# Does transpiration respond to short term nitrogen deficiency?

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## Abstract

To test the hypothesis that the short term effect of N-derivation on plants is an increase in transpiration rates, as plants attempt to increase the delivery rate of $NO_3^-$ to the root surface, the effects of removal or addition of N to the roots or shoots of wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.) on transpiration was determined. N was supplied to the roots in the form of $NO_3^-$ solution and as foliar sprays of either 20 mM $NaNO_3^-$ or the nitric oxide (NO) donor sodium nitroprusside (150 µM). Over short time periods (~ 1 h) deprivation of $NO_3^-$ led to increased transpiration rates. This stimulation of $NO_3^-$ uptake persisted for up to 3 d but the magnitude of the stimulation declined from 3 h after removal of $NO_3^-$. This up-regulation of transpiration by N-deficit was preventable through the application of $NO_3^-$ and nitroprusside containing foliar sprays. Plants transferred to solutions containing a range of $NO_3^-$ concentrations demonstrated decreased transpiration at the higher $NO_3^-$ concentrations. We concluded that plants up-regulated their transpiration in order to increase bulk-flow of water to roots in an attempt to enhance $NO_3^-$ uptake.

## Introduction

Environmental stresses are the most important factors limiting plant productivity. The most serious of these stresses is water shortage (Neill et al., 2003b). Water shortage occurs when the rate of transpiration; the outward flux of water through a plant's stomata during $CO_2$ uptake, is greater than the amount of water taken up by the plant (Mata and Lamattina, 2003). If the lack of water is severe, this could result in detrimental effects on the plant's metabolism and physiology, which is why plants have in place mechanisms to deal with water stress situations. Among these mechanisms is tight control of stomatal

conductance. More than 95% of the water that moves through plants exits through the stomatal pores (Neill et al., 2003b). Stomata control conductance by either opening or closing the stomatal pore in response to signals from the root system, which informs the shoot of the soil water status (Comstock, 2003). This results in optimization of water use efficiency (WUE) as less water is lost during $CO_2$ influx (Neill et al., 2003b).

One of the signals of water stress from the root system is abscisic acid (ABA). ABA is a ubiquitous plant hormone that regulates many aspects of plant development especially when plants are under stressful conditions (Swamy and Smith, 1999). ABA has been shown to induce stomatal closure within approximately 5 min of ABA application, so one can presume that stomatal responses to ABA application take place without any changes in gene expression (Pei and Kuchitsu, 2005). In conditions of drought stress ABA is synthesized in the roots (as the roots of the plant are the first to detect any changes in the soil water status) and exported to the shoot in the xylem vessels via the transpiration stream where it accumulates in the leaves in the vicinity of the stomata (Neill et al., 2003b). At the stomata ABA initiates a complex web of signaling events. Upon arrival at the guard cells ABA is detected by receptors as yet to be identified (Comstock, 2003), and induces increases in cytosolic $Ca^{2+}$ which activates the slow-activating sustained (S-type) of rapid transient (R-type) anion channels that mediate anion release from the guard cells, resulting in depolarization of the membrane (Schroeder et al., 2001). Depolarization promotes $K^+$ efflux via outward-rectifying $K^+$ ($K^+$ out) channels and inhibits $K^+$ influx from $K^+$ uptake ($K^+$ in) channels (Pei and Kuchitsu, 2005).The net result is that loss of solutes from the guard cells reduces the turgor and volume of the guard cells resulting in their shrinkage and stomatal closure (Wilkinson, 1999).

Research has shown that nitric oxide (NO) is an important endogenous intermediate in the ABA- induced stomatal closure signaling process (Mata and Lamattina, 2002; 2003; Crawford and Guo, 2005). NO is a short –lived gaseous free radical that was first described as a toxic compound but now, it is recognized to be an important active member both in animal and plant physiology (Mata and Lamattina, 2002). It functions in processes such as growth, disease resistance, stress tolerance, programmed cell death and

or addition of N to the roots or shoots of wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.) had on transpiration and stomatal conductance.

## Materials and methods

*Cultivation of plants*

Wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.) plants were grown from seeds in a greenhouse, in trays containing vermiculite. Plants were watered daily by overhead irrigation for 2 weeks, and then transferred into a phytotron chamber set at mean day/night temperatures of 25/18 °C with a light intensity of 450µmol $m^{-2} s^{-1}$ and a photoperiod of 14 h. Plants were established in aerated hydroponic solutions in 20 L tanks with Long Ashton nutrient mediums prepared according to Hewitt (1966), with modifications to contain 2 mM $NaNO_3^-$. Nutrient solutions were renewed once or twice weekly depending on the size of the plants.

*Water flux measurements*

An hour before measurements, ten 6-week old wheat plants were transferred into separate 5 L buckets containing aerated hydroponic solution; 5 plants in buckets with 2 mM $NO_3^-$, 5 with solution without $NO_3^-$ and 5 buckets that contained water but no plants were used as controls. Transpiration rates were determined gravimetrically by weighing buckets every hour for the first 3 h, then every 2 h for 6 h and then every 4 h there after. The difference in the weights after every measurement is the amount of water lost i.e. amount transpired. Leaf areas were measured with a Li-Cor leaf area meter (Li-3100, Li-Cor, Inc, Lincon Nebraska, USA) after harvesting the plants and used to express transpiration relative to leaf size.

