Iso-osmotic regulation of nitrate accumulation in lettuce (*Lactuca sativa* L.)

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ABSTRACT

Concerns about possible health hazards arising from human consumption of lettuce and other edible vegetable crops with high concentrations of nitrate have generated demands for a greater understanding of processes involved in its uptake and accumulation in order to devise more sustainable strategies for its control. This paper evaluates a proposed iso-osmotic mechanism for the regulation of nitrate accumulation in lettuce (*Lactuca sativa* L.) heads. This mechanism assumes that changes in the concentrations of nitrate and all other endogenous osmotica (including anions, cations and neutral solutes) are continually adjusted in tandem to minimise differences in osmotic potential of the shoot sap during growth, with these changes occurring independently of any variations in external water potential. The hypothesis was tested using data from six new experiments, each with a single unique treatment comprising a separate combination of light intensity, N source (nitrate with or without ammonium) and nitrate concentration carried out hydroponically in a glasshouse using a butterhead lettuce variety. Repeat measurements of plant weights and estimates of all of the main soluble constituents (nitrate, potassium, calcium, magnesium, organic anions, chloride, phosphate, sulphate and soluble carbohydrates) in the shoot sap were made at intervals from about 2 weeks after transplanting until commercial maturity, and the data used to calculate changes in average osmotic potential in the shoot. Results showed that nitrate concentrations in the sap increased when average light levels were reduced by between 30 and 49 % and (to a lesser extent) when nitrate was supplied at a supra-optimal concentration, and declined with partial replacement of nitrate by ammonium in the external nutrient supply. The associated changes in the proportions of other endogenous osmotica, in combination with the adjustment of shoot water content, maintained the total solute concentrations in shoot sap approximately constant and minimised differences in osmotic potential between treatments at each sampling date. There was, however, a gradual increase in osmotic potential (*ie* a decline in total solute concentration) over time largely caused by increases in shoot water content associated with the physiological and morphological development of the plants. Regression analysis using normalised data (to correct for these time trends) showed that the results were consistent

with a 1:1 exchange between the concentrations of nitrate and the sum of all other endogenous osmotica throughout growth, providing evidence that an iso-osmotic mechanism (incorporating both concentration and volume regulation) was involved in controlling nitrate concentrations in the shoot.

Key words: ammonium, hydroponics, irradiance, iso-osmotic control, lettuce, nitrate, osmotic potential, regulation mechanisms, shoot sap.

INTRODUCTION

The maintenance of essential plant processes requires an adequate supply of nitrogen (N) throughout growth. Most agricultural crops favour nitrate as their primary N source because, unlike ammonium, it can be taken up in relatively large quantities without significant adverse effects (Barker and Mills, 1980). Once absorbed, much of the nitrate is reduced and assimilated into organic forms of N in the cytoplasm of both root and shoot cells, with the remaining nitrate stored in their vacuoles (Martinoia et al.,1981; Granstedt and Huffaker, 1982). This accumulated nitrate is often used as a temporary reserve of N, which allows a near-constant concentration of nitrate in the cytoplasm to maximise nitrate reduction and assimilation (Miller and Smith, 1996; van der Leij et al., 1998), and buffers the plant against any short-term spatial and temporal fluctuations in external supply (Burns, 1994). In addition, endogenous nitrate also helps to maintain the internal cation-anion balance (van Beusichem et al., 1985, 1988), acts as an osmoticum for the maintenance of turgor (Mott and Steward, 1972) and, during reduction, is intimately involved in the control of pH within the plant (Raven and Smith, 1976; van Beusichem et al., 1988), amongst other plant processes (Marschner, 1995, p231-239).

The extent to which crops accumulate nitrate varies between species, with lettuce and spinach particularly prone to generating high concentrations in their leaves (Maynard et al., 1976; Corré and Breimer, 1979; Santamaria, 2006). However, agricultural and environmental factors which increase the rate of uptake relative to that of reduction can also affect nitrate concentrations in all crops to a greater or lesser extent. This most often occurs when poor light restricts growth, especially at higher levels of nitrate supply (Maynard et al., 1976; Burns et al., 2003). Concerns about possible health hazards arising from human consumption of nitrate in lettuce and spinach (particularly when grown under protected conditions) have led to the introduction of legislation setting maximum limits on the nitrate contents of these crops (European Commission, 1997 and 2006). This, in turn, has generated demands for a greater understanding of the processes involved in nitrate uptake and accumulation in order to devise more sustainable strategies for its control.

Several mechanisms have been proposed for regulating the rates of uptake and reduction of nitrate within plants (see reviews by Forde and Clarkson, 1999; Tischner, 2000; Walch-Liu et al., 2005), but their relative importance and effectiveness in controlling nitrate accumulation at a whole-plant level are still unclear. One popular hypothesis is based on the assumption that net uptake of nitrate is controlled by negative feedback effects from nitrate concentrations in the roots which, in turn, control the nitrate concentration in the shoot. Such a mechanism is often believed to operate as a nitrate homeostat. Two models based on these principles, with regulation either by feedback inhibition of nitrate influx (Cárdenas-Navarro et al., 1998 and 1999a) or by concentration-dependent efflux (Scaife, 1989; Scaife and Schloemer, 1994), have both been shown to provide a good qualitative description of diurnal fluctuations in nitrate accumulation in tomato and spinach respectively. However, at a whole-plant level, such representations are probably over-simplistic, especially for crops such as lettuce which reduce most endogenous nitrate in their shoots; in particular, it is difficult to visualise a mechanism whereby nitrate in the shoot can act as a signal for controlling its uptake by the roots when it is largely excluded from the phloem, and cannot readily be translocated back to the roots. Furthermore, these models ignore the contributions of amino acids (and other nitrate reduction products), which are also known to exert feedback control over nitrate uptake (Muller and Touraine, 1992). Assimilates such as amino acids recycle freely between the roots and shoots, and are much more likely to be involved in integrating the effects of shoot demand for N, and controlling the uptake of nitrate and other nutrients by the roots (Cooper and Clarkson,1989; Marschner et al., 1997).

In contrast, other research has highlighted the role of nitrate as an osmoticum in many plant species, and especially in crops such as lettuce (Blom-Zandstra and Lampe, 1985; Behr and Wiebe, 1988; Blom-Zandstra et al., 1988; Drews et al., 1995; McCall and Willumsen, 1999; Buwalda and Warmenhoven, 1999). Nitrate is one of several soluble plant constituents which help to maintain the cell turgor needed for tissue expansion by reducing the osmotic potential of the vacuolar sap (Mott and Steward, 1972; Palmer et al., 1996; McIntyre, 1997; Andrews et al., 2005). Reducing the light intensity increases the nitrate content of lettuce leaves and decreases the contents of sugar and organic anions (mostly carboxylates), without causing significant changes to the concentration of total solutes present (Blom-Zandstra and Lampe, 1985; Blom-Zandstra et al., 1988; McCall and Willumsen, 1999). This has led to the hypothesis that shoot nitrate concentration (as opposed to nitrate uptake by the roots) may actually be regulated either directly by leaf osmotic potential or indirectly through its effect on turgor. Such a mechanism is most accurately described as iso-osmotic regulation (in which different endogenous solutes are used to maintain a constant osmotic

potential at a similar external water potential) to distinguish it from responses to those of water stress or salinity (Wyn Jones and Gorham, 1982). Two different models in which decreases in nitrate concentration are assumed to be proportional to corresponding increases in sugar concentration have been developed by Seginer et al. (1998 and 1999) and Zhang et al. (2004) to test this hypothesis. Both models (and their subsequent variants) gave a good description of the changes in nitrate concentration for independent data with lettuce over time. However, while these results may appear to support an iso-osmotic control or turgor maintenance regulatory mechanism, the assumption that sugars provide an appropriate surrogate for *all* of the solutes which are normally used to adjust osmotic potential when the concentration of nitrate changes is unlikely. Further work is therefore needed to examine the relationships between nitrate and other important osmotica within a plant in order to evaluate this assumption.

The objective of the current work was to examine the interactions of nitrate with all of the other important soluble constituents (including all anions, cations and neutral solutes most commonly present as osmotica in shoot sap) during the growth of lettuce in order to test whether the responses are consistent with an endogenous iso-osmotic mechanism for the regulation of nitrate accumulation in its tissues. Six separate experiments were carried out with the plants grown hydroponically in a glasshouse, each with a single treatment consisting of a specific combination of light level, N source, and concentration of nitrate in the nutrient supply. Plants were sampled destructively at intervals throughout growth from about 2 weeks after transplanting until commercial maturity to determine the effects of the treatments on the concentrations of the individual osmotica and their contribution to the concentration of total solutes in shoot sap over the course of each experiment. The results were used to measure interactions between the concentrations of nitrate and the sum of the other (residual) solutes to determine whether there was a consistent quantitative relationship between the two. The experiments provide a comprehensive dataset for the evaluation of the proposed iso-osmotic mechanism at a resolution not previously available throughout the major part of the lifetime of a crop.

MATERIALS AND METHODS

Plant Material and Growing Systems

Experiments were carried out on butterhead lettuce (*Lactuca sativa L.*, cv. Vegas) grown hydroponically under glass with different light intensities, nitrogen sources and nitrogen concentrations. The experiments were conducted in small-scale re-circulating Nutrient Film

Technique (NFT; Graves 1983) systems similar to that described by Broadley et al. (2003). Each consisted of a series of four parallel gullies (110 mm wide by 40 mm deep, and spaced at 220 mm centres) constructed on a gradient of approximately 1 in 60 mm (*ie* 1.67%) over a 5.2 m length. The gullies were fitted with adjustable lids each with 45 mm circular holes drilled at 220 mm intervals to provide individual planting locations within each. The 22 plants grown in each gully were treated as a separate replicate in the experiments described below. All NFT systems had their own dedicated nutrient supply, provided by 0.2 m^3 of nutrient solution which was continuously pumped from a storage tank to the top end of the gullies (at a rate of 1.1 ± 0.1 dm^3 min^{-1} per gully). The nutrient solution then flowed down the gradient within the gullies, before draining back under the influence of gravity into the storage tank, where it mixed with the residual solution still present. During operation, the depth of nutrient solution within the gullies remained between 10 and 20 mm throughout. Use of these systems allowed the plants to be grown to maturity at a density similar to that used commercially for glasshouse lettuce.

