Nitrate signalling to stomata and growing leaves: interactions with soil drying, ABA, and xylem sap pH in maize

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Abstract

Increasing the nitrate (N) concentration in the rooting substrate above deficiency decreased stomatal conductance and leaf growth rate compared with sufficient N in maize seedlings (Zea mays L.) growing in drying substrate. Novel effects were detected when N in the non-deficient range was supplied directly to the xylem of detached shoots: concentrations above 2.0 mol m\(^{-3}\) KNO\(_3\) reduced transpiration, and concentrations above 12 mol m\(^{-3}\) KNO\(_3\) reduced leaf growth rate. Evidence is provided that the novel effects of N on transpiration and growth were mediated by pH-based ABA redistribution. ABA at 0.05 mol m\(^{-3}\), whilst ineffective alone, sensitized leaf growth to increases in KNO\(_3\) concentration (from 3.0 mol m\(^{-3}\)), and the capacity of higher concentrations of ABA to reduce growth was enhanced by KNO\(_3\). Transpiration was sensitively reduced by KNO\(_3\), ABA, or buffers adjusted to pH 6.7–7.0 (compared with buffers adjusted to pH 5.0) alone. Nevertheless, a synergistic effect of KNO\(_3\) and either ABA or buffers adjusted to pH 6.7–7.0 was observed. Buffers of pH 5.6 supplied to detached shoots alleviated the depression of transpiration caused by 12 mol m\(^{-3}\) KNO\(_3\). Buffers adjusted to pH 6.7 increased the sensitivity of growth to KNO\(_3\). Xylem sap extracted from intact seedlings growing in drying soil exhibited an initial increase in N concentration, followed by a decrease at progressively lower soil water potentials. The importance for novel N signalling above deficiency is discussed with reference to the generality of fluctuations in soil and xylem N concentration within this range.

Key words: Abscisic acid (ABA), apoplast, leaf growth, nitrate, pH, root, soil drying, stomatal guard cells, transpiration, xylem.

Introduction

Both soil water deficit and soil nitrate (N) deficiency can induce stomatal closure and cause reductions in leaf growth rates in plants (McDonald and Davies, 1996). Both environmental cues generate signals within plants that carry information about soil drying and soil nutrient levels from the roots to the stomata and growing leaves in the shoot. However, little is known about the responses of plants, and signals generated within them, as soil and xylem N concentrations increase above the deficient range, and what published information that there is is often conflicting (Morgan, 1986). It can be argued that more research should be focused on this area. In developed agriculture most plants are exposed to a fluctuating range of non-deficient N concentrations in the soil, and xylem sap N concentrations can vary sensitively in response to changes in the amount and form of N in the soil (Andrews, 1986a, b; Peuke et al., 1996), especially in species which assimilate a significant proportion of the N that they take up in the leaf rather than in the root (Lexa and Cheeseman, 1997). Soil water content also affects the amount of N that enters the root and/or partitions into the xylem, and whilst some research has demonstrated a sensitive reduction in xylem N concentration as soil dries or becomes flooded (Gollan et al., 1992; Bahrun et al., 2002; Jackson et al., 2003), others show that xylem N concentrations increase as soil dries, dependent on the species and/or the sap extraction method used (Goodger et al., 2005; Jia and Davies, 2007). It might also be expected that aerial...

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Abbreviations: ABA, abscisic acid; cv., cultivar; gs, stomatal conductance; N, nitrate; NRA, nitrate reductase activity; PPFD, photosynthetic photon flux density; RWC, relative water content.

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conditions giving rise to reductions in transpirational water flux could increase N concentrations within the xylem, if N uptake/assimilation in the root remains unperturbed (Shaner and Boyer, 1976a, b; Smith, 1991).

Upon exposure of roots to drying soil, concentrations of the plant hormone abscisic acid (ABA) are increased in the xylem sap flowing from the roots to the shoots. ABA can be a potent inhibitor of leaf and stem growth rates, and induces the closure of stomatal pores in leaves (Zhang and Davies, 1990, 1991). In several species soil drying alkalizes the xylem sap flowing to the shoot (Wilkinson, 2004), which causes ABA to accumulate in the xylem and leaf apoplast even in the absence of de novo synthesis, such that there is increased delivery to guard cells or growing cells within the leaf (Wilkinson and Davies, 1997; Bacon et al., 1998; Wilkinson et al., 1998). Under well-watered conditions a more acidic pH prevents such accumulations, as the pH gradient over the membranes of the cells of the stem and leaf is sufficient to remove ABA from the apoplast, and it becomes stored or broken down within the symplast (Davies et al., 2002; Wilkinson and Davies, 2002). An acidic apoplastic pH is thus associated with open stomata and rapidly growing leaves in some species.

More recently it has been suggested that nutrient levels in the soil may also impact on a range of long-distance signalling systems (McDonald and Davies, 1996). Limiting N supply reduces the transport of cytokinins within the xylem (Rahayu et al., 2005). Lowered xylem cytokinin concentrations can increase stomatal sensitivity to xylem ABA (Radin et al., 1982; Fusseder et al., 1992), perhaps explaining some of the evidence for N deficiency-induced increases in tissue ABA sensitivity (McDonald and Davies, 1996). It has also been shown that reductions in xylem N concentration can alkalize xylem sap (Kirkby and Armstrong, 1980; Dodd et al., 2003; and see Gollan et al., 1992; Schurr et al., 1992), with the implication that root- or leaf-sourced ABA is better able to access the guard cells or the growing cells under these circumstances (see above).

