Nitrate and Ammonium Interactions in Maize

By

Jessey George

Thesis submitted in fulfilment of the requirements for the degree of

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I dedicate this thesis

in loving memory of my mother

AMMINI GEORGE

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Abstract

Nitrogen (N) is one of the major mineral nutrients required by a plant for its growth and development. Nitrate (NO_3^-) and ammonium (NH_4^+) are the predominant forms of N available to plants in agricultural soils. Plants have the ability to absorb both these forms efficiently from the soil solutions. With soil solution concentrations of NH_4^+ being much lower (on average 10%) than NO_3^-, contribution of these small amounts of NH_4^+ to the overall N budget of crop plants is often overlooked. This research focussed on the contribution of this NH_4^+ in the nitrogen economy of maize plants. The study also investigated whether NH_4^+ has any effect on uptake and utilization of other nutrients, and most importantly, NO_3^-.

Growth of maize inbred line B73 was increased when one-third of total nitrogen was supplied as NH_4^+ with low NO_3^-, but not for another inbred line Gaspe Flint. Further investigations on B73 found a 20% increase in plant growth when supplied with 10% NH_4^+ along with sufficient NO_3^-. Ammonium being a cheaper N source and the low energy and carbon skeleton requirement for its assimilation has contributed in increased shoot dry matter accumulation in these plants. A corresponding increase in total N, total free amino acids and sugars in the leaves of these plants were observed. A positive correlation was seen between transcript levels of putative high affinity NO_3^- and NH_4^+ transporters. This together with an increased activity of N assimilatory enzymes suggested that small amounts of NH_4^+ can increase the uptake and assimilation of N in these plants. 10% NH_4^+ in the nutrient solution does not inhibit the NO_3^- uptake capacity in plants but when the concentration was increased to 50% there is a reduction in NO_3^- uptake capacity for plants growing in low N. This indicates that high concentration of NH_4^+ limit the absorption of NO_3^- which is an important signalling molecule for various metabolic activities in plants. Reduction in NO_3^-

uptake capacity of plants grown in 10% NH_4^+ at sufficient N was correlated with higher total free amino acids in the roots, particularly glutamine and asparagine. This reduction in NO_3^- uptake capacity when grown in small amounts of NH_4^+ is a long term effect caused by the products of N assimilation and could be reversed by moving plants to solely NO_3^- treatments. Higher concentrations of amino acids in the roots of these plants suggests that NH_4^+ that enters the root gets first into the assimilatory pathway in the cytosol prior to the assimilation of NH_4^+ formed by the reduction of NO_3^- in the plastids.

This study showed that small amounts of NH_4^+ improve plant growth and lead to major changes in N uptake and assimilation processes. Based on these effects and the fact that plants in the field always have a small amount of N available as NH_4^+, it is recommended that NH_4^+ be added to the experimental nutrient solutions with maize and the effect be explored in other major plant species.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Jessey George

June, 2014

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List of Abbreviations

%, percent

AMT, ammonium transporters

ANOVA, analysis of variance

B, boron

C, carbon

Ca, calcium.

CHL, chloride transporter

Cu, copper

DAE, days after emergence

Fe, Iron

GHA, γ-glutamyl hydroxamate

GOGAT, glutamate synthase

GS, glutamine synthetase

HATS, high affinity transport system

K, potassium.

LATS, low affinity transport system

Mg, Magnesium

Mn, manganese

Mo molybdenum

N, nitrogen

NH_4^+, ammonium

NiR, nitrite reductase

NO_2^-, Nitrite

NO_3^-, nitrate

NR, nitrate reductase

NRT, nitrate transporters

NUE, nitrogen use efficiency

NUpE, nitrogen uptake efficiency

NUtE, nitrogen utilization efficiency

P, Phosphorus

PCR, polymerase chain reaction

Q-PCR, quantitative real time polymerase chain reaction

S, sulphur

Zn, zinc

Chapter 1: Introduction and literature review

1. 1 INTRODUCTION

Nitrogen (N) is one of the major mineral nutrients that are required by a plant for its growth and development. It is a major component of chlorophyll (major pigment responsible for photosynthesis in plants), amino acids (the building blocks of proteins), and nucleotides (the building blocks of nucleic acids). Nitrogen deficiency in plants leads to decreased growth and yellowing of leaves resulting in yield reduction. Although nitrogen is present in all soils, it is often not present in sufficient quantities for plants to achieve maximal growth and yield and hence farmers apply large amounts of N fertiliser. Unfortunately N fertiliser recovery by crops is often poor leading to unnecessarily high costs of cultivation and also environmental hazards (Canfield et al., 2010, Diaz and Rosenberg, 2008). So it is very important that the nitrogen use efficiency (NUE) of crops is increased obtain optimum yield and improve economic and environmental sustainability.

Nitrate (NO_3^-) and ammonium (NH_4^+) are the predominant forms of N available to plants in agricultural soils (Wolt, 1994). Nitrate concentrations are generally 10 times that of NH_4^+ and this ratio is consistent for the pool of N available to plants across soil types (Marschner, 2011). Even though most plants prefer a combination of NH_4^+ and NO_3^-, the balance between plant NH_4^+ and NO_3^- use appears to vary depending on environmental factors such as soil pH and temperature (Clarkson and Warner, 1979, Falkengren-Grerup, 1995, Haynes and Goh, 1978, Macduff et al., 1987, Marschner et al., 1991). Plant N preference may also be related to energetic costs and pH effects of uptake and assimilation of either NH_4^+ or NO_3^- (Bartelheimer and Poschlod, 2013, Britto and Kronzucker, 2013, Glass et al., 2002). The issue is complicated by NH_4^+ inhibition of NO_3^- uptake (Kronzucker et al., 1999, Rufty et al., 1982).

With soil solution concentrations of NH_4^+ being so much lower than NO_3^-, the contribution of NH_4^+ to the overall N budget of crop plants is often overlooked. This research focussed on the effect of small amounts of NH_4^+ in the soil solution on the performance of maize (*Zea mays* L.) and the reasons may be for this effect. The study also investigated whether NH_4^+ has any effect on uptake and utilization of other nutrients and the apparent inhibition of NO_3^- uptake by NH_4^+. The amino acid content distribution in plants tissues was also measured and the effect of N treatments on their distribution was determined.

1.2 LITERATURE REVIEW

1.2.1 Nitrogen in the soil

Nitrogen in its gaseous form (N_2) constitutes 78% of the atmosphere, but it is unavailable to most plants who can utilize this nitrogen only if it is fixed in the soil either in organic forms (amino acids) or inorganic forms (NH_4^+ or NO_3^-) by lightning or by bacteria. Industrially fixed N (fertilisers) are the dominant form used to increase N in agricultural soils (Tilman, 1999). Although NH_4^+ or NO_3^- are the major forms in which agricultural plants take up nitrogen, studies have shown that some plants in the arctic tundra regions and in temperate forests (where organic N is the main form of N) can absorb simple amino acids in the absence of inorganic N (Chapin et al., 1993, Farrell et al., 2013, Holst et al., 2012, Inselsbacher and Näsholm, 2012).

About 90% of the total N in most soils is present in the organic matter produced by microbial decomposition of plants and animal residues (Rosswall, 1976). This includes large amounts of humus, nucleic acids, amino acids, amides, vitamins, hormones etc. The decomposition of organic matter plays an important role in the availability of NH_4^+ and NO_3^- and the factors that influence this are climate, vegetation and topography (Haynes, 1986). During decomposition organic N is mineralised to NH_4^+, and it is then nitrified to NO_3^- by nitrifying bacteria. Ammonium is positively charged and it binds with negatively charged clay particles thus making it not easily leachable. Nitrate on the other hand is present as free ions and more easily lost through leaching and runoff.

In well aerated, less acidic soils, NH_4^+ is rapidly nitrified to NO_3^-, hence NO_3^- is the predominant form of N present in most agricultural soils and preferred N form for most cultivated crops (Haynes, 1986, Tills and Alloway, 1981). On the other hand, in areas of the world where soil conditions are unsuitable for growth of nitrifying bacteria, such as in tundra and boreal ecosystems, NH_4^+ is the major source of N (Keeney, 1980). McKane et al. (2002) conducted experiments on various plant species in arctic tundra region and showed that most prefer NH_4^+ and glycine as their N source during their initial growth stages. Research with mycorrhizal plants in some boreal forests has demonstrated that they can use organic N as their N source (Näsholm et al., 1998) as this forms the major N reserve in these soils. Under submerged, often anaerobic conditions in wetland soils, where main crop is rice, mineral N is predominantly available as NH_4^+ and the main N source for paddy rice (Islam and Islam, 1973).

The way plants have adapted to different environmental conditions plays a major role in their preference for NH_4^+ and NO_3^-. In different ecosystems, environmental conditions result in different proportions of NH_4^+ and NO_3^-, and native plants have adapted to these conditions (Boudsocq et al., 2012, Britto and Kronzucker, 2013). Concentrations of NH_4^+ and NO_3^- in soils vary with management practices such as fertilization and grazing

(White et al., 1987), soil pH (Bigg and Daniel, 1978, Vessey et al., 1990) and soil temperatures (Dong et al., 2001).

Although NO_3^- is the most abundant form of N in agricultural soils NH_4^+ is always present in small amounts (10% of the NO_3^- concentration) (Marschner, 2011). Due to the predominance of NO_3^- most agricultural crops prefer it as their N source. In forest soils, NH_4^+ is the most abundant form of N and most plants in this ecosystem have a preference for NH_4^+ (Kronzucker et al., 1997). Species specific preferences to either form of N have been established in some grassland (Weigelt et al., 2003) and alpine communities (Miller and Bowman, 2003).

1.2.2 N fertilizer use and its environmental and economic impact

The substantial increase in global population since 1900 has increased demand for food requiring the application of substantially more N fertilisers. Of the total N applied only 33%-50% of it actually ends up in cereal grains (Raun and Johnson, 1999). A large proportion of the rest is either lost to the atmosphere or as surface run off into water bodies allowing algal blooms to grow which depletes the oxygen in water affecting the living organisms and causing dead zones (Beman et al., 2005, Heisler et al., 2008, Mee, 2006). There is also leaching into ground water thereby contamination of wells which can lead to health issues of high nitrate in drinking water. The emission of nitrous oxide into the atmosphere from fertilisers is a major contributor to greenhouse gas emissions from agriculture (Sistani et al., 2011). In addition to this, the cost of cultivation for farmers is unnecessarily high due to underutilisation of applied N fertilisers. Nitrogen fertiliser costs fluctuate with the price of natural gas used to produce them and N fertilisers now are second only to fuel as input cost for most farmers (Mueller et al., 2011).

Increasing the nitrogen use efficiency (NUE) of cereals is an important avenue towards increasing crop yield in an economically and environmentally sustainable manner. NUE is most commonly defined as the grain yield per unit of N supplied (Moll et al., 1982). There are two components of NUE , one is the efficiency of plants in acquiring N from the soil, or nitrogen uptake efficiency (NUpE); the other is the ability of plants to use the absorbed N to produce grain, or; nitrogen utilization efficiency (NUtE) (Hirel et al., 2007). Research is now focussed on improving agronomic practices to improve NUE (fertilizer management, irrigation practices etc.) and developing hybrids and cultivars with higher nitrogen use efficiency (Atkinson et al., 2005, Raun and Johnson, 1999).

1.2.3 NO_3^- and NH_4^+ uptake in plants

1.2.3.1 Mechanism of uptake

Both NO_3^- and NH_4^+ enter into root apoplast by diffusion or mass flow and into symplast by active transport through plasma membrane (Crawford and Glass, 1998). The anion NO_3^- is believed to be mainly transported across membranes via symport with protons (Glass and Siddiqi, 1995a, McClure et al., 1990). There are three distinctive transport systems which operate in plants for the uptake of NO_3^-. They are the inducible high affinity transport system (iHATS), which is induced by the presence of NO_3^-, constitutive high affinity transport system (cHATS), which operates even in the absence of NO_3^- (Forde, 2000, Aslam et al., 1993) and low affinity transport system (LATS) (Crawford and Glass, 1998, Glass et al., 2002). When NO_3^- concentration in the medium exceeds approximately 250 µM LATS mediated transport dominates and shows a linear relationship with external concentration (Glass et al., 2002, Crawford and Glass, 1998). For HATS NO_3^- uptake the *Km* value is found to be 9.3 µM in lettuce (Swiader and Freiji, 1996), but ranges up to 224 µM in maize (Pace and McClure, 1986). Studies have shown iHATS are up regulated on introduction to NO_3^- (Aslam et al., 1992, Aslam et al., 1993, Siddiqi et al., 1990) and are down regulated on continuous exposure to NO_3^- (Glass and Siddiqi, 1995a).

Ammonium being a cation can be passively taken up in plant roots following electrochemical gradient (Smith and Walker, 1978). Studies on the affinity to NH_4^+ suggests that uptake of NH_4^+ in species such as rice and *Lemna* shows a biphasic pattern, with a saturable high affinity transport system (HATS) operating at low NH_4^+ concentrations (Wang et al., 1994) and a linear low affinity transport system (LATS) at higher external concentrations (Ullrich et al., 1984, Wang et al., 1993b). Studies have shown that the electrochemical gradient at high external concentrations of NH_4^+ is energetically downhill (Ullrich, 1992). However in agricultural soils the NH_4^+ concentration is generally low and it is likely that NH_4^+ uptake is mainly through HATS (Wolt, 1994). The *Km* value for influx of NH_4^+ via HATS was found to be as low as 1 µM in *Spartina* (Bradley and Morris, 1990) and as high as 190 µM in rice (Wang et al., 1993b).

1.2.3.2 Nitrogen transporters in plants

Two gene families, namely NRT1/PTR, recently renamed the NPF family (Léran et al., 2014) and NRT2 (MFS super family) play important roles in NO_3^- uptake (Tsay et al., 2007, Chrispeels et al., 1999, Crawford and Glass, 1998, Forde, 2000). In Arabidopsis 53 genes have been identified that belong to NPF family. Out of these genes only 16 have been identified and functionally characterised to be NO_3^- transporters (Krapp et al., 2014, Léran et al., 2014, Tsay et al., 2007). The NPF family of transporters have 12 putative transmembrane domains connected by short peptide loops and mediate proton coupled active transport (Chen et al., 2008). In maize a total of 17 *NRT* genes HATS and LATS) have been identified (Plett et al., 2010).

The first NO_3^- transporter, called CHL1, was identified in a T-DNA tagged chlorate resistant mutant of *Arabidopsis thaliana* (Tsay et al., 1993) which is otherwise called NRT1.1. The NRT1 transporters are thought to be responsible for LATS NO_3^- uptake, except NRT1.1 (Liu et al., 1999) that has been shown to have dual affinity for NO_3^- and can also act as high affinity transporter when the external concentration of NO_3^- decreases (Sun et al., 2014, Parker and Newstead, 2014). Switching of NRT1.1 from low affinity to high affinity transport is due to phosphorylation of a single residue Thr 101 (Liu and Tsay, 2003). This transporter is also thought to act as a sensor regulating the expression of primary NO_3^- response genes based on external NO_3^- levels (Ho et al., 2009, Krouk et al., 2010). The xylem loading of NO_3^- absorbed by root is carried out by NRT1.5 which is a low affinity bidirectional (influx and efflux) NO_3^- transporter (Lin et al., 2008). NRT1.7 is thought to be involved in the remobilization of NO_3^- from. older leaves to younger leaves (Fan et al., 2009). Another transporter, NRT1.8, is found to be involved in xylem unloading of NO_3^- (Li et al., 2010), and NRT1.9 mediates phloem NO_3^- transport (Wang and Tsay, 2011). Recently two NRT1 transporters namely NRT1.11 and NRT1.12 have been reported to be involved in transfer of NO_3^- from the xylem to phloem in petioles (Hsu and Tsay, 2013). Recently it was found that certain NRT1 transporters can also transport abscisic acid (ABA) (Kanno et al., 2012) and glucosinolates (Nour-Eldin et al., 2012).

The NRT2 family of transporters are high affinity NO_3^- transporters in plants. The first NRT2 transporters were identified in *Aspergillus nidulans* (Unkles et al., 1991, Brownlee and Arst, 1983) and *Chlamydomonas reinhardtii* (Quesada et al., 1994). Close homologs of these NO_3^- transporters have been identified in Arabidopsis (Orsel et al., 2002), maize (Santi et al., 2003, Quaggiotti et al., 2004), barley (Vidmar et al., 2000, Tong et al., 2005) and wheat (Yin et al., 2007). Studies have shown that NRT2 in barley (Tong et al., 2005) and in Arabidopsis (Okamoto et al., 2006) require co-expression of another protein

(NAR2/NRT3) to facilitate their HATS NO_3^- transport function. *NRT2.1* and *NRT2.3* genes encode NO_3^-/nitrite (NO_2^-) specific transporters whereas *NRT2.2* encode NO_3^- specific transporters (Kotur et al., 2013, Quesada et al., 1998). Studies have suggested that HATS transcript levels are negatively regulated at high N (Garnett et al., 2013, Liu et al., 2009, Okamoto et al., 2003, Santi et al., 2003). Of all NRT2 family of proteins, NRT2.1 is the main component of high affinity NO_3^- uptake in plants (Li et al., 2007, Okamoto et al., 2006). The expression of *NRT2.1* was absent in the roots of NH_4^+ fed plants indicating the substrate affinity for *NRT2.1* (Zhuo et al., 1999). It has been found that *AtNRT2.4* in Arabidopsis was highly expressed in N starved plants indicating a role in uptake of NO_3^- at low N levels (Kiba et al., 2012). In maize, *ZmNRT2.5* expression was found only in low N treatment in a lifecycle experiment in maize (Garnett et al., 2013). Together this suggests these two NO_3^- transporters may play critical role in N acquisition under low N conditions.

Two distinct families of AMTs exist in plants, namely AMT1 and AMT2 (Koegel et al., 2013, Loqué and von Wirén, 2004). The heterologous expression in yeast or *Xenopus* oocytes indicates that they are high affinity NH_4^+ transporters (Ninnemann et al., 1994, Gazzarrini et al., 1999). The first NH_4^+ transporter gene in plants, AMT1, was identified in Arabidopsis and encodes a high affinity transporter (Ninnemann et al., 1994). Since then several studies have isolated homologues of AMT1 and AMT2 from Arabidopsis (AtAMT1.1 to AtAMT1.5 and one AMT2) (Gazzarrini et al., 1999), 3 in tomato (Lauter et al., 1996) and 10 in rice (Sonoda et al., 2003). In rice AMT2 family consists of three subfamilies namely AMT2, AMT3 and AMT4 (Suenaga et al., 2003), and the transporters appear to be functioning as HATS in plants. Sohlenkamp (2002) showed that when concentration of NO_3^- decreased *AtAMT2* transcript levels in roots, but did not affect transcript levels in the shoots indicating that a role of internal N status in regulating the uptake of NH_4^+. Functional characterisation was carried out on *AMT1.1A* and *AMT1.3* genes in maize (Gu et al., 2013), and it was found that they are the main contributors to high affinity NH_4^+ transport.

1.2.3.3 Assimilation of NO_3^- and NH_4^+

The inorganic N that enters t roots has to be first incorporated into organic N to be further metabolised by the plant. In most plants NO_3^- which enters into the roots can be assimilated in the roots or transported to the shoots in the xylem. In herbaceous plants, NO_3^- assimilation takes place mainly in leaves, whereas in woody plants, it mostly occurs in the roots (Andrews, 1986, Faure et al., 2001). On the other hand, the NH_4^+ entering the roots is mostly assimilated in the roots (Murphy and Lewis, 1987). The sources of NH_4^+ in plants are from uptake by the root system, production during NO_3^- reduction, deamination of N compounds and by catabolism of amino acids (Lea et al., 2007).

Major enzymes involved in N assimilatory pathway are NO_3^- reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT). The first step in the assimilation of NO_3^- is the conversion of NO_3^- to NO_2^- catalysed by the enzyme NO_3^- reductase (NR) (Beevers and Hageman, 1969). The NO_2^- produced in cytoplasm enters plastids in the roots or chloroplasts in the leaves where it is converted to NH_4^+ by enzyme nitrite reductase (NiR). The NH_4^+ then enters the GS/GOGAT cycle to form glutamine and glutamate. Two isoforms of GS exist in plants namely, cytoplasmic glutamine synthetase (GS1) and plastidic/chloroplastic glutamine synthetase (GS2) (McNally et al., 1983). GS1 catalyses the assimilation of NH_4^+ absorbed in roots and GS2 is responsible for the assimilation of NH_4^+ formed by the reduction of NO_3^- or during photorespiration. About 95% of the NH_4^+ that enters the roots of the plants is assimilated in cytoplasm by cytoplasmic GS1/ GOGAT cycle. Ammonium formed by reduction of NO_3^- enters plastid/chloroplasts and GS2/GOGAT pathway to form glutamine and glutamate which are the precursors for synthesis of other amino acids (Woo et al., 1982). Energetic cost involved in uptake and assimilation of NO_3^- is greater than that required for absorption and assimilation of NH_4^+ (Bloom et al., 1992). This is mainly due to the fact that NO_3^- must first be reduced to NO_2^-, a process that requires the transfer of two electrons and then to NH_4^+, a process that requires transfer of six electrons (Bloom et al., 1992).

Amino acids are the precursors of protein synthesis but are also considered to be the currency of N exchange in plants (Coruzzi and Bush, 2001). They are synthesised in roots or leaves and are transported to other developing organs of plants via xylem and phloem. The pools of amino acids in the plants are regulated by N uptake and assimilation, pH regulation and availability of sugars (low sugars inhibit N assimilation) (Stitt et al., 2002). Plants grown in NH_4^+ or a combination of NO_3^- and NH_4^+ have more amino acids than plants fed with only NO_3^- perhaps resulting from the preferential uptake of NH_4^+ compared to NO_3^- and faster incorporation of NH_4^+ into the organic form of N (Atanasova, 2008, Causin and Barneix, 1993). The proportions of individual amino acids also vary depending on the form of N (Loqué and von Wirén, 2004). It was found that when maize plants were supplied with urea, which usually breaks down into NH_4^+, the major amino acids accumulated in plants was glutamine (Pavlík et al., 2010). Plants have a tendency to maintain glutamate homeostasis in plant as it is involved in both assimilation and reassimilation of NH_4^+ in plants (Forde and Lea, 2007, Walker et al., 1984). It has been suggested that as long as NH_4^+ assimilation is actively taking place in plants, glutamate content in the plant tissue is stable because this amino acid plays a central role in plant nitrogen metabolism (Forde and Lea, 2007).

1.2.3.4 Regulation of nitrogen uptake

Plants have evolved mechanisms to regulate activity of N uptake systems to maintain plant N concentration. External concentration of both NO_3^- and NH_4^+ (Stitt, 1999, Crawford, 1995, Tsay et al., 2011) and plant's internal N status (Liu et al., 2009, Imsande and Touraine, 1994) act as signals for controlling N uptake in plants. Nitrate uptake is determined by a regulatory mechanism which is activated by N demand of the plant, thus when plants are deprived of N they show a higher NO_3^- uptake rate compared to plants with a continuous supply of N (Doddema and Telkamp, 1979). There is considerable temporal variation in NO_3^- uptake capacity of plants based on demand of the plants and supply of N in the nutrient medium (Garnett et al., 2013). The 'primary nitrate response' is induced by N starvation followed by N resupply. This transient response lasts several hours and results in increased high affinity NO_3^- uptake capacity and a corresponding increase in the NRT2 protein in the roots (MacKown and McClure, 1988).

Amino acid concentration inside the plant acts as another regulator of N uptake in plants. It has also been suggested that amino acids translocated from the shoot to root via the phloem may act as an indicator of N nutritional status of the plant thereby regulating the NO_3^- uptake from the nutrient medium (Cooper and Clarkson, 1989, Muller and Touraine, 1992).

Feedback regulation of NH_4^+ similar to that of NO_3^- is also observed in plants (von Wirén et al., 1997). In Arabidopsis it was observed uptake capacity of NH_4^+ increased when plants were deprived of N and decreased on resupply of N, potentially due to negative feedback regulation by tissue concentration of glutamine (Gazzarrini et al., 1999, Lanquar et al., 2009, Yuan et al., 2007).

For both NO_3^- and NH_4^+, feedback regulation by the N status of plants is more visible in HATS than in LATS (Lejay et al., 1999, Wang et al., 1993a). Molecular studies on both

NRT2s and *AMTs* have shown that transcript levels of these genes either increase or decrease in response to changes in N status (Gazzarrini et al., 1999, Lejay et al., 1999).

Both NO_3^- and NH_4^+ uptake are diurnally controlled. Their uptake increases during the day, reaches maximum at the end of light period and then it decreases (Gazzarrini et al., 1999, Glass et al., 2002). However, in a study in barley and maize, supply of sucrose increased NO_3^- uptake by 38% in the dark grown seedlings indicating role of sugars in the regulation of NO_3^- uptake (Sehtiya and Goyal, 2000). A direct correlation with the diurnal patterns of N uptake and transcript levels of *NRT2* and *AMT1* genes have been found in some studies (Lejay et al., 1999, Von Wirén et al., 2000).

The pH of nutrient medium can affect NO_3^- and NH_4^+ uptake, and the optimal pH range for the uptake of NO_3^- and NH_4^+ seems to be species specific. Doddema and Telkemp (1979), in their experiments with Arabidopsis showed that the optimum pH for NO_3^- uptake was 8.0. In contrast to this finding, barley plants showed maximum NO_3^- uptake at a pH of 4.0 (Rao and Rains, 1976). Glass and co-workers (1990) demonstrated in barley that NO_3^- influx was higher within a pH range of 4.5-6.5 than at pH 7.5. *Eucalyptus* seedlings showed no difference in uptake was seen between pH 4.0 and 6.0 (Garnett and Smethurst, 1999). As with NO_3^-, there is a range of reported responses of NH_4^+ uptake to pH. Optimum pH for NH_4^+ uptake for *Typha latifolia* and soybean was found to be 6.5 and 6.0 respectively, whereas, Garnett and Smethurst (1999) showed that NH_4^+ uptake at pH 4.0 was twice that at pH 6.0 in *E. nitens*. Raun et al. (2007) in experiments with tea plants showed that the uptake of NH_4^+ was not affected by pH.

Soil temperature also has a great influence on the uptake of NH_4^+ and NO_3^- by plants. Several studies have demonstrated that NO_3^- uptake is sensitive to low temperature while NH_4^+ uptake is insensitive to low temperature (Clarkson and Warner, 1979, Macduff et al., 1987, Macduff and Jackson, 1991). In *Lolium perenne* at low temperature, 85% of N absorbed was in the form of NH_4^+ (Clarkson et al., 1986).

1.2.4 Factors affecting plant preference for different nitrogen sources

In agricultural soils and well aerated soils, nitrification increases the availability of NO_3^- as major form of N. However some plants are able to slow down (Lata et al., 2004, Subbarao et al., 2007b, Subbarao et al., 2007a) or increase (Hawkes et al., 2005, Lata et al., 2000) nitrification and alter the relative amounts of NO_3^- and NH_4^+ available in the soil. It is hypothesised that plants that inhibit nitrification have a greater preference for NH_4^+ than NO_3^- (Boudsocq et al., 2012).

Uptake and assimilation of NO_3^- requires 12 ATP molecules in contrast to two ATP required for NH_4^+ assimilation, which suggests that plant growth may be more energy limited under NO_3^- nutrition than NH_4^+ (Bloom et al., 1992). Although the absorption and assimilation of NH_4^+ conserves energy (Slasac et al., 1987), NH_4^+ when used at high concentration as the sole source of N it can be toxic to plants affecting their growth and development (Gerendás et al., 1997, Kronzucker et al., 2001). The absorption and assimilation of NH_4^+ releases H^+ ions into the nutrient medium making it acidic which reduces root growth (Raven and Michelis, 1979). In contrast, NO_3^- uptake and assimilation increases pH of the medium because of the release of hydroxyl ions in to the growth medium (Raven and Smith, 1976).

Studies have shown that in NO_3^- fed plants the absorption of NO_3^- by plants also enhances absorption of cations K^+, Mg^{2+} and Ca^{2+}, but plants grown only with NO_3^- may show deficiencies of phosphate and sulphate, as well as some trace elements, (Jackson and Williams, 1968). Increased cation uptake by NO_3^- fed plants may also be due to favourable conditions produced by rise in rhizosphere pH during NO_3^- uptake and assimilation. Kirkby and Knight (1967) in their studies demonstrated that organic anions are formed during reduction of NO_3^- in plants and that in order to maintain the ionic balance, uptake of NO_3^- should also be accompanied by inorganic cations which provide counter ions for organic anions as well as NO_3^-.

Conversely, NH_4^+ absorption facilitates the uptake of phosphate and sulphate but limits the absorption of some cations like K^+, Ca^{2+} and Mg^{2+} (Gahoonia et al., 1992). Ammonium in the growth solution also helps in the absorption of most micronutrients from soil solution (Kirkby and Mengel, 1967, Riley and Barber, 1971). This is because uptake and assimilation of NH_4^+ acidifies the growth medium which facilitates the absorption of micronutrients (Hageman, 1984). The limitation in absorption of K^+, Mg^{2+} and Ca^{2+} in NH_4^+ fed plants may be due to the competition for these ions at the site of their uptake by NH_4^+ ions or the H^+ ions produced during NH_4^+ uptake and assimilation (Cox and Reisenauer, 1973). In contrast, a study by Rayar and Van Hai (1977) in soybean found that NH_4^+ up to 500 µM enhanced uptake of K^+, Mg^{2+} and Ca^{2+}, but at higher concentrations it limited the uptake of these ions due to competition. The increased uptake of P in maize with NH_4^+ is thought to be due to change in the pH of growth medium during NH_4^+ assimilation (Miller et al., 1970).

Studies have shown that most agricultural crops respond better when N is supplied as a combination of NH_4^+ and NO_3^- (Below and Gentry, 1987, Gentry, 1992, Haynes and Goh, 1978, Schrader et al., 1972, Bernardo et al., 1984). Cox and Reisenauer (1973) demonstrated that maximum dry matter was obtained when wheat plants were grown in a combination of NH_4^+ and NO_3^+. The response to particular form of N varies from species to species (Glass and Siddiqi, 1995b, Haynes and Goh, 1978). It was found in maize that when plants were given NO_3^- and NH_4^+ in various proportions, maximum dry matter was obtained when the proportion was 50/50 compared to either form alone (Schrader et al., 1972). However, in tomato optimum yield was obtained when NO_3^- and NH_4^+ were applied in 3:1 ratio and higher proportion of NH_4^+ in the nutrient solution decreased the yield (Bloom et al., 1993).

1.2.5 Inhibition of NO_3^- uptake by NH_4^+

Ammonium inhibition of NO_3^- uptake has been reported in many studies (Lee et al., 1992, Mackown et al., 1982, Muller and Touraine, 1992, Munn and Jackson, 1978, Rufty et al., 1982). Extensive efforts have been made to understand the inhibitory effect of NH_4^+ on NO_3^- uptake and two main theories have been developed to explain the inhibition of NO_3^- uptake by NH_4^+. The first is that a short term inhibition may be due to the direct effect of NH_4^+ on plasma membrane due to membrane depolarisation leading to inhibition of NO_3^- influx (Glass et al., 1985, Ingemarsson et al., 1987, Lee and Drew, 1989, Mackown et al., 1982). Alternatively it has been suggested that this inhibition may be caused by stimulation of NO_3^- efflux (Ayling, 1993, Deane-Drummond and Glass, 1983, Jackson et al., 1976). It has been suggested that the internal concentration of NO_3^- plays an important role in the stimulation of NO_3^- efflux in the presence of NH_4^+ (Aslam et al., 1993). On the other hand, in barley it was shown that the reduction in NO_3^- uptake was primarily due to the inhbition of influx and only a minor contribution of stimulation of efflux was observed when the NO_3^- uptake was measured in the presence of NH_4^+ (Kronzucker et al., 1999). Studies on cotton plants showed that inhibition of NO_3^- by NH_4^+ depended on root concentration of N (Aslam et al., 2001). This inhibitory effect may also be due to cytoplasmic accumulation of NH_4^+ (Glass et al., 2007). Another potential source for inhibition of NO_3^- uptake is NH_4^+ toxicity whereby, high NH_4^+ decouples electron transport.

Another cause of inhibition of NO_3^- uptake is the long term effect of N assimilatory products on NO_3^- uptake. In soybean seedlings it was observed that NO_3^- uptake was inhibited by the phloem-translocated amino acids (Muller and Touraine, 1992). Major amino acids that inhibited uptake in this study were alanine, glutamic acid, aspartic acid, arginine and asparagine. Taylor and Bloom (1998) in their studies on maize seedlings suggested that with preferential uptake of NH_4^+, the inhibition of NO_3^- uptake in their study is by the products of NH_4^+ assimilation. In their study an enhanced H^+ extrusion was observed from roots which were associated with increased NH_4^+ assimilation. Similarly, it was demonstrated in maize and barley that tissue concentration of amino acids asparagine and glutamine regulated NO_3^- uptake (Lee et al., 1992).

1.3 AIM & OBJECTIVES

Although NO_3^- predominates as the N source in agricultural soils there is always a small amount of NH_4^+ (10% of NO_3^-) present in these soils. The contribution of this small amount of NH_4^+ to the growth of plants is unknown and no studies have yet looked at the significance of this small amount of NH_4^+ in the nitrogen uptake of plants. Therefore, the aim of work presented in this thesis is to understand the contribution of 10% NH_4^+, similar to the concentration found in most agricultural soils, in the N budget of maize plants.

The research objectives of this thesis were:

i) to quantify the effect of small amounts of NH_4^+ on the growth and nitrogen budget of maize inbred lines B73 and Gaspe Flint;

ii) to determine why 10% NH_4^+ may have a disproportional influence on maize growth;

iii) to understand how small amounts of NH_4^+ affect the uptake of NO_3^- in plants;

iv) to understand the distribution of amino acids in different plant parts and how this is affected by nitrogen availability and small amounts of NH_4^+.

Chapter 2 is an investigation of the response of two maize inbred lines, Gaspe Flint and B73, to varying concentrations of NH_4^+ both at low and high total N levels.

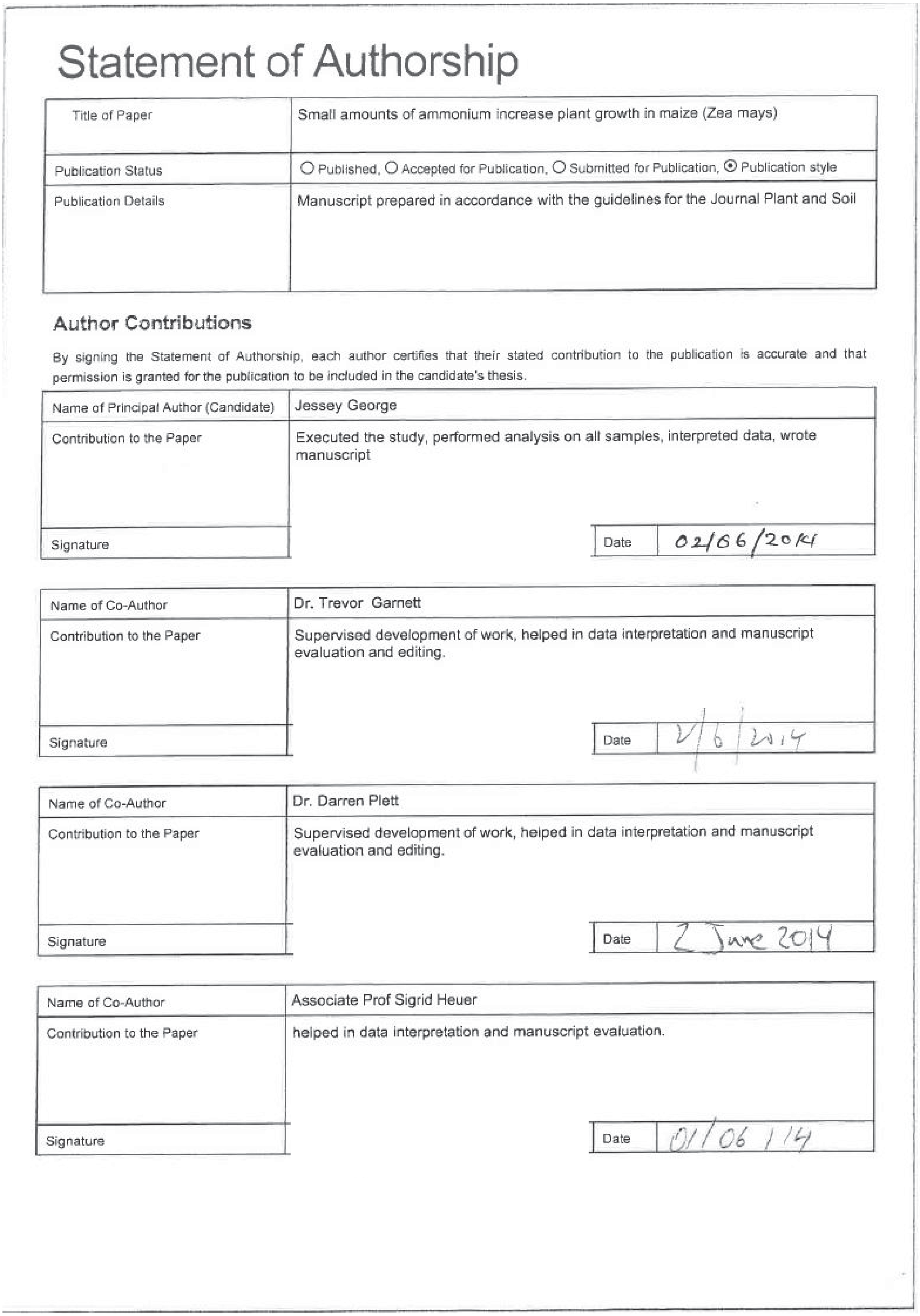
The effect of 10% NH_4^+ on maize growth and the total N budget of maize are studied in chapter 3. This study also looked at the cause of increased plant growth in maize.

Chapter 4 examined the effect of different NH_4^+ concentrations on the uptake of NO_3^- by the maize inbred line B73 and how the NO_3^- uptake capacity of plants responded when grown in very small amounts of NH_4^+.

In chapter 5 the distribution of amino acids in different plant parts of the maize inbred line B73 in response to 10% NH_4^+ at both low and sufficient levels are measured.

Chapter 6 gives the broad overview of the findings along with discussions on the points of interest in this study and proposal for future directions.

Chapter 2: Small amounts of ammonium (NH_4^+) increase plant growth in maize (Zea mays L.).



Statement of Authorship

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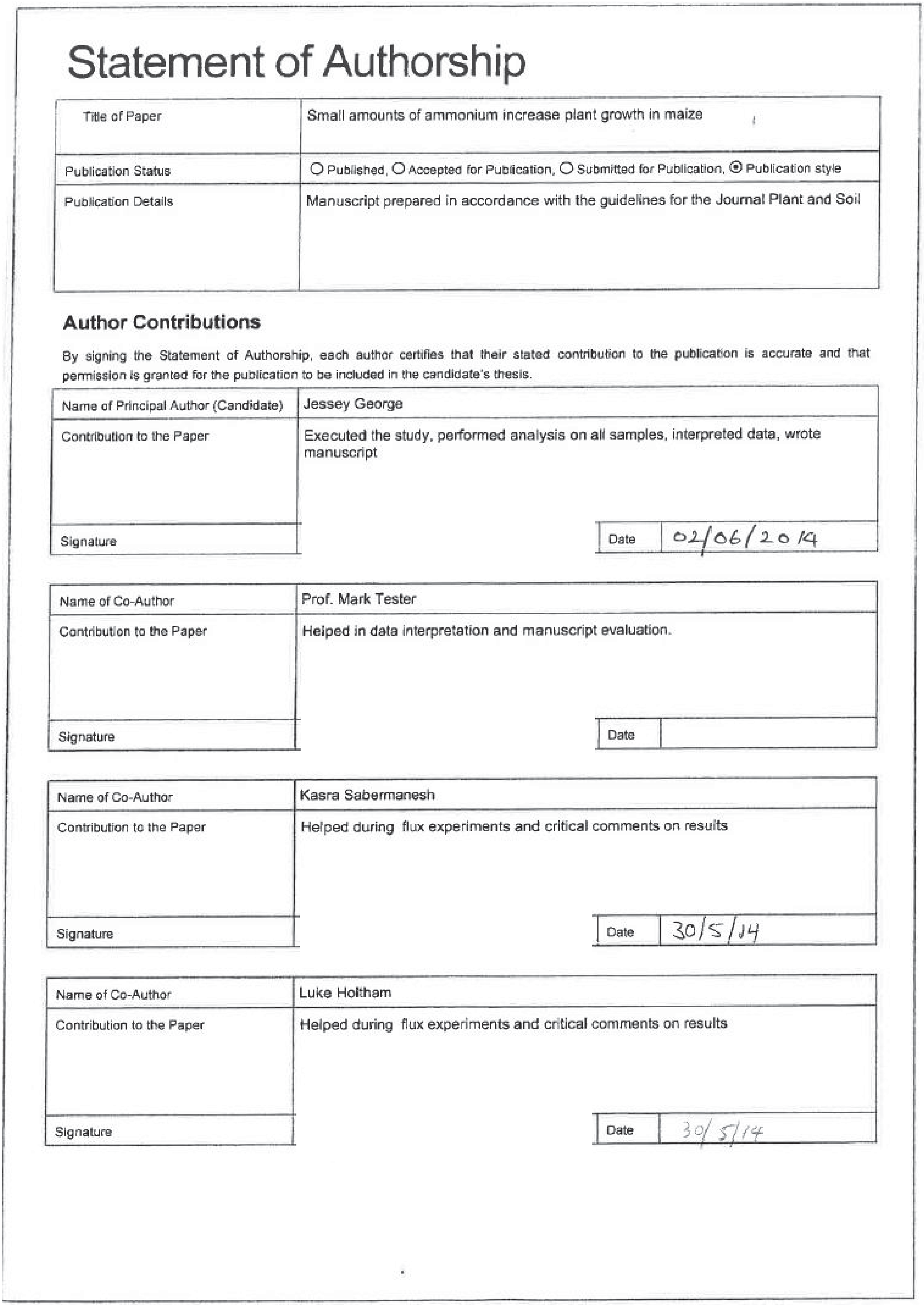

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Contribution to the Paper	Helped during flux experiments and critical comments on results		
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Name of Co-Author	Luke Holtham		
Contribution to the Paper	Helped during flux experiments and critical comments on results		
Signature		Date	30/5/14

Title

Small amounts of ammonium (NH_4^+) increase plant growth in maize (*Zea mays* L.)

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ABSTRACT

Background and Aims: Nitrate (NO_3^-) and ammonium (NH_4^+) are the predominant forms of nitrogen (N) available to plants in agricultural soils. Nitrate concentrations are generally 10 times that of NH_4^+ and this ratio is consistent across a wide range of soil types, thus the possible contribution of NH_4^+ to overall N budget of crop plants is often overlooked. The objective of this study was to quantify the importance of small amounts of NH_4^+ in the growth and total N uptake of maize.

Methods: Maize inbred lines Gaspe Flint and B73 were grown hydroponically for 30 days at reduced (0.5 mM) and sufficient (2.5 mM) levels of NO_3^-. Ammonium was added at 0.05 mM and 0.25 mM to both levels of NO_3^-.

Results: Small amounts of NH_4^+ improved plant growth in B73 but not in Gaspe Flint. Total nitrogen uptake, macronutrient (S & P) and micronutrient uptake were increased with NH_4^+ addition in B73. Although the NH_4^+ uptake capacity was higher than NO_3^- flux capacity for both maize lines, Gaspe Flint plants responded to the NO_3^- in the medium and showed a similar NH_4^+ flux capacity in both low N treatments and in sufficient N treatments irrespective of NH_4^+ concentration.

Conclusion: Small amounts of NH_4^+ supplied along with NO_3^- can increase maize plant growth but this response varies between maize genotypes.

Key words: Nitrate, Ammonium, macronutrients, micronutrients, uptake capacity, biomass

INTRODUCTION

Nitrogen (N) is one of the major nutrients required by plants, and growth and yield in plants are affected by N limitation. Plants absorb N mainly in the form of NO_3^- and NH_4^+ from soil solution (Glass et al. 2002). Nitrate is the dominant form present in agricultural soils and hence is the focus of most research on N uptake (Marschner 2011). Although the NH_4^+ concentration in the soil solution is only in small amounts compared to NO_3^- this ratio is consistent in most agricultural soils (Wolt 1994) meaning that it could be a significant contribution to plant N uptake.

Although NO_3^- is the major form of N available to plants, many previous studies have demonstrated that a combination of NO_3^- and NH_4^+ are beneficial for plant growth (Bloom et al. 1993; Cox and Reisenauer 1973; Lewis et al. 1989). Previous studies with maize showed a positive growth response when plants were supplied with a mixture of NO_3^- and NH_4^+ (Below and Gentry 1987; Schrader et al. 1972; Smiciklas and Below 1992). These studies were conducted using relatively high proportions of NH_4^+, the lowest was 25 % NH_4^+ by in maize (Schrader et al. 1972). There have not been any published studies looking at the effects of NH_4^+ in the concentration range found in agricultural soils.

There are a number of possible reasons for increased plant growth with a mixture of NO_3^- and NH_4^+. Nitrate first has to be reduced into nitrite (NO_2^-) in cytosol and then it enters plastids where NO_2^- gets converted to NH_4^+ before it is converted to amino acids (Bloom et al. 1992). This extra processing, as it were, means that NO_3^- assimilation is a more energy consuming process compared to NH_4^+ and this is one of the possible causes of increased growth in the presence of NH_4^+ (Clarkson 1985; Haynes and Goh 1978; Schrader et al. 1972).

