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# Functional role of ammonium and nitrate in regulating transpiration for mass-flow acquisition of nutrients in *Phaseolus vulgaris* L.

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# DECLARATION

I, Mandilakhe Naku hereby, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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# ABSTRACT

Transpiration serves in leaf cooling, maintaining turgor pressure, promoting xylem transport of nutrient solutes from roots to shoots and delivering mobile soil nutrients to root surfaces. Soil availability of nitrogen can modulate transpiration rates, consequently powering nutrient delivery to the root surfaces (‘mass-flow'). Although such knowledge on N-regulation of transpiration is available, it remains unknown, however, whether it is $NO_3^-$ or $NH_4^+$ that regulates transpiration. Given that both nitrogen forms co-occur in soils, it is not known how they interact at varying ratios in modulating stomatal behaviour. To test the functional role of $NO_3^-$ and $NH_4^+$ in regulating water fluxes for mass-flow nutrient acquisition, *P. vulgaris* L. plants were grown with $NO_3^-$ or $NH_4^+$ placed at one of four distances behind a nylon mesh, which prevented direct root access to nitrogen, whilst control plants intercepted the nitrogen source (Chapter 3). Day- and night-time stomatal conductance and transpiration, measured using Infra-Red Gas Analyser (IRGA) declined in $NO_3^-$ fed plants with the increased distance behind a nylon mesh, with maximum water fluxes at the closest distance (ca. 0 mm), demonstrating a regulatory role of $NO_3^-$ on stomata closure. An opposite trend was displayed by $NH_4^+$ -fed plants, which indicated the incapacity of $NH_4^+$ to down-regulate water fluxes and ammoniacal syndrome at high concentrations.

To test how different $[NO_3^-]$ and $[NH_4^+]$ regulate day- and night-time stomatal conductance and transpiration (Chapter 4), *P. vulgaris* was fed with six concentrations (0, 0.25, 0.5, 1, 2, 4 and 8 mM) of each nitrogen form. A biphasic trend emerged, as postulated in previous studies (Wilkinson *et al*., 2007; Matimati *et al.*, 2013), characterized by an increase in stomatal conductance and transpiration as $[NO_3^-]$ increased, attaining a maximum before declining with higher $[NO_3^-]$. Plants displayed 2-fold higher photosynthetic rates, 2.2-fold higher stomatal conductance and 2.3-fold higher transpiration rates at 4 mM than at 0.25 mM of $[NO_3^-]$. The lowest $[NO_3^-]$ up-regulated night-time stomatal conductance and transpiration, indicating that $NO_3^-$ -fed plants opened their stomata at night-time, but reduced night-time water loss at higher $[NO_3^-]$. $NH_4^+$-fed plants had the incapacity to regulate day- and night-time water fluxes, but rather displayed wilting and stress known as ‘ammoniacal syndrome'. Thus, under $NO_3^-$ deprived soil conditions *P. vulgaris* may be opportunistic in their water uptake, transpiring more when water is available in order to draw nutrients through ‘mass-flow'.

This thesis explored and confirmed the functional role of $NO_3^-$ in regulating day- and night-time water fluxes as a mechanism for increasing ‘mass-flow' acquisition of N and possibly other nutrients, signalling a down-regulation of day-time and night-time water fluxes when $[NO_3^-]$ is replete (Chapter 3 & 4). Where both $NO_3^-$ and $NH_4^+$ are present in soils, it is the $[NO_3^-]$ and not $[NH_4^+]$ that regulated stomatal conductance and transpiration. Since organic nitrogen forms such as amino acids also occur in soils, there is a need for further work on their role in stomatal behaviour. Using amino acids laced with $15^N$ isotopes as a nitrogen source can allow their acquisition and role on stomatal behaviour to be discovered. Current trends in research are focussed around developing real-time in-situ sensing of soil nitrogen status to promote enhanced nitrogen and water use efficiency in agricultural systems. This thesis provides the vital literature on stomatal regulation by $[NO_3^-]$.

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# DEDICATION

This thesis is dedicated to my late grandmother Agnes Ntombomzi Naku, my mother Zukiswa Naku, my brothers Akhona Naku and Anathi Naku for their love, wisdom, prayers and unconditional support throughout my academic career. Without you this thesis may never have been completed.

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# ABBREVATIONS AND SYMBOLS

| | |
|---|---|
| ABA | abscisic acid |
| AIC | akaike information criterion |
| ANOVA | analysis of variance |
| $C_i$ | intercellular $CO_2$ mole fractions |
| $De$ | diffusion coefficient |
| DM | dry mass |
| $E$ | transpiration |
| $E_{night}$ | night-time transpiration |
| $g_s$ | stomatal conductance |
| $g_{night}$ | night-time stomatal conductance |
| LSD | least significant difference |
| PAR | photosynthetically active radiation |
| PVC | polyvinyl chloride |
| $Rd$ | respiration dark |
| TDM | total dry mass |
| $WUE_i$ | photosynthetic water use efficiency |

# CHAPTER 1

## 1.0 INTRODUCTION

### 1.1 Background to the research problem

Terrestrial water fluxes are dominated by transpiration, which contributes 80 to 90 % of terrestrial evapotranspiration (Jashechko *et al.,* 2013*)*. As plants open their stomata to assimilate $CO_2$ during photosynthesis, there is an unavoidable loss of water vapour to the surrounding unsaturated air (Kramer and Booyer, 1995). Furthermore, plants transpire more than 90% of their root acquired water (Raven and Johnson, 2001), these large amounts of transpirational water fluxes serve many functional roles, such as leaf cooling (Nobel, 1999), maintaining of turgor pressure (Rygol *et al*., 1993), powering solute transport from roots to shoots *via* xylem and delivering nutrients through the soil to the root surface through mass-flow (Barber, 1995; Cramer *et al*., 2008; Cramer *et al*., 2009; Christman *et al*., 2009; Matimati *et al*., 2013). The large amounts of transpirational water fluxes suggest a major functional role, possibly in powering the movement of water and dissolved nutrients through the soil to the roots by mass-flow (Cramer *et al*., 2008; Matimati *et al.*, 2013). This notion may be supported by the substantial night-time transpiration observed in photosynthetically inactive $C_3$ and $C_4$ plants (Caird *et al*., 2007; Kupper *et al*., 2012). Evidence of substantial night-time transpiration has discredited the long-held assumption that stomata remain closed during the night in $C_3$ plants (Cirelli, 2014). In addition, regulation of transpiration rates in response to nutrient availability (e.g. Cramer *et al*., 2008; Cramer *et al*., 2009; Matimati *et al*., 2013) has been consistent with the adaptive stimulation of nutrient supply to plant roots through mass-flow (Raven, 2008).

Availability of soil nutrients to roots is mainly controlled by the rate at which nutrients move through the soil towards the root surface (Barber, 1962). A plant may acquire soil nutrients in three ways: 1) as roots proliferate and get in contact with nutrients (root interception) or 2) diffusion of soil nutrients towards the root surface and 3) delivery of dissolved soil nutrients to the root surface in response to transpiration (mass-flow) (Barber, 1962). Interception only occurs when roots are get in contact with nutrient sources, which facilitates nutrient assimilation (Matimati *et al.*, 2013). Thus, root interception of soil nutrients increases with proliferation of roots in the soil (Kage, 1997). Diffusion occurs when dissolved soil nutrients move along a concentration gradient towards the roots, driven by a net influx of ions at the root surface (Cernusak *et al*., 2011). Mass-flow occurs when water and dissolved soil nutrients transported to the root surface, driven by transpirational water flux (Cernusak *et al*., 2011; Chapman, 2012; Matimati *et al.*, 2013). Transpiration driven mass-flow is effective in delivering mobile soil nutrients to root surfaces, particularly $NO_3$, K and S (Silberbush and Barber, 1983; Barber and Silberbush, 1984, Silberbush 2002). Mass-flow acquisition of nutrients is vital for plants that are struggling to intercept nutrients as a result of poor root density (Cramer *et al.,* 2008). Thus, mass-flow has no direct role to influence on acquisition of nutrients across the plasma membrane. It is the concentrated region at the root surface that may enhance membrane nutrient transport (Dalton *et al.*, 1975; Fiscus, 1975; Fiscus and Kramer, 1975).

Transpiration may be regulated by the concentration of nutrients in the cytosol, particularly N (Wilkinson *et al*., 2007), which consequently modulates the mass-flow acquisition of mobile soil nutrients (Cramer *et al*., 2008; Matimati *et al*., 2013). But a key research question has emerged regarding the N-form that signals the regulation of transpiration for mass-flow acquisition of nutrients. Assimilation of N as $NO_3^-$ may signal the regulation of transpiration, potentially through mechanisms such as root hydraulic conductance and stomatal conductance (Cramer *et al*., 2009). $NO_3^-$ may modulate root hydraulic conductance through its control of aquaporins, and may induce stomata closure through nitric oxide (NO) as a reduction product of nitrate reductase (NR) (Cramer *et al.*, 2009). In contrast, N uptake as $NH_4^+$ did not modulate root hydraulic conductance or increase the expression of the aquaporins in *P. vulgaris* (Guo *et al*., 2007). It remains unclear, however, whether plants fertilized with ammoniacal-N fertilizers, such as $NH_4^+SO_4$ and $NH_4$+Cl modulate their transpiration rates. Previous studies suggest that $NH_4^+$ taken up by roots is converted into amino acids in the roots (Miller and Cramer 2004), and has no role in regulating stomatal closure. In addition, plants supplied with $NH_4^+$ possess a lower water use efficiency (*WUE)* ( Raven *et al*., 2004; Guo *et al*., 2007a,b) , and $NH_4^+$ toxicity in plants causes symptoms such as growth inhibition, wilting and other traits of water stress (Cramer and Lewis, 1993; Chaillou and Lamaze, 2001), known as ‘ammoniacal syndrome’. Thus, the effects of nutrient availability, particularly N forms ($NO_3^-$ and $NH_4^+$) in regulating transpiration for nutrient uptake through mass-flow is poorly understood. This study investigates the functional role of two common N forms ($NO_3^-$ and $NH_4^+$)

in regulating transpiration rates, which is inextricable from mass-flow acquisition of nutrients

### 1.2 Statement of research problem

The functions of transpiration are well documented (Cramer *et al*., 2009). These include cooling of leaves (Nobel, 1999), maintaining leaf turgor pressure (Rygol *et al*., 1993), driving root to shoot ascension of solutes through the xylem and powering mass-flow, the delivery of nutrients to the root surface (Barber, 1995; Cramer *et al*., 2008; Cramer *et al*., 2009; Christman *et al*., 2009; Matimati *et al*., 2013). Recent studies using urea, concluded that nitrogen modulates transpiration rates and consequently mass-flow nutrient acquisition by plants (Matimati *et al.*, 2013). Despite knowledge on N regulation of transpiration, it remains unknown whether all forms of N can regulate transpiration given that urea releases both $NO_3^-$ and $NH_4^+$. In addition, the mechanism involving N regulation of transpiration is not well-known. $NO_3^-$ has been found to increase root hydraulic conductivity (Carvajal *et al*., 1996; Clarkson *et al*., 2000), which often results in a high rate of transpiration. An uptake of N as $NO_3^-$ may induce stomatal closure (Gloser *et al*., 2007; Gorska *et al*., 2008 Cramer *et al*., 2009). In contrast, plants supplied with $NH_4^+$ generally display lower water use efficiency than those acquiring $NO_3^-$ and may display wilting and other symptoms of water stress (Crammer and Lewis, 1993; Chaillou and Lazame, 2001; Raven *et al*., 2004; Guo *et al*., 2007). Thus, the role of $NH_4^+$ in plant water fluxes is still obscure. A knowledge gap also exists on how water-use efficiency varies in plants in response to different N-forms, especially under varying environmental conditions. Such information is vital for nutrient and water management in plant production. Further, atmospheric $CO_2$ controls stomatal opening and consequently water use efficiency. In the wake of predicted increases in global $CO_2$ levels (Siegenthaler and Oeschger, 1978), it remains unclear how *WUE* and nutrient acquisition in crops will respond, especially when exposed to varying rates of N fertilizers. Studies on N-regulation of transpiration are critical in order to untangle the complex relationships between nutrient acquisition and plant water use and to improve our interpretation of plant physiological responses to the environment.

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## 1.3 Hypotheses

It is hypothesized that both $NO_3^-$ and $NH_4^+$ indirectly signal for the increase in rates of transpiration, when N is limiting, for increasing its mass-flow acquisition by the roots. The hypothesis is based on the suggestion that N-deprived plants may increase their stomatal conductance, for the mass-flow delivery of nutrients to the roots (Cramer *et al*. 2008; Matimati *et al*., 2013). Specific hypotheses are that:

1. A limitation in both $NO_3^-$ and $NH_4^+$ must increase the rate of both day- and night-time stomatal conductance and transpiration in *P. vulgaris* to facilitate nutrient acquisition through mass-flow, and down-regulate stomatal conductance and transpiration when N is replete and;
2. Plants that lack a direct access to $NO_3^-$ and $NH_4^+$ rich soil patches must display higher water fluxes than plants with direct root access to N.
3. Plants that fed with increasing [$NH_4^+$] and [$NO_3^-$] should initially up-regulate day- and night-time water flux for mass-low delivery of the limiting N, then down-regulate these fluxes as foliar [N] exceeds tolerable levels.
4. Increased proportion of $NO_3^-$ : $NH_4^+$ should down-regulate transpiration because of the regulatory role of the $NO_3^-$.

## 1.4 Aim and objectives of the research

The primary aim of the study was to determine whether $NH_4^+$ or $NO_3^-$ regulate the rate of transpiration and, consequently, mass-flow acquisition of nutrients by *P. vulgaris*. Specific objectives of the study were:

1. To determine the functional role of different N forms ($NH_4^+$ and $NO_3^-$) on plant water fluxes and mass-flow acquisition of nutrients in *P. vulgaris*.
2. To evaluate plant water fluxes and mass-flow acquisition of nutrients in *P. vulgaris* when supplied with N as $NH_4^+$ and/or $NO_3^-$ at varying distances from the roots.
3. To test the biphasic regulation of transpiration and stomatal conductance in *P. vulgaris* when supplied with varying [$NH_4^+$] and [$NO_3^-$].
4. To test the biphasic regulation of transpiration and stomatal conductance in *P. vulgaris* when supplied with N as mixture of $NH_4^+$ and $NO_3^-$ at varying propotions.

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# CHAPTER 2

## 2.0 LITERATURE REVIEW

### 2.1 Introduction

Terrestrial plants transpire *ca.* 95% and only retain 5% of their root-acquired water (McElron *et al*., 2001; Raven and Johnson, 2001). Plants can transpire more than 100 moles of water when they assimilate a mole of $CO_2$ during photosynthesis (Hack *et al*., 2006). Such water fluxes have commonly been considered as wasteful, yet an inevitable consequence of assimilating $CO_2$ through their stomata (Cowan and Troughton, 1971, Monteith, 1988; Kramer and Boyer, 1995). Transpiration, however, serves in leaf cooling (Nobel, 1999), maintaining turgor pressure (Rygol *et al*., 1993), possibly driving solutes from the root to shoot through xylem (Campbell *et al.*, 1999; Strasburger, 1998), and powering nutrients from the soil towards root surfaces (Barber, 1995; Cramer *et al.*, 2008; Cramer *et al*., 2009; Christman *et al*., 2009; Matimati *et al*., 2013). The high transpiration fluxes are a potential mechanism of delivering nutrients to root surfaces from nutrient-poor soils through mass-flow (Cramer *et al*., 2008; Matimati *et al*., 2013). Likewise, night-time transpiration in species that partially close their stomata at night may be construed as further evidence for transpiration-driven mass-flow of nutrients to root surfaces (Matimati *et al*., 2013).