Investigations into the effects of non-deficient soil N on shoot physiology and chemical signalling are relatively few, and are largely limited to comparisons between excessively high and deficient (rather than optimal) levels of N in the soil around intact plants, under relatively long-term soil-drying regimes. In these reports, high N reduced stomatal conductance ($g_s$) and transpiration in dry soil (Morgan, 1986; Liu and Dickmann, 1992; Karrou and Maranville, 1995; but see Bennett et al., 1986), whilst leaf area remained higher than in low N plants (Morgan, 1986). High N reduced shoot water potential in dry soil in several cases, often affected root/shoot ratio (Morgan, 1986), and Liu and Dickmann (1992) demonstrated that it increased leaf ABA concentrations compared with low N plants. However, other lines of research have determined that increasing the supply of N to the soil and to detached tissues can also increase xylem and/or apoplastic pH (Mengel et al., 1994; Hoffman and Kosegarten, 1995).

This is proposed to occur via a separate and distinct mechanism to that whereby xylem sap is alkalized under N deficiency and/or soil drying (Wilkinson and Davies, 2002; Wilkinson, 2004). It is argued that increasing the N supply above deficiency may also control transpiration and growth via a pH-mediated effect on ABA distribution.

The effect of supplying a wide range of N concentrations to the rooting medium of intact maize (Zea mays L.) plants growing in wet and drying conditions, on several aspects of shoot physiology, is investigated here. In addition, by supplying a range of N concentrations directly via the xylem sap to detached shoots severed from the roots 1–2 cm above a subcrown internode, it has been possible to examine the effects of N on transpiration and growth in the absence of coincidental and potentially confounding effects on root water uptake, root/shoot ratios and root-sourced cytokinin and ABA production/mobilization. The effect of N in combination with ABA and a range of xylem sap pH, on transpiration and growth in this system, is also examined to determine whether these signals interact.

**Materials and methods**

**Measurements in intact plants**

Approximately 15 seeds of maize (cv. ZP677) were sown in 4.0 l pots containing John Innes No. 2 commercial potting compost, assumed to contain a nitrate concentration of 75 mg l$^{-1}$, or in N-free vermiculite (LBS Horticulture, Lancashire, UK). Plants were raised in a greenhouse with supplemental lighting (provided by 600 W sodium Plantastar lamps, Osram, Germany; photoperiod 16 h, light intensity approximately 600 μmol m$^{-2}$ s$^{-1}$, with a day/night temperature of approximately 28–35/14–20 °C), and watered daily to the drip point until the initiation of nutrient treatments. Five to six days after sowing, KNO$_3$ was supplied to the compost as a solution from 8.0 mol m$^{-3}$ to 24 mol m$^{-3}$ and to the vermiculite in a macro-nutrient medium (2.0 mol m$^{-3}$ MgSO$_4$, 3.0 mol m$^{-3}$ CaCl$_2$, 2.0 mol m$^{-3}$ KH$_2$PO$_4$, 1.0 mol m$^{-3}$ K$_2$HPO$_4$) from 2.0 mol m$^{-3}$ to 20 mol m$^{-3}$. Over the experimental period 100–200 ml (dependent on external conditions) of nutrient medium (or water) was supplied per pot daily for 3–4 d (well-watered treatments), after which time the soil/vermiculite was allowed to dry for a further 2–4 d. Measurements of $g_s$ and leaf growth rate commenced 1–2 d after initiation of N addition (7 d after sowing, when the third main leaf had emerged and was expanding at a constant rate).

The N concentrations in the growth medium after 3–7 d of treatment have been calculated to approximate 75 (water only controls) to 315 (24 mol m$^{-3}$ KNO$_3$ treatments) mg N l$^{-1}$ soil in the experiments in John Innes, and 0–150 mg l$^{-1}$ in the vermiculite experiments. A maize crop in the field has been calculated to experience a range of N concentrations in the soil from approximately 90 to 160 mg l$^{-1}$ (at fertilizer addition rates of 80–250 kg ha$^{-1}$, assuming an additional 150 kg ha$^{-1}$ or 60 mg l$^{-1}$ N accrues over the growing season from natural processes; 0.5–1.0 kg m$^{-3}$ d$^{-1}$). A typical nursery-raised horticultural crop has been calculated to experience a range of N concentrations in the soil from approximately 50 to 400 mg l$^{-1}$ (600 mg l$^{-1}$ for tomato), assuming a 7.0–14 mol m$^{-3}$ N liquid feed (21 mol m$^{-3}$ for tomato) is supplied to spent compost with a residual level of 20 mg l$^{-1}$, as 150 ml d$^{-1}$ to a 4.0 l pot over 7 d (as occurs in the summer at the
height of the growing season). Commercial potting composts contain 50–100 mg l\(^{-1}\) N (loam-based such as John Innes) or 100–200 mg l\(^{-1}\) N (peat-based). Therefore, the range that has been supplied to maize in pots is equivalent to the range found in the field and to that supplied to nursery-raised plants. It is possible that maize in the field could actually access more than the calculated maximum of 160 mg l\(^{-1}\) as a result of root access to deeper soil than is available to the plants in pots, and for this reason up to 315 mg l\(^{-1}\) N (24 mol m\(^{-3}\) liquid feed) has been used in these experiments.

