RHODES UNIVERSITY

Photosynthetic and growth response of C_3 and C_4 subspecies of *Alloteropsis semialata* to nitrogen-supply.

Thesis

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Torches are made to light, jewels to wear,
Dainties to taste, fresh beauty the use,
Herbs for their smell, and sappy plants to bear;
Things growing to themselves are growth's abuse:
Seeds spring from seeds and beauty breedeth beauty;
Thou wast begot; to get is thy duty.

Upon the earth's increase why shouldst thou feed,
Unless the earth with thy increase be fed?
By law of nature thou art bound to breed,
That thine may live when thyself art dead;
And so, in spite of death, thou dost survive,
In that thy likeness still is left alive.

From: Venus and Adonis

(By William Shakespeare)

ABSTRACT

The greater photosynthetic nitrogen use efficiency (PNUE) of C_4 compared with C_3 plants may explain the relative success of C_4 grasses in nutrient poor environments. This study compared the responses in photosynthetic parameters, leaf nitrogen and biomass allocation between the C_3 and C_4 subspecies of *Alloteropsis semialata* supplied soil nitrogen at three levels. Photosynthesis was assessed by means of CO_2 response curves and the leaf nitrogen content assayed. Plants were destructively harvested, leaf areas determined and the dry biomass of functional plant components was measured. Results confirmed that the higher PNUE of C_4 plants allowed them to accumulate more biomass than C_3 plants at the high nitrogen level, despite smaller leaf areas. The greater productivity of C_4 plants enabled them to invest more in storage and sexual reproduction than in leaves when compared to the C_3 plants. In contrast the C_3 plants invested biomass in less efficient and more nitrogen demanding leaves and bigger root systems. PNUE and photosynthetic rates were not significantly affected by nitrogen-limitation in either subspecies and the major response was a decrease in biomass accumulation and an increase in biomass allocation to roots. This altered root to shoot ratio was accompanied by a lowered allocation to sexual reproduction in the C_4 subspecies, but an unaltered allocation to leaves, while in the C_3 subspecies there was a decrease in leaf allocation. In a further experiment, the C_4 subspecies was supplied three levels of nitrogen provided as nitrate, or alternatively as ammonium plus nitrate, and leaves were excised to within 5 cm of the ground at the start of treatment. Prior to flowering, photosynthesis was assessed by means of CO_2 response curves and the plants were destructively harvested. Leaf areas and the dry biomass of functional plant components were determined, and at levels of nitrogen supply higher than those found in savanna soils the rate of photosynthesis was increased. Leaf re-growth was reduced by severe nitrogen limitation and co-provision of nitrate and ammonium had no significant effect other than increased tillering. Both subspecies of *Alloteropsis semialata* are adapted to nutrient poor environments and maintain photosynthetic rates by reducing leaf area. The C_4 subspecies is likely to show greater resilience in disturbance-prone environments by exploiting its higher PNUE to allocate greater resources to storage and sexual reproduction, while the C_3 subspecies is usually found in environments with closed canopies which favour vegetative growth, and allocate greater resources to leaves and roots.

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CHAPTER 1

GENERAL INTRODUCTION

It has been estimated that C_4 photosynthesis contributes about 21% of global primary productivity (Lloyd and Farquhar, 1994). The significance of C_4 grasses is undeniable; their evolution enhanced productivity in hot, frequently dry, nutrient poor tropical and subtropical regions (Sage, 1999), and they account for 30% of global cereal production (Brown, 1999; Osborne and Beerling 2006). Most C_4 plants are grasses (Linder and Rundell, 2005) and approximately half of the 10,000 known grass species have the C_4 photosynthetic pathway with C_4 grasses and sedges dominating nearly all grasslands in the tropics, subtropics and warm temperate zones. They also form a major biotic component of arid landscapes in these areas (Sage, 2004).

C_3 photosynthesis and the evolution of the C_4 syndrome

To fully understand C_4 photosynthesis one first has to understand how C_3 photosynthesis takes place and the inefficiencies implicit in the C_3 pathway at current ambient CO_2 levels. In C_3 plants the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) in the first step of the photosynthetic carbon reduction (PCR) cycle to form 3-phosphoglycerate (PGA), it also catalyses the oxygenation of RuBP which leads to the formation of PGA and 2-phosphoglycolate (PGly) in a competing reaction. PGly is used to generate CO_2 in the photorespiratory (or the photosynthetic carbon oxidation - PCO) cycle. Collectively, the oxygenation of RuBP and the metabolism of PGly is called photorespiration. Carbon can be exported from the PCR cycle to produce carbohydrates like starch and sucrose which leads to an increase in biomass. PGA is also used to regenerate RuBP in a process involving many enzyme- mediated steps. The regeneration of RuBP requires energy in the form of adenine triphosphate (ATP) and reduced nicotinomide adenine dinucleotide phosphate (NADPH) which are produced by the electron transport system in the thylakoids

of the chloroplasts. Participating in the electron transport system there are three discrete multi-protein complexes:

1. The photosytem II complex (PS II-LHC).
2. The cytochrome *bf* complex.
3. The photosytem I complex (PS I-LHC).

These complexes are linked by mobile pools of plastoquinone and plastocyanine (von Caemmerer, 2000).

The oxygenation of RuBP producing PGly is considered a wasteful side reaction of rubisco: it uses active sites that would otherwise be used in carboxylation, it consumes RuBP, and releases previously fixed CO_2 and NH_3. Oxygenation can therefore be considered a design flaw in C_3 photosynthesis that reduces the performance of rubisco under a specific set of conditions (Sage, 1999; Griffiths, 2006). Rubisco probably evolved 3 billion years ago when the CO_2 content of the atmosphere was many times greater than it is now and the O_2 content was insignificant, and in such an atmosphere oxygenation would have been inconsequential (Sage, 1999; Zhu *et al.* 2004). CO_2 proxy estimates and correlations with temperatures indicate that atmospheric CO_2 partial pressures of over 4 000 ppm occurred in the Ordovician period when the first land plants evolved, dropping to levels of below 500 ppm during late-Devonian and Carboniferous glacial periods (Royer, 2005). In the Devonian Period (~375 million years ago) the evolution of seed bearing plants, along with the advent of arborescence and the appearance of multi-storied forests, lead to the development of more extensive and deeper weathered soils which resulted in the substantial reduction in atmospheric CO_2 partial pressures (Algeo and Scheckler, 1998). Atmospheric CO_2 levels were subsequently raised to between 1 000 and 3 000 ppm in the Mesozoic and then declined to present day levels, although CO_2 levels today are probably higher than they have been for the last 25 million years (Royer, 2005). Photorespiration is brought about by an increase in the ratio of O_2 to CO_2 (Zhu *et al.*, 2004), and rubisco oxygenase activity only becomes significant when the soluble O_2 concentration exceeds a level that is ten times greater than that of the CO_2 concentration (Sage, 2004). This arises when the atmospheric partial pressure of O_2 is 100 times that of CO_2 at a temperature of 30°C, due to the different solubility of O_2 and CO_2. Furthermore, increasing temperatures

result in greater rates of photorespiration because of the greater solubility of O_2 than CO_2 at higher temperatures, and the reduction of rubisco specificity for CO_2. In the current atmosphere, photorespiration inhibits photosynthesis by over 30% when temperatures exceed 30°C (Sage 2004).

It is widely held that it was the declining atmospheric CO_2 concentration of the Late Miocene that was the major contributing factor to bring about the expansion of C_4 grasslands (Ehleringer *et al.*, 1997; Kellogg, 1999; Sage, 2004; Osborne and Beerling, 2006). However, Osborne and Beerling (2006) point out that recent palaeo-CO_2 reconstructions indicate that CO_2 concentrations were low for at least 15 million years before the Late Miocene, and the declining CO_2 concentration was not the direct trigger for the expansion of C_4 plants. It is more likely that environmental and biotic factors promoted the dominance of C_4 plants in the Late Miocene, although the C_4 physiology is an adaptation to low CO_2 concentrations and the declining CO_2 levels of the Oligocene may have selected for the first C_4 plants (Keeley and Rundel, 2003; Osborne and Beerling, 2006).

C_4 photosynthesis

C_4 photosynthesis overcomes the problems associated with photorespiration by concentrating CO_2 in those cells containing rubisco and lowering oxygenase activity. The C_4 mechanism involves the carboxylation of phosphoenolpyruvate (PEP) and the shuttling of four- and three-carbon acids between two specialized compartments – the mesophyll tissue, and bundle sheath cells (or the functionally similar mestome sheath) which surround the vascular tissue and in which rubisco is restricted. As a result, the intercellular CO_2 concentrations in the mesophyll of 100 – 200 μmol mol^{-1} rises in the bundle sheath to between 1 000 and 3 000 μmol mol^{-1} (Sage 1999) which has been shown experimentally by Kanai and Edwards (1999), thus elevating the CO_2 concentration up to 10-fold above air levels (von Caemmerer and Furbank, 2003). These CO_2 concentrations in the bundle sheath ensure that rubisco functions at saturating levels of CO_2 and photorespiration is reduced to a minimum.

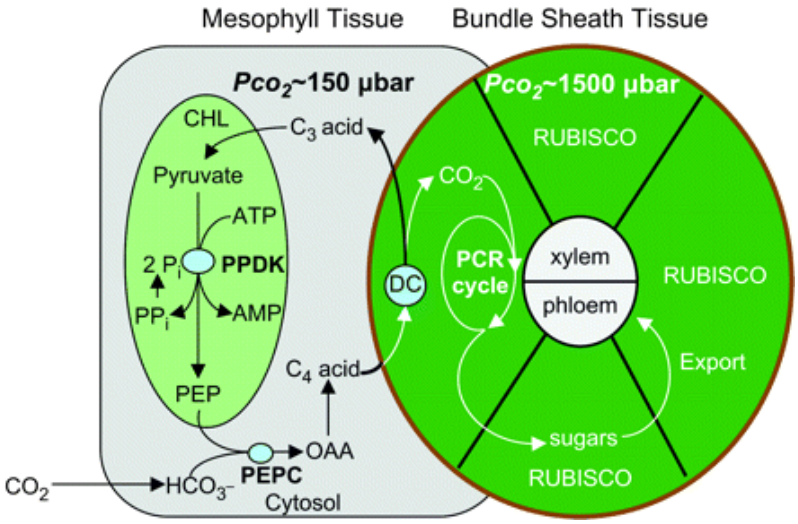

Fig. 1.1: A schematic diagram of the generalized C_4 photosynthetic pathway showing the coupling of the C_4 cycle that originates in the mesophyll and the C_3 cycle in the bundle sheath which is dependent on CO_2 released during decarboxylation. Arrows indicate the path of molecular diffusion. *Abbreviations*: decarboxylating enzyme (DC), PEP carboxylase (PEPC), photosynthetic carbon reduction (PCR), pyruvate phosphate dikinase (PPDK), oxaloacetic acid (OAA). (After Sage and McKown, 2006)

C_4 photosynthesis evolved independently at least 31 times (Kellogg, 1999; Sage 2004), and is not a single metabolic pathway. In most C_4 plants it has been found to require the specialized organization of leaf cells around the vascular bundles known as 'Kranz anatomy' in order that the functioning of the mesophyll cells can be coordinated with that of the bundle-sheath cells. The enzyme phosphoenolpyruvate carboxylase (PEP carboxylase) and other existing enzymes in the cytosol of the mesophyll cells form four-carbon acids by fixing inorganic CO_2 which then diffuse into the bundle-sheath cells where they are decarboxylated. Chloroplasts containing rubisco in the bundle sheath cells complete the C_3 photosynthetic pathway. The walls of bundle sheath cells are relatively impervious to the diffusion of gases, and as a result the C_3 cycle is almost entirely dependent on the four-carbon acid decarboxylation for CO_2. The decarboxylation of the four-carbon acid results in the production of a three-carbon acid which diffuses back into the mesophyll where PEP carboxylase is located; if necessary the three-carbon acid is converted to pyruvate and then phosphorylated to regenerate PEP. No new enzymes are required for the C_4 mechanism to take place, and all those involved have important roles in

C_3 plants. For example, PEP carboxylase is important in stomatal function, pH balance, nitrogen assimilation, carbohydrate metabolism and osmotic regulation (Sage, 1999).

Since C_4 photosynthesis has polyphyletic origins, it is not surprising that the only enzymatic step common to all versions of the pathway is the initial carboxylation by PEP carboxylase to form oxaloacetic acid (OAA). Three decarboxylation enzymes have been isolated, and it is the relative abundance of these enzymes that is used in the identification of three subtypes. These enzymes are: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PCK). If NADP-ME is used, OAA is converted to malate which then diffuses into the bundle sheath and is decarboxylated to pyruvate which diffuses back into the mesophyll cells where phosphorylation yields PEP. If NAD-ME is used, OAA is transaminated to aspartate which diffuses into the bundle sheath. Pyruvate results from decarboxylation which is transaminated to alanine and diffuses back to the mesophyll where it is converted back into pyruvate to be phosphorylated to PEP. Plants containing PCK form PEP during decarboxylation which returns directly to the mesophyll where it undergoes carboxylation by PEP carboxylase (Sage, 2004). However, von Caemmerer and Furbank (2003) point out that the classification of species into the three subtypes is not as straight-forward as has been suggested, and that particularly in the NADP-ME subtypes, there is some variability in the C_4 acids and decarboxylase enzymes that are used.

When C_4 plants are compared to ecologically similar C_3 species, the C_4 species will exhibit higher rates of photosynthesis at low CO_2 and high temperatures, but they will also use light, water and nitrogen more efficiently (Sage, 1999). The greater efficiency of nitrogen use arises from the reduced requirement for the enzyme rubisco, although this will be partially offset by the nitrogen requirements for the decarboxylase enzymes.

The importance of nitrogen in photosynthesis and plant growth

Field and Mooney (In: Givnish, 1986) suggest that worldwide, nitrogen is considered one of the mineral nutrients most limiting to plant growth. Even though it is the most abundant element in the atmosphere, it can only become available to plants as a result of the recycling of organic forms of the element and is subject to either leaching from the soil or

conversion into unavailable atmospheric nitrogen by denitrifying bacteria. Nitrogen is a limiting resource in many ecosystems, and from an evolutionary perspective, the problem of nitrogen limitation has two components:

- How much nitrogen does an individual plant require?
- How should that plant allocate a given nitrogen pool between reproduction, leaves, roots and stems to achieve maximum fitness?

The relationship between photosynthetic rate and leaf nitrogen has been well established; Bolton and Brown (1980) showed a linear increase in photosynthetic rates with increasing leaf nitrogen in both C_3 and C_4 wild grass species. They suggest that the enhanced photosynthetic rates were due to increased levels of rubisco in C_3 grasses which they found constitute between 30% and 50% of soluble leaf protein, while in C_4 grasses high levels of leaf nitrogen were probably due to increases of both rubisco and the CO_2 concentrating enzymes of the C_4 cycle. Rubisco constituted a smaller portion of the leaf protein than in the C_3 grass species studied. Further studies by Sage and Pearcy (1987) have shown that at identical leaf nitrogen levels (determined as organic nitrogen per unit area), C_4 species have higher photosynthetic rates than C_3 species, although at any given applied nitrogen level the C_3 species had a higher leaf nitrogen than the C_4 species. They showed that C_3 species can equal the photosynthetic rates of C_4 species with a greater allocation of nitrogen to the leaves. This results in C_4 species having a greater photosynthetic nitrogen use efficiency (PNUE - net rate of leaf CO_2 assimilation per unit leaf N), with a greater nitrogen availability for allocation to leaf and root production, giving C_4 plants an advantage in terms of growth and competition. However, these conclusions were drawn from plants adapted to high nutrient availability where photosynthetic rates respond strongly to increases in nitrogen levels.

In C_3 species, rubisco can account for up to 30% of the total leaf nitrogen (Evans, 1989; Long, 1999). At a CO_2 concentration of 10-100 times that found in C_3 species at the site of the rubisco activity, C_4 species would only require between 13% and 20% of the amount of the rubisco in a C_3 leaf to achieve the same photosynthetic rates at a temperature of 30°C. In addition to the benefit of lower rubisco requirements in C_4 species due to the high CO_2 concentration at the site of rubisco activity, the rubisco of the C_4 plants has a higher catalytic turnover rate (k_{cat}) than that of C_3 species (Long, 1999; Sage, 2002; Ghannoum *et*

al. 2005). The specificity of rubisco for CO_2 decreases as temperatures increase and this can also lead to greater differences in nitrogen use efficiency in C_4 species relative to C_3 species at high temperature. Selection has favoured rubisco with greater specificity for CO_2, but a reduced catalytic turnover rate in C_3 plants, while in C_4 plants the rubisco functions at higher CO_2 concentrations and the forms of rubisco that have evolved have a lower specificity but higher catalytic turnover rates (Long, 1999; Zhu *et al.* 2004). The higher catalytic turnover rates of rubisco in C_4 plants contribute to the higher PNUE. The benefit of the lower requirement for rubisco and higher catalytic turnover rate in C_4 leaves is partially offset by the nitrogen requirement of the C_4 cycle (mainly PEP carboxylase). But because of the very high catalytic turnover rate of the PEP carboxylase, low enzyme concentrations are required and only account for 5% of leaf nitrogen (Long 1999).

In summary, C_4 species have a lower nitrogen requirement than C_3 species because of lower rubisco requirements and greater catalytic turnover rates leading to higher PNUE, and this difference in nitrogen requirement is enhanced with increasing temperatures. Furthermore, in environments where nitrogen is a limiting resource, this higher PNUE may confer a competitive advantage (Li, 1993, Long, 1999).

The greater nitrogen use efficiency of C_4 plants

The greater PNUE of C_4 plants has been demonstrated in many species, but the effect of nitrogen limitation on different species reveals a range of different responses. Vos *et al.* (2004) report that when maize (C_4) was supplied with a range of nitrogen levels, the rate of leaf appearance, the duration of leaf expansion and the number of leaves were not affected. The total leaf area increased by 30% when nitrogen supply was increased from severe limitation to non-limiting, with leaf nitrogen concentration per unit area increased three- to four-fold. In potato (C_3), leaf area varied three- to four-fold across the same range of nitrogen levels, but only small differences in leaf nitrogen concentration were found.

Hocking and Meyer (1991) carried out an investigation into the effect of nitrogen nutrition in maize (C_4) and wheat (C_3) at ambient and raised carbon dioxide levels. They found that at ambient carbon dioxide levels, the nitrogen use efficiency (NUE - defined as weight of

biomass produced per unit of nitrogen in the plant) was higher in maize than wheat at all levels of nitrogen nutrition. In wheat an increase in the supply of nitrogen decreased in NUE by 52%, while in maize it was decreased by only 34%. Carbon dioxide enrichment had no effect on the NUE in maize, but in wheat it was raised to levels similar to that of maize. Relative nitrogen accumulation rates were similar in the two species, while maize accumulated a greater proportion of its nitrogen in the roots relative to the shoots at all levels of nutrition.

Most comparisons between C_3 and C_4 plants have been made using agricultural crops or weeds and Sage and Pearcy (1987) stress the importance of comparisons being made between species with similar growth forms and from similar ecosystems. C_4 species from low nutrient environments may have a lower PNUE than C_3 species from high nutrients environments, but when species are compared from the same nutrient environments the C_4 plants have the higher PNUE.

Long (1999) points out that the exploitation of the higher NUE by C_4 species, does not simply result in better performance than C_3 species in nitrogen poor environments. He suggests two contrasting strategies could exist. The C_4 species could either:

a. Produce a greater leaf area with the same amount of nitrogen and in so doing enhance whole-plant photosynthetic rates, or
b. Produce the same leaf area and partition a greater portion of the available nitrogen to root development and therefore be able to exploit a larger volume of soil and possibly obtain more nitrogen.

A number of studies have shown the differing responses of the three subtypes of C_4 plants to different levels of nitrogen nutrition. Bowman (1991) hypothesized that NADP-ME species may have higher photosynthesis and growth rates at a given leaf nitrogen concentration than NAD-ME and PCK species, and suggested that the use of a larger number of amino acid intermediates in the C_4 pathway by NAD-ME and PCK species accounted for this. In an investigation of a number of *Panicum* species with either NADP-ME or NAD-ME pathways, Bowman (1991) found that at high nitrogen levels the NADP-ME species had higher NUE than the NAD-ME species, but at low levels of nitrogen there was no difference. Thus, he assumed that NADP-ME subtypes have a lower nitrogen requirement for a given amount of growth than the NAD-ME subtypes. Bowman

(1991) also found that nitrogen supply had a greater effect on biomass accumulation in NADP-ME species than NAD-ME species and accounted for this in terms of the higher NUE. However, there were no consistent differences in photosynthetic rate either between subtypes or treatments indicating that the NAD-ME species of *Panicum* had maintained photosynthetic rates by the allocation of greater amounts of their nitrogen budget to the leaves, and compensating for their lower PNUE. These findings could not be confirmed by Taub and Lerdau (2000) who found substantial interspecific variation in PNUE in three species of NAD-ME and three species of NADP-ME subtypes, with only *Eleusine indica* conforming to a low PNUE. In a subsequent study by Ghannoum *et al.* (2005) where 13 NAD-ME and 14 NADP-ME C_4 grasses were grown under adequate and/or deficient nitrogen supplies, it was found that average PNUE and NUE were significantly greater in NADP-ME species than in NAD-ME species. Photosynthetic rates were similar in the two subtypes and this was achieved by NADP-ME grasses having less leaf nitrogen and rubisco having a higher catalytic turnover rate (k_{cat}).

The ecological significance of the greater PNUE of C_4 grass species has only been studied to a limited degree (Sage and Kubien, 2003). It has been found that in temperate grasslands of North America, the greater PNUE of C_4 grasses allow them to dominate in areas where soils are low in available nitrogen and where they become superior competitors. When nitrogen enrichment takes place, the advantage in PNUE is offset, and C_3 species can match the photosynthetic potential of the C_4 species resulting in these grasslands being dominated by C_3 species. According to Sage and Kubien (2003), in tropical soils there is a reduction in nitrogen availability following the establishment of a C_4-dominated sward. This is partially due to the high C:N ratios of C_4 grasses compared with C_3 grasses, which results in reduced decomposition rates and slower nitrogen mineralization. Other factors include the reduction of microbial activity since these soils are typically acidic with low moisture levels (Lewis, 1986; Miller and Cramer, 2004) and it is suggested that root exudates from many grasses can also reduced microbial activity, as a result available nitrogen is predominantly in the form of ammonium ions (Bate and Gunton, 1982; Lewis, 1986). Increased frequency of fires leads to the volatilization of nitrogen reducing the availability nitrogen, which is then likely to increase the competitiveness of the C_4 grasses and the reduced likelihood of woody C_3 plants and grasses becoming established (Sage and Kubien, 2003). However, Wand *et al.* (2001) point out that African grasses have been poorly studied in the past, and

these grasses may show responses that are unique, particularly those that originate in areas of low nutrient availability.

The efficient utilization of nitrogen is only one of the important factors to have influenced the evolution of C_4-dominated tropical savannas and temperate grasslands, and may have been particularly important in the African context. Knapp and Medina (1999) suggest that the selection of the C_4 photosynthetic pathway has favoured a number of traits, which include:

a. Greater water use efficiency owing to the ability of C_4 plants to maintain photosynthetic rates at low internal carbon dioxide levels.
b. Fire tolerance, dependent on a perennial tufted growth form and a large below-ground biomass, capable of storing nutrient resources.
c. Greater rates of photosynthesis at high light intensities and the reduction of temperature-dependent photorespiration.
d. Greater efficiency in the use of nitrogen in photosynthesis.

Knapp and Medina (1999) point out that those traits which seem to have enabled C_4 grasses to dominate tropical savannas are insufficient in explaining their dominance. They suggest that although the C_4 pathway may facilitate certain aspects of survival and growth in environments with the characteristics of tropical savannas, it is their capacity to assimilate CO_2 under low atmospheric partial pressures that provides them with a competitive advantage over C_3 plants. Changing levels of ambient CO_2 and the associated global warming can thus be expected to have a considerable impact on the distribution of C_4 plants in the future.

The impact of global change on C_4 plants

The distribution of C_4 grass species is largely dependent on the minimum growing-season temperature. At a daytime temperature range of between 20°C and 28°C, C_3 dominance is replaced by C_4 dominance under current atmospheric conditions (Ehleringer *et al*., 1997), with the optimum temperature of light-saturated photosynthesis in C_4 grass species typically about 10°C higher than that of C_3 grass species (Long, 1999). It has been reported

that the photosynthesis of C_3 species is generally increased by between 20 - 50% with a doubling of atmospheric CO_2, while for C_4 species the increase is only between 0 - 25% leading to an increase in biomass accumulation, with smaller differences expected in natural settings (Ghannoum *et al.*, 2000; Sage and Kubien, 2003). In a meta-analysis of wild C_3 and C_4 grasses by Wand *et al.* (1999), it was found that the increase in biomass accumulation due to elevated CO_2 in C_3 and C_4 species was 44% and 33% respectively. Sage and Kubien (2003) suggest that the lower response by C_4 species to CO_2 enrichment can be accounted for by the relatively low CO_2 saturation point of the net rate of CO_2 fixation, but at elevated temperatures these plants can develop significant CO_2 sensitivity. The CO_2 saturation point increases with temperature and only once the operating intercellular CO_2 concentration falls below the CO_2 saturation point, will the C_4 species become sensitive. Furthermore, at elevated temperatures stomatal conductance often declines due to a lowering of the relative humidity and this reduces the intercellular CO_2 concentration. CO_2 sensitivity in C_3 species is also increased with temperature and they remain sensitive at higher ambient CO_2 levels than C_4 species (Ghannoum *et al.*, 2000; Sage and Kubien, 2003; Ainsworth and Long, 2005).

Photosynthetic acclimation or down-regulation occurs when plants are grown at high CO_2 levels for any length of time. This is a loss of sensitivity to CO_2 which is largely brought about by the production of regulatory signals in response to raised carbohydrate levels and result in the reduction of photosynthetic enzyme synthesis, principally rubisco (Ghannoum *et al.*, 2000; Sage and Kubien, 2003; Ainsworth and Long, 2005). Photosynthetic acclimation to elevated CO_2 is accentuated by low levels of available nitrogen which is associated with reduced leaf nitrogen (Ghannoum and Conroy, 1998, Ainsworth and Long, 2005). Sage and Kubien (2003) suggest that the greater PNUE of C_4 species is the key feature that may lead to these plants maintaining their competitiveness at the high ambient CO_2 levels that are predicted. In most soils, nitrogen is limiting and those plants with a greater PNUE can maintain greater productivity and avoid a situation where the nitrogen supply rate to the plant is less than the carbon supply rate from photosynthesis, resulting in an accumulation of carbohydrates and reduction in photosynthetic capacity (Wand *et al.*, 1999; Ghannoum *et al.*, 2000; Sage and Kubien, 2003; Ainsworth and Long, 2005). Hence, the study of the nitrogen-response in C_3 and C_4 plants is imperative in determining the plant response to future climate change.

AIM OF THIS INVESTIGATION

The greater PNUE may have conferred a competitive advantage on C_4 grass species and this could account for the expansion of C_4 grasslands, particularly in the African context where soils are typically low in nitrogen. The competitive advantage of a species can be defined in terms of its growth potential and its ability to accumulate biomass, as well as its reproductive output. As nitrogen availability directly affects the photosynthetic parameters it is relevant to assess the nutrient effects on photosynthetic parameters and the subsequent downstream effects on growth, allocation and reproduction. It is also important to assess the significance of the form in which nitrogen is made available to the plant, either as nitrate ions, or as co-provision as ammonium and nitrate ions.

However, if generalizations are to have any level of validity the choice of species to be investigated is of significance. According to Westoby (1999) the criteria on which species might be compared fall under three categories: (a) habitat, (b) ecological strategy or (c) phylogeny. He proposed that phylogenetically related species share recent common ancestry and are therefore more likely to have similar traits; furthermore phylogenetic niche conservatism leads to similar trait combinations because they have been subjected to similar forces of natural selection.

In this investigation, the response to different levels of nitrogen supplied to *Alloteropsis semialata* (R. Br.) Hitchc. was investigated. *A. semialata* is the only known species that has two subspecies one of which has a C_4 photosynthetic pathway – *A. semialata* (R. Br.) Hitchc. subsp. *semialata*, and the other a C_3 pathway – *A. semialata* (R. Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell. This would mean that any study of its responses would eliminate the likelihood that differences in phylogeny may have, and avoid the problems associated with differing growth capacity. The two subspecies also share similar environmental preferences having been collected growing naturally in close proximity to each other by Barrett *et al.* (1983) and Liebenberg and Fossey (2001); consequently the major factor that is likely to affect the response of these two subspecies to different levels of nitrogen supply will be their respective photosynthetic pathways.