To test the effects of different $NO_3^-$ concentrations on plants, 4-week old sunflower plants (6 plants per 20 L tank) were supplied with three different $NO_3^-$ concentrations: 0, 0.1, 1 and 5 mM $NaNO_3^-$. Solutions were changed from 2 mM $NO_3^-$ 30 min before initiation of measurements. Stomatal conductances were determined from the youngest fully expanded leaves every hour for the first 2 h and then every 2 h there after, using a Decagon steady state diffusion leaf porometer (SC-1, Decagon Devices, Inc, Washington,

hormone responses (Crawford and Guo, 2005).There are three main sources of NO in plants; nonenzymatic sources, nitric oxide synthase-like enzymes (NOS) and nitrate reductase (NR), (Neill et al., 2003a). Mata and Lamattina, (2003) have shown that ABA-induced NO synthesis and stomatal closure requires NR. NR is a key enzyme of $NO_3^-$ assimilation in higher plants converting $NO_3^-$ to $NO_2^-$ which in turn is converted to $NH_4^+$ and on to amino acids (Mata and Lamattina, 2003). NO is generated as a byproduct of the decomposition of $NO_3^-$ in a reaction that is catalyzed by the enzyme NR (Neill et al., 2003a). Neill et al., (2003b) showed that NO activates the synthesis of cGMP (cyclic guanosine monophosphate) and subsequently cADPR (cyclic Adenosine diphosphate ribose) both of which are required for ABA- and NO-induced stomatal closure.

Supply of certain mineral nutrients can strongly influence transpiration and stomatal conductance in a plant (Clarkson et al., 2000). Deficiencies of nutrients such as $NO_3^-$ and phosphorus (P) have been shown to cause partial or complete stomatal closure even when water supply to the plant is adequate (Carvajal et al., 1996). Chapin et al., (1988) showed that nitrogen (N) deprivation resulted in decreased stomatal conductance within 2 days. Stomatal closure due to N-deprivation results from chemical signals from the root system since there is no change in leaf water potential (Chapin et al., 1988). N-deficiency can increase stomatal sensitivity to ABA by reducing the transport of cytokinins within the xylem (Radin et al., 1982). It also increases access of ABA to stomata by alkalizing xylem sap (Dodd et al., 2003).

$NO_3^-$ and other nutrients are carried to the roots by transpiration driven bulk-flow (Marquez et al., 2005). Nitrate will diffuse faster towards the root surface if influx of water at the root surface is increased (Clarkson et al., 2000). Thus under N-deficient conditions it would be easier for the plant to increase the rate at which $NO_3^-$ diffuses to the root surface than expanding the root surface for nutrient absorption (Clarkson et al., 2000).We hypothesized that the short term effects of N-deprivation on plants would be an increase in transpiration rates, as plants up-regulate transpiration in an attempt to increase the delivery rate of $NO_3^-$ to the root surface. We determined the effects that the removal

USA). One set of measurements was also taken after the plants had been put in the dark (night-time measurements) for 1 h.

To determine the effect of supplying N through foliar applications, sprays were applied to 5-week old sunflower plants in four 20 L tanks (6 plants per tank). One tank was supplied with a 2 mM $NO_3^-$ and the other three tanks were supplied with solutions without $NO_3^-$ 1 h before spraying the plants. Of the plants without $NO_3^-$, 6 were sprayed with a 20 mM $NaNO_3^-$ solution, 6 with a 150 µM SNP (sodium nitroprusside) solution (NO donor) and the other 6 (control plants) were sprayed with water. All the spray solutions contained 0.01% [v/v] Tween-80 as a surfactant. Transpiration rate measurements were obtained using a Decagon steady state diffusion leaf porometer. Measurements were taken every hour for the first 2 h and then every 4 h there after from 8 am to 6 pm then again at 8 am the next day.

## Results

$NO_3^-$ deprived plants lost more water daily compared to the plants supplied with 2 mM $NO_3^-$ solutions over the 3-day period. Differences in water loss between the two treatments were seen within an hour of plants being deprived of $NO_3^-$ (Fig.1). On day 1 the difference in water loss between $NO_3^-$ deprived plants and those supplied with 2mM $NO_3^-$ solutions increased to the highest Hedge's g value of 3.0 just 5 h after plants had been deprived of $NO_3^-$ (Fig. 2). After this the difference in water loss of the two treatments gradually became smaller with time to a Hedge's g value of 2.0 at the end of day 2 (33 h after $NO_3^-$ deprivation) and 1.7 at the end of day 3 (42 h after $NO_3^-$ deprivation).

Increasing the concentrations of $NO_3^-$ over a short time period reduced the stomatal conductance ($g_s$) of sunflower plants (Fig. 3). Initially all the plants reduced their $g_s$ as the plants adjusted to $NO_3^-$ concentration changes from 2 mM $NO_3^-$ solutions for the first 2 h after nutrient changes. Plants that were changed to nutrient solutions with lower $NO_3^-$ concentrations (0, 0.1 and 1 mM $NO_3^-$) had sharper reductions in $g_s$ than the 5 mM $NO_3^-$ supplied plants. Two hours after the nutrient change, the 0 mM $NO_3^-$ supplied plants up-regulated their $g_s$ to a high of 371 mmol $m^{-2}$ $s^{-1}$ at 2 pm (4 h later), then $g_s$ reduced to 347