Another reason for increased plant growth may be related to higher uptake capacity of NH_4^+ relative to NO_3^- when both N sources are present. It has been seen in previous studies when both forms of N are available, plants take up NH_4^+ preferentially over NO_3^- (Clarkson et al. 1986; Gazzarrini et al. 1999; Glass et al. 2002; Hatch and Macduff 1991) and total N uptake of these plants was increased. The reason for preferential uptake of NH_4^+ may be that when NH_4^+ is taken by roots it gets assimilated directly in the roots by enzymes cytosolic glutamate synthetase (GS 1) and glutamate synthase (GOGAT) resulting in faster incorporation of inorganic N into organic N.

This positive growth effect of NH_4^+ could also be due to effects on other aspects of plant nutrition, given that in some cases NH_4^+ can improve phosphorus (P), sulphur (S) (Kirkby 1968) and micronutrient (Blair et al. 1970; Jeong and Lee 1996; Kirkby and Mengel 1967; Riley and Barber 1971; Thomson et al. 1993) nutrition of plants. The absorption of NO_3^- by plants can enhance the absorption of cations like K^+, Mg^{2+} and Ca^{2+}, but can lead to slower uptake of phosphate, sulphate and some trace elements (Jackson and Williams 1968). Iron deficiency has been observed in plants grown solely with NO_3^- compared to plants grown with both NH_4^+ and NO_3^- (Zou et al. 2001). On the other hand, when NH_4^+ is supplied to plants it can increase the uptake of iron from the nutrient solution and remobilization of iron inside plant (Marschner et al. 1987; Zou et al. 2001).

Most studies have focussed on effect of simultaneous supply of NO_3^- and NH_4^+ either in equal concentrations or in higher ratios and not on the effect of small quantities. Therefore the aim of this study was to explore and quantify effects of small amounts of NH_4^+ on maize growth. Two maize inbred lines were used in this study, Gaspe Flint and B73. Gaspe Flint is a short stature maize inbred line with a life cycle of 60 days, and because of its small stature is highly suited to growth in controlled environments. B73 was chosen because it is the source of the reference maize genome sequence and it has been used in a large number of physiological studies. We investigated whether small amounts of NH_4^+ when offered along with NO_3^-, both at low and sufficient total N supply would increase plant growth in these inbred lines and attempted to find the cause of this effect, if there was any. We assessed plant N, C, macro- and micro-nutrient contents to determine the effect of the added NH_4^+.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds were bubbled overnight in water, and then placed on filter paper moistened with 0.5 mM $CaCl_2$ and placed in an incubator at 28°C. The germinated seedlings were transplanted into a climate controlled growth chamber providing a day/night temperature of 26/22°C and a photoperiod of 14 h. The photon flux density in the growth chamber was approximately 450 µmol m^{-2} s^{-2} at average leaf height. Plants were grown on mesh collars in tubes as explained in Garnett *et al.* (2013). Johnson's modified nutrient solution (Johnson et al. 1957) was used containing (in mM) 0.8 K, 0.1 Ca, 0.5 Mg, 1 S, 0.5 P and (in µM) 2 Mn, 2 Zn 25 B, 0.5 Cu, 0.5 Mo, and 200 Fe (as FeEDTA and FeEDDHA). Iron was supplemented twice weekly with the addition of Fe $(NH_4)_2(SO_4)_2.6H_2O$ (8 mg L^{-1}), and this was the NH_4^+ source for 0.05 mM NH_4^+ in all the treatments. $(NH_4)_2SO_4$ was used as the NH_4^+ supplement to provide the extra 0.2 mM NH_4^+ in the 0.25 mM NH_4^+ treatments. Solution pH was monitored every second day and maintained between 5.9 and 6.0 by adding 1M HCl or 1M NaOH. Nitrate and NH_4^+ concentrations in the solutions were monitored using NO_3^- and NH_4^+ electrodes (TPS, Springwood, Australia) and maintained at the target concentration of ± 5%. Nutrient solutions were replaced 23 days after emergence (DAE) and the plants were grown for 30 d. Another experiment was also conducted in a similar manner but the N treatments used in this experiment were 0.75 mM NO_3^- alone in one treatment and 0.5 mM NO_3^- + 0.25 mM NH_4^+ in the other treatment. Table 1 shows various treatments in the two experiments and their final harvest day after emergence.

Table 1: The NO_3^- and NH_4^+ treatments and duration of experiments.

Experiment	***Treatments***			***Experimental duration***
		NO_3^- (mM)	**NH_4^+ (mM)**	(DAE)
Experiment 1	LN+LA	0.50	0.05	30
	LN+HA	0.50	0.25	
	SN+LA	2.50	0.05	
	SN+HA	2.50	0.25	
Experiment 2	LN+0A	0.75		23
	LN+HA	0.50	0.25	

Plant biomass & Chemical analysis

In Experiment 1 plant biomass was measured on the 10th, 17th, 23rd and 30th DAE. In Experiment 2 plants were grown for 23 DAE. In both the experiments, roots and shoots were separated, roots blotted with paper towel before fresh biomass was weighed. Plant parts were then dried at 40°C for 7 d to obtain dry weights. Dry matter was ground to a fine powder and shoot tissues were analysed for N and C using a mass spectrometer (Sercon, Cheshire, UK). Shoot macro- and micro-nutrient content were determined following acid digestion using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES; ARL 3580B, ARL, Lausanne, Switzerland)

Flux measurement

The unidirectional fluxes into roots were measured 23 DAE in Experiment 1 using ^{15}N labelled NO_3^- and NH_4^+. On the day of sampling, plants were transferred to a controlled environment room with matching growth conditions and into matching growth solutions. Plants were then moved to a nutrient solution containing 100 µM $^{14}NO_3^-$ or $^{14}NH_4^+$ for 5 min. and then to flux solutions containing either 100 µm NO_3^- or NH_4^+ labelled with ^{15}N (^{15}N 10%) for 10 min. Roots were then rinsed in unlabelled 100 µM NO_3^- or NH_4^+solution for 2 min to wash off ^{15}N from root surface and apoplast. The flux timing of 10 min was chosen to minimize any efflux or transport to shoots based on study by Kronzucker *et al.* (1995). Roots were then separated from shoots, blotted and weighed. Plant parts were dried in an oven at 40°C for 7 d. After measuring the dry matter content the roots were ground to a fine powder and the total N and ^{15}N in plant samples were determined with an isotope ratio mass spectrometer (Sercon, Cheshire, UK). Unidirectional NO_3^- influx was calculated based on ^{15}N content of the root.

Statistical analysis

Statistical analysis of biomasses, total N, fluxes and macro and micro nutrient content were carried out using two-way analysis of variance (ANOVA) in Graph Pad Prism software (Version 6.00, 1992-2012 GraphPad Software, Inc).

RESULTS

Plant biomass

Experiment 1

When compared to Gaspe Flint plants (Figure 1A) the shoot biomass of B73 (Figure 1B) was higher on last sampling day for plants grown in all treatments. However, in B73, but not Gaspe Flint, shoot biomass was higher in LN+HA than in LN+LA. The shoot biomass of both Gaspe Flint and B73 showed no response to increased concentrations of NH_4^+ in the medium when grown with sufficient N (Figure 1A & B). Increasing concentration of NH_4^+ showed no change in root biomass of Gaspe Flint and B73 throughout the growth period both at LN+HA and SN+HA (Figure 1C & D) except on 23 DAE where we saw a higher root biomass for plants grown in SN+LA compared to SN+HA. In Gaspe Flint and B73, the root: shoot showed no difference both in LN+LA and LN+HA throughout the plant growth. However, on 10 DAE, the root: shoot ratio of plants in SN+HA was lower than that of SN+LA for Gaspe Flint and on 30 DAE the root: shoot were similar for plants in all the treatments (Figure 1E). The root: shoot of Gaspe Flint and B73 plants was at its maximum value when plants were younger. In B73 a higher root: shoot was seen for plant grown in LN+LA and LN+HA compared to plants in both sufficient N treatments on 10 DAE. (Figure 1F), thereafter this ratio decreased for plants in all treatments and at final harvest a decrease was observed in the root: shoot of plants in LN+HA compared to LN+LA.

Experiment 2

This experiment was conducted to determine whether it was the extra N contributed by the added NH_4^+ that lead to increased biomass of B73 plants in LN+HA compared to LN+LA in experiment 1. Plants were tested for their response to NH_4^+ at low N levels but the total N level was kept constant at 0.75 mM using NO_3^- alone in one treatment and 0.5 mM NO_3^- and 0.25 mM NH_4^+ in the other treatment. It was observed that plants grown in a mixture of NO_3^- and NH_4^+ (LN+HA) had higher shoot dry matter (Figure 2A) than plants grown in NO_3^- alone treatment (LN+0A). No difference in root dry matter was observed between treatments (Figure 2B). The root: shoot of B73 plants was higher with NH_4^+ on 10 DAE (Figure 2C). Thereafter the root: shoot decreased significantly in plants treated with NH_4^+ and was lower than plants grown only with NO_3^- and this trend was consistent throughout the growing period.

Tissue N% and total N

On 17 DAE there was no difference in N % in both Gaspe Flint and B73 shoots (Figure 3A & B). However on 30 DAE, the tissue N% of Gaspe Flint plants grown in SN+LA was higher than that of plants grown in LN+LA and LN+HA (Figure 3A). In contrast, on 17 DAE, B73 plants grown in SN+HA had higher N concentration in shoots than plants in SN+LA (Figure 3B). On 30 DAE the shoot N % was higher in plants grown in LN+HA compared to plants in LN+LA, and no difference was observed with NH_4^+ in both sufficient N treatments (Figure 3B).

It was observed that the total N uptake of Gaspe Flint plants was significantly higher in both sufficient N treatments than at low N on 30 DAE (Figure 3C). No response of added NH_4^+ was observed in Gaspe Flint plants both at low N and at sufficient N treatments. For B73 it was observed that plants grown in LN+HA had a higher total N compared to LN+LA on 30 DAE (Figure 3D). However, no difference in total N content was observed in sufficient N treatments with added NH_4^+. On 30 DAE Gaspe Flint plants had a lower total N content in their shoots than B73 at both N levels (Figure 3C & D). The net N uptake showed no difference with added NH_4^+ in both low and sufficient N treatments in Gaspe on 17 and 30 DAE (Figure 3E). However, B73 plants grown in LN+HA had higher net uptake than LN+LA and no difference was seen between SN+LA and SN+HA (Figure 3F). The C: N data indicates that plants grown in LN+LA had higher carbon in their shoots compared LN+HA in both Gaspe Flint and B73 (Figure 3G & H). However, in B73 the lowest C: N was measured in plants grown in SN+HA.

In the second experiment B73 plants grown in NO_3^- and NH_4^+ (LN+HA) had higher tissue N concentration and total N in shoots (Figure 4A & B) than plants grown only in NO_3^- (LN+0A). The net uptake relative to root dry matter content also showed an increase in

LN+HA compared to LN+0A (Figure 4C). However, no difference in C: N was observed between treatments (Figure 4D).

Nitrate and ammonium influx

In order to better understand plant growth response, high-affinity uptake capacity was measured for plants in experiment 1 on 23 DAE using ^{15}N labelled 100 µM NH_4^+ or NO_3^- solutions. In Gaspe Flint it was observed that NO_3^- flux capacity decreased with increasing N concentration (Figure 5A). The lowest NO_3^- flux capacity was seen in plants grown in SN+HA. The NH_4^+ flux capacity in Gaspe Flint also responded to N concentration, showing a twofold higher NH_4^+ uptake capacity at low N irrespective of the NH_4^+ supply (Figure 5A). However, in B73 plants the NO_3^- flux capacity was lower only for plants grown in SN+HA (Figure 5B). In comparison, B73 NH_4^+ flux capacity decreased as N content in the medium was increased and a further reduction in NH_4^+ uptake capacity was observed with higher NH_4^+ in SN+HA. The NH_4^+ flux capacity of B73 plants was greater than that of Gaspe Flint plants and NO_3^- uptake capacity was higher for Gaspe Flint.

Macro and Micronutrient uptake

Macro and micro nutrient contents of maize shoots were measured under various NH_4^+ and NO_3^- treatments in experiment 1. No differences in macronutrient contents were observed in Gaspe Flint with addition of NH_4^+ in LN+HA but in SN+HA potassium (K) concentration decreased in both Gaspe Flint (Figure 6A) and B73 plants (Figure 6b). Sulphur (S) and phosphorus (P) content in shoot tissues of B73 plants that were grown in LN+HA were higher than that in LN+LA (Fig 6B). On the other hand, increasing NH_4^+ content from 0.05 mM to 0.25 mM appears to have enhanced the uptake of most micronutrients in both lines at low NO_3^- levels except manganese (Mn) (Figure 6C & D). Of all the micronutrients, iron (Fe) uptake showed the greatest increase (75 mg/kg) for B73 plants in LN+HA compared to LN+LA (Figure 6C & D).

In experiment 2 we observed that plants grown in a mixture of $NH_4^+$and NO_3^- had higher concentrations of P and S compared to plants grown only in NO_3^- (Figure 7A). However, a decrease in Ca concentration was observed in these plants. All micronutrient except Mn and Zn concentration were increased when plants were grown in a mixture of NO_3^- and NH_4^+ (Figure 7B).

DISCUSSION

Results obtained in the first experiment indicate that for B73, shoot growth increased with small amounts of added NH_4^+, but this was not observed in Gaspe Flint (Figure 1B). This difference in response suggests that maize lines vary in their response to small amounts of NH_4^+. This experiment provided some extra N from the added NH_4^+ and this may have contributed to the increase in plant biomass in the case of B73. However, increase in plant biomass of B73 plants in LN+HA compared to the same N concentration in the form of NO_3^- alone in LN+0A (Experiment 2) suggested that increase in plant growth observed in B73 plants in LN+HA in Experiment 1 was not due to increase in total N (Figure 2A & B), but specifically the added NH_4^+.

A number of hypotheses have been put forward to explain the increase in plant biomass with a mixture of NO_3^- and NH_4^+. One is that assimilation of NH_4^+ requires less energy than NO_3^- assimilation (Bloom et al. 1992). When plants are simultaneously supplied with NH_4^+ the N requirement of plant is partially met by NH_4^+ that gets assimilated in the roots. Therefore, less NO_3^- is required to be assimilated in the shoots which again conserve more energy. This extra energy could be available for increased shoot growth.

Another important factor that determines growth is the C metabolism in plants. It is well documented that N assimilation is very closely related to C metabolism because C skeletons produced by photosynthesis are used for amino acid synthesis during N assimilation (Kaiser and Förster 1989; Pace et al. 1990). This theory is supported by the low C: N ratio of plants in LN+HA compared to LN+LA.

Another possible reason for increased plant growth may be that N uptake increases with the addition of NH_4^+. This may be due to higher absorption capacity and assimilation of NH_4^+ in the roots by NH_4^+ assimilating enzymes glutamine synthetase (GS1) and glutamate synthase (GOGAT). This would result in faster incorporation of N into organic form which may have facilitated the increase in total N content of B73 plants in both experiments and a corresponding increase in plant growth with added NH_4^+. Glass et al (2002) found that 50% of total N in tomato plants was from NH_4^+ even though it was only 10% of the total N in the growth medium. Moreover, assimilation of NH_4^+ in roots may have resulted in rapid translocation of root fixation products, mainly amino acids, to shoots resulting in better shoot growth which was shown in earlier studies on maize (Cramer et al. 1993). This agrees with studies in sorghum (Lewis et al. 1982)and in hydroponically grown maize (Alexander et al. 1991; Gentry 1992) where higher concentrations of NH_4^+ in growth solution increased the total N uptake.

Tissue N concentration in Gaspe Flint plants grown at low N with 0.05 mM NH_4^+ was higher than for B73 plants grown in the same treatment (Figure 2A & B) indicating that even at low concentrations of N Gaspe Flint plants can take more N. This is supported by higher net uptake in Gaspe Flint plants compared to B73. In this treatment, tissue N concentration for B73 plants was low enough to suggest N deficiency (Reuter et al. 1997).

The above effect can be further substantiated by higher NH_4^+ uptake capacity of B73 plants compared to its NO_3^- uptake capacity as seen in figure 3A and 3B. In plants two main uptake systems exists for NO_3^- and NH_4^+ in plants. They are the saturable high affinity transport system (HATS) and non- saturable low affinity transport system (LATS). In our study the HATS uptake capacity of both NH_4^+ and NO_3^- were measured as HATS uptake plays a major role in the uptake of N in maize (Garnett et al. 2013). Our finding that NH_4^+ flux capacity was higher than NO_3^- flux capacity in both inbred lines agrees with previous work (Glass et al. 2002; Hole et al. 1990; Lee and Rudge 1986; Teyker et al. 1988). Other studies also showed that even when concentration of NO_3^- is ten times more than NH_4^+, plants have the tendency to absorb NH_4^+ more rapidly than NO_3^- (Gessler et al. 1998).

Our results showed that NO_3^- flux capacities of Gaspe Flint plants decreased as N levels in the growth solution increased indicating that Gaspe Flint plants alter their uptake capacities based on total N supply. This result is expected as a number of studies show that nutrient deprivation augments the transport capacity of the deficient ion (Clarkson et al. 1983; Cogliatti and Clarkson 1983; Lefebvre and Glass 1982). This also matches well with the result obtained in Gaspe Flint life cycle experiment where it was observed that plant NO_3^- uptake capacity was regulated by the demand and supply of NO_3^- (Garnett et al. 2013). All these reports indicate that the response is specific to the deficient nutrient (Lee 1982). Given this it is unusual that Lee & Rudge (1986) found that deprivation of NO_3^- augments the transport system for NH_4^+. Our results also support this theory as we see a higher NH_4^+ uptake capacity for plants that were grown in low NO_3^-. As evidenced by higher measured uptake capacity for NO_3^- and NH_4^+, especially NO_3^-, Gaspe Flint plants appear to be better at capturing N. On the other hand, in B73, NO_3^- uptake capacity was lower only for plants in SN+HA and showed no response to N in the medium but showed a response to NH_4^+ present in the sufficient N treatment. However, NH_4^+ uptake capacity of B73 plants decreased as N in the nutrient medium was increased. This suggests that NH_4^+ uptake capacity in Gaspe Flint is dependent on NO_3^- in the medium whereas in B73, NH_4^+ uptake capacity decreased as N in the medium increased.

Gentry and co-workers proposed that the increased growth in wheat cultivar Inbar with equimolar concentrations of NH_4^+ and NO_3^- was due to increased N and K uptake compared to cultivar Len (Gentry et al. 1989). However, our results show that when plants were grown in LN+HA (in which the proportion of NO_3^- and NH_4^+ was 2:1) both P and S concentrations in B73 plants were increased, but not in Gaspe Flint. The enhanced P uptake in plants supplied with NH_4^+ may be due to acidification at the root surface caused by absorption of NH_4^+. It is known that acidification of nutrient medium enhances uptake of micronutrients and also P in plants (Miller et al. 1970). We propose that better nutrient absorption in B73 plants may be a positive factor in better plant growth compared to Gaspe Flint. Micronutrient concentrations in this study also show a similar trend in both inbred lines where Fe, Cu, Mo. and Zn increased with increasing NH_4^+ at low and high N levels, and similar results were found in beans (Thomson et al. 1993). Even though pH of the nutrient solution was maintained at 5.9 we cannot rule out the effect of changed apoplastic pH affecting uptake of these micronutrients since acidification increases availability of micronutrients (Sarkar and Wyn Jones 1982).

Previous studies have observed that plants grow better with a mixture of NO_3^- and NH_4^+ rather than solely NO_3^- as the N source. Our study looked at the contribution of a small amount of NH_4^+ when supplied with low N and sufficient N levels and found that adding NH_4^+ with NO_3^- at low N levels improved growth in B73 with a corresponding increase in the total N content and also higher uptake of some essential nutrients. Previous studies have also shown varied responses to mixed nutrition of NO_3^- and NH_4^+ between different cultivars or inbred lines of the same plant species (Feil 1994; Gentry et al. 1989; Heberer and Below 1989). The different responses seen between lines in this study are in agreement with the earlier reports on two wheat cultivars Inbar and Len where only the former showed an increase in tillering when grown in mixed N (Gentry et al. 1989; Wang and Below 1992).

That variation exists in response of plants to addition of small amounts of NH_4^+ to NO_3^- medium is interesting and further study on various maize inbred lines will give a thorough understanding on the reasons for this variation. Our results are consistent with NH_4^+ stimulating growth being because of less energy requirement or improved macro and micronutrient nutrition improving growth. Furthers studies are being carried out to understand more fully the reasons for this growth stimulation.

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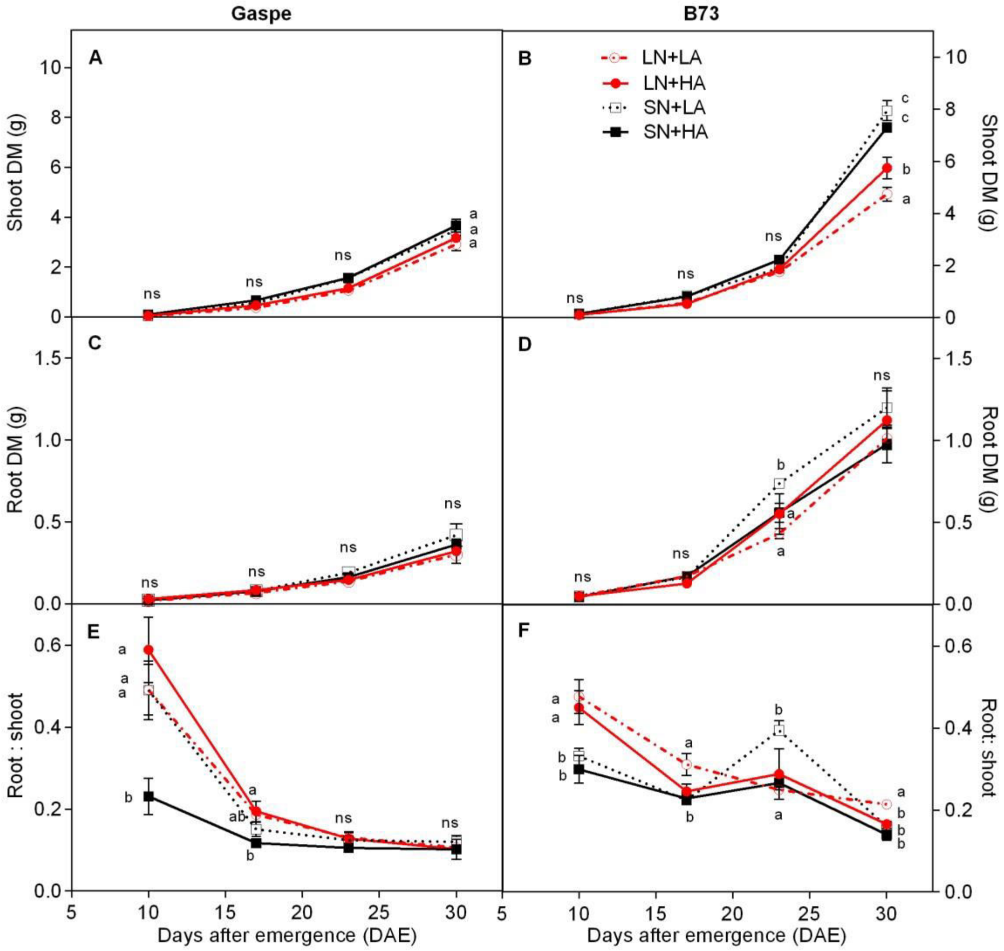


Figure 1: Shoot dry matter, root dry matter and root to shoot ratio of maize inbred lines Gaspe Flint (A, C &E) and B 73 (B, D & F) grown in 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+LA), 0.5 mM NO_3^- + 0.25 mM NH_4^+,(LN+HA) 2.5 mM NO_3^- + 0.05 mM NH_4^+ (SN+LA) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+HA). Values are mean ± SEM (n=6). Statistical analysis used a two way analysis of variance. Significant differences at P value <0.05 are represented by different letters for each day of harvest.

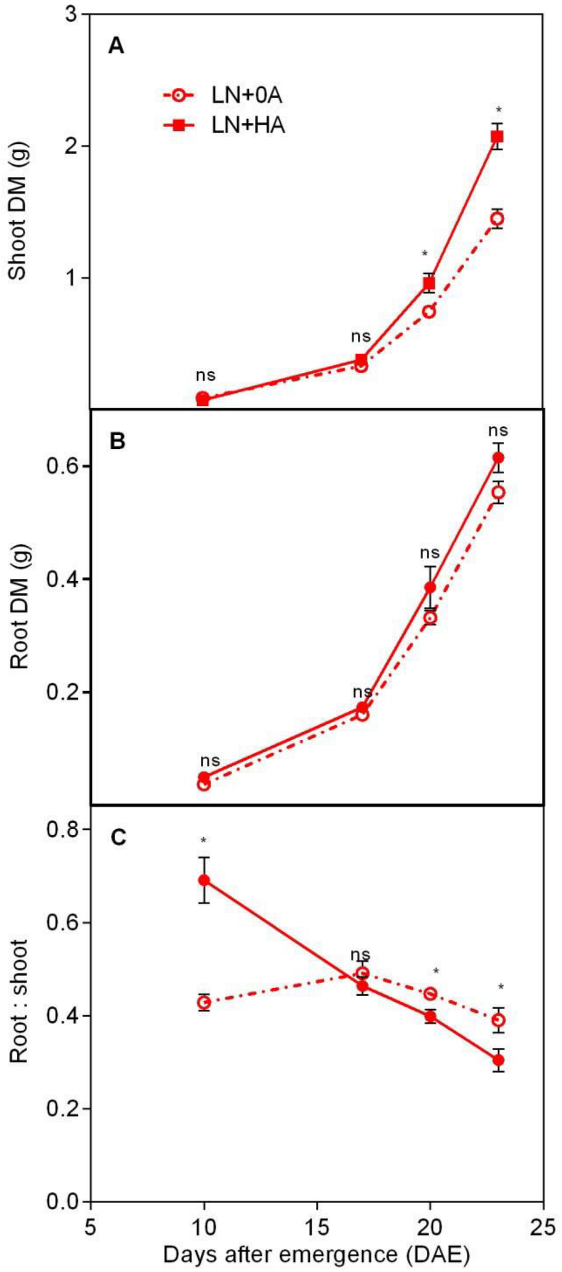


Figure 2: Shoot dry matter (A), root dry matter (B) and root to shoot ratio (C) of maize inbred line B73 grown in 0.75 mM NO_3^- (LN+0A) & 0.5 mM NO_3^- + 0.25 µM NH_4^+ (LN+HA). Values are mean ± SEM (n=8). Statistical analysis used a two way analysis of variance. The symbol * represents significances between treatments on each day of harvest ($P<0.05$).

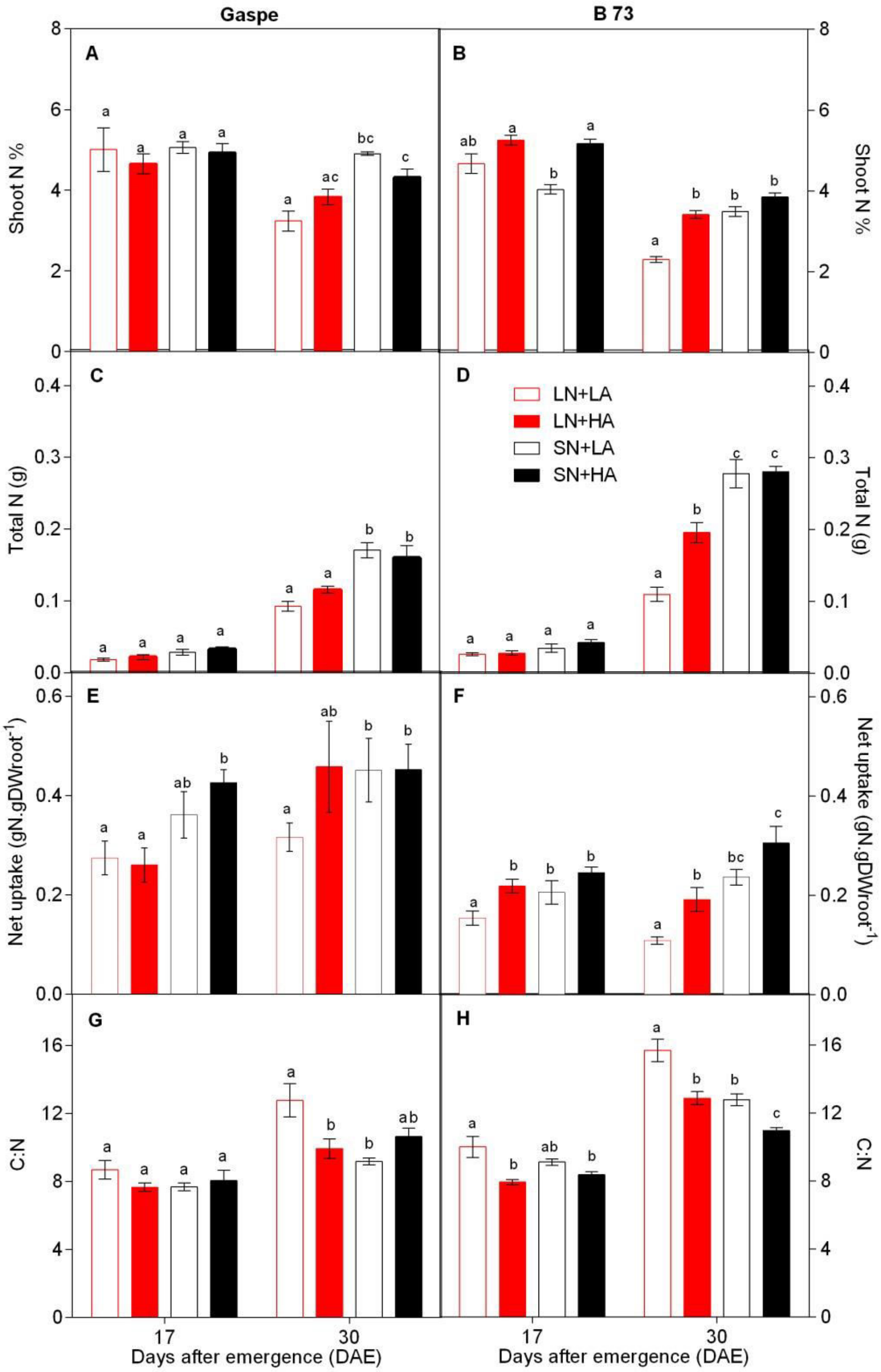


Figure 3: Shoot N%, total N content, net uptake and C:N ratio of the maize inbred lines Gaspe flint (A, C, E & G) and B 73 (B, D, F & H) grown in 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+LA), 0.5 mM NO_3^- + 0.25 mM NH_4^+, (LN+HA) 2.5 mM NO_3^- + 0.05 mM NH_4^+ (SN+LA) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+HA). Values are mean ± SEM (n=6). Statistical analysis used a two way analysis of variance. Significant differences at P value <0.05 are represented by different letters for each group of bars.

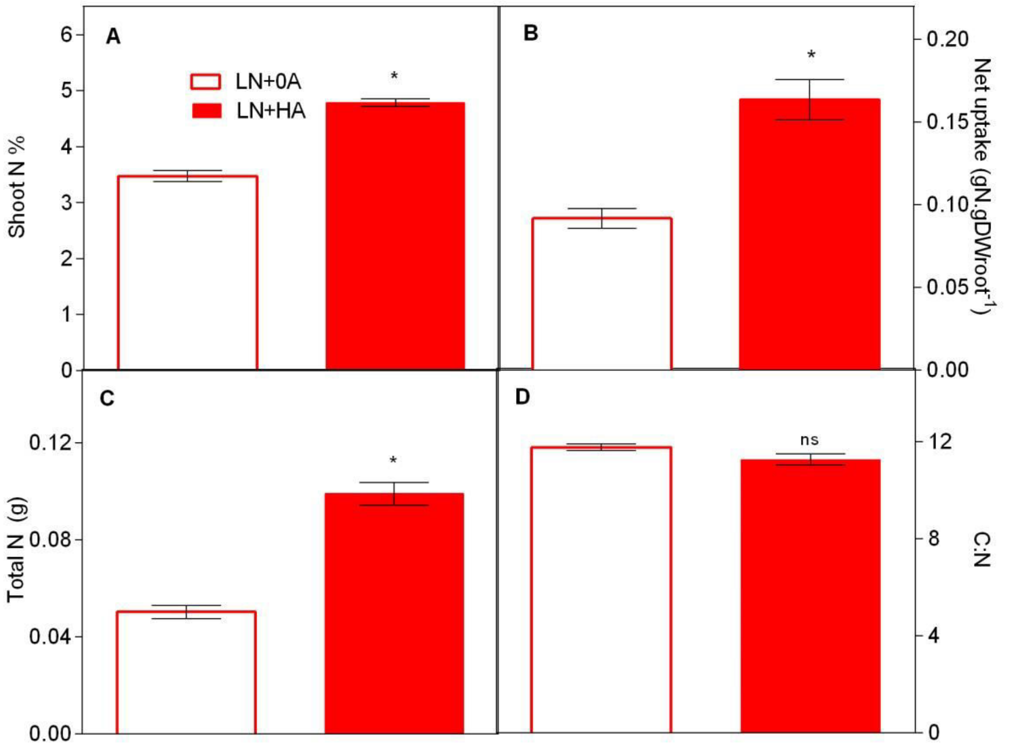


Figure 4: Shoot N% (A) Net uptake (B) total N (C) and C:N (D) of the maize inbred line B73 grown in 0.75 mM NO_3^- (LN+0A) & 0.5 mM NO_3^- + 0.25 µM NH_4^+ (LN+HA) on 23 DAE. Values are mean ± SEM (n=8). Statistics analysis used a paired t test. The symbol* represents significances between treatments ($P<0.05$).

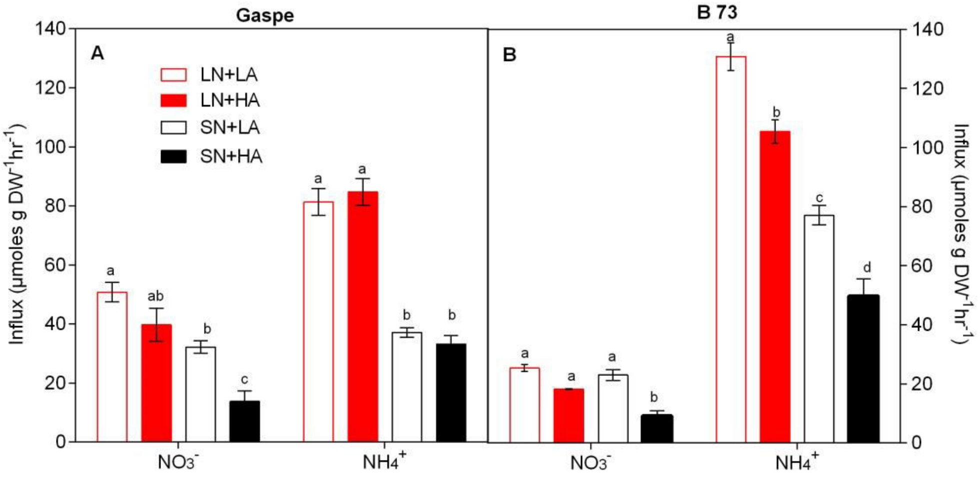


Figure 5: Ammonium and nitrate flux capacities measured at 100 µM ^{15}N concentration in maize inbred lines Gaspe Flint (A) and B 73 (B) grown in 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+LA), 0.5 mM NO_3^- + 0.25 mM NH_4^+, (LN+HA) 2.5 mM NO_3^- + 0.05 mM NH_4^+ (SN+LA) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+HA) on 23 DAE. Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Significant differences at P value <0.05 are represented by different letters for each group of bars.

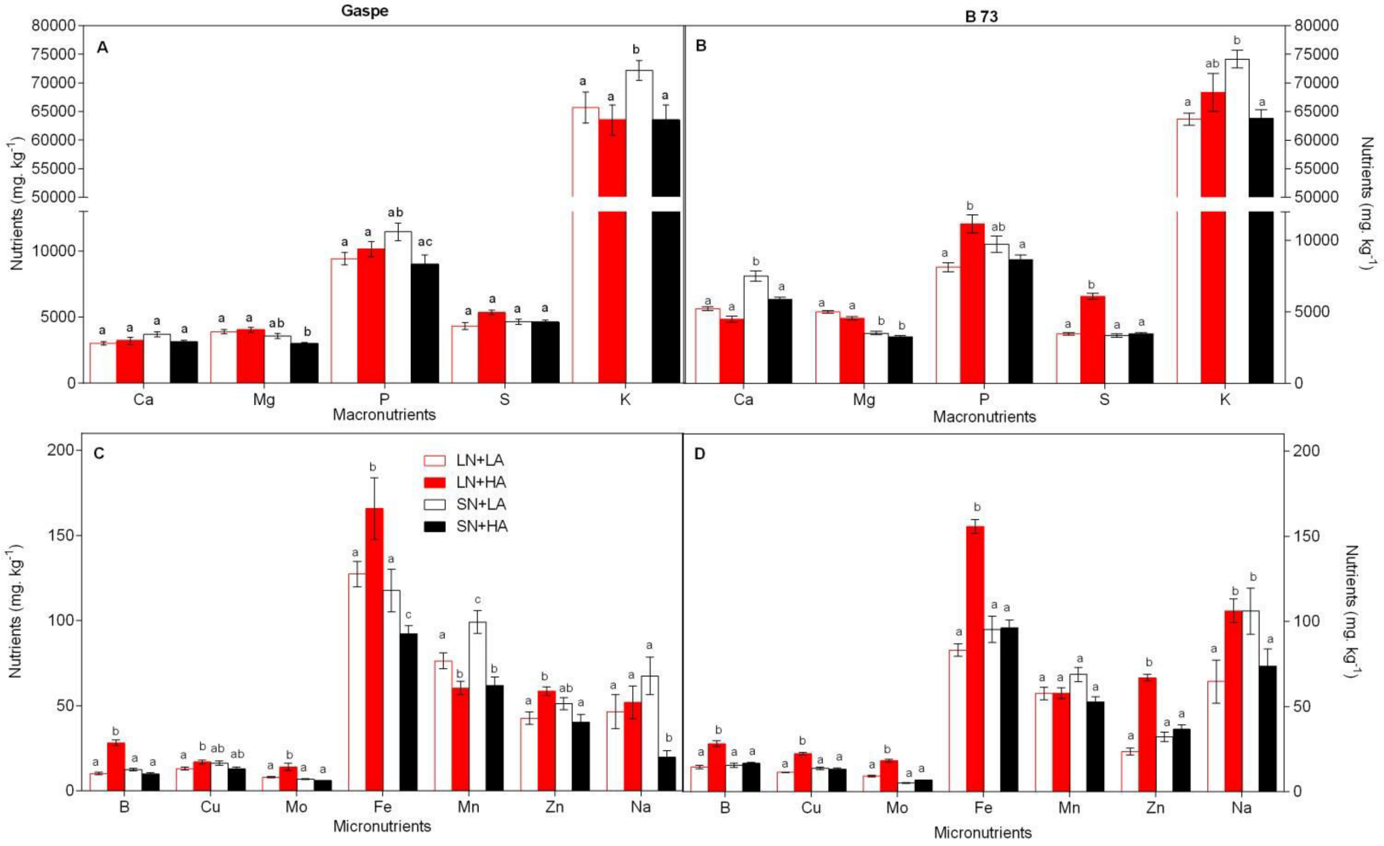


Figure 6: Macronutrient and micronutrient concentration in the shoots of maize inbred lines Gaspe Flint (A & C) and B 73 (B & D) grown in 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+LA), 0.5 mM NO_3^- + 0.25 mM NH_4^+, (LN+HA) 2.5 mM NO_3^- + 0.05 mM NH_4^+ (SN+LA) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+HA) on 30 DAE. Values are mean ± SEM (n=6). Statistical analysis used a two way analysis of variance. Significant differences at P value <0.05 are represented by different letters for each group of bars.

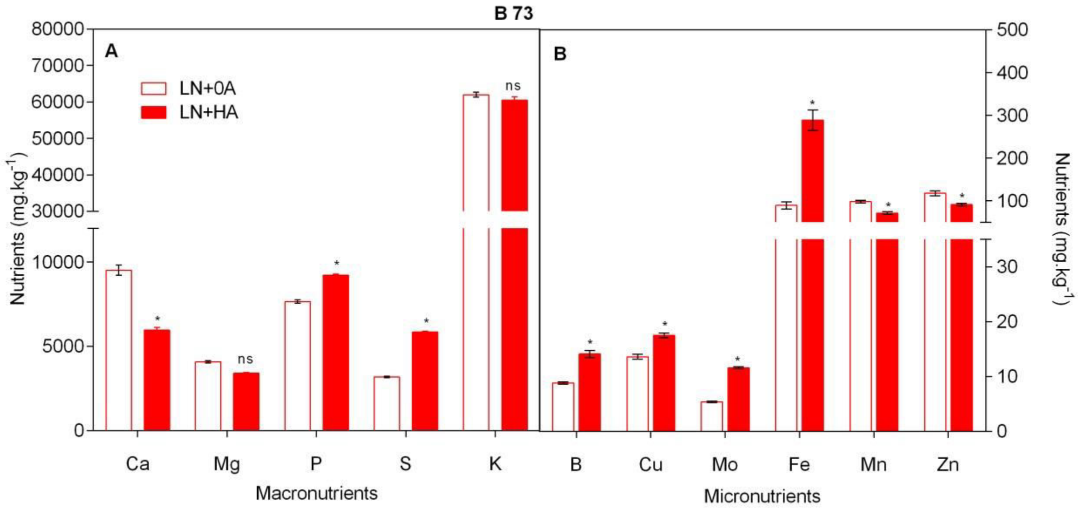
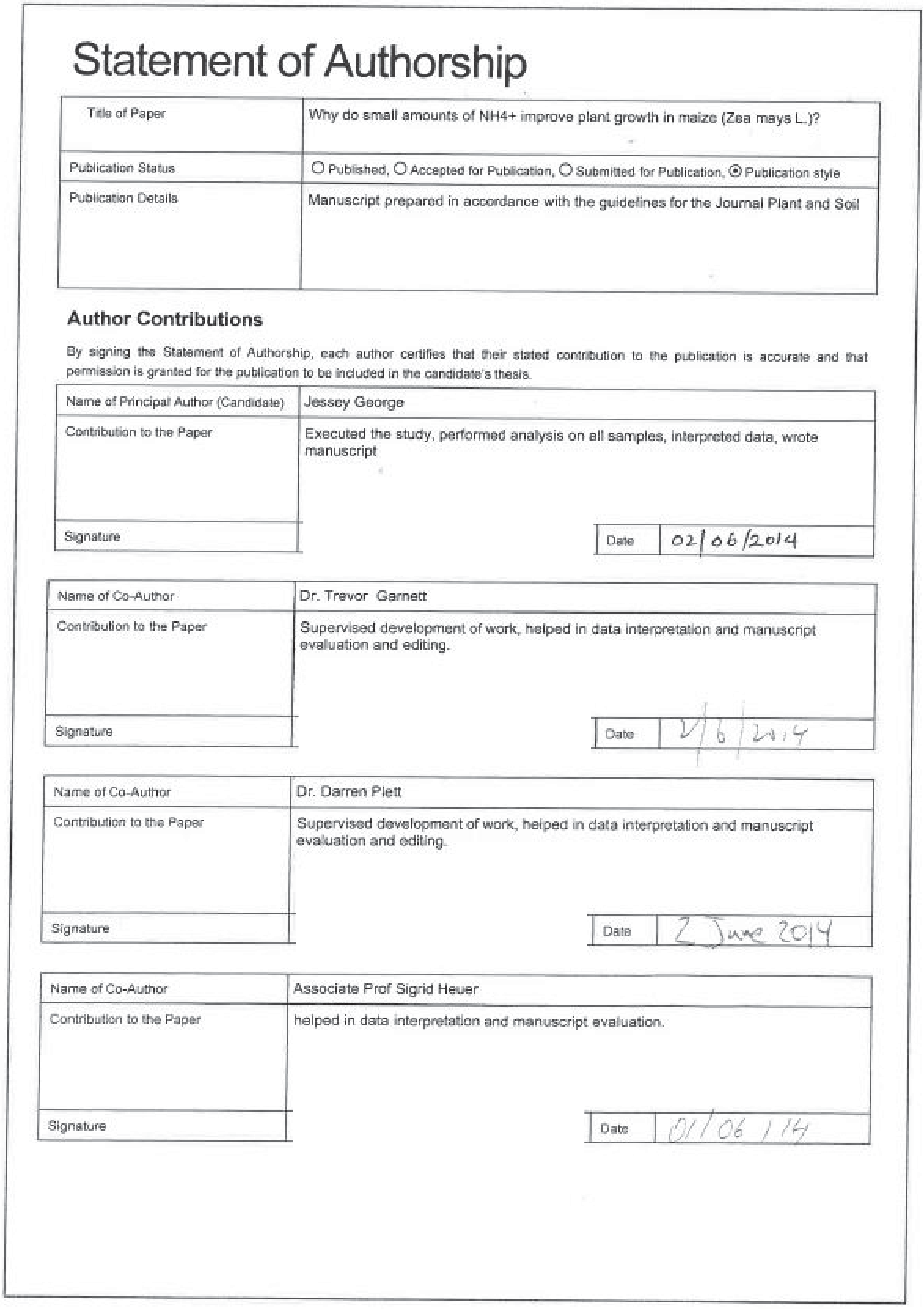


Figure 7: Macronutrient (A) and micronutrient (B) concentration in the shoots of maize inbred line B 73 grown in 0.75 mM NO_3^- (LN+0A) & 0.5 mM NO_3^- + 0.25 µM NH_4^+ (LN+HA) on 23 DAE Values are mean ± SEM (n=8). Statistical analysis used a two way analysis of variances. The symbol * represents significances between treatments within each group of bars (P<0.05).