This investigation attempts to determine whether a C_4 subspecies of *Alloteropsis* (*A. semialata* (R. Br.) Hitchc. subsp. *semialata*) can exploit their PNUE to produce:

1. A greater leaf biomass than C_3 subspecies so as to increase their competition for light, and with their higher photosynthetic rates achieve greater whole plant photosynthetic productivity.

2. A similar leaf biomass to C_3 subspecies and invest more resources in root biomass favouring competition for soil resources.

3. A similar leaf biomass and root biomass and invest more resources in either underground storage favouring survival in environments subject to disturbance (like fire, frost or herbivory), or reproduction thus potentially enhancing the C_4 subspecies' fitness.

However, these predictions represent the extremes of a continuum with intermediate responses being likely. These aims are addressed in the chapters of this thesis as follows: The impact of the different levels of nitrogen supply on the PNUE and photosynthesis on the two subspecies of *A. semialata* is discussed in Chapter 2, and the downstream effects on growth, allocation and reproduction, in Chapter 3. The difference in response to the co-provision of ammonium and nitrate to that of nitrate alone by the C_4 subspecies is discussed in Chapter 4, and an overall synthesis in Chapter 5.

Introduction to *Alloteropsis semialata*

Alloteropsis semialata (R. Br.) Hitchc. subsp. *semialata* is widely distributed through the eastern and northern parts of South Africa extending through Africa to India, Southeast Asia and Australia, while *A. semialata* (R. Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell occurs in southern Africa (Ueno and Sentoku, 2006). *A. semialata* is a non-dominant grass of the mesic 'sourveld' (Ellis 1974), and where the vegetation is typically savanna (Scholes and Walker 1993). Ellis (1974) reports that the C_4 subspecies has been collected from lower altitudes and higher temperatures where rainfall was 775 mm or less, while the C_3 subspecies was restricted to areas with higher altitude and lower temperatures in the north, but reaches the coast near Pondoland and southwards to East London. In the Grahamstown district only the C_3 subspecies is endemic.

Fig. 1.2: Tussocks of *A. semialata* (R. Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell growing at Jameson Dam, near Grahamstown. June 2006.

Fig. 1.3: Tillers of the C_3 subspecies (left) and the C_4 subspecies (right) of *A. semialata*, with the C_4 subspecies having flowered (Photograph by B.S.Ripley, 2006).

According to Gibbs Russell *et al.* (1991), the C_4 subspecies of *A. semialata*, found in Southern Africa, is of the NADP-ME biochemical subtype, which would be consistent with the notion that this subspecies is characteristic of regions with wetter climates and nutrient-

poor soils (Knapp and Medina, 1999) and it has been suggested that the C_4 subspecies (*A. semialata* (R. Br.) Hitchc. subsp. *semialata*) found in Australia has the PCK biochemical subtype (Gibbs Russell *et al.*, 1991). Liebenberg and Fossey (2001) found the C_3 subspecies of *A. semialata* to be diploid while the C_4 was polyploid, and Ripley *et al.* (2007) characterized the photosynthetic pathways of the *semialata* subspecies using plants collected from semi-natural open grasslands surrounding Middleburg (Mpumalanga, South Africa) and the *eckloniana* subspecies from plants collected outside Grahamstown. Three independent tests were performed; photosynthetic carboxylation efficiency (CE), CO_2 compensation point (Γ) and ^{13}C isotope discrimination, all of which were in the ranges typical of C_4 and C_3 plants (Table 1.1). This shows that these plants were from populations that were true C_3 and C_4 plants. However, this is an important clarification as Ueno and Sentoku (2006) questioned the photosynthetic type of *A. semialata semialata* based on enzymatic activities.

Table 1.1: Photosynthetic characterization of *A. semialata* subspecies from Ripley *et al.* (2007) are shown for Γ, CE and $\delta^{13}C$ isotopic ratios of well watered C_3 and C_4 subspecies. Italicized ranges indicate typical values reported for C_3 and C_4 species.

Subspecies	**CO_2 Compensation Point** (μmol mol^{-1})	**Carboxylation Efficiency** (mmol m^{-2} s^{-1})	**$\delta^{13}C$ isotope ratio** ($^{0}/_{00}$)
C_4	3.7 $\pm$ 2.7 *(<10)*	237 $\pm$ 56 *(210 to 480)*	-11.6 $\pm$ 0.5 *(-10 to -15)*
C_3	42.5 $\pm$ 6.5 *(41 to 45)*	116 $\pm$ 34 *(55 to 130)*	-26.0 $\pm$ 0.5 *(-24 to -30)*

CHAPTER 2

NUTRIENT SUPPLY AND PHOTOSYNTHESIS

INTRODUCTION

The primary physiological effect of the C_4 syndrome is the elevation of CO_2 concentrations at the site of rubisco activity in the chloroplasts of the bundle sheath cells. This has two profound effects on the process of photosynthesis. Firstly, it enables the rubisco to operate at a near maximum rate and secondly it minimizes the competitive inhibition of the oxygenase activity of rubisco resulting in practically eliminating the PCO cycle with the corresponding loss of energy associated with photorespiration. This allows C_4 species to achieve potentially higher rates of photosynthesis than C_3 species at temperatures above 25°C (Ehleringer *et al.* 1997), even though the decarboxylate cycle is an energy requiring process and C_3 species characteristically have significantly higher rubisco concentrations.

As mentioned in Chapter 1, there have been a number of comparative studies showing the effect of different levels of soil nitrogen supply on photosynthesis in C_3 and C_4 species in which a striking response of photosynthetic rate to nitrogen supply level was observed. The response leveled off in some species while in other species, the response was linear with increasing leaf nitrogen concentrations, accounted for by the large portion of leaf nitrogen invested in rubisco. Bolton and Brown (1980) found that the rate of photosynthesis for *Panicum maximum* (C_4) increased four-fold with an increase in leaf nitrogen from 1% to the maximum value of 4.3% while in *Festuca arundinacea* (C_3) at the highest level of nitrogen supply, leaf nitrogen was slightly higher, but photosynthetic rates were half that of *P. maximum*. Vos *et al.* (2005) showed a strong relationship between photosynthetic rate and leaf nitrogen expressed per unit area, regardless of whether leaf nitrogen differences were a result of soil nitrogen supply or leaf age. In an investigation into the ecologically similar agricultural weeds *Chenopodium album* (C_3) and *Amaranthus retroflexus* (C_4), Sage and Pearcy (1987) found that for a given applied nitrogen level, the C_3 species had a greater leaf nitrogen per unit area such that the light saturated rate of

photosynthesis for the two species were similar. They showed that C_4 photosynthesis does not automatically enable C_4 plants to have greater rates of photosynthesis than C_3 plants. Greater allocation of nitrogen to the leaves of C_3 plants allowed similar photosynthetic rates and compensated for the lower PNUE in relation to the C_4 plants. However, Sage and Pearcy (1987) suggest that this greater cost of photosynthesis in C_3 plants could limit allocation to other plant processes such as root or leaf production. Sage *et al.* (1987) found that *C. album* invested 1.3 to 2.4 as much nitrogen in rubisco as *A. retroflexus* at an equal leaf nitrogen concentration.

Li (1993) investigated the photosynthetic response to different levels of nitrogen supply in closely related C_3 and C_4 *Cyperus* species which were morphologically and ecologically similar. It was found that with increased nitrogen supply there was a significant increase in leaf nitrogen, but the leaf nitrogen concentrations were not different between C_3 and C_4 species. As leaf nitrogen increased, so did the rates of photosynthesis, but for any given leaf nitrogen concentration, the rate of photosynthesis of the C_4 *Cyperus* species was generally higher than that for the C_3 species. Thus, the C_4 species had a greater PNUE than the C_3 species, and this was due to the photosynthetic rates rather than to leaf nitrogen concentrations.

Bowler and Press (1996) have also shown that the leaf nitrogen concentration of fast and slow growing grasses increased with increased levels of nitrogen supply. Increased leaf nitrogen and carboxylation efficiency, and the rate of CO_2 saturated photosynthesis (A_{max}), in the fast growing species showed the greater response to high levels of nitrogen supply than did the slow-growing species. Ghannoum and Conroy (1998) investigated the effect of nitrogen deficiency and CO_2 enrichment on the growth response of wild C_3 and C_4 *Panicum* grasses. It was found that leaf nitrogen concentration increased with an increased level of nitrogen supply, and that high nitrogen increased the rate of CO_2 assimilation to a greater degree in C_4 species than C_3 species; the rate of CO_2 assimilation in *Panicum coloratum* (C_4) was nearly twice that of *Panicum laxum* (C_3) at the high level of nitrogen supply. In *Panicum antidotale*, a C_4 species, nitrogen stress reduced the carboxylation efficiency and CO_2 saturated assimilation rate (Ghannoum and Conroy, 1998), and it is suggested that in the C_4 species, nitrogen stress reduces rubisco activity more than that of PEP carboxylase resulting in the delivery of CO_2 to the bundle-sheath exceeding the rate of fixation by rubisco.

The conversion of intercepted light into biomass by means of photosynthesis depends largely on the components of the chloroplasts which in C_3 plants contain up to 75% of the organic nitrogen found in the leaf (Field and Mooney, 1986; Poorter and Evans, 1998). Most of this organic nitrogen is contained in the light-harvesting chlorophyll-protein complexes (LHC), the electron transport and (nicotinomide adenine dinucleotide phosphate) $NADP^+$-reducing components of the thylakoids, and the CO_2 assimilating enzyme rubisco in addition to the other enzymes required for CO_2 assimilation in the stroma (Lawlor, 2002). In C_4 plants there are the additional enzymes of the C_4 cycle, notably PEP carboxylase, but it is the rubisco which constitutes by far the largest amount of leaf protein, and is most likely to be compromised under conditions of nitrogen stress. Hence the fundamental differences in the physiology of C_3 and C_4 photosynthesis will place differing demands on these nitrogen containing components, so that the response to different levels of nitrogen supply is expected to be different.

The measurement of CO_2 assimilation

Because of the high concentration of rubisco in the chloroplasts of C_3 plants, the rate at which carboxylation catalyzed by rubisco takes place could be described by either its RuBP-saturated or RuBP-limited rate. RuBP supply is linked to the regeneration of RuBP, which in turn is linked to the supply of ATP and NADPH by the electron transport chain. The RuBP-saturated rate of CO_2 assimilation (A) is dependent on the maximum rubisco activity (V_{cmax}). This rate of assimilation is called the rubisco-limited rate, and is dependent on the amount of rubisco protein present in the chloroplast and its catalytic turnover rate. At high levels of intercellular CO_2 partial pressure (C_i), A is limited by the supply of RuBP, which is dependent on the rate of RuBP regeneration. The rate of RuBP regeneration is in turn based on the rate of electron transport. At a given level of irradiance, electron transport is referred to as 'J' and the maximum electron transport rate is the J_{max}, which depends on the amount of thylakoid protein present. A is dependent on the regeneration of RuBP and the electron transport chain, and is called the RuBP regeneration-limited or electron-transport-limited rate (von Caemmerer, 2000).

In C_4 plants, C_4 acids are generated by the fixation of CO_2 by PEP carboxylase in the mesophyll which then diffuses into the bundle-sheath cells where they are decarboxylated.

The released CO_2 is used in the carboxylation of RuBP by rubisco. At low CO_2 partial pressures the CO_2 assimilation rate has a linear relationship to the maximum PEP carboxylase activity (V_{pmax}), while at high CO_2 partial pressures the CO_2 assimilation rate is dependent on either the maximal rubisco activity (V_{cmax}), or the rate of PEP regeneration. In C_4 plants, the energy requirements for the regeneration of RuBP in the bundle-sheath are the same as in C_3 plants, although there is the additional cost of energy in the form of ATP for the regeneration of PEP from pyruvate in the mesophyll, but there is no net NADPH requirement by the C_4 cycle. In C_3 photosynthesis, RuBP regeneration can be limited by the rate of electron transport, while in C_4 photosynthesis there is the possibility that PEP regeneration may also be limited by the activity of enzymes like pyruvate phosphate dikinase (PPDK) in the C_4 cycle (von Caemmerer, 2000).

CO_2 response curves

A is affected by nutrition and CO_2 response curves have been useful in analyzing the photosynthetic capacities of plants subjected to different levels of nitrogen nutrition (von Caemmerer, 2000). CO_2 response curves are constructed when A is plotted as a function of C_i (Fig. 2.1). In C_3 plants it has been shown that different nitrogen levels bring about changes in V_{cmax} and J_{max}, while in C_4 plants V_{pmax} and V_{cmax} are affected. von Caemmerer (2000) suggests that in the CO_2 response curves of C_3 plants, there is convincing evidence to indicate that the initial slope of the curve (dA/dC_i), referred to as the apparent carboxylation efficiency (CE), closely correlates with the rubisco activity. The asymptote of the response curve which corresponds to the assimilation rate at saturated CO_2 partial pressures (A_{max}) is limited by the supply of RuBP initially, but ultimately becomes phosphate limited when it is dependent on the rate at which triose phosphate is utilized in the synthesis of starch and sucrose (von Caemmerer, 2000; Long and Bernacchi, 2003). Triose phosphate is the product of first the phosphorylation and then the reduction of PGA by ATP and NADPH. The CO_2 partial pressure at which assimilation is zero (x intercept) is referred to as the CO_2 compensation point (Γ). Γ could occur when the stomata are completely closed and represents equilibrium between CO_2 uptake in photosynthesis and its release by mitochondrial respiration and photorespiration.

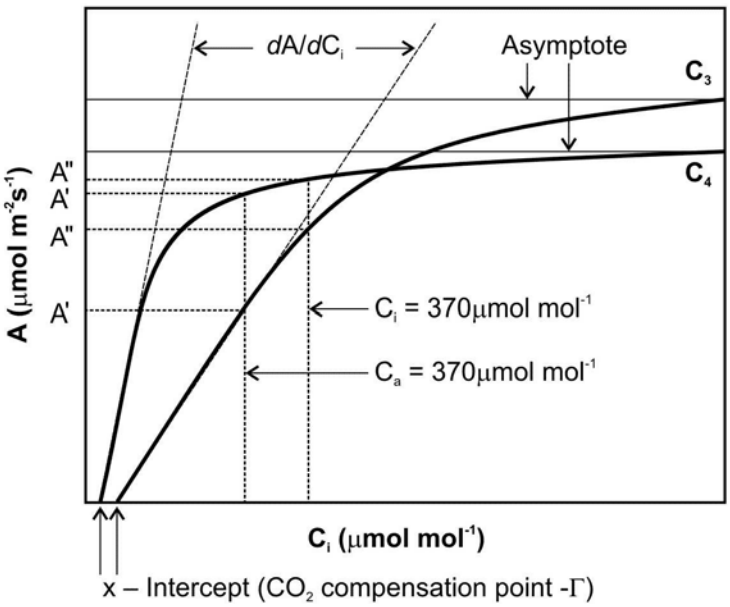

Fig. 2.1: Typical CO_2 response curves for C_3 and C_4 plants showing the initial slope of the curve (dA/dC_i) referred to as the apparent carboxylation efficiency, the asymptote which corresponds to the assimilation rate at saturated CO_2 partial pressures, and x – intercept referred to as the CO_2 compensation point. A′ is the rate of photosynthesis when the air surrounding the leaf has a CO_2 partial pressure (C_a) of 370 μmol mol^{-1}, and A″ is the rate of photosynthesis when the intercellular CO_2 partial pressure (C_i) is 370 μmol mol^{-1} – these values were used in the calculation of the stomatal limitation (see Methods of this Chapter).

In C_4 plants the CO_2 response curve saturates abruptly. The initial slope of the curve (CE) is proportional to PEP carboxylase activity and the asymptote of the response curve or A_{max} and is proportional to rubisco activity which is difficult to distinguish from the PEP regeneration limitation. When PEP carboxylase activity is very low there is a reduction in the CE because rubisco in the bundle sheath is not fully saturated with CO_2. PEP regeneration at high irradiance can be limited by the enzymes of the C_4 cycle or by the capacity of the electron transport chain of the chloroplast. The Γ is characteristically lower than that of C_3 plants owing to limited photorespiration as a consequence of the high CO_2 partial pressures in the bundle-sheath cells.

Table 2.1: The components of C_3 and C_4 photosynthetic biochemistry assessed by the photosynthetic parameters derived from CO_2 response curves.

Photosynthetic parameter	**In C_3 plants**	**In C_4 plants**
Apparent carboxylation efficiency (CE)	Rubisco limited	PEP carboxylase limited
CO_2 saturated assimilation rate (A_{max})	RuBP regeneration limited Electron transport limited Phosphate limited	Rubisco limited PEP regeneration limitation C_4 Cycle enzyme limited Electron transport limited
CO_2 compensation point (Γ)	Mitochondrial respiration Photorespiration	Mitochondrial respiration Photorespiration

Practical implications of constructing CO_2 response curves

To provide greatest insight into the biochemistry of photosynthesis, one would ideally aim at measuring the A as a function of CO_2 partial pressure at the site of carboxylation – the chloroplast of C_3 plants and the cytoplasm of those mesophyll cells containing PEP carboxylase in C_4 plants (von Caemmerer, 2000). This would ensure that the resistance to the diffusion of CO_2 would have no effect on the assimilation rate. However, it has become common practice to calculate the CO_2 partial pressure in the substomatal cavities, as this is most practical and eliminates the most important variability. According to Long and Hällgren (1993) the stomata are considered the most significant limiting factor to the diffusion of CO_2, as stomatal conductance varies when the stomata themselves respond to CO_2. The CO_2 partial pressure in the substomatal cavities is referred to as the intercellular CO_2 partial pressure (C_i) and the rate at which CO_2 diffuses through the stomata, is the leaf diffusive conductance of CO_2. It is different from that conductance known as the stomatal conductance which is used to refer to diffusion from airspaces within the leaf to the leaf's outer surface (Long and Hällgren, 1993; Long and Bernacchi, 2003). The leaf diffusive conductance (g_l) can be calculated by measuring the flux of water vapour and atmospheric humidity, or more precisely the mesophyll-intercellular air interface, assuming that the intercellular air space is saturated. The value for water vapour flux when applied to leaf

diffusive conductance can be corrected for molecular size and loss of CO_2 resulting from the mass flow of gasses out of the leaf due to the development of a pressure gradient. Once measurements of the CO_2 concentration surrounding the leaf (C_a) and A are obtained the C_i can be calculated as follows:

$$C_i = C_a - A/g_1$$

Photosynthetic nitrogen use efficiency

In a survey of 137 plants, Field and Mooney (1986) found that A was highly correlated with leaf nitrogen concentration, and they found that this correlation applied when both parameters were based on either a leaf area or leaf dry mass. The strong relationship between A and leaf nitrogen when mass-based is positively correlated to the mass per unit leaf area or specific leaf area (SLA), and these leaf traits are linked to leaf lifespan (Reich *et al.*, 1999). This was confirmed in a survey of over 250 000 plants worldwide by Wright *et al.* (2004). Leaves with a low SLA are likely to achieve greater lifespans as they can resist physical weathering and are protected from herbivory (Poorter and Garnier, 1999; Reich *et al.*, 1999). Since low SLA is associated with greater tissue density and the allocation of greater amounts biomass to structural components rather than metabolic activities, low mass-based leaf nitrogen is associated with low SLA and results in low photosynthetic rates. There is a lack of correlation between area-based leaf nitrogen and leaf lifespan as, while the mass-based leaf nitrogen decreases with decreasing SLA, there is minimal or no change in the area-based leaf nitrogen (Reich *et al.*, 1999). SLA tends to change in response to variation in light intensity or nutrient availability during growth, and climate alters the relationships among leaf traits largely by altering SLA (Poorter and Garnier, 1999; Reich *et al.*, 1999; Wright *et al.*, 2004). The area-based relationship between A and leaf nitrogen is functionally significant as it gives insight into resource-harvesting since both light interception and CO_2 assimilation are both intrinsically area based phenomena. Field and Mooney (1986) refer to PNUE when calculated from area-based measurements of leaf nitrogen and A as the potential PNUE and it is an index of performance under defined conditions that allows direct comparison among species. They define A as the measured photosynthetic rate under saturating light intensity and optimal temperature, relative humidity and the CO_2 concentration typical of normal air, and by

expressing PNUE as the ratio of A to leaf nitrogen (provided that they are both expressed on an area basis), one eliminates the variable of SLA.

The key research questions

The objective of this investigation is to determine whether the availability of nitrogen can have an effect on primary productivity in the C_3 and C_4 subspecies of *Alloteropsis semialata*. The specific questions are:

1. Is the rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} and PNUE of the C_4 subspecies greater than that of the C_3 subspecies, and will the relative C_3 and C_4 rates increase with increased availability of nitrogen?

2. What effect will the availability of nitrogen have on the photosynthetic parameters of: (a) CE (b) A_{max}, and (c) the Γ, and how will these differ between C_3 and C_4 subspecies?

METHODS

Growth and treatment of plants

A collection of individually potted plants were established from genets of the C_4 subspecies (*A. semialata* (R. Br.) Hitchc. subsp. *semialata*) and the C_3 subspecies (*A. semialata* (R. Br.) Hitchc. subsp. *eckloniana* (Nees) Gibbs Russell). These genets were collected in February 2004 from grasslands surrounding Middelburg (Mpumalanga Province) and Grahamstown (Eastern Cape Province) respectively. Tillers of each subspecies were obtained from these potted plants, the roots of which were thoroughly washed with tap water to remove any adhering soil. The tillers were dried with absorbent

paper, moribund biomass removed, weighed, and planted in 20 L pots filled with washed river sand. Three tillers of each subspecies were planted in each pot and they were planted on the 13th to the 18th of May 2005. The pots were established and maintained in a clear polythene tunnel where average day/night temperatures were 31°C – 17°C, and humidity ranged between 25% and 64%.

Table 2.2: Average daily temperatures in the tunnel measured in the shade during the period in which plants were established and maintained.

Growth period	0 – 4 weeks	4 – 8 weeks	8 – 12 weeks	12 – 16 weeks	16 – 20 weeks	20 – 23 weeks
Daily temp. (°C)	23.4	21.9	24.7	26.3	28.1	31.9

Pots receiving the same treatments were randomly placed in 6 rows within the tunnel with the C_4 subspecies alternating with the C_3 subspecies (Fig. 2.2), and were regularly moved about to prevent an edge effect from occurring.

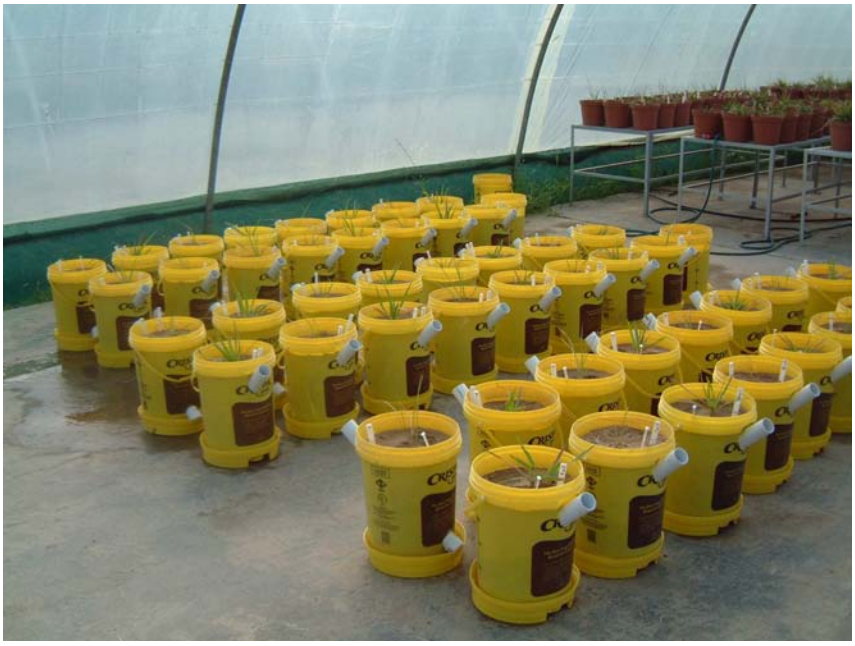

Fig. 2.2: Potted *A. semialata* placed in 6 rows within the tunnel with the C_4 subspecies alternating with the C_3 subspecies.

The pots received one of three levels of nitrogen treatment and the first treatment was applied on the 8th of July 2005. There were 6 – 8 replicates of each treatment. The pots

were watered twice weekly with Long Ashton's solution (Hewitt, 1966) and any seepage from the pots was collected in trays below the pots and reapplied. Three different levels of nitrogen were used with nitrogen in the form of nitrate (one third potassium nitrate and two thirds calcium nitrate) which was calculated on the assumption that the plants would use all nitrogen available:

1. High nitrogen: equivalent to 7 g N m^{-2} yr^{-1}; the nitrogen levels found in nutrient rich soils at Nylsvlei.
2. Medium nitrogen: equivalent to 3.5 g N m^{-2} yr^{-1}; the nitrogen levels found in nutrient poor soils at Nylsvlei.
3. Low or zero nitrogen: No additional nitrogen added.

Nylsvlei is a savanna research site, situated north of Pretoria. It has two main soil types; nutrient-poor soils which support broad-leafed savanna and nutrient-rich soils which support fine-leafed savanna. In the nutrient poor soils, net nitrogen mineralization rates of 3.5 g N m^{-2} yr^{-1} have been recorded, with approximately twice this rate in the nutrient-rich sites (Scholes *et al.*, 1997).

Nitrogen content of the sand

Prior to treatment, a 100 g sample of sand was taken from each pot at a depth of 10 cm and total nitrogen analysed (Matrocast Laboratories, Cape Town), and then again just prior to the final harvest a 100 g sample of sand was obtained. The second sample was obtained by sub-sampling cores extracted from diagonally fitted root in-growth tubes using a barrel auger and from which all root tissue was removed by sifting (see Chapter 3 for a full description of the root in-growth tubes), and total nitrogen was analysed (Bemlab, Somerset West).

Determination of the photosynthetic parameters and PNUE

After 12 weeks (in the last week of September) gas exchange measurements were carried out. Five plants were randomly selected from each subspecies and treatment. CO_2 response (A/Ci – CO_2 assimilation vs. intercellular CO_2 concentration) curves were measured

according to Long and Bernacchi (2003) and were carried out on the mid-leaf section of the youngest fully expanded leaf using an integrated portable photosynthesis system (LI 6400-40 LI-COR, Inc., Nebraska, USA). Cuvette CO_2 concentrations ranged between 50 and 1600 μmol mol^{-1} for the C_3 subspecies and between 20 and 1600 μmol mol^{-1} for the C_4 subspecies. The cuvette block temperatures were set at 25°C as this is the C_3/C_4 crossover temperature for current ambient CO_2 concentrations (see Introduction of this Chapter), and in the 4 week period leading to measurements being taken the average daily temperature was 24.7°C. Vapour pressure deficit (VPD) was less than 2.2 kPa and the light provided by an LED light source within the cuvette was set at 2000 μmol m^{-2} s^{-1}. Care was taken at the beginning of the CO_2 response curve to ensure that stomatal conductance had stabilised and photosynthetic rate had acclimated to cuvette conditions where CO_2 concentration was set at 380 μmol mol^{-1} for at least 15 minutes. At the end of each measurement the leaf was harvested, oven-dried to constant mass at 50-60°C and ground to a homogenous powder using a ball mill (800M mixer / mill, Glen Creston, Middlesex, UK), nitrogen content was determined using a mass spectrometer (PDZ Europa 20-20, Cheshire, UK).

The response curves were then corrected for CO_2 leakage according to the method suggested by Long and Bernacchi (2003) and individually fitted using a non-linear regression by means of the program Statistica version 7 (© Statsoft, Inc., Tulsa, USA) and the monomolecular function given by Causton and Dale (1990). Each replicate yielded $r^2 > 0.98$ and fitted parameters (a, b and c) were used to calculate values of A for series of fixed C_i values for which averages were then obtained.

The photosynthetic parameters and stomatal limitation (*ls*) were calculated from the fitted CO_2 response curves using the method suggested by Long and Bernacchi (2003), averages found and results recorded as percentages. The *ls* is frequently used to quantify the limitation imposed on A by the combined conductance of the stomata and leaf boundary layer (g_l). It was calculated as the difference between A at an atmospheric or ambient CO_2 concentration - A′, and the predicted A that would be obtained if the if the mesophyll had free access to CO_2 at an ambient concentration -A″ (i.e. if $C_i = C_a$), as the percentage of A″. The potential PNUE was calculated according to Field and Mooney (1986).

