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Morphological, Anatomical and Physiological Responses Related to Differential Shoot *vs.* Root Growth Inhibition at Low Temperature in Spring and Winter Wheat

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Several morphological, anatomical and physiological changes and their relationship with differential root *vs.* shoot growth inhibition at low temperature (5°C) were studied in spring and winter wheat cultivars. Root : shoot ratios, expressed either as a function of root and shoot fresh weight or as a function of root and leaf areas, increased at low temperature and this increment was more pronounced in spring cultivars than in winter ones. Although winter cultivars developed relatively smaller root systems at 5°C, this characteristic was counterbalanced by a lower stomatal frequency and increased thickness of epidermal cell walls in leaves unfolded at this temperature, relative to spring cultivars. Likewise, at 5°C a decrease in the osmotic potential of shoots and roots was observed in parallel with sugar accumulation; this decrease was more marked in winter cultivars. These results indicate a differential morpho-anatomical and physiological plasticity of winter and spring cultivars during development at low temperature. The possible association between these changes and plant water economy at low temperatures is discussed.

Key words: Spring wheat, winter wheat, *Triticum aestivum*, low temperature, root : shoot ratio, root surface area, stomatal frequency, osmotic potential.

INTRODUCTION

A range of physiological and morphological adjustments occur in plants grown at low temperatures (Levitt, 1980; Pollock and Eagles, 1988). In many species, low temperatures are associated with increased root : shoot (R : S) ratios (Körner and Larcher, 1988; Sharrat, 1991). Since low soil temperatures reduce root hydraulic conductivity (Kramer, 1983; Fennell and Markhart III, 1997) and subsequently induce water deficit in shoots (Fennell *et al.*, 1990), the changes in R : S ratio have been considered as a response to overcome water limitation (Wilson, 1988).

In a previous study, changes in shoot and root growth rates as well as carbohydrate accumulation were compared in six wheat cultivars ranging from winter to spring genotypes cultivated at either 25 or 5°C. At 5°C, relative growth rate (RGR) of shoots was similarly reduced in all cultivars, but RGR of roots was more affected in winter wheats. This resulted in smaller R : S ratios in winter cultivars than in spring cultivars (Equiza *et al.*, 1997).

If increased R : S ratios are indeed an important factor for plant water economy at low temperatures, it is not clear how winter cultivars can cope with relatively smaller root systems than spring cultivars, unless other characteristics that may enable them to improve water uptake or reduce water losses are developed in parallel. However, root and shoot biomasses are not necessarily correlated with their respective exposed areas. The relationship between root and shoot area is considered a critical factor with respect to the functional relationship between the plant parts and the environment (Brouwer, 1983). In the words of van Noorwijk and de Willigen (1987), 'the most meaningful way to express R : S ratios may be on the basis of the size of their interface with the environment; for example, the ratio of root area to leaf area'.

In addition, several morphological and anatomical changes, such as increased leaf, epidermis and cell wall thickness, lower leaf and specific leaf areas and altered stomatal frequency, have been found in different species during growth at low temperatures (Huner *et al.*, 1981; Körner and Larcher, 1988; Boese and Huner, 1990). These changes have been associated with cold acclimation and the acquisition of freezing tolerance (Fowler *et al.*, 1981), but no causal relationships have been established (Huner *et al.*, 1981). Thus, a link between altered R : S ratios and other morpho-anatomical changes has not yet been established.

Winter cultivars accumulate more soluble sugars in shoots and roots than spring cultivars during cold acclimation (Tognetti *et al.*, 1990; Hurry *et al.*, 1995; Equiza *et al.*, 1997). Higher soluble sugar content leads to a decreased osmotic potential, which in turn favours water uptake by roots and retention by shoots (Hare *et al.*, 1998).

The present work was undertaken to: (1) assess changes in R : S ratios (expressed as a function of root and shoot fresh weight or as a function of root and shoot areas) at low temperature in spring and winter cultivars; (2) compare several morpho-anatomical and physiological characteristics of these cultivars during growth at low temperature,

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including specific leaf area, leaf thickness, stomatal frequency, total soluble sugars and osmotic potential of roots and shoots; (3) relate these morpho-anatomical and physiological characteristics to the changes in R:S ratios and discuss the possible ecophysiological significance of these responses.

MATERIALS AND METHODS

Plant material and growth conditions

The following wheat cultivars were used: Buck Patacón and San Agustín INTA (spring types), and ProINTA Pincén and ProINTA Puntal (winter types). In some experiments the intermediate types, Buck Yapeyú and ProINTA Oasis, were included.

Seeds were germinated in plastic pots (10 cm diameter × 30 cm deep) in vermiculite soaked with half strength Hoagland's solution (Hoagland and Arnon, 1950). Seedlings were grown under controlled environmental conditions at 25 ± 2°C, 250 μmol photon m^{-2} s^{-1} (PAR), 50–60 % relative humidity and 14 h photoperiod. After 7 d the seedlings were thinned to ten per pot. On the eighth day, when the first leaf was fully expanded, half of the pots were transferred to a chamber at 5 ± 1°C; all other conditions were the same as previously. The remaining control pots were kept at 25°C.

After transfer to 5°C the temperature of the root medium declined at approx. 2°C h^{-1}, reaching the temperature of the cold chamber after 10 h.

Growth measurements

Ten seedlings, randomly selected from two pots of each cultivar, were harvested every 2 d from pots at 25°C, while those exposed to 5°C were harvested every 7 d. In each case, plants were compared at similar phenological stages, as recommended by Krol *et al.* (1984) for studies on plants growing at different temperatures. Harvests continued in both treatments until the third leaf was completely expanded. Relative growth rates were estimated from the slopes of linear regressions of the natural logarithm of fresh weight *vs.* time (Hunt, 1978). Although dry matter accumulation is normally used in plant growth analysis, fresh weight was used here (see Discussion). R:S ratios were obtained from individual plants and results of variation in R:S ratio during cold treatment were expressed as the ratio of change in R:S ratio of plants at 5°C in relation to plants at 25°C at equivalent developmental stages.