To raise plants for the experiments, pelleted seeds of lettuce (supplied by Pinetree de Ruiter Seeds, UK) were sown directly into tapered rockwool cubes (approximately 37 by 37 by 40 mm) wetted with de-ionised water in shallow trays. The seeds were allowed to germinate at 15 ^oC in the dark, before the trays were transferred to the glasshouse. After about 2 weeks (at the 1 to 2 true leaf stage), lettuce seedlings were selected for uniform size and appearance and transferred to the NFT systems, by inserting their rockwool cubes into the planting holes in the adjustable lids so that they rested on the bottom of the gullies in full contact with the flowing nutrient solution. The developing plants remained in the NFT systems during a conditioning phase of about 2 weeks (see Table 1 for details) until the start of each experiment, when the initial plant samplings were made. Thus the plants were provided with nutrient solutions of the same composition throughout both the conditioning stage and subsequent experiment, so that they were completely adjusted to their experimental nutrient supply before any measurements were taken.

Glasshouse temperatures during conditioning and experiment were maintained between 25 ^oC and 10 ^oC (± 3 ^oC) during the day and night respectively, using automatic venting, fans and heating. Supplementary lighting was not used, except where required for one experimental treatment (see below). Light intensity above the crop canopy was measured at 5 minute intervals using two solarimeters positioned at opposite ends of the gullies, and was recorded automatically using Squirrel 1201 data loggers (Grant Instruments, UK). Data from the two solarimeters were averaged and used as representative estimates of the changes in light intensity throughout each experiment.

Experimental Treatments

Six different experiments (referred to as T1 to T6) are described in this paper, each representing a separate treatment. A combination of one of three light intensities (high, medium or low) and one of four nutrient solutions NS1, NS2, NS3 and NS4 were used continuously throughout each experiment, see Table 2. Experiments T1 to T4 were conducted simultaneously between late April and either late June or early July 2003 (depending on maturity date) in the same glasshouse compartment. Experiments T5 and T6 were carried out simultaneously from early March to mid May 2004 in two other compartments, both with a low light transmission factor. The plants in Experiment T5 were also given supplementary lighting from 400 W sodium vapour lamps between 0900 and 1700 hours, increasing daytime irradiance (compared with that in Experiment T6) without changing the day length. Over the course of the experiments light intensities averaged 7.0, 4.9 and 3.6 MJ m^{-2} d^{-1} for the high, medium and low irradiance levels respectively. However, natural variations in external light (both within and between each 24-hour period) produced some fluctuations in accumulated irradiance within the three glasshouses as shown in Fig. 1.

The four nutrient solutions differed in either their N source or concentration, see Table 3. Solutions NS1 and NS2 both contained nitrate at 4 mol m^{-3} as the sole N source, but NS2 also included chloride at 2 mol m^{-3} as an additional competitive anion; solution NS3 contained both nitrate (at 3 mol m^{-3}) and ammonium (at 1 mol m^{-3}), providing the maximum ammonium concentration in solution culture which can be considered relevant for soil-grown plants (Marschner, 1995, p.249). Solution NS4 contained the same macro nutrients as NS1, but at double their concentration (*ie* with nitrate at 8 mol m^{-3}). Other macronutrients were maintained at non-limiting levels in all nutrient solutions (see Table 3). Micronutrients in all nutrient solutions were supplied at the following concentrations (mmol m^{-3}) using: 100.0 as FeNaEDTA, 30.0 as H_3BO_3; 10.0 as $MnSO_4$; 1.0 as $ZnSO_4$; 3.0 as $CuSO_4$; and 0.5 as Na_2MoO_4. All solutions were made up in deionised rainwater. The initial conductivities of solutions NS1 to NS4 were 767, 806, 783 and 1399 µS cm^{-1} respectively. The solutions were maintained close to pH 6.0 throughout each experiment by regular additions of dilute $Ca(OH)_2$ (experiment T3) or H_2SO_4 (all other experiments). All solutions were replaced at weekly intervals during early plant development stages and twice a week thereafter to minimise nutrient depletion. However, regular analysis of the nutrient solutions showed that towards the end of the experiment, ammonium concentrations in NS3 had tended to decline to relatively low levels compared with those of nitrate each time the solution was replaced.

Sampling Strategy and Plant Measurements

Plants were sampled destructively at intervals throughout each experiment, with a total of four plants (one per replicate) taken at about 0900 hours on each date, using a systematic sampling approach to minimise any positional effects. This involved selecting the last plant (excluding the guard) from opposite ends of alternate gullies. Plant spacing was maintained by relocating the guard plants to occupy the newly vacated planting positions on each occasion. The first sampling was taken at the end of the conditioning phase and was considered to represent the start of each experiment (see Table 1 for details). The second sampling was made about a week later, with subsequent samplings at approximately two per week thereafter until commercial maturity. The plants were considered mature when they had produced a well-defined heart, and their total shoot fresh weights had exceeded 320 g (for plants grown at high and medium light) or 230 g (for those grown at low light). This ensured that the mature heads all exceeded minimum EU standards for butterhead lettuce after trimming (European Commission, 2001), whilst allowing for a reduced head weight for plants grown under the equivalent of winter light levels. Because the growth rates of the plants differed between experiments, there were more samplings for some experiments than for others. Further details of the experimental schedules, initial and final fresh weights of shoots and sampling frequency are given in Table 1.

The shoots of the sampled plants were cut off at the base of the stem at the junction with the rockwool cubes, and the latter (together with their associated roots) discarded. After measuring the fresh weight of each whole shoot, numbers of expanded leaves were counted. Overhead photographs of representative plants were also taken for each treatment. The shoots were oven dried at 80-90 ^{o}C for between 24 and 48 hours, and the dry weight measured. Shoot water content (g g^{-1} DM) was calculated from the difference between fresh and dry weight. The dried shoot was milled to pass through a 1.0 mm sieve and used for subsequent chemical analysis. As the amount of dry material was insufficient to allow all analytical determinations to be made on individual plants (particularly when they were young), the shoot material from all four replicates was pooled at each sampling, and homogenised before analysis.

Nitrate was determined colorimetrically on water extracts (prepared by shaking 100 mg of the pooled shoot material with 25 cm^3 for 30 minutes) by Flow Injection Analysis (FIASTAR 5012, FOSS Tecator, Sweden). Total N concentrations were measured directly by IR analysis following combustion of 100mg samples using a CN2000 Analyser (LECO Corporation, Michigan, USA). Corresponding organic N concentrations were calculated by

difference. The measurements of nitrate and organic N assume that ammonium and nitrite concentrations were insignificant. This is consistent with evidence that ammonium assimilation occurs almost entirely in the roots (Engels and Marschner, 1993), and that nitrite rarely accumulates in intact plants under normal conditions (Marschner, 1995, p.233). Potassium, calcium and magnesium concentrations were measured on 100 mg sub-samples after digestion with 2 cm^3 H_2SO_4/H_2O_2 (containing 0.1% Se) at 330^oC for 1.75 hours and dilution to 50 cm^3, using ICP/OES (JY Instruments, France). Chloride, phosphate and sulphate concentrations were determined on water extracts (prepared as for nitrate analysis, above) using ICP/OES. Water soluble carbohydrates (SCH) were extracted from 100 mg sub-samples into between 10 and 20 cm^3 of boiling water for 2 hours, centrifuged and diluted to 50 cm^3. SCH was determined colorimetrically (at 490 nm) in the extracts by the phenol – sulphuric acid method using glucose standards (Dubois et al., 1956). Molar concentrations of SCH were calculated by assuming that their average molecular weight was 360, and that there were no changes in the proportions of the individual soluble carbohydrates between treatments, or during growth (following the method of Veen and Kleinendorst, 1985 and 1986).

Soluble organic anion concentrations were calculated as the difference between those for total cations and inorganic anions present using a simple charge balance equation, (after Houba et al., 1971; van Beusichem et al., 1988), assuming that all organic anions were both divalent and fully dissociated. This assumption was based on an approximation of data from Blom-Zandstra and Lampe (1985), who showed that virtually all of the organic acids in lettuce were divalent (mostly consisting of malic acid). They also used pK_a data for each of the organic acids present to calculate that no more than 6% were likely to remain undissociated at the pH of lettuce sap.

Concentrations of each of the soluble mineral and organic constituents in the dry material were converted into mmol kg^{-1} of shoot water using water content data. The concentration of total solutes at each sampling date was calculated as the sum of the concentrations of the above individual soluble constituents, following Blom-Zandstra and Lampe (1985) and van Beusichem et al. (1988). The differences between the total solute concentration and the corresponding nitrate concentration were also calculated. For the purposes of this paper, this difference is referred to as the ‘residual solutes concentration’, and is used to examine the relative changes in nitrate and the sum of all other osmotica present during the course of the experiments.

Shoot osmotic potentials (π in MPa) were not measured directly because of practical difficulties in extracting representative samples of sap from whole shoots. Instead, they were calculated using the following state equation:

$$\pi = - R.T.c$$

where c is the concentration of total solutes (mol kg^{-1} water), T is the temperature in K, and R is the gas constant (0.008315 MPa K^{-1} mol^{-1}). This equation assumes that shoot sap behaves as an ideal solution, and that each of its ionic constituents are fully dissociated (*ie* that the osmotic coefficients of each approximate to unity; see Wyn Jones and Gorham, 1982; Nobel, 2005). For the purposes of these calculations, the average temperature in the glasshouse at 0900 hours (*ie* the time of each sampling) was assumed to be 293 K (*ie* 20 ^{o}C). Independent measurements on detached leaf blades of lettuce have shown that this method gives a reliable estimate of osmotic potential (Blom-Zandstra and Lampe, 1985).

Estimation of Errors

Statistical analyses were all conducted in Genstat (Version 9.1, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Standard errors of differences (SEDs) of shoot fresh and dry weights, and water contents were estimated using a standard analysis of variance of the replicated measurements (ANOVA) after log-transformation of the data to stabilise the variances across each of the datasets. However, as there were no replicate measurements for the mineral and organic constituents in the shoots, a polynomial model (either quadratic or cubic, whichever gave the best fit) was fitted to sampling date for the combined data of each constituent from all six experiments (treatments), and an ANOVA used to estimate SEDs from the error variability of each fitted model for significance testing. Where necessary, these analyses were performed on log-transformed data to stabilise the variances across each dataset. The significance of any treatment effects was assessed relative to Experiment T1, the control treatment. Unless otherwise stated, statistical significance was determined at the 95 % confidence level.

