recent varieties have had any significant impact on their nitrate accumulating traits.

Effects of seasonal lettuce type

REML analysis on the complete diversity set revealed no significant differences in nitrate-N concentration between the accessions which are grown predominantly in summer (day neutral types) and those grown in winter (short day types), even when the data from the two experiments were analysed together. To eliminate the possibility that an underlying effect of seasonal type could have been confounded by differences between morphotypes, further analyses were carried out using only the butterhead accessions. This morphotype was used because it not only represented the largest proportion (50%) of the diversity set, but also included a similar number of summer and winter production types.

Results for both experiments showed that while the average nitrate-N concentration for the day neutral butterhead cultivars was not significantly different from that for the short day types, the average water concentration of the latter was significantly larger. Analysis of parallelism also revealed different positive relationships between the concentrations of nitrate-N and water for the two lettuce types in both experiments, with the best fit to the data described by two parallel lines; R_{adj}^2 = 38.3 % (P=0.002) in the winter experiment and 36.2 % (P=0.003) in the summer experiment, see Figure 7. These differences between the two seasonal types explains why the corresponding relationships for all butterhead cultivars were more variable than for the other morphotypes in Figure 4. Table 6 shows the intercept for the day neutral types was slightly greater than that for the short day types in both experiments, suggesting that the latter maintained a higher concentration of water at any given nitrate concentration, while the common slope of both lines indicates that the process of volume regulation was effectively identical for both seasonal types within each experiment (*ie* the unit changes in concentration of water and nitrate are essentially the same for each).[13] Furthermore, the slope of these lines was greater in the summer experiment than that in the winter one, much as observed across all morphotypes (cf Tables 6 and 4).

The slopes of the corresponding relationships between the concentrations of nitrate-N and assimilated-C were negative in both experiments, again reflecting the exchange of nitrate with soluble organic compounds as part of the osmotic control mechanisms.[13] Table 6 shows there was no significant difference in the relationship for the two seasonal types in the winter experiment, with both fitting the same regression line between nitrate-N and assimilated-C concentrations (R_{adj}^2 = 76.2 %; $P<0.001$), see Figure 8A. In the summer experiment, on the other hand, there were separate parallel relationships for the day neutral and short day types (R_{adj}^2 = 68.2 %; $P<0.001$), with the former maintaining a slightly higher average assimilated-C concentration for any given nitrate-N concentration (Figure 8B). This may indicate that the day neutral types have a somewhat greater relative capacity for assimilating C in the summer when light levels are greater. Nevertheless, the common slope of these lines indicates that both seasonal types are likely to use similar proportions of soluble organic assimilates for the exchange with nitrate.

Statistical analyses showed that there were also strong relationships between the concentrations of nitrate-N and total-N, with no significant differences between day neutral and short day types within each experiment (R_{adj}^2 = 86.7 %; in winter and R_{adj}^2 = 57.3 % in summer; both significant at $P<0.001$). Table 6 shows that variations in nitrate-N accounted for 81.7 % of the variations in total-N concentrations across all butterhead cultivars in winter and 90.3 % of the variation in summer. The corresponding relationships between nitrate-N and assimilated-N were less clear-cut, with no significant relationship detected for the winter experiment. There was, however, a weak negative relationship in the summer experiment, with both seasonal types following a single regression line between the concentrations of nitrate-N and assimilated-N (R_{adj}^2 = 25.4 %; P=0.007), see Table 6. The slope of this line was significantly different from zero, which would suggest that lower nitrate accumulators amongst these butterhead cultivars were slightly better at assimilating N, at least in the summer. However, any effect was quite weak because relative changes in nitrate-N concentrations were greater than those for assimilated-N. Thus these results confirm that the differences in nitrate concentration were likely to arise more from differences in nitrate uptake than from those in nitrate reduction, much as was observed for the other morphotypes in the diversity set (see above).

The relationships between nitrate concentration and shoot weights also differed for the two seasonal types, see Table 6. In the winter experiment, nitrate-N concentrations in all butterhead accessions increased with fresh weight. The data were best described by two parallel lines (R_{adj}^2 values of 41.5 %; significant at P=0.001), with the day neutral types maintaining a higher average concentration across the weight range. However, there were no corresponding relationships between nitrate-N concentration and shoot dry weight, suggesting that the associations with fresh weight were probably due to the relatively larger water concentrations in the day neutral types (see Table 6). In the summer experiment, on the other hand, the relationships between nitrate-N concentration and both shoot fresh and dry weight were broadly similar. There were pronounced differences in the behaviour of the two seasonal types for both relationships, with analyses of parallelism showing that the data were best described by individual regression lines with different slopes and intercepts in each case; R_{adj}^2 = 34.8% for fresh weight ($P=0.009$) and 24.1 % for dry weight ($P=0.037$). While nitrate-N concentrations in the short day types tended to increase slightly with both shoot fresh and dry weight, those in the day neutral types tended to decline slightly with increasing plant size, with the slope in the dry weight relationship significantly different from zero. These negative relationships between nitrate-N concentration and both fresh and (especially) dry weight for the summer production types were unexpected, and help to explain the greater variability observed amongst the butterhead cultivars in the equivalent relationships for all morphotypes (see above). Comparisons showed that the variances for all three variates were generally a little greater for the day neutral types than for the short day types, but otherwise there were no obvious statistical reasons for the apparent aberrant behaviour of the former. Further studies will therefore be needed to verify whether these effects are real.

Comparisons between seasons

REML analysis of the combined nitrate concentration data from the two experiments confirmed the significant effect of genotype ($P<0.001$), but showed a much smaller effect of environment (season), with $P=0.039$ for concentrations in the DM, and not significant for those in the FM. This contrasts with the previous study which showed a highly significant effect of season when subset A of the population was sampled at an earlier stage of growth, irrespective of whether the nitrate concentration was expressed on a DM or FM basis.[28] The relatively small effect of environment in the current experiments is a little surprising given that accumulated light levels in the 7 or 10 day period immediately prior to each sampling were between 7 to 8 times greater in the summer experiment than in the winter one. However it may have arisen because of the large proportion of leaves within the hearts of most of these mature accessions. These inner leaves would have been largely protected against differences in ambient light, and may have tended to dampen the net effects of the seasonal differences in light levels on the shoot as a whole.

The REML analysis also showed that there was a significant interaction between genotype and environment ($P<0.001$), implying that there were differences in the pattern of nitrate concentrations across the population between winter and summer seasons. The main cause of these interactions was the larger range of nitrate concentrations in the summer experiment than in the winter one (see Table 2). However, there were also changes in the ranking of some accessions between the two seasons, with the behaviour of the *L. serriola* accessions particularly noticeable in this respect (see Figure 2). Nitrate-N concentrations in the tissue DM of these wild relatives were more or less evenly distributed across the concentration range in the winter, but in summer they had substantially lower concentrations than any of the *L. sativa* cultivars. Different contrasting behaviour was also observed when nitrate concentrations were expressed on a FM basis, with two of these *L. serriola* types (accessions 4 and 12) generating the highest nitrate concentrations of the whole diversity set in the winter, whereas their concentrations were more or less evenly distributed within the intermediate range in the summer. Some seasonal changes in ranking were also observed amongst some *L. sativa* accessions, although these were generally less dramatic.

Despite these differences, nitrate concentrations in the two seasons were highly correlated (R_{adj}^2 values of 38.2 and 28.8 % on a DM and FM basis respectively; both significant at $P<0.001$). Analyses of parallelism also revealed that there was some partitioning in both relationships, with the data best described by a series of parallel lines (one for each morphotype), as shown in Table 7 and Figure 9. Such parallel relationships increased the R_{adj}^2 values to 60.7 and 61.6 % for concentrations in the DM and FM respectively ($P<0.001$).

There were no significant improvements in the fit by allowing the slopes of the regression line for each morphotype to vary independently. These data show that there was actually considerable consistency in ranking of nitrate concentration for accessions within each morphotype, confirming the observations made in a previous study by Burns *et al,*[28] where plants in subset A of the population were sampled at an earlier stage of growth. Such conservation of rankings amongst lettuces of the same morphotype is consistent with a non-crossover type of G x E interaction.[34] This suggests that contrary to previous suggestions,[33] screening for nitrate accumulation amongst lettuce cultivars within the same morphotype does not necessarily have to be conducted at the same time of year as that in which the plants are normally grown, provided that suitable representative cultivars of each morphotype are always included as controls.

