Effects of soil resistance to root penetration on leaf expansion in wheat.

by

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ERRATA

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p. IV (ABSTRACT): second paragraph, sentence before last: In leaf 5, cell size, *number of files and number of cells per file were all reduced* at high R_s.

p. 10, line 1: units for r_w should read : g g^{-1} d^{-1}.

p. 59, third paragraph, line 7: after "the number of cell files" INSERT: "*and the number of files per cell*"
were significantly reduced.

p. 78. Table I, first column: units for E should be: mm d^{-1}.

p. 82, second paragraph, line 3: after "cellochron age", INSERT: instead of as a function of spatial location (compare left and middle panels in Fig. 2).

p. 105, verso: Legend of Fig 8 should read: b) average cell width in abaxial layer and c) number of cells in that layer, ...

Statement

Three of the chapters of this thesis are being submitted for publication in scientific journals:

Chapter 2:	Submitted to:	Annals of Botany.
	Title:	Effects of soil resistance to root penetration on the anatomy of mature leaves in wheat. Composition, number and size of epidermal cells.
	Authors:	G.T.S. Beemster and J. Masle.
Chapter 3:	Submitted to:	Plant Physiology.
	Title:	Effects of soil resistance to root penetration on leaf expansion in wheat (*Triticum aestivum* L): Kinematic analysis of cell partitioning and expansion.
	Authors:	G.T.S. Beemster, J. Masle, R.E. Williamson and G.D. Farquhar.
Chapter 4:	Submitted to:	Annals of Botany.
	Title:	The role of apical growth around the time of leaf initiation in determining leaf width at maturity in wheat plants with impeded roots.
	Authors:	G.T.S. Beemster and J. Masle.

Fruitful discussions with, and comments of the co-authors of the above papers have been thankfully incorporated in this work.

With this exception, the work reported in this thesis is my own.

G.T.S. Beemster.

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Abstract

Typically, increasing soil resistance to root penetration (R_s) results in lower rates of leaf appearance, the formation of fewer tillers, slower leaf expansion and reduced sizes of mature leaves in seedlings of *Triticum eastivum* L. cv. Egret. The cellular basis of these observations at the whole plant scale was investigated in this work.

The reduced sizes of mature leaf blades were associated with smaller length and width of epidermal cells, a decrease in the number of cell files constituting the blade and a shift in the relative proportions of epidermal cell types. Marked differences in the magnitude of the effect of R_s on these cellular characteristics were observed between leaves. In leaf 1 the number of cell files constituting the leaf was unaffected by R_s; the reduced leaf width was mainly due to the cells being narrower. In leaf 3 the number of cell files per blade was reduced by high R_s; the reduction of cell width was much smaller and often not significant. In leaf 5, both cell size and number of files were reduced at high R_s. In all leaves the effect of R_s on the number of cells within a file was relatively small, indicating that the reduction in leaf length was largely due to the cells being shorter.

Slower leaf elongation rates were associated with a reduction of relative rates of cell expansion and partitioning throughout the growth zone in leaf 1. In leaves 3 and 5 slower elongation rates were associated with a reduction of the number of dividing cells in each file of the intercalary meristem and with a smaller length of cells entering the elongation only zone. The smaller length of cells exiting the division zone was associated with increased rates of cell partitioning, while the relative rates of cell elongation were unaffected.

The rate of leaf primordia initiation at the apical meristem was reduced by high R_s and the primordia grew slower after their initiation. When compared at the same plastochron age, most developmental parameters in the apex were not significantly affected by R_s. An exception to this was the circumference of leaf primordia nodes which, at a given plastochron age, was smaller at high than low R_s.

Differences between leaves in the cellular basis of the reduced leaf expansion at high R_s are discussed in relation to the timing of the stress with respect to the leaf developmental stage. Spatial and temporal patterns of cell division and cell expansion throughout the development of the leaf are discussed in relation to the realisation of characteristics of the mature blade.

List of symbols:

A and A'	CO_2 assimilation rate and net assimilation rate over 24 h period (mol C m^{-2} s^{-1})
a	Leaf area (m^2)
c	cellochron *i.e.* time interval during which a new cell is added to a cell file in the elongation only zone (h)
E	leaf elongation rate (m s^{-1})
F	number of cells passing a given point of the elongation only zone per unit of time ("flux", cells h^{-1})
HI	Haun index
h_i, h_a	height of a leaf primordium measured from its base to its tip and of the apex relative to the base of leaf 5 (µm)
L and *W*	Leaf length and width, respectively (mm)
$l(x)$ and $l^*(x)$	local length of sister cells and elements, respectively (mm)
l_i	length of the emerged part of the blade (mm)
l_f and l^*_f	length of mature sister cells and elements, respectively (µm)
L_{sd}, L_{ad}, L_{el}	length of the zone of symmetrical division, asymmetrical division, and elongation only , respectively (mm)
$n(x)$	number of cells along a file between location x_{sd} and a further location x
N_{sd}, N_{ad}, N_{el}	number of sister cells along a file in the zone of symmetrical division, asymmetrical division, and elongation only, respectively
PI	plastochron index
$p_{sd}(x)$ and $p_{ad}(x)$	local relative rates of symmetrical and asymmetrical partitioning, respectively (cells $cell^{-1}$ h^{-1})
$\phi_{p,i}$, $\phi_{p,x}$	proportion of fresh transverse walls within an interval *i*, of the division zone, and between the base of the leaf and a location *x*, respectively
ϕ_{tr}	proportion of sister cells associated with a trichome in the zone of asymmetrical division
ρ and ρ_l	total cell density and density per cell type (cells m^{-2})
r_l and r_w	relative rate of leaf area expansion (m^2 m^{-2} d^{-1}) and dry-weight accumulation, respectively (g g^{-1} d^{-1})
r_{max}	maximum local relative cell elongation rate in the elongation only zone (µm µm^{-1} h^{-1})
$r_{sd}(x)$, $r_{el}(x)$	local relative cell elongation rates in the zone of symmetrical division and in the elongation only zone, respectively (µm µm^{-1} h^{-1})
σ	ratio of total plant mass to leaf area (g m^{-2})
σ_l	ratio of leaf blades mass to area (g m^{-2})
$\overline{t_c}$	average cell cycling time in the zone of symmetrical division (h)
$t(x)$	time taken for a cell to be displaced from x_{sd} to a particular further location x
x	location along the growth zone
x_{sd}, x_{ad}, x_{el}	location from the base of the leaf (x_0) of the distal end of the zone of symmetrical division, asymmetrical division, and elongation only (mm) respectively
R_s	soil resistance to root penetration (MPa)

$\rho(x)$	cell density at location x (cells m^{-1})
$v(x)$	local *v*elocity of displacement along a cell file (m h^{-1})

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Introduction.

The effects of unfavourable root environments on plant growth.

It is widely recognised that soil conditions during crop growth are of critical importance for achieving maximal yields. It is for this reason that often costly investments in soil treatments such as mechanical cultivation, fertilisation, irrigation and sometimes sterilisation are an integrated part of modern agricultural practice.

In natural ecosystems, the composition of plant species growing under otherwise similar climatic conditions, varies greatly depending on soil characteristics. This is due to differences between species in their capacity to adapt to unfavourable root environments.

What are the characteristics of the root environment that determine its suitability for growth?

Soils are usually not homogenous throughout the root environment; large spatial variation in soil physical and biochemical properties are more the rule than the exception. A great number of characteristics of physical, chemical and biological nature determine the suitability of soils for supporting plant growth.

Physical characteristics.

Soil water potential.

Transpirational water losses of crops can be as high as 8 mm/day for irrigated sorghum and corn crops (Unger, Eck and Musick 1981). Whether the resultant reduction of water content of a soil becomes limiting to plant growth is determined by soil water potential (force required to extract water from the soil), which is determined by soil texture (sand, silt, clay and organic matter content), bulk density and water content. The water potential at which plants start to wilt depends on evaporative demand (Denmead and Shaw 1962), but transpiration rates are already affected well above the wilting point, indicating that mild water stress already occurs at higher water potentials (Unger, Eck and Musick 1981).

Soil mechanical resistance to root penetration (R_s).

The resistance encountered by roots when growing into a particular soil is higher if the soil is densely packed or has a low soil water content (Barley, Farrell and Greacen 1965; Taylor and Ratliff 1969). Over a wide range of combinations of soil density and soil water content, the rate of both root growth (Barley, Farrell and Greacen 1965; Taylor and Ratliff 1969; Goss 1977); shoot growth (Masle and Passioura 1987; Passioura and Gardner 1990) and yield of crops (see references in Bowen (1981)) are negatively affected by soils with a high resistance to root penetration (R_s). A measure for assessing the resistance encountered by roots can be obtained using a soil penetrometer, a device that measures the force required to drive a cone of known dimensions into the soil. The values obtained by this method are proportional to, but overestimations of the actual the resistance encountered by the roots (Bengough and Mullins 1990). The reduction of both root and shoot growth is over a wide range of combinations of soil densities and water potentials directly proportional to R_s, and occurs before limitations of water, nutrient or carbohydrates can be detected (Masle and Passioura 1987; Passioura 1988), indicating that soil strength itself is probably directly sensed by the roots.

Soil temperature.

In temperate and colder climates, temperature plays a major role in determining the geographical distribution of plant species (Langridge and McWilliam 1995). Soil temperature affects most root functions (growth, water and nutrient uptake) and thereby plant growth (Voorhees 1981).

Soil aeration.

Respiration of roots and soil (micro) organisms requires a constant diffusion of O_2 into and CO_2 out of the soil. If the soil were sealed off at the surface, oxygen would be depleted within a few days (Cannell and Jackson 1981). Both root and shoot growth are sensitive to O_2 concentrations around the roots (Grable and Siemer 1968; Cannell and Jackson 1981; Neuman and Smith 1991). Diffusibility of a soil is determined by the fraction of air filled porosity and the thickness of the water film surrounding the roots (Grable and Siemer 1968)

Chemical characteristics.

Mineral availability.

A complex system of interrelated equilibria between fixed and ionically bound pools of ions and their concentration in the soil solution determines availability for uptake by the root system (Sumner and Boswell 1981).

Salinity.

Excessive concentrations of soluble salts accumulate in the root environment under conditions when evapotranspiration exceeds irrigation and precipitation. This can result in reduced crop productivity from osmotic stress (total soluble salt concentration (Slatyer 1967)), from toxicity or nutrient imbalances when specific solutes are excessive, and from deterioration of the soil structure in case of excess sodium (Hoffman 1981).

pH.

The pH is probably the most commonly measured soil chemical characteristic in agricultural practice. Acid soils are believed to be restricting plant growth, due to direct toxic effects of the pH to the roots and through its effects on the levels of certain minerals in the soil solution, causing either toxic levels (Al or Mn), or growth limiting levels of other minerals (Ca, Mg and Mo) to occur (Gerdemann 1974; Moore 1974).

Biological characteristics

Micro-organisms in the rhizosphere (bacteria, actinomycetes, fungi, nematodes and viruses) could roughly be subdivided into pathogens, symbionts and organisms that have no direct interaction with plants.

Pathogens.

A wide range of pathogenic micro-organisms has been described, some of which cause only minor stress symptoms, while others result in severe growth reductions or plant death. Many pathogenic micro-organisms are specific to a limited host range, but others affect a much greater variety of plant species.

Symbionts.

Some symbiontic micro-organisms are beneficial for the plant species they form a symbiosis with, usually by improving nutrient availability for the plant. Two examples in this class are mycorrhizae, which generally increase the nutrient uptake capacity of the roots (Gerdemann 1974) and *Rhizobium* bacteria that form nodules on most leguminosae roots, enabling them to reduce atmospheric N_2 into organic nitrogen compounds (Werner 1992).

Interactions/commonalities in the way various factors in soil environment affect plant growth.

The above illustrates the wide range of factors affecting the suitability of a soil for plant growth. However, many of those characteristics affect plant growth indirectly, through interactions with other factors. We already mentioned this aspect for pH, mycorrhizae and rhizobium bacteria, which affect plant growth mainly through effects on nutrient availability, and for salinity, which affects plant growth primarily by reducing the water potential of the soil solution.

Soil characteristics that result in reduced root volume, such as high R_s, lack of aeration and root pathogens, reduce the amount of potentially available water and nutrients. Under conditions where water and nutrients become limiting, plants with reduced root systems are likely to be affected sooner and more severely than plants with a more prolific root system.

Despite the great number of soil characteristics that affect plant growth, there is only a limited number of physiological reasons why they do so. These are related to effects on the primary functions of the root system in relation to whole plant growth and development:

1. Restricted uptake of nutrients can result in one or more nutrients becoming limiting for growth. Which aspect of plant growth is affected, depends greatly on the physiological function in the plant of the particular ion(s) whose uptake is limiting. Excellent books have been written on symptoms resulting from specific mineral deficiencies and estimates of critical levels of many ions in plant tissue have been established (see Bould, Hewitt and Needham (1983)). Nutrient availability undoubtedly is an aspect of the root environment that is of major importance in

relation to plant growth and development. However, the physiological basis of the effects on plant growth and development are very specific for each individual ion and the study of these is an area of research that falls outside the scope of this thesis.

2. Restricted water uptake. Water constitutes more than 80% of most plant tissues (Slatyer 1967), and is therefore a direct requirement for plant growth. However, most of the water taken up by the roots is transpired from the leaves, thus creating a constant water flow from the roots to the shoots by which minerals and metabolic components are transported. Restricted water availability primarily results in stomatal closure, which inhibits photosynthesis and transpiration. More severe water stress can reduce the water supply to growing regions of the plant thereby restricting the driving force for growth. Finally low water potentials in general may affect the activity of enzymatic processes critical for functioning of the metabolism of individual cells and the plant as a whole.
3. Soil resistance to root penetration. Soil resistance to root penetration reduces stomatal conductance and plant growth rates. It has been shown that in the short term this effect is not mediated by plant water relations, mineral uptake or carbohydrate status of the plant (Blackman and Davies 1985; Gollan, Passioura and Munns 1986; Masle and Passioura 1987; Saab and Sharp 1989; Masle 1990; Passioura and Gardner 1990; Gollan, Shurr and Schulze 1992).

Agronomical and ecological importance.

The increasing mass of agricultural tractors and equipment and their frequent usage under situations that are too wet, means that soil compaction, destruction of soil structure and increases in mechanical impedance are becoming an ever increasing problem in agricultural practice (Bowen 1981). Data from (Carter *et. al.* 1965; Carter and Tavernetti 1968) cited by (Bowen 1981) show that increasing soil strength in the range of 0 to 2.5 MPa penetrometer resistance reduced cotton yield from 3600 to 1200 kg/ha.

Wheat grain yield in dryland cropping in Australia is greatly determined by the amount of soil water available for the crop throughout the growing season (Hamblin and Kyneur 1993). Many years of low rainfall, therefore, have brought many Australian farmers to the brink of bankruptcy.

In the western United States, crop production is limited by salinity on about a quarter of the 20 million ha of irrigated land, and about half of that irrigated acreage was actually considered to be threatened by salinity (Wadleigh (1968), cited by Hoffman (1981)). Moreover, worldwide survey of 24 countries showed that over 50 million of a total of 756 million ha cultivated land and 91 million ha of irrigated land is affected by salinity (Salhevet and Kamburov (1976), cited by Hoffman (1981)).

Over 1.5% of the US energy budget is spent on tillage practices, aimed at loosening soil for root penetration, bury the residue from previous crops, provide a suitable environment for seed germination, improve water infiltration, provide aeration and control weeds (Sumner and Boswell 1981).

Acid soils are an enormous problem under circumstances where annual rainfall exceeds evapotranspiration and tens of millions of tons of lime are used yearly in agriculture to correct for low soil pH (Adams 1981). Acid rain is the deposition of atmospheric pollutants of acidic nature, most importantly SO_2, NO_x and HCl, resulting from western industrialisation. It has been implicated as a possible cause of massive damage to forests in Germany and several other European countries, possibly though acidification of the soil (Mason 1992).

These figures are not complete or even comprehensive, but are numbers that illustrate the significance of soil characteristics from an agronomical and ecological perspective.

Topic of the thesis.

In this thesis I am going to investigate the effects of soil resistance to root penetration (R_s) on leaf development and leaf area expansion in wheat.

R_s was chosen as the experimental treatment, because of its wide relevance to natural conditions. In relation to root growth it has been shown that soil strength should be regarded as a property that has a general influence, rather than as a limiting condition in unusual soils (Barley, Farrell and Greacen 1965), and it is likely that a similar statement could be made for shoot growth. It is thought that R_s is one of the soil characteristics

sensed by the roots, of plants growing on drying soils, causing reduced growth rates and stomatal conduction (Passioura 1988; Passioura and Gardner 1990).

I already indicated that R_s is determined for any particular soil by soil density and soil water content. Research into the effects of R_s is experimentally difficult because the soil density and water content change together and it is therefore hard to directly link effects on plant growth of changes in either bulk density and water content *per se* directly to R_s when it is not specifically measured. A difficulty with soil stress as an experimental treatment in general is to obtain steady-state conditions. Especially in field situations it is very hard to obtain reproducible results, because it is virtually impossible to maintain a constant level of stress due to varying soil and environmental conditions.

Earlier work has defined a set of conditions in which these limitations could be largely overcome. Masle and Passioura (1987) and Masle (1990) defined a set of combinations of soil bulk density and water content for which the effects on plant growth are directly associated with R_s, *i.e.* not due to limited oxygen diffusibility or soil water potential (Masle and Passioura 1987; Masle 1990; Passioura and Gardner 1990). Moreover, amounts of nutrients which need to be added to the soil in order for them to be non-limiting for growth under these conditions have been established (Masle, unpublished data).

With the same set of soil conditions, the effects of R_s on whole plant and leaf growth response (Masle and Passioura 1987; Masle and Farquhar 1988; Masle 1990; Masle, Farquhar and Gifford 1990; Masle 1992), photosynthetic characteristics and carbohydrate metabolism (Masle and Farquhar 1988; Masle 1990; Masle, Farquhar and Gifford 1990; Masle 1992) and water relations (Masle and Passioura 1987; Masle 1990; Passioura and Gardner 1990) have already been well documented through earlier work.

This background work was mostly done with wheat, which as a grass is especially suitable for leaf developmental studies. The reasons for this are that, in contrast to dicotyledonous leaves, growth is essentially unidirectional and leaves elongate at an approximately constant rate during the period during which up to 90% of the blade is formed (Ong and Baker 1985). During this phase the processes cell division, expansion and maturation occur in separate zones at the base of the leaf (Figure 1). The spatial

organisation of the growth zone of grass leaves is very similar to the situation found in root tips. As a result of this, experimental methods that were originally developed to investigate cell division and elongation rates in root tips (Erickson and Sax 1956a; Erickson and Sax 1956b; Green 1976) are very suitable for the analysis of growth kinematics in grass leaves (Boffey, Sellden and Leech 1980; Volenec and Nelson 1981; Schnyder, Nelson and Coutts 1987). Many physiological aspects of leaf expansion in general and in response to various environmental stresses have been investigated using this approach.

Environmental conditions.

All experiments reported in this thesis were done under the same conditions, with one of the cultivars (Egret) on which most information had been obtained.

The conditions during the experiment were set as: 18/15 °C day/night temperature, 600 $\mu E.m^{-2}.s^{-1}$ photosynthetically active irradiance, 11 h photo- and thermoperiod and a relative humidity of 80% day and night. These conditions are realistic for wheat and allow reasonably low rates of water loss i.e. minimal diurnal variation in R_s. The 11 h duration of the photo-period encertains a fairly long period of vegetative development. The somewhat high value for the relative humidity prevents excessive evaporation from the soil and transpiration from the leaves, thus enabling maintenance of a more constant level of soil water

Underlying mechanisms.

Decrease root and shoot growth.

Roots growing in a soil with a high mechanical resistance to root penetration (R_s) generally elongate slower (Barley 1962; Barley, Farrell and Greacen 1965; Taylor and Ratliff 1969; Russel and Goss 1974; Atwell 1988; Masle 1992), but have an increased diameter (Goss 1977; Russel and Goss 1974; Wilson and Robards 1977; Atwell 1988; Masle 1992). Branching is often reported to be affected, but conflicting results have been obtained as to whether high R_s increases or decreases the number of lateral roots (Goss 1977; Wilson and Robards 1977; Atwell 1988; Masle 1990; Gordon *et. al.* 1992; Masle

1992). The increased diameter is for the greatest part due to an increase in the amount of cortical tissue, while the stele is relatively unaffected. The increased amount of cortical tissue is mainly associated with increasing cell diameter and sometimes also with an increase of the number of cortical cell files (Goss 1977; Wilson and Robards 1977; Atwell 1988). Reduced root length is associated with a reduction in cell length (Wilson and Robards 1977; Atwell 1988). Root elongation rates of plants grown hydroponically and in vermiculite are also reduced by low water potentials of the applied nutrient solution (Sharp, Silk and Hsiao 1988; Spollen and Sharp 1991; Zhong and Läuchli 1993).

The overall rate of leaf area expansion of the plant is a result of a small number of developmental processes:

1) The rate of leaf initiation and emergence per axis (stem/branch).
2) Rate and duration of expansion of individual leaves, which together determine the mature size of newly formed leaves.
3) Formation of new growth axes (tillers/branches) from axillary meristems.

Leaf appearance rate of wheat plants growing in soils with low soil water potential or high R_s are reduced (Masle and Passioura 1987; Masle 1990; Masle 1992). Leaves elongate more slowly (Masle and Passioura 1987; Saab and Sharp 1989) and although the duration of leaf elongation is increased by high R_s, this does not compensate completely for the reduced elongation rate and mature leaves are smaller in both length and width (Masle and Passioura 1987; Masle 1990; Masle 1992). In this thesis, I will study this reduction in leaf elongation rate and final size of mature leaves in more detail.

Growth analysis.

The basis of differences in plant growth rates can be analysed by decomposing the overall plant growth rate into a number of characteristics relating to photosynthetic rates and parameters quantifying dry matter distribution using the identity:

$$r_w = \frac{A'}{\sigma},$$ (Equation 1; (Masle, Farquhar and Gifford 1990))

with r_w the relative growth rate on a mass basis ($g.m^{-2}.d^{-1}$) A' the net assimilation rate ($g.m^{-2}.d^{-1}$) and σ ($g.m^{-2}$) the ratio of plant mass to leaf area. If A' is determined in terms of a units of carbon, it relates to assimilation rate measured in gas exchange systems A (mol CO_2 $m^{-2}s^{-1}$) according to:

$$A' = lA(1-\phi), \qquad \text{(Equation 2; (Masle, Farquhar and Gifford 1990))}$$

with (l, a fraction of light period per 24h) and ϕ the proportion of carbon fixed in the shoot that is subsequently lost by the shoot at night and by the roots during day and night.

σ can be further decomposed into γ, the root:shoot ratio a measure for dry weight partitioning between roots and shoots, and σ_l, the ratio of leaf dry weight per unit leaf area ($g.m^{-2}$).

$$\sigma = (\gamma + 1)\sigma_l, \qquad \text{(Equation 3; (Masle, Farquhar and Gifford 1990))}$$

Relative growth rate.

The reduction of seedling growth in response to high R_s is especially notable during the first few days after germination (Masle and Passioura 1987; Masle 1990; Masle 1992). Thereafter, differences in relative growth rates, in terms of mass (r_w) and leaf area (r_l) gradually become more similar between plants grown at low and high R_s, sometimes to the extent that the difference is no longer significant (Masle 1990; Masle 1992). This is especially true for r_l, which appears to be less sensitive to high R_s than r_w after about two weeks after sowing (Masle 1990; Masle 1992).

Dry weight partitioning

Root growth is at first more affected by R_s than shoot growth, *i.e.* plants growing at high R_s initially have a lower root to shoot ratio than plants growing at low R_s. However, shortly after germination, shoot growth becomes more reduced by high R_s than root growth (Masle 1990; Masle, Farquhar and Gifford 1990; Masle 1992). Root to shoot ratio remains constant over the first 16 days after sowing at high R_s, whereas at low R_s

this ratio gradually decreases and at about two weeks after sowing it becomes lower than at high R_s (Masle, Farquhar and Gifford 1990). The amount of carbon in the leaf per unit leaf area is higher in plants growing at high R_s (Masle and Farquhar 1988; Masle, Farquhar and Gifford 1990).

Assimilation rate.

Net assimilation rate was increased by 20% over the period between 13 and 20 days after sowing when plants were grown at high R_s (Masle 1992). However, this latter response was not representative for wheat in general, because in most other genotypes assimilation rates were not significantly affected or decreased by high R_s (Masle 1992).

The increased A' for the cultivar Egret is associated with increased photosynthetic rates per unit leaf area during the light period (Masle and Farquhar 1988; Masle, Farquhar and Gifford 1990). This occurs despite lower intercellular CO_2 concentrations (Masle and Farquhar 1988; Masle 1990) that are caused by higher stomatal resistance at high R_s (Masle and Passioura 1987; Masle 1990; Masle, Farquhar and Gifford 1990; Masle 1992). This suggests a greater biochemical photosynthetic capacity per unit leaf area at high R_s. In support of this hypothesis a 25% increase in ribulose biphosphate carboxylase (Rubisco) activity per unit leaf area was found in the leaves of plants growing at high R_s (Masle and Farquhar 1988). Part of the increased photosynthetically bound carbon however is lost, due to a 10 - 12% increase of fraction of fixed carbon that is lost by respiration (Masle, Farquhar and Gifford 1990).

Transpiration and water use efficiency.

As a result of the increased stomatal resistance in leaves of plants growing at high R_s, transpiration rates per unit leaf area are greatly reduced (Masle 1992). Because assimilation rates per unit leaf area during the light period (when most transpiration occurs) are somewhat increased, the amount of water transpired per unit of carbon accumulated, water use efficiency (*WUE*), is greatly increased by high R_s (Masle and Farquhar 1988; Masle 1992).

Cellular level.

Biophysical view.

The physical description of cell expansion put forward by Lockhart (Lockhart 1965a; Lockhart 1965b) plays a central role in our understanding of cell and organ expansion. According to this model the cell wall is a viscous material, which stretches elastically, but resists irreversible deformation unless the tension within it exceeds a certain threshold. Above this threshold, the material stretches irreversibly at a rate that is dependent on the amount of tension exerted on the tissue. In its most general form the Lockhart equation (Equation 1 (Passioura and Fry 1992) describes the relationship between volumetric expansion rate ($d\ln(V)/dt$), water relations of a single cell or an expanding tissue (the water potential outside the cell or in the xylem, Ψ_o, and the osmotic pressure inside the cell or expanding tissue, π), cell wall mechanical properties (extensibility (m) and threshold pressure for irreversible expansion, Y) and hydraulic conductance of cell wall(s) and membrane(s) (K).

$$\frac{d\ln(V)}{dt} = \frac{mK}{m+K}(\Psi_o + \pi - Y) \qquad \text{(Equation 1).}$$

In single-cell experimental models, it has been shown that the hydraulic conductance is not a limiting factor for cell expansion (Zhu and Boyer 1992). This has led many researchers to adopt the simplified version of the Lockhart equation which excludes the role of hydraulic conductance (equation 2).

$$\frac{d\ln(V)}{dt} = m(P - Y) \qquad \text{(Equation 2).}$$

Turgor.

The formulation of the Lockhart equation (especially when formulated as in equation 2) suggests expansion rates to be determined by turgor (assuming m and Y are relatively invariable). However, in experiments in which the turgor pressure of expanding cells or

tissues is manipulated, this only leads to transient changes in the rate of expansion (Passioura and Fry 1992; Zhu and Boyer 1992). Moreover, turgor pressure is virtually constant along the growth zone of expanding maize roots, while local rates of expansion varied up to five-fold in the same locations (Spollen and Sharp 1991; Tomos and Pritchard 1994). The current consensus is that although turgor is required as a driving force, it does not in itself determine the rate of expansion growth except for short term (minutes) transient changes.