Chapter 3: Why do small amounts of ammonium (NH_4^+) increase plant growth in maize (Zea mays L.)?



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Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Dr. Trevor Garnett		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation and editing.		
Signature		Date	2/6/2014

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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation and editing.		
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Contribution to the Paper	Helped in data interpretation and manuscript evaluation.		
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Contribution to the Paper	Amino acid, organic acid and sugar analysis		
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Name of Co-Author	Luke Holtham		
Contribution to the Paper	Helped during experiment and critical comments on results		
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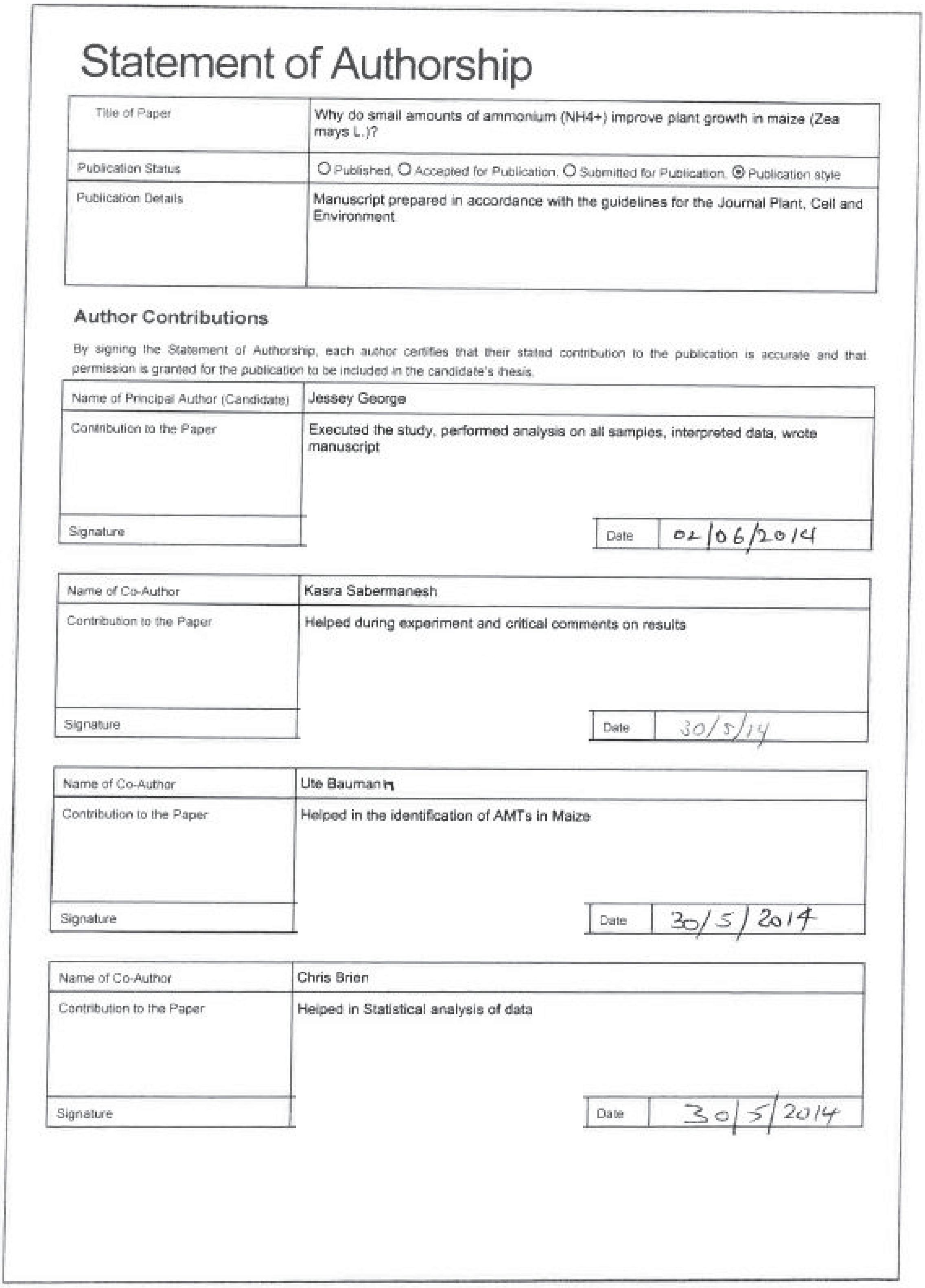

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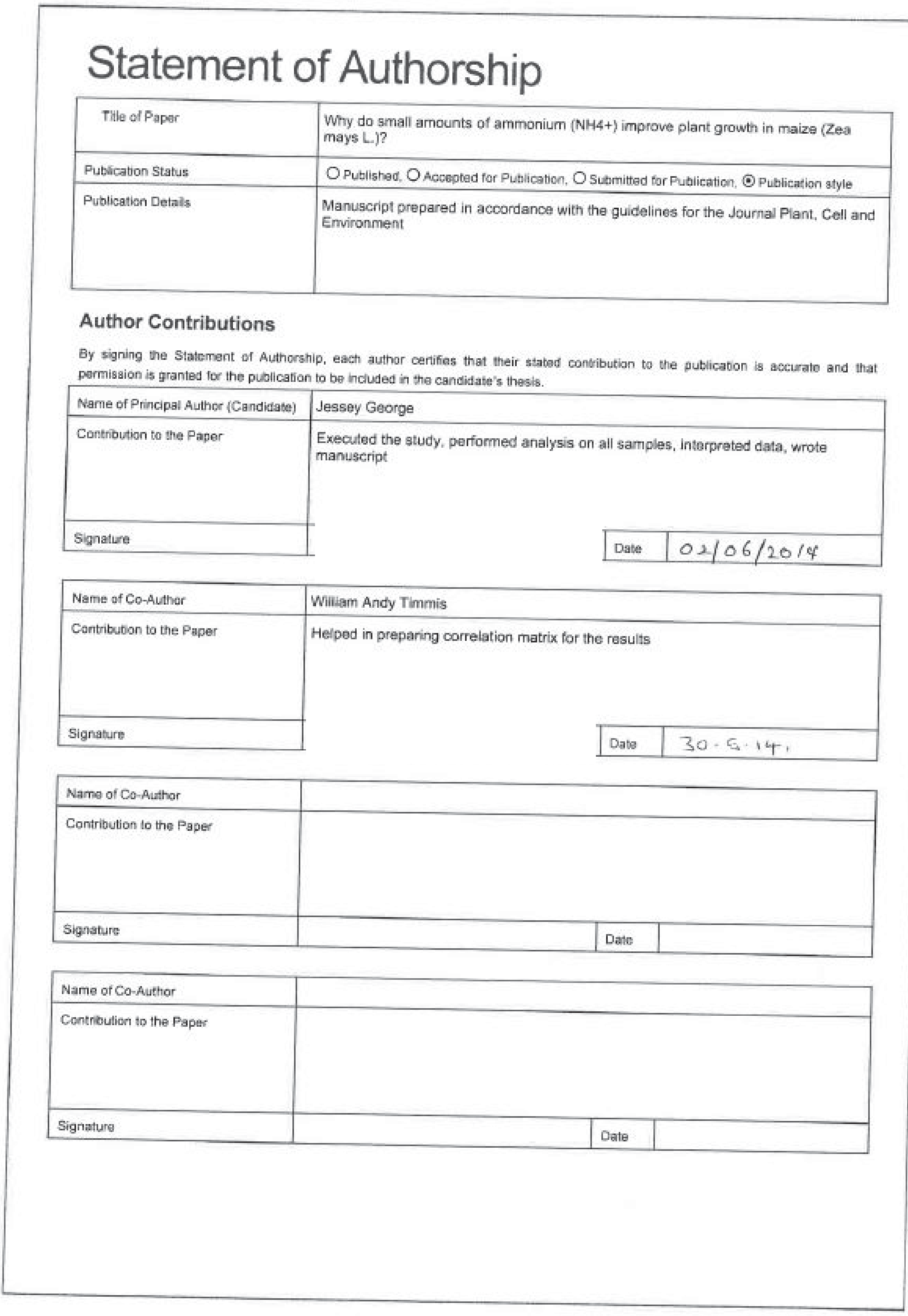
Contribution to the Paper	Helped during experiment and critical comments on results
Signature	Date 30/5/14

Name of Co-Author	Ute Baumann
Contribution to the Paper	Helped in the identification of AMTs in Maize
Signature	Date 30/5/2014

Name of Co-Author	Chris Brien
Contribution to the Paper	Helped in Statistical analysis of data
Signature	Date 30/5/2014



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Name of Principal Author (Candidate)	Jessey George		
Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	William Andy Timmis		
Contribution to the Paper	Helped in preparing correlation matrix for the results		
Signature		Date	30-5-14.

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Title

Why do small amounts of ammonium (NH_4^+) improve plant growth in maize (*Zea mays* L.)?

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ABSTRACT

Nitrate (NO_3^-) and ammonium (NH_4^+) are predominant forms of nitrogen (N) available to plants in agricultural soils. NO_3^- concentrations are generally 10 times that of NH_4^+ and this ratio is consistent across a wide range of soil types. With soil solution concentrations of NH_4^+ being so much lower than NO_3^-, the contribution of NH_4^+ to the overall N budget of crop plants is often overlooked. The objective of this study was to quantify the importance of very small amounts of NH_4^+ in maize growth. Experiments were carried out using maize inbred line B73 grown hydroponically at reduced (0.55mM) and sufficient (2.75mM) levels of NO_3^- with and without substitution of 10% of the NO_3^- with NH_4^+. Small amounts of NH_4^+ did improve growth under sufficient N and this coincided with an increase in total N uptake, total free amino acids in the roots and sugars in the youngest emerged blade. A negative correlation between total amino acid concentration and NO_3^- uptake capacity was observed, supporting a role for amino acid concentration in the roots acting as a signal for regulation of NO_3^- uptake in plants. These results suggest a small amount of NH_4^+ (10%) plays an important role in stimulating maize growth and leads to major changes in N uptake and assimilation processes.

Key Words: nitrate, ammonium, nitrogen uptake, nitrogen assimilation, amino acid, organic acid

INTRODUCTION

Nitrogen (N) is the major mineral nutrient taken up by plants in large quantities. Plant growth and crop yields are dramatically affected by its limitation (Hirel, Le Gouis, Ney & Gallais, 2007, Xu, Fan & Miller, 2012). Nitrate (NO_3^-) and ammonium (NH_4^+) are the predominant forms of N available to plants in agricultural soils (Glass, Britto, Kaiser, Kinghorn, Kronzucker, Kumar, Okamoto, Rawat, Siddiqi, Unkles & Vidmar, 2002). Although NO_3^- is the most dominant form of N that is present in most agricultural soils, there is always small amount of N present in the soils as NH_4^+ (Wolt, 1994). NO_3^- concentrations are generally 10 times that of NH_4^+ and this ratio is consistent in the pool of N available to plants in soil solution (Miller & Hawkins, 2007, von Wirén, Gazzarrini, Gojont & Frommer, 2000). Plants have the ability to absorb both these forms efficiently from the soil solutions depending on their availability.

Studies in the past have demonstrated that a combination of NO_3^- and NH_4^+ increases growth in most plants compared to NO_3^- alone (Below & Gentry, 1987, Cox & Reisenauer, 1973, Schrader, Domska, Jung & Peterson, 1972, Warncke & Barber, 1973). In one study Maize plants obtained greater growth when the proportion of NO_3^- and NH_4^+ was 50/50 in comparison to either form alone (Schrader *et al.*, 1972). Another study showed that maximum yield was obtained in maize when NH_4^+ in the solution was 75% of total N (Barker & Bradfield, 1963). Yet in another study, the beneficial effect on plant growth was achieved only when provided with equal concentrations of NO_3^- and NH_4^+, and growth decreased when the proportion of NH_4^+ increased beyond this (Schortemeyer & Feil, 1996). Despite these studies of the effects of NH_4^+ on plant growth there have been limited studies determining the effects of small amounts of NH_4^+, amounts similar to that present in agricultural soils.

There are a number of reasons that a combination of NO_3^- and NH_4^+ can increase plant growth. Firstly, NH_4^+ is a reduced form of N, and its assimilation is energetically cheaper, consuming only two ATP molecules compared to 12 for NO_3^- assimilation (Bloom, Sukrapanna & Warner, 1992). Assimilation of this cheaper form of N conserves sugars leaves more carbon available for shoot growth. Increased N uptake in plants may be another cause of the enhanced plant growth. In rice the presence of NO_3^- in growth medium along with NH_4^+ increased uptake of NH_4^+ and resulted in higher N content (Kronzucker, Siddiqi, Glass & Kirk, 1999) compared to plants that were grown in identical concentration of NO_3^- alone. Therefore, the higher uptake capacity and assimilation of readily available NH_4^+ may be a positive factor in increasing the plant growth in maize. Experiments on barley revealed that, compared to NO_3^- alone, provision of NO_3^- and NH_4^+ simultaneously increased expression of GS genes which facilitate the assimilation of N in plants (Lopes & Araus, 2008). Studies have shown that plants fed with NH_4^+ or a combination of NO_3^- and NH_4^+ also had higher concentration of total free amino acids than solely NO_3^- fed plants (Allen & Smith, 1986, Causin & Barneix, 1993). The abundance of free amino acids in these plants may therefore act as an indication of high N status of plants (Cooper & Clarkson, 1989, Coruzzi & Bush, 2001, Lee & Rudge, 1986). While plant N nutritional status act as a regulator of NO_3^- and NH_4^+ uptake in plants (Xu, Tsai & Tsai, 1992).

Increase in N uptake with both NO_3^- and NH_4^+ may be reflected in changes to the NO_3^- and NH_4^+ transport systems. Studies have demonstrated that for most plants there exists at least two different uptake systems for NO_3^- and NH_4^+ (Crawford & Glass, 1998). They are a saturable high affinity transport system (HATS) that operates for low concentration of NO_3^- and NH_4^+ in the medium and a non- saturable low affinity transport system (LATS) when their concentration is high in the nutrient medium. Nitrate uptake is predominantly mediated by a group of transporters called NO_3^- transporters (NRTs) and

NH_4^+ uptake by NH_4^+ transporters (AMTs). There are two families of NO_3^- transporters in higher plants namely *NRT1* renamed as the *NPF* family (Léran, Varala, Boyer, Chiurazzi, Crawford, Daniel-Vedele, David, Dickstein, Fernandez & Forde, 2014) and NRT2 (Glass, Brito, Kaiser, Kronzucker, Kumar, Okamoto, Rawat, Siddiqi, Silim & Vidmar, 2001). NRT1 family of transporters are low affinity NO_3^- transporters with the exception of NRT1.1 (CHL1) which is a dual affinity transporter that can act as high affinity transporter when phosphorylated (Liu, Huang & Tsay, 1999). In maize, a total of 17 *NRT* genes (HATS and LATS) have been identified (Plett, Toubia, Garnett, Tester, Kaiser & Baumann, 2010). Two families in *AMT* have been revealed by phylogenetic studies on plants, namely *AMT1* and AMT2 in sorghum (Koegel, Ait Lahmidi, Arnould, Chatagnier, Walder, Ineichen, Boller, Wipf, Wiemken & Courty, 2013). All these transporters are HATs and no LATs have been identified yet. Functional characterisation was carried out on *ZmAMT1.1A* and *ZmAMT1.3* in maize (Gu, Duan, An, Zhang, von Wirén & Yuan, 2013), and they are considered to be major components in high affinity NH_4^+ transport system in maize roots.

Simultaneous absorption of both N forms may have a beneficial effect on intracellular pH. It is known that NO_3^- assimilation produces OH^- ions which increases intracellular pH whereas assimilation of NH_4^+ releases H^+ ions which decreases the intracellular pH (Raven & Smith, 1976). It may be that the positive growth effects of a small amount of NH_4^+ is through ameliorating negative effects of increased internal pH resulting from assimilation of NO_3^- as the sole nitrogen source. The simultaneous supply of both N forms to plants may help maintain the cation-anion balance in plants leading to a beneficial effect of small amounts of NH_4^+ when supplied with NO_3^-. It has been observed that in plants grown with NH_4^+ as the sole N source there is a depletion of inorganic cations inside the plants that may reduce plant growth (Britto & Kronzucker, 2005, Cox & Reisenauer, 1973). On the other hand, an increase in phosphorus (P) uptake has been reported in plants grown with NH_4^+ (Riley & Barber, 1971, Zeng, Liu, Kinoshita, Zhang, Zhu, Shen & Xu, 2012). Molybdenum (Mo) content increased in tomato plants grown in some NH_4^+ than that in NO_3^- alone (Smart & Bloom, 1993) Thus the supply of both NO_3^- and NH_4^+ simultaneously may help plants in acquiring a balance of anions and cations.

Preliminary studies in our laboratory suggested that even small amounts of NH_4^+ have substantial effects on plant growth. The present study aims to substantiate this observation and attempt to explain how this effect comes about. In order to test the above hypotheses we grew the plants hydroponically under two levels of N low NO_3^- (0.55 mM) and sufficient NO_3^- (2.75 mM) with and without the substitution of 10% of the NO_3^- with NH_4^+. We looked at the effect of 10% NH_4^+ on NO_3^- and NH_4^+ uptake systems of plants by measuring uptake capacity using ^{15}N labelled NO_3^- and NH_4^+ and by measuring the transcript abundance of several major NO_3^- and NH_4^+ transporter genes. Measurements were also taken of the N content, C: N ratio, amino acids, organics acids and sugar content in these plant to try to discover how 10% NH_4^+ along with NO_3^- may contribute to increased growth.

MATERIALS & METHODS

Plant material and growth conditions

The inbred maize line B73 was grown in a hydroponic growth solution containing two total N concentrations: low (0.55 mM) and sufficient N (2.75 mM). Plants were grown in four treatments namely 0.55 mM NO_3^- (LN), 0.5 mM NO_3^- with 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- with 0.25 mM NH_4^+ (SN+A). The seeds were aerated overnight in water and then placed on a filter paper moistened with 0.5 mM $CaCl_2$ solution and germinated in an incubator at 28°C. Germinated seedlings were transplanted to one of eight 120 L ebb and flow hydroponic system with fill and drain cycles of 15 min in a climate controlled growth chamber providing a day/night temperature of 26/22°C and a photoperiod of 14 h. Photon uptake density in the growth chamber was approximately 550 µmol m^{-2} s^{-2} at average plant height. Plants were grown on mesh collars in tubes as explained in Garnett et al. (2013). Nutrient solution used was Johnson's modified nutrient solution which contained (in mM) 1.8 K, 0.6 Ca, 0.5 Mg, 1 S, and 0.5 P. Both treatment solutions contained (in µM) 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 200 Fe (as FeEDTA and FeEDDHA) (Johnson, Stout, Broyer & Carlton, 1957). Iron was supplemented twice weekly with the addition of $FeSO_4$ (8 mg l^{-1}). $(NH_4)_2SO_4$ was used as NH_4^+ supplement to the NH_4^+ treatments. Solution pH was monitored daily and maintained between 5.9 and 6.0. NO_3^- and NH_4^+ concentrations in the solutions were monitored using NO_3^- and NH_4^+ electrodes (TPS, Springwood, Australia) and maintained at the target concentration ± 5%. Nutrient solutions were changed weekly and temperature in growth solution was maintained at 22°C using a refrigerated chiller.

Plant harvests, root traits & chemical analysis

Plants were harvested 16, 24, 29 and 36 days after emergence (DAE). Fresh samples for all the assays and RNA extraction were harvested into liquid N between 11am and 1pm on the harvesting day and stored at -80°C. Roots and shoots were separated and fresh weights recorded. Plant parts were dried at 40°C for 7 days to obtain the dry weights and the dry matter was ground to a fine powder. The shoot tissue was analysed for tissue N% using a mass spectrometer (Sercon, Cheshire, UK). Shoot macro- and micro-nutrient content was determined using inductively coupled plasma optical emission spectrometry (ICP-OES. ARL 3580B, ARL, Lausanne, Switzerland). Plants from each treatment were also collected for root morphological analysis using Win-Rhizo. Pro root image analysis software (V.2005b, Regent Instruments, Quebec, Canada).

Uptake measurement

The unidirectional fluxes of NO_3^- and NH_4^+ into roots were measured on all four harvest days using ^{15}N labelled NO_3^- and NH_4^+. The flux capacities of both NO_3^- and NH_4^+ were measured at 100 µM and 1000 µM. On the day of sampling plants were transferred to a solution identical to the uptake solution but with ^{14}N NO_3^- or ^{14}N NH_4^+ for 5 min. Plants were then exposed to solution containing either 100 µM or 1000 µM labelled with 10% enriched ^{15}N for 10 min. Roots were then rinsed in ^{14}N solution for 2 min to remove ^{15}N from the root surface and apoplast. The flux timing of 10 min was chosen to minimize any efflux or transport to shoots based on study by Kronzucker *et al.* (1995). Roots were then blotted, separated and the biomass measured. Plant parts were dried at 40°C for 7 d. After measuring the dry weights roots were ground to a fine powder and total N and ^{15}N in plant samples were determined with an isotope ratio mass spectrometer (Sercon, Cheshire, UK). Unidirectional NO_3^- and NH_4^+ influx capacities were calculated based on ^{15}N content of the root.

Glutamine synthetase and NO_3^- reductase activity assay

Fresh root and leaf samples were homogenised in a mortar and pestle in liquid N and stored at -80°C. Glutamine synthetase was assayed using a biosynthetic reaction by the quantification γ- glutamyl hydroxamate (GHA) formed during the reaction with glutamine (O'Neal & Joy, 1973)). NO_3^- reductase activity was measured in freshly ground root and youngest expabded blade samples that were stored in -80°C freezer Long & Oaks (1990).

Amino acids and organic acid determination

Amino acids, organic acids and sugars were measured on ground root and youngest fully emerged blade (YEB) samples stored at -80°C. Approximately 100 mg of ground samples were measured and freeze dried. Tissue amino acid concentration was determined using liquid chromatography electrospray ionization-mass spectrometry, as described by Boughton et al.(2011), once the samples had been derivatized following the method of Cohen & Michaud (1993). Organic acids and sugars in the freeze dried samples were determined using gas chromatography-mass spectrometry as described in Roessner et al. (2001).

NO_3^- and NH_4^+ in the tissues

NO_3^- was extracted from 20 mg of the fresh root and shoot tissues in 1 mL deionized water at 95-100°C in a water bath for 20 min. Nitrate concentration in the extract was determined calorimetrically after scaling down the reagents for 20 mg samples as described in Cataldo et al (1975).

Approximately 100 mg of fresh tissue was homogenized in a mortar and pestle using 1.2 mL of 10 mM formic acid. Ammonium was determined in the supernatant according to the OPA method described in Szczerba *et al.* (2008).

Realtime-QPCR

RNA from the root and YEB tissues were extracted using EZ-10 Spin column total RNA mini preps super kit (Biobasic, Ontario, Canada) according to the manufacturer's instructions. RNA integrity was measured on 1.8% agarose gel before cDNA synthesis using 1 µg total RNA with oligo (dT) using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Q-PCR was carried out on synthesised cDNA according to the method described in Burton *et al.* (2008). In this method the amount of each amplicon in each cDNA was quantified with respect to a standard curve of expected amplicon. Four control genes (*ZmGapDh*, *ZmActin*, *ZmTubulin* and *ZmElF1*) were utilized for the calculation of normalising factor. The normalization was carried out as detailed in Vandesompele *et al.* (2002) and Burton *et al.* (2004). Q-PCR primers for all *NRT* genes were taken from Garnett et al. (2013) and *AMT* primers were designed from the closest homologues of sorghum AMT genes (Koegel *et al.*, 2013). Q-PCR products were verified by sequencing, agarose gel electrophoresis and melt curve analysis to confirm that a single product was being amplified. All primer sequences and QPCR product information for the control genes, *NRT* genes and *AMT* genes can be found in Table S1.

Statistical Analysis

Statistical analysis of biomass, total N, flux capacity and amino acid data were analysed using two-way analysis of variance in Graph Pad Prism software (Version 6.00, 1992-2012 GraphPad Software, Inc). Two sided correlation analysis was done using Genstat (GenStat Sixteenth Edition, Version. 16.2.011713, VSN International Ltd.).

RESULTS

10 % NH_4^+ increased shoot dry matter and total N uptake of maize plants supplied with sufficient N

Maize plants were grown in low NO_3^- (LN), low NO_3^- with 10% NH_4^+ (LN+A), sufficient NO_3^- (SN) and sufficient NO_3^- with 10% NH_4^+ (SN+A). Plants supplied with 10% NH_4^+ at sufficient N (SN+A) accumulated more shoot dry matter over the growing period than plants grown only with NO_3^- (SN) (Figure 1A), but no difference in shoot dry matter was observed with NH_4^+ in the low N treatments. Roots of plants grown in low N were smaller compared to sufficient N but did not change with the addition of 10 % NH_4^+ (Figure 1B). Initially the root: shoot of plants in all treatments was the same (Figure 1C). However, on 24 DAE the root: shoot ratios of plants in both the sufficient N treatments were lower compared to the low N treatments, the lowest being SN+A, and this was maintained in later harvests. Reductions in root morphology measurements (root length, surface area, volume and diameter) were observed for LN+A (Supporting information Figure S1).

At the first harvest, 16 DAE, shoot N concentrations in plants grown at sufficient N (SN & SN+A) were higher compared to low N treatments (LN & LN+A) (Figure 2A). Although the N concentration in shoot tissues in all treatments decreased over time, a higher N concentration was still observed in plants that were grown in SN+A compared to SN on 36 DAE. Similarly, total N uptake in the shoots of plants grown in SN+A was higher on 36 DAE (Figure 2B). No difference in the C: N ratio was observed between plants in any treatments (Figure 2C). When the net N uptake relative to root dry matter was calculated a similar increase to that of total N uptake was observed for plants in SN+A on 36 DAE (Figure 2D). There was a striking drop in the net uptake relative to root size from 24 DAE in both the sufficient N treatments.

High affinity NO_3^- uptake capacity and transcript levels of high affinity NO_3^- transporters were repressed in SN+A

Because no increase in shoot dry matter and total N content was observed with 10% NH_4^+ in the low N treatments the remainder of the chapter will focus on the sufficient N treatments. Low N treatment results are shown in the supplementary data. NO_3^- uptake capacity, at 100 µM (HATS) was variable over time and, for both treatments, showed a peak at day 24 and drop at day 29 (Figure 3A & B). On day 29 no difference in uptake capacity was seen between treatments. A smaller HATS NO_3^- uptake capacity was observed in SN+A compared to SN (Figure 3A) and, likewise, smaller NO_3^- uptake capacity was observed in LN+A plants compared to LN plants (Supporting information Figure S2). LATS NO_3^- uptake capacity was obtained by subtracting HATS uptake capacity from LATS + HATS uptake capacity at 1000 µM external concentration (Supporting information Figure S3A &

B). Nitrate uptake capacity in LATS range showed similar patterns to HATS but the reduction in LATS NO_3^- uptake capacity in SN+A plants was not as prominent as in HATS capacity (Figure 3B). In all treatments NH_4^+ uptake capacities were greater than NO_3^- uptake capacities (Figure 3). Although NH_4^+ uptake capacity at HATS did not show a temporal variation like NO_3^- uptake capacity, NH_4^+ uptake capacity at HATS were greater for plants in SN compared to SN+A (Figure 3B). NH_4^+ uptake capacities at LATS also show no difference between treatments but showed a large decrease on 29 DAE (Figure 3D).

10% NH_4^+ in the sufficient N treatment decreased transcript levels of all *ZmNRT2* (Fig 4A, B &C). Transcript levels of *ZmNRT2.1* and *2.2* were lower for plants in LN+A compared to LN on 16 and 36 DAE (Supporting information Figure S5). *ZmNRT2.5* transcript levels remained lower throughout the growing period for plants in SN+A and show a peak on 29 DAE in SN plants. On 16 DAE no difference in the transcript levels of *ZmNRT3.1* was observed between treatments but transcript levels of this gene showed an increase at the last harvest and SN levels were higher at this point. (Figure 4D). No effect of NH_4^+ was observed in the transcript levels of *ZmNRT1* family members except on 36 DAE where a decrease was observed for *ZmNRT1.1A* and *ZmNRT1.1B* transcript levels for plants grown in SN+A compared to SN (Supporting information Figure S4A & B). On 36 DAE the transcript levels of *ZmNRT1.5A* were higher in both low N treatments compared to sufficient N treatments (Supporting information Figure S4C).

The transcript levels of *ZmAMT1.1A* was higher for the SN treated plants compared to SN+A on all days except at 24 DAE (Figure 5A). *ZmAMT1.1B* and *ZmAMT1.3* (Figure 5B & C)) show a similar trend in their transcript levels although only the transcript levels of *ZmAMT1.1B* are lower with NH_4^+ present (Figure 5B). A drop in the transcript levels were observed on 24 DAE for all the *ZmAMT1*s except *ZmAMT3.1* for plants in LN and LN+A and after which their transcript levels increased (Supporting information Figure S6). Compared to *ZmAMT1*, all the *ZmAMT2* (Figure 5D, E & F) were expressed at a much lower level. Out of the three *ZmAMT2*s only *ZmAMT3.2* showed a similar expression pattern to *ZmAMT1*s (Supporting information Figure S6E). It can be seen that on 36 DAE the transcript levels of all *ZmAMT* were lower in SN+A than for plants grown in SN except for *ZmAMT3.1* which showed no difference across plant growth (Figure 5D).

The root GS activity and total free amino acids in the roots were increased in plants grown with 10% NH_4^+

A similar general trend in YEB GS activity was observed for both treatments but on 36 DAE an increase in the GS activity was observed for plants grown without NH_4^+ (Figure 6A). A higher root GS activity was seen for plants in SN+A compared to SN on 24 and 36 DAE (Figure 6B). Similar results were also observed for plants in LN compared to LN+A on 16 and 36 DAE (Supporting information Figure S7B). The YEB NR activity was highest on 16 DAE (Figure 6C) and showed a dramatic drop in subsequent measurements. At one time point YEB NR activity was higher without NH_4^+ and there was an increase in root NR activity observed for plants grown in SN+A on 29 and 36 DAE (Figure 6D).

The total free amino acid concentration was highest in the roots and YEB of SN+A plants (Figure 7). In the roots, a peak in total free amino acid was seen on 16 and 29 DAE for plants grown in SN+A and in the YEB it was observed on 29 DAE compared to SN (Figure 7A). A higher concentration of free amino acids in roots of plants in SN+A was observed on all days except on 36 DAE (Figure 7B). However, in low N treatments no difference in total amino acid concentration was observed between treatments (Supporting information Figure S8). Glutamine and asparagine content showed a similar pattern to total amino acids in the roots (Figure 8A & E). The concentrations of each of these four amino acids were higher in roots of plants that were grown in SN+A but not in the shoots except for glutamine on 29 DAE. Unlike glutamine and asparagine (Figure 8A & E) glutamate and aspartate (Fig 8C & F) concentration in the roots did not show temporal variation. The profiles of most other amino acids showed a similar concentration pattern as the total amino acids (Supporting information Figure S9).

10% NH_4^+ increased the root organic acid and shoot soluble sugars in plants

The fold change in the major organic acids like 2-oxo-gluterate, pyruvate, malate and citrate in SN+A relative to SN are depicted in figure 9. It can be seen that, organic acid 2-oxoglutaric acid and pyruvate showed a several fold increase in the roots of plants in SN+A on 24 DAE (Figure A & C). On the other hand, citrate and malate showed a similar concentration pattern in the roots but no difference was observed between treatments (Figure 9E & G). However, in the shoots no differences in any of the organic acids were observed (Figure 9B, D, F & H). The fold changes of other minor organic acids and fatty acids relative to their measurement on 16 DAE for LN treatments are represented in supplementary information figure S10.

It can be observed from figure 10 that the sugars namely glucose, fructose and trehalose in the roots of plants in SN+A was lower compared to SN on 16 and 24 DAE and increases on 29 and 36 DAE (Figure 10A, C & E). On the other hand, in the YEB of these plants sugars were higher on 16 and 24 DAE and reduced by 29 and 36 DAE (figure 10B, D & F). We observed a 4 fold increase in glucose and 2 fold increase in fructose content in the YEB of plants in SN+A plants compared to SN on 16 DAE (Figure 10B & D), and no differences were observed on 36 DAE between treatments. Similarly, trehalose (Figure 10C) was also higher in SN+A on 16 DAE and decreased during the subsequent harvesting days. However, sucrose content in the roots (Figure 10G) showed no difference between treatments but in the YEB it was higher on 29 DAE for plants in SN+A (Figure 10H). More sugars and sugar phosphates were measured (Supplementary information Figure S11).

A higher accumulation of root NO_3^- was observed on 24 DAE in the LN and SN plants compared to plants in LN+A and SN+A (Figure S11B). On the other hand, replacing 10% of NO_3^- with NH_4^+ did not result in increased accumulation of NH_4^+ in any of the tissues (Supporting information Figure S11C & D).

The effects of NH_4^+ on macro and micronutrients were not dramatic. There was an increase in S concentration of shoot tissues for plants grown in LN+A and SN+A compared to LN and SN respectively (Supporting information Figure S12A). However, a reduction in K concentration was observed for plants grown in SN+A. In the micro nutrient data we observed that boron (B) and molybdenum (Mo) concentrations were higher for plants in SN+A compared to SN (Supporting information Figure S12B).

Comparison of correlation between amino acids, flux capacities and transporters in the plants grown in SN and SN+A

Correlations between all measured parameters are presented in Figure 11. A positive correlation exists between all amino acids in SN and SN+A, but cysteine shows a weak negative correlation with all amino acids in SN+A. A strong negative correlation between flux capacities and all amino acids in the roots of plants grown in SN+A was observed, but not in SN. Similarly, NO_3^- flux capacities and HATS NH_4^+ flux capacities were positively correlated with *NRT2* transcript levels in SN+A, but not in SN. *AMT1*s in SN+A are positively correlated and in SN it is negatively correlated. Shoot and root N%, shoot GS and NR activities and net N uptake relative to root dry matter show strong positive correlations with amino acids in SNA but not in SN however root: shoot is positively correlated with all amino acids in both SN and SN+A. Again strong negative correlations exist between roots and shoot N% and GS and NR activities in the shoot.

DISCUSSION

Conservation of energy may have contributed to better plant growth with 10% NH_4^+ at sufficient N

Plants grown with sufficient N but added NH_4^+ were able to accumulate more shoot dry matter. These results are in line with the findings that plant growth is improved with a combination of NO_3^- and NH_4^+ rather than either source alone (Haynes & Goh, 1978, Roosta & Schjoerring, 2007, Schrader *et al.*, 1972). Further to this, our results show that NH_4^+ as low as 10% of total N in nutrient solution can make a substantial difference in plant growth. One of the main reasons for increased plant growth with added NH_4^+ may be the lower energy requirement for uptake and assimilation of NH_4^+. Nitrate assimilation requires 12 ATP molecules compared to 2 ATP for NH_4^+ assimilation (Bloom, 1997). A portion of N requirement is met by NH_4^+ requiring the plant to assimilate less NO_3^-. This matches well with the studies that have shown that when plants are supplied with sufficient nutrients, especially N, the shoot retains carbohydrates, which stimulates shoot dry matter accumulation and decreases the root: shoot (Ericsson, 1995). This is true in our experiment where we see a higher root: shoot ratio of plants grown in low N (Figure 1c).

Plants in SN+A had higher glucose and fructose compared to plants in SN indicating the influence of NH_4^+ on sugar concentration. This supports the suggestion by Coruzzi and Bush (2001) that uptake of N is essentially linked to plant's overall C status and photosynthetic activity. Better carbon metabolism in the leaves is supported by concentration of sugars in YEB of these plants. Earlier studies have also shown that limited supply of N reduces sugar content in pot grown *Nicotiana plumbaginifoliaco* compared to plants grown in hydroponics with ample supply of N (Ferrario-Méry, Thibaud, Betsche, Valadier & Foyer, 1997). With soybean, it has been shown that when nutrient medium contained either a 3: 1 ratio of NO_3^- and NH_4^+ or NH_4^+ as sole source, the total free soluble sugars were higher compared to NO_3^- alone (Chaillou, Vessey, Morot-Gaudry, Raper, Henry & Boutin, 1991).

NH_4^+ increases the uptake and assimilation of N in plants

Another reason for increased growth of plants in SN+A may be that the provision of small amounts of NH_4^+ along with NO_3^- increases uptake of N and hence the N nutritional status of plants. With 10% NH_4^+ we observed an increase in shoot N concentration by 36 DAE and a subsequent increase in total nitrogen uptake in shoots (Fig 3B). This has to be put in the context of tissue N levels, which for SN plants suggests that they do have sufficient N. However, when the medium contains small amount of NH_4^+, plants have more N in their tissues due to the preferential uptake of NH_4^+ compared to NO_3^-. Moreover, we see a higher concentration of amino acids in these plants, which is generally an indication of higher N nutritional status of the plants (Cooper & Clarkson, 1989).

Ammonium uptake capacity of plants was consistently higher than NO_3^- uptake capacity. When tobacco when plants was grown in 1mM NH_4NO_3, there was slower NO_3^- uptake but this was balanced by faster uptake of NH_4^+ (Matt, Geiger, Walch-Liu, Engels, Krapp & Stitt, 2001). This may be because when both NO_3^- and NH_4^+ are present in the nutrient solution, plants have the tendency to absorb NH_4^+ faster than NO_3^-. In a study with tomato it was observed that with a 10% NH_4^+: 90% NO_3^- nutrient solution, NH_4^+ contributed 50% of the total N uptake by plants (Glass *et al.*, 2002). As soon as NH_4^+ enters the roots it is converted to glutamine by GS and then to glutamate by glutamate synthase (GOGAT) (Bernard & Habash, 2009). Glutamine and glutamate are the precursors of other amino acids and nitrogenous compounds (Miflin & Lea, 1977). This suggests faster incorporation of N into the metabolism in 10% NH_4^+ treated plants grown in sufficient NO_3^-. In poplar (Man, Boriel, El-Khatib & Kirby, 2005) and tobacco (Fuentes, Allen, Ortiz-Lopez & Hernández, 2001) it was found that GS activity is one of the key components in the regulation of plant productivity. Our GS activity results suggest that NH_4^+ assimilation is faster in plants grown in SN+A compared to SN. Higher NO_3^- reductase activity in the roots of plants in SN+A suggests that NH_4^+ has also increased NO_3^- assimilation in the roots of these plants. A higher concentration of glutamine and glutamate in the roots of SN+A treated plants is consistent with an increased rate of N assimilation.

It is known that NH_4^+ used as sole source of N decreases essential organic acids such as 2-oxo-gluterate, malate, pyruvate and citrate levels, affecting the internal pH status of the plants and also affecting N assimilation and amino acid synthesis (Goodchild & Givan, 1990, Hoffmann, Milde, Desel, Hümpel, Kaiser, Hammes, Piippo, Soitamo, Aro & Gerendás, 2007). Reduction of NO_3^- releases 1 mole OH^- equivalent per mole of NO_3^-. The pH homeostasis inside the cell is achieved by the production of organic acids such as malate. On the other hand when NH_4^+ is also co-supplied with NO_3^- the release of H^+ ions during the uptake and assimilation of NH_4^+ maintains pH homeostasis in plants thus decreasing production of organic acids (Raven & Smith, 1976). However in our study we found that the supply of very small amounts of NH_4^+ did not decrease organic acid levels instead led to a significant increase in the root concentration of important organic acids such as 2-oxo-gluterate and pyruvate, which are essential for the synthesis of amino acids from NH_4^+ (Figure 10).

Another growth effect of NH_4^+ on plant nutrition can be a depletion of inorganic cations. Britto and Kronzucker argued that uptake of NH_4^+ may inhibit the uptake of other cations such as potassium (K) and calcium (Ca) (Britto & Kronzucker, 2005). However, cation depletion depends on the growth condition and plant species (Lang & Kaiser, 1994, Roosta & Schjoerring, 2007). In our results K^+ decreased with 10% NH_4^+ at sufficient N. However, this decrease did not drop K levels to anywhere near that associated with K deficiency (Reuter, Robinson & Dutkiewicz, 1997). On the other hand, S in the plants was increased in NH_4^+ treated plants. Overall, it would appear that cation-anion balance was not a cause of the growth effects observed in plants with added NH_4^+.

Great temporal variations exists in the NO_3^- uptake capacity and amino acid concentration in the plants

Nitrate uptake capacity showed great temporal variation during the growth period. Nitrate uptake capacities did not differ between treatments on 29 DAE (Figure 3A). However, measurements over time revealed important treatment differences such as decrease in NO_3^- uptake capacity for plants grown in SN+A on all other days of harvest, and increase in uptake capacity on 24 & 36 DAE. Variations in NO_3^- uptake capacity are thought to reflect changing N demand of plants as previously described in maize (Garnett *et al.*, 2013). Few studies have examined NO_3^- uptake capacity across the lifecycle other than the study on maize (Garnett *et al.*, 2013) and one on oilseed rape (Malagoli, Lainé, Le Deunff, Rossato, Ney & Ourry, 2004). This temporal variability has to be taken into consideration when uptake studies on NO_3^- and N metabolism are performed.

The increase in NO_3^- uptake capacity on 24 DAE corresponded to a reduction in total free amino acids in roots and shoots in both sufficient N treatments (Figure 3A, B and Figure 8A). Similar results can also be seen with glutamine and asparagine concentrations and NO_3^- uptake capacities suggesting a role of these amino acids in regulation of NO_3^- uptake in plants. It was proposed that there is a pool of amino acid that circulates between roots and shoots depending on the N status of plants which may act as a signal for NO_3^- uptake regulation (Cooper & Clarkson, 1989, Muller & Touraine, 1992). External application of amino acids can increase internal amino acid concentration and result in decreased NO_3^- uptake capacity (Breteler & Arnozis, 1985). However, compared to glutamine and asparagine, concentrations of glutamate and aspartate were less responsive in roots of plants grown in SN+A. It has been suggested that as long as NH_4^+ assimilation is actively taking place in plants, glutamate content in plant tissue is stable (Walker, Givan & Keys, 1984). It has also been suggested that plants maintain glutamate homeostasis because it plays a central role in amino acid metabolism because it is involved in both assimilation and reassimilation of NH_4^+ (Forde & Lea, 2007). Stitt and his co-workers showed that glutamate concentrations remained constant in tobacco leaves irrespective of growth conditions (Stitt, Müller, Matt, Gibon, Carillo, Morcuende, Scheible & Krapp, 2002). Therefore, glutamine and asparagine appear to be more involved in the regulation of NO_3^- uptake than glutamate and aspartate.

Unlike NO_3^- uptake capacity, NH_4^+ HATS uptake capacity did not show any temporal variation based on the amino acid concentration in roots of plants. However, it is to be noted that NH_4^+ uptake capacity was also lower for SN+A plants compared to SN plants indicating the role of plant N content in the regulation of NH_4^+ uptake. The decrease in both NO_3^- and NH_4^+ uptake capacities with 10% NH_4^+ at sufficient N may imply that when maize plants were supplied with small amounts of NH_4^+ the plants are better able to meet N demand.

A negative correlation between NRT 2 transcript levels and amino acids were observed with 10 % NH_4^+ at sufficient N

The transcript levels of *ZmNRT2.1* and *ZmNRT2.2* were positively correlated with NO_3^- uptake capacity in both SN and SN+A. However compared to SN treatment plants, the transcript levels were lower in SN+A and a corresponding reduction in NO_3^- uptake capacity was also observed. Similarly a study in tobacco showed that NO_3^- increased the expression of *NRT2* genes but was repressed when reduced forms of N were supplied to plants (Quesada, Krapp, Trueman, Daniel-Vedele, Fernandez, Forde & Caboche, 1997). However, *ZmNRT2* transcript levels are correlated with NO_3^- uptake capacity, and was associated with changing N demand of the plants (Garnett *et al.*, 2013). Decrease in *ZmNRT2* transcript levels in SN+A could be because the small amount of NH_4^+ increased plant tissue N, thus reducing the demand for N compared to SN. The regulation of *ZmNRT2* transcript levels can be further explained by the model put forward by Krouk *et al.* (2006) where they showed that transcripts are controlled by the feedback regulation through tissue content of N metabolites and repression by high external NO_3^- availability. This is consistent with the total N content in the shoots and the amino acid concentration in the roots of SN+A plants compared to SN plants. Although *ZmNRT3.1* is not directly involved in transport of NO_3^-, they have been shown to be essential for functioning of NRT2.1/2.2 (Yong et al., 2011) which may explain why their transcript levels showed a profile similar to what is seen for *ZmNRT2s*. The positive correlation of *ZmNRT2.1* and *ZmNRT2.2* with uptake capacity of plants suggests that they have a major role in NO_3^- uptake of maize plants. In Gaspe Flint *ZmNRT2.5* transcripts were high only in low N treatments and not in sufficient N (Garnett *et al.*, 2013). However, in our results a higher transcript levels of *ZmNRT2.5* was observed in plants grown in SN compared SN+A indicating that plants in SN need more N compared to SN+A.