Generally plants acquire soil nutrients in three ways: i) when roots proliferate and get in contact with nutrients (interception), ii) diffusion of soil nutrients towards the root surface and iii) delivery of dissolved soil nutrients to the root surface in response to transpiration (Barber, 1962). Although transpiration-driven mass-flow has no direct role in the uptake of nutrients across the plasma membrane, the concentration of nutrients around the root surface may enhance diffusion and active uptake of soil nutrients (Dalton *et al*., 1975; Fiscus, 1975; Fiscus and Kramer, 1975).Thus, the concentration of nutrients in the cytosol, particularly N may regulate transpiration (Wilkinson *et al*., 2007), and modulate mass-flow acquisition of mobile soil nutrients (Cramer *et al*., 2008; Matimati *et al*., 2013). Although the role of N in modulating transpiration was confirmed, the mechanisms signalling the regulation of transpiration by different N forms (e.g. $NO_3^-$ , $NH_4^+$ and amino acids) remain unclear. Since N regulates transpiration, it should consequently control the mass-flow acquisition of mobile nutrients (Cramer *et al*., 2008; Matimati *et al.*, 2013). Chapter 2 reviews the effects of N forms ($NO_3^-$, $NH_4^+$ and amino acid) on acquisition of soil nutrients, ii) Adaptations that regulate rate of transpiration, iii) N-forms on regulatory mechanism of transpiration, iv) N-forms in regulating night-time transpiration and v) N-forms on *WUE* for implications in agriculture.

### 2.2 Acquisition of $NH_4^+$, $NO_3^-$ and amino acids

Nitrogen in the soil exists as a complex mixture of both organic and inorganic forms, and, in addition to seasonal and day-time changes, is also characterised by an extremely heterogeneous distribution (Miller and Cramer, 2004). The dominant N form exists as complex organic molecules that are converted to $NH_4^+$ by soil bacteria and fungi through mineralisation (Miller and Cramer, 2004). When oxidised, $NH_4^+$ can be converted *via* $NO_2^-$ to $NO_3^-$ through a process known as nitrification (Miller and Cramer, 2004). Thus, nitrogen is acquired mainly as $NH_4^+$, $NO_3^-$, uncharged $NH_3$ in equilibrium with charged $NH_4^+$ forms, and organic forms such as cationic amino acids such as L-arginine (L-Arg) or L-lysine (L-Lys) (Svennerstam *et al*., 2008). The acquisition of soil nutrients including $NH_4^+$, $NO_3^-$ and amino acids by terrestrial plants occurs through diffusion, mass-flow delivery and root interception, depending on root system of the plant species, nutrient concentration and nutrient mobility within the soil (Barber, 1962).

Plants acquire water and nutrients through root interception when their roots proliferate and become denser, and consequently get in contact with nutrient solutes within the soil (Barber, 1962). Nutrient availability, particularly $NO_3^-$ at the rhizosphere enhances root growth by influencing root elongation and branching through $NO_3^-$ signaling to gene expression of root growth (Linkhor *et al*., 2002; Tian *et al* ., 2005). This is supported by the fact that $NO_3^-$ enhances root growth, whilst $NH_4^+$ and amino acids do not enhance root growth (Zhang and Rengel, 1999). Thus, $NO_3^-$ enhances root growth, which aids the root systems to acquire immobile nutrients (S, K and $NH_4^+$) easily through interception (Miller and Cramer, 2004). In addition, a highly proliferated root system may also play a significant role in intercepting mobile and leaching nutrients i.e. ($NO_3^-$) which move to deeper areas of the soil where water is greater at depth (Kudoyavora, 2015). There is a suggestion that lateral roots proliferate on localized patches within soil patches of higher N, particularly $NO_3^-$ (Drew and Saker, 1975), resulting in stimulatory effects on the number and location of lateral roots (Malamy and Ryan, 2001), but not initiated by amino acids or $NH_4^+$ (Leyser and Filter, 1998).

Acquisition of other soil nutrients by the roots varies depending on the availability of N-forms ($NH_4^+$ or $NO_3^-$ or amino acids) within the soil (Guo *et al.*, 2007a). In some species, $NH_4^+$, $NO_3^-$ and amino acids may negatively or positively influence the rate of acquisition of soil nutrients at the root surface. This varies with species, developmental stage, nutrient concentration, soil moisture or soil water status and temperature (Wallace, 1986). Amino acids levels, particularly proline correlates with drought-resistance in plants (Sigh *et al*., 1972). Barley grown under water stress conditions had high accumulation of proline (Aspinall and Paleg, 1981). Such amino acid levels significantly alter modulation of water and nutrient acquisition in relation to plant water potential (Rai, 2002). Thus, amino acids possibly play a significant role in signaling the modulation of plant water fluxes under water stress conditions. On the contrary, other studies found no correlation between proline levels and drought resistance (Singh *et al*., 1972; Waldren and Teare, 1974, Hanson *et al.*, 1977, 1979). Thus, the effect of amino acids on plant water fluxes remains poorly understood. $NH_4^+$ increased the acquisition of N, this displayed by higher foliar N concentration (Schrader *et al*., 1972). Nutrient acquisition increased with $NH_4^+$ supply in barley (Lewis, 1983) and rice (Qian *et al*., 2004), but not in $NO_3^-$ fed plants. In contrast, $NO_3^-$ nutrition increased acquisition soil nutrients in *P. vulgaris* (Guo *et al*., 2007b). $NO_3^-$ seems to play a significant role in stimulating the acquisition of water, and consequently the delivery of dissolved soil nutrients to roots through mass-flow (Guo *et al*., 2007b). Some species supplied with a mixture of $NH_4^+$ and $NO_3^-$ solution, however, had a higher water and nutrient acquisition compared to sole $NH_4^+$ or $NO_3^-$ nutrition (Guo *et al*., 2007b). The significance of combined N nutrition relies on the ratio of the N forms. For example, a mixed $NO_3^-$ and $NH_4^+$ at a ratio of 50/50 strongly increased the total acquisition of N more than a sole $NO_3^-$ and $NH_4^+$ nutrition in rice (Qian et al., 2004). Foliar accumulation of N, K+, $NO_3$- and $Ca_2^+$ increased when the $NO_3^-$ was higher than $NH_4^+$ in the ratio, for example $NO_3^-$ (7.5 mM) : $NH_4^+$ (2.5 mM) than $NO_3^-$ (2.5 mM) : $NH_4^+$ (7.5 mM) (Helali *et al*., 2010). Therefore, the role of different N forms in regulating transpiration and mass-flow movement of soil nutrients remains unclear.

Diffusion, the movement of nutrients from regions of higher concentration towards regions of lower region, contributes to nitrogen acquisition in plants (Jones, 2005). N acquisition through diffusion depends on a concentration gradient and a diffusion coefficient (De) (Miller and Cramer, 2004). Diffusion coefficient for $NO_3^-$ is greater than form $NH_4^+$ and amino acids, and De for amino acid is strongly varying in water (Miller and Cramer, 2004). For example $NO_3$- De is c.a. $1 \times 10^{-10}$ $m^{2\ s^{-1}}$ c.a, $NH_4^+$ is c.a.10-fold to 100-fold less, and amino acids (lysine, glycine and glutamate) is $1 \times 10^{-12}$, $1 \times 10^{-11}$, $1 \times 10^{-11}$ $m^2\ s^{-1}$, respectively (Silberbush and Baber, 1984; Owen and Jones, 2001) . It is believed that De is determined by differences in nutrient mobility or leaching through the soil. It is suggested that diffusion on nutrient acquisition contributes greater in $NO_3^-$ more than in $NH_4^+$, due to poor mobility in $NH_4^+$ and $NH_4^+$ binding to clay particles (Xu *et al*., 2011). The effectiveness of diffusion in contributing to root nutrient acquisition relies on ionic form, viscosity of water, temperature, soil moisture, tortuosity and the soil buffer capacity (Miller and Cramer, 2005; Chapman *et al.*, 2012).

Transpiration modulates nutrients acquisition from the soil to the root surface through ‘mass-flow’ (Barber, 1962). Mass-flow plays an important role in driving acquisition of mobile nutrients ($NO_3^-$ and K) through the soil towards the root surface (Oyewole *et al*., 2014). Nutrient acquisition through mass-flow is more effective and clear in the soil more than in a hydroponic system (Oyewole *et al*., 2014). Moreover, mass-flow acquisition of nutrients is vital for plants that are struggling to intercept nutrients as a result of poor root density (Cramer *et al.,* 2008). Thus, mass-flow has no direct role to influence on acquisition of nutrients across the plasma membrane. It is the concentrated region at the root surface that may enhance membrane nutrient transport (Dalton *et al.*, 1975; Fiscus, 1975; Fiscus and Kramer, 1975).

### 2.3 The effects of N-forms on regulatory mechanism of plant water fluxes.

Although environmental conditions can determine the rate of transpiration (Collatz *et al*., 1991), recent studies have shown that nutrient availability, particularly N, controls transpiration and consequently mass-flow acquisition of nutrients (Matimati *et al*., 2013). Generally, mass-flow acquisition of soil nutrients depends on fluxes of water, nutrient concentration and nutrient mobility (Barber, 1995). The availability of immobile nutrients such as P and S at root surfaces is limited, and hence the role of transpiration in their acquisition is inadequate (Barber 1995; Kramer and Boyer, 1995). A limited availability of P at the root surface may rapidly increase root hydraulic conductivity (Radin and Eidenbock, 1984; Carvajal *et al.*, 1996) probably due to increased aquaporin expression (Clarkson *et al.*, 2000). An extra Phosphorus fertiliser on plants has no effect on transpiration, but plants transpired adequately in moist soil (Fidelibus *et al*., 2001). Furthermore, increased P concentration at root surface seems to increase *WUE* in many species ( Raven *et al.*, 2004), but this will mainly focus to the relief of photosynthesis from P- limitation (Jacob and Lawlor, 1991), rather than any P- induced decrease in transpiration (Cramer *et al.,* 2009). It is, however, the $K^+$ ion as the main osmotic solute in plants that may regulate transpiration (Mengel and Arneke, 1982). As $K^+$ ions move into guard cell through protein pump of the cell membrane to increase turgidity, turgid guard cells change their stomatal opening, resulting in water vapour movement to the atmosphere (Bower, 2008). In addition, nitrogen potentially regulates transpiration and consequently the acquisition of other nutrients, as was the case in *P. vulgaris* (Matimati *et al*., 2013) and *Erharta. calycina* (Cramer *et al*., 2008).

Whilst N is now known to regulate transpiration and mass-flow nutrient acquisition, there is limited knowledge on the role of other N forms, such as $NH_4^+$ and organic N (amino acids). Comparing their mobility, $NO_3^-$ is regarded as more mobile in the soil than $NH_4^+$ (Miller and Cramer, 2004). Acquisition of N as $NO_3^-$ is possibly the signal that regulates the rate of transpiration (Matimati *et al*., 2013), exerting its effects on root hydraulic conductivity and stomata conductance (reviewed by Cramer *et al.*, 2009). A limited availability of $NO_3^-$ at the root surface is known to rapidly increase aquaporin- mediated root hydraulic conductivity (Carvajal *et al.*, 1996: Clarkson *et al*., 2000; Gloser *et al*., 2007; Gorska *et al*., 2008). The root $NO_3^-$, without a reduction product (NO) can modulate aquaporin expression, which is an adaptive characteristic that has functional significance in the mass-flow delivery of $NO_3^-$ to the root surface when the root of N-deprived plants are exposed to fertile soil patches (Gorska *et al*., 2008). As $NO_3^-$ fluxes into the root exceed the capacity of nitrate reductase enzymes, $NO_3^-$ is transported to shoots and is reduced by nitrate reductase (NR) enzymes to produce NO and $NO_2$ (Desikan *et al*., 2002; Neill *et al*., 2008). NO is known as the main signal that has functional significance in resulting in stomatal closure (Neill *et al*., 2008). This explains why stomatal conductance in *P. vulgaris* began to decline when nitrogen source was beyond 10 mm from the root surface (Matimati *et al.*, 2013). Plants supplied with $NO_3^-$ fertiliser their xylem/ apoplastic pH will generally be more alkaline, resulting in the accumulation of abscisic acid (ABA) in the apoplast (Mengel *et al*., 1994; Mühling and Lauchli, 2001; Jia and Davies, 2007). ABA has a vital functional role in regulating stomatal closure (Wilkinson *et al*., 2007).

Unlike $NO_3^-$, acquisition of N as $NH_4^+$ does not increase the expression of root aquaporins or alter root hydraulic conductance for regulation of transpiration (Gou *et al.*, 2007a). Despite an observed incapacity of $NH_4^+$ in regulating root hydraulic conductance, however, *Oryza sativa* has increased its root hydraulic conductance when low [$NH_4^+$] was available at the rhizosphere (Gao *et al*., 2010). In some species, roots readily assimilate $NH_4^+$ into amino acids (Miller and Cramer, 2004) and $NH_4^+$ does not appear to elicit the closing of stomata (Cramer *et al.*, 2009). This interpretation may suggest a lack of evidence that emerged on $NH_4^+$ regulation of transpiration through root hydraulic conductance and stomata aperture. Nevertheless, it remains poorly understood how plants supplied with $NH_4Cl$ or $(NH_4)_2SO_4$ fertilisers, regulate their rate of transpiration. For example, Goodger and Schachtman (2010) suggested how plants supplied with $NH_4^+$ might control their rates of transpiration. $NH_4^+$-supplied plants, however, generally show lower *WUE* than those acquiring $NO_3^-$ (Raven *et al*., 2004; Guo *et al*., 2007a, b) and indicating wilting and other symptoms of water stress (Cramer and Lewis, 1993; Chaillou and Lamaze, 2001), which is known as ammoniacal syndrome (Chaillou and Lamaze, 2001). Such water stress may be caused by a lack of co-ordinated regulation of root hydraulic conductance and $g_s$ (Cramer *et al*., 2009). High soil [$NH_4^+$] is associated with agriculture and is rare in natural ecosystems. As a result plants may lack a mechanism to respond to these particular conditions.

Plants seem to control their transpiration in response to availability of amino acids in the soil, depending to the nature of amino acid (Rai and Sharma,1992). For example glycine, alanine, leucine, threonine, lysine, arginine, proline, tryptophan and phenylalanine induced endogenous ABA which consequently down-regulated transpiration through stomata closure (Rai and Sharma, 1992) This notion is supported by a notable lower value of $g_s$ in proline-fed plants compared to plants fed without proline (control) (Rajagopal, 1981). Furthermore, availability of exogenous proline at the root surface enhanced permeability of aquaporin in *Vinca* sp (Rai and Sharma, 1992), which consequently increases nutrient entry across cell membrane of the root system. These findings suggest that amino acid may possibly regulate transpiration and mass-flow acquisition of soil nutrients, although there is no further knowledge raised on regulatory mechanism of transpiration in response to amino acid.

## 2.4 Adaptations that regulate rate of transpiration

Is plant water flux a design trait of plants or rather a design error, whereby plants unavoidably lose water vapour as their stomata open for carbon assimilation? It appears that plants possess several biological traits that may enhance water fluxes (Yates *et al*., 2010). Such biological traits ensure water flux into leaf tissue to encounter the demands for water imposed by transpiration (Cramer *et al.*, 2009). A typical example of such a trait is the extent and density of venation in leaves, which dictates the hydraulic conductivity of leaf tissue (Brodribb *et al.*, 2007). In some species, aquaporins as transport channels in the mesophyll cells have functional significance in up-regulating water fluxes (Kaldenhoff and Fischer, 2006; Sakurai *et al.*, 2008). For example, *Julgans regia* displayed variable leaf hydraulic conductance, which possibly enhanced leaf gas exchanges while buffering leaf water status in response to ambient light fluctuations (Cochard *et al*., 2007). Such light-mediated activation of leaf acquaporins resulted in a rapid (< 1h) and spectular 400% increase in hydraulic conductivity.

Most plants seem to lack traits that might reduce transpiration, such as leaf hair and sunken stomata, which may be taken as mechanisms that promote transpiration, although such traits generally influence both water and $CO_2$ fluxes (Cramer *et al*., 2009). An evolution that occurs in leaf traits may not necessarily exert the same influence on water loss and $CO_2$ assimilation (Cramer *et al.*, 2009). For instance, stomatal crypts have a great impact on water conservation than on $CO_2$ fluxes (Roth-Nebelsick, 2007). Further, although small leaves are usually described as an adaptive trait to ensure close coupling of leaf and air temperature (McNaughton and Jarvis, 1983), they may promote the rate of transpiration (Parkhurts and Loucks 1972; Roth-Nebelsick, 2007) through decreased boundary layer (Nobel, 1999; Yates *et al*., 2010). Higher stomatal density and small leaves in sclerophyllous shrubs, for example, display a coupling between the leaf surface and the atmosphere, which promotes transpiration in winter, when evaporative demand is low and tight control of transpiration in summer (Yates *et al*., 2010). Thus, such enhanced water loss may be important for plants growing in nutrient poor soils for powering mass-flow acquisition of nutrients in winter while water is sufficient (Yates *et al*., 2010). This may illustrate why small leaved species mostly grow in nutrient-limited Mediterranean-type ecosystems in which nutrients are sufficient for acquisition in the cold and wet winter period (Cramer *et al*., 2009).

