The effect of root cooling on hormone content, leaf conductance and root hydraulic conductivity of durum wheat seedlings (Triticum durum L.).

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\textbf{Summary}

Root cooling of 7-day-old wheat seedlings decreased root hydraulic conductivity causing a gradual loss of relative water content during 45 min (RWC). Subsequently (in 60 min), RWC became partially restored due to a decrease in transpiration linked to lower stomatal conductivity. The decrease in stomatal conductivity cannot be attributed to ABA-induced stomatal closure, since no increase in ABA content in the leaves or in the concentration in xylem sap or delivery of ABA from roots was found. However, decreased stomatal conductance was associated with a sharp decline in the content of cytokinins in shoots that was registered shortly after the start of root cooling and linked to increases in the activity of cytokinin-oxidase. This decrease in shoot cytokinin content may have been responsible for closing stomata, since this hormone is known to maintain stomatal opening when applied to plants. In support of this, pre-treatment with synthetic cytokinin benzyladenine was found to increase transpiration of wheat seedlings with cooled roots and bring about visible loss of turgor and wilting.

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\textbf{KEYWORDS}
Aba; Cytokinins; IAA; Leaf extension growth; Root cooling; Water relations

\textbf{Abbreviations:} IAA, indole-3-acetic acid; Aba, abscisic acid; Ba, benzyladenine; Ipa, isopentenyladenosine; RWC, relative water content

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Introduction

There is much published evidence supporting the view that transfer of chemical signals in xylem sap from stressed roots influences shoot growth and behaviour (Jackson, 1993; Hartung et al., 2002). Decrease of stomatal conductivity by ABA exported from roots is a much-researched example and an important one since limiting water loss from the foliage is an adaptive reaction to many unfavourable root environments. For example, cooling of the root zone often occurs under natural conditions in spring and crops such as *Triticum durum* can experience a sharp decline in soil temperature in the field. This disturbs their water relations, since low temperatures decrease root hydraulic conductivity thereby lowering leaf water potentials (Fennell and Markhart, 1998). This in turn can close stomata, thereby restricting water loss. However, it has been shown that abscisic acid (ABA) may not be implicated in stomatal closure induced by low temperatures experienced by whole plants (Wilkinson et al., 2001). Instead it is thought that sensitivity of stomata to Ca**+** ions increases under these conditions, leading to their closure. However, in case of local decline in root zone temperature, this mechanism seems unlikely since the leaves themselves do not experience the cooler temperature. Thus, when stomata close in response to cooler root temperatures they must be responding to some signal transferred from stressed roots to the leaves. We have shown previously that root cooling rapidly decreases the concentration cytokinin in shoots of wheat seedlings (Kudoyarova et al., 1998). Since cytokinins can influence stomatal conductivity (Tal and Imber, 1971), a decrease in delivery of cytokinins from chilled roots in xylem sap might be important for stomatal regulation. Accordingly, we have now examined a possible involvement of cytokinins in the control of stomatal conductivity of wheat seedlings with cooled roots and have incorporated studies of interactions with the hormones ABA and auxin (IAA).

Materials and methods

Spring durum wheat (*Triticum durum* L., cv. Bezenchukskaya 139) plants were grown in water culture. Seeds were germinated in darkness on distilled water supplemented with 50 µM CaCl2 at a temperature of 24 °C for 3 d. Three-day-old seedlings were then transferred to half-strength Hoagland–Arnon nutrient medium and grown under light intensity of 400 µmol m⁻² s⁻¹ PAR and a 14-h photoperiod for 8 d and a day/night air temperature of 26/20 °C. The corresponding temperature of nutrient medium was 22/18 °C. The root-zone temperature of 7-d-old plants was cooled to about 6 °C for 15 min by adding ice to the nutrient medium. Thirty minutes prior to root cooling, the leaves of some plants were immersed in 100 mM benzyladenine (BA) for 1 min. An analogue inductive electromechanical position sensor was used to monitor extension growth. Output signal from the sensor was tracked continuously and registered with a chart recorder.