Root surface area and leaf area determinations

Plants from each temperature were harvested at the second and third expanded leaf stages for the assessment of root surface area and leaf area. Root surface area was determined by the titration method described by Carley and Watson (1966), which is based on the cation exchange capacity of roots and provides a practical estimation of root interface with the soil environment (Böhm, 1979). Briefly, roots were washed with distilled water and air-dried for 8–10 min at room temperature to drain the excess water while retaining the original fresh volume. They were then immersed in a 3 N HCl solution (15 s). After draining on a glass to remove excess acid (5 min), they were transferred to a beaker containing 25 cm^3 distilled water. The contents of the beaker were stirred to wash the acid from the roots and then allowed to stand (10 min). This solution was titrated with 0·3 N NaOH to pH 7 using an automated Orion titrimeter. This method delivers relative data of the root surface area, since the titration value is expressed in ml NaOH (Böhm, 1979).

Individual leaf blade areas were measured with a leaf area meter (LI 3000A; LiCor, Lincoln, NE, USA). Percentage dry matter (% DM) of individual leaves was determined by drying in an oven at 70°C to a constant weight. Specific leaf area, expressed either as the ratio of leaf area to fresh weight (SLA-FW) or leaf area:dry weight (SLA-DW) were calculated for the third leaves.

Leaf anatomy

Free-hand transverse sections were made on the central portion of fixed third leaves. Samples were stained with 1 % safranin. Leaf thickness was determined at a magnification of ×100.

Plastic imprints were made from the central portion of first, second and third expanded leaf surfaces. Stomatal frequency was estimated at 15 different sectors of each adaxial and abaxial leaf surface with a plaid of 1 mm^2 at a magnification of ×120. Length of epidermal cells between stomata (ECS), whole stomatal length (SL) and width (SW) were measured on the abaxial surface of the third leaf (×400).

Quantitation of soluble sugars

Total soluble sugars were extracted from fully expanded first leaves as in Santoiani *et al.*, (1993). Reducing sugars and sucrose plus fructan were determined as in Equiza *et al.* (1997).

Osmotic potential determinations

Roots were washed quickly in distilled water, surface dried on filter paper and frozen at −20°C. Frozen roots were ground with a mortar and pestle at 0°C. The resulting powder was placed in a disposable syringe; after pressing, the sap obtained was used for osmolality determination in a WesCor osmometer (Kikuta and Richter, 1992). Plants at the third expanded leaf stage were used for shoot osmolality determinations, and samples were prepared in the same way as for roots.

The data obtained were converted to osmotic potential as:

$$\Psi_0 = -RTC_s i$$

where R is 8·2 × 10^{-6} MPa $mmol^{-1}$ K^{-1}; T is 298 K; and C_s is solute concentration (mmol l^{-1}). The coefficient of the solute (i) was assumed to be 1·0 (Meyer and Boyer, 1981).

No corrections were made for possible effects of apoplastic dilution on osmotic potentials.

Relative water content determination

Relative water content (RWC) was computed as:

$$\text{RWC} = 100 \times (\text{fresh weight} - \text{dry weight}) \times (\text{turgid weight} - \text{dry weight})^{-1}$$

by using 5 cm segments excised from the central portion of the third expanded leaf. Turgid weight was determined from rehydrated segments floated on distilled water for 6 h at 15°C in the dark, at the beginning of the light period. Previous estimations indicated that possible sugar degradation and/or interconversion within the 6 h had a negligible effect on RWC.

Statistical analysis

A completely randomized design with three replicates was used. All variables were determined on ten plants in each replicate. Differences between cultivars were analysed by Tukey's test.

RESULTS

Shoot and root fresh weight

Relative growth rates of shoots from plants grown at 5°C were reduced by about 75–80 % relative to those grown at 25°C, regardless of the cultivar assayed. Relative growth rates of roots from spring cultivars were 55–60 % lower at 5°C, while in winter cultivars reductions of about 75 % were observed (Table 1).

As a consequence of this differential root *vs.* shoot growth inhibition at 5°C, the R:S ratios, expressed on a fresh weight basis, were substantially increased in spring cultivars in relation to plants maintained at 25°C and assayed at comparable developmental stages. Small changes were observed in winter cultivars (Fig. 1).

Leaf and root areas

In order to analyse shoot and root responses to low temperature on the basis of the size of their interface with the environment, R:S ratios were calculated as a function of leaf and root areas. The final area of the second leaf, which emerged at 25°C but expanded at 5°C, was not significantly different at any temperature regardless of the cultivar assayed. The final area of the third leaf, which emerged and expanded completely at 5°C, was reduced by about 20 % at 5°C in winter cultivars. However, when the third leaf of these cultivars completed expansion at 5°C (reaching the third leaf stage), tillering was initiated. Thus, at both the second and third leaf stages the total leaf area was unaffected by cold treatment and there were no significant differences between cultivars (Table 2).

Root surface area of plants cultivated at 5°C increased 2–2·5-fold in spring cultivars at the second and third expanded leaf stages, respectively, while winter cultivars showed an increase of 1·3–1·7-fold (Table 2).

Thus, R:S ratios expressed as a function of root and leaf areas increased at low temperature, and this increment was on average 1·6-fold higher in spring than in winter cultivars (Table 2).

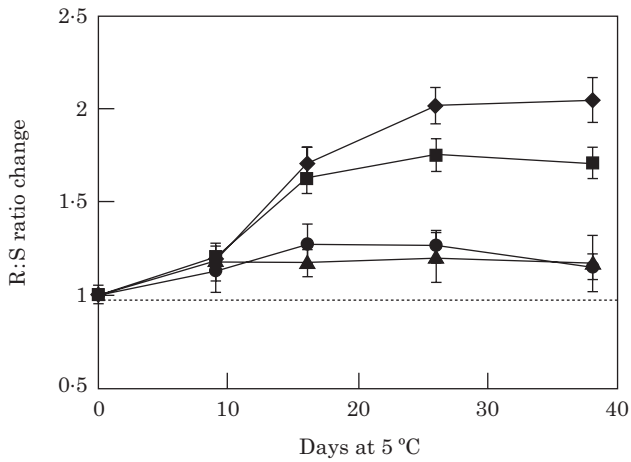

FIG. 1. Changes in R:S ratio, expressed on a fresh weight basis, of plants at 5°C in relation to plants at 25°C at equivalent developmental stages. Symbols correspond to different cultivars: Patacón (◆); San Agustín (■); Puntal (●); Pincén (▲). Each point is the mean of three replicate experiments.