Table 2.3: The monomolecular function and equations used to calculate the photosynthetic parameters, stomatal limitation and photosynthetic nitrogen use efficiency.

$$A = a\,(1 - e^{b-c.Ci})\ (\mu mol\ CO_2\ m^{-2}\ s^{-1})$$

$$CE = a\,c\,e^{b}\ (\mu mol\ CO_2\ m^{-2}\ s^{-1})$$

$$A_{max} = a\ (\mu mol\ CO_2\ m^{-2}\ s^{-1})$$

$$\Gamma = b/c\ (\text{x intercept})\ (\mu mol\ mol^{-1})$$

$$ls = \frac{(A'' - A')}{A''} \times 100\ (\%)$$

$$PNUE = A/N_{leaf}\ (\mu mol\ CO_2\ mol^{-1}\ N\ s^{-1})$$

Abbreviations: CO_2 assimilation rate (A), fitted parameters of the monomolecular equation (a, b, c and e) according to Causton and Dale (1990), intercellular CO_2 partial pressure (C_i), apparent carboxylation efficiency (CE), the CO_2 saturated rate of assimilation (A_{max}), the CO_2 compensation point (Γ), stomatal limitation (*ls*), A at an ambient CO_2 concentration of 370 μmol mol^{-1}(A′), A when C_i is equal to ambient CO_2 concentration (A″), the potential photosynthetic nitrogen used efficiency (PNUE) and the leaf nitrogen concentration measured on an area basis (N_{leaf}).

As soil nitrogen did not increase with increasing nitrogen supply (see Results), it was assumed that all the supplied nitrogen was taken up by the plants. This ignores the minor depletion of available nitrogen that was likely to have occurred because of immobilization by soil organisms (Lewis, 1986). The allocation of nitrogen to leaves for the whole plant was then calculated as a percentage of the total nitrogen taken up using leaf nitrogen analysis for leaves harvested at 12 weeks and total leaf mass (see Chapter 3 for calculation of total leaf mass). As the plants that received the zero nitrogen supply treatment must have been entirely dependent on the re-allocation of stored nitrogen reserves, having been grown from re-planted tillers, their nitrogen contents were subtracted from those of leaves from 3.5 and 7 g N m^{-2} y^{-1} treatments to account for re-allocation.

$$\text{N allocated to leaves} = \frac{\text{leaf N}}{(\text{N supplied} + \text{sand N}) - \text{N re-allocated}} \times \frac{100}{1}\ \%$$

(Where sand N is the nitrogen content of the sand before to treatment)

In an attempt to avoid the re-allocation of nitrogen from senescent leaves and under-ground storage, seeds of both subspecies were collected from established plants (180 from the C_3 subspecies and 80 from the C_4 subspecies), sterilized in a 0.3% sodium hypochorite solution for 10 minutes, washed in distilled water, and germinated on moist absorbent paper in a controlled environment. Seedlings were then transplanted into washed sand where they received the same treatment. However, those plants receiving zero nitrogen and 3.5 g N m^{-2} y^{-1} did not establish.

Statistical Analyses

All statistical comparisons were made by means of the program Statistica version 7 (© Statsoft, Inc., Tulsa, USA). All parameters, except sand nitrogen content were compared between subspecies, treatment, and the interaction between subspecies and treatment using factorial ANOVA with a Type III model for the calculation of the sum of squares as cell size was unbalanced (Quinn and Keough, 2002). Sand nitrogen content prior to treatment and after treatment was compared using one-way ANOVA. CE data were arcsine transformed as they were heteroscedastic and variance was not homogeneous. Where main effects were significant at the 95% confidence level Tukey *post-hoc* comparisons were made, and averages were given ± standard error.

RESULTS

Sand nitrogen content

There was no significant difference in the nitrogen content of the sand between treatments (Table 2.4), prior to treatments ($F_{(2,9)} = 0.2$, $p < 0.7$), or after the application of treatments ($F_{(2,15)} = 0.6$, $p < 0.5$). Treatments did increase sand nitrogen, but this was not significant. This implies that, except for minor nitrogen loses due to soil organisms; the plants took up all supplied nitrogen to a minimum sand content of *ca.* 0.25 mg N g^{-1}.

Table 2.4: The nitrogen content of the sand before treatment, and prior to the final harvest. All values are means ± standard error (n = 3 to 6).

Nitrogen supply (g N m^{-2} y^{-1})	Nitrogen content before treatment (mg N g^{-1})	Nitrogen content prior to final harvest (mg N g^{-1})
7.0	0.13 ± 0.03	0.23 ± 0.03
3.5	0.12 ± 0.03	0.28 ± 0.03
0.0	0.15 ± 0.03	0.25 ± 0.03

Photosynthetic rate and PNUE

A at an ambient CO_2 concentration of 370 μmol mol^{-1} was significantly higher in the C_4 subspecies than in the C_3 subspecies (Table 2.6) and was not affected by nitrogen supply (Fig. 2.3a). Similarly the PNUE was significantly higher in the C_4 subspecies than in the C_3 subspecies (Table 2.6), and it too was not affected by nitrogen supply (Fig. 2.3b). The average value of A for the C_4 subspecies was 20.0 ± 1.4 μmol m^{-2} s^{-1} and PNUE was 0.6 ± 0.04 μmol CO_2 mol^{-1} N s^{-1}, while the average value of A for the C_3 subspecies was 12.5 ± 1.0 μmol m^{-2} $s^{-1}$and PNUE 0.4 ± 0.05 μmol CO_2 mol^{-1} N s^{-1}.

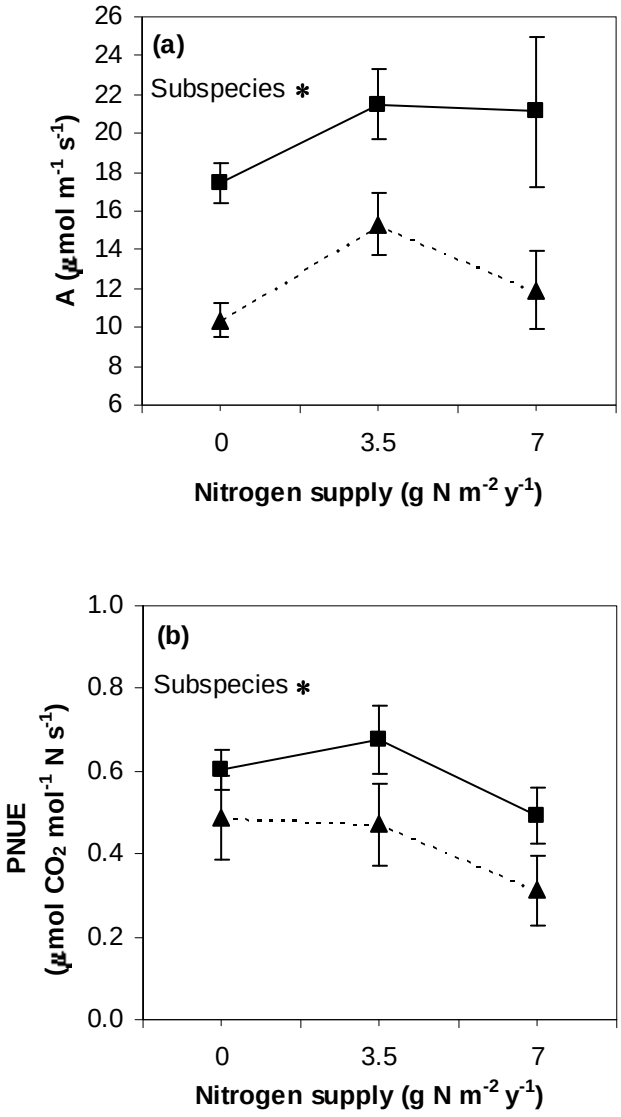

Fig. 2.3: (a) The rate of photosynthesis at an ambient CO_2 concentration of 370 µmol mol^{-1} – A and (b) the potential photosynthetic nitrogen use efficiency (PNUE) of *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors (n = 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The percentage leaf nitrogen was not significantly different for the C_4 and C_3 subspecies (Fig. 2.4a). However, the percentage nitrogen decreased linearly with a decreasing nitrogen supply and there was a significant difference between those plants receiving high and low nitrogen (Table 2.6, Fig. 2.4b). When leaf nitrogen was expressed on a leaf area basis, although the same general trend was evident, there was no significant

difference between subspecies, nor was the difference between treatments significant, which is possibly because of changes in the SLA in the C_4 subspecies, but not in the C_3 where SLA was not different across treatments (see Chapter 3). As leaf nitrogen was not different between subspecies the difference in PNUE was therefore due to the difference in A rather than leaf nitrogen concentration.

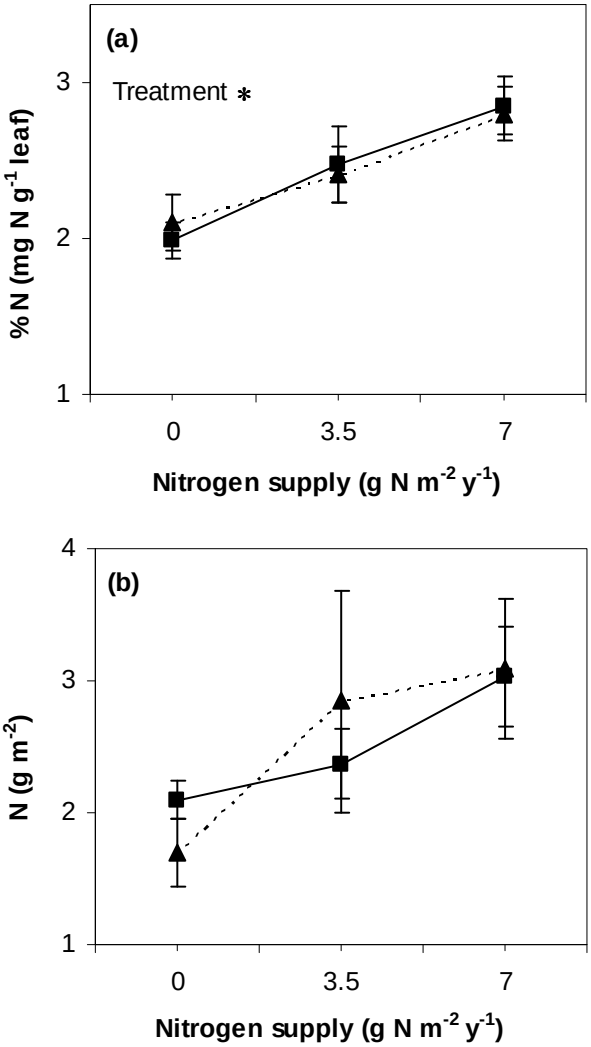

Fig. 2.4: (a) Percentage leaf nitrogen and (b) leaf nitrogen per unit leaf area of *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors (n = 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The allocation of nitrogen to leaves

The average leaf nitrogen for the C_3 subspecies was significantly higher than that for the C_4 subspecies ($F_{(1,24)} = 9.8$, $p < 0.005$). Calculated for the whole plant at 12 weeks, the C_3 subspecies allocated a significantly greater percentage of its nitrogen budget to the leaves than the C_4 subspecies ($F_{(1,24)} = 13.3$, $p < 0.001$); an allocation that was nearly twice that of the nitrogen allocated to leaves than the C_4 subspecies (Table 2.5), and is was consistent with the lower PNUE of the C_3 subspecies. There was no significant difference in the allocation of nitrogen to leaves with increased nitrogen supply from 3.5 to 7 g N m^{-2} y^{-1} for either subspecies (Table 2.5) and this too is consistent with the finding that the PNUE is unaffected by nitrogen supply.

Table 2.5: The mass of nitrogen supplied to the two subspecies of *A. semialata* and the percentage allocated to leaves at 12 weeks. All values are means ± standard error (n = 6 or 8).

Subspecies	Nitrogen supply (g N m^{-2} y^{-1})	Total N supplied (mg)	Leaf N (mg)	% N allocated to leaves
C_3	7.0	412.2	187.1 ± 19.8	47.8 ± 9.1
	3.5	206.1	99.6 ± 17.1	53.9 ± 9.3
	0.0	0.0	21.3	–
C_4	7.0	412.2	71.0 ± 19.8	18.2 ± 2.8
	3.5	206.1	61.8 ± 17.1	33.8 ± 3.7
	0.0	0.0	23.3	–

CO_2 response curves

CO_2 response curves constructed for the two subspecies and three levels of nitrogen-supply were typical for C_3 and C_4 plants. The C_4 curves had steeper initial slopes, saturated at lower C_i values and had lower CO_2 compensation points than that for the C_3 curves (Fig. 2.5).

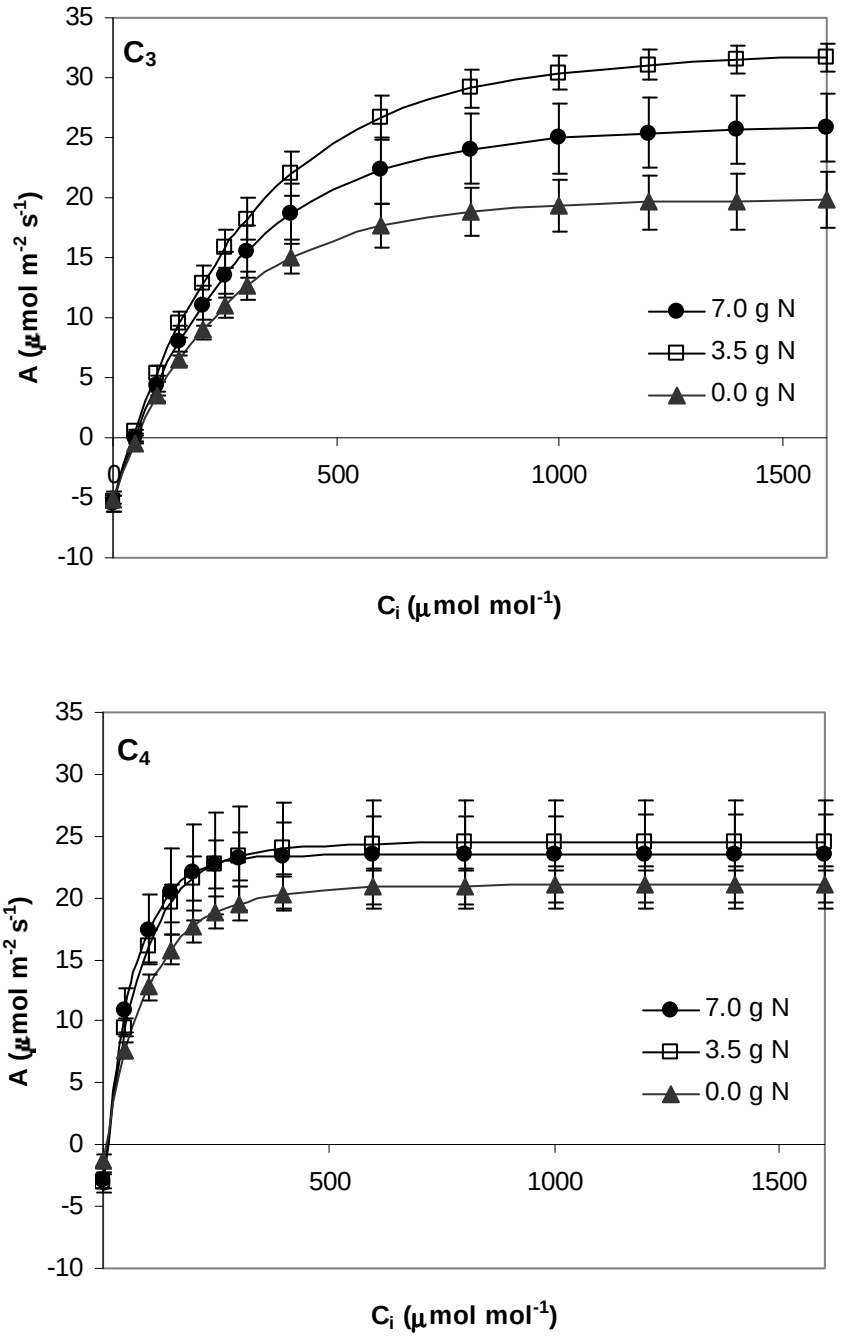

Fig. 2.5: Fitted CO_2 response curves of net assimilation (A) for the C_3 and C_4 subspecies of *A. semialata* at three different levels of nitrogen supply (7.0 g N m^{-2} y^{-1}, 3.5 g N m^{-2} y^{-1} and 0.0 g N m^{-2} y^{-1}). All are mean values with vertical bars representing standard errors. (n = 5)

CE was significantly higher in the C_4 subspecies than the C_3 subspecies (Table 2.6, Fig. 2.6). For the C_4 subspecies values ranged between 234 – 395 mmol m^{-2} s^{-1} (Average = 325 ± 111 mmol m^{-2} s^{-1}) and for the C_3 subspecies between 108 – 127 mmol m^{-2} s^{-1} (Average = 118 ± 35 mmol m^{-2} s^{-1}). In the C_3 subspecies no

treatment effect was observed and the availability of nitrogen had no effect on CE. However, in the C_4 subspecies a trend can be identified with CE decreasing as nitrogen supply decreased with an interaction effect that was significant at the 90% confidence level (Table 2.6). The lowest value of CE at zero nitrogen supply was higher than that of the C_3 subspecies, but not significantly so (Fig. 2.6).

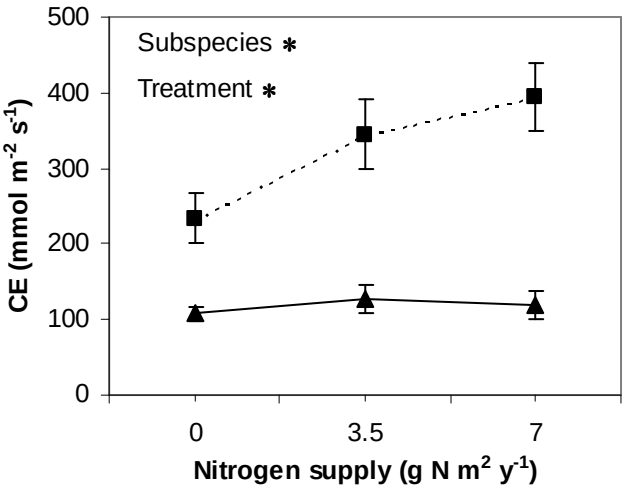

Fig. 2.6: Apparent carboxylation efficiency (CE) calculated from CO_2 response curves for *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors (n = 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The A_{max} showed no significant difference between subspecies: the A_{max} for the C_4 subspecies was not affected by treatment, while there was an apparent treatment effect for the C_3 subspecies although no significant interaction effect (Table 2.6). The highest A_{max} was observed for the C_3 subspecies when supplied the medium nitrogen level and there was a significant difference in the RuBP regeneration rate between low and medium nitrogen supply, a difference of 38% (Fig. 2.7).

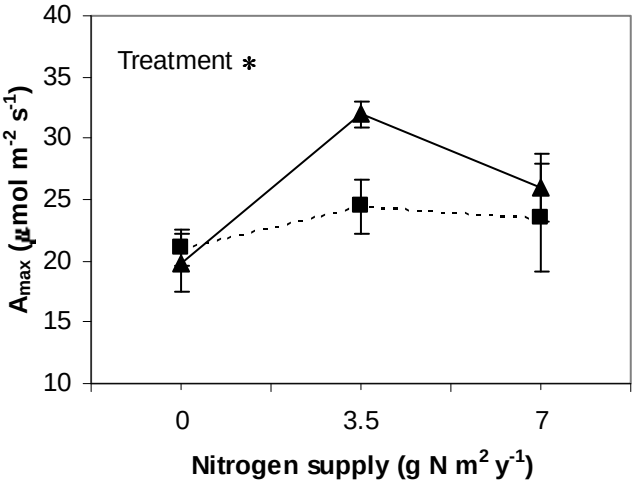

Fig. 2.7: The CO_2 saturated rate of photosynthesis (A_{max}) calculated from CO_2 response curves for *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The values for Γ were significantly different between subspecies but not between treatments (Fig. 2.8, Table 2.6). For the C_3 subspecies the values were between 45 – 55 μmol mol^{-1} (Average = 51 ± 8 μmol mol^{-1}), and for the C_4 subspecies they were between 5 – 9 μmol mol^{-1} (Average = 7 ± 5 μmol mol^{-1}). These values are consistent with those of typical C_3 and C_4 plants (Table 1.1).

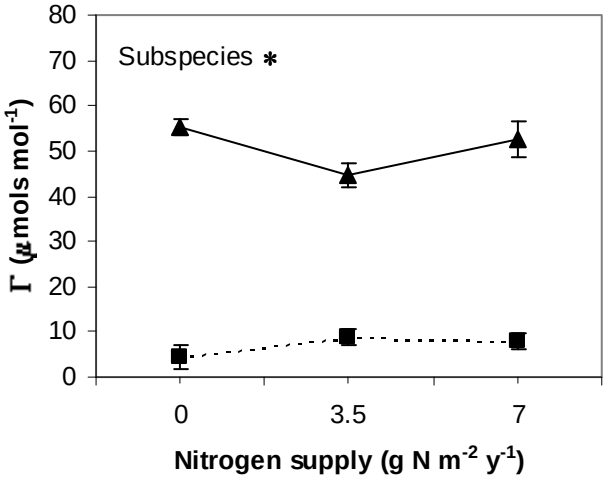

Fig. 2.8: CO_2 compensation points (Γ) calculated from CO_2 response curves for *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors (n = 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The values for stomatal limitation (*ls*) were significantly different between subspecies but not between treatments (Fig. 2.9, Table 2.6). *ls* calculated for the C_3 subspecies was between 27% and 34% (Average = 30 ± 7%) and for the C_4 subspecies between 9% and 13% (Average = 11 ± 6%). These *ls* values were consistent with those of typical C_3 and C_4 plants (Ripley *et al.*, 2007).

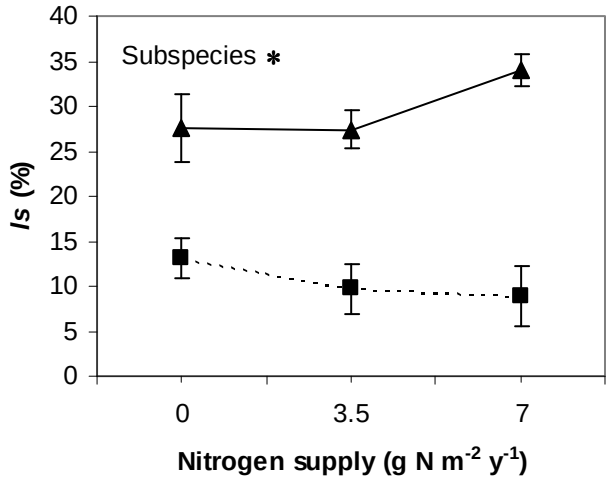

Fig. 2.9: Percentage stomatal limitation (*ls*) calculated from CO_2 response curves for *A. semialata* at three different levels of nitrogen supply. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors (n = 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Table 2.6: Factorial ANOVA results for photosynthetic parameters of *A. semialata* compared between subspecies and nitrogen supply treatments, and the interaction of subspecies and nitrogen supply treatments. Where n.s. indicates no significant difference and n = 5.

Parameter	**Significant difference**	**F**	**d f**	**Approx. p <**
A	Subspecies	19.7	1,24	0.0002
	Treatment	2.4	2,24	0.12 (n.s.)
	Subspecies *treatment	0.3	2,24	0.77 (n.s.)
PNUE	Subspecies	6.2	1,24	0.021
	Treatment	2.5	2,24	0.12 (n.s.)
	Subspecies *treatment	0.2	2,24	0.87 (n.s.)
N %	Subspecies	0.0	1,24	0.98 (n.s.)
	Treatment	9.2	2,24	0.0011
	Subspecies *treatment	0.1	2,24	0.88 (n.s.)
CE	Subspecies	63.4	1,24	0.0001
	Treatment	4.0	2,24	0.045
	Subspecies *treatment	2.8	2,24	0.13 (n.s.)
A_{max}	Subspecies	1.9	1,24	0.19 (n.s.)
	Treatment	4.5	2,24	0.022
	Subspecies *treatment	1.4	2,24	0.27 (n.s.)
Γ	Subspecies	421.0	1,24	0.001
	Treatment	1.1	2,24	0.36 (n.s.)
	Subspecies *treatment	4.1	2,24	0.03
ls	Subspecies	70.9	1,24	0.001
	Treatment	0.6	2,24	0.58 (n.s.)
	Subspecies *treatment	2.0	2,24	0.17 (n.s.)

Abbreviations: Photosynthetic rate at an ambient CO_2 concentration of 370 μmol mol^{-1} (A), photosynthetic nitrogen used efficiency (PNUE), leaf nitrogen concentration calculated as a percentage (N %), apparent carboxylation efficiency (CE), the CO_2 saturated rate of photosynthesis (A_{max}), the CO_2 compensation point (Γ), stomatal limitation (*ls*).

DISCUSSION

Photosynthetic rate and PNUE

It is well established that the rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} is higher for C_4 species than for C_3 species at any given nitrogen level. This applies to C_4 grass species as well as C_4 agricultural crops and weeds, and it has also been found that there is an increase in CO_2 assimilation with increased nitrogen supply (Bolton and Brown, 1980; Sage and Pearcy, 1987; Li, 1993; Vos *et al*., 2005). In this experiment the rate of photosynthesis for the C_4 subspecies was found to be nearly twice that of the C_3 subspecies, although, neither subspecies showed any increase in photosynthetic rate with increased nitrogen supply (Fig. 2.3a). Wand *et al*., (2001) points out that African grasses, particularly those of the nutrient poor eastern parts of South Africa have been poorly studied and unique responses can be expected. A similar conservative response of photosynthetic rate to different levels of nitrogen supply has been shown in potato (Vos and Biemond, 1992), where under nitrogen limitation leaf size is 'downregulated' in order to maintain the nitrogen concentration and photosynthesis. Wand *et al*., (2001) found that South African C_4 grasses generally have been found to have comparatively lower leaf nitrogen concentrations and have lower photosynthetic rates leading to lower growth potentials than those found in the northern hemisphere. These low growth potentials and the conservative response of photosynthesis to nitrogen limitation, shown here, are likely to be adaptations to nutrient poor environments.

The greater PNUE found for C_4 subspecies than for the C_3 subspecies (Fig. 2.3b) is consistent with the findings in a range of previous studies where C_3 and C_4 species were compared (Bolton and Brown, 1980; Sage and Pearcy, 1987; Li, 1993; Simiomi *et al*. 2004). In the C_4 subspecies of *A. semialata* the higher PNUE was due to higher rates of photosynthesis rather than lower leaf nitrogen concentrations which were similar for the C_3 and C_4 species (Fig. 2.5). Li (1993) found that this was also the case in a number of C_4 species of the genus *Cyperus*. The greater cost of C_3 photosynthesis in terms of nitrogen, at the whole plant level, can be seen with the greater allocation of available nitrogen at medium and high levels of supply to leaves, by the C_3 subspecies (Table 2.5). This is

consistent with what was found by Sage and Pearcy (1987) who suggested that C_4 plants are able to allocate a greater fraction of their nitrogen budget to other parts of the plant and for functions other than photosynthesis.

The lack of variation in the PNUE at different levels of nitrogen nutrition found in this investigation (Fig.2.3b) has been previously reported by Bolton and Brown (1980) at similar levels of nitrogen supply in an investigation using two species of the grass *Panicum*; *P. maximum* and *P. milioides*, and the C_3 grass, *Festuca arundinacea*. However, Sage and Pearcy (1987) found that for the agricultural weeds *Chenopodium album* (C_3) and *Amaranthus retroflexus* (C_4) there was a curvilinear increase in the PNUE with increased leaf nitrogen which may be due to their adaptation to nutrient rich environments.

Photosynthetic parameters

The CE values obtained for the C_3 and C_4 subspecies of *A. semialata* in this investigation were within the range cited for C_3 and C_4 plants (Table 1.1). Increased CE and A_{max}, have been shown in C_3 and C_4 plants when grown at increasing levels of nitrogen supply and with increase in leaf nitrogen (Sage *et al.*, 1987; Bowler and Press, 1996; and Ghannoum and Conroy, 1998). In this experiment there was an increase in leaf nitrogen with increased nitrogen supply for both subspecies when calculated on a percentage basis (Fig. 2.4a), and this increase in available nitrogen resulted in the C_4 subspecies having increased CE values with increasing levels of nitrogen supply (Fig. 2.6) and A_{max} remained unaffected. In the C_3 subspecies A_{max} increased while the CE was constant. In the C_3 subspecies A_{max} was highest at the medium level of nitrogen supply (Fig.2.7) and declined at high nitrogen levels which may be accounted for by the earlier onset of senescence observed in these plants. For both subspecies these increased parameters (CE in the C_4 and A_{max} in the C_3) had little effect on the photosynthetic rate at ambient CO_2 concentrations, for the C_4 subspecies CO_2 saturation occurs close to ambient CO_2 concentrations and in the C_3 subspecies ambient CO_2 concentration was well below A_{max}. The decline in CE for the C_4 subspecies with nitrogen limitation indicates a reduced PEP carboxylase activity and may be linked with the greater nitrogen demand for the enzymes associated with carboxylation in the C_4 cycle. However, the value for the CE in these plants remains higher than those for the C_3 subspecies at high and medium levels of nitrogen supply. In the

C_4 subspecies nitrogen limitation had no effect on the PEP regeneration and rubisco activity which are associated with A_{max}, in contrast to the findings for *Panicum antidotale* by Ghannoum and Conroy (1998) where the reduction in rubisco activity exceeded that of PEP carboxylase activity under conditions of nitrogen limitation. Maintaining the level of PEP regeneration and rubisco activity under conditions of nitrogen limitation by the C_4 subspecies supports the hypothesis that *A. semialata* is adapted to poor nutrient environments. The significant decrease in A_{max} with severe nitrogen limitation in the C_3 subspecies is associated with decreased RuBP regeneration and probably indicates a limitation of the electron transport system, or sink limitation of photosynthesis mediated via phosphate transport.