Finally, analyses of parallelism were also carried out using nitrate concentration data for the butterhead cultivars to check if seasonal type had any effect on the G x E interactions. The results shown in Figure 10 reveal that concentrations of nitrate-N in the shoot DM for the winter experiment were strongly correlated with those in the summer (R_{adj}^2 = 33.3 %; P=0.002), with no statistical advantage in partitioning the relationship between day neutral and short day types. The relatively low slope of this line (0.667±0.189 g g^{-1} of nitrate-N) reflects the G x E interaction (caused largely by the lower nitrate accumulators exhibiting relatively higher concentrations in the winter experiment), but the absence of separate relationships for these two seasonal types (Figure 10A) again implies these were of the non-crossover type. In contrast, a similar analysis of parallelism for nitrate concentrations in the FM showed that these data were best described by two parallel lines, one for the day neutral types and another for the short day types (R_{adj}^2 = 46.4 %; P<0.001), see Figure 10B. The slope of these lines was 0.409±0.183 g g^{-1} of nitrate), with intercepts of 2780±887 mg kg^{-1}

FM for the summer production types and 2115±783 mg kg^{-1} FM for the winter cultivars. Thus, on average, short day types accumulated relatively more nitrate in the FM in the summer experiment than the corresponding day neutral types, and *vice versa*. These separate lines arose because the two seasonal types exhibited different relationships between the tissue concentrations of nitrate-N and water in both experiments (see Figure 7A and B). It follows that where lettuce accessions are to be screened for the concentration of nitrate in the FM, the two seasonal types should be kept separate to avoid the risk of crossover G x E interactions. The alternative is to screen for nitrate concentrations in the DM, where such differences between seasonal types appear to be smaller, and the risks of complicating crossover interactions are likely to be less.

SUMMARY AND CONCLUSIONS

Effects of Genotype

- There was a highly significant effect of genotype on nitrate accumulation across a lettuce diversity set (consisting of of 45 *L. sativa* and three *L. serriola* accessions) grown hydroponically in both winter and summer conditions. Average nitrate concentrations varied between morphotypes, and were higher in butterhead and leaf lettuce, and lower in crisphead and cos cultivars in both seasons.

- There was a strong positive relationship between the concentrations of nitrate-N and water in the DM for each morphotype. The slopes of these relationships were the same for all morphotypes, showing that the process of volume regulation (part of the osmotic control process automatically invoked when nitrate concentrations vary) was essentially the same for each, despite differences in their ‘residual’ water concentrations. Day neutral and short day butterhead cultivars (used commercially for summer or winter glasshouse production respectively) also exhibited similar parallel relationships between nitrate and water concentration, with the short day types maintaining relatively higher water concentrations in their tissues.

- A consequence of these relationships with water concentration is that expressing nitrate concentration on a shoot DM or FM basis had relatively little effect on the ranking of individual accessions provided that accessions within each morphotype were compared separately.
- There was also a strong negative relationship between the concentrations of nitrate-N and assimilated-C in the DM for each morphotype. The slopes of these relationships were the same for all morphotypes, showing that all use a similar fraction of soluble assimilated-C as a replacement for nitrate in an additional mechanism for osmotic control (concentration regulation).
- Variations in concentration of nitrate-N across all accessions were more strongly associated with those of total-N than with those of assimilated-N. This implies that genotypic effects on nitrate accumulation were caused more by differences in nitrate uptake than from differences in their capacity to chemically reduce nitrate.
- Relationships between nitrate-N concentration in the tissue DM and shoot fresh or dry weight were weak and inconsistent. However, there was some evidence that day neutral butterhead cultivars used for summer production may have behaved differently to short day types normally grown in the winter, but this requires further investigation.
- A comparison of nitrate-N concentrations in the DM at maturity with those from an earlier sampling (using a subset of the population) confirmed that morphological changes in head development during maturation can mask underlying genotypic effects, provided these are not confounded by substantial changes in intercepted light throughout this process.

- A comparison of nitrate-N concentrations for groups of accessions comprised predominantly of either newer or older cultivars provided no evidence that recent selection methods have produced lower nitrate-accumulating varieties.

Effects of Environment (season) and Genotype x Environment Interactions

- Average nitrate-N concentrations in all morphotypes were greater in winter than in summer due to lower accumulated light levels. These seasonal differences were larger for the *L. serriola* accessions than for the *L. sativa* cultivars.
- There is evidence that the main effects of environment (season) were smaller when plants were sampled at maturity than at an earlier stage in growth. This may have been caused by the larger biomass of the mature plants which dampened the net effects of seasonal differences in light.
- There were also significant interactions between genotype and environment which were caused largely by the wider range of nitrate concentrations under summer conditions. These were manifested in a series of parallel relationships between the nitrate-N concentrations in the two seasons (one for each morphotype), showing that there was no differential effect of morphotype on the G x E interactions.
- Despite these interactions, there was considerable consistency in the ranking of accessions within the same morphotype between the winter and summer seasons, confirming previous observations that the G x E interactions were predominantly of the non-crossover type.[28] There was also a similar consistency in ranking of nitrate concentrations for day neutral and short day butterhead cultivars used commercially for either summer or winter glasshouse production respectively, provided they were expressed on a DM basis.

- It follows that screening for nitrate accumulation amongst lettuce cultivars or breeding lines within the same morphotype or seasonal type does not necessarily have to be restricted to the same time of year in which the plants are normally grown, provided that suitable representative cultivars of each type are always included as controls.

- Taken overall, the natural variation in shoot nitrate accumulation within and between different morphotypes and seasonal types, and the associated variation in key related traits, suggest that breeding for nitrate content of lettuce is likely to be a viable approach for the production of low accumulating cultivars.

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Table 1. Lettuce accessions (accn) used in the summer and winter screens. Accessions 4, 12 and 27 are from the *Lactuca serriola* L. species, and all others are from the *Lactuca sativa* L. species.

Accn no.	**Source or Warwick accession number**	**Cultivar**	**Morphotype (sub-type)**	**Seasonal type**
1	In-house (LJ01004)	Saladin	Crisphead (Iceberg)	summer
2	In-house (LJ01003)	Iceberg	Crisphead (Batavian)	summer
3	Tozer Seeds	Cobham Green	Butterhead	summer
4	HRIGRU 005095	PI251247*	Wild relative	summer
5	HRIGRU 002932	Bath	Cos/Romaine	summer
6	HRIGRU 001861	Batavia Blonde de Paris	Crisphead (Batavian)	summer
7	HRIGRU 005492	Lobjoit's Green Cos	Cos/Romaine	summer
8	HRIGRU 005048	Ambassador	Butterhead	winter
9	HRIGRU 001228	Red Grenoble	Crisphead (Batavian)	summer
10	HRIGRU 004435	Merveille des Quatres Saisons	Butterhead	summer
11	HRIGRU 012675	Little Gem	Cos/Romaine	summer
12	HRIGRU 006355	PI281876*	Wild relative	summer
13	HRIGRU 001206	Bloody Warrior	Cos/Romaine	summer
14	HRIGRU 001735	New Chicken	Stem lettuce	summer
15	HRIGRU 001683	Romanie de Benicardo	Cos/Romaine	summer
16	Tozer Seeds	Lollo Rossa	Leaf (curled)	summer
17	Tozer Seeds	Lollo Biondo	Leaf (curled)	summer
18	Tozer Seeds	Lilian	Butterhead	summer
19	Clause	Carioca	Crisphead (Batavian)	summer
20	Pinetree de Ruiter	Vegas	Butterhead	summer
21	Elsoms Seeds	Kennedy	Butterhead	summer
22	HRIGRU 001066	Webb's Wonderful	Crisphead (Batavian)	summer
23	HRIGRU 001474	Waldmann's Dark Green	Leaf (curled)	summer
24	HRIGRU 004480	Chinese Stem Lettuce	Stem lettuce	summer
25	HRIGRU 10,001405	Little Gem (pre-selection sucrine)	Cos/Romaine	summer
26	In-house (LJ04044)	Grand Rapids	Cos/Romaine	summer
27	In-house (LJ03050)	UC96US23 98G343†	Wild relative	summer
28	Enza	Abel	Butterhead	winter
29	Rijk Zwaan	Coronel	Butterhead	winter
30	Rijk Zwaan	Lorely	Butterhead	winter
31	Rijk Zwaan	Montel	Butterhead	winter
32	Rijk Zwaan	Wendel	Butterhead	winter
33	Rijk Zwaan	Wynona	Butterhead	winter
34	Rijk Zwaan	Varinka	Butterhead	winter
35	Rijk Zwaan	Hillary	Butterhead	winter
36	Nunhems	Cortina	Butterhead	summer
37	Nunhems	Novita	Butterhead	winter
38	Nunhems	LM2727	Butterhead	winter
39	Nunhems	Patrick	Butterhead	winter
40	S & G (Syngenta)	Josephine	Butterhead	winter
41	S & G (Syngenta)	Sputnik	Butterhead	winter
42	Nunhems	Peter	Butterhead	summer
43	Nunhems	Michael	Butterhead	summer
44	Rijk Zwaan	Atlantis	Butterhead	summer
45	Pinetree de Ruiter	Hawaii	Butterhead	summer
46	Rijk Zwaan	Berwick	Leaf (curled)	winter
47	Nunhems	Charita	Leaf (curled)	summer
48	Nunhems	Mirata	Leaf (curled)	summer

* Source: USDA † Source: Richard Michelmore, UC Davis

Table 2. Summary of the shoot data in the summer and winter experiments.