Conductivity (K).

In higher plants, the path for water movement into expanding cells/tissue is longer than for isolated cells and the hydraulic resistance in these organisms may be substantial. Indeed, the existence of significant water potential gradients between the xylem and enlarging cells of elongating organs implies significant frictional resistance to water movement from the xylem into the enlarging tissues (Boyer 1993; Nonami and Boyer 1993). Low water potentials in the root medium can result in local inversion of these gradients in elongating soybean stems, effectively starving expanding cells for water. This results in total growth inhibition for about two days, after which the gradient was re-established and growth continued at a lower rate than the non-stressed control (Nonami and Boyer 1989).

Cell wall rheology (m and Y).

Current understanding of cell wall extensibility is based on knowledge of the biochemical composition of the primary cell wall (see eg. Brett and Waldron (1990), Carpita and Gibeaut (1993)). In short, the cell wall consists of great numbers cellulose microfibrils embedded in the so called cell wall matrix. The cellulose microfibrils are extremely long, ca 10 nm wide bundles of 30 to 100 cellulose molecules, with a very high tensile strength, whereby they determine to a great extent the strength of the cell wall as a whole. In elongating cylindrical cells/organs the direction of elongation is thought to be determined by the alignment of the cellulose microfibrils, which functions as a "hoop reinforcement" thus preventing increase in diameter to a much greater extent than expansion in length (Green 1980). The matrix of the cell wall, in which the cellulose microfibrils are embedded, consists of a variety of polysaccharides, proteins and phenolic compounds. Recent models (Passioura and Fry 1992; Cosgrove 1993) suggest a critical

role for the matrix polymers that cross link the network of cellulose microfibrils in cell wall expansion, thereby preventing cell wall expansion. It is thought that certain enzymes (notably XET and expansins) in expanding cell walls cut or loosen these crosslinking polymers, allowing the microfibrils to move apart and thereby the wall to expand (Smith and Fry 1991; McQueen-Mason, Durachko and Cosgrove 1992; Potter and Fry 1994; Taylor *et. al.* 1994). Other enzymes (such as peroxidases) have been implicated with cross-linking of the microfibrils, thereby stiffening the cell wall and reducing cell expansion (MacAdam, Nelson and Sharp 1992; MacAdam, Sharp and Nelson 1992). The effect of ABA on leaf expansion (see below) may be mediated by its effects on the activity of one or more of these enzymes.

Metabolic view.

Because the reduction of shoot growth rates to high R_s were shown to be unrelated to soil water potential, soil aeration and nutrient supply, it was hypothesised that growth of the shoot is primarily reduced in response to a hormonal message induced in the roots at high R_s. Unfortunately no research has been done directly addressing the relationship between R_s and ABA metabolism.

However, it has been shown that the concentration of ABA in the xylem increases in response to both high soil density (Tardieu *et. al.* 1992) and low soil water contents (Zhang and Davies 1990b; Tardieu, Zhang and Davies 1992; Bano *et. al.* 1993). This suggests that ABA could be involved in the hormonal message in response to high R_s. This increased xylem ABA concentration is believed to cause the reduction of stomatal conductance (Zhang and Davies 1990b; Shurr, Gollan and Schulze 1992; Tardieu, Zhang and Davies 1992; Tardieu *et. al.* 1992) and leaf elongation rates in response to a number of root stresses in many species (Quarrie and Jones 1977; Van Volkenburg and Davies 1983; Saab *et. al.* 1990; Zhang and Davies 1990a; Saab, Sharp and Pritchard 1992; Blum and Sinnema 1995). In maize it was shown that ABA accumulation plays a direct role in both the maintenance of primary root elongation and the inhibition of shoot elongation at low water potentials (Saab *et. al.* 1990). It has been shown that ABA reduces the extensibility of expanding cell walls, m (Cleland 1986), which could be the mechanism underlying its effect on leaf expansion.

There is some evidence from work with saline stressed wheat and barley, that the ABA concentration in the xylem sap cannot always account fully for the observed reduction of leaf expansion rate in response to root stress (Munns 1992). There are indications that other hormones such as cytokinins (Bano *et. al.* 1993; Itai and Vaadia 1965; Itai and Vaadia 1971) and ethylene (Apelbaum and Yang 1981; Dunlap and Molina 1993) could also be involved in root signals that mediate growth response and stomatal conductance of the leaves in response to adverse conditions in the rhizosphere. Moreover, the concentration of various minerals in the xylem sap, as well as its pH affect the response to ABA (Amzallag, Lerner and Poljakoff-Mayber 1992; Shurr, Gollan and Schulze 1992; Bernstein, Läuchli and Silk 1993), it has been hypothesised that this is due to effects on compartmentation of ABA in the leaf (Davies and Zhang 1991).

Growth rates of leaves at high R_s become limited by carbohydrate levels in the growing regions once the seed reserves are depleted. However, at similar carbohydrate levels, leaf growth rates are lower at high than at low R_s (Masle, Farquhar and Gifford 1990), implying that the sensitivity of the growing tissues of the leaf to carbohydrate status is reduced by R_s. It is thought that this reduced sensitivity of the leaves to carbohydrate levels in the growing regions is mediated by a chemical signal from the roots, possibly ABA, and that it increases the availability of carbohydrates for root growth (Masle, Farquhar and Gifford 1990).

Commonalities with other soil stresses.

Many commonalities exist in the response of plant growth to salinity, low soil water potentials and high soil mechanical resistance to root penetration: Reduced leaf elongation rates occur in response to salinity (Termaat, Passioura and Munns 1985; Bernstein, Läuchli and Silk 1993; Zakharin 1993), drying soil (Passioura 1988; Randall and Sinclair 1988; Passioura and Gardner 1990) and low water potential in hydroponic solutions (Van Loo 1992); Leaf emergence rates are reduced by drying soil (Randall and Sinclair 1988) and low water potentials in hydroponic solutions (Van Loo 1992); Stomatal conductance is reduced in drying soil (Sharp and Davies 1979; Zhang and Davies 1990b; Tardieu *et. al.* 1992; Bano *et.al.* 1993;); Root / shoot ratio is increased by salinity (Zakharin 1993), low water potentials in vermiculite (Sharp, Silk and Hsiao 1988; Saab, Sharp, Pritchard and Voetberg 1990) and drying soil (Sharp and Davies

1979; Passioura 1988) because root growth is less affected than shoot growth; Finally as with high R_s, ABA levels in the xylem sap have been shown to increase in plants growing in drying (Gollan, Passioura and Munns 1986; Passioura 1988; Zhang and Davies 1990a; Zhang and Davies 1990b; Tardieu *et. al.* 1992; Tardieu, Zhang and Davies 1992; Bano *et. al.*1993) or saline soils (Munns 1992).

Functionally, the existence of a common response mechanism to high R_s, and low water potential seems an attractive possibility, because many commonalities exist between the two factors. It was already pointed out that R_s is determined by soil water potential (for any given soil density) (Bengough and Mullins 1990; Passioura and Gardner 1990) and that the reduction of root growth due to high R_s limits the amount of available water in the rhizosphere. The response to high R_s may in fact be interpreted as an anticipation to possible water limiting conditions resulting from this restricted root system (Masle and Farquhar 1988). The commonalities between these responses to different soil characteristics makes that many of the results obtained by investigating any one of these stresses are likely to be of significance to our understanding of the others. Moreover, they may indeed be of more general consequence to plant growth response to adverse physical soil conditions than is often realised.

Research strategy.

The aim of this thesis is to analyse some of the cellular aspects of the overall leaf growth in response to R_s. As the previous section illustrates, many of the processes that are involved in leaf area expansion operate at the cellular level. Therefore a sensible approach to resolve the mechanisms by which leaf expansion is affected by R_s is to investigate the cellular basis of the growth reduction observed at the whole plant scale. For this we will analyse the cellular basis of differences in mature leaf dimensions, the kinematics of cell division and expansion in elongating leaves and the relationships between apical characteristics and early events of leaf formation.

For practical reasons we will focus on the epidermal cell. In contrast to the mesophyll, the epidermis is continuous (*i.e.* no intercellular airspaces), and being the outermost cell layer, the epidermis is easily accessible. Physiologically, the epidermis is also very interesting, because it forms the barrier between the plants internal and external

environment. Through regulation of stomatal conductance the epidermis plays a crucial role in the regulation of photosynthesis and transpiration . The epidermis has been implicated as the tissue that determines expansion rates in both leaves and stems (Green 1980; Kutschera, Bergfeld and Schopfer 1987; Kutschera 1989; Sauter, Seagull and Kende 1993; Hara 1995). Green (1980) emphasises the importance of the outer epidermal wall from a biophysical perspective in relation to changes in shape, such as those that occur during the process of leaf initiation at the apex (Green 1980). Moreover, the initial periclinal cell divisions that are involved in the formation of new leaf primordia at the apex, are largely restricted to the surface layer of cells in monocotyledonous plants (Esau 1977).

Literature on leaf development and expansion in grasses.

The developmental process of grass leaves in general and wheat leaves in particular, comprises of two distinct phases, the early development at the apex and the phase of rapid linear expansion, respectively. These have traditionally been investigated as more or less separate events. So far the relationship between developmental processes during those two phases have hardly been explored. The reason for this is that most researchers traditionally focussed their attention on either the process of leaf initiation at the apex or leaf expansion after emergence of the tip of the leaf from the whorl of enclosing sheaths.

Leaf initiation at the apex.

Cereal leaves are initiated down the side of the apex, adhering to a distichous phyllotaxis (alternating between opposite sides of the shoot; (Williams 1974)). The onset of leaf initiation is marked by a shift in the direction of growth in the region where the new primordium will emerge. The cytoskeleton plays a central role in this process, through its effects on the polarity of both cell division and expansion.

The organisation of the cytoskeleton determines the orientation of mitotic division and subsequent cell plate formation (for a recent review, see Baskin and Cande (1990)). The earliest morphologically recognisable event of leaf initiation is an increase in the proportion of periclinal divisions. In *Triticum aestivum* these first periclinal divisions occur in the outermost cell layer of the apex (Foard 1971).

It has been widely accepted that the direction of cell expansion is governed by the alignment of cellulose microfibrils in the primary cell wall of expanding cells (Carpita and Gibeaut 1993). A great body of research has shown that the pattern of cellulose deposition in the primary cell wall is correlated with the organisation of microtubule arrays in the cytoplasm (Hardham, Green and Lang 1980; Gunning and Hardham 1982). It has been suggested that the microtubule arrays determine the orientation of the cellulose deposition in the cell wall, by guiding the movements of the cellulose synthetase molecules in the plasma membrane (Gunning and Hardham 1982). A reorientation of both microtubules and cellulose microfibrils prior to the emergence of leaf primordia has been shown during leaf formation in *Graptopetalum paraguayense* (Hardham, Green and Lang 1980). The shift in the direction of cell expansion is not a direct consequence of the change in the orientation of cell division, but both processes are partly independent. Evidence for this comes from the observation that a protrusion forms on the side of gamma radiated *Triticum aestivum* apices in which cell division is completely inhibited (Foard 1971). The mechanism responsible for the reorientation of the cytoskeleton in the location of the future leaf primordium is currently not resolved. One plausible explanation is that the cytoskeleton responds in reaction to local stretching of the apical tissue caused by the growth of previously formed primordia (Jesuthasan and Green 1989).

The initial protrusion enlarges through continued cell division and expansion, and also through lateral expansion of the periclinal cell division activity (Steevens and Sussex 1989). As a result of this, the primordium completely encircles the apex and becomes hood shaped (Williams 1974). Clonal analysis in *Nicotiana* and *Zea mays* has shown that in those species a total of 100 to 200 cells from different histological layers of the apex contribute to the formation of the leaf primordium (Poethig 1984a; Poethig 1984b).

In wheat, the rate of initiation of leaf primordia by the apex is linearly dependent on the temperature in the range between 10 and 20 °C (Miglietta 1989). Beyond this range, the responsiveness to higher temperatures decreases and maximum rates occur around 25 °C (Friend, Helson and Fisher 1962). Besides temperature, leaf initiation rates are dependent on light intensity, photoperiods, nutrient supply and ambient CO_2

concentration (Friend, Helson and Fisher 1962; Austin and Jones 1975; Milthorpe and Moorby 1979; Masle, unpubl.)

Early growth of leaf primordia.

After a limited period, during which cell division occurs throughout the newly initiated leaf primordium, cell division ceases in the tip of the primordium and the cells in this region start elongating, causing a sudden increase in leaf expansion rates 2 - 3 days before emergence (Williams 1960).

Initiation of the vascular system occurs very early during the development of the leaf. In *Triticum aestivum* provascular strands are initiated in a characteristic order. The median provascular strand is initiated first, approximately 1 plastochron after inception of the primordium, when it is only 4 cells high. A succession of laterals is initiated during the next four plastochrons. The median and first order lateral strands are initiated in the disk of insertion of the primordium and develop both acropetally into the primordium and basipetally to connect with the rest of the vascular system. Lower order laterals are initiated later and up in the primordium, and further development is solely in basipetal direction (Sharman and Hitch 1967). It has been demonstrated in stems of various species that canalised flows of auxin through the undifferentiated mass of ground tissue mediates the initiation and further development of procambial strands that connects new leaves with other parts of the plant (Sachs 1984a; Sachs 1984b). It is likely that the presence of a functional vascular system is a prerequisite to the increased influx of water, minerals and carbohydrates needed for the increased rates expansion occurring during the phase of rapid leaf expansion.

Growth during the phase of rapid leaf expansion .

Structure and functioning of the growth zone.

After emergence of the tip of the leaf from the whorl of leaves surrounding the apex, leaf elongation rates are approximately linear for some days until a final decline sets in (Kemp 1980; Paolillo and Sorrells 1992). In wheat and barley 90% of the leaf is formed during the phase of linear growth (Ong and Baker 1985). During this phase, cell division is restricted to a short zone (< 0.5 cm) at the base of the leaf. Cells produced by this

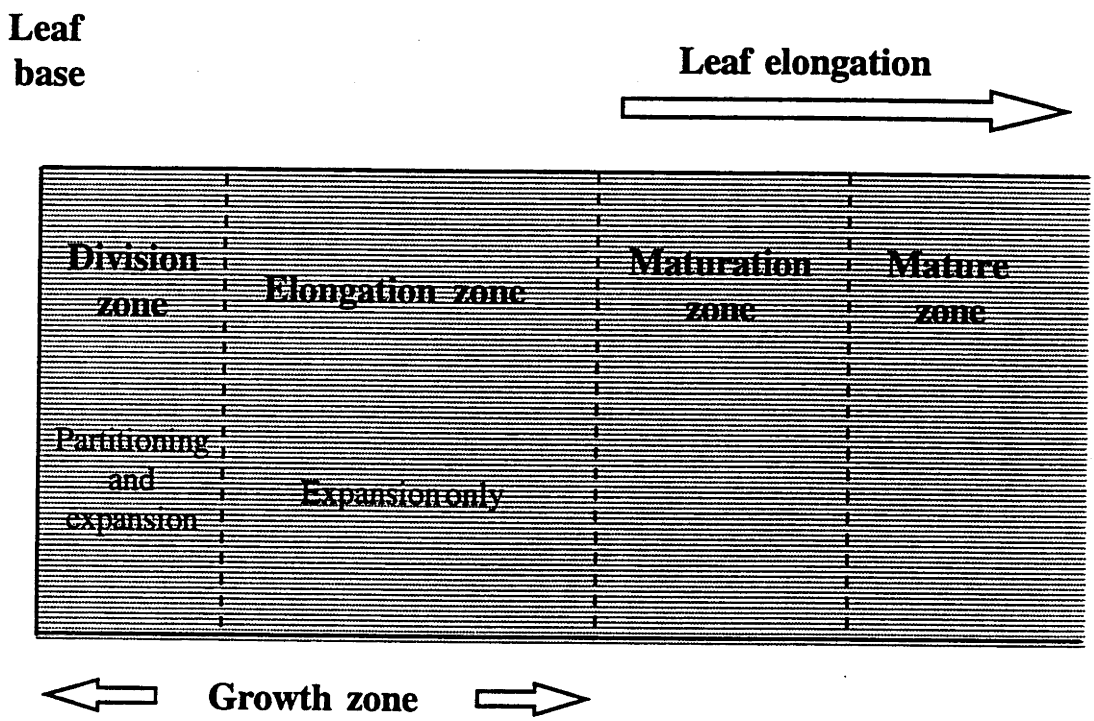

Figure 1. Spatial organisation of the basal region of a grass leaf during the phase of rapid leaf expansion. Definition of each zone is based on the occurrence or absence of the processes of partitioning, expansion and maturation in it.

meristem move through a zone of constant length in which all cells are elongating, until they reach the end of the growth zone where they have attained their final length (Figure 1; Boffey, Sellden and Leech (1980)). Due to the fact that cell division and expansion are longitudinally oriented, the shape of the leaf becomes elongate, with cells arranged in files along its longitudinal axis. This organisation is typical for all grasses. The length of the growth zone varies from 13 mm in a slow growing genotype of *Festuca arundinacea* (Volenec and Nelson 1981) to 90 mm in *Zea mays* (Meiri, Silk and Läuchli 1991). For wheat the length of the growth zone varies between 15 and 70 mm, depending on leaf position (Kemp 1980). Due to the constant increase in cell length as cells are moving through the elongation zone, the velocity at which cells are displaced away from the base increases throughout this zone. When final cell length is reached at the distal end of the elongation zone, velocity becomes equal to leaf elongation rate (Erickson 1976). In all cases, the growth zone is shorter than the length of the encircling whorl of sheaths of older leaves. Although cells no longer grow in locations distal to the growth zone, they often undergo further physiological development (eg. chloroplast development (Leech 1985; Dean and Leech 1982) and secondary wall deposition (MacAdam, Volenec and Nelson 1989)) before maturity. For this reason, a "maturation zone" is indicated in Figure 1.

Perturbations of the leaf developmental program.

In a number of grasses, the rate of leaf primordia initiation at the apex is affected by temperature (Friend, Helson and Fisher 1962; Manupeerapan *et. al.* 1992), light intensity (Friend, Helson and Fisher 1962), photoperiod (Friend, Helson and Fisher 1962; Nicholls and May 1963) and soil water potential (Nicholls and May 1963; Husain and Aspinall 1970). There is often a direct relationship between the rate of primordium initiation and leaf emergence ((Gallagher 1979; Ong and Baker 1985; Hay and Kemp 1990; Miglietta 1991; Masle, unpublished data), which has prompted Hay and Kemp (1990) to suggest that primordium initiation is the process that controls leaf development. During the early developmental stages in grasses there is a gradual increase in the size of the apex (Rösler 1928; Abbe and Phinney 1951; Abbe, Phinney and Baer 1951; Mitchell and Soper 1958; Nicholls and May 1963; Greyson, Walden and Smith 1982; Manupeerapan, Davidson, Pearson and Christian 1992) which is related mainly to an increased cell number (Rösler

1928; Abbe, Randolph and Einset 1941; Abbe, Phinney and Baer 1951; Mitchell and Soper 1958). Often there is a correlation between the size of the apical meristem (or in some cases the size of the sub-apical meristem) and the size of leaves initiated by it. Both ontogenetic differences in mature leaf size (Abbe, Randolph and Einset 1941) and differences in leaf size in response to environmental factors such as light intensity (Mitchell and Soper 1958; Friend, Helson and Fisher 1962), temperature (Friend, Helson and Fisher 1962) and nutrient supply (Allsopp 1954) have been correlated to the size of some part of the apex. It has been suggested for the environmental factors light intensity and temperature, that this response is mediated through the supply of carbohydrates or hormones (Mitchell and Soper 1958; Friend, Helson and Fisher 1962) to the meristematic tissues in the apex. Interestingly, the formation of different types of leaves in heterophyllic plants has also been related to changes in the size of the (sub)apical meristem (Wardlaw 1952; Allsopp 1954; Franck 1976; Bruck and Kaplan 1980).

Leaf elongation rate (E) during the phase of linear elongation is a function of the length of the growth zone (l_{gz}) and local relative rates of cell expansion ($r(x)$) according to $E = \int_0^{l_{gz}} r(x)dx$. Differences in E due to differences in l_{gz} have been found in response to leaf position (partly offset by lower expansion rates) (Kemp 1980), N supply (Kemp 1980) light intensity (Schnyder and Nelson 1989) and between genotypes (Schünmann, Ougham and Turk 1994). High concentrations of NaCl in solution culture and low soil water potentials both shortened the growth zone and reduced maximum relative cell expansion rates (Bernstein, Läuchli and Silk 1993; Bernstein, Silk and Läuchli 1993; Spollen and Nelson 1994). Most studies into local expansion rates in the growth zone have been based on a sub-division of the growth zone in abstract elements. The length of these elements usually is in the order of a few millimetres. Only a small number of investigations have been published that addressed differences in E on a cellular level. It was shown that genotypic variation in leaf expansion rate in tall fescue is associated with a higher rate of cell production in the division zone and greater mature cell length (as a result of a longer elongation zone which was partly offset by lower elongation rates) in the fast growing genotype (Volenec and Nelson 1981; Volenec and Nelson 1983). Higher elongation rates due to increased levels of N fertilisation in the same species were also associated with higher cell production rates, while mature cell length was unaffected

(Volenec and Nelson 1983). Unfortunately, the process of cell division was not investigated in any detail, so it is not clear whether these higher rates of cell production in the division zone were the result of shorter cell cycling times or of a greater population of dividing cells. Moreover, as Green (1976) pointed out, cell division in itself (the instantaneous act of forming a new cell wall) does not generate growth, but in the division zone of expanding organs cell division and expansion often co-occur. A full understanding of growth in the division zone in response to genetic or environmental factors therefore requires analysis of these two processes separately.

Outline.

This thesis is organised in 6 chapters, according to the approach outlined before, describing whole plant response, anatomical basis of differences in size of mature leaves, kinematics of cell division and expansion during the phase of rapid leaf expansion and early development of leaf primordia in the apical meristem, in that order. This allows the reader a gradually deepening insight into the cellular processes that underlie observations made in earlier sections.

The first results chapter (Chapter 2) covers the analysis of the effects of R_s on whole plant growth. This chapter places the effects of R_s on the development of individual leaves in perspective to the overall reduction of plant growth both in terms of mass and leaf area. Moreover, the effect of R_s on final leaf dimensions through its effects on rate and duration of leaf expansion will be established in this chapter. In the remaining results chapters the underlying cellular basis for these observations will be explored.

In chapter 3, the cellular basis of the effect of R_s on mature leaf dimensions is explored, by determining total number, types and sizes of epidermal cells constituting mature leaves. From this, the relative roles of cell division and expansion in determining mature leaf dimensions can be deduced.

Differences in leaf elongation rate are investigated with respect to the processes of cell division and elongation (Chapter 4). This knowledge about the process of leaf elongation can be integrated over the duration of its occurrence to show functional

relationships between the processes of cell division and expansion during the phase of rapid leaf expansion and the final dimensions of the leaf as quantified in chapter 3.

In the last results chapter (Chapter 5) the relationship between early development of a leaf primordium and apical characteristics is explored. In this chapter the extent to which these very early stages of leaf development are determining further development will be addressed.

The final discussion chapter is devoted to a general discussion of the results. An attempt is made to describe the leaf developmental program from initiation to maturity and show how its progress is affected by R_s and ontogeny. Furthermore, the importance of the results as a basis for further cell biological and plant physiological research will be discussed.

Chapter 3, 4 and 5 are presented as a sequence of 3 manuscripts as they have been submitted. Therefore, each chapter contains introduction, detailed methods and discussion of results. As a consequence, there may be a little repetition in the content of these sections between successive chapters, for which I apologise.

Innovative aspects.

Several aspects of this work can be qualified as innovative and therefore deserve to be given some extra attention at this stage.

The central theme of this work is the relationship between growth and development on scales that have traditionally been associated with completely different research disciplines. Cell division and expansion being traditionally a cell biology topic, whereas most research into whole plant growth responses to environmental conditions has always been mainly of interest to plant and crop physiologists.

As a consequence of our focus on the cellular level, existing methods based on the kinematic approach (Goodwin and Stepka 1945; Erickson and Sax 1956a; Erickson and Sax 1956b; Green 1976; Silk and Erickson 1979; Gandar 1980), which facilitate determination of local cell expansion rates in the growth zone of grass leaves during the phase of linear expansion have been modified. These modifications accommodate more

cell level oriented results. Most important aspects of this modification are the calculation of local cell division rates and accurate determination of the size of the population of cells in individual parts of the growth zone.

A final unique aspect of this thesis is its integrating nature, addressing in the same experimental system the effect of stress on the whole development of a leaf, from initiation to maturity and from the cellular to the whole plant scale.

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Chapter 2: Whole plant growth response to high R_s.

Abstract.

The results presented in this chapter show that the whole plant growth responses of wheat seedlings to variations in soil resistance (R_s) obtained in this work have been consistent between experiments and are in agreement with the literature.

Typically, a strong reduction of growth in mass and area occurs very early in the development of the plant. After 12 days, relative growth rates (r_w; $g.g^{-1}.d^{-1}$ and r_l; $m^2.m^{-2}.d^{-1}$) became similar between R_s treatments up to R_s = 4.2 MPa. Under more severe root impedance (R_s = 6.6 MPa) the treatment on which of the work presented in the next chapters was done, r_w and r_l remained reduced for longer.

The overall reduction of leaf growth was due to lower leaf appearance rates, slower leaf expansion and reduced final leaf sizes. The cellular basis of these effects will be investigated in the rest of this thesis.

Introduction.

In this chapter we present typical features of the growth response to high R_s at the whole plant and leaf scale under the standard conditions outlined in the introduction. Plants described in this chapter were sampled from the same experiments as those used for investigations at the cellular level (Chapters 3, 4 and 5). There were 3 main experiments (See table I): Experiment 1, for analysis of the cellular basis of the difference in mature leaf blades (Chapter 3); Experiment 2: kinematic analysis of leaf expansion (Chapter 4) and Experiment 3 in which the growth and dimensions of the apex were analysed (Chapter 5). The purpose of this chapter is two-fold: firstly to give a general description of our experimental system and present the nature and magnitude of the overall growth response to R_s which will be analysed in more detail in the core of this thesis; secondly, to enable quantitative links to be made between parameters at the whole leaf and cellular scale.

Materials and Methods

Experimental conditions.

All experiments were conducted in controlled growth cabinets set to the standard environmental conditions described in the introduction chapter. The combinations of soil bulk density and water potential used to create a range of R_s in various experiments are summarised in Table 1. In all experiments the soil was fertilised before packing into pots with 2200 mg/kg dry soil finely ground super phosphate and 357 mg/kg dry soil NH_4NO_3. The only exception to this was that 2 of the five replicates analysed in experiment 1, (run 1) received a total amount of N, equivalent to 357mg/kg NH_4NO_3 (123 mg N) in the form of mixtures of KNO_3 + NH_4NO_3 and $Ca(NO_3)_2$ + NH_4NO_3, respectively. Growth analysis showed no significant effect of these nutrient treatments (data not shown) and all plants were bulked for further analysis, regardless of nutrients applied. The soil was packed into cylindrical PVC pots 200 mm high and 87 mm in diameter, using a piston of equal diameter to the internal pot diameter. This piston was fitted to an arbor press and moved by a torsion wrench set to apply a given pressure. Soil resistance was estimated by penetrometer resistance (R_s) measured with a 60° cone penetrometer of 2.0 mm diameter There were slight differences in the R_s achieved for a given setting of the wrench between experiments (see Table I), possibly due to variations in the distribution of soil aggregate sizes and of organic debris.

Leaf expansion.