The values for Γ found in this investigation were within the range cited for C_3 and C_4 plants (Table 1.1), and the significantly higher values measured in the C_3 subspecies than those measured for the C_4 subspecies are due to the high levels of photorespiration characteristic of C_3 plants. Γ is proportional to both mitochondrial respiration and photorespiration; both of which can generate CO_2 in the cell. In C_4 plants photorespiration is suppressed even though the enzymes are present within the cell. Any value measured for the CO_2 compensation point for C_4 plants would largely indicate the level of mitochondrial respiration in that species (Ghannoum *et al.*, 1998; von Caemmerer, 2000). It is worth noting that the lack of variation with different levels of nitrogen supply is consistent with the findings of Bolton and Brown (1980).

Farquhar and Sharkey (1982) explain *ls* as the contribution of stomatal resistance to the total resistance to CO_2 assimilation, and that in C_4 plants, despite generally lower intercellular CO_2 partial pressures, *ls* is low because of the early CO_2 saturation of the assimilation rate. In *A. semialata* the *ls* of the C_4 subspecies is close to one third that of the C_3 subspecies and this may account for the greater water use efficiency than can usually be seen in C_4 plants, and may represent a water-conservation strategy to enable survival in arid environments (Long, 1999) .

CONCLUSIONS

- The rate of photosynthesis at ambient CO_2 concentrations in the C_4 subspecies was nearly twice that of the C_3 subspecies while the PNUE was a third greater, and the greater PNUE of the C_4 subspecies was due to the higher photosynthetic rates, rather than a lower leaf nitrogen concentration.

- The allocation of nitrogen to leaves by the C_4 subspecies was half of that allocated to leaves by the C_3 subspecies in those plants receiving medium and high levels of nitrogen supply.

- Both subspecies demonstrated a conservative response to variations in nitrogen supply with regard to rate of photosynthesis and the potential PNUE, with photosynthesis maintained even under conditions of severe nitrogen limitation.

- Variation in the levels of nitrogen availability had little effect on the photosynthetic parameters. Severe nitrogen limitation negatively affected CE which correlates with the PEP carboxylase activity in the C_4 subspecies, while in the C_3 subspecies severe nitrogen limitation negatively affected the A_{max} which correlates with the electron transport system. However this had no effect on photosynthetic rates at ambient CO_2 concentrations.

- Both subspecies had Γ and *ls* that are typical for their respective photosynthetic pathways. Both subspecies were unaffected by variation in nitrogen supply and severe nitrogen limitation had no effect on mitochondrial respiration and photorespiration.

It is therefore apparent that nitrogen supply had little effect on the rate of photosynthesis and the photosynthetic parameters, and this suggests that the response to nitrogen supply was at the whole plant level, rather than at the leaf level.

CHAPTER 3

GROWTH AND BIOMASS ALLOCATION

INTRODUCTION

According to Valladares (1999) fitness, defined in Darwinian terms, is the measure of an individual's relative contribution to the gene pool of the next generation. It is therefore a measure of the adaptation of a species to its environment in terms of reproductive success and its survival to reproductive maturity. However, Valladares (1999) goes on to point out that fitness is a very difficult phenomenon to measure and it can only be done directly by measuring fecundity. Its measurement is only ever in relative terms through parameters like longevity, size or growth which must be dependent on primary production. Plant productivity is not only limited by leaf photosynthesis. Respiratory energy consumption and dissipation influence productivity, as does the quantity and proportion of light interception (Li, 1993). To maximize the proportion of intercepted light a plant can increase its total leaf area and therefore achieve a greater productivity at the whole plant level. As mentioned in Chapter 1, Long (1999) suggested that C_4 plants can exploit their higher PNUE than that found in C_3 plants in one of two ways: either by producing a greater leaf area with the same amount of nitrogen in order to achieve greater productivity and a greater ability to compete for light, or produce the same leaf area and allocate greater resources to root development exploiting a greater volume of soil and favouring the C_4 plants in competition for limiting soil resources.

Bloom *et al.* (1985) proposed that plants optimize allocation both in the short-term and the long-term in order to maximize primary production. This was explained in terms of an economic analogy whereby investment in growth results in a compounding of the investment, particularly if growth occurs in the production of new leaves or roots. Since growth is a measure of net resource gain from the environment it can be related to the plant's fitness. Bloom *et al*. (1985) further point out that plants can adjust allocation patterns so that all resources have an equal limitation on growth and there is homeostatic response to imbalances in resources. This means that allocation is adjusted between roots

or shoots such that if a plant is nutrient stressed it will increase allocation to roots, or if light were limiting then allocation to shoot growth would be enhanced. In other words, plants allocate more resources to organs that capture the most limiting resource, and this phenomenon is referred to as the "optimal allocation theory", by Weiner (2004).

The alteration of allocation patterns is referred to as "phenotypic plasticity" by Sultan and Bazzaz (1993), who showed that when *Polygonum persicaria* was grown at different levels of nutrient supply, plants were able to maintain the same canopy leaf surface area relative to their total biomass or leaf area ratio (LAR) and percentage leaf nitrogen. The lack of variation in LAR and leaf nitrogen was referred to as "functional homeostasis". They found that this can be accounted for by alterations in biomass allocation or phenotypic plasticity, like increased root to shoot ratios which was used to maintain plant growth despite severe nutrient limitation, and which contributes to the maximizing of fitness. However, McConnaughay and Coleman (1999), in defining ontogenetic drift as the change of a trait in a predictable way as a function of plant growth or development, have suggested that plasticity in biomass allocation patterns may be the result of ontogenetic drift coupled with plasticity in growth rates which they refer to as "apparent plasticity", as opposed to "true plasticity" in allocation patterns. Only when the relationship between the biomass accumulation of the plant components is isometric and linear as plants grow and develop, can ontogenetic drift be ignored (McConnaughay and Coleman, 1999).

In an investigation into the comparative growth and biomass allocation of a C_3 and C_4 species, Sage and Pearcy (1987) found that over a range of levels of nitrogen supply, the relative growth rate (RGR) increased with increasing nitrogen supply. However, the RGR in the C_4 species (*Amaranthus retroflexus*) was 20% greater at high nitrogen than the C_3 species (*Chenopodium album*), but was similar at low nitrogen. Nitrogen deficiency brought about a decrease in the relative biomass allocation to leaves for both species, although, the decrease was greater in the C_3 species. A deficiency of nitrogen resulted in an increase in allocation to roots for both, and increased allocation to shoots in the C_4 species only. These differences were accounted for by the greater PNUE of the C_4 species. Tilman and Wedin (1991) investigated the differential biomass allocation patterns in five perennial grass species (three C_3 and two C_4) that are said to be dominant at different times during secondary succession on abandoned fields in the North American prairies. This investigation was carried out on nitrogen-poor sandy soil, where a gradient was established

by the addition of different amounts of nitrogen rich top soil and ammonium nitrate fertilizer. It was found that for all five grass species, with increasing soil nitrogen, the total plant biomass increased along with their vegetative growth rate. But with decreasing soil nitrogen, the biomass allocation patterns changed and the root to shoot ratios increased. The C_4 grasses had significantly greater portions of their biomass allocated to roots (~80%) at lower soil nitrogen levels than the C_3 grasses, and the C_4 species also had the greatest allocation to root biomass at all nitrogen levels.

Poorter and Nagel (2000) state that biomass is not a fixed attribute of a plant and that it has long been known that allocation to roots increases with decreasing nutrient or water availability. They refer to plants having a "functional equilibrium" whereby the plant shifts allocation patterns towards the shoots if carbon gain by the shoot is impaired by a low level of above-ground resources such as light or CO_2. Allocation patterns are shifted to the roots if below-ground resources such as water or nutrients are limited.

The economic analogy presented by Bloom *et al*. (1985) proposes that allocation to reproduction is analogous to a high-risk investment. Reproductive structures have high resource costs and divert plant reserves away from roots or leaves where they would provide a greater resource return on investment. It is suggested that in general, allocation to sexual reproduction is greater in resource-rich environments rather than in resource-poor environments where survival is tenuous.

Reekie and Bazzaz (1987) suggest that reproductive output is crucial in the measurement of a plant's fitness. However, they find that even among genotypes of the same species allocation to flowers and fruits may not reflect the true carbon costs of reproduction and it is necessary to consider not only the reproductive output but also the resource allocation to reproduction, like the allocation to stems or ancillary and supporting structures. Sultan and Bazzaz (1993), measuring mass and number of fruits produced by *Polygonum persicaria* noted a 75% decrease in reproductive output of plants grown in extremely low nutrient conditions compared with those grown under ample nutrient conditions. They propose that phenotypic plasticity in response to nutrient levels might have evolved owing to the fitness advantage conferred by the ability of a plant to exploit occasional pulses of nutrients that occur in seasonal and disturbed environments. Tilman and Wedin (1991) found that in

perennial grasses, there is a decrease in allocation to reproductive tissue (seeds and flowering stems) with decreasing soil nitrogen levels. They found that the lowest allocation was made by the C_4 grasses, which, along with their high allocation to roots and low vegetative reproduction rates and low vegetative growth rate, enables them to dominate the most nitrogen poor environments. The species of grass used in this investigation were tussock forming grasses and vegetative reproductive rate was determined by the allocation to rhizomes, but did not assess the rate of tiller production within the tussock.

Weiner (2004) argues for caution in the interpretation of plasticity in allocation patterns. He suggests that the relationship between structure and function is not always straightforward, as many organs have multiple functions and the unit of allocation, usually biomass, is not necessarily the unit of function and he gives the example of surface area. Weiner (2004) also points out that although plasticity in allocation patterns may reflect flexibility in strategies resulting from selection pressures, many allocation patterns follow allometric trajectories and are more likely to be a function of plant size. However, as Poorter and Nagel (2000) state, if one's intention is to determine the functioning of the plant in its environment at any particular moment, then it is the "actual" allocation of biomass in connection with its "actual" physiology that will determine its performance. Furthermore, the growth rate of a plant is complicated by the fact that it is seldom exponential and depends on ontogeny as well as growth conditions (Poorter and Garnier, 1999).

The key research questions

The objective of this investigation is to evaluate whether the availability of nitrogen can have an effect on the growth, allocation or biomass partitioning and reproductive effort in the C_3 and C_4 subspecies of *A. semialata,* and to test whether the hypothesis suggested by Long (1999) mentioned above is valid for this species. The specific questions are:

1. How do the C_3 and C_4 subspecies differ in their growth rate and the allocation of biomass to roots, leaves, culms, corms and flowers?

2. How are the growth rates and the allocation of biomass affected by nitrogen supply?

METHODS

Tillers of the C_3 and C_4 subspecies of *A. semialata* were planted in pots filled with washed river sand, and the plant pot culture is described in the section on "Growth and treatment of plants" of the Methods in Chapter 2.

Determination of leaf growth rate

The leaf growth rate was calculated from allometric determination of leaf area and the specific leaf area (SLA) using leaves harvested after 23 weeks of treatment. To calculate the allometric leaf area, leaves of plants grown in washed sand for 7 weeks, were removed from the leaf sheath at the ligule, and the length and width (at widest point) of each apical leaf and each non-apical leaf was measured. Only the live portions of the leaves were measured and leaves that were more than 50% dead were considered dead. The projected leaf area of each leaf was then determined using image analysis software (WinDIAS, Delta-T Devices, Cambridge, U.K.). A total of 130 leaves from the C_3 subspecies and 68 leaves from the C_4 subspecies were measured. The leaf area was then plotted against the length multiplied by width and an equation fitted using linear regression analysis (Fig. 3.1). The r^2 values from the regression analysis were 0.99 and 0.96 for C_3 and C_4 subspecies respectively. Differences between leaves form different treatments were not significant and so data for treatments were combined.

For the C_3 leaves:

Leaf area (cm^2) = leaf length (mm) x maximum width (mm) x 0.0076

For the C_4 leaves:

Leaf area (cm^2) = leaf length (mm) x maximum width (mm) x 0.0070

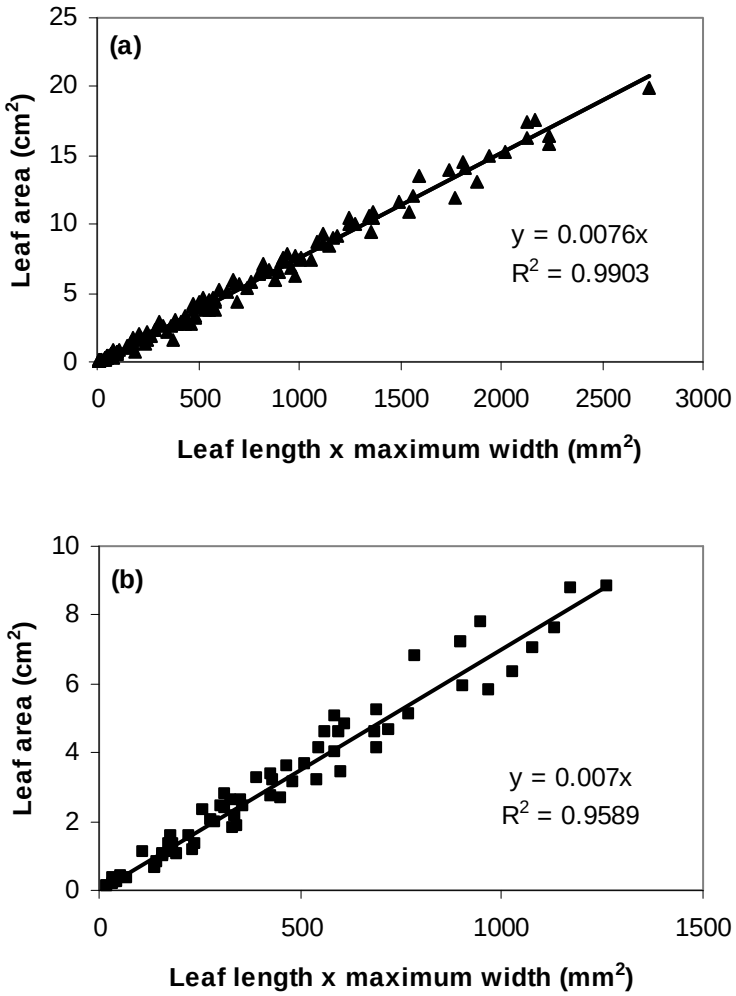

Fig. 3.1: Leaf area in relation to the product of leaf length and maximum width for (a) the C_3 subspecies and (b) the C_4 subspecies showing fitted equations using linear regression analysis, and correlation coefficients.

In the week prior to the initiation of treatments, the area of each leaf on all the tillers in each pot was measured to give the total canopy area for the pot. Treatments were then assigned to each subspecies so that the total leaf area of all plants receiving the same treatment was the same, and there was no bias favouring any one treatment. Subsequent allometric measurements were made every four weeks until the plants were harvested. When the plants reached a size that made measuring all the leaves impractical, then a minimum of 9 emerging (or partially expanded) and 15 fully expanded and extended leaves were measured from each pot; measurements were made on adjacent leaves from three randomly chosen portions of the whole plant. The number of emerging and fully expanded leaves was then counted and the area was multiplied by that number to give the total canopy area.

Relative leaf growth rate (RLGR) starting at 4 weeks was calculated using total leaf areas according to Poorter and Garnier (1999), using the following formula:

$$RLGR\ (mm^2\ cm^{-2}) = \frac{\ln M_2 - \ln M_1}{t}$$

$$= \frac{\ln M_2 / M_1}{t}$$

(Where $\ln M_1$ and $\ln M_2$ are the natural logarithms of leaf biomass in grams and t is time in weeks)

Root biomass sampling

The pots were fitted with diagonally placed 50 mm PVC tubing (Fig. 3.2) into which 22 holes with a diameter of 35 mm were drilled, the total surface area of which was 38.5% of the below ground surface area of the tube, and similar to those used by Johnson *et al.* (2001)

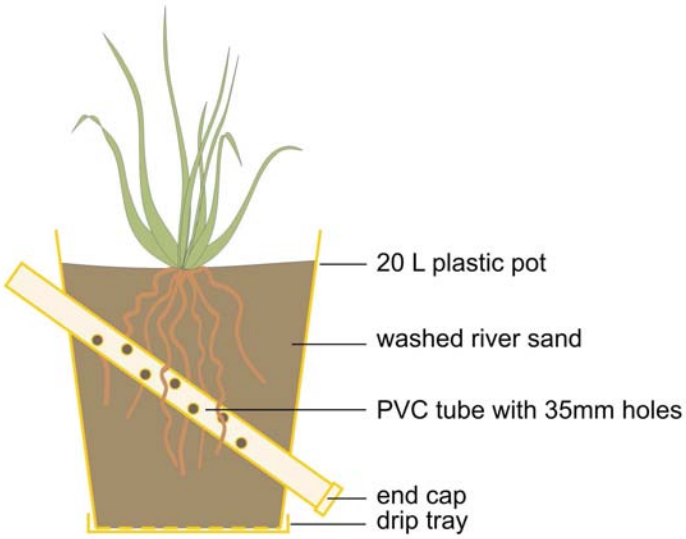

Fig. 3.2: Diagram of a pot with a diagonally fitted 'root in-growth tube'.

The lower end of each tube was be closed by a PVC end cap into which a 3 mm hole is drilled to prevent the accumulation of water or nutrient solution, these tubes will be referred to as 'root in-growth tubes'. In-growth tubes were diagonally positioned such that

both superficial and deep roots and sand could be sampled. Sampling was done using a barrel auger with dimensions which fitted to the internal diameter of the in-growth tubes. The leading edge of the barrel auger was sharpened ensuring that roots were cleanly severed, and root sampling took place at 12 weeks and again at 20 weeks after the initiation of treatments.

Determination of biomass

The plants were harvested prior to and after 23 weeks of nutrient treatment, on the 16th of December 2005. The sand was washed from the roots and the number of tillers counted. A sub-sample of three tillers from each plant was used to determine the projected leaf area as above, using image analysis software (WinDIAS, Delta-T Devices, Cambridge, U.K.). Combined tillers from each pot were then separated into the following functional components (Fig. 3.3):

1. **Leaf lamina** – that portion of the leaf from the ligule to its tip.
2. **Leaf sheath** – from the point it arises from the culm to the ligule and indicated by the presence of chlorophyll.
3. **Culm** – flower supporting stems.
4. **Inflorescence** – consisting of digitally arranged racemes made up of spikelets.
5. **Corm** – including the non-photosynthetic base of the culm, the base of leaf sheaths, and the rhizome.
6. **Root** – removed at the point of attachment to the base of the rhizome.
7. **Moribund biomass** – senescent leaves and leaf sheaths.

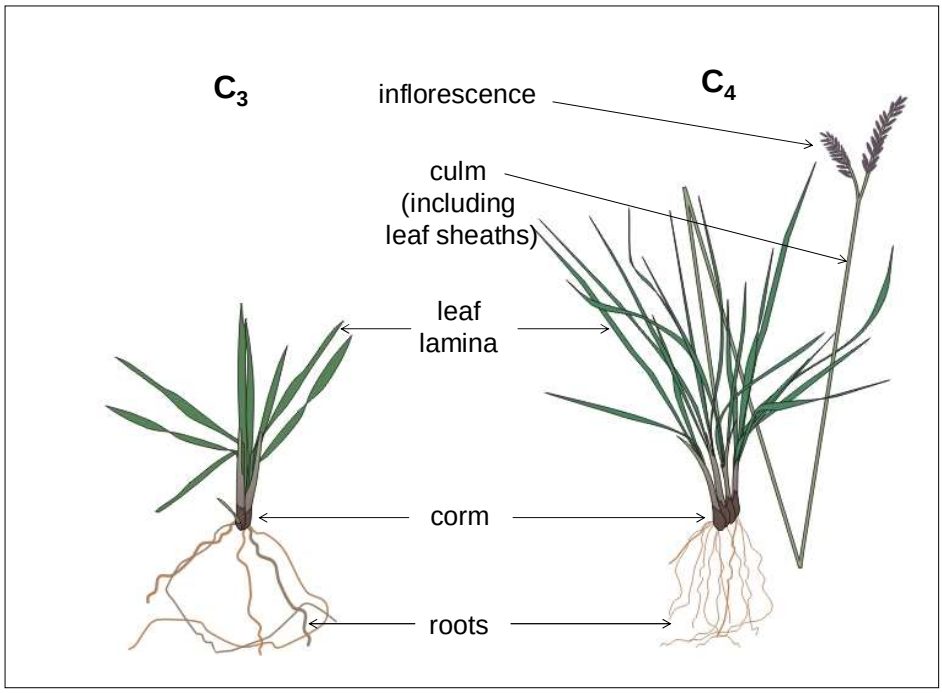

Fig. 3.3: Diagrams of the C_3 and C_4 subspecies of *A. semialata* showing the functional components.

The plants were then oven-dried to constant weight at 50-60°C and the respective components weighed to determine the biomass allocation. The relative biomass of plant components were expressed as a percentage of the whole plant dry weight (excluding moribund biomass). Senescent leaves were retained on the plant and decomposition would have been limited. Consequently the relative senescent leaf and leaf sheath biomass, and the moribund biomass, was expressed as a percentage of the whole plant (including moribund biomass) and was considered proportional to leaf turnover and was based on Sultan and Bazzaz, 1993, who used the ratio of senescent leaves to live leaves. The leaf sheathes were not considered to be important photosynthetic organs (Poorter and Nagel, 2000) and their biomass was assumed to be proportional to the culm.

Specific leaf area (SLA) was determined for each subspecies and treatment by dividing the projected leaf area by leaf dry weight, for the sub-sample of three isolated tillers. The total leaf area at harvest was calculated by multiplying the total leaf mass by the SLA. From the calculated leaf area for the whole plant the leaf area ratio (LAR) was determined for each subspecies and treatment by dividing the leaf area by total dry weight.

Allometric analysis

The ontogenetic differences between the C_3 and C_4 subspecies of *A. semialata* were compared using data from the final harvest since ontogenetic drift is at least partially related to the size of the plant (Poorter and Nagel, 2000). By combining the different nutrient treatments, the relationship between various plant components and plant size was determined according to McConnaughay and Coleman (1999), and the allometric trajectory could be determined, but it was not possible to determine whether the nitrogen treatments affected the plant ontogeny as the experimental design of this investigation did not allow for frequent destructive harvests.

Statistical Analyses

All statistical comparisons were made by means of the program Statistica version 7 (© Statsoft, Inc., Tulsa, USA). Leaf biomass accumulation and relative growth rates were compared between subspecies, treatment and the interaction between subspecies and treatment using repeated measures factorial ANOVA. All other biomass parameters, except LAR and SLA for subspecies were compared factorial ANOVA with a Type III model for the calculation of the sum of squares, as cell size was unbalanced (Quinn and Keough, 2002). LAR and SLA for subspecies were compared using one-way ANOVA. Where data were heteroscedastic and variance not homogeneous, they were transformed. Log transformations were used for leaf area production, canopy leaf area and biomass, LAR, SLA, biomass accumulation, number of tillers, mean tiller mass and root to shoot ratio. Arcsine transformations were used for the percentage leaf biomass, corm, inflorescence and flower.

Where main effects were significant at the 95% confidence level, Tukey *post-hoc* comparisons were made, and averages were given ± standard error. Allometric trajectories were compared between subspecies using a general linear model (GLM) with plant size as a continuous predictor, as plant masses from each treatment only partially overlapped, and between treatment interactions were not included in the analysis (Poorter and Nagel, 2000).

RESULTS

Growth and biomass accumulation

Leaf area production

The rate of leaf area production was different between C_3 and C_4 subspecies at the high level of nitrogen supply (Fig. 3.4, Table 3.1).

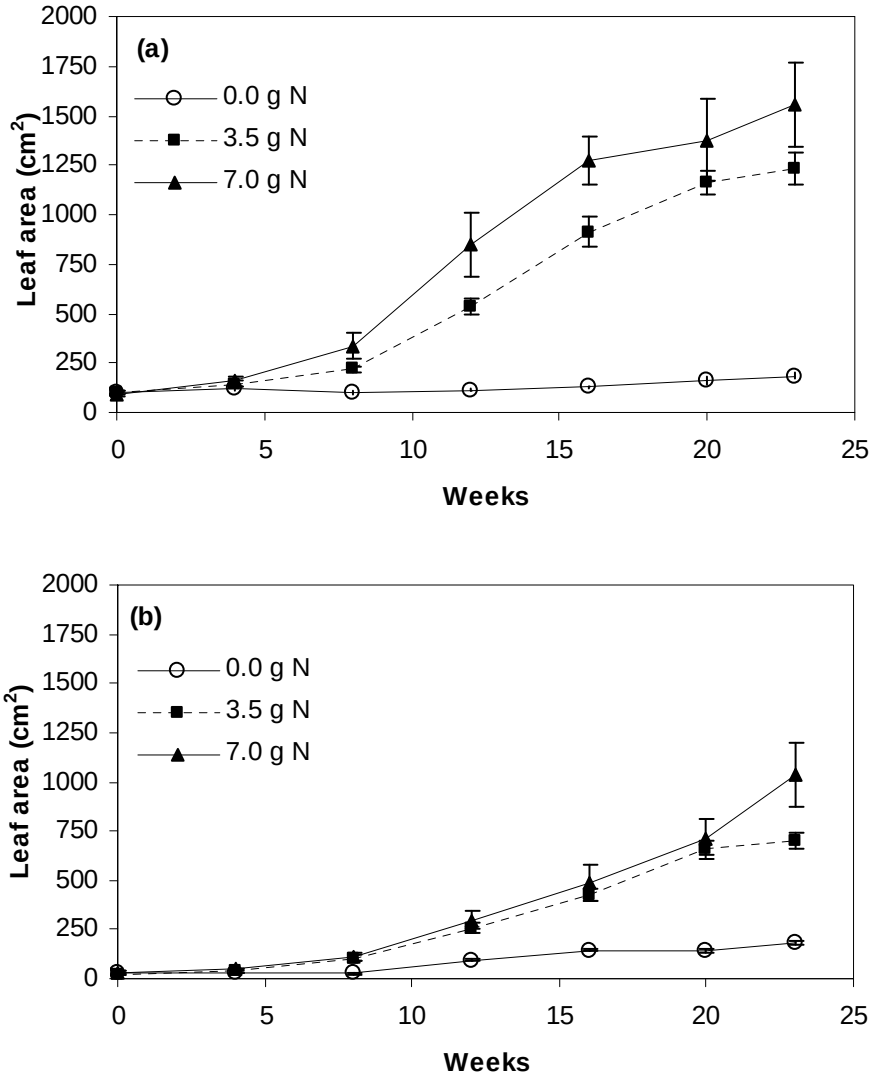

Fig. 3.4: Canopy leaf area of *A. semialata* over the period from prior to the initiation of treatments to the final harvest for (a) the C_3 subspecies and (b) the C_4 subspecies (where indicated nitrogen levels were supplied as g N m^{-2} y^{-1}) All mean values with vertical bars representing standard errors ($n \geq 6$).

By week 12 the C_3 plants receiving 7 g N m^{-2} y^{-1} had produced 54% of their final leaf areas at the time of harvest, compared to the C_4 plants which had produced only 29%. These values increase by week 20 when the C_3 plants had produced 88% of final leaf area as opposed to only 69% in the C_4 plants. The difference was less evident at the medium level of nitrogen supply where the C_3 plants had produced 43% of their final leaf areas by week 12, compared with 37% for the C_4 plants, and no difference by week 20. The least difference was seen for zero nitrogen supply; 62% and 51% of final leaf area was produced by the C_3 and C_4 plants by week 12, with 86% and 80% of leaf area by week 20, respectively. By the final harvest the canopy leaf area (Fig. 3.5) was significantly higher in the C_3 subspecies at the medium and high nitrogen levels and there was a significant interaction effect (Table 3.1), indicating the different response to different levels of nitrogen supply by the two subspecies, with the C_3 subspecies showing a greater increase in leaf area with increasing nitrogen supply.