\(G_w\) was measured daily at 14.30 h on the abaxial surface of the second, third, and/or fourth leaf approximately 2.0 cm from the tip, in three plants per pot in each of three pots per treatment (nine replicates per treatment) using a porometer (AP4; Delta-T Devices, Cambridge, UK). The leaf with the highest \(G_w\) was determined on each day of the experiment, and that leaf was used for measurements. This was usually the third leaf or days 3–4 after the initiation of \(\text{KNO}_3\) addition, and the fourth leaf on days 5–7, such that only fourth leaf \(G_w\) was determined in drying soil. The length of the most rapidly expanding leaf at each stage of the experiment was measured several times a day in nine different plants from three different pots per treatment, to establish a cumulative increase in leaf growth over time. The third leaf was measured on days 3–5 after the initiation of \(\text{KNO}_3\) addition, the fourth leaf on days 4–7, and the fifth leaf on days 5–7. This usually meant that only the growth rates of the fifth leaf were measured in drying soil. Soil volumetric water content (\(\theta_v, \text{m}^3\text{m}^{-3}\)) was determined in each of three pots per treatment daily using a soil-moisture probe (ML2X; Delta-T Devices, Cambridge, UK) and converting the microvolt readings obtained using a two-point calibration from field capacity and oven-dried soil. Leaf relative water content (RWC) was measured in a separate experiment, at three different soil N concentrations over 3 d (day 1 being well-watered, and then allowing the soil to dry over the following 2 d). Leaf 2 (days 1 and 2) or leaf 3 (day 3) was removed from the shoot and immediately weighed. The leaves were placed in 5.0 ml of \(\text{H}_2\text{O}\), refrigerated overnight, and weighed again the following day. They were then placed in a drying oven for a further 5 d, and a dry weight was obtained. Leaf RWC (\% maximum fresh weight)=\((\text{initial weight–dry weight})/\text{maximum fresh weight–dry weight})\times100.

In some of the experiments xylem sap was collected from the detached shoot (cut 1.0 cm above the soil/vermiculite), of two to four replicates from each treatment at approximately 16.00 h on days 3–7 from the initiation of N addition, by quickly sealing it into a drying chamber. Approximate 10–20 μl of sap was collected between approximately −0.3 MPa and −1.0 MPa over approximately 5 min. The sap was quickly frozen for subsequent determinations of N concentration. Xylem sap was collected from the shoot rather than the root in order to sample sap closer to the sites of interest in the leaves, and because recent work has determined that using the root pressure vessel to pressurize both soil and root can negate soil drying-induced increases in xylem sap ABA concentration (Megat Wahab, 2007), presumably by diluting xylem sap contents, including nitrate, with soil water. The concentrating effect on sap constituents of using natural root pressure slowly to exude sap at a cut shoot stump has also been highlighted (Goodger et al., 2005). Concentrations of xylem transported substances collected from the shoot under short-term pressurization have been determined to be within the same range as those measured in sap collected using other techniques (Sobeth et al., 2004).

**Analysis of xylem sap nitrate concentration**

Xylem sap N concentrations were determined using a standard range laboratory N test kit from The Nitrate Elimination Co., Inc. (ML, USA) with minor modification. A 50 μl aliquot of diluted xylem sap (×80) was mixed with 200 μl of reaction buffer (25 mol m\(^{-3}\) \(\text{K}_2\text{HPO}_4\), 0.025 mol m\(^{-3}\) EDTA, pH 7.5, 0.33 mol m\(^{-3}\) NADH, 0.05 U of nitrate reductase YnaR1). After incubation for 30 min at room temperature, 200 μl of 1% sulphanilamide in 3 N HCl and 200 μl of 0.02% \(\text{N}_{-}\)naphthyl ethylenediamine were sequentially added to the reaction mixture solution. After incubation for a further 10–20 min, the absorbance at 540 nm was read using a spectrophotometer (Ultrspec 2100 pro; Biochrom Ltd, Cambridge, UK). N content was calculated based on a standard curve.