Variate	Expt	Statistic			
		Minimum	**Maximum**	**Average**	**Median**
Fresh weight (g)	winter	36.5	349.0	144.94	142.15
	summer	107.2	553.4	209.59	196.97
Dry weight (g)	winter	1.23	16.74	5.737	5.406
	summer	4.70	31.72	10.379	9.107
Water concentration (g g^{-1} DM)	winter	16.88	32.09	24.978	24.685
	summer	6.829	24.508	20.173	21.213
Nitrate concn (mg kg^{-1} FM)	winter	2289	6422	4023.9	3956.5
	summer	2896	5488	4279.5	4249.5
Nitrate-N concn (g kg^{-1} DM)	winter	11.26	31.18	23.493	24.500
	summer	7.46	30.45	20.642	21.135
Total-N concn (g kg^{-1} DM)	winter	49.08	69.93	60.319	61.560
	summer	31.30	62.57	54.111	55.005
Assimilated-N concn (g kg^{-1} DM)	winter	32.94	41.43	36.827	36.490
	summer	22.61	40.55	33.468	33.195
Assimilated-C concn (g kg^{-1} DM)	winter	310.7	387.8	341.13	338.30
	summer	345.0	429.6	371.94	365.75

Table 3. Regression data for the concentration of nitrate (mg kg^{-1}) in FM against the corresponding concentration of nitrate-N (g kg^{-1}) in DM for each lettuce morphotype in the winter and summer experiments; all lines with the same slope are parallel.

Expt	**Lettuce type**	**Number**	**slope**	**± se**	**intercept**	**± se**
winter	butterhead	24	116.4	15.2	1204	401
	cos	7	116.4	15.2	1398	352
	crisphead	6	116.4	15.2	858	334
	leaf	6	116.4	15.2	1055	412
	stem	2	116.4	15.2	1707	417
	wild relatives	3	116.4	15.2	2780	445
summer	butterhead	24	138.2	11.4	1280	266
	cos	7	138.2	11.4	1288	235
	crisphead	6	138.2	11.4	1300	239
	leaf	6	138.2	11.4	1431	304
	stem	2	138.2	11.4	1847	301
	wild relatives	3	138.2	11.4	2880	206

Table 4. Regression data for the concentration of nitrate-N (g kg^{-1} DM) against the concentrations of water (g g^{-1} DM), assimilated-C (g kg^{-1} DM), total-N (g kg^{-1} DM) and assimilated-N (g kg^{-1} DM) for each lettuce morphotype in the winter and summer experiments; all lines with the same slope are parallel.

Variate	**Expt**	**Lettuce type**	**slope**	**± se**	**intercept**	**± se**
Water concn.	winter	butterhead	0.668	0.165	8.22	4.40
		cos	0.668	0.165	5.15	4.03
		crisphead	0.668	0.165	0.84	4.64
		leaf	0.668	0.165	6.55	4.68
		stem	0.668	0.165	4.92	4.27
		wild relatives	0.668	0.165	12.01	3.70
	summer	butterhead	1.376	0.229	-7.18	5.02
		cos	1.376	0.229	-9.74	4.75
		crisphead	1.376	0.229	-9.06	4.66
		leaf	1.376	0.229	-4.70	4.99
		stem	1.376	0.229	-5.76	4.58
		wild relatives	1.376	0.229	-2.99	2.70
Assimilated-C	winter	butterhead	-0.2299	0.0192	102.41	6.42
		cos	-0.2299	0.0192	100.09	6.71
		crisphead	-0.2299	0.0192	102.92	7.11
		leaf	-0.2299	0.0192	100.46	6.42
		stem	-0.2299	0.0192	99.77	6.98
		wild relatives	-0.2299	0.0192	104.37	6.83
	summer	butterhead	-0.2448	0.0410	111.4	14.9
		cos	-0.2448	0.0410	110.7	15.6
		crisphead	-0.2448	0.0410	112.5	15.9
		leaf	-0.2448	0.0410	114.2	15.1
		stem	-0.2448	0.0410	111.2	15.7
		wild relatives	-0.2448	0.0410	110.4	17.0
Total-N	winter	butterhead	0.8941	0.0630	-30.93	4.01
		cos	0.8941	0.0630	-29.84	3.61
		crisphead	0.8941	0.0630	-31.86	3.63
		leaf	0.8941	0.0630	-28.81	3.83
		stem	0.8941	0.0630	-29.69	3.63
		wild relatives	0.8941	0.0630	-28.81	3.88
	summer	butterhead	0.815	0.109	-24.34	6.32
		cos	0.815	0.109	-24.48	5.77
		crisphead	0.815	0.109	-23.84	5.69
		leaf	0.815	0.109	-20.20	6.06
		stem	0.815	0.109	-23.30	5.91
		wild relatives	0.815	0.109	-20.02	4.23
Assimilated-N	winter	butterhead	-0.391	0.362	40.5	13.7
		cos	-0.391	0.362	34.4	13.1
		crisphead	-0.391	0.362	33.3	13.8
		leaf	-0.391	0.362	38.2	12.9
		stem	-0.391	0.362	32.1	13.2
		wild relatives	-0.391	0.362	37.7	13.0
	summer	butterhead	-0.645	0.209	45.38	7.36
		cos	-0.645	0.209	39.98	7.27
		crisphead	-0.645	0.209	39.39	7.13
		leaf	-0.645	0.209	43.90	6.52
		stem	-0.645	0.209	39.64	7.36
		wild relatives	-0.645	0.209	26.52	5.99

Table 5. Regression data for the concentration of nitrate-N (g kg^{-1} DM) against fresh and dry weights (g) of the shoots for each lettuce morphotype in the winter and summer experiments; all lines with the same slope are parallel.

Variate	**Expt**	**Lettuce type**	**slope**	**± se**	**intercept**	**± se**
Fresh weight	winter	butterhead	0.0180	0.0125	23.22	1.99
		cos	0.0180	0.0125	17.85	2.44
		crisphead	0.0180	0.0125	17.32	2.00
		leaf	0.0180	0.0125	21.47	2.75
		stem	0.0180	0.0125	13.48	4.58
		wild relatives	0.0180	0.0125	20.32	3.64
	summer	butterhead	0.00397	0.00853	22.06	1.76
		cos	0.00397	0.00853	17.04	2.55
		crisphead	0.00397	0.00853	17.07	2.47
		leaf	0.00397	0.00853	23.33	2.76
		stem	0.00397	0.00853	16.94	4.45
		wild relatives	0.00397	0.00853	8.60	2.85
Dry weight	winter	butterhead	0.090	0.319	25.29	1.91
		cos	0.090	0.319	19.87	2.54
		crisphead	0.090	0.319	18.31	2.02
		leaf	0.090	0.319	23.89	2.68
		stem	0.090	0.319	17.13	5.19
		wild relatives	0.090	0.319	23.08	4.30
	summer	butterhead	-0.154	0.163	24.01	1.55
		cos	-0.154	0.163	19.70	2.41
		crisphead	-0.154	0.163	19.45	2.35
		leaf	-0.154	0.163	26.18	2.57
		stem	-0.154	0.163	21.82	4.58
		wild relatives	-0.154	0.163	12.09	3.79

Table 6. Regression data for the concentration of nitrate-N (g kg^{-1} DM) against the concentrations of water (g g^{-1} DM), assimilated-C (g kg^{-1} DM), total-N (g kg^{-1} DM) and assimilated-N (g kg^{-1} DM) and the fresh and dry weights (g) of the shoots for the summer and winter seasonal types of butterhead lettuce in the winter and summer experiments; all lines with the same slope are parallel.