Transcript profile of all *ZmAMT1* and *ZmAMT3.2* were similar to *ZmNRT1.1A*. This is consistent with the finding in maize that *ZmAMT1.1* expression increased in plants supplied with NH_4^+ for 24 hours and was down regulated after that (Gu *et al.*, 2013). This is also in agreement with the results obtained in Arabidopsis where *AtAMT1.1* gene was down-regulated as soon as NH_4^+ was resupplied to starved plants (Gazzarrini, Lejay, Gojon, Ninnemann, Frommer & von Wiren, 1999, Lanquar, Loqué, Hörmann, Yuan, Bohner, Engelsberger, Lalonde, Schulze, von Wirén & Frommer, 2009, Rawat, Silim, Kronzucker, Siddiqi & Glass, 1999, Yuan, Loque, Kojima, Rauch, Ishiyama, Inoue, Takahashi & von Wiren, 2007). On the other hand, *OsAMT1.1* was found to be an NH_4^+ responsive gene in rice (Ranathunge, El-kereamy, Gidda, Bi & Rothstein, 2014) since transcript levels increased when grown in NH_4^+. However, our plants were grown in steady state conditions whilst these other studies were primary NO_3^- response studies so it is difficult to compare transcriptional responses of individual genes.

Our results show that even a small amount of NH_4^+ has a major effect on plant metabolism resulting in increased growth. Associated with this increased growth we observed substantial effects on sugar concentration, organic acid concentration and amino acid concentration in response to replacement of 10% NO_3^- with NH_4^+. Many of the metabolites that we measured play key roles in major metabolic pathways such as TCA cycle and glycolysis indicating the complexity and fundamental importance of NH_4^+ on plant growth. This importance is emphasised by transcript analysis showing that high affinity NO_3^- transporters are down regulated by feeding plants even with only 10% of total N. All results reinforce that the small amount of NH_4^+ present in most agricultural soil can play major role in plant growth and various metabolic activities in plants as it is the cheaper source of N. Therefore it appears prudent to include a small amount of NH_4^+ in experimental nutrient solutions and to understand whether the effects on plant growth described here are relevant to field-grown crop plants.

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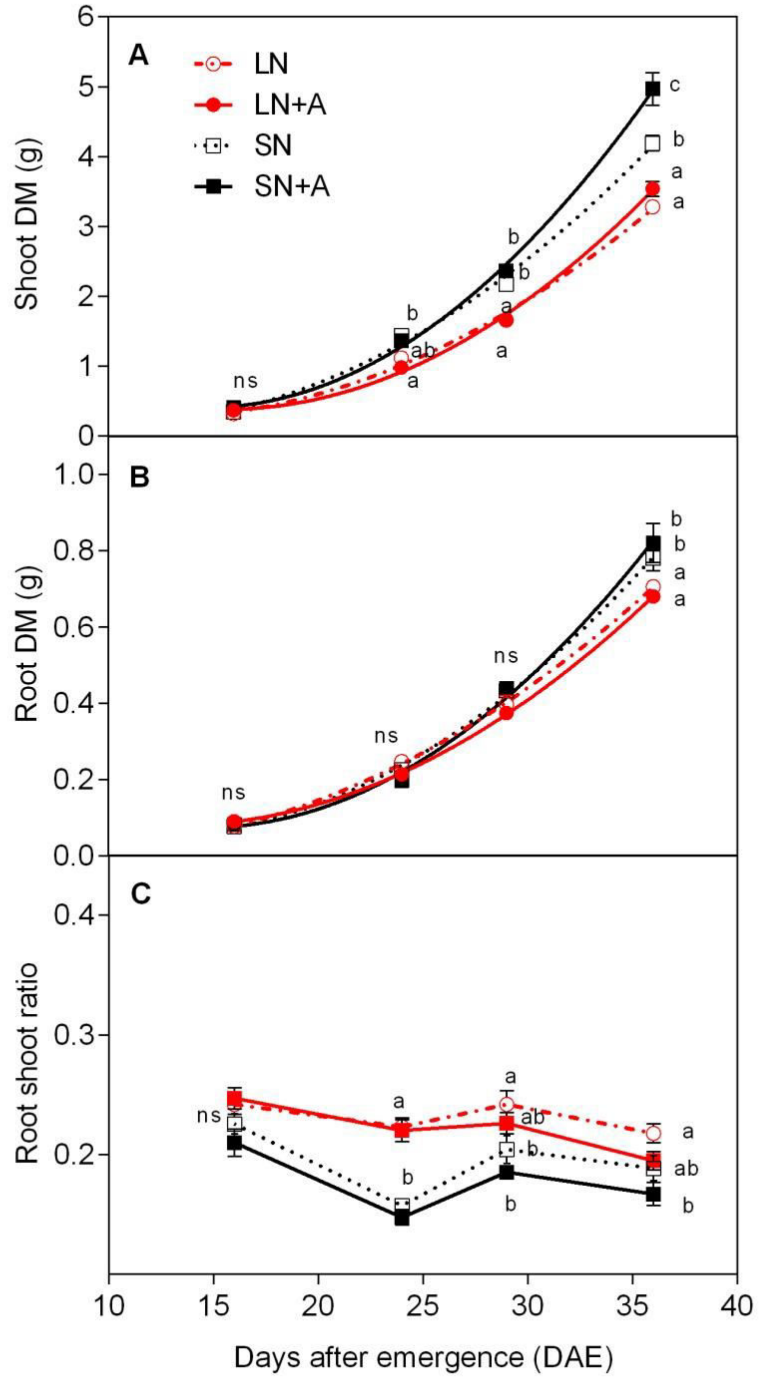


Figure 1: Shoot dry weight (A), root dry weight (B) and root: shoot (C) of maize inbred line B73 plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=16). Statistical analysis used a two way analysis of variance. Different letters represents significances at $P<0.05$ on each day of harvest.

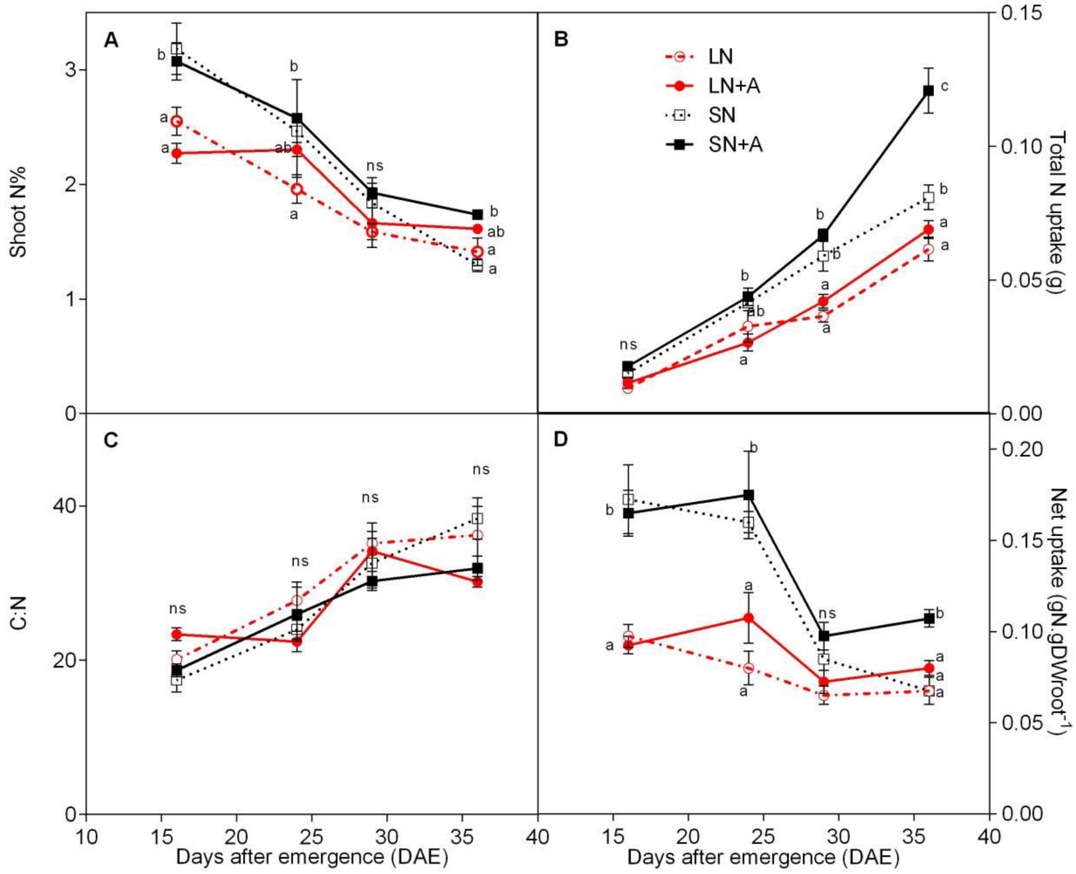


Figure 2: Shoot N% (A), total N uptake in the shoots (B), C:N ratio (C) and net N uptake (D) of maize inbred line B73 plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Different letters represents significances at $P<0.05$ on each day of harvest.

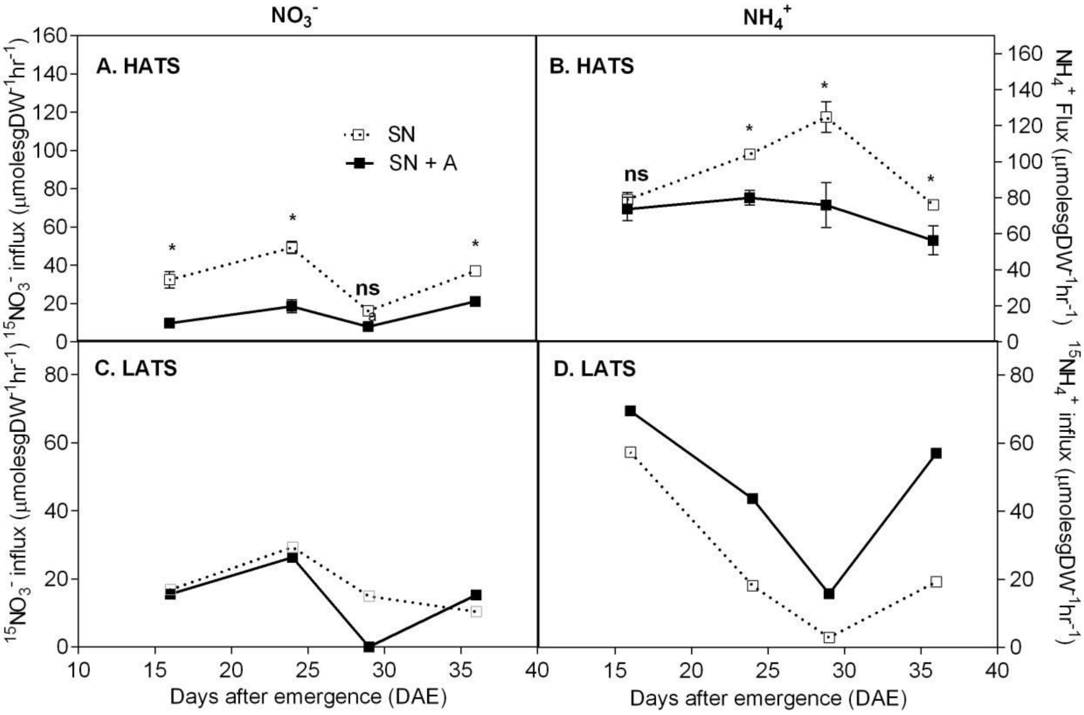


Figure 3: Nitrate and ammonium flux capacities of maize inbred line B73 plants grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents the significances at P <0.05 on each day of harvest.

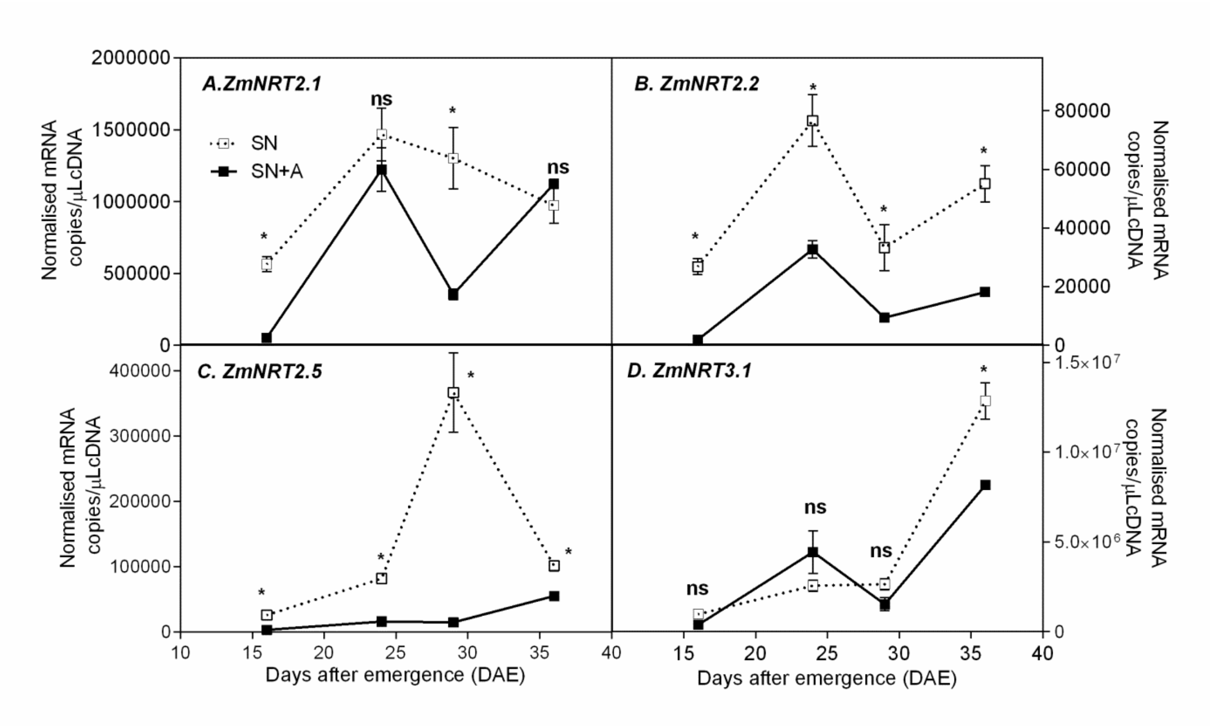


Figure 4: Root transcript levels of putative high affinity NO_3^- transporters of maize inbred line B73 grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). End point is normalised against control genes as described in the text. Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P < 0.05$ on each day of harvest.

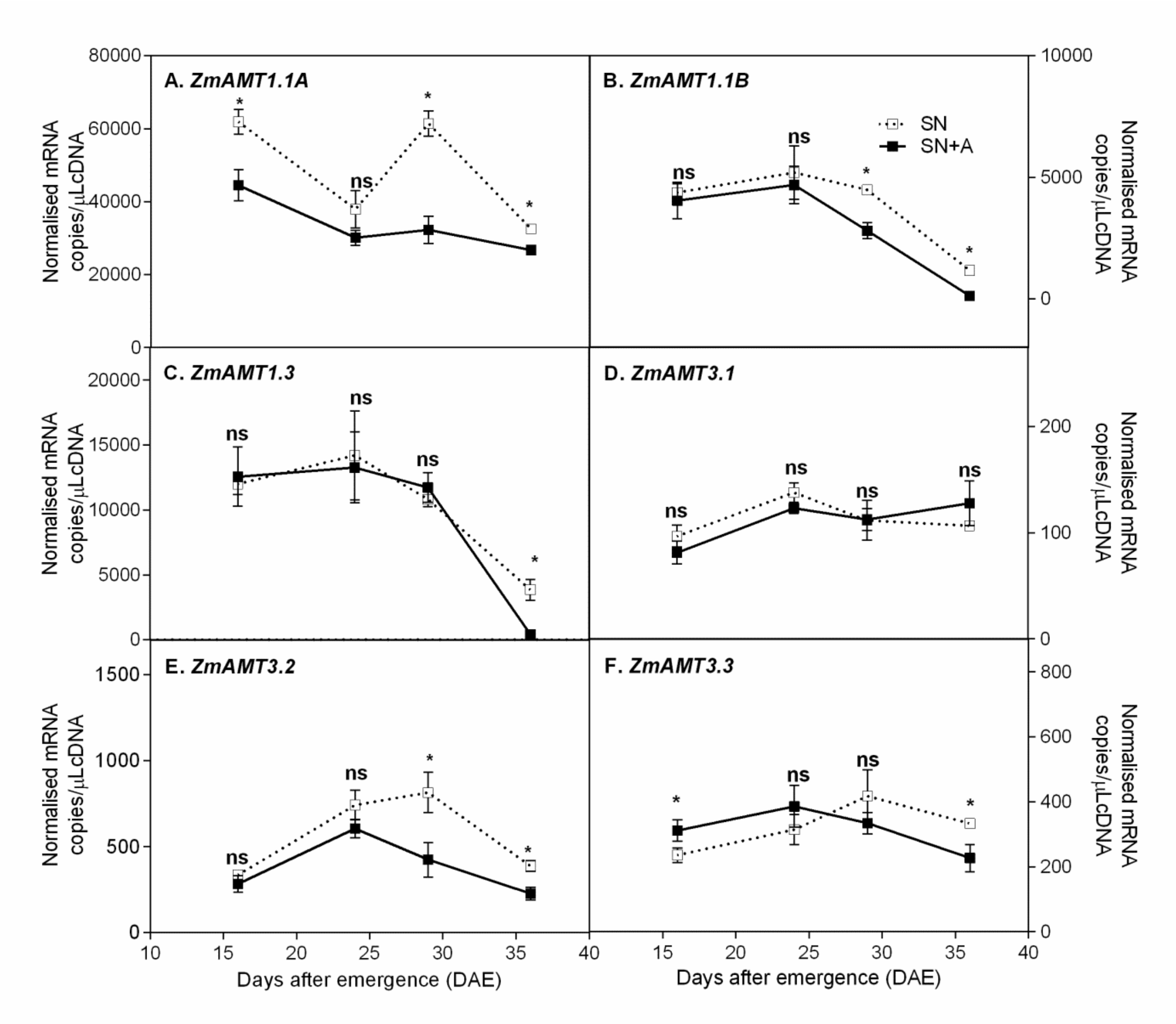


Figure 5: Root transcript levels of high affinity NH_4^+ transporters of maize inbred line B73 grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). End point is normalised against control genes as described in the text. Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P<0.05$ on each day of harvest.

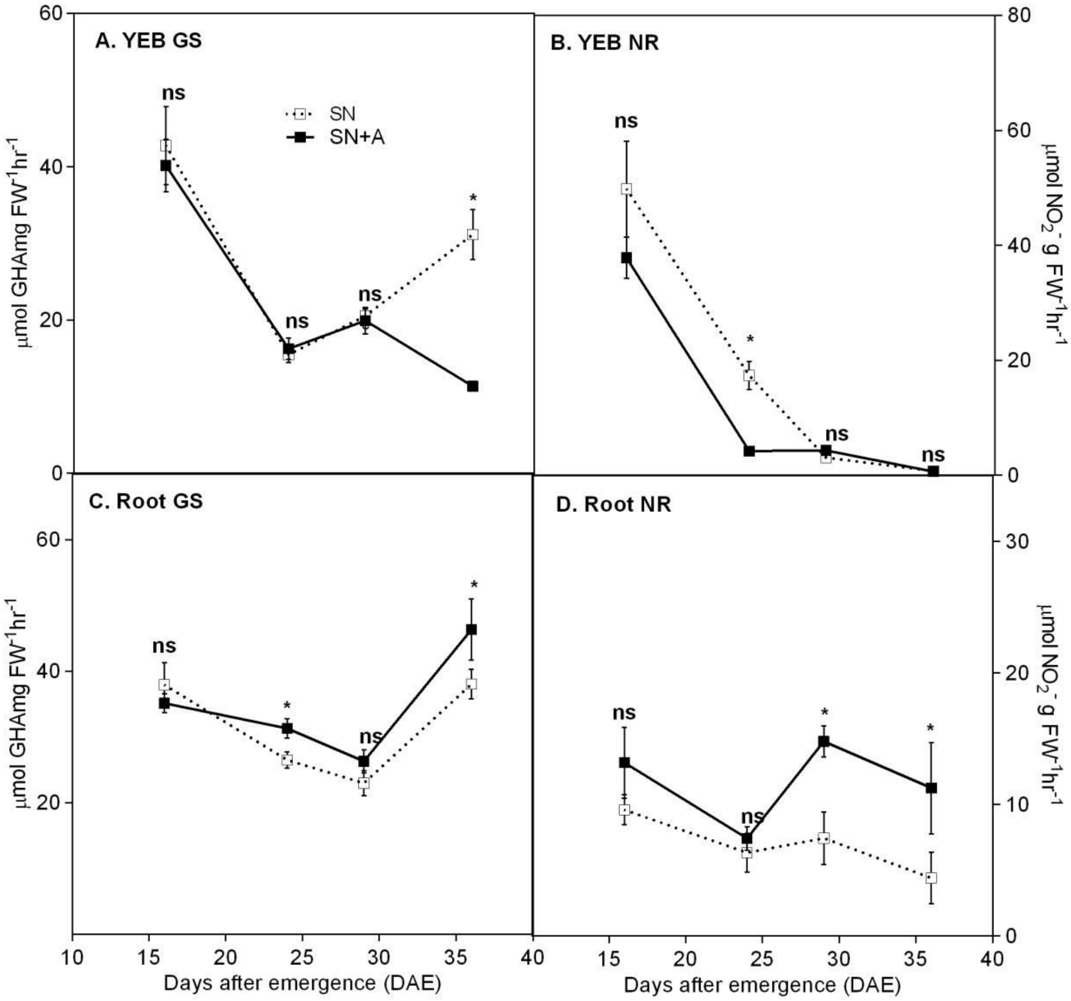


Figure 6: The activities of enzymes glutamine synthetase (GS) and nitrate reductase (NR) in YEB (A & B) and in roots (C & D) of maize inbred line B 73 grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. The symbol * represents the significances at P<0.05 on each day of harvest.

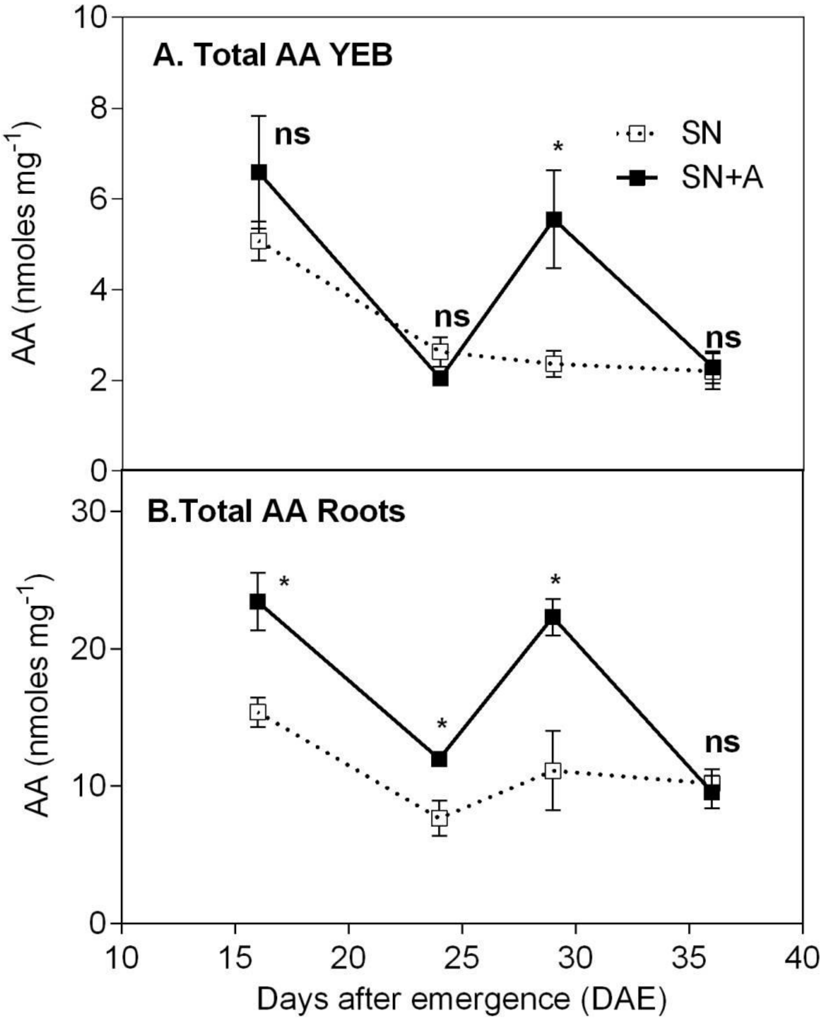


Figure 7: Total amino acids in YEB (A) and roots (B) of maize inbred line B73 grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at P<0.05 on each day harvest.

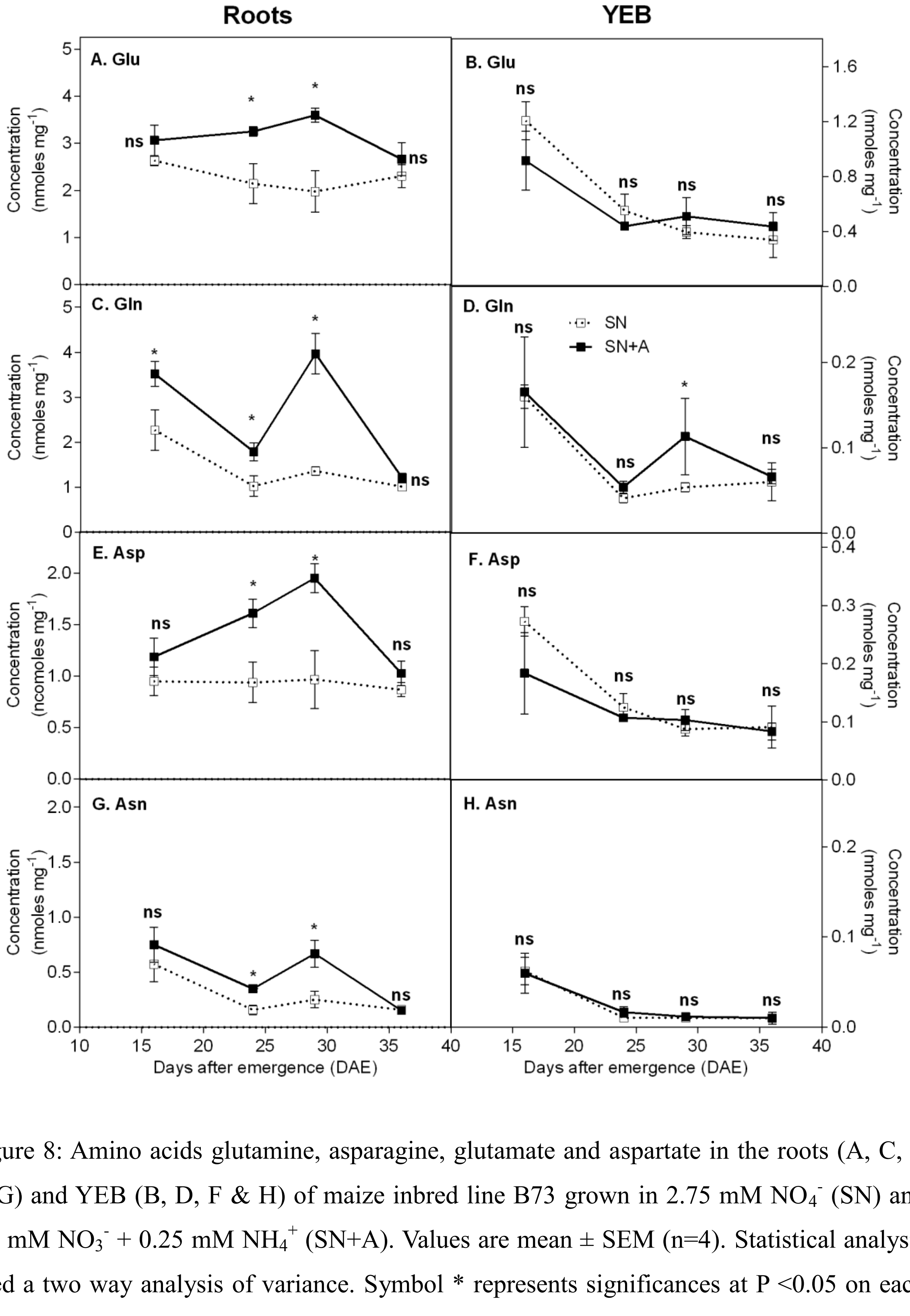


Figure 8: Amino acids glutamine, asparagine, glutamate and aspartate in the roots (A, C, E & G) and YEB (B, D, F & H) of maize inbred line B73 grown in 2.75 mM NO_4^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P < 0.05$ on each day of harvest.

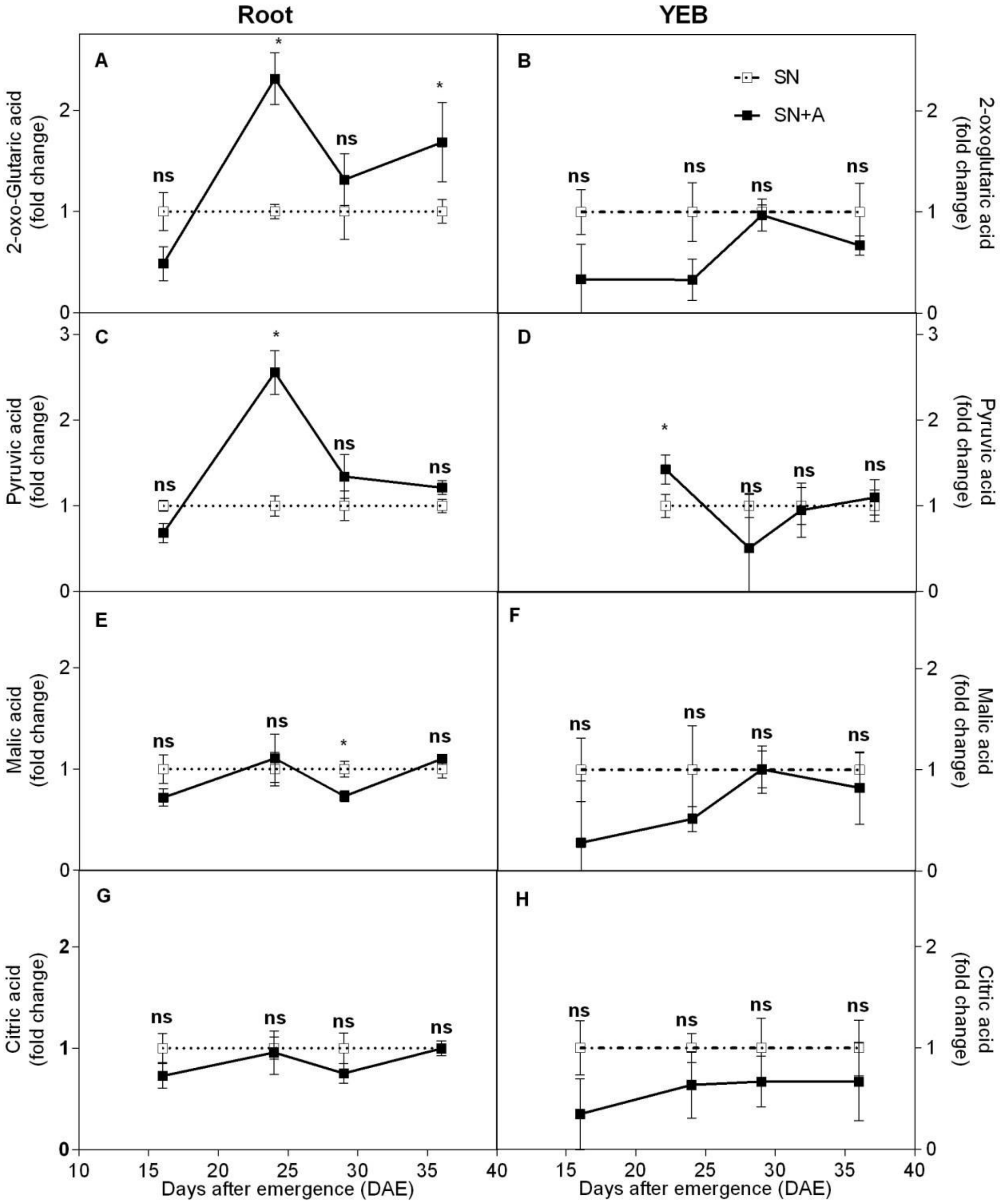


Figure 9: Major organic acids in the roots and the YEB of maize inbred line B73 grown in 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at P<0.05 on each day of harvest.

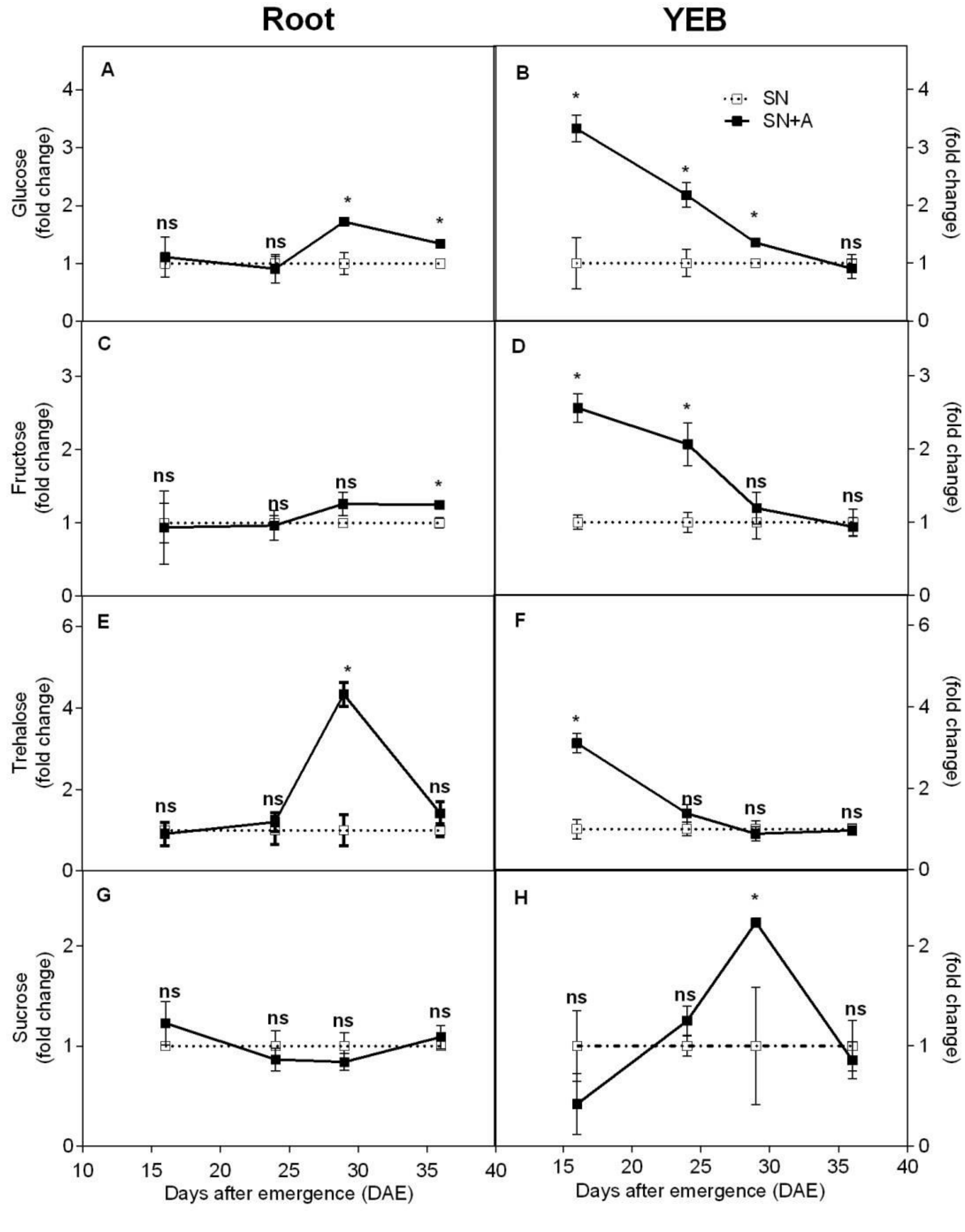
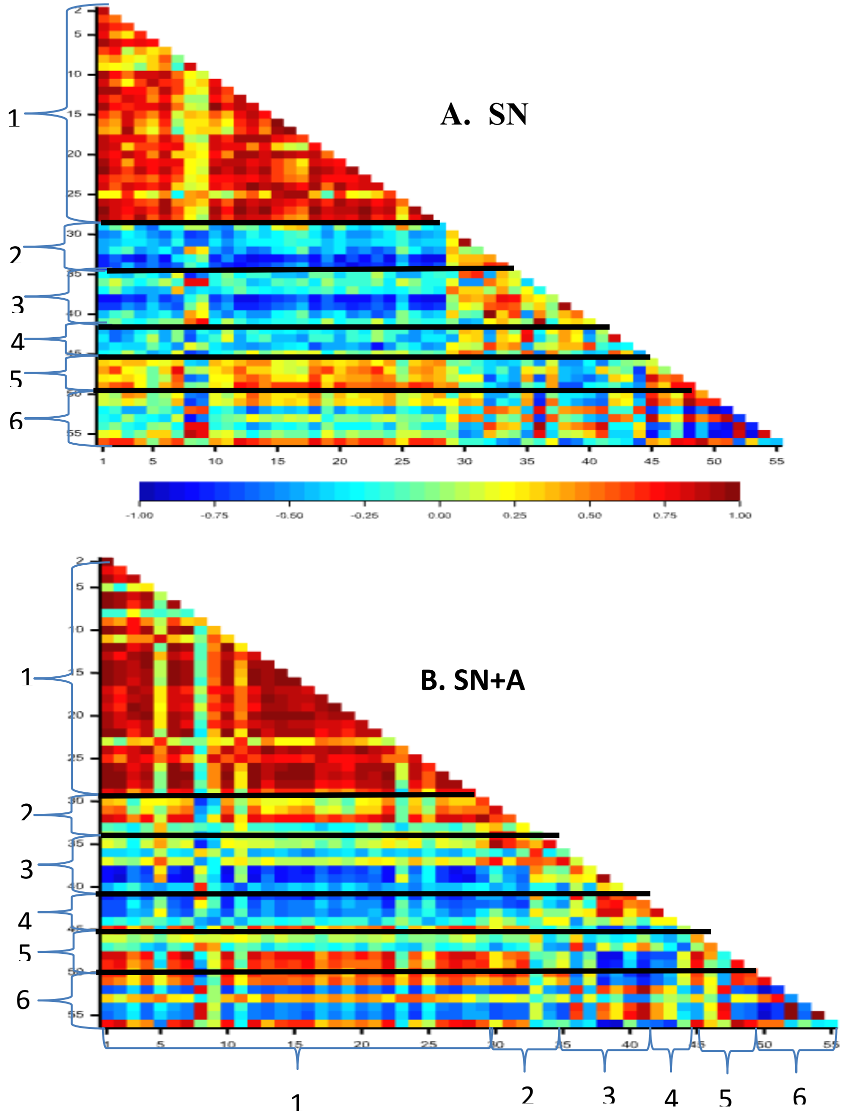


Figure 10: Sugars glucose, fructose, trehalose, and sucrose in the root (A, C, E & G) youngest fully emerged blades (YEB) (B, D, F & H) of maize inbred line B73 grown in 2.75mM NO_3^- (SN) and 2.5mM NO_3^- + 0.25mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P<0.05$ on each day of harvest.



1
1. Total_AA
2. Ala
3. Arg
4. Asn
5. Asp
6. Beta_Alanine
7. Citrulline
8. Cysteine
9. GABA
10. Gln
11. Glu
12. Gly
13. His
14. Homoserine
15. Ile
16. Leu
17. Lysine
18. Met
19. Orn
20. Phe
21. Pro
22. Ser
23. Tau
24. Thr
25. Trp
26. Tyr
27. Val
28. Hydroxy proline

2
29. AMT1.1A
30. AMT1.1B
31. AMT1.3
32. AMT3.1
33. AMT3.3
34. AMT3.2

3
35. NRT1.1A
36. NRT1.1B
37. NRT1.5A
38. NRT2.1
39. NRT2.2
40. NRT2.5

4
41. NRT3.1
42. NO3__100
43. NO3_1000
44. NH4_100
45. NH4_1000

5
46. Root_NR
47. Root_GS
48. Shoot_NR
49. Shoot_GS

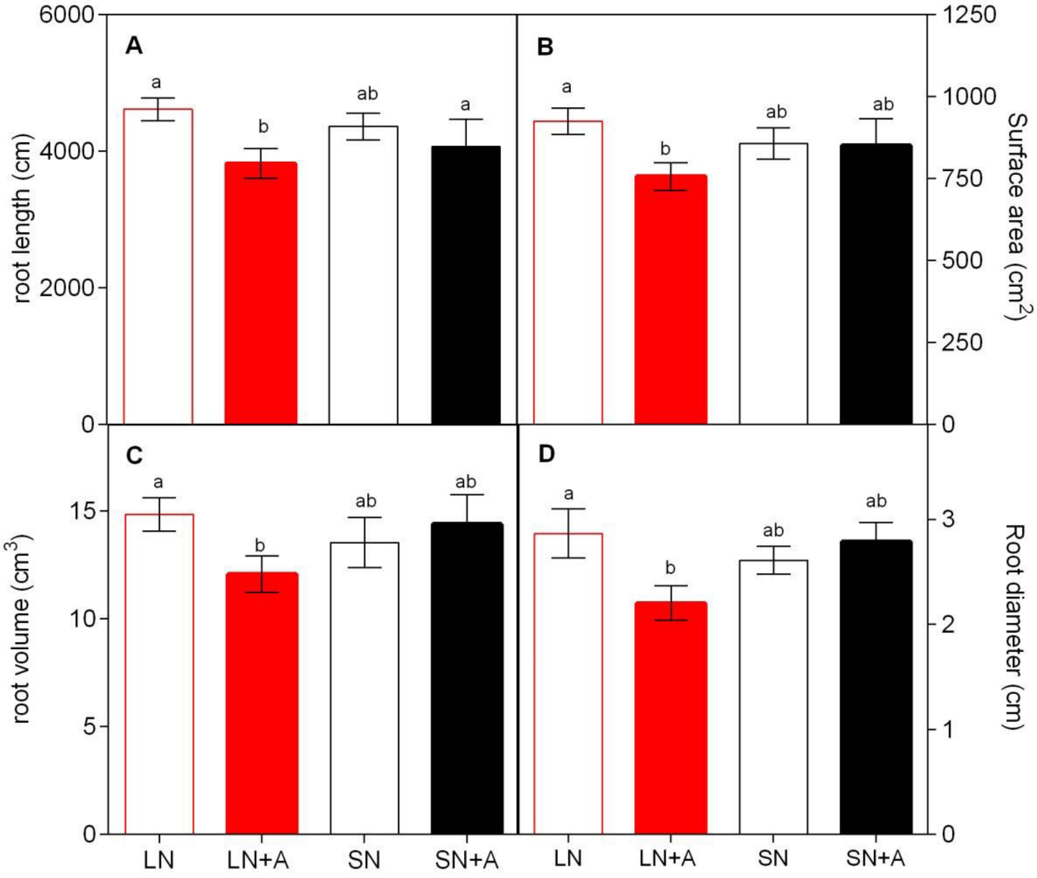
6
50. Root_N%
51. Shoot_N%
52. TotalNUptake
53. NetUptake
54. Root_DM
55. Shoot_DM
56. Root: Shoot

Figure 11: Two sided correlation drawn between different parameters in the plants grown in SN and SN+A. The matrix represents all amino acids (1), *ZmAMT* (2), *ZmNRT* (3), NO_3^- and NH_4^+ flux capacities (4) NR and GS activities (5) and growth parameters (6). Darker blue (negative) and red (positive) represents significant correlation at $p<0.05$

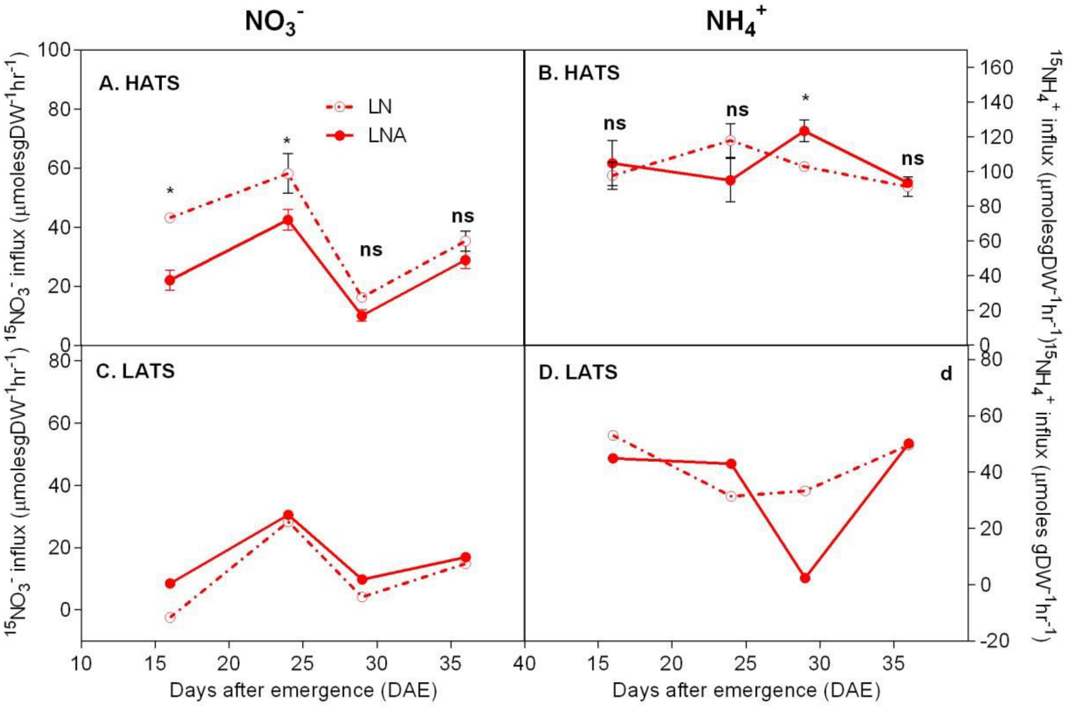
Supplementary Data

Supporting Information Table S1: Q-PCR primers for assay for maize gene expression along with the Q-PCR product size (bp).