**Transpiration and growth bioassays in detached shoots**

Effects of exogenous N concentration, ABA concentration, and buffers adjusted to a range of pH values were tested on the rate of transpirational water loss through stomata (a measure of stomatal openness) and the rate of extension growth of the third leaf of greenhouse-cultivated maize seedlings (7–8-d-old, raised at approximately 30 °C, supplemental lighting as above) detached from the roots at a subcrown internode. The subcrown internode was developed by deep-sowing the seeds (approximately 25 per 10.0 l pot) 6.0 cm below the surface of the compost (John Innes No. 2). This allows the removal of the root system such that bathing solutions (15–20 cm long shoots immersed to a depth of approximately 1.0 cm) can directly access the transpiration stream whilst leaving the elongation zones of the leaves intact and undamaged. Seedlings were detached from the roots after the plants had been kept in the dark for approximately 30 min, and the subcrown internode was recut approximately 2.0 cm below the crown under water before immediate transfer to solution, in order to prevent embolism. Treatment solutions were 5.0 ml of water ± \(\text{KNO}_3\) and/ or ABA at the appropriate concentration, or 5.0 ml of buffer (1.0–3.0 mol m\(^{-3}\) \(\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4\) at the volume ratios required to achieve the correct pH) ± additions. N concentrations used were equivalent to the range detected in the xylem sap of the plants grown in well-watered and drying N-limiting, N-sufficient, and N-supplemented soils as described above. ABA concentrations used were equivalent to those detected in xylem sap extracted by various methods from intact maize plants relatively early in a soil drying cycle (Zhang and Davies, 1990, 1991). Concentrations were chosen (0.05–1.0 mmol m\(^{-3}\)) that incipiently reduced transpiration in the bioassay, but which did not completely close stomata, such that they still had the capacity to close further. The vials containing the seedlings were placed under lights (as above, PPFD approximately 500 μmol m\(^{-2}\) s\(^{-1}\)) at 28–30 °C. Vials containing seedlings were weighed and the length of the third leaf was measured as soon as possible after transfer to solution (within 30 min), and every hour (approximately) thereafter for up to 6 h. At the end of the experiment total leaf area was measured in a leaf area meter (Li-3000A; Li-Cor Inc., Lincoln, NE, USA). Water loss was converted from weight to mmol, and expressed on a per unit leaf area per second basis. Leaf extension was calculated as a cumulative increase in leaf length (cm) at approximately 1 h intervals. Means and standard errors from five to seven replicates were determined.

**Results**

**Effects of \(\text{KNO}_3\) concentration and substrate drying on \(g_w\), leaf growth, xylem sap N concentration, and leaf RWC in intact plants**

As expected (Fig. 1), increasing the \(\text{KNO}_3\) concentration supplied to maize plants growing in N-free vermiculite from 2.0 to 20 mol m\(^{-3}\) (calculated as 15–150 mg N 1\(^{-1}\)
vermiculite on days 4 and 5 after initiation of N addition) increased leaf growth rates [(A) third leaf, (B) fourth leaf], when the vermiculite was either well-watered (Fig. 1A) or allowed to dry (Fig. 1B), and increased gs of the second and/or third leaf when supplied with up to 10 mol m⁻³ KNO₃ (75 mg N l⁻¹ vermiculite) (Fig. 1C). However, 20 mol m⁻³ KNO₃ did not increase gs any more than 10 mol m⁻³ could in well-watered vermiculite, and significantly decreased it compared with gs at 10 mol m⁻³ KNO₃ in drying vermiculite (Fig. 1C). On this basis, a substrate N concentration of 75 mg l⁻¹ is assumed to be optimal for leaf growth. The xylem sap N concentration measured on day 5 after the initiation of N addition increased as expected with applied KNO₃ concentration (Fig. 1B, C).

Figure 2 shows that 10 mol m⁻³ and 20 mol m⁻³ KNO₃ supplied as supplemental liquid feeds to maize in John Innes No. 2 compost (to give calculated totals of 150 mg and 225 mg nitrate l⁻¹ soil from day 3 after the initiation of N addition) decreased the extension rate of the fourth leaf 3 d and 4 d after the start of N addition (1 d and 2 d after the start of soil drying) compared with the rates detected in seedlings supplied with water only (assumed to be receiving 75 mg l⁻¹ N from the compost itself). This effect could also be observed in well-watered soil within
48 h of treatment (data not shown). Leaves were approximately 1.2 cm shorter 4 d from the start of N addition, and 2 d after the start of soil drying, in the presence of N at concentrations greater than sufficiency. Similarly, supplemental liquid feeds of N of 16 mmol and 24 mol m$^{-3}$ N (235 and 315 mg N l$^{-1}$ soil from 3 d after initiation of N addition) significantly reduced the growth of the fifth leaf compared with plants supplied with water only (75 mg N l$^{-1}$ soil), 3 d after the initiation of soil drying (data not shown). However, $g_s$ was more sensitive than leaf growth to soil N concentration as soil dried. N supplied at 16 mol m$^{-3}$ and 24 mol m$^{-3}$ reduced $g_s$ compared with the water-only controls very early in the soil drying cycle (1 d after the initiation of soil drying), whilst there was no effect of supplemental N on $g_s$ in well-watered soil (Fig. 3). All three supplemental liquid N concentrations (8.0, 16, and 24 mol m$^{-3}$) reduced $g_s$ compared with water-only controls as soil dried further.

As expected, in well-watered soil (day 3) xylem sap N concentration increased with soil N concentration (Table 1). On day 4, a small reduction in soil water content consistently increased xylem sap N concentration, at least in the control 75 mg l$^{-1}$ soil N treatment. On subsequent days of further soil drying, xylem sap N concentration decreased again in the 75 mg l$^{-1}$ and 235 mg l$^{-1}$ soil N treatments, such that it was lower than it had been in well-watered soil on day 6 (4 d after the initiation of soil drying). Soil drying did not significantly affect the xylem sap N concentration (which remained high) when the soil was supplied with a heavy N load (315 mg l$^{-1}$).