mmol $m^{-2} s^{-1}$ at 4 pm. The plants in the other treatments continued to reduce $g_s$; the 5 mM $NO_3^-$ supplied plants at a faster rate than the 0.1mM $NO_3^-$ and 1mM $NO_3^-$ plants to reach 184 mmol $m^{-2} s^{-1}$ at 4 pm. The 0.1mM and 1mM $NO_3^-$ plants had similar $g_s$ however; the 0.1 mM $NO_3^-$ plants had slightly higher $g_s$. Night-time transpirational water loss mirrors the water loss during the day-time (Fig. 4). The 0 mM $NO_3^-$ supplied plants had the greatest transpirational water loss at night (70 mmol $m^{-2} s^{-1}$) and 5 mM $NO_3^-$ supplied plants had the lowest transpirational water loss at night (50 mmol $m^{-2} s^{-1}$), (Fig. 4B).This trend was similar to that observed during the day-time when 0 mM $NO_3^-$ supplied plants had the highest $g_s$ (301mmol $m^{-2} s^{-1}$) and 5 mM $NO_3^-$ supplied plants had the lowest $g_s$ (263 mmol $m^{-2} s^{-1}$) transpirational water loss (Fig. 4A). Both the day-time and night-time $g_s$ showed a negative linear relationship with an increase in $NO_3^-$ concentration with $r^2$ = 0.8232 for day-time $g_s$ and $r^2$ = 0.689 for night-time $g_s$ (Fig. 4).

Application of foliar sprays to 0 mM $NO_3^-$ supplied plants reduced the $g_s$ below that of 0 mM $NO_3^-$ supplied plants without foliar spray application (Fig.5). At time 0 h the 2 mM $NO_3^-$ supplied plants had higher $g_s$ than plants supplied with foliar spays. Within 1 h the 2 mM $NO_3^-$ supplied plants were transpiring less than the plants subjected to foliar sprays. Both the 2 mM $NO_3^-$ supplied plants and 0 mM $NO_3^-$ supplied plants had a slight spike in $g_s$ at time 6 h (2 pm) probably because plants were photosynthesizing at an optimum level, thus stomatal apertures were wide for maximum $CO_2$ uptake. The plants supplied with SNP and $NaNO_3^-$ foliar sprays showed an increase in $g_s$ at time 10 h (6 pm, when the day-time temperature had started to decrease), more so in the plants sprayed with SNP which suggests that effects of NO on stomata may be temperature dependent.

## Discussion

It is well known from previous work that the withdrawal of $NO_3^-$ from the plant rhizosphere results in a decease in stomatal conductance ($g_s$), (Radin and Ackerson, 1981; Chapin et al., 1988; Clarkson et al., 2000; Dodd et al., 2003). The decrease in $g_s$ has been attributed to long distant chemical signaling since the leaf water potential does not change (Chapin et al., 1988). $NO_3^-$-deficiency reduces the transport of cytokinins within the xylem and this can increase stomatal sensitivity to xylem ABA (Radin et al., 1982;

Wilkinson et al., 2007). Dodd et al., (2003) also showed that $NO_3^-$-deprivation caused xylem sap to become alkaline with the implication that ABA has better access to the guard cells. Reduction in photosynthesis through feedback inhibition also causes a decrease in $g_s$ due to reduced accumulation of sugar in stomatal guard cells when $NO_3^-$ deficiency is prolonged (Chapin et al., 1988; Taiz and Zeiger, 2002).

Over a short time period (within 1 h), the withdrawal of $NO_3^-$ from plant rhizospheres resulted in an increase in the amount of water lost ($g_s$) by plants (Fig. 1, 2). The expansion of the root system is the usual response reported for plants experiencing essential nutrient deficiencies, but since $NO_3^-$ diffuses freely and more rapidly (than increasing the root surface) toward root surfaces, increasing absorption rates should increase the delivery rate of $NO_3^-$ to root surfaces (Clarkson et al., 2000). Hence the up-regulation in transpiration by the $NO_3^-$ deprived plants may be an attempt to promote the movement of $NO_3^-$ to the roots of the plants. However, during the 3-day-period, as $NO_3^-$ -deficiency was prolonged, the plants began to down-regulate transpiration and the rate of water loss from the $NO_3^-$ deprived plants was reduced to levels similar to those of plants supplied with 2 mM $NO_3^-$ solutions (Fig. 2). This probably occurred because the long distance chemical signals (Radin et al., 1982), xylem sap alkalization (Wilkinson et al., 2007) and reduced photosynthesis start to take effect on the $NO_3^-$ deprived plants.

The transpiration rate of $NO_3^-$ deprived plants was more sensitive to temperature change than plants supplied with 2 mM $NO_3^-$ solutions, especially on day 1 where the greatest difference in water loss between the two treatments was shown at 12 noon at a maximum temperature of 25°C (Fig.2). The temperature-dependent effects on the amount of water loss by $NO_3^-$ deprived plants supports previous observation by Radin, (1990), where cotton plants that had been subjected to prolonged $NO_3^-$ deprivation increased their transpiration rates markedly to rates approaching those of the fully nourished plants as the temperature was increased from 10°C to 30°C.