RESULTS

Plant Appearance

Overhead photographs indicated that medium and low light plants (T5 and T6 respectively) produced larger expanded leaves than those of the control plants (T1) grown under high light. These plants also initiated new leaves at a slower rate (against time), but the differences became insignificant when expressed on a shoot fresh or dry weight basis. Leaf initiation rates (against time) were similar for all four experiments at the high light level (T1 to

T4), but were significantly greater for T3 (with the ammonium + nitrate supply) when shoots of the same size were compared, indicating a smaller average leaf weight. This was consistent with evidence from the overhead photographs, which showed a somewhat more compact growth habit for these plants. Similar effects of ammonium nutrition on reduced leaf size (but not leaf numbers) have been observed with young sugar beet plants by Raab and Terry (1994). Leaves in this experiment (T3) were also darker green and had a 'tougher' appearance than in all other experiments (much as observed by Scaife et al., 1986; and Abd-Elmomiem et al., 1996); these plants also showed evidence of slight tipburn towards maturity, in agreement with other reports for ammonium-fed plants (Scaife et al., 1986; Gunes et al., 1995).

By the time of commercial maturity, all plants had formed well-defined hearts, although those produced under medium and low light conditions were less compact than for those at the high light level. There was no evidence of bolting in any of the treatments, although in experiment T3, smaller wrapper leaves left more of the inner heart leaves partially visible. This, and the associated effects on leaf colour and appearance, reduced the overall quality of the lettuce heads in this treatment.

Shoot Growth and Water Contents

Shoot dry weights increased with time throughout at absolute growth rates which differed between each experiment, see Fig. 2A. Medium and low light levels (T5 and T6) consistently reduced the rate of dry matter production relative to that at high light, with the effects of either N source or concentration of nitrate in the nutrient supply having a smaller effect. In general, partial replacement of nitrate with ammonium (T3) reduced the absolute growth rate slightly compared with the control (T1), whereas doubling the concentration of nitrate (T4) had a small opposite effect. There was no effect of adding chloride to the nutrient solution (T2) on shoot dry weight. Compared with those in the control, plants in T4 reached the same final shoot dry weight 3 days earlier, whereas those in the slower growing experiments (T3 and T5) took a further 7 and 12 days respectively; and plants in T6 never reached the same final size as those in the control. ANOVAs carried out on the log transformed dry weight data revealed highly significant main treatment effects of both sampling date and experiment, and of the interactions between the two (all at $P<0.001$). Re-plotting the dry weight data on a log scale (see Fig. 2B), showed that the largest differences in relative growth rate (RGR; the slope of these curves) between treatments occurred after about day 12, when the curves for T1 to T4 (all grown at high light) tended to diverge.

Shoot fresh weights followed similar patterns to those for dry weight, except that those for plants grown with both ammonium and nitrate (T3) increased relatively more slowly than for T1, T2, T4 and T5, see Fig.2C and D. As a result, net fresh weight increases in T3 were intermediate between those for the medium and low light levels (T5 and T6 respectively). This difference occurred because the moisture contents of the shoots in T3 were initially lower, and did not increase as quickly as those in the control plants during the course of the experiments, see Fig. 2E. In contrast, shoot water contents at lower light levels (especially for T6) were slightly greater than in the control, differences which became statistically significant when plants of the same dry weight were compared, see Fig.2F.

Changes in Composition of Shoot Sap

Nitrate

An ANOVA showed there were highly significant effects of experiment and the interactions between sampling date and experiment on nitrate accumulation. Smaller average concentrations were observed where the nitrate supply was partially replaced with ammonium (T3), whereas larger concentrations occurred at lower light intensities (T5 and T6), and when more nitrate was used in the nutrient supply (T4). Graphs of the changes in nitrate concentration in the shoot sap against time in Fig. 3A show how these responses varied during growth. The variations for the control experiment (T1) did not appear to follow any consistent pattern, with concentrations fluctuating between *ca* 33 and 53 mmol kg^{-1} water throughout, whereas other experiments showed more consistent trends. For example, despite the conditioning stage, partial replacement of nitrate with ammonium (T3) did not affect the shoot nitrate concentration prior to the start of the experiment (*ie* by the initial sampling). However, the nitrate concentrations steadily declined thereafter, reaching a minimum of *ca* 10 mmol kg^{-1} water (about 25 % of the starting value) during the following 9 to 12 days, before partially recovering towards the end of the experiment. Likewise, nitrate concentrations were not only consistently higher in the medium and low light experiments, but tended to increase towards maturity. Doubling the concentration of nitrate in the external supply (T4) also tended to increase nitrate accumulation relative to the control (T1) in the early part of the experiment, whereas the addition of chloride (T2) had a consistently small but non-significant depressive effect.

In addition, effects of short-term natural fluctuations in light intensity were superimposed on the above trends, contributing additional ‘noise’ to the longer-term variations in shoot nitrate concentration. This resulted in certain similarities in the patterns of variation between experiments carried out in the same year. Thus, for example, the pronounced increases in

nitrate concentration in T5 and T6 between 23 and 27 days (Fig.3A) can be attributed to a period of reduced light intensity between 21 and 24 days (*cf* Fig. 1). Similarly the short-lived dip in nitrate accumulation in T1 and T2 between 16 and 20 days were largely the result of enhanced increases in accumulated radiation between 13 and 18 days. These similarities between concurrent experiments were not evident when comparisons were made for plants at the same dry weight (Fig. 3B), and suggests that short term variations in nitrate concentration are more dependent on time-based changes in the aerial environment than on the size of the plant. As a result subsequent graphs showing changes in concentration of other soluble solutes during the course of the experiments are plotted against time, rather than dry weight.

Other anions

Apart from nitrate, the most prevalent anions initially present in shoot sap were in the organic form (largely as dissociated organic anions, see Blom-Zandstra and Lampe, 1985), at which time concentrations ranged from 53 to 73 mmol kg^{-1} water. Thereafter, their concentrations declined somewhat erratically throughout the experiments at rates which varied between each (data not shown). On average, organic anion concentrations declined more rapidly in medium and (especially) low light conditions (T5 and T6 respectively) compared with the control. Differences between the experiments at the highest light intensity were smaller. There was also no evidence of lower shoot organic anion concentrations in T3 where ammonium was included in the N supply, in contrast to other results where plants were fed with ammonium N (*eg* Kirkby, 1968; Breteler and Smit, 1974; van Beusichem et al., 1988). Presumably the continued uptake of nitrate (albeit at a reduced rate) in our experiment T3 was sufficient to counteract any decrease in organic anion concentration arising from the associated ammonium uptake, even though the latter was still large enough to suppress shoot nitrate accumulation significantly (*cf* Fig. 3).

Initial chloride and phosphate concentrations in the shoot sap were between 10 and 30 mmol kg^{-1} water, but tended to decline gradually during the experiments. Highest chloride concentrations were found in T2 (where additional chloride was included in the nutrient supply), and these undoubtedly contributed to the small associated reductions in shoot nitrate in this experiment relative to the control (*cf* Fig. 3). Average chloride concentrations (over all samplings) were also significantly higher in T3, with the combined nitrate and ammonium supply. In contrast, lower chloride concentrations were found in plants grown at reduced light levels (T5 and T6), and, to a lesser extent, in those supplied with a higher nitrate concentration in the nutrient supply (T4), conditions which all favour uptake of nitrate over that of chloride. Concentrations of phosphate, on the other hand, showed little

evidence of treatment effects, whereas corresponding sulphate concentrations were low in comparison with the other anions, remaining below 3 mmol kg^{-1} water throughout.

Cations

Concentrations of potassium, easily the most prevalent cation, ranged from *ca* 150 to 175 mmol kg^{-1} water at the initial sampling, but declined steadily in all experiments thereafter. However, during the early stages of the experiments, the decline in its concentration was more pronounced in T3, where both ammonium and nitrate were present in the nutrient supply, although subsequently concentrations tended to stabilise (at least partially). Potassium concentrations were consistently lower in low and medium light conditions throughout the experiments, whereas those in T4 (which had extra potassium as well as nitrate in the nutrient supply) were marginally higher than the control, particularly in the middle part of the experiment. Initial calcium and magnesium concentrations were about 10 and 5% of those for potassium respectively, did not decline to the same relative extent during growth, and showed only small treatment effects.

Soluble carbohydrates

Initial SCH concentrations ranged from *ca* 30 to 42 mmol kg^{-1} water. Thereafter concentrations for the control remained approximately constant, whereas those for plants grown under medium and low light conditions (T5 and T6 respectively) tended to decline throughout the experiments. In contrast, partial replacement of nitrate with ammonium (T3) consistently increased SCH concentrations, producing a broad peak between 9 and 16 days, before gradually declining thereafter.

Residual solutes

The concentration of residual solutes represents the sum of all soluble ions and neutral compounds in the shoot sap apart from nitrate. Its value ranged from 295 to 337 mmol kg^{-1} water at the start of the experiments, and tended to decline during growth, see Fig.4A. The rate of decline was slightly greater for plants grown under low and medium light conditions (T5 and T6 respectively). Differences between the other experiments (including the control), which were all grown at the high light level, were small and non-significant.

Total solutes and osmotic potential

Concentrations of total solutes ranged from 343 to 377 mmol kg^{-1} water at the start of the experiments, but generally declined fairly smoothly thereafter, see Fig 4B. The rate of decline was slightly greater under low and medium light conditions, but the concentrations in

these treatments only became significantly different from the control as the plants approached maturity. Plants grown in experiments at the high light level all generated similar trends in concentrations of total solutes in all experiments. Fig. 4B also shows the corresponding changes in osmotic potential (see right hand ordinate for its scale), which gradually increased (*ie* became less negative) throughout each experiment. Values ranged from an average of *ca* -0.9 MPa at the start to about -0.6 MPa at maturity depending on experiment, and were of a similar size to other isolated measurements for lettuce (Behr and Wiebe, 1988; McCall and Willumsen, 1998 and 1999), and other vegetative crops (Veen and Kleinendorst, 1986). Note that, because the osmotic potential decreases in proportion to the increases in the concentration of total solutes, changes in the former are always the converse of the latter.