Variate	**Expt**	**Seasonal type**	**slope**	**± se**	**intercept**	**± se**
Water concn	winter	summer	0.951	0.242	3.99	5.84
		winter	0.951	0.242	-1.49	6.87
	summer	summer	1.398	0.385	-5.80	8.21
		winter	1.398	0.385	-8.97	8.58
Assimilated-C	winter	both	-0.2299	0.0266	102.43	8.88
	summer	summer	-0.3077	0.0446	136.6	16.4
		winter	-0.3077	0.0446	132.3	16.0
Total-N	winter	both	0.8173	0.0666	-26.06	4.23
	summer	both	0.903	0.160	-29.43	9.26
Assimilated-N	winter	both	NS*	NS*	NS*	NS*
	summer	both	-0.831	0.280	51.88	9.82
Fresh weight	winter	summer	0.1282	0.0307	10.17	4.05
		winter	0.1282	0.0307	5.71	4.76
	summer	summer	-0.0446	0.02460	32.87	5.110
		winter	0.1086	0.03403	4.22	5.657
Dry weight	winter	both	NS*	NS*	NS*	* NS
	summer	summer	-1.276	0.549	35.57	5.16
		winter	1.724	0.953	9.83	6.81

* No significant relationship

Table 7. Regression data for the concentrations of nitrate in the winter experiment against those in the summer experiment. The data for nitrate-N (g kg^{-1} DM) and for nitrate (mg kg^{-1} FM) are given separately; all lines with the same slope are parallel.

Variate	**Lettuce type**	**slope**	**± se**	**intercept**	**± se**
Nitrate-N (g kg^{-1} DM)	butterhead	0.761	0.120	8.42	2.81
	cos	0.761	0.120	6.72	2.47
	crisphead	0.761	0.120	4.91	2.52
	leaf	0.761	0.120	5.91	3.20
	stem	0.761	0.120	4.20	3.18
	wild relatives	0.761	0.120	16.96	2.17
Nitrate (mg kg^{-1} FM)	butterhead	0.672	0.126	1225	567
	cos	0.672	0.126	1237	515
	crisphead	0.672	0.126	478	522
	leaf	0.672	0.126	675	641
	stem	0.672	0.126	874	668
	wild relatives	0.672	0.126	2778	606

Legends to Figures

Figure 1. Accumulated solar radiation from the time of transplanting for each experiment. Key: — — — winter experiment; ——— summer experiment.

Figure 2. Mean shoot nitrate-N concentrations (g kg^{-1} DM) and SEDs for the 48 lettuce accessions in the diversity set for: (a) the winter experiment; and (b) the summer experiment.

Figure 3. Comparison of mean nitrate concentrations (mg kg^{-1} FM) and mean nitrate-N concentrations (g kg^{-1} DM) in the shoots of different morphotypes in the diversity set for: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: ● ———— butterhead; ▲ - - - - - - - cos; ■ —— - - —— crisphead; Δ —— —— leaf lettuce; □ – – – – stem lettuce; ○ – – – – – wild relatives.

Figure 4. Relationship between mean concentrations of nitrate-N (g kg^{-1} DM) and water (g g^{-1} DM) in the shoots of different morphotypes in the diversity set for: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: see legend to Figure 3.

Figure 5. Relationship between mean concentrations of nitrate-N (g kg^{-1} DM) and assimilated-C (g kg^{-1} DM) in the shoots of different morphotypes in the diversity set for: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: see legend to Figure 3.

Figure 6. Relationship between mean concentrations of nitrate-N (g kg^{-1} DM) and total-N (g kg^{-1} DM) in the shoots of different morphotypes in the diversity set for: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: see legend to Figure 3.

Figure 7. Relationship between mean concentrations of nitrate-N (g kg^{-1} DM) and water (g g^{-1} DM) in the shoots of day neutral and short day butterhead accessions: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: ◊ ——— day neutral types; ♦ —— —— short day types

Figure 8. Relationship between mean concentrations of nitrate-N (g kg^{-1} DM) and assimilated-C (g kg^{-1} DM) in the shoots of day neutral and short day butterhead accessions: (a) the winter experiment; and (b) the summer experiment. Key to symbols and lines: see legend to Figure 6. Note: the two lines in the winter experiment are superimposed.

Figure 9. Comparison of mean nitrate concentrations in the shoots for the winter experiment with those for the summer experiment for different morphotypes in the diversity set: (a) nitrate-N (g kg^{-1} DM); and (b) nitrate (mg kg^{-1} FM). Key to symbols and lines: see legend to Figure 3.

Figure 10. Comparison of mean nitrate concentrations in the shoots for the winter experiment with those for the summer experiment for day neutral and short day butterhead accessions: (a) nitrate-N (g kg^{-1} DM); and (b) nitrate (mg kg^{-1} FM). Key to symbols and lines: see legend to Figure 7. Note: the two lines in the winter experiment are superimposed.

Fig 1

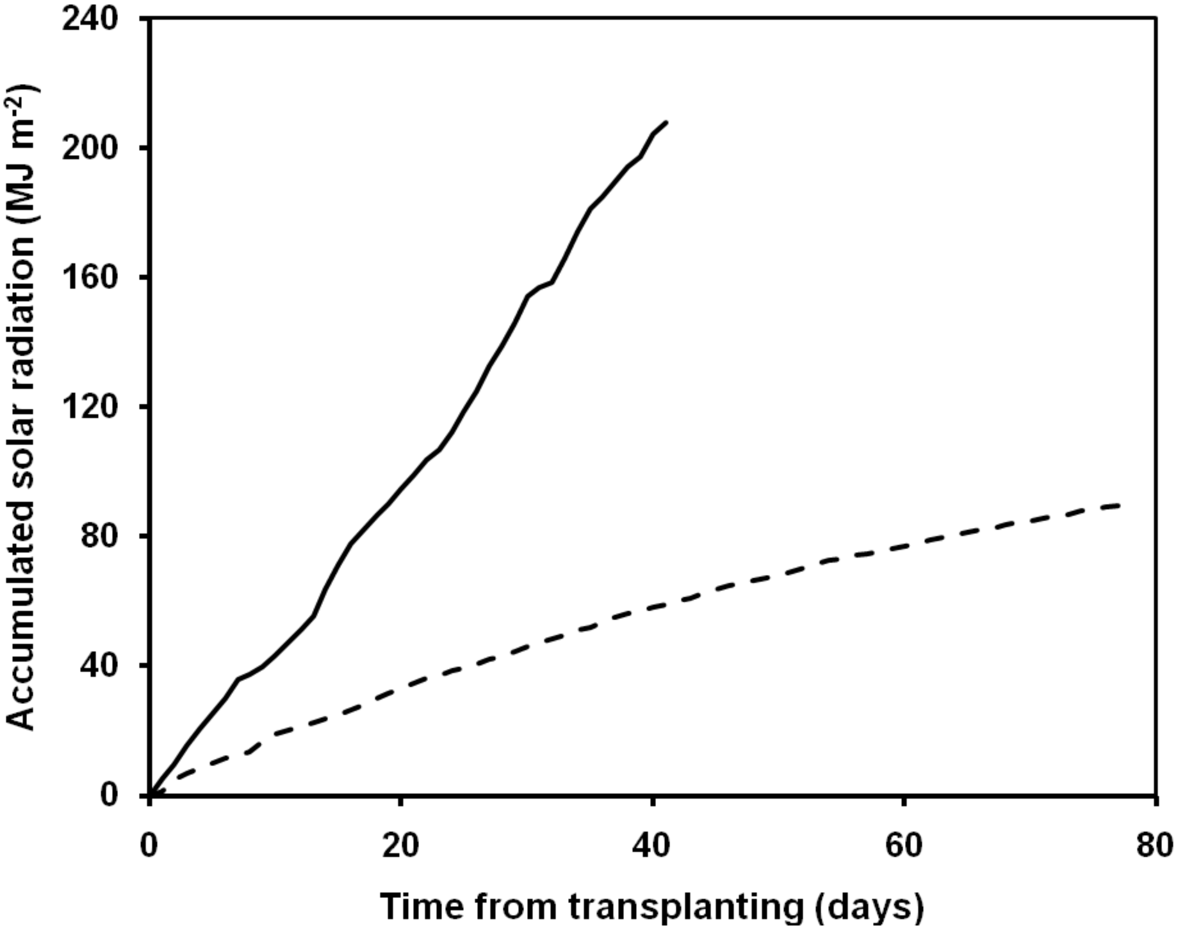
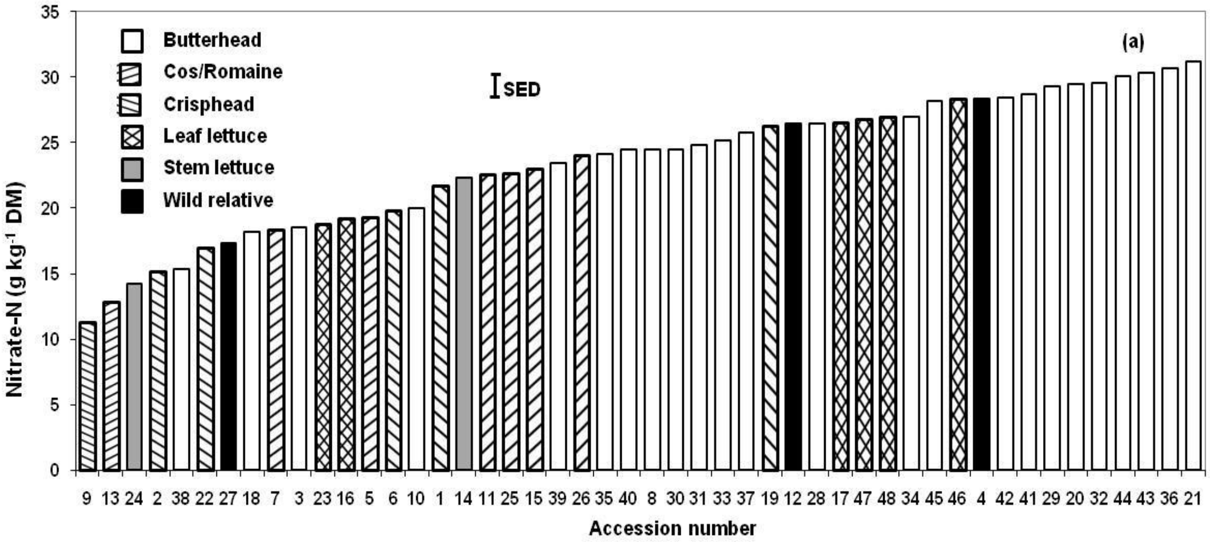