### 2.5 The effects of N forms in regulating night time transpiration.

Terrestrial plants, particularly $C_3$ and $C_{4,}$ possess incomplete stomata closure in night-time (Scholz *et al*., 2007). An observed incomplete stomata closure in $C_3$ and $C_4$ plant species in night-time possibly causes substantial night-time transpiration ($E_{night}$) (Caird *et al*., 2007). Although it is believed that transpiration occurs during photosynthesis when plants open their stomata to release water vapour and assimilate carbon dioxide [i.e. Water use efficiency] (Hack *et al*., 2006), substantial $E_{night}$ occurs with the absence of $CO_2$ assimilation and contribute ca. 30% to terrestrial transpiration ($E_T$) (Caird *et al*., 2007). A question is raised on the functional role of night-time transpiration, if leaf cooling and turgor pressure maintenance are not demanded by plants in night-time (Howard and Donavan, 2007). It is suggested that night-time transpiration may have functional role in driving mass-flow acquisition of soil nutrients to root surface (Caird *et al*., 2007). Night-time transpiration and mass-flow nutrient acquisition seem to fluctuate due to environmental factors, particular nutrient availability in the ecosystem (Scholtz *et al.,* 2007).

Whilst nutrient availability is known to control $E_{night}$, some studies suggested that the rate of $E_{night}$ may increase at a low nutrient availability (Ludwig *et al*., 2006; Scholtz *et al*., 2007; Matimati *et al*., 2013). Other studies suggested that N and P availability had no effect on the $E_{night}$ (Howard and Donavan, 2007). These differences could possibly be affected by various nutritional concentrations supplied, or different genetic responses from plant species (Matimati *et al.*, 2013). A recent study on *Aspalathus linearis* concluded that both day- and night-time transpiration had a functional role in promoting nutrients delivery in nutrient poor sand *via* mass-flow (Matimati *et al*., 2013). In addition, the study conducted on saplings of hybrid aspen (*P. tremula* L. × *P. tremuliodes* Michx.) displayed an increase in $E_{night}$ with low N treatment, which was associated with restricted uptake and delivery of N (Kupper *et al*., 2012). Therefore, if substantial $E_{night}$ is an adaptive process for acquisition of soil nutrition *via* mass-flow, then a limited nutrient availability, particularly N may possibly increase $E_{night.}$

## 2.6 Effects of N forms on water use efficiency and implications in agriculture.

Globally, water is a major limiting natural resource, and *ca*. 70% of water is used in agriculture for irrigation to achieve good plant production (Oweis and Hachum, 2006; Lambers *et al*., 2007). Plants transpire ca. 95 % and retain only 5% of root acquired water (McElron *et al*.,2001; Raven and Johnson, 2001). High rates of transpiration are viewed as a cost on *WUE*, because changes of *WUE* are associated with changes of gas exchange perimeters including transpiration rate, stomatal conductance, intrecellular $CO_2$ and photosynthetic rate. *WUE* at a leaf level is defined as a ratio of net assimilation of $CO_2$ to net assimilation of transpired water (moles of $CO_2$ to kg of transpired water), and thereafter known as instantaneous water use efficiency or $WUE_i$ (Shangguan *et al*., 2000). A reduced photosynthetic capacity at the mesophyll level may lead to greater *WUE*i. Manipulation of *WUEi* can be a mechanism to improve overall crop water use (Cramer *et al*., 2009). However, *WUEi* may be significantly manipulated by nutrient availability, particularly P and N at deprived levels (not deficiency) (Wilkinson *et al*., 2007; Matimati *et al*., 2013) through stomatal conductance and root hydraulic conductance (Raven *et al.*, 2004). This notion is supported by observed higher value of *WUEi* at a lower availability of N source supplied in *P. vulgaris* (Matimati *et al*., 2013). $NH_4^+$ nutrition increased $WUE_i$ more than $NO_3^-$ nutrition in Rice (Guo *et al*, 2007 b), white clover and wheat (Hong-Jensen and Schjoerring, 1997; Yin and Raven, 1998), but not amino acids and $NO_3^-$ nutrition. $NO_3^-$ nutrition increased $WUE_i$ nutrition in tomato more than $NH_4^+$ (Clausen, 2002). We believe that $WUE_i$ response in plants vary depending on crop species. Now, it seems that there is no concrete implication on N-forms regulation of *WUEi* due to variable responses.

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# CHAPTER 3

## 3.0 $NH_4^+$ AND $NO_3^-$ REGULATION OF DAY- AND NIGHT-TIME TRANSPIRATION IN *P. VULGARIS* L.

### 3.1 Abstract

Transpiration powers acquisition of soil nutrients towards the root surface through mass-flow. Nitrogen may regulate transpiration and possibly mass-flow acquisition of soil nutrients. Despite such knowledge on N regulation of transpiration, it remains unknown whether both inorganic N forms can regulate transpiration. The study investigates the hypothesis that both $NO_3^-$ and $NH_4^+$ indirectly signal for the increase in rates of transpiration, when N is limiting, for increasing mass-flow acquisition by roots. *P. vulgaris* was grown in troughs designed to create a N-availability gradient by restricting roots from intercepting a slow-release $NO_3^-$ or $NH_4^+$, placed at one of four distances behind a 25-µm mesh from which nutrients could move by diffusion or mass-flow (dubbed 'mass-flow' treatment). Control plants had direct access to $NO_3^-$ and $NH_4^+$ in their root zone through interception, mass-flow and diffusion (dubbed 'interception' treatment). $NO_3^-$-fed 'mass-flow' plants closest to the N source had 2-fold higher stomatal conductance ($g_s$), 2.6-fold higher transpiration ($E$), 1.8-fold higher night-time stomatal conductance ($g_{\mathrm{night}}$), and 1.5-fold higher night-time transpiration ($E_{night}$) than $NO_3^-$-fed 'interception' plants, despite comparable values of photosynthetic rate ($A$). $A$, $E$ and $g_s$ of 'mass-flow' plants declined with increasing distance from the N source to values even lower than those of 'interception' plants. Unlike $NO_3^-$-fed mass-flow plants, $NH_4^+$ didn't regulate day- and night-time transpiration but rather displayed toxicity symptoms. Thus, under $NO_3^-$ availability plants may be opportunistic in their water uptake, transpiring more when the water is available, in order to acquire $NO_3^-$ and other nutrients through mass-flow.

### 3.2 Introduction

Terrestrial plants transpire up to 95% of root acquired water and retaining less than 5% for cell expansion and growth (McElron *et al*., 2013). Consequently, transpiration has been construed as serving other functions, such as cooling of leaves (Nobel, 1999), maintaining turgor pressure (Rygol *et al*., 1993), driving the xylem transport of solutes from roots to shoots (Campbell *et al*., 1999), and powering nutrients through the soil towards the root surfaces through mass-flow (Barber 1995; Carmer *et al*., 2008; Cramer *et al*., 2009; Matimati *et al*., 2013).

Although there are other edaphic factors that influence plant water fluxes, nutrient availability, particularly N may regulate transpiration and modulate mass-flow acquisition of other soil nutrients (Cramer *et al*., 2008, Matimati *et al*., 2013). This notion is supported by a remarkable decrease of transpiration rate (*E*) in *E. calycina* (Cramer *et al*., 2008), *Randia canthodies* (Zhu *et al*., 2014) and *P.vulgaris* (Matimati *et al.*, 2013) when N was supplied. Despite this response, which was also triggered by P supply in *R. canthodies*, it was N that regulated transpiration-driven mass-flow of soil nutrients towards the rhizosphere ( Cramer *et al*., 2008; Matimati *et al*., 2013).

N-regulation of stomatal conductance and transpiration are known, but the pending question is whether both N-forms ($NO_3^-$ and $NH_4^+$) regulate transpiration possibly for enhancing mass-flow of soil nutrients in nutrient-deficient plants. $NO_3^-$ possibly regulates transpiration through controlling: i) root hydraulic conductivity through aquaporins, and 2) shoot hydraulic conductivity through the stomatal aperture (Cramer *et al*., 2009). For example, low $NO_3^-$ increased water fluxes and nutrient acquisition in *P. vulgaris* L (Guo *et al*., 2007a), and in *E. cyclina* (Cramer *et al*., 2008) whilst $NO_3^-$–replete plants down regulated their water fluxes (Cramer *et al*., 2008; Matimati *et al*., 2013). Likewise, urea which releases precursors of $NO_3^-$ and $NH_4^+$, increased water fluxes and nutrient acquisition in *P. vulgaris* when the roots had limited access, but decreased when roots intercepted much of the fertilizer. On the contrary, $NH_4^+$ seems to play no significance in regulating plant water fluxes (Cramer *et al*., 2009). Excessive levels cause $NH_4^+$ toxicity or ‘ammoniacal syndrome’ (Cramer and Lewis, 1993; Challiou and Lamaze, 2001), which may be related to failure in regulating water fluxes. $NH_4^+$ toxicity is noted from suppression of plant growth, leaf chlorosis and reduction of shoot: root ratio. Role of $NH_4^+$ in transpiration requires testing, given that $NH_4^+$ is converted to amino acids which have no significance in regulating stomatal closure.

Therefore, the main objective of the study was to determine whether both $NH_4^+$ and $NO_3^-$ regulate plant water fluxes and consequently mass-flow acquisition of nutrients in *P. vulgaris*. The specific objectives were: i) to determine whether $NH_4^+$ and $NO_3^-$ regulate day- and night-time stomatal conductance and transpiration consequently affecting the mass-flow acquisition of nutrients in *P. vulgaris*, and ii) to evaluate whether varying availability of $NH_4^+$ and $NO_3^-$, placed at different distances from the roots affects plant water fluxes and mass-flow acquisition of nutrients in *P. vulgaris*. It was hypothesized that both $NO_3^-$ and $NH_4^+$ indirectly signal for the increase in rates of transpiration, when N is limiting, for increasing mass-flow nutrient acquisition by roots. This is based on the fact that N-deprived plants may increase their water fluxes, for the mass-flow delivery of nutrients (Cramer *et al*., 2008; Matimati *et al*., 2013).

## 3.3 Research design and methodology

### *3.3.1 Plant cultivation*

A total of 72 PVC 6-litre troughs were partitioned with PVC plates into two compartments, one for growing plants with volume of 4 litres and second part for nutrient supply with volume of 2 litres. Each PVC plate had a 30 $cm^2$ window covered with a 20 µm nylon mesh to prevent roots from directly accessing the nutrients, while allowing nutrients to move between compartments. The plant and nutrient compartments were filled with 4 and 2 kg of sterile acid-washed and rinsed (pH 7) sand respectively. Two seeds of *P. vulgaris* cv. Star 2000 (Stark Ayres, Rondebosch, Cape Town) were sown in each pot at a depth of 50 mm equidistant from the edges at 50 mm from the PVC plate. After wetting the sand, a 6.5 $cm^3$ core was excavated at either 0, 9, 18 or 27 mm from the PVC plate in the nutrient compartment using a 9-mm diameter cork borer. To avoid rapid volatilisation, 5 g of slow-release $NH_4Cl$ or $KNO_3$ (Haifa Chemicals, Brackenfell, South Africa) fertilisers were placed in the soil cores at varying distances and lightly covered with sand at the top, thus creating a gradient of N availability.

All plants were grown for about 62 days in a temperature- and relative humidity-controlled greenhouse at the Bellville Campus of Cape Peninsula University of Technology (33.9324° S, 18.6406° E). The positions of the troughs were regularly rearranged within a glasshouse in every second day to create uniform growing conditions for cultivated plants. An ideal temperature range between 18°C - 25 °C was maintained. Plants received a 200 ml of N-free Long Ashton nutrient solution (Hewitt, 1952) containing 2.4 mM $PO4^{3-}$, 2 mM K, 4 mM Ca, 1.5 mM Mg, 3.5 mM $SO4^{2-}$, 0.1 mM FeEDTA, 0.02 mM Mn, 0.14 mM $H_3BO_3$, 4.2 mM Na, 4 mM Cl, 0.003

mM Cu, 0.0002 mM Mo and 0.002 mM Zn twice per week. Soil moisture was monitored at hourly intervals using $ECH_2O$-5TE moisture sensors and EM50 data loggers (Decagon Devices, Pullman, USA). Irrigation supply was maintained in the range of 0.15 and 0.2 g $H_2O$ $g^{-1}$ DW sand, which was maintained with the aid of estimates of moisture content from moisture sensors.

### *3.3.2 Gas exchange analysis*

All plants were watered prior to gas exchange measurements. Photosynthetic rate ($A$), stomatal conductance ($g_s$), transpiration rate ($E$), respiration dark ($Rd$), night-time stomatal conductance ($g_{night}$) and transpiration rate ($E_{night}$) of the third fully expanded leaves of each plant were measured using a Licor 6400-02B cuvette connected to a Li6400XT portable gas exchange system. Gas exchange parameters were measured after equilibration of the leaf in the cuvette (*ca*. 5 min) at a saturating photosynthetically active radiation (PAR) level of 1200 µmol quanta $m^{-2}$ $s^{-1}$ with 400 µmol $L^{-1}$ $CO_2$ and flow rate of 500 µmol $s^{-1}$. Average leaf temperature and relative humidity were 25$^0$C and 65 % respectively during the measurements.

### *3.3.3 Biomass measurement*

After harvesting and oven-drying (70˚C for 48 hours) the shoot and roots, their biomasses were weighed. The harvesting involved gently excavating and washing the roots under running water and then blotting them with paper towel and the plants were separated into shoots and roots before oven-drying. Dried shoots of each plant were milled in a Wiley mill using a 0.5 mm mesh (Arthur H. Thomas Co. Philadelphia, CA, USA) to enable analysis of tissue nutrient concentrations and for mass spectrometry ($^{15}N/^{14}N$, $^{13}C/^{12}C$).

### *3.3.4 Foliar elemental and isotope analysis*

The milled leaf material was ashed at 480 ˚C for 8 hours before dissolving with a 1:1 (v/v) of HCl (Kalra, 1998) to measure foliar nutrient concentration. Performing an assessment of the element concentration in solution, an inductively coupled plasma atomic emission spectrometry was used. Foliar [N] and $^{15}N$ /$^{14}N$ isotope ratios (expressed as $\delta^{15}N$) were determined using mass spectrometry to verify the N source used by plants. Atmospheric $N_2$ fixation gave a $\delta^{15}N$ signature closest to the natural abundance values of almost zero whilst urea-N should show enriched $\delta^{15}N$ signature due to losses of N through volatilization (Högberg, 1990, Högberg and

Johannisson 1993). Between 1.900 and 2.000 mg of ground leaf sample was weighed into a 5 mm × 9 mm tin capsule. The tin capsule was combusted in thermo flash EA 1112 series elemental analyser coupled to a delta plus XP isotope ratio mass spectrometer *via* a thermo Finnigan conflo control unit. International Atomic Energy Authority standard was used to determine the isotope values.

### 3.3.5 Data analysis

One-way analyses of variance (ANOVA) and post-hoc Tukey's HSD tests were performed using Statistica (version 10 Statsoft Inc., Tulsa, OK, USA) to evaluate content between the fertiliser treatments.

## 3.4 Results

### 3.4.1 Plant biomass in response to $NO_3^-$ and $NH_4^+$ availability

Total biomass of $NO_3^-$-fed plants decreased with the increase in distance from the N-source (i.e.0, 9, 18 and 27 mm distances), behind nylon mesh. Plants which had direct root-access to $NO_3^-$ (interception) had significantly ($p<0.001$) higher total biomass than plants that accessed $NO_3^-$ at the same distance (ca. 0 mm) just behind a nylon mesh ('mass-flow') plants (Fig 3.1a).

