PHYSIOLOGICAL AND METABOLIC FACTORS DETERMINING NITROGEN USE EFFICIENCY OF TOMATO SEEDLINGS GROWN WITH ELEVATED DISSOLVED INORGANIC CARBON AND DIFFERENT NITROGEN SOURCES

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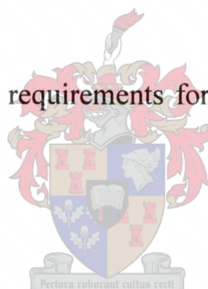

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

The aim of this study was to determine (1) the influence of elevated dissolved inorganic carbon (DIC) on the nitrogen use efficiencies (NUE) of tomato seedlings grown with different nitrogen sources, (2) how changes in the regulation and activities of nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPc), carbonic anhydrase (CA) and subsequent changes in metabolites would account for observed changes in NUE, and (3) to what extent elevated DIC contributed to the carbon budget of plants grown with different nitrogen sources. *Lycopersicon esculentum* cv. F144 seedlings were grown in hydroponic culture (pH 5.8) with 2 mM of either NO_3^- or NH_4^+ and the solutions were aerated with either 0 ppm or 5000 ppm CO_2 concentrations. The similar NUEs of NH_4^+-fed plants grown with either root-zone CO_2 concentration were largely due to their similar RGRs and N uptake rates. Elevated root-zone DIC had an initial stimulatory effect on NH_4^+ uptake rates, but it seems as if this effect of DIC physiological processes was cancelled out by the toxic effect of unassimilated NH_4^+. The NUE for NO_3^--fed plants supplied with 5000 ppm root-zone CO_2 was higher relative to 0 ppm root-zone CO_2 and it was possibly due to the higher relative growth rates for similar N uptake rates of 5000 ppm compared to 0 ppm root-zone CO_2. Nitrate-fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 had higher *in vivo* NR and *in vitro* NR and PEPc activities. These increases in enzymes activities possibly lead to increases in organic acid synthesis, which could have been used for biomass accumulation. This would account for the increased relative growth rates of NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2. The increasing root-zone CO_2 concentrations resulted in the $\delta^{15}N$ values of NH_4^+-plants becoming more positive indicating an absence of enzymatic discrimination. This may have been due to the inhibitory effect of DIC on NH_4^+ uptake, causing plants to utilise both internal isotopes equally. The $\delta^{13}C$ studies showed that PEPc contributed equally to both NO_3^-- and NH_4^+-fed plants over the long term. From this it can be concluded that the lower NUE of NH_4^+-compared to NO_3^--fed plants grown with 5000 ppm root-zone CO_2 was due to increased N uptake and exudation of organic

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compounds into the nutrient solution. Experiments with $\delta^{13}C$ also showed that at increasing root-zone CO_2 concentrations, PEPc made a bigger contribution to the carbon budget via the anaplerotic reaction.

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UITTREKSEL

Die doel van hierdie studie was om (1) die invloed van verhoogde opgeloste anorganiese koolstof dioksied (DIC) op die stikstofverbruiksdoeltreffenheid (NUE) van plante wat op verkillende stikstofbronne gekweek is, te bepaal. (2) Veranderinge in die regulering van nitraat reduktase (NR), fosfo-enolpirovaatkarboksilase (PEPc) en karboonsuuranhidrase (CA) is bestudeer en gekorreleer met waargeneemde verskille in NUE. (3) 'n Beraming van die mate waartoe verhoogde DIC bydra tot die koolstofbegroting van plante, gekweek op verskillende stikstofbronne, word bespreek. *Lycopersicon esculentum* cv. F144 saailinge is in waterkultuur (pH 5.8) met 2 mM NO_3^- of NH_4^+ gekweek en die oplossings is alternatiewelik met 0 ppm of 5000 ppm CO_2 belug. Die NUEs van plante gekweek met NH_4^+ en belug met albei CO_2 konsentrasies was vergelykbaar grootliks as gevolg van hul ooreenkomstige relatiewe groeitempo's en N opname. DIC het aanvanklik NH_4^+ opname gestimuleer, maar enige latere stimulerende effek van DIC op fisiologiese prosesse was klaarblyklik uitgekanselleer deur NH_4^+ toksiteit veroorsaak deur vertraagde assimilasie. Die NUE van plante gekweek met NO_3^- en 5000 ppm CO_2 was hoër as dié van plante gekweek met NO_3^- en 0 ppm CO_2. Dit is moontlik gekoppel aan hoër relatiewe groeitempo's teenoor onveranderde N opname tempo's. Plante gekweek met NO_3^- en 5000 ppm CO_2 het hoër *in vivo* NR en *in vitro* NR en PEPc aktiwiteite getoon as plante gekweek met NO_3^- en 0 ppm CO_2. Bogenoemde toenames in ensiem aktiwiteite word verbind met biomassa toename deur verhoogde organiese suur sintese. Dit bied 'n moontlike verklaring vir die hoër relatiewe groeitempo's van plante gekweek met NO_3^- en 5000 ppm CO_2 teenoor plante gegroei met NO_3^- en 0 ppm CO_2. Die $\delta^{15}N$ waardes van plante gekweek met NH_4^+ en 5000 ppm CO_2 was meer positief as dié van plante gekweek met NH_4^+ en 0 ppm CO_2 wat gedui het op die afwesigheid van ensiematiese diskriminasie. Dit kon as gevolg gewees het van die vertragende effek van DIC op NH_4^+ opname wat daartoe sou lei dat die plante beide isotope eweveel inkorporeer. Eksperimente met $\delta^{13}C$ het getoon dat PEPc oor 'n lang tydperk eweveel begedra het tot die koolstofbegroting van plante gekweek met beide NO_3^- and NH_4^+. Hiervan kan

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afgelei word dat die laer NUE van plante gekweek met NH_4^+ en 5000 ppm CO_2 in vergelyking met dié van plante gekweek met NO_3^- en 5000 ppm CO_2 die gevolg was van verhoogde NH_4^+ opname en uitskeiding van aminosure in die voedingsoplossing. Eksperimente met $\delta^{13}C$ het ook getoon dat verhoogde DIC konsentrasies die bydrae van PEPc tot die plant se koolstofbegroting laat toeneem.

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"The woods are lovely, dark and deep,

But I have promises to keep,

And miles to go before I sleep,

And miles to go before I sleep."

- Robert Frost

Stopping by woods on a snowy evening

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LIST OF ABBREVIATIONS

$\delta^{13}C$	carbon isotopic ratio
$\delta^{15}N$	nitrogen isotopic ratio
°C	degrees centigrade
^{14}C	radio-labelled carbon
$^{14}CO_2$	radio-labelled carbon dioxide
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
CA	carbonic anhydrase (EC 4.2.1.1)
dH_2O	distilled water
DIC	dissolved inorganic carbon
DTT	1,4-dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
Fd_{red}	reduced ferredoxin
FW	fresh weight
G6PDH	glucose 6-phosphate dehydrogenase (EC 1.1.1.49)
NADH-GOGAT	NADH-glutamate synthase (EC 1.4.1.14)
Fd-GOGAT	Fd- glutamate synthase (EC 1.4.1.13)
GS	glutamine synthetase (EC 6.3.1.2)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IP	inhibitor protein

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LSD	least significant difference
NAD	nicotinamide adenine dinucleotide, oxidised form
NADH	ß-nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NED	N-1-naphtylethylenediamine dihydrochloride
NR(A)	nitrate reductase (activity) (EC 1.6.6.1)
NUE	nitrogen use efficiency
OPPP	oxidative pentose phosphate pathway
PEP	phosphoenolpyruvate
PEPc	phosphoenolpyruvate carboxylase (EC 4.1.1.31)
6PGDH	6-phosphogluconate dehydrogenase (EC 1.1.1.43)
PK	protein kinase
PP	protein phosphatase
PVPP	polyvinylpolypyrrolidine
SE	standard error
TCA cycle	tricarboxylic acid cycle
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol

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Chapter 1

1.1 General introduction

Less energy intensive methods are needed to produce food and with respect to the conservation of energy expensive nitrogen fertiliser, one of the important focus points would be an increase in nitrogen use efficiency (NUE) of crops (Lewis, 1986). There are various ways of defining NUE. It can be defined as the relationship between the nitrogen content of a plant either as N taken up from the solution or soil and gain in biomass, or total N in the plant tissue and gain in biomass (Small, 1972). NUE can also be defined as yield production per unit N available in the soil (Moll *et al.,* 1982). This comprises of two primary components, (1) the efficiency of absorption (uptake) and (2) the efficiency with which the N absorbed is utilized to produce yield. Growth rate and NUE generally decrease with increasing nitrogen availability (Small, 1972), and it has been found that NO_3^--fed plants generally have a higher NUE than NH_4^+-fed plants. In this study NUE was defined as N taken up from the solution and gain in biomass because plants were harvested before reaching the reproductive stage.

Nitrate and NH_4^+ are the major sources of inorganic nitrogen taken up by the roots of higher plants. Nitrate has to be reduced to NH_4^+ to be able to be incorporated into organic structures. Most of the NH_4^+ has to be incorporated into organic compounds in the roots in contrast with NO_3^- which is readily mobile and can also be stored in the vacuoles of roots, shoots and storage organs (Marschner, 1995). For assimilation of NH_4^+ there is a high demand for carbon skeletons and carbon flow between sucrose synthesis and amino acid synthesis appears to be regulated via cytosolic protein kinases, which modulate the activity of two key enzymes, sucrose-P synthase and phosphoenolpyruvate carboxylase (PEPc), by phosphorylation (Champigny & Foyer, 1992). These two enzymes respond to phosphorylation in opposite ways with PEPc activated and sucrose-P synthase inactivated. In this way photosynthate is partitioned away from sucrose synthesis to amino acid synthesis. High PEPc activity is needed to replenish

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the TCA cycle intermediates because of the drain of carbon skeletons during amino acid synthesis (Marschner, 1995).

Incorporation of dissolved inorganic carbon (DIC) serves this anaplerotic function by providing intermediates for the TCA cycle through the activity of PEPc, which is responsible for re-fixation of respiratory CO_2 (Vuorinen & Kaiser, 1997). DIC comprises a pH-dependent combination of CO_2, HCO_3^- and CO_3^{2-} in solution (Norstadt & Porter, 1984). Although the assimilation of DIC through PEPc activity in the root is responsible for only a small contribution to the C budget of the whole plant, the rate of assimilation is a significant proportion of the C budget of the root. DIC assimilation could occur at rates equivalent to 30% of the rate of respiration in plant roots exposed to 5000 ppm root-zone CO_2 (Cramer & Lips, 1995). Positive effects of root-zone DIC on plant growth have been reported previously (Vapaavuori & Pelkonen, 1985), although, Cramer & Richards (1999) found that growth effects on plants grown with elevated root-zone DIC were most readily seen in plants growing under high irradiances, salinity stress or high shoot temperatures. Elevated root-zone DIC has been found to lead to an increase in NO_3^- uptake compared to ambient DIC (Cramer *et al.,* 1993), whereas NH_4^+ uptake was decreased or unchanged with elevated DIC compared to ambient DIC (Cramer *et al.,* 1996). Cramer *et al.* (1993) found that elevated root-zone DIC led to a larger proportion of root derived carbon being allocated to organic acids in NO_3^--fed maize plants, whereas in NH_4^+-fed maize plants more carbon was allocated to amino acids (aspartate, asparagine, glutamate, glutamine). Elevated root-zone DIC was found to stimulate nitrate reductase (NR) activity *in vitro* and *in situ* in barley plants (Cramer *et al.,* 1996). Cramer *et al.* (1999) found that PEPc activity *in vitro* was unaffected by the supply of elevated root-zone DIC, but the authors used 360 ppm root-zone CO_2 for the ambient CO_2 treatment, whereas in the present study 0 ppm root-zone CO_2 was used.

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As elevated root-zone DIC influences the uptake and partitioning of NO_3^- and NH_4^+ into organic compounds as well as influencing the activity of two key enzymes such as NR and PEPc, one would expect an additional influence on nitrogen use efficiency (NUE) because NUE is determined by the assimilation and partitioning of nitrogen. Therefore the relationship between NUE, the uptake of N and to what extent NR and PEPc influence NUE was investigated using tomato seedlings grown in hydroponic culture at 360 ppm and 5000 ppm root-zone CO_2 with 2 mM of either NO_3^- or NH_4^+. Additionally, the contribution of DIC to the total carbon budget of plants grown in hydroponics at 0, 5000 and 10000 ppm root-zone CO_2 with 2 mM of either NO_3^- or NH_4^+ were investigated as well as the fate of DIC taken up over a 24 h period.

The hypotheses that were set out to test in this study were:

Hypothesis 1: Elevated root-zone DIC and different nitrogen sources have an effect on NUE.

2: NR and PEPc play a role in determining NUE under different root-zone DIC concentrations and different nitrogen sources.

3: Metabolites such as organic and amino acids play a role in determining NUE and their concentrations are influenced by different root-zone DIC concentrations and different nitrogen sources.

4: Root-zone DIC contributes to the carbon budget when applied at different concentrations in combination with different nitrogen sources.

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1.2 Literature review

1.2.1 Interaction between nitrogen and carbon metabolism in roots

Carbon and nitrogen metabolism are linked because they must share organic carbon and energy supplied directly from photosynthetic electron transport and CO_2 fixation, or from respiration of fixed carbon via glycolysis, the oxidative pentose phosphate pathway (OPPP), the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain. The integration of these important metabolic processes must involve extensive regulation between the pathways (Huppe & Turpin, 1994). The primary assimilation of inorganic nitrogen into amino acids requires carbon skeletons in the form of ketoacids, which are intermediates of respiratory metabolism (Ireland, 1990), and energy in the form of ATP and reductant provided by respiration of stored and/or translocated photosynthate (Andrews, 1986; Lee, 1980).

1.2.1.1 *Carbon requirements for nitrogen assimilation*

Inorganic nitrogen assimilated from the environment requires carbon skeletons for the synthesis of amino acids. The high demand of carbon skeletons for NH_4^+ assimilation in roots is reflected not only in higher activities of phosphoenolpyruvate carboxylase (PEPc) as compared with NO_3^--fed plants but also in the approximate doubling of the rates of O_2 consumption per unit root weight (Marschner, 1995). 2-Oxoglutarate from the TCA cycle is used for net glutamate synthesis while the synthesis of other amino acids requires carbon skeletons of which most are intermediates in respiratory pathways (Ireland, 1990). The provision of 2-oxoglutarate is of key importance for the synthesis of glutamate (Huppe & Turpin, 1994). However, to date, the exact enzymatic origin of 2-oxoglutarate for plant NH_4^+ assimilation remains uncertain because a variety of 2-oxoglutarate-synthesizing enzymes and isozymes exist in several sub-cellular compartments within the same plant cell. The main candidates are isocitrate dehydrogenases and aspartate aminotransferases (AspAT) (Gálvez *et al.,* 1999). Two different isocitrate

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dehydrogenase activities, depending on cofactor (NAD or NADP) specificity, co-exist in the cell and catalyse the oxidative decarboxylation of isocitrate to form 2-oxoglutarate. Production of 2-oxoglutarate by an isocitrate dehydrogenase allows for net glutamate synthesis via the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle, whereas an AspAT origin leads to the synthesis of aspartate instead of glutamate, and requires oxaloacetate as carbon-skeleton input (Gálvez *et al.,* 1999).

Ammonium uptake is toxic to plant function and therefore it must be rapidly assimilated into non-toxic organic compounds (Gálvez *et al.,* 1999). The toxic effects of NH_4^+ nutrition are caused by the unassimilated NH_4^+ ion. The NH_4^+ ion leads to dissipation of pH gradients across membranes (Bloom, 1997) such as thylakoids, inner mitochondrial membranes or tonoplast membranes. For this reason NH_4^+ assimilation has a large requirement for carbon skeletons for amino acid synthesis, which are provided by the TCA cycle and the removed intermediates have to be replenished by increased activity of PEPc (Marschner, 1995). With NH_4^+- compared to NO_3^--nutrition the net carbon fixation in roots is up to 3-fold higher in rice and tomato (Ikeda *et al.,* 1992) and about 5-fold higher in maize (Cramer *et al.,* 1993). Studies of dark CO_2 fixation in plants show that anaplerotic carbon is required to replace TCA cycle intermediates consumed in biosynthesis and the onset of nitrogen assimilation in plant tissues results in a large stimulation of dark CO_2 fixation (Basham *et al.,* 1981; Van Quy *et al.,* 1991). There is a linear relationship between the rate of nitrogen assimilation and anaplerotic carbon fixation (Van Quy *et al.,* 1991). If oxaloacetate were depleted, it would negatively affect the provision of carbon skeletons for amino acid synthesis. The carboxylation of PEP (from glycolysis) and HCO_3^- by cytosolic PEPc to form oxaloacetate serves as an anaplerotic reaction in the TCA cycle. The oxaloacetate is quickly reduced to malate by cytosolic malate dehydrogenase, which is transported to the mitochondrion where it enters the TCA cycle. The observed increase in PEPc activity during

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nitrogen assimilation is an indication of the significance of anaplerotic PEP carboxylation to supply ketoacids (Van Quy *et al.*, 1991).

1.2.1.2 *Energy requirements for nitrogen assimilation*

The carbon requirements for amino acid synthesis are independent of the form of inorganic nitrogen assimilated and the major differences between the assimilation of inorganic NO_3^- and NH_4^+ are the energy costs associated with the reduction of NO_3^- to NH_4^+ (Huppe & Turpin, 1994). Compartmentalization of NO_3^- assimilatory pathway enzymes increases the complexity of integrating and controlling nitrogen and carbon metabolism during assimilation. How energy requirements are met depends on the type of tissue and its physiological circumstances (Huppe & Turpin, 1994). The energy requirement for reducing the nitrogen source has to be met before respiratory carbon flow can be activated to provide the necessary carbon skeletons for nitrate assimilation (Vanlerberghe *et al.*, 1991).

The reduction of NO_3^- to NH_4^+ is mediated by nitrate reductase, which involves the two-electron reduction of NO_3^- to NO_2^-, and nitrite reductase, which transforms NO_2^- to NH_4^+ in a six-electron reduction (Marschner, 1995). The assimilation of NO_3^- into glutamine in higher plants requires reduced ferredoxin (Fd_{red}) for nitrite reductase and ferredoxin-dependent glutamate synthase (Fd-GOGAT) activities in the root plastid. In heterotrophic tissues ferredoxin-$NADP^+$ oxidoreductase uses NADPH as a substrate for the reduction of Fd (Redinbaugh & Campbell, 1998). One possible source of the NADPH required for Fd reduction in maize root plastids is the oxidation of glucose by the oxidative pentose phosphate pathway (OPPP) enzymes, glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.43) (Redinbaugh & Campbell, 1998). The enzymes of this pathway, namely

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G6PDH, 6PGDH, transketolase, and transaldolase were found present in both root plastids and the cytosol (Emes & Fowler, 1979).

The assimilation of NH_4^+ is mediated by the GS-GOGAT pathway (Blevins, 1989). Glutamine synthetase (GS, EC 6.3.1.2) combines NH_4^+ with glutamate to form glutamine. This reaction requires the hydrolysis of one ATP and involves a divalent cation such as Mg^{2+}, Mn^{2+}, or Co^{2+} as a cofactor. Plants contain two classes of GS, GS1 in the cytosol and GS2 in root plastids or shoot chloroplasts (Grossman & Takahashi, 2001). GS1 is likely to be involved in the assimilation of external NH_4^+ (Grossman & Takahashi, 2001). GOGAT catalyses the transfer of the amide group from glutamine to 2-oxoglutarate, yielding two molecules of glutamate. Plants contain two types of GOGAT: one accepts electrons from NADH, the other accepts electrons from Fd (Grossman & Takahashi, 2001). NADH-GOGAT (EC 1.4.1.14) located in plastids of heterotrophic tissues such as roots is involved in assimilation of NH_4^+, while NADH-GOGAT in vascular bundles of developing leaves assimilate glutamine translocated from the roots. Fd-GOGAT (EC 1.4.1.13) found in chloroplasts serves in photorespiratory metabolism, while Fd-GOGAT found in root plastids presumably functions to incorporate the glutamine generated during NO_3^- assimilation (Grossman & Takahashi, 2001). The heterotrophic tissues obtain the majority of ATP and NADH from the mitochondrial electron transport chain and some from glycolysis (Huppe & Turpin, 1994), while Fd is provided by OPPP (Redinbaugh & Campbell, 1998).

1.2.2 CO_2 transport across root membranes

Dissolved inorganic carbon (DIC) is a pH dependent combination of CO_2, HCO_3^- and CO_3^{2-} in solution. The solubility of CO_2 increases from pH 5 and up because, in addition to the CO_2, a proportion of the DIC is soluble in water and exists as HCO_3^-. Using the equations of Golterman

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& Clymo (1969), the HCO_3^- proportion of DIC is calculated to be 4 % and 82 % at pH 5 and 7, respectively (Cramer *et al.*, 1996). Carbonic anhydrase (CA) catalyses the reversible hydration of CO_2 to HCO_3^-, which is the inorganic substrate for PEPc. Inorganic carbon can enter the plant either as CO_2 or HCO_3^-, depending on the pH (Van der Westhuizen & Cramer, 1998). Carbon dioxide readily diffuses through membranes and available evidence indicates that CO_2 is the major form of inorganic carbon translocated across the plasmamembrane of roots (Raven and Newman, 1994). The uptake of CO_2 as HCO_3^- across membranes may require either a symport with H^+, an antiport with an OH^- or, alternatively, HCO_3^- in the cell wall may trap H^+ to yield CO_2, which could diffuse into the cell (Van der Westhuizen & Cramer, 1998).

These three uptake mechanisms were proposed for photosynthetic exogenous HCO_3^- assimilation by aquatic plants and unicellular systems (Lucas, 1983). It has recently been found that the cyanobacterium *Synechococcus* sp. strain PCC 7942 has an ATP-binding cassette transporter involved in HCO_3^- uptake which appears to be the first primary-active HCO_3^- transporter (Omata *et al.,* 1999), although Na^+/HCO_3^- cotransporters and HCO_3^-/anion exchangers have been characterized in mammals (Omata *et al.,* 1999) and the HCO_3^-/anion exchanger has also been previously proposed by Lucas (1983). The seagrass *Zostera marina* have two possible systems for utilising HCO_3^- as inorganic carbon source, the first being an ATPase-based HCO_3^- uptake system and the second mechanism relies on extracellular/surface-bound CA for HCO_3^- acquisition (Beer & Rehnberg, 1997). To date no plasmamembrane HCO_3^- transporter like that of *Synechococcus* sp. strain PCC 7942 and *Zostera marina* has been found in terrestrial plants suggesting that CO_2 diffusion across the root plasmamembrane is the most likely mechanism of inorganic carbon uptake by roots.