Table 2 shows that supplying supplemental liquid N to intact maize plants (giving rise to concentrations of 188 mg and 300 mg N l$^{-1}$ substrate and above), which also increased xylem sap N concentrations, decreased $g_s$ (Figs 1C, 3) and leaf growth rates (Fig. 2) compared with an optimal N concentration (of approximately 75 mg N l$^{-1}$ soil or vermiculite) in drying soil, without affecting leaf RWC (Table 1).
Effect of soil nitrate (N) concentration and soil drying in intact maize plants on soil volumetric water content (θv) and xylem sap N concentration

Treatments were 100–200 ml (dependent on external conditions) of water only or 16 mol m⁻³ and 24 mol m⁻³ KN0₃ supplied daily for 3 d, giving calculated soil N concentrations of 75, 235, and 315 mg l⁻¹ 3–6 d after the start of N addition. Soil drying was initiated 3 d after the start of N addition. Results are means (n=4–7 for soil volumetric water content, n=2–3 for xylem N concentration) ±SE. Significant differences between treatments are shown by the different letters.

<table>
<thead>
<tr>
<th>Days from start of N addition</th>
<th>Soil N concentration (mg l⁻¹)</th>
<th>Soil water content (θv, m³ m⁻³)</th>
<th>Xylem sap N concentration (mol m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (well-watered)</td>
<td>75</td>
<td>0.454±0.005 a</td>
<td>9.0±1.4 a</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>0.463±0.007 a</td>
<td>13.4±0.8 b</td>
</tr>
<tr>
<td></td>
<td>315</td>
<td>0.466±0.013 a</td>
<td>20.5±2.0 c</td>
</tr>
<tr>
<td>4 (drying)</td>
<td>75</td>
<td>0.388±0.017 b</td>
<td>21.0±2.9 c</td>
</tr>
<tr>
<td></td>
<td>235</td>
<td>0.400±0.008 b</td>
<td>16.6±7.4 abc</td>
</tr>
<tr>
<td></td>
<td>315</td>
<td>0.422±0.020 b</td>
<td>13.2±7.2 abc</td>
</tr>
<tr>
<td>5 (drying)</td>
<td>75</td>
<td>0.331±0.017 c</td>
<td>13.4±6.6 abc</td>
</tr>
<tr>
<td></td>
<td>235</td>
<td>0.339±0.007 c</td>
<td>9.1±2.8 a</td>
</tr>
<tr>
<td></td>
<td>315</td>
<td>0.332±0.012 c</td>
<td>23.7±6.4 c</td>
</tr>
<tr>
<td>6 (drying)</td>
<td>75</td>
<td>0.284±0.010 d</td>
<td>3.9±1.3 d</td>
</tr>
<tr>
<td></td>
<td>235</td>
<td>0.279±0.014 d</td>
<td>6.8±2.0 ad</td>
</tr>
<tr>
<td></td>
<td>315</td>
<td>0.290±0.006 d</td>
<td>24.6±1.2 c</td>
</tr>
</tbody>
</table>

Effect of soil nitrate (N) concentration and soil drying around roots of intact maize plants, on volumetric soil water content (θv) and leaf relative water content (RWC)

Treatments were 200 ml of water only or 10 mol m⁻³ and 20 mol m⁻³ KN0₃ supplied daily for 3 d, giving calculated soil N concentrations of 75, 188, and 300 mg l⁻¹ 3–5 d after the start of N addition. Soil drying was initiated 3 d after the start of N addition. Results are means (n=4 for θv, n=6 for leaf RWC) ±SE. Significant differences between treatments on each day of the experiment are shown by the different letters.

<table>
<thead>
<tr>
<th>Days from start of N addition</th>
<th>Soil N concentration (mg l⁻¹)</th>
<th>Soil water content (θv, m³ m⁻³)</th>
<th>Leaf RWC (% maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (well-watered)</td>
<td>75</td>
<td>0.444±0.0009 a</td>
<td>97.72±0.19 a</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>0.445±0.014 a</td>
<td>97.48±0.18 a</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.447±0.002 a</td>
<td>97.35±0.10 b</td>
</tr>
<tr>
<td>4 (drying)</td>
<td>75</td>
<td>0.407±0.013 a</td>
<td>98.43±0.15 a</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>0.392±0.010 a</td>
<td>98.39±0.09 a</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.385±0.009 a</td>
<td>98.26±0.05 a</td>
</tr>
<tr>
<td>5 (drying)</td>
<td>75</td>
<td>0.337±0.008 a</td>
<td>97.6±0.16 a</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>0.324±0.014 a</td>
<td>97.7±0.18 a</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.296±0.007 b</td>
<td>97.3±0.16 a</td>
</tr>
</tbody>
</table>

Supplied to the xylem, by transpiring less, as expected (Fig. 4A). KN0₃ at 20 mol m⁻³ as found in the xylem sap of plants growing in soil supplied with supplemental N (Table 1), had little effect on transpiration in the absence of ABA, although it exacerbated the effect of ABA to close stomata when these compounds were supplied in combination. Transpiration in cv. ZP677 was more sensitive to N than that in cv. Earligold (compare Fig. 4A and B); 12 mol m⁻³ KN0₃ reduced transpiration in both the presence and absence of ABA. Figure 4C shows that KN0₃ concentrations as low as 2.0 mol m⁻³ (equivalent to those found in the xylem sap of plants growing in substrate supplied with optimal N concentrations, Fig. 1B; Table 1) significantly reduced transpiration in cv. ZP677 compared with that in shoots supplied with water only.

N supplied from 2.0 to 20 mol m⁻³ to detached shoots of cv. ZP677 had no effect on the bulk leaf ABA concentration of the leaves harvested 6 h later (data not shown), implying that de novo ABA synthesis was not induced.