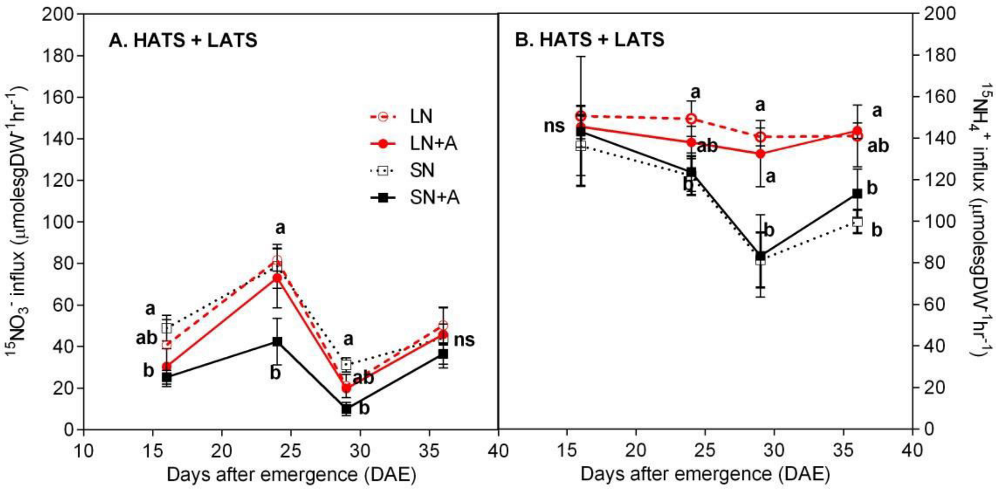
Gene	**Gene ID**	**Forward Primer (5’->3’)**	**Reverse primer (5’->3’)**	**Q-PCR Product size (bP)**
ZmNRT1.1A	GRMZM2G086496	CCTCCAGCAAGAAGAGCAAG	GACACCGAGAAGGTGGTCA	238
ZmNRT1.1B	GRMZM2G161459	GTCATCAGCGCCATCAACCT	GGGTCACACCGTGTGCCAAA	282
ZmNRT1.5A	GRMZM2G044851	CGTATGTTGTTCTTGTCTTCTTG	GTGCTATCGTCGTCAATGG	104
ZmNRT2.1	GRMZM2G010280	CGACGAGAAGAGCAAGGGACT	GGCATATTCGTACATACAAAGAGGT	183
ZmNRT2.2	GRMZM2G010251	CGACGAGAAGAGCAAGGGACT	AGGTGAACATGGATGATGGAT	166
ZmNRT2.5	GRMZM2G455124	GCATCGTCCCGTTCGTCTC	CCGTCTCCGTCTTGTACTTGG	129
ZmNRT3.1	GRMZM2G179294	GCATCCACGCCTCTCTCAAG	TCAGCAACGACAGCCACTCAT	177
ZmAMT1.1A	GRMZM2G175140	CCAGCAGCCAGGTGTAAAA	CGACTCCCAAGTAGCCAAG	161
ZmAMT1.1B	GRMZM2G118950	TGAACATCATGCTGACCAAC	AGTGCCTGCCGATGAAGC	114
ZmAMT1.3	GRMZM2G028736	TGGACTCGACGTACCTGCTCT	AAGAAGTGCTTGCCGATGAAG	217
ZmAMT3.1	GRMZM2G335218	CCAGGCTCACCAAGGACAG	CACGGCGATGGAGGAGTC	143
Zm AMT3.2	GRMZM2G338809	GCGTGGATGCTGTTCGTG	GTAGCCGCCGCAGTAGTC	117
ZmAMT3.3	GRMZM2G043193	CGCCTCCAAATCAAACGCATCC	GAGCAGCAGGACCAGGAAGG	98
ZmGAPDh	GRMZM2G077927	GACAGCAGGTCGAGCATCTTC	GTCGACGACGCGGTTGCTGTA	114
ZmActin	GRMZM2G126069	CCAATTCCTGAAGATGAGTCT	TGGTAGCCAACCAAAAACAGT	156
ZmTubulin	GRMZM2G152466	GAGGACGGCGACGAGGGTGAC	CAAAGCGGGGGAATAAAGTCT	186
ZmElF1	GRMZM2G154218	GCCGCCAAGAAGAAATGATGC	CGCCAAAAGGAGAAATACAAG	220



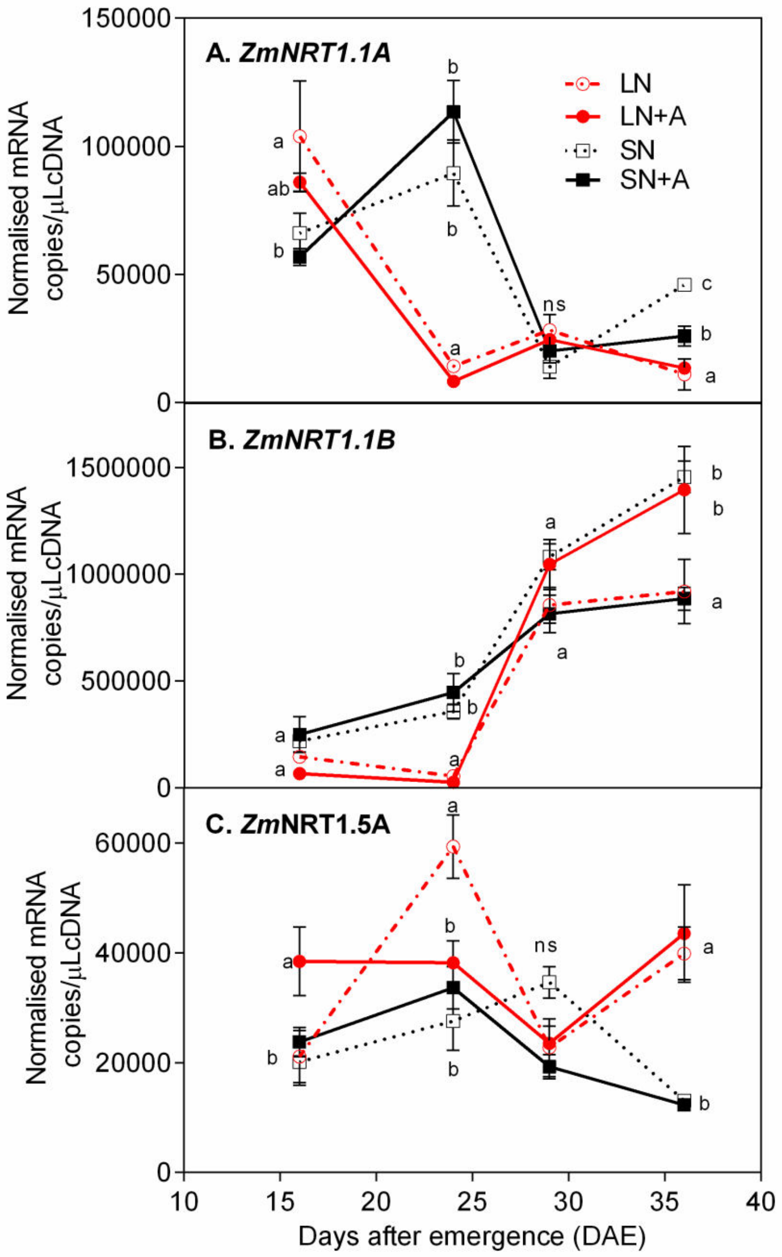
Supporting Information Figure S1: Root parameters of maize cultivar B 73 harvested on 34 days after planting. Root length (A), root surface area (B) root volume (C) and average root diameter (D). The plants were grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Different letters represents significances at $P<0.05$.



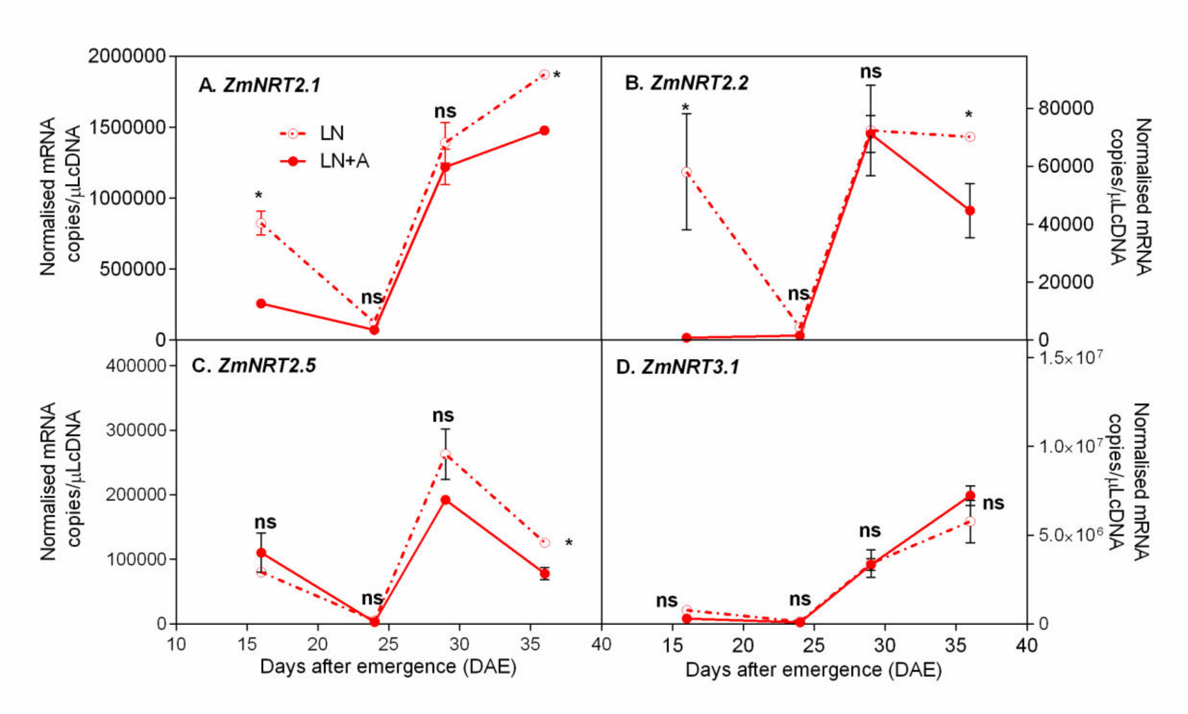
Supporting Information Figure S2: Nitrate and ammonium fluxes of maize inbred line B73 plants grown in 0.55 mM NO_3^- (LN) and 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P < 0.05$ on each day of harvest.



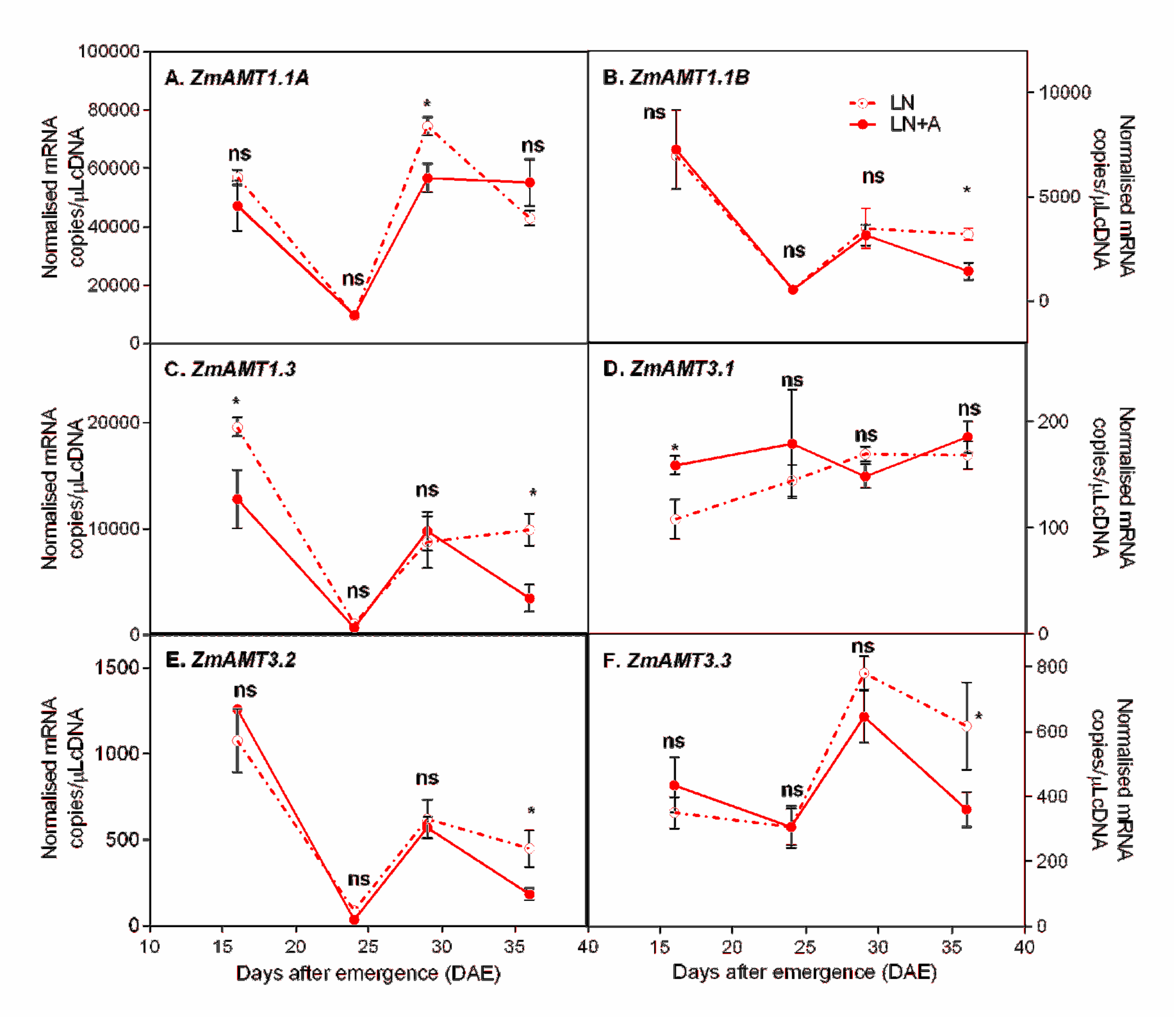
Supporting Information Figure S3: Low affinity nitrate and ammonium flux capacities of maize inbred line B73 plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis used a way analysis of variance. Different letters represents significances at $P<0.05$ on each day of harvest.



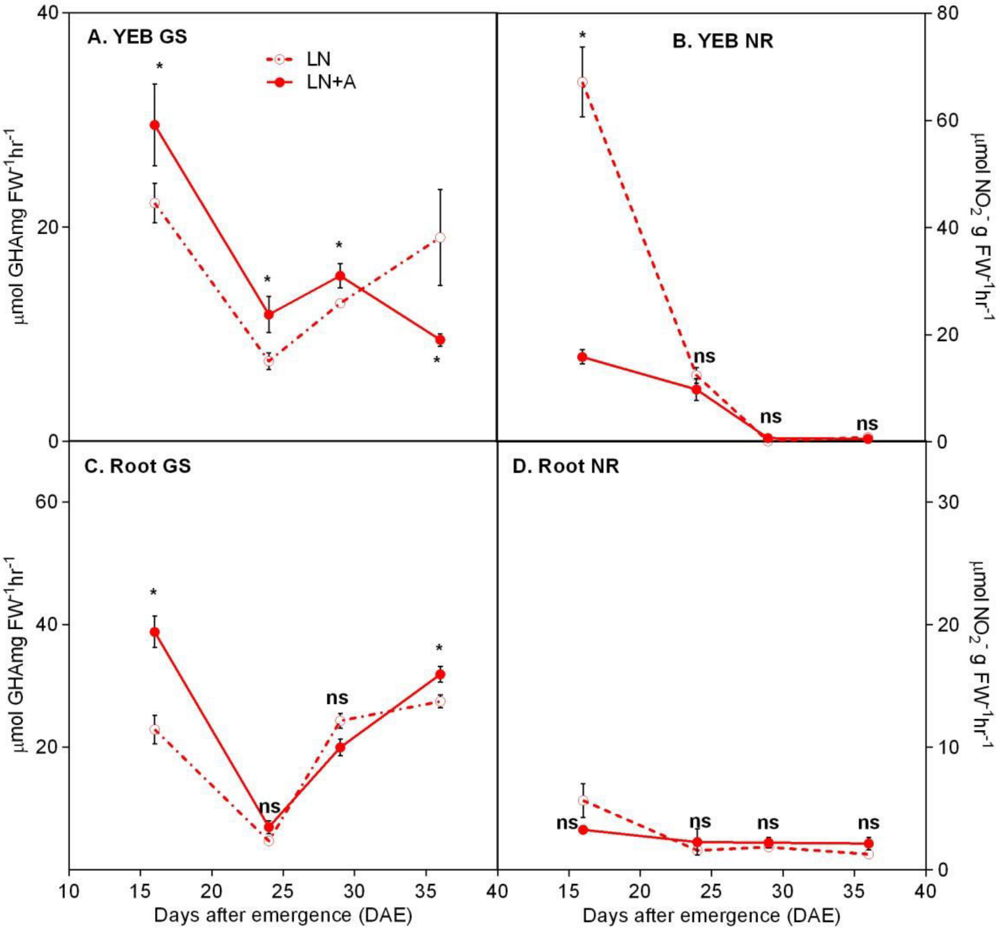
Supporting Information Figure S4: Root transcript levels of putative low affinity NO_3^- transporters of maize inbred line B73 grown in 0.55mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). End point is normalised against control genes as described in the text. Statistical analysis used a two way analysis of variance. Different letters represents significances at P <0.05 on each day of harvest.



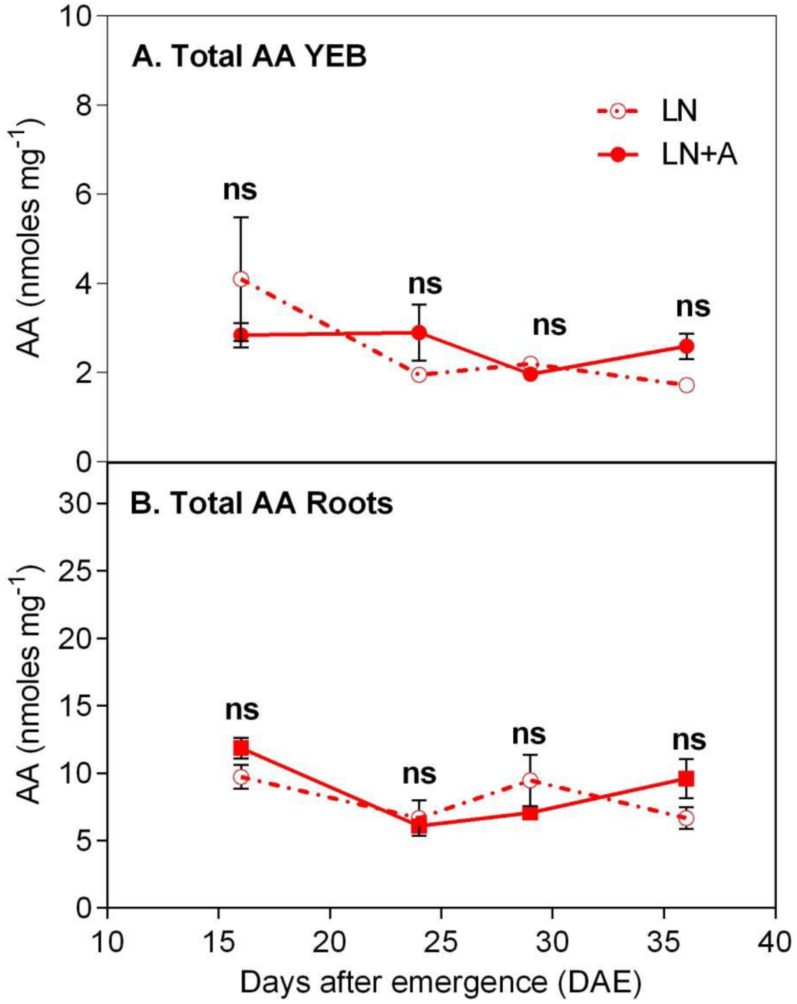
Supporting Information Figure S5: Root transcript levels of putative high affinity NO_3^- transporters of maize inbred line B73 grown in 0.55 mM NO (LN) and 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A). Values are mean ± SEM (n=4). End point is normalised against control genes as described in the text. Statistical analysis used a two way analysis of variance. Ssymbol * represents significances at P<0.05 on each day of harvest.



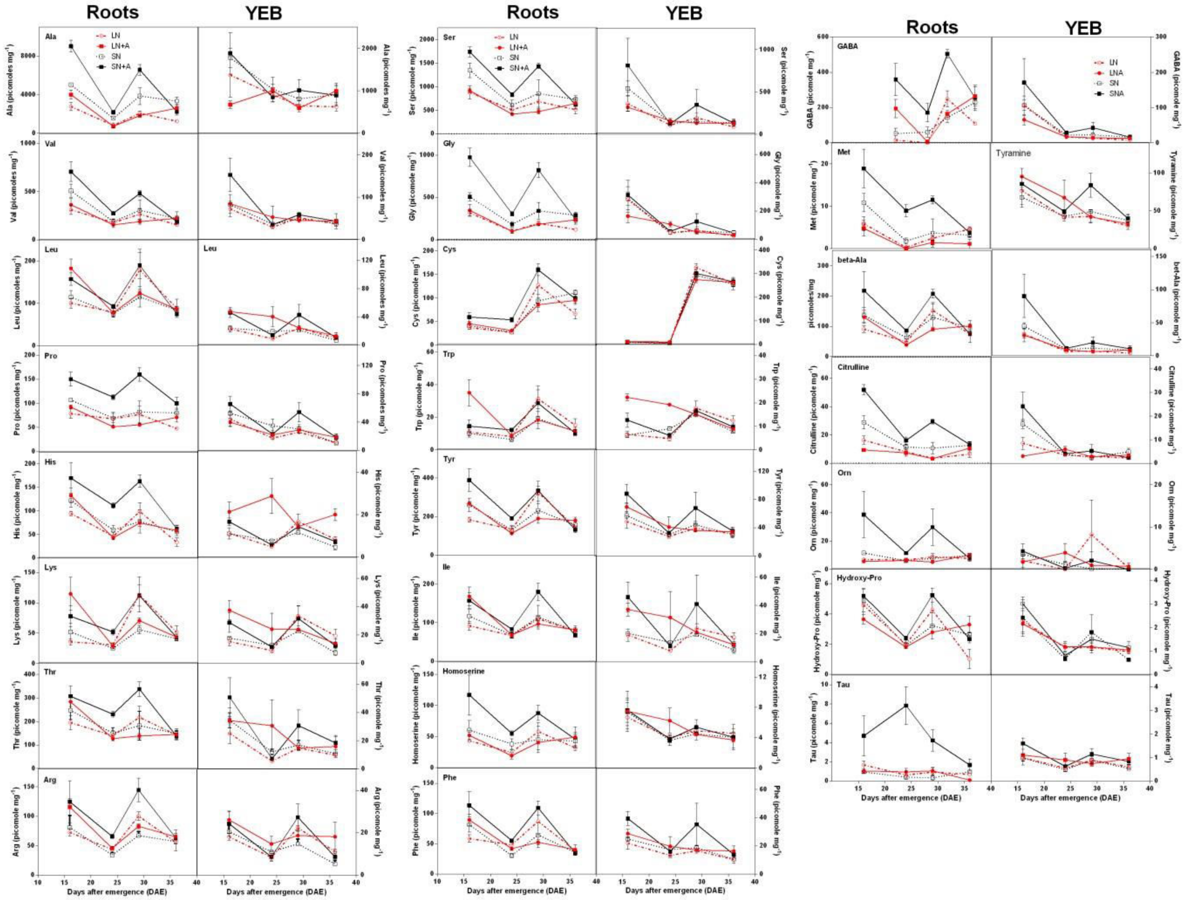
Supporting Information Figure S6: Root transcript levels of putative high affinity NH_4^+ transporters of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) and 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A). Values are mean ± SEM (n=4). End point is normalised against control genes as described in the text. Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P<0.05$ on each day of harvest.



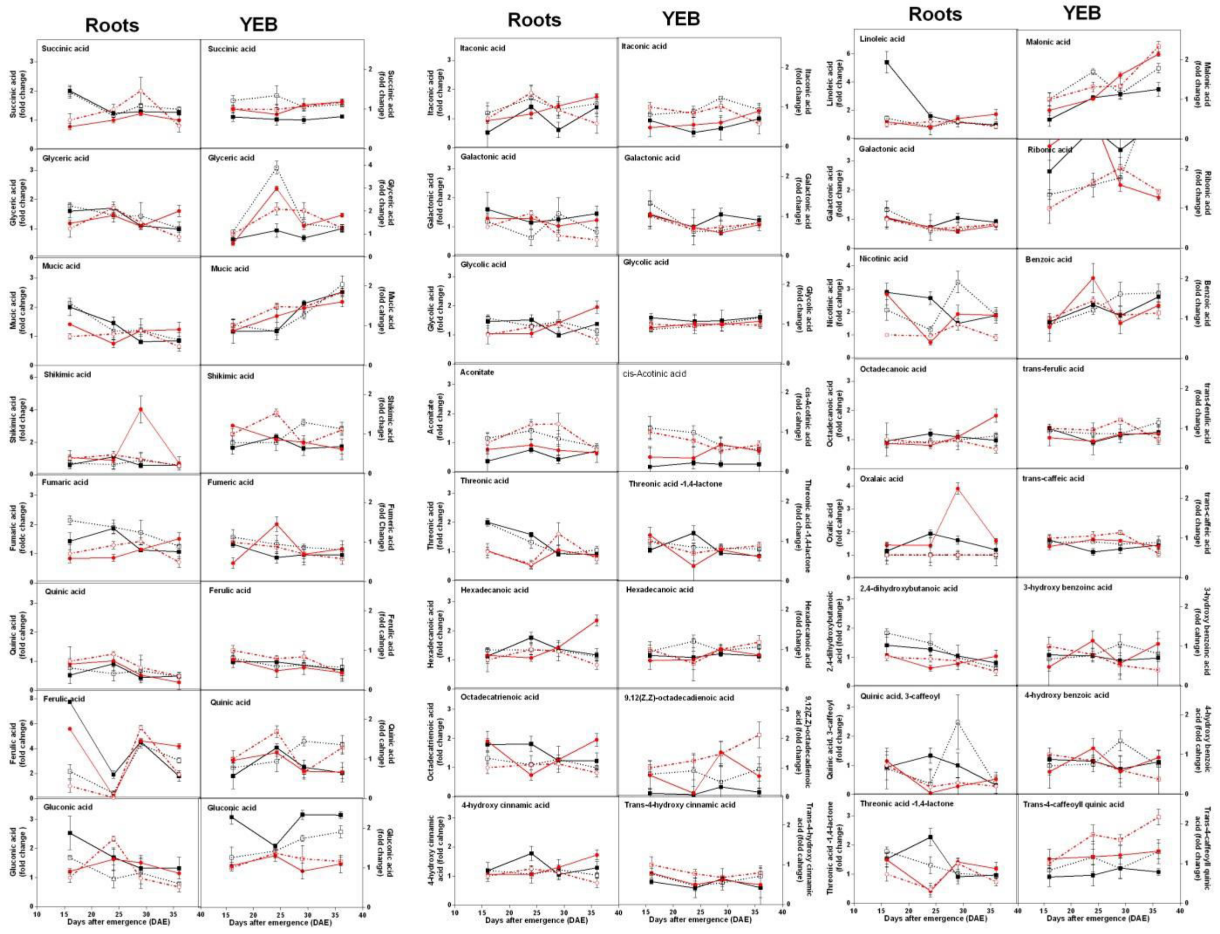
Supporting Information Figure S7: The activities of enzymes glutamine synthetase (GS) and nitrate reductase (NR) in YEB (A & B) and roots (C & D) of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) and 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance. Symbol * represents significances at $P<0.05$ on each day of harvest.



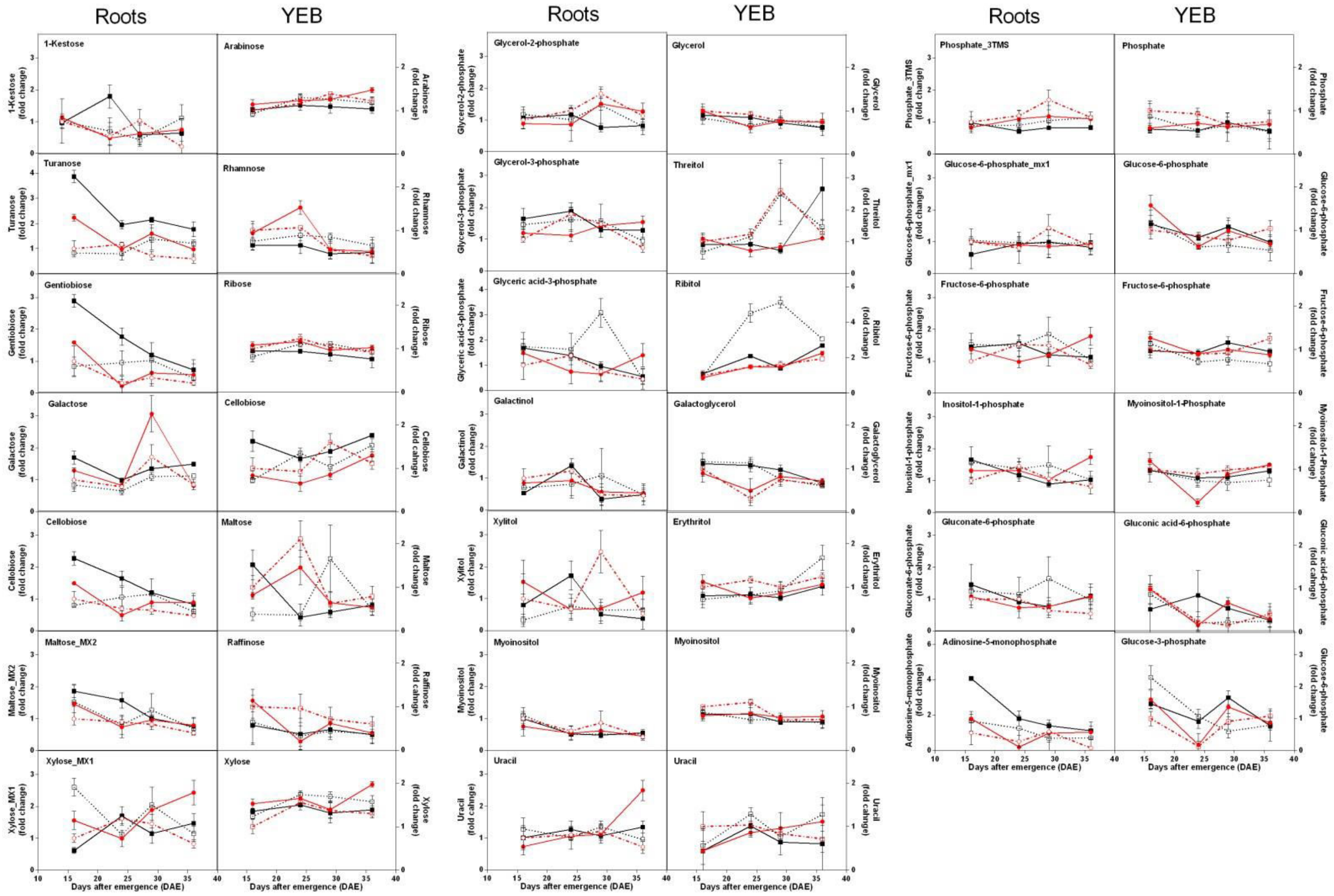
Supporting Information Figure S8: Total amino acids in YEB (A) and roots (B) of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) and 0.5 mM NO_3^- + 0.05 mM NH_4^+ (LN+A). Values are mean ± SEM (n=4). Statistical analysis used a two way analysis of variance.



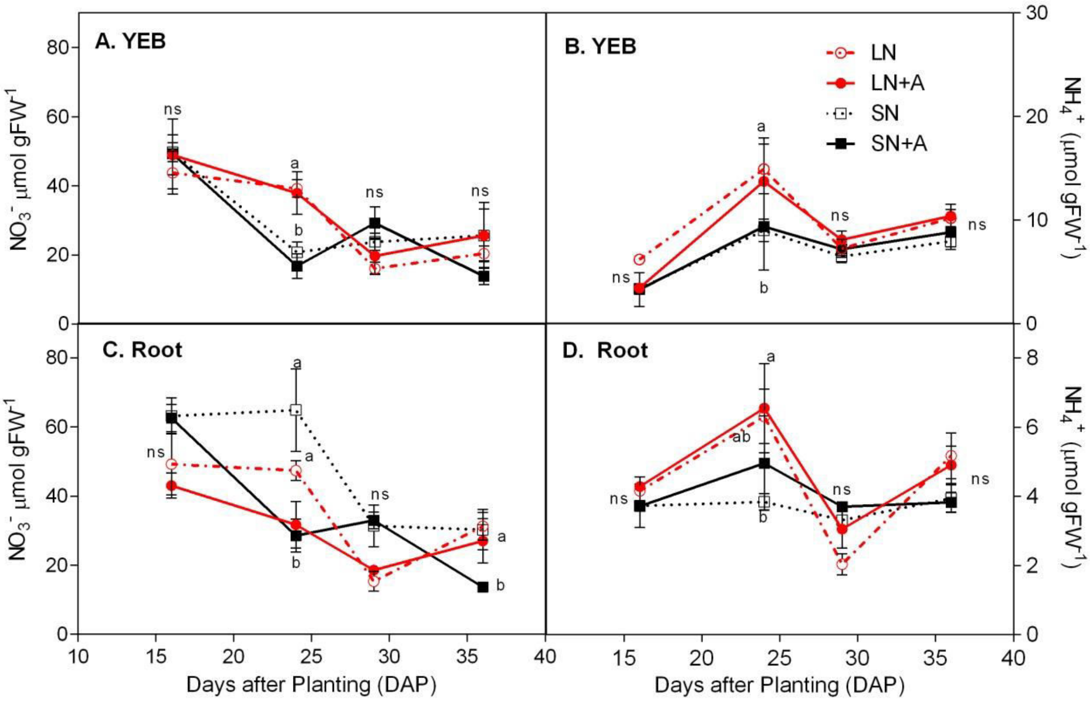
Supporting Information Figure S9: Concentration of individual amino acids in roots and YEB of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4).



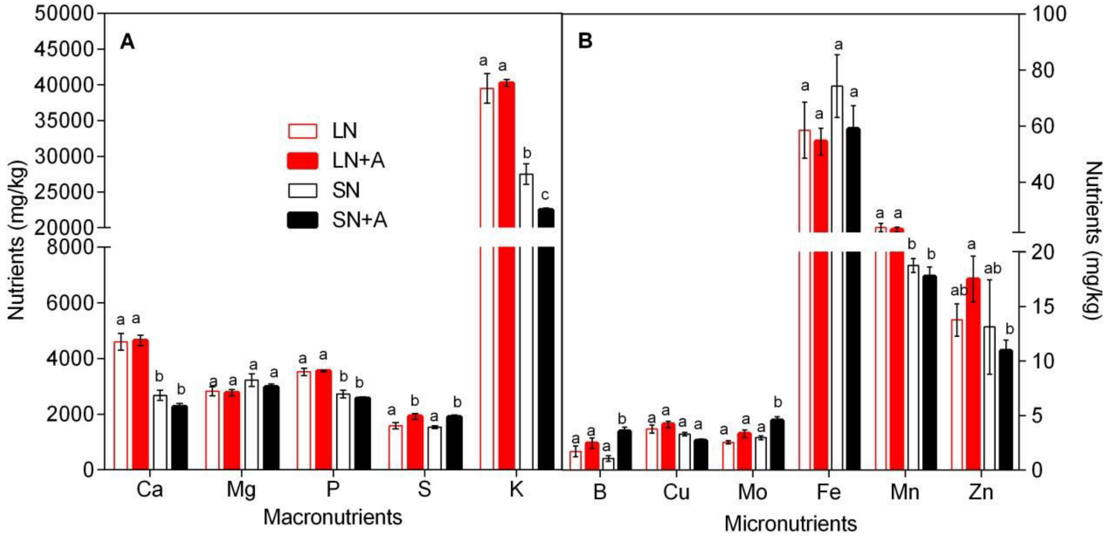
Supporting Information Figure S10: Organic acids in roots and YEB of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are fold changes with reference to LN on day 16.



Supporting Information Figure S11: Sugars and esters in roots and YEB of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are fold changes with reference to LN on day 16.



Supporting Information Figure S12: Ammonium and nitrate concentrations in YEB (A & B) and roots (C & D) of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are means ± SEM (n=4). Significant differences between treatments at P<0.05 are represented by different letters.



Supporting Information Figure S13: Macronutrient and micronutrient concentration in the shoots of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) taken on 36 days after emergence. Values are mean ± SEM (n=4). Significant differences between treatments at $P<0.05$ are represented by different letters.

Chapter 4: Long and short term effect of ammonium (NH_4^+) on nitrate (NO_3^-) uptake capacity.

Statement of Authorship

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Publication Status	○ Published, ○ Accepted for Publication, ○ Submitted for Publication, ● Publication style
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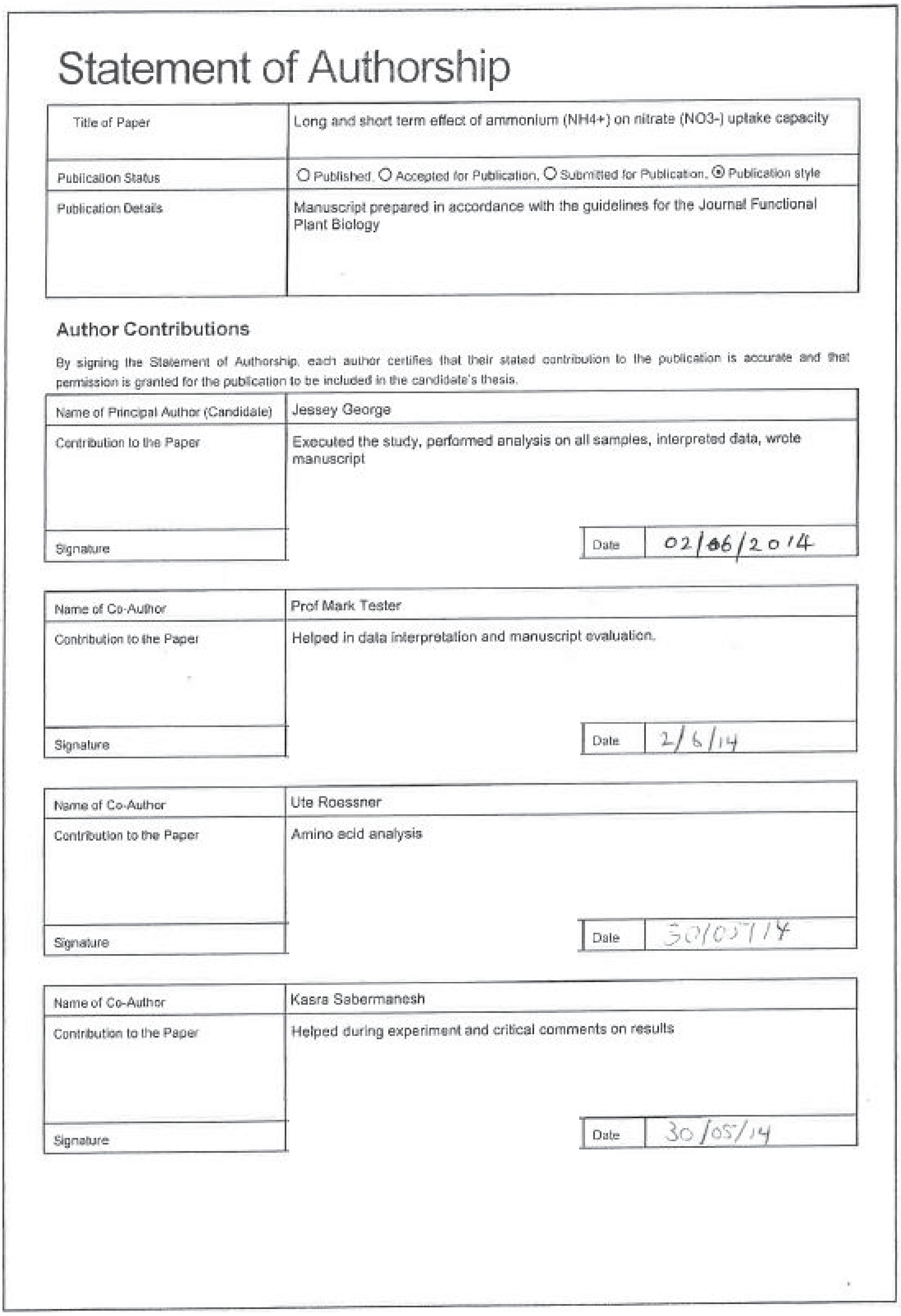
By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jessey George		
Contribution to the Paper	Performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Dr. Trevor Garnett		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	2/6/2014

Name of Co-Author	Dr. Darren Plett		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation.		
Signature		Date	2 June 2014

Name of Co-Author	Associate Prof Sigrid Heuer		
Contribution to the Paper	Helped in data interpretation and manuscript evaluation.		
Signature		Date	01/06/14



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Author Contributions

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Name of Principal Author (Candidate)	Jessey George		
Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Prof Mark Tester		
Contribution to the Paper	Helped in data interpretation and manuscript evaluation.		
Signature		Date	2/6/14

Name of Co-Author	Ute Roessner		
Contribution to the Paper	Amino acid analysis		
Signature		Date	30/05/14

Name of Co-Author	Kasra Sabermanesh		
Contribution to the Paper	Helped during experiment and critical comments on results		
Signature		Date	30/05/14

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Author Contributions

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Name of Principal Author (Candidate)	Jessey George		
Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Luke Holtham		
Contribution to the Paper	Helped during experiment and critical comments on results		
Signature		Date	30/05/14

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Title

Long and short term effects of ammonium (NH_4^+) on nitrate (NO_3^-) uptake capacity

Authors

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ABSTRACT

Our previous studies have shown that plants grown in small amounts of NH_4^+ along with sufficient NO_3^- have decreased NO_3^- uptake capacity. In order to test the hypothesis that it was not NH_4^+ itself, but rather downstream metabolites of NH_4^+ within the plants that were responsible for reduction in NO_3^- uptake capacity, maize inbred line B73 was grown in low NO_3^- with (LN+A) or without (LN) NH_4^+ and sufficient NO_3^- with (SN+A) or without (SN) NH_4^+. Some plants were also switched between NH_4^+ and non-NH_4^+ treatments to check the long term effect of NH_4^+ on NO_3^- uptake capacity. Nitrate flux capacities were measured at 0%, 10% and 50% external NH_4^+concentration in the flux solution. Our results showed that, regardless of growth treatment, high affinity (HATS) NO_3^- flux capacity was not decreased by 10% NH_4^+ in flux solution, but at 50% NH_4^+ in flux solution the NO_3^- flux capacities of plants grown in low N treatments were smaller. This suggests a short term inhibition by NH_4^+ from interactions with the NO_3^- assimilatory pathway. Our results are also consistent with the diminished NO_3^- uptake capacity of plants grown with small amounts of NH_4^+ being the long term effect of downstream metabolites of NH_4^+, particularly pools of glutamine and asparagine in roots. Reduction in NO_3^- flux capacity that is commonly reported may be an artefact of the measurement protocols and of less importance under more realistic nutrient regimes.

Key words: Amino acids, glutamine synthetase activity, glutamine, and asparagine, inhibition

INTRODUCTION

Nitrogen (N) is one of the major nutrients whose deficiency most frequently limits plant growth. Nitrate (NO_3^-) and ammonium (NH_4^+) are two major forms of N available to plants in most agricultural soils (Glass, Britto *et al.* 2002). However, NO_3^- is the dominant source of N since the concentration of NO_3^- in the soil solution is approximately 10 times that of NH_4^+ (Wolt 1994). As a result researchers have generally ignored the contribution of these small amounts of NH_4^+ in N economy of plants.

Studies have shown that a combination of NO_3^- and NH_4^+ lead to better plant growth than either N source alone (Haynes and Goh 1978; Schrader, Domska *et al.* 1972). Recent results from our laboratory found that there was an increase in shoot dry matter in plants grown with even small amounts (10%) of NH_4^+ under sufficient N nutrition. Along with this growth stimulus we observed a diminished NO_3^- flux capacity in the plants grown under this N regime (George, Sabermanesh *et al.* 2014). Exactly how NO_3^- uptake is affected by NH_4^+ is unclear.