The relative concentrations of most of the individual osmotica changed during growth, with only potassium maintaining an approximately constant proportion of the total solute concentration (from 37 to 48%) between treatments throughout. However, the combined contribution of nitrate, potassium, organic anions and SCH also remained approximately constant, accounting for between 79 and 88% of the total, depending on treatment. Including chloride in the equation increased the total proportions and reduced their range to between 83 and 90%, largely by removing the bias introduced by experiment T2, where additional chloride was included in the nutrient supply. These results show that no single osmotica was entirely responsible for correcting the osmotic potential for changes in nitrate concentration during growth. It follows, therefore, that the stabilisation of osmotic potential across treatments at each sampling date is an integrated effect involving all of the soluble constituents within the shoot sap.

Effects of time on the Relationships between Residual Solutes and Nitrate

The proposed existence of such an integrated process to minimise any differences in osmotic potential between treatments implies that all increases in nitrate concentration in shoot sap should be accompanied by corresponding mole for mole decreases in those of the residual solutes, and *vice versa*. From this it follows that there should be a clear 1:1 negative relationship between the two across all treatments at each sampling date. This was tested by plotting graphs of these relationships for selected sampling dates (at 13, 16, 20, 23 and 27 days after the start of each experiment) in Fig. 5. These dates were selected as they were the only ones common to all experiments. The Figure shows separate lines for each sampling date, with each line spanning the range of concentrations of nitrate and residual solutes found in the six treatments.

The lines in Fig. 5 were fitted using a sequential linear regression approach (analysis of parallelism) in Genstat (Version 9.1, Lawes Agricultural Trust, Rothamsted Experimental Station, UK), in which three separate alternative models were constructed sequentially to describe the relationship between residual solutes and nitrate. These were: a single line for all treatments and all selected sampling dates; parallel lines where a different intercept is allowed for each selected harvest date; and different lines where separate intercepts and slopes are allowed for each selected sampling date. The changes in residual deviance between these nested models were assessed using an accumulated analysis of variance table to determine whether the second or third step significantly improved the overall fit to the data so as to identify which of the three models gave the best statistical description of the results.

The results showed there was a significant negative correlation between the concentrations of residual solutes and those of nitrate when data for all samplings were pooled (R^2= 0.577; P<0.001). However, there was also clear evidence of significant additional effects of sampling date, with the regression analysis showing that the data were best described by a series of parallel lines each with a slope of -1.122 (±0.120) mmol (mmol nitrate)$^{-1}$, and different intercepts for each experiment (P<0.001), as shown in Fig. 5. There was no additional statistical improvement to the fit by allowing the slopes of the lines to vary between sampling dates. These results show that the slope of all of the lines did not deviate significantly from -1.0, the value expected for a simple 1:1 exchange of nitrate by the other osmotica if a perfect instantaneous osmotic balance is to be maintained. The analysis also shows that this balance was maintained over time, despite the values of the intercepts on the residual solutes axis declining with sampling date, see Table 4. This displacement of the regression lines over successive sampling dates reflects the gradual decline in the concentration of total solutes during the course of the experiments (*cf* Fig. 4B).

Effects of Treatment on the Relationships between Residual Solutes and Nitrate

Fig. 5 and Table 4 describe how the relationships between the concentrations of nitrate and alternative residual solutes changed over time (*ie* between selected sampling dates), assuming that all treatments behave consistently. This section tests this assumption by examining whether the equivalent relationships also apply to all treatments (including those of increasing the concentration of nitrate or adding ammonium to the external supply). To do this, data from all treatments and *all* sampling dates were included. The relationships were evaluated using the same sequential linear regression approach described in the previous section, but with the selected sampling date replaced by treatment number.

Initial comparisons showed that concentrations of residual solutes and nitrate were negatively correlated when data for all experiments were included (R^2=0.301; P<0.001). However, there was clear evidence of significant treatment effects, with regression analysis showing that the data were best described by a series of parallel lines with a common slope of -1.884 (±0.521) mmol (mmol nitrate)$^{-1}$, and different intercepts for each experiment, see Fig. 6A. Plants fed with higher concentrations of nitrate (T4) had a larger intercept on the residual solutes axis than the control, whereas all other experiments had smaller values for the intercept. Although the common slope of these lines is again not significantly different to -1.0, this is largely because of the large size of its standard error. As a result, this analysis does not provide convincing evidence for a 1:1 relationship between the concentrations of residual solutes and nitrate. The large size of this standard error is likely to have been caused (at least in part) by underlying changes in the relationships over time (*cf* Fig. 5) which were, in turn, driven by the gradual decline in the total solute concentration during the course of the experiments (*cf* Fig. 4B).

In an attempt to correct for these time trends, the concentrations of nitrate and residual solutes were both normalised by expressing them as a fraction of the concentration of total solutes at each sampling date. Values of the latter used for this purpose were estimated from separate linear regression equations fitted to the data for each of the experiments in Fig. 4B in order to smooth out any short-term variations over time. The results of this normalisation are plotted in Fig. 6B. Regression analysis showed that the normalised concentrations of residual solutes and nitrate were highly correlated (R^2=0.689; P<0.001) and that there were no significant differences between experiments. As a result, these normalised data were best described by a single line of slope -0.937 (±0.085) and intercept 0.989 (±0.016). Neither the slope nor the intercept of this line is significantly different from -1.0 and 1.0 respectively and, as the standard errors of both are small, the relationship is indicative of a 1:1 exchange of nitrate with the residual solutes throughout growth, even though the concentration of total solutes declined over time. These results confirm that there was no significant effect of experiment (*ie* of a specific treatment, including that with both ammonium and nitrate in the supply) on this relationship, and are therefore consistent with the 1:1 relationships from the earlier analysis in which data for all treatments were grouped at each selected sampling date.

Relationships between Solute and Water Contents in Shoot Dry Matter

The influence of treatment (experiment number) on the relationships between solute concentrations (either nitrate or total solutes, both expressed as mmol g^{-1} DM) and water

content in the shoot dry matter was examined using the sequential regression analysis in the same way as described in the last section, but without normalising the data. Results for the nitrate data showed that the relationships followed a series of parallel lines ($P<0.001$), see Fig 7A. Plants grown under medium and low light conditions (T5 and T6), or with higher nitrate concentrations in the supply (T4) gave larger intercepts on the nitrate axis (*ie* less negative) than the control (T1), whereas additional chloride (T2) or partial replacement of nitrate with ammonium (T3) in the nutrient solution reduced the value of this intercept (making it more negative), see Table 5. A corresponding sequential regression analysis for the concentrations of total solutes (again expressed on a dry weight basis) against shoot water content showed that these relationships also followed a series of parallel lines ($P<0.001$), as illustrated in Fig.7B. However, with this dataset, the values of the intercept for medium and low light conditions were slightly less than that for the control, whereas partial replacement with ammonium significantly reduced it; adding chloride to the supply had no effect, see Table 5.

DISCUSSION

Growth and N Assimilation

Fig. 2A and B show that shoot dry matter accumulation at medium and low light levels was significantly less than that in the control. However, when dry weights were expressed in terms of effective day degrees (Scaife et al., 1987), there were no differences in the relative rates of growth between any of these treatments (data not shown). This indicates that the combined effects of temperature and reduced light in these experiments were energy related, and entirely independent of N supply. Fig. 2A also shows that doubling the nitrate supply induced a small increase in growth rate over the control, whereas partial replacement of nitrate with ammonium slightly reduced it. Although a small amount of ammonium in the nutrient supply can be beneficial (Barker and Mills, 1980; Savvas et al., 2006), the reduced rate of dry matter accumulation from the ammonium in T3 is consistent with previous observations for lettuce (Raynal Lacroix, 1994; Abd-Elmoniem et al., 1996; Demšar and Osvald, 2003) and for a number of other crops (Kirkby, 1968; van Beusichem et al., 1988; Raab and Terry, 1994), when its external concentration typically exceeds 10 to 15 % of the total N supply. However, despite the resulting small differences in plant size in these treatments, weight for weight there were no differences in the organic N concentrations in the shoot dry matter (data not shown). This indicates that dry matter accumulation and N assimilation changed more or less in tandem during the course of the experiments. From this we conclude that supplying N at 4 mol m^{-3} in many of the experiments may have

restricted growth slightly compared to the 8 mol m^{-3} treatment, but no plants were likely to have suffered significant N deficiency as a result. It also follows that the reduced quality of the ammonium-fed plants was caused by the direct effects on other endogenous processes (*eg* restricted cation uptake, poor internal pH control, mild ammonia toxicity) induced by the presence of ammonium ions in the supply (Barker and Mills, 1980; Marschner, 1995, p.47-50) rather than by a shortage of N *per se*.

Effects of Treatments on Internal Solutes

Nitrate concentrations in shoot sap were larger in plants grown under medium and low light conditions, despite slightly higher water contents which would have tended to increase dilution. This effect of light is consistent with previous reports, and is well documented, particularly for salad crops (Cantliffe, 1972; Maynard et al., 1976; Burns et al., 2003). Partial replacement of nitrate with ammonium in the nutrient solution reduced nitrate accumulation in shoot sap (by up to 75%), an effect also observed in previous studies where ammonium was used as either the sole or a partial source of N for lettuce (Scaife et al., 1986; Hähndel and Wehrmann, 1986a; van der Boon et al., 1988; Steingröver et al., 1993; McCall and Willumsen, 1998). This reduction occurred despite an associated decline in shoot water content (by up to about 30 % compared to the control), which would otherwise have tended to increase sap concentrations. Similar reductions in water contents (or increases in dry matter content) have been observed when ammonium was an important component of the N supply for lettuce (Scaife et al., 1986; Raynal Lacroix, 1994; Savvas et al., 2006) and other crops (Raab and Terry, 1994). In contrast, adding chloride to the nitrate supply had only a small depressive effect on nitrate accumulation, because of the greater selectivity of this crop for nitrate. This agrees with other results for lettuce grown with an adequate nitrate supply (Hähndel and Wehrmann, 1986b; Blom-Zandstra and Lampe, 1985; van der Boon et al.,1988; McCall and Willumsen, 1998). In general, substantial chloride replacement of nitrate only occurs when the supply of the latter is either withheld or substantially reduced (Blom-Zandstra and Lampe, 1983; Glass and Siddiqi, 1985; Veen and Kleinendorst, 1986), even in halophytic plants which generally tolerate higher chloride levels (Steinstra, 1986).