TABLE 1. *Relative growth rates (RGR, d^{-1}) ± s.e. of shoots and roots of seedlings cultivated at 25 or 5°C, estimated on a fresh weight basis*

	RGR					
	Shoots			Roots		
Cultivar	25°C	5°C	5:25	25°C	5°C	5:25
Patacón	0·191 ± 0·008	0·047 ± 0·002	0·25	0·134 ± 0·009	0·054 ± 0·002	0·40
San Agustín	0·204 ± 0·006	0·055 ± 0·006	0·27	0·112 ± 0·010	0·051 ± 0·003	0·45
Puntal	0·184 ± 0·009	0·041 ± 0·002	0·22	0·117 ± 0·013	0·030 ± 0·002	0·26
Pincén	0·183 ± 0·011	0·036 ± 0·002	0·20	0·089 ± 0·008	0·024 ± 0·002	0·27

TABLE 2. *Relationship between total leaf area and root surface area at the second expanded leaf stage (2 L) and third expanded leaf stage (3 L) of plants cultivated at 25 or 5°C*

	Total leaf area (cm^2)		Root surface area (ml)		R:S ratio	
Cultivar	25°C	5°C	25°C	5°C	25°C	5°C
Patacón (2 L)	$12{\cdot}7 \pm 1{\cdot}1^{ab}$	$12{\cdot}2 \pm 1{\cdot}5^{a}$	$2{\cdot}3 \pm 0{\cdot}3^{a}$	$4{\cdot}8 \pm 0{\cdot}9^{b}$	$0{\cdot}18 \pm 0{\cdot}02^{a}$	$0{\cdot}40 \pm 0{\cdot}05^{b}$
San Agustín (2 L)	$14{\cdot}7 \pm 1{\cdot}1^{b}$	$13{\cdot}1 \pm 1{\cdot}4^{a}$	$2{\cdot}5 \pm 0{\cdot}4^{a}$	$4{\cdot}7 \pm 0{\cdot}6^{b}$	$0{\cdot}17 \pm 0{\cdot}02^{a}$	$0{\cdot}36 \pm 0{\cdot}03^{b}$
Puntal (2 L)	$10{\cdot}5 \pm 0{\cdot}6^{a}$	$10{\cdot}2 \pm 0{\cdot}6^{a}$	$2{\cdot}1 \pm 0{\cdot}2^{a}$	$2{\cdot}8 \pm 0{\cdot}4^{a}$	$0{\cdot}20 \pm 0{\cdot}02^{a}$	$0{\cdot}28 \pm 0{\cdot}04^{a}$
Pincén (2 L)	$14{\cdot}5 \pm 1{\cdot}3^{b}$	$12{\cdot}4 \pm 1{\cdot}4^{a}$	$2{\cdot}3 \pm 0{\cdot}3^{a}$	$2{\cdot}9 \pm 0{\cdot}4^{a}$	$0{\cdot}16 \pm 0{\cdot}03^{a}$	$0{\cdot}24 \pm 0{\cdot}03^{a}$
Patacón (3 L)	$21{\cdot}3 \pm 2{\cdot}6^{a}$	$21{\cdot}4 \pm 2{\cdot}1^{a}$	$2{\cdot}8 \pm 0{\cdot}2^{a}$	$8{\cdot}3 \pm 1{\cdot}0^{c}$	$0{\cdot}14 \pm 0{\cdot}02^{a}$	$0{\cdot}39 \pm 0{\cdot}07^{b}$
San Agustín (3 L)	$20{\cdot}9 \pm 1{\cdot}9^{a}$	$19{\cdot}0 \pm 0{\cdot}6^{a}$	$3{\cdot}0 \pm 0{\cdot}4^{a}$	$6{\cdot}5 \pm 1{\cdot}2^{bc}$	$0{\cdot}14 \pm 0{\cdot}01^{a}$	$0{\cdot}37 \pm 0{\cdot}04^{b}$
Puntal (3 L)	$17{\cdot}6 \pm 2{\cdot}7^{a}$	$19{\cdot}6 \pm 2{\cdot}9^{a}$	$2{\cdot}4 \pm 0{\cdot}5^{a}$	$4{\cdot}0 \pm 0{\cdot}5^{a}$	$0{\cdot}14 \pm 0{\cdot}02^{a}$	$0{\cdot}21 \pm 0{\cdot}04^{a}$
Pincén (3 L)	$21{\cdot}1 \pm 2{\cdot}4^{a}$	$22{\cdot}4 \pm 2{\cdot}4^{a}$	$2{\cdot}6 \pm 0{\cdot}4^{a}$	$4{\cdot}6 \pm 0{\cdot}7^{a}$	$0{\cdot}12 \pm 0{\cdot}02^{a}$	$0{\cdot}21 \pm 0{\cdot}04^{a}$

For each phenological stage, means within the same column with different superscripts are significantly different at $P < 0{\cdot}05$ (Tukey's test).

TABLE 3. *Specific leaf area, expressed as leaf area:fresh weight (SLA-FW) or as leaf area:dry weight (SLA-DW), of third leaves from wheat cultivated at 25 or 5°C*

	SLA-FW ($cm^2\ g^{-1}$ f.wt)		SLA-DW ($cm^2\ g^{-1}$ d.wt)	
Cultivar	25°C	5°C	25°C	5°C
Patacón	$53{\cdot}3 \pm 4{\cdot}3^{a}$	$44{\cdot}5 \pm 2{\cdot}4^{a}$	$557{\cdot}9 \pm 59{\cdot}5^{a}$	$405{\cdot}2 \pm 25{\cdot}4^{b}$
San Agustín	$52{\cdot}5 \pm 1{\cdot}8^{a}$	$43{\cdot}0 \pm 2{\cdot}9^{a}$	$582{\cdot}2 \pm 13{\cdot}9^{a}$	$401{\cdot}5 \pm 44{\cdot}9^{b}$
Puntal	$51{\cdot}6 \pm 2{\cdot}7^{a}$	$40{\cdot}3 \pm 2{\cdot}9^{a}$	$497{\cdot}2 \pm 22{\cdot}0^{a}$	$215{\cdot}9 \pm 24{\cdot}5^{a}$
Pincén	$50{\cdot}5 \pm 2{\cdot}5^{a}$	$40{\cdot}7 \pm 2{\cdot}3^{a}$	$500{\cdot}2 \pm 49{\cdot}6^{a}$	$223{\cdot}1 \pm 17{\cdot}6^{a}$

Means within the same column with different superscripts are significantly different at $P < 0{\cdot}05$ (Tukey's test).