Many studies have demonstrated that reduction in NO_3^- uptake capacities of plants in the presence of NH_4^+ is a rapid effect which becomes visible as soon as plants are exposed to NH_4^+ (Aslam, Travis *et al.* 2001; Doddema and Telkamp 1979; Garnett, Shabala *et al.* 2001; Muller and Touraine 1992). In experiments using corn it was found that presence of NH_4^+ in the nutrient solution resulted in a marked decrease of NO_3^- influx (MacKown, Volk *et al.* 1982; Mackown, Jackson *et al.* 1982; Warncke and Barber 1973). In contrast, Deane-Drummond and Glass (1983) showed that efflux of NO_3^- from barley roots was increased in the presence of NH_4^+ and this was the cause of reduced NO_3^- influx. This contradicts results in maize where there was no efflux of already accumulated NO_3^- when plants were moved to a solution containing NH_4^+ (Ayling 1993; Ingemarsson, Oscarson *et al.* 1987; Lee and Drew

1989; Mackown, Jackson *et al.* 1982). However, a study on barley roots observed that NH_4^+ inhibited both NO_3^- influx and efflux (Kronzucker, Glass *et al.* 1999).

The reduction in NO_3^- uptake capacity may also be due to the long term effect caused by the products of NH_4^+ assimilation. Taylor and Bloom demonstrated that when both NH_4^+ and NO_3^- are present in nutrient medium, NH_4^+ uptake is much higher than NO_3^- uptake along the length of maize roots (Taylor and Bloom 1998). They also indicate that with the preferential uptake of NH_4^+ compared to NO_3^-, inhibition of NO_3^- influx is by the products of NH_4^+ assimilation, which was indicated by extrusion of more H^+ ions from the roots. Similar results were also observed in maize by Lee and his co-workers (1992) who demonstrated that amino acids, asparagine and glutamine in roots rather than the substrate NH_4^+ were involved in the inhibition of NO_3^- uptake. In soybean seedlings it was observed that NO_3^- uptake was inhibited by the amino acid translocated through phloem (Muller and Touraine 1992). Major amino acids that inhibited uptake in this study were alanine, glutamic acid, aspartic acid, arginine and asparagine. Similarly a study on barley identified glutamine as the main down regulator of *HvNRT2* transcript levels where *HvNRT2* are genes that encode the high affinity NO_3^- transporters in plants (Vidmar, Zhuo *et al.* 2000).

Studies have used a range of NH_4^+ concentrations ranging from µM to mM to test inhibition of NO_3^- uptake. Lee and Drew (1989) used external NH_4^+ concentration ranging from 0.005 - 50 mM and found that at 0.005 mM concentration there was slight increase in NO_3^- influx in barely while at all other external concentrations NO_3^- influx was reduced. Inhibition of NO_3^- uptake was observed in *Eucalyptus nitens* at 100 µM ammonium nitrate (Garnett, Shabala *et al.* 2001). In wheat increasing external concentration of NH_4^+ from 12.5% to 50% progressively decreased NO_3^- uptake while NH_4^+ uptake increased (Minotti, Williams *et al.* 1969). A higher concentration of 10 mM NH_4^+ was used in cotton plants where the inhibitory effect was most prominent in plants containing high concentrations of NO_3^- (Aslam, Travis *et al.* 2001).

As mentioned above we have observed a reduction in NO_3^- uptake capacity in the absence of external NH_4^+ in flux solution for plants grown with 10% NH_4^+ at sufficient N levels. We hypothesised that the reduction in NO_3^- flux capacity in plants treated with small amounts of NH_4^+ is a long term effect mainly due to the adaptation of plants to NH_4^+ assimilation and inhibition of NO_3^- influx by its assimilatory products. We wanted to test this and also test whether there is any short term effect of NH_4^+ on plants with various NO_3^- and NH_4^+ treatments, and what concentration of external NH_4^+ is required to have this effect. Therefore, in the current study we looked at high affinity (HATS) and low affinity (LATS) NO_3^- flux capacities in the presence and absence of external NH_4^+ in flux solution. Maize inbred line B73 was grown in a hydroponic solution containing two N concentrations: low (0.55 mM) and sufficient N (2.75 mM) for three weeks and NO_3^- flux capacity was measured both at 0.5 mM (HATS) and 2.5 mM (LATS). The effect of external NH_4^+ concentration on NO_3^- flux capacity was tested at three levels of NH_4^+ in the flux solution; 0%, 10% and 50% to determine at what concentration of NH_4^+ the NO_3^- uptake capacity is reduced. We also determined the long term effects of NH_4^+ or its assimilatory products on NO_3^- uptake capacity by switching plants between ammonium and no-ammonium treatments under both low and sufficient N regimes. Amino acid contents in roots and youngest expanded blade (YEB) were measured in order to test the hypothesis that it is the pools of amino acid in plants that are responsible for reduction in NO_3^- uptake capacity.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds were aerated overnight in RO water and then placed on a filter paper moistened with 0.5 mM $CaCl_2$ solution and germinated in an incubator at 28°C. After two days the germinated seedlings were transplanted into eight 120 L ebb and flow hydroponic systems with fill and drain cycles of 15 min in a climate controlled growth chamber providing a day/night temperature of 26/22°C and a photoperiod of 14 h. Photon flux density in the growth chamber was approximately 550 µmol m^{-2} s^{-2} at average plant height. Plants were grown on mesh collars in tubes as explained in Garnett et al. (2013). plants were grown in 0.55 mM NO_3^- (LN), 0.5 mM NO_3^- with 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- with 0.25 mM NH_4^+ (SN+A) The nutrient solution used was Johnson's modified nutrient solution containing (in mM) 1.8 K, 0.6 Ca, 0.5 Mg, 1 S, and 0.5 P. Both treatment solutions contained (in µM) 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 200 Fe (as FeEDTA and FeEDDHA) (Johnson, Stout *et al.* 1957). Iron was supplemented twice weekly with the addition of $FeSO_4$ (8 mg l^{-1}). $(NH_4)_2SO_4$ was used as the NH_4^+ supplement to NH_4^+ treatments. Solution pH was monitored daily and maintained between 5.9 and 6.0. Nitrate and NH_4^+ concentrations in the solutions were monitored using NO_3^- and NH_4^+ electrodes (TPS, Springwood, Australia) and maintained at the target concentration of ± 5%. Nutrient solutions were changed weekly. Fresh samples for all the assays were harvested into liquid N between 11am and 1pm on the harvesting day and stored at -80°C. Some plants were switched from LN to LN+A, LN+A to LN, SN to SN+A and SN+A to SN on 22 days after emergence (DAE) and were allowed to grow in the switched treatments for 2day before doing the flux experiment as outlined below. Various treatments are summarised in Table 1.

Table 1. Various NO_3^- and NH_4^+ treatments

Name	0 – 22 DAE		22 – 24 DAE	
	NO_3^- (mM)	NH_4^+ (mM)	NO_3^- (mM)	NH_4^+ (mM)
LN	0.55	0	0.55	0
LN→LN+A	0.55	0	0.50	0.05
LN+A	0.50	0.05	0.50	0.05
LN+A→LN	0.50	0.05	0.55	0
SN	2.75	0	2.75	0
SN→SN+A	2.75	0	2.50	0.25
SN+A	2.50	0.25	2.50	0.25
SN+A→SN	2.50	0.25	2.75	0

Flux measurement

Unidirectional NO_3^- fluxes in the roots were measured 24 DAE using ^{15}N labelled NO_3^-. The fluxes were measured for both non-switched and switched plants. Nitrate$^-$ fluxes were measured at HATS concentration of 500 µM and LATS concentration of 2500 µM. These concentrations were chosen to allow estimation of the contribution of the HATS and the LATS to the NO_3^- uptake capacity. Fluxes were measured at three concentrations of NH_4^+: 0, 10% and 50% relative to NO_3^- in the flux solution. On the day of sampling, plants grown in the nutrient solutions were transferred to an identical nutrient solution to that in the growth medium containing ^{14}N NO_3^- with 0%, 10% or 50% ^{14}N NH_4^+ for 5 minutes. Plants were then transferred to solution containing either 0.5 mM or 2.5 mM of NO_3^- labelled with 10% enriched ^{15}N NO_3^- with 0%, 10% or 50% ^{14}N NH_4^+ for 10 minutes. Roots were then rinsed in identical solutions containing but with ^{14}N nitrogen for 2 minutes to remove ^{15}N from the root apoplast. The flux timing of 10 minutes was chosen to minimize any efflux or transport to shoots based on the study by Kronzucker *et al.* (1995). Roots were then separated from shoots, blotted and weighed. Plant parts were dried in an oven at 40°C for 7 days. After measuring the dry matter content roots were ground to a fine powder and the total N and ^{15}N in the plant samples were determined with an isotope ratio mass spectrometer (Sercon, Cheshire, UK). Unidirectional NO_3^- influx was calculated based on ^{15}N content of the root.

Glutamine synthetase activity assay

Fresh root and leaf samples were homogenised in a mortar and pestle in liquid N and stored at -80°C. Glutamine synthetase was assayed using a biosynthetic reaction by quantification of γ- glutamyl hydroxamate (GHA) formed during the reaction with glutamine. This method is adapted from the method described by O'Neal and Joy (1973). Samples were incubated at 30°C for 30 min and absorbance was measured at 540 nm.

Amino acids determination

Fresh roots and youngest expanded blades (YEB) were harvested into liquid nitrogen on the day of flux experiment (24 DAE) and stored in -80°C freezer. Amino acids were measured on ground root and youngest fully expanded blade (YEB) samples that were stored in -80°C freezer. Approximately 100 mg of aliquots were taken from the ground fresh sample and freeze dried. Tissue amino acid concentration was determined using liquid chromatography electrospray ionization-mass spectrometry, as described by Boughton *et al.* (2011), once the samples had been derivatized following the method of Cohen & Michaud (1993).

Statistical Analysis

Data were analysed using two-way analysis of variance using Graphpad Prism software. (Version 6.00, 1992-2012 GraphPad Software, Inc). The heat map was drawn using the fold changes in relation to the data for plants in LN for individual amino acid concentrations, NO_3^- flux capacities, root GS activity, YEB GS activity, root and shoot NO_3^- and NH_4^+ concentrations using Genesis software (Version.1.7.6, Sun Microsystems Inc.).

RESULTS

Plants in sufficient N treatments accumulated more dry matter than plants in low N

Shoot biomass was measured 24 DAE. Shoot dry matter in all the sufficient N treatments (black bars) was higher than in low N treatments (red bars) (Figure 1A), but no difference was observed with 10% NH_4^+ both at low N and sufficient N. No effect of switching was observed in the biomass data. However, the root biomass of SN+A plants was lower than for plants in all the other treatments except for plants in SN+A→SN (Figure 1B). The root: shoot of plants in all the sufficient N treatments was lower than those in low N treatments (Figure 1C). The root: shoot of plants in SN+A and SN+A→SN were the lowest from all other treatments.

No difference in N concentration was observed between switched and non-switched plants both in low N and sufficient N treatments, but N concentration was higher in all sufficient N treatments (Figure 2A). It can also be seen that the total N uptake is much higher in all the sufficient N treatments compared to low N treatments (Figure 2B). Root N concentration was also decreased when plants were grown in SN+A→SN (Figure 2C). A reduction in shoot N concentration and total N content was observed for plants in SN+A→SN. However it can be observed that plants grown in SN+A had the highest net uptake and decreased when these plants were switched to SN for two days (Figure 2D).

HATS NO_3^- uptake capacity reduced when external NH_4^+ concentration in the flux solution was increased in the low N treatments

The HATS NO_3^- uptake capacities were lower for plants grown in sufficient N treatments at all external NH_4^+ concentrations (Figure 3; all black bars) compared to low N treatments (Figure 3; all red bars). The HATS NO_3^- flux capacity for plants grown in 10% NH_4^+ (SN+A) (Figure 3; bars 7, 15 & 23) was lower than for plants in SN (Figure 3; bars 5, 13 & 21), at all concentrations of NH_4^+ in the flux solution (relative to NO_3^-). At 50% NH_4^+ in flux solution concentration no difference in NO_3^- flux capacities were observed between low N treatment (bars 17-20) and plants in SN (bar 21) and SN+A→SN (bar 24). However, NO_3^- flux capacity of plants switched from SN→SN+A (Figure 3 bars 6 & 14) was lower than that in SN plants (Figure 3 bars 5 & 13) both at 0% and 10% external NH_4^+. This flux capacity was similar to the flux capacities of plants in SN+A (bars 7, 15 and 23 in figure 3). Conversely, SN+A→SN plants (Figure 3 bars 8, 16 & 24) showed a higher NO_3^- uptake capacity compared to SN+A plants (Figure 3 bars 7, 15 & 23) and were equal to flux capacities of plants in SN (bars 5, 13 and 21 in figure 3).

There was no reduction in HATS NO_3^- uptake capacity of plants in low N treatments when there was 10% NH_4^+ in the flux solution (Figure 3 bars 9-12) compared to no external NH_4^+ (Figure 3 bars 1-4). Presence of 50% external NH_4^+ reduced HATS NO_3^- flux capacity for plants grown in low N (Figure 3 bars 17-20) compared to no external NH_4^+ (Figure 3 bars 1-4) in the flux solution. However, in sufficient N treatments no difference was observed between flux capacities of plants at any of the external concentration of NH_4^+ in flux solution (Figure 3; bars 5, 13 &21, bars 6, 14 & 22, bars 7, 15 & 23 and bars 8, 16 & 24). LATS NO_3^- uptake capacity was calculated by subtracting the mean uptake capacity at 0.5 mM NO_3^- concentration from the uptake capacity at 2.5 mM (Figure S1). In general, it was observed that LATS NO_3^- uptake was lower compared to HATS NO_3^- uptake capacity in all the treatments. It can be seen that plants grown in low N treatments had lower LATS flux capacity compared to their corresponding sufficient N treatment. The trend shows that LATS flux capacities were higher for plants grown with 10% NH_4^+ in the medium (Figure S1; bars 3, 6, 7, 11, 15, 18, 19 and 23) compared to plants without NH_4^+ (Supplementary information Figure S1; bars 1, 4, 5, 8, 9, 12, 13, 16, 17, 21 and 24).

Switching of plants between NH_4^+ and non- NH_4^+ treatments and vice versa increased the GS activity but reduced the amino acid content in the roots.

Glutamine synthetase activity (GS) in the YEB of plants grown in SN and SN+A was higher than for those in LN and LN+A (Figure 4A). When plants were switched between NH_4^+ and non-NH_4^+ treatments it was observed that the GSA in the YEB of plants in low N treatments increased. On the other hand, plants grown in SN+A had higher amino acid concentration in YEB compared to all other treatments (Figure 4B). Free amino acids in the YEB of plants grown in LN+A were higher than that in LN, whereas total free amino acids decreased in the YEB of plants grown in treatments LN+A→LN, SN→SN+A and SN+A→SN when compared to plants in LN+A, SN and SN+A respectively (Figure 4B). In roots a higher GSA was observed for plants grown in SN+A compared to plants in LN, LN+A and SN treatments (Figure 4C). Similar to the YEB results, when plants were moved from LN→LN+A , LN+A→LN and SN→SN+A there was a two fold increase in the root GSA, whilst there was a small decrease in root GSA of plants in SN+A→SN. Conversely, no decrease in total amino acid was observed for plants in LN→LN+A compared to LN. However, in roots reduction in total free amino acid is seen only for plants in SN+A→SN compared to SN+A (Figure 4D).

Glutamate contents in roots showed no treatment differences except for SN+A and SN+A→SN plants (Figure 5A & E). Plants in SN+A had higher glutamate and aspartate contents in roots compared to plants moved from SN+A and grown in SN for 2 d (SN+A→SN). The concentration of glutamine and asparagine were higher in roots of plants grown in SN+A and SN→SN+A compared to SN and SN+A→SN respectively (Figure B & G). Similar to roots, it can be observed in Figure 5D that YEB of plants in SN+A had the highest glutamine content compared to all treatments. Here we also see a higher concentration of glutamine in plants grown in LN+A compared to LN. However, when plants were moved between NH_4^+ and non-NH_4^+ treatments there was a large reduction in concentration of all four amino acids for all plants in switched treatments regardless of which way they were switched (LN→LN+A , LN+A→LN and SN→SN+A, SN+A→SN). Asparagine concentration in the YEB of plants grown in SN and SN+A were higher than those in LN (Figure 5D). Glutamate (Figure 5B) and aspartate (Figure 5F) concentrations did not show any difference between treatments in non-switched plants (LN, LN+A, SN & SN+A) and switched plants (LN→LN+A, LN+A→LN and SN→SN+A & SN+A→SN). However, there was a reduction in the concentration of glutamine, asparagine, glutamate and aspartate in YEB of switched plants. The concentrations of all other amino acids are presented in the supplementary information (Figure S2). It was observed that the highest concentrations of most AA were higher in plants that were grown in SN+A compared to all other treatments.

Switching of plants increased the accumulation of NO_3^- and decreased the NH_4^+ concentration in the roots

Nitrate content in YEB and roots of plants in both SN and SN+A was higher than that in LN and LN+A (Figure 6A & C) but compared to SN, SN+A had a lower NO_3^- concentration. Switching plants from LN→LN+A and LN+A→LN increased the root NO_3^- content (Figure 6B). On the contrary, there was a reduction in YEB NO_3^- concentration of plants in SN→SN+A & SN+A→SN (Figure 6A). Ammonium concentrations were always higher in YEB than in roots irrespective of treatments and less variation was observed between treatments for NH_4^+ compared to NO_3^-. Ammonium concentration in the YEB of LN+A and SN+A plants was higher than other treatments (Figure 6B). Plants in LN+A had significantly higher root NH_4^+ concentration than LN plants. However, when plants were switched between treatments (LN→LN+A, LN+A→LN, SN→SN+A & SN+A→SN). NH_4^+ concentration was reduced in roots (Figure 6D) compared to non-switched plants (LN, LN+A, SN & SN+A).

Switching of plants between NH_4^+ and non – NH_4^+ treatments changes the amino acid profiles and GS activities in the plants

The heat map in figure 7A shows that most of the individual amino acid concentrations in roots of plants in LN→LN+A and LN+A→LN was lower than that of LN and LN+A plants respectively. Conversely, in the sufficient N treatments the concentration of only a few amino acids were decreased between switched (SN→SN+A & SN+A→SN) and non-switched plants (SN and SN+A). Root GS activity and root NO_3^- concentrations are clustered together, as are glutamine and asparagine. Glutamate and aspartate content are present in two separate clusters. All the fluxes and amino acids lie in two separate clusters showing strong negative correlation between them. In the shoot heat map we can see that most of the amino acids are reduced by switching plants regardless of which way they are switched (the alternate red coloured boxes in Figure 7B). It can be observed that amino acids that showed low concentrations are clustered together and in the roots not much variation in these amino acids were observed by switching.

DISCUSSION

In this study there was no growth increase at harvest (24 DAE) in plants supplied with 10% NH_4^+. However, in our earlier study the growth stimulation in plants supplied with 10% NH_4^+ only became evident at 36 DAE when shoot dry matter was higher for plants grown in sufficient N with 10% NH_4^+ (George, Sabermanesh *et al.* 2014). Twenty four DAE was chosen as the harvest day for this study because in our earlier study the highest NO_3^- flux capacity was observed on this day. Although all sufficient N treatments had higher N content compared to low N concentration, the net N uptake of plants grown in sufficient N treatments with 10% NH_4^+ was the highest. This indicates that plants grown with 10% NH_4^+, along with sufficient N, were able to capture more N from the nutrient solution than plants grown in other treatments.

The reduction in NO_3^- flux capacity in plants grown in sufficient N treatments (SN) compared to low N (LN) treatments is consistent with the bulk of the literature which describes NO_3^- flux capacity being reduced with increasing N content (Imsande and Touraine 1994; von Wirén and Merrick 2004). Many studies have also suggested that free amino acid content in plants acts as an indicator of N nutritional status of plants, and it is amino acid levels that lead to reduced flux capacity (Cooper and Clarkson 1989; Imsande and Touraine 1994; Oaks, Aslam *et al.* 1977; Rodgers and Barneix 1993). Molecular studies revealed that this effect is due to the down regulation of NO_3^- transporters at the mRNA level when N content in the plants are higher (Krapp, Fraisier *et al.* 1998; Quesada, Krapp *et al.* 1997). Further reduction in NO_3^- flux capacity when plants were grown in 10% NH_4^+ at sufficient N levels (SN+A) compared to SN plants is also consistent with our earlier study (George, Sabermanesh *et al.* 2014). In that case it was correlated with an increase in amino acid levels and this was also observed in the current study, supporting the hypothesis that increased amino acids reduces NO_3^- uptake capacity. Even a very small amount of NH_4^+ in the nutrient solution led to increased amino acid concentration and N content of plants and hence there is a further reduction in NO_3^- uptake capacity in sufficient N treatments with small amounts of NH_4^+.

Unlike plants grown with SN+A, plants grown with LN+A showed no decrease in NO_3^- uptake capacity compared to LN. This can be explained by low N content of low N treated plants compared to sufficient N treatments. This has been described in Gaspe flint maize plants which were grown in low N medium and increased their uptake capacity to meet N demand (Garnett, Conn *et al.* 2013). Plants in LN+A increased their uptake capacity as 10% NH_4^+ present in this treatment was not enough to meet N demand. This suggests that long term effect of NH_4^+ on NO_3^- uptake capacity is visible only when plants are grown in sufficient N.

Plants grown without NH_4^+ in the growth solution (LN) showed no reduction in their NO_3^- uptake capacity when there was 10% NH_4^+ relative to NO_3^- in the flux solution. Similar results were also observed in barley which was grown in 10 mM NO_3^- and the fluxes were measured with 1 mM NH_4^+ (10% relative to NO_3^-) in the external solution (Kronzucker, Glass *et al.* 1999). In this experiment, when concentration of NH_4^+ in the flux solution was 50% NO_3^- uptake capacity of plants in LN was reduced 10-15% compared to uptake capacity of plants in no NH_4^+ in the flux solution. This shows there is concentration dependence to the short term effect of NH_4^+ on NO_3^- uptake capacity. Similarly, NO_3^- uptake capacity of barley started to reduce when concentration of NH_4^+ in the solution was 50% or more relative to NO_3^- (Deane-Drummond and Glass 1983).

Many theories have been put forth regarding the mechanism of short term inhibition of NH_4^+ on NO_3^- uptake. The most common theory is plasma membrane depolarization by NH_4^+ which reduces the driving force for NO_3^- uptake in plants (Ullrich 1992; Zhou, Theodoulou *et al.* 1998). However, plasma membrane depolarization also results from potassium (K) treatments (Newman, Kochian *et al.* 1987). Therefore plasma membrane depolarization alone cannot be the reason for short term inhibition of NO_3^- uptake by NH_4^+.

Deane-Drummond and Glass (1983) showed that when plants were grown in low N the exposure of plants to NH_4^+ reduced net NO_3^- uptake because of the increase in efflux of NO_3^- by NH_4^+. A contrasting result was observed in maize where they saw no efflux of the already accumulated NO_3^- from the roots when plants were exposed to NH_4^+ (Mackown, Jackson *et al.* 1982). However, the reduction in NO_3^- uptake that we see in our results is not due to increased efflux of NO_3^- because the flux timing was chosen to minimise any possible efflux (Kronzucker, Siddiqi *et al.* 1995). Therefore, the reduction in NO_3^- uptake capacity may be due to NH_4^+ 'short circuiting' the N assimilatory pathway. This means that as plants preferentially absorb NH_4^+ compared to NO_3^-, NH_4^+ is assimilated in the GS/GOGAT pathway prior to the NH_4^+ produced by the reduction of NO_3^-. This reduces the NO_3^- uptake capacity of plants. However, plants can overcome this short term effect by increasing total GS activity in these plants. Similar results were seen for plants that were grown in LN+A compared to SN+A indicating it is the N content of plants that determines the short term effect of NH_4^+ on NO_3^- uptake capacity.

Moving plants between NH_4^+ and no-NH_4^+ treatments had no effect on NO_3^- flux capacities of plants grown in low N treatments. This can be explained again by lower N content of plants where these plants need much more N than supplied (as either NO_3^- or NH_4^+) and thus have a higher NO_3^- uptake capacity. However, plants moved from SN+A→SN and SN→SN+A and grown in that treatment for 2 days had altered flux capacities such that they were the same as that of the plants already growing in these treatments. We see a corresponding reduction in amino acids in the roots of plants in SN+A to SN but no increase in total amino acid concentration in SN to SN+A was observed (Figure 3D). Therefore, the question arises as to whether it is the total amino acids or the level of some particular amino acids that effect in NO_3^- uptake capacity of those plants grown in small amount of NH_4^+. In maize the intracellular pool of amino acids, especially higher concentration of glutamine and asparagine, decreased absorption of N sources from the nutrient medium (Lee, Purves *et al.* 1992). These two amino acids were also found to inhibit NR activity (NRA) in maize both at low and high NO_3^- supply (Sivasankar, Rothstein *et al.* 1997). The increase in glutamine and asparagine content in roots of plants grown in SN+A or plants in SN→SN+A suggests their involvement in the reduction in uptake capacity. Similarly low concentration of glutamine was observed in plants growing in SN and plants moved from SN+A to SN which coincided with an increase in flux capacities of plants in these treatments. Other studies also showed that plants supplied with glutamine as their N source had increased glutamine content in roots and a corresponding decrease in NO_3^- uptake capacity (Lee, Purves *et al.* 1992; Muller and Touraine 1992). Similarly, in barley it was observed that higher concentration of glutamine contributed to the reduction in NO_3^- uptake capacity which was confirmed by increase in NO_3^- uptake capacity when glutamine synthetase inhibitor methionine sulfoximine was used in the treatment (Vidmar, Zhuo *et al.* 2000). In this study a significant decrease in transcript levels of *HvNRT2* was observed in response to a higher glutamine concentration and a corresponding decrease in the NO_3^- influx.

A correlation was observed between amino acid content in roots and increase in GS activity for plants grown in SN+A. This may be due to activity of glutamine synthetase enzyme in cytosol (GS1) which is responsible for primary assimilation of NH_4^+ absorbed up by roots (Oliveira and Coruzzi 1999). The increased activity of GS1 in roots of plants grown in SN+A may have increased total amino acid pools in roots. This amino acid pool in roots may regulate the uptake of NO_3^- into plants. Of all the switch treatments, moving plants from SN to SN+A resulted in no decrease in total free amino acids in roots of these plants suggesting that the 10% NH_4^+ in sufficient N treatments contributed to the production of more amino acids by increasing GS activity (Figure 4B). This suggests that when there is sufficient N in the medium addition of small amounts of NH_4^+ increases the activity of GS1 enzyme in roots (Hirel, Bouet *et al.* 1987). However, an increase in the GS activity of the switched plants in low N correlated with the reduction in total free amino acid contents in roots as well as in YEB of those plants. This can be explained by the earlier studies in Arabidopsis where they reported that there is an antagonistic effect of amino acids on GS activity where higher concentration of amino acids decreases GS activity and vice versa (Oliveira and Coruzzi 1999). Therefore increase in GS activity in these plants may be due to release of this antagonistic effect by amino acids levels. This theory can be further substantiated by our work which found higher root NO_3^- accumulation when plants were switched between NH_4^+ and no- NH_4^+ treatments. This suggests that activity of GS is increased in the switched plants due to the low levels of amino acids and greater NO_3^- accumulation in the plants.

It can be concluded from this study that when plants are grown in 10% NH_4^+ along with sufficient level of NO_3^-, the reduction in NO_3^- flux capacity is due to the long term effect of high concentration of total free amino acids, particularly glutamine and asparagine in roots of these plants. However, when 50% NH_4^+ was supplied externally in the flux solution, NO_3^- uptake capacity was reduced in low N treatments due to the short term effect by the 'short circuiting' of NH_4^+ in the N assimilatory pathway. Both the short term and long-term effect of NH_4^+ can be rapidly reversed by moving plants to a no-NH_4^+ medium.

Although we see a reduction in the NO_3^- uptake capacity of plants grown in 10 % NH_4^+ at sufficient NO_3^- levels, total N uptake and shoot N concentration in these plants were higher indicating that uptake capacity is not an important factor in determining the actual N uptake of plants. Rather this represents the nutritional status of plants where we see a systemic regulation based on N content inside the plants (Glass, Britto *et al.* 2002; Imsande and Touraine 1994). We also see an increase in plant growth with small amounts of NH_4^+ on later stages of growth (George, Sabermanesh *et al.* 2014). Therefore, reduction of NO_3^- flux capacity by small amounts of NH_4^+ with sufficient N (NO_3^-) appears to be an artefact and is not important unless we are measuring the fluxes.

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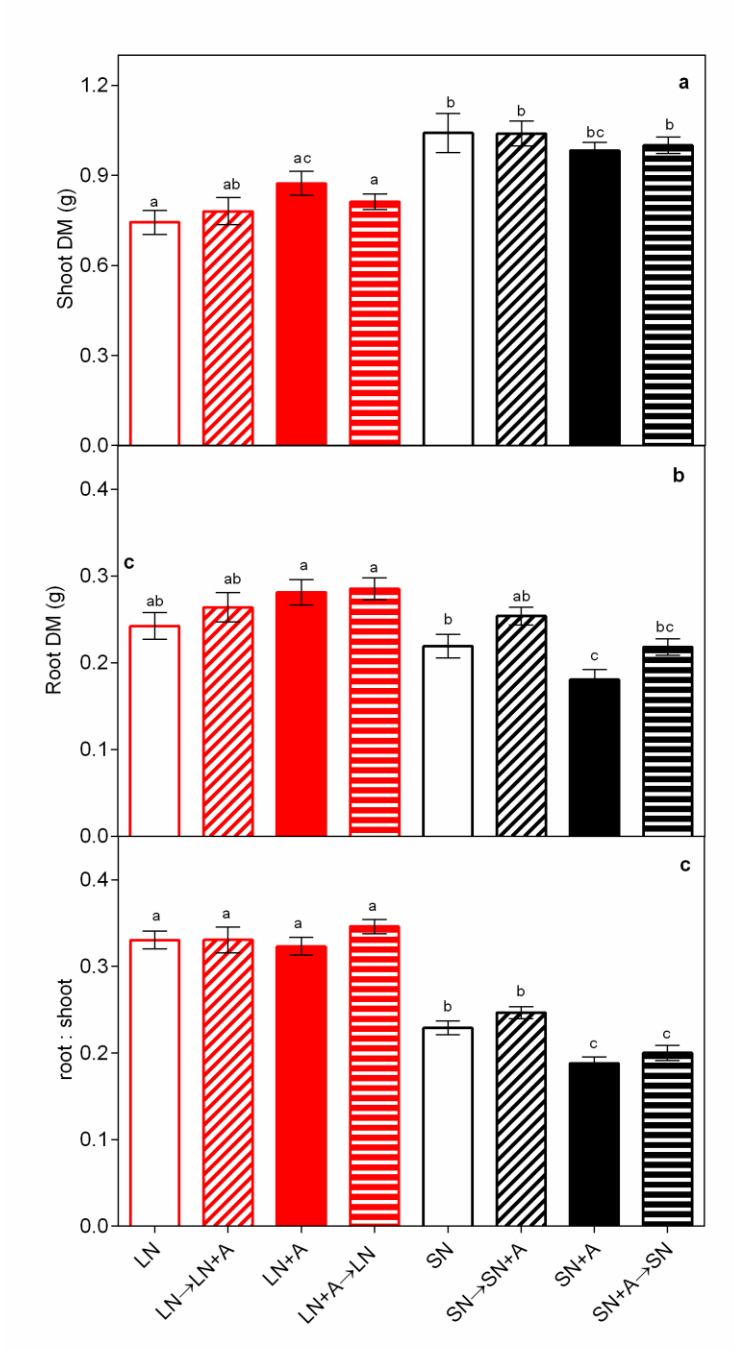


Figure 1: Dry matter accumulation in roots and shoots and the root: shoot of plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when they are switched between ammonium and no-ammonium treatments on 22 days after emergence. The data were collected on 24 DAE. Values are means ± SEM where n=4. Statistical analysis used an ordinary one way analysis of variance. Significant differences at $P<0.05$ are represented by different letters.

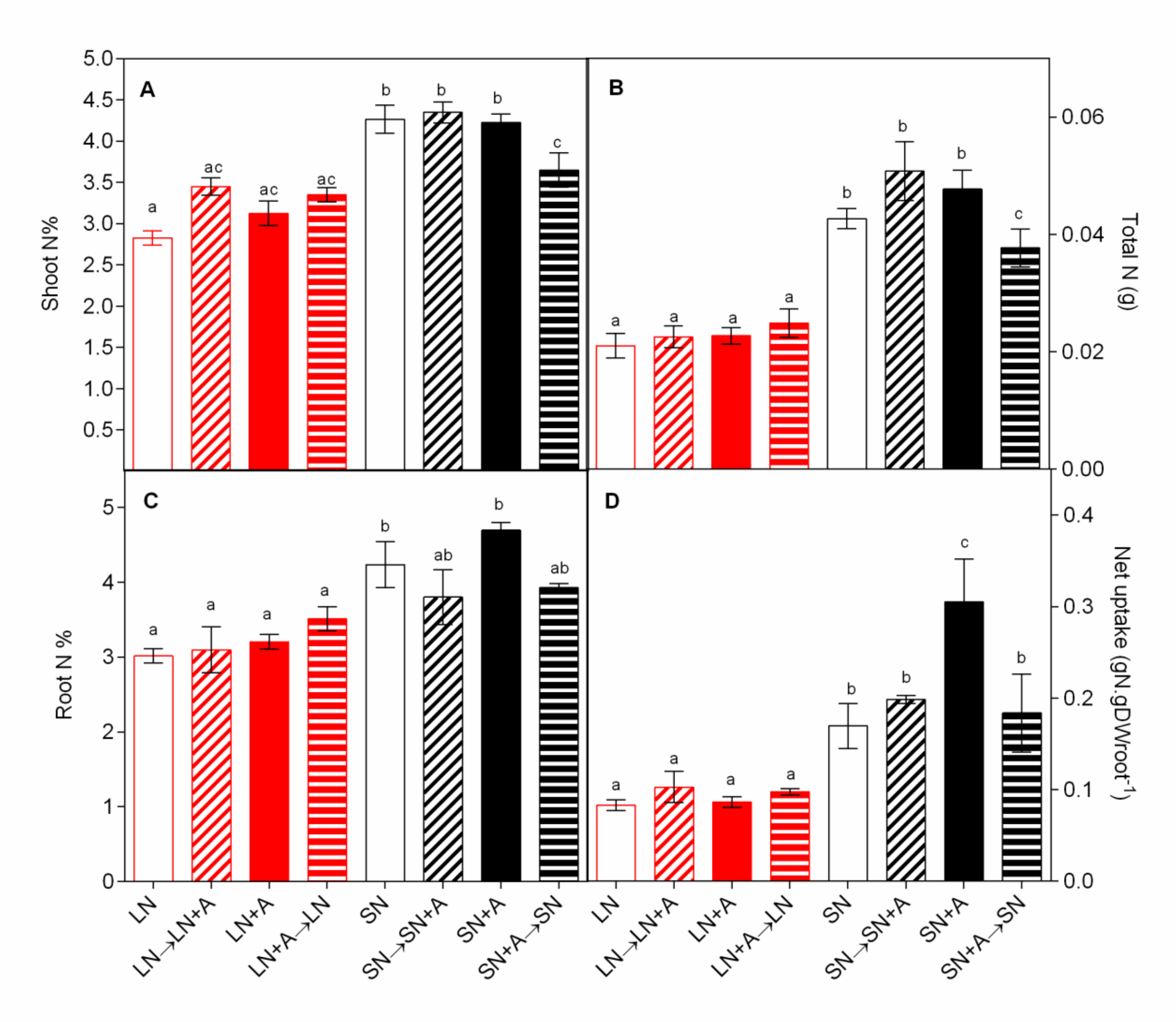


Figure 2: Tissue N concentration in the shoot (A), the total N (B), tissue N concentration in the root (C), and net uptake (D) in the plants that were taken grown in in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) at low N (red bars) and sufficient N (black bars) and when there were switched between NH_4^+ and no-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM (n=4). Statistical analysis used an ordinary one way analysis of variance. Significant differences at P<0.05 are represented by different letters.

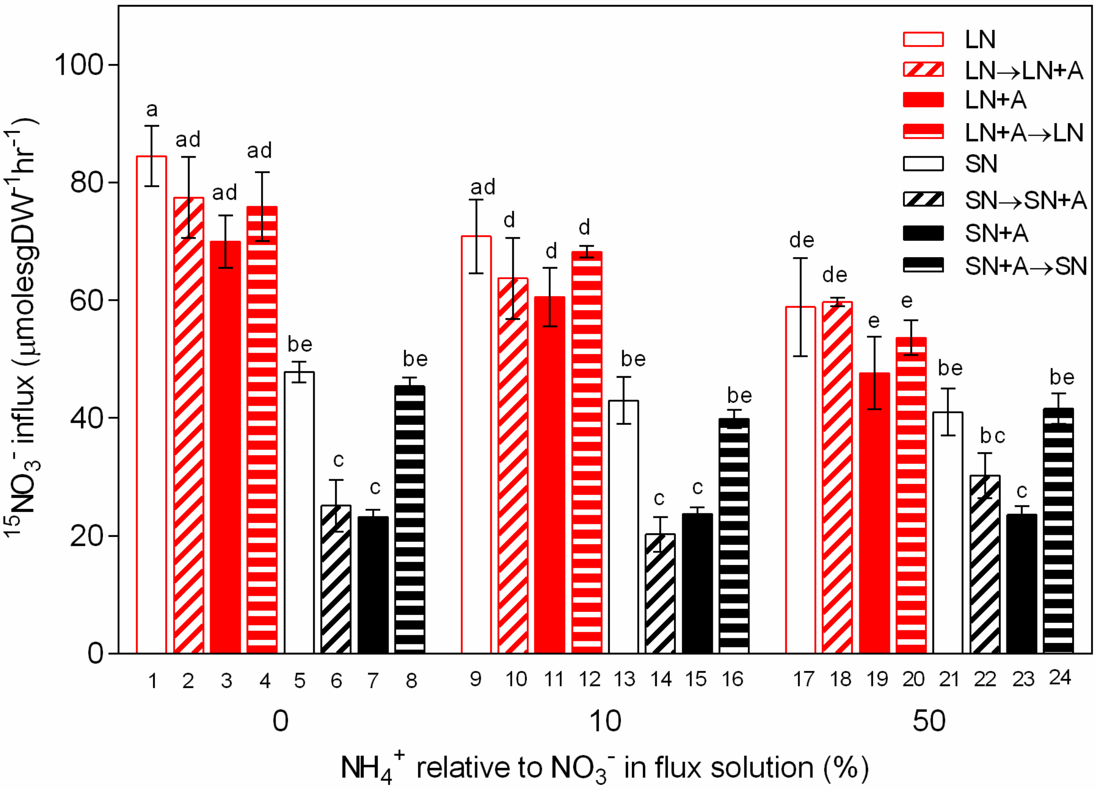


Figure 3: Nitrate uptake capacity measured at 500 µM for plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between NH_4^+ and non- NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM where n=4. Statistical analysis used an ordinary one way analysis of variance. Significant differences at $P<0.05$ are represented by different letters for each group of bars.

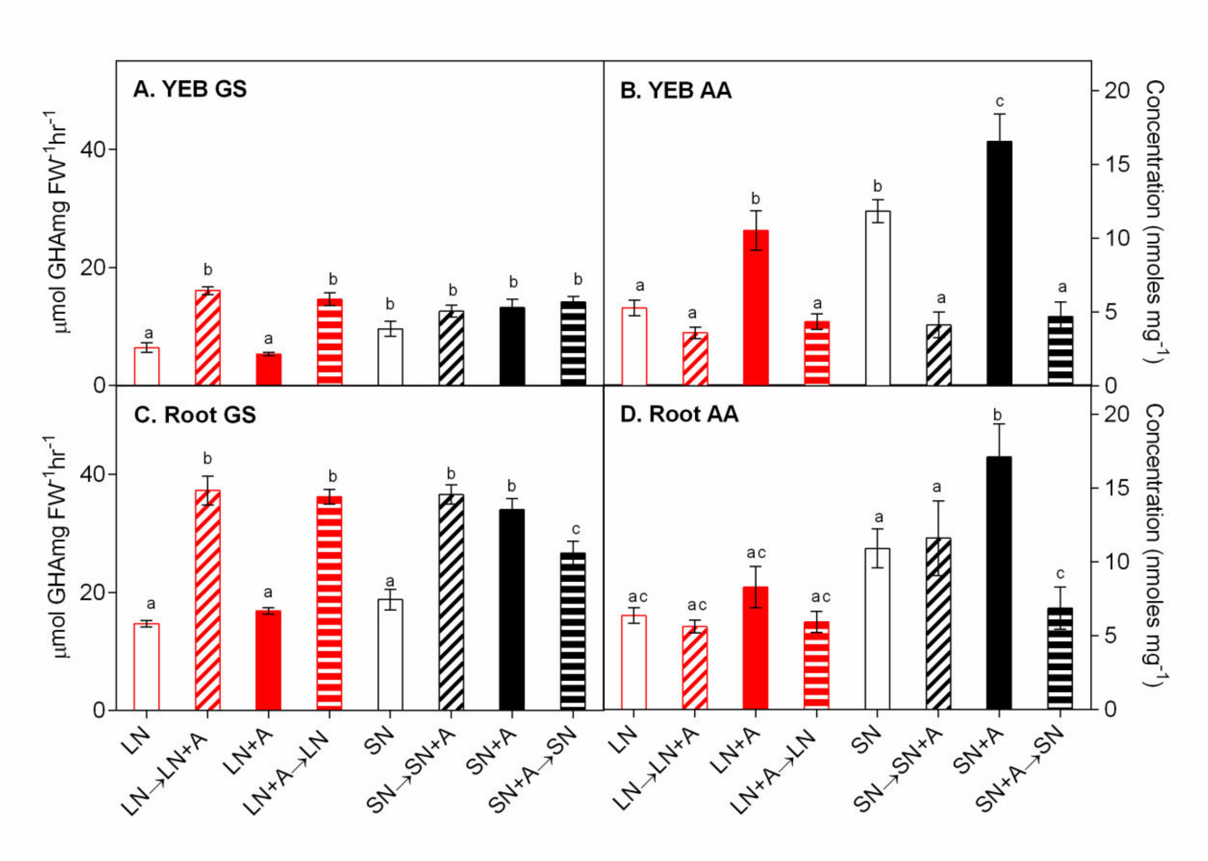


Figure 4: Glutamine synthetase activity and amino acid in the YEB (A & B) and root (C & D) of plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between $NH_4^+$and non-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM where n=4. Statistical analysis used an ordinary one way analysis of variance. Significant differences at $P<0.05$ are represented by different letters.

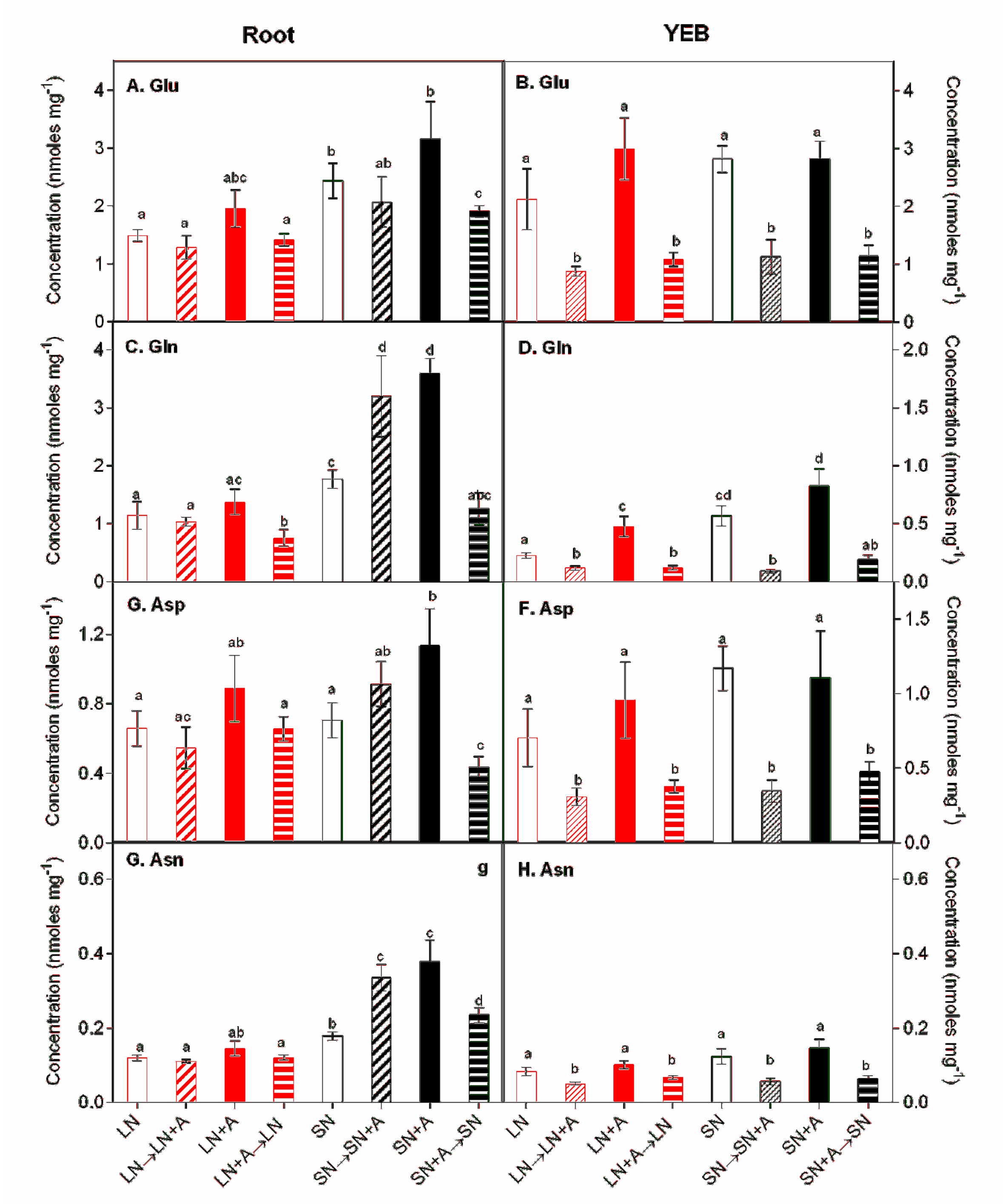


Figure 5: Glutamine (A & B), asparagine (C & D), glutamate (E & F) and aspartate (G & H) in the roots of plants grown in in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between $NH_4^+$and non-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM where n=4. Statistical analysis used an ordinary one way analysis of variance. Significant differences at P<0.05 are represented by different letters.