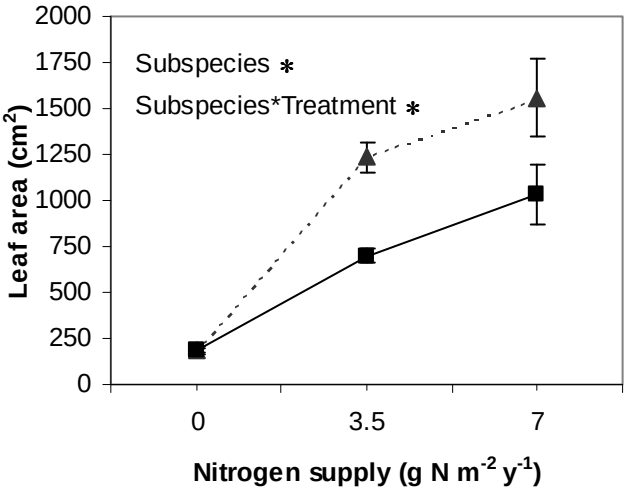

Fig. 3.5: Canopy leaf area of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Whole-plant investment of carbon in leaf area is shown by the leaf area ratio (LAR) and his was lower in the C_4 subspecies that in the C_3 subspecies (Fig. 3.6a). The average LAR across all treatments for the C_4 subspecies was half that of the C_3 subspecies; 14.3 ± 0.5 cm^2 g^{-1} for the C_4 subspecies, and 30.1 ± 1.5 cm^2 g^{-1} for the C_3. There was a treatment effect which was significant for both the subspecies (Table 3.1) but in the C_4 subspecies a reduction in LAR only occurred at low nitrogen supply. The specific leaf area (SLA) was significantly lower in the C_4 subspecies than the C_3 subspecies and there

was a significant, although not homologous, interaction effect (Fig. 3.6b, Table 3.1). This was due to the greater investment of carbon in leaf area in the C_4 subspecies than the C_3 subspecies. In the C_3 subspecies SLA was constant at all levels of nitrogen supply but in the C_4 subspecies SLA increased with increasing nitrogen availability. The mean SLA for C_4 subspecies was 96.0 ± 1.9 $cm^2 g^{-1}$ and for the C_3 subspecies it was 124.0 ± 3.5 $cm^2 g^{-1}$.

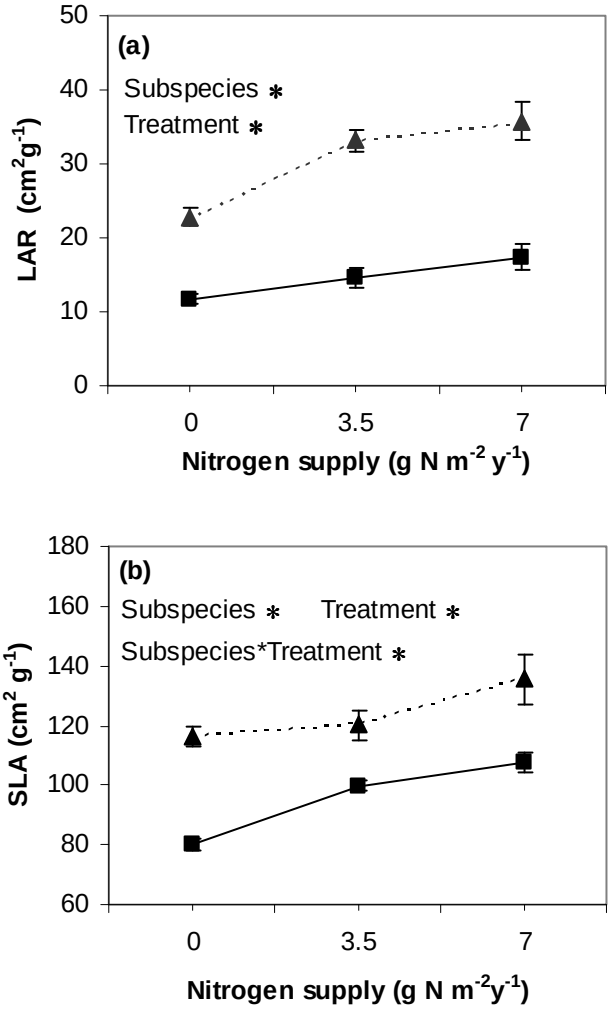

Fig. 3.6: (a) Leaf area ratio – LAR, (b) and specific leaf area - SLA of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Relative leaf growth

The relative leaf area growth rate (RLGR) for the C_4 subspecies was significantly higher than that of the C_3 subspecies (Fig. 3.7, Table 3.1). This was a result of the C_4 plants starting with almost zero leaf area due to the leaves of the transplanted tillers senescing at

the beginning of the experiment. While the C_3 plants maintained more of the original leaf area and hence had a lower RLGR. C_4 plants receiving low nitrogen supply had significantly lower growth rates than those receiving medium and high nitrogen supply prior to 16 weeks (Fig. 3.7b, Table 3.1), while the C_3 plants receiving low nitrogen supply had significantly lower growth rates prior to 20 weeks (Fig. 3.7a, Table 3.1). There was a significant interaction effect between subspecies and treatment (Table 3.1) resulting from the decreased growth rate for both subspecies at all levels of nitrogen supply except at zero supply for the C_3 plants where the growth rate increased over this period (Fig. 3.7).

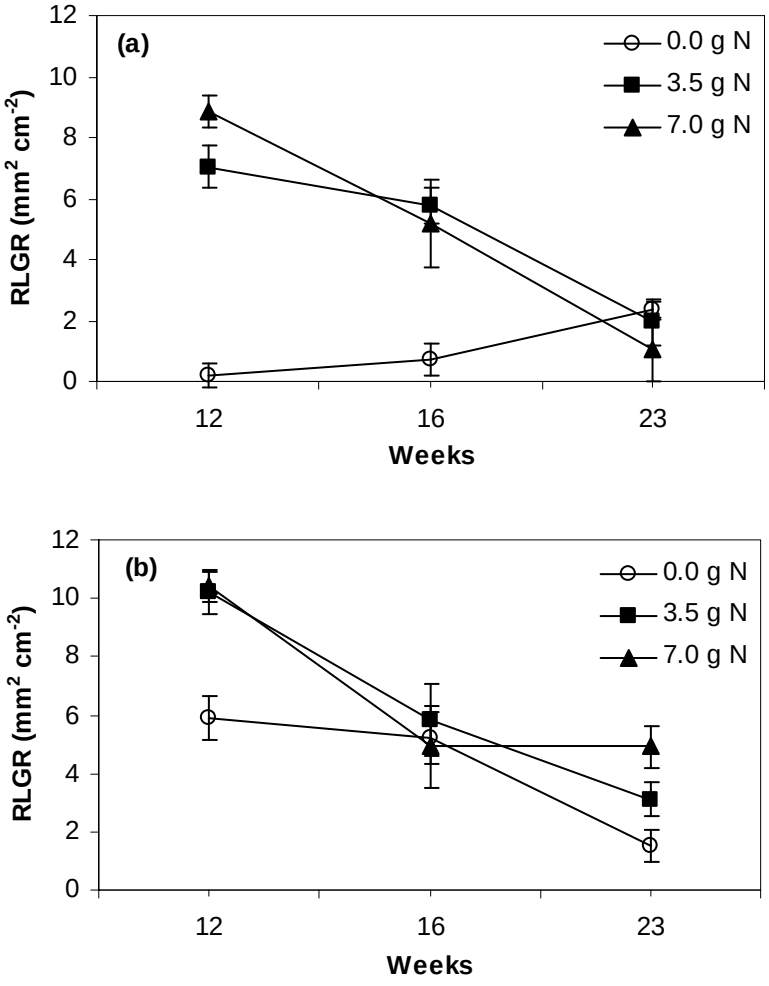

Fig. 3.7: Relative leaf area growth rate (RLGR) of *A. semialata* over the period from 12 weeks after the initiation of treatments to the final harvest for (a) the C_3 subspecies and (b) the C_4 subspecies (where indicated nitrogen levels were supplied as g N m^{-2} y^{-1}). All mean values with vertical bars representing standard errors ($n \geq 6$). Subspecies, Treatment and Subspecies*Treatment showed significant differences at the 95% confidence level (Tukey *post-hoc*).

Root Growth

It was found that there was no correlation between the biomass of the roots sampled and the final harvested root biomass (at 12 weeks $r^2 = 0.09$ and at 20 weeks $r^2 = 0.16$). It was concluded that the root mass sampled from the in-growth tube, at 12 and 20 weeks, was an inadequate method for quantifying root growth, although it did illustrate the rapid growth in biomass experienced by the C_4 plants receiving medium and high nitrogen treatment after 12 weeks (Fig. 3.8).

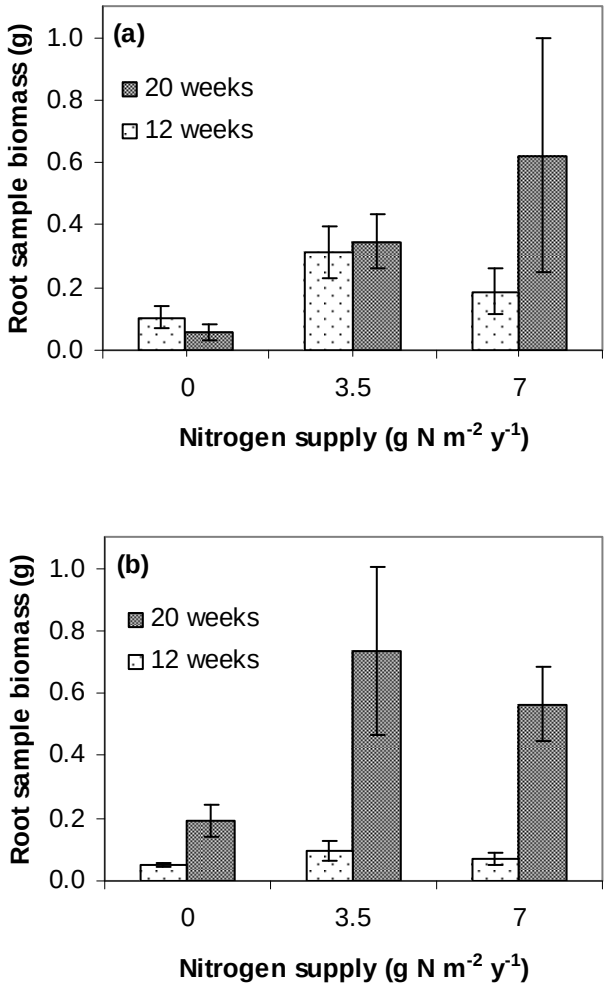

Fig. 3.8: Sampled root biomass for *A. semialata* at three different levels of nitrogen supply, 12 and 20 weeks after the initiation of treatments for (a) the C_3 subspecies and (b) the C_4 subspecies. All mean values with vertical bars representing standard errors ($n \geq 6$).

Biomass accumulation

As a check to ensure that the initial mass of the tillers at planting, did not affect the final biomass, the initial mass was correlated to the final mass and no correlation was found for

either subspecies and at any of the treatment levels (r^2 for the C_4 subspecies was 0.00, 0.27 and 0.25, and the r^2 for the C_3 subspecies was 0.00, 0.10 and 0.09 for the high, medium and low nitrogen treatments respectively).

There was a greater accumulation of biomass in the C_4 subspecies than in the C_3 subspecies over the period of the experiment (Fig. 3.9). Both subspecies responded significantly to an increase in the supply of nitrogen (Table 3.1), and at medium and high levels of nitrogen supply the C_4 plants had accumulated 25 and 30% more biomass respectively, than the C_3 plants, while at zero nitrogen supply the C_4 plants had 48% more biomass.

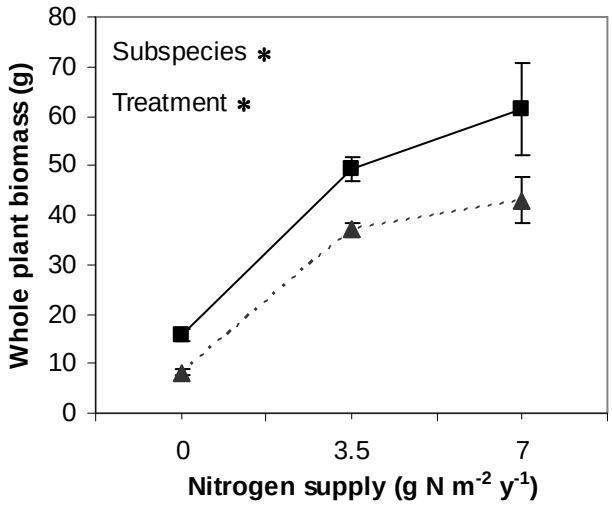

Fig. 3.9: Whole plant biomass excluding moribund material of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Table 3.1: Factorial ANOVA results for leaf area and growth parameters of *A. semialata* that were compared between subspecies and nitrogen supply treatments and the interaction of subspecies and nitrogen supply treatments for plants harvested at 23 weeks. Where n.s. indicates no significant difference and n = 6 or 8.

Parameter	**Significant difference**	**F**	**d f**	**Approx. p <**
Leaf area production	Subspecies	105.3	1,38	0.0001
	Treatment	85.4	2,38	0.0001
	Subspecies*Treatment	2.78	2,38	0.075 (n.s.)
Canopy leaf area	Subspecies	17.4	1,38	0.0001
	Treatment	230.1	2,38	0.0001
	Subspecies*Treatment	4.4	2,38	0.0198
LAR	Subspecies	162.67	1,38	0.0001
	Treatment	18.08	2,38	0.0001
	Subspecies*Treatment	0.9	2,38	0.42 (n.s.)
SLA	Subspecies	77.7	1,138	0.0001
	Treatment	18.3	2,138	0.0001
	Subspecies*Treatment	4.9	2,138	0.0086
RLGR	Subspecies	20.9	3,34	0.0001
	Treatment	15.2	6,68	0.0001
	Subspecies*Treatment	3.8	6,68	0.002
Biomass accumulation (living material)	Subspecies	32.3	1,38	0.0001
	Treatment	174.1	2,38	0.0001
	Subspecies*Treatment	2.6	2,38	0.092 (n.s.)

Biomass Partitioning

The biomass of the plants was partitioned into tillers, and the biomass of the tillers was partitioned into leaves, roots, corms and flowers (consisting of culms and inflorescences) on a proportional basis, resulting in characteristically different patterns of biomass investment for the C_3 and C_4 subspecies.

Tiller number

The number of tillers produced from the initial three that were planted was significantly different between treatments and there was an interaction effect between subspecies and treatment (Table 3.2). More tillers were produced at the higher levels of nitrogen supply, and there was a greater response by the C_3 subspecies to increased nitrogen levels than the C_4 subspecies, with a larger number of tillers produced by both subspecies at both the high and medium nitrogen levels (Fig. 3.10). There was a significant difference between the mass of the individual tillers in the C_3 and C_4 subspecies, the average mass for the C_3 subspecies tiller was 1.2 ± 0.2 g, and for the C_4 subspecies tiller 3.4 ± 0.2g, there was no significant difference in tiller mass between treatments.

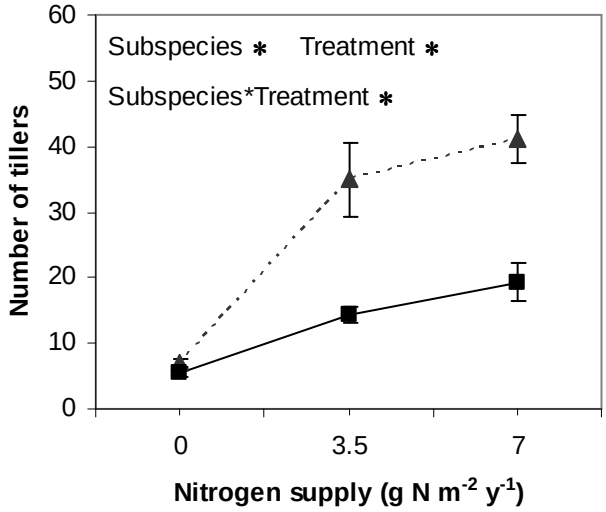

Fig. 3.10: The number of tillers produced by *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Leaf biomass

At the whole plant level both the C_3 and C_4 subspecies produced a significant increase in total leaf biomass (or canopy leaf biomass) with the increased nitrogen supply (Fig. 3.11a, Table 3.2). There was also a significant interaction between subspecies and treatment effects (Table 3.2) for leaf biomass and the C_3 subspecies had a greater response to the availability of nitrogen than the C_4 subspecies. This greater response by the C_3 subspecies is confirmed by the relative leaf biomass (Fig. 3.11b), which was calculated as a percentage of the whole plant biomass. There was a significant interaction effect between subspecies

and treatment (Table 3.2) and the C_3 subspecies had a greater percentage biomass at high and medium nitrogen levels than the C_4 subspecies (with a difference of 12% and 14% respectively); showing greater allocation by the C_3 subspecies to leaves at these levels. At low nitrogen supply the allocation was reduced in the C_3 subspecies by 9% from the medium nitrogen supply, but was still 5% higher than the C_4 subspecies. There was no significant difference in the relative leaf biomass at all levels of nitrogen supply in the C_4 subspecies indicating that the carbon allocation was unaffected by different levels of nitrogen availability.

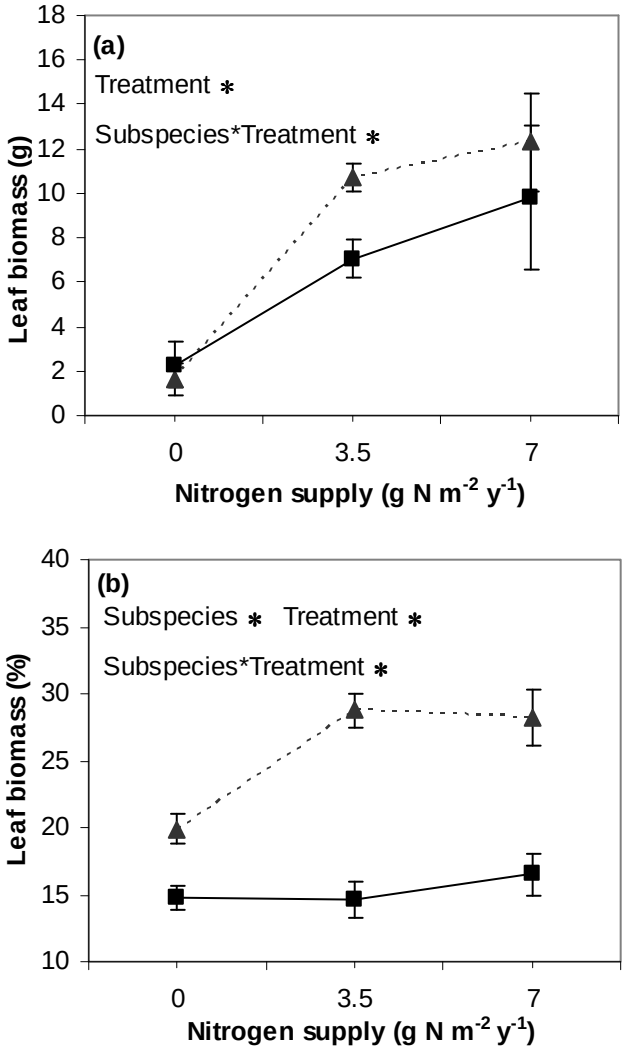

Fig. 3.11: (a) Canopy leaf biomass and (b) percentage leaf biomass of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

At the beginning of treatment application, all senescent leaf and leaf sheath material, the moribund biomass, was removed from the plants and thus moribund biomass that accumulated during the 23 week treatment period represents leaf turnover for that period. The C_4 subspecies showed a significantly lower leaf turnover than the C_3 subspecies (Fig. 3.12, Table 3.2), and this is associated with a lower SLA. For both subspecies, the low nitrogen treatment produced the greater turnover rate probably due to the re-allocation of nitrogen to new leaves.

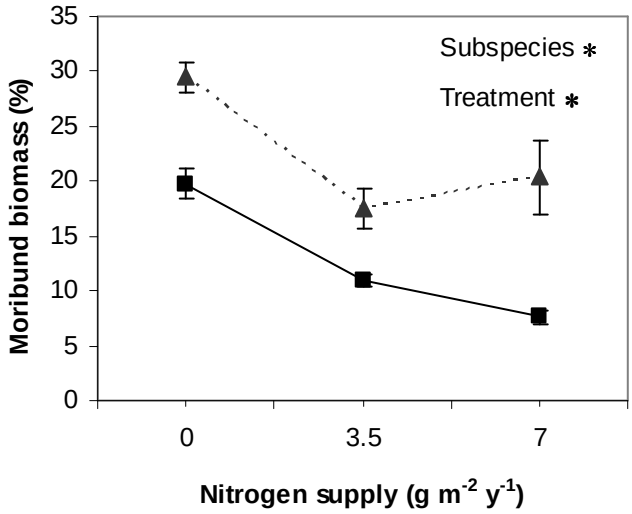

Fig. 3.12: Percentage senescent leaf and leaf sheath biomass, the moribund biomass of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Root biomass

Limited nitrogen availability resulted in a significantly greater allocation of biomass to root growth in both subspecies (Fig. 3.13a, Table 3.2). At the medium and high nitrogen supply the C_3 subspecies allocated significantly more of its biomass to root growth than the C_4 subspecies; in the C_3 approximately half of the biomass could be attributed to the roots (48% and 45% respectively), whereas in the C_4 subspecies only about a third of the biomass could be attributed to roots (35% and 34% respectively). At low nitrogen supply, allocation by the C_4 subspecies was equal to that of the C_3 subspecies at high and medium nitrogen supply. However, the partitioning of biomass to below-ground and above-ground

plant components was not significantly different between subspecies. This was evident from the similar root to shoot ratios for the C_3 and C_4 subspecies (Fig. 3.13b). Root to shoot ratios of those plants receiving a low nitrogen supply was significantly higher (Table 3.2) than those plants receiving medium and high nitrogen supply.

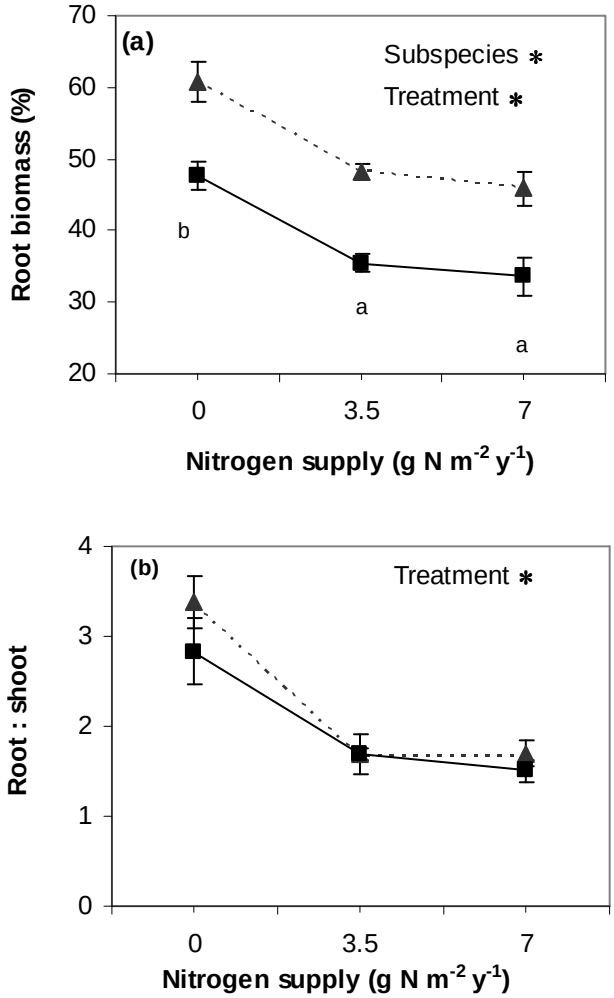

Fig. 3.13: (a) Percentage root biomass and (b) below-ground to above-ground biomass ratio of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Corm biomass

The C_4 subspecies made a significantly greater allocation of biomass to the development of corms than the C_3 subspecies (Fig. 3.14, Table 3.3). This occurred at all levels of nitrogen supply and there was no treatment effect for either subspecies. The average biomass

allocation across all treatments for the C_4 subspecies was 26 ± 1.0% and for the C_3 subspecies it was 15 ± 1.0%. Thus, if corms represent energy storage then the C_4 subspecies had greater energy storage than the C_3 subspecies.

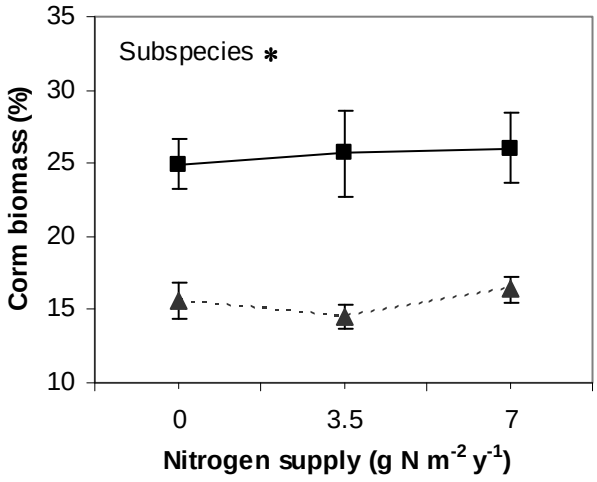

Fig. 3.14: Percentage corm biomass at three different levels of nitrogen supply of *A. semialata* at 3 different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Flower biomass

Biomass allocation to inflorescences was significantly different between subspecies (Fig. 3.15a, Table 3.3), with flowering only occurred in 9% of the C_3 plants, while in the C_4 plants 96% flowered, and data were heteroscedastic. The above-ground culm may be considered an ancillary or supporting structure of reproduction since its growth only occurs when the plant is about to flower. For the culm plus inflorescence (the flower) there was a significant difference between both subspecies and treatment, although Tukey *post-hoc* showed no significance at the 95% confidence level for treatment (Fig. 3.15b, Table 3.3).

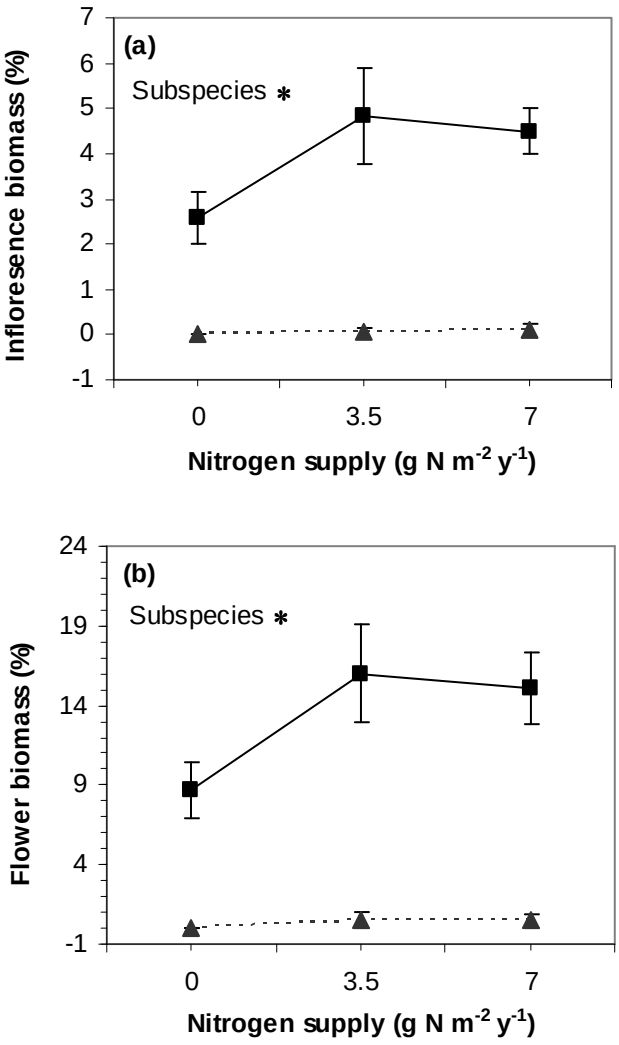

Fig. 3.15: (a) Percentage inflorescence biomass and (b) percentage flower biomass of *A. semialata* at three different levels of nitrogen supply for plants harvested at 23 weeks. The C_3 subspecies represented by: ▲ and the C_4 subspecies by: ■. All mean values with vertical bars representing standard errors ($n \geq 6$). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Table 3.2: Factorial ANOVA results for biomass allocation of *A. semialata* that were compared between subspecies and nitrogen supply treatments and the interaction of subspecies and nitrogen supply treatments for plants harvested at 23 weeks. Where n.s. indicates no significant difference and n = 6 or 8.