TABLE 4. *Dry matter content (DM) of leaf 2 and leaf 3 from wheat cultivated at 25 or 5°C*

	% DM L2		% DM L3	
Cultivar	25°C	5°C	25°C	5°C
Patacón	$8{\cdot}5 \pm 0{\cdot}3^{a}$	$11{\cdot}2 \pm 1{\cdot}8^{a}$	$8{\cdot}9 \pm 0{\cdot}5^{a}$	$11{\cdot}6 \pm 0{\cdot}9^{a}$
San Agustín	$8{\cdot}9 \pm 0{\cdot}4^{a}$	$11{\cdot}4 \pm 0{\cdot}9^{a}$	$8{\cdot}8 \pm 0{\cdot}4^{a}$	$11{\cdot}4 \pm 0{\cdot}3^{a}$
Puntal	$9{\cdot}2 \pm 0{\cdot}2^{a}$	$13{\cdot}8 \pm 1{\cdot}3^{b}$	$8{\cdot}8 \pm 0{\cdot}3^{a}$	$17{\cdot}4 \pm 1{\cdot}7^{b}$
Pincén	$8{\cdot}8 \pm 0{\cdot}3^{a}$	$14{\cdot}6 \pm 1{\cdot}6^{b}$	$9{\cdot}3 \pm 0{\cdot}9^{a}$	$18{\cdot}2 \pm 1{\cdot}2^{b}$

Means within the same column with different superscripts are significantly different at $P < 0{\cdot}05$ (Tukey's test).

Specific leaf area (SLA-FW and SLA-DW)

SLA-FW from third leaves developed at 5°C decreased about 15–20 % in all cultivars analysed (Table 3). Since fresh weight is related to the tissue volume, a decrease in SLA-FW values represents an increase in leaf thickness (Lu and Neumann, 1999).

SLA-DW of third leaves at 5°C decreased about 30 % in spring cultivars and about 56 % in winter cultivars (Table 3). A decrease in SLA-DW might indicate transient dry matter deposition and/or an increased cell wall thickness (Lu and Neumann, 1999).

Percentage dry matter of second leaves increased about 1·3-fold during cold treatment in spring cultivars and about 1·5–1·6-fold in winter types, relative to control plants at 25°C. Percentage dry matter of third leaves also increased about 1·3-fold in spring cultivars, but in winter cultivars the increase was about 2-fold. Thus, in cold-treated winter cultivars, dry matter content of third leaves was 25 % higher than that of second leaves (Table 4). Intermediate responses were observed in cultivars Buck Yapeyú and ProINTA Oasis both for SLA-DW and % DM (data not shown).

Leaf thickness

Leaf thickness of third leaves developed at 5°C was about 1·5-fold higher than those developed at 25°C, regardless of the cultivar analysed. Although no significant differences in leaf thickness were observed between cultivar types, winter cultivars appear to have thicker epidermal cell walls than spring cultivars (Fig. 2).

Stomatal frequency

Stomatal frequency of third leaves decreased at 5°C in both leaf surfaces, relative to controls at 25°C. The adaxial surface showed 20 % fewer stomata, regardless of the cultivar type. However, significant differences were observed between cultivars for the abaxial surface: stomatal frequency was 20–25 % lower in spring cultivars, but 50 % lower in winter cultivars (Fig. 3).

On the abaxial surface, the length of epidermal cells between stomata increased 1·5-fold in spring wheats and 1·9-fold in winter wheats at 5°C. No significant differences were found in length or width of stomata (Table 5).

Soluble sugar accumulation and osmotic potential

The kinetics of soluble sugar accumulation in roots, including reducing sugar and sucrose plus fructan, is shown in Fig. 4. Differences in the maximum accumulation level were observed between spring and winter cultivars. In both cultivar types, sugar concentration increased 3-fold during the first week at 5°C. After this, concentrations in spring