240
200
160
120
80
40
0
Accumulated solar radiation (MJ m-2)
0
20
40
60
80
Time from transplanting (days)

Fig 2


(a)
Butterhead
Cos/Romaine
Crisphead
Leaf lettuce
Stem lettuce
Wild relative
SED
Nitrate-N (g kg^{-1} DM)
0
5
10
15
20
25
30
35
9 13 24 2 38 22 27 18 7 3 23 16 5 6 10 1 14 11 25 15 39 26 35 40 8 30 31 33 37 19 12 28 17 47 48 34 45 46 4 42 41 29 20 32 44 43 36 21
Accession number

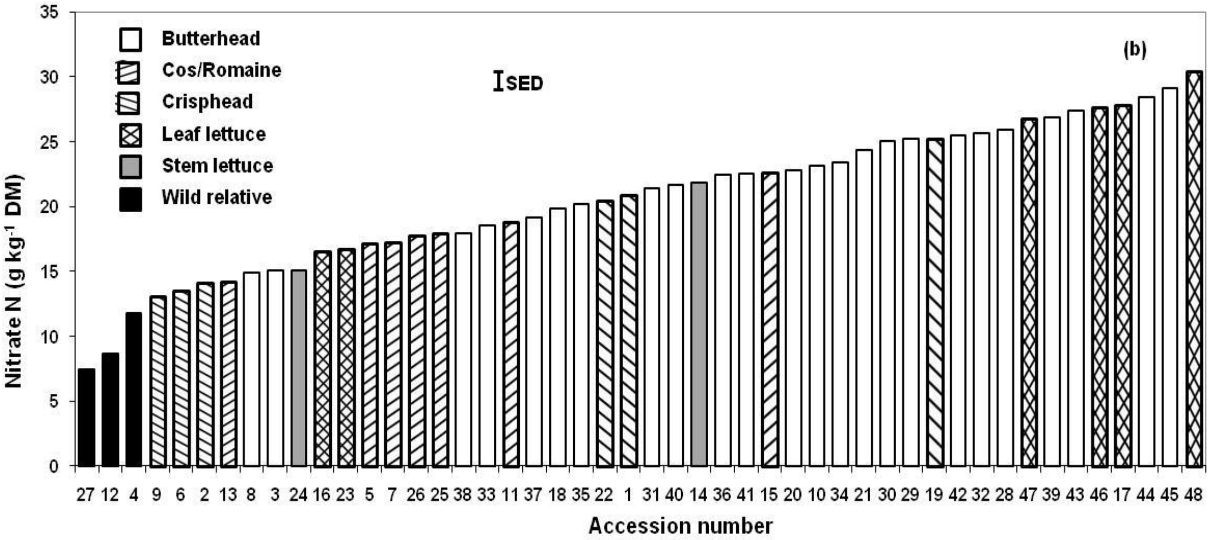
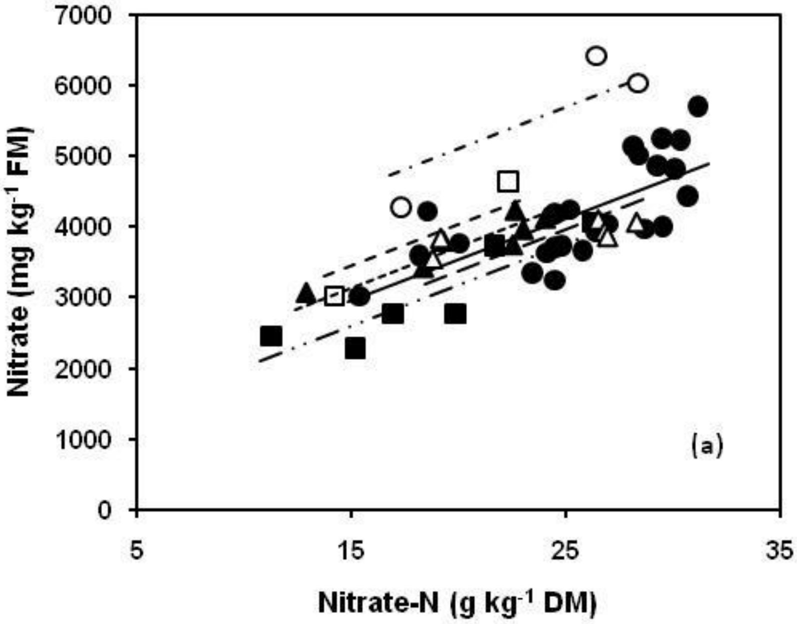

(b)
Butterhead
Cos/Romaine
Crisphead
Leaf lettuce
Stem lettuce
Wild relative
SED
Nitrate N (g kg^{-1} DM)
0
5
10
15
20
25
30
35
27 12 4 9 6 2 13 8 3 24 16 23 5 7 26 25 38 33 11 37 18 35 22 1 31 40 14 36 41 15 20 10 34 21 30 29 19 42 32 28 47 39 43 46 17 44 45 48
Accession number

Fig 3


7000
6000
5000
4000
3000
2000
1000
0
Nitrate (mg kg^{-1} FM)
5
15
25
35
Nitrate-N (g kg^{-1} DM)
(a)