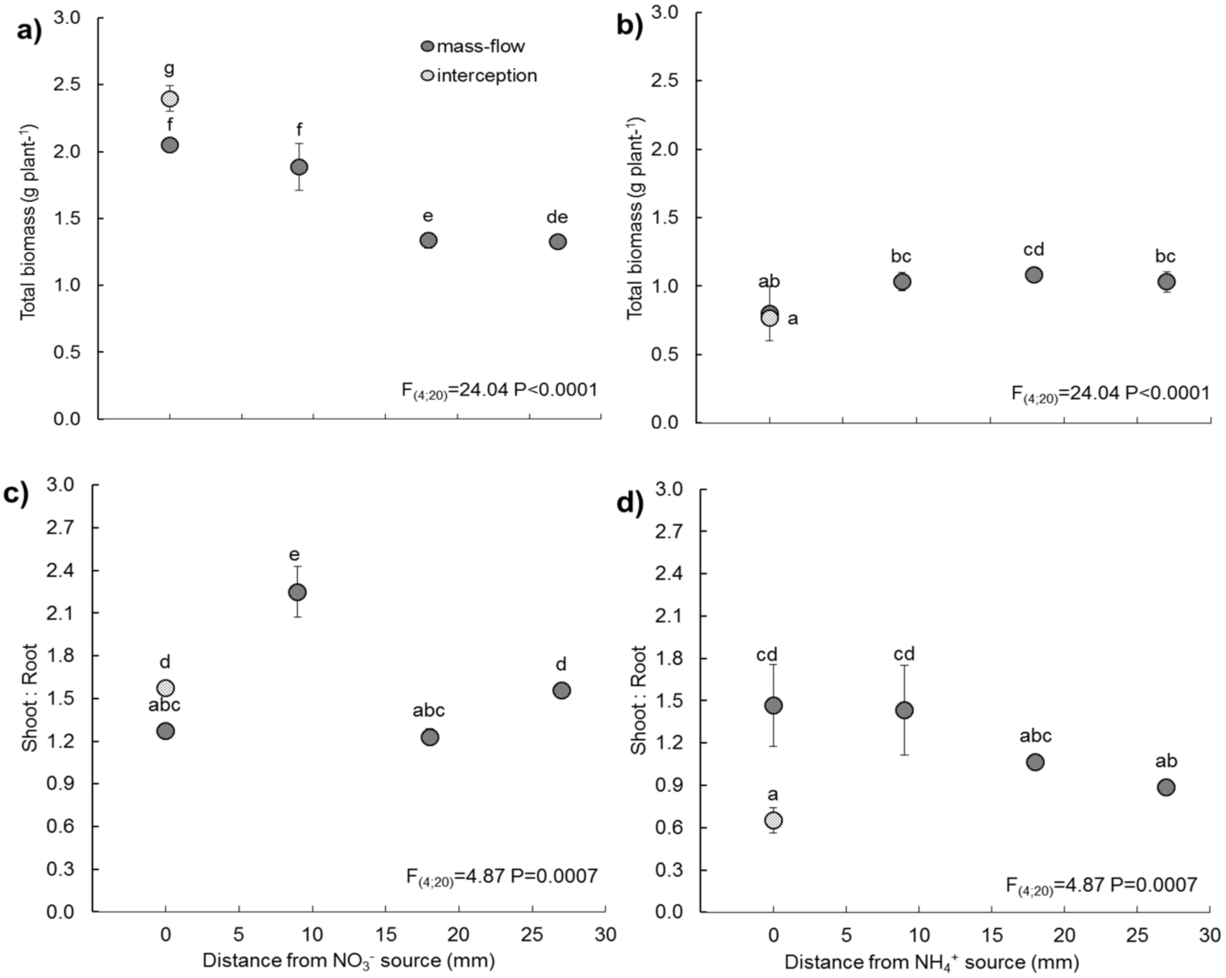

Fig.3.1. Variation in total biomass of *Phaseolus.vulgaris* plants fed with $NO_3^-$ (a) and $NH_4^+$ (b) with the fertilizer placed at varying distances. The respective shoot: root ratios for $NO_3^-$ (c) and $NH_4^+$ (d) plants. Dark circles and bars represent mean (n=6) ± standard errors for “mass-flow” plants, whilst lighter circles represent “interception” plants. Means with different letters are significantly different after a one-way ANOVA and a post-hoc Turkey’s HSD.

Unlike in $NO_3^-$-fed plants, total biomass in $NH_4^+$-fed plants did not significantly vary at increased distances from the N-source behind a nylon mesh, but were all higher than for plants that had direct root-access to $NH_4^+$(Fig 3.1b). Thus, $NH_4^+$-fed plants were smaller than $NO_3^-$-fed plants, this was displayed by their root biomass (data not presented; Fig 3.2). Shoot: root ratios did not display discernible trends in $NO_3^-$-fed plants (Fig 3.1c), but showed a significant decline ($p<0.001$) with distance for $NH_4^+$-fed plants (Fig 3.1d). Despite having the same total biomass, ‘mass-flow’ $NH_4^+$-fed plants (ca. 0 mm) had a two-fold higher shoot: root ratio than ‘interception’ plants, which had direct root-access to $NH_4^+$.

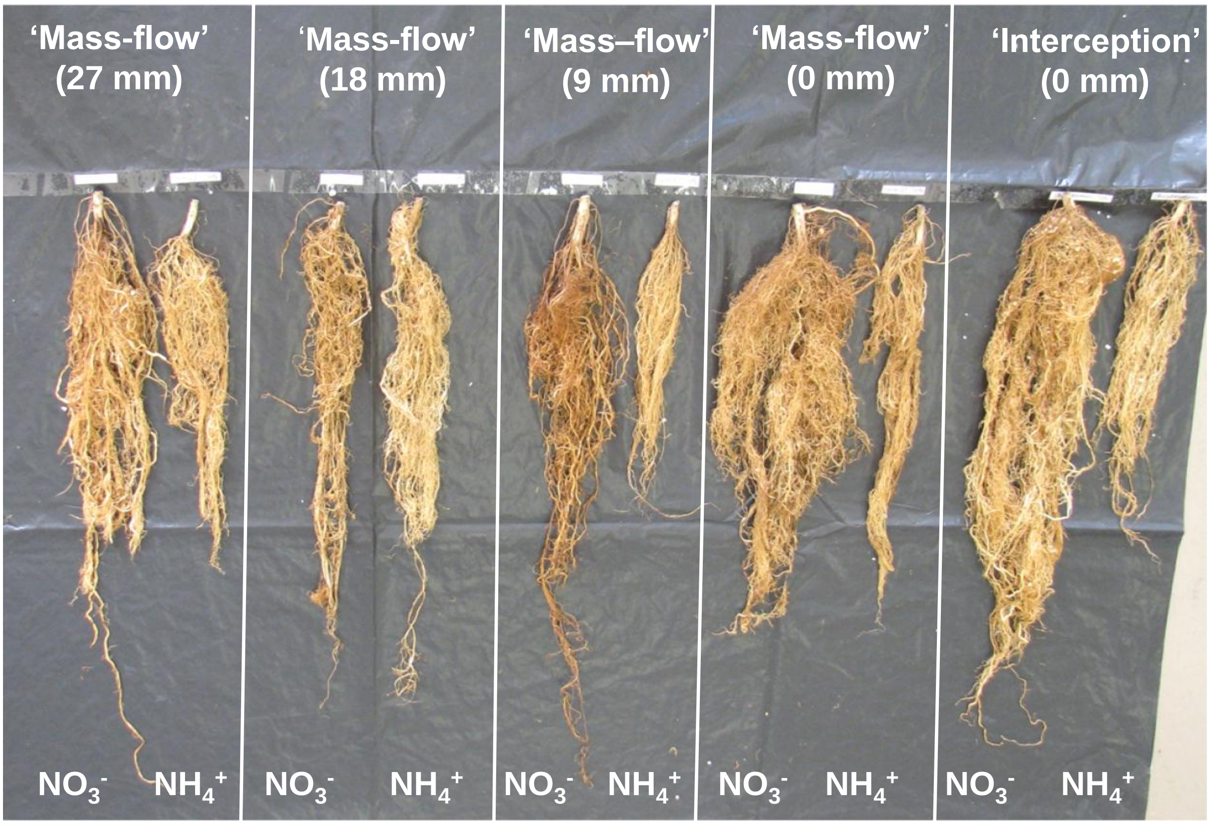

Fig.3.2. Roots of *Phaseous vulgaris* plants that accessed either $NO_3^-$ or $NH_4^+$ at different distances (0, 9, 18 and 27 mm) from behind a nylon mesh by ‘diffusion’ and ‘mass-flow’ or directly by root ‘interception’, diffusion and mass-flow.

### *3.4.2 Gas exchange response to $NO_3^-$ and $NH_4^+$ availability*

In both $NO_3^-$- and $NH_4^+$-fed plants photosynthetic rates (*A*), stomatal conductance ($g_s$) and transpiration rates (*E*) varied with the N-form and its proximity to the roots (Fig 3.3). For $NO_3^-$- fed ‘mass-flow’ plants, *A*, $g_s$ and *E* decreased with the increased distance from the N-source, whilst $NH_4^+$-fed plants had increased values with distance from N-source.

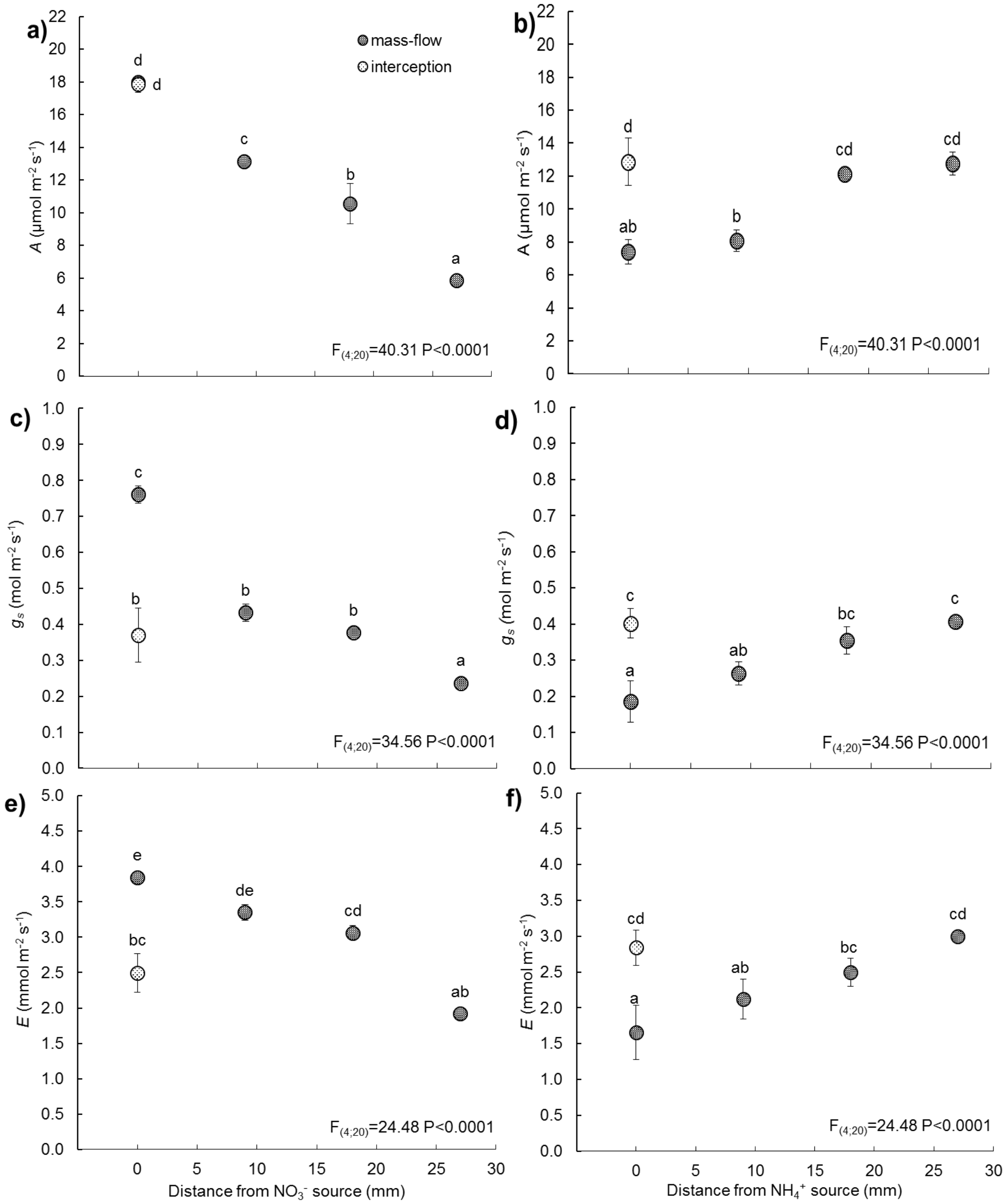

Fig.3.3. Correlation of photosynthetic rate (*A*), stomatal conductance ($g_s$), and transpiration rate (*E*), with $NH_4^+$ and $NO_3^-$ at varying distances of mass-flow and interception in *Phaseolus vulgaris*. Each circle and bar represent a mean ±SE (n=6). Different letters on each circles showed significant differences after one-way ANOVA with post-hoc Turkey's HSD.

Despite having the same *A* values, $NO_3^-$- fed plants that intercepted N had lower *E* and $g_s$ values than plants that accessed N from behind a nylon mesh ('mass-flow', 0 mm). In contrast, $NH_4^+$-fed plants with direct access to N (interception) had higher *A*, $g_s$ and *E* than plants that accessed N from behind a nylon mesh ('mass-flow', 0 mm). The highest values of *A*, $g_s$ and *E* were attained for $NO_3^-$-fed 'mass-flow' plants at the most proximal distance (0 mm) to a nylon mesh whilst in $NH_4^+$-fed plants had their highest *A*, $g_s$ and *E* at distances distal to the N source.

Respiration dark (*Rd,* µmol $m^{-2}$ $s^{-1}$), night-time stomatal conductance ($g_{night}$) and night-time transpiration ($E_{night}$) in $NO_3^-$-fed 'mass-flow' plants decreased as the distance from $NO_3^-$-source increased (Fig 3.4). At the most proximal distances (ca. 0 mm), 'interception' plants, which had direct access to $NO_3^-$ had lower *Rd,* $g_{night}$ and $E_{night}$ than 'mass-flow' plants (0 mm). *Rd,* $g_{night}$ and $E_{night}$ in $NH_4^+$-fed plants declined with increased distances from the $NH_4^+$-source behind a nylon mesh (mass-flow plants; Fig 3.4b, d & f). Both $NO_3^-$- and $NH_4^+$-fed plants displayed similar patterns, but different magnitudes, in their respective $g_s$ and $g_{night}$ values as well as trends in *E* and $E_{night}$ (Fig 3.4 c-f).

### 3.4.3 Foliar N and Carbon Isotope response to $NO_3^-$ and $NH_4^+$ availability.

Although $NO_3^-$- fed plants had insignificant differences in foliar [N], $NH_4^+$-fed plants accumulated foliar N at increased distances from the $NH_4^+$-source, this coinciding with their reduced biomasses (Fig 3.5 a & b). 'Interception' plants, which had direct access to $NH_4^+$ had the same foliar [N] as 'mass-flow' plants that acquired $NH_4^+$ from the nearest distance behind the nylon mesh (ca. 0 mm).

Values of $\delta^{15}N$ in the fertilizers used were 0.1 ‰ in $NH_4^+$ and 0.2 ‰ $NO_3^-$. Plants that had direct 'interception' of $NH_4^+$ attained higher $\delta^{15}N$ than mass-flow plants closest to the N-source (0 mm) (Fig 3.5c & d). Unlike in $NH_4^+$-fed plants, the $NO_3^-$-fed plants closest to the N-source (0 mm) had more enriched values (positive) than 'interception' plants. Generally, $NO_3^-$-fed and $NH_4^+$-fed plants displayed contrasting response trajectories as the N-source became distant and less accessible. $NO_3^-$-fed plants had higher $\delta^{15}N$ values when the N-source was close, whilst $NH_4^+$-fed plants had higher $\delta^{15}N$ as $NH_4^+$ fertilizer became distant.