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1.2.3 Influence of elevated CO_2 on growth

Growth effects on plants grown with elevated DIC are most readily seen in plants growing under high irradiances, salinity stress or high shoot temperatures (Cramer & Lips, 1995). At high light intensities photosynthetic rate, stomatal conductance and water use efficiency are lower in plants supplied with elevated DIC than in plants supplied with ambient DIC (Cramer & Richards, 1999). Carbon supplied through the xylem by elevated DIC may allow photosynthesis to supply sufficient carbon while maintaining relatively low stomatal conductance. Under high light intensities reduction of photoinhibition and photorespiration is important in determining growth, especially when temperatures are high and stomata are partially closed, thereby limiting the availability of inorganic carbon for photosynthesis. The transport of organic carbon through the xylem could possibly reduce photoinhibition and photorespiration (Cramer & Richards, 1999). In an experiment with salinity stressed tomato plants the dry weights of plants grown with salinity elevated DIC were significantly larger than that of plants grown with ambient DIC (Cramer & Lips, 1995). These results indicated that root incorporation of HCO_3^- might have a significant influence on plant growth under stress conditions. As the amount of HCO_3^- incorporated compared to photosynthesis was small, Cramer & Lips (1995) concluded that the influence of elevated DIC on plant growth was probably mediated through the anaplerotic provision of carbon to the root or through some secondary factor such as pH or nutrient availability of CO_2 on root physiology as found previously by Enoch & Olesen (1993).

1.2.4 Influence of elevated CO_2 on N uptake and metabolism

Elevated DIC was found to lead to increased NO_3^- uptake compared to ambient DIC (Cramer *et al.* 1993) whereas NH_4^+ uptake was decreased or unchanged with elevated DIC compared to ambient DIC (Cramer *et al.* 1996). This increase of NO_3^- uptake by elevated DIC was due to increased incorporation of the reduction products of NO_3^- into amino acids or a direct stimulatory

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effect on NO_3^- uptake, which is independent of nitrate reductase (NR) activity (Cramer *et al.,* 1996). NO_3^- is actively taken up and can be mediated by an OH^-:NO_3^-, an HCO_3^-:NO_3^- exchange mechanism (Hodges, 1973) or a NO_3^-:H^+ mechanism (McClure *et al.*, 1990ab). Current studies favour the model of NO_3^- uptake mediated by a H^+ symport. The higher pH inside the root cells favours the formation of HCO_3^- from incoming CO_2 in the presence of CA. The possibility of exchange of cytoplasmic HCO_3^- for other anions such as NO_3^- exists, which results in an increased uptake of NO_3^- under enriched DIC conditions (Cramer *et al.*, 1996). Where it was previously hypothesised that DIC brought about changes in electrochemical conditions across the root plasmalemma resulting in increased anion uptake and decreased cation uptake (Cramer *et al.,* 1996), it is now thought that these changes are mediated by mechanisms other than changes in electrochemistry across the root plasmalemma (Cramer *et al.,* 1999). In roots of plants supplied with NO_3^- there was an increase in organic acid synthesis in plants supplied with elevated DIC, but amino acids for both treatments were the same. These authors concluded that in plants supplied with NO_3^-, carbon incorporated by root PEPc was mostly incorporated into organic acids to maintain ionic balance in cells and xylem sap. The amount of organic acids formed seems to be the most strongly affected by the elevated root-zone CO_2. A large proportion of the labelled organic acid was found in the stem and leaf tissue, which indicates that carbon was translocated from the root to the shoot, which could have resulted in reduced growth of the root. Elevated root-zone CO_2 increased PEPc activity, which could provide carbon skeletons for amino acid synthesis in these plants (Cramer & Lips, 1995).

Ammonium uptake was inhibited by elevated DIC as was mentioned earlier. Ammonium assimilation requires carbon skeletons from the TCA cycle for amino acid synthesis (Schweizer & Erismann, 1985). When elevated root-zone DIC is supplied, oxaloacetate produced from PEPc does not necessarily enter the TCA cycle, but could be reduced to malate and translocated to the

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shoot (Cramer & Lips, 1995) or be aminated to aspartate and asparagine (Cramer *et al.,* 1993). These processes consume DIC and may divert carbon away from the synthesis of glutamate, which is the acceptor for NH_4^+ during NH_4^+ assimilation thereby resulting in a decrease in NH_4^+ uptake (Van der Westhuizen & Cramer, 1998). The increased synthesis of amino acids could also down regulate NH_4^+ uptake as has been shown previously by Causin and Barneix, (1993); Feng *et al.,* (1994); Glass *et al.,* (1997). Although the mechanism of NH_4^+ uptake remains unknown, a model was proposed analogous to the K^+ HATS whereby NH_4^+ enters the cell via a symporter driven by the transmembrane H^+ gradient (Taylor & Bloom, 1998).

PEPc and CA have a high affinity for CO_2, which leads to the dark incorporation of HCO_3^- in the roots (Edwards & Walker, 1983). Incorporation of DIC may serve an anaplerotic function by providing intermediates for the TCA cycle through the activity of PEPc, which plays a role in re-fixation of respiratory CO_2 (Vuorinen & Kaiser, 1997). The assimilation of DIC through PEPc activity in the root is responsible for only a small contribution to the carbon budget of the whole plant, but DIC assimilation could occur at rates equivalent to 30% of the rate of respiration in plant roots exposed to 5000 ppm CO_2 (Cramer & Lips, 1995) providing carbon skeletons for the assimilation of nitrogen (Cramer & Lewis, 1993) and other metabolic processes where carbon skeletons are required. In tomato plants the *in vivo* assimilation of DIC into acid-stable products was increased 10-fold by elevated root-zone DIC (Cramer & Lips, 1995). According to Enoch and Olesen (1993) it is possible that some of the DIC taken up is translocated to the shoot by the transpiration stream where it is photosynthetically assimilated, but in experiments conducted by Cramer & Lips on *Lycopersicon esculentum* it was found that only a small proportion of the ^{14}C label from a 1 h pulse with $NaH^{14}CO_3$ was located in organic products in the shoot (Cramer and Lips, 1995) indicating that not much of the inorganic carbon was translocated from the root. Cramer & Richards (1999) reported that organic carbon derived

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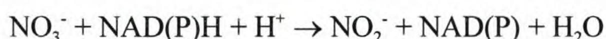
from DIC incorporation and subsequent translocation in xylem from the roots to the shoots may provide a source of carbon for the shoots, especially under conditions where low stomatal conductance may be advantageous, such as salinity stress, high shoot temperatures and high light intensities.

1.2.5 Control mechanisms of key enzymes involved

1.2.5.1 *Nitrate reductase*

Nitrate reductase (NR) (EC 1.6.6.1) is a cytosolic protein catalysing the following two-electron transfer step:



$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD(P) + H_2O$

This constitutes the first step of amino acid synthesis. Three forms of the enzyme are found, namely NADH-NR, which is the major form in photosynthetic tissues, NAD(P)H-NR which occurs in all tissues and especially roots, and NADPH-NR which is found in fungi. NR is a homodimer (Warner & Kleinhofs, 1992) containing a molecule of FAD, MoCo (molybdenum) and cytochrome b_{557} (Hoff *et al.,* 1992).

NR activity is affected by several factors such as NO_3^- availability, pH, light/dark, inhibitor proteins (IP), phosphorylation and the rate of photosynthesis (Kaiser & Huber, 1994). NR is regulated at the gene level by effective regulation of NR gene expression contributing to control of NR protein levels and at post-translational level the NR protein is modified by reversible protein phosphorylation, which provides a more rapid regulation of NR activity. The relative contribution of NR synthesis and of NR phosphorylation and dephosphorylation to the overall diurnal modulation of NR may vary between species and may depend on nitrate supply (Kaiser *et al.,* 1999). NR is active in the dephosphorylated form and partially inactivated in the phosphorylated form. NR phosphorylation not only controls the catalytic activity of NR, but also

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acts as a signal for NR protein degradation, with phosphorylated NR probably being a better substrate for protein degradation than the dephosphorylated form (Kaiser & Huber, 1997), indicating a link between phosphorylation status and level of NR protein. Dephosphorylation of NR *in vitro* is inhibited by divalent cations such as Mg^{2+} (Kaiser & Huber, 1994), which are required for protein kinase (PK) activity and inactivation of protein phosphatases (PP) thus retaining phosphorylated NR in the inactive state (Kaiser & Huber, 1994). The NR-PK is Ca^{2+}-dependent and metabolite regulated (G6P) and phosphorylates NR, this step is a prerequisite but not sufficient for inactivation (Spill & Kaiser, 1994). Kaiser & Huber (1994) found that AMP could stimulate NR-PP while P*i* inactivates PK, thus preventing NR from being phosphorylated. Complete inactivation requires the binding of an IP to phosphorylated NR (Glaab & Kaiser, 1995). It is assumed that the IP binds directly to the regulatory phosphorylation site of NR and this is supported by the finding that 14-3-3's bind to certain phosphopeptides using a rapid centrifugal filtration assay (Bachmann *et al.,* 1996). Divalent cations bind to the 14-3-3's and induce a conformational change required for ligand binding (Athwal *et al.,* 1998) and this could explain the requirement for Mg^{2+} for maintenance of the inactive form of NR (Kaiser *et al.,* 1999).

NR is activated by cytosolic acidification, which stimulates PP and/or inhibits PK; conversely NR is inactivated by cytosolic alkalisation, which should inhibit PP and stimulate PK. Feeding weak acids or bases to plant tissue can therefore activate or inactivate NR respectively (Kaiser & Brendle-Behnish, 1995). In contrast to this, Mengel proposed that increased cytosolic pH would stimulate NR activity in roots because NO_3^- reduction leads to OH^- production. This would stimulate organic anion synthesis such as the synthesis of malate to buffer the increase in pH and these processes have a promoting effect on NRA (Mengel *et al.*, 1983).

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1.2.5.2 *Phosphoenolpyruvate carboxylase*

PEPc (EC 4.1.1.31) is a ubiquitous cytosolic enzyme catalysing the 'irreversible' β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO_3^- and Me^{2+} to yield oxaloacetate and P*i* (Chollet *et al.,* 1996). It is a two-step reaction with the initial, reversible formation of carboxyphosphate and the enolate of pyruvate followed by the irreversible carboxylation of the latter (Lepiniec *et al.,* 1994). PEPc has been widely researched ever since it was discovered to be the enzyme responsible for initial fixation of atmospheric CO_2 during C_4 photosynthesis and Crassulacean acid metabolism. PEPc activity is ubiquitous in plants, widely distributed in bacteria, but so far has not been found in animals, yeast or fungi (Lepiniec *et al.,* 1994). It plays an important role 'anaplerotic function' to replenish intermediates (oxaloacetate and malate) of the TCA cycle and so providing carbon skeletons for nitrogen assimilation and amino acid biosynthesis (Melzer & O'Leary, 1987).

PEPc is a homoterameric enzyme whose activity is sensitive to various allosteric metabolite effectors such as glucose-6-phosphate, L-malate, ions, pH and temperature . The phosphorylation of PEPc is a covalent process that influences its affinity for L-malate and its catalytic activity and it has been found that non-photosynthetic PEPc's from C_3 and C_4 plants undergo regulatory phosphorylation similar to their C_4 and CAM photosynthetic counterparts (Lepiniec *et al.*, 1994). PEPc from several plant tissues can be phosphorylated *in vitro* by exogenous PEPc kinases and Ca^{2+} dependent kinases and it has been found that C_3 PEPc is phosphorylated by an endogenous protein kinase (Zhang *et al.*, 1995) and dephosphorylated by protein phosphatases type 2A1 (PP 2A) (Chollet *et al.*, 1996). PEPc has a pH optimum of between 7.5 and 8.5 (Lepiniec *et al.*, 1994).

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Root PEPc in plants reaches higher values in plants fed with NH_4^+ than in plants fed with NO_3^- (Schweizer & Erismann, 1985). PEPc activity of NO_3^- fed plants remained constant, which indicated that NO_3^- was not being assimilated in the root. PEPc activity increases during ammonium feeding after N starvation. The increase of root PEPc activity that depends on *de novo* protein synthesis contributes to the replenishment of carbon skeletons for continuous supply of ammonium in roots. Ammonium nutrition can ameliorate the inhibition by malate and thereby increase the *in situ* PEPc activity in roots compared to nitrate nutrition (Koga & Ikeda, 1997). Glutamine is the primary amino acid produced during ammonium assimilation in roots and it rapidly increases in roots when ammonium is supplied (Oaks & Hirel, 1985).

1.2.5.3 *Carbonic anhydrase*

The biological demand for CO_2, HCO_3^- or H^+ (in non-green tissues) frequently exceeds the uncatalysed equilibrium between CO_2 and HCO_3^- (Raven & Newman, 1994). CA (EC 4.2.1.1) is a ubiquitous enzyme that catalyses the reversible hydration of CO_2 (Rengel, 1995). Enzyme activity was found mainly located in the stroma of chloroplasts (87% of total cellular activity), but significant activity (13%) was also found in the cytosol of *Solanum tuberosum* leaves (Rumeau *et al.,* 1996). In a study on *Zea mays* root tips it was found that *in vivo* CA activity, which provides PEPc with HCO_3^-, was more than 200 times higher than that of PEPc *in vivo* (Chang & Roberts, 1992) indicating that HCO_3^- concentration was not the rate-limiting step for PEPc activity. Ohki (1976) found that photosynthesis, respiration, chlorophyll content and carbonic anhydrase activity were correlated with zinc nutrition in cotton. The carbonic anhydrase activity was found to increase as zinc status improved from deficiency to adequacy indicating a close relationship between enzyme activity and zinc concentration and this concurred with results found by Tobin (1970).

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Chapter 2

The influence of root-zone dissolved inorganic carbon on nitrogen use efficiencies and enzyme activities of tomato seedlings

Running title: DIC influence on N metabolism

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2.1 Summary

- The effects of elevated root-zone dissolved inorganic carbon (DIC) and NO_3^- or NH_4^+ nutrition on the growth and nitrogen use efficiency (NUE) of tomato seedlings were studied.
- Plants were hydroponically grown and the solutions aerated with 0 or 5000 ppm root-zone CO_2.
- High root-zone DIC concentration increased the NUE of NO_3^--fed plants, but not those of NH_4^+-fed plants. *In vitro* nitrate reductase activity (NRA) was higher in roots and lower in leaves of plants grown with 5000 compared to 0 ppm root-zone CO_2. With NO_3^- nutrition the activity of phosphoenolpyruvate carboxylase (PEPc) in roots was higher at high DIC concentrations while with NH_4^+ the opposite was true.
- Increased NRA was due to increased enzyme levels and not inhibitor protein binding or phosphorylation. Increased PEPc activity could be due to stimulation of NO_3^- and inhibition of NH_4^+ uptake by high DIC concentrations. The phosphorylation status was

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unaltered by DIC concentration. The lack of response of the NUE of NH_4^+-fed plants to DIC was ascribed to competition for carbon skeletons in the roots of these plants.

Key words: dissolved inorganic carbon, growth, nitrate reductase, nitrogen use efficiency, phosphoenolpyruvate carboxylase.

2.2 Introduction

Soils have dissolved inorganic carbon concentrations (DIC) ranging between 2000 and 5000 μmol mol^{-1} which can rise up to 200 000 μmol mol^{-1} under special circumstances due to the accumulation of respiratory CO_2 produced by the biological components of soils (Norstadt & Porter, 1984) compared to 360 μmol mol^{-1} in hydroponic solutions. Elevated root-zone DIC has effects on physiological processes including photosynthesis (Cramer & Richards, 1999), respiration (Van der Westhuizen & Cramer, 1998), NO_3^- uptake (Cramer *et al.,* 1996), partitioning of C and N to organic and amino acid synthesis (Cramer & Lewis, 1993) and growth (Cramer & Richards, 1999). Positive effects of root-zone DIC on plant growth have been reported previously (Vapaavuori & Pelkonen, 1985), although, Cramer & Richards (1999) found that growth effects on plants grown with elevated root-zone DIC were most readily seen in plants growing under high irradiances, salinity stress or high shoot temperatures.

Incorporation of root-zone DIC serves an anaplerotic function by providing intermediates for the TCA cycle through the activity of PEPc, which is responsible for re-fixation of respiratory CO_2 (Vuorinen & Kaiser, 1997) and in this way provides carbon skeletons for amino and organic acid synthesis. DIC assimilation could occur at rates equivalent to 30% of the rate of respiration in plant roots exposed to 5000 ppm root-zone CO_2 (Cramer & Lips, 1995). Cramer *et al.* (1993) found that root-zone DIC led to a larger proportion of root derived carbon being

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allocated to organic acids in NO_3^--fed maize plants, whereas in NH_4^+-fed maize plants more carbon was allocated to amino acids (aspartate, asparagine, glutamate, glutamine). DIC fixation might increase the assimilation of NH_4^+ into amino acids in the roots as a consequence of the improved supply of anaplerotic carbon (Cramer *et al.,* 1993). In NO_3^--fed tomato plants the *in vivo* assimilation of DIC into acid-stable products was increased 10-fold by elevated root-zone DIC concentrations (Cramer & Lips, 1995).

Inorganic carbon can enter the plant either as CO_2 or HCO_3^-, depending on the pH. Uptake of HCO_3^- may require either a symport with H^+, an antiport with an OH^- or, alternatively, HCO_3^- in the cell wall may trap H^+ to yield CO_2, which could diffuse into cells (Lucas, 1983). The biological demand for CO_2, HCO_3^- or H^+ (in non-green tissues) frequently exceeds the uncatalysed equilibrium between CO_2 and HCO_3^- (Raven & Newman, 1994). Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyses the reversible hydration of CO_2 (Rengel, 1995). Enzyme activity was found mainly located in the stroma of chloroplasts (87% of total cellular activity), but significant activity (13%) was also found in the cytosol of *Solanum tuberosum* leaves (Rumeau *et al.,* 1996). In a study on *Zea mays* root tips it was found that *in vivo* CA activity, which provides PEPc with HCO_3^-, was more than 200 times higher than that of PEPc *in vivo* (Chang & Roberts, 1992).

Cramer *et al.* (1999) found that the activity of PEPc *in vitro* in tomato plants was at least an order of magnitude greater than PEPc activity *in vivo*. This indicated that the concentration of PEPc was not a limiting factor for the assimilation of DIC from the root medium, but rather that other processes regulated or limited the activity of this enzyme *in vivo*. Protein phosphorylation results in a decrease in the sensitivity of PEPc to allosteric inhibitors such as malate (Jiao & Chollet, 1991) and an increase in catalytic activity. Koga & Ikeda (1997) found that root PEPc

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activity gradually increased upon transfer to NH_4^+ nutrition and reached higher values in NH_4^+-fed wheat, barley and tomato plants compared to NO_3^--fed plants. They concluded that NH_4^+ nutrition possibly alleviated malate inhibition thereby increasing root PEPc activity compared to NO_3^- nutrition and that the increase in root PEPc activity was dependent on *de novo* synthesis thus contributing to the replenishment of carbon skeletons for NH_4^+ assimilation.

Elevated root-zone DIC has lead to an increase in NO_3^- uptake compared to ambient root-zone DIC (Cramer *et al.,* 1996) whereas NH_4^+ uptake was decreased or unchanged with elevated root-zone DIC compared to ambient root-zone DIC (Cramer *et al.,* 1996). Nitrate uptake has been increased by elevated root-zone DIC due to increased incorporation of the reduction products of NO_3^- into amino acids or a direct stimulatory effect on NO_3^- uptake (Cramer *et al.,* 1996). Ammonium assimilation requires carbon skeletons from the TCA cycle for amino acid synthesis (Schweizer & Erismann, 1985). When elevated root-zone DIC is supplied, oxaloacetate produced from PEPc does not necessarily enter the TCA cycle, but could be reduced to malate and translocated to the shoot (Cramer & Lips, 1995) or be aminated to aspartate and asparagine (Cramer *et al.,* 1993) diverting carbon away from the synthesis of glutamate resulting in a decrease in NH_4^+ uptake (Van der Westhuizen & Cramer, 1998).

Elevated root-zone DIC stimulated nitrate reductase (NR) activity *in vitro* and *in situ* in barley plants (Cramer *et al.,* 1996). NR is the first enzyme of NO_3^- assimilation and is affected by several factors including NO_3^- availability, pH (Kaiser & Brendle-Behnisch, 1995), light/dark, inhibitor proteins (IP) (Glaab & Kaiser, 1995) and the rate of photosynthesis (Kaiser & Brendle-Behnisch, 1991). NR is active in the dephosphorylated form and partially inactivated in the phosphorylated form (Glaab & Kaiser, 1995). Dephosphorylation of NR *in vitro* is inhibited by divalent cations such as Mg^{2+}, which are required for protein kinase (PK) activity and

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inactivation of protein phosphatases (PP) thus retaining phosphorylated NR in the inactive state (Kaiser & Huber, 1994). Complete inactivation additionally requires the binding of an IP to phosphorylated NR (Glaab & Kaiser, 1995). NR is activated by cytosolic acidification, which stimulates PP and/or inhibits PK; conversely NR is inactivated by cytosolic alkalisation, which should inhibit PP and stimulate PK. Feeding weak acids or bases to plant tissue can therefore activate or inactivate NR respectively (Kaiser & Brendle-Behnisch, 1995).

As elevated root-zone DIC influences NO_3^- and NH_4^+ uptake and partitioning into organic compounds through the activity of two key enzymes, namely NR and PEPc, one would expect an additional influence on nitrogen use efficiency (NUE) because NUE is determined by the assimilation and partitioning of nitrogen. NUE can be defined as the relationship between the nitrogen content of a plant either as N taken up from the solution or soil and gain in biomass, or total N in the plant tissue and gain in biomass. Growth rate and NUE generally decrease with increasing nitrogen availability (Small, 1972), and it has been found that NO_3^--fed plants generally have a higher NUE than NH_4^+-fed plants (Martins-Loução & Cruz, 1999).

The aim of this study was to determine how changes in root-zone DIC concentrations would influence NUE and how these changes may be related to changes in NR, PEPc and CA activities. The hypothesis was that elevated root-zone DIC could lead to a decrease in cytosolic pH, which could inhibit CA activity as well as inhibit PK and/or activate PP and so lead to changes in NR and PEPc phosphorylation status and subsequent activation.

Abbreviations: CA, carbonic anhydrase; IP, inhibitor protein; NED, N-1-naphtylethylenediamine dihydrochloride; NR(A), nitrate reductase (activity); NUE, nitrogen use efficiency; PEPc, phosphoenolpyruvate carboxylase; PK, protein kinase; PP, protein phosphatase

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2.3 Materials and Methods

2.3.1 *Growth conditions*

Seedlings (14 d old) of *Lycopersicon esculentum* (L.) cv. F144 grown on a 1:1 mixture of vermiculite and compost were transferred to hydroponic culture after rinsing the roots in distilled H_2O. The hypocotyls of the plants were wrapped in black closed-cell foam rubber and inserted through collars in the lids of 22 l hydroponic tanks with eight plants per tank. The tanks were completely opaque and contained 20 l Long Ashton nutrient medium (Hewitt, 1966) modified to contain 2 mM of either $NaNO_3$ or NH_4Cl as a nitrogen source and 0.09 mM FeEDTA as an iron source. The nutrient medium was changed weekly and the pH of the medium was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. Plants were grown in a temperature controlled (minimum 15°C, maximum 25°C) greenhouse at the University of Stellenbosch during spring (September and October). Nutrient solutions were strongly aerated with ambient air (360 ppm CO_2) or with air containing elevated root-zone CO_2 (5000 ppm CO_2) produced by enriching ambient air with CO_2 from a cylinder of industrial grade CO_2 (Afrox, Cape Town, South Africa). Plants grown for the *in vitro* NR, PEPc and CA assays were aerated with 0 ppm instead of 360 ppm root-zone CO_2 to accentuate the differences between low and elevated root-zone CO_2 treatments. Carbon dioxide was removed from the air by passing ambient air through 2 M NaOH and a column (4 cm diameter and 30 cm length) containing 4-8 mesh soda lime (Saarchem, Krugersdorp, South Africa). The CO_2 concentration was monitored continuously using an ADC Mk3 (Analytical Development Corporation, Hoddeston, England) infrared gas analyser (IRGA). To prevent diffusion of CO_2 from the root-zone and the consequent enrichment of atmosphere around the shoots, the lids of hydroponic tanks were sealed with closed-cell foam rubber around the rim and clamped onto the tanks. The air-space between the surface of the nutrient solution and the lid was maintained under a partial vacuum to ensure that net air flow was inwards. Plants were used for experiments when the biomass was *ca.* 6 g.