The number of leaves on main stem was counted and the length of the emerged part of the 2 youngest blades were measured with a ruler daily just after the onset of the light period. In addition, in experiment 2 total leaf length was also measured from the soil surface to the leaf tip at the beginning and the end of the light period. From these data the main stem foliar stage was determined according to Haun (1973). The Haun scale method is based on the observation of appearance of new leaves on the main stem which is defined in wheat by the emergence of the blade above the ligule of the previous leaf. The interval between the emergence of 2 successive leaves represents a developmental unit. Each developmental unit is subdivided into decimal fractions corresponding to the ratio of the length of the emerged part of the blade of the youngest emerged leaf (l_n) to

the length of the previous blade (l_{n-1}), and the foliar stage is defined as: $n+\frac{l_n}{l_{n-1}}$. In practice the tip of a new leaf appears from the whorl of the older leaves around the time when the ratio $\frac{l_n}{l_{n-1}}$ approaches unity. Under constant environmental conditions the relationship between foliar stage thus defined and time is linear (Haun 1973). Leaf appearance rate was calculated as the slope of the relationship between main stem foliar stage and time after sowing. In experiment 2 the elongation of leaf 2 was recorded using an LVDT (linear velocity displacement transducer). This device enables automatic measurements of leaf expansion rates at time intervals of only a few seconds.

Growth analysis.

In experiment 1, 6 plants per treatment were harvested on day 12 and 19 after sowing. Leaves were cut above the crown and blades and sheaths were separated. Blade and sheath fresh weights were determined and the area of individual main stem leaves as well as the total leaf area (a) were measured using an electronic leaf area meter (LiCor, LI-3000). Length (L) and width (W) of all individual leaves were also measured using a ruler. A ratio of 0.57, independent of R_s and time, was found between measured leaf area (a) and the leaf area calculated from the product of leaf dimensions. ($0.57a = \sum_0^i L_i W_i$). This factor was used to estimate leaf area from measurements of lengths and widths of all leaves at three other dates (day 10, 14 and 17, respectively). The pots were covered with plastic and stored in a cold room (-2 °C) until root washing. The leaves, sheaths, roots and the seed were then oven dried for 48 h at 60 °C for dry weight measurements. Growth parameters were calculated and statistical analysis was performed using the ANOVA routine of the statistical package Genstat 5 (Version 2.1; Lawes Agricultural Trust (Rothamsted Experimental Station)).

Tillering.

In experiment 3, two harvests were done in which 5 plants were harvested during the phase of linear elongation of leaf 5 (*i.e.* 2 - 3 days after emergence of the tip of that leaf from the whorl of older leaves (day 24 and 29 at low and high R_s, respectively). Five

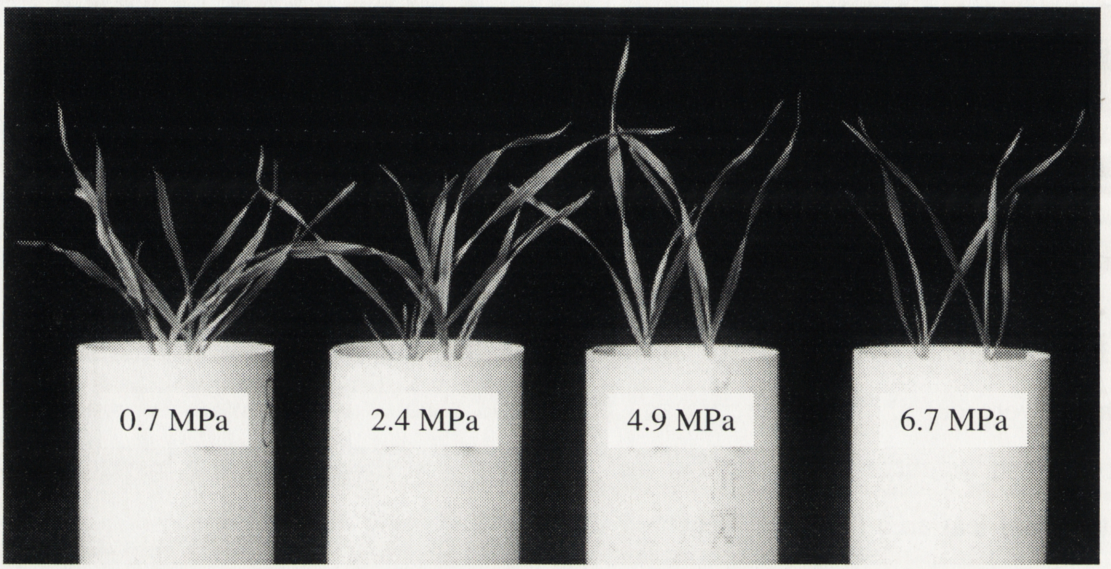


Figure 1. The effect of R_s on the growth of young wheat seedlings at 15 days after sowing.

Table I. Overview of soil conditions and type of observations made in the different experiments described in this thesis.

Soil conditions					
Density ($g.cm^{-3}$)	Water content (g H_2O g^{-1} dry soil)	R_s (MPa) Experiment 1 (Chapter 3) run 1	run 2	Experiment 2 (Chapter 4)	Experiment 3 (Chapter 5)
1.12	0.24	0.9	0.9	0.5	0.6
1.25	0.24	2.8	-	-	-
1.37	0.24	4.2	-	-	-
1.42	0.22	6.6	7.7	7.5	7.0
Observations in each experiment					
whole plant and leaf growth		+	+	+	+
anatomy mature leaf		leaf 1&3	leaf 5	-	leaf 5
kinematics of leaf expansion		-	-	leaf 1, 3 & 5	leaf 5
apical dimensions		-	-	-	+

other plants were harvested when leaf 5 was fully expanded, *i.e.* just after the emergence of leaf 7 (day 28-31 and day 34 - 37 at low and high R_s, respectively). The total number of visible tillers was determined on all plants and the area of tiller blades was measured separately from that of main stem blades.

Results.

Whole plant response.

Whole plant leaf growth was negatively correlated with R_s. Figure 1 shows a photograph of representative plants of each R_s treatment at day 15, *i.e.* midway between the two harvests. Both leaf area and total dry weight were reduced with increasing R_s at both harvests ($p < 0.001$; Figure 2 and 3). The decrease of both leaf area and total dry weight in response to an increase of R_s from 0.9 to 2.8 MPa is much smaller than for further increases.

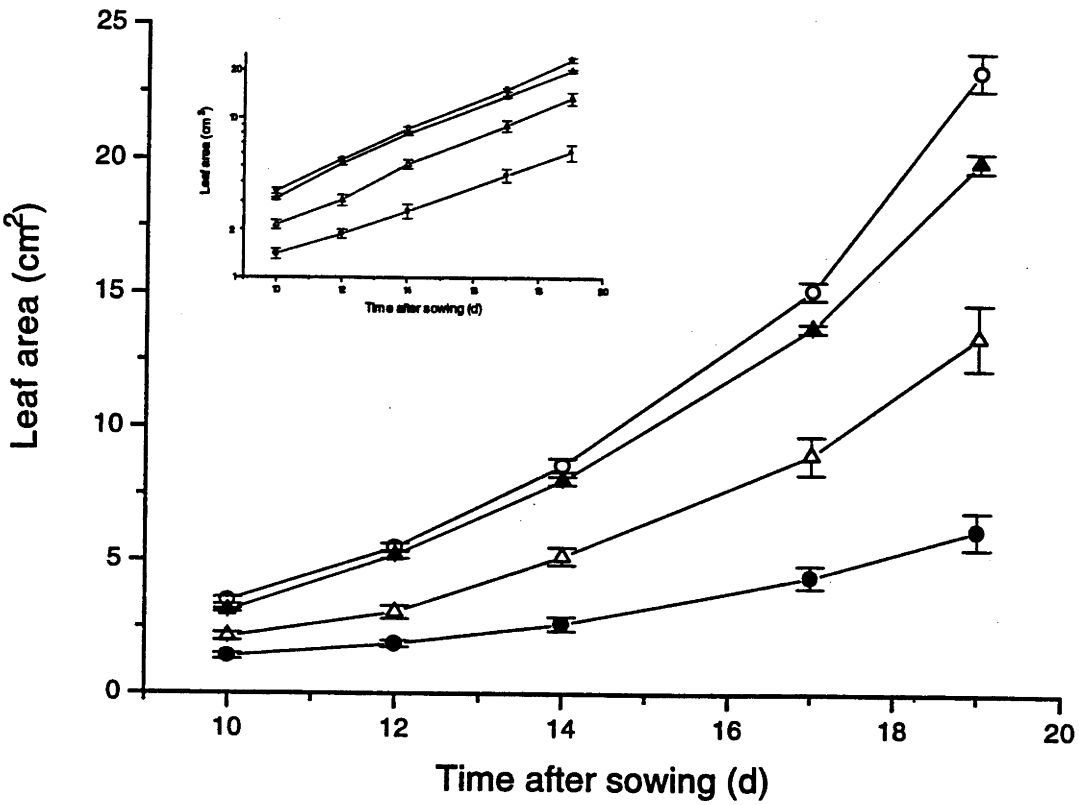
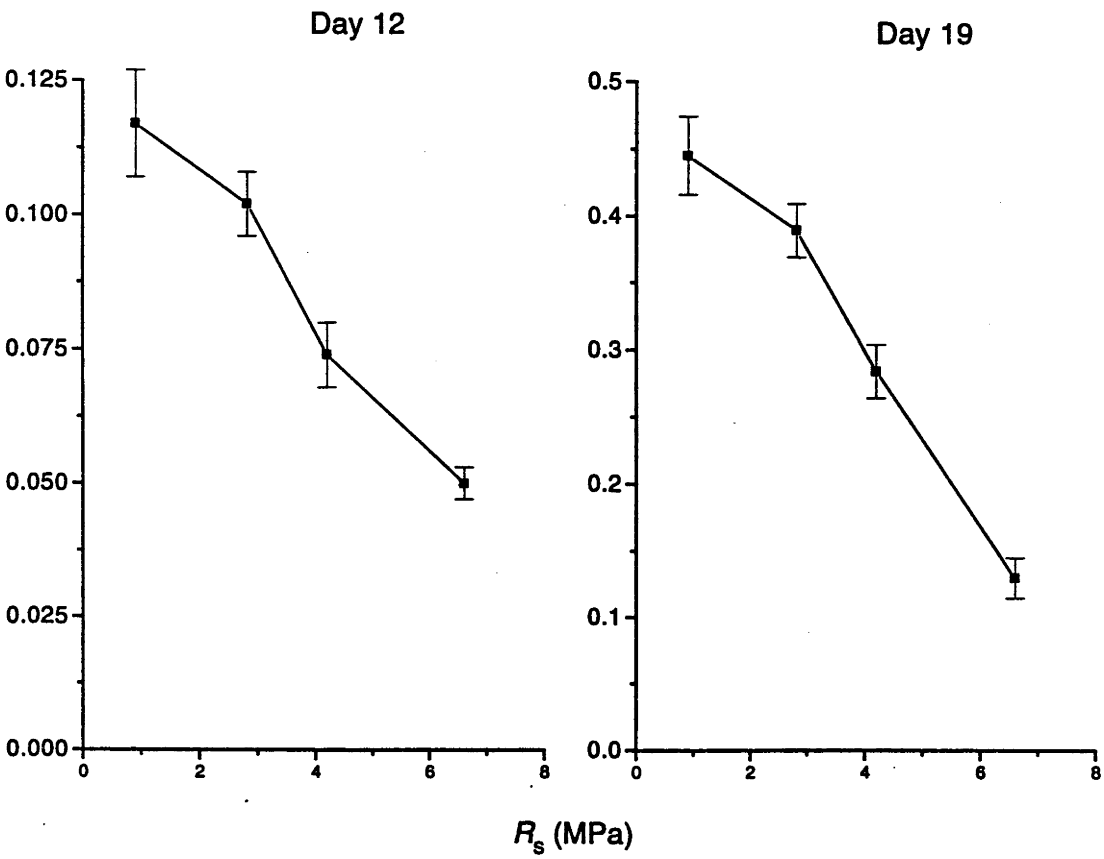


Figure 2. Averages and standard errors (n = 6) of leaf area as a function of time after sowing. Symbols denote R_s = 0.9 (open circles), 2.8 (solid triangles), 4.2 (open triangles) and 6.6 MPa (solid circles), respectively. Insert: same data plotted on an exponential scale.



ıre 3. Averages and standard errors (n = 6) of total plant dry weight as a function of day 12 and 19, respectively.

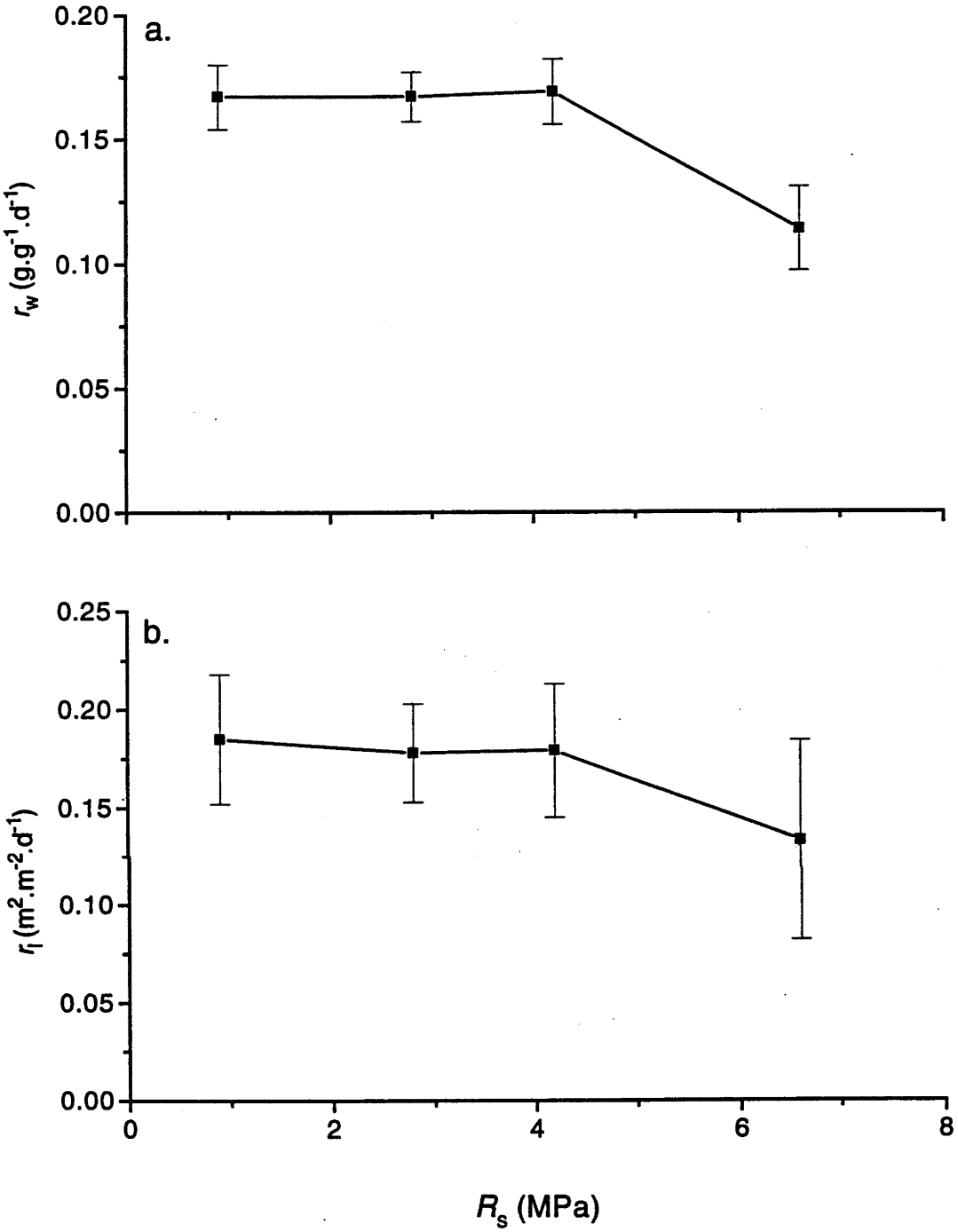


Figure 4. Relative growth rates in terms of dry weight (r_w; a) and leaf area (r_l; b) between day 12 and 19 as a function of R_s. Error bars denote standard errors (n = 6).

Table II. Number and leaf area of tillers and main stem leaf area at low and high R_s, respectively, of plants at similar developmental stages. Significance of the differences were determined with ANOVA analysis of variance. Asterixes denote significance level of the differences: * $0.10 < p < 0.05$, ** $0.05 < p < 0.01$.

	Number of main stem leaves	R_s (MPa)		Difference (%)
		0.7	7.0	
Number of tillers	5	4.0 (0.6)	3.4 (0.2)	-15 *
	7	9.8 (0.7)	7.8 (0.9)	-20 **
Leaf area of tillers	5	20.5 (4.4)	12.2 (2.2)	-40 *
	7	84.3 (6.3)	48.6 (7.0)	-42 **
Leaf area main stem	5	38.1 (3.8)	30.1 (1.9)	-21 **
leaves	7	60.4 (3.3)	45.4 (3.7)	-25 **
Fraction of leaf	5	0.33 (0.04)	0.28 (0.03)	-5 *
area in tillers	7	0.58 (0.01)	0.51 (0.02)	-7 **

Growth analysis.

Relative rates of dry weight accumulation (r_w, $g.g^{-1}.d^{-1}$) and leaf area expansion (r_l, $m^2.m^{-2}.d^{-1}$) between day 12 and 19 were not significantly affected by an increase of R_s from 0.9 to 4.2 MPa (Figure 4). However, a further increase from 4.2 to 6.6 MPa caused significant decreases of both parameters r_w ($p = 0.012$) and r_l ($p = 0.071$).

As was outlined in the introduction differences in plant growth rate can be decomposed according to the identity $r_w = \frac{A'}{\sigma}$.

The net assimilation rate (A') was unaffected by an increase in R_s from 0.9 to 2.8 MPa (Figure 5a). At R_s = 4.2 MPa, A' was somewhat increased compared to the lower R_s

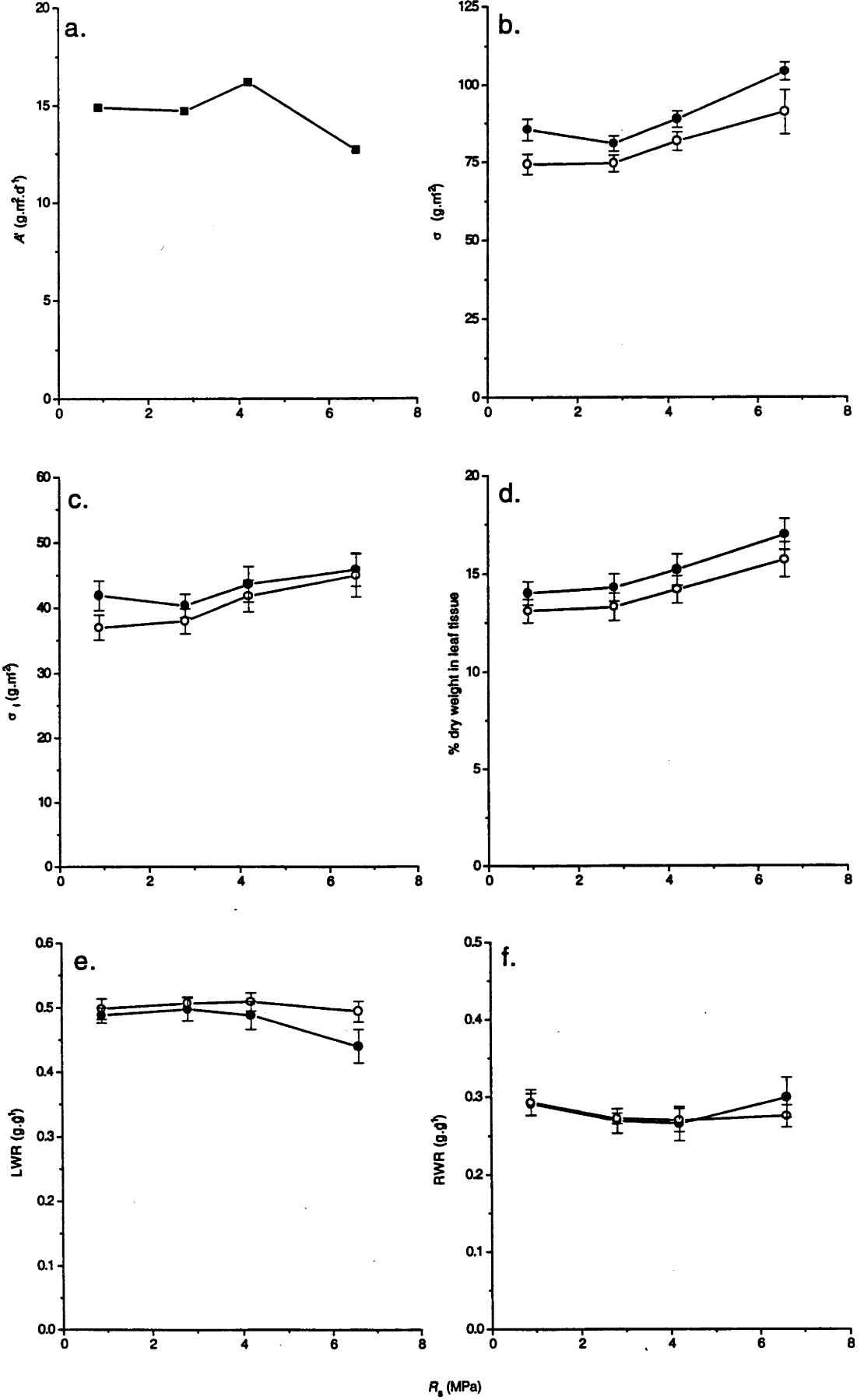


Figure 5. Averages and standard errors (n = 6) of: a) *A'*; b) σ; c) σ_l; d) % dry weight blade; e) *LWR* and f) *RWR*. Solid and open symbols denote day 12 and 19, respectively. *A'* was calculated over the whole of the period between day 12 and 19 using the method described by Evans (1972).

Table III. Comparison of average seed mass, R_s and length of mature blade or whole leaf (sheath + blade) between different experiments. Difference between R_s treatments (in percent of the value of low R_s) is indicated in brackets. Seeds used for the experiment described in chapter 5 were from a different batch of seeds than the other experiments. The seeds used in all experiments were close to the median size for the particular batch of seed.

		Experiment 1				Experiment 2		Experiment 3	
		run 1		run 2		(Kinematics)			
seed mass (mg)		24 - 28		24 - 28		32 - 34		42 - 44	
blade	leaf 1	116	92 (-21)	119	86 (-28)	-	-	98	98 (0)
length	leaf 3	189	158 (-17)	187	155 (-17)	-	-	231	198 (-14)
(mm)	leaf 5	-	-	220	161 (-27)	-	-	254	235 (-8)
whole leaf	leaf 1	-	-	-	-	140	106 (-24)	133	132 (-1)
length	leaf 3	-	-	-	-	222	175 (-21)	254	219 (-14)
(mm)	leaf 5	-	-	-	-	-	-	275	260 (-6)

treatments. This increase was not reflected in r_w because it was cancelled by a comparable increase in the total plant dry weight per unit leaf area, σ (Figure 5b). However, at R_s = 6.6 MPa A' and σ was increased, resulting in lower r_w.

The parameter σ can be decomposed into $\sigma = (\gamma + 1)\sigma_l$, were γ is the ratio of root to shoot dry weight and σ_l the leaf mass per unit leaf area ($g.m^{-2}$). Parameter σ decreased from day 12 to 19 ($p < 0.001$; Figure 5), which was mainly due to a decrease in σ_l ($p < 0.001$; Figure 5c). The distribution of dry matter between roots and shoots was little different at these 2 dates (Figure 5e and f). Higher σ with increasing R_s ($p < 0.001$; Figure 5b) was mainly due to higher σ_l ($p < 0.001$; Figure 5c), and at the highest R_s level there was also a decrease in the fraction of total dry matter allocated to leaves on day 12 (Figure 5e).

Leaf development.

The lower rate of leaf area expansion with increasing R_s was at least partly due to a decrease in the rate of leaf appearance ($p < 0.001$; Figure 6). Furthermore, high R_s also

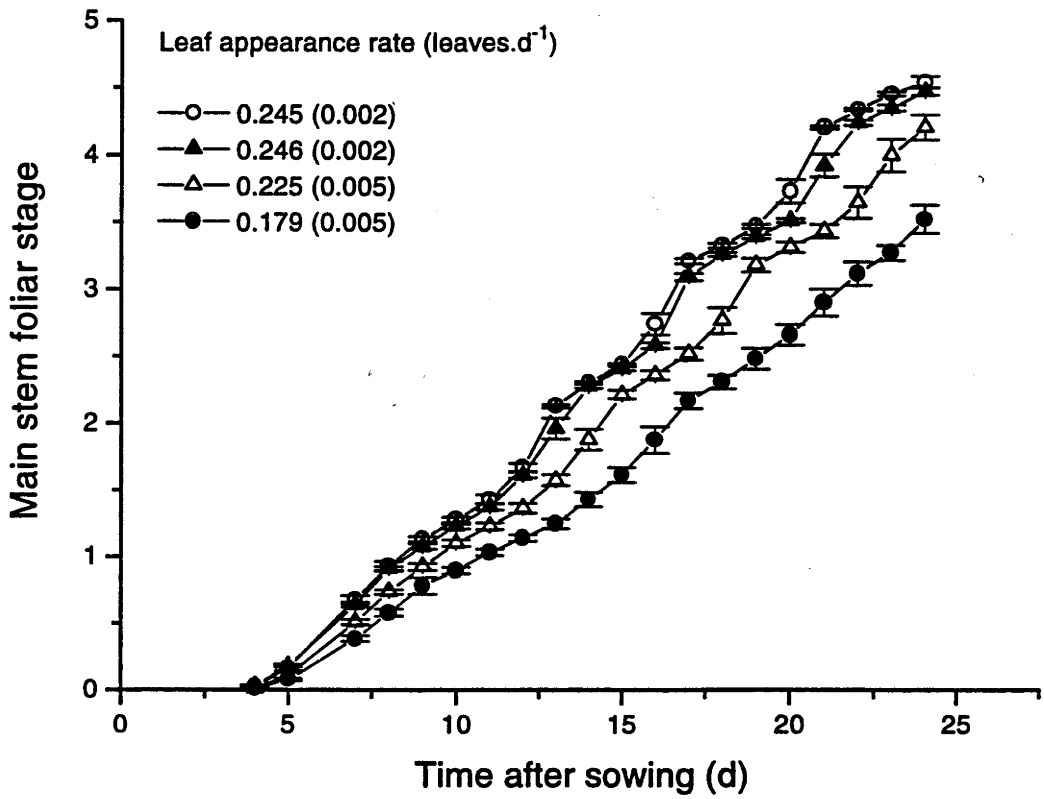


Figure 6. Averages and standard errors (n = 6) of foliar stage according to Haun (1973) as a function of time after sowing. The average leaf emergence rate (= slope of the curves; leaves/day) and standard errors are indicated. Same symbols as in Fig. 2.

Table IV. Maximum leaf elongation rates (mm/h) during the light and dark period in leaf 1 and 3 derived from measurements of leaf length with a ruler (leaf 1 and 3). Leaf 2 data were derived from LVDT data of leaf 2 shown in Figure 8. The last column indicates reduction (%) of leaf elongation rates due to an increase in R_s from 0.5 to 7.5 MPa.