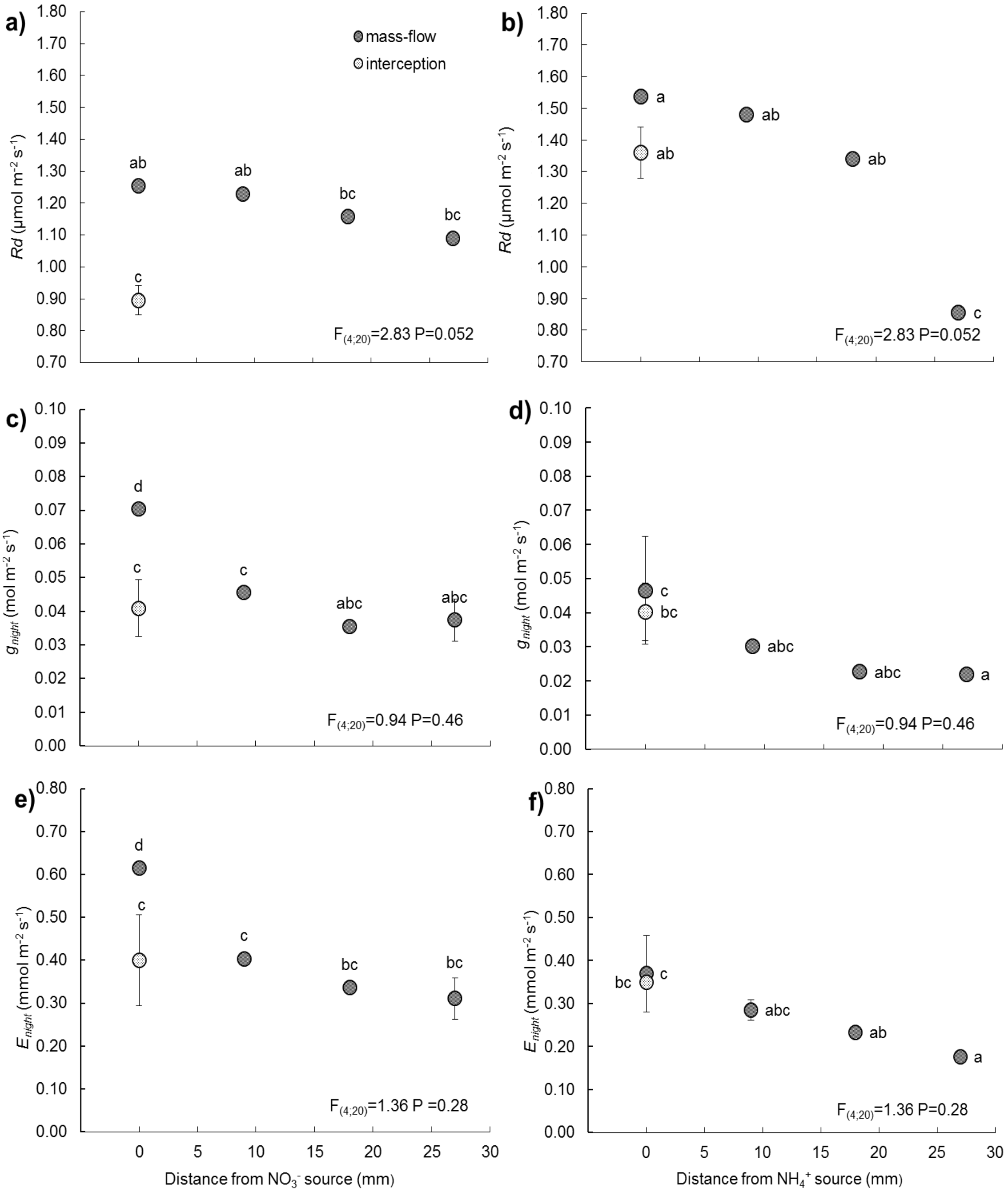

Fig.3.4. Respiration dark (*Rd*), night-time stomatal conductance ($g_S$) and transpiration (*E*) of *Phaseolus vulgaris* plants acquire $NO_3^-$ and $NH_4^+$ nutrition at varying distances (0, 9, 18 and 27 mm) behind nylon mesh dubbed ‘ mass-flow plant’ , or intercepting nutritional sources directly dubbed as ‘ interception’ plants. ‘Mass-flow plants’ represented by solid filled circles and ‘interception plants’ are represented by light filled circles. Each circle and bar represent a mean ±SE (n=6). Different letters on each circle showed significant differences after one-way ANOVA with post-hoc Turkey’s HSD.

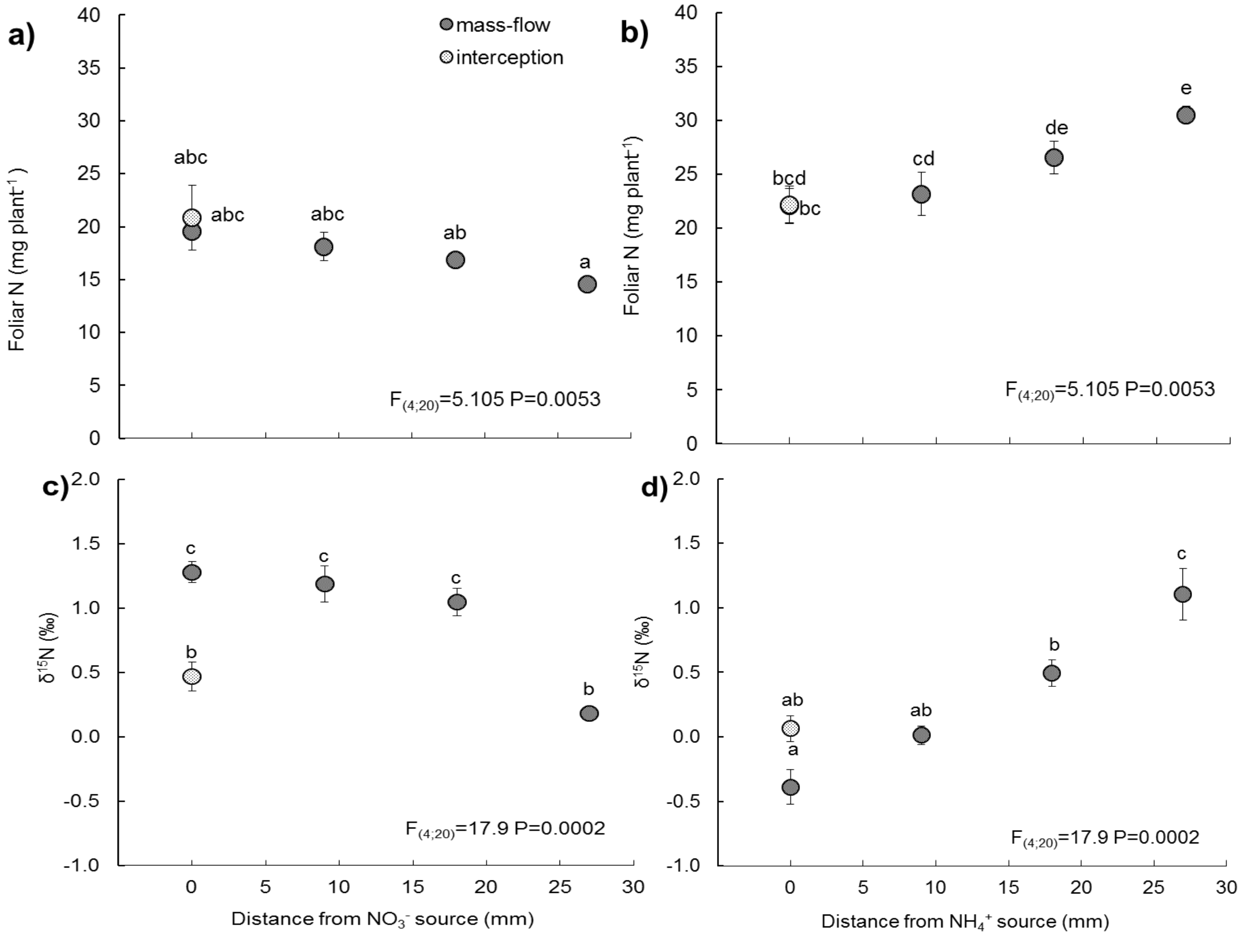

Fig.3.5. A variation of the foliar N and $\delta^{15}$N with distance from the $NO_3^-$ and $NH_4^+$ sources in *Phaseolus vulgaris* accessing a slow-release fertiliser either by bar represents a mean ± SE (N=6). Means with different letters showed significant differences after a one-way ANOVA with post hoc Tukey's HSD.

## 3.5 Discussion

Evidence emerged that accessibility to soil N by *P. vulgaris*, either as $NH_4^+$ or $NO_3^-$, partially regulated photosynthetic rates and day- and night-time water fluxes ($g_s$, $g_{night}$, $E$, $E_{night}$ and *WUE*), consequently affecting mass-flow acquisition of nutrients. Contrasting trends were displayed by $NO_3^-$ and $NH_4^+$, suggesting that $NH_4^+$-fed plants lacked a mechanism to control their day-time water fluxes (discussed further). Plants that had direct 'interception' of $NO_3^-$ or $NH_4^+$ (*ca.* 0 mm) responded differently from plants accessing $NO_3^-$ or $NH_4^+$ past a mesh screen from the closest distance (*ca.* 0 mm). Day- and night-time water fluxes measured from $NO_3^-$-fed plants indicated the role of $NO_3^-$ in stomatal regulation.

The experimental set-up used was effective in varying N-availability to the roots, thus allowing the functional role of $NO_3^-$ and $NH_4^+$ to be explored. The significance of supplied $NO_3^-$ and $NH_4^+$ to the plants was revealed by the absence of effective nodules on roots and the sterile sand used. The observed gradual decline in photosynthesis and total biomass as the N-source became distant points to a gradual decline in availability of soil N from $NO_3^-$-fed plants, however, explains a regulatory role of N-availability particularly $NO_3^-$. Furthermore, the stunted root growth (Fig 3.2), wilting of leaves (data not shown) and indistinguishable differences in total biomass for $NH_4^+$-fed plants is consistent with the symptoms of ammoniacal syndrome as described in previous studies (Chaillou and Lamaze, 2001; Miller and Cramer, 2004), showing morphological effects of N fertilizer as $NH_4^+$. However, the increase in photosynthetic rates in $NH_4^+$-fed plants distant from the fertilizer serves as evidence for a gradual decline in availability of the ‘potentially toxic’ $NH_4^+$ fertilizer. $NH_4^+$ toxicity arises from accumulation of amino acids in tissues of plants (Miller and Cramer, 2004) without capacity to down-regulate its uptake. Soil N availability may also regulate plant water fluxes in *P. vulgaris* (Matimati *et al*., 2013), as was demonstrated in this study with the $NO_3^-$-fed plants.

Evidence for $NO_3^-$ regulation of water fluxes emerged from this study, which suggests that modulation of $g_s$, $E$, $g_{night}$ and $E_{night}$ were possibly for powering the acquisition of nutrients through ‘mass-flow’ from inaccessible, but proximal, fertile soil zones. Firstly, the closest (ca. 0 mm) ‘mass-flow’ plants had 2-fold higher $g_s$, 1.6-fold higher $E$, 1.8-fold higher $g_{night}$, and 1.5-fold higher $E_{night}$ compared to ‘interception’ plants (ca. 0 mm), which directly accessed $NO_3^-$ from the rhizosphere, despite having the same photosynthetic rates (Fig 3.3 a). This suggests that *P. vulgaris* elevated its water fluxes to power the inaccessible, but mobile $NO_3^-$ to its root surfaces without compromising on photosynthetic activity. These plants were not limited by water and, therefore, variation in water fluxes was not a consequence of differences in the available water. These data are corroborated by previous reports that N regulates transpiration, resulting in greater water flux when N availability is restricted, but not deficient (Cramer *et al*, 2008; Cramer *et al*., 2009; Matimati *et al*., 2013). Secondly, the decline in $g_s$, $E$, $g_{night}$ and $E_{night}$ in $NO_3^-$-fed mass-flow plants demonstrates a regulatory role of N as previously discussed (Cramer *et al.,* 2009; Matimati *et al.*, 2013). In brief, increasing [$NO_3^-$] above sufficiency must have rapidly elicited concentration-dependent stomatal closure (Wilkinson *et al*. 2007), potentially mediated by changes in xylem pH, ABA signalling (Wilkinson *et al.*, 1998) and nitric oxide (NO) signalling (Desikan *et al.*, 2005; Neill *et al.*, 2008). This trend of $NO_3^-$-fed plants contrasted that of $NH_4^+$-fed plants, which appeared as lacking the ability to down regulate their N intake (Cramer *et al.*, 2009).

Unlike $NO_3^-$, the incapacity of $NH_4^+$ to down-regulate $g_s$, $E$, $g_{night}$ and $E_{night}$ when in excess suggests failure of $NH_4^+$ to elicit stomatal closure, to reduce the toxic accumulation of amino acids (Cramer *et al.*, 2009), which led to the observed ammoniacal syndrome. At the closest distance from $NH_4^+$ source (*ca.* 0 mm), 'interception' plants with direct root access to $NH_4^+$ had 1.9-fold higher $A$, 2- fold higher $g_s$ and 1.8-fold higher $E$ than 'mass-flow' plants (*ca.* 0 mm from $NH_4^+$), indicating the incapacity of the directly acquired $NH_4^+$ to elicit stomatal closure. Despite having the same total biomass, 'mass-flow' plants closest to $NH_4^+$-source (*ca.* 0 mm) invested more in shoot biomass by 2.5-fold compared to 'interception' plants (*ca.* 0 mm) (Fig 3.1 b & d; Fig 3.2). It is possible that the increased root biomass helped distal 'mass-flow' plants in countering uncoordinated regulation of root hydraulic conductance and $g_s$, which characterize the ammoniacal syndrome (Cramer *et al.*, 2009).

Since no effective nodules were observed, the increasingly more positive $\delta^{15}N$ values increasing from -0.4‰ to 1‰ with distance from $NH_4^+$-source in 'mass-flow' plants, suggests use of a more enriched N source following volatilization of $NH_3$ (Högberg, 1990; Högberg and Johannisson, 1993). The fact that foliar [N], $\delta^{15}N$ increased along with stomatal conductance and transpiration for 'mass-flow' plants (Fig 3.5 b & d & Fig 3.3 d & f) is consistent with the 'mass-flow' delivery of $NH_4^+$ from a distal source. Despite having the same total biomass, the unexpectedly higher $g_s$ and $E$ in $NH_4^+$-fed 'interception' plants (*ca.* 0 mm) than 'mass-flow' plants (*ca.* 0 mm), may be linked to their extensive root system (Fig 3.2) and enhanced $A$ (Fig 3.3b). Thus, root hydraulic conductivity in response to $NH_4^+$-N requires further scrutiny, given that previous studies indicated that $NH_4^+$ may not alter the expression of root aquaporins or root hydraulic conductance (Guo *et al.*, 2007b). Contrasting $NH_4^+$, root-acquired $NO_3^-$ increases aquaporin-mediated root hydraulic conductivity

(Carvajal *et al*., 1996; Clarkson *et al*., 2000; Gloser *et al*., 2007; Gorska *et al*., 2008) and, when it is in excess of the capacity of root nitrate reductase, it is reduced to NO in leaves (Cramer *et al.*, 2009) or it can alter xylem sap pH (Mengel *et al*., 1994; Mühling and Lauchli, 2001), resulting in increased sensitivity of guard cells to ABA, which stimulates stomatal closure (Wilkinson, 2004; Wilkinson *et al.*, 2007; Jia and Davies, 2007).

Unlike $NO_3^-$-fed 'mass-flow' plants (*ca*. 0 mm), which had more positive $\delta^{15}N$, 'interception' plants (*ca*. 0 mm) had $\delta^{15}N$ values closer to those of the fertilizer (0.2‰), suggesting that 'mass-flow' was less important in 'interception' plants. Unlike $NH_4^+$, $NO_3^-$ is less-prone to isotopic enrichment in the soil, which possibly explains the same $\delta^{15}N$ values obtained across the 'mass-flow' distances, except the furthest (*ca*. 27 mm). $NO_3^-$-fed 'mass-flow' plants furthest from N-source must have reduced their metabolism due to N-limitation, as noted from decreased *A, gs* and *E.* This possibly explains the less-positive foliar $\delta^{15}N$ values. These data are consistent with the notable down-regulation of foliar [N] in 'mass-flow 'plants in response to N-limitation (Cramer *et al*., 2008; Matimati *et al*., 2013).