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2.3.2 *Relative growth rates and nitrogen use efficiencies*

The plants were grown in 1 l bottles in a controlled environment chamber (Controlled Environments LTD., Winnipeg, Canada) with a relative humidity of 60%, light/dark temperature of 25°/18°C and a 14 h light period. The nutrient solutions were aerated either with ambient air (360 ppm CO_2) or air containing elevated root-zone CO_2 (5000 ppm) and the pH was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. There were four treatments comprised of either 2 mM $NaNO_3$ or NH_4Cl combined with either 360 ppm or 5000 ppm CO_2. The air-space between the surface of the nutrient solution and the lid was maintained under partial vacuum.

The fresh weights of the seedlings were determined regularly over the course of 15 d by carefully blotting the roots of the seedlings and weighing. In a preliminary trail it was shown that this procedure did not significantly reduce the biomass accumulation of the plants. The RGRs were calculated from linear regression of the logarithms of the fresh weights versus time. Fresh nutrient solution was supplied after each weighing and samples of the nutrient solution retained for analysis of the N content. The NO_3^- and the NH_4^+ concentrations of the samples were determined according to the methods of Cataldo *et al.* (1975) and Solorzano (1969), respectively. After 15 d the plants were harvested and fresh weights of the shoots and roots determined after which the plants were dried in an oven at 80°C for 48 h and reweighed.

For total N determination the oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, California, USA). The digestion was carried out with 0.05 g of milled plant material in a digestion block (Gerhardt, Germany) with 3 ml 3.4% (w/v) salicylic acid in concentrated sulphuric acid, 1 ml of distilled H_2O and a selenium pellet (Saarchem). The samples, including titriplex V standards, were digested at room temperature for 2 h, at 200°C for 1 h, 270°C for 1 h and at 370°C until they were clear. The concentration of

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NH_4^+ was determined on the diluted digest according to the method of Solorzano (1969).

2.3.3 *NO_3^- uptake*

Nitrate uptake was measured using eight replicate plants per treatment. The plants were transferred to bottles containing 300 ml Long Ashton nutrient solution (pH 5.8) with 0.2 mM $NaNO_3$ and were pre-incubated for 12 h at an irradiance of *ca.* 1200 µmol m^{-2} s^{-1}. The hypocotyls of the plants were wrapped in closed-cell foam rubber and inserted through the lids of the bottles. The roots were aerated with either 360 ppm or 5000 ppm root-zone CO_2. The roots receiving 5000 ppm root-zone CO_2 had columns containing soda lime attached to the lids of the bottles to trap the CO_2 released from the solution and thus prevent enrichment of the atmosphere surrounding the shoots. The bottles were placed in a water-bath and the temperature of both the water and the surrounding air maintained at 20°C.

After pre-incubation three plants of each treatment were harvested and divided into roots, stems and leaves, weighed, quenched in liquid nitrogen and stored at -80°C. These samples were later used to determine the initial tissue NO_3^- concentrations. The remaining plants were supplied with 300 ml fresh Long Ashton nutrient solution (pH 5.8) with 1 mM $NaNO_3$ and incubated for a further 6 h. Sub-samples of 1 ml were taken at the start of the experiment, and after 1, 3 and 6 h for determination of NO_3^- uptake rates measured by NO_3^- depletion. Thereafter the plants were harvested and divided into roots, stems and leaves, weighed, quenched in liquid nitrogen and stored at –80°C.

The *in vivo* NRA was calculated from the difference between NO_3^- taken up from the nutrient medium and the concentrations of NO_3^- in the plant tissue after the uptake period, less the initial concentrations in the plant tissue. The NO_3^- concentration in the nutrient solutions and

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the plant tissue were measured using the method of Cataldo *et al.* (1975). Tissue NO_3^- was extracted by vacuum infiltrating a homogenous sample of tissue (*ca.* 0.3 g) in 10 ml distilled water and extracting in a water bath at 80°C for 2 h. Each extract was mixed and sub-samples of 1 ml centrifuged at 1300 g for 5 min after which the NO_3^- concentration was determined.

2.3.4 *Nitrate reductase activity (in vitro)*

Eight replicate plants from each treatment were harvested, divided into root and leaf material, quenched in liquid nitrogen and stored at –80°C until assaying for *in vitro* NRA. The enzyme was assayed according to a modification of the method of Kaiser and Huber (1997). The frozen tissue was homogenised with acid-washed sand in a pre-cooled mortar and pestle in 4 ml g^{-1} FW extraction buffer containing 100 mM HEPES-KOH (pH 7.6), 3 mM DTT, 10 μM FAD (Sigma Chemical Co., St Louis, Missouri, USA), 2 mM EDTA, 10% (v/v) glycerol, 2% (w/v) casein, 2.5% (w/v) PVPP and 1 μM sodium molybdate. The homogenate was centrifuged at 16 000 g and 4°C for 5 min and then 100 μl of the supernatant incubated with 5 μl of either 1) 200 mM $MgCl_2$, 2) 300 mM EDTA or 3) a mixture of 100 mM AMP, 200 mM KH_2PO_4 and 300 mM EDTA for 10 min at 30°C. Assaying with $MgCl_2$ provided an estimate of NR activity *in vivo* while assaying with EDTA allowed estimation of the activity of the phosphorylated NR without the IP while the maximum activity (equivalent to total NR protein) of the enzyme was assayed with the AMP, KH_2PO_4 and EDTA mixture. The incubation period was reduced from 30 min used by Kaiser and Huber (1997) to 10 min to avoid degradation of NR activity over the longer time period (data not shown). Reaction medium (900 μl) consisting of 100 mM HEPES-KOH (pH 7.6), 1 mM DTT, 10 μM FAD, 20 mM KNO_3, and 0.2 mM NADH was added to the supernatant and incubated for 5 min at 30°C in a water bath after which the reaction was stopped by addition of 125 μl of 0.5 M zinc acetate. The samples were centrifuged for 1 min in a microfuge and 1 ml of a 1:1 mixture of 1% (w/v) sulphanilamide in 1.5 M HCl and 0.01% (w/v)

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NED added to 300 µl of reaction medium and the absorbance determined at 540 nm after 15 min. The NRA was calculated from the amount of NO_2^- formed.

2.3.5 *Phosphoenolpyruvate carboxylase activity (in vitro)*

Eight replicate plants from each treatment were harvested, divided into roots and leaves, quenched in liquid nitrogen and stored at –80°C until assayed for PEPc activity according to a modification of the method of Coombs (1987). The frozen tissue was homogenised with acid-washed sand in a pre-cooled mortar and pestle in 4 ml g^{-1} FW extraction buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 20% (v/v) glycerol, 5 mM NaF, 20 µM leupeptin (Sigma), 2% (w/v) casein and 2% (w/v) PVPP. The homogenate was centrifuged at 25 000 g for 15 min at 4°C and 75µl of the supernatant added to 500 µl of reaction medium consisting of 50 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 5 mM NaF, and 3 mM PEP (Sigma). Malate sensitivity, which gives an indication of phosphorylation status of the enzyme, was determined by the addition of malate to a final concentration of 0.8 mM to the reaction medium (Foyer *et al.,* 1998). Total extractable PEPc activity was measured in the absence of malate (Foyer *et al.,* 1998). The reaction was initiated by addition of 50 µl of 11.7 mM $NaH^{14}CO_3$ (specific activity of 0.98 µC*i* µmol^{-1}) and incubated at 30°C. From this reaction mixture 50 µl samples were added to 250 µl of 20 % (v/v) of saturated dinitrophenylhydrazine in 2 M HCl. The samples were left to stand overnight in a fume hood after which 2 M NaOH was added to neutralise the pH of the samples. The samples were counted on a LS 1801 liquid scintillation counter (Beckman Instruments Inc., Fullerton, California, USA) with 2 ml of Readygel (Beckman).

2.3.6 *Carbonic anhydrase activity (in vitro)*

Eight replicate plants from each treatment were harvested, divided into roots and leaves,

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quenched in liquid nitrogen and stored at –80°C until assayed for CA activity according to a modification of the method of Makino (1992). The frozen tissue was homogenised with acid-washed sand in a pre-cooled mortar and pestle in 4 ml g^{-1} FW extraction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM DTT, 0.5 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton-X100 (Sigma), 2% (w/v) casein and 2% (w/v) PVPP. The homogenate was centrifuged at 15 000 g for 5 min at 4°C and the supernatant kept on ice until addition to the reaction cuvette. Reaction buffer (4 ml) consisting of 20 mM Tris (pH 8.3) was added to the temperature controlled (3±0.5°C) reaction cuvette together with 500 µl of supernatant. After stabilisation of the pH, 2 ml of the reaction buffer saturated with CO_2 was added to the reaction cuvette and the rate of pH change was measured between pH 8.5 and 7.9 (Corning, New York, USA). The observed rate of change of pH was converted to equivalent µmol H^+ generated by comparison with a calibration established by titrating the reaction buffer between pH 8.5 and 7.9 with HCl.

2.3.7 *Statistical analysis*

Results were subjected to analysis of variance to determine the significance of differences between the responses to the applied factors. Where analysis of variance was performed, *post-hoc* Fisher's projected least significant difference (LSD) tests (95%) were conducted to determine the differences between the individual treatments using Statgraphics Ver. 7.0 (1993). Where percentage data were used these were arcsine transformed (Zar, 1984) prior to statistical analysis. When only two treatments were compared a Student's t test was used.

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2.4 Results

2.4.1 *Relative growth rates and nitrogen use efficiencies*

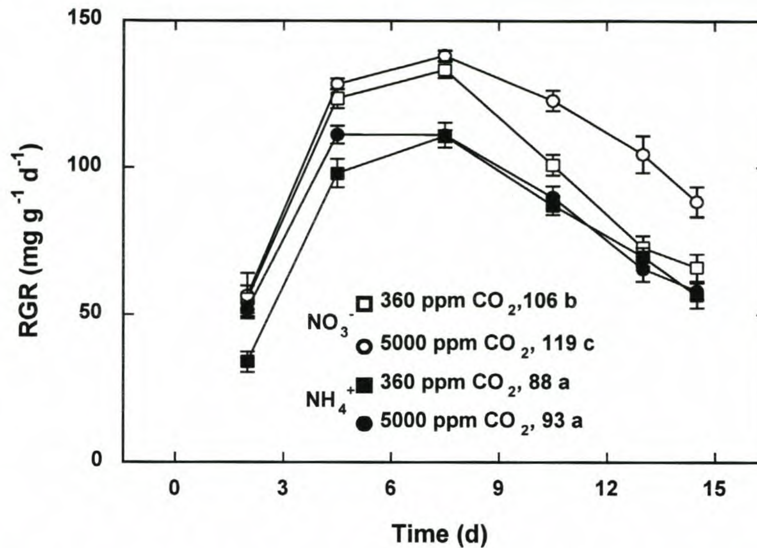

Figure 1 Comparison of the RGR (mg g^{-1} d^{-1}) over the growth period of 15 d for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing either 360 ppm or 5000 ppm root-zone CO_2. Mean RGR values for the 15 d period are given in the legend and different letters next to the values indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

The RGRs of NO_3^--fed plants grown with 360 ppm and 5000 ppm root-zone CO_2 were initially the same, but after 8 d the plants grown with 5000 ppm had higher RGR values than those of plants grown with 360 ppm root-zone CO_2 (Fig. 1). The RGRs of NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 were higher than those of NH_4^+-fed plants grown with 360 ppm root-zone CO_2 for the first 8 d of the experiment, after which no differences between RGRs could be discerned. The mean RGR over 15 d was *ca.* 1.1-fold higher for NO_3^--fed plants grown with 5000 ppm root-zone CO_2 compared to those grown with 360 ppm root-zone CO_2 (Fig. 1). In plants supplied with NH_4^+ nutrition the root-zone CO_2 concentration did not influence the mean RGR.

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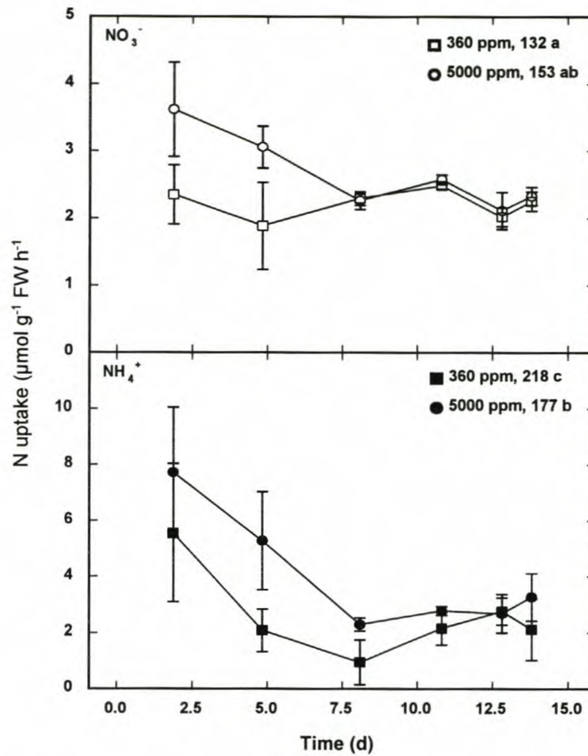

Figure 2 Comparison of the N uptake (µmol g^{-1} plant FW h^{-1}) over the growth period of 15 d for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing either 360 ppm or 5000 ppm root-zone CO_2. Mean N uptake rates (µmol g^{-1} root DW h^{-1}) for the last day of the long-term experiment are given in the legend and different letters next to the values indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

Expression of N uptake per plant fresh weight does not directly take into account the root specific uptake, but also represents whole plant growth and components such as shoots, which do not have a direct contribution to uptake. The expression of N uptake per gram root weight would therefore give a more accurate indication of the uptake since it represents directly the surfaces across which uptake proceeds. Nitrate uptake rates, expressed per total plant fresh weight, were initially higher for plants grown with 5000 ppm root-zone CO_2 compared to 360 ppm root-zone CO_2, but after 8 d no difference could be discerned between the uptake rates for the two different root-zone CO_2 concentrations (Fig. 2). Ammonium uptake rates were initially higher for plants grown with 5000 ppm root-zone CO_2 compared to plants grown with 360 ppm root-zone CO_2, but after 11 days no differences between the uptake rates for the two root-zone CO_2

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concentrations could be distinguished (Fig. 2). The NH_4^+ uptake rates were initially *ca.* 2.1-fold higher than the NO_3^- uptake rates for both root-zone CO_2 concentrations but after 8 d the uptake rates of the plants supplied with NO_3^- and NH_4^+ were similar (Fig. 2). No significant difference was found between the NO_3^- uptake rates (expressed per gram root dry weight over the last day of the long-term growth experiment) of plants grown at 360 ppm root-zone CO_2 compared to 5000 ppm root-zone CO_2 (Fig. 2). The NH_4^+ uptake rate (expressed per gram root dry weight) for plants grown with 360 ppm root-zone CO_2 was *ca.* 1.7-fold higher than the NO_3^- uptake rate and *ca.* 1.2-fold higher than the NH_4^+ uptake rate of plants grown with 5000 ppm root-zone CO_2 (Fig. 2).

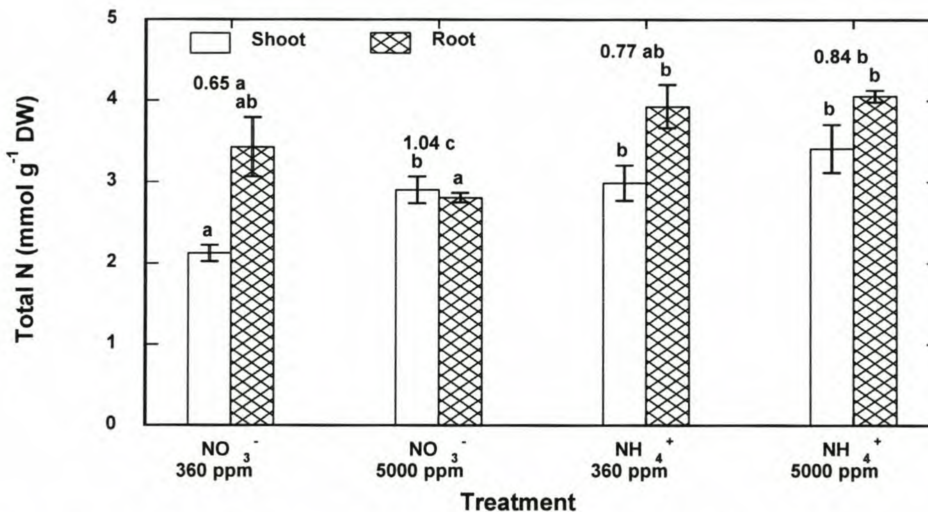

Figure 3 Comparison of the total N concentration (μmol g^{-1} DW) in roots and shoots of tomato seedlings grown for 15 d on either 2 mM NO_3^- or NH_4^+ combined with either 360 ppm or 5000 ppm root-zone CO_2. Error bars indicate the SE of the mean (n=6). Shoot to root ratios for total N are given above the bars. Different letters indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs were tested separately.

In NO_3^--fed plants, there was a *ca.* 1.4-fold increase in the concentration of total N of the shoots of plants grown with 5000 ppm compared to 360 ppm root-zone CO_2 (Fig. 3). Elevated DIC had no effect on the total N in the roots of NO_3^--fed plants. There were no significant differences due to variable root-zone CO_2 concentration in the total N concentration of shoots or roots in NH_4^+-fed plants. The total N shoot: root ratios for NO_3^--fed plants were *ca.* 1.6-fold

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higher with 5000 ppm root-zone CO_2 compared to 360 ppm root-zone CO_2 (Fig. 3), but root-zone CO_2 did not change the distribution of total N within NH_4^+-fed plants.

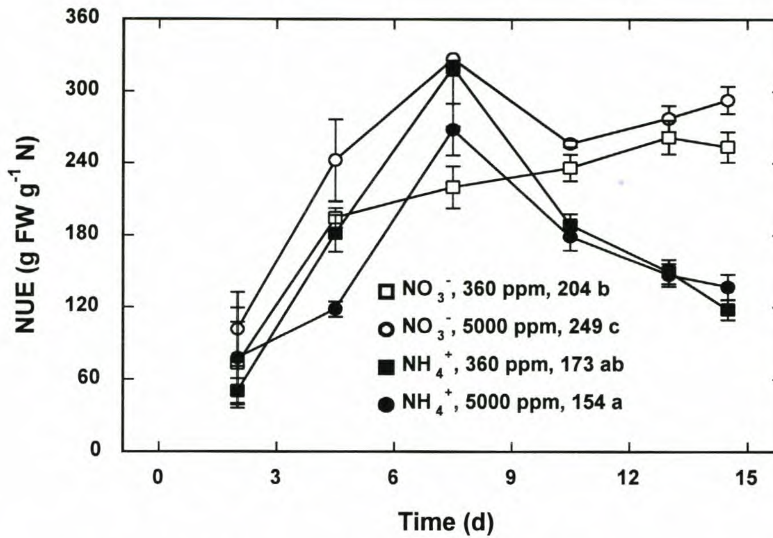

Figure 4 Comparison of NUE (g plant FW g^{-1} N) over the growth period of 15 d for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ combined with either 360 ppm or 5000 ppm root-zone CO_2. Mean NUE values for the 15 d period are given in the legend and different letters next to the values indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

The NUEs for all treatments showed an increase during the first 8 d of the growth experiment, after which there was a decline in NUE for NH_4^+-fed plants while the NUE of the NO_3^--fed plants remained stable (Fig. 4). Plants supplied with NO_3^- nutrition combined with 5000 ppm root-zone CO_2 maintained a higher NUE throughout the growth period than plants supplied with 360 ppm CO_2. The mean NUE over the 15 d period was *ca.* 1.2-fold higher for NO_3^--fed plants grown at 5000 ppm compared to 360 ppm root-zone CO_2. The NUE of NH_4^+-fed plants grown at 5000 ppm root-zone CO_2 was initially lower than that of plants grown at 360 ppm root-zone CO_2, but after 8 d this difference was eliminated and elevated DIC had no effect on the mean NUE of NH_4^+-fed plants.

2.4.2 *Nitrate reductase (in vivo and in vitro)*

Both the i*n vivo* NRA and the uptake rate of NO_3^- were found to be *ca.* 1.6-fold higher in plants receiving 5000 ppm than in those receiving 360 ppm root-zone CO_2 and NRA was correlated with NO_3^- uptake rate over the short term. The uptake rate of NO_3^- (μmol g^{-1} FW h^{-1}) was 25.3 ± 2.1 for plants grown with 5000 ppm root-zone CO_2 compared to 16.1 ± 1.3 for plants grown with 360 ppm (P=0.007). The *in vivo* NRA (μmol g^{-1} FW h^{-1}) was 57 ± 6.7 for plants grown with 5000 ppm root-zone CO_2 compared to 36.2 ± 3.7 for plants grown with 360 ppm (P=0.026).

Table 1. *In vitro* NRA (μmol g^{-1} FW h^{-1}) for the different activation states of NR expressed as percentages in leaves and roots after 15 d of tomato plants grown with 2 mM NO_3^- and aerated with air containing either 0 ppm or 5000 ppm root-zone CO_2. SE (±) of the mean is given next to the values (n=8). Different letters next to values indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs as well as activities and percentage activities were tested separately.

Character	**NRA (umol g^{-1} FW h^{-1})**		**Activation status (%)**	
	0 ppm CO_2	5000 ppm CO_2	0 ppm CO_2	5000 ppm CO_2
Leaf				
$MgCl_2$	13.13 ± 0.63 b	10.77 ± 0.35 a	69 ± 2.26 a	70 ± 3.69 a
EDTA	18.74 ± 1.03 d	15.36 ±1.02 bc	97 ± 2.31 b	98 ± 1.57 b
AMP+KH_2PO_4+EDTA	19.40 ± 1.41 d	15.62 ± 0.84 c	100	100
Root				
$MgCl_2$	6.16 ± 0.44 a'	8.93 ± 0.70 bc'	63 ± 0.44 a'	67 ± 0.70 b'
EDTA	7.84 ± 0.40 b'	10.80 ± 0.75 d'	81 ± 0.40 c'	82 ± 0.75 c'
AMP+KH_2PO_4+EDTA	9.71 ± 0.43 cd'	13.19 ± 0.88 e'	100	100

In vitro root NRA's determined after pre-incubation with $MgCl_2$, EDTA or AMP were all significantly higher for plants grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 (Table 1). In contrast, *in vitro* leaf NRA's determined after pre-incubation with $MgCl_2$, EDTA or AMP were significantly lower for plants grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2. In the roots NRA assayed after pre-incubation with $MgCl_2$ had the lowest activity, while assaying after pre-incubation with AMP had the highest activity for both root-zone CO_2 concentrations. Root NR phosphorylation status (expressed as percentage of

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activity assayed after pre-incubation with AMP) was significantly higher for plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Table 1).