		R_s (MPa)		
		0.5	7.5	Difference (%)
Leaf 1	light	1.56	0.74	-52
	dark	0.94	0.56	-40
Leaf 3	light	2.07	1.22	-41
	dark	1.28	0.92	-28
Leaf 2	light	1.55	0.80	-48
	dark	0.95	0.55	-42

reduced the number of tillers of plants harvested during the linear expansion of leaf 5 and shortly after emergence of leaf 7 by 15 and 20%, respectively ($p = 0.066$; Table II). The reduction in leaf area at high R_s compared to low R_s, was greater for the tillers than for main stem leaves, resulting in a decrease of the fraction of the total leaf area contributed by the tillers (Table II). The fact that total leaf area of the tillers was reduced more than the number of tillers suggests that the reduction in tiller leaf area was associated with a decrease in both the number of tillers and the average area per tiller.

The length and width of mature leaves were consistently reduced by high R_s (data shown in Chapter 3 and table III). The magnitude of the reduction of mature leaf length, was very similar between the first two experiments, but was smaller in experiment 3. A probable cause for this was loosening of the soil early in the experiment, when harvesting plants for analysis of early development of leaf 5, from the same pots in which plants harvested later for analysis of leaf 5 at maturity. Differences in the amount of seed reserves (Table III) may have been a further cause for the differences between successive experiments.

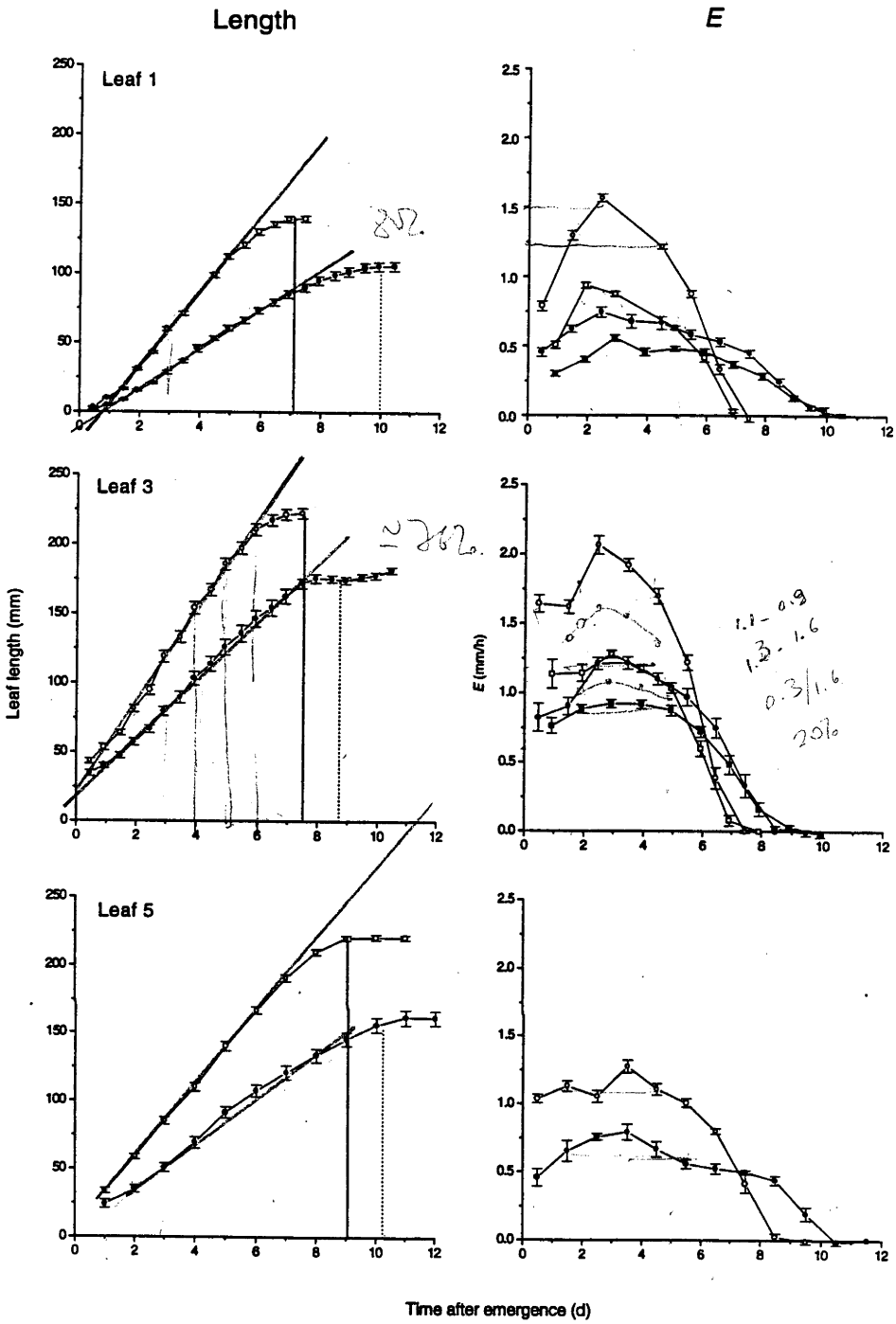


Figure 7. Averages and standard errors (n = 35 (leaf 1 & 3) and 5 (leaf 5), respectively) of length (left hand panels) and elongation rates (*E*; right hand panels) of leaf 1, 3 and 5 (top to bottom) as a function of time after sowing. Data for leaf 1 and 3 were obtained form experiment 2 and leaf 5 data from experiment 1, run 2, respectively. Symbols: circles: average leaf elongation rate during the light period; squares: averages elongation rates during the dark period. Leaf 5 data are average over 24 h. Open symbols denote 0.5 / 0.9 MPa and solid symbols 7.5 / 7.7 MPa, respectively.

Leaf elongation was slower at high R_s, but proceeded for longer, the net effect being still a shorter final leaf length (Figure 7). Leaf elongation rates during both light and dark period reached a maximum two to three days after emergence before gradually decreasing (Figure 7). Due to the longer duration of leaf elongation and lower rates at high R_s, the curves of leaf elongation rates *vs* time were flatter (Figure 7), *i.e.* maximum elongation rate was maintained for a longer period of time. The difference in duration of leaf expansion at low and high R_s became gradually smaller in successive leaves, especially between leaf 1 and 3. Leaf elongation rates were more affected by high R_s during the light period than during the dark period (Fig. 8 and Table IV).

Discussion.

Our results show a clear effect of R_s on overall plant growth and leaf growth in particular. Plants growing at high R_s have a smaller mass and leaf area at any time after sowing. The reduced leaf area can be attributed to slower leaf appearance rate, slower leaf expansion rate and smaller final leaf size and the formation of fewer tillers. These results are consistent with earlier observations (Masle and Passioura 1987; Masle 1990; Masle, Farquhar and Gifford 1990; Masle 1992).

In the range 0.9 and 4.2 MPa much of the effect of R_s occurred at early stages of development. After two weeks growth the relative growth rates (r_w and r_l) were indeed all similar. The significant differences in total plant mass and leaf area on day 12 and 19, imply that r had been affected earlier and that the absolute growth rates, *i.e.* the increase in mass or leaf area per unit of time, were inversely related to R_s. Masle (1992) reported the same pattern for the growth response to an increase of R_s from 1.5 to 5.5 MPa, in a range of wheat and barley genotypes. At the highest R_s level in our experiment (6.6 MPa), the treatment that was used for the microscopic studies presented in the next 3 chapters, r_w and r_l between day 12 and 19 were still slower than on looser soil.

The variation in r was analysed using the identity $r_w = \frac{A'}{\sigma}$ (Masle, Farquhar and Gifford 1990). Up to R_s = 2.8 MPa, both A' and σ were similar. At R_s = 4.2, A' was in fact increased. (Masle and Farquhar 1988; Masle, Farquhar and Gifford 1990) reported a

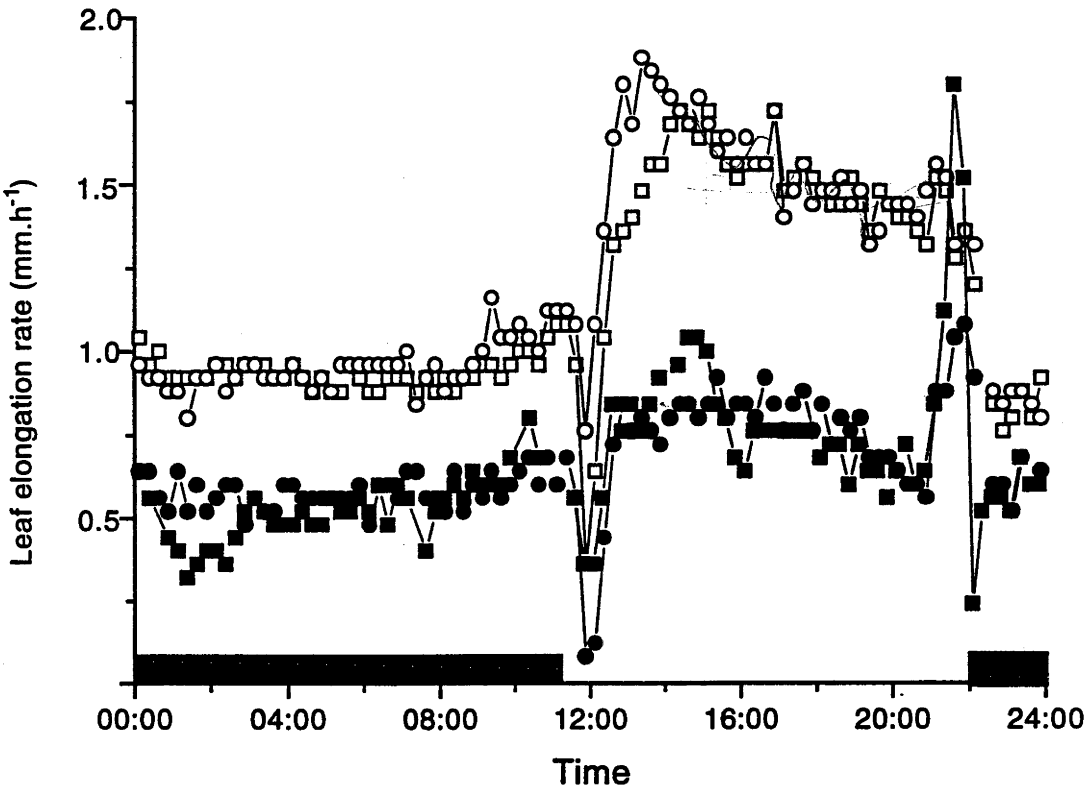


Figure 8. Leaf elongation rates of individual leaves determined with LVDT measurements in the third day after emergence of the blade tip from the sheath of leaf 1 at $R_s = 0.5$ (open symbols) and $R_s = 7.5$ MPa (solid symbols), respectively. Different symbols indicate individual plants. The dark bar on the axis indicates the dark period.

similar response for the same genotype in the same range of R_s and showed that the increase in A' was due to an increased photosynthetic capacity. This increase was not reflected in r, because σ was increased by a similar proportion. The decrease in r_w at R_s = 6.6 MPa was due to a decrease in A' and a relatively greater increase in σ. This level of soil impedance is higher than those compared in earlier experiments. It is possible that such severe impedance causes damage to the photosynthetic machinery or severe stomatal closure. Unfortunately, no data on photosynthetic capacity or stomatal conductance were obtained in the present work.

Leaf elongation rates during the light period were higher than those in the dark. The 3 °C difference between the day and night temperatures (18 and 15 °C, respectively) was too small to account for these differences (See table IV). It is obvious that the rates of leaf expansion during the dark period depend upon the amount of assimilates stored during the light period and that there is therefore no unique relationship between day and night elongation rates. More interesting is the fact leaf elongation rates were more affected by R_s in the light than in the dark. At high R_s carbohydrate contents in the growing region of wheat plants are higher (Masle 1990; Masle, Farquhar and Gifford 1990). It has been proposed that a chemical signal (possibly ABA) originating in impeded roots, and transported in the xylem, causes slower leaf growth at high R_s. It may be that in the dark, when transpiration rates would be much smaller, a smaller amount of this inhibitory compound is transported to the leaves.

The overall growth response to high R_s presented in this chapter was seen in all our experiments and was similar to the response described in earlier studies for the same genotype under similar conditions (Masle and Passioura 1987; Masle, Farquhar and Gifford 1990). There is considerable genetic variation in the magnitude of the growth reduction with increasing R_s and possibly in the underlying mechanisms (Masle 1992). However, a reduction in the rate of leaf appearance and elongation and in final leaf size have been found in a number of genotypes and also species (Masle, Farquhar and Gifford 1990; Masle 1992).

The whole plant growth response to R_s, as presented in this chapter, gives rise to a number of questions about the underlying cellular processes that are being addressed in the remainder of this thesis:

1. Why are mature leaves smaller? Smaller leaf size can be brought about by either smaller cells or a smaller number of them. The effects of R_s on the numbers of cells per leaf and their sizes will be analysed in Chapter 3.
2. What is the cellular basis of the reduced leaf elongation rates? Using the kinematic approach (Erickson and Sax 1956a; Erickson and Sax 1956b; Gandar 1980; Volenec and Nelson 1981) the effects of R_s on the dynamics of cell division and expansion during the phase of linear leaf expansion will be examined (Chapter 4).
3. What are the effects of R_s on apical development and on the expansion of young leaf primordia, and what are the consequences on further development and leaf dimensions at maturity? Microscopic analysis of apical dimensions during the first two plastochrons of leaf 5 development will be presented in Chapter 5.

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Chapter 3

Composition, number and size of epidermal cells in mature blades.

Abstract.

Wheat seedlings (*Triticum aestivum* L.) were grown on soils with contrasted resistances to root penetration (R_s). High R_s reduced the rates of leaf appearance and expansion. Although the duration of expansion was increased, mature leaves were smaller. Underlying changes in leaf anatomy were investigated on cleared mature leaves, focusing on the epidermes. Three leaves were analysed: leaves 1 and 3 which started their development in the embryo, and leaf 5 which was initiated on the seedlings, after imposition of contrasted soil conditions. In all leaves, high R_s caused a reduction in mature cell sizes, lengths and widths, and a shift in the relative proportions of functionally different cell types, with a decrease in the relative proportions of stomata and associated cell types (interstomatal and sister cells) and an increase in the proportions of unspecialised elongated epidermal cells and of trichomes. In leaves 3 and 5 the number of cellular files across the blade was also reduced, while in leaf 1 it was similar at the two R_s. These differences between leaves are attributed to differences in their developmental stage when root stress was first perceived. Remarkably, R_s had no effect (leaf 1) or relatively small effects (leaves 3&5) on the number of cells per file, suggesting that this parameter is either largely insensitive to environmental perturbations, or is programmed at the outset before stress was perceived at the apex. The next two chapters will address the effects of R_s on the genesis of these mature leaves attributes.

Introduction.

Soils with high resistance to root penetration (R_s) reduce plant growth. Many studies have concentrated on root responses (eg. Barley,1962; Taylor and Ratliff, 1969; Goss, 1977). However, leaf growth is also severely affected (Masle and Passioura, 1987; Masle, 1990). In fact, in the long term (scale of a few weeks) carbon accumulation in wheat seedlings with impeded roots may be more reduced in leaves than in roots (Masle et al., 1990; Masle, 1992). Leaf emergence is delayed; blades expand more slowly, over a longer period of time and mature blades are both narrower and shorter (Masle and Passioura, 1987; Masle, 1990&1992).

The aim of the present study was to examine the cellular bases of these whole leaf growth responses. The present paper reports observations of the anatomy of mature blades from wheat plants grown on soils with contrasted R_s. Effects of R_s on epidermal cell dimensions and numbers, and on the organisation of these cells into files were investigated. The next chapter addresses effects on the kinetics of cell partitioning and cell expansion in the epidermis of elongating blades. Reasons for focusing on epidermal cells were two-fold: firstly leaf expansion is constrained by the extensibility of the epidermis (Kutschera et al., 1987; Kutschera,1989). Secondly, in wheat, mesophyll cells are lobed in an irregular fashion so that tracing the contour of neighbouring cells is difficult and not always reliable.

Grass leaves are convenient experimental systems for anatomical studies. Most of the leaf length is generated in a basal growth zone, physically well defined and organised into cellular files parallel to the leaf axis. New cells are formed by transverse divisions at the base of the leaf and displaced to more distal positions by the formation and expansion of cells at more basal locations. A few millimetres from the base of the leaf, cells loose their ability to divide and only expand, reaching their mature size while still enclosed in the sheath of the preceding leaf (Boffey et al., 1980; Volenec and Nelson, 1981). Cell differentiation follows a basipetal gradient and continues well after the cell has reached its final dimensions (Boffey et al., 1980).

Materials and Methods.

Growth conditions.

Wheat plants (*Triticum aestivum* L. cv. Egret) were grown in cylindrical PVC pots (200 mm high; 87 mm in diameter). Four seeds (24 - 28 mg) were sown in each pot at constant depth (15 mm). The soil used for this experiment was a silty loam made of 5% coarse sand, 40% fine sand, 31% silt, 19% clay and 4.5% organic matter and was part of the batch of soil used by Masle and Passioura (1987) in earlier experiments on growth responses to R_s. This soil was fertilised before use by addition of 2.2 g finely ground super phosphate per kg dry soil and 123 mg nitrogen provided as a mixture of KNO_3 and NH_4NO_3, or of $Ca(NO_3)_2$ and NH_4NO_3 or as NH_4NO_3 alone. After thorough mixing, soil water content was brought to 0.24 or 0.22 g H_2O per g dry soil and homogeneously packed to a bulked density of 1.12 and 1.42 g cm^{-3}, respectively, resulting in contrasted soil resistances to penetration of 0.9 and 6.6 MPa penetrometer resistances, respectively, in a first run (experiment a, see below), and 0.9 and 7.7 MPa, in a second run (experiment b).

The pots were placed in a growth chamber providing a photo- and thermo-period of 11h with day/night temperatures of 18/15 °C, 600 µmol quanta m^{-2} s^{-1} irradiance, and a relative humidity of 80%. After germination the soil was covered with a layer of white plastic beads in order to limit soil water evaporation and therefore variations in R_s. Each pot was weighed daily, and lost water was added 1 to 3 times a day in order to maintain soil water content within 1% of its original value. Ten days after sowing, seedlings were thinned to 2 per pot. All three nutrient regimes ensured plentiful nutrient supply (data not shown) and gave similar plant growth and morphology. Results below are therefore from bulked data.

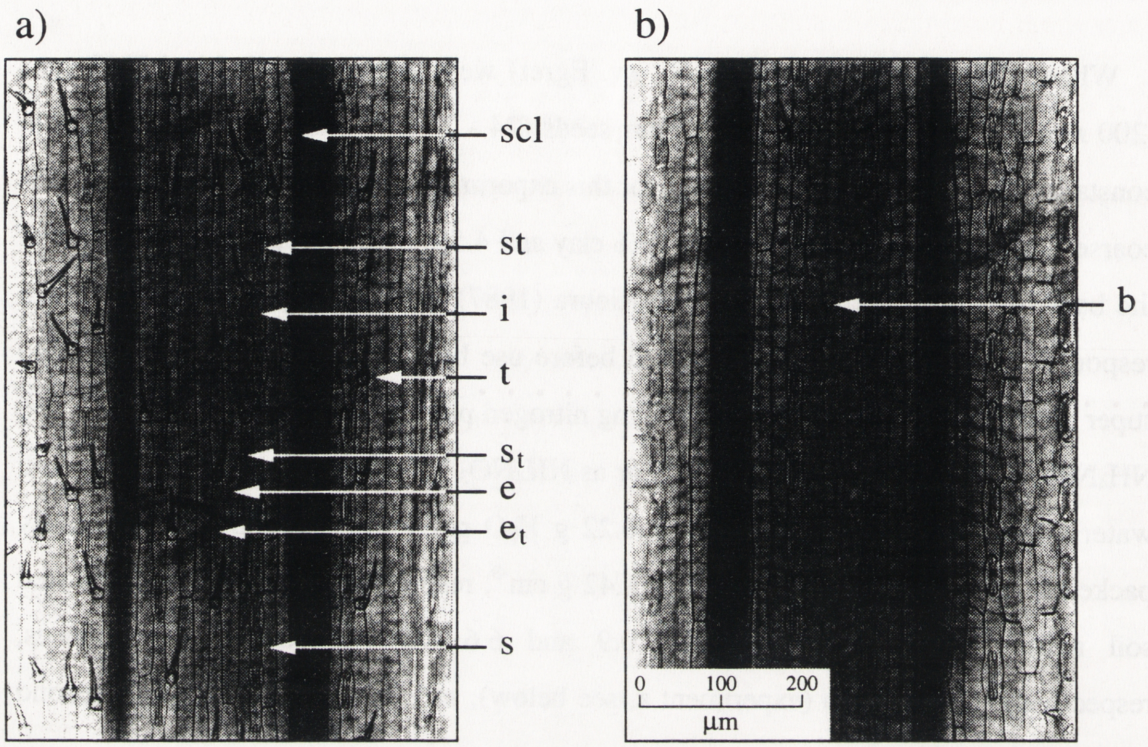

Figure 1. Typical anatomy of the abaxial (a) and adaxial (b) epidermis of a wheat leaf. Symbols denote the various epidermal cell types which were distinguished in the present study: *b*, bulliform cell; *e*, elongated, non specialised cell; *st*, stomata; *i*, interstomatal cell; *s*, sister cells; *t*, trichomes; *scl*, schlerenchyma cell. Subscript *t* for elongated and sister cells denotes association with a trichome.

Leaf preparation.

In a first run, fully expanded blades of leaf 1 and 3 were harvested on 5 plants at each R_s. As their analysis revealed unexpected leaf position effects, a second batch of plants were grown under the same conditions (run 2) from which a third leaf, leaf 5, was harvested. Leaf 1 and 3 start their development in the wheat embryo (Hayward, 1938) while leaf 5 is initiated after germination ie, in this experiment, several days after the roots had experienced low or high soil resistance. Upon harvest, blade length (*L*) and area (*a*) were measured. The whole blade was then cleared for light microscopy, using a modification of the technique described by Clarke (1959). Leaves were immersed in 70 % boiling methanol for about 30 minutes until all chlorophyll was removed, and then in lactic acid until analysis. The cleared leaves were mounted on a light microscope (Zeiss axioscope) fitted with a Panasonic video camera (model WV-CL 702E). Maximum leaf width (*W*) was measured mid-way along the blade and morphometric analysis of the epidermis was performed on video images (total magnification 190x) using the morphometric program MTV (Garr Updegraff/Datacrunch, 1991) on a PC equipped with a video-card.

Morphometric analysis of blade epidermis.

The epidermis of a wheat leaf contains a variety of cells. Files of different cell types are arranged in a regular pattern (Percival, 1921; Stebbins and Shah, 1960; Esau, 1977; Silvy, 1982) which is closely associated with the location of veins in the mesophyll (Fig. 1). Between two adjacent veins, there were usually two stomatal rows, comprising stomatal complexes (guard cells and associated subsidiary cells, label *st* in Fig.1) separated by inter-stomatal cells (*i*). Adjacent to these files were files of wider "sister cells" (*s*) so-called because they derive from the same "mother cells" as the subsidiary cells (Tomlinson, 1974; Tomlinson,1994). The two inner files of sister cells, located between two adjacent stomatal rows were separated by several files of unspecialised, long and narrow "elongated" cells (*e*) on the blade abaxial side, enlarged and shorter "bulliform" cells (*b*) on the adaxial side. One or two outer files above larger veins were made of sclerenchymatous cells (*scl*). A number of shorter elongated cells and also of

sister cells were associated with trichomes, especially at high R_s (cells labelled e_t and s_t, respectively in Fig. 1).

The effects of R_s or leaf position on blade anatomy were therefore analysed in terms of total number of files across the blade, number of files of the various types described above, cell density (total number of cells per unit area, and number of cells of a given type), and cell dimensions. To allow for possible temporal or ontogenetic variations in the effects of R_s on leaf histogenesis the above parameters were determined at several locations along and across the blade, on 3 replicated leaves. For these leaves, blade width, number of veins, number and width of cellular files were determined at 10 locations evenly distributed along the blade. At one of these locations (distance $0.4L$ from the ligule) cell dimensions were measured in three different interveinal areas selected on one side of the mid-vein (second area from the blade mid-rib and margin, and area between the central veins). At each of 3 other locations along the blade (0.2, 0.6 and 0.8) measurements were restricted to the second interveinal area from the mi-rib. The length and greatest and smallest widths of 10 cells of each type were measured. For the two other replicates, cell dimensions were measured at one location only (second interveinal area from mid-rib and distance $0.4L$ from ligule). Cell densities were also determined at that same location, for all 5 replicates. A series of overlapping images of a 3 mm long segment were printed with a video-graphic printer (Sony, model UP-811). Individual cells were included in the counts when their basal transverse wall was within the segment. Sclerenchyma cells located above the veins were omitted because their contours were often hard to follow. Cells of this type contributed little to the total epidermal area and their proportions relative to files of other types were little affected by R_s. The width of the observed segment was estimated by averaging the widths measured at 5 equidistant points along its length, and used to calculate the total area to which cell counts had to be referred. The total number of cells per file was also counted along one file of bulliform cells, sister cells and stomatal/interstomatal cells in 5 replicated leaves per treatment. The three files were selected close to the mid-rib, among files which could be unambiguously traced from the ligule to the tip of the blade. All parameters were

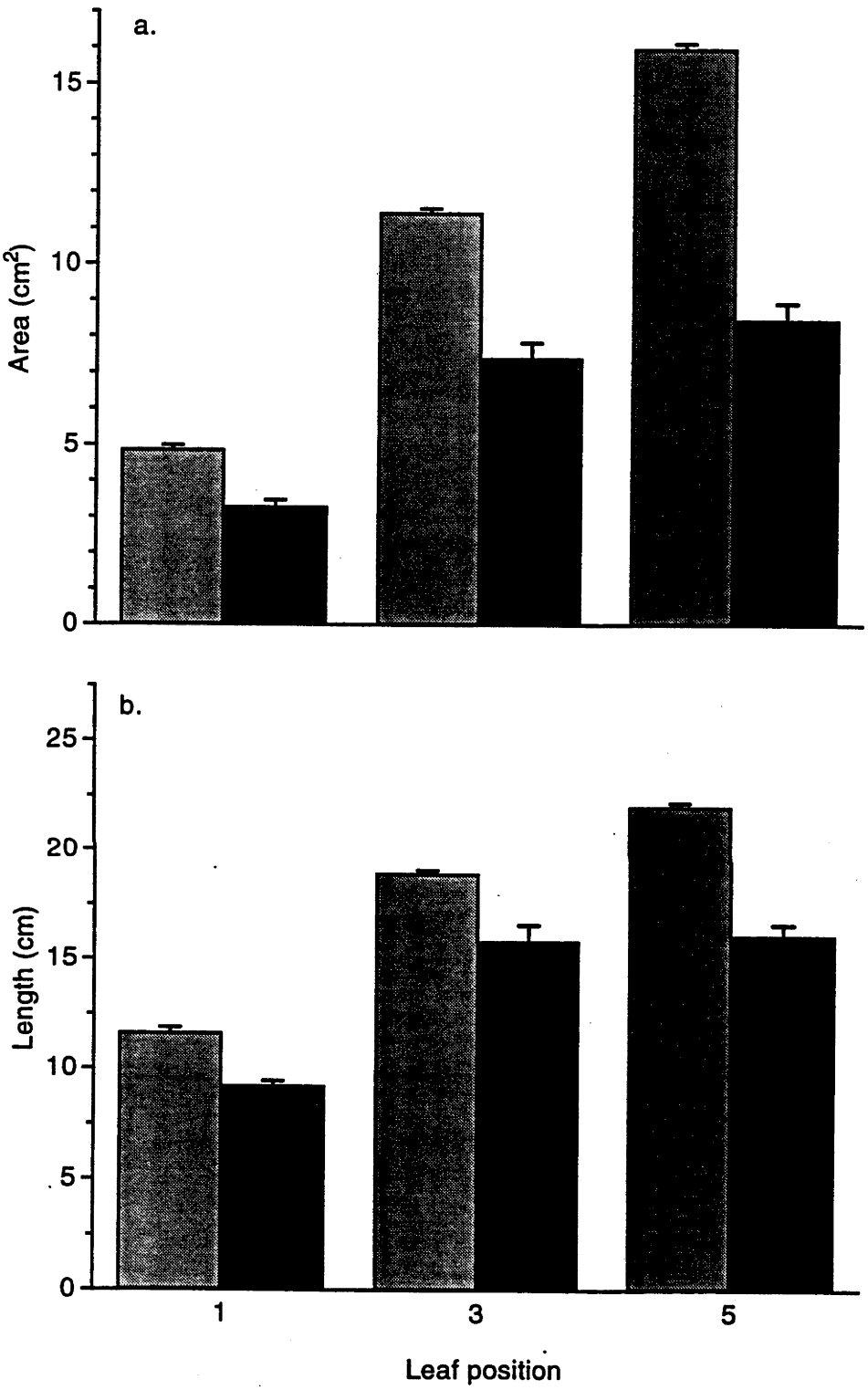

Figure 2. Variations in the size of mature blades with soil resistance and leaf position: a) blade area; b) blade length. Leaves grown at low and high R_s are described by grey and black bars, respectively. Error bares denote the standard error of the mean (n=5).

determined on the two epidermes. When the effects of soil conditions or leaf position were similar for each epidermis, data are presented for the abaxial epidermis only.