The transpiration rates of the plants were dependent on the concentration of $NO_3^-$ supplied; the higher the concentration of $NO_3^-$ the lower the transpiration rate. This may

be related to the flux of $NO_3^-$ to the shoot and the potential synthesis of NO in the leaves. The activities of the enzyme NR are induced by its own substrate $NO_3^-$ (Cramer and Miller, 2004) and thus an increase in $NO_3^-$ concentration may result in reduced transpiration rates due to increased NR-dependent synthesis of NO, which induces stomatal closure. NR-dependent synthesis of NO is induced by very small concentrations of $NO_3^-$ (Cramer and Miller, 2004) possibly explaining why even low concentrations of $NO_3^-$ were able to reduce the transpiration rate of the sunflower plants. This small increase in $NO_3^-$ was enough to activate NR resulting in the production of NO, which induces stomata to close and subsequently reduce transpiration. Wilkinson et al., (2007) also showed that the control of maize transpiration rates is concentration dependent. Their results showed that increasing concentrations of $KNO_3^-$ supplied to detached shoots deprived of N, significantly reduced the rate of transpiration compared to that of shoots supplied with water only within 5 h of application. Wilkinson et al., (2002) showed that increasing the $NO_3^-$ supplies above deficiency controlled transpiration via a pH-mediated (alkalization of xylem sap) effect on ABA distribution, but via a different mechanism to that where by xylem sap is alkalized under $NO_3^-$ deficiency (Wilkinson et al., 1998; Wilkinson and Davies, 2002).

It is generally accepted that at night, when there is no photosynthesis and therefore no demand for $CO_2$, stomatal apertures are kept small and this prevents unnecessary water loss (Taiz and Zeiger, 2002; Snyder et al., 2003). However because plants do not completely shut stomata at night there is significant night-time water loss (Howard and Donovan, 2007).The pattern of night-time $g_s$ mirroring day-time $g_s$ (Fig. 4) supports observations by Snyder et al., (2003), where they showed positive correlations between night-time $g_s$ and daytime $g_s$ for $C_3$ and $C_4$ plant species, and that plants did not regulate their stomata at night for increased $g_s$ at night, as observed by Howard and Donovan, (2007) on work done on 6 different *Helianthus* species. Thus the night-time transpiration is only higher in plants that have higher daytime potential for transpiration and have enough water available to them to be able to express this potential. Contrary to the initial prediction based on habitat differences; that night-time transpiration would be greater in wetter habitats than in habitats that are water limited (Snyder et al., 2003). One of the

physiological benefits of night-time transpiration is enhanced nutrient acquisition as continued transpiration at night means that the total daily bulk-flow of water to the roots is increased (Clarkson et al., 2000; Mc Donald et al., 2002).

The application of SNP and $NaNO_3^-$ foliar sprays prevented the up-regulation of transpiration by $NO_3^-$ -deprived plants; however the $NaNO_3^-$ foliar spray reduced $g_s$ more than the SNP foliar spray (Fig. 5). This is probably because plants were able to access more NO from the decomposition of $NO_3^-$ in the reaction catalyzed by the enzyme NR (Neill et al., 2003), compared to the amount of NO donated by the small amount of SNP (150 μM) sprayed on the leaves. The induction of stomatal closure by the application of SNP in several different plants has been shown to be time- and concentration dependent (Mata and Lamattina, 2001; Neill et al. 2003; Kolla and Raghavendra, 2007). The results show that the application of SNP could also be temperature-dependent (Fig. 5). The activities of the enzyme NR are concentration dependent since its activities are induced by its own substrate $NO_3^-$ (Cramer and Miller, 2004). Since plants supplied with $NaNO_3^-$ showed a similar change in $g_s$ at lower temperatures, it is possible that the production of NO from the decomposition of $NO_3^-$ by NR is also temperature-dependent.

There are three main groups of plants in regards to where $NO_3^-$ is assimilated in the plant: plants that assimilate $NO_3^-$ in leaves, plants that assimilate $NO_3^-$ in roots and plants that can significantly assimilate $NO_3^-$ both in the leaves and roots (Marquez et al., 2005). Sunflower (Wilkinson, 1990) and wheat (Cramer and Lewis, 1993) can assimilate $NO_3^-$ both in the leaves and roots, although a large proportion of $NO_3^-$ is generally assimilated in the shoots of these plants. This flux of $NO_3^-$ to the shoot may act as the signal that allows plants to detect the N-status of the root and allows stomata to respond rapidly to changes in N-availability. The $NO_3^-$ arriving in the leaves may be converted to NO within the guard cells resulting in a signaling cascade which closes stomata. In the absence of available $NO_3^-$, NR activity is likely to decline disabling this signaling mechanism.

## Conclusions

This study shows that the short term effects of N-deprivation on plants, is an increase in $g_s$, this leading to an increase in plant transpiration. The high transpiration rates caused by N-deprivation are reversible through application of $NO_3^-$ and nitroprusside containing foliar sprays, and decreases in transpiration due to $NO_3^-$ application are concentration dependent and possible even temperature dependent. Thus plants regulate transpiration in response to N-availability, enabling plants to control the rate of flow of water and consequently nutrients through the soil towards the root surface. The optimization of $CO_2$ gain per water lost (water use efficiency, WUE) should be considered in the light of the role played by water in acquiring nutrients. We can no longer consider water loss from plants as merely a by-product of photosynthesis. It is an essential process for nutrient acquisition that responds to nutrient availability and therefore optimization of WUE must also depend on nutrient status.

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## Figure Legends

Fig. 1.

The amount of water lost by wheat plants supplied with 2 mM $NO_3^-$ solutions and plants supplied with solutions without $NO_3^-$ at time 0 h on day 1, over a 3-day period. Water loss was determined gravimetrically over time, after 1 h, 2 h and 4 h. Symbols and error bars indicate the means ± S.E. (n = 5).