Parameter	**Significant difference**	**F**	**d f**	**Approx. p <**
Number of tillers	Subspecies	32.2	1,38	0.0001
	Treatment	79.1	2,38	0.0001
	Subspecies*Treatment	3.7	2,38	0.0338
Mean tiller mass (living material)	Subspecies	72.7	1,38	0.0001
	Treatment	0.3	2,38	0.74 (n.s.)
	Subspecies*Treatment	0.5	2,38	0.63 (n.s.)
Canopy leaf biomass	Subspecies	1.5	1,38	0.24 (n.s.)
	Treatment	191.0	2,38	0.0001
	Subspecies*Treatment	8.8	2,38	0.0008
Percentage leaf biomass	Subspecies	86.2	1,38	0.0001
	Treatment	7.4	2,38	0.0066
	Subspecies*Treatment	5.8	2,38	0.0066
Percentage moribund biomass	Subspecies	49.8	1,38	0.0001
	Treatment	27.4	2,38	0.0001
	Subspecies*Treatment	1.7	2,38	0.21 (n.s.)
Root to shoot ratio	Subspecies	2.2	1,38	0.16 (n.s.)
	Treatment	27.6	2,38	0.0001
	Subspecies*Treatment	0.3	2,38	0.74 (n.s.)
Percentage root biomass	Subspecies	54.0	1,38	0.0001
	Treatment	28.4	2,38	0.0001
	Subspecies*Treatment	0.21	2,38	0.98 (n.s.)
Percentage corm biomass	Subspecies	47.1	1,38	0.0001
	Treatment	0.3	2,38	0.78 (n.s.)
	Subspecies*Treatment	0.2	2,38	0.86 (n.s.)
Percentage inflorescence biomass	Subspecies	149.0	1,38	0.0001
	Treatment	3.0	2,38	0.062 (n.s.)
	Subspecies*Treatment	1.5	2,38	0.25 (n.s.)
Percentage flower biomass	Subspecies	155.8	1,38	0.0001
	Treatment	3.3	2,38	0.048
	Subspecies*Treatment	1.3	2,38	0.28 (n.s.)

Allometric trajectory

There was a proportional and linear increase in the biomass of leaves roots and corms with increased whole plant biomass to within 82%, irrespective of nitrogen treatment (Fig. 3.16b and Fig. 3.17). This suggests that the allometric relationship was unaltered by nutrient treatment, and different treatments (indicated by different symbols in Fig. 3.16 to Fig. 3.18) shifted the parameter along the trajectory with 3.5 and 7 g N m^{-2} y^{-1} treatments always resulting in higher total plant biomass than the 0 g N m^{-2} y^{-1} treatment. The allometric relationships were significantly different between the C_3 and C_4 subspecies for all parameters (Table 3.3).

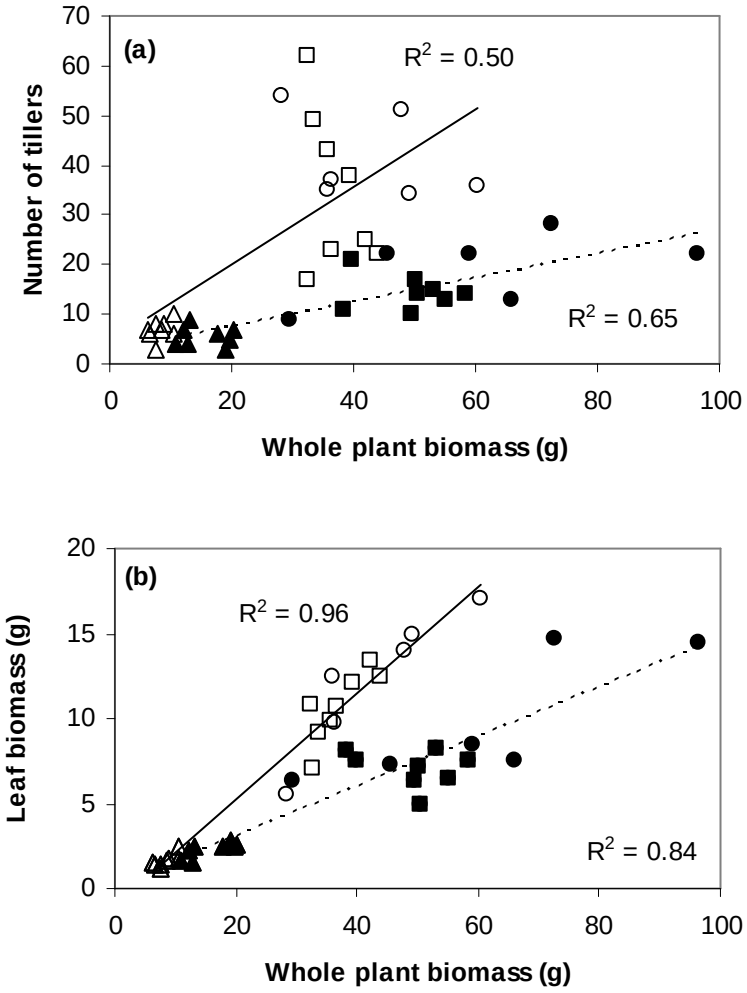

Fig. 3.16: Allometric analysis of (a) number of tillers produced and (b) leaf biomass for the C_3 subspecies (open symbols) and the C_4 subspecies (closed symbols) of *A. semialata* at three levels of nitrogen supply, indicated as: 7 g N m^{-2} y^{-1}: ○ and ●, 3.5 g N m^{-2} y^{-1}: □ and ■, and 0 g N m^{-2} y^{-1}: Δ and ▲.

The allometric relationships were weaker for the number of tillers produced and flower mass (Fig. 3.16a and Fig. 3.18a). However, whole plant biomass accounted for 76% of variation in the number of flowers produced by the C_4 subspecies (Fig. 3.18b), and accounted for 68% of the variation in inflorescence mass. Thus, the number of flowers per plant was most affected by nitrogen supply, since most C_4 plants flowered and the flowers produced by plants receiving medium and high levels of nitrogen supply had flowers of similar biomass.

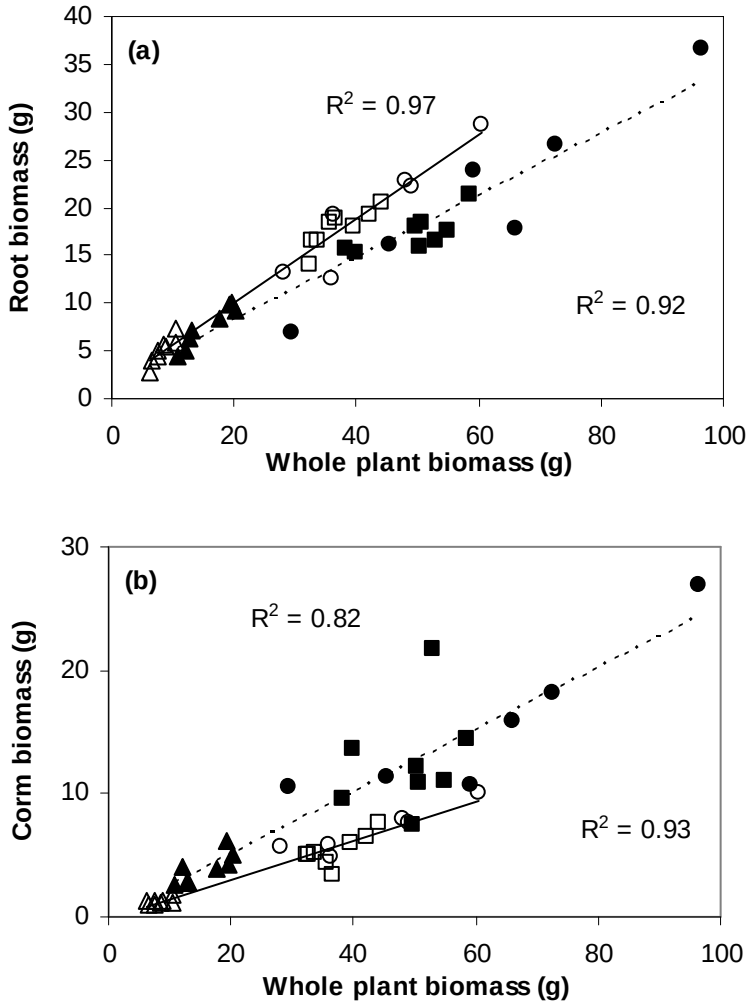

Fig. 3.17: Allometric analysis of (a) root biomass and (b) corm biomass for the C_3 subspecies (open symbols) and the C_4 subspecies (closed symbols) of *A. semialata* at three levels of nitrogen supply, indicated as: 7 g N m^{-2} y^{-1}:○ and ●, 3.5 g N m^{-2} y^{-1}: □ and ■, and 0 g N m^{-2} y^{-1}: Δ and ▲.

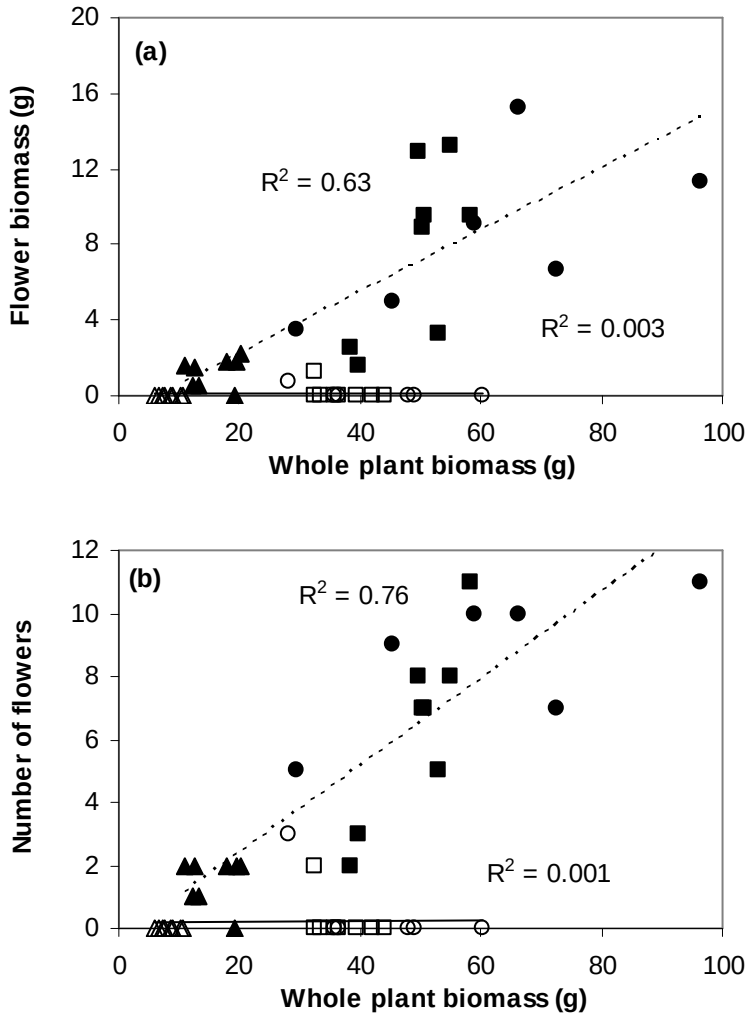

Fig. 3.18: Allometric analysis of (a) flower biomass and (b) the number of flowers produced for the C_3 subspecies (open symbols) and the C_4 subspecies (closed symbols) of *A. semialata* at three levels of nitrogen supply, indicated as: 7 g N m^{-2} y^{-1}: ○ and ●, 3.5 g N m^{-2} y^{-1}: □ and ■, and 0 g N m^{-2} y^{-1}: Δ and ▲.

Table 3.3: Linear effects model for the partitioning of biomass to tillers, leaves, roots, corms, inflorescences and flowers in C_3 and C_4 subspecies of *A. semialata.* Analysis accounted for the interaction of subspecies with plant size and df = 1,40.

Parameter	Significant difference	F	Approx. p <
Number of tillers	Whole plant biomass	41.5	0.0001
	Subspecies*whole plant biomass	11.2	0.0002
Leaf biomass	Whole plant biomass	477.3	0.0001
	Subspecies*whole plant biomass	63.3	0.0001
Root biomass	Whole plant biomass	643.0	0.0001
	Subspecies*whole plant biomass	13.9	0.0006
Corm biomass	Whole plant biomass	153.0	0.0001
	Subspecies*whole plant biomass	8.3	0.0007
Inflorescence biomass	Whole plant biomass	29.7	0.0001
	Subspecies*whole plant biomass	29.4	0.0001
Flower biomass	Whole plant biomass	23.34	0.0001
	Subspecies*whole plant biomass	22.8	0.0001
Number of flowers	Whole plant biomass	38.9	0.0001
	Subspecies*whole plant biomass	37.2	0.0001

DISCUSSION

Productivity and leaf area

In Chapter 2 it was shown that the rate of photosynthesis at ambient concentrations of CO_2 for the C_4 subspecies of *A. semialata* was nearly twice that of the C_3 subspecies (Fig. 2.4a), although neither subspecies showed any increase in photosynthetic rate with increased nitrogen supply. As the area-based leaf nitrogen concentrations of the C_4 and C_3 subspecies was the same (Fig. 2.5b), different rates of photosynthesis resulted in the C_4 subspecies having a PNUE that was a third greater than for the C_3 subspecies (Fig. 2.4b), which was unaffected by nitrogen supply. This was associated with the lower leaf area production by the C_4 subspecies (Fig. 3.4) leading to a smaller canopy leaf area at the final harvest than

the C_3 subspecies (Fig. 3.5). In addition to a smaller leaf area, the C_4 subspecies partitioned less biomass into leaves than the C_3 subspecies (Fig. 3.11b) despite the higher carbon cost of each leaf evident from the lower SLA in the C_4 subspecies (Fig. 3.6b). Although the C_4 subspecies produced a smaller leaf area (Fig. 3.4), these plants had larger relative growth rates at all levels of nitrogen supply (Fig. 3.7), and accumulated a greater biomass than the C_3 subspecies (Fig. 3.9). This shows that the greater efficiency of C_4 photosynthesis at the leaf scale, translates into a smaller investment into leaf canopy at the whole plant level, but with greater whole plant productivity. This contrasts with the hypothesis proposed by Long (1999) whereby the greater efficiency of C_4 photosynthesis enables the production of a greater leaf canopy to enhance whole plant productivity.

Under conditions of severe nitrogen limitation both subspecies had reduced leaf area production (Fig. 3.4) leading to smaller canopy leaf area (Fig. 3.5), with a corresponding decline in whole plant productivity. In the C_4 subspecies this reduced leaf area was associated with a reduction in the cost of leaves as shown by a lower SLA (Fig. 3.6b) and greater leaf turnover (Fig. 3.12); this was consistent with the findings by Rudman *et al*. (2001) where in two C_4 grasses, *Panicum coloratum* and *Cenchrus ciliaris*, the SLA decreased with decreasing nitrogen supply. Partitioning of biomass to leaves by the C_3 subspecies was also reduced by severe nitrogen limitation, with allocation by the C_3 subspecies reduced to that of the C_4 subspecies (Fig. 3.11b), where allocation was not reduced. Thus, the lower rates of photosynthesis and lower PNUE of the C_3 subspecies were likely to have a greater impact on whole plant productivity, than in the case of the C_4 subspecies, and this was reflected in faster growth rates (Fig. 3.7) and greater final biomass accumulation in the C_4 subspecies (Fig. 3.9). Reduced canopy leaf area was also associated with greater leaf turnover rates (Fig. 3.12) for both subspecies, and this reduced leaf area along with the re-allocation of nitrogen from senescing leaves was likely to account for the conservative response of photosynthetic rates and PNUE to nitrogen supply ('Potato-type response'; Vos and Biemond, 1992), and contrasts with the response of maize which maximises light interception at the cost of PNUE and leaf photosynthesis ('Maize-type response'; Vos *et al.*, 2005).

Biomass partitioning

Both subspecies had a similar allocation of biomass to underground components (Fig. 3.13b). Although, the C_3 subspecies invested more biomass in roots than the C_4 subspecies (Fig. 3.13a), this was offset by an increased biomass allocation to enlarged below-ground corms in the C_4 subspecies (Fig. 3.14). These corms were developed from vegetative culms and leaf bases which probably function as storage organs (Donald and Ripley, 2006), the greater investment in root biomass by the C_3 subspecies (Fig. 3.13a) could potentially be due to the alternative use of the roots for storage. In terms of the economic analogy presented by Bloom *et al*. (1985), the diversion of resources to storage rather than growth results in a loss of "compound interest" along with those costs associated with the translocation and conversion into compounds suitable for storage. However, the fitness benefits of storage may outweigh these costs in the future, by increasing survivorship or re-growth following disturbance.

Severe nitrogen limitation in both subspecies resulted in an increased allocation of biomass to under-ground components (Fig. 3.13b) and in the C_4 subspecies this had no effect on the allocation to corms (Fig. 3.14). Increased allocation to roots when nutrients are limited has been has been known for a long time (Berendse *et al.* 1999; Poorter and Nagel, 2000) and this increased allocation has been found to be as appropriate in cultivated grasses (Hocking and Meyer, 1991) and wild grasses (Tilman and Wedin, 1991; Ghannoum and Conroy, 1998; Ghannoum *et al*. 2005). According to the "Optimal allocation theory" (Bloom *et al.*, 1985), plants adjust their allocation so that all resources are equally limited, and they will make a greater allocation to organs that capture the most limiting resource in order to maintain their "functional equilibrium". Thus plants grown under low nutrient conditions will increase allocation to roots at the expense of leaves and shoots to increase nutrient uptake, and it is suggested that this trade-off is as a result of the diversion of carbohydrates into the root system in response to low nitrogen supply (Levang-Brilz and Biondini, 2002).

The greater biomass accumulated by the C_4 subspecies can also be accounted for by the greater investment in sexual reproduction. The total flower biomass, consisting of the inflorescence and supporting culm, was a major component of C_4 biomass (Fig. 3.15b), while for the C_3 subspecies it was insignificant, and that seed which is produced by the C_3 subspecies was found to have limited viability when compared with the C_4 subspecies.

In the additional experiment mentioned in Chapter 2, where seeds were collected from established plants and germinated. The germination rate for the seeds of the C_3 subspecies was found to be 23%, while that for the C_4 subspecies was 75%.

Low nitrogen supply resulted in a reduced allocation of biomass to flowers and it seems that these savings in biomass enabled the C_4 plants to maintain allocation to leaves (Fig. 3.11b) and enhance allocation of biomass to roots (Fig. 3.13a), in contrast with the C_3 plants where allocation of biomass to leaves was reduced (Fig. 3.11b) in order to enhance that to roots (Fig. 3.13a). The reduction of reproductive effort under conditions of severe nitrogen limitation has been show in previous investigations by Tilman and Wedin (1991), Sultan and Bazzaz (1993) and Sultan (2001), although the extent to which this reduction occurs varies in species with different ecological strategies. In the C_3 subspecies larger numbers of tillers were produced at high and medium levels of nitrogen supply than in the C_4 subspecies (Fig. 3.10) which suggests a predominance of vegetative reproduction. This represents a possible strategy employed by the C_3 subspecies to compensate for limited sexual reproduction, since vegetative reproduction has lower investment requirements, and along with the production of more tillers there would be no loss of productivity that is associated with investment in sexual reproduction.

The growth and phenology of the C_3 and C_4 subspecies of *A. semialata* has been monitored over two years in a common garden experiment by Ripley *et al*. (2007), who found that in each year all twenty C_4 plants flowered over the November-December period, which is likely to coincide with rising average daily temperatures. While, none of the C_3 plants flowered over the whole experiment (Ibrahim, *et al*., 2006), which suggests an alternative trigger mechanism was involved in reproductive culm initiation, like increased nutrient availability which could follow fire. Consequently, the final destructive harvest of the plants in this experiment, which occurred during mid-December, was timed to coincide with the annual phase of sexual reproduction. It was also seen that by the time that the plants were harvested, the relative leaf growth rate had declined to near zero for both subspecies, and were not significantly different (Fig. 3.7, Table 3.1). This indicates that both species had reached the end of their vegetative growth phase and suggests that differences in biomass allocation between the C_3 and C_4 subspecies represents an ecologically significant difference in allocation strategy, rather than an ontogenetic response to nitrogen treatment (Coleman *et al.*, 1994).

Increased biomass accumulation with increased nitrogen supply has been shown in C_3 and C_4 species of *Panicum* (Bowman, 1990; Ghannoum and Conroy, 1998) and a number of other grass genera (Bowler and Press, 1996; Rudman *et al*. 2001; Ghannoum *et al*. 2005). Similar increases in biomass accumulation with increased nitrogen supply were shown in this experiment, and allometric analysis indicates that the primary effect on leaves, roots and corms was a shift along a single allometric trajectory. Hence, nitrogen supply determined biomass partitioning to these components primarily through its effect on plant size (Poorter and Nagel, 2000; Müller *et al*., 2000; Cahill, 2003) and resulted in apparent plasticity, or plasticity in their growth rates (Weiner, 2004), rather than as a direct consequence of nitrogen availability, in contrast to the findings of Shipley and Meziane (2002) and Grechi *et al*. (2007). The allometric relationship for tillering and flowering were less direct, and there was considerable overlap at medium and high levels of nitrogen supply along the trajectory. This lack of allometric relationship for tillering and flowering could result from the plants having to reach a minimum size before these functions are initiated (Sultan, 2001; Weiner, 2004). The differences in biomass allocation patterns between subspecies result from differences in allometric trajectories (Table 3.3), and since the allometric relationships associated with optimal allocation are fundamental aspects of a genotype's strategy, and result from natural selection (Müller *et al*., 2000; Weiner, 2004).

Differences in the partitioning of biomass in the C_3 and C_4 subspecies of *A. semialata* support the idea of a mechanistic link between the photosynthetic type of a plant and the ecological strategy employed. They also suggest an additional strategy to those proposed by Long (1999) whereby higher photosynthetic rates and greater availability of nitrogen due to higher PNUE, enables C_4 plants to make greater investments in storage and sexual reproduction and at the expense of roots and leaves. While in C_3 plants greater investments of biomass in leaves and tillers suggests a predominance of vegetative growth and reproduction. However, these findings were from experiments where tillers were replanted and which have the ability to re-allocated resources, this would not occur during the establishment of seedlings and the response to different levels of nitrogen availability may be different. The same experimental design was attempted using germinated seeds, but establishment, especially at low nitrogen supply, was unsuccessful (data not shown) and suggests that recruitment may require levels of nutrient availability above that which is normally found in mesic grasslands.

Ecological implications

The C_3 and C_4 subspecies of *A. semialata* co-occur in the mesic grasslands of South Africa described as 'sourveld' (Ellis 1974). The range of the C_3 subspecies is restricted to areas of higher altitude which are characterized by lower temperatures and less rainfall, while the C_4 subspecies occurs at lower altitudes where temperatures and rainfall are higher (Ellis, 1974). Soils in these mesic grasslands are typically nutrient poor due to the relatively high rainfall which is associated with leached and infertile soil (Scholes and Walker, 1993), therefore it seems likely that both subspecies of *A. semialata* are adapted to low nutrient soils.

In the context of the African grasslands, one of the most important factors that determine vegetation structure is fire (van Wilgen *et al.*, 2003; Keeley and Rundel, 2005), this is significant because of the seasonal nature of rainfall with warm moist growing seasons which are associated with high biomass production and providing adequate fuel for fires. The frequency of fire is dependent on rainfall and in grasslands with higher rainfall fire is more frequent, because of their greater fuel loads (Scholes and Walker, 1993; Keeley and Rundel, 2005). With the high proportion of C_4 grasses occupying these habitats, there is usually an accumulation of above-ground detritus owing to the slow decomposition rate, a consequence of the high C:N ratio, and this detritus adds to the fuel load enhancing flammability (Knapp and Medina, 1999). The C_4 subspecies of *A. semialata* occupies habitats that are likely to experience a greater frequency of fire than the C_3 subspecies, since they are moist and a greater fuel load is likely to accumulate. Not all C_4 grasses found in the grasslands of southern Africa are tolerant of fire and Uys *et al.*, (2004) found that from a range of sites in South Africa that bunch grass species or ones forming tussocks are well adapted to recover after fire, although not able to tolerate shading. Bond and Keeley (2005) suggest that resprouting is the most effective plant trait for the survival of fires in grassy ecosystems where frequency is high. The C_4 subspecies of *A. semialata* maintains a high corm biomass over a range of levels of nutrient supply (Fig. 3.12) which is likely to function as a storage organ and this may enable resprouting after fire. Fires are often patchy in nature and recruitment from neighbouring unburned habitats is important (Scholes and Walker, 1993) in re-establishing the vegetation. The C_4 subspecies of *A. semialata* invests extensively in sexual reproduction (Fig. 3.15b), producing large amounts of seed, which seems to be initiated by the seasonal rise in daily temperatures during the November-

December period. This may be an additional strategy to enhance survival in fire prone environments. Conditions after fire where high light intensity is coupled with warm moist conditions and additional nutrient availability is likely to be advantageous to a species associated with basal meristems and fast growth rates (Keeley and Rundel, 2005).

The C_3 subspecies of *A. semialata* occur in habitats where conditions are cooler and more arid. Primary production in these habitats is limited and less biomass accumulates reducing the fuel load, this will lead to a reduction in the frequency of fires (Scholes and Walker, 1993; Keeley and Rundel, 2005). Under these conditions it seems likely that an ecological strategy favouring vegetative reproduction along with leaf and root growth may be advantageous. The closed canopies of these ecosystems will facilitate greater competition for light with little need for resprouting, greater below-ground competition for limited resources and little opportunity for seedling recruitment. When fires do occur, the pulse of additional nutrients may provide the trigger necessary for the initiation of flowering, as conditions post-fire favour seed germination.

CONCLUSIONS

- Biomass accumulation in the C_4 subspecies was greater than that of the C_3 subspecies across all levels of nitrogen supply and associated with a photosynthetic rate in the C_4 subspecies that was nearly twice that of the C_3 subspecies. Severe nitrogen limitation compromised growth in both subspecies, but had a greater impact on the C_3 subspecies.

- The C_3 subspecies made a greater allocation to leaf biomass than the C_4 subspecies. Severe nitrogen limitation had little effect on the partitioning of biomass to leaves in the C_4 subspecies, but allocation was greatly reduced in the C_3 subspecies. In the C_4 subspecies biomass allocation was maintained but with a reduction in canopy leaf area.

- In both subspecies partitioning of biomass to roots was increased with the reduction of nitrogen supply. This increased allocation was associated with a reduction in the allocation of biomass to leaves by the C_3 subspecies in conditions of severe nitrogen limitation. The C_3 subspecies made a greater investment in root biomass than the C_4 subspecies at all levels of nitrogen supply.

- The smaller biomass allocation made by the C_4 subspecies to roots enabled them to make a greater biomass allocation to corms than the C_3 subspecies. This allocation could form an energy storage strategy to enhance survival in disturbance-prone environments.

- The smaller biomass allocation made by the C_4 subspecies to leaves enabled them to make a greater biomass allocation to sexual reproduction, which could further enhance survival in disturbance-prone environments. This allocation was strongly influenced by nitrogen availability in conditions of severe nitrogen limitation when there was a significant reduction in allocation. The lack of allocation to sexual reproduction by the C_3 subspecies was possibly compensated for by greater allocation to vegetative reproduction in the form of tillering which may enhance survival in environments with closed canopies.

- Alterations in biomass allocation patterns which occurred as a result of nitrogen limitation were largely determined by plasticity of growth rates. The differences in biomass allocation patterns between the C_3 and the C_4 subspecies are a result of different allometric trajectories which are correlated to differences in photosynthetic mechanisms and give rise to different ecologic strategies.

CHAPTER 4

CO-PROVISION OF NITROGEN IN THE FORM OF AMMONIUM AND NITRATE

INTRODUCTION

Most of the nitrogen which is present in soil is in the form of organic nitrogen and it was shown at Nylsvlei that on nutrient rich soils (broad leafed savanna) the total ecosystem nitrogen is about 350 g N m^{-2}, of which only 8% is found in the soil (Scholes and Walker, 1993). In a process called mineralization, organic nitrogen is converted into ammonium ions, by soil micro-organisms, which are then oxidized via nitrite ions to nitrate ions in the process called nitrification by the bacteria, *Nitrosomonas spp.* and *Nitrobacter spp.* respectively (Miller and Cramer, 2004). In acid soils nitrification is limited and with a reduction of pH from 6 to one of 5, the rate of nitrification declines rapidly, and below a pH of 5 it is negligible. The response to acidity seems to depend on the strains of bacteria involved and nitrification has been shown to occur, to some degree, in soils with a pH of 4. Nitrification is also limited by anaerobic conditions, soil temperatures below 5^{o}C and above 40^{o}C and a lack of soil water (Miller and Cramer, 2004). Savanna soils are characteristically acidic and subject to long periods when soil moisture is lacking. Bate and Gunton (1982) report higher levels of ammonium than nitrate at Nylsvlei. This is supported by the assertion by Lewis (1986) that in acid soils, ammonium is the predominant form of inorganic nitrogen and that, in many grasslands of the world; ammonium is more abundant than nitrate. Root exudates of grasses which suppress nitrification, and may also play an important role in maintaining a greater abundance of ammonium (Bate and Gunton 1982). As ammonium ions are positively charged they are less likely to be leached from the soil where the particles are negatively charged, unlike nitrate ions which are negatively charged and are highly mobile within the soil (Miller and Cramer, 2004).