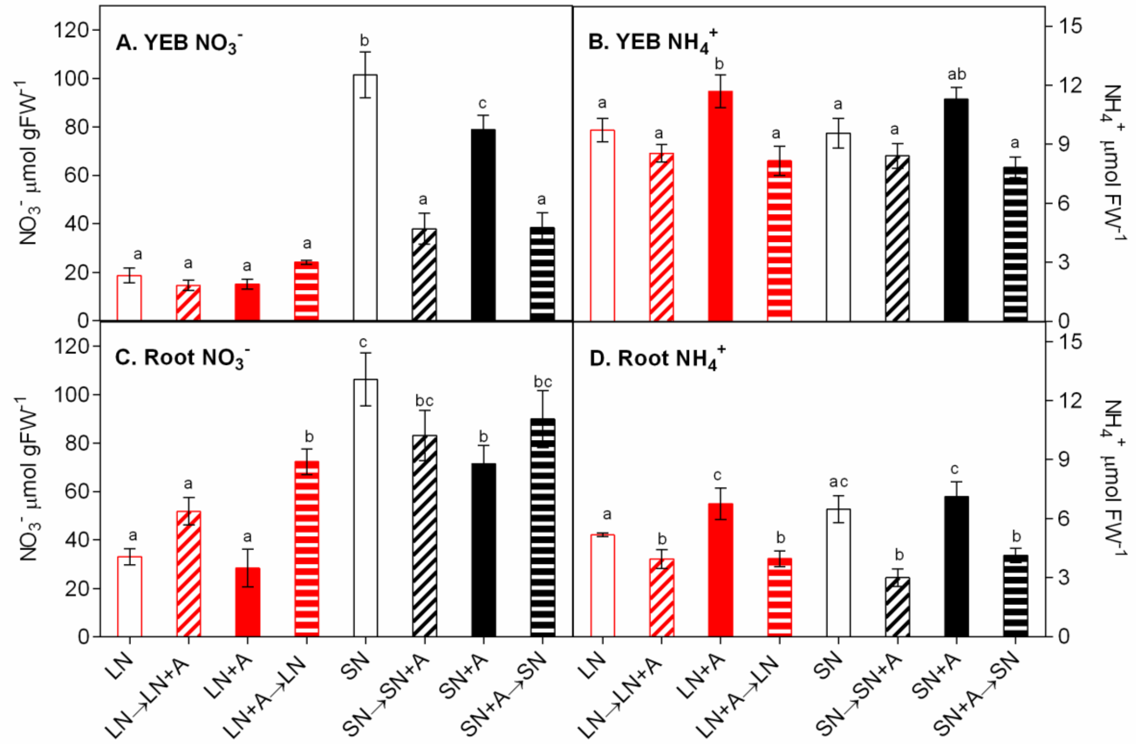


Figure 6: Nitrate and NH_4^+contents in roots and YEB of plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between $NH_4^+$and non-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM where n=4. Statistical analysis used an ordinary one way analysis of variance. Significant differences at P<0.05 are represented by different letters.

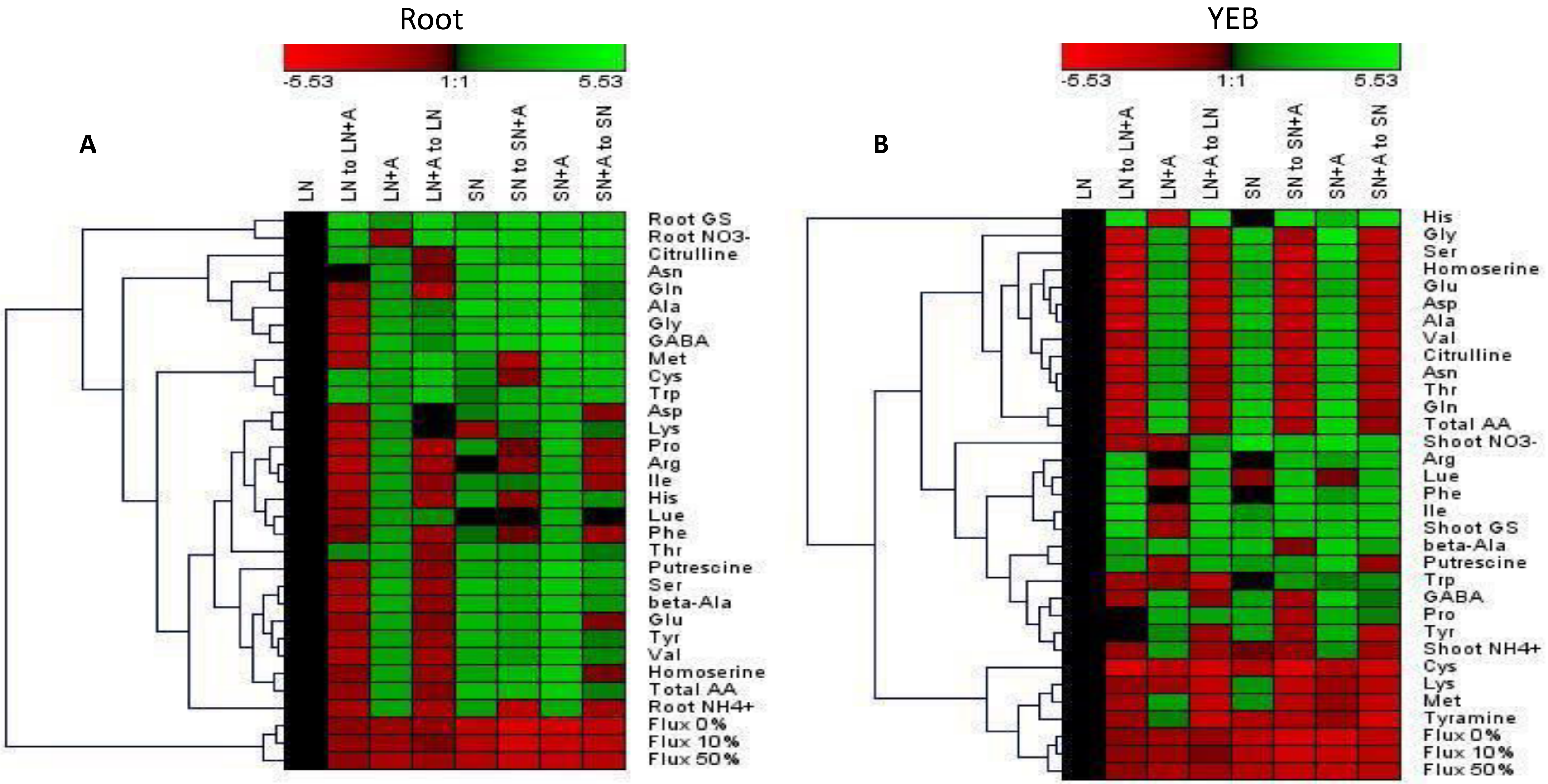


Figure 7: Various amino acid concentration in the roots and YEB of plants grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^- (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25mM NH_4^- (SN+A) and when there were switched between NH_4^+ and no-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are fold changes relative to the measurements in LN.

Supplementary Figures

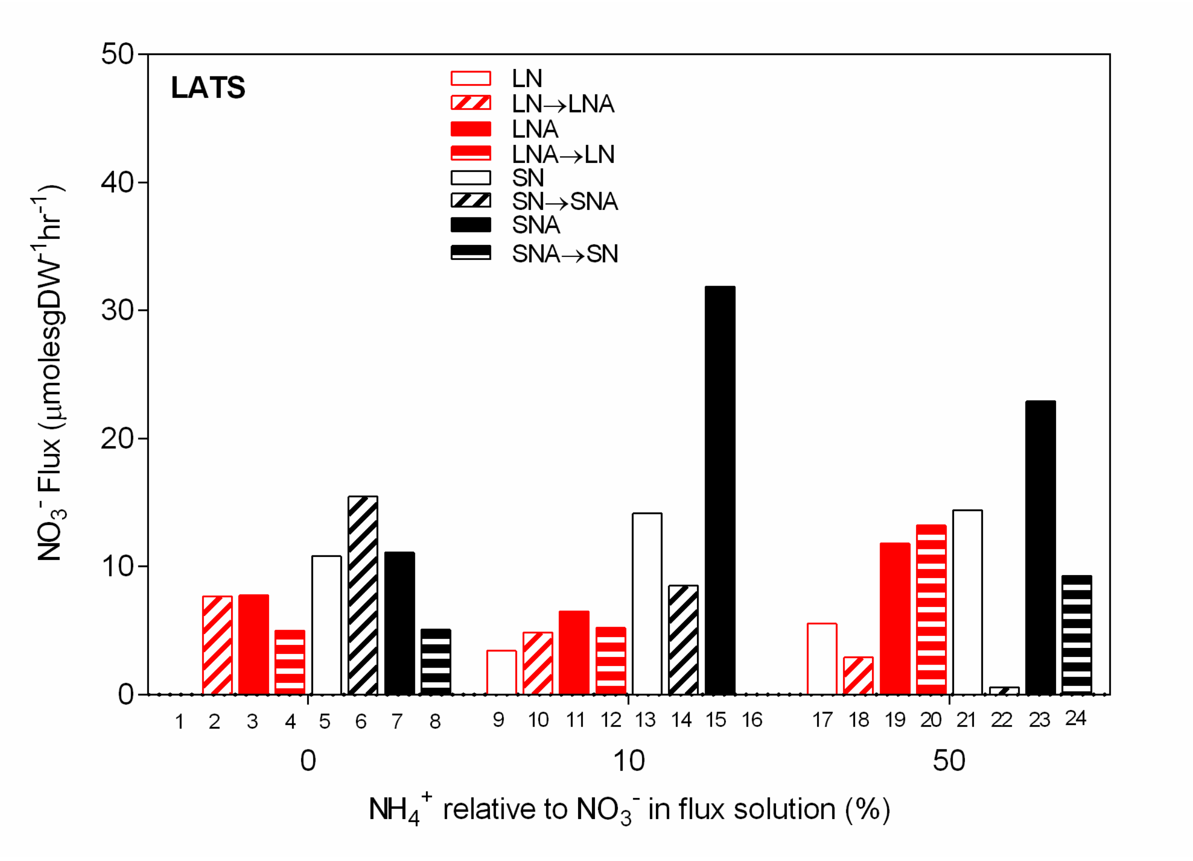


Figure S1: Nitrate Uptake capacity measured at 2500 µM of plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between $NH_4^+$and non-NH_4^+ treatments on 22 DAE. The flux solution contained 0%, 10% and 50% NH_4^+ relative to NO_3^- . The data were collected on 24 DAE. Values are means ± SEM where n=4. Significant differences at P<0.05 are represented by different letters.

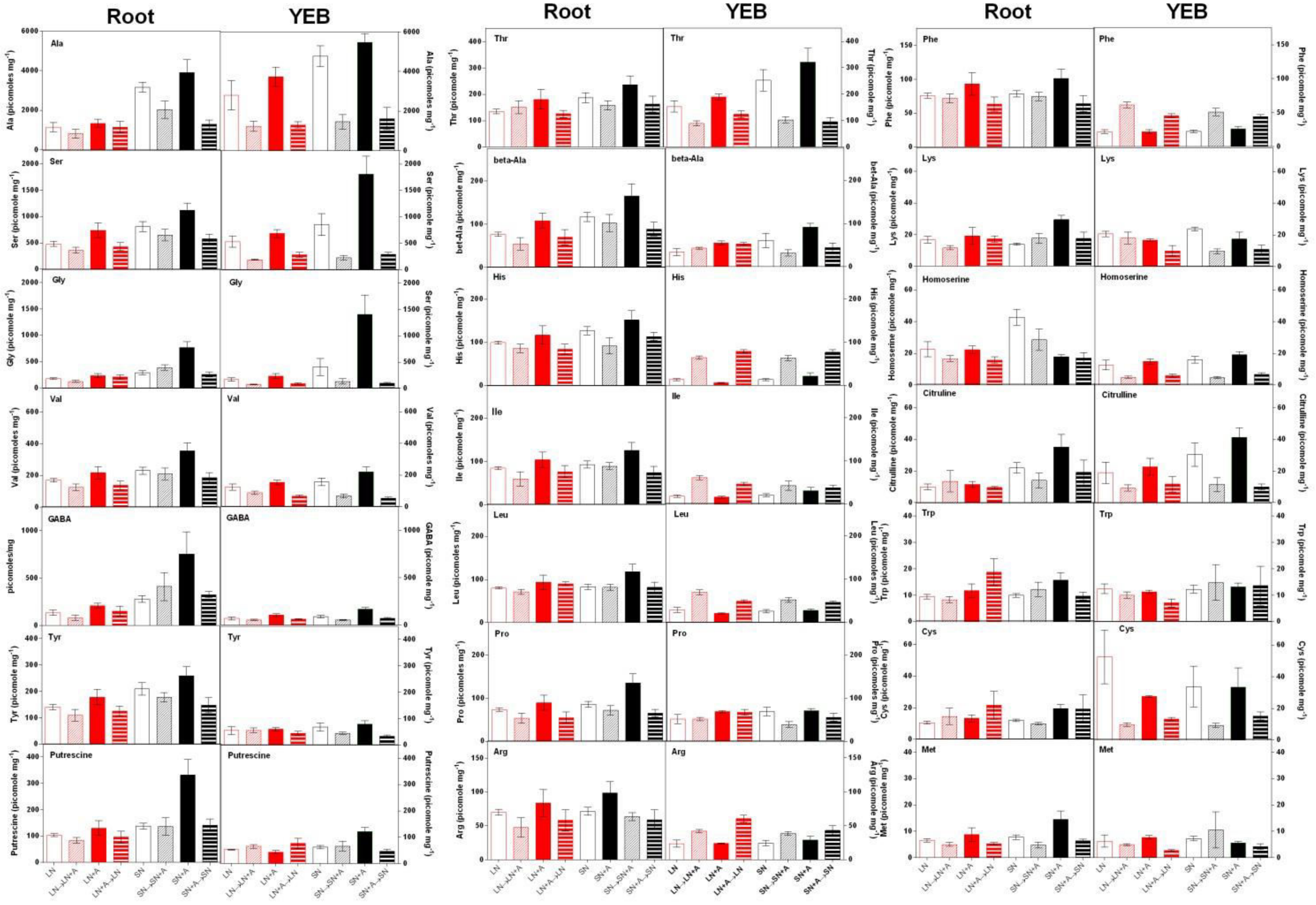
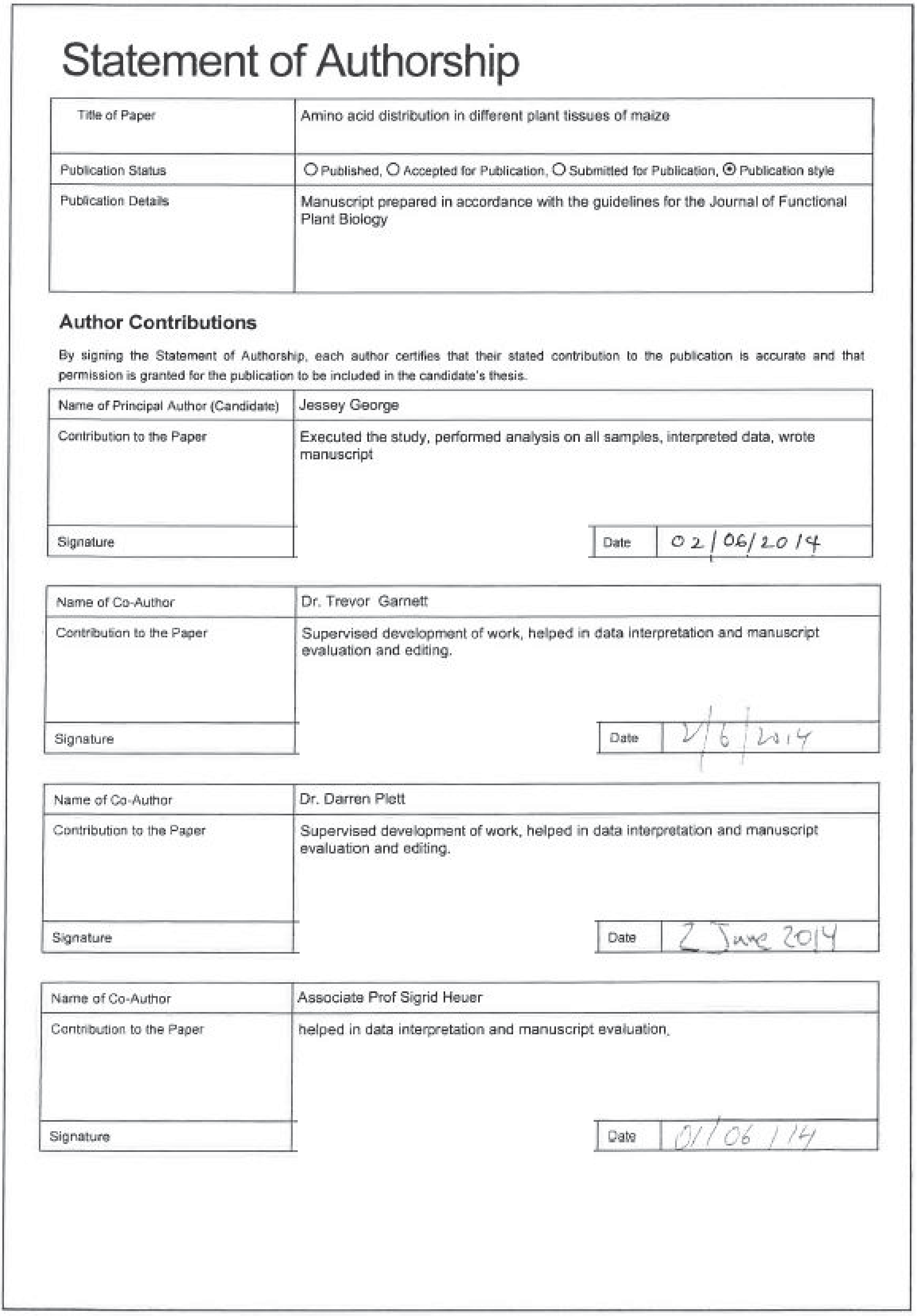


Figure S2: Concentration of all amino acids in roots and YEB of plants grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A) and when there were switched between $NH_4^+$and non-NH_4^+ treatments on 22 DAE. The data were collected on 24 DAE. Values are means ± SEM where n=4.

Chapter 5: Amino acid distribution in different plant tissues of maize (Zea mays L.).



Statement of Authorship

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Author Contributions

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Name of Principal Author (Candidate)	Jessey George		
Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Dr. Trevor Garnett		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation and editing.		
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Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation and editing.		
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Contribution to the Paper	helped in data interpretation and manuscript evaluation.		
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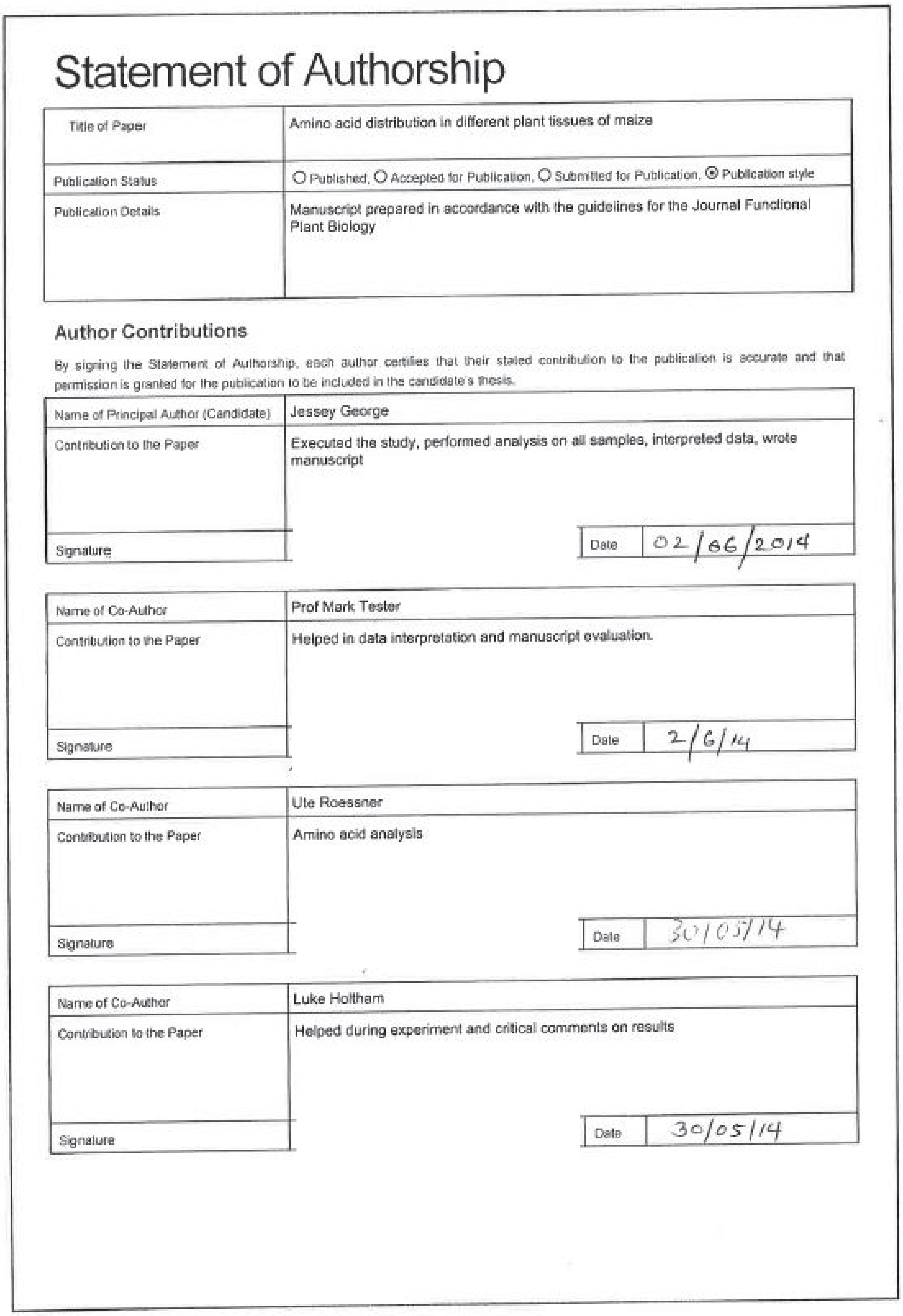

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Author Contributions

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Contribution to the Paper	Executed the study, performed analysis on all samples, interpreted data, wrote manuscript		
Signature		Date	02/06/2014

Name of Co-Author	Prof Mark Tester		
Contribution to the Paper	Helped in data interpretation and manuscript evaluation.		
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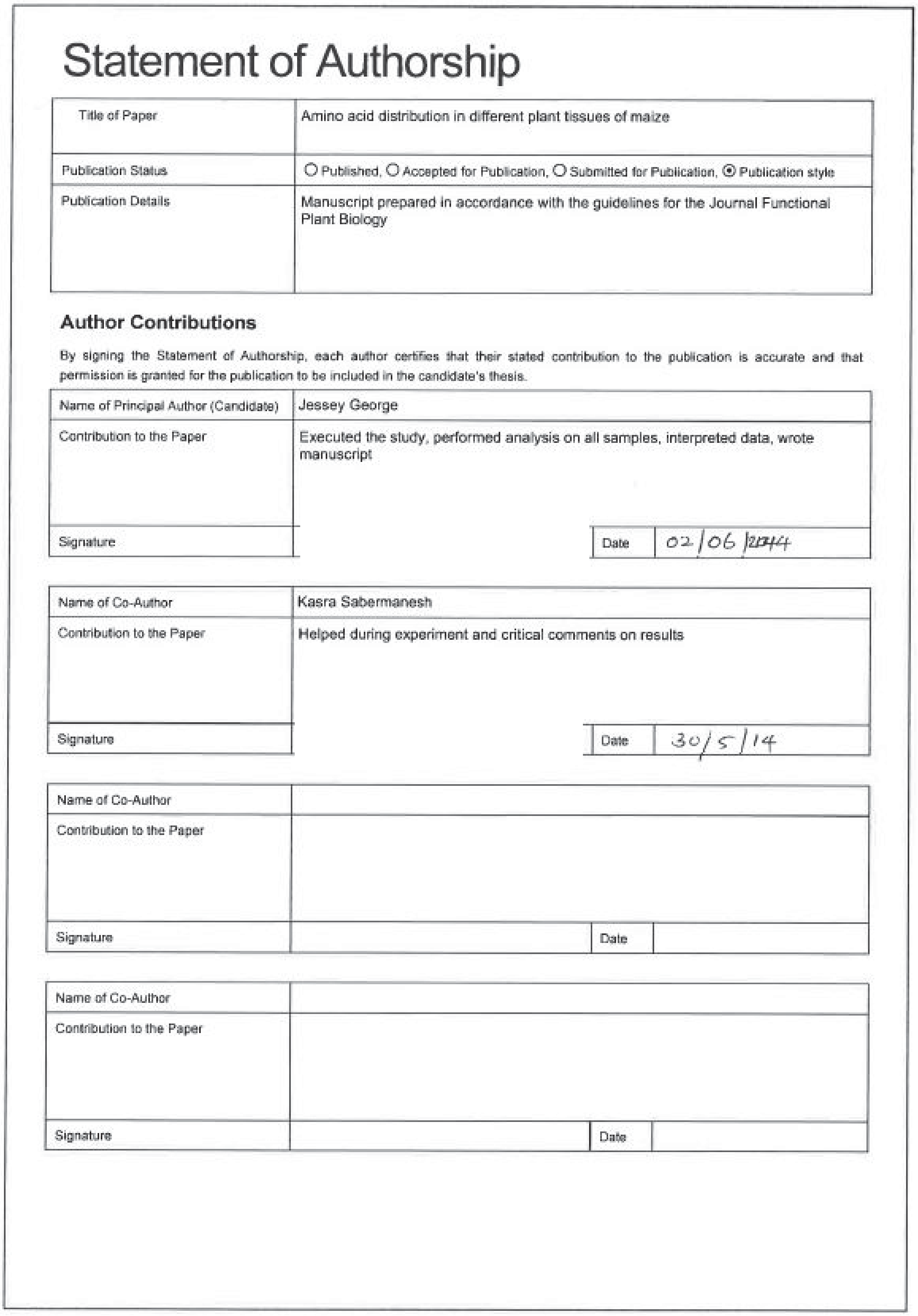
Name of Co-Author	Ute Roessner		
Contribution to the Paper	Amino acid analysis		
Signature		Date	30/05/14

Name of Co-Author	Luke Holtham		
Contribution to the Paper	Helped during experiment and critical comments on results		
Signature		Date	30/05/14



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Contribution to the Paper	Helped during experiment and critical comments on results		
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Title

Amino acid distribution in different plant tissues of maize

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ABSTRACT

Most studies of amino acids in plant tissue report amino acid concentrations in roots and shoots or root and a single representative leaf such as youngest expanded blade (YEB). There are few reports of how these amino acids are distributed in various shoot tissues. Here we measured the amino acid concentrations (and subsequently calculated contents) in various plant tissues of maize inbred line B73. Plants were grown in low and sufficient levels of NO_3^- with and without 10% NH_4^+ to better understand how tissue levels change with N availability. The highest amino acid content was in the youngest leaf and lowest was observed in the oldest leaf. Both stem and roots also had much higher amino acid content than old leaves. The total amino acid content in roots, stem and youngest leaf of plants grown in 10% NH_4^+ with sufficient N was highest of the N treatments. Glutamate was the amino acid that was measured in highest levels in the roots and youngest leaf of plants in all the treatments. However, plants that were grown in sufficient N with 10% NH_4^+ had higher glutamine content in their roots compared to glutamate. It was also observed that older leaves had very low contents of amino acids compared to YEB and youngest leaf. Contents of amino acids like glutamine, arginine and histidine were found to be in very low in leaves compared to their contents in the root and stem. This study showed that, although the YEB (leaf 4 in this study) had lower levels of amino acids than the youngest leaf (leaf 5), it still correlates more closely with leaf N status and is more useful for distinguishing between N treatment effects, than the whole shoot.

Key words: amino acids, asparagine, glutamine, youngest expanded blade, glycine, serine

INTRODUCTION

Nitrogen (N) is one of the major nutrients required by plants for growth and development. Plants absorb N mainly in the form of nitrate (NO_3^-) and ammonium (NH_4^+). Nitrate is the dominant form of N present in most agricultural soils with the NH_4^+ concentration generally being 10% of the NO_3^- concentration (Wolt, 1994). After NO_3^- is taken up by plants it is either assimilated in the roots or translocated to the shoots in the xylem via transpiration stream (Andrews, 1986). On the other hand, NH_4^+ is primarily assimilated in the cytosol of the roots unless it is supplied at high concentrations (Murphy & Lewis, 1987). The first products of organic N assimilatory pathway are the amino acids glutamine and glutamate. These amino acids are the precursors of other amino acids and nitrogenous compounds such as proteins (Oaks, 1994). Amino acids have been referred to as the currency of N exchange in plants (Coruzzi & Bush, 2001).

Although there are many published studies reporting amino acid concentrations in root and shoots, or roots and representative shoot tissues (i.e. the youngest expanded blade) there is little published information on the distribution of amino acid in various tissues including root and shoot. In order to know the N status of a plant, most researchers measure YEB N content rather than the whole shoot as this should better reflect the current N status of the plant (Reuter & Robinson, 1997). Whole shoot analysis would misrepresent the N status of younger leaves because of a dilution effect by the old tissue where N is remobilised to younger tissue (Mae & Ohira, 1981, Masclaux-Daubresse, Daniel-Vedele, Dechorgnat, Chardon, Gaufichon & Suzuki, 2010). However, it is unknown whether information is lost when only YEB is measured and answering this query is the basis for this study. N remobilisation can greatly reduce N levels in older, but the rate of remobilisation is reduced when N levels are higher (Ono, Terashima & Watanabe, 1996), hence we included N treatments in our investigation.

Plants grown with NH_4^+ generally have greater tissue free amino acid content than NO_3^- fed plants (Causin & Barneix, 1993) because of preferential and faster uptake and assimilation of NH_4^+. Ammonium assimilation entails the incorporation of NH_4^+ into organic N. Our earlier studies have shown that 10% NH_4^+ along with NO_3^- in the nutrient solution increased shoot dry matter content in maize at certain stages of development and this was associated with an increase in the total free amino acid in roots of these plants (George, Sabermanesh, Holtham, Roessner, Bauman, Brian, Timmins, Heuer, Tester, Plett & Garnett, 2014). The experiments are focussed on the influence of NH_4^+.

This study quantified the distribution of individual amino acids in different plant tissues at the V5 stage in maize inbred line B73. We analysed the response to 10% NH_4^+ at low N and sufficient N level to clarify whether N supply affected the AA distribution.

MATERIALS AND METHODS

Plant material and growth conditions

Maize inbred line B73 was grown in a hydroponic growth solution containing two total N concentrations: low (0.55 mM) and sufficient N (2.75 mM). Plants were grown in four treatments namely: 0.55 mM NO_3^- (LN), 0.5 mM NO_3^- with 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.5 mM NO_3^- with 0.25 mM NH_4^+ (SN+A). Seeds were aerated overnight in RO water then placed on a filter paper moistened with 0.5 mM $CaCl_2$ solution and germinated at 28°C. Germinated seedlings were transplanted to one of eight 120 L ebb and flow hydroponic systems with fill and drain cycles of 15 min in a climate controlled growth chamber providing a day/night temperature of 26/22°C and a photoperiod of 14 h.

Photon flux density in the growth chamber was approximately 550 µmol m^{-2} s^{-2} at the average leaf height. Plants were grown on mesh collars in tubes as explained by Garnett et al (2013). The nutrient solution used was Johnson's modified nutrient solution which contained (in mM) 1.8 K, 0.6 Ca, 0.5 Mg, 1 S, and 0.5 P. Both treatment solutions contained (in µM) 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 200 Fe (as FeEDTA and FeEDDHA) (Johnson, Stout, Broyer & Carlton, 1957). Iron was supplemented twice weekly with the addition of $FeSO_4$ (8 mg l^{-1}). $(NH_4)_2SO_4$ was used as the NH_4^+ supplement to the NH_4^+ treatments. Solution pH was monitored daily and maintained between 5.9 and 6.0. NO_3^- and NH_4^+ concentrations in the solutions were monitored using a NO_3^- and NH_4^+ electrodes (TPS, Springwood, Australia) and maintained at the target concentration of ± 5%, nutrient solutions were changed weekly. The fresh samples were harvested into liquid N between 11am and 1pm 24 d after emergence (DAE) and stored at -80 °C. The plant parts harvested were: the oldest leaf (Leaf 1), second oldest leaf (Leaf 2), third oldest leaf (Leaf 3), YEB (Leaf 4), youngest leaf (Leaf 5), stem and root.

Amino acid determination

Approximately 100 mg of ground samples were measured and freeze dried. Tissue amino acid concentration was determined using liquid chromatography electrospray ionization-mass spectrometry, as described by Boughton et al. (2011), once the samples had been derivatized following the method of Cohen & Michaud (1993).

Statistical Analysis

Statistical analysis of all the data was completed using two-way analysis of variance using GraphPad Prism software. (Version 6.00, 1992-2012 GraphPad Software, Inc).

RESULTS

No effect of N treatment for fresh weights of leaf 1–4 or roots was observed (Figure 1). A significant increase in stem biomass was observed for the plants grown in LN+A, SN and SN+A compared to LN. There was an increase of biomass for the youngest leaf (leaf 5) in LN+A compared to LN plants and the biomass of youngest leaves in SN and SN+A were higher than that for plants in LN and LN+A (Figure 1).

It was observed that plants grown in both sufficient N treatments (SN and SN+A) had higher shoot N concentration and total N compared to plants that were grown in low N treatments (LN and LN+A) (Figure S1A & B). Root N concentration was also higher in both sufficient N treatments and no difference was observed with 10% NH_4^+ at both low and sufficient N levels (Figure S1C). However net N uptake was higher for plants in SN+A compared to all other treatments and SN had higher net uptake compared to both the low N treatments (Figure S1D).

The highest total amino acid concentration was in the youngest leaf, and all other parts showed not much variation except for leaf 1 (Figure 2A). Compared to all other treatments, SN+A had higher total free amino acid concentration in all the plant parts (Figure 2A) except in leaf 2 and 3. However, in the stem and in YEB (Leaf 4) a higher amino acid concentration was measured in LN+A compared to LN plants. We can also see that oldest leaf (Leaf 1) had more amino acids concentration in LN and SN+A than leaf 2 and 3 (Figure 2A). Total free amino acid contents were generally higher in roots, stems and youngest leaves of plants across the treatments compared to older leaves (Figure 2B). Amino acid content in older leaves was lower compared to YEB and the youngest leaf. Similar to concentration, the highest content of total free amino acids was measured for plants grown in SN+A compared to other treatments in most plant parts. However, plants in

LN+A and SN+A had higher amino acid content compared to LN and SN, respectively, in the stem and the youngest leaf (Leaf 5). Although fewer differences between treatments in amino acid content were observed in older leaves the highest content of total amino acids in Leaf 1 and Leaf 2 was in LN plants and in leaf 3 it was highest in the SN plants.

Individual amino acids were grouped together in subsequent figures according to their biosynthetic pathways. Majority of amino acids derived from α-ketogluterate are in roots and stem and only proline and glutamate had higher content in the youngest leaf (Figure 3). We can also see that all these amino acids were higher in stem of plants grown in LN+A compared to LN (Figure 3). Glutamate was generally the amino acid with highest content in roots and youngest leaf (Leaf 5) except for roots of plants in SN+A where higher glutamine content was observed (Figure 3A). No difference in the glutamate content was measured between NH_4^+ and non- NH_4^+ treatments both at low and sufficient levels of N in all plant parts except in the stem where the AA content of plants in LN+A was higher compared to LN (Figure 3B). Proline content of youngest leaf of plants in SN and SN+A was higher than those in low N treatments and the highest was seen in SN+A (Figure 3D). Arginine content was higher in roots and stem compared to leaves (Figure 3C). Histidine content in stems of plants grown in LN+A, SN and SN+A was higher than in LN and no treatment difference was seen in all the other plant parts (Figure 3E). Glutamine concentration in the leaves also showed similar trend like its total contents (Figure S3A).

However no variation was observed with glutamate content between treatments Majority of amino acids derived from oxaloacetate are found in the root and stem except for asparagine, aspartate and threonine which also were high in youngest leaf (Leaf 5) (Figure 4). The highest content of asparagine was observed in root, stem and youngest leaf of plants grown in SN+A (Figure 4A). However, the concentration of asparagine (nmoles/mg) in the oldest leaf is higher than in the younger leaves (Figure S3C). Majority of aspartate was found to be in root, stem and youngest leaf (Figure 4B). It was higher in stem of plants in LN+A compared to LN and an increase was seen in the youngest leaf of plants in sufficient N treatments compared to low N treatments. Isoleucine and threonine contents were higher in stem of plants in LN+A compared to LN and in the youngest leaf of plants in SN+A compared to SN (Figure 4C & D). Higher lysine and methionine contents were measured in the stem of plants in LN+A, SN and SN+A compared to LN (Figure 4E & F).

Amino acids derived from pyruvate (Figure 5) were higher in root and stem, while alanine (Figure 5A) was also found in young leaves, especially in plants grown in SN and SN+A compared to LN and LN+A (Figure 5A). Valine (Figure 5B) and leucine (Figure 5C) were measured in low content in leaves.

Of the amino acids derived from 3-phosphoglycerate (Figure 6), serine and glycine contents were higher in stem and youngest leaf compared to other plant parts (Figure 6A & B). A higher concentration of cysteine was observed in YEB (Leaf 4) and the youngest leaf (Leaf 5) (Figure 6C).

The contents of tyrosine, phenylalanine and tryptophan (those derived from phosphenolpyruvate), were higher in roots and stem than in leaves (Figure 7A, B & C) and there was almost no tryptophan in leaves. Tyrosine content was higher in the youngest leaf (leaf 5) of plants in sufficient N treatments than in low N treatments (Figure 7A). It can be observed that the root to stem ratio of tyrosine is higher compared to phenylalanine and tryptophan. A significant increase in these amino acids can be seen in the stem of plants in LN+A compared to LN.

Most of the secondary amino acids measured (Figure S2) showed a similar pattern of tissue distribution to primary amino acids. Putrescine (Figure S2A) and GABA (Figure S2B) were measured in higher content in roots when NH_4^+ was supplied. Higher concentration of citrulline was found in youngest leaf and highest content was in the plants grown in SN+A (Figure S2C). Beta-alanine (Figure S2D) and homoserine (Figure S2E) had higher contents in the roots, stem and youngest leaf. Treatment difference was observed in the youngest leaf for citrulline and beta-alanine and root for homoserine. Not much variation in tyramine content was observed between different plant parts except for youngest leaf of plants in SN+A which showed a higher content compared to all other plant parts (Figure S2F)

DISCUSSION

This study was done to discover whether we are missing important variation, especially with regards to N treatment effects, if we only analyse a representative sample from the shoot, such as the YEB. We observed that total amino acid content is higher in the youngest leaf than in other plant parts. It should also be noted that, as well as having the highest levels, treatment differences were more prominent in the youngest leaf (Leaf 5) and less so in the youngest expanded blade (YEB). The youngest leaf was expected to have higher amino acids given that it is the largest sink for N and other nutrients and assimilates (Mae & Ohira, 1981).

Other than the youngest leaf, root and stem also showed high accumulation of amino acids. Higher content of total free amino acids in stem could be due to translocation of amino acids from roots and senescing leaves to developing leaves (Riens, Lohaus, Heineke & Heldt, 1991). Most of the individual amino acids also showed higher contents in stem and roots than leaves. Other than leaves root is also the site for N assimilation and all NH_4^+, absorbed by plant are assimilated in roots (Andrews, 1986, Murphy & Lewis, 1987). This may have contributed to the higher content of amino acids in the roots. The higher content of amino acids in the stem of these plants is supported by a study which showed that 40% of grain protein is developed from the amino acids from the stem (Simpson & Dalling, 1981). Amino acids Gln, Arg, His, Ile, Val, Leu, Tyr, Phe and Trp showed very low contents in leaves compared to stem indicating that taking measurements in YEB as the representative organ for the leaves may not give us a good understanding of the plant N status in relation to these amino acids. Amino acids synthesised in the roots are transported to the shoots in xylem via the transpiration stream and amino acids from N remobilization of senescing plant parts takes place through the phloem (Riens *et al.*, 1991). The stem in our study consisted of the leaf sheath and the leaf primordia which may also be a sink for the amino acids.

It was expected that older leaves of plants grown in sufficient N treatments would retain more N than low N treatments (Ono *et al.*, 1996). However, it was observed in this study that old leaves appeared to have lost most of their amino acids irrespective of the N treatment and have very low amino acid contents. Although amino acid content in older leaves is low compared to younger leaves the concentration data showed that amino acid concentration, especially asparagine, was higher in older leaves. This is consistent with protein degradation being higher in senescing leaves, and as asparagine is one of the major transport forms of N (Thomas, 1978). It could be the product of this degradation used for the translocation of remobilised N to the young sink leaves (Joy, 1988). Lower amino acid concentration in leaf 2 and 3 compared to the youngest leaf may be due to the stabilization of N in the form of protein in fully expanded leaves (Atilio & Causin, 1996). It was also found in rice that substantial amounts of N is lost from fully expanded young leaves as they act as a supplier of remobilised N (Mae & Ohira, 1981).

The concentration of N provided to plants in various treatments also appeared to affect the distribution of amino acids in different plant parts. Compared to low N treatments the sufficient N treatments had higher amino acid contents in the root, stem, YEB and youngest leaf indicating that taking measurements on YEB can give adequate information on the N status of the plants based on treatment. However, we can see that treatment differences in the content of many amino acids (glutamine, arginine, asparagine etc.) were not visible in YEB indicating that analysing YEB is useful when we are only looking at the total amino acid content and information on individual amino acid content may be missing from analysis of YEB alone. The contents of these amino acids were also very low in the older leaves compared to roots and stem, also indicating they would not provide representative data for amino acid analysis of shoots.

The form of N in the growth medium plays an important role in determining the constituents of free amino acids in plants (Atanasova, 2008, Causin & Barneix, 1993). In this study, a higher content of total free amino acids and glutamine were observed in plants grown with small amounts of NH_4^+ in the medium. Our earlier studies showed that amino acid concentrations in plants increase when they are supplied with a small amount of NH_4^+ (10%) at sufficient N levels (George *et al.*, 2014). Similarly, other groups have shown that high tissue amino acid contents when there is sufficient N in the medium and also when there is simultaneous supply of both NO_3^- and NH_4^+ compared NO_3^- alone (Atanasova, 2008, von Wirén & Merrick, 2004). Earlier studies have also shown that when plants are grown with NH_4^+, glutamine and asparagine make up the majority of free amino acids in roots and that they are the primary compounds used for transport of N inside the plants (Atanasova, 2008, Lea, Sodek, Parry, Shewry & Halford, 2007). Pate and his co-workers (1981) showed that asparagine formed the major amino acids in most plant parts. Although, our results do not show dominance of asparagine in all plant parts, roots, stem and young leaves of the plants grown in SN+A had nearly two-fold higher asparagine than all other treatments. It was also seen that proline, serine and glycine distribution in different plant parts were changed when NH_4^+ was supplied in the medium. This change was more visible in the youngest leaf and stem. This indicates that taking measurements on the YEB may miss important information on treatment effects.

Serine and glycine contents were very high in the youngest leaves of plants in SN+A compared to all other treatments. Serine and glycine are inter-convertible amino acids and are involved in the glycolytic pathway (Ongun & Stocking, 1965). They are also produced during photorespiration in photosynthetic leaves (Bourguignon, Rebeille & Douce, 1998) and are two important N metabolites in photosynthetic carbon metabolism (Wallsgrove, Keys, Lea & Miflin, 1983). A small amount of NH_4^+ increases the levels of these amino acids in younger leaves which may facilitate in synthesis of more photosynthetic carbon. Again, this indicates that if only the YEB was taken for measuring AA no difference in their content would have been observed between different treatments and important information on serine and glycine metabolism would be missed.

We have seen in this study that distributions of amino acids in different plant tissues vary considerably. This study showed that YEB is a good representative part to be taken from the plant shoot for quantifying amino acids in shoot, but that some treatment effects may only be discovered by looking at the younger leaves.