Statistical analysis.

The effect of R_s on the relative proportions of files of various types and on cell densities was analysed by log-linear modelling of contingency tables, using the statistical package Genstat 5 (version 2.1; Lawes Agricultural Trust (Rothamsted Experimental Station)). All other data were analysed with ANOVA analysis of variance using the same statistical software.

Results.

Whole leaf dimensions. High R_s significantly reduced the area of all mature blades ($p < 0.001$; Fig. 2a) by ca 35% in leaf 1 and 3, and 47% in leaf 5. This reduction in area reflected a decrease in both leaf length (21, 16 and 27% for leaf 1, 3, and 5, respectively (Fig. 2b)) and leaf width (Fig. 3). Maximum blade width measured at a distance of $0.4L$ from the ligule was decreased by 30, 20 and 38% in leaf 1, 3, and 5, respectively (Fig. 3). This reduction was representative of that measured at other locations within the basal two-thirds of the blade but was greater than reduction at more distal locations (Fig. 3). Soil resistance modified leaf shape with blades being almost rectangular at high R_s (eg leaf 1, Fig. 3), while having a marked triangular shape at low R_s. The greater response of leaf 5 compared to the two other leaves may only be apparent as the high R_s for leaf 5 was 7.7 MPa penetrometer resistance cf 6.6 for leaf 1 and 3 (see methods). The elongation rates of leaf 1 and 3 on the plants from which leaf 5 was sampled also responded more to R_s than those from the first run, on which the data in Figs 2&3 were obtained (Table I).

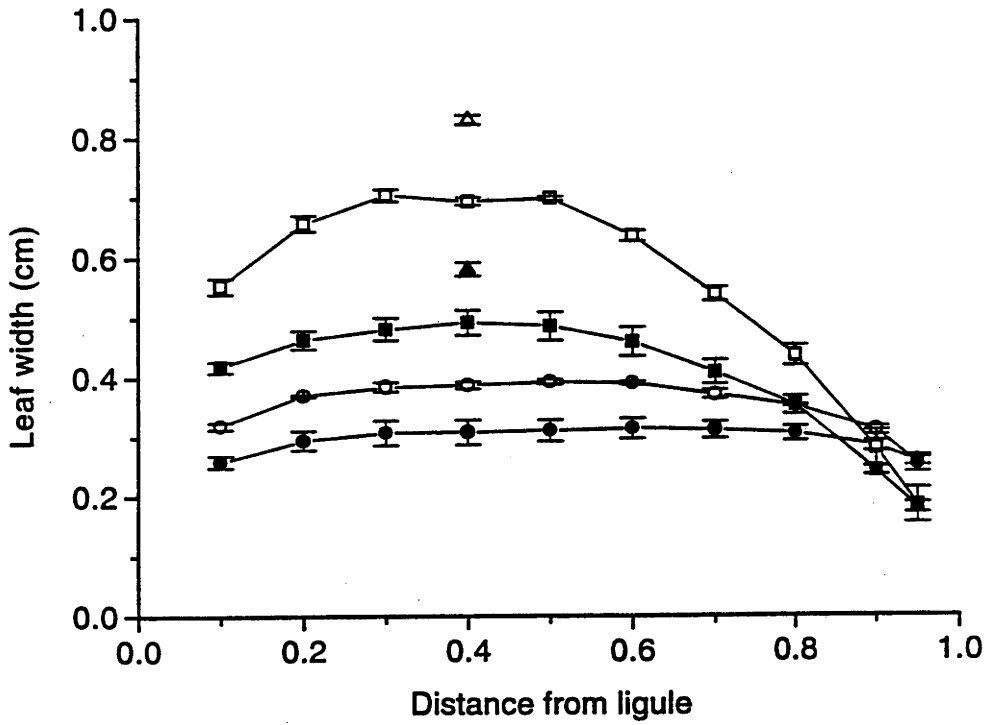

Figure 3. Variations in the width of mature blades with soil resistance (open and solid symbols for low and high R_s, respectively), leaf position (circles, squares and triangles for leaf 1, 3 and 5, respectively), and distance from the ligule expressed as a proportion of total blade length .

Table I. Comparison of leaf elongation rates ($mm.d^{-1}$) between plants grown in two successive runs for analysis of leaf 1 and 3 (run 1) and leaf 5 (run 2). Numbers are the slope of the regression line fitted to leaf length *vs* time data, during the linear phase of leaf elongation.

	Leaf 1		Leaf 3		Leaf 5	
R_s	low	high	low	high	low	high
run 1	24.1	14.2	31.5	23.7	-	-
run 2	24.2	12.9	31.9	21.7	25.8	15.4

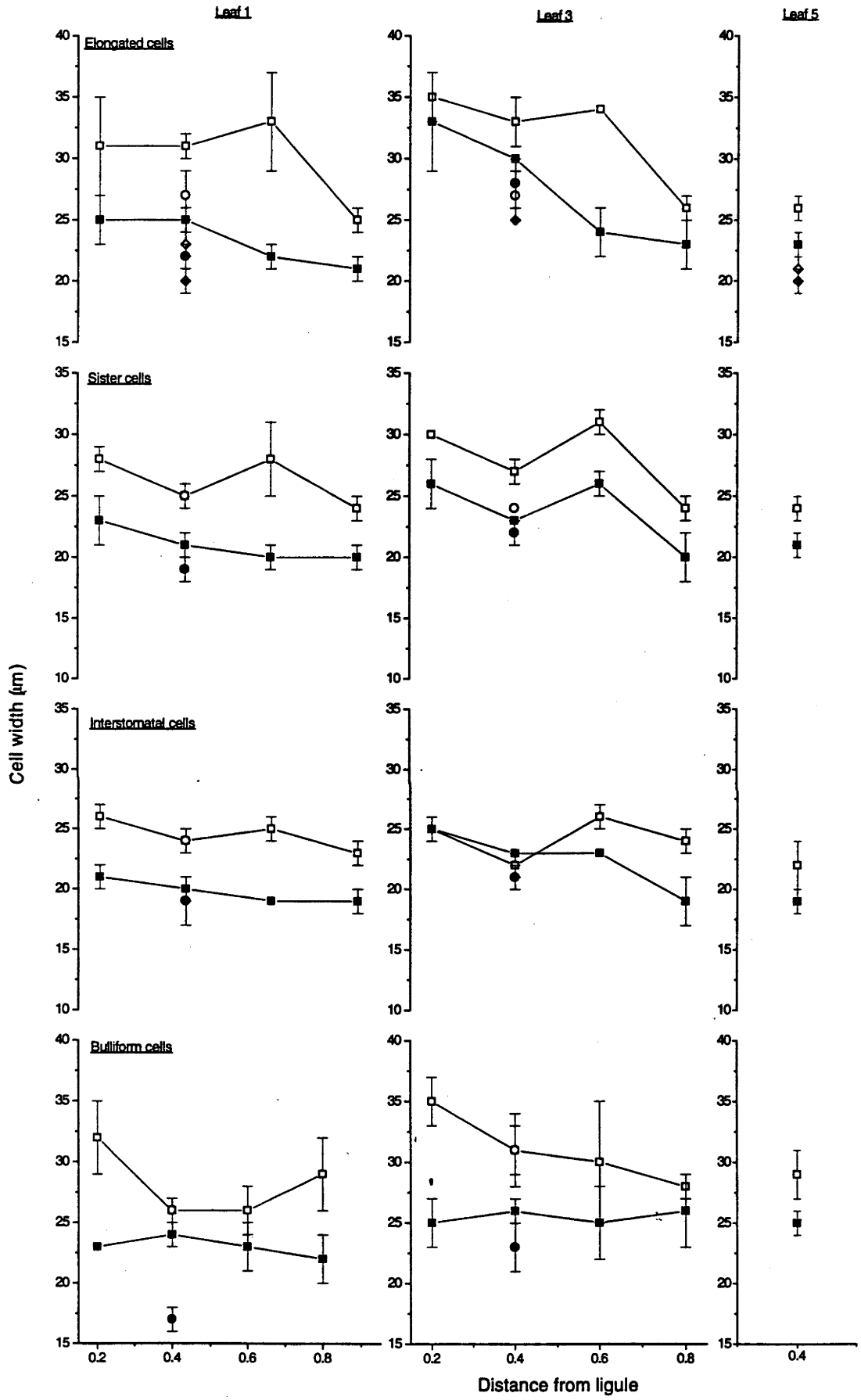
Leaf 1
Leaf 3
Leaf 5
Elongated cells
Sister cells
Interstomatal cells
Bulliform cells
Cell width (µm)
Distance from ligule
0.2
0.4
0.6
0.8

Figure 4. Effects of soil resistance (open and closed symbols for low and high R_s, respectively) on epidermal cell widths in leaf 1, 3 and 5. Data are shown for various cell types for the abaxial epidermis, except for bulliform cells (present on the adaxial epidermis only). Widths of elongated cells, sister cells and interstomatal cells on the adaxial side of the blade showed a similar pattern of variation. Data points (□, ■) correspond to mean values (± s.e., n=3 or 5 leaves) at four equidistant locations along the blade for leaves 1 and 3, one location only for leaf 5, all within the second interveinal area from the mid-rib. At position 0.4*L* widths are also shown for cells located in the second interveinal area from the blade margins (○, ●). For elongated cells, data refer to cells which were not associated with trichomes except at one location where cells associated with trichomes are also shown ((◊, ♦) to be compared with (▫, ▪)).

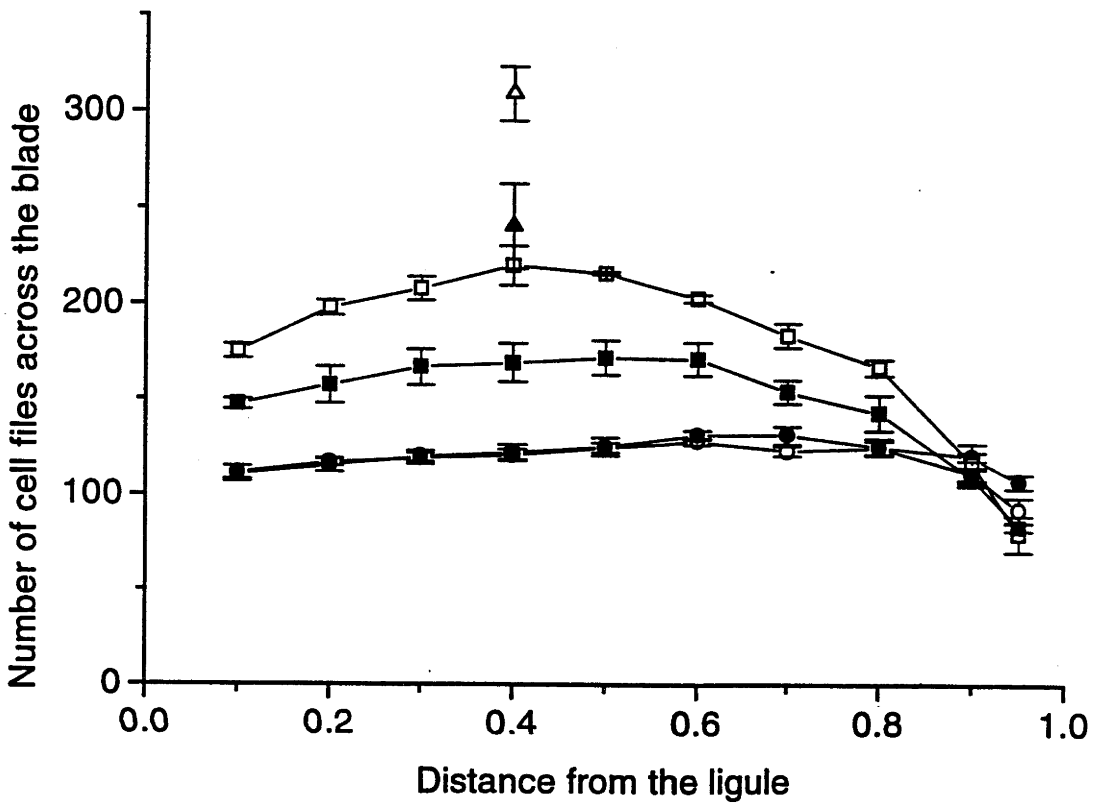

Figure 5. Variations in the number of cellular files constituting the abaxial epidermis of mature blades as a function of soil resistance, leaf position and location along the blade. Symbols are as in Fig.3.

Number of cell files; cell width. However, there were striking true leaf effects on the cellular bases of the overall changes in blade dimensions with R_s described above. In leaf 1, blades from plants grown at high R_s were narrower only because cells, of all types, were much narrower (Fig. 4). The number of cellular files constituting the blade was indeed similar at the two R_s (Fig. 5). In leaf 3 and 5, in contrast, blades grown at high R_s were made of fewer cell files (Fig. 5). Cells within these files were also narrower, but somewhat less than in leaf 1; the reduction in cell width was spatially uneven across and along the blades and not always significant (Fig. 4).

Figure 4 shows that there were significant spatial variations in cell width within a blade, especially at low R_s, with cells becoming narrower towards the tip of the blade or its margins (circles in Fig. 4) compared to more central locations. There were also variations associated with cell type and formation of trichomes. For example, elongated cells tended to be wider than sister cells. More noticeably, elongated cells which, within a file, were preceded and followed by a trichome were significantly narrower than those preceded and followed by another elongated cell (comparison of diamonds and squares in Fig. 4). However, when expressed in relative terms, the effects of R_s on cell width were of similar magnitude at different locations on the blade and for different cell types.

There was a high correlation between number of cell files and number of veins across the blade ie the number of cell files between two veins varied little with either R_s or leaf position (overall mean number = 11.7). However, variation in R_s caused qualitative differences, in the relative proportions of different types of files: the number of "ground tissue" files (Esau, 1977), made of elongated, bulliform and sclerophyllous cells, was increased by high R_s while the number of stomatal rows and associated files of sister cells remained unaffected. This shift was especially marked for the adaxial epidermis (Fig. 6) where files of stomata and sister cells represented 46% of all cell files compared with 50 % at low R_s ($p<0.001$)). Furthermore, high R_s caused a significant increase in the number of files with trichomes which represented 27% of all files with elongated cells and 56% of all files with sister cells, as opposed to 12% and 37%, respectively at low R_s.

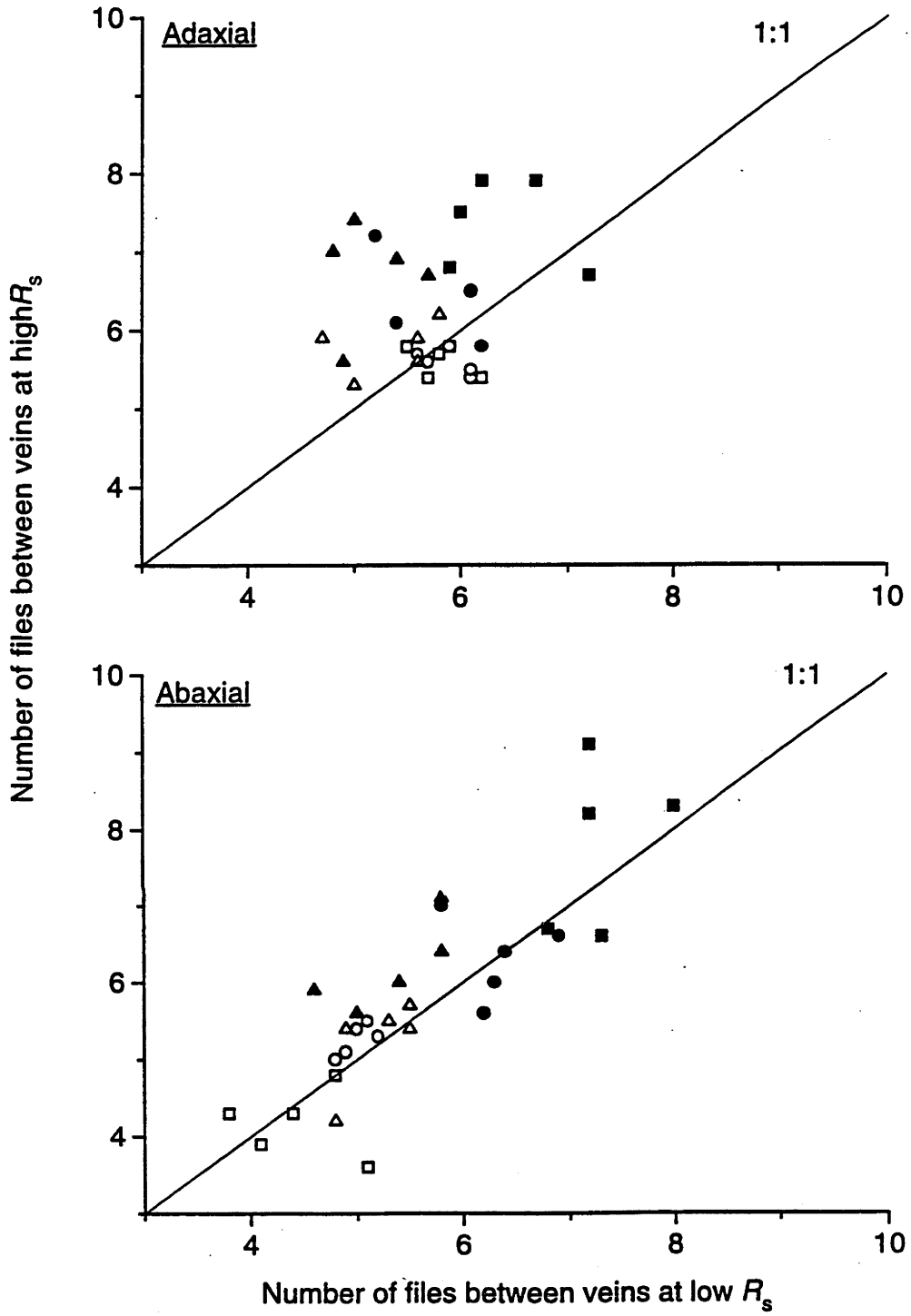

Figure 6. Effects of soil resistance on the average number of cellular files between adjacent veins in the adaxial and abaxial epidermis. Data points correspond to individual blades of leaf 1 (□, ■), leaf 3 (○, ●) and leaf 5 (△, ▲). Open symbols represent the number of stomatal files and related files of sister cells, solid symbols represent the number of all other files.

Table II. Total number of cells counted along single files from ligule to blade tip on the abaxial and adaxial epidermis. Numbers in brackets denote standard errors of the means (n=5 leaves). Asterixes denote statistically significant differences between R_s ($p < 1\%$ (***); $1\% < p < 5\%$ (**); $5\% < p < 10\%$ (*))

	Cells	Interstomatal			Sister			Bulliform		
	R_s	low	high		low	high		low	high	
	Epidermis									
Leaf 1	Abax	370 (13)	353 (14)	-	338 (13)	386 (12)	*	-	-	-
	Adax	528 (19)	519 (14)	-	475 (15)	456 (10)	-	354 (9)	314 (7)	**
Leaf 3	Abax	705 (6)	583 (34)	***	557 (10)	520 (35)	-	-	-	-
	Adax	898 (20)	784 (42)	***	760 (11)	667 (25)	***	559 (16)	503 (36)	***
Leaf 5	Abax	-	-		968 (13)	982 (13)	-	-	-	-
	Adax	-	-		-	-	-	974 (13)	826 (16)	***

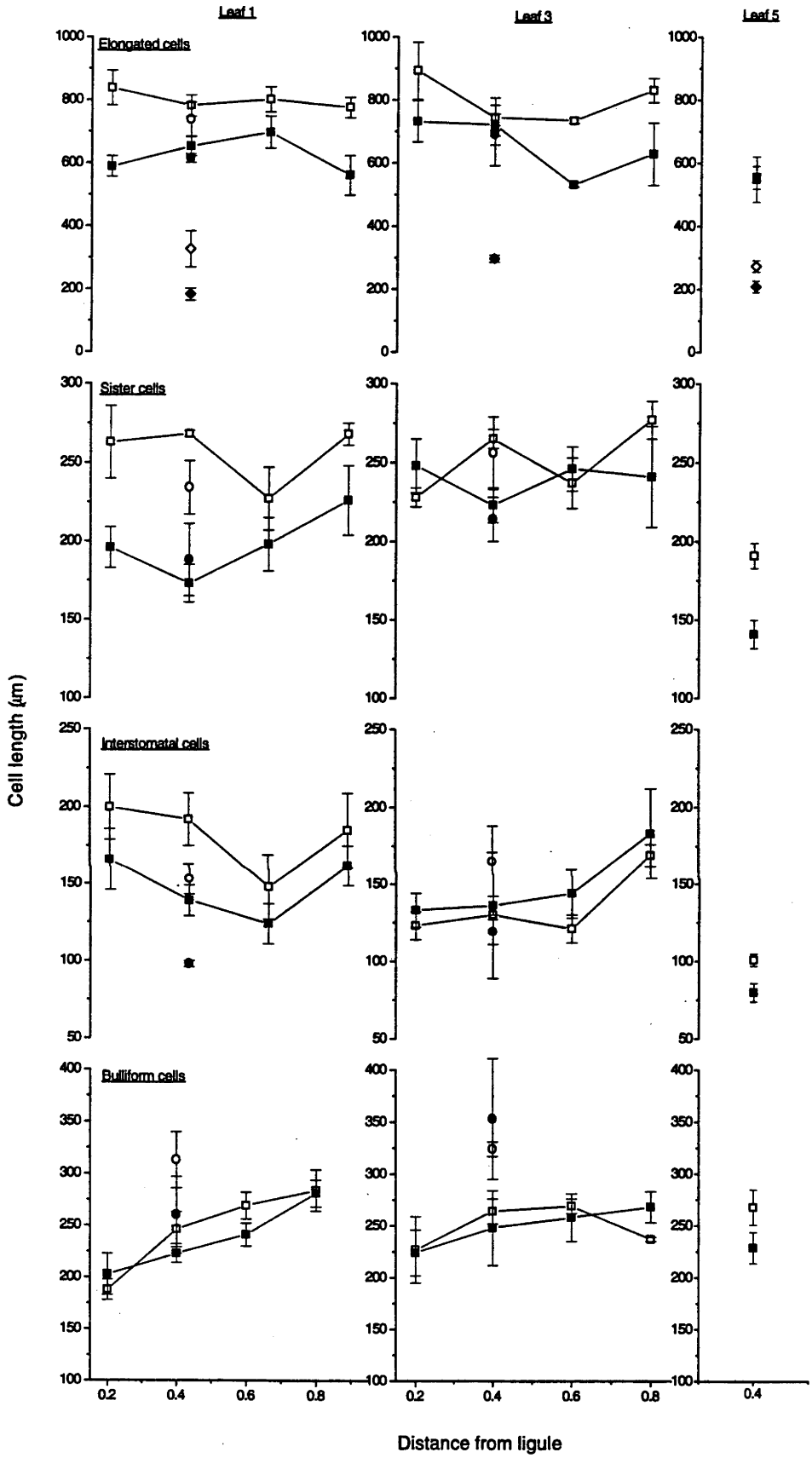

Figure 7. Effect of soil resistance on epidermal cell lengths in leaves 1, 3 and 5 for the same cell types and same leaves as in Fig. 4. Symbols are as in Fig.4

The number of cell files and veins across the blade increased from leaf 1 to 3 to 5 (Fig. 5). These variations bore no relation to variations in cell width, for which there was no consistent ranking between leaves (Fig. 4). They were large enough to cause a significant increase in blade width of successive leaves (Fig. 3), even when cell widths were decreased (as from leaf 3 to 5, at low R_s (Fig. 4)).

Cell length; number of cells along a file. Mature cell lengths are described in Fig. 7. They were spatially even more variable than cell widths, in an apparently more or less random fashion rather than according to a positional gradient along or across the blade. Elongated cells were 2 to 4 times longer than other cells except when associated with a trichome (Fig. 7) and cells of all types were longer on the abaxial than adaxial epidermis (data not shown). In leaf 1 high R_s caused a marked reduction in cell length which was of similar magnitude for all cell types (20-25%) apart from bulliform cells (0-10% depending on location along the blade). At the one location of leaf 5 where cell dimensions were measured, cell lengths were also reduced (Fig. 7). In contrast, in leaf 3, only elongated cells were on average shorter at high than low R_s.

The total cell numbers per file in the mature blade are shown in Table II. Consistent with the differences in length between cell types shown in Fig.7, the number of cells counted along adjacent files of different type varied. Remarkably, however, in all three types of files shown in Table II, cell number was unaffected (in leaf 1 and 5), or only slightly decreased (about 10%, in leaf 3) by high R_s.