Nitrogen is also made available to plants via wet and dry deposition. Wet deposition occurs when ionizing radiation, e.g. lightning, converts atmospheric dinitrogen into ammonia which dissolves in rain drops to form ammonium ions. Oxides of nitrogen released during

vegetation fires or as a result of industrial pollution form nitric acid which dissociates in the rain to form nitrates. Airborne dust and ash constitutes dry deposition, and it contains both organic and inorganic nitrogen as ammonium and nitrate. Combined deposition rates vary considerably in different areas and can range between 2 and 19 kg N ha^{-1} y^{-1} (Lewis, 1986). Bate and Grunton (1982) report that at Nylsvlei the total deposition rate was 1.12 kg N ha^{-1} y^{-1}, of which 77% was in the form of ammonium. However, Scholes and Walker (1993) have shown some reservations about the findings by Bate and Gunton (1982) and suggest that the high ammonium levels in their findings may be artefacts due to the use of the specific ion electrode for ammonium determination. They suggest that a more realistic value would be closer to those values found at a study site 200 km southeast of Nylsvlei with the total deposition rate of 2.5 kg N ha^{-1} y^{-1}, of which ammonium deposition was 58 ± 2%. Scholes and Walker (1993) also report that in many hundreds of *in situ* mineralization tests performed at Nylsvlei, no significant ammonium was detected while large amounts of nitrate were detected. This would indicate that ammonification was taking place and that ammonium was not being accumulated because the rate of nitrification was equal to the rate of mineralization. However, the *in situ* measurement of mineralization relies on the measurement of the rate of inorganic nitrogen accumulation in soil from which root uptake is prevented. To achieve this, a 50 mm diameter stainless steel cylinder is knocked into the soil to a depth of 250 mm isolating the soil from the surrounding roots, and which is likely to prevent root exudates from influencing microbial activity and prevent any lateral movement of ions. It seems that there is a greater likelihood that both nitrate and ammonium are present in savanna soils, although ammonium may not constitute as much as 77% of the inorganic nitrogen as suggested by Bate and Gunton (1982).

Most plants are able to absorb both ammonium and nitrate from soil surrounding the roots; however, nitrate absorbed from the soil can be concentrated to far higher levels than those found in the soil. This transport has been shown to be an active, energy driven process, over almost the whole range of nitrate concentrations found in soils while efflux is passive. The uptake of each nitrate ion is coupled with the movement of two protons down an electrochemical potential gradient (Fig. 4.1), which is dependent on ATP enabling the enzyme ATPase to transfer protons across the plasma membrane and maintain the H^+ gradient (Miller and Cramer, 2004).

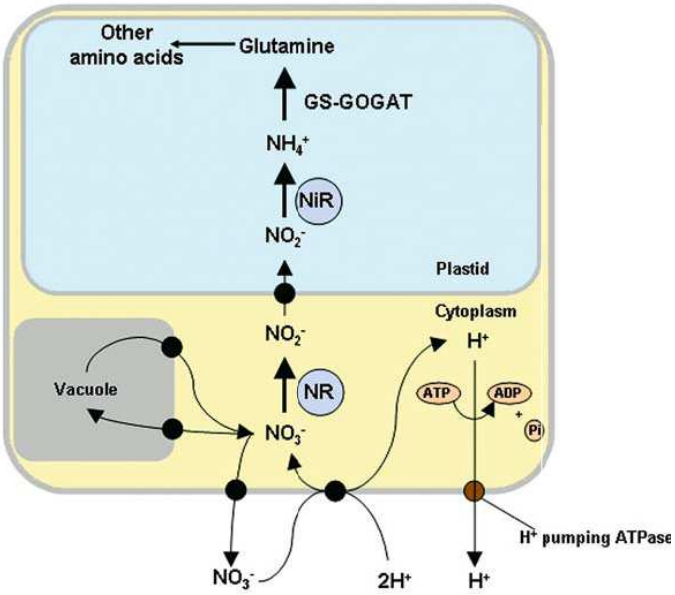

Fig. 4.1: Schematic diagram of nitrate uptake and assimilation of plant cells. *Abbreviations*: nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate-2-oxoglutarate aminotransferase (GOGAT).
(After Miller and Cramer, 2004)

Uptake also only occurs in the presence of transporter proteins, referred to as the nitrate permeases, which transport the nitrate across the plasma membranes of epidermal and cortical cells of the roots (Lewis, 1986; Miller and Cramer, 2004). The uptake of nitrate from the soil has also been widely reported to be associated with an increase in pH (Lambers *et al*. 1998; Britto and Kronzucker, 2002). This is associated with the generation of hydroxyl ions during its reduction, some of which are excreted, and some neutralized by the formation of organic acids, mainly malic acid, from sugars (Lambers *et al*. 1998). Nitrate appears to be non-toxic to plants and large concentrations can accumulate in their tissues (particularly by members of the Chenopodiaceae). The assimilation of nitrate requires its prior reduction to ammonium ions, this occurs in two stages, initially to nitrite ions via the enzyme nitrate reductase (NR), which is then further reduced to ammonium ions via the enzyme nitrite reductase (NiR), these enzymes are found in both the roots and leaves. The reduction of nitrate (Fig. 4.1) is an energy consuming process requiring, requiring approximately 15% of plant-available energy when it occurs in the roots with an additional 2 to 5% of available energy for ammonium assimilation. This represents a significant loss of energy, and constitutes the greatest disadvantage of nitrate as a source of

nitrogen to the plant, compared with ammonium where reduction is obviated (Lambers *et al*. 1998).

The uptake of ammonium has been shown to be both active and passive, and more rapid than that of nitrate (Murphy and Lewis, 1987). There is evidence that ammonium transport into plant cells occurs by means of transporter proteins and this is mediated by the co-transport of protons, as in the case of nitrate, but with a lower energy requirement. It has also been suggested that ammonium uptake can occur through potassium channels in the plasma membrane and like the uptake of potassium ions, it is driven by the negative membrane potential. There is evidence that the utilization of these potassium channels form an important route for ammonium uptake by roots, despite the disruption of their activity in potassium transport (Miller and Cramer, 2004). The uptake of ammonium is associated with a decrease in soil pH and it has been suggested that this occurs due to the efflux of protons from the plant in response ammonium absorption and compensating for the charge imbalance (Lewis, 1986; Britto and Kronzucker, 2002), this acidification of the rhizosphere is probably one of the major causes of toxicity due to ammonium nutrition in plants (Miller and Cramer, 2004). Ammonium uptake also depends on the availability of carbohydrates in the roots to a greater extent than for nitrate uptake. These carbohydrates provide the carbon skeletons for the synthesis of amino acids from the ammonium which would otherwise become toxic (Marschner, 1995). The high photosynthetic rates of C_4 plants allow these plants to meet the requirement for carbohydrate, and in so doing avoid ammonium ions accumulating in plant tissues, which can occur in C_3 plants (Cramer and Lewis, 1993; Miller and Cramer, 2004). Associated with the higher demand of carbohydrates in ammonium fed plants, is the reduction in root to shoot ratios, which has been shown in both C_3 and C_4 plants (Cramer and Lewis, 1993).

Miller and Cramer (2004) report that many studies have shown that plants benefit from a mixture of both nitrate and ammonium, although the optimal ratio and concentration of the nitrate and ammonium may vary in different species of plant, the age of the plant and the pH of the growth medium. Britto and Kronzucker (2002), reviewing the co-provision of ammonium and nitrate, suggest that it induces a “synergistic growth response”. This growth response can exceed maximal growth rates when supplied with nitrogen as ammonium or nitrate alone by as much as 40 to 70% in solution culture, although somewhat less in soil. Herberer and Below (1989) found that in two varieties of spring

wheat that were hydroponically grown, co-provision resulted in the increased production of dry biomass of 36% and 76% more than when nitrate was provided alone. They found that co-provision had no effect on the partitioning of biomass, although it did increase tillering. Seminal in the attempts to explain this synergistic growth response is the stimulation of the biochemical response, e.g. maximizing the synthesis of the plant growth-promoting hormone –cytokinin. Another important effect is that the increase in pH of the rhizosphere associated with nitrate nutrition is ameliorated by the decrease in pH associated with ammonium nutrition. Furthermore, bacterial activity can rapidly convert ammonium in the soil to nitrate and this too can have an acidifying effect, preventing the soil pH from deviating from its optimum (Miller and Cramer, 2004). In rice the efflux of nitrogen is also considerably reduced with the co-provision of ammonium and nitrate, and Kronzucker *et al*. (1999) found that the combined nitrogen uptake can be up to 75% higher than when the same concentration of nitrogen is supplied in the form of either ammonium or nitrate. Using maize xylem sap analysis, Murphy and Lewis (1987) showed that far more nitrogen reached the shoot with co-provision than either nitrate or ammonium alone. There is also significantly greater allocation of nitrogen to the shoot with co-provision, and Britto and Kronzucker (2002) suggest that possibly the most important synergistic response of co-provision of ammonium and nitrate is the enhanced translocation of nitrogen to the shoot with the obvious implications for photosynthesis and productivity.

Much of the translocation via the xylem is un-metabolized nitrate as its reduction is driven by photochemical energy and occurs largely in the leaves, the remainder consists mainly of the product of ammonium assimilation. Root assimilation of ammonium has been shown to be enhanced by the presence of nitrate and is explained by the induction of the GS-GOGAT pathway of the proplastids of the roots by nitrate (Fig. 4.1). This opens up a pathway not available to ammonium assimilation in the absence of nitrate. The GS-GOGAT pathway is believed to be the major pathway for ammonium assimilation (Lewis, 1986; Miller and Cramer, 2004). Glutamine synthetase (GS) catalyses the ATP-dependent amination of glutamate, amination being the addition of the amide group ($-NH_2$). The amination of glutamate produces glutamine and the enzyme glutamine:oxoglutarate aminotransferase (GOGAT) catalyses the transfer of the amide group from glutamine to 2-oxoglutarate to produce two molecules of glutamate. The presence of nitrate could possibly also be responsible for the alleviation of ammonium toxicity through its ability to be reduced in the

shoot, thus moderating the differential allocation of carbon between the roots and shoots and improving electron flow between photosystems I and II (Britto and Kronzucker, 2002).

The key research questions

It seems likely that *A. semialata* grows naturally in environments where both nitrate and ammonium are to be found in the soil. Although the C_3 subspecies would be more likely to be adapted to soils with higher nitrate levels that those of the C_4 subspecies, since the C_3 subspecies is endemic in areas with less rainfall and where soils would be less leached than the soils on which the C_4 subspecies is endemic (Chapter 3). The aim of this section of the investigation is to determine whether the co-provision of ammonium and nitrate induces a synergistic growth response that exceeds that with the supply of nitrate alone for *A. semialata*, and whether the supply of nitrogen in excess of that which is typically found in savanna soils affects photosynthesis and growth and allocation to vegetative plant components. The specific questions are:

1. What effect will nitrogen, when in the form of either nitrate or ammonium plus nitrate, have on the rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1}, and the photosynthetic parameters of: (a) CE (b) A_{max}, and (c) the Γ, when supplied at different levels?

2. What effect will nitrogen, when in the form of either nitrate or ammonium plus nitrate, have on the accumulation of biomass and its allocation to tillers, roots, leaves, and corms, when supplied at different levels?

3. Will the supply of nitrogen have a different effect on the C_3 subspecies from the effect on the C_4 subspecies of *A. semialata*, when in the form of nitrate or ammonium plus nitrate and supplied at different levels?

METHODS

Growth and treatment of Plants

In a separate experiment tillers of the same clone of the C_4 subspecies were obtained from the collection of potted genets – the same genets that were used as the source of tillers for the previous experiment. Tillers of the C_3 subspecies were obtained from a single genet that was collected from grasslands outside of Grahamstown. The roots of these tillers were thoroughly washed with tap water to remove any adhering soil and the shoot trimmed to 10 cm above the base of the corm. The tillers were then planted in sand and sprayed with a commercial organic fungicide (Dithane® M45, Rohm & Haas Co., Philadelphia, U.S.A.) and after one week, on the 15th and 16th of March 2006, they were removed from the sand and individually planted in 7 L pots filled with washed river sand. Clones from single genets were used in order to avoid variation due to genotypic variation and the tillers were planted so that only 5 cm of shoot emerged from the sand with each tiller planted to exactly the same depth. Only one tiller was planted per pot since the pots had approximately one third the capacity of the pots used in the previous investigation. The pots were placed on drip trays and maintained in the same clear polythene tunnel used in the previous investigation. A randomized block design was used with C_3 and C_4 subspecies alternating and the blocks were rotated every week to prevent an edge effect from occurring.

The potted tillers were watered with tap water for 4 weeks, after which pots received one of five treatments, the first of which was on the 13th April 2006. As in the previous investigation the pots were watered twice weekly with Long Ashton's solution (Hewitt, 1966) and any seepage from the pots was collected in the trays and reapplied. Three levels of nitrogen were used with nitrogen in the form of either nitrate (one third potassium nitrate and two thirds calcium nitrate), or ammonium nitrate and which were calculated on the assumption that the plants would use all nitrogen available:

1. High nitrogen (nitrate): equivalent to 10 g N m^{-2} y^{-1}.
2. High nitrogen (ammonium nitrate): equivalent to 10 g N m^{-2} y^{-1}.
3. Medium nitrogen (nitrate): equivalent to 5 g N m^{-2} y^{-1}.
4. Medium nitrogen (ammonium nitrate): equivalent to 5 g N m^{-2} y^{-1}.

5. Low or zero nitrogen: no additional nitrogen added.

The high nitrogen level was chosen because it was higher than that found in nutrient rich soils at Nylsvlei (Scholes *et al.*, 1997). The medium nitrogen level was mid-way between 3.5 and 7 g N m^{-2} y^{-1}, as these levels of nitrogen supply elicited little difference in response in most of those parameters that were measured in the previous experiment. Prior to the application of the first treatment, shoots of all tillers were again trimmed to 5 cm above the level of the sand so that all the leaves were removed although leaf sheaths remained. This was carried out so that no re-allocation from senescent leaves could take place (in contrast with the previous experiment) and the effect of re-allocation of nitrogen between C_3 and C_4 plants in different nutrient treatments would be the same. There were 8 replicates of each treatment for each subspecies.

Determination of leaf photosynthesis, leaf area and biomass

After 21 weeks of treatment on the 8th of September 2006, four plants of the C_4 subspecies were randomly selected from each treatment except the high nitrogen (ammonium nitrate) treatment where five plants were selected, and the rate of photosynthesis and photosynthetic parameters were determined as in the previous experiment (Chapter 2). Due to undetermined factors C_3 plants in this experiment performed poorly, became chlorotic, and many died. Plants of the C_4 subspecies showed none of these symptoms and hence research aims were restricted to a C_4 subspecies by treatment comparison.

The following week (after 22 weeks of nutrient treatment), before inflorescences had been produced, 6 plants of the C_4 subspecies were randomly selected from each treatment and destructively harvested and separated into plant components as in the previous experiment (Chapter 3). The projected leaf areas were found for the whole plants and used to determine specific leaf area (SLA) leaf area ratio (LAR).

Statistical Analyses

All statistical comparisons were made by means of the program Statistica version 7 (© Statsoft, Inc., Tulsa, USA) as in the previous experiment. Those plants supplied with ammonium plus nitrate were compared with those supplied with nitrate alone and between the three different levels of supply, along with the interaction of these treatments, using factorial ANOVA, where low nitrogen was represented as low nitrogen (nitrate) and repeated as low nitrogen (ammonium nitrate). Where data were heteroscedastic and variance not homogeneous, they were appropriately transformed, with log transformation for A and A_{max}, and arcsine transformation for CE. Where main effects were significant at the 95% confidence level, Tukey *post-hoc* comparisons were made, and averages were given ± standard error. Allometric trajectories were compared between those plants supplied with ammonium plus nitrate were compared with those supplied with nitrate alone with plant size as in Chapter 3, using a general linear model (GLM) with plant size as a continuous predictor.

RESULTS

Photosynthetic rate and the photosynthetic parameters

The rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} of those plants which received nitrogen at a level equivalent to 10 g N m^{-2} y^{-1} was significantly higher than those receiving medium and low levels of nitrogen supply for both plants receiving the co-provision of ammonium and nitrate, and those receiving nitrate alone (Fig. 4.2). The greatest increase in rate was observed for those plants which received the co-provision of ammonium and nitrate, although not significantly so.

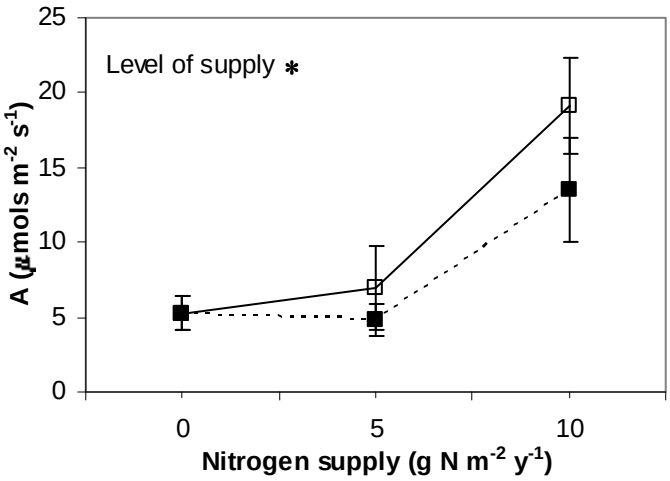

Fig. 4.2: Rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} (A) for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■, and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 4 or 5). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The CO_2 response curves reflected the enhanced rate of photosynthesis at nitrogen levels equivalent to 10 g N m^{-2} y^{-1} (Fig. 4.3a and b), with significantly higher carboxylation efficiency (CE) and CO_2 saturated photosynthetic rate (A_{max}) for the high levels of nitrogen supply (Table 4.1 and 4.2). A_{max} for the co-provision of ammonium plus nitrate exceeded that for nitrate alone, although not significantly. The CO_2 compensation point (Γ) was not significantly different for the different treatments (Table 4.1) with the lowest value of 12.1 ± 5.5 μmol mol^{-1} within the range for C_4 plants (Table 1.1), and the higher values having standard errors of up to 23.8 μmol mol^{-1}.

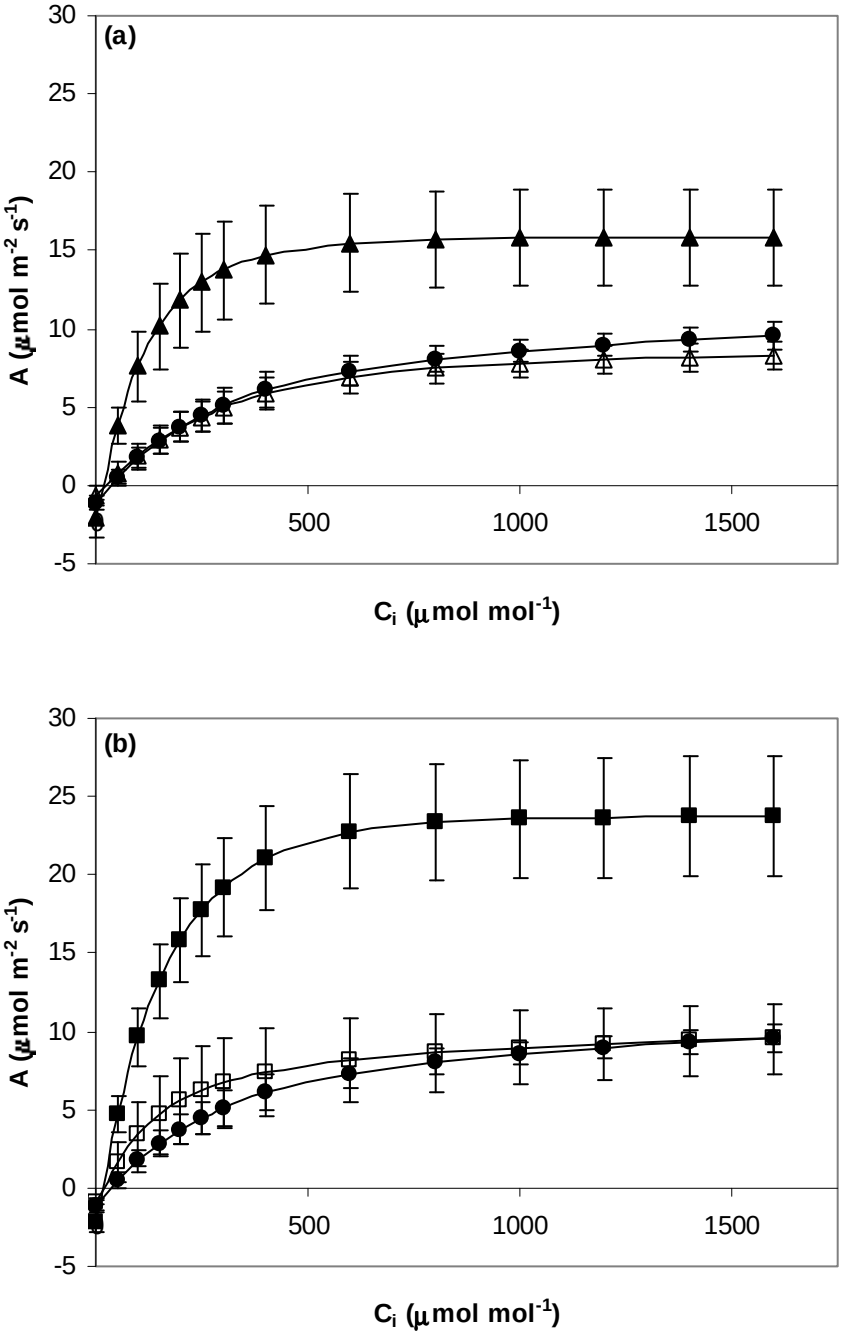

Fig. 4.3: Fitted CO_2 response curves of net assimilation (A) for the C_4 subspecies of *A. semialata*. (a) Nitrate provision at 5 g N m^{-2} y^{-1} represented by: Δ, and 10 g N m^{-2} y^{-1} by: ▲. (b) The co-provision of nitrate plus ammonium at 5 g N m^{-2} y^{-1} by: □, and 10 g N m^{-2} y^{-1} by: ■. Zero nitrogen supply shown in (a) and (b) by: • All mean values with vertical bars representing standard errors (n = 4 or 5).

Table 4.1: Photosynthetic parameters for the C_4 subspecies of *A. semialata* are shown for apparent carboxylation efficiency (CE), the CO_2 compensation point (Γ) and the CO_2 saturated photosynthetic rate (A_{max}) at the indicated levels of nitrogen supplied as nitrate or co-provided as ammonium nitrate. Within each column, values assigned the same letter (Tukey *post-hoc*) represent homologous groups at the 95% confidence level. All values are means ± standard error (n = 4 or 5).

Form of nitrogen	**N supply ($g\ N\ m^{-2}\ y^{-1}$)**	**CE ($mmol\ m^{-2}\ s^{-1}$)**	**A_{max} ($\mu mol\ m^{-2}\ s^{-1}$)**	**Γ ($\mu mol\ mol^{-1}$)**
Nitrate	10	147 ± 61 [b]	15.8 ± 3.1 [b]	12.1 ± 5.5 [a]
	5	31 ± 5 [a]	8.5 ± 0.9 [a]	29.7 ± 21.7 [a]
Ammonium Nitrate	10	152 ± 30 [b]	23.7 ± 3.8 [b]	17.4 ± 5.5 [a]
	5	61 ± 30 [a]	10.0 ± 2.1 [a]	41.0 ± 23.8 [a]
	0	35 ± 8 [a]	11.8 ± 2.9 [a]	45.3 ± 19.0 [a]

Table 4.2: Factorial ANOVA results for the rate of photosynthesis and photosynthetic parameters for the C_4 subspecies of *A. semialata* compared between the form of nitrogen and level of nitrogen supply, and the interaction between nitrogen supply treatments. Where n.s. indicates no significant difference and n = 4 or 5.

Parameter	**Significant difference**	**F**	**d f**	**Approx. p <**
A	Form	0.7	1,19	0.42 (n.s.)
	Level of Supply	11.2	2,19	0.001
	Form*level of supply	0.2	2,19	0.82 (n.s.)
CE	Form	0.4	1,19	0.57 (n.s.)
	Level of Supply	10.9	2,19	0.001
	Form*level of supply	0.1	2,19	0.87 (n.s.)
A_{max}	Form	1.06	1,19	0.32 (n.s.)
	Level of Supply	6.8	2,19	0.006
	Form*level of supply	0.5	2.19	0.63 (n.s.)
Γ	Form	0.6	1,19	0.7 (n.s.)
	Level of Supply	1.8	2,19	0.2 (n.s.)
	Form*level of supply	0.1	2,19	0.95 (n.s.)

Abbreviations: Photosynthetic rate at an ambient CO_2 concentration of 370 $\mu mol\ mol^{-1}$ (A), apparent carboxylation efficiency (CE), the CO_2 saturated rate of photosynthesis (A_{max}), the CO_2 compensation point (Γ).

Biomass accumulation and partitioning

The total biomass accumulation was not significantly affected by either the level of nitrogen supply or the provision of nitrate versus the co-provision of nitrate plus ammonium, over the period of the experiment (Table 4.3). However, the graph below (Fig. 4.4) indicates a trend for greater biomass accumulation at the higher levels of nitrogen supply and greater biomass accumulation with the co-provision of ammonium plus nitrate, which correlates with the greater rates of photosynthesis that were measured. This seems to indicate that seasonal variation in growth is important as the biomasses recorded in this experiment were much lower than in the previous experiment where the plants were grown for a similar period of time, but harvested in December, three months later in the year (see Chapter 2).

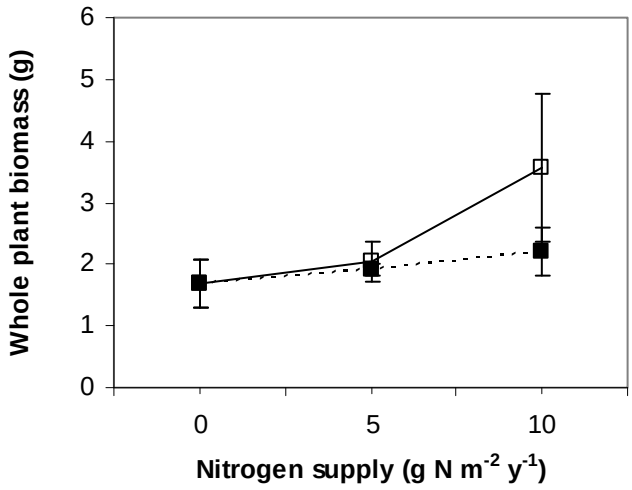

Fig. 4.4: Total biomass accumulated excluding moribund tissue for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■ and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 6). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Those plants receiving high nitrogen (10 g N m^{-2} y^{-1}) as ammonium plus nitrate produced significantly more tillers than all other treatments and there was a significant interaction effect (Table 4.3), although the variance of data was not homogeneous due to the limited growth of those plants receiving zero nitrogen (Fig. 4.5).

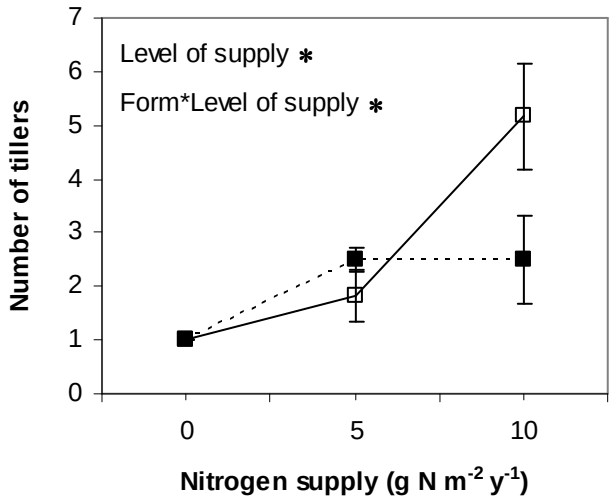

Fig. 4.5: The number of tillers produced for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■ and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 6). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

The percentage leaf biomass of those plants subjected to severe nitrogen limitation was significantly lower than those receiving nitrogen (Table 4.3), whether as nitrate or as ammonium plus nitrate (Fig. 4.6).