This study quantified, possibly for the first time, night-time stomatal conductance and transpiration in *P. vulgaris* in response to $NO_3^-$ or $NH_4^+$ accessibility. When roots are constrained from directly accessing proximal $NO_3^-$ sources, *P. vulgaris* elicits both day and night-time stomatal conductance which increases 'mass-flow' delivery of N, signalling down-regulation of stomatal conductance when $NO_3^-$ is replete or as the N-source becomes distant. $NH_4^+$-fed plants, however, lacked the mechanism of down-regulating their stomatal conductances, but rather displayed water-stress symptoms ('ammoniacal syndrome') when $NH_4^+$ became replete. It is likely that water fluxes of urea-fed *P. vulgaris* in previous studies (Matimati *et al*., 2013) were also regulated by the $NO_3^-$ but not $NH_4^+$, which are both by-products of urea. Thus, under $NO_3^-$ availability plants may be opportunistic in their water uptake, transpiring more when the water is available, in order to acquire $NO_3^-$ and other nutrients through mass-flow.

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# CHAPTER 4

## 4.0 BIPHASIC $NO_3^-$ REGULATION OF DAY- AND NIGHT-TIME WATER FLUXES IN *P. VULGARIS* L IN A HYDROPONIC SYSTEM.

### 4.1 Abstract

Plant water fluxes respond to nutrient availability through root aquaporins and leaf stomata. Although the functional role of root aquaporin and stomata in controlling day-time and night-time water fluxes is known, the nutritional regulation of plant water fluxes remains largely unclear. To test the biphasic (up- and down-regulation) of day- and night-time water fluxes by $NO_3^-$ or $NH_4^+$, *P. vulgaris* plants were fed with $KNO_3$ or $NH_4Cl$ fertilisers at one of seven concentrations or as five varying proportion. Photosynthetic rate (*A*), stomatal conductance ($g_s$) and transpiration rate (*E*) in $NO_3^-$-fed plants increased with the increase in [$NO_3^-$], attaining the highest value at 4 mM [$NO_3^-$]. $NO_3^-$-fed plants with the highest water fluxes at 4 mM [$NO_3^-$] had 2-fold higher *A*, 3-fold higher $g_s$ and 2.8-fold higher *E*, 1.9-fold lower $g_{night}$ and 2.2-fold lower $E_{night}$ than at the lowest [$NO_3^-$] (*ca.* 0.25 mM). Unlike in $NO_3^-$-fed plants, *A*, $g_s$ and *E* in $NH_4^+$-fed plants declined with the increase in [$NH_4^+$], attaining the highest value at 0.25 mM [$NH_4^+$].$NH_4^+$-fed plants at lowest concentration (0.25 mM) had 4-fold higher $g_s$ and 3.5-fold higher *E* compared to plants at the highest [$NH_4^+$] (*ca.* 8 mM). Plants fed with $NO_3^-$ and $NH_4^+$ as proportions of 100%, 75%$NO_3^-$:25% $NH_4^+$ and 50%$NO_3^-$:50$NH_4^+$ displayed lower *A, $g_s$* and *E* than in plants fed with 0%$NO_3^-$:100%$NH_4^+$.$NO_3^-$-fed plants regulated their day- and night-time water flux in response to availability of limiting [$NO_3^-$], whilst day- and night-time water flux in $NH_4^+$-fed plants was not regulated by [$NH_4^+$]. However, $NH_4^+$-fed plants displayed ammoniacal syndrome. In conclusion, a limiting availability of [$NO_3^-$] regulates day-night-time water flux possibly through root aquaporin and leaf stomata.

### 4.2 Introduction

Plant water fluxes respond to nutrient availability through functional traits such as root aquaporins and leaf stomata (Cramer *et al*., 2009), which responds primarily to [N] in their rhizosphere (Chapter 3) (Cramer *et al*., 2008; Matimati *et al*., 2013). Root aquaporins are plasma membrane intrinsic proteins that facilitate water fluxes by increasing root hydraulic conductivity and are strongly expressed in the exodermis, endodermis and vascular tissue of several species (Maurel *et al*., 2008). Stomata are small pores surrounded by guard cells that regulate gas exchange between plants and the atmosphere (Takahashi *et al*., 2015). Although the functional role of root aquaporin and stomata in controlling day-time and night-time water fluxes is known (Chaumont *et al*., 2005; Maurel *et al*., 2008), the nutritional regulation of plant water fluxes remains largely unclear (Cramer *et al*., 2009; Matimati, 2013).

Whilst $C_3$ and $C_4$ plants are expected to close their stomata at night when $CO_2$ assimilation is absent, substantial night-time conductance ($g_{\mathrm{night}}$) and transpiration ($g_{\mathrm{night}}$) in some species have now been reported (Caird *et al*., 2007). High $g_{\mathrm{night}}$ approximating 50% of day-time stomatal conductance ($g_s$), $E_{\mathrm{night}}$ of *ca*. 15 - 30% of day-time transpiration ($E$) have been reported (Caird *et al*., 2007; Howard and Donovan, 2007). It is suggested that $g_{\mathrm{night}}$ may play a functional role of delivering nutrients to root surfaces through mass-flow (Chapter 3) (Daley and Philips, 2006; Ludwig *et al*., 2006; Caird *et al*., 2007; Scholz *et al*., 2007). Night-time opening of stomata is also perceived as a stress response of plants to drought (e.g. Cavender – bares *et al*., 2007; Philips *et al*., 2010; Zeppel *et al*., 2012). Currently, there is no empirical evidence supporting the mechanism regulating night-time stomatal opening (de Dios *et al*., 2013).

Although the mechanism is unclear, nutrient availability, particularly nitrogen, may potentially regulate $g_s$ and $E$ (Cramer *et al*., 2008; Matimati *et al*., 2013; Chapter 3) as well as $g_{night}$ and $E_{night}$ (Ludwig *et al*., 2006; Cirelli, 2014). The N regulation of day- and night-time water fluxes was demonstrated by high $g_{\mathrm{s}}$ and $E$ values in N-limited *E. calycina* (Cramer *et al*., 2008) and *P. vulgaris* (Matimati *et al*., 2013), but not deficiency. Increased $g_{night}$ and $E_{night}$ observed in N-limited *Helianthus* spp. (Caird *et al*., 2007) and *P. vulgaris* (Chapter 3), suggested stomatal regulation by N. Thus, N has capacity to regulate day- and night-time stomatal fluxes.

Roles of $NO_3^-$ and $NH_4^+$ in controlling day- and night-time water fluxes is unclear, although $NO_3^-$ is linked to the regulation of day- and night-time water fluxes (Gloser *et al*., 2007; Guo *et al*., 2007; Wilkinson *et al*., 2007; Cramer *et al*., 2009). Increased $NO_3^-$ availability in the rhizosphere may rapidly increase aquaporin-mediated root hydraulic conductance in herbaceous species (Clarkson *et al*., 2000; Gloser *et al*., 2007; Gorska *et al*., 2008; Cramer *et al*., 2009). $NO_3^-$ acts as a hydraulic signal that rapidly coordinates response at the whole plant level (Gorska *et al*., 2008). Root hydraulic conductivity increased when $NO_3^-$ availability was abundant and declined when $NO_3^-$ supply was limiting, but not deficient (Carvajal *et al*., 1996; Clarkson *et al*., 2000). Supra-optimal [$NO_3^-$] in the rhizosphere elicited stomatal closure (Wilkinson *et al*., 2007), due to alteration of xylem pH, ABA signalling (Wilkinson *et al*., 1998) and nitrate oxide (NO) signalling (Desikan *et al*., 1998; Neill *et al*., 2008). Thus, [$NO_3^-$] regulation of stomata is biphasic, comprising of concentration-dependant up-regulation and down-regulation phases (Wilkinson *et al*., 2007). Contrary to $NO_3^-$, $NH_4^+$ had no role in regulating root hydraulic conductivity, but rather suppressed acquaporin expression in *P. vulagris*, thus down-regulating water uptake (Guo *et al.,* 2007a).

The proposed 'biphasic' model of N-regulation of stomatal conductance (Wilkinson *et al*., 2007) has not been empirically tested under hydroponic systems. Previous work has used urea-N, which supplied both $NO_3^-$ and $NH_4^+$ (Cramer *et al*., 2008; Matimati *et al*., 2013). Moreover, night-time water fluxes were not monitored. To test the biphasic (up- and down-regulation) of day- and night-time water fluxes by $NO_3^-$ or $NH_4^+$, it was hypothesized that: i) plants fed with increasing [$NO_3^-$] and [$NH_4^+$] should initially up-regulate $g_s$, $g_{night}$, $E$ and $E_{night}$ for 'mass-flow' delivery of the limiting N, then down-regulate these fluxes as foliar [N] exceeds tolerable levels ii) at the same rhizosphere [N], higher proportions of $NO_3^-$ :$NH_4^+$ should down-regulate $g_s$ and $E$, because of the regulatory effect of the $NO_3^-$.

## 4.3 Materials and methods

### *4.3.1 Growth and treatment*

*P. vulgaris* cv. Star 2000 (Stark Ayres, Cape Town) seeds were germinated in vermiculite under controlled greenhouse conditions at the Cape Peninsula University of Technology (CPUT) (33.9324° S, 18.6406° E). Fourteen 'rough-tot' troughs with six planting holes (200 mm x 300 mm) on the lids were used for growing *P. vulgaris* in a deep water culture system under greenhouse conditions at the CPUT (33.9324° S, 18.6406° E). To test the role of [N] and N-form on regulation of water fluxes, either [$NO_3^-$] or [$NH_4^+$] at one of seven levels (0 mM; 0.25 mM; 0.5 mM; 1 mM; 2 mM; 4 mM or 8mM), were allocated to the troughs and similar-sized 10-d old *P. vulgaris* seedlings planted in each hole using square pieces (9 $cm^2$) of OTG 10mm Pilates Mat (Sportsmans Warehouse, Cape Town) for anchorage. Each trough had 68 L of N-free Long Ashton solution (Hewitt, 1966), with the different [$NO_3^-$] and [$NH_4^+$] added as $KNO_3$ and $NH_4Cl$ respectively. The solution was aerated from the base of the trough using airstones connected to Dophin 7500 double 2 outlet pumps. To test the hypothesis that varying the ratios of $NO_3^-$: $NH_4^+$ at the same concentration of N controls water fluxes, a similar deep water culture system was set, but with either 2 mM or 4 mM supplied as proportions of 0% $NO_3^-$:100% $NH_4^+$; 25% $NO_3^-$ :75% $NH_4^+$; 50% $NO_3^-$ :50% $NH_4^+$; 75% $NO_3^-$ : 25% $NH_4^+$ and 100% $NO_3^-$:0% $NH_4^+$.

### *4.3.2 Gas exchange measurement*

Photosynthetic rate ($A$), day-time stomatal conductance ($g_s$) and transpiration ($E$), night-time stomatal conductance ($g_{\mathrm{night}}$) and transpiration ($E_{\mathrm{night}}$) were measured using a 6400-02B chamber with LED light source interfaced with a Li-6400 XT photosynthesis system. $A$, $g_s$ , $E$, $g_{\mathrm{night}}$ and $E_{\mathrm{night}}$ were measured after equilibration in the cuvette (*ca*. 5 min) at a 400 µmol $L^{-1}$ $CO_2$ and flow rate of 500 µmol $s^{-1}$. Since greenhouse temperatures were maintained at 30$^{o}$C, the leaf temperature was set at 30$^{o}$ C, and relative humidity was averaged at 57 % during the measurements. LED lights were switched off during measurements of $g_{\mathrm{night}}$ and $E_{\mathrm{night}}$ at night-time from 21H00 - 01H00 SSAT. In plants with varying ratios of both $NO_3^-$ and or $NH_4^+$, $A$, $g_s$ and $E$ were measured 12 hours after adding N.

### *4.3.3 Plant biomass measurement*

After 21 d from planting, plants were gently removed and their roots rinsed to remove salts and dried using paper towel. Roots and shoots were separated before oven drying them in paper bags until there was no further weight loss at 60$^{o}$C for *ca.* 48hrs cut separately into shoot and root system. Root measurements were done. Both shoot and root were put in the oven and then dried at 70 C for 48 h (Matimati *et al*., 2013).

### *4.3.4 Data analysis*

Analyses of variance (ANOVA) and post-hoc Tukey's HSD tests on total biomass, shoot: root weight ratio, A, $g_s$, $E$, $g_{\mathrm{night}}$ and $E_{\mathrm{night}}$ were done in Statistica Version 13.2 (Dell Software, Texas, USA).

## 4.4 Results.

### *4.4.2 Plant biomass measurements*

Total biomass and shoot: root ratios of $NO_3^-$-fed plants increased as [$NO_3^-$] increased, reaching their maximum biomass at 2 mM, whilst $NH_4^+$-fed plants had reduced biomass as [$NH_4^+$] increased (Fig 4.1). $NH_4^+$-fed plants, however, increased their shoot: root portioning of biomass as the [$NH_4^+$] increased, attaining a maximum at 1 mM.

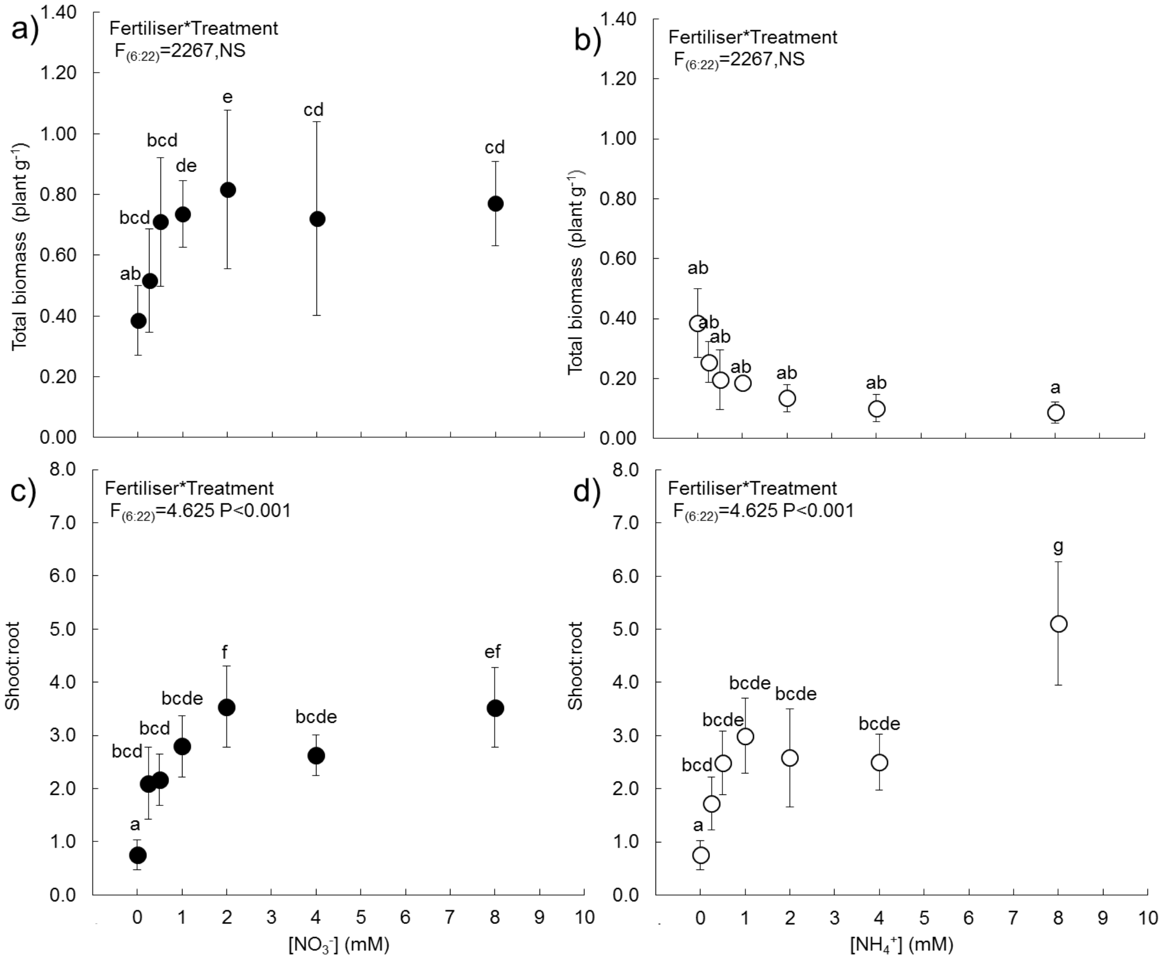

Fig.4.1. Total biomass of *Phaselous vulgaris* plants fed with different (a) [$NO_3^-$] and (b) [$NH_4^+$] and their respective shoot: root ratios for (c) $NO3^-$ and (d) $NH_4^+$ fed plants. Circles and bars represent mean (n=6) ± standard errors. Means with different letters are significantly different after a one-way ANOVA and a post-hoc test using Turkey’s HSD.