2.4.3 *Phosphoenolpyruvate carboxylase*

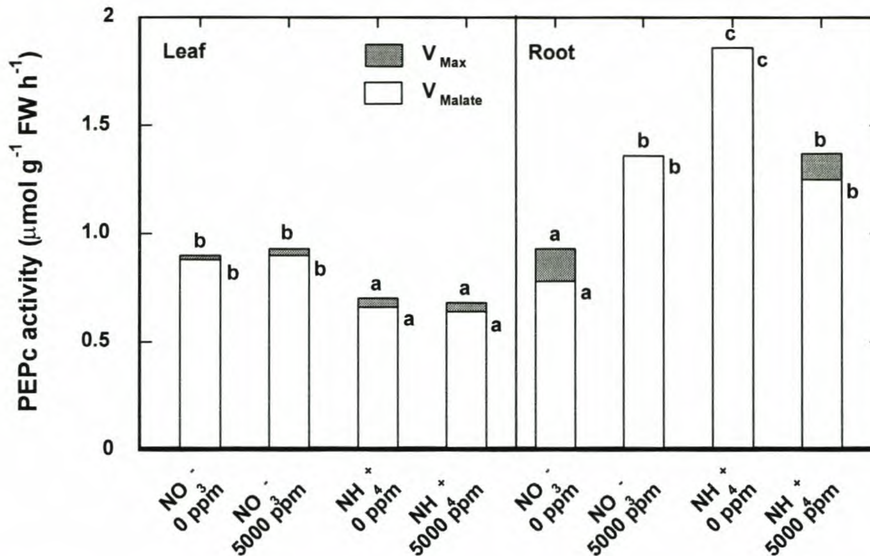

Figure 5 Comparison of total PEPc activity (µmol g^{-1} FW h^{-1}) and activity when assayed with malate (µmol g^{-1} FW h^{-1}) for leaves and roots of tomato plants grown on either 2 mM NO_3^- or NH_4^+ combined with either 0 ppm or 5000 ppm root-zone CO_2 after 15 d. Error bars indicate the SE of the mean (n=8). Different letters above the bars indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs were tested separately.

Total PEPc activity (phosphorylated and dephosphorylated) in the leaves was *ca.* 1.3-fold higher in NO_3^--fed plants compared to NH_4^+-fed plants grown with both 0 and 5000 ppm root-zone CO_2 (Fig. 5). Nitrate-fed plants had a *ca.* 1.4-fold higher total and *ca.*1.7-fold higher phosphorylated root PEPc activity at 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2. Ammonium-fed plants had a *ca.* 0.7-fold lower total and phosphorylated root PEPc activity at 5000 ppm root-zone CO_2 compared to 0 ppm 5000 ppm root-zone CO_2. No significant differences in total and phosphorylated root PEPc activity were found between NO_3^-- and NH_4^+-grown plants at 5000 ppm root-zone CO_2, however, at 0 ppm root-zone CO_2 NH_4^+-grown plants had *ca.* 2-fold higher

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total and *ca.* 2.4-fold higher phosphorylated root PEPc activities than NO_3^--grown plants.

2.4.4 *Carbonic anhydrase*

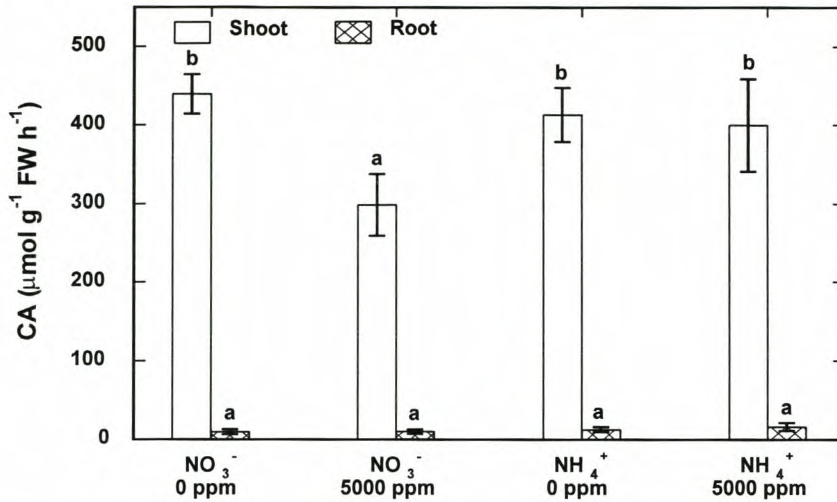

Figure 6 Comparison of CA activity (µmol g^{-1} FW h^{-1}) for leaves and roots of tomato plants grown on either 2 mM NO_3^- or NH_4^+ combined with either 0 ppm or 5000 ppm root-zone CO_2 after 15 d. Error bars indicate the SE of the mean (n=8). Different letters above the bars indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs were tested separately.

Leaf CA activity was *ca.* 0.7-fold lower in NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Fig. 6). The concentration of root-zone CO_2 had no significant effect on leaf CA activity of NH_4^+-grown plants. Root CA activity was only *ca.* 3% of the CA activity found in leaves. No significant difference in root CA activity could be discerned for either N source or root-zone CO_2 concentration. CA activity was more than 400-fold and 7-fold higher than total PEPc activity in the leaves and roots, respectively.

2.5 Discussion

The higher RGRs of NO_3^-- compared to NH_4^+-fed plants (Fig. 1) may have been be due to competition for carbon skeletons in the root of plants grown with NH_4^+ nutrition (Cramer and Lewis, 1993). This was thought to occur due to limited carbon skeleton availability in C_3 plants

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and the fact that NH_4^+ is largely assimilated in the roots, while NO_3^- assimilation occurs predominantly in the shoots (Andrews, 1986). The increased RGR of NO_3^--fed plants compared to NH_4^+-fed plants grown with either root-zone CO_2 concentration (Fig. 2) was not associated with increased N uptake, but may have been due to the toxic effect of accumulated NH_4^+ (Cramer & Lewis, 1993). The initial higher NO_3^- uptake rates expressed per total plant fresh weight of NO_3^--fed plants grown with 5000 ppm root-zone CO_2 compared to plants grown with 360 ppm root-zone CO_2 could have been because an increased uptake with 5000 ppm root-zone CO_2 only occurred when the tissue NO_3^- concentrations were low (Fig. 2). Ammonium is assimilated more rapidly than NO_3^- (Smart & Bloom, 1993) and this may have accounted for the initial higher NH_4^+ compared to NO_3^- uptake rates (Fig 2). The seedlings might have had enough organic carbon present in the cells to assimilate the NH_4^+ taken up during the rapid growth phase over the first five days (Fig 1). After *ca.* five days the growth rate reached a constant phase. The seedlings probably could not assimilate and utilise the NH_4^+ taken up and therefore the uptake of NH_4^+ was inhibited. A possible reason for the inhibition of NH_4^+ uptake in roots aerated with 5000 ppm root-zone CO_2 could have been that the root-zone CO_2 was converted to HCO_3^- and fixed anapleroticaly by PEPc to provide carbon skeletons for the synthesis of amino acids such as aspartate and asparagines, thereby diverting the carbon skeletons away from the TCA cycle and glutamate synthesis, which is needed for NH_4^+ assimilation (Van der Westhuizen & Cramer, 1998). The increased synthesis of amino acids could also have downregulated NH_4^+ uptake as shown previously by Causin & Barneix, (1993); Feng *et al.,* (1994); Glass *et al.,* (1997).

DIC leads to a shift in carbon and nitrogen partitioning and the higher total N concentrations for shoots and higher total N shoot: root ratios found for NO_3^--fed plants grown with 5000 ppm compared to 360 ppm root-zone CO_2 (Fig. 3) may have been due to greater translocation of reduced N, as found previously by Cramer and Lips (1995). Shoot total N

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concentrations of NH_4^+-fed plants grown with 360 ppm root-zone CO_2 were higher than those of NO_3^--fed plants possibly due to a greater uptake of NH_4^+ and translocation of reduced N in plants supplied with NH_4^+ nutrition (Fig. 3).

The initial increase in NUE for NO_3^-- and NH_4^+-fed plants grown at either root-zone CO_2 concentration was due to the almost linear increase in RGR and initially high N uptake rates (Fig. 1, 2, 4). The stable NUE of NO_3^--grown plants after *ca.* 8 d was due to NO_3^- uptake rates remaining constant (Fig. 4). Decreased NUE of NH_4^+-grown plants after *ca.* 8 d (Fig. 4) was possibly due to the toxic effect (Cramer and Lewis, 1993) of accumulated NH_4^+. The higher mean NUEs of NO_3^--fed plants grown with 5000 ppm root-zone CO_2 compared to plants grown with 360 ppm root-zone CO_2 indicated better growth with elevated root-zone DIC for similar N uptake rates (Fig. 4).

Tomato plants predominantly reduce NO_3^- in the shoots (Andrews, 1986) and in this study it was found that plants grown with both 0 and 5000 ppm CO_2 had higher NO_3^- reduction in the shoots compared to the roots (Fig 3, Table 1). The predominant site of NO_3^- reduction is also dependent on the concentration of NO_3^- supplied (Andrews, 1986) and would lead to a shift in the contribution made by the roots and the shoots, respectively. Seeing as a low concentration of NO_3^- (2 mM) was used in this study it may have encouraged increased root reduction of NO_3^-. From this it can be concluded that the decreased leaf NRA with 5000 ppm compared to 0 ppm root-zone CO_2 may have represented down-regulation of NRA in response to elevated root NRA. The root NRA assayed after $MgCl_2$ pre-incubation, which provides an estimate of *in vivo* NRA, was higher for plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Table1) allowing greater root participation in the reduction of NO_3^-. The larger proportion of NRA expressed after $MgCl_2$ pre-incubation at 5000 ppm root-zone CO_2 indicated a small reduction in inhibition by IP-

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binding combined with phosphorylation relative to 0 ppm root-zone CO_2. NRA assayed after EDTA pre-incubation, representing NR activity without inhibition by IP binding and thus reflecting the phosphorylation status of the NR, was higher in roots of plants grown with 5000 ppm compared to 0 ppm root-zone CO_2. However, there was no difference between plants supplied with 5000 or 0 ppm root-zone CO_2 in the proportion of NR inactivated by phosphorylation alone (Table 1). Root NRA assayed after AMP pre-incubation, which represents maximum NR activity (dephosphorylated) was higher in plants supplied with 5000 ppm compared to 0 ppm root-zone CO_2 which indicated an increase in NR protein. This is corroborated by the fact that the plants receiving 5000 ppm root-zone CO_2 had *ca.* 1.6-fold higher NO_3^- uptake rates expressed per gram root fresh weight than those of plants receiving 360 ppm root-zone CO_2 and consequently higher NRA *in vivo*. Aeration with 5000 ppm root-zone CO_2 could have increased NR protein concentration and led to a small activation of NR by a decrease in cytosolic pH.

The reduction and assimilation of NO_3^- in the shoots of tomato plants could account for the higher total PEPc activity in leaves of NO_3^--fed plants compared to NH_4^+-fed plants grown with 0 and 5000 ppm root-zone CO_2 (Fig. 5). The higher total leaf PEPc activity and lower total root PEPc activities of NO_3^- compared to NH_4^+-fed plants grown with 0 ppm root-zone CO_2 (Fig. 5) were similar to results found by Schweizer and Erismann (1985). From the results it could be concluded that root-zone CO_2 did not have an effect on the total or phosphorylated leaf PEPc activity of NO_3^--fed plants. However, in the roots an increase in total and phosphorylated PEPc activity was found for NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2. This contradicts a lack of response of PEPc activity to elevated root-zone CO_2 reported previously for tomato (Cramer *et al.*, 1999). This may have been due to Cramer *et al.* (1999) using 360 ppm root-zone CO_2, whereas in the present study 0 ppm root-zone CO_2 was used.

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Another possibility for the contradictory results may have been the different methods used for assaying the enzyme. In this study there were difficulties with background NADH oxidation and therefore a PEPc assay using ^{14}C instead of NADH was chosen. Decreased total root PEPc activity of NH_4^+-fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 was probably due to inhibition of NH_4^+ uptake by 5000 ppm root-zone CO_2 (Fig 2). The PEPc of NH_4^+-fed plants measured after *ca.* four weeks was adequate to sustain an NH_4^+ assimilation rate, which would ensure no accumulation of NH_4^+. It remains to be evaluated whether PEPc activity at the early stages of NH_4^+ uptake (see Fig 2) would be adequate. The lack of difference between leaf phosphorylated PEPc activities for plants grown with either N form and root-zone CO_2 concentration indicated that protein concentration rather than phosphorylation status was important in determining the activity of leaf PEPc. According to Koga and Ikeda (1997) the increase in root PEPc activity that contributes to the replenishment of carbon skeletons for the continuous supply of NH_4^+ in roots is dependent on *de novo* protein synthesis.

The decrease in leaf CA activity of NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Fig. 6) could have been due to an increased translocation of N to the shoot in NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Fig. 3). The only supporting result found was that of Khan *et al,* (1996) showing that increased concentrations of $NaNO_3$ inhibited leaf *in vitro* CA activities. Another possibility was that organic acids derived from root-zone DIC could have been translocated in the xylem (Cramer & Richards, 1999) and decarboxylated in the shoot to be re-assimilated by photosynthesis (Arteca and Poovaiah, 1982) and the resulting increased CO_2 concentration in the shoot could have been responsible for the depression of CA activity. The higher leaf CA activities of NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 compared to NO_3^--fed plants grown with 5000 ppm root-zone CO_2 (Fig. 6) could have been due to the stimulating effect of NH_4^+ on *in vitro* CA activity as found by

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Mohammad *et al.,* (1997). The extremely low CA activities in roots compared to leaves could have been due to the high concentration of CO_2 in soils (Norstadt and Porter, 1984). The higher *in vitro* CA activity compared to *in vitro* PEPc activity found in this study concurred with *in vivo* results of Chang and Roberts (1992) that CA activity was more than 200 times higher than that of PEPc *in vivo*. Since both PEPc (Chollet *et al.,* 1996) and CA (Rumeau *et al.,* 1996) are located in the cytosol, it was concluded that HCO_3^- availability was not a limitation for PEPc activity. If CA in the roots is not needed for HCO_3^- provision, it may be concluded that the CA is not required. However, CA catalyses the reversible hydration reaction (Rengel, 1995) and an interesting possibility is that CA may facilitate the release CO_2 from roots. Since the measurements of CA and PEPc activities in this study were on whole tissue extracts it is not possible to rule out the possible role of CA in provision of HCO_3^- for PEP carboxylation in certain tissues.

2.6 Conclusions

Nitrate-grown plants had higher RGRs and NUEs than NH_4^+-grown plants at 5000 ppm root-zone CO_2 due to increase in biomass for the amount of N taken up. Nitrate and NH_4^+ uptake was influenced initially by root-zone CO_2 after which the uptake rates maintained steady-state. Changes in NUE were associated with modifications in the coarse control of NR and PEPc as well as small changes in fine control of NR.

2.7 Acknowledgements

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Chapter 3

An investigation into the contribution of dissolved inorganic carbon to the partitioning of C and N in tomato plants

Running title: DIC influence on N and C partitioning

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3.1 Summary

- This study investigated the extent of uptake and allocation of $NaH^{14}CO_3$ into labile pools of leaves, stems and roots over 24 h after a 1 h pulse.
- NO_3^-- and NH_4^+-fed plants grown with either 0 or 5000 ppm root-zone CO_2 were fed with 0.93 MBq $NaH^{14}CO_3$. The soluble and insoluble fractions of roots, stems and leaves as well as the nutrient solutions were analysed to determine in which organic form and proportion ^{14}C was present.
- The $DI^{14}C$ incorporation in the roots was *ca.* 10-fold higher in NH_4^+-fed compared to NO_3^--fed plants grown with both root-zone CO_2 concentrations. Ammonium-fed plants grown with 5000 ppm root-zone CO_2 had the highest organic and inorganic exudation of incorporated ^{14}C. Plants retained up to 86 % of incorporated $DI^{14}C$ after 24 h.
- The greater $DI^{14}C$ incorporation by NH_4^+-fed plants was probably in response to the carbon requirements for NH_4^+ assimilation. The large exudation of NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 was probably due to the high $DI^{14}C$ incorporation of these plants.

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Keywords: dissolved inorganic carbon, NO_3^-, NH_4^+, carbon partitioning, nitrogen partitioning

3.2 Introduction

Most soils have higher dissolved inorganic carbon (DIC) than hydroponic solutions due to the accumulation of respiratory CO_2 produced by the biological components of soils. DIC comprises a pH-dependent combination of CO_2, HCO_3^- and CO_3^{2-} in solution (Norstadt & Porter, 1984). Incorporation of DIC serves an anaplerotic function by providing intermediates for the TCA cycle through the activity of phosphoenolpyruvate carboxylase (PEPc), which is responsible for re-fixation of respiratory CO_2 (Vuorinen & Kaiser, 1997). The assimilation of DIC (as HCO_3^-) through PEPc activity in the root is responsible for only a small contribution to the carbon budget of the whole plant, but DIC assimilation could occur at rates equivalent to 30% of the rate of respiration in plant roots exposed to 5000 ppm CO_2 (Cramer & Lips, 1995). DIC fixed by PEPc provides carbon skeletons for both amino acid (Schweizer & Erismann, 1985, Vuorinen & Kaiser, 1997) and organic acid synthesis (Cramer *et al.,* 1993).

Elevated DIC supplied to the root-zone and the subsequent uptake thereof has effects on physiological processes including photosynthesis (Cramer & Richards, 1999), respiration (Van der Westhuizen & Cramer, 1998), NO_3^- uptake (Cramer *et al.,* 1996), partitioning of C and N to organic and amino acid synthesis (Cramer & Lewis, 1993) and growth (Cramer & Richards, 1999). Positive effects of root-zone DIC on plant growth have been reported previously (Vapaavuori & Pelkonen, 1985), although, Cramer & Richards (1999) found that growth effects on plants grown with elevated DIC were most readily seen in plants growing under high irradiances, salinity stress or high shoot temperatures.

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Elevated root-zone DIC leads to a shift in partitioning of carbon and nitrogen into organic and amino acid synthesis of plants grown with different nitrogen sources (Cramer & Lewis, 1993). Nitrate uptake can be increased by elevated DIC due to increased incorporation of the reduction products of NO_3^- into amino acids or a direct stimulatory effect on NO_3^- uptake (Cramer *et al.,* 1996). Ammonium uptake was decreased or unchanged with elevated root-zone DIC compared to ambient root-zone DIC (Cramer *et al.,* 1996). The higher rates of ^{14}C incorporation in roots of NH_4^+-fed plants compared to NO_3^--fed plants is correlated with increased PEPc activity in the presence of NH_4^+ nutrition compared to NO_3^- nutrition in the roots (Schweizer & Erismann, 1985; Arnozis *et al.,* 1988). Cramer *et al.* (1993) found that root-zone DIC led to a larger proportion of root derived carbon being allocated to organic acids in NO_3^--fed maize plants to maintain ionic balance in the xylem sap, whereas in NH_4^+-fed maize plants more carbon was allocated to amino acids (aspartate, asparagine, glutamate, glutamine). This was due to the carbon requirements for amino acid synthesis during NH_4^+ assimilation (Cramer *et al.,* 1993). Vuorinen *et al.,* (1992) used labelled organic and amino acids and found the label incorporated into protein and insoluble components.

Previous studies regarding the uptake and partitioning of $DI^{14}C$ were done using chase periods of 1 to 6 h and did not take into account the amount of carbon lost through exudation and respiration over a 24 h period. The aim of this study was to investigate the extent of $NaH^{14}CO_3$ taken up into labile pools of leaves, stems and roots over a 1 h pulse and how these pools changed over a chase period of 24 h. The partitioning of incorporated ^{14}C to the insoluble fractions or loss through respiration and root exudation was followed and the root exudation products were analysed to determine in what form carbon was exuded from the roots.

Abbreviations: DIC; dissolved inorganic carbon

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3.3 Materials and Methods

3.3.1 *Growth conditions*

Seedlings (14 d old) of *Lycopersicon esculentum* (L.) cv. F144 grown on a 1:1 mixture of vermiculite and compost were transferred to hydroponic culture after rinsing the roots in distilled H_2O. The hypocotyls of the plants were wrapped in black closed-cell foam rubber and inserted through collars in the lids of 22 l hydroponic tanks with eight plants per tank. The tanks were completely opaque and contained 20 l Long Ashton nutrient medium (Hewitt, 1966) modified to contain 2 mM of either $NaNO_3$ or NH_4Cl as a nitrogen source and 0.09 mM FeEDTA as an iron source. The nutrient medium was changed weekly and the pH of the medium was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. Plants were grown in a temperature controlled (minimum 15°C, maximum 25°C) greenhouse at the University of Stellenbosch during spring (September and October). Nutrient solutions were strongly aerated with CO_2-free air (0 ppm CO_2) or with air containing elevated root-zone CO_2 (5000 ppm CO_2) produced by enriching ambient air with CO_2 from a cylinder of industrial grade CO_2 (Afrox, Cape Town, South Africa). Carbon dioxide was removed from the air by passing ambient air through 2 M NaOH and a column (4 cm diameter and 30 cm length) containing 4-8 mesh soda lime (Saarchem, Krugersdorp, South Africa). The CO_2 concentration was monitored continuously using an ADC Mk3 (Analytical Development Corporation, Hoddeston, England) infrared gas analyser (IRGA). To prevent diffusion of CO_2 from the rhizosphere and the consequent enrichment of atmosphere around the shoots, the lids of hydroponic tanks were sealed with closed-cell foam rubber around the rim and clamped onto the tanks. The air-space between the surface of the nutrient solution and the lid was maintained under a partial vacuum to ensure that net air flow was inwards. Plants were used for experiments when the biomass was *ca.* 6 g.

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3.3.2 *DI^{14}C labeling*

The seedlings were pre-treated overnight in 300 ml containers in a Long Ashton nutrient solution with 2 mM of either $NaNO_3$ or NH_4Cl and aerated with 0 ppm or 5000 ppm CO_2. The seedlings were then transferred to fresh 300 ml Long Ashton solution containing 2 mM of either $NaNO_3$ or NH_4Cl. $NaHCO_3$ containing 0.093 MBq $NaH^{14}CO_3$ was added to the nutrient solutions, a 1 ml sub-sample taken from each container and then aeration was discontinued. Solutions were aerated for 15 s every 15 min over a period of 1h. The plants were removed from the nutrient solutions, the roots rinsed in two volumes of 2 mM $CaSO_4$ and transferred to fresh nutrient solutions containing 2 mM of either $NaNO_3$ or NH_4Cl. The roots of three plants from each treatment were blotted dry and the plants were divided into leaf, stem and root components, weighed, quenched in liquid N_2 and stored at -18°C. This procedure was repeated after 6, 12 and 24 h. Sub-samples of the nutrient solutions were taken at each harvest and stored at -18°C.