Fig. 2.

The "effect size" (Hedge's g) was used to express the difference between the wheat plants supplied with 2mM $NO_3^-$ and the plants supplied with solutions without $NO_3^-$. Hedge's g was calculated as $g = t\sqrt{(n_1 + n_2) / (n_1 n_2)}$, where $t$ is the value given by the Student's t test for the differences between the two groups and $n$ is the number of samples (n = 5) in each treatment.

Fig. 3.

The stomatal conductance ($g_s$) of 4-week-old sunflower plants supplied with three different $NO_3^-$ concentrations (0, 0.1, 1 and 5 mM $NO_3^-$) over a period of 8 hours. Measurements were taken from the youngest fully expanded leaves using a Decagon steady state diffusion leaf porometer. Symbols and error bars indicate the means ± S.E. (n = 6).

Fig. 4.

The stomatal conductance ($g_s$) of 4-week-old sunflower plants supplied with three different $NO_3^-$ concentrations (0, 0.1, 1 and 5 mM $NO_3^-$) during the day (A) and at night (B). A Decagon steady state diffusion leaf porometer was used to take measurements from the youngest fully expanded leaves. Each data point during the day represents the average of 10 measurements and at night, each data point represents a single measurement. The error bars indicate S.E.

Fig. 5.

Stomatal conductance ($g_s$) of 5-week-old sunflower plants subjected to foliar sprays of 20 mM $NaNO_3^-$ and 150 μM SNP (sodium nitroprusside) and water as a control. Plants subjected to foliar sprays were supplied with nutrient solutions deprived of $NO_3^-$ to determine the effects of sprays on $g_s$. The foliar sprays included 0.01% [v/v] Tween-80 as a surfactant. Symbols and error bars indicate the means ± S.E. (n = 6).

Figures

Fig. 1.