Cell densities; cell indices. The overall effect of variations in cell length and width and in the relative proportions of files of various types described above was reflected in variations in cell densities (number of cells per unit area). Soil resistance was the main source of variation of cell densities. Figure 8 shows that total densities were significantly greater at high than low R_s. This effect was especially marked in leaf 1 (68% increase on

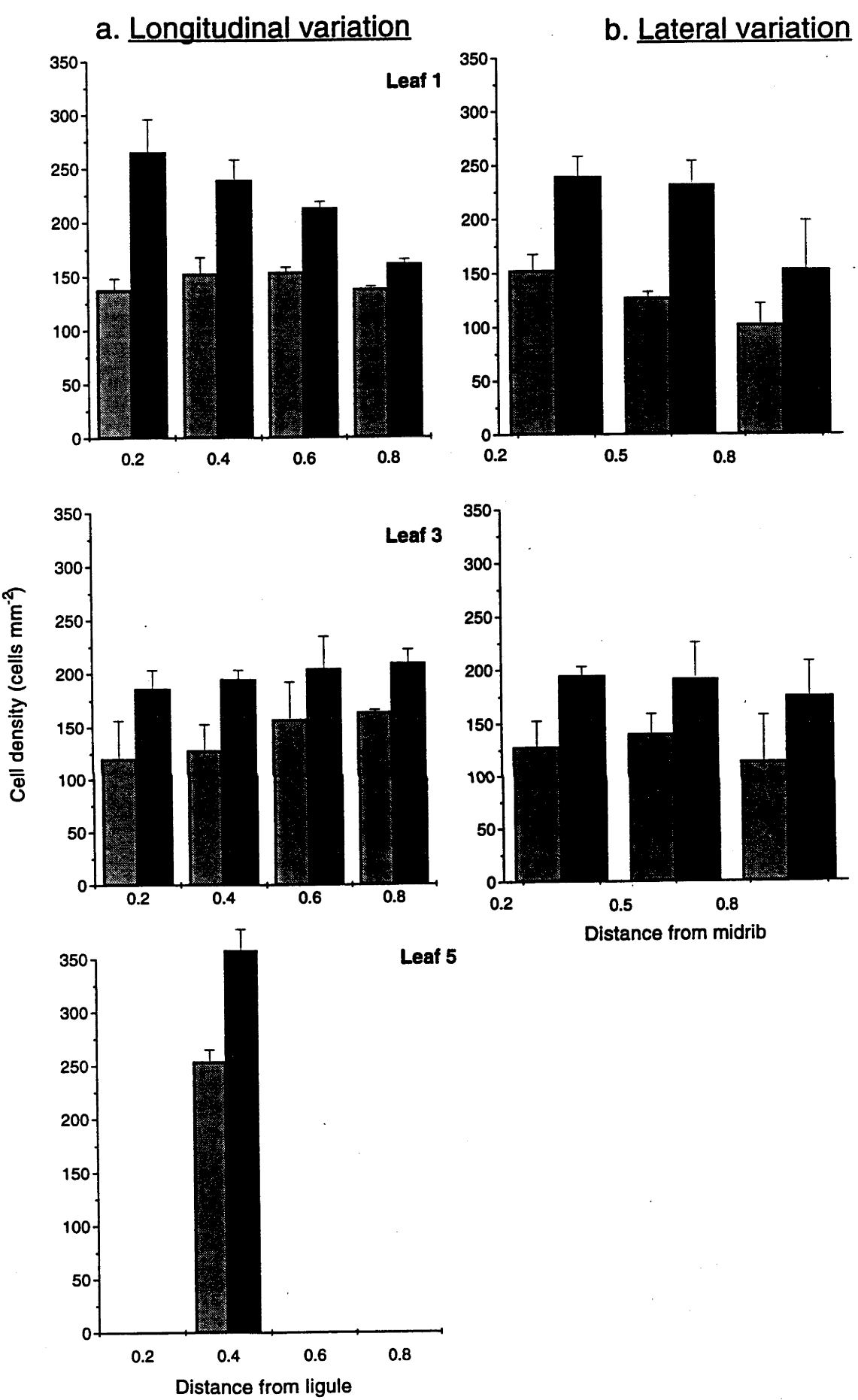
a. Longitudinal variation
b. Lateral variation
Leaf 1
Leaf 3
Leaf 5
Cell density (cells mm-2)
0
50
100
150
200
250
300
350
0.2
0.4
0.6
0.8
0.5
Distance from midrib
Distance from ligule

Figure 8. Total epidermal cell densities for leaf 1, 3 and 5 at low and high R_s (grey and black bars, respectively); a) longitudinal variation within the second interveinal area from the mid-rib; b) lateral variation at a distance $0.4L$ from the ligule. Labels on the x-axis refer to the location of counts, described by the distance from the ligule in panel a) (0 = ligule; 1 = tip of the leaf) and by the distance from the mid-rib in panel b) (0 = mid-rib; 1 = $0.5W$).

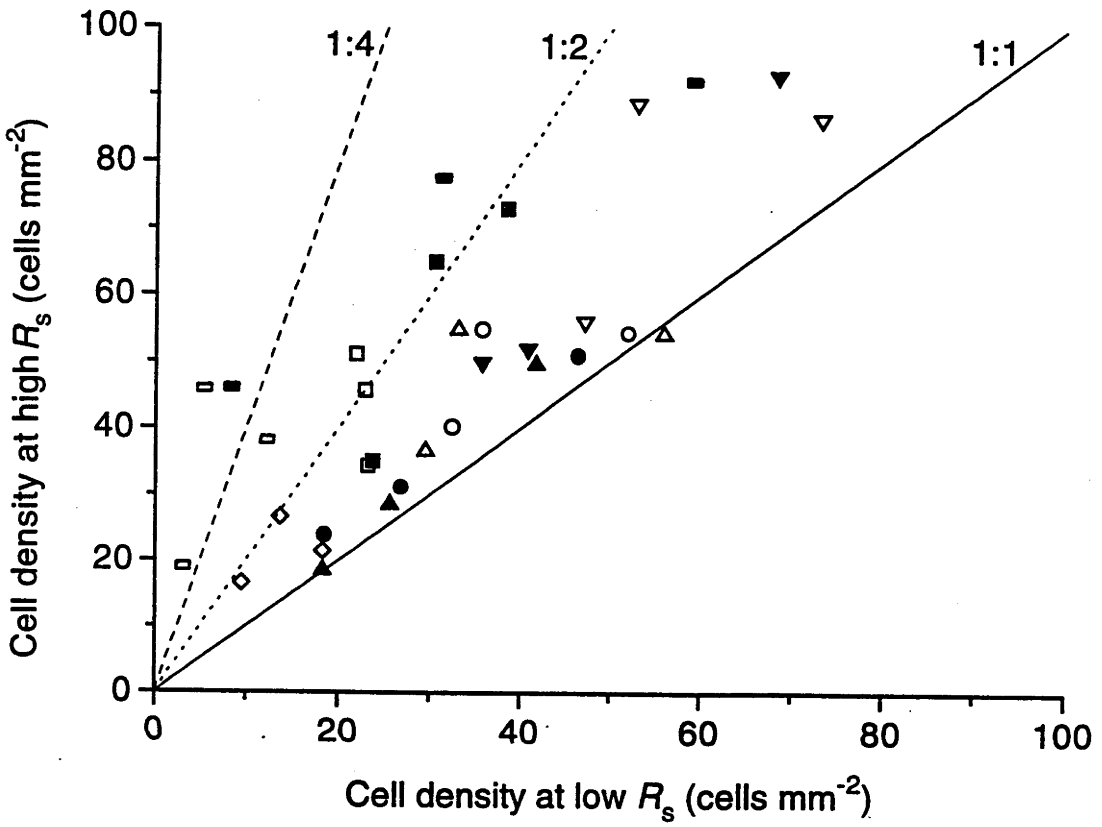

Figure 9. Effects of R_s on the densities of various epidermal cell types in the adaxial and abaxial epidermes (open and solid symbols, respectively); ▭ trichomes ; ▯ elongated cells; o interstomatal cells; ▽ sister cells; △ stomata; ◊ bulliform cells. Data points represent average densities (n=5) for each leaf position (1, 3 and 5) at location 0.4 *L* along the blade and in the second inter-veinal area from the mid-rib.

average as opposed to 54 and 38% in leaf 3 and 5, respectively) and, in that leaf, was greater towards the base of the blade and in central files compared to more distal or marginal locations. In leaf 3, cell densities were spatially more uniform. Densities of trichomes and of elongated cells were the most increased (100 to 200%) while the densities of stomata and interstomatal cells were less affected (25 % at the most) (Fig. 9). The increase in the density of sister cells was intermediate.

Changes in the relative proportions of various cell types with R_s can be quantified by comparing cell indices in which the number of cells of a given type is weighed by the abundance of these cells relatively to the entire population (Fig. 10). By definition, these indices take values ranging between 0 and 1 and the total of the indices calculated for a given sample equals 1. Figure 10a shows that indices for trichomes and elongated cells were significantly increased at high R_s while indices for stomata and derived cells were reduced. These shifts were not solely caused by the large increase in trichome number. When trichomes were excluded from the total number of cells for the calculation of cell indices (Fig. 10b), there was still a clear trend to an increase in elongated cell indices and a decrease in stomatal indices. Within a leaf, sister cells were the most abundant (ca 30% of all cells). As expected from the genesis of the stomatal complex (Tomlinson, 1974), the densities of sister cells, stomata, and interstomatal cells were in a stable ratio close to a 2:1:1 ratio. Elongated cells were in similar or slightly lower number than stomata and interstomatal cells while, on the adaxial side of the blade, bulliform cells were significantly fewer.

Discussion

Whole leaf growth responses to variations in soil mechanical resistance have been well documented (Masle and Passioura, 1987; Masle, 1992). The present study confirms that high R_s severely restricts leaf expansion and mature leaf size in wheat seedlings and provides new information on some of the anatomical modifications underlying these

metabolic effects of high R_s. Earlier studies with the same experimental system have shown that in an initial period, reduced leaf expansion at high R_s could not be explained by reduced assimilates, nutrients or water supply, and that signal(s) elicited in the roots were acting as primary trigger (Masle and Passioura, 1987; Masle, 1990; Masle et al., 1990). The nature of these signals and the cascade of metabolic events that they generate in leaf meristems are still speculative. Abscisic acid and related compounds or ethylene appear as likely candidates (Apelbaum and Yang, 1981; Parker and Ford, 1982; Zhang and Davies, 1990; Tardieu et al., 1992; Spollen et al., 1993). However, Masle et al. (1990) also provided evidence that in a second period, leaf growth becomes carbon limited, but with a lower responsiveness to sugar concentrations than at low R_s. From their study, we can infer that, in the present experiment, such a limitation would have developed only after leaf 1 was fully expanded but while leaf 3 and 5 were expanding.

The size of bulliform cells was the least reduced by high R_s while that of elongated cells was the most (Figs 3&7). For this latter type this size reduction was to a large extent related to the increased frequency of trichome formation (Fig. 9). Trichomes are small, hair bearing cells produced by a final asymmetrical division of some meristematic cells (Stebbins and Shah, 1960). Such asymmetrical divisions were seen at the distal end of the leaf meristem in files of both sister cells and elongated cells (chapter 4). Trichomes were derived from the smallest daughter cell while the biggest daughter cell differentiated into non-specialised elongated or sister cells which were much shorter and narrower than those produced by symmetrical division (Figs 4&7). Such pairs of cells were scattered along whole files, with no apparent pattern, and no consistency between files. Adjacent cells in neighbouring files were not necessary smaller than cells preceding or following them in the file. Together these observations indicate that asymmetrical partitioning was associated with a shorter cell cycle than symmetrical partitioning or with locally prolonged meristematic activity. Both interpretations would lead to smaller cell sizes at maturity and it is known that division rates in leaf meristems vary both spatially and temporally (eg Poethig and Szymkowiak, 1995 and also chapter 4).

As a result of reduced cell sizes, blades grown at high R_s were characterised by increased cell densities. Following from the above, this was mostly due to an increased density of trichomes and of elongated cells; however, the densities of sister cells and bulliform cells were also increased. Stomatal densities were the least increased (adaxial epidermis), or even not significantly different from those at low R_s (abaxial epidermis, leaves 1 and 3). Earlier studies with the same genotype had shown that, on a leaf area basis, stomatal conductance was decreased by high R_s (Masle and Passioura, 1987), and that photosynthetic capacity was increased to such an extent that the rate of CO_2 assimilation was enhanced (Masle and Farquhar, 1988). The present data show that the former effect was the direct result of reduced stomatal aperture. They also suggest that the increased capacity for photosynthesis may only reflect an increased amount of photosynthetic machinery per unit area following from higher mesophyll cell densities, rather than changes in the activity of photosynthetic enzymes.

As a whole these effects of high R_s on the anatomy of mature leaves are typical of the syndrome associated with water shortage. Reduced cell sizes leading to increased cell densities, increased frequency of trichome formation, reduced stomatal index- have also been reported in a number of experiments in response to drought (Zalenski, 1904; Yapp, 1912; McCree and Davis, 1974; Quarrie and Jones, 1977; Zagdanska and Kozdoj, 1994) or to treatments that influence the water status of developing leaves (Jones, 1985; Ristic and Cass, 1991). These similarities reinforce the proposition formulated by (Masle and Farquhar, 1988)that plants at high R_s may evolve an early warning system in anticipation of a more likely shortage of water, which induces a number of morphological and physiological adaptations enabling conservation of water. Masle and Passioura (1987) suggested that plants growing at high R_s are reminiscent of bonsai plants. The present study does not give support to that analogy; in their comparative study of a number of bonsai plants Körner et al. (1989) indeed found that mature leaves were made of fewer and often larger cells. In our wheat plants, high R_s had totally (leaf 1) or partially (leaf 3 and 5) opposite effects.

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Introduction

Growth of young wheat plants is sensitive to soil resistance to root penetration (R_s) (Masle and Passioura, 1987; Masle, 1990; Masle, 1992). As R_s increases, leaf growth is reduced (Masle and Passioura, 1987; Masle et al., 1990); so is root growth, although to a lesser extent (Masle, 1992). Leaves appear more slowly, expand more slowly (in terms of both area and dry weight), and reach a smaller mature size (Masle, 1992). In the short term, these responses could not be attributed to lower water potential, nor to reduced carbohydrate or nutrient supply (Masle and Passioura,1987; Masle, 1990), therefore suggesting that a chemical signal originating from the roots is acting as the primary trigger of decreased leaf growth. Longer term experiments showed a positive relationship between growth rate and carbohydrate concentration in the leaves (Masle et al., 1990). Because a given growth rate was associated with higher carbohydrate concentrations in leaves grown at high R_s, it was concluded that increased R_s caused the sensitivity of the growing leaf to carbohydrate to be reduced .

To gain a better understanding of the underlying mechanisms we decided to investigate the cellular bases of the observed differences in whole leaf growth. As a first step, we concentrated on leaf anatomy and histogenesis. Changes in the epidermal anatomy of mature leaves (cell types, cell dimensions and number) were reported in the previous chapter. The decreased size of mature leaf blades was attributed to smaller average cell sizes and to differences in the number and types of epidermal cells. The respective contributions of changes in cell number *vs* cell size were also shown to be dependent on leaf position.

The present paper describes the effects of soil resistance on the kinetics of cell elongation and cell partitioning in expanding leaves. These were analysed using the kinematic approach (Erickson and Sax,1956a&b; Green, 1976; Silk and Erickson,1979; Gandar, 1983; Silk, 1984). This approach is based on the continuity equation, commonly

fact that measurements were made on individual cells within a single file of cells, which could be either sister cells (cells in files adjacent to stomatal rows) or trichomes. Also local partitioning rates were estimated using the distribution of newly formed cell walls instead of the number of cells in mitosis, as commonly used in studies on the kinetics of cell division.

Materials and Methods.

Growth conditions

Wheat plants (*Triticum aestivum* L. cv. Egret) were grown in pots in a controlled growth chamber with 18/15 °C day/night temperature, 600 µmol quanta m^{-2} s^{-1} irradiance, 11 h photo- and thermoperiod and a relative humidity of 80%, day and night. Calibrated seeds (32 to 34 mg) were sown at constant depth in soils with low or high mechanical resistance to penetration. The same soil as described by Masle and Passioura (1987) (silty loam with 5% coarse sand, 40% fine sand, 31% silt, 19% clay and 4.5% organic matter) was fertilised with 2.2 g finely ground superphosphate and 357 mg NH_4NO_3 per kg dry soil and homogeneously packed into cylindrical PVC pots (200 mm high and 87 mm diameter) so as to achieve a penetrometer resistance of 0.5 and 7.5 MPa. These contrasted soil resistances were obtained by varying both soil bulk density and soil water content as in Masle and Passioura (1987) and Masle et al. (1990). These parameters were set as 1.10 - 1.15 g cm^{-3} and 0.24 g H_2O g^{-1} dry soil, respectively, at low R_s and 1.42 g cm^{-3} and 0.22 g H_2O g^{-1} dry soil at high R_s. Pots were watered at least daily so as to maintain these two parameters as close as possible around their initial values.

Morphometric measurements

Leaf length was measured twice a day (just after the onset and just before the end of the light period). Leaf 1, 3, and 5 on the main stem were harvested 2 to 3 days (at low and high R_s, respectively) after emergence from the sheath of the previous leaf (or above

the soil surface for leaf 1). Leaves were harvested at a similar stage of development for both treatments, during the period when elongation is approximately linear with respect to time. In order to avoid roots becoming pot-bound and also to restrict variations of soil water content between waterings, it was necessary to work on young plants i.e. on the first leaves. However, since leaf 1 and 3 are in wheat initiated in the embryo, their histological features will partly reflect seed characteristics. Leaf 5 was therefore also analysed as representative of leaves which have undergone their whole development at low or high R_s. Leaves were immediately immersed in boiling methanol until all chlorophyll was removed, and then transferred to lactic acid for clearing and storage.

Five leaves were analysed at each position (1, 3, and 5). The cleared leaves were mounted on a light microscope (Zeiss axioscope) fitted with a Panasonic video camera (model WV-CL 702E). A file of sister cells which could easily be traced through the basal 35 mm of the leaf epidermis was then selected. The lengths of all cells along that file were measured from video-images using the morphometric program MTV (Garr Updegraff/ Datacrunch, 1991). Trichomes were measured separately from the sister cells with which they were associated. In the division zone, the location of recently formed (thinner) cell walls was also recorded. Within each file, expanding cells could be separated into three groups, defining three contiguous segments: a) a first segment at the base of the leaf, where cell partitioning takes place, and gives rise to two daughter cells of similar size. This segment, typically 3 to 5 mm long was identified by the presence of thin cell walls, interpreted as "fresh" cell walls, reflecting recent divisions and was characterised by an independence of cell size on location along the file. b) a middle segment, much shorter, characterised by daughter cells of unequal dimensions produced by a final asymmetrical division of a cell, with the bigger daughter cell being identifiable as a sister cell, and the smaller, distal cell, as a trichome. In these two first segments the processes of elongation and of partitioning co-occur. c) a distal segment, where cells expand but no longer undergo division; these cells have thickened walls and increase in length from the proximal to the distal end of the segment, where maximal, final length is achieved. These three segments as a whole constitute the growth zone, beyond which cells have stopped expanding and only undergo physiological maturation. In the

remainder of the text, these three segments will be referred to as: zone of symmetrical division, zone of asymmetrical division, and zone of elongation only, respectively, and the location of their distal end as x_{sd}, x_{ad}, and x_{el}, respectively.

Kinematic analysis

Kinematic methods described in the literature (e.g. Scott et al., 1967; Volenec and Nelson, 1981; Silk et al., 1989; Schnyder et al., 1990) are based on measurements along files consisting of a single cell type. To enable calculations on composite files including both sister cells and trichomes, it was necessary to introduce the concept of "elements". An element was defined by the combination of a sister cell and, if present, its associated trichome. The lengths of mature elements so defined were used to calculate cell density and fluxes (eqs 2 and 3 below, respectively).

a) elongation zone.

For each leaf, the individual lengths of successive cells (sister cells or trichomes) or of elements were averaged over 0.5, 1.0 and 2.0 mm intervals in the basal (x_{sd} + 2) mm of the leaf, the next 8 mm, and the remaining, most distal part of the growth zone, respectively (data set 1). A second set of 0.5 mm-spaced data was generated for the elongation only zone of each leaf using the fitted parameters of a Richards function to the individual cell lengths plotted as a function of location along the blade. The function used was as in Morris and Silk (1992) but modified to describe cell (or element) length distributions rather than the distribution of velocities, according to

$$\frac{l_f}{(1+e^{-k(x-x')})^{1/n}} \quad \text{or} \quad \frac{l_f^*}{(1+e^{-k(x-x')})^{1/n}} \quad \text{(eq.1)},$$

where l_f and l_f^* denote the length of mature cells and mature elements, respectively, x is the location along the blade, and n, k, and x' are fitting parameters. Although also affecting the spread of the curve, parameter n is mainly related to the position of the inflection point (higher n for higher inflection point). For any given n, parameter k

determines the total spread of the curve along the x-axis (lower k values being associated with greater spread) (Morris and Silk, 1992). Mature cell (or element) length was taken as the average cell (element) length over a 10 mm segment distal to the growth zone (from location (x_0 +25mm) to location (x_0 +35 mm), where x_0 denotes the base of the leaf).

These two data sets were used to calculate time *vs* location relationships in the elongation only zone using the following method:

1. Cell density distribution, $\rho(x)$, was defined as the inverse of elemental length $l^*(x)$

$$\rho(x) = \frac{1}{l^*(x)} \qquad \text{(eq.2).}$$

2. The average cell density $\bar{\rho}(x)$ between two locations, x-0.5dx and x+0.5dx was estimated by averaging the local densities calculated at these two locations.

3. The number of cells in the interval dx was then calculated as $\bar{\rho}(x)\,dx$.

4. The flux of sister cells through any point distal to x_{sd}, F, was obtained using

$$F = \frac{E}{l_f^*} \qquad \text{(eq. 3),}$$

where E is the leaf elongation rate calculated from the several measurements of leaf lengths made prior to leaf harvest, during the phase where elongation is linear with respect to time.

5. The cellochron (c), the time for a cell to be displaced by one position in the elongation zone, is the inverse of F (Silk et al., 1989). The time, $t(x)$, taken for a cell to be displaced from x_{sd} to a particular further location, x, was calculated as

$$t(x) = c.n(x) \qquad \text{(eq. 4),}$$

where $n(x)$ is the total number of sister cells between locations x_{sd} and x (Silk et al., 1989).

Local relative cell elongation rates, $r(x)$, in the elongation only zone were calculated for each cell type (sister cell or trichome) as:

$$r(x) = \frac{d(\ln(l(x))}{dt} \qquad \text{(eq. 5)},$$

where time t is related to location, x and flux, F, as described by equations 2-4 above.

The number of cells per file in the elongation only zone, N_{el}, was taken as the number of cells between x_{ad} and the location where the cell length calculated with the fitted Richards function reached 95% of l_f. The residence time of a cell in the elongation only zone, T_{el}, was calculated as

$$T_{el} = c.N_{el} \qquad \text{(eq. 6)}$$

The contribution, E_{el}, of the elongation only zone to the overall leaf elongation rate (E) is given by the integral $\int_{x_{sd}}^{x_{el}} r(x).dx$. It may also be written as

$$E_{el} = F.(l_f^* - l_{ad}^*) \qquad \text{(eq. 7). This latter formula was the one used to calculate } E_{el}.$$

b) Division zone

Zone of symmetrical division. The *average* partitioning rate ($\overline{p_{sd}}$) is equal to the ratio of the rate at which sister cells are produced to the number of cells in the zone of symmetrical division, N_{sd}, ie

$$\overline{p_{sd}} = \frac{F}{N_{sd}} \qquad \text{(eq. 8, Green (1976)).}$$

The inverse of $\overline{p_{sd}}$ represents the *average* cell cycling time ($\overline{t_c}$), i.e. the time elapsed between two successive cell divisions:

$$\overline{t_c} = \frac{N_{sd}}{F}$$ (eq. 9, Green (1976)).

Local symmetrical partitioning rates at any location x along the division zone, $p_{sd}(x)$, were calculated as the average partitioning rate over intervals of n_i cells around the cell at location x using eq. 8 with n_i instead of N_{sd} and F corrected by the proportion ($\phi_{p,i}$) of all newly formed cross-walls, found in this interval of i cells:

$$p_{sd}(x,i) = \frac{\phi_{p,i}}{n_i} \cdot F$$ (eq.10).

In the present study, the interval, i, was 21 cells; inspection of the data showed that this number allowed a good compromise between resolution and smearing of local variations in $\phi_{p,i}$.

In order to calculate *local* cell elongation rates in the zone of symmetrical division, local velocity ($v(x)$) was calculated according to Gandar and Rasmussen (1991) as follows:

If y(x) is the rate of cell production per unit length per unit time, then the cumulative rate of cell production up to x is

$Y(x) = \int_{x_0}^{x} y(s).ds$, where s indicates all values between x_0 and x. This quantity (Y) is a flux; so

$$Y(x) = \frac{v(x)}{l(x)}$$ (Silk et al., 1989).

At $x=x_{sd}$, $Y_{sd}=F$, so that

$$\frac{Y(x)}{Y(x_{sd})} = \frac{v(x)}{l(x)} \cdot \frac{1}{F} \quad .$$

We write $\dfrac{Y(x)}{Y(x_{sd})} = \dfrac{\int_{x_0}^{x} newcellwalls\,.ds}{\int_{x_0}^{x_{sd}} newcellwalls\,.ds}$ as $\phi_{p,x}$.

It then follows that

$$v(x) = \phi_{p,x} \cdot F \cdot l(x) \qquad \text{(eq. 11)} \qquad (14).$$

Parameter $\phi_{p,x}$ increases from 0 at x_0 to 1 at x_{sd} ; $l(x)$ was estimated as the average cell length in an interval of 11 cells around location x.

Local relative cell elongation rates were then calculated over intervals of 20 cells around location x (total length=dx), as

$$r(x) = \frac{dv}{dx} \quad \text{(eq.12; Silk et al., 1989)},$$

where dv is the difference in the velocities calculated at the extremities of the interval. In order to reduce the noise in $r(x)$, local velocities $v(x)$ were first smoothed using a running average of 20 cells.

The total elongation generated in the zone of symmetrical division, $E_{sd} = \int_{x_0}^{x_{sd}} r(x).dx$ was more simply calculated as

$$E_{sd} = F.l_{sd} \qquad \text{(eq.13)},$$

where l_{sd} is the cell length at the x_{sd} .

Table I. Average leaf elongation rate, E, over the 48 h preceding leaf harvest and corresponding fluxes of sister cells, F, calculated from these values and from the average mature elemental lengths, l_f^*. For each variable, the two first columns show average values for the two R_s; the third column gives the difference (Δ) between these values expressed as the percentage of the low R_s mean). Asterixes denote statistically significant differences ($p < 1\%$ ***; $1\% < p < 5\%$ **; $5 < p < 10\%$ * from ANOVA analysis of variance with $n = 5$).

Leaf	E (m.m.d^{-1})			F (cells.d^{-1})			l_f^* (µm)		
	R_s (MPa)		Δ (%)	R_s (MPa)		Δ (%)	R_s (MPa)		Δ (%)
	0.5	7.5		0.5	7.5		0.5	7.5	
1	26.2	17.2	-34 ***	98	81	-17 **	268	214	-20 ***
3	30.2	24.0	-21 **	131	112	-14 **	231	215	-7 *
5	27.2	20.4	-25 **	120	115	-4	228	178	-22 ***

growth zone. To nevertheless enable calculation of average local rates over the sample of leaves harvested for a treatment, local partitioning and elongation rates calculated for individual leaves were first averaged over 0.5 mm intervals.

Fitting of the modified Richards function was done with the PC program Microcal Origin (version 3.5; Microcal Software Inc.) and the statistical package Genstat 5 (version 2.1; Lawes Agricultural Trust (Rothamsted Experimental Station)) was used for ANOVA analysis of variance of all calculated parameters.

Results.

Whole leaf elongation rate, E, was always significantly slower for plants grown on soils with high R_s compared to low R_s ($p < 0.001$; I). In leaf 1 and 3 the number of cells moving out of the elongation zone per unit of time (F) was smaller and these cells were shorter (Table I); in leaf 5 cell flux was not affected by R_s, ie the reduction in E was proportional to that in l_f. A fraction of 7 to 10% of the overall leaf elongation rate was generated in the zone of symmetrical division, 4 to 6% in the zone of asymmetrical division, and the remaining 85 to 90% in the elongation only zone. Soil resistance had a significant negative effect on both E_{sd} and E_{el} in all leaves; the only exception was in leaf 1 for which the overall side-wall expansion occurring in the zone of symmetrical division was similar at the two R_s (data not shown)..

The overall elongation generated in the growth zone, E, (or part of it, eg E_{sd}) is the integral of local relative elongation rates, $r(x)$, over the whole growth zone (or that part):

$$E = \int_{0}^{L_{gz} \text{ or } L_{sd}} r(x)dx$$ (see Methods).

Parameters relating to cell division do not figure in this expression in keeping the point made by other workers (eg Green, 1976) that cell partitioning does not generate

Table II. Average length (mm) and standard error (n = 5) of: the growth zone as a whole (L_{gz}), the zone of symmetrical division (L_{sd}), the zone of asymmetrical division (L_{ad}) and the elongation only zone (L_{el}). Asterisques denote statistically significant differences as in Table I.