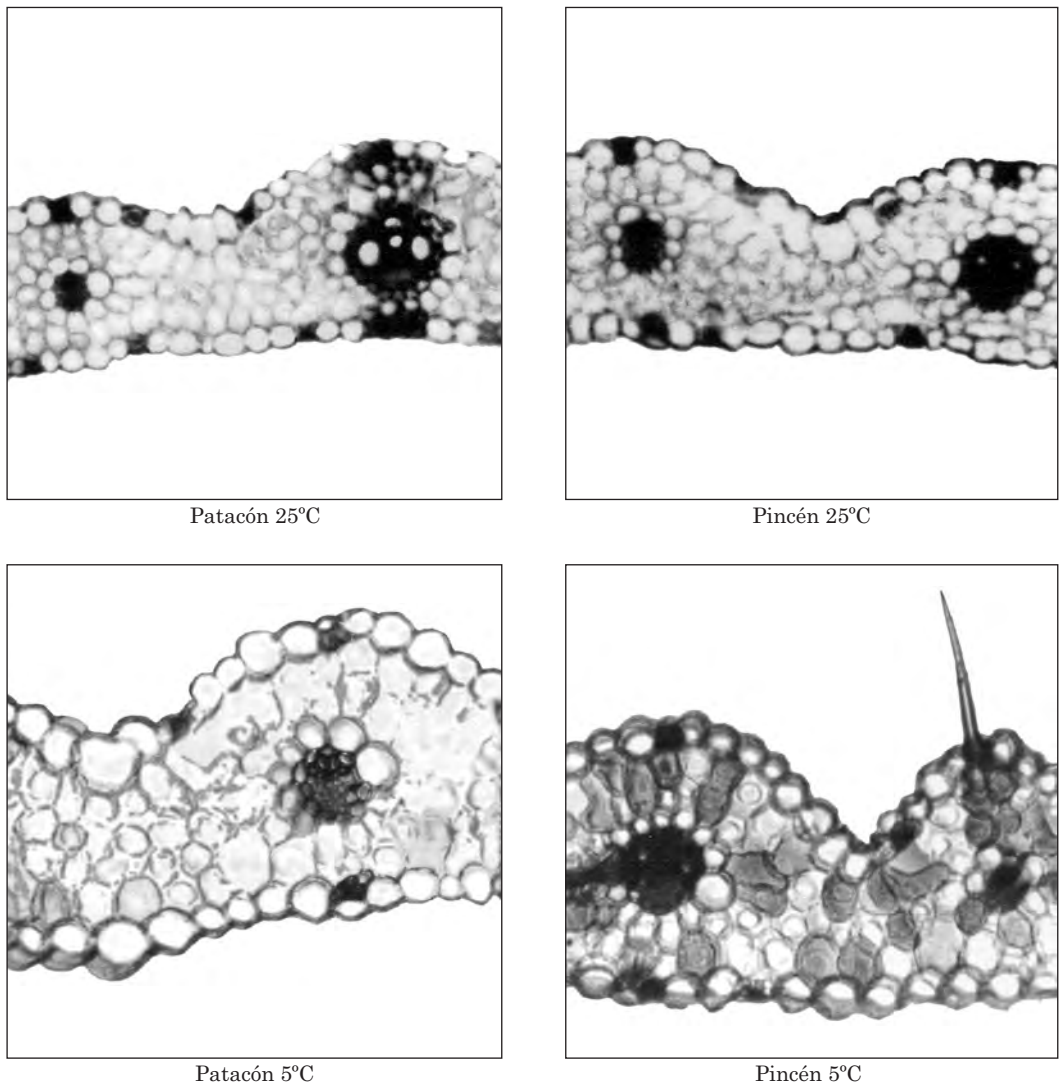

FIG. 2. Leaf cross-sections of the third leaves of plants grown at 25 or 5°C. ×100.

TABLE 5. *Length of epidermal cells between stomata (ECS), stomatal length (SL) and stomatal width (SW) of third leaves expanded at 25 or 5°C*

	ECS (μm)		SL (μm)		SW (μm)	
Cultivar	25°C	5°C	25°C	5°C	25°C	5°C
Patacón	176·4 ± 11·9[a]	266·3 ± 17·6[a]	71·2 ± 2·8[a]	67·9 ± 4·6[a]	31·2 ± 1·9[a]	34·6 ± 1·4[a]
San Agustín	173·4 ± 16·7[a]	254·2 ± 22·9[a]	73·6 ± 1·9[a]	74·8 ± 2·9[a]	31·9 ± 1·2[a]	33·4 ± 2·1[a]
Puntal	170·0 ± 10·1[a]	306·1 ± 15·0[b]	69·1 ± 2·4[a]	67·0 ± 2·1[a]	30·6 ± 0·8[a]	30·6 ± 1·3[a]
Pincén	163·9 ± 14·2[a]	316·0 ± 31·5[b]	68·5 ± 3·5[a]	68·9 ± 3·1[a]	32·4 ± 1·0[a]	34·1 ± 0·7[a]

Means within the same column with different superscripts are significantly different at $P < 0{\cdot}05$ (Tukey's test).

cultivars plateaued while winter cultivars continued to accumulate sugars over 4 weeks, reaching concentrations 5-times higher than plants grown at 25°C (Fig. 4).

An increase in osmolality occurred in parallel with sugar accumulation. Osmolality of root sap from spring wheats reached its maximum value after 2 weeks at low temperature, while it continued to increase for several more weeks in winter wheats (Fig. 5).

Shoot responses were similar to those observed in roots: at the third expanded leaf stage sugar content of cold-treated plants was 3- to 4-fold higher in spring wheats and 9-fold higher in winter ones, than in control plants.

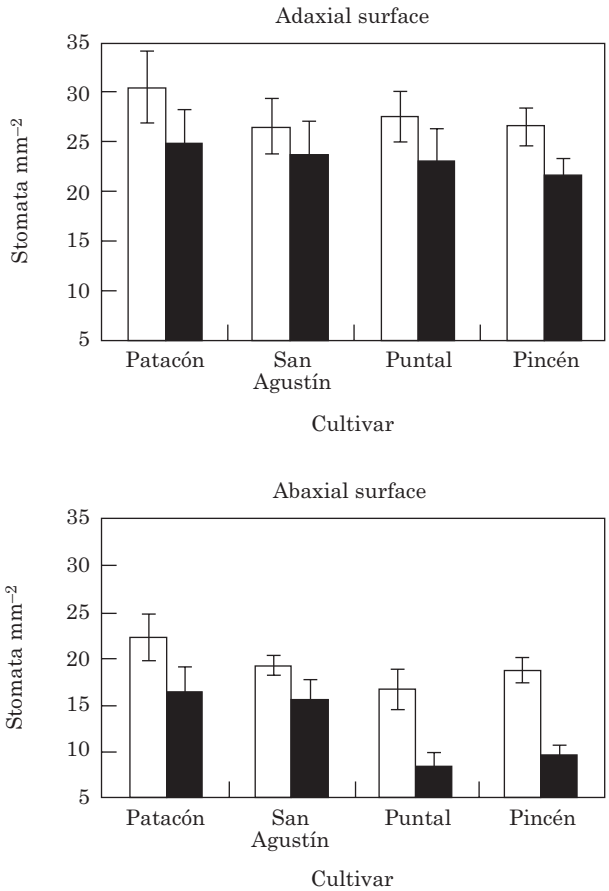

FIG. 3. Stomatal frequency of third leaves of plants grown at 25 (□) or 5°C (■). Measurements were made at 15 different sectors of each leaf surface with a plaid of 1 mm^2.

Osmotic potential of shoots was lower than that of roots, irrespective of growth temperature and cultivar, and showed the same trends as for roots when comparing cultivars (Table 6). Intermediate responses were observed in cultivars Buck Yapeyú and ProINTA Oasis, both for sugar accumulation and osmotic potential (data not shown).

The increase in sugar content at the last harvest in cold-treated plants accounted for only 20–40 % of the decrease in osmotic potential of roots, or 50–75 % in shoots. Thus, other solutes must also have accumulated during the cold period.

Relative water content (RWC)

Both at 25 and 5°C the relative water content of the third leaf was about 96 %, and there were no significant differences among cultivars (Table 7). RWC of roots, determined in a similar manner as for leaves, was close to 100 % in every case (data not shown).