Samples for hormone analysis were collected prior to and after the cooling treatment. Cytokinin extraction and separation by TLC, ABA, and IAA extraction by solvent partitioning and immunoassays were performed with the help of specific rabbit antibodies raised against ABA, IAA, and zeatin riboside (Veselov et al., 1992; Kudoyarova et al., 1998). Reliabilities of immunoassay for IAA and ABA were enabled by both specificity of antibodies and purification of hormones according to a modified scheme of solvent partitioning (Veselov et al., 1992). Anti-cytokinin antibodies had high immunoreactivity towards zeatin, its riboside, 9N-glucoside and nucleotides, which were separated by TLC prior to immunoassay (Veselov et al., 1999). The antibody showed low cross reactivity to dihydrozeatin and isopentenyladenine (iPA) and their derivatives. Reliability of the hormone immunoassays was confirmed by dilution tests, chromatographic examination of the distribution of immunoreactivity, and comparison of the results of immunoassay against that of physicochemical assays (LC-MS, Veselov et al., 1999).

The rate of transpiration was measured by weight loss. This involved placing 20 seedlings with their roots in a vessel containing 50 ml of nutrient medium, and weighing it before and after 15-min of transpiration. The difference reflected the rate of transpiration (evaporation was prevented by covering each vessel with foil). The results of a typical experiment comprising about 10 replicates are presented as means ± standard errors.

Relative water content (RWC) was determined by floating leaf pieces (about 2 cm in length) on distilled water for 3–4 h at 22 °C in darkness. The turgid weight (TW) was taken after blotting and dry weight determined after 24 h at 80 °C. Fresh weight (FW), dry weight (DW), and TW were used to determine RWC according to the formula: RWC=[(FW−DW)/(TW−DW)] × 100%.

Measurement of xylem sap flow and its collection was carried out as follows: Excised roots with the mesocotyl still attached were fixed to a calibrated glass capillary using pressure-tight silicone rubber
compressed by a screw. These silicone seals were 15-mm-long and individually prepared for each plant with a central hole individually matched to the diameter of the mesocotyl. The root medium was aerated and with an applied suction of 0.02 MPa to induce xylem sap to flow into the capillary. After 15 min, xylem sap was collected, weighed, and analysed for hormone content. Stomatal conductivity was measured by means of a diffusion porometer (Mk3, Delta-T Devices, UK).

For estimation of cytokinin-oxidase activity, shoots were homogenised in 0.1 M Tris-buffer (pH 7.0) and centrifuged at 12,000 g for 30 min. To separate the sample from endogenous hormones, an equal volume of saturated solution of ammonium sulphate was added to supernatant, and the suspension was centrifuged and the pellet re-suspended in one of the buffers indicated in Table 1. Thirty nanograms of the cytokinin iP A were added as a substrate to the mixture, which was then incubated for 1 h at 37 °C. Sufficient cold ethanol was added to each sample to raise the concentration to 60% (v/v), the resulting suspension was incubated at 4 °C for 40 min and then centrifuged at 5000 g for 10 min. The quantity of iP A lost by degradation was determined by means of an immunoassay. The absence of conversion of iP A to zeatin or dihydrozeatin derivatives was confirmed using immunoassays employing the corresponding antibodies. The presence of imidazole and Cu2+ accelerated the rate of the iP A disappearance, while 1,3-diphenylurea inhibited the process. These tests indicated that the decrease in iP A content is specific to its conversion to adenosine catalysed by cytokinin-oxidase (Hare and Van Staden, 1994).

**Results**

Sudden cooling of the roots of wheat seedlings resulted in an immediate cessation of leaf growth as measured by a highly sensitive position sensor (Fig. 1). It also reduced leaf relative water content (RWC) (Table 1, without BA date) and slowed the flow of xylem sap from detached roots subjected to a standard negative pneumatic pressure of 0.02 MPa (Fig. 2).

Transpiration measured as weight losses during first 15 min after the start of cooling was lower than in control by 20% (Table 1, without BA date). This slightly slower transpiration persisted during the subsequent 30 min followed by further drop in transpiration to 50% of that of control plants in association with partial recovery in RWC from 88% to 92%. Pre-treatment of plants with BA (30 min prior to root cooling) increased transpiration rates (which were already slightly higher with BA than without it at time zero) and prevented the decline in transpiration (Table 1) seen in chilled seedlings not given BA. In association with this effect, BA also lowered leaf RWC prior to root chilling. Further decline in RWC content continued in BA-treated plants following root cooling and, unlike plants with no BA, RWC did not increase at the end of the experiment. The large amount of water loss

![Figure 1. Effect of cooling the roots of 7-d-old wheat seedlings to approximately 6 °C on elongation of the youngest emerged leaf measured by an analogue inductive electromechanical position sensor.](image-url)

**Table 1.** Effect of cooling the roots to approximately 6 °C on leaf RWC, transpiration, and stomatal conductivity of 7-d-old wheat seedlings that did or did not receive a 1 min foliar pre-treatment of 100 mM BA