	Leaf	R_s (MPa)		Difference (Δ, %)
		0.5	7.5	
L_{gz}	1	25.3 (1.4)	23.8 (1.7)	-6
	3	27.0 (1.3)	24.0 (1.0)	-9
	5	26.3 (1.8)	21.0 (2.4)	-20 **
L_{sd}	1	1.6 (0.2)	1.8 (0.1)	9
	3	3.4 (0.1)	2.0 (0.1)	-40 ***
	5	4.3 (0.4)	2.5 (0.2)	-42 ***
L_{ad}	1	1.2 (0.3)	1.5 (0.1)	25
	3	1.6 (0.6)	1.4 (0.3)	-10
	5	1.5 (0.4)	1.5 (0.3)	6
L_{el}	1	22.4 (1.1)	20.5 (1.7)	-9
	3	21.7 (1.8)	20.8 (1.0)	-4
	5	20.5 (1.8)	17.0 (2.4)	-17

Table III. Average number of cells in a file of sister cells and standard error (n = 5) in: the growth zone as a whole (N_{gz}), the zone of symmetrical division (N_{sd}); the zone of asymmetrical division (N_{ad}) and the elongation only zone (N_{el}).

	Leaf position	R_s (MPa)		Difference (Δ, %)
		0.5	7.5	
N_{gz}	1	349 (17)	355 (18)	2
	3	396 (18)	358 (18)	-10 *
	5	457 (11)	414 (16)	-9 *
N_{sd}	1	77 (9)	79 (9)	3
	3	144 (8)	95 (9)	-34 **
	5	198 (16)	133 (10)	-32 ***
N_{ad}	1	58 (11)	70 (5)	20
	3	48 (15)	57 (8)	18
	5	52 (13)	72 (11)	39
N_{el}	1	212 (11)	207 (15)	-3
	3	204 (26)	206 (13)	1
	5	207 (13)	208 (20)	0

growth. Rates of cell partitioning become important only if they influence one of the terms of the above equation. Examples of such an influence would be if L_{gz} were related to cell number rather than having a fixed length or if values of r reflect cell size rather than position within a growth zone of fixed dimensions. By analysing changes in cell partitioning rates and in L_{gz}, as well as cell elongation rates, we can analyse these processes.

Size of the growth zone

The total length of the growth zone (L_{gz}) was similar in leaves at a particular R_s and relatively little affected by R_s (Table II). There was a systematic trend for L_{gz} to be shorter at high R_s but this reduction in size was only significant in leaf 5 ($p=0.034$). However, leaf position and R_s affected the relative sizes of the division zone and elongation only zone (Table II). At low R_s the division zone was nearly three times longer in leaf 5 than in leaf 1 and in both leaf 3 and 5 was 40% shorter at high than low R_s, while being unchanged in leaf 1. In contrast, the elongation zone tended to be shorter in higher order leaves and, although consistently shorter at high than low R_s, was relatively less sensitive to soil conditions with the reduction in L_{el} being statistically non significant (Table II). The length of the zone where asymmetrical division took place varied little.

The size of each individual zone within the growth zone can be described in terms of length as above, or in terms of the number of cells along a file in that zone (Table III). When this is done, the above conclusions still hold. However, the size of the elongation zone (N_{el}) now emerges as a remarkably conserved characteristic, which was independent of leaf position and soil conditions. Secondarily and consistent with the increased frequency of trichomes observed in mature blades grown at high R_s (see below and Chapter 3) the number of cells in the zone of asymmetrical division was in all leaves greater at high R_s.

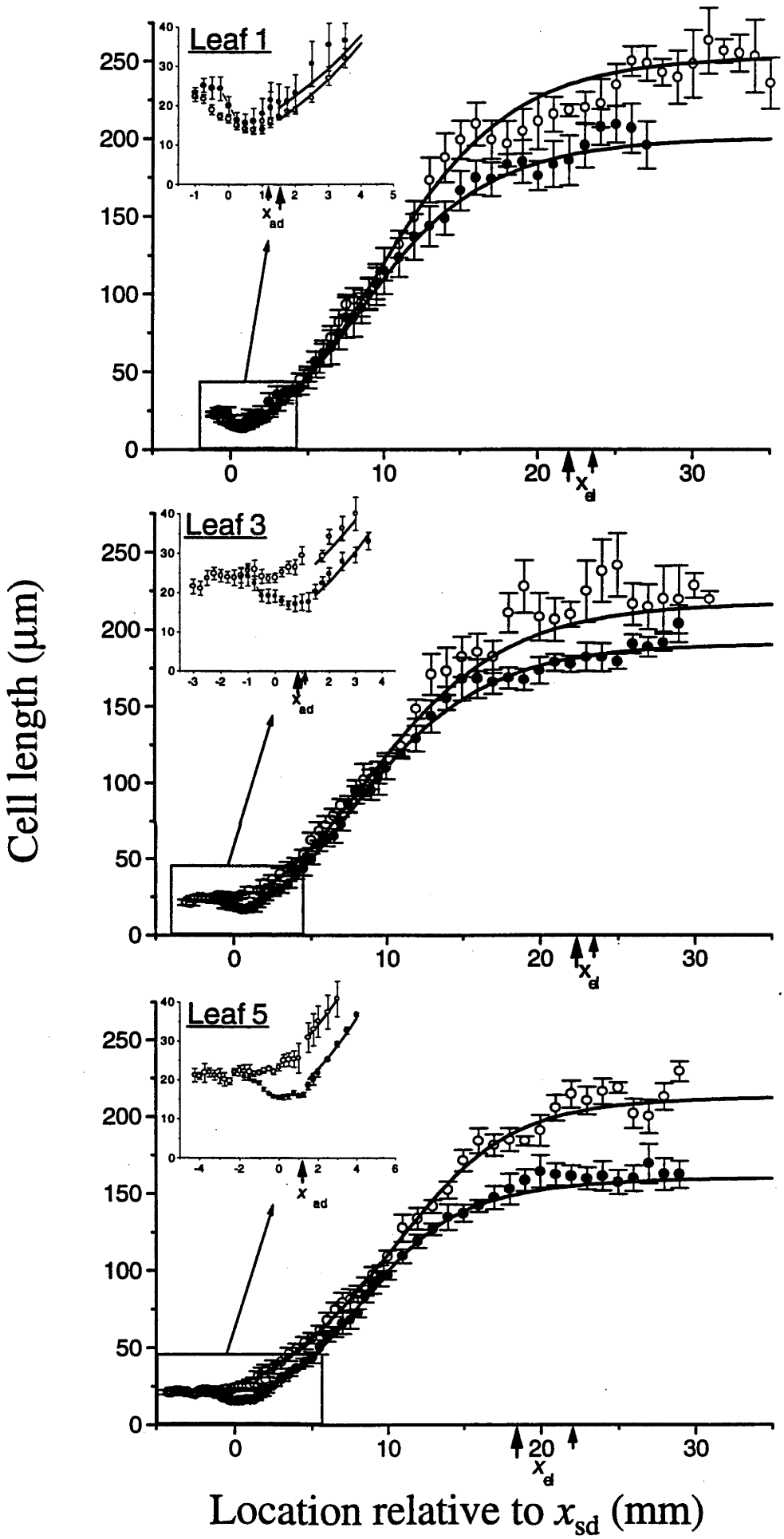

Leaf 1
Leaf 3
Leaf 5
Cell length (μm)
Location relative to x_{sd} (mm)
x_{ad}
x_{ed}

Figure 1. Relationship between average cell length and distance from x_{sd} (location 0) along a file of sister cells in leaf 1, 3 and 5, at low and high R_s (open and closed circles, respectively). Data points denote averages of individual cell lengths over 0.5, 1, or 2 mm intervals, depending on location along the growth zone (see Methods). Data for 4 to 5 leaves were averaged at each position, and the error bars denote standard errors of the mean. Solid lines describe fitted values from the Richards function (see Methods). Vertical arrows on the x-axis indicate in the inset the location of x_{ad} and in the main panel, that of x_{el} at low and high R_s, respectively (small and large arrows, respectively).

Cell length distribution

The above data imply significant variations of the rate of cell expansion, with both leaf position and R_s. Given the variation in L_{sd}, cell length distributions and derived kinetic parameters were compared with respect to position from x_{sd} rather than from the base of the leaf (Figs1-6). The distributions of cell lengths along the growth zone of leaf 1, 3, and 5, are shown in Fig.1. In all three leaves, meristematic cells at the base of the division zone were of similar length at low and high R_s. In leaf 3 and 5 cell length remained stable throughout the division zone at low R_s, while at high R_s it steadily declined, reaching a minimum value around x_{sd} (see insets in Fig. 1). In those leaves, sister cells at the base of the elongation zone were 25 to 30% shorter at high than low R_s. The difference at the end of the elongation zone was relatively smaller (15 to 25%, Table I) despite the elongation zone being slightly shorter. This was because cells were displaced through the elongation zone more rapidly at low than high R_s (shorter T_{el}, see Fig 2, right panel). In leaf 1 (inset Fig. 1a) a different pattern was observed: within 0.5 mm into the division zone, meristematic cells were in fact significantly longer at high than low R_s. However, their size declined sharply at the distal end of the division zone to the same length as cells in unstressed leaves. The cell length distributions observed at the two R_s progressively diverged in the elongation zone. Within about 12mm of x_{sd}, cells became significantly smaller at high R_s and mature cells were about 20% shorter, as for leaf 3 and 5.

Cell lengths of trichomes were also measured. The frequency of sister cells associated with trichomes in mature blades was much greater at high than at low R_s ($p < 0.001$) in leaves 3 and 5 (94 vs 70%, and 79 vs 45%, respectively), while being similar in leaf 1 (80%). In all leaves trichomes were much smaller than the associated sister cells (4 to 8 μm at x_{ad} against 15 to 25 μm for sister cells and 22 to 27 μm compared to 150 to 250 μm at the mature stage) and were only 5 to 10% shorter at high than at low R_s ($p<0.001$).

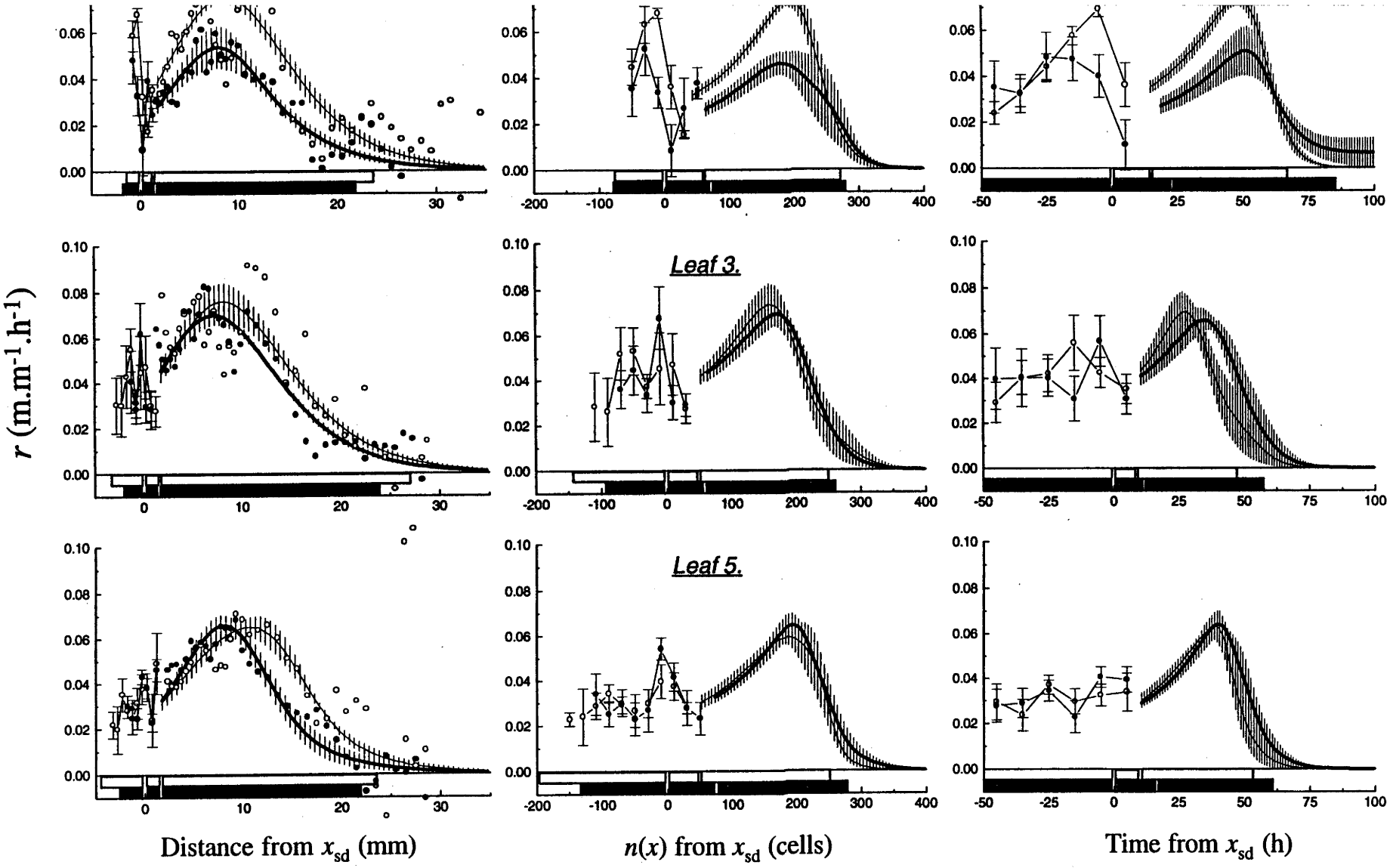

r (m.m-1.h-1)
Leaf 3.
Leaf 5.
Distance from xsd (mm)
n(x) from xsd (cells)
Time from xsd (h)

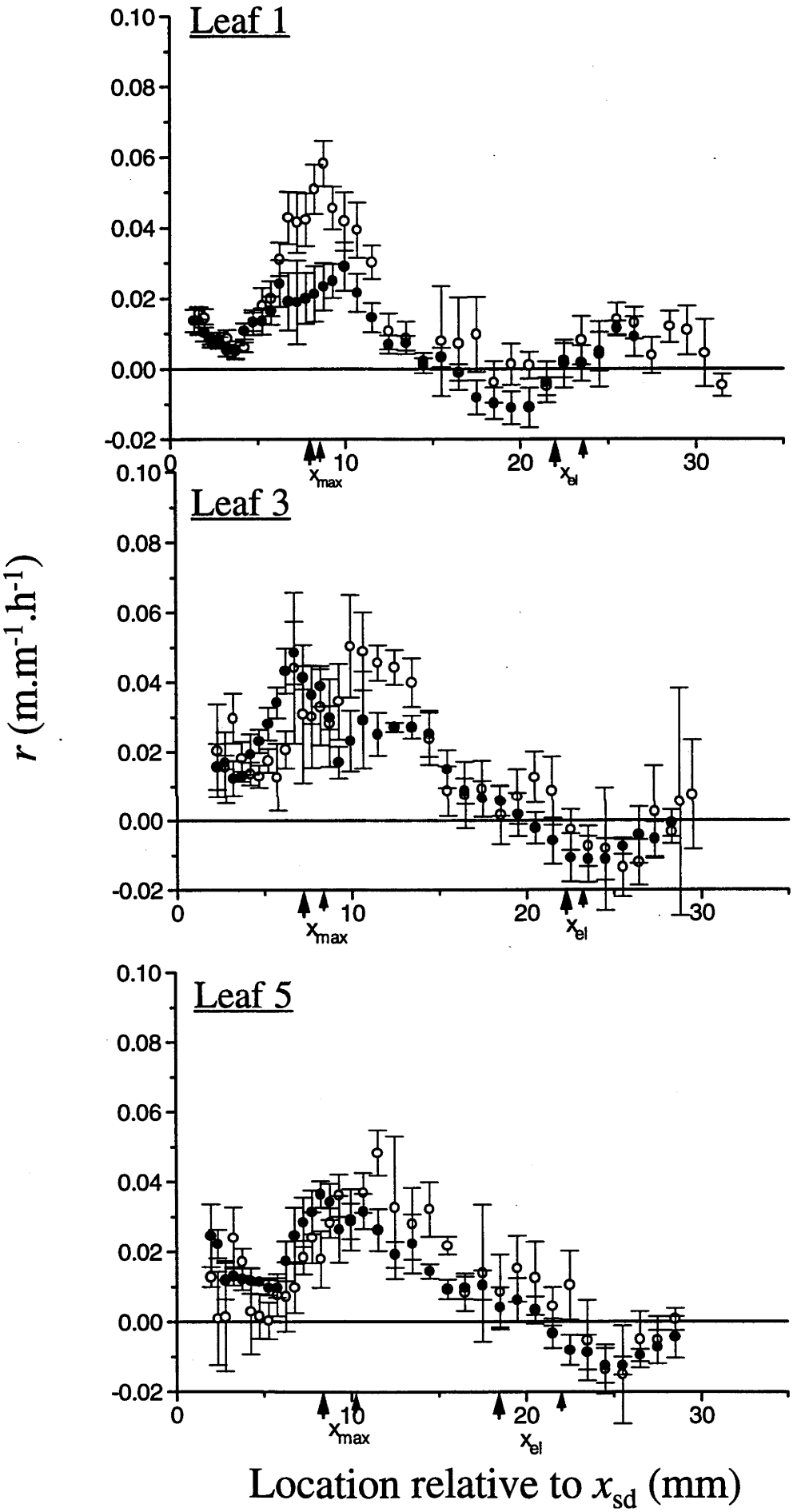
Figure 2. Local average relative cell elongation rates of sister cells along the growth zone of leaf 1, 3 and 5 plotted as a function of: physical distance from x_{sd} (left panel), rank of the cell along the file (middle panel) and time since the cell moved out of the division zone (right panel). In the zone of symmetrical division, data points (open and solid symbols for low and high R_s, respectively) represent moving averages of raw data and associated standard errors. For the remainder of the growth zone, moving averages are only shown in the left panel; the lines (thin and thick line at low and high R_s, respectively) describe rates derived from fitting cell lengths distributions by the Richards function (see Methods). The two horizontal bars on the x-axis describe for low and high R_s leaves (open and solid bar, respectively) the length of the growth zone (left panel), the corresponding number of cells (middle panel), and time for a cell to move through it (right panel). These bars are subdivided into 3 segments corresponding, from left to right, to: the zone of symmetrical division (negative abscissae), the zone of asymmetrical division (from 0 to the first vertical mark) and finally the zone of elongation only.

Cell elongation rates

Figure 2 describes the relative rates of cell expansion along the growth zone. In the elongation zone, there was a good correlation between the relative cell elongation rates (r) calculated from the fitted Richards function and the values obtained with the averaging method (both are on the left panel in Fig. 2). The data obtained with this function could therefore be confidently used to compare $r(x)$ values between treatments. For each leaf the spatial and temporal patterns of cell elongation are shown (leaft and right panel, respectively). The middle panel shows the variation in r as a function of cell rank ($n(x)$) along the file; in the elongation zone, this is equivalent to measuring cell age in terms of number of cellochrons. The three axes are inter-related as expressed by equations 2-4.

Elongation rates of non-proliferative cells. Local variation in $r_{el}(x)$ along the elongation only zone was important (Fig. 2, left panel) and followed a consistent pattern for all leaves and treatments, similar to the spatial pattern reported in other studies of leaf and root meristems (Erickson and Sax, 1956b; Volenec and Nelson, 1981; Morris and Silk, 1992): rates increased sharply from x_{sd} towards a location (x_{max}), 7 to 10 mm distal to x_{sd}, where it reached its maximum (r_{max}). The maximum rate, r_{max}, was maintained in a short region only, of about 2 mm, beyond which a steady decline occurred. At low R_s, r_{max} had similar values for all leaves, of 7-7.5% per hour, but was achieved 2.5 mm further into the elongation zone in leaf 5 than in leaves 1 and 3.

High R_s caused a reduction in relative cell elongation rates in all leaves. But the magnitude of this effect and its spatial characteristics varied with leaf position. In leaf 1, r_{el} was significantly slower at high R_s throughout the elongation zone (20% decrease in r_{max} ($p<0.05$)). In leaf 5, this was only the case in the distal half of the elongation zone with rates at more proximal locations being in fact greater at high R_s than in the control. Leaf 3 showed an intermediate pattern. In these two leaves high R_s did not affect the value of maximum elongation rate but caused a displacement of x_{max} towards the base of the leaf, of 1.2 and 1.8 mm for leaf 3 and 5, respectively (Fig. 2a). An average


Leaf 1
Leaf 3
Leaf 5
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
0
10
20
30
x_{max}
x_{el}
r (m.m^{-1}.h^{-1})
Location relative to x_{sd} (mm)

ıre 3. Local relative elongation rates of trichomes associated with the sister cells
ribed in Fig. 2 for leaf 1, 3 and 5. Open and closed symbols represent low and high
ıta, respectively. Rates were calculated with the averaging technique described in
ıods. Vertical arrows on the x-axis indicate the location of x_{max} and x_{el} at low and
R_s (small and big arrow, respectively).

displacement of 0.8 mm also occurred in leaf 1, but this was not statistically significant. However, when expressed relatively to the length of the elongation zone, the location where maximum elongation rate occurred was in all leaves remarkably insensitive to soil conditions, being at half-way along the elongation zone in leaf 5, and 35 to 39% along in leaf 1 and 3.

In all leaves the spread of the distribution of cell elongation rates at high R_s was increased relative to that at low R_s when rates were plotted as a function of cell rank, ie cellochron age (middle panel in Fig. 2). Given the near constancy of the total number of cells in the elongation zone, this lateral displacement reflects greater local cell densities (shorter cells) at high R_s. In leaf 1 and 3 low R_s rates were even less spread compared to high R_s rates when cell age was measured in chronological time (right panel): this further relative displacement of the two distributions is a measure of increase in the cellochron ($c=1/F$) at high compared to low R_s. In leaf 5 where the cellochron varied little with R_s, the temporal patterns of cell elongation were similar at the two R_s: maximum rates occurred about 40 h after the cell had moved out of the zone of symmetrical division and approached zero 12 to 18 h later at low and high R_s, respectively (difference not statistically significant, see horizontal bars on the x-axis in Fig. 2 (right panel)). This means that for this leaf, the offset of the spatial distributions of elongation rates mostly reflected the shorter length of the elongation zone in leaves grown at high R_s. In contrast, in leaf 1, cells elongated more slowly at high R_s, to the point where more time was required to move a cell through the elongation zone (53 and 65 h at low and high R_s, respectively (Fig. 2, right panel)), despite this zone being shorter.

The relative elongation rates of the trichomes associated to the sister cells described in Fig. 2 are shown in Fig. 3. These rates initially decreased with increasing distance from x_{sd} in the basal part of the elongation only zone. At more distal locations, local rates showed a similar pattern of spatial variation as for sister cells, and reached a maximum at approximately the same location (ie around x_{max}). Maximum relative elongation rates were roughly 50% lower for trichomes than for the associated sister cells and in leaves 1

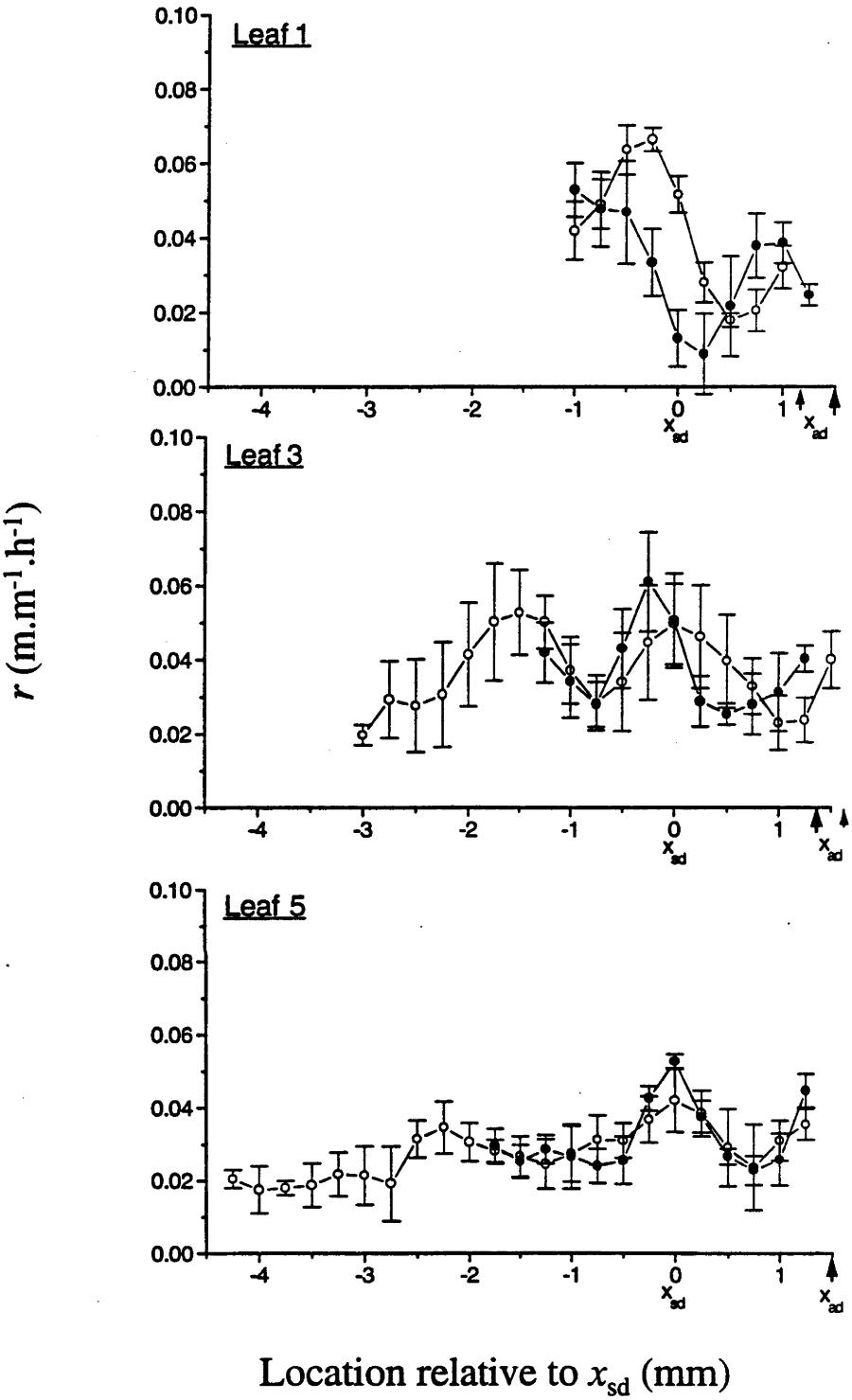


Figure 4. Local average relative elongation rates in the division zone of leaf 1, 3 and 5 (same data as in Fig. 2, left panel, negative abscissae, but plotted on an expanded scale). Symbols as in Fig. 3. Vertical arrows on the x-axis indicate the location of the end of the zone of asymmetrical division (x_{ad}) , at low and high R_s (small and large arrow, respectively).

and 3 elongation stopped closer to the leaf base, at about two-thirds along the elongation zone (Fig. 3). The effects of R_s however, were, in terms of direction and magnitude, quite similar for the two cell types.