Relationship between absorbing and transpiring organs

When the relationship between absorbing and transpiring organs was expressed in terms of fresh weight, spring cultivars had a larger root system than winter ones, at comparable shoot sizes (Fig. 6A). Nevertheless, when this relationship was expressed in terms of root surface areas relative to the total number of stomata per plant, all cultivars seemed to follow a unique relationship, with a higher slope at 5°C than at 25°C (Fig. 6B).

DISCUSSION

In a previous paper, the relationship between root and shoot growth inhibition at low temperature and carbohydrate accumulation was compared in several wheat cultivars (Equiza *et al.*, 1997). In that work, as well as in the present study, growth was analysed on a fresh weight basis. Although dry weight accumulation is normally used for growth measurement, at low temperature fresh weight accumulation reflects more accurately the organ growth in terms of size. This is because during cold acclimation an important fraction of the dry weight increment corresponds to transient deposition of many solutes, including non-structural carbohydrates, proteins, lipids, amino acids, etc (Levitt, 1980), while cell water content is not affected (Tanino *et al.*, 1990; Tognetti and Pontis, 1992). Thus, dry weight accumulation does not describe adequately the growth pattern defined as an irreversible increment of mass and size (Salisbury and Ross, 1992) and its use, in these cases, should be avoided.

Although all cultivars showed similar reductions in shoot relative growth rate at low temperature, root relative growth rate was reduced significantly more in winter cultivars than in spring ones. Consequently, R:S ratio increase was significantly lower in winter cultivars (Fig. 1). To analyse the differences in root and shoot partitioning in an ecophysiological sense, R:S ratios were also expressed as a function of root and leaf areas, as recommended by

TABLE 6. *Total soluble sugars and osmotic potential of shoots from wheat cultivars cultivated at 25 or 5°C until the third leaf stage*

	Total soluble sugars (μmol g^{-1} f.wt)		Osmotic potential (MPa)	
Cultivar	25°C	5°C	25°C	5°C
Patacón	27·3 ± 1·6^a	81·0 ± 8·2^a	−1·17 ± −0·01^a	−1·34 ± −0·02^b
San Agustín	27·7 ± 5·7^a	110·7 ± 11·2^b	−1·19 ± −0·02^a	−1·38 ± −0·03^b
Puntal	28·4 ± 5·1^a	269·4 ± 31·2^c	−1·20 ± −0·02^a	−2·16 ± −0·01^a
Pincén	30·5 ± 2·8^a	300·7 ± 33·1^c	−1·16 ± −0·03^a	−1·97 ± −0·02^a

Means within the same column with different superscripts are significantly different at $P < 0{\cdot}05$ (Tukey's test).

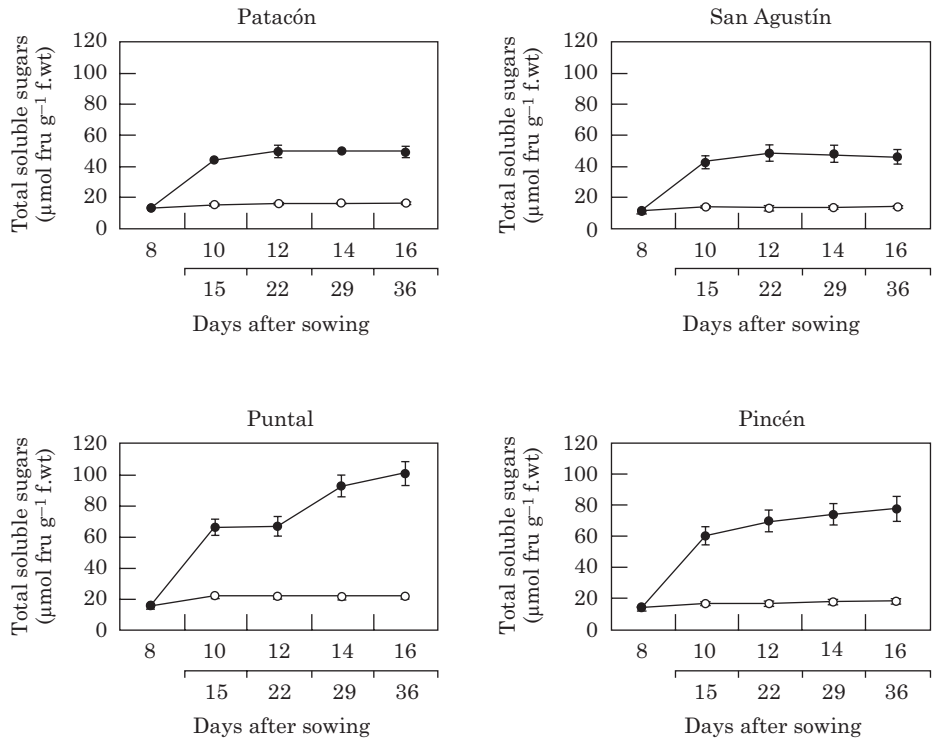

FIG. 4. Total soluble sugar (reducing sugar and fructan, including sucrose) contents of roots from plants cultivated at 25°C (○, days 8–16) or at 5°C (●, days 8–36). Each point is the mean of three replicate experiments.