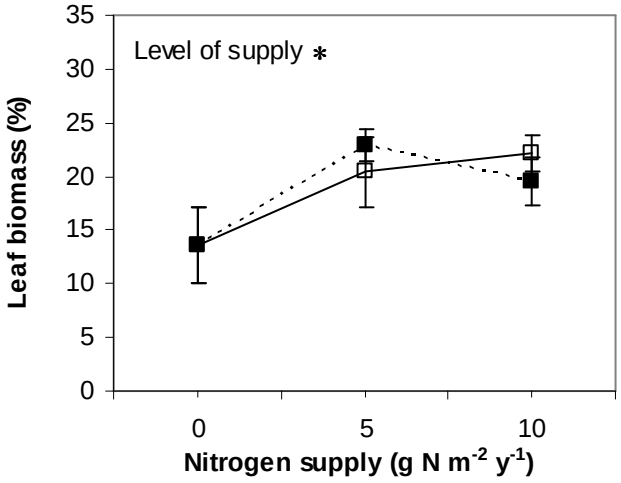

Fig. 4.6: Percentage leaf biomass for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■ and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 6). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Canopy leaf area deceased significantly with decreasing nitrogen availability (Fig. 4.7a). Whole plant investment in leaf area was also affected by severe nitrogen limitation and this was shown by the LAR, which was a significantly lower for those plants receiving zero nitrogen (Fig. 4.7b, Table 4.3). However, there was no significant interaction effect for either canopy leaf area or LAR. Investment of carbon in leaf area in terms of the SLA was not significantly different for different treatments and the mean SLA for all treatments was found to be 99.1 ± 1.9 cm^2 g^{-1}, while in the previous experiment it was found to be 96.0 ± 1.9 cm^2 g^{-1} (Chapter 3).

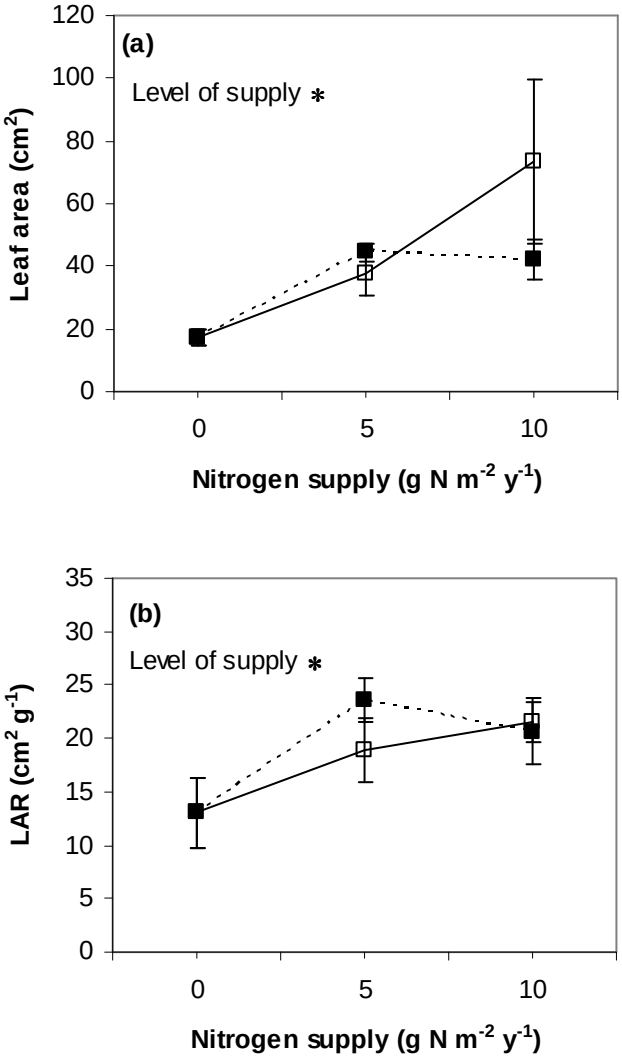

Fig. 4.7: (a) Canopy leaf area, and (b) Leaf area ratio – LAR for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■ and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 6). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Investment in below-ground biomass by those plants subjected to severe nitrogen limitation was higher than for the other treatment, this was borne out by the significantly greater below-ground biomass to above-ground biomass ratio for those plants (Fig. 4.8a, Table 4.3). The percentage root biomass was also significantly higher for the plants subjected to severe nitrogen deficiency (Fig. 4.8b, Table 4.3). However, the percentage corm biomass shows no significant difference for different treatments (Table 4.3).

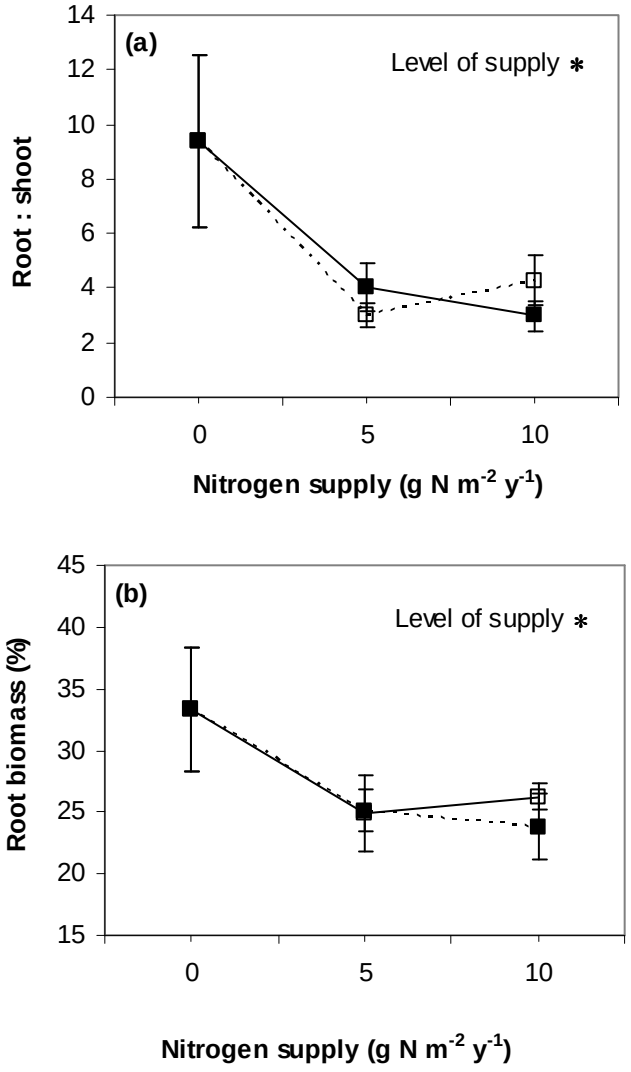

Fig. 4.8: (a) Below-ground to above-ground biomass ratio and (b) the percentage root biomass for the C_4 subspecies of *A. semialata* at three different levels of nitrogen supply. Nitrate supply represented by: ■ and the co-provision of nitrate plus ammonium by: □. All mean values with vertical bars representing standard errors (n = 6). Significant differences at the 95% confidence level (Tukey *post-hoc*) represented by: ∗.

Table 4.3: Factorial ANOVA results for the biomass parameters for the C_4 subspecies of *A. semialata* compared between the form of nitrogen and level of nitrogen supply, and the interaction between nitrogen supply treatments. Where n.s. indicates no significant difference and n = 4 or 5.

Parameter	**Significant difference**	**F**	**d f**	**Approx. p <**
Whole plant biomass	Form	1.4	1,19	0.3 (n.s.)
	Level of Supply	2.4	2,19	0.12 (n.s.)
	Form*level of supply	0.9	2,19	0.44 (n.s.)
Number of tillers	Form	2.1	1,19	0.2 (n.s.)
	Level of Supply	12.9	2,19	0.001
	Form*level of supply	4.9	2,19	0.014
Canopy leaf area	Form	0.8	1,19	0.4 (n.s.)
	Level of Supply	6.3	2,19	0.01
	Form*level of supply	1.5	2,19	0.3
Percentage leaf biomass	Form	0.001	1,19	0.98 (n.s.)
	Level of Supply	5.1	2,19	0.013
	Form*level of supply	0.1	2,19	0.66 (n.s.)
LAR	Form	0.3	1,19	0.59 (n.s.)
	Level of Supply	5.6	2,19	0.009
	Form*level of supply	0.6	2,19	0.58 (n.s.)
SLA	Form	1.9	1,19	0.19 (n.s.)
	Level of Supply	0.1	2,19	0.91 (n.s.)
	Form*level of supply	0.5	2,19	0.62 (n.s.)
Below-ground to above-ground ratio	Form	0.3	1,19	0.61 (n.s.)
	Level of Supply	5.3	2,19	0.02
	Form*level of supply	1.0	2,19	0.4 (n.s.)
Percentage root biomass	Form	0.1	1,19	0.8 (n.s.)
	Level of Supply	3.9	2,19	0.032
	Form*level of supply	0.1	2,19	0.92 (n.s.)
Percentage corm biomass	Form	0.1	1,19	0.72 (n.s.)
	Level of Supply	0.1	2,19	0.94 (n.s.)
	Form*level of supply	0.6	2,19	0.59 (n.s.)

Allometric trajectory

The biomass of roots and corms along with leaves receiving ammonium plus nitrate increased with a corresponding increase in whole plant biomass to within 70%, irrespective of the level of nitrogen supply (Fig. 4.9b and Fig. 4.10). This suggests that the allometric relationship was unaltered by nutrient treatment, but there was a greater degree of overlap between different treatments (indicated by the different symbols in Fig. 4.9 and Fig. 4.10) than was seen in the previous experiment described in Chapter 3.

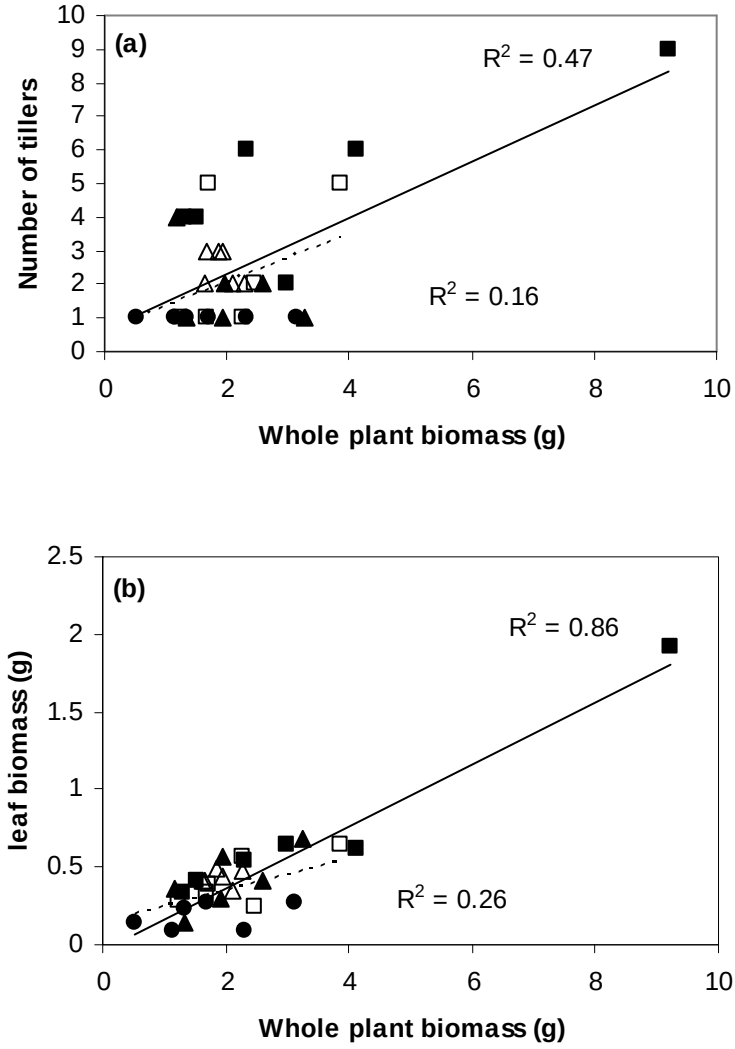

Fig. 4.9: Allometric analysis of (a) number of tillers produced and (b) leaf biomass for the C_4 subspecies of *A. semialata*. Nitrate supply represented by open symbols and the co-provision of nitrate plus ammonium by closed symbols, indicated as: 10 g N m^{-2} y^{-1}: □ and ■, 5 g N m^{-2} y^{-1}: Δ and ▲, and 0 g N m-2 y-1: ●.

The allometric relationships were weaker for the number of tillers produced and leaf biomass for those plants treated with nitrate only (Fig. 4.9a and b), and the allometric relationships were only significantly different for corm biomass between different forms of nitrogen supply (Table 4.4).

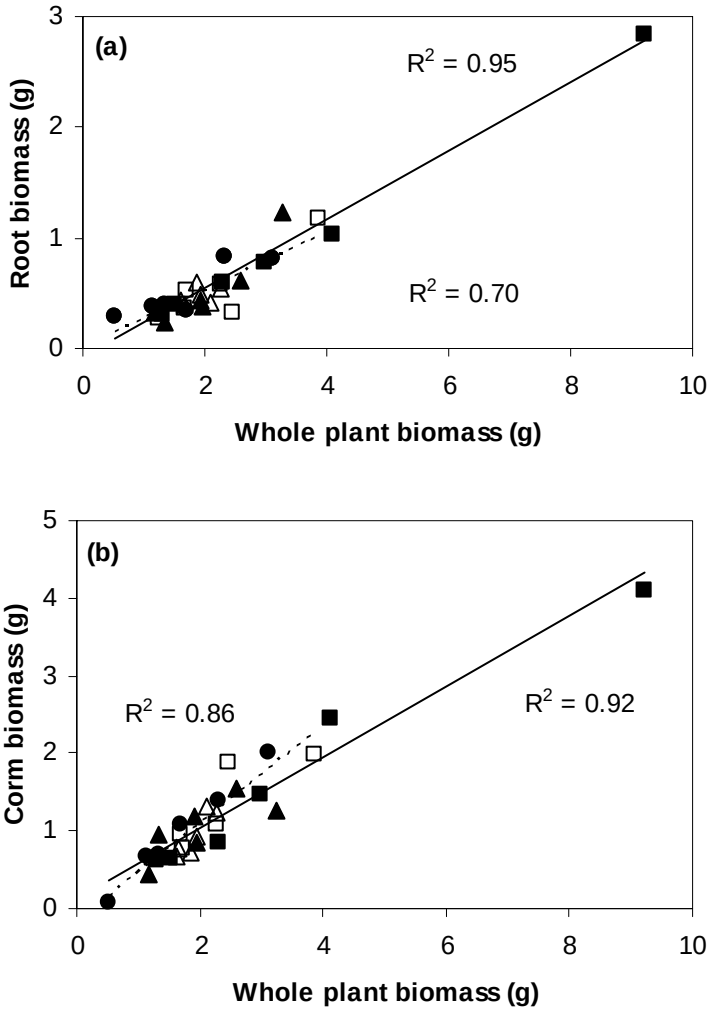

Fig. 4.10: Allometric analysis of (a) root biomass and (b) corm biomass for the C_4 subspecies of *A. semialata*. Nitrate supply represented by open symbols and the co-provision of nitrate plus ammonium by closed symbols, indicated as: 10 g N m^{-2} y^{-1}: □ and ■, 5 g N m^{-2} y^{-1}: Δ and ▲, and 0 g N m-2 y-1: ●.

Table 4.4: Linear effects model for the partitioning of biomass to tillers, leaves, roots and corms in the C_4 subspecies of *A. semialata.* Analysis accounted for the interaction form of nitrogen with plant size.

Parameter	**Significant difference**	**F**	**Approx. p <**
Number of tillers	Whole plant biomass	8.5	0.0007
Leaf biomass	Whole plant biomass	35.0	0.0001
Root biomass	Whole plant biomass	151.4	0.0001
Corm biomass	Whole plant biomass	181.9	0.0001
	Form*whole plant biomass	5.0	0.033

DISCUSSION

Photosynthetic rate and the photosynthetic parameters

In the C_4 subspecies of *A. semialata* an increase in the rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} was only observed when the level of nitrogen supply exceeded that of nutrient-rich savanna soils (Fig. 4.2). Increased photosynthetic rates have been shown in a number of investigations (Bolton and Brown, 1980; Sage and Pearcy, 1987; Li, 1993; Vos *et al*., 2005) and in those investigations the plants were exposed to very high levels of nitrogen supply, higher than those found in savanna soils. The apparent, but not significant, increase in photosynthetic rate with co-provision of ammonium plus nitrate (Fig. 4.2, Table 4.2) may have been due to the reported enhanced translocation of nitrogen to the shoot (Britto and Kronzucker, 2002). CE increased similarly with both co-provision of ammonium plus nitrate and nitrate alone (Table 4.1), and although A_{max} increased with increased nitrogen supply, those plants supplied with high levels of nitrate plus ammonium had higher A_{max} values than those plants supplied with nitrate alone (Table 4.1). However, since photosynthesis occurred closer to the asymptote of the CO_2 response curve in the nitrate fed plants than those receiving both ammonium and nitrate; the higher A_{max} values by those plants receiving ammonium plus nitrate had little effect on photosynthetic rates. It is also worth noting that neither the CE, nor A_{max} was significantly

different between zero nitrogen supply and that of 5 g m^{-2} y^{-1} irrespective of the form of supply. This in part confirms the findings reported in Chapter 2 where photosynthetic rates did not vary with different treatments.

Total biomass accumulation and partitioning

The greater biomass accumulation with the co-provision of nitrate plus ammonium has been widely reported (Weissman, 1964; Heberer and Below, 1989; Britto and Kronzucker, 2002), although this effect is most accentuated in hydroponically grown plants. In this experiment no statistically significant increase in biomass accumulation was observed (Table 4.3), although a trend for greater accumulation was obvious (Fig. 4.4). This lack of statistical significance may have been as a result of the absence of a nitrification inhibitor to the sand, but it may also be due to the plants being harvested in September before allocation to reproduction had taken place and prior to the plants entering a major growth phase. Heberer and Below (1989) found that in two varieties of wheat, the co-provision of nitrate plus ammonium resulted in an increase in grain yield and in the number of tillers, and they suggest that the increased rate of tillering can account for the increased grain yields with co-provision. The significantly larger number of tillers produced by *A. semialata* with high levels of ammonium plus nitrate (Fig. 4.5, Table 4.3) could indicate the potential for increased biomass accumulation with time, if one considers the number of tillers produced by a genet as an indication of the potential for growth. However, it could also indicate an increased allocation to reproductive effort, which not only confirms the findings for the C_4 subspecies of *A. semialata* in Chapter 3, but also corresponds with the enhancement of nitrogen translocation to the shoot with co-provision.

In this experiment the leaves were removed from tillers prior to the first treatment. This was carried out to prevent the re-allocation of resources from old leaves to new, while in the previous experiment only dead leaves were removed so that leaf turnover could be monitored, but non-senescent leaves were left intact allowing re-allocation. The likely result of preventing the re-allocation of resources was the low percentage leaf biomass of those plants subjected to severe nitrogen limitation compared to those receiving nitrogen, whether as nitrate or as ammonium plus nitrate (Fig.4.6). LAR is an indication of whole plant investment in leaf area and the lower LAR at zero nitrogen supply (Fig. 4.7b) further

supports this hypothesis. In the previous experiment, where the leaves were not excised, the LAR was little affected by severe nitrogen limitation (Chapter 3). The carbon cost of leaf area indicated by the SLA was not affected by either the level of nitrogen supply or the form of nitrogen supplied (Table 4.3), and largely confirms the findings for the C_4 subspecies of *A. semialata* in the previous experiment (where the mean SLA = 95.9 cm^2 g^{-1}), and this subspecies was found to have little plasticity in its functional attributes (Chapter 3).

The increased investment in root biomass in conditions of severe nitrogen deficiency (Fig. 4.8b) confirms the findings in Chapter 3, where it was found that *A. semialata* is able to adjust its allocation patterns so that all resources are equally limited, making a greater allocation to organs that capture the most limiting resource and maintaining "functional equilibrium". The lack of significant difference in allocation to the corm between different treatments (Table 4.3) is also consistent with the findings in the previous experiment (Chapter 3). In the C_4 subspecies, energy storage within the corm may confer a competitive advantage in environments subject to disturbance and a lack of plasticity in an attribute of such importance is not likely to be affected by different levels of nitrogen supply, or the form of the nitrogen supplied.

Allometric analysis of leaf, root and corm biomass (Fig. 4.9b and 10) shows strong relationships to plant size, while the poorer relationship shown for tillering (Fig. 4.9a) is consistent with the findings in the previous experiment. The overlap for the different treatments, particularly at the medium and high levels of nitrogen supply, was probably due to the low growth rates during the period of the experiment and supports the finding in the previous experiment where the C_4 subspecies of *A. semialata* accumulated most biomass during the flowering period of November-December (Chapter 3). The significantly different trajectory brought about by co-provision of ammonium and nitrate for corm biomass (Table 4.4) may result from the trend for greater biomass accumulation and suggests the significance of storage in this subspecies.

CONCLUSIONS

- The supply of nitrogen at levels higher than those found in savanna soils resulted in an increase in the rate of photosynthesis at an ambient CO_2 concentration of 370 μmol mol^{-1} and at these levels of nitrogen supply the rate of photosynthesis was higher with the co-provision of nitrate plus ammonium than with nitrate alone, although not significantly so.

- CE and A_{max} are both increased by a supply of nitrogen that is higher than that found in savanna soils. The co-provision of ammonium plus nitrate had no effect on the CE, but resulted in a higher A_{max} than with nitrate alone at these levels of nitrogen supply. However, this had no significant effect on ambient photosynthetic rates since photosynthesis operates at a point below A_{max}.

- Co-provision of ammonium plus nitrate at levels higher than those found in savanna soils resulted in a greater biomass accumulation and the production of a significantly larger number of tillers than either the provision of nitrate alone, or lower levels of nitrogen supply.

- Severe nitrogen limitation resulted in a significantly lower partitioning of biomass the leaf tissue and leaf area, and a greater partitioning of biomass to roots. The co-provision of ammonium plus nitrate did not affect biomass allocation.

- Partitioning of biomass to corm was not affected by either nitrogen limitation or co-provision of ammonium plus nitrate.

CHAPTER 5

CONCLUSIONS

The principal objective of this investigation was to test the hypothesis proposed by Long (1999) who suggested that C_4 species exploit their higher PNUE by not simply having greater productivity at the whole plant level than C_3 species, but by making a greater allocation to those organs that capture the most limiting resource. Two contrasting ecological strategies were hypothesized whereby the C_4 species could either produce a greater leaf area with the same amount of nitrogen and in so doing enhance whole plant photosynthetic rates and conferring a greater ability to compete for light, or produce the same leaf area and partition a greater portion of the available nitrogen to root development and therefore be able to exploit a larger volume of soil, which would confer a competitive advantage over C_3 species in nitrogen poor environments. Two additional strategies are suggested which may be appropriate *A. semialata*. These are to produce a similar leaf biomass and root biomass, but invest more resources in either underground storage favouring survival in environments subject to disturbance (like fire, frost or herbivory), or reproduction, thus potentially enhancing the C_4 subspecies' fitness. While in nitrogen deficient environments the C_4 subspecies may exploit the higher PNUE by produce greater root biomass than the C_3 subspecies, but not at the expense of leaf biomass.

PNUE and leaf area

For the C_3 and C_4 subspecies of *A. semialata* it was found that the C_4 subspecies had higher photosynthetic rates at ambient concentrations of CO_2 than the C_3 subspecies. The photosynthetic rate of the C_4 subspecies was nearly twice that of the C_3, and for both subspecies the rate of photosynthesis was unaltered by different levels of nitrogen supply while these levels did not exceed the range found in savanna soils. When C_4 plants were supplied with nitrogen levels in excess of those found in savanna soils the rate of photosynthesis increased, and the form in which the nitrogen was supplied (whether as ammonium plus nitrate or nitrate alone) made no significant difference to photosynthetic rates. The C_3 and C_4 subspecies had the same leaf nitrogen concentrations, and when

calculated as a percentage, leaf nitrogen declined with reduced nitrogen supply, but when calculated on an area-basis it remained the same across all treatments. As the photosynthetic rates of the C_4 subspecies were greater than those of the C_3 subspecies the PNUE was found to be a third greater than for the C_3 subspecies. Associated with a higher PNUE the C_4 subspecies produced a smaller leaf area than the C_3 subspecies with a lower investment of nitrogen and biomass into leaf area, this resulted in the C_4 subspecies having a smaller canopy leaf area than the C_3 subspecies. Despite the C_4 subspecies producing a smaller leaf area, these plants were able to achieve greater relative growth rates and accumulate greater biomass than the C_3 subspecies at the levels of nitrogen supply between zero and the equivalent to 7 g m^{-2} y^{-1}. The C_4 subspecies was therefore capable of achieving greater whole plant productivity than the C_3 subspecies with a smaller investment into leaf canopy, as a result of the greater efficiency of C_4 photosynthesis at the leaf scale.

Severe nitrogen limitation brought about reduced leaf area production and resulted in a smaller canopy leaf area for both subspecies, which led to a corresponding decline in whole plant productivity and a decline in biomass accumulation. The partitioning of biomass to leaves by the C_4 subspecies was unaltered by severe nitrogen limitation; however, the higher nitrogen costs of leaves in the C_3 subspecies was associated with a stronger limitation by nitrogen than in the C_4 subspecies. Thus, the lower rates of photosynthesis and higher PNUE of the C_3 subspecies were likely to have a greater impact on whole plant productivity, than in the case of the C_4 subspecies, and this was reflected in faster growth rates and greater final biomass accumulation in the C_4 subspecies. Reduced canopy leaf area was also associated with greater leaf turnover rates for both subspecies, and this reduced leaf area along with the re-allocation of nitrogen from senescing leaves was likely to account for the conservative response of photosynthetic rates and PNUE to nitrogen supply in both subspecies.

Biomass partitioning

Allocation of biomass to underground components was the same for both subspecies, although the C_3 subspecies invested more biomass in roots than the C_4 subspecies, while the lower investment in roots by the C_4 subspecies was offset by an increased biomass allocation to corms. Severe nitrogen limitation resulted in an increased allocation of

biomass to roots by both subspecies, but had no effect on allocation to corms by the C_4 subspecies. The greater biomass accumulation by the C_4 subspecies resulted in a greater investment in sexual reproduction than in the C_3 subspecies, where investment was insignificant. In the C_4 plants severe nitrogen limitation led to a reduction in the allocation of biomass to flowers which offset allocation to roots, while maintaining allocation to leaves. In the C_3 plants allocation to roots was offset by reduced allocation to leaves with a corresponding reduction in whole plant productivity. Tillering increased with increased nitrogen supply in both subspecies, but the C_3 subspecies produced larger numbers of tillers than in the C_4 subspecies at medium and high levels of nitrogen supply. This suggests a possible strategy to compensate for limited sexual reproduction in the C_3 subspecies.

Allometric analysis showed that an increase in biomass accumulation corresponded with increased nitrogen supply and this affected allocation to leaves, roots and corms which was shifted along a single allometric trajectory. Thus the nitrogen supply determined biomass partitioning to these components through its effect on plant size and resulted in apparent plasticity, or plastic growth rates. The relationship between plant size and flowering and tillering was less direct, with greater overlap at medium and high levels of nitrogen supply along the allometric trajectory. This lack of allometric relationship may result from the plants having to reach a minimum size before flowering and tillering is initiated. The different biomass allocation patterns for the C_3 and C_4 subspecies of *A. semialata* was the result of different allometric trajectories and if it is accepted that the allometric relationships are associated with optimal allocation, these allocation patterns would have resulted from natural selection, and suggest a mechanistic link between the photosynthetic type and the ecological strategy employed.

Ecological strategies

Both subspecies of *A. semialata* are endemic to mesic grasslands of South Africa and are likely to be adapted to infertile soils. The frequency of fires is greater in those habitats occupied by the C_4 subspecies of *A. semialata* than the C_3 subspecies, and in post-fire habitats competition for light and soil nutrients would be limited. However, the high corm biomass of the C_4 subspecies of *A. semialata*, suggests a likely function as a storage organ which may enable resprouting after fire, and the extensive investment in sexual

reproduction and the production of large amounts of seed may promote recruitment, since fires are typically patchy in nature and recruitment from neighbouring unburned habitats is important in vegetation re-establishment. In contrast the C_3 subspecies of *A. semialata* occur in habitats where fires are less frequent, and the closed canopies which result, will facilitate greater competition for light and greater below-ground competition for limited resources. Under these conditions there would be little need for resprouting and seedling recruitment would be limited, while vegetative reproduction along with leaf and root growth may confer a greater competitive advantage.

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