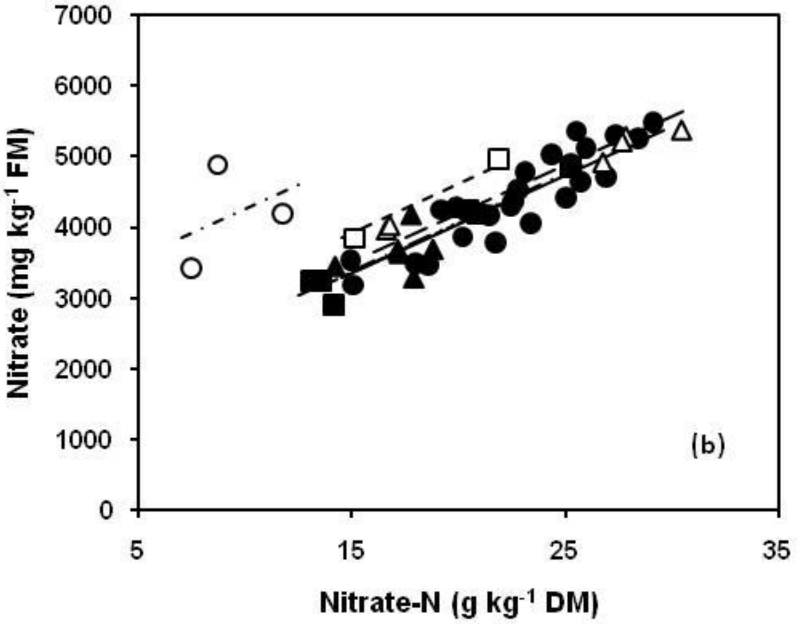
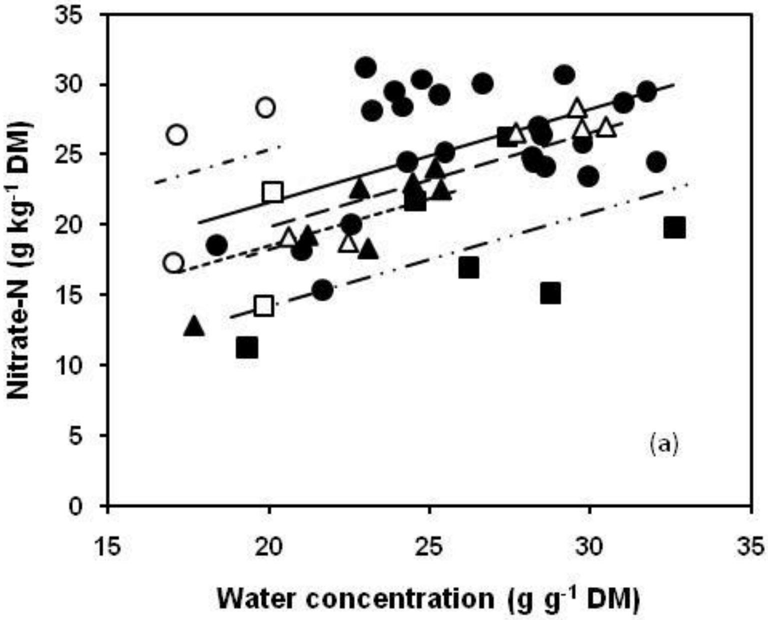

7000
6000
5000
4000
3000
2000
1000
0
Nitrate (mg kg^{-1} FM)
5
15
25
35
Nitrate-N (g kg^{-1} DM)
(b)

Fig 4


Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
15
20
25
30
35
Water concentration (g g^{-1} DM)
(a)

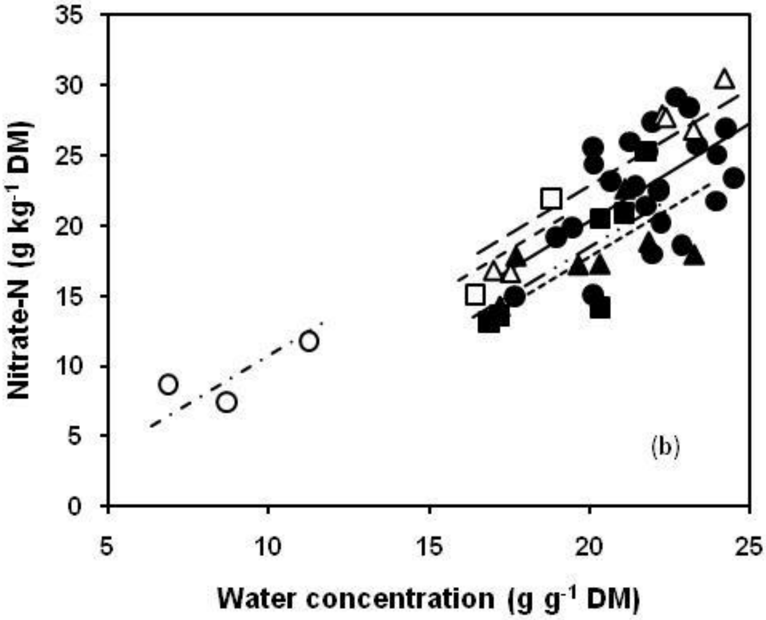
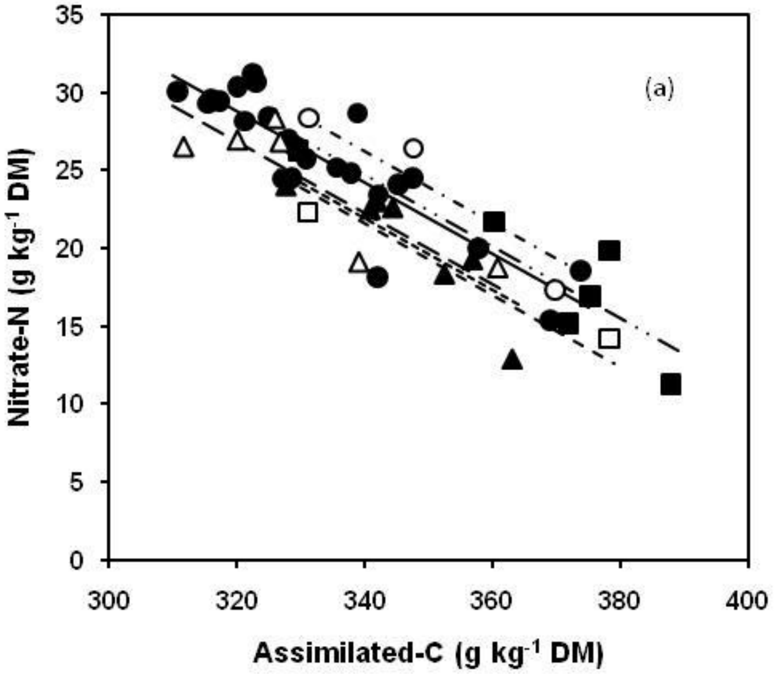

Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
5
10
15
20
25
Water concentration (g g^{-1} DM)
(b)

Fig 5


(a)
Nitrate-N (g kg^{-1} DM)
Assimilated-C (g kg^{-1} DM)
0
5
10
15
20
25
30
35
300
320
340
360
380
400

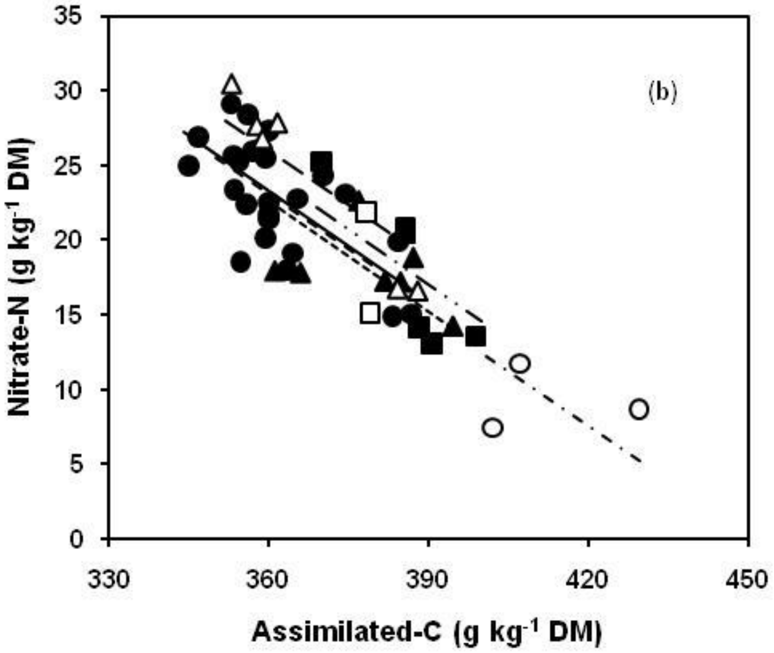

(b)
Nitrate-N (g kg^{-1} DM)
Assimilated-C (g kg^{-1} DM)
0
5
10
15
20
25
30
35
330
360
390
420
450