<table>
<thead>
<tr>
<th>Time of cooling (min)</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without BA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RWC (%)</td>
<td>94.8±0.3</td>
<td>91.7±0.4</td>
<td>89.4±0.4</td>
<td>88.3±0.2</td>
<td>92±0.4</td>
</tr>
<tr>
<td>Transpiration (mg seedling⁻¹ h⁻¹)</td>
<td>43±3.3</td>
<td>33.6±2.7</td>
<td>35.8±2.4</td>
<td>32.1±2.6</td>
<td>22±1.6</td>
</tr>
<tr>
<td>Stomatal conductance (mM m⁻² s⁻¹)</td>
<td>121±18</td>
<td>114±22</td>
<td>109±11</td>
<td>—</td>
<td>59±10</td>
</tr>
<tr>
<td><strong>With BA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RWC (%)</td>
<td>92.7±0.5</td>
<td>91.2±0.5</td>
<td>89.4±0.5</td>
<td>89.3±0.1</td>
<td>89.2±0.2</td>
</tr>
<tr>
<td>Transpiration (mg seedling⁻¹ h⁻¹)</td>
<td>49.9±4.7</td>
<td>47.4±4</td>
<td>48.9±4.7</td>
<td>38.1±4.6</td>
<td>37.6±3</td>
</tr>
<tr>
<td>Stomatal conductance (mM m⁻² s⁻¹)</td>
<td>169±11</td>
<td>148±10</td>
<td>148±22</td>
<td>—</td>
<td>114±17</td>
</tr>
</tbody>
</table>

Means of 10 replicates and 10 seedlings per replicate with standard errors.
associated with the relatively fast transpiration of BA-treated plants with cooled roots caused visible wilting of leaves.

Stomatal conductance was lowered by 26% a 30 min after root cooling (Table 1) and by 60 min conductance was only 48% of the initial value. In contrast, conductance was much higher in BA-treated plants with stomatal conductance of cooled plants enhanced to levels similar to uncooled control plants (Table 1). Thus, while root cooling alone closed stomata, BA pre-treatment overrode this effect.

Root cooling decreased cytokinin concentration in shoots by two thirds within 15 min (Fig. 3), while that of ABA remained at control levels. The treatment also more than tripled the concentration of IAA in the shoots. The effects of cooling on hormone in the roots varied. ABA and cytokinins were largely unchanged while IAA concentrations decreased markedly (93% decrease). In vitro measurement of the ability of the protein fraction recovered from leaves to degrade the cytokinin substrate iPA showed that root cooling increased this activity by 56% (from 410 to 640 pmol iPA mg⁻¹ of protein h⁻¹).

ABA and cytokinin concentration in xylem sap was not significantly affected by the treatment, but their overall delivery rate (concentration × sap flow rate) from roots declined by more than 50% due to reduced transpiration flow (Table 2). On the assumption that, in planta, hormone concentrations of xylem sap would be diluted in proportion to sap flow rate, these delivery rates are assumed to reflect the delivery rates from roots to shoots of intact plants.

Discussion

Cessation of leaf growth similar to that registered in our experiments with root cooling have also been observed after other root perturbations, such as salinity, illumination, and increases in air temperature (Munns et al., 2000; Ben Hai and Tardieu, 1996). This fast inhibition of growth can only be due to an inhibition of cell elongation, which is particularly sensitive to changes in water supply. Thus, one likely cause of the much slower leaf

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### Table 2. ABA and cytokinin concentration in xylem sap of wheat seedlings and their rate of delivery from roots following their cooling during 15 min

<table>
<thead>
<tr>
<th></th>
<th>Temperature of nutrient medium (°C)</th>
<th>Concentration of hormone in xylem sap (nM)</th>
<th>Delivery of hormone from root to shoot (pmol h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>11.9 ± 0.3</td>
<td>0.76 ± 0.04</td>
</tr>
<tr>
<td>Root cooling</td>
<td>6</td>
<td>13.7 ± 0.8</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Zeatin+zeatin riboside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>78 ± 12</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Root cooling</td>
<td>6</td>
<td>85 ± 9</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

Means of 10 replicates with standard errors.
elongation is the onset of increased leaf water deficit generating a loss of turgor in potentially expanding cells. This possibility is supported by our finding of a rapid loss of RWC in leaves of plants within 15 min of cooling the roots and, within a similar time frame, by decrease in water flow from detached cooled roots when subjected to a known driving force. This slower water flow is interpreted as the outcome of reduced root hydraulic conductivity that may be a direct consequence of low temperature on cell membranes (Fennell and Markhart, 1998). This loss of leaf hydration occurred despite some slowing of the rate of water loss by transpiration and evidence of partial stomatal closure as indicated by diffusion porometry. Thus, a lowered root hydraulic conductance is seen as the overriding influence tending to dehydrate the leaves in the short term when the roots are cooled.