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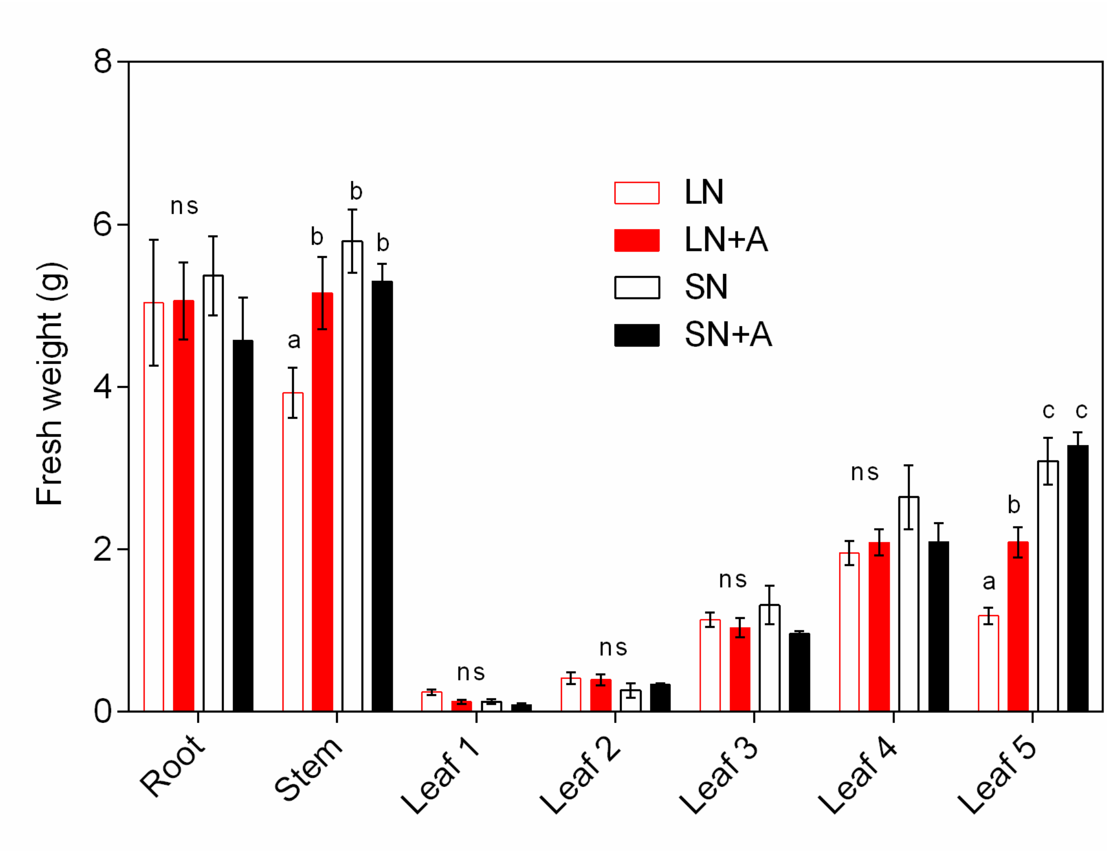

Figure 1: Fresh weight of different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are mean ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at P value <0.05 are represented by different letters for each group of bars.

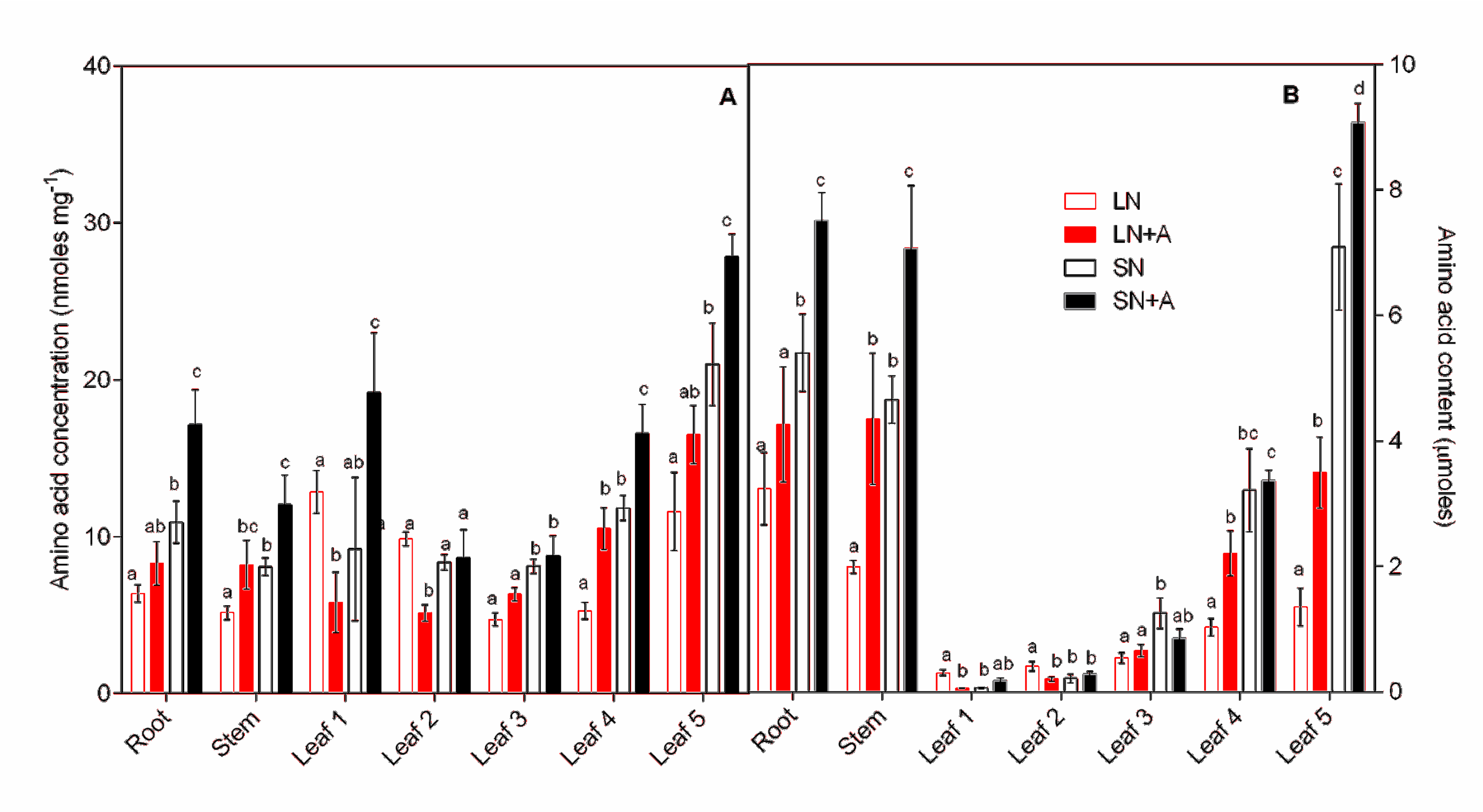

Figure 2: Total amino acid content (A) and total amino acid concentration (B) in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at $P<0.05$ are represented by different letters for each group of bars.

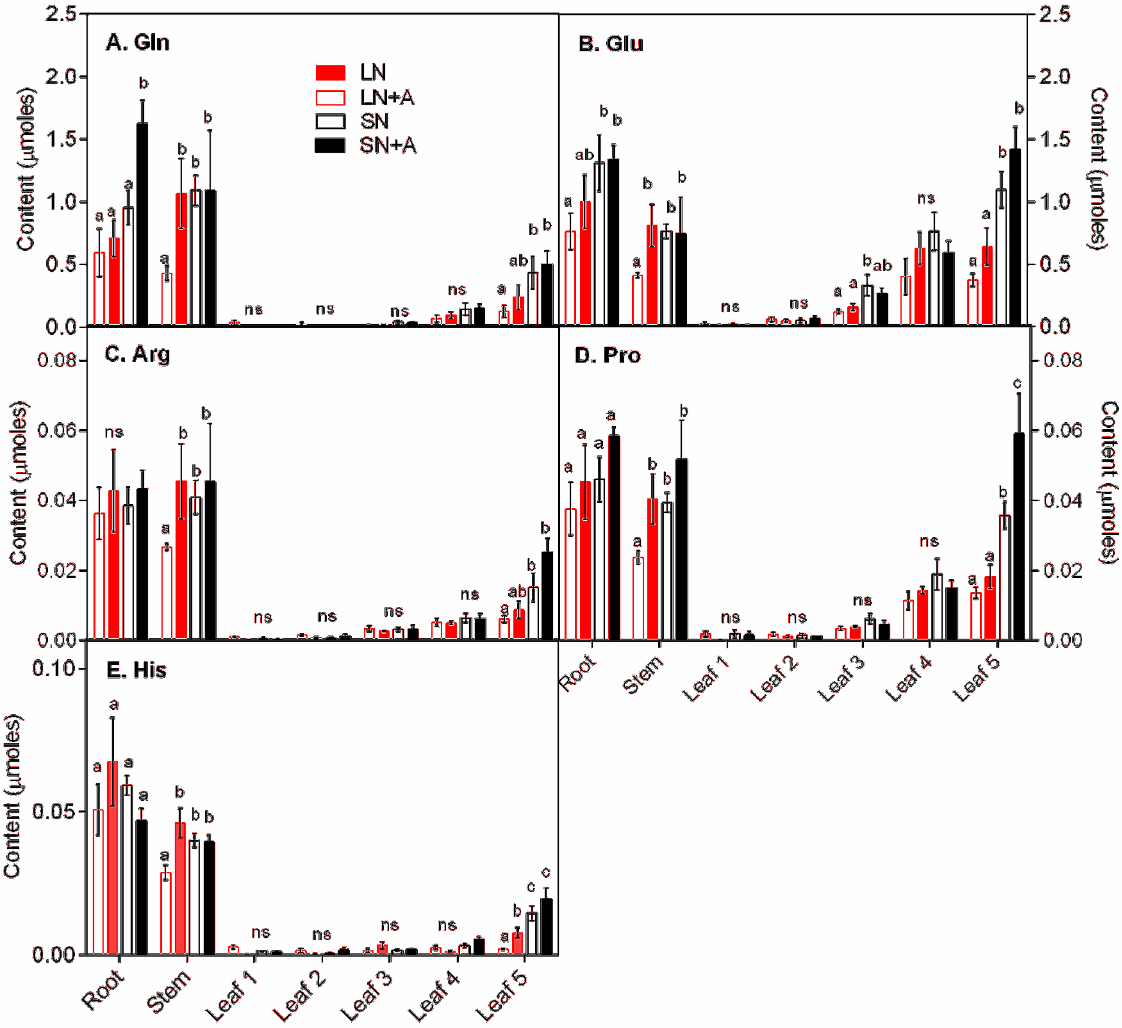

Figure 3: Amino acids in this figure are derived from α-ketogluterate. Glutamine (A), aspartate (B), arginine (C) proline (D) citrulline (E) and histidine (f) content in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). These amino acids are derived from organic acid α-ketogluterate. Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at P<0.05 are represented by different letters for each group of bars.

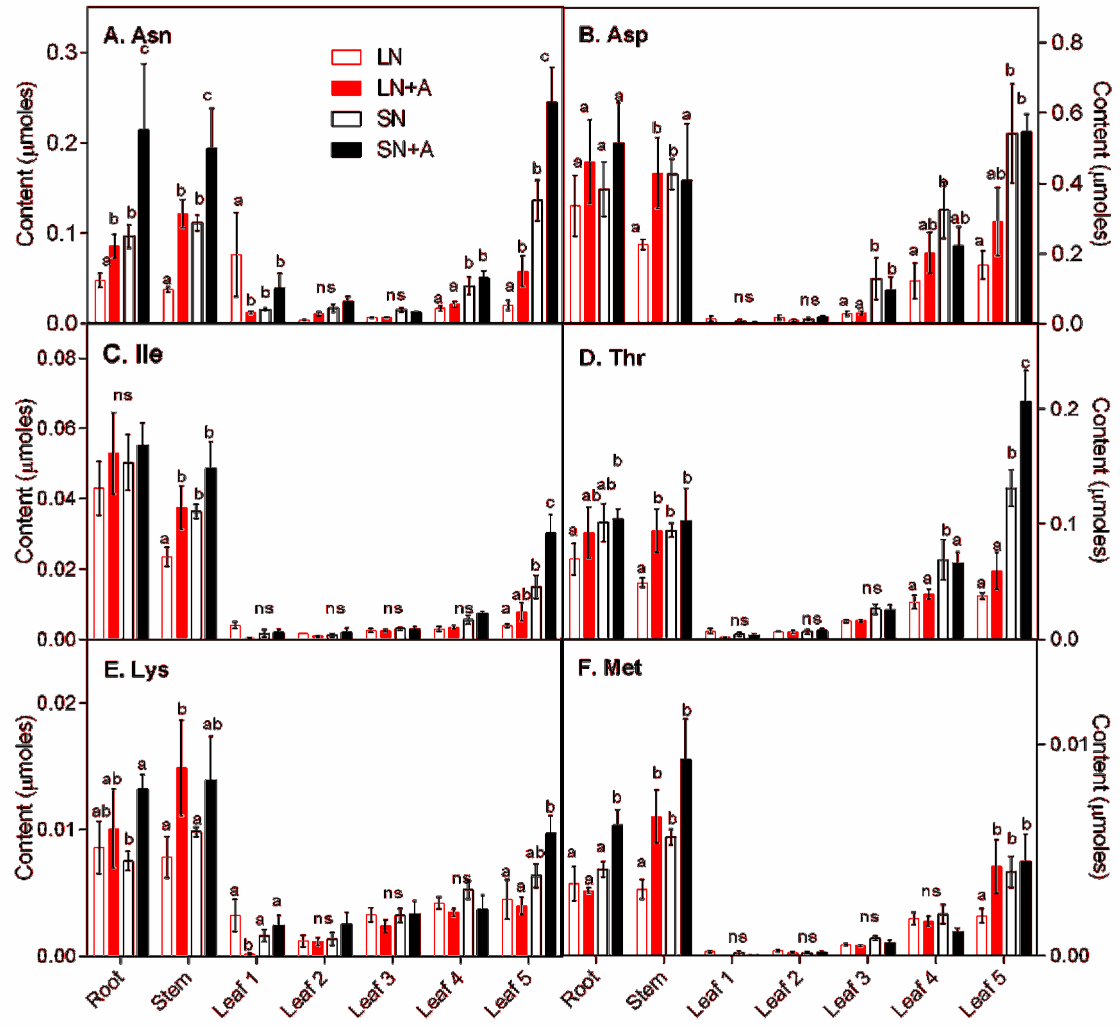

Figure 4: Asparagine (A), aspartate(B), isoleucine (C), threonine (D), lysine (E) and methionine (F) contents in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN), 0.50 mM NO_3^- + 0.05 mM NH_4^- (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^- (SN+A). These amino acids are derived from organic acid oxaloacetate. Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at P<0.05 are represented by different letters for each group of bars.

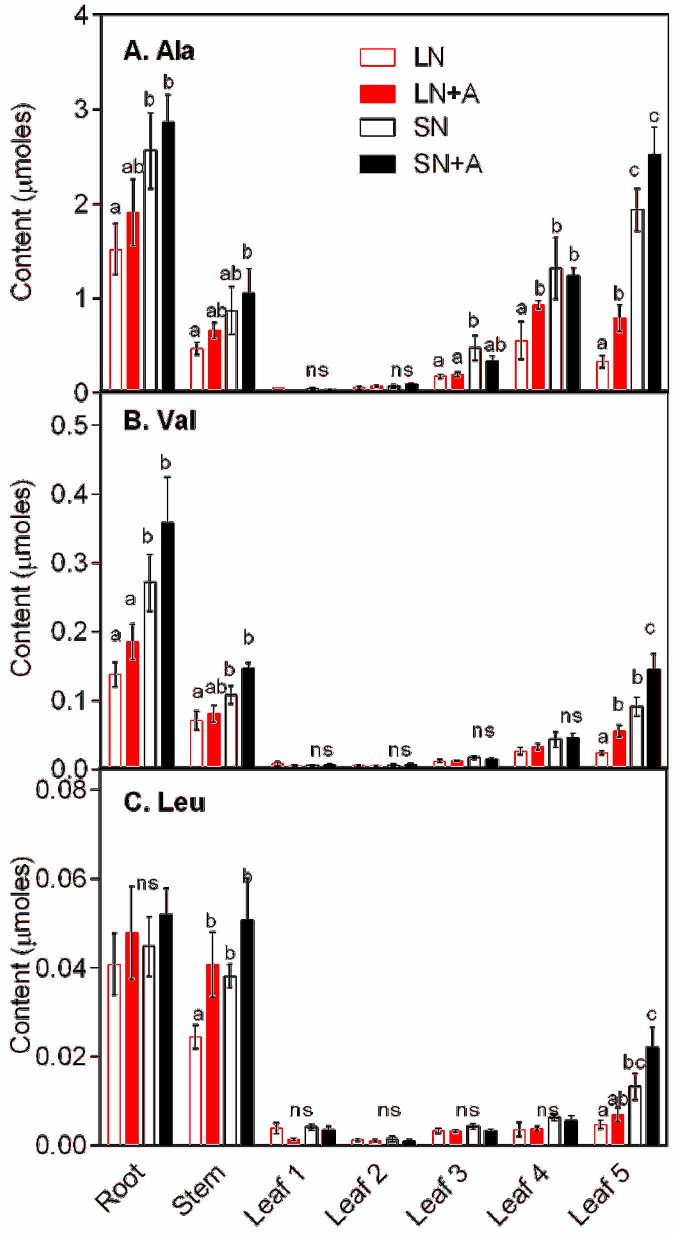

Figure 5: Alanine (A), valine (B), and leucine (C) contents in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). These amino acids are derived from the organic acid pyruvate. Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at P<0.05 are represented by different letters for each group of bars.

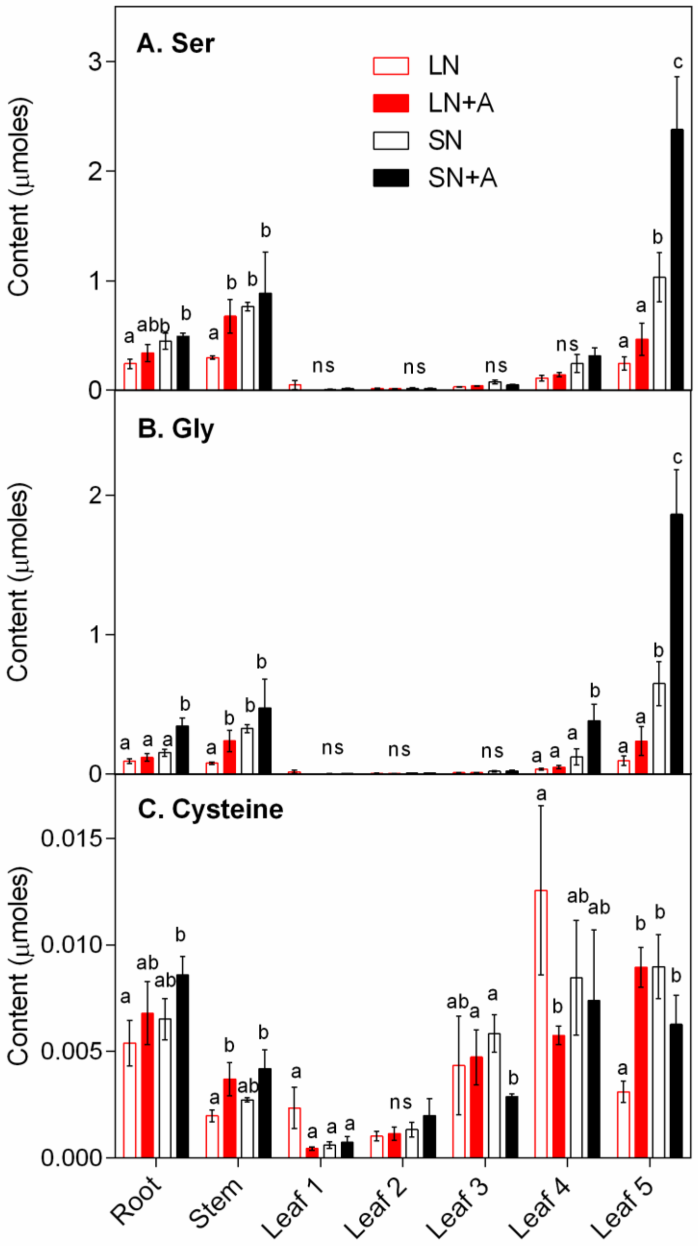

Figure 6: Serine (A)), glycine (B) and cysteine (C) contents in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). These amino acids are derived from 3-phosphoglycerate. Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at P<0.05 are represented by different letters for each group of bars.

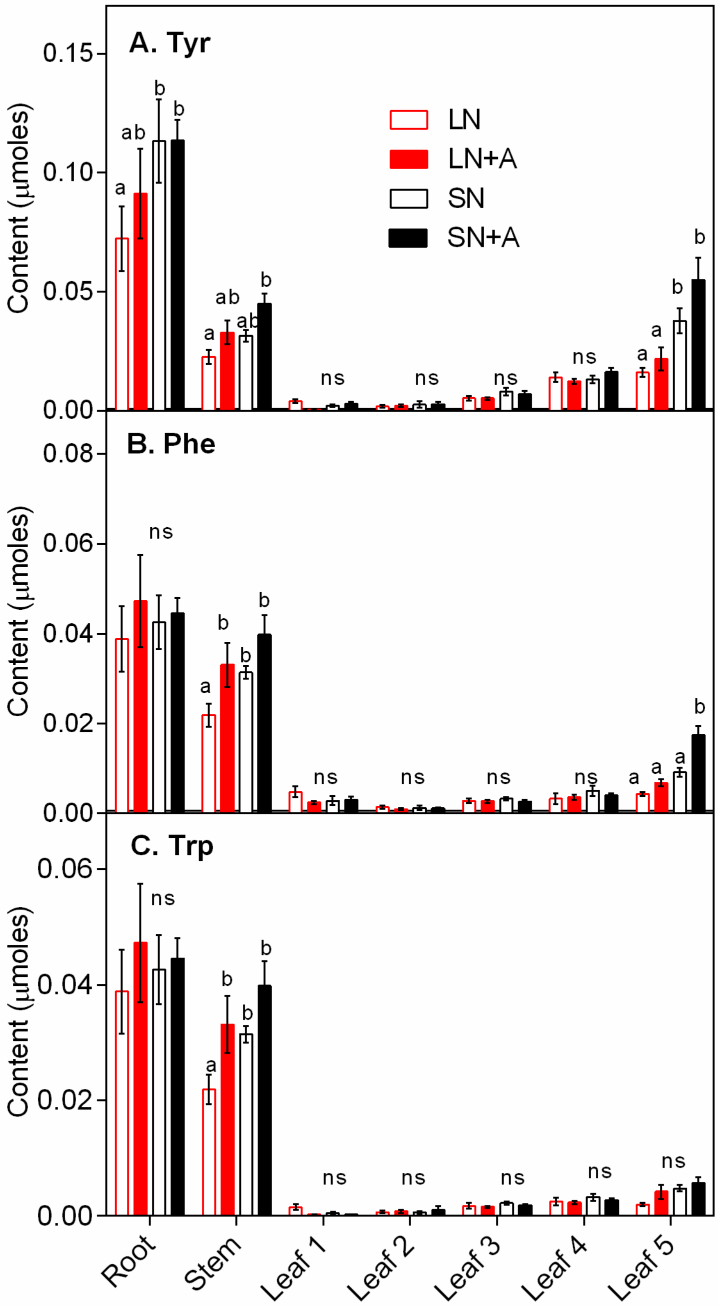

Figure 7: Tyrosine (A), phenylalanine (B), and tryptophan (C), contents in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). These amino acids are derived from the organic acid phosphenolpyruvate. Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at $P<0.05$ are represented by different letters for each group of bars.

Supplementary Figures

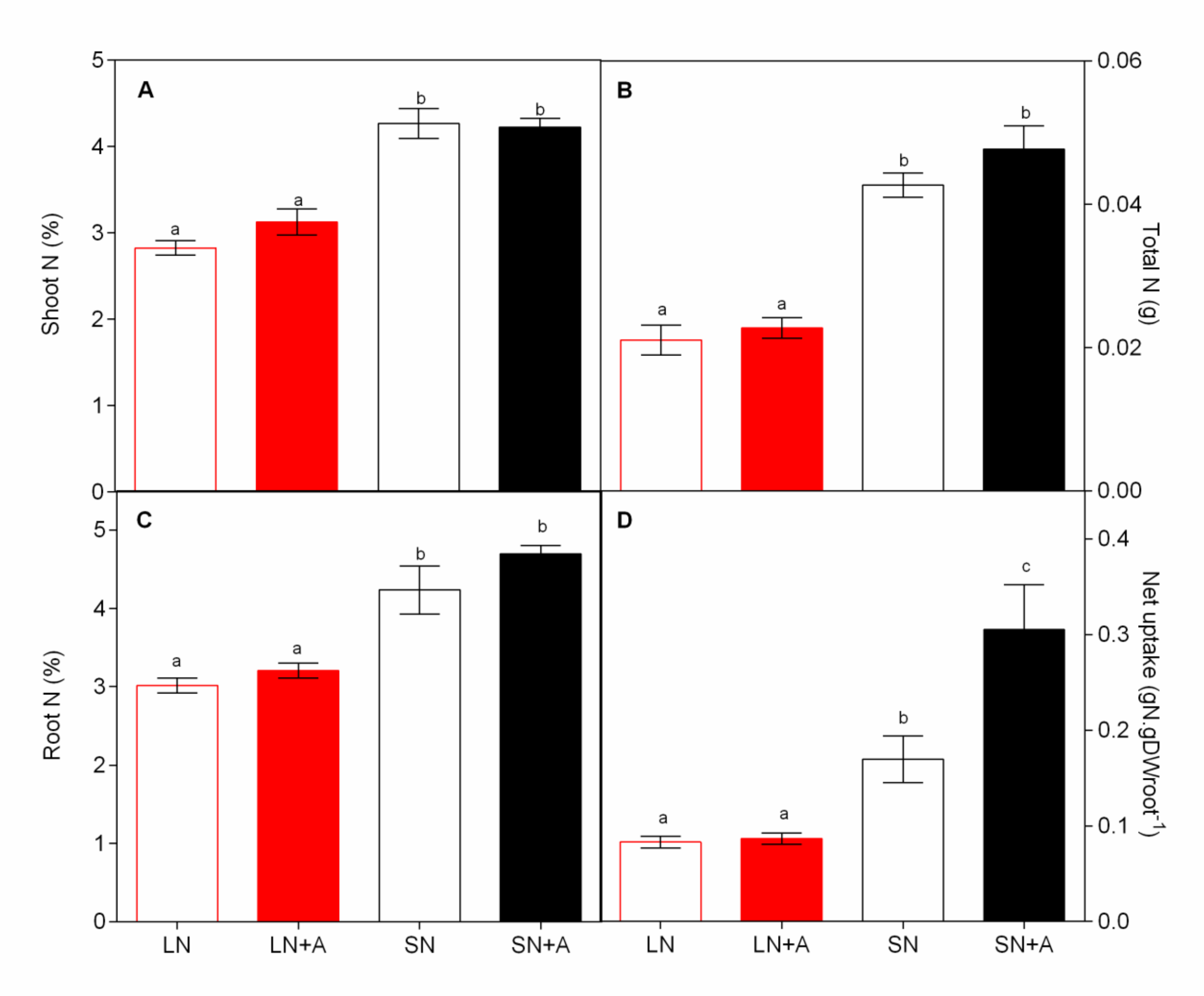

Figure S1. Shoot N concentration (A), total N (B), roots N concentration (C) and net uptake relative to root dry matter (D) of plants grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are means ± SEM (n=4). Statistical analysis used a one way analysis of variance. Significant differences at $P<0.05$ are represented by different letters.

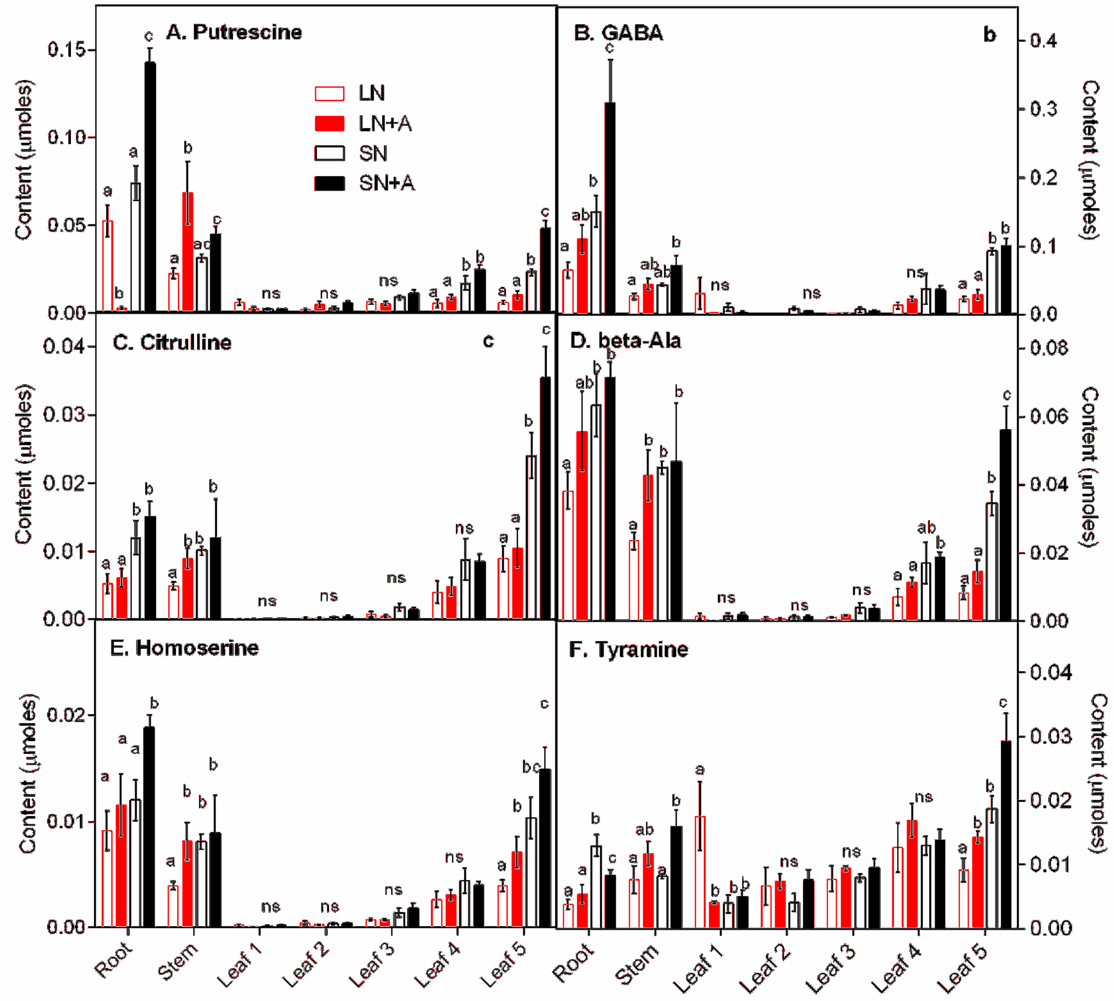

Figure S2. Putrescine (A), GABA (B), citulline (C), bet-alanine (D), homoserine (E) and tyramine (F) contents in the different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A), 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at $P<0.05$ are represented by different letters for each group of bars.

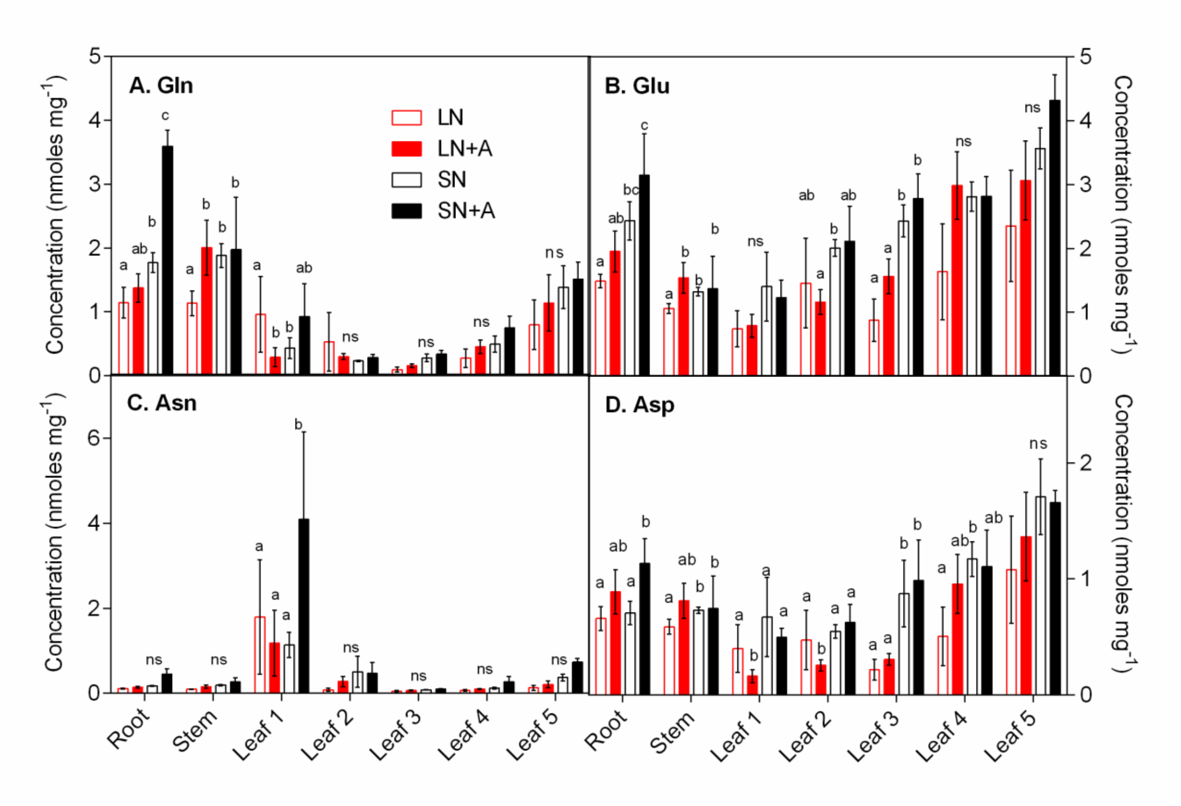

Figure S3. Glutamine (A), asparagine (B), glutamate (C) and aspartate (D) concentration in different plant parts of maize inbred line B73 grown in 0.55 mM NO_3^- (LN) , 0.50 mM NO_3^- + 0.05 mM NH_4^+ (LN+A) , 2.75 mM NO_3^- (SN) and 2.50 mM NO_3^- + 0.25 mM NH_4^+ (SN+A). Values are means ± SEM (n=4). Statistical analysis for treatment difference used a two way analysis of variance. Significant differences between treatments at $P<0.05$ are represented by different letters for each group of bars.

Chapter 6: General discussion & Future directions

Among the numerous studies that focus on the effect of a combination of NO_3^- and NH_4^+ on plant growth, relatively few have looked at plant responses to small concentrations of NH_4^+ (relative to NO_3^-) found in most agricultural soils. Therefore, this dissertation examined in maize the influence of 10% NH_4^+ to total N budget on vegetative growth, N uptake and N metabolism.

6.1 ADVANCES IN KNOWLEDGE FROM THIS STUDY

The first study investigated the effect of different proportions of NO_3^- and NH_4^+ on plant growth and showed that a combination of NO_3^- and NH_4^+ could increase plant growth in maize but variation existed between the two studied genotypes as explained in chapter 2. It has been well documented that maize plants achieve optimal growth and yield under mixed nutrition of NO_3^- and NH_4^+ (Below and Gentry, 1987, Gentry, 1992, Schrader et al., 1972, Smiciklas and Below, 1992, Alexander et al., 1991, Haynes and Goh, 1978, Wiesler, 1997). In this study we found that when plants were grown with low N the maize inbred line B73, but not Gaspe Flint, had a higher shoot dry matter when a small proportion of NO_3^- was replaced with NH_4^+. Increase in dry matter for B73 was accompanied by an increase in shoot N concentration as well as total N content in these plants. Phosphorus (P), sulphur (S) and most micronutrient concentrations were also increased in B73 plants grown in a mixture of NO_3^- and NH_4^+. Similar results have also been reported for wheat where the line Inbar grew better than Len in a mixture of NO_3^- and NH_4^+ due to the increased N in these plants (Gentry et al., 1989). Therefore, this study informed us that maize cultivars can vary in their response to small amounts of NH_4^+ and further investigation could be done on various maize cultivars as to understand this response. Based on the result on chapter 2 we chose to dissect the B73 response in subsequent studies.

In Chapter 3 we further showed that even 10% NH_4^+ can play a major role in the total N budget of maize plants B73 plants were grown in solutions with 10% of NO_3^- replaced with NH_4^+ both at low N and sufficient N levels and showed that 10% NH_4^+ at sufficient N levels increased shoot dry matter content in 36 day old plants. A corresponding increase in total N content and net N uptake were observed in these plants. This result agrees with studies in the past that plants supplied with NH_4^+ increased total N content in plants (Cox and Reisenauer, 1973, Kronzucker et al., 1999). However, a major down regulation of high affinity NO_3^- transporters even with just 10% of N as NH_4^+ at sufficient N was observed indicating a feedback regulation by higher N nutritional status of the plants as explained in Krouk et al (2006). The results also showed that small amounts of NH_4^+ along with sufficient NO_3^- can increase the concentration of various primary metabolites such as amino acids, reducing sugars like glucose and fructose and organic acids like, 2-oxogluterate, pyruvate, citrate and malate. Most of these metabolites are involved in the major metabolic pathways such as the tricarboxylic acid cycle and Krebs cycle indicating the importance and complexity of NH_4^+ impact on plant growth.

Much of the research on N uptake and metabolism are done in growth solution using only NO_3^- with no NH_4^+ is added to it. Based on our findings we recommend that small amounts of NH_4^+ should be included in growth solutions, firstly because it better reflects field conditions, and secondly because it changes growth and greatly modifies N metabolism. The major impact on N metabolism suggest that if plants are grown solely on NO_3^-, the results may not relate well to field experiments.

This study also showed that NH_4^+ uptake capacity exceeded NO_3^- uptake capacity under all N treatments indicating preferential uptake of NH_4^+ when both forms of N are available. Similar result was also obtained in a recent study in maize which showed that even when plants are adequately supplied with N the NH_4^+ uptake capacity exceeded NO_3^- uptake capacity (Gu et al., 2013). It was also observed that large temporal variation existed in NO_3^- uptake capacity of plants between harvests indicating that this must be taken into account if flux measurements between treatments are taken at single time points. For example, in our experiments, NO_3^- uptake capacities of plants were decreased by small amounts of NH_4^+ at DAE 24 but not DAE 29. Similar results were also reported in a maize experiment where NO_3^- flux capacity measured across the lifecycle showed temporal variation based on the supply and demand of N (Garnett et al., 2013). Here, in chapter 3-5, temporal variation was also observed in amino acid concentration in plants which showed a negative correlation with uptake capacity suggesting a feedback regulation of amino acids on NO_3^- uptake in plants. A pool of amino acids circulates between root and shoot, which act as signal for N uptake regulation (Cooper and Clarkson, 1989, Muller and Touraine, 1992). This temporal variation in NO_3^- uptake capacity and amino acid concentration needs to be taken into consideration when investigating N uptake and N metabolism in plants.

Many theories have been put forth to describe the inhibition in NO_3^- uptake by NH_4^+. Ammonium enhances plasma membrane depolarization, consequently decrease the proton motive force for NO_3^- (Ullrich, 1992, Lee and Drew, 1989). In our study plants grown in a low N medium showed decreased NO_3^- uptake capacity in the presence of NH_4^+ in the flux solution when the solution concentration of NH_4^+ was 50% of the NO_3^- concentration (Chapter 4). This short-term effect may be due to NH_4^+ “short circuiting” the process of NO_3^- assimilation, meaning that NH_4^+ taken up by the roots may enter the N assimilatory pathway (GS/GOGAT) prior to the NH_4^+ formed by the reduction of NO_3^-. This may slow down NO_3^- assimilation in plants which in turn may reduce NO_3^- uptake in plants. Plants can adapt to this by increasing glutamine synthetase (GS) activity in these plants, as GS is the enzyme involved in the assimilation NH_4^+ absorbed in the root and also NH_4^+ produced by reduction of NO_3^- in the root and shoot. Many studies of inhibition effect used short-term measurements where plants were exposed to NH_4^+ after not previously being exposed to NH_4^+(Deane-Drummond and Glass, 1983, Lee and Drew, 1989). As in our experiments, the reduction in NO_3^- uptake could be due to short circuiting and be alleviated with time as was observed when plants were switched to NH_4^+ for 48 hours. The inhibition of NO_3^- flux capacity that is commonly reported may be an artefact of measurement protocols and NH_4^+ toxicity and of less importance under more realistic nutrient regimes.

At sufficient N levels there was no short-term effect of NH_4^+ on NO_3^- uptake capacity at any external concentration of NH_4^+ in the flux solution. However, plants grown in sufficient N without NH_4^+ had lower NO_3^- flux capacity compared to low N treatments and a further reduction was observed for plants grown in 10% NH_4^+ at sufficient N levels irrespective of NH_4^+ concentrations in the flux solution (Chapter 4). The inhibition of NO_3^- uptake capacity in the sufficient N treatment without NH_4^+ may be due to higher N status of plants compared to low N treatments. A further decrease in NO_3^- uptake capacity in plants treated with 10% NH_4^+ at sufficient N levels may be related to feedback regulation due to the higher concentration of root amino acids, especially glutamine and asparagine. These amino acids were present in high concentration in the roots of plants grown in 10% NH_4^+ at sufficient N level. Previous studies have also suggested that tissue concentration of these amino acids may regulate NO_3^- uptake capacity (Breteler and Arnozis, 1985, Muller and Touraine, 1992, Lee et al., 1992, Padgett and Leonard, 1996). Although there was a reduction in the NO_3^-uptake capacity for plants grown in 10% NH_4^+ with sufficient N (NO_3^-), there was higher N uptake and better plant growth in these plants. This suggests that the reduction in NO_3^- uptake capacity in plants grown in small amounts of NH_4^+ at sufficient N is due to the feedback regulation of N levels in plants.

In many previous experiments amino acid concentrations were measured in roots and shoots or a representative part of the shoot, such as the youngest expanded blade (YEB). However, it was unclear whether amino acid concentrations differ greatly between particular plant parts which could lead to discrepant conclusions. Therefore, we measured the distribution of amino acids in different plant tissues in maize. We found higher concentrations of most amino acids in the root, stem and youngest leaf. The high and low N treatment differences were observed in the YEB and youngest leaf. The effect of NH_4^+ was more prominent in the youngest leaf compared to the YEB. However the YEB is a better option as it is easy to access compared to the whole shoot. If the whole shoot is taken for measuring amino acid contents, the result will be diluted by the very low amino acid contents in the older leaves. Amino acids in the stem, roots and youngest leaf accounted for majority of amino acids in the plants, while the older leaves had very small amounts of amino acids. It was also found that the plants grown in 10% NH_4^+ at sufficient N levels had the highest content of most amino acid in the roots, youngest leaf and stem compared to all other treatments. This study showed that YEB is a good representative part to be taken from the plant shoot for quantifying individual amino acids, but some treatment effects may only be discovered by looking at younger leaves.

6.2 FUTURE DIRECTIONS

Small amounts of NH_4^+ improved plant growth in maize inbred line B73, but not in Gaspe Flint. Further investigations are required on a panel of diverse inbred lines to get a better understanding about the reasons for variations between genotypes in response to small amounts of NH_4^+. To understand this genotypic difference a study of metabolic responses and molecular mechanism underlying the response to small amounts of NH_4^+ (similar to that discussed in chapter 3) could be completed in a wider range of genotypes. This may more completely reveal the mechanism(s) behind the growth response to a small amount of NH_4^+ and more importantly better understand the N uptake and assimilation processes.

The results from this study were obtained during the initial vegetative growth stages of the plants. It will be interesting to investigate the NH_4^+ response later in the life cycle and how this affects the yield. This study was only done in maize; given the magnitude of growth and metabolism effects it would be pertinent to investigate these effects in other agricultural crops growing in similar soils with the persistent low levels of NH_4^+. The positive effect on growth, total N uptake and activity of N assimilatory enzymes of small amounts of NH_4^+ was only seen when sufficient NO_3^- was present in nutrient solution. This increased growth was associated with increases in tissue levels of amino acids, sugars and organic acids. We also observed a high total N uptake in plants supplied with 10% NH_4^+ compared to NO_3^- alone treatments. This indicates that both N uptake efficiency and N utilization efficiency in plants may be improved by small amounts of NH_4^+ when supplied with sufficient N. It could be explored whether the uptake and assimilation responses are connected or whether they are separate responses.

A transcriptome analysis using microarray or RNAseq or a QTL mapping approach in a population derived from genotypes differing in their response to NH_4^+ would help us in identifying genes that are up or down regulated in the presence and absence of 10% NH_4^+. This may identify candidate genes that regulate N uptake in the presence of small amounts of NH_4^+ and those which contributed to the increased assimilation of N. These genes may be used in transgenic or breeding approaches in efforts to improve the nitrogen use efficiency in plants.

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