In contrast to the responses of both nitrate and other individual osmotica, Fig 4B shows there was little or no treatment effect on the concentration of total solutes in shoot sap, except possibly towards the end of the medium and low light experiments. From this we conclude that the concentrations of the alternative individual osmotica are adjusted endogenously to compensate for differences in nitrate concentration so as to minimise treatment effects on the osmotic potential of the sap. Similar inferences can be made from more restricted or shorter-term measurements in other reports for lettuce. For example,

differences in total solute concentration were small irrespective of the light level (Blom-Zandstra and Lampe, 1985; McCall and Willumsen, 1999), N source or concentration (McCall and Willumsen, 1998 and 1999), or cultivar type (Behr and Wiebe, 1988), despite considerable associated variations in nitrate content. Taken together, these findings provide evidence that iso-osmotic control is likely to be involved in the regulation of nitrate accumulation in the shoot sap of this crop.

Fig 4B also shows there was a gradual decline in total solute concentration in all treatments over the course of growth. Such changes have not previously been reported for lettuce, possibly because of the restricted timescale of these earlier studies. Our results suggest that much of this decline was caused by a dilution effect from the associated increase in the water content of the shoots, because the concentrations of total solutes in the sap declined consistently with water content at a similar rate for all treatments (data not shown). This effect may have been associated with gradual changes in shoot architecture and plant development during growth. For example, the effects of changing proportions of leaf to stem tissue, and increases in self-shading of mature leaves by newly developing ones can affect the water relations of a plant, including its osmotic and water potentials (Wyn Jones and Gorham, 1982). In addition, the gradual development of a heart (which often has a relatively lower concentration of minerals such as nitrate, potassium and calcium in relation to sugars, see Drews et al., 1997) as a lettuce plant matures may also influence its average osmotic potential over time.

Relevance to the Iso-osmotic Control Hypothesis

Previous studies have provided evidence in favour of some form of osmotic control during the accumulation of nitrate by lettuce. For instance, Blom-Zandstra and Lampe (1985) showed that the sum of the concentrations of organic anions plus glucose and sucrose declined approximately linearly with increase in nitrate, while the osmotic potential remained constant. Equivalent graphs with average slopes close to -1.0 mol (mol nitrate)$^{-1}$ were also presented by Blom-Zandstra et al. (1988) for two different lettuce cultivars grown at three light levels. From this, they inferred that organic anions and sugars together were the only soluble constituents needed for maintaining constant osmotic potential following changes in nitrate concentration. Buwalda and Warmenhoven (1999), on the other hand, found slopes of only -0.60 and -0.68 mol (mol nitrate)$^{-1}$ for the same relationships in two experiments with lettuce plants grown with limited P nutrition, implying that other solutes must also have been involved for osmotic potential to be maintained.

This is confirmed by our results which show that, despite considerable differential effects on the relative proportions of many of the individual endogenous solutes, there were no significant treatment differences in their combined concentrations at each sampling date. As a result, differences in of osmotic potential between treatments were essentially eliminated, providing strong evidence for *concentration regulation* as an integral part of an iso-osmotic mechanism for controlling nitrate concentrations in lettuce. This is further supported by the negative linear relationships between concentrations of nitrate and residual solutes, each of which had a slope not significantly different from -1.0 on a mole for mole basis. Although the changes in the total solute concentration induced gradual displacement of the proportionate relationships over successive time intervals (Fig. 5), correcting for these changes (as in Fig. 6B) caused the individual parallel lines to collapse on to a single line which closely approximated to that for 1:1 replacement. Thus these results are also quantitatively consistent with an underlying iso-osmotic regulation mechanism for controlling nitrate accumulation in the shoot, in which concentrations of all available solutes are adjusted in tandem, minimising differences in their combined concentration and their associated osmotic potential.

The differences in water content of the shoot between treatments and their changes over time indicate that the plants continually adjusted the volumes of their cells according to conditions throughout growth, by the process of *volume regulation* (Wyn Jones and Gorham, 1982; Nobel, 2005). In effect, the substantial reduction in shoot water content when the nitrate supply was partially replaced with ammonium, and the smaller increase in water contents when plants were grown under lower light conditions (Fig. 2E and F) provided an additional (fine tuning) mechanism for minimising short-term changes in the total solute concentrations in the sap (Fig. 4B). Our results also show that there were parallel linear relationships between the concentrations of either nitrate or total solutes (both in the shoot dry matter) and its water content, with the intercepts of the lines changing between experiments (Fig.7). Reduced light levels had the largest effect on the nitrate relationships (increasing the intercept, making it less negative), whereas the presence of ammonium in the nutrient supply caused the largest change (a reduction) in the intercept for the total solutes relationships (Table 5). However, despite these differences, the apparent constancy of the slope within each set of relationships would suggest that any incremental changes in amount of either nitrate or total solutes to those of shoot water remained essentially the same for each across all experiments.

Previous studies by Cárdenas-Navarro et al. (1999b) and Dapoigny et al. (2000) also highlighted similar positive linear relationships between nitrate content of the shoot (when

expressed on a dry matter basis) and its water content. As with our data, their results suggest that the slopes of these relationships were independent of a wide range of factors across each of their experiments, whereas the intercepts varied with both cultivar and growing conditions. In addition, equivalent *negative* relationships between nitrate and dry matter contents were demonstrated across populations of cultivars (Maynard et al., 1976; Reinink et al., 1987; Reinink and Eenink, 1988), partly because of the associated water content effects and partly because SCH and starch tended to accumulate to a greater extent in the low-nitrate plants. Cárdenas-Navarro et al. (1999b) suggested that such water content relationships were a reflection of homeostasis of endogeneous nitrate in the sap, with changes in amounts of nitrate resulting from associated changes in the size of the shoot water reservoir in which the nitrate concentration is regulated. However, our results suggest that this interpretation may be too simplistic, because there were still substantial variations in nitrate concentrations between treatments, even when these were expressed on a shoot water basis (Fig. 3). In contrast, treatment differences in the concentrations of total solutes were much smaller (Fig. 4B). It is therefore more likely that the changes in water content contribute to a homeostatic effect on *all* solutes (not just nitrate) within the shoot, in order to stabilise the average osmotic potential of its sap. Such a response would be entirely consistent with the colligative nature of the effects of endogenous solutes on shoot water relations (Wyn Jones and Gorham, 1982; Nobel, 2005). From this and the data above, we conclude that the maintenance of a constant osmotic potential is the result of at least two integrated strategies involving changes both to the contents of individual solutes present depending on their availability (concentration regulation) and to the average water content of the shoot (volume regulation), and that the combined effects of both these processes play a central role in the regulation of nitrate accumulation in the shoots of lettuce.

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Table 1. Summary of the experimental schedules and sampling frequenies*.

Detail	Expt T1	Expt T2	Expt T3	Expt T4	Expt T5	Expt T6
Sowing date	22 April	22 April	22 April	22 April	4 March	4 March
Transplant date	8 May	8 May	8 May	8 May	17 March	17 March
Start of expt:						
date	27 May	27 May	27 May	27 May	7 April	7 April
shoot fresh wt	5.59 g	5.61 g	5.89 g	5.11 g	3.74 g	2.20 g
No. of samplings	9	9	11	8	10	10
End of expt:						
date	26 June	26 June	3 July	23 June	14 May	14 May
shoot fresh wt	324.4 g	326.0 g	324.0 g	342.0 g	337.2 g	235.4 g

* Experiments T1 to T4 were carried out in 2003, and experiments T5 and T6 in 2004.

Table 2. The combinations of light levels and nutrient solutions used in the experiments. Actual cumulative irradiances are illustrated in Fig. 1, and details of the nutrient solutions are given in Table 3.

Treatment	Light level	Mean irradiance (MJ m^{-2} d^{-1})	Nutrient solution
T1	high	7.0	NS1
T2	high	7.0	NS2
T3	high	7.0	NS3
T4	high	7.0	NS4
T5	medium	4.9	NS1
T6	low	3.6	NS1

*T1 = Control treatment

Table 3. Concentrations of macronutrient salts (mol m^{-3}) in the nutrient solutions. Micronutrient concentrations were identical for all solutions, and are given in the text.

Chemical constituent	Solution NS1	Solution NS2	Solution NS3	Solution NS4
$Ca(NO_3)_2$	2	2	1.5	4
K_2SO_4	1	0	1	2
KH_2PO_4	0.5	0.5	0.5	1
$MgSO_4$	0.4	0.4	0.4	0.8
$(NH_4)_2SO_4$	0	0	0.5	0
KCl	0	2	0	0

Table 4. Regression data for the relationships between the concentrations of residual solutes and nitrate (both in mmol kg^{-1} water) at selected sampling dates.

Sampling number	Days after start of each experiment	Slope	± se	Intercept	± se
		mmol (mmol nitrate)$^{-1}$		mmol kg^{-1} water	
1	13	-1.122	0.120	317.59	8.12
2	16	-1.122	0.120	310.60	7.43
3	20	-1.122	0.120	295.09	7.63
4	23	-1.122	0.120	280.88	8.38
5	27	-1.122	0.120	260.77	8.82

Table 5. Regression data for shoot concentrations of nitrate and total solutes (both in mmol g^{-1} DM) against water content (g g^{-1} DM) for each experiment (treatment).

Solute	Experiment	Slope	± se	Intercept	± se
		(mmol g^{-1} water)		(mmol g^{-1} DM)	
Nitrate	T1	0.07973	0.00848	-0.653	0.179
	T2	0.07973	0.00848	-0.769	0.179
	T3	0.07973	0.00848	-0.813	0.143
	T4	0.07973	0.00848	-0.477	0.180
	T5	0.07973	0.00848	-0.434	0.190
	T6	0.07973	0.00848	-0.170	0.202
Total solutes	T1	0.0857	0.0157	4.218	0.331
	T2	0.0857	0.0157	4.213	0.331
	T3	0.0857	0.0157	2.867	0.264
	T4	0.0857	0.0157	4.375	0.333
	T5	0.0857	0.0157	3.699	0.351
	T6	0.0857	0.0157	4.049	0.373

Legends to figures:

Figure 1. Cumulative light intensities for the different light levels in the experiments. Key: high light, solid thin line; medium light, broken line; and low light, solid thick line.

Figure 2. Changes in shoot weights and water contents: (A) and (B) dry weight with time; (C) and (D) fresh weight with time; (E) water content with time; and (F) water content with dry weight. The ordinates in (B), (D) and (F) have been log transformed in order to show the standard error of differences (SEDs) and associated degrees of freedom (df). Key to symbols: open squares, experiment 1 (control); solid squares, experiment 2; solid circle, experiment 3; open circle, experiment 4; open triangle, experiment 5; solid triangle, experiment 6.