KN0₃ concentration, ABA, and leaf growth: Leaf growth in detached maize cv. ZP677 shoots was less sensitive to both ABA and KN0₃ supplied to the xylem than transpiration (compare Fig. 5 with Fig. 4B, C). KN0₃ at 12 mol m⁻³, which reduced transpiration (Fig. 4B), did not reduce leaf growth rate (Fig. 5A). ABA at 0.05 mol m⁻³ which reduced transpiration (Fig. 4B), did not reduce leaf growth rate (Fig. 5A). However, a combination of both 0.05 mmol m⁻³ ABA and 12 mol m⁻³ KN0₃ significantly reduced leaf growth rate. Higher concentrations of both ABA (0.1 mmol m⁻³) and KN0₃ (20 mol m⁻³) were required to reduce leaf growth rate independently of one another than were required to reduce transpiration (Fig. 5B, C). Combinations of ABA, and KN0₃ from 10 mol m⁻³ significantly reduced leaf growth rate (Fig. 5D, and see Fig. 5A).

In some cases there was a slight increase in growth rate, particularly in the presence of the lower range of KN0₃ concentrations (2.0–5.0 mol m⁻³), compared with water-only controls (data not shown). This seemed to indicate that shoots were unable to grow at the maximal rate in the presence of water only in some experiments.

pH: Figure 6 shows that buffers adjusted to pH 7.0 reduced both transpiration (Fig. 6A) and leaf growth (Fig. 6B) compared with the rates detected when buffers adjusted to pH 5.0 were supplied to the xylem of detached shoots.

KN0₃ concentration, pH, and transpiration: Figure 7 shows that when 3.0 mol m⁻³ and 12 mol m⁻³ KN0₃ were supplied to detached maize shoots in buffers adjusted to pH 6.7, there was a synergistic effect of alkaline pH and KN0₃ to reduce transpiration even further compared with KN0₃-only controls. Buffers supplied at a pH of 5.6 significantly relieved 12 mol m⁻³ (but not 3.0 mol m⁻³) KN0₃-depressed transpiration rates (Fig. 7). However, buffers adjusted to an even more acidic pH (5.0) could not alleviate N-inhibited transpiration (not shown).

KN0₃ concentration, pH, and leaf growth rate: In a similar manner to their effect on transpiration, buffers adjusted to pH 6.7 containing 3.0 mol m⁻³ (but not 12 mol m⁻³) KN0₃ had a synergistic effect on leaf growth rate: this...
was slowest when these treatments were applied to the shoots in combination (Fig. 8). Figure 8 shows that KNO₃-inhibited leaf growth was not relieved by supplying the N in pH 5.6 buffer.

Discussion

Increasing the vermiculite N concentration from deficient to optimal increased both leaf growth rate and $g_s$ in intact maize plants. This occurred in both the presence and absence of soil drying. As described in the Introduction, previous research has shown that reduced xylem cytokinin concentrations and/or an increase in xylem sap pH could increase stomatal sensitivity to xylem ABA as N becomes deficient. Low N can be associated with an increase in root nitrate reductase activity (NRA), which produces malate that may then accumulate in the xylem flowing from the root. It has been suggested (Wilkinson, 1999, 2004; Wilkinson and Davies, 2002) that the negative charge carried by the malate anion (or other carboxylate by-products of NRA) can increase the pH of the xylem (Kirkby and Armstrong, 1980; Patonnier et al., 1999), which influences stomatal aperture via an effect on ABA distribution. Malate concentrations increased in the xylem of *Ricinus communis* L. plants as the soil N concentration was reduced (Peuke and Jeschke, 1995). Apoplastic malate has been shown to be associated with stomatal closure (Hedrich et al., 1994; Schell, 1997; Patonnier et al., 1999; Goodger et al., 2005), but not with reduced growth rates to date.

Little emphasis has previously been placed on potential N signalling above the deficient range, which is surprising given that plants fertilized at standard rates often exhibit xylem N concentrations that fluctuate within this range. For example, Bahrun et al. (2002) measured xylem sap N

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**Fig. 4.** The effect on the mean transpiration rate ($n=7$, A, B; $n=6$, C, ±SE) of feeding solutions of KNO₃ (20 mol m$^{-3}$ A, 12 mol m$^{-3}$ B, 2–20 mol m$^{-3}$ C) and/or ABA (0.1 mmol m$^{-3}$ A, 0.05 mmol m$^{-3}$ B) to de-rooted maize (cv. Earligold A, or cv. ZP677 B, C) seedlings via the subcrown internode. (C) The effects of a range of KNO₃ concentrations on transpiration in the absence of ABA.
Fig. 5. The effect on the mean cumulative increase in length of the third leaf (n=7, A, B; n=6, C, D, ±SE), of feeding solutions of KNO₃ (12 mol m⁻³ A, 20 mol m⁻³ B, 2–20 mol m⁻³ C, D) and/or ABA (0.05 mmol m⁻³ A, 0.1 mmol m⁻³ B, D) to de-rooted maize (cv. ZP677) seedlings via the subcrown internode. (C, D) The effects of a range of KNO₃ concentrations on growth in the presence (D) and absence (C) of ABA.