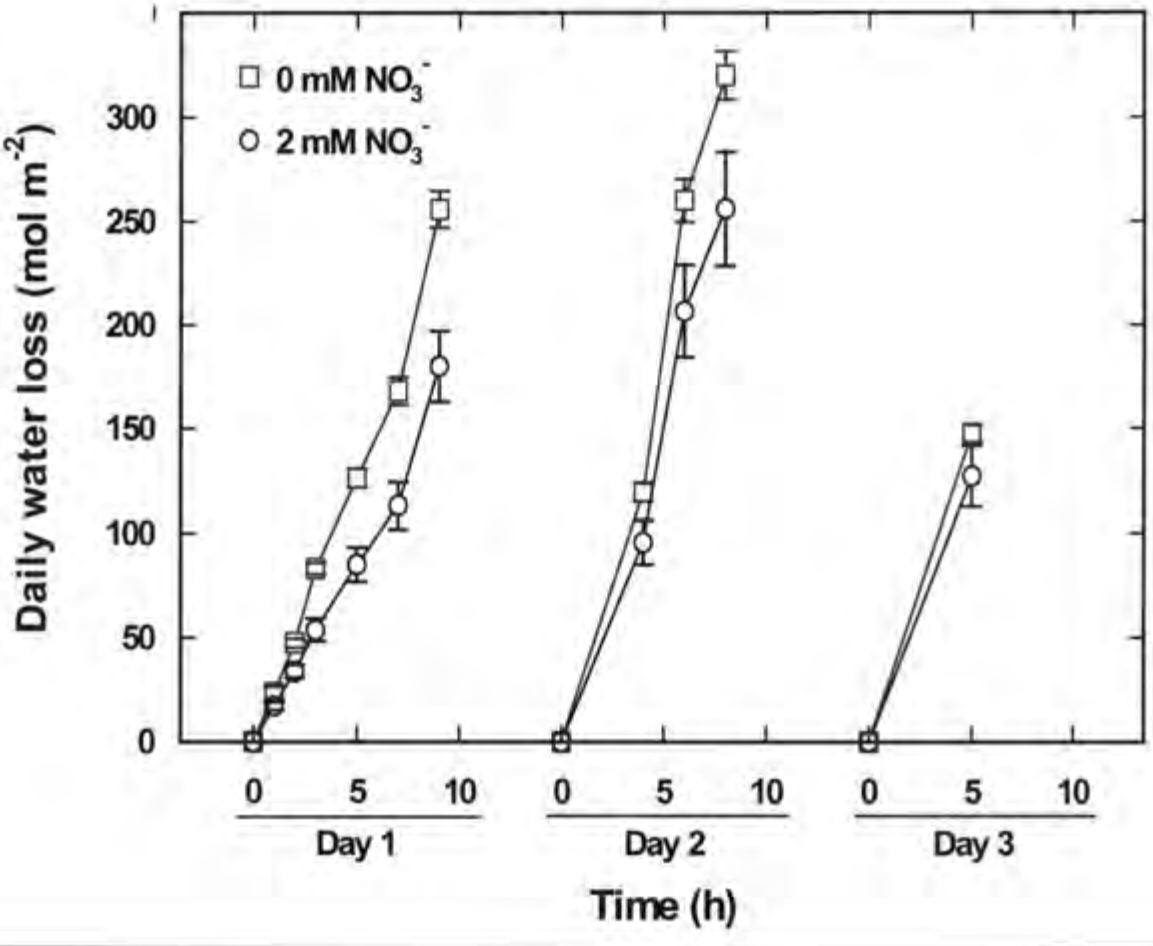


**Fig. 2.**

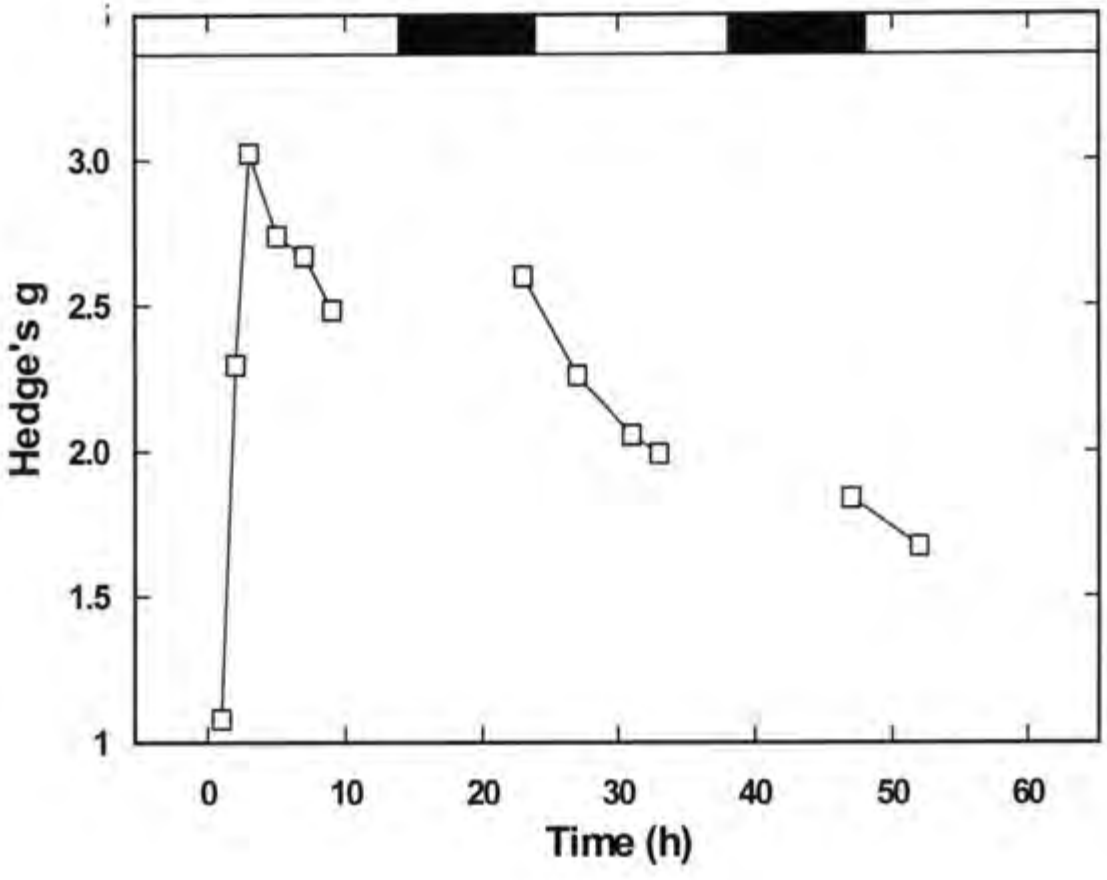


Fig. 3.