### *4.4.2 Gas exchange measurements.*

Photosynthetic rate (*A*), stomatal conductance ($g_s$) and transpirational rate (*E*) in $NO_3^-$-fed plants increased with the increase in [$NO_3^-$], attaining the highest value at 4 mM [$NO_3^-$] (Fig 4.2). $NO_3^-$-fed plants with the highest water fluxes at 4 mM [$NO_3^-$] had 2-fold higher *A*, 3-fold higher $g_s$ and 2.8-fold higher *E* than at the lowest [$NO_3^-$] (*ca.* 0.25 mM). Despite having the same *A* as N-free plants, $NO_3^-$-fed plants at 0.25 mM, had 3-fold higher $g_s$ and 2.2 -fold higher $E$. Unlike in $NO_3^-$-fed plants, $g_s$ and $E$ values in $NH_4^+$-fed plants declined with higher [$NH_4^+$], displaying the highest values when [$NH_4^+$] was lowest (*ca.* 0.25 mM) (Fig 4.2). $NH_4^+$-fed plants at lowest concentration (0.25 mM) had 4-fold higher $g_s$ and 3.5-fold higher $E$ compared to plants at the highest [$NH_4^+$] (*ca.* 8 mM). Despite the indistinguishable $A$, the lowest [$NH_4^+$] (*ca.* 0.25 mM) had 2-fold higher $g_s$ and 1.4 -fold higher $E$ than N-free plants. The $A$, $g_s$ and $E$ of $NO_3^-$-fed *P. vulgaris* displayed curvilinear trajectories, comprising of an up-ward then a down-ward phase (Fig 4.2), whilst trajectories of the $NH_4^+$-fed plants displayed asymptotic decline in $g_s$ and $E$ with higher [$NH_4^+$].

Trends in day- and night-time water fluxes totally different for both $NO_3^-$-fed and $NH_4^+$-fed *P. vulgaris.* Contrary to the curvilinear trajectories, night-time stomatal conductance ($g_{night}$) and transpiration ($E_{night}$) in $NO_3^-$ fed plants declined with the increased [$NO_3^-$] (Fig 4.3). $NO_3^-$-fed plants with the highest water fluxes at 0.25 mM [$NO_3^-$] had 1.9-fold higher $g_{night}$ and 2.2 fold higher $E_{night}$ than at the highest [$NO_3^-$] (*ca.* 8 mM).

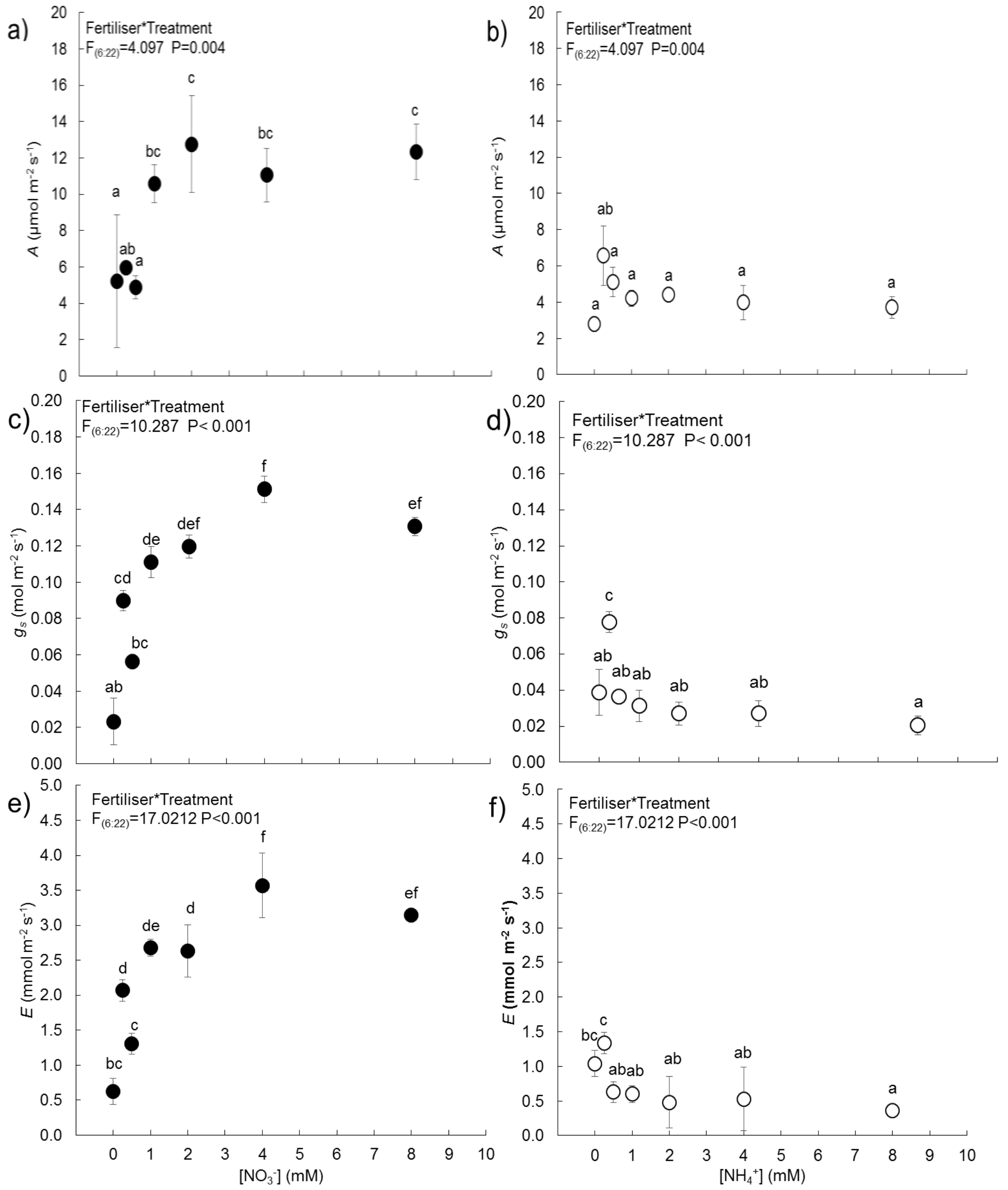

Fig.4.2. Correlation of photosynthetic rate (*A*), stomatal conductance ($g_s$), and transpiration rate (E), with $NO_3^-$ and $NH_4^+$ at varing concentration in *Phaseolus vulgaris*. Each circle and bar represent a mean± SE (n=6). Different letters on each circle showed significant differences after one way Anova with post-hoc Turkeys HSD.

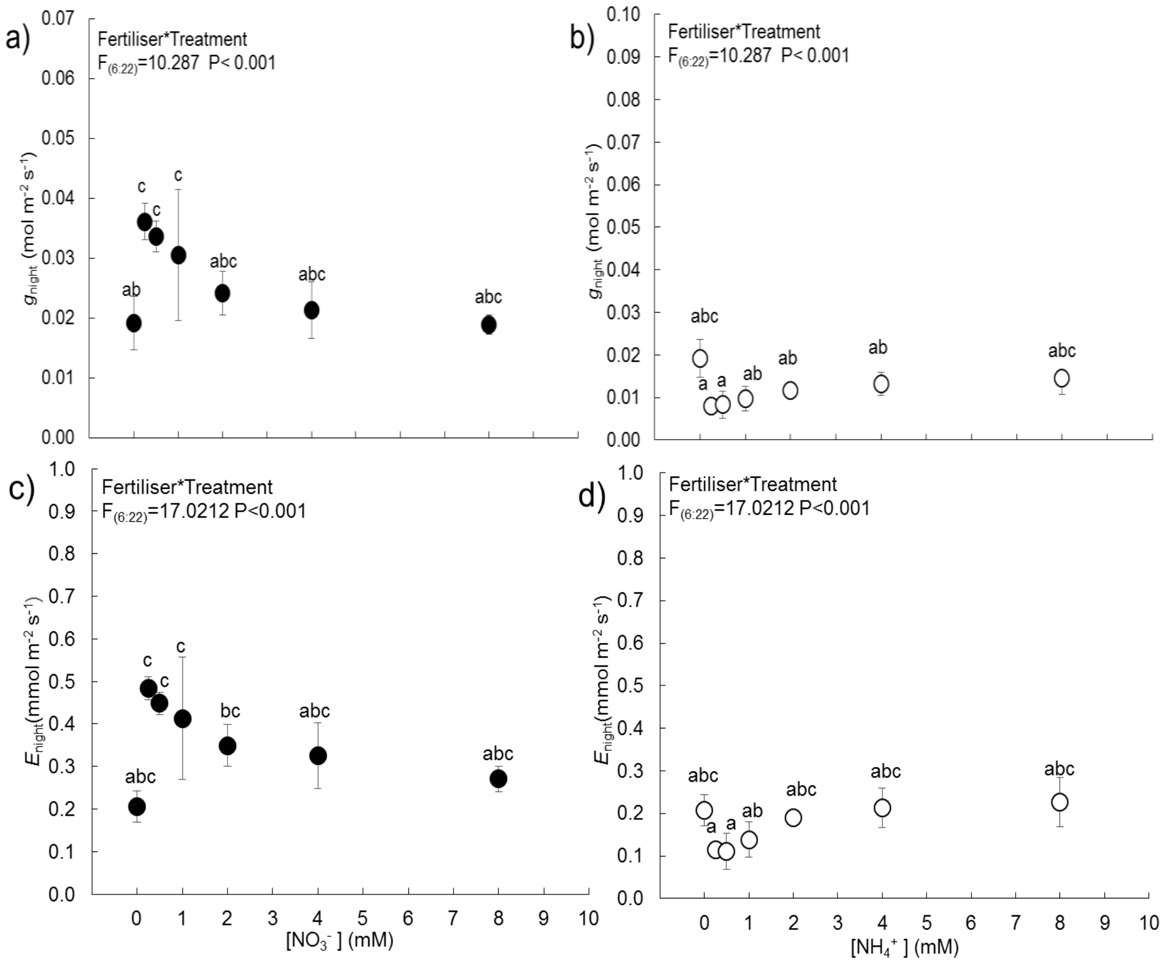

Fig.4.3. Night-time stomata conductance ($g_{night}$) and transpiration rate ($E_{night}$) of *Phaseolus vulgaris* fed with $NO_3^-$ and $NH_4^+$ nutrition at varying concentrations in a hydroponic system. Each circle and bar represents a mean ± SE (N=6). Means with different letters showed significant differences after a one way ANOVA with post hoc Tukey's HSD.

Plants fed with $NO_3^-$ and $NH_4^+$ as proportions of 100% $NO_3$: 0% $NH_4^+$, 75%$NO_3^-$:25% $NH_4^+$ and 50%$NO_3^-$:50$NH_4^+$ displayed lower *A, $g_s$* and *E* than in plants fed with 100%$NH_4^+$: 0%$NO_3$. Trends in *A*, $g_s$ and *E* were similar in both plants fed with mixture of $NO_3^-$ and $NH_4^+$ at 2 mM and 4 mM, but different magnitudes. Plants that were solely $NH_4^+$-fed without $NO_3^-$ had the highest *A, $g_s$* and *E.*

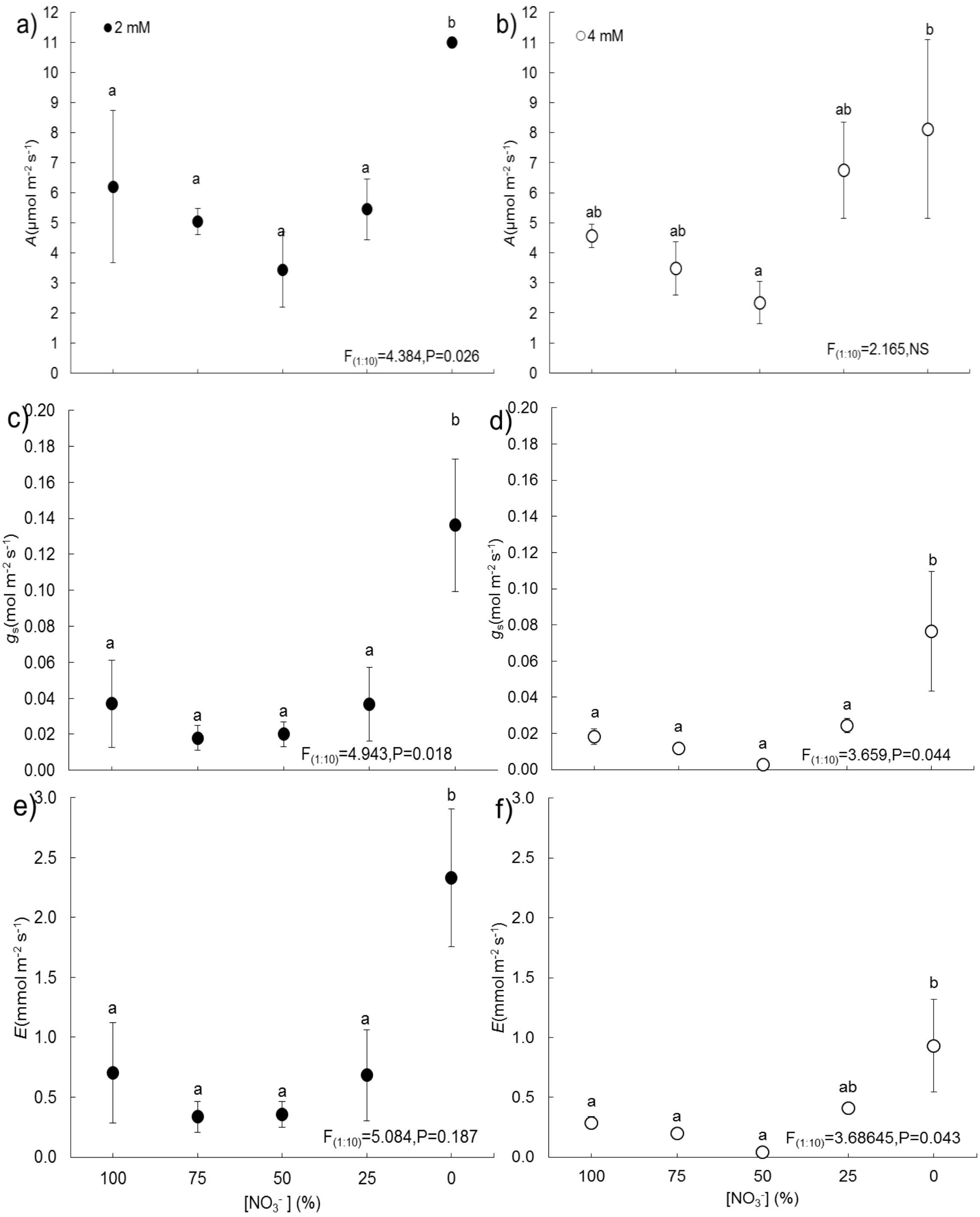

Fig.4.4. Variation in photosynthetic rate (A), stomatal conductance ($g_s$) and transpiration rate (*E*) of *Phaselous vulgaris* plants fed with either sole $NO_3^-$ (a) or $NH_4^+$ and mixture of $NO_3^-$ and $NH_4^+$ in varying ratios at 2 and 4 Mm concentration. Dark circles and bars represent mean (n=3) ± standard errors for sole $NO_3^-$ or $NH_4^+$-fed plants, or mixture of $NO_3^-$ and $NH_4^+$-fed plants. Means with different letters are significantly after a one-way ANOVA and a post-hoc Turkey's HSD.

## 4.5 Discussion

The proposed ‘biphasic’ model of N-regulation of stomatal conductance (Chapter 3) (Wilkinson *et al*., 2007) was tested and verified with empirical evidence from hydroponically grown *P. vulgaris*. Many lines of evidence emerged in support of the $NO_3^-$ regulation of plant water fluxes (discussed further). No evidence was noted in $NH_4^+$-fed plants, which supported our hypothesis that it is not $NH_4^+$ but $NO_3^-$ that regulated water fluxes as previously suggested (Cramer *et al.*, 2009), based on a review of literature. At the same rhizosphere [N], it emerged those higher proportions of $NO_3^-$ :$NH_4^+$ down-regulated $g_s$ and $E$, because of the regulatory effect of the $NO_3^-$.