Fig 6

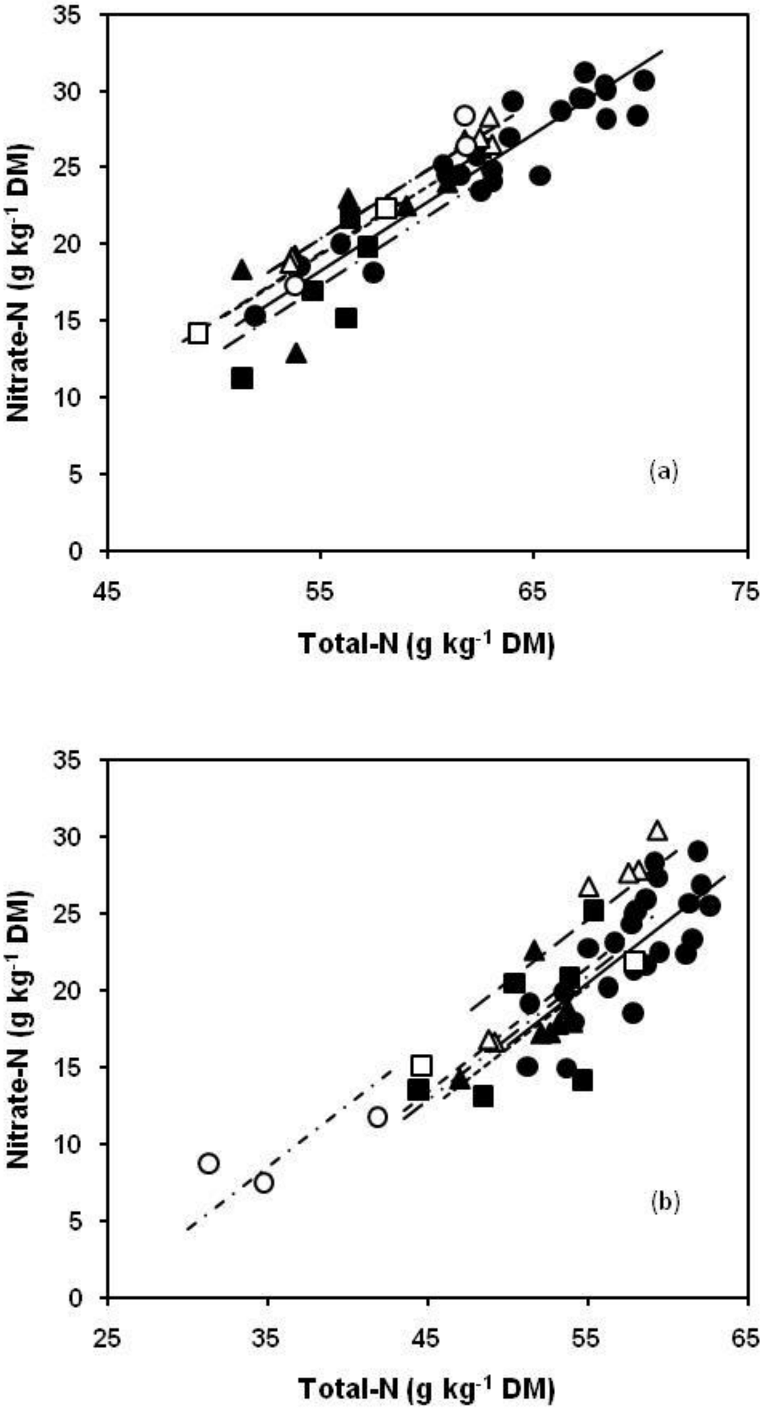

Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
45
55
65
75
Total-N (g kg^{-1} DM)
(a)
Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
25
35
45
55
65
Total-N (g kg^{-1} DM)
(b)

Fig 7

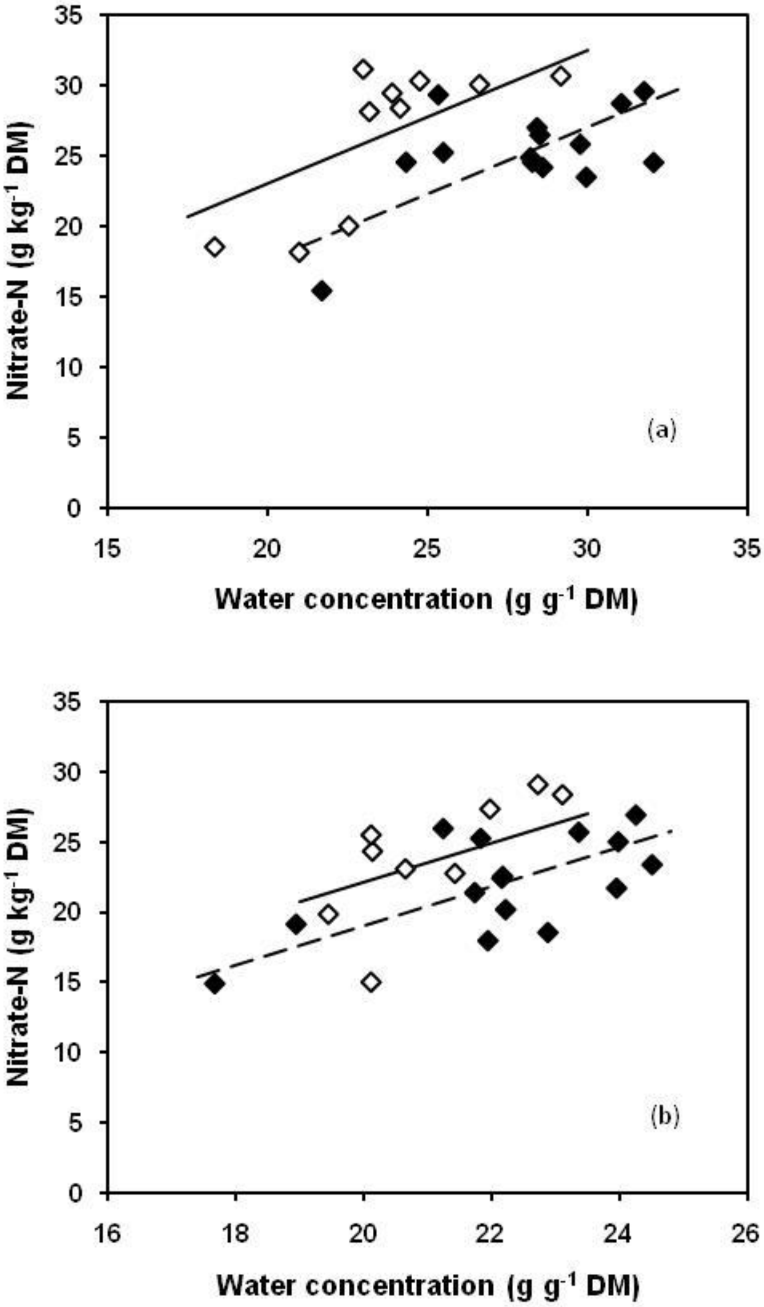

Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
15
20
25
30
35
Water concentration (g g^{-1} DM)
(a)
Nitrate-N (g kg^{-1} DM)
35
30
25
20
15
10
5
0
16
18
20
22
24
26
Water concentration (g g^{-1} DM)
(b)