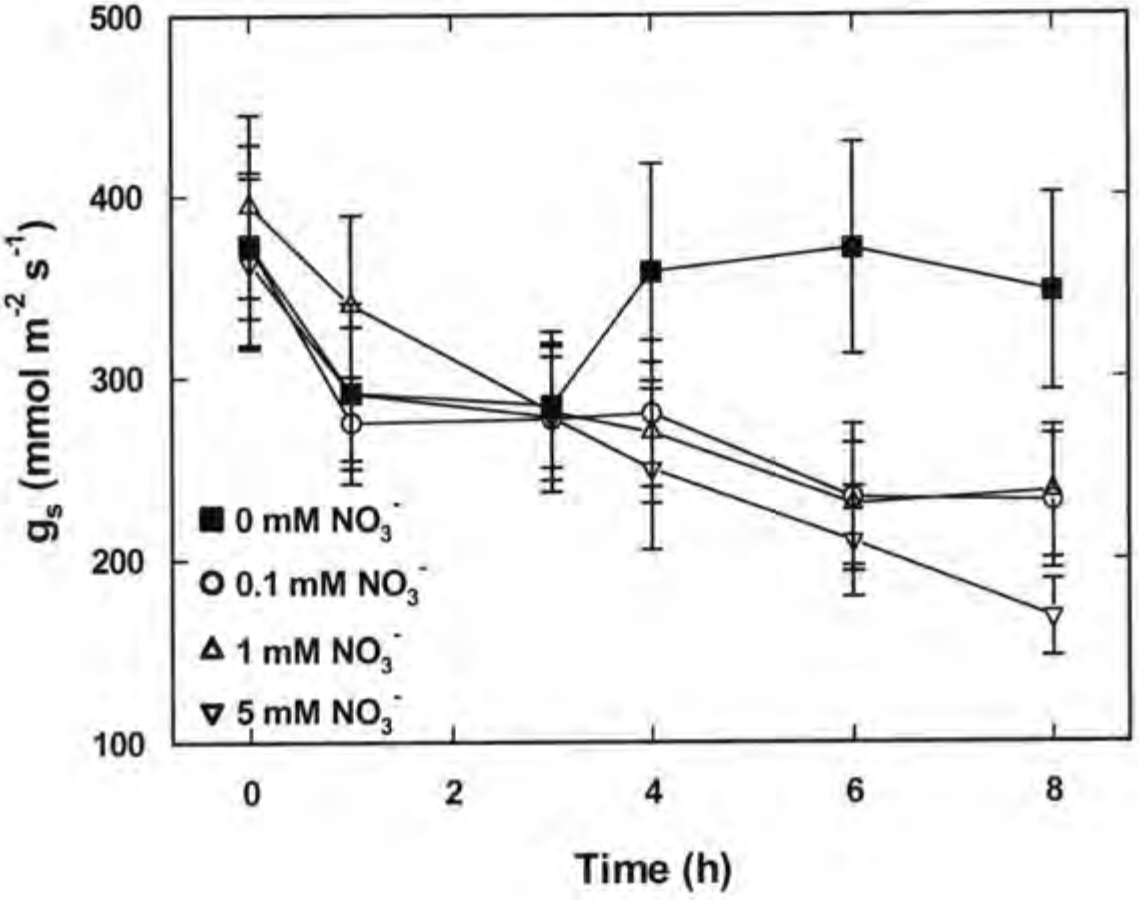
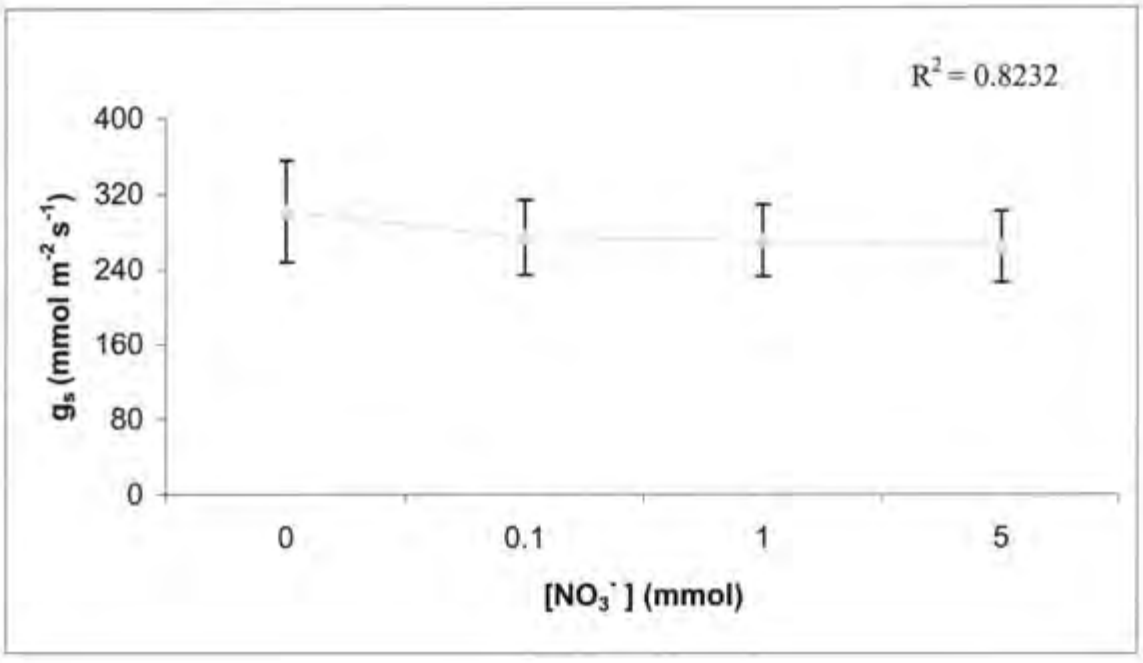

500
400
300
200
100
$g_s$ (mmol $m^{-2}$ $s^{-1}$)
0 mM $NO_3^-$
0.1 mM $NO_3^-$
1 mM $NO_3^-$
5 mM $NO_3^-$
0
2
4
6
8
Time (h)

**Fig. 4.**

**A**


R² = 0.8232
400
320
240
160
80
0
gs (mmol m-2 s-1)
0
0.1
1
5
[NO3-] (mmol)