The plant components were homogenised in a mortar and pestle in 50 ml of cold 80 % (v/v) ethanol and stored at -18°C for 48h prior to extraction for 60 min at 45°C. The samples were filtered through Whatman no 1 filter paper (Whatman International Ltd, Maidstone, England) and the filtrate was made up to volume (75 ml). A subsample of 500 μl from each soluble fraction was acidified with 50 μl of 0.3 M HCl and stood in a fume hood for 12 h on a shaker. Then 2 ml of Readygel (Beckman Instruments Inc., Fullerton, California, USA) was added, the samples mixed and counted on a LS 1801 scintillation counter (Beckman). The residue was dried in an oven for 48h at 80°C and the weights of the insoluble fraction determined. The biscuits of the insoluble fraction were finely ground in a mortar and pestle and a sub-sample of 100 mg weighed out and acidified with 500 μl of 0.3 M HCl, stood on a shaker in a fume hood for 12 h. Afterwards 2 ml of Soluene-350 (Packard Instrument BV Chemical Operations, Groningen, The Netherlands) was added and samples shaken for a further 12 h. 4 ml of 96% (v/v) ethanol was added and the samples were mixed. A sub-sample of 100 μl was taken

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and counted with 2 ml of Readygel (Beckman) on a LS 1801 liquid scintillation counter (Beckman). Samples of 75 ml of the soluble fractions were evaporated and made up to 20 ml. Sub-samples of 1 ml were loaded onto 1 ml Dowex 50W-X8 and Dowex 1W-X8 (Sigma) columns in 1 ml disposable syringes and further eluted, collected and counted in the same way as the nutrient solutions.

Sub-samples of 20 ml of the nutrient solutions were evaporated and made up to 5 ml. The samples were separated into basic, acidic and neutral fractions using ion exchange resins prepared according to Atkins & Canvin (1971). Samples were loaded onto 1 ml Dowex 50W-X8 and Dowex 1W-X8 (Sigma, St Louis, Missouri) columns in 1 ml disposable syringes and eluted with 25 ml 50% (v/v) ethanol. The elute was collected as the neutral fraction. The basic fraction was eluted from the Dowex 50W-X8 column with 10 ml 2 M HCl and the acidic fraction was eluted from the Dowex 1W-X8 column with 10 ml of 6 M formic acid. Subsamples of 1 ml were taken of each fraction and counted with 2 ml Readygel (Beckman) on a scintillation counter. The basic fraction mainly consists of amino acids, the acidic fraction mainly of organic acids and monophosphate esters and the neutral fraction mainly of sugars (Atkins & Canvin, 1971) and will be referred to in the text as amino and organic acids and the neutral fraction, respectively.

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3.4 Results

3.4.1 $^{14}CO_2$ *incorporation into soluble fractions*

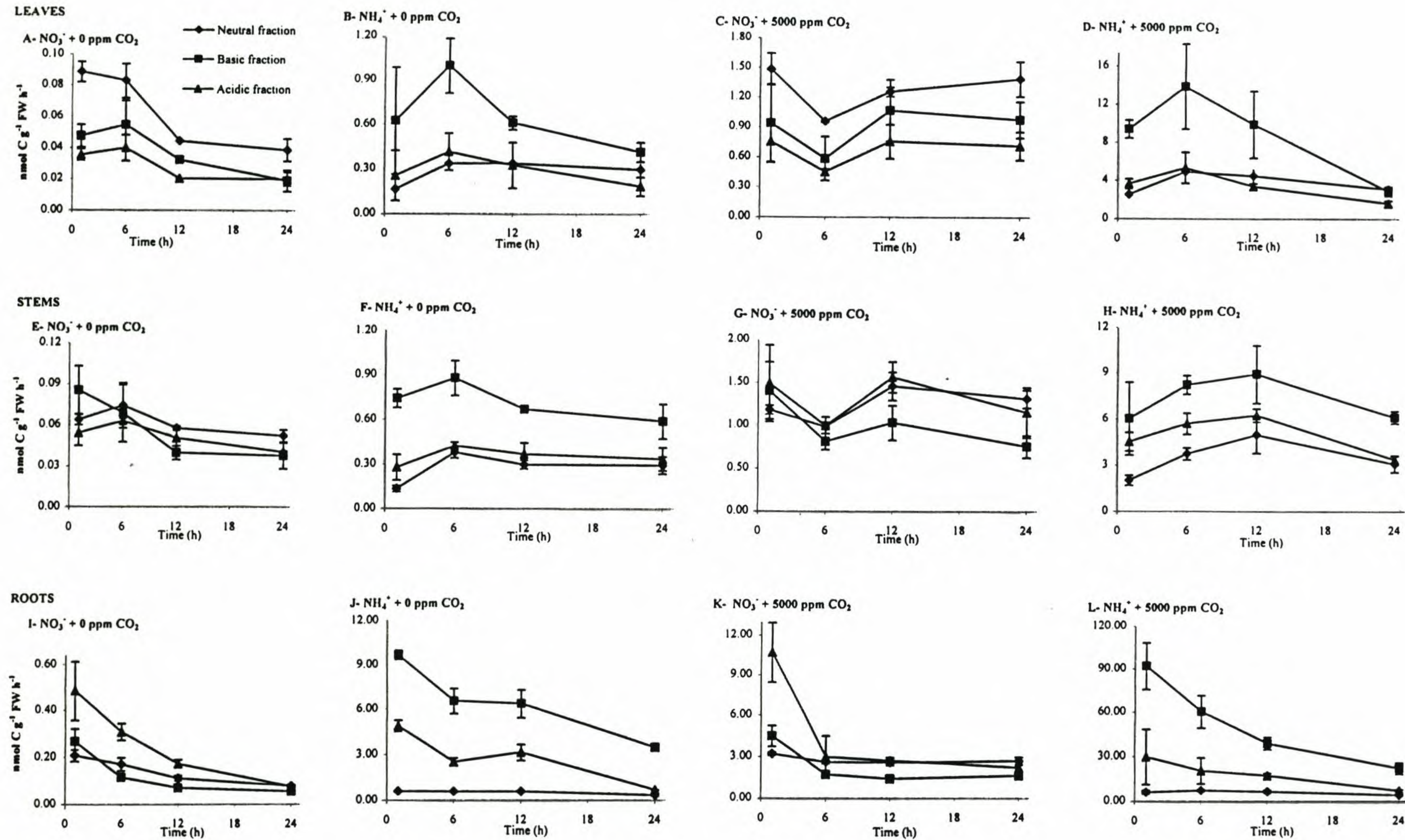

Figure 1. Incorporation of $^{14}CO_2$ (nmol C g^{-1} FW h^{-1}) supplied to roots for 1 h and traced over 24 h into acid-stable organic products (80% ethanol soluble) comprised of the neutral fraction, the basic fraction and the acidic fraction in roots, stems and leaves of four-week-old tomato plants. The tomato plants were grown with 2 mM NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. Error bars indicate SE of the means (n=3).

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Ammonium-fed plants grown with 0 and 5000 ppm root-zone CO_2 concentrations incorporated ca. ten-fold more $^{14}CO_2$ than NO_3^--fed plants grown with 0 and 5000 ppm root-zone CO_2 (Fig 1 A to L). In NH_4^+-fed plants grown with either 0 or 5000 ppm root-zone CO_2 ca. 10- or 4-fold more ^{14}C labelled organic carbon was localized in the stems and leaves than in NO_3^--fed plants, respectively (Fig 1 A to L). The predominant labelled compound in NO_3^--fed plants was the neutral fraction and when these NO_3^--fed plants were grown with 5000 ppm root-zone CO_2 the labelled neutral fraction was ca. 17- to 20-fold more for roots, stems and leaves than when grown with 0 ppm root-zone CO_2 (Fig 1A, C, E, G, I, K). Irrespective of the CO_2 concentration used, NO_3^--fed plants had the ^{14}C label predominantly incorporated into organic acids in the roots, whereas in leaves the major labelled fraction consisted of the neutral compounds (Fig 1 A, C, E, G, I and K). Amino acids were the major labelled compound for NH_4^+-fed plants (Fig 1 B, D, F, H, J, L). Furthermore, NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 had between 8- and 16-fold more labelled amino acids than plants grown with 0 ppm root-zone CO_2 (Fig 1 B, D, F, H, J, L). The stems and leaves of NH_4^+-fed plants grown with either root-zone CO_2 concentration had a peak in the labelled amino acid fraction after 6 to 12 h, after which there was a decrease in the fraction. Irrespective of the treatment, the roots had ca. 10-fold higher ^{14}C incorporation into the soluble fractions than the stems and leaves (Fig 1).

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3.4.2 *Carbon to nitrogen ratios*

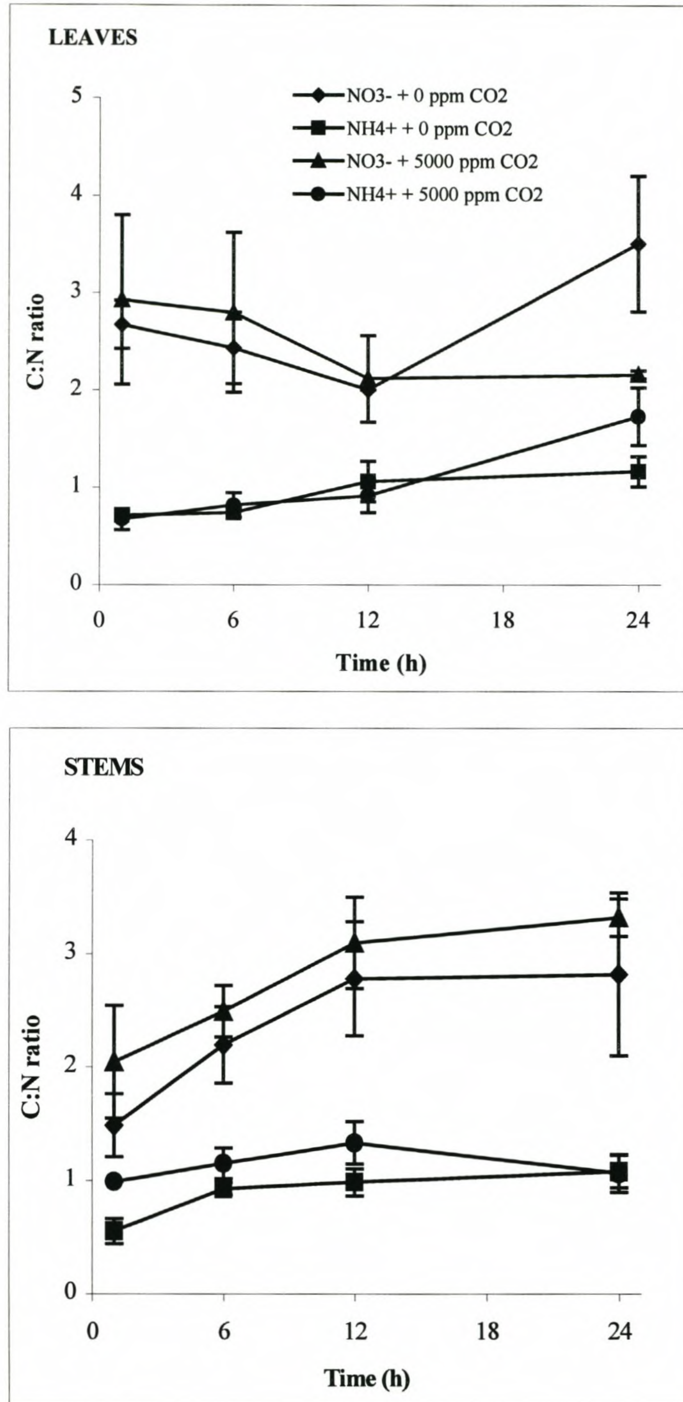

Figure 2 The C:N ratios (ratio of neutral and acidic fraction to basic fraction) of root assimilated $^{14}CO_2$ of the acid-stable organic products (80% ethanol soluble) of the leaves and stems of four-week-old tomato plants grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. Error bars indicate SE of the means (n=3).

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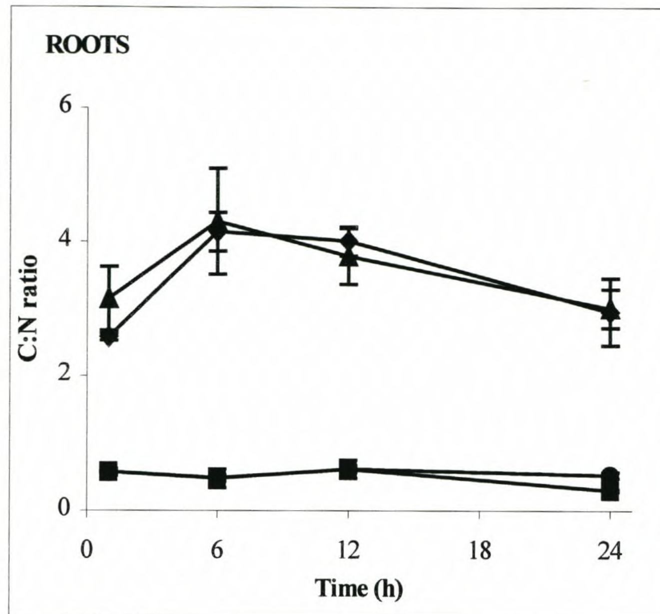

Figure 2 The C:N ratios (ratio of neutral and acidic fraction to basic fraction) of root assimilated $^{14}CO_2$ of the acid-stable organic products (80% ethanol soluble) of the roots of four-week-old tomato plants grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. Error bars indicate SE of the means (n=3).

The C:N ratio was *ca.* 2.5-fold lower for the leaves, stems and roots of NH_4^+-fed plants compared to NO_3^--fed plants for both root-zone CO_2 concentrations over the 24 h period (Fig 2). The C:N ratio of NH_4^+-fed plants grown with both root-zone CO_2 concentrations increased *ca.* 2-fold in leaves over 24 h and increased in stems for the first 12 h. However, in roots the C:N ratios of the NH_4^+-fed plants grown with both root-zone CO_2 concentrations remained constant (Fig 2).

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3.4.3 $^{14}CO_2$ *incorporation into insoluble fractions*

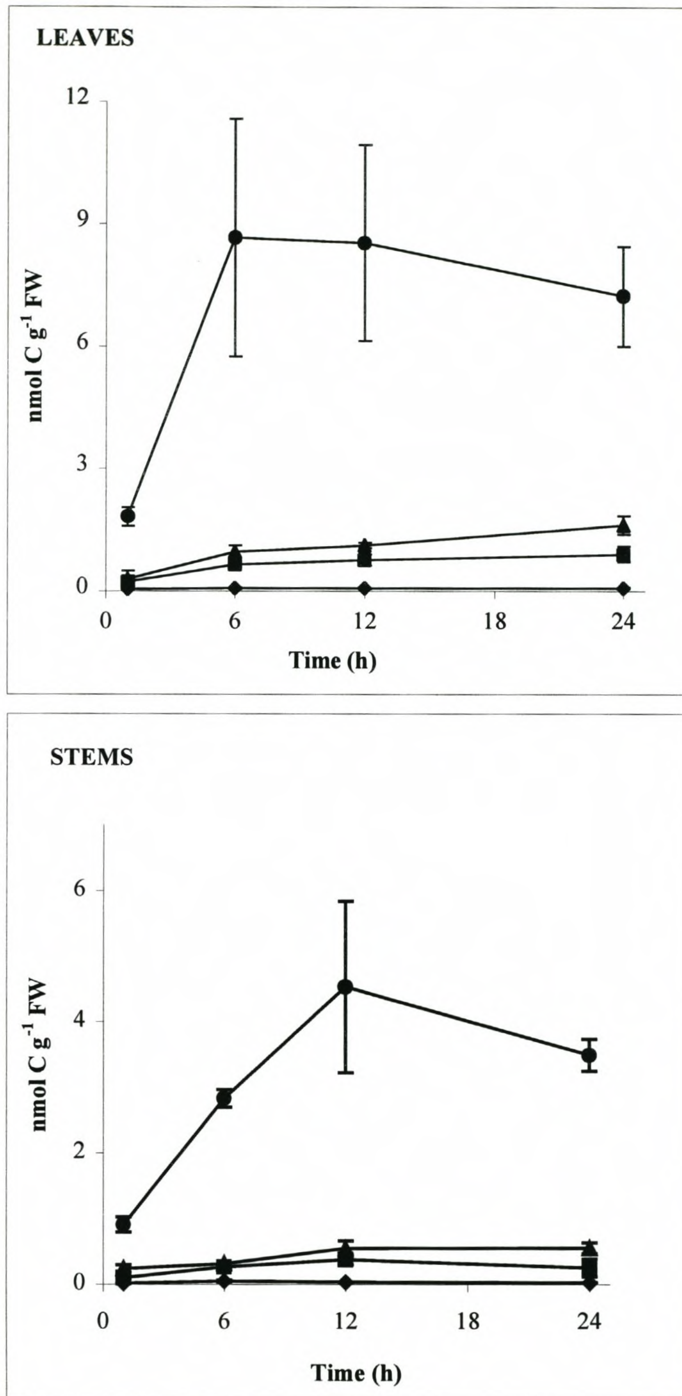

Figure 3 Incorporation of $^{14}CO_2$ into acid stable organic products (80% ethanol insoluble) in leaves and stems of four-week-old tomato plants grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. The ^{14}C was supplied to the roots for 1 h followed by a chase period of up to traced over 24 h Error bars indicate SE of the means (n=3).

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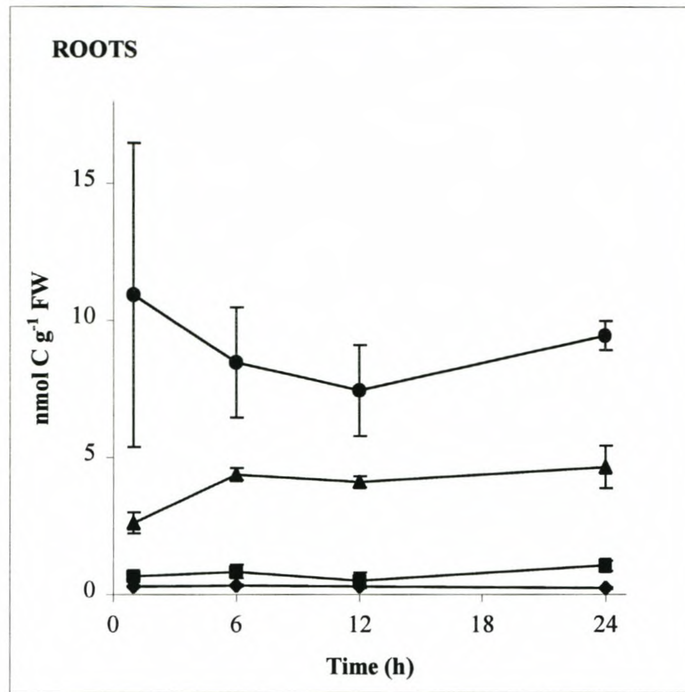

Figure 3 Incorporation of $^{14}CO_2$ into acid stable organic products (80% ethanol insoluble) in roots of four-week-old tomato plants grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. The ^{14}C was supplied to the roots for 1 h followed by a chase period of up to traced over 24 h Error bars indicate SE of the means (n=3).

The amount of ^{14}C incorporated into the leaves, stems and roots insoluble fractions was higher for NH_4^+-fed plants compared to NO_3^--fed plants grown with 0 and 5000 ppm root-zone CO_2, respectively and had values ranging from 2.3-fold for the roots to 11-fold for the leaves (Fig 3). The amount of ^{14}C incorporated into the NO_3^--fed insoluble fractions of roots, stems and leaves was higher when grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 (Fig 3).

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3.4.4 *Organic and $^{14}CO_2$ release of incorporated $^{14}CO_2$ over time*

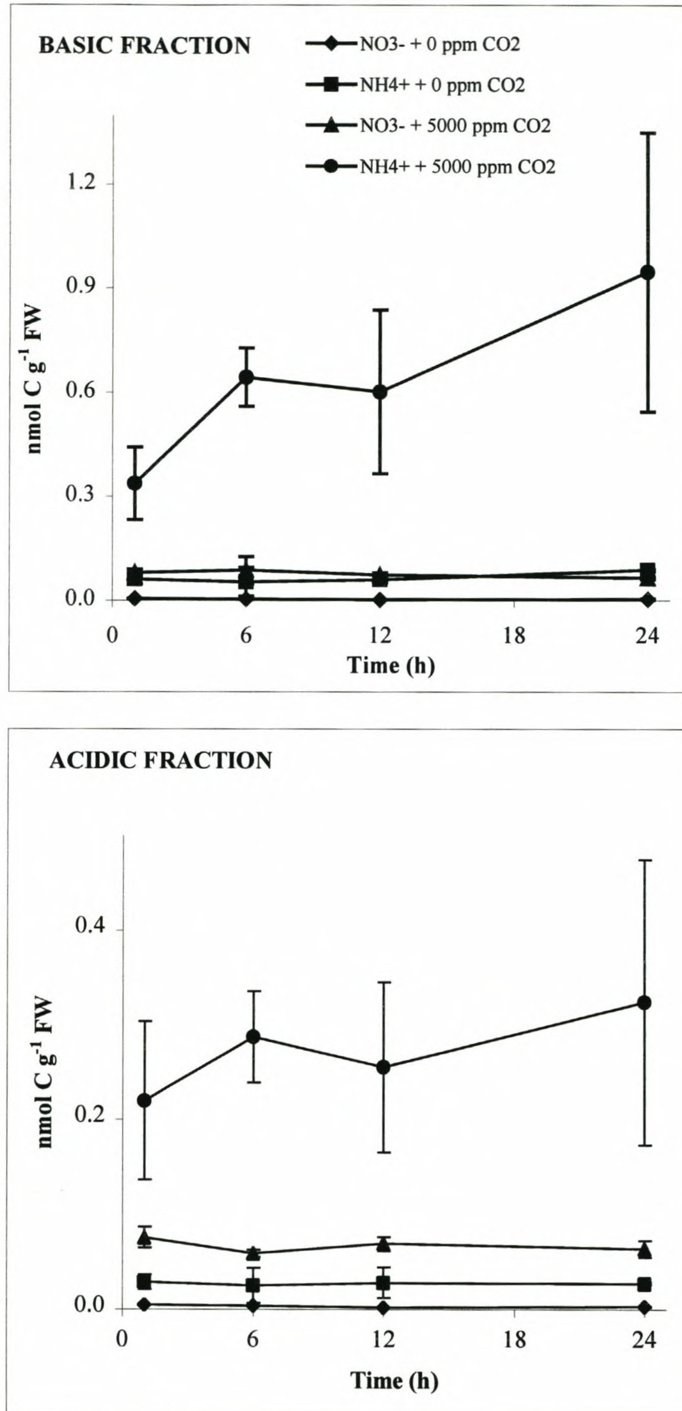

Figure 4 Exudation of acid-stable organic ^{14}C products consisting of basic and acidic fractions over 24 h from the roots of four-week-old tomato plants supplied with $^{14}CO_2$ for 1 h. These plants were grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. Error bars indicate SE of the means (n=3).