Fig 8

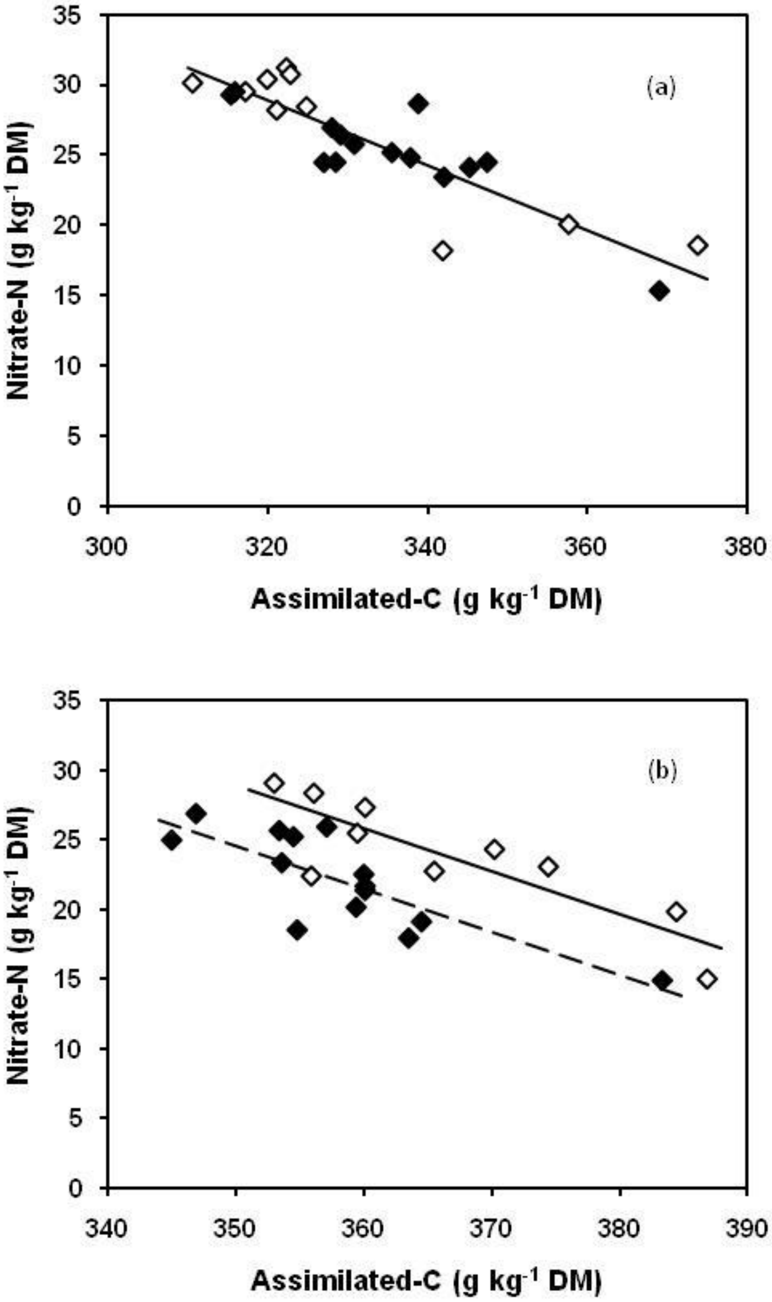
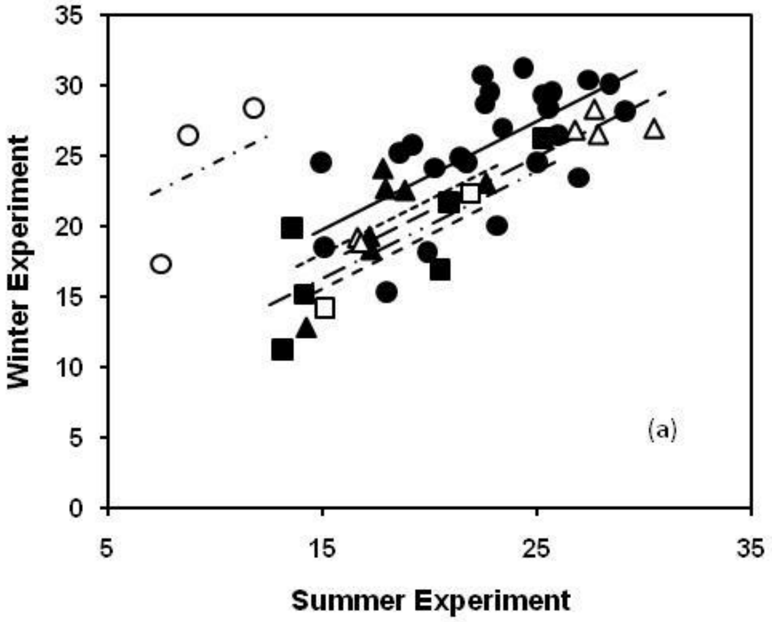

(a)
Nitrate-N (g kg⁻¹ DM)
35
30
25
20
15
10
5
0
300
320
340
360
380
Assimilated-C (g kg⁻¹ DM)
(b)
Nitrate-N (g kg⁻¹ DM)
35
30
25
20
15
10
5
0
340
350
360
370
380
390
Assimilated-C (g kg⁻¹ DM)

Fig 9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30
25
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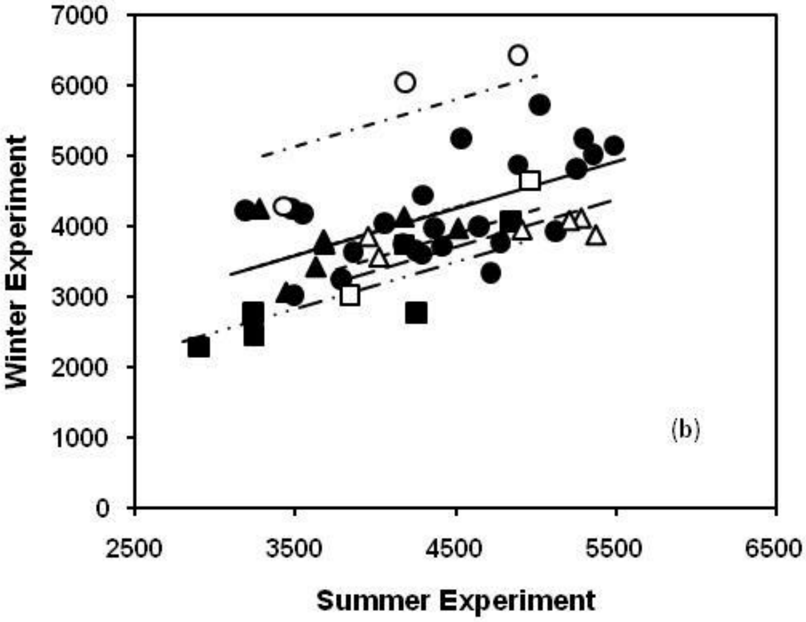
15
10
5
0
5
15
25
35
Winter Experiment
Summer Experiment
(a)


7000
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3000
2000
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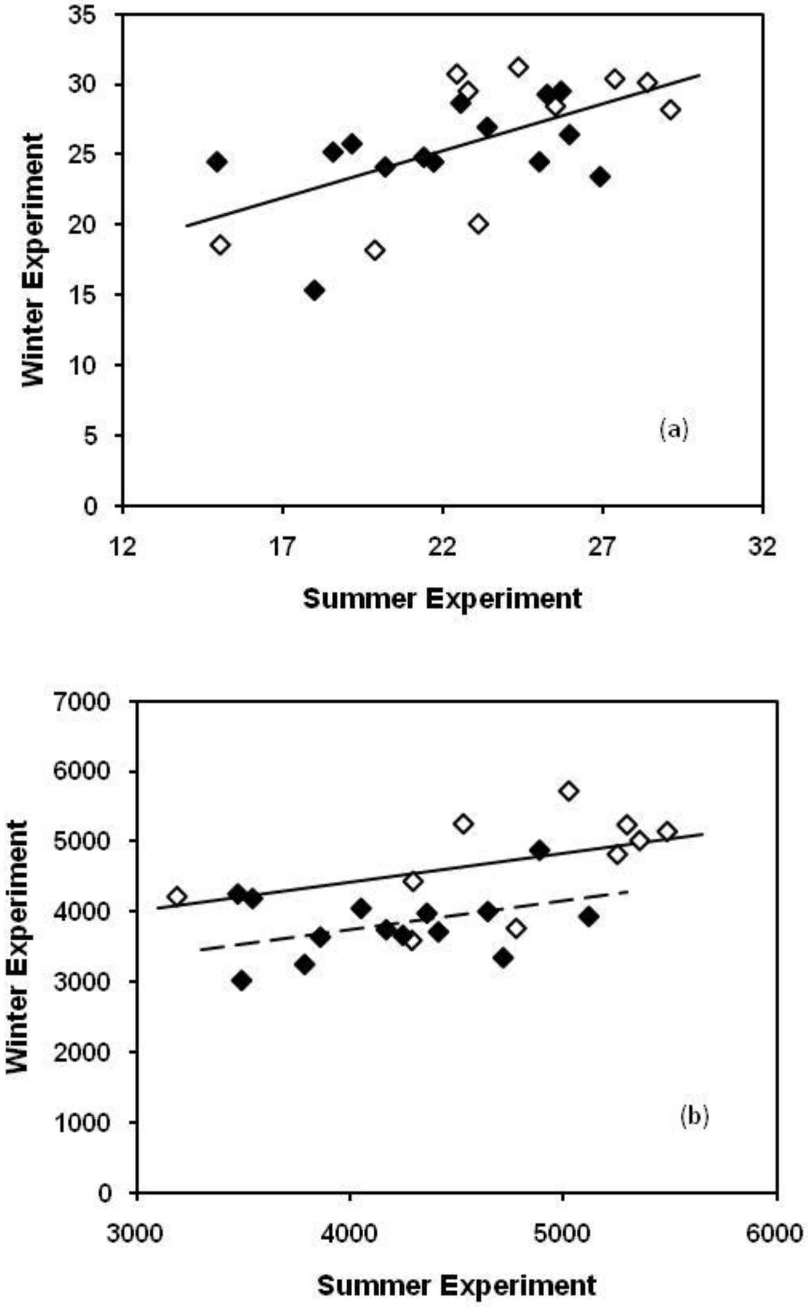
2500
3500
4500
5500
6500
Winter Experiment
Summer Experiment
(b)

Fig 10


35
30
25
20
15
10
5
0
Winter Experiment
12
17
22
27
32
Summer Experiment
(a)
7000
6000
5000
4000
3000
2000
1000
0
Winter Experiment
3000
4000
5000
6000
Summer Experiment
(b)