**B**

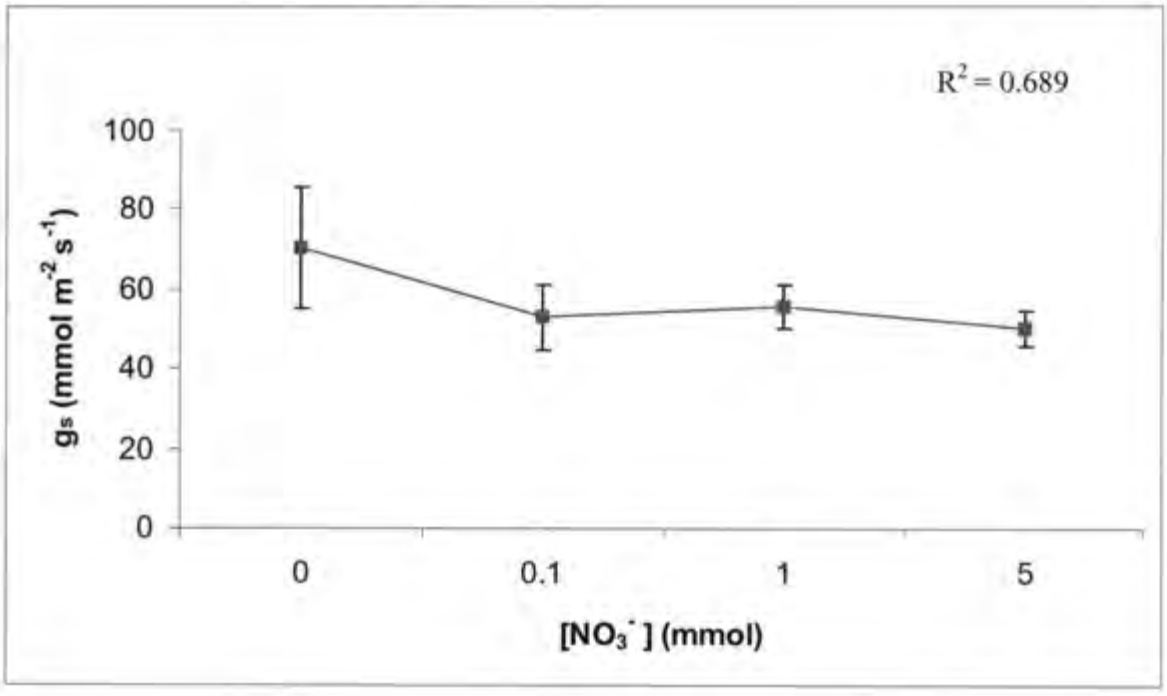
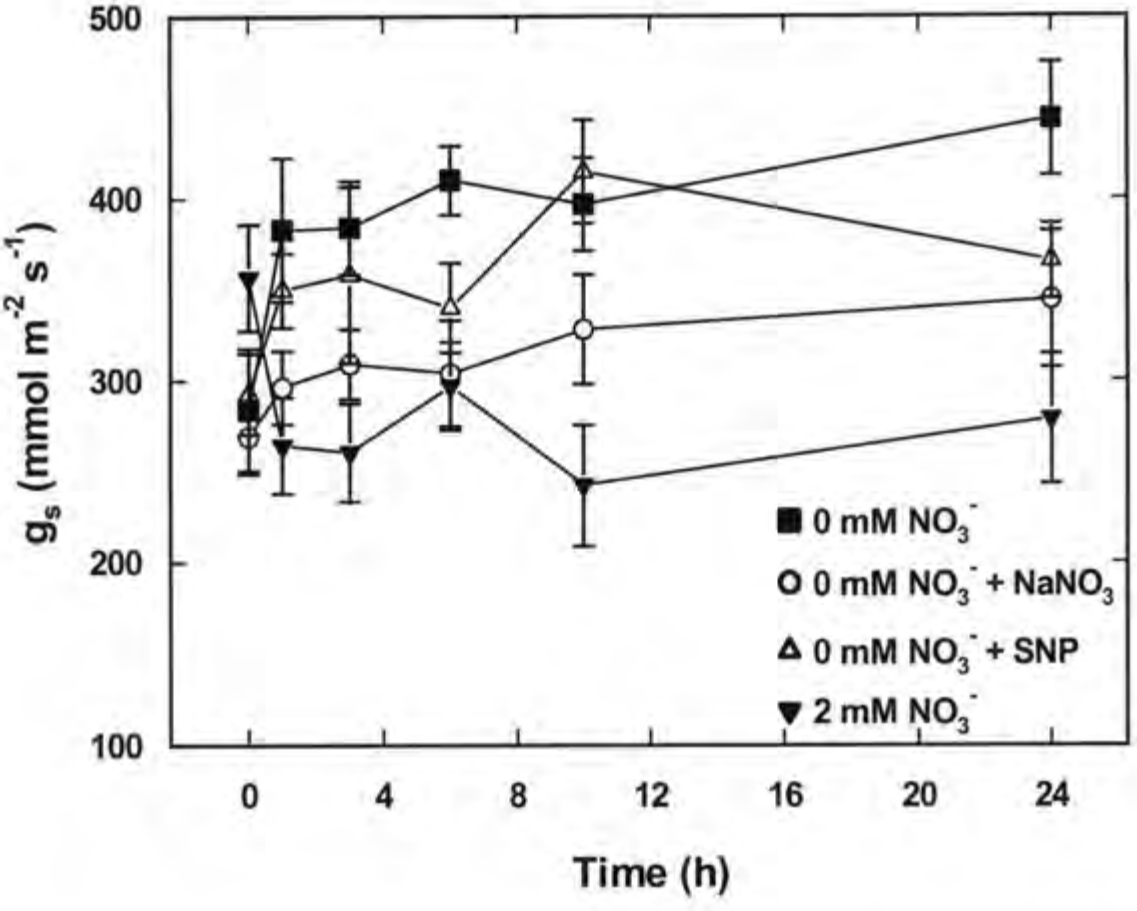

R² = 0.689
100
80
60
40
20
0
gs (mmol m-2 s-1)
0
0.1
1
5
[NO3-] (mmol)

Fig. 5.


500
400
300
200
100
$g_s$ (mmol $m^{-2}$ $s^{-1}$)
0
4
8
12
16
20
24
Time (h)
0 mM $NO_3^-$
0 mM $NO_3^-$ + $NaNO_3$
0 mM $NO_3^-$ + SNP
2 mM $NO_3^-$