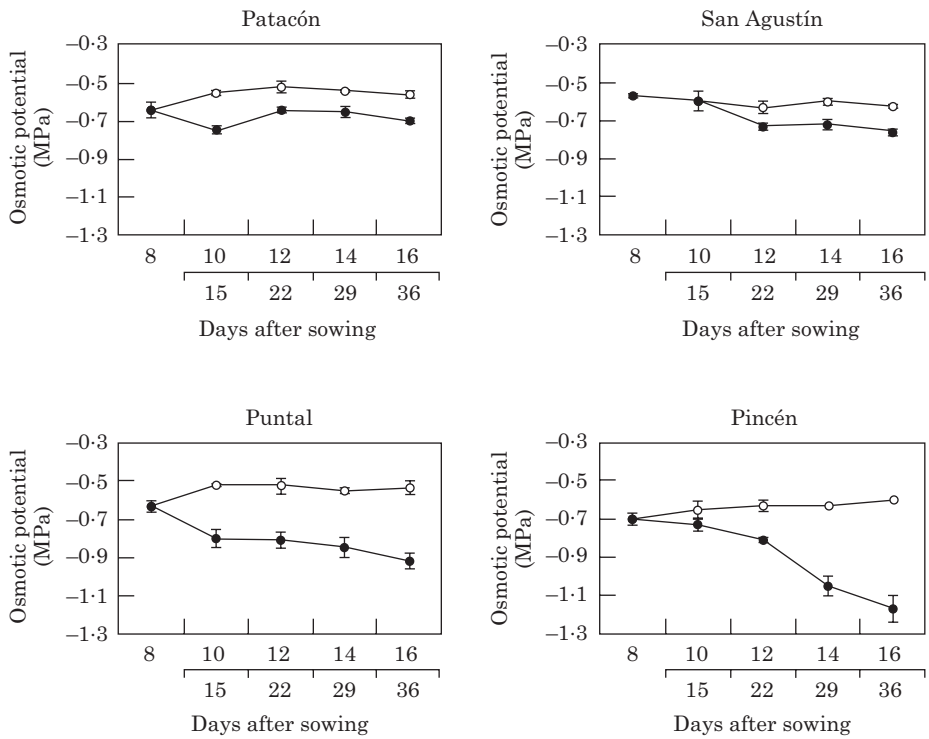

FIG. 5. Osmotic potential of roots from plants cultivated at 25°C (○, days 8–16) or at 5°C (●, days 8–36). Each point is the mean of three replicate experiments.

TABLE 7. *Relative water content (RWC) of third leaves of different wheat cultivars grown at 25 or 5°C*

Cultivar	RWC (%)	
	25°C	5°C
Patacón	94·5 ± 1·8	96·1 ± 1·9
San Agustín	95·8 ± 1·3	97·3 ± 0·9
Puntal	94·1 ± 1·5	96·5 ± 2·2
Pincén	95·4 ± 2·4	97·4 ± 1·2

Values are means of ten plants from three replicates ± s.e.

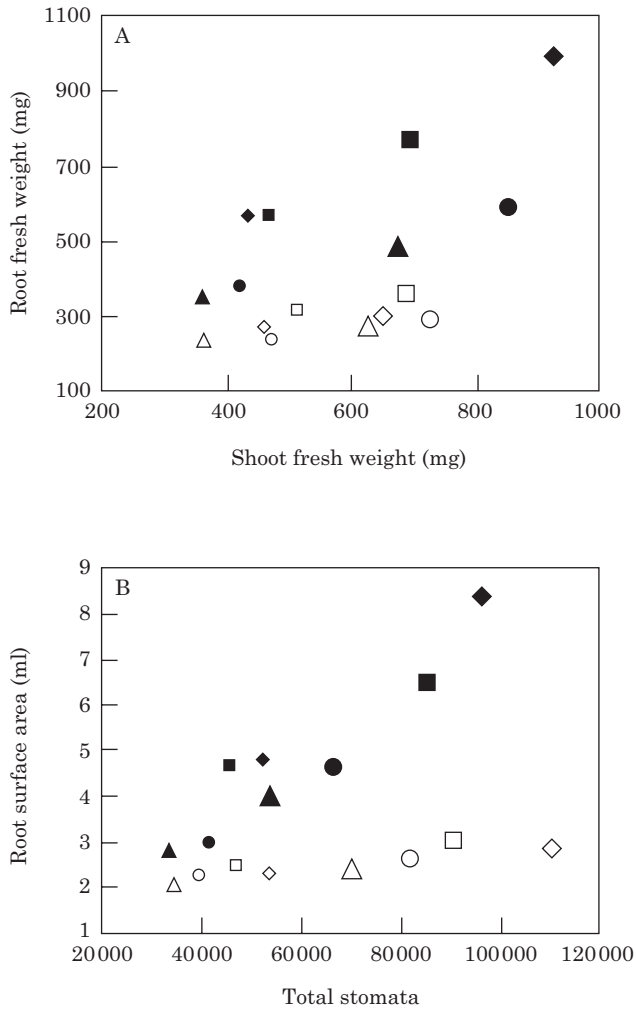

FIG. 6. Relationship between absorbing and transpiring organs at contrasting temperatures, expressed as either root fresh weight *vs.* shoot fresh weight (A) or as root surface area *vs.* total stomata per plant (B). Data were obtained from plants cultivated at 25°C (open symbols) or 5°C (closed symbols) and harvested at the second expanded leaf stage (smaller symbols) and third expanded leaf stage (larger symbols). Different symbols correspond to different cultivars: Patacón (◆); San Agustín (■); Puntal (●); Pincén (▲). Each point is the mean of three replicate experiments.

Brouwer (1983) and van Noorwijk and de Willigen (1987). The titration method was selected to assess root surface areas because this method in widely used in cereals to evaluate root surface areas in relation to root absorptive capacity (Kapulnik *et al.*, 1985; Jacoud *et al.*, 1999). Although it only gives relative values of the root surface area, this method may provide a better indication of the real absorptive area than other methods (Böhm, 1979). The results reported here indicate that, at a given developmental stage, root surface area of cold-treated plants was greater and this increment was about 60 % higher in spring cultivars than in winter ones. No significant changes in total leaf area, relative to control plants, were observed in either cultivar type. Thus, the differences between spring and winter cultivars in root *vs.* shoot growth at low temperature were maintained when expressed either in terms of organ size or the size of the organ interface with the environment.

Increased R:S ratios have been interpreted as a response to overcome restrictions to water absorption which, in the case of low temperatures, might be related to increased water viscosity and increased root resistance to water transport. Since the increase in R:S ratio in response to low temperatures was smaller in winter than in spring cultivars, we investigated whether winter cultivars developed other morphological, anatomical and physiological changes at low temperatures which could compensate for the relatively smaller root systems.