Figure 3. Changes in shoot nitrate concentrations: (A) with time; and (B) with shoot dry weight. The ordinates in (A) and (B) have been log transformed in order to show the SEDs and df. Key to symbols: see legend to Figure 2.

Figure 4. Changes in shoot solute concentrations with time: (A) residual solutes; and (B) total solutes. The average osmotic potential is also given on the right hand ordinate of (B). The ordinates in (A) and (B) have been log transformed in order to show the SEDs and df. Key to symbols: see legend to Figure 2.

Figure 5. Relationships between the concentrations of residual solutes and nitrate for selected sampling dates. Key to symbols: open square, sampling 1 (at 13 days); solid square, sampling 2 (at 16 days); open circle, sampling 3 (at 20 days); solid circle, sampling 4 (at 23 days); open triangle, sampling 5 (at 27 days). Key to regression lines: ——— sampling 1; —— —— sampling 2; - - - - sampling 3; —— - —— sampling 4; —— - - —— sampling 5.

Figure 6. Relationships between the concentrations of residual solutes and nitrate for each treatment: (A) original data; and (B) after normalisation to remove the effects of time trends. Key to symbols: see legend to Figure 2. Key to regression lines in (A): ——— experiment 1; - - - - experiment 2; ——— experiment 3; —— —— experiment 4; —— - —— experiment 5; —— - - —— experiment 6.

Figure 7. Relationships between the concentrations of (A) nitrate; and (B) total solutes (both in shoot dry matter) respectively against shoot water content for each treatment. Key to symbols: see legend to Figure 2. Key to regression lines: see legend to Figure 6.

Figure 1.

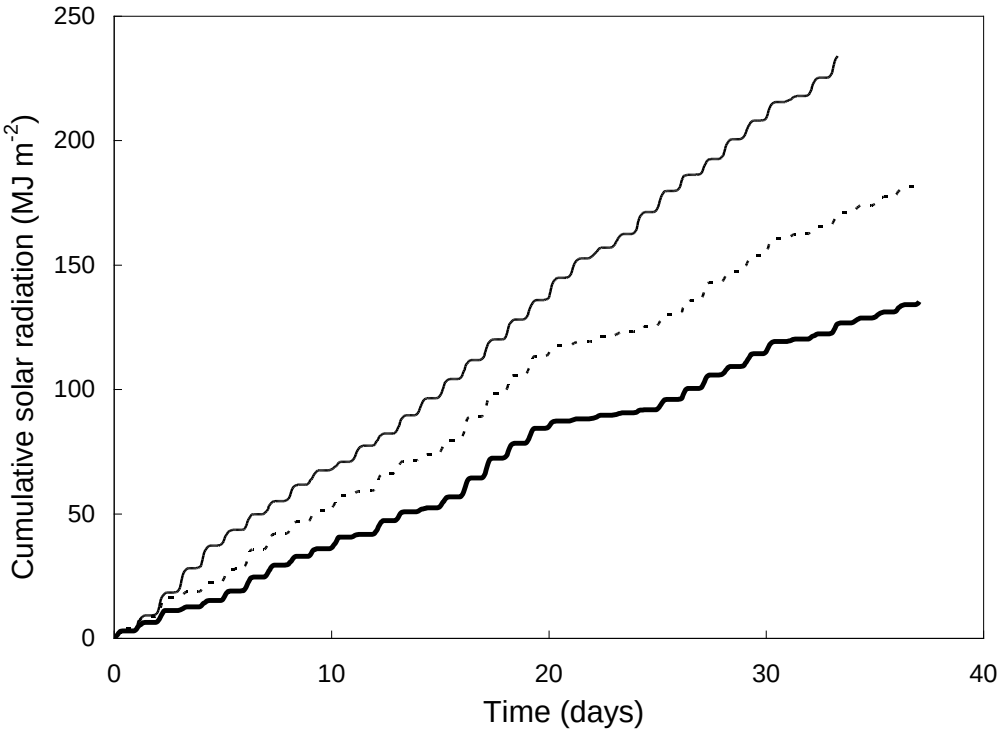

250
200
150
100
50
0
Cumulative solar radiation (MJ m-2)
0
10
20
30
40
Time (days)

Figure 2

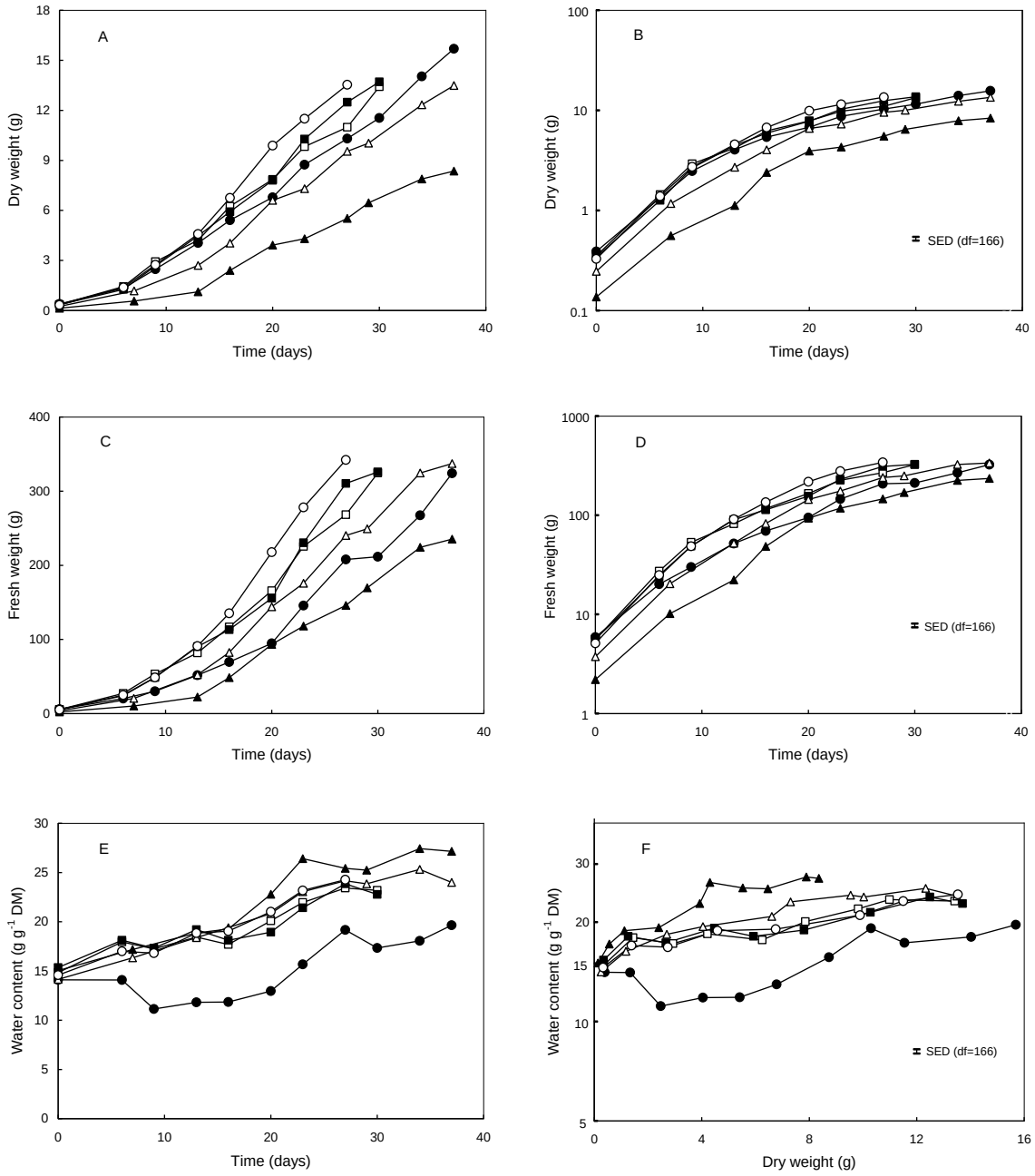

A
Dry weight (g)
Time (days)
B
Dry weight (g)
SED (df=166)
Time (days)
C
Fresh weight (g)
Time (days)
D
Fresh weight (g)
SED (df=166)
Time (days)
E
Water content (g g-1 DM)
Time (days)
F
Water content (g g-1 DM)
SED (df=166)
Dry weight (g)

Figure 3.