Firstly, absence of nodulation from *P.vulgaris* indicated that supplied $NO_3^-$ and $NH_4^+$ fertilisers were the likely sources of plant-accessible N. Secondly, the plants were hydroponically grown and had sufficient water, thus, water limitation was not a factor controlling water fluxes. The observed gradual increase in photosynthesis and biomass accumulation in $NO_3^-$-fed plants with the increase in [$NO_3^-$], indicated that photosynthesis and biomass accumulation were up-regulated by high availability of $NO_3^-$ source. However, excessive [$NH_4^+$] caused ‘ammonical syndrome’ characterised by decreased photosynthesis and total biomass, and higher shoot: root ratios. ‘’Ammonical syndrome’’ was associated with reduced water potential and suppression of photosynthesis as the result of reduced stomatal conductance in $NH_4^+$-fed plants (Cramer and Lewis, 1993).

The hypothesis that $NO_3^-$ regulates day- and night-time plant water flux was supported by three lines of evidence. Firstly, the significantly higher $A$, $g_{s\ \mathrm{and}}$ $E$ in the highest $NO_3^-$-fed plants (e.g.8 mM) compared to N-free plants confirming the role of [$NO_3^-$] in regulating water fluxes. These findings concur with Chapter 3 that restricted $NO_3^-$ availability at a proximal distance (ca. 0 mm) behind a 20 µ mesh up-regulated the day-time water fluxes in *P. vulgaris*. Secondly, the 2-fold lower $A$, 2.2-fold lower $g_s$ and 2.3-fold lower $E$ than $NO_3^-$-fed plants at 8 mM, $NO_3^-$-fed plants (0.25 mM) indicates that increased [$NO_3^-$] elicited stomatal closure. Similarly, the reduced $A$, $g_s$ and $E$ in plants fed with sole $NO_3^-$ or a mixture of $NO_3^-$ and $NH_4^+$ compared to sole $NH_4^+$ supports the down-regulation of water fluxes by $NO_3^-$.Thirdly, increased $g_{\mathrm{night}}$ and $E_{\mathrm{night}}$ were observed in plants fed with $NO_3^-$ at a lower concentration ( e.g. 0.25

mM), indicating that $NO_3^-$-fed plants opened their stomata at night, resulting in substantial water fluxes at limiting [$NO_3^-$], but not deficiency (Chapter 3). $NO_3^-$ must have controlled stomatal conductance (Chapter 3; Fig 4.2) and root hydraulic conductivity as previously suggested (Gloser *et al*., 2007).

Unlike $NO_3^-$, $NH_4^+$ had no capacity to regulate day- and night-time water fluxes in *P. vulgaris* under water sufficient conditions. The low $A$, $g_s$, $E$, $g_{night}$ and $E_{night}$ in $NH_4^+$-fed plants were due to ammonium toxicity ('ammoniacal syndrome') and not $NH_4^+$ down-regulating water fluxes. To support this statement, plants that had the lowest [$NH_4^+$] ca. 0.25 mM had the highest $g_s$ and $E$, and reduced ammoniacal syndrome (pictures not displayed). The incapability of $NH_4^+$ to regulate water fluxes may be due to $NH_4^+$ toxicity, when in excess. In previous studies, suppression of mRNA aquaporin expression occurred when *P. vulgaris* plants were fed with $NH_4^+$, resulting in lower water uptake compared to $NO_3^-$-fed plants (Guo *et al*., 2007a). The observed higher $g_s$, $E$, $g_{night}$ and $E_{night}$ in plants fed with sole $NH_4^+$ nutrition (0% $NO_3^-$ : 100% $NH_4^+$) than other ratios at 2 and 4 mM (Fig 4.4) which seem as an ideal [$NH_4^+$] to feed *P. vulgaris* hydroponically. However, the observed high magnitudes of day-time water fluxes in $NH_4^+$ fed plants indicated that $NH_4^+$ lacked a mechanism to elicit stomatal closure (Cramer *et al*., 2009). Thus, $NH_4^+$ may not be a key signalling N-form involved in plant water fluxes, despite the speculation of a potential regulatory role (Goodger and Schachtman, 2009). Stomatal opening and closure may be mediated by cytosolic pH and/or phytohormones concentrations, e.g. ABA (Wilkinson *et al*., 1998). $NH_4^+$ absorbed by roots is transported as electro-neutral amino acids such as glutamine (Miller and Cramer, 2004; Goodger and Schachtman, 2009). The transportation of $NH_4^+$ as amino-acids may partly explain the very low xylem [$NH_4^+$], but high concentrations of glutamine when plants are grown on $NH_4^+$ only (Gollan, 1992). Because of glutamine concentrations, the pH of the cytosol does not become alkaline, and the flux of protons is at its highest, and the low levels of ABA are insufficient to reduce stomatal conductance (Goodger and Schachtman, 2009).

In conclusion, Chapter 4 explored the notion that develops the understanding on nutrient regulation of water fluxes in plants, particularly the biphasic regulation of water fluxes by $NO_3^-$ and the lack of similar regulation by $NH_4^+$ of water flux (Cramer *et al*., 2009). The functional role of $NO_3^-$ in regulating water fluxes implies that *P. vulgaris* control their root acquaporins and stomata in response to availability [$NO_3^{-}$'-]

in the rhizosphere, to loose water continuously (day and night) for delivering soil nutrient through mass-flow (Chapter 3). Furthermore, $NO_3^-$- stomatal regulation seems to be a strategic adaptive trait to optimize *WUE* which possibly maintains plant survival or productivity. Thus, $NO_3^-$ -fed plants with an improved *WUE*, however, produces better yield in response to $NO_3^-$ availability, but not sole $NH_4^+$. *P. vulgaris* down-regulated their water fluxes when they fed with a mixture of $NO_3^-$ and $NH_4^+$, indicating a regulatory role of $NO_3^-$ to elicit stomata closure. However, *P. vulgaris* didn't regulate water fluxes when they fed with sole $NH_4^+$ nutrition, but rather displayed water-stress symptoms ('ammoniacal syndrome') when root [$NH_4^+$] is excessive.

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# CHAPTER 5

## 5.0 SYNTHESIS

### 5.1 Summary

Terrestrial plants loose more than 90% of their root acquired water (Raven and Johnson, 2001), which serves in leaf cooling (Nobel, 1999), maintain leaf turgidity (Rygol *et al*., 1993), powering solute transport from roots to shoots *via* xylem and delivering nutrients through the soil to the root surface through mass-flow (Barber, 1995; Cramer *et al*., 2008; Cramer *et al*., 2009; Christman *et al*., 2009; Matimati *et al*., 2013). The inextricable link between large amounts of water fluxes by nutrient-limited plants suggest a major functional role of transpiration, possibly in powering the acquisition of water and dissolved nutrients through the soil to the root surfaces by mass-flow (Chapter 3 and Chapter 4) (Cramer *et al*., 2008; Matimati *et al.*, 2013). This notion has been supported by the substantial night-time transpiration observed in photosynthetically inactive $C_3$ and $C_4$ plants (Caird *et al*., 2007; Kupper *et al*., 2012). In addition, regulation of transpiration rates in response to nutrient availability (Chapter 3, Cramer *et al*., 2008; Cramer *et al*., 2009; Matimati *et al*., 2013) has been consistent with the adaptive stimulation of nutrient supply to plant roots through mass-flow (Raven, 2008).

The current study explored a hypothesis that mass-flow nutrient acquisition is controlled by water fluxes, and that water fluxes is possibly regulated by nutrient availability. Despite a poor understanding on $NO_3^-$ and $NH_4^+$ in regulation of water fluxes for mass-flow acquisition of nutrients, the current study showed that day- and night-time water fluxes in *P. vulgaris* is regulated by [$NO_3^-$ ] either under xeric and mesic conditions (Chapter 3 & 4). When $NO_3^-$ source was distant from a nylon mesh, there was a limiting [$NO_3^-$] in the rhizosphere, and plants up-regulated their water fluxes to acquire nutrients from their remote source in the soil. Beyond that distance, $NO_3^-$ possibly remained poorly accessible and plants had to down-regulate their water fluxes to conserve water and as a response to $NO_3^-$ limitation (Radin *et al*., 1981 & 1982). Overall, plants may possibly up-and down-regulate their water fluxes in response to $NO_3^-$ availability in the soil, with a purpose of driving acquisition of soil nutrients through mass-flow. Unlike $NO_3^-$, $NH_4^+$ lacks the capacity to regulate day- and night-time water fluxes in *P. vulgaris* grown in sand or hydroponically (Chapter 3 & 4). The reduced photosynthesis, stomatal conductance and transpiration noted in

$NH_4^+$ - fed plants characterized ‘ammoniacal syndrome’ (discussed further). Despite a lack of evidence emerged on *WUE*, other studies suggested that $NH_4^+$ may also reduce *WUE* in $NH_4^+$ fed plants (Cruiz *et al*., 2008).

$NO_3^-$ regulate day- and night-time water fluxes through N-linked regulatory mechanisms (Wilkinson *et al*., 1998, 2004 & 2007; Clarkson *et al*., 2000; Desikan *et al*., 2005; Gloser *et al*., 2007; Cramer *et al*., 2009) occurring in both roots and shoots. Acquisition of N as $NO_3^-$ was possibly the signal that regulate day- and night-time water fluxes in Chapter 2 & 3, exerting its effects on root hydraulic conductivity (not displayed in the study) and on stomatal conductance as previously reported by others (discussed below). Increasing [$NO_3^-$] in the rhisophere is known to rapidly up-regulate aquaporin-mediated root hydraulic conductivity (Carvajal *et al*., 1996; Clarkson *et al*., 2000; Gloser *et al*., 2007; Gorska *et al*., 2008). [$NO_3^-$] in the rhizosphere regulate aquaporin expression (Gorska *et al*. 2008), which is probably an adaptive trait that has a significant impact in the mass-flow acquisition of $NO_3^-$ towards the rhizosphere when the roots of $NO_3^-$-limited plants are exposed to fertile soil patches (Gorska *et al*., 2010). Indeed $NO_3^-$-fed plants up-regulated their water fluxes as distance from the $NO_3^-$-source was decreased (Chapter 3), whilst in hydroponic system $NO_3^-$-fed plants elevated their day-time water flux as [$NO_3^-$] increased (Chapter 4). $NO_3^-$ root uptake in excess of capacity of the nitrate reductase (NR) enzyme for reduction may possibly be transported to the shoots where nitrous oxide (NO) is produced. NO has a functional significance in stomatal regulation, which possibly explains the functional role of $NO_3^{--}$ in regulating day- and night-time water flux in *P. vulgaris*. High availability of $NO_3^-$ in the rhizosphere induces xylem/apoplast pH to be more alkaline (Jia and Davies, 2007), resulting in the accumulation of ABA in the apoplast, which enhances stomata closure (Wilkinson and Davies, 1997). $NO_3^-$ down-regulation of stomatal conductance through NO, increased xylem/apoplastic pH and ABA, however, may possibly reduce water fluxes, and consequently improve *WUE* under water deficit conditions (Wilkinson, 2004).

Contrary to $NO_3^-$ , $NH_4^+$ lacked a mechanism to regulate day-and night-time water fluxes in *P. vulgaris* under dry and well-watered conditions (Chapter 3 & 4). $NH_4^+$ supply may not increase the expression of root aquaporins or alter root hydraulic conductance as potential mechanism of water flux regulation (Guo *et al*., 2007a). $NH_4^+$ is taken up by roots and readily assimilated into amino acid (building blocks of proteins) (Miller and Cramer, 2004) and $NH_4^+$ does not appear to elicit stomatal responses (Cramer *et al*., 2009). Nevertheless, plants that are fed with $NH_4Cl$, as a common fertilizer, did not regulate their day- and night-time water fluxes. $NH_4^+$- fed plants generally displayed lower *WUE* than those fed with $NO_3^-$ (Raven *et al*. 2004; Guo *et al*., 2007a & b) and may possess leaf chlorises and other symptoms of water stress (Cramer and Lewis 1993; Chaillou and Lamaze 2001, Chapter 4). In natural ecosystems high soil [$NH_4^+$] are rare, compared to agricultural soils and plants may thus lack the mechanisms to respond to these variations in soil [$NH_4^+$].

$NO_3^-$ regulation of day- and night-time water fluxes in *P. vulgaris*, explains that plants may evolve their adaptive traits (e.g. stomata conductance and aquaporin-mediated root hydraulic conductivity) in response to $NO_3^-$ availability under water deficit conditions. $NO_3^-$ regulated stomata aperture may possibly minimize water fluxes in order to increase *WUE* and consequently improve crop production. High rate of transpirational water fluxes in plants fed with $NO_3^-$, however, encourages those plants have poor dense root system to acquire nutrient through mass-flow from their distant source in the soil to the root surface, where different species compete for soil nutrients.  High [$NO_3^-$] in the rhizosphere increases biomass accumulation and shoot: root ratio in *P. vulgaris* plants, however, this interpretation suggest that the increased [$NO_3^-$] in the soil induce plant growth and plant production. Plants fed with $NO_3^-$ at sufficient level have a large root system (Chapter 3, Figure 3.2), which is significant to acquire less mobile nutrients (such as P and $NH_4^+$) from the soil .

As the global climate change occurs, $NO_3^-$ regulation of water fluxes raises key questions to resolve in future. The $NO_3^-$ regulation provides implications as follows: the first implication focuses on physiologic perspective, that $NO_3^-$ signals the mechanism of water flux regulation, such as stomatal closure, which acts to conserve water. This notion fulfills a gap which emerged on N-form that is responsible to regulate water fluxes for mass-flow acquisition of soil nutrients. This study also support an existing model on proposed mechanism for the biphasic regulation of water flux by $NO_3^-$ , and the incapacity of $NH_4^+$ regulation of water flux (Cramer *et al*., 2009). Secondly, the $NO_3^-$ regulation of water fluxes may have implications for controlling intrinsic water use efficiency (*$WUE_i$*) which may possibly maintain plant survival or productivity under conditions of limited water supply, and therefore an improved *WUE* will produce better crop yield. This notion recommends that plants grow in an ecosystem where $NO_3^-$ nutrition is limiting, but not deficiency, closes their stomata to reduce water flux, and consequently improve *WUE*. Although it is known that $NO_3^-$ down-regulates water flux and consequently increases *WUE,* further physiological research is needed to understand better $NO_3^-$ regulation of whole plant water flux and plant-level *WUE*.

### 5. 2 Conclusion

This thesis explored and confirmed the notion that $NO_3^-$ nutrition regulates both day- and night-time water flux which increases ‘mass-flow’ acquisition of N and other soil nutrients, signaling down-regulation of day-time water flux when root [$NO_3^-$] is limiting, but not deficiency (Chapter 3 & 4). Day-time water fluxes in *P. vulgaris* increased with the increased root [$NO_3^-$], whilst night-time water flux increased as root [$NO_3^-$] decreased (Chapter 3 & 4). However, *P. vulgaris* didn’t regulate day- and night-time water fluxes when they were fed with $NH_4^+$ nutrition , but rather displayed water-stress symptoms (‘ammoniacal syndrome’) when root [$NH_4^+$] is excessive. Thus, under $NO_3^-$ deprived conditions plants may be opportunistic in their water uptake, transpiring more when water is available in order to enhance the acquisition of nutrients through mass-flow.

### 5. 3 Further research study

Since organic nitrogen forms such as amino acids also found in soils, there is need for further investigation on their role in regulating transpiration and mass-flow acquisition of soil nutrients. Using amino acids laced with $^{15}N$ isotopes as a nitrogen source can allow their acquisition and role on stomatal regulation to be explored. Current trends in research are focussed around developing real-time *in-situ* sensing of soil nitrogen status to promote enhanced nitrogen and water use efficiency in agricultural systems. This thesis provides the vital literature on stomatal regulation by [$NO_3^-$]. $NO_3^-$ regulation of transpiration and mass-flow nutrient acquisition, however, also initiates a future study on the functional role of $NO_3^-$ in regulating whole plant transpiration of $C_4$ and $C_3$ species.

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