There were remarkable morphological and anatomical differences between winter and spring cultivars in terms of SLA and stomatal frequency at 5°C. Reductions in SLA-FW were observed in the third leaves of all cultivars, indicating a higher volume per unit leaf area. This was also observed in cross-sections of these leaves, which increased in thickness at low temperature in both spring and winter cultivars. Although no differences were found between cultivars in SLA-FW, there were differences between them in SLA-DW. A lower SLA-DW in cold-treated winter cultivars could be ascribed either to accumulation of solutes and/or to increased cell wall thickness (Levitt, 1980; Lu and Neumann, 1999). It is interesting to note that in spring cultivars the percentage DM values of second leaves, whose expansion began at 25°C and was completed at 5°C, were similar to those of third leaves, which emerged and expanded completely at 5°C. However, percentage DM values of third leaves from winter cultivars were about 25 % higher than those of second leaves. Since it has previously been shown that, after transferring plants from 25 to 5°C, soluble sugars accumulate both in leaves already developed at the time of transfer, as well as in leaves which are unfolded at low temperature (Tognetti *et al.*, 1990), differences in percentage DM between leaves 2 and 3 in winter cultivars might be due to an increase in structural components, like cell walls. Cross-sections of winter cultivar leaves developed at 5°C showed thicker epidermal cell walls. However, further work is required to investigate structural and non-structural carbohydrate partitioning as well as leaf anatomy in contrasting cultivars at low temperature.

Stomatal frequency was affected by the cold treatment. Although the number of stomata was reduced on both leaf surfaces, greater differences were observed on the abaxial side, in which stomatal frequency was differentially reduced among cultivars. The increased length of epidermal cells

TABLE 8. *Summary of morphological, anatomical and physiological characteristics in wheat plants developed at low temperatures*

	Response (in relation to control temperature)	
	Spring cvs.	Winter cvs.
Root : shoot ratio (f.wt)	+	+
Root : shoot ratio (area)	+	+
Leaf thickness	+	+
Epidermal cell wall thickness	+	+
Stomatal frequency (adaxial surface)	−	−
Stomatal frequency (abaxial surface)	−	−
Total stomata (stomatal frequency × leaf area)	−	−
Osmotic potential	−	−

+, increased value; −, decreased value. Size of symbols indicates magnitude of response.

between stomata could account for differences in stomatal frequency. It should be noted that in winter cultivars blades are folded along the central midrib at a closer angle than in spring ones, both at 25 and 5°C, which results in the abaxial side being more exposed to the environment. A lower stomatal frequency on this exposed abaxial surface may therefore contribute to a significant reduction in water loss.

The contribution of sugar accumulation to a possible osmotic adjustment was also evaluated. The osmotic potential decreased in parallel with sugar accumulation and this decrease was steeper in winter cultivars. Since RWC remained unaffected by temperature, and close to saturation in all cases, lowered osmotic potential of winter wheats indicates a higher degree of osmoregulation both in root and shoots.

The results presented here indicate a differential morpho-anatomical and physiological plasticity between winter and spring cultivars at low temperatures, but it is still unclear what, if any, ecophysiological significance these changes may have. By comparing all these modifications in both cultivar types it appears that winter cultivars develop smaller root systems than spring ones, but also develop leaves which are more xeromorphic (Table 8). Moreover, when root surface areas of cold-treated plants were plotted against the total number of stomata per plant, all cultivars seemed to follow a unique relationship, with a higher slope at 5°C than at 25°C (Fig. 6B). Thus, an apparent functional equilibrium between absorbing and transpiring areas seems to be established at each temperature, irrespective of cultivar type. Winter cultivars develop smaller root systems, but this is counterbalanced by a lower transpiring area. In accordance with this, preliminary observations indicate that water loss by both stomatal and cuticular transpiration in detached third leaves from plants developed at 5°C is lower than in leaves from control plants, and this effect is more pronounced in winter than in spring cultivars (Equiza, Orioli and Tognetti, unpubl. res.).

Some anatomical and morphological changes, such as increased leaf, epidermis and cell wall thickness, lower leaf and specific leaf areas and altered stomatal frequency, have been suggested to have an adaptive value in relation to increased freezing tolerance (Fowler *et al.*, 1981), but no satisfactory explanations of the mechanisms involved have been proposed to date. Huner (1985) strongly supported the idea that some of the changes caused by low temperatures might be related to the actual plant functioning at these temperatures without consequences on freezing tolerance. Furthermore, it has been shown that the correlation between changes in plant morphology at low temperatures and freezing tolerance is coincidental and should not be used as a reliable selection criterion for winter hardiness (Gray *et al.*, 1997). Although these authors favour the hypothesis that plant morphology may be related to an interaction between light intensity and temperature, we emphasize that the anatomical and morphological changes described in the present work are more likely to be associated with water economy of these plants during growth at low temperature.

This hypothesis is also supported by several pieces of evidence from the literature on cold-acclimation. In 1973, Anderson and McNaughton analysed 17 populations of 12 vascular plant species and found that wild ecotypes adapted to colder regions tolerated the water stress induced by decreasing root temperature from 20 to 3°C better than ecotypes from warmer regions. It has also been shown that in a non-freezing resistant plant—maize—xeromorphic characteristics like a thick epidermal layer, deep sunken guard cells and low stomatal frequency are induced by reducing the temperature from 30 to 14°C (Stamp *et al.*, 1984). Furthermore, the changes described in the present work should favour the maintenance of high water contents, while it is known that freezing resistance is enhanced by water stress (Cloutier and Siminovitch, 1982). Recent work by Stefanowska *et al.* (1999) with cold-acclimated rape also favours the link between morphological changes and control of water loss at low temperatures.

In studies conducted in controlled environmental conditions in which cool temperate species were subjected to both low root and shoot temperature, no water deficits were observed (Woodward and Friend, 1988; Tognetti and Pontis, 1992). This is also the case in the present work. However, water stress can be induced by lowering root zone temperature while maintaining shoots in a warm environment (Fennel and Markhart, 1990). These temperature differentials may also occur in natural environments. In temperate regions, during winter and early spring, root zone temperatures display relatively small diurnal fluctuations and remain generally low while air temperatures may fluctuate more widely. Thus, shoots may frequently be exposed to higher temperatures than roots (McMichael and Burke, 1996). It has been shown that due to low soil temperatures and high irradiance, the temperature of leaves may exceed that of roots by 20°C (Körner and Larcher, 1988). Under these conditions—which are not uncommon—water supply by roots might fail to fulfil the atmospheric demand (Kramer, 1983). Therefore, the morpho-anatomical and physiological responses reported in this work might be regarded as adaptations leading to

avoidance of water deficits during periods in which soils remain cold while air and leaf temperatures fluctuate widely.

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