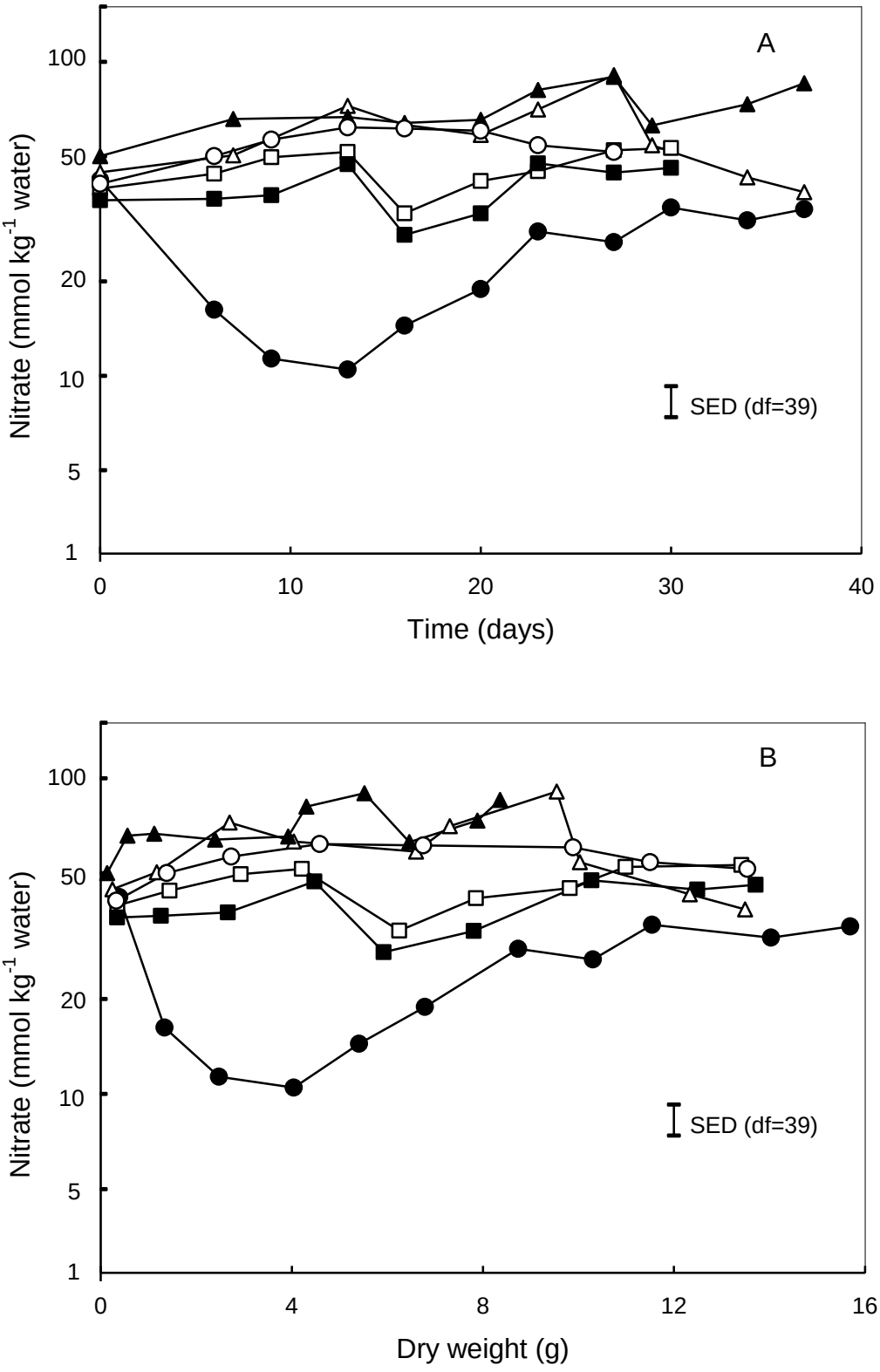
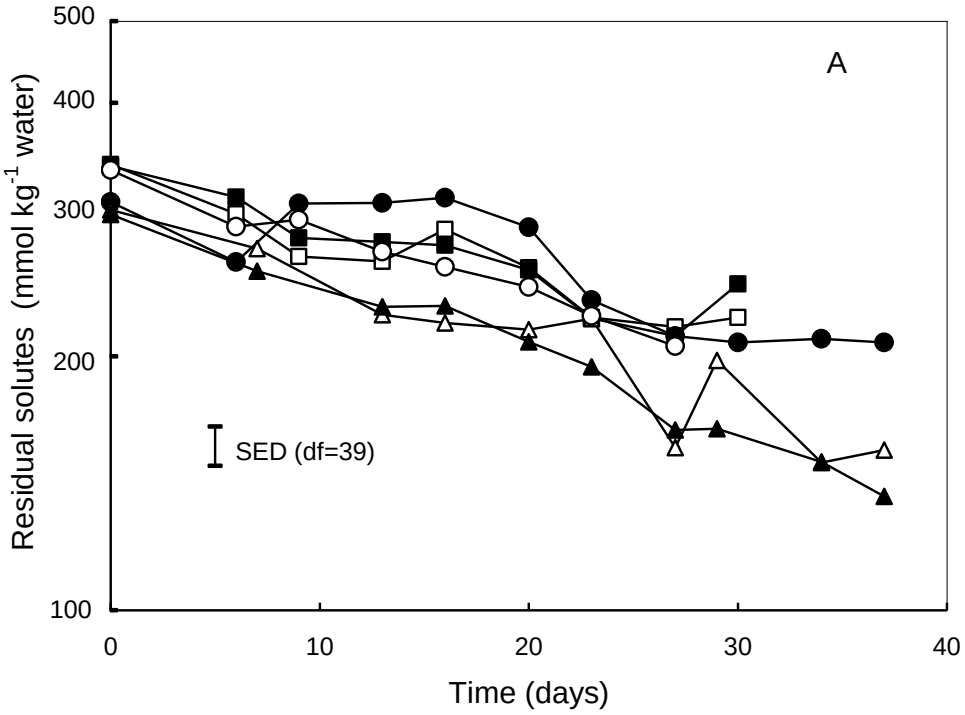

A
100
50
20
10
5
1
Nitrate (mmol kg-1 water)
SED (df=39)
0
10
20
30
40
Time (days)
B
100
50
20
10
5
1
Nitrate (mmol kg-1 water)
SED (df=39)
0
4
8
12
16
Dry weight (g)

Figure 4.


A
Residual solutes (mmol kg-1 water)
500
400
300
200
100
SED (df=39)
0
10
20
30
40
Time (days)

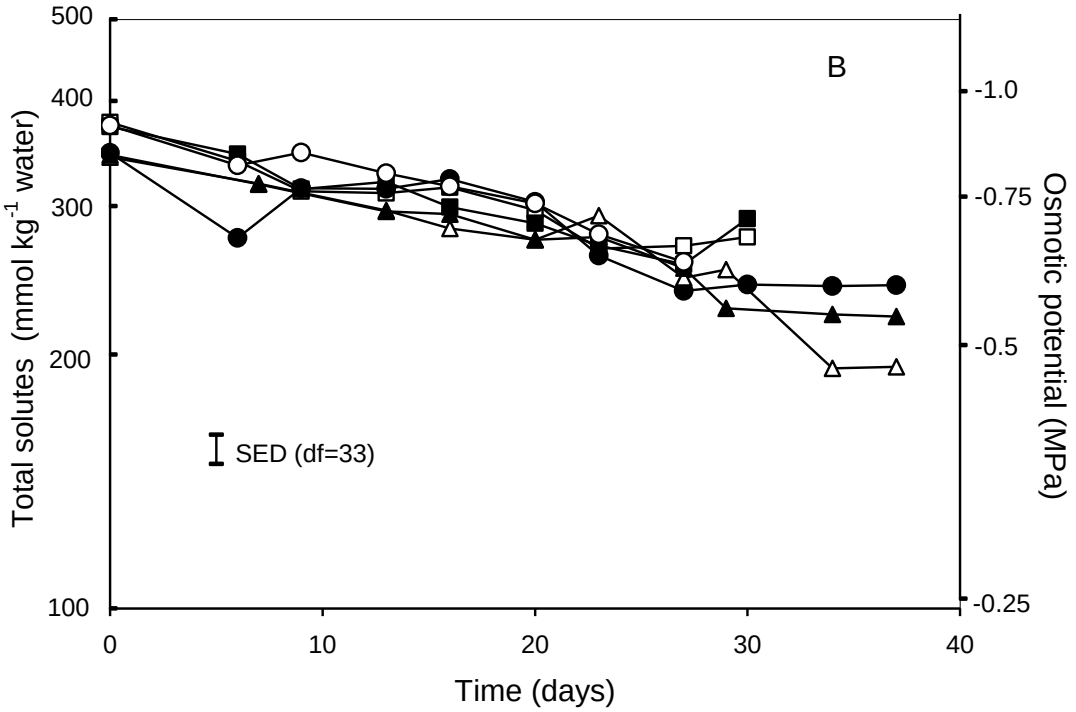

B
Total solutes (mmol kg-1 water)
500
400
300
200
100
SED (df=33)
-1.0
-0.75
-0.5
-0.25
Osmotic potential (MPa)
0
10
20
30
40
Time (days)

Figure 5.

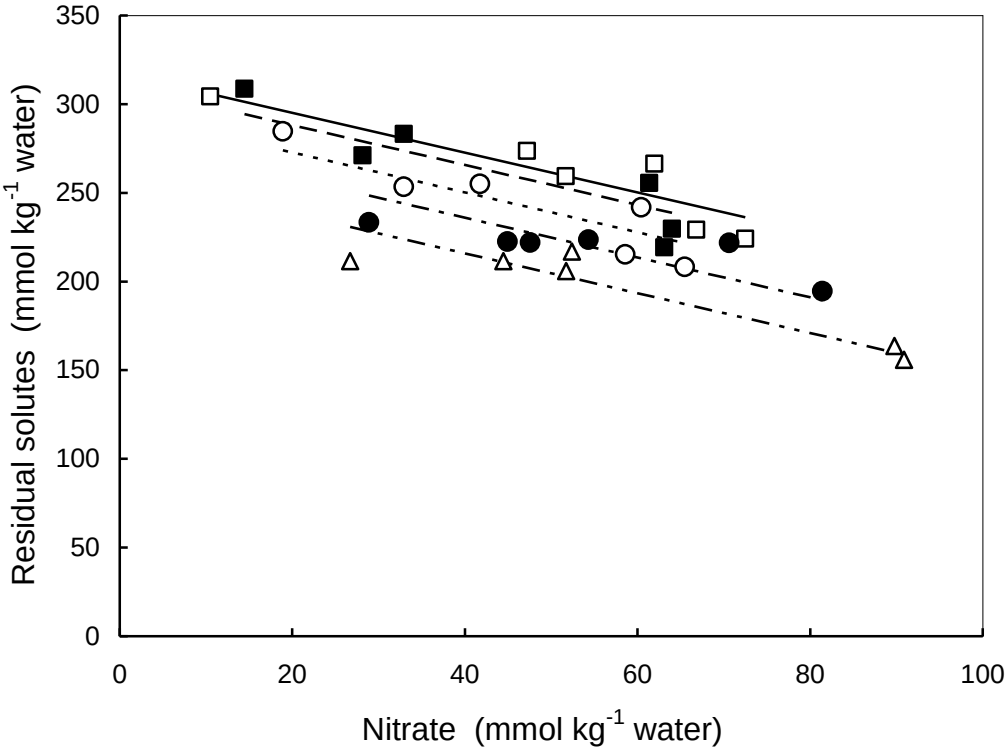
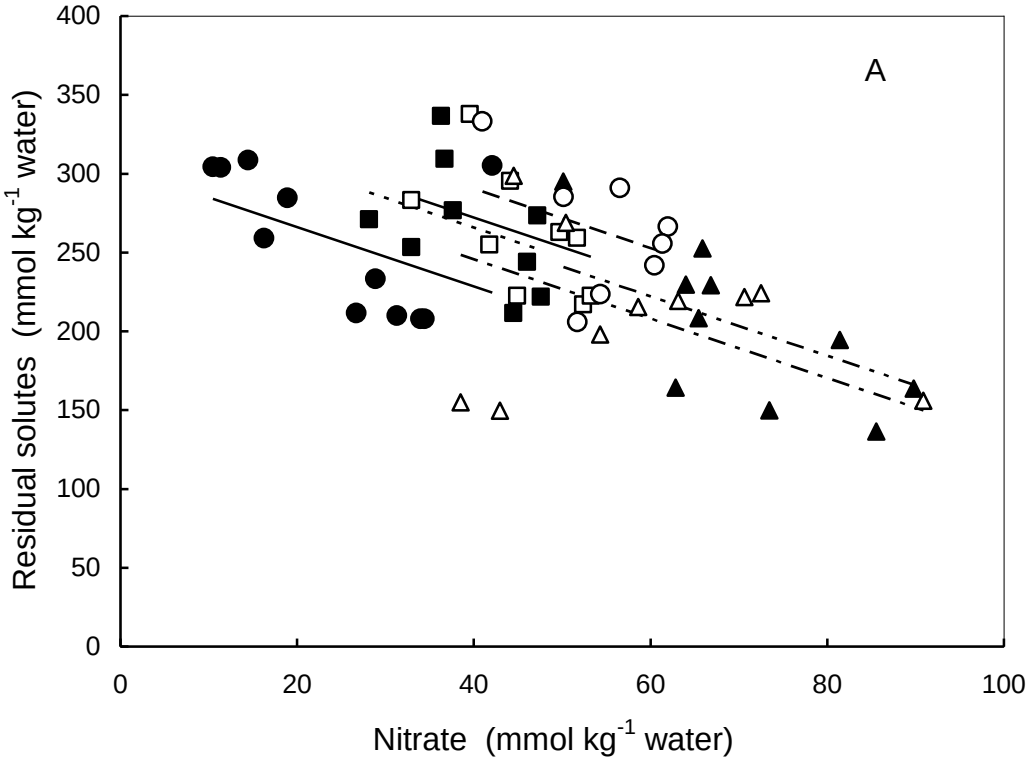