Fig. 6. The effect of feeding buffer solutions (3.0 mol m⁻³ K₂HPO₄/KH₂PO₄) of different pH values to de-rooted maize (cv. Earligold) seedlings via the subcrown internode, on the mean rates (±SE) of (A) transpirational water loss and (B) cumulative increases in length of the third leaf (n=7).
concentrations in maize that fluctuated between 0.5 and 10 mol m$^{-3}$. Goodger et al. (2005) detected xylem sap N concentrations of 2.4–6.0 mol m$^{-3}$ in well-watered maize plants, and of 1.7–12 mol m$^{-3}$ in plants growing in drying soil, dependent on the sap extraction method used. Figure 1 and Table 1 show that, here, optimal xylem N concentrations fell between 0.6 mol and 7.1 mol m$^{-3}$, where both $g_s$ and leaf growth rate was maximal in intact plants, and the highest concentration of N detected in xylem sap extracted from plants growing in non-supplemented (drying) soil was 21 mol m$^{-3}$. Detached shoot bioassays determined that a significant proportion of the upper end of the N concentration range, detected by us and by others to occur in intact plants, could potentially impact on shoot physiology in a novel manner.

Several data sets have described variations in xylem N concentrations in response to changes in the environment other than those simply resulting from fluctuations in soil nitrate availability (see Introduction; Lexa and Cheeseman, 1997). In maize, Shaner and Boyer (1976a, b) and Bahrun et al. (2002) detected soil drying-induced decreases, whilst Goodger et al. (2005) detected soil drying-induced increases in N concentration under some circumstances. Here an initial significant increase in xylem sap N concentration was detected, followed by a decrease as soil dried further (Table 1). A concentration of N within the xylem sap may occur not only in response to increasing soil N availability/concentration (Andrews, 1986a, b), which may be an issue in soil becoming depleted of moisture, but also as a result of a reduction in the flux of water in the xylem with the transpiration stream (Smith, 1991). Acquisition of soil N by root uptake processes is often only reduced by very severe soil water deficit, that also affects shoot water relations (Brewitz et al., 1996). Consistent with this, in our hands, soil drying only reduced xylem N concentrations at the lowest soil moisture contents measured. However, in species in which changes in soil water content have been reported to induce more sensitive reductions in xylem N concentration (see above), environmental modification of NRA distribution between root and shoot may be involved (Brewitz et al., 1996; Lexa and Cheeseman, 1997). An increase in the proportion of N that is reduced by the root would tend to decrease xylem N concentrations (Peuke et al., 1996). It is likely that xylem N concentration is regulated via a complex interplay of factors, which varies between species (Jia and Davies, 2007) and as edaphic and aerial conditions change (Brewitz et al., 1996; Lexa and Cheeseman, 1997; Jackson et al., 2003), and that a large proportion of this variation will fall within the non-deficient range.

Figures 1–3 show that increasing soil N above an optimum or sufficient concentration (which also increased the xylem N concentration, Fig. 1B, Table 1) no longer increased leaf growth rate or $g_s$, but started to reduce both of these variables, at least in drying soil. The response of stomata and growing leaves to increasing N concentration from deficient to sufficient to supra-optimal is therefore biphasic (Fig. 1C; and compare Figs 1C and 3 to see the

Fig. 7. The effect on the mean transpiration rate ($n=5, \pm SE$) of feeding solutions of KNO$_3$ to de-rooted maize (ZP677) seedlings via the subcrown internode, in water or in buffers adjusted to pH 5.6 or pH 6.7 (1.0 mol m$^{-3}$ K$_2$HPO$_4$/KH$_2$PO$_4$).

Fig. 8. The effect on the mean total increase in leaf length after 5.25 h ($n=5, \pm SE$) of feeding solutions of KNO$_3$ to de-rooted maize (ZP677) seedlings via the subcrown internode, in water or in buffers adjusted to pH 5.6 or pH 6.7 (1.0 mol m$^{-3}$ K$_2$HPO$_4$/KH$_2$PO$_4$).
effects of the full range of soil N concentrations used). This effect is represented stylistically in Fig. 9. The non-deficient inhibitory part of the response (e.g. Fig. 2, leaf growth, Fig. 3, gₜ) was not mediated by an effect of N on root–shoot ratio, shoot water potential (Table 2), or root-sourced changes in ABA or cytokinin biosynthesis, as similar effects were seen when N was supplied directly via the xylem to detached shoots of maize cv. ZP677 (Figs 4, 5). Nor was it a result of de novo ABA synthesis within the shoot, although all of these effects may have a role in vivo (Liu and Dickmann, 1992) which may intensify the effect of N on stomata and growing leaves observed here. Instead, it is proposed that, as xylem N concentrations increase from deficient to optimal to supra-optimal, there is a biphasic effect on pH (impacting on gₜ and growth), which decreases from deficient to optimal N, and then increases again as N increases further (Fig. 9). The pH increase above the deficient range may reduce transpiration and growth by concentrating root- and shoot-sourced ABA within the apoplast (Davies et al., 2002; Wilkinson and Davies, 2002; Wilkinson, 2004). It has been demonstrated elsewhere in several species that increasing the N load in the xylem concomitantly increases its pH (Mengel et al., 1994; Hoffman and Kosegarten, 1995). Mengel et al. (1994) proposed that the N-induced increase in apoplastic pH was a result of the removal of protons from this compartment. N is taken up from the leaf apoplast into the cells of the leaf via NO₃⁻/H⁺ co-transport over the plasma membrane (Ullrich, 1992), and it is later assimilated by NRA. In support of this, Hoffman and Kosegarten (1995) determined that, in response to an increased N supply to detached sunflower (Helianthus annuus L.) leaves (0–2.5 mol m⁻³), the pH increase detected was more marked in the leaf apoplast (pH 5.7 to 6.4 within 1.0 h) than in the stem xylem sap. Xylem alkalization as N becomes deficient is thought to occur by other means (see above).