After about 45 min a gradual recovery in leaf RWC began in parallel with a more marked decline in transpiration. These signs of recovery are presumably the result of further stomatal closure that could arise for several reasons. The importance of reduced transpiration in restoring some normality to leaf water relations is indicated by experiments in which pre-treatment with BA interfered with this process by enhancing transpiration. The consequence was a further lowering of leaf RWC of plants with chilled roots and prevention of a partial recovery of RWC by untreated chilled plants that commenced about 1 h after chilling began.

The decline in transpiration in plants with cooled roots may have more than one cause. The well-known increase in foliar ABA resulting from leaf dehydration was expected to be involved since this is believed to close stomata in wilting plants. However, other mechanisms must operate because leaf ABA concentrations were not increased by root cooling as was found by Wilkinson et al. (2001) when whole plants were chilled. Our results also show that stomatal closure is not explained by an increase in the export of ABA from the chilled roots. On the contrary, ABA delivery was found to decrease substantially by root chilling. This finding resembles those obtained with flooded plants. Here too, the stress on the roots caused a decline in root hydraulic conductivity that created water deficits in shoots. These were subsequently reduced by stomatal closure despite a decreased delivery of ABA in xylem sap (Jackson, 2002). Thus, unlike roots in dry soil, roots cooled to 6–7 °C failed to generate an ABA signal. Smith and Dale (1988) reported a fast increase in ABA content in shoots of plants with roots cooled to 12 °C. In our case, approximately 6 °C is likely to be too low to sustain an increase in ABA synthesis. A more likely root signal regulating stomata in plants with cooled roots could involve cytokinins (Fujieda et al., 1992). Our results seem to be in accordance with this possibility. Cytokinins have long been known to maintain more open stomata (Tal and Imber, 1971) and a decline in the content of endogenous cytokinin in the leaf may well favour stomatal closure and diminished transpiration. Our analyses revealed rapid decreases in both foliar cytokinins and their rate of delivery from roots where much cytokinin formation is believed to take place. The involvement of cytokinins in control of transpiration may be further supported if it can be shown that when decline in endogenous hormone content is overcome using the exogenous cytokinins, stomatal closing is suppressed. In such work, applying cytokinins through leaves rather than through roots is important to exclude the possible effect of hormone on root hydraulic conductivity. Such leaf treatment, using BA, slowed the decline in transpiration in plants with cooled roots thereby implicating cytokinins in the control of stomatal conductance.

The question arises by which mechanism cytokinin content was reduced in shoots of plants with cooled roots. Cytokinin content in shoots was decreased by two thirds in only 15 min after the start of root cooling, while their delivery from roots was only halved. Clearly, changes in cytokinin content in shoots cannot be entirely explained by the decreased export of cytokinin from cooled roots, since the effect is both too swift and too large. It therefore seems to be unlikely that reduced delivery alone was the reason. Changes in the activity of enzymes that metabolise cytokinins is a more likely option for decreasing shoot cytokinins. In accordance with this view, cytokinin-degrading activity of protein extracts from leaves was much greater when plants had their roots cooled for only 15 min. The question remains concerning what kind of signal coming from cooled roots might change the ability of the shoot to degrade its cytokinins? The enzyme activity might be triggered by a root hydraulic signal. There is evidence that dehydration may influence hormone metabolism (Quin and Zeevart, 1999) and this could apply to cytokinins. On the other hand, IAA is known to promote the activity of cytokinin-oxidase (Hare and Van Staden, 1994, Werner et al., 2003). The accumulation of IAA that we observed in shoots of wheat seedlings might therefore be at least partially responsible for the increase in activity of cytokinins oxidase. Auxin accumulation in shoots is a probable outcome of reduced export from shoots to cooled roots. If this is correct, a corresponding
sharp decrease of IAA in cooled roots would be expected and we confirmed this by direct measurement.

Whatever the mechanism that brings about decreased cytokinin concentrations in plants with cooled roots, the decline in shoots is extreme, occurs in only a few minutes, and its impact on stomatal apertures appears important for the normalisation of plant water relations.

Acknowledgements

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References