Residual solutes (mmol kg-1 water)
Nitrate (mmol kg-1 water)
0
50
100
150
200
250
300
350
0
20
40
60
80
100

Figure 6.


A
Residual solutes (mmol kg-1 water)
Nitrate (mmol kg-1 water)
0
50
100
150
200
250
300
350
400
0
20
40
60
80
100

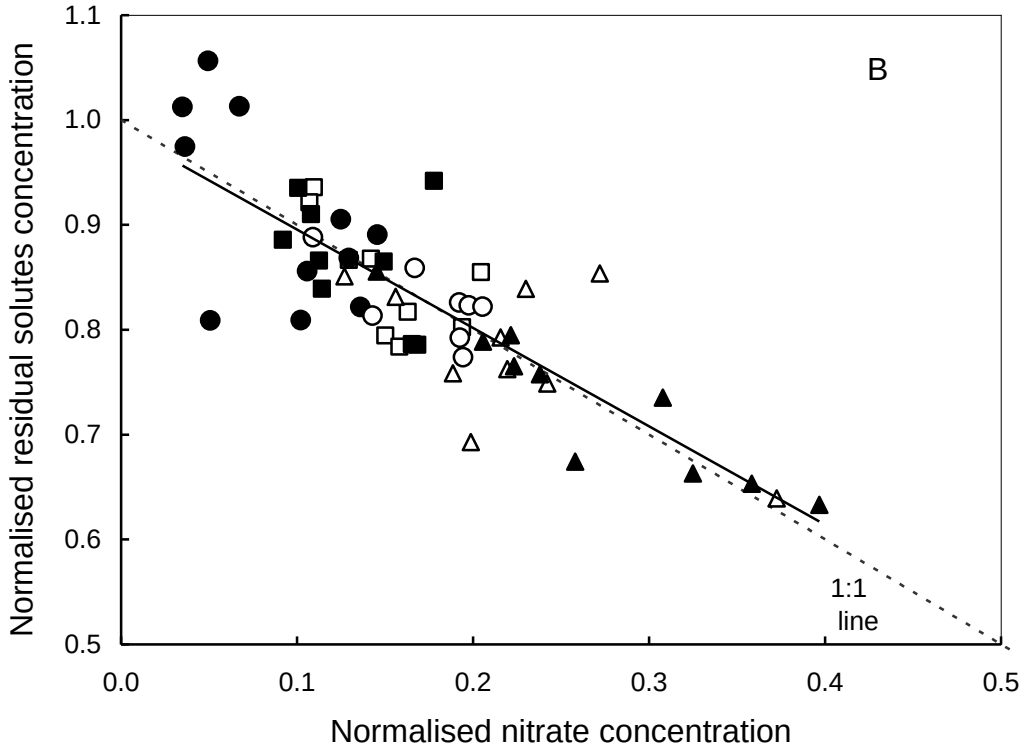
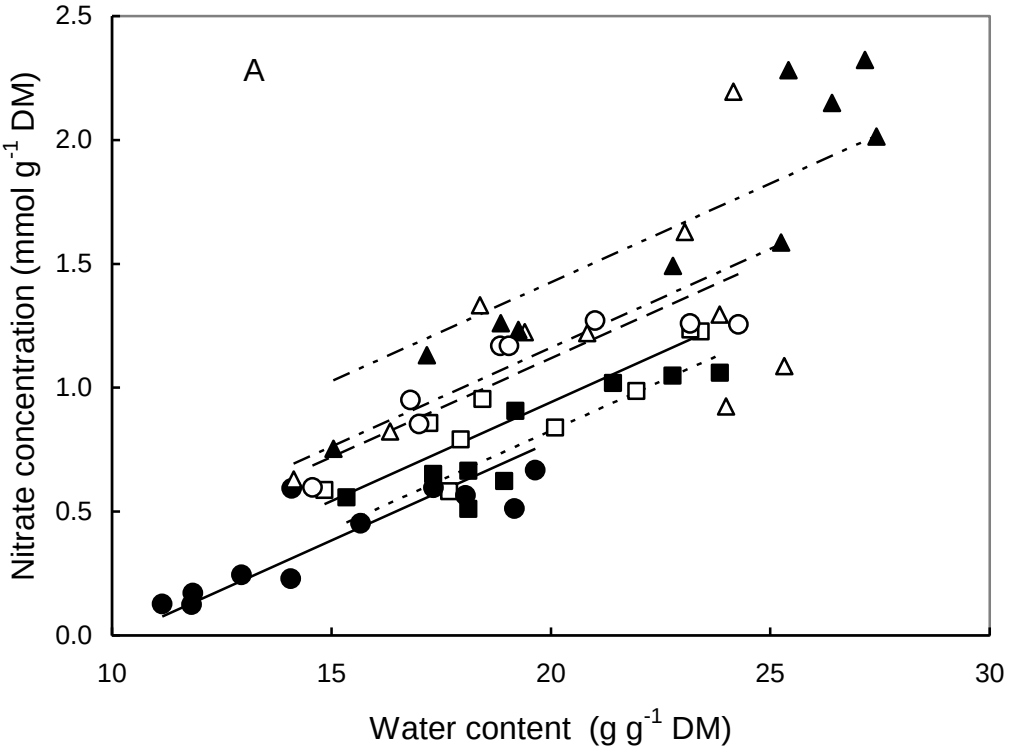
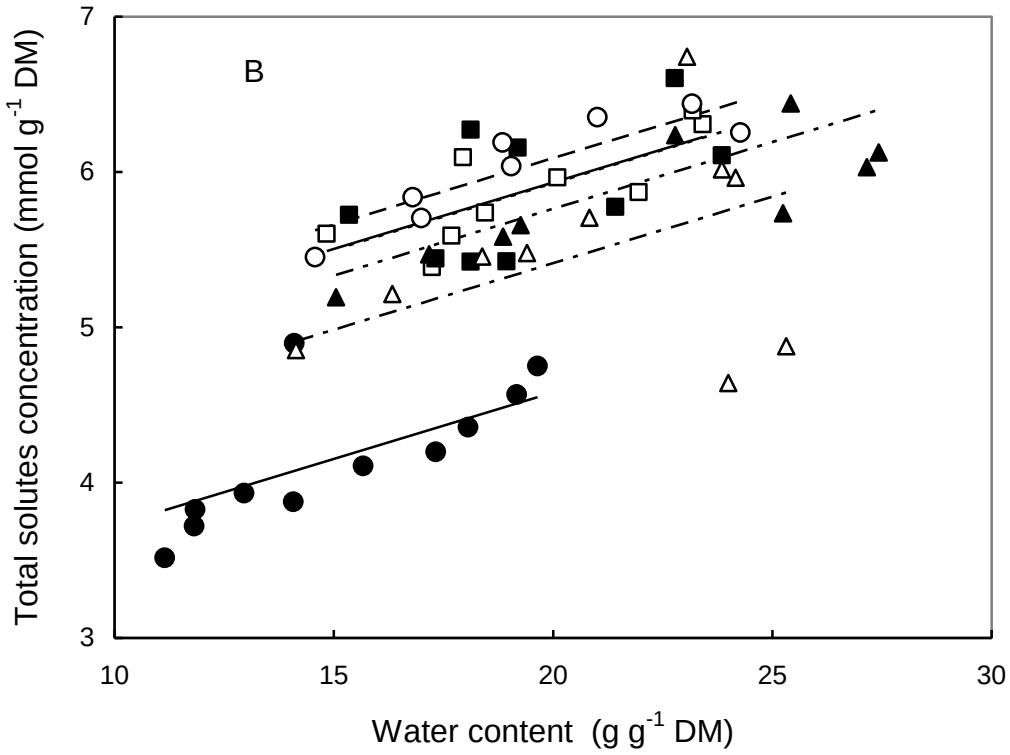

B
Normalised residual solutes concentration
Normalised nitrate concentration
1:1
line
0.5
0.6
0.7
0.8
0.9
1.0
1.1
0.0
0.1
0.2
0.3
0.4
0.5

Figure 7.


A
Nitrate concentration (mmol g-1 DM)
Water content (g g-1 DM)
2.5
2.0
1.5
1.0
0.5
0.0
10
15
20
25
30


B
Total solutes concentration (mmol g-1 DM)
Water content (g g-1 DM)
7
6
5
4
3
10
15
20
25
30