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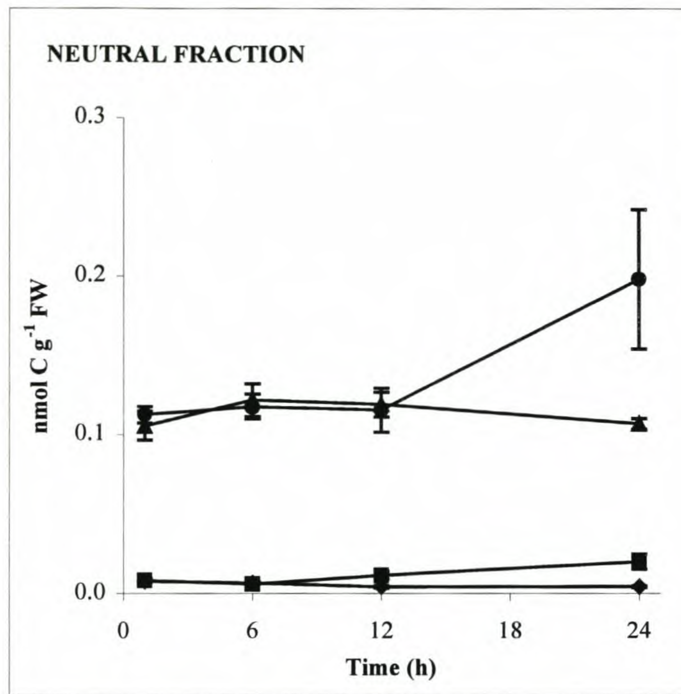

Figure 4 Exudation of acid-stable organic ^{14}C products consisting of neutral fractions over 24 h from the roots of four-week-old tomato plants supplied with $^{14}CO_2$ for 1 h. These plants were grown with 2 mM either NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2. Error bars indicate SE of the means (n=3).

Ammonium-fed plants grown with 5000 ppm root-zone CO_2 had the highest root exudation of incorporated ^{14}C over the 24 h period (Fig 4). The amount of incorporated ^{14}C lost through root exudation as amino acids, organic acids and neutral compounds was *ca.* 10- fold higher for NH_4^+-fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Fig 4). The NH_4^+-fed plants grown with both root-zone CO_2 concentrations exuded ^{14}C labelled amino acids 3- or 5-fold more than ^{14}C labelled organic acids and neutral compounds, respectively (Fig 4). The amount of incorporated ^{14}C lost through root exudation in the form of these water-soluble fractions was *ca.* 21-fold higher when NO_3^--fed plants were grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 (Fig 4). Nitrate-fed plants exuded ^{14}C labelled neutral compounds 2-fold more than ^{14}C labelled organic and amino acids when grown with either root-zone CO_2 (Fig 4). The amount of ^{14}C exudation in the form of neutral compounds was the same for NO_3^-- and NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 for the first 12 h (Fig 4).

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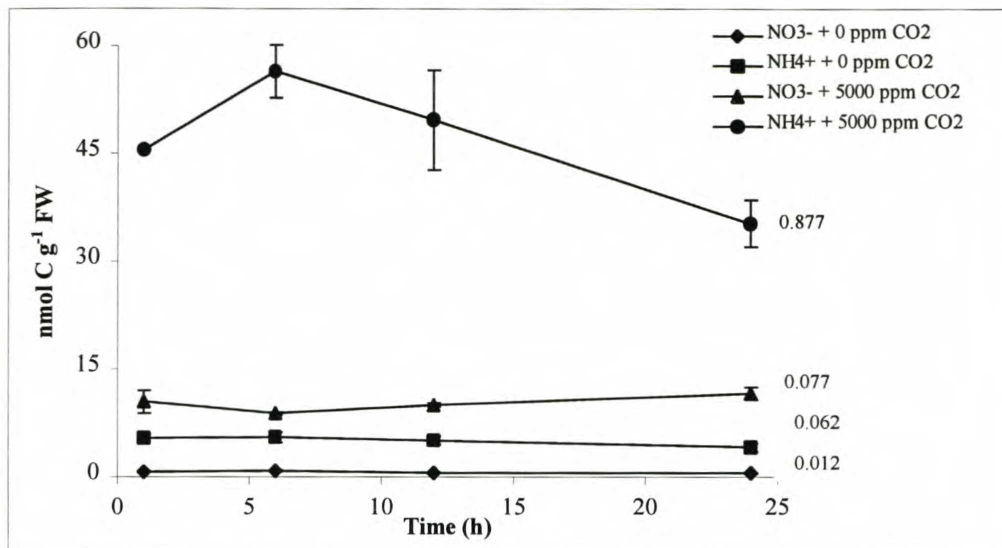

Figure 5. The total $^{14}CO_2$ incorporated into the soluble, insoluble and root exudation fractions (^{14}C taken up per g plant FW) by the roots during a 1 h pulse of ^{14}C followed by a chase period of 24 h. These 4-week-old plants were grown with 2 mM either NO_3^- or NH_4^+ and the root solutions aerated with 0 or 5000 ppm root-zone CO_2. The values on the graph are the rates of inorganic $^{14}CO_2$ loss (nmol CO_2 g^{-1} FW h^{-1}) calculated from the slope of the graph over the 24 h period. Error bars indicate SE of the means (n=3).

Figure 5 shows the total organic amount (soluble + insoluble + root exudation) of root incorporated ^{14}C for the different treatments over the 24 h and a decrease in the total would signify a loss of inorganic ^{14}C via respiration from either the roots or the leaves. Irrespective of the DIC concentration, NH_4^+-fed plants had consistently higher rates of inorganic ^{14}C loss compared to NO_3^--fed plants (Fig 5). With both nitrogen forms, 5000 ppm root-zone CO_2 resulted in greater rates of $^{14}CO_2$ loss relative to 0 ppm root-zone CO_2 (Fig 5).

3.5 Discussion

This work confirms that elevated DIC supplied to plant roots in hydroponic culture results in enhanced CO_2 fixation through PEPc and thereby probably contributes significantly to the carbon balance of such roots. Under normal growth conditions the PEPc reaction is probably closer to saturation with CO_2 than in hydroponic culture. Consequently PEPc probably makes little contribution to respiratory CO_2 fixation in hydroponic culture. In soils, the endogenous CO_2

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concentrations are usually between 2000 and 5000 μmol mol^{-1} due to the accumulation of respiratory CO_2 produced by the biological components of soil (Norstadt & Porter, 1984). Therefore, when hydroponic cultures are aerated with atmospheric air it will not be representative of naturally occurring conditions and CO_2 re-fixation may take place at rates far slower than what it normally does. Even though a significant portion of carbon is released via respiration, only a very small portion is re-fixed. This is probably due to the overwhelming flow of CO_2 out of the root due to the concentration gradient. Supplying the roots with elevated CO_2 would result in more respiratory CO_2 being re-fixed because the high root-zone CO_2 concentration would inhibit loss of respiratory CO_2.

This study indicated for the first time that the products of PEPc fixation were retained in the plant and not rapidly consumed in the respiratory pool and that regardless of N source, plants grown with 0 ppm root-zone CO_2 concentration retained *ca.* 66 % of their incorporated ^{14}C, whereas with 5000 ppm root-zone CO_2 NO_3^--fed plants retained *ca.* 86 % and NH_4^+-fed plants retained *ca.* 77% of their incorporated ^{14}C after 24 h. The fate of the $DI^{14}C$ supplied was dependent on the form of nitrogen on which the plants were grown (Fig 1). PEP carboxylation may serve as a source of anaplerotic carbon during NH_4^+ assimilation to compensate for the loss of TCA cycle intermediates to amino acid synthesis (Schweizer & Erismann, 1985, Vuorinen & Kaiser, 1997). PEP carboxylation could also serve as a source of carbon for organic acid synthesis in NO_3^--fed plants to maintain ionic balance in the xylem sap (Cramer *et al.,* 1993). Ammonium nutrition resulted in greater $DI^{14}C$ incorporation than NO_3^- nutrition and this was due to the more rapid uptake of NH_4^+ compared to NO_3^- (Murphy & Lewis, 1987) and the subsequent requirement of carbon skeletons for amino acid synthesis.

The diversion of incorporated ^{14}C into organic acids in NO_3^--fed plants grown with both root-zone CO_2 concentrations (Fig 1) which can subsequently be exported to the shoots (Cramer

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& Richards, 1999) was consistent with results of Cramer *et al.* (1993). This was supported by the higher C:N ratios (Fig 2) compared to NH_4^+-fed plants indicating a shift from nitrogenous to non-nitrogenous compounds. The greater proportion of organic acids in roots, stems and leaves of NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 was probably due to an increased DIC supply. Nitrate uptake is increased with elevated DIC (Cramer *et al.,* 1996) and the increased NO_3^- uptake and subsequent translocation to the shoot for reduction (Andrews, 1986) would require a greater proportion organic acid synthesis to maintain the ionic balance. This increased organic acid synthesis is the result of DIC fixation by PEPc (Cramer *et al.,* 1993). The decrease in the organic acid fraction over 24 h in roots of NO_3^--fed plants grown with 0 ppm root-zone CO_2 (Fig 1) may have been due to translocation of organic acids from the roots to the shoots where it could be decarboxylated to form carbohydrates (Cramer & Richards, 1999) which would account for the greater proportion of neutral fraction seen in the shoots (Fig 1), exudation of 2 % of the root organic acids into the nutrient solution (Fig 4) as well as a loss of 42 % of the total incorporated ^{14}DIC as $^{14}CO_2$ via respiration (Fig 5). The decrease in the leaf, stem and root organic fractions of NO_3^--fed plants grown with 5000 ppm root-zone CO_2 (Fig 1) may have been due to an increased synthesis of carbohydrates and amino acids at the expense of organic acids (Cramer *et al.,* 1993) and subsequent partitioning of these amino acids to the cell walls, as can be seen in the insoluble fraction (Fig 3).

The increase in ^{14}C incorporation in NH_4^+-fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Fig 1) was probably due to more carbon availability at 5000 ppm root-zone CO_2. The peak in the labelled amino acid fraction after 6 to 12 h was most possibly due to a rapid increase in labelled amino acids from the roots to the shoots. The decrease in the amino acid fraction of NH_4^+-fed roots grown with 0 and 5000 ppm root-zone CO_2 (Fig 1) was probably due to an increased partitioning to the insoluble fraction, an increase in root amino acid and carbohydrate exudation and respiratory loss. The decrease in the labelled amino acid fraction in

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the leaves may have been due to the breakdown of the amino acids to carbohydrates via respiration (Bryce & Thornton, 1996). This usually happens when plants are starved for starch and sugars such as NH_4^+-fed plants, which utilise most of their available carbon skeletons for amino acid synthesis. The carbohydrates may have been partitioned to the insoluble fraction (Fig 3) which would account for the increase of the insoluble fraction seen for NH_4^+-fed plants grown with both root-zone CO_2 concentrations or could have been translocated to the roots for exudation into the nutrient solution (Fig 4) as the was case with NH_4^+-plants grown with 5000 ppm root-zone CO_2.

Ammonium-fed plants grown with 5000 ppm root-zone CO_2 exuded more ^{14}C labelled organic compounds most probably because they assimilated far more ^{14}C than plants grown on other treatments (Fig 1). As found previously by Cramer & Van der Westhuizen (2000), elevated root-zone CO_2 concentrations resulted in greater net exudation of neutral compounds, organic acids and amino acids (Fig 4). This was possibly due to greater PEPc CO_2 re-fixation, which could result in an increased organic and amino acid synthesis. The increased loss of incorporated $DI^{14}C$ as inorganic carbon over 24 h for NO_3^-- and NH_4^+-fed plants grown with 5000 ppm relative to 0 ppm root-zone CO_2 was not concurrent with the respiratory rates found by Cramer & Van der Westhuizen (2000). This was because Cramer & Van der Westhuizen measured respiration as $^{14}CO_2$ efflux over a 3 h period, whereas in this study the loss of $DI^{14}C$ was measured over 24 h and this was representative of the cycling of root incorporated $DI^{14}C$. Plants grown with 5000 ppm root-zone CO_2 lost more $DI^{14}C$ relative to plants grown with 0 ppm root-zone CO_2 (Fig 5) probably due to the increased ^{14}C fixation and incorporation found with 5000 ppm CO_2 and therefore the $DI^{14}C$ loss of plants grown with 5000 ppm root-zone CO_2 may have been proportionately similar to plants grown with 0 ppm root-zone CO_2.

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Plants grown with 5000 ppm root-zone CO_2 exuded more organic ^{14}C than plants grown with 0 ppm root-zone CO_2 (Fig 4) due to the increased ^{14}C fixation and incorporation found with 5000 ppm CO_2. Ammonium-fed plants exuded *ca.* 3 % of their total $DI^{14}C$ taken up, whereas NO_3^--fed plants exuded ca. 2 % of the total incorporated $DI^{14}C$ over the 24 h period (Fig 4). Exudation of low-molecular-weight (LMW) compounds, such as organic acids, can directly mobilize mineral nutrients in the rhizosphere (Marschner, 1995). Precise data on LMW root exudates are difficult to obtain as under nonsterile conditions, especially in nutrient solutions, microorganisms may utilize a major part of it as a carbon source, and under sterile conditions the amounts released are considerably lower (Marschner, 1995). Organic acids in root exudates are not only important for mobilizing phosphorus, but also micronutrients and heavy metals such as copper, lead and cadmium (Marschner, 1995). The neutral compounds exuded by NO_3^--fed plants have only minor direct effects on the mobilization of mineral nutrients and it is thought that phloem-derived sugars lead to elevated concentrations of sugars in the apoplasm and, despite an effective retrieval system mechanism of plasma membrane-bound uptake systems, release of sugars into the external solution cannot be prevented (Jones & Darrah, 1993, Marschner, 1995). The ^{14}C labelled amino acid exudation of NH_4^+-fed plants might have been due to the plants trying to overcome the down regulation of NH_4^+ uptake due to increased amino acid synthesis (Causin & Barneix, 1993; Feng *et al.,* 1994; Glass *et al.,* 1997) or could have been due to the release of amino acids into the external solution in the same way as for sugars (Marschner, 1995).

3.6 Conclusions

This study indicated for the first time that up to 86 % of the products of PEPc fixation were retained in the plant after 24 h and not rapidly consumed in the respiratory pool as previously thought. The $DI^{14}C$ incorporated by the roots was higher in NH_4^+-fed compared to NO_3^--fed plants and higher for plants fed with 5000 ppm compared to 0 ppm root-zone CO_2 due to the

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availability of carbon. The assimilated ^{14}C was incorporated into organic acids, the neutral fraction and most notably to amino acids. The greater exudation of ^{14}C labelled organic carbon and loss of inorganic carbon at 5000 ppm root-zone CO_2 concentrations relative to 0 ppm root-zone CO_2 was proportionately similar due to the increased ^{14}C incorporation found with 5000 ppm CO_2.

3.7 Acknowledgements

We would like to thank the National Research Foundation for financial support.

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Chapter 4

An investigation into the contribution of dissolved inorganic carbon to the carbon budget of tomato plants

Running title: Contribution of DIC to C budget

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4.1 Summary

- The effect of dissolved inorganic carbon (DIC) concentrations on the $\delta^{13}C$ and $\delta^{15}N$ values of tomato plants were measured and the contribution of phosphoenolpyruvate carboxylase-fixed carbon to the overall carbon budget calculated.
- The tomato seedlings were hydroponically grown with NO_3^- or NH_4^+ nutrition and solutions aerated with either 0, 5000 or 10000 ppm root-zone CO_2.
- The $\delta^{13}C$ values for both NO_3^-- and NH_4^+-fed plants were 0.6‰ more positive at higher DIC concentrations. The $\delta^{15}N$ values of NO_3^--fed plants were unchanged by DIC whereas in NH_4^+-fed plants the values were 1.5‰ higher. A small (< 4 %) proportion of the total plant carbon was derived from PEPc, which increased with DIC concentration.
- The more positive $\delta^{13}C$ values may have been due to inhibition of loss of internal CO_2, which would inhibit respiration and result in the incorporation of both isotopes. The more negative $\delta^{15}N$ values may have been due to the discrimination of glutamate synthetase against the heavier isotope, which resulted in the stems having lighter $\delta^{15}N$ values than the root.

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Key words: $\delta^{13}C$, $\delta^{15}N$, dissolved inorganic carbon, phosphoenolpyruvate carboxylase contribution

4.2 Introduction

Soils have higher dissolved inorganic carbon (DIC) than hydroponic solutions due to the accumulation of respiratory CO_2 produced by the biological components of soils and the physical constraints on diffusion within the soil. DIC comprises a pH-dependent combination of CO_2, HCO_3^- and CO_3^{2-} in solution (Norstadt & Porter, 1984). Root respiration produces inorganic carbon, which equilibrates with that in the soil resulting in relatively high tissue DIC concentrations. Incorporation of DIC serves an anaplerotic function by providing intermediates for the TCA cycle through the activity of phosphoenolpyruvate carboxylase (PEPc) (Vuorinen & Kaiser, 1997), and in this way provides carbon skeletons for amino and organic acid synthesis. The assimilation of DIC (as HCO_3^-) through PEPc activity in the root is responsible for only a small contribution to the carbon budget of the whole plant, but DIC assimilation could occur at rates equivalent to 30% of the rate of respiration in plant roots exposed to 5000 ppm CO_2 (Cramer & Lips, 1995). These authors estimated the rate of DIC re-fixation from assimilation of exogenous ^{14}C, but the limitation with this technique is that it underestimates the activity of the pathway, which may also incorporate endogenous CO_2 released by root respiration. Furthermore it only provides an indication of net CO_2 fixation while some of the fixed ^{14}C would be respired to some extent (Cramer *et al.,* 1993).

In C_3 plants, most isotopic change during carboxylation is caused by Rubisco, which has a CO_2 discrimination value of about -29‰, (Roeske & O'Leary, 1984) while a small proportion of carbon is fixed by PEPc as well, which has a CO_2 discrimination value of about -5.7‰ (Farquhar & Richards, 1983). In C_3 plants, carbon isotopic ratios of plants are not only influenced by enzymatic carboxylation and the $\delta^{13}C$ values of their CO_2 source, but also the

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subsequent discrimination associated with stomatal diffusion and the ratios of internal to external CO_2 partial pressures (Le Roux-Swarthout *et al.*, 2001). Discrimination against the heavier carbon isotope has been demonstrated to be negatively correlated to water use efficiency (WUE) in several species (Scartazza *et al.*, 1998) due to the greater depletion of leaf intercellular CO_2 when stomata are closed to a greater extent in plants exhibiting high WUE (Farquhar *et al.*, 1989).

Variability in the contribution of PEPc to leaf $\delta^{13}C$ values during leaf development has been demonstrated in studies where PEPc activity was high during the heterotrophic stages of leaf development and declined as the leaf attained greater photoautotrophy (Blanke & Ebert, 1992). Heterotrophic tobacco plants were found to be enriched in ^{13}C relative to the carbon sources in their growth medium and the authors concluded that the anaplerotic activity of PEPc was responsible for the ^{13}C enrichment which is commonly observed where heterotrophic inputs to growth are large, such as in very young leaves (Le Roux-Swarthout *et al.*, 2001). This concurs with results found by Terwilliger & Huang (1996) showing that heterotrophic tomato and tobacco leaves were enriched in ^{13}C compared to adjacent photoautotrophic leaves. The advantage of using ^{13}C values is that the relative contribution of ^{13}C to plant total carbon can be calculated because PEPc has a known discrimination factor against ^{13}C (Farquhar & Richards, 1983).

Elevated root-zone DIC has been shown to increase NO_3^- uptake compared to ambient root-zone DIC, whereas NH_4^+ uptake was decreased or unchanged with elevated root-zone DIC compared to ambient root-zone DIC (Cramer *et al.*, 1996). Nitrate reductase (NR) and glutamine synthetase (GS) have N discrimination values of about 15‰ and 17‰, respectively (Handley & Raven, 1992; Yoneyama *et al.*, 1993). These steps cause the inorganic nitrogen inside the cell to become enriched in ^{15}N relative to the nitrogen assimilated into organic compounds. Ammonium

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is assimilated immediately in the root (Bloom, 1988), therefore organic nitrogen in shoots and roots is the product of a single assimilation event and little variation in $\delta^{15}N$ is observed when NH_4^+ is the nitrogen source (Evans, 2001). Variability in the assimilation of NO_3^-, however, causes significant intra-plant variation in $\delta^{15}N$ probably because NO_3^- is assimilated to variable extents in both roots and shoots (Evans, 2001). The $\delta^{15}N$ of leaves can be greater than roots because the available NO_3^- is enriched in ^{15}N relative to root organic nitrogen due to fractionation during assimilation (Evans *et al.,* 1996). Organ-specific loss of nitrogen, different patterns of nitrogen assimilation, and reallocation of nitrogen can cause further intra-plant variations in $\delta^{15}N$. Efflux of organic nitrogen from roots could also alter their $\delta^{15}N$ (Robinson *et al.,* 1998).

The aim of this study was to investigate to what extent elevated root-zone DIC contributed to the carbon budget of tomato seedlings using the anticipated changes in discrimination for C as a result of anaplerotic CO_2 fixation via PEPc in the roots. Furthermore, the influence of changes in DIC concentration on the utilization of different nitrogen sources was also determined. Plants were grown hydroponically on different nitrogen sources in combination with root-zone CO_2 sources of increasing concentrations that had a different isotopic signal than atmospheric CO_2. The $\delta^{13}C$ and $\delta^{15}N$ values of the plants for the different treatments were measured and the relative contribution of PEPc to the carbon budget for each treatment was calculated.

Abbreviations: DIC, dissolved inorganic carbon; PEPc, phosphoenolpyruvate carboxylase

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4.3 Materials and Methods

4.3.1 *Growth conditions*

Seedlings (14 d old) of *Lycopersicon esculentum* (L.) cv. F144 grown on a 1:1 mixture of vermiculite and compost were transferred to hydroponic culture after rinsing the roots in distilled H_2O. The hypocotyls of the plants were wrapped in black closed-cell foam rubber and inserted through collars in the lids of 22 l hydroponic tanks with eight plants per tank. The tanks were completely opaque and contained 20 l Long Ashton nutrient medium (Hewitt, 1966) modified to contain 2 mM of either $NaNO_3$ or NH_4Cl as a nitrogen source and 0.09 mM FeEDTA as an iron source. The nutrient medium was changed weekly and the pH of the medium was maintained at 5.8 by adjusting the pH with HCl or NaOH daily. Plants were grown in a temperature controlled (minimum 15°C, maximum 25°C) greenhouse at the University of Stellenbosch during winter (July and August) spring (September and October). Nutrient solutions were strongly aerated with either 0 ppm CO_2, 5000 ppm CO_2, or 10000 ppm CO_2. Carbon dioxide was removed by passing ambient air through 2 M NaOH and a column containing soda lime and CO_2 was supplied at elevated levels by enriching ambient air with CO_2. Plants were grown on a CO_2 source (Afrox, Cape Town, South Africa) from either fossilfuel (plants grown during July and August) or from sugarcane fermentation (plants grown during September and October) with a different isotopic signal from ambient atmospheric CO_2. The CO_2 source from fossilfuel had a $\delta^{13}C$ value of –19.12‰ whereas the CO_2 source from sugarcane fermentation had a $\delta^{13}C$ value of –10.91‰. The CO_2 concentration was monitored continuously using an ADC Mk3 (Analytical Development Corporation, Hoddeston, England) infrared gas analyser (IRGA). To prevent diffusion of CO_2 from the rhizosphere and the consequent enrichment of atmosphere around the shoots, the lids of hydroponic tanks were sealed onto the tanks with closed-cell foam rubber around the rim and clamped onto the tanks. The air-space between the surface of the nutrient solution and the lid was maintained under a partial vacuum to ensure that net air flow was inwards. Plants were

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harvested when the biomass was *ca.* 6 g and fresh weights of the leaves, stems and roots determined after which the plants were dried in an oven at 80°C for 48 h and reweighed.