Our data support the hypothesis that the non-deficient N signal acts via a pH-based ABA redistribution mechanism. Under some circumstances a combination of ABA and N is required to elicit an effect on shoot physiology, where neither compound is as effective alone (Figs 4A, 5A). That supplying the N in relatively acidic buffer (pH 5.6) restored KNO₃-depressed transpiration, whilst supplying the N in relatively alkaline buffer exacerbated the KNO₃-induced reductions in both growth and transpiration, strongly supports the possibility that N exerts its effects via an alkalinization of the xylem/apoplastic sap (Figs 7, 8). In related work, Jia and Zhang (1997) detected large reductions in the amount of ABA transported out of maize leaves in the phloem when buffers of pH 7.4 as opposed to pH 5.5 were injected into the xylem, implying that ABA accumulated in the leaf when sap was more alkaline. Sauter and Hartung (2002) demonstrated that artificial sap perfused through maize stems became enriched by stores of ABA from the stem parenchyma when its pH was buffered to more alkaline values (7.0 as opposed to 6.0). It is not known whether the resultant changes in leaf and stem ABA concentrations would have been sufficient to affect shoot physiology, although this seems likely given that, in this study, buffers adjusted to pH 6.7–7.0 supplied to detached maize shoots reduced transpiration and leaf growth, compared with buffers adjusted to pH 5.0 (Figs 6–8), or with water-only controls.

Consistent with the hypothesis that xylem N concentration and pH are positively correlated, and affect shoot physiology by increasing the penetration of ABA to its target cells, Schurr et al. (1992) determined that correlations between xylem sap N concentrations and stomatal sensitivity to xylem ABA were positive in sunflower plants. Jia and Davies (2007) determined a synergistic effect of xylem N and ABA on gₜ in detached leaves of Commelina communis L. The same authors determined that additional substrate N exacerbated an effect of soil water deficit to depress gₜ in intact tomato (Lycopersicon esculentum L.). It is unlikely that the nitrate molecule itself acts directly on guard cells to reduce stomatal aperture (Guo et al., 2003; Jia and Davies, 2007).

It must be noted, however, that more acidic buffers (pH 5.0) did not reverse the effect of KNO₃ to depress transpiration and growth (not shown), as might also have been expected. Whilst acidic pH is often associated with more open stomata in intact plants or detached shoot tissue assumed to contain an endogenous supply of ABA.
(Fig. 6A), seemingly there is a separate, direct, and opposing effect of pH on guard cell ion transport and stomatal aperture when the stomata are isolated from the rest of the plant (Wilkinson and Davies, 1997; Roelfsema and Prins, 1998) or inadequately supplied with ABA (Wilkinson et al., 1998). Alternatively, the failure of acidic buffers to reverse the effect of KNO₃ may be based on the fact that N-induced alkalization is probably localized around the target cells (the stomata and growing cells) in the leaf apoplast, and may be largely unreflected in the xylem further down the transpiration stream (Hoffman and Kosegarten, 1995). Whether or not such a localized response can be reversed by supplying buffers at a point distant from this cannot be established until the pH of the apoplast closer to the proposed sites of action of ABA has been measured. Nevertheless, these results indicate that some amplification and reversal do seem possible.

It must also be noted that part of the effect of KNO₃ to close stomata and reduce leaf growth may be transposed via a non-pH-based effect of malate. An increase in leaf N concentration can increase NRA in mesophyll cells (Andrews, 1986a, b), which can increase malate synthesis (Lips, 1997). If this malate can access the guard cell apoplast, it may induce stomatal closure by directly increasing the activity of anion efflux channels at the guard cell plasma membrane (Hedrich et al., 1994; Patonnier et al., 1999). To date, there is no evidence that malate affects leaf growth, although this must remain a possibility.

These data clearly indicate that increases in xylem N concentration above the deficient range, mirroring changes in soil N or being the result of internal plant processes, can act as sensitive chemical signals from the root, transposed by changes in apoplastic pH, that can control shoot physiology in such a way as to conserve water (Fig. 9). This would obviously be adaptive in plants growing in drying soil or air. In addition, during periods of high temperature and/or high radiant load, N uptake from the leaf apoplast into leaf mesophyll cells can increase (during periods of high photosynthetic activity; Andrews, 1986a, b). As described above, it is the uptake of NO₃⁻ into the symplast and the concurrent removal of a proton from the apoplast that is believed to be the basis for N-induced apoplastic alkalization (see above). A pH- and ABA-based feedback effect to keep stomata closed (and growth rates depressed) would be adaptive under these circumstances, when the driving force for water loss is high. Such a phenomenon may be a contributory factor to the midday closure of stomata commonly detected in field studies (Tardieu and Davies, 1992).

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References