4.3.2 *Mass spectrometer determinations*

For $\delta^{13}C$ and $\delta^{15}N$ determination the oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, California, USA). Between 2.100 and 2.200mg of each sample was weighed into an 8 by 5 mm tin capsule (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{13}C$ and $\delta^{15}N$ values for the carbon and nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to the CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one International Atomic Energy Agency standard - $(NH_4)_2SO_4$.

The $\delta^{13}C$ value of the CO_2 gas supplied to the roots was determined by attaching a 180 by 6 mm O.D. pyrex glass tube to a vacuum line. The line was evacuated after which it was closed off from the pump by means of a valve. A small amount of the sample gas was introduced into the line via another valve. The CO_2 introduced was frozen down into the pyrex tube by applying a dewar flask containing liquid nitrogen to the lower part of the pyrex tube, after which the tube was flame sealed and burnt off. The tube was then attached to the mass spectrometer inlet and the delta value of the gas measured by comparison with a gas of known $\delta^{13}C$.

The carbon isotopic ratio of a sample is usually expressed as $\delta^{13}C$ = [R(sample)/R(standard)-1] x 1000 where $\delta^{13}C$ is the isotope ratio in delta units relative to a standard based upon CO_2 derived from limestone from the Pee Dee formation in South Carolina,

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and R(sample) and R(standard) are the absolute isotope ratios of the sample and standard, respectively (Ehleringer & Rundel, 1989). $\delta^{13}C$ values so calculated are expressed in parts per thousand. Isotopic ratios of samples are expressed as $\delta^{15}N$ = R(sample)/R(standard) x 1000, where $\delta^{15}N$ is the isotope ratio relative to the atmospheric air standard, and R(sample) and R(standard) are the molar ratios of the heavier to the lighter isotope. The value for R(standard) is 0.0036765 (Evans, 2001).

4.3.3 *Statistical analysis*

Results were subjected to analysis of variance to determine the significance of differences between the responses to the treatments. Where percentage data were used these were arcsine transformed (Zar, 1984) prior to statistical analysis. Where analysis of variance was performed, *post-hoc* Fisher's projected least significant difference (LSD) tests (95%) were conducted to determine the differences between the individual treatments using Statgraphics Ver. 7.0 (1993).

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4.4 Results

Unless stated otherwise, the CO_2 source used to aerate the hydroponic solutions for the respective treatments was from sugarcane fermentation.

4.4.1 *Growth results*

Table 1 Comparison of DW (g) and S:R of tomato plants treated with 2 mM either NO_3^- or NH_4^+ and aerated with air containing either 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). SE (±) of the mean is given next to the values (n=6). Different letters next to values indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs were tested separately.

Character	**DW (g)**				**S:R**
	Leaf	Stem	Root	Plant	
NO_3^- 0 ppm CO_2	0.33 ± 0.03 b	0.14 ± 0.02 c	0.08 ± 0.01 b	0.55 ± 0.05 c	6.04 ± 0.22 a
5000 ppm	0.42 ± 0.01 c	0.18 ± 0.01 d	0.07 ± 0.01 b	0.67 ± 0.02 d	8.37 ± 0.77 b
10000 ppm	0.57 ± 0.04 d	0.23 ± 0.01 e	0.11 ± 0.01 c	0.91 ± 0.06 e	7.75 ± 0.55 b
NH_4^+ 0 ppm CO_2	0.20 ± 0.01 a	0.07 ± 0.00 a	0.05 ± 0.00 a	0.32 ± 0.01 a	5.92 ± 0.18 a
5000 ppm	0.28 ± 0.02 b	0.10 ± 0.01 b	0.06 ± 0.01 ab	0.44 ± 0.03 b	6.30 ± 0.45 a
10000 ppm	0.34 ± 0.02 b	0.11 ± 0.00 b	0.07 ± 0.01 b	0.51 ± 0.03 bc	6.38 ± 0.25 a

The leaf and stem dry weights of NO_3^--fed plants increased significantly with increasing root-zone CO_2 concentration, whereas in roots the only increase in dry weight was when plants were grown with 10000 ppm compared to 5000 ppm root-zone CO_2 (Table 1). The plant dry weight of NO_3^--fed plants showed the same pattern as the leaves and stems with an increase in dry weight concurrent with an increase in root-zone CO_2 concentration (Table 1). Ammonium-fed plants showed a significant increase in dry weight of leaves and stems when plants were grown with 10000 ppm and 5000 ppm CO_2 compared to 0 ppm root-zone CO_2, whereas in roots a significant increase was found when plants were grown with 10000 ppm compared to 0 ppm

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root-zone CO_2 (Table 1). A significant increase in plant dry weight of NH_4^+-fed plants was noted in plants grown with 5000 ppm and 10000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2. Nitrate-fed plants had significantly larger plant dry weights than NH_4^+-fed plants for all root-zone CO_2 concentrations (Table 1).

The NO_3^--fed plants had significantly higher shoot to root ratios when grown with 5000 ppm and 10000 ppm root-zone CO_2 concentrations compared to 0 ppm root-zone CO_2 (Table 1). The increase in root-zone CO_2 concentration did not have any significant effect on the shoot to root ratio of NH_4^+-fed plants and NO_3^--fed plants had significantly higher shoot to root ratios than NH_4^+-fed plants when grown with 5000 and 10000 ppm root-zone CO_2 (Table 1).

4.4.3 $\delta^{13}C$

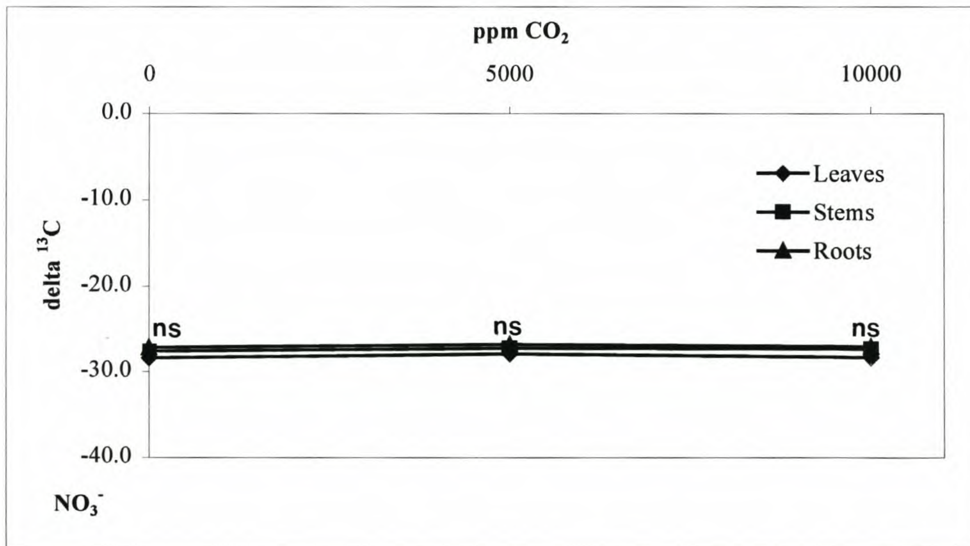

Figure 1 A Comparison of the $\delta^{13}C$ values for tomato seedlings grown on 2 mM NO_3^- and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from fossilfuel). The letters ns next to the plots indicate 'no significant differences' between the treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

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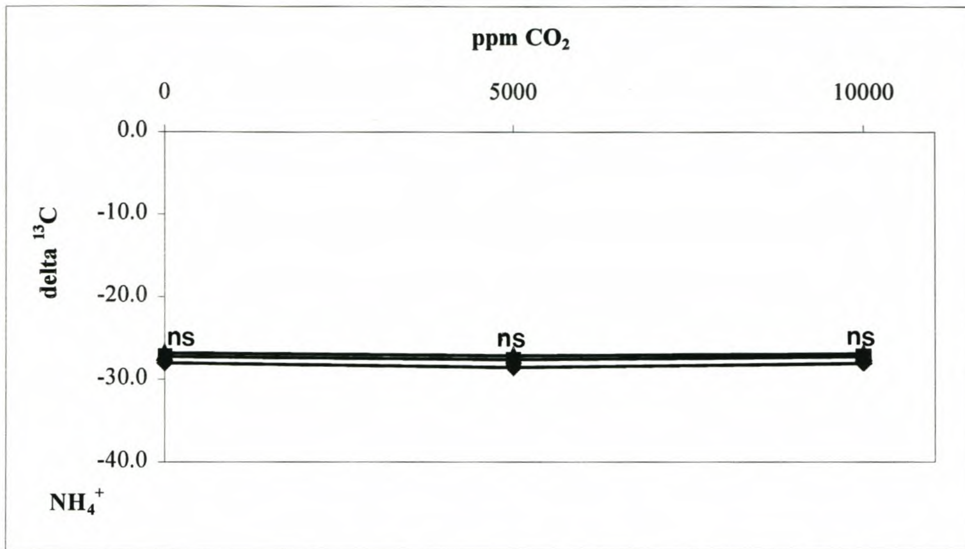

Figure 1 B Comparison of the $\delta^{13}C$ values for tomato seedlings grown on 2 mM NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from fossilfuel). The letters ns next to the plots indicate 'no significant differences' between the treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

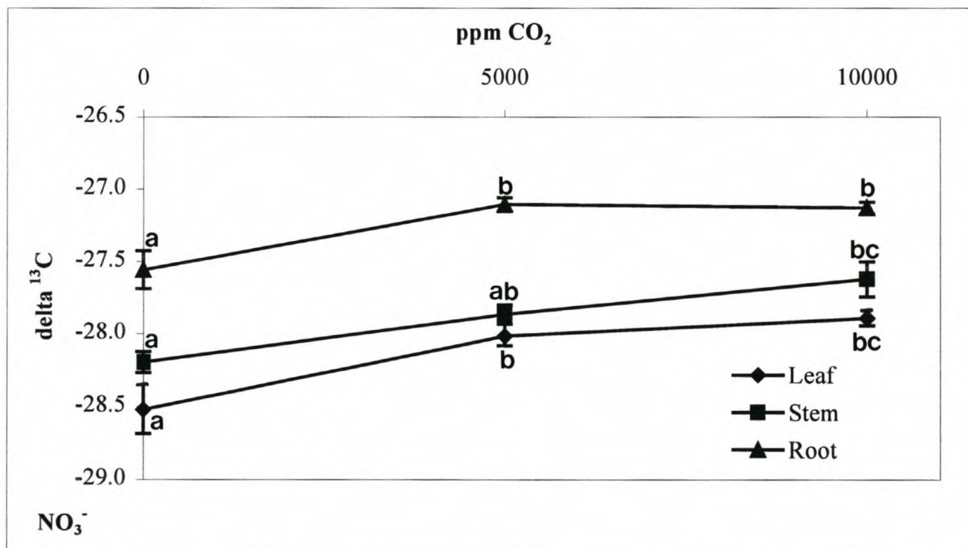

Figure 2 A Comparison of the $\delta^{13}C$ values for tomato seedlings grown on 2 mM NO_3^- and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

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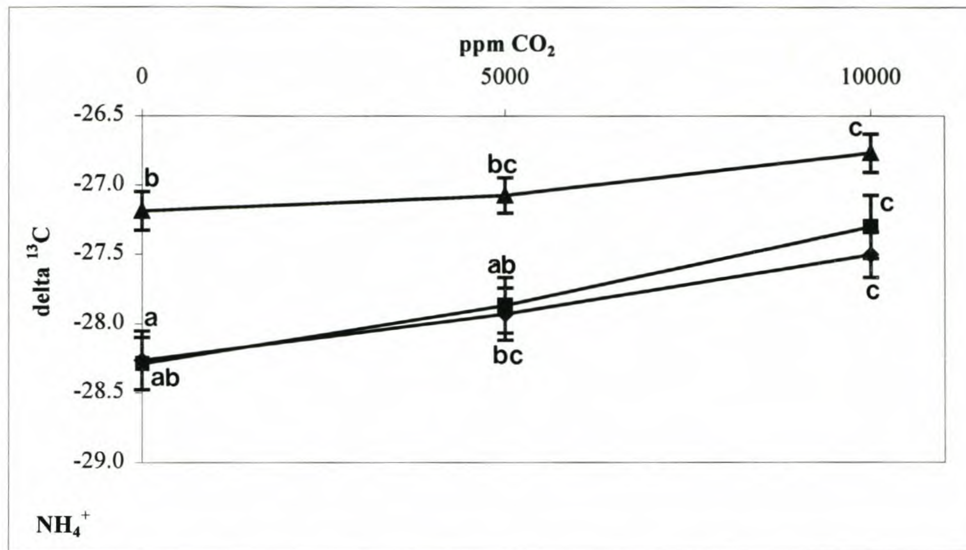

Figure 2 B Comparison of the $\delta^{13}C$ values for tomato seedlings grown on 2 mM NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

No significant differences were found in the $\delta^{13}C$ values of leaves, stems and roots of NO_3^- and NH_4^+-fed plants grown with 0, 5000 and 10000 ppm CO_2 from fossilfuel (Fig 1 A and B). The $\delta^{13}C$ values of the roots of NO_3^--fed plants grown with CO_2 from sugarcane fermentation were more positive than those of the leaves and the stems. The roots were therefore enriched in ^{13}C (Fig. 2 A) and a similar trend could be seen for the $\delta^{13}C$ values of NH_4^+-fed plants grown with CO_2 from sugarcane fermentation (Fig 2 B). A significant increase in $\delta^{13}C$ values was found in leaves and roots of NO_3^--fed plants grown with 5000 and 10000 ppm root-zone CO_2 from sugarcane fermentation compared to 0 ppm root-zone CO_2 (Fig 2 A) and for the stems a significant increase in $\delta^{13}C$ was found when grown with 10000 ppm compared to 0 ppm root-zone CO_2 (Fig 2 A). The $\delta^{13}C$ values of leaves, stems and roots of NH_4^+-fed plants increased significantly when plants were grown with 10000 ppm compared to 0 ppm root-zone CO_2 from sugarcane fermentation (Fig 2 B).

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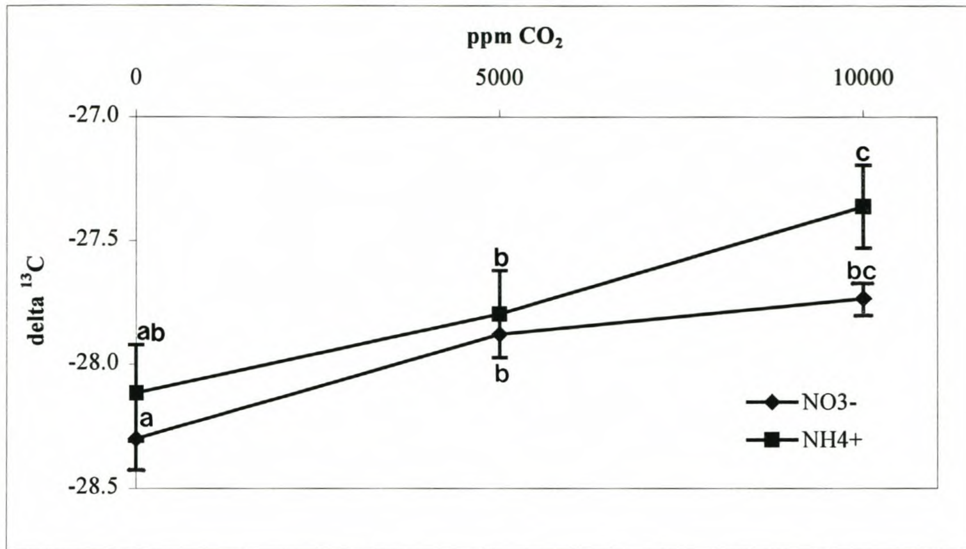

Figure 2 C Comparison of the total plant $\delta^{13}C$ values for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

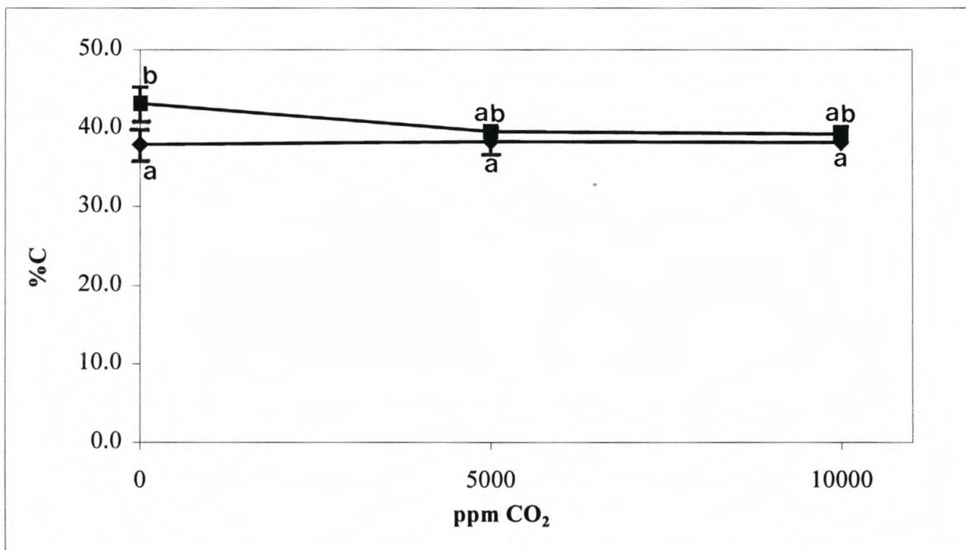

Figure 2 D Comparison of C calculated as a percentage of the total plant dry weight for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

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The total plant $\delta^{13}C$ for NO_3^-- and NH_4^+-fed plants both increased significantly when plants were grown with 10000 ppm compared to 0 ppm root-zone CO_2, but no significant difference could be discerned between the $\delta^{13}C$ values of NH_4^+- compared to NO_3^--fed plants (Fig 2 C). The percentage carbon of the total plant weight showed no change for NO_3^-- or NH_4^+-fed plants grown with increasing root-zone CO_2 concentration (Fig 2 D).

Table 2 Percentage contribution of PEPc to the carbon budget of tomato plants treated with 2 mM either NO_3^- or NH_4^+ and aerated with air containing either 0 ppm, 5000 ppm, or 10000 ppm root-zone CO_2 from sugarcane fermentation. SE (±) of the mean is given next to the values (n=6). Different letters next to values indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Different organs were tested separately

Character	**% PEPc contribution**			
	Leaf	Stem	Root	Plant
NO_3^- 0 ppm CO_2	0.00 a	0.00 a	0.00 a	0.00 a
5000 ppm	2.05 ± 0.26 bc	1.35 ± 0.27 b	1.91 ± 0.20 b	1.73 ± 0.21 b
10000 ppm	2.55 ± 0.20 bc	2.36 ± 0.50 bc	1.81 ± 0.17 b	2.32 ± 0.22 bc
NH_4^+ 0 ppm CO_2	0.00 a	0.00 a	0.00 a	0.00 a
5000 ppm	1.37 ± 0.77 b	1.73 ± 0.82 b	0.49 ± 0.55 b	1.31 ± 0.72 b
10000 ppm	3.12 ± 0.67 c	4.04 ± 0.94 c	1.79 ± 0.60 b	3.11 ± 0.70 c

Using the mass balance equation from Terwilliger & Huang (1996) the proportional contribution of PEPc fixed CO_2 was calculated. The equation used was: $\delta^{13}C_{PLANT} = p\delta^{13}C_{PEPc\ fixed\ CO2} + (1 - p)\delta^{13}C_{GROWTH\ MEDIUM}$ where p was the proportional $\delta^{13}C$ contribution made by PEPc and the growth medium. Therefore at 0 ppm root-zone CO_2 PEPc made a negligible contribution to the fractional $\delta^{13}C$ enrichment. Organic carbon derived from photosynthesis will have a different $\delta^{13}C$ compared to root-derived organic carbon and re-fixation of CO_2 from shoot-derived organic carbon will result in an under-estimation of root utilization of inorganic carbon. Although root CO_2 fixation may not be important quantitatively, Cramer & Lips (1995) found it to be very important stochiometrically. There was no significant increase in the percentage contribution of

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PEPc in the leaves, stems and roots of NO_3^--fed plants grown with 10000 ppm compared to 5000 ppm root-zone CO_2, whereas significant increases could be seen when 5000 ppm was compared to 0 ppm root-zone CO_2 (Table 2). However, a significant increase was found in the percentage contribution of PEPc in the leaves and stems of NH_4^+-fed plants grown with increasing root-zone CO_2 concentration, but for roots this was only true when the percentage contribution of PEPc at 5000 ppm was compared to 0 ppm root-zone CO_2. No significant difference in the percentage contribution of PEPc to leaves, stems, roots or the whole plant was found between NO_3^-- and NH_4^+-fed plants (Table 2).

4.4.4 $\delta^{15}N$

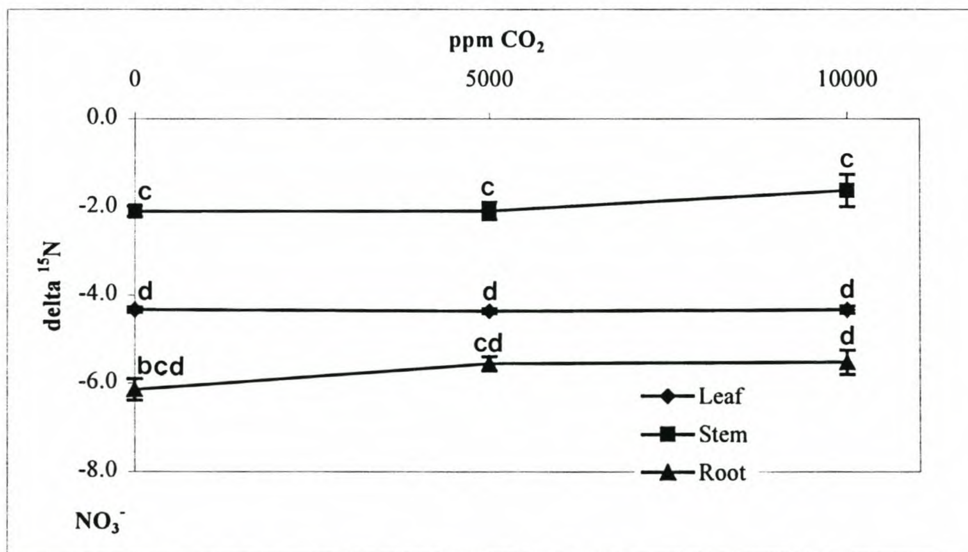

Figure 3 A Comparison of the $\delta^{15}N$ values for tomato seedlings grown on 2 mM NO_3^- and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

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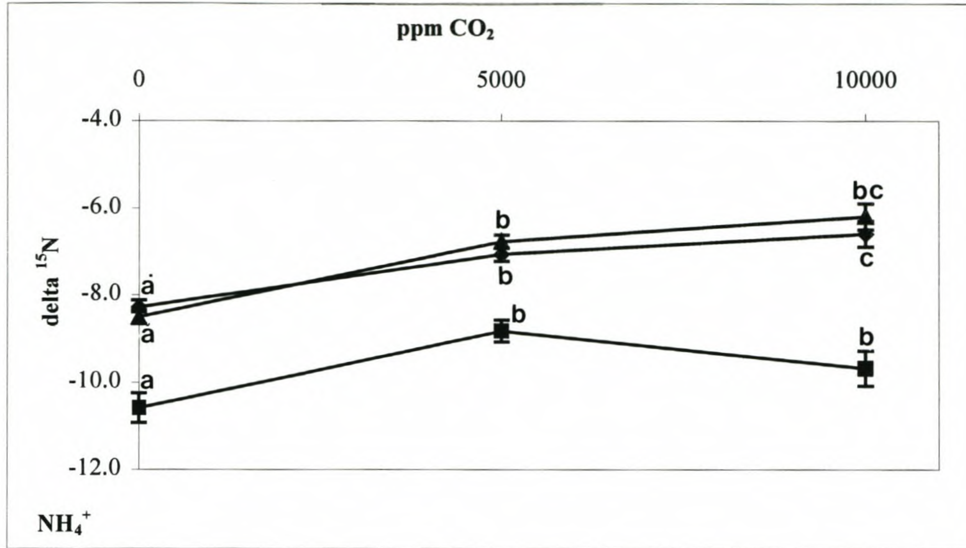

Figure 3 B Comparison of the $\delta^{15}N$ values for tomato seedlings grown on 2 mM NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6). Different organs were tested separately.

Increasing root-zone CO_2 concentrations had no effect on the $\delta^{15}N$ values of leaves, stems and roots of NO_3^--fed plants (Fig 3 A). The leaf $\delta^{15}N$ values of NH_4^+-fed plants increased significantly with increases in root-zone CO_2 concentration, while the $\delta^{15}N$ values of the stems and roots of NH_4^+-fed plants increased significantly when plants were grown on 5000 ppm compared to 0 ppm root-zone CO_2 (Fig 3 B).

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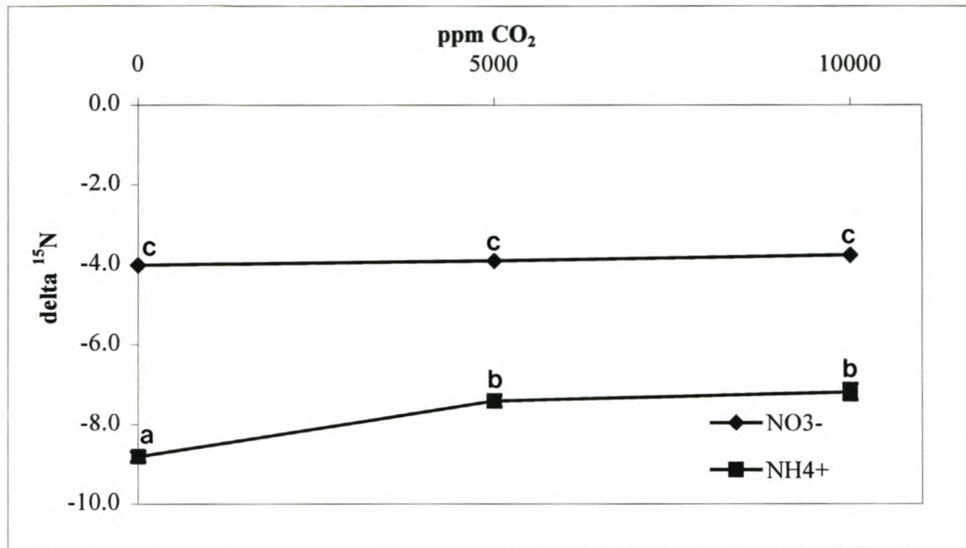

Figure 3 C Comparison of the total plant $\delta^{15}N$ values for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

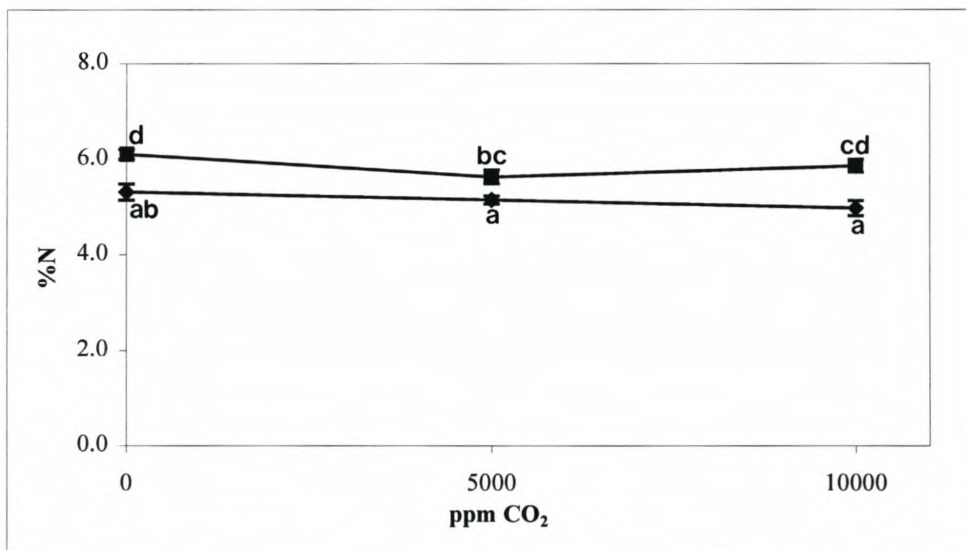

Figure 3 D Comparison of N calculated as a percentage of total plant dry weight for tomato seedlings grown on either 2 mM NO_3^- or NH_4^+ and aerated with air containing 0 ppm, 5000 ppm or 10000 ppm root-zone CO_2 (from sugarcane fermentation). Different letters next to the plots indicate significant differences between treatments tested using analysis of variance (ANOVA) with post-hoc LSD tests. Error bars indicate the SE of the mean (n=6).

No significant difference was found between the $\delta^{15}N$ values of NO_3^--fed plants grown on increasing root-zone CO_2 concentrations, whereas with NH_4^+ nutrition there was a significant

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increase in $\delta^{15}N$ values when plants were grown with 5000 ppm and 10000 ppm compared to 0 ppm root-zone CO_2 (Fig 3 C). The $\delta^{15}N$ values of the NO_3^--fed plants were significantly more positive than those of NH_4^+-grown plants at all root-zone CO_2 concentrations (Fig 3 C). The nitrogen as a percentage of the plant dry weight remained unchanged for NO_3^--fed plants grown with increasing root-zone CO_2 concentrations, whereas with NH_4^+-fed plants there was a significant decrease in percentage nitrogen for plants grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 (Fig 3 D).

4.5 Discussion

The increase in plant dry weight of NO_3^--fed plants with greater root-zone CO_2 (Table 1) may have been due to the xylem translocation of the root incorporated elevated DIC as organic carbon to the leaves, where it could provide a source of carbon for photosynthesis under high light intensities as found by Cramer & Richards (1999), although it could also be linked to an increased supply of reduced carbon for growth. The increased translocation of DIC-derived organic carbon at increasing root-zone CO_2 concentrations would account for the concurrent dry weight increase in the stems and leaves of plants grown with increasing concentrations of root-zone CO_2. The significantly higher shoot to root ratios for NO_3^--fed plants grown with 5000 and 10000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 were probably due to these above mentioned increases in dry weights. The increase in plant dry weight for NH_4^+-fed plants grown with 5000 and 10000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 may have been due to inhibition of NH_4^+ uptake at elevated DIC concentrations (Cramer *et al.,* 1996). By avoiding excessive uptake of NH_4^+ the plants might be able to avoid the toxic effects of NH_4^+ and the carbon drain associated with ammonium assimilation that are deleterious to growth. Another possibility might be that the plants excrete the amino acids formed during NH_4^+ assimilation to avoid toxicity.

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The $\delta^{13}C$ value of the CO_2 source from fossilfuel was similar to that of C_3 plants and when this gas was used, no changes in $\delta^{13}C$ values of NO_3^-- and NH_4^+-fed plants were found when grown on increasing root-zone CO_2 concentrations (Fig 1 A and B). However, significant changes in the $\delta^{13}C$ values of NO_3^-- and NH_4^+-fed plants were found when grown on increasing root-zone CO_2 concentrations from sugarcane fermentation (Fig 2 A and B). From this it can be concluded that the changes in the $\delta^{13}C$ values were due to the fixation of CO_2 by PEPc and not due to changes in WUE. The more positive $\delta^{13}C$ values of roots of NH_4^+-fed plants grown with 0 ppm and 10000 ppm root-zone CO_2 compared to the $\delta^{13}C$ values of roots of NO_3^--fed plants grown on the same root-zone CO_2 concentration was probably due to NH_4^+ being assimilated more rapidly than NO_3^- (Smart & Bloom, 1993) and consequently greater incorporation of root-zone DIC by PEPc to drive the anaplerotic reaction (Vuorinen & Kaiser, 1997) supplying carbon skeletons for amino acid synthesis (Cramer & Lewis, 1993) (Fig 2 A and B). Because the uptake of NH_4^+ is more rapid than NO_3^- it will utilise both C isotopes to a greater extent than would NO_3^- uptake. Therefore, NO_3^- uptake would use less DIC and allowing discrimination against the heavier isotope, the lighter isotope will be favoured. A possible explanation for the more positive $\delta^{13}C$ values for plants grown with both nitrogen sources at increasing root-zone CO_2 (Fig 2 C) was that the high external CO_2 concentrations could inhibit the loss of internal CO_2 through inhibiting respiration (Van der Westhuizen & Cramer, 1998). Therefore, both isotopes will be incorporated and this would result in the $\delta^{13}C$ values of the plants becoming more positive.

A small proportion (<4%) of the carbon in the plant material was derived from root PEPc activity and this proportion was similar to that reported earlier for ^{14}C derived studies (Cramer and Lips, 1995). The increase in the percentage contribution of PEPc for NH_4^+-fed stems, leaves and whole plants grown with 10000 ppm compared to 5000 ppm root-zone CO_2 indicated an increased translocation of amino acids from the roots to the stems and leaves, meaning that at a higher root-zone CO_2 concentration more of the stem and leaf carbon was contributed by the root

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PEPc activity (Table 2). The NH_4^+ taken up is predominantly assimilated in the roots (Andrews, 1986), drawing carbon skeletons from the TCA cycle to form amino acids (Schweizer and Erismann, 1985). In turn, the carbon skeletons from the TCA cycle can be rapidly depleted and is therefore dependent on the anaplerotic reaction of PEPc, which refixes respiratory CO_2 (Vuorinen & Kaiser, 1997).

A possible reason for the stable $\delta^{15}N$ values of NO_3^--fed plants grown at increasing root-zone CO_2 concentrations (Fig 3 C) could be that as NO_3^- is taken up there is discrimination against the heavier isotope and the lighter isotope is incorporated into organic compounds in the roots (Fig 3 A). The heavier NO_3^- isotope is either effluxed into root solution or into the xylem sap. The xylem sap's $\delta^{15}N$ value becomes more positive and the leaves in turn discriminate against the heavier isotope (Fig 3 A). In NH_4^+-fed plants GS discriminates against the heavier ^{15}N (Yoneyama *et al.*, 1993), which results in the stem having a more negative $\delta^{15}N$ value than the root because it receives the lighter isotopes from the root (Fig 3 B). Amino acids formed in the roots of NH_4^+-fed plants at increasing root-zone CO_2 concentrations were probably translocated to the shoots to enter photorespiration which would result in the lighter isotope diffusing out and the heavier isotope assimilated resulting in the leaves of NH_4^+-fed plants having more positive $\delta^{15}N$ values than the stems (Fig 3 B). Another possibility was that the $\delta^{15}N$ values became more positive for NH_4^+-fed plants grown at increased root-zone CO_2 concentrations (Fig 3 C) due to inhibition of NH_4^+ uptake, which would result in less DIC taken up because less carbon skeletons for amino acid synthesis would be required. The decrease in nitrogen as a percentage of total plant dry weight for NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 compared to 0 ppm root-zone CO_2 (Fig 3 D) was probably due to the inhibition of NH_4^+ uptake by elevated root-zone CO_2 (Cramer *et al.,* 1996).

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4.6 Conclusions

The $\delta^{13}C$ values of plants grown on both nitrogen sources followed similar trends at increasing root-zone CO_2 concentrations and it may be concluded that nitrogen had no effect on the $\delta^{13}C$ values of the plants. The increasing root-zone CO_2 concentrations caused the $\delta^{15}N$ values of NH_4^+-fed plants to become more positive and indicated an absence of enzymatic discrimination. A possible explanation for this was the inhibitory effect of DIC on NH_4^+ uptake, which leads to the plants utilising both internal isotopes equally. A small proportion of the total plant carbon was derived from PEPc but as the root-zone CO_2 increased, the percentage contribution by PEPc increased significantly indicating a greater demand for carbon skeletons.

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Chapter 5

General conclusions

This chapter aims to integrate the flux between DIC-derived carbon and inorganic N uptake and the subsequent partitioning. Furthermore it will illucidate how integration of these processes contribute to NUE. The table below (Table 1) represents the NUE for NO_3^- and NH_4^+ nutrition and the factors that influence the relationship between NUE expressed as C allocation per unit N taken up. This includes factors for assimilating N and processes controlling utilization of carbon, which would primarily be photosynthetically acquired carbon as well as carbon from root anaplerosis. Although some background work has been done on short-term NO_3^- and NH_4^+ uptake and DIC partitioning, the long-term NUE was investigated in this study.

Table 1 A summary of the effect of elevated DIC on the short-term (6 h) and long-term (*ca.* 15 days) physiological processes of four week old tomato seedlings grown with 2 mM NO_3^- or NH_4^+ and aerated with 0 or 5000 ppm root-zone CO_2.

Physiological process	Influence
NUE - NO_3^-	increased
NUE - NH_4^+	no change
NO_3^- uptake (short-term) (long-term)	increased initial increase, thereafter rates similar
NH_4^+ uptake	decreased after an initial increase
NR activity NR protein levels inhibitor protein binding phosphorylation	increased increased decreased no change
PEPc activity PEPc protein levels phosphorylation	increased with NO_3^-, decreased with NH_4^+ increased with NO_3^-, decreased with NH_4^+ increased with NO_3^-, decreased with NH_4^+
Amino acid synthesis	increased most notably with NH_4^+
Organic acid synthesis	increased most notably with NO_3^-
% PEPc contribution	increased with NO_3^- and 5000 ppm CO_2, ↑ with NH_4^+ and 5000 ppm CO_2, vastly increased with NH_4^+ and 10000 ppm CO_2

A higher NUE for NO_3^--fed plants relative to NH_4^+-fed plants was found when grown with 5000 ppm and 360 root-zone CO_2 (Chapter 2, Fig 4) indicating that NO_3^--fed plants had higher relative growth rates (RGR) and biomass (Chapter 2, Fig 1) per N taken up than NH_4^+-fed plants did. This supported previous results for plants supplied with NH_4^+ nutrition, which accumulated less biomass over the growing period than plants supplied with NO_3^- nutrition (Cramer & Lewis, 1993). The toxic effects of NH_4^+ nutrition are partially caused by the unassimilated NH_4^+ ion. The NH_4^+ ion leads to dissipation of pH gradients across membranes (Bloom, 1997) such as thylakoids, inner mitochondrial membranes or tonoplast membranes. Ammonium nutrition is also costly in terms of carbon skeletons required from the TCA cycle for amino acid synthesis and these carbon intermediates have to be replenished by increased activity of PEPc (Schweizer & Erismann, 1985). The depletion of root carbohydrates in roots supplied with NH_4^+ nutrition has previously been found to strongly inhibit root growth in wheat (Cramer and Lewis, 1993). Nitrate-fed plants invested their DIC-derived carbon into growth, which would lead to increased NUEs. However, in Chapter 4, Table 1 an increase in plant dry weight was found for NH_4^+-fed plants grown with 5000 and 10000 ppm root-zone compared to 0 ppm root-zone CO_2, whereas in Chapter 2 a decrease was found in plant dry weight (data not shown). These differences may be attributed to different physiological stages of the plants brought about by seasonal variation.

The increase in the retention of $DI^{14}C$ of plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 is an indication of the increased incorporation of $DI^{14}C$ and assimilation into organic soluble and insoluble products (Chapter 3, Fig 1). The small percentage contribution (Chapter 4, Table 2) made by PEPc for NO_3^-- and NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 is supported by results found by Cramer & Lips (1995). However, even though root PEPc makes only a small contribution to the carbon budget this contribution could be quite significant over the long term for the plant carbon status if the retention of $DI^{14}C$ after only a 1 h pulse is 86 %

after 24 h. In addition, the function of PEPc is most probably stronger associated with the anaplerotic top-up of the TCA cycle and not the carbon budget *per se*.

The contribution of PEPc activity is dependent on nitrogen source. The increase in total and phosphorylated root PEPc activity found for NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Chapter 2, Fig 6) may have been due to the requirement of carbon skeletons for amino acid synthesis (Schweizer & Erismann, 1985, Vuorinen & Kaiser, 1997) or organic acid synthesis (Cramer *et al.,* 1993) for ionic balance of the xylem sap and subsequent translocation to the shoots (Chapter 2, Fig 3). Another possibility may have been that there was an increase in anaplerotic PEPc activity to the TCA cycle due to the increased loss measured in organic acid exudation of plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 (Chapter 3, Fig 4 b). The diversion of incorporated ^{14}C into organic acids in NO_3^--fed plants (Chapter 3, Fig 1) grown with both root-zone CO_2 concentrations was supported by the higher C:N ratios (Chapter 3, Fig 2) compared to NH_4^+-fed plants indicating a shift from nitrogenous to non-nitrogenous compounds. The greater proportion of organic acids in NO_3^--fed plants grown with 5000 ppm compared to 0 ppm root-zone CO_2 may have been translocated to the shoots to be decarboxylated to be used for photosynthesis or may have been used directly for respiration (Cramer & Richards, 1999) and in this way contributed to growth.

Furthermore, the percentage contribution of PEPc over a long growth period was similar for NO_3^-- and NH_4^+-fed plants (Chapter 4, Table 2). From this it can be concluded that the lower NUE's for NH_4^+-fed plants was due to their investing their carbon acquired from DIC to amino acids to overcome toxic effects. The toxic effects of NH_4^+ nutrition are caused by the unassimilated NH_4^+ ion. The NH_4^+ ion leads to dissipation of pH gradients across membranes (Bloom, 1997) such as thylakoids, inner mitochondrial membranes or tonoplast membranes. Using carbon derived from DIC to reduce the ion to an organic form would ameliorate this

effect. Nitrate-fed plants invested their DIC-derived carbon into growth, which would lead to increased NUE's. The similar PEPc activities (Chapter 2, Figure 5) and percentage contribution of PEPc for NO_3^- and NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 was probably due to the inhibition of NH_4^+ uptake over the long term by elevated DIC (Chapter 2, Fig 2). The $\delta^{13}C$ values of NO_3^-- and NH_4^+-fed plants grown with increasing root-zone CO_2 concentrations (Chapter 4, Fig 2 C) was representative of the ratio of carbon fixed by the roots via PEPc activity to carbon fixed by the shoots via photosynthesis. The similar $\delta^{13}C$ values for NO_3^-- and NH_4^+-fed plants grown with increasing root-zone CO_2 concentrations indicated that the ratio of carbon originating from the roots relative to carbon originating from the shoots was the same and therefore that the contributions made by root PEPc and photosynthesis was equal for both nitrogen sources. This lack of difference in photosynthetic contribution between NO_3^-- and NH_4^+-fed plants are supported by results of Cramer & Lewis (1993) reporting on the lack of difference in photosynthetic rates of wheat plants grown with 4 mM NO_3^- and NH_4^+ and results of Lewis *et al.* (1986) using barley grown on 2 mM nitrogen. Therefore, it can be concluded that it was not the amount of carbon assimilated that influenced the NUEs. The lower NUEs of NH_4^+- compared to NO_3^--fed plants grown with 5000 ppm root-zone CO_2 were due to the higher N uptake rates of NH_4^+-fed plants compared to NO_3^--fed plants (Fig 2, Chapter 2) and increased exudation of amino acids into the nutrient solution (Fig 4, Chapter 4).

Previous results have indicated that NO_3^- uptake was stimulated and NH_4^+ uptake inhibited by elevated root-zone DIC concentrations (Cramer *et al.,* 1996). This contrasts with the findings presented in this thesis for which at least three possible explanations can be offered: 1) the previous work was done using other genotypes, 2) the previous work was done over a shorter incubation time, 3) as was shown in the current work, the expression of data can have a profound effect. This aspect warrants further investigation. Since the percentage PEPc contribution was also similar, it can be concluded that these plants had similar total carbon budgets derived from

root carbon fixation. However, the carbon partitioning differed dramatically between NO_3^-- and NH_4^+-fed plants grown with 0 ppm and 5000 ppm root-zone CO_2 with NO_3^--fed plants favouring incorporation of $DI^{14}C$ into organic acids (Chapter 3, Fig 1), which may have been translocated to the shoots for leaf assimilation of NO_3^- (Andrews, 1986), especially under high DIC conditions. Ammonium uptake resulted in increased incorporation of $DI^{14}C$ into amino acids to overcome the toxic effect of NH_4^+ (Chapter 3, Fig 1). The more positive $\delta^{15}N$ values of NH_4^+-fed plants grown with 5000 and 10000 ppm compared to 0 ppm root-zone CO_2 (Chapter 4, Fig 3 C) supports results found previously that NH_4^+ uptake is inhibited by elevated DIC, because the increase in $\delta^{15}N$ could possibly be ascribed to the plants having to utilize the internal NH_4^+ and therefore both isotopes equally.

5.1 Conclusions

Looking at the short-term experiments it seems as if NO_3^--fed plants acquired more carbon at 5000 ppm root-zone CO_2 than NH_4^+-fed plants did for similar N uptake rates. However, the $\delta^{13}C$ studies showed that PEPc contributed equally to both NO_3^-- and NH_4^+-fed plants over the long term. From this it can be concluded that NO_3^-- and NH_4^+-fed plants grown with 5000 ppm root-zone CO_2 had similar carbon budgets and the factor that influenced NUE was the more rapid uptake of NH_4^+ compared to NO_3^- and exudation of amino acids into the root environment by NH_4^+-fed plants.

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