Elongation rates of proliferative cells. The rates of expansion of meristematic cells in the zone of symmetrical division were significant, comparable to the rates calculated for non-proliferative cells (Fig. 2). Because the division zone was physically very short compared to the elongation only zone, the spatial variation of r_{sd} with respect to distance from x_{sd} (rates for negative abscissae in Fig. 2) are shown in more detail on an expanded scale in Fig. 4. In leaf 1, r_{sd} was high at the base of the meristem, similar to the maximum rates calculated in the elongation zone, and similar at high and low R_s. At high R_s it declined rapidly as x_{sd} was approached while remaining high at low R_s over most of the division zone. In contrast, in leaf 3 and 5, r_{sd} was insensitive to R_s and, especially in leaf 5, more uniform spatially and through time than in leaf 1 (Fig.2, panel b&c).

Cell partitioning rate.

Figure 5 describes the local rates of cell partitioning in the zones of symmetrical and asymmetrical division (p_{sd} and p_{ad}, respectively (eqs 10 and 15)). There is a small overlap of the p_{sd} and p_{ad} distributions around x_{sd} because a few fresh walls were occasionally observed beyond the location where the first asymmetrical division occurred, and also because of some smearing related to the use of moving averages over 21 cells (see Methods). In all leaves the overall rate of partitioning (sum of p_{sd} and p_{ad}) was maximum in this short region of about 1mm around x_{sd}. At low R_s, partitioning rates decreased markedly from leaf 1 to 5, whereas at high R_s there was no consistent ontogenetic effects. This reflects a strong and unexpected interaction between R_s and leaf position in the response of cell partitioning rate: in leaf 1, high R_s caused a reduction in both p_{sd} and p_{ad}, except at the very base and very tip of the division zone, whereas in leaf 3 and 5, these rates were in fact enhanced. This shift in the effects of R_s with leaf position is reflected in the respective durations of the average cycling time in the zone of

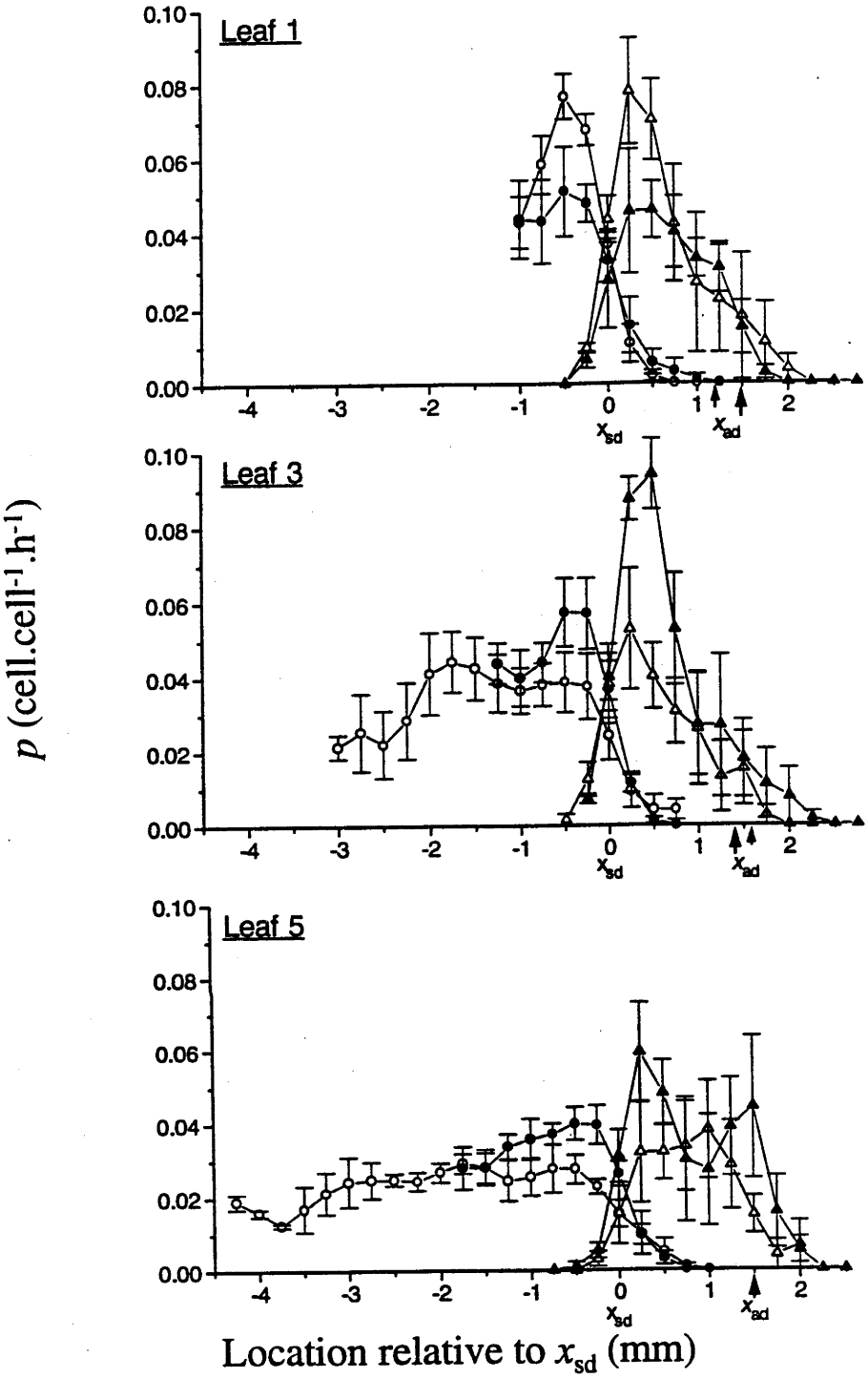


Figure 5. Local relative rates of symmetrical and asymmetrical partitioning in the division zone (p_{sd} and p_{ad}, represented by circles and triangles, respectively). Open and solid symbols denote low and high R_s rates, respectively.

Table IV. Average cell cycle duration ($\overline{t_c}$, (h)) and standard errors (into brackets, n = 5).

Leaf position	R_s (MPa)		Difference (Δ%)
	0.5	7.5	
1	19.1 (2.0)	24.7 (4.1)	30
3	26.7 (2.1)	20.6 (2.5)	-23 *
5	39.7 (3.1)	27.9 (2.0)	-30 **

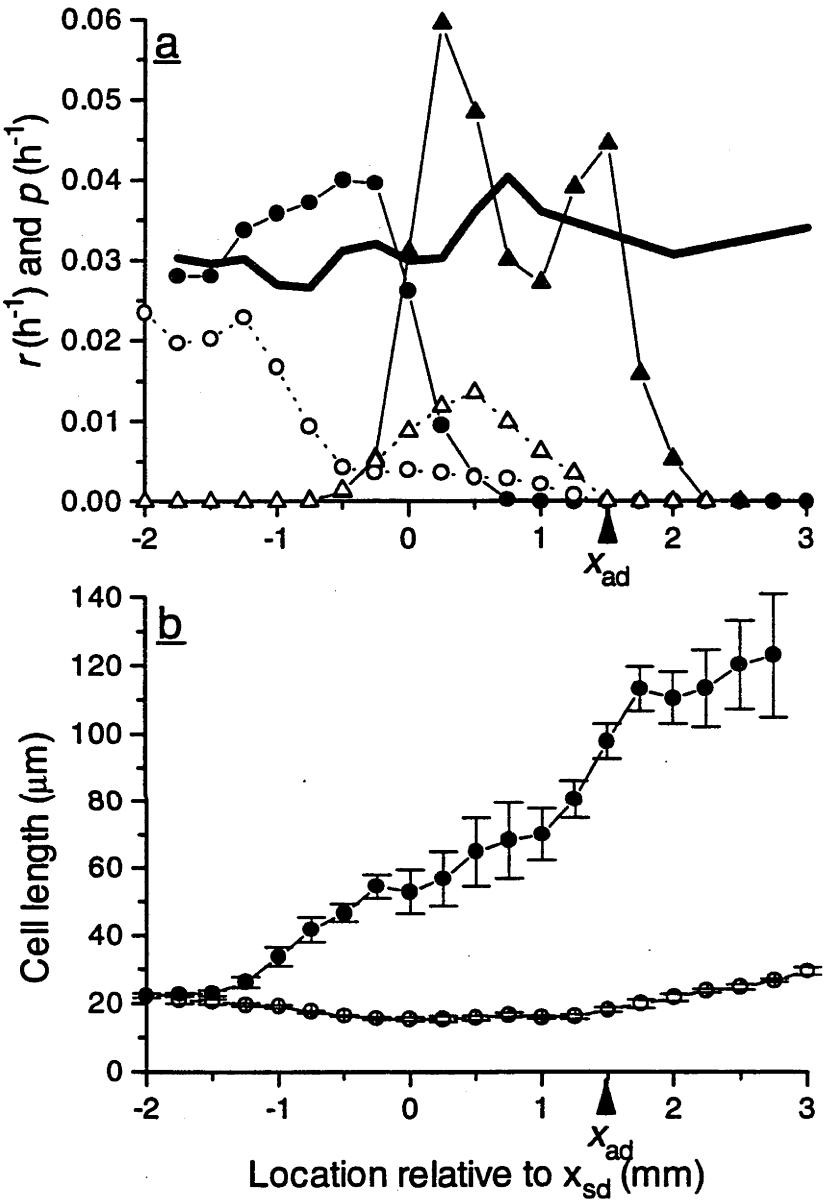


Figure 6. a.) Local relative cell elongation rate (thick line); rates of symmetrical and asymmetrical partitioning (circles and triangles, respectively) in the division zone and in the basal region of the elongation only zone. Open symbols and dotted lines are for sist cells, closed symbols and solid lines for elongated cells, respectively. b.) Corresponding cell length for the two cell types. Data are for leaf 5 at high R_s.

symmetrical division, $\overline{t_c}$ (the reciprocal of $\overline{p_{sd}}$) (Table IV). In leaf 1, high R_s caused $\overline{t_c}$ to increase by 5 h, whereas in leaf 3 and 5, $\overline{t_c}$ was shortened by 6 and 12 h, respectively.

In the steady-state, cell flux F (shown in Table I as E/l_f^*) is constant troughout the elongation zone, and represents the overall rate of cell production by the division zone (eq. 9). It may be seen from eq. 9 that the greater cell flux in leaf 3 and 5 compared to leaf 1 (Table I) reflects the fact that the decrease in $\overline{p_{sd}}$ in these leaves (Fig. 5) was relatively smaller than the increase in the number of meristematic cells, N_{sd} (Table III). Similarly, the smaller flux at high R_s was in leaf 1 due to lower $\overline{p_{sd}}$, while in leaf 3 it was driven by the decrease in N_{sd}, which was relatively greater than the increase in $\overline{p_{sd}}$.

Comparison of different cell types

Since adjacent cell files do not slide with respect to each other (Freeling et al., 1988), local relative elongation rates at a given location along the growth zone are the same for all cell files. However, Fig. 6a shows with leaf 5 at high R_s taken as an example, that both the size of the division zone, and the rates of cell partitioning vary between adjacent cellular files of different types. Symmetrical partitioning was restricted to a much shorter segment in files of elongated cells than in files of sister cells. In addition, the local rates of partitioning, p_{sd} (x), were much lower and declined rapidly towards more distal locations, well below the elongation rates at the same location, whereas in files of sister cells partitioning rates remained high and comparable to the local elongation rates. As a result the length of elongated cells increased from the base of the meristem to the distal end of the division zone while that of neighbouring sister cells decreased (Fig. 6b). Interestingly, asymmetrical division occurred at a similar location in the two types of file. This location also coincided with the site where guard mother cells were initiated in stomatal files (data not shown).

Discussion.

Methodology

Quantitative analysis of growth kinematics based on the continuity equation relies on the fundamental assumption that the leaf growth pattern is constant in time (time-invariant fields of velocity and cell production rate (Goodwin and Stepka, 1945; Erickson and Sax, 1956b; Silk and Erickson, 1979). There is little doubt that, to a first approximation, the leaves analysed in the present study fulfilled that requirement. They were sampled about 2-3 d after emergence from the enclosing sheath, ie about half-way through the phase where elongation was linear with respect to time. Furthermore, Schnyder et al. (1990) showed in *Lolium perenne*, another grass, that the spatial distribution of epidermal cell lengths in the growth zone, the length of that zone, and therefore its number of cells, all remained mostly unchanged from at least leaf tip emergence to the time of transition between blade and sheath growth, i.e. for at least 2-3d before and after our sampling time.

Kinematic calculations based on cell length distributions along an extending axis have always been based on the analysis of cell files containing a single type of cells (Volenec and Nelson,1983; Gandar and Hall, 1988; Silk et al., 1989). The analysis of composite files of sister cells interspersed with a variable number of trichomes depending on leaf position and R_s, required consideration of "structural elements" made of a sister cell and the associated trichome (when present) to enable us to determine the kinematic parameters of the two cell types individually (Figs 2&3).

We found that trichomes elongate at an approximately 50% slower rate than sister cells (Figs 2&3). Because, as already noted, cells in adjacent files do not slide relatively to one another, the formation of trichomes will slow local elongation rates in adjacent files too. If the lower elongation rate in positions lateral to trichomes is not compensated for elsewhere, an increase in the frequency of trichomes will affect r and the mature length of cells of other types.

Cell length distribution

The length of sister cells (and of all other cell types, except for trichomes) varied greatly along the growth zone. Within a single leaf, the largest cells at any given position along the blade were more than twice as long as the smallest ones. Ignoring the presence of trichomes, local variation in length from cell to cell is largely generated in the division zone: cell partitioning produces two daughter cells of half the size of the mother cell; these expand in an approximately synchronised fashion, before dividing again. This next division takes place at approximately the same time for both daughter cells, resulting in small clusters of cells of similar length. Variation in cell length from cluster to cluster is to some extent related to time elapsed since last division. Over larger spatial scales, as already emphasised by Green (1976) variability in cell length (Fig. 1) is also generated by spatial variation along the division zone in the ratio of the local rates of cell elongation *vs* cell partitioning. The present study shows that such variation is large in the growth zone of wheat leaves (comparison of Figs 2-4). For example, in leaf 1 at high R_s, partitioning rates remained stable over the distal region of the zone of symmetrical division (abscissae -1 to 0 in Fig.5) while elongation rates declined (Fig. 4); as a result, cell lengths decreased over that region (inset of Fig. 1). The variability in length between neighbouring cells which is generated in the division zones is maintained in the elongation only zone and, consequently, in the mature zone.

Practically, local variation in cell length necessitated smoothing of cell length distributions to enable kinematic calculations (methods reviewed by Silk (1984)). Use of the Richards function in the elongation only zone, allows good estimation of r_{max} and x_{el}. However, Morris and Silk (1992) found that the distribution pattern of relative elemental elongation rates in the growth zones of some plants cannot be adequately described by the Richards function. In addition to this, the present study shows that individual cell types can behave differently. Results derived from the use of the Richards function should therefore always be compared to those obtained with an independent smoothing method. Averaging individual cell lengths over short, contiguous intervals provides a

simple smoothing procedure, not involving any *a priori* assumptions about the data. In the present study, the relationship between position and cell length (as well as the derived elongation rates) thus obtained agreed closely with the curve obtained by fitting individual data points by the Richards function (see Fig. 2, left panel).

Growth kinetics

It was showed in Chapter 3 that at high R_s leaves elongate more slowly and reach a smaller final size although the duration of elongation is increased, especially in the first leaves. The present analysis sheds some light on the cellular bases of these observations, and shows that they vary with leaf position. In leaf 1 and 3, leaf elongation is slower because cell flux into the mature part of the blade (F), and length of these fresh mature cells (l_f and l_f^*) are both reduced. In leaf 5, slower E is mostly due to shorter cells; F is little reduced. Analysis of mature leaves (Chapter 3) also showed that the total number of sister cells along a file in the mature blade was unaffected by R_s; this was also observed on the mature leaves harvested upon termination of the present experiment (data not shown). This implies that reduction in mature blade length is directly proportional to that in mature sister cells length and secondly that the overall duration of leaf elongation will increase proportionally to the reduction in cell flux. From the F values shown in Table I and N_f values obtained from the ratio of final blade length to average cell length, we calculate that the linear phase of elongation was 1.5 , 1.3, and 0.3 day longer at high R_s for leaf 1, 3, and 5, respectively.

Understanding the effects of R_s on whole leaf characteristics therefore comes down to addressing two questions: Why are mature cells shorter? Why is cell production rate reduced? Our results show that the answer to these questions varies with leaf position. In leaf 5, once cells became non proliferative they expanded at the same relative rate (r_{el}), for the same duration (T_{el}) at the two R_s. Reduction in their final size at high R_s was therefore brought about by reduction in cell length upon entry into the elongation zone (Fig. 1). Since there was no difference between R_s treatments in cell lengths at the very base of the meristem (l_0) nor in the rate of cell elongation through the division zone (Fig1 and 4, respectively), the smaller cell size at x_{sd} and x_{ad} in high R_s leaves can be

attributed solely to a reduction in cell length at partitioning. With l_0 and r_{sd} being unchanged, the increase in partitioning rates (Fig. 5) is a direct measure of that reduction. Applying eq. 8, we calculate that $\overline{p_{sd}}$ was for leaf 5, 42% greater at high than low R_s. This value is similar to the difference between shortest cells in the respective division zones (data not shown).

The other effect of high R_s in leaf 5 was to decrease the size of the population of proliferative cells (N_{sd}, Table III), ie the number of divisions per leaf founder cell. This decrease in N_{sd} was, in relative terms, similar to the increase in $\overline{p_{sd}}$, so that the overall cell production rate, F, remained unchanged. From inspection of the N_{sd} and $\overline{p_{sd}}$ values in the other leaves, we consider this match between variation of the parameters in leaf 5 as a coincidence rather than the reflection of a direct relationship between cell cycle duration and number of proliferative cells.

The interpretation of the slower elongation of leaf 1 is quite different. In that leaf, the length of cells moving into the elongation zone was similar at the two R_s (Fig.1). The reduced size of mature cells therefore reflected the much slower elongation rates once the cell had become non-proliferative (Fig. 2). High R_s prolonged the duration of cell expansion (Fig. 2, right panel), but this effect was too small to offset that of the slower elongation rates. High R_s also reduced the rate of elongation of meristematic cells except at the very base of the meristem (Figs 2& 4). Although this had no direct consequence on final cell lengths, the absence of reduction in r_{sd} while p_{sd} was decreased explains why meristematic cell length increased and became higher than at low R_s in the basal part of the meristem. In the distal part of the division zone, cell length decreased faster at high than at low R_s, because r_{sd} was there relatively more reduced than p_{sd} (Figs 4&5). This pattern of cell length distribution was also seen in roots under water stress (Silk, 1992). The reduction in cell partitioning rates is the opposite of the effect of R_s in leaf 5. Since the size of the population of meristematic cells was unaffected by R_s, reduction in p_{sd} was the cause of lower cell flux (Table I).

In summary, in leaf 5, high R_s reduced leaf elongation rate by reducing the number of divisions per founder cell and by reducing the length at which proliferative cells were partitioned, which was associated with enhanced rates of cell partitioning. In contrast, in leaf 1, leaf elongation rate was reduced because of slower relative cell expansion and cell partitioning rates. In leaf 3, N_{sd} was reduced and p_{sd} was enhanced while the rates of cell expansion were not (r_{sd}), or only little (r_{el}) affected, as in leaf 5. It is worth reemphasising at this point that cell partitioning *per se* does not generate growth. However the profile of cell lengths in the division zone is determined by the relative variations of the partitioning and expansion rates (Clowes, 1961; Green, 1976), which may thereby influence the profile of cell lengths in the mature blade (eg leaf 3 and 5).

The differences we observed in the response of successive leaves to R_s could reflect differences in the stage of development these leaves had reached when first affected by stress. Three leaf primordia are initiated in the embryo of wheat seeds. Differences in R_s were experienced by roots from germination, and their effects on leaf elongation were first detected when leaf 1 was about 20mm long. By that time the leaf meristem was formed. At the same time, leaf 3 had just been initiated and it would be several days before the initiation of leaf 5, so that the formation of the meristem (number of formative divisions, see Chapter 3) and number of proliferative divisions per founder cell (N_{sd}, Table III)) in those leaves could be affected . An alternative, or additionnal explanation is that these differences between leaves reflect time-dependent changes in the responsiveness of the apical and leaf meristems to stress-induced root signals or in the nature of their metabolic effects. The trigger of slower leaf expansion at high R_s has been attributed to some kind of hormonal compound(s) (Masle and Passioura, 1987; Masle, 1990), with ABA or related compounds being the most likely candidates as under drought (Masle and Farquhar, 1988). However, there is also evidence that in the longer term (days) leaf development becomes carbon limited at high R_s and that its sensitivity to sugar concentrations is altered (Masle et al., 1990).

There has been a lot of discussion as to whether stress affects leaf growth via cell expansion or cell division. This study demonstrates that high R_s may affect the two sets of processes. Being based on the measurements of individual cell lengths along the growth zone rather than on the displacement of marks like most other kinematic studies, with identification of proliferative *vs* non proliferative cells, the present analysis provides new information, at the cellular level, especially for the meristem. Stress effects on the number of proliferative cells could be separated from those on cell cycle duration. A decrease in the number of divisions per founder cell under stress is consistent with effects of water stress in root meristems (Silk, 1992) but a shortening of the cell cycling time was a surprise. Data on the effects of growth conditions on cell division in intact higher plants are scarce. Furthermore most studies compare mitotic indexes or cell production rates (eg MacAdam et al., 1989; Silk, 1992) which confound variations in number of dividing cells and cell cycle duration. However Powell et al. (1986a, b) found that toxic Zn levels significantly lengthened the cell cycle in one cultivar of *Festuca rubra*, while having no effect in another one. They also reported contrasting responses of cell sizes in the meristem. These observations led Francis (1992) to suggest that environmental stress can effect specific aspects of the cell cycle, which are linked to cellular mechanisms for tolerating stress. These mechanisms vary with genotype; our observations indicate that they are also developmentally regulated and vary between leaves.

Measurements of individual cell lengths enabled us to show that, although a minimum longitudinal expansion has to occur before partitioning takes place, there is no single threshold size at which cells divide, even for a given genotype. Cell size in the division zone varied between leaves, R_s treatments and with position in the division zone (Fig. 1). This suggests to us that rather than being related to cell size *per se* as has sometimes been suggested from observations on lower organisms (eg John et al., 1993), the occurrence of partitioning may be dependent on a minimum amount of metabolic machinery. Haber's experiments with irradiated seedlings clearly showed that elongation can continue in the absence of mitosis (Foard and Haber, 1961; Haber 1962; Haber and Foard, 1963).

We were also able to calculate local cell partitioning and cell expansion rates in the division zone. Thin walls provided us with lasting, and ordered, markers of recent cell divisions, enabling us to work with larger cell samples. From the total number of thin walls per file of sister cells in the division zone and the cell flux out of this file at x_{sd} we indeed estimate that the time needed to thicken a new wall ranged from 4 to 6h for leaf 1&3 and leaf 5, respectively (equivalent to 15 to 25% of the average cell cycle duration). Since this time was in all leaves similar at the two R_s, we are confident in the reliability of the R_s effects on the p_{sd} and r_{sd} distributions shown in Figs 4&5 and conclude that cell partitioning and cell expansion rates of meristematic cells are not directly related. For example, high R_s enhanced p_{sd} in leaf 5 while having no detectable effect on r_{sd}. Furthermore, comparison of Figs. 2 and 4 shows that there was no constant relationship between expansion rates in the division and elongation zones, suggesting that the regulation of wall expansion in dividing cells differs from that in non-proliferative cells. Baskin et al. (1994) recently identified a mutant of *Arabidopsis* with impaired expansion only in root cells which had ceased dividing. There are important spatial gradients in the elongation rates of both groups of cells as well as in the rates of partitioning. Future work should aim at relating these gradients to gradients in cell metabolic status, especially with respect to carbohydrates and putative stress-induced root signals.

The present study shows that interpretations of growth responses inferred from characteristics of mature leaves/cells alone are dangerous. We saw that differences in mature cell length does not necessarily reflect differences in rates of wall expansion or duration of cell expansion in the elongation only zone (eg leaf 5). Similarly, the number of sister cells per file in the mature blade was in all leaves independent of R_s, despite large, and furthermore variable, differences in meristem size and rates of partitioning. The number of cells in files of other type was also not (stomata) or little (eg bulliform cells) affected by R_s (Chapter 3). In the light of the variability and complexity of the effects of R_s on the growth kinetics at the cellular level, the relative invariance of the final number of cell along mature files is fascinating. This observation bears on the still unresolved issue of the relationships between the control of overall organ shape and size and of cellular characteristics (see Haber 1962, and more recently Green, 1994).

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Chapter 5

Determination of mature leaf dimensions by growth at the apex.

Abstract

High soil resistance to root penetration (R_s) slows down leaf growth and reduces mature leaf size. The aim of the present study was investigate to what extent some these effects are generated at the primordial stage in relation to apical characteristics. The relationships between apex growth, timing of leaf 5 initiation and expansion rate of the young leaf primordium are analysed for plants grown at contrasted $_{Rs}$.

High R_s reduced the rates of apex and leaf development but did not appear to have immediate effects on the pattern of development of the newly initiated phytomers. During an initial short period, the leaf and node development were related to plastochronic age, according to similar relationships at the two R_s. Effects on developmental patterns were first detected on radial phytomer expansion during plastochron 2. The ontogenetic pattern of longitudinal leaf development was affected later, at the post-primordial stage. Differences in the number of formative divisions and in the number of proliferative cells along the intercalary meristem that were reported in earlier chapters are generated at the primordial and post-primordial stage. They do not appear to be related to the size of the apical dome at leaf initiation nor to the size and number of meristematic cells initially recruited to the leaf primordium. Further studies towards the understanding of the effects of *R*s on the kinematics of leaf growth and mature leaf anatomy should concentrate on the development of the young leaf from plastochrone 1 to 5.

Introduction.

Leaf growth is restricted when roots encounter a high resistance (R_s) to penetration through the soil. This has been shown for several species and has been well documented for wheat (Masle and Passioura, 1987&1990; Masle et al., 1990; Masle, 1992). During the early stages of seedling growth, high R_s decreases the rate of leaf area expansion by slowing down the rate of leaf appearance and elongation and by reducing the dimensions of mature leaves (Masle and Passioura, 1987). A mechanistic understanding of these whole leaf responses requires to identify which of the many developmental processes involved in leaf formation are affected by R_s and to analyse how their integration in time and space is modified. The effects of R_s on the anatomy of mature blades and on the kinetics of cell partitioning and cell elongation in expanding leaves were reported in previous chapters. It was shown that the size and structure of the leaf meristem and its functioning during the phase of linear elongation following leaf emergence (rate of cell partitioning, rate of surface expansion of meristematic and non-meristematic cells, relationships between these rates) were all sensitive to R_s. However, the degree to which these parameters were affected or even the direction of the response varied between successive leaves. Although time-dependent adaptation to stress could not be excluded, comparison of leaf 1 which starts its development in the embryo and of leaf 5 which is initiated after germination showed that these leaf effects partly reflected differences in the developmental stages of successive leaves when root impedance first occurred. In leaf 5, high R_s reduced the number of proliferative cells in the leaf intercalary meristem which was made of fewer files, with fewer cells. These cells partitioned at a faster rate and at a smaller size. Remarkably, R_s did not affect the rate of cell expansion, nor the duration of expansion once the cell had become non proliferative. These results suggested that for leaves which fully developed after the onset of root impedance, variation in expansion rate and mature leaf size were mostly caused by effects of R_s on early events of leaf ontogeny. The aim of the present study was to test this hypothesis further by analysing the effects of R_s on apex growth, timing of leaf initiation and expansion rate of the young leaf primordium. Early research into apical development showed that the size of the apex gradually increases with plant age (see references in Clowes, 1961). Some authors (eg Abbe et al., 1941; Wardlaw, 1952) have suggested a causal relationship between this ontogenetic increase in apex size and the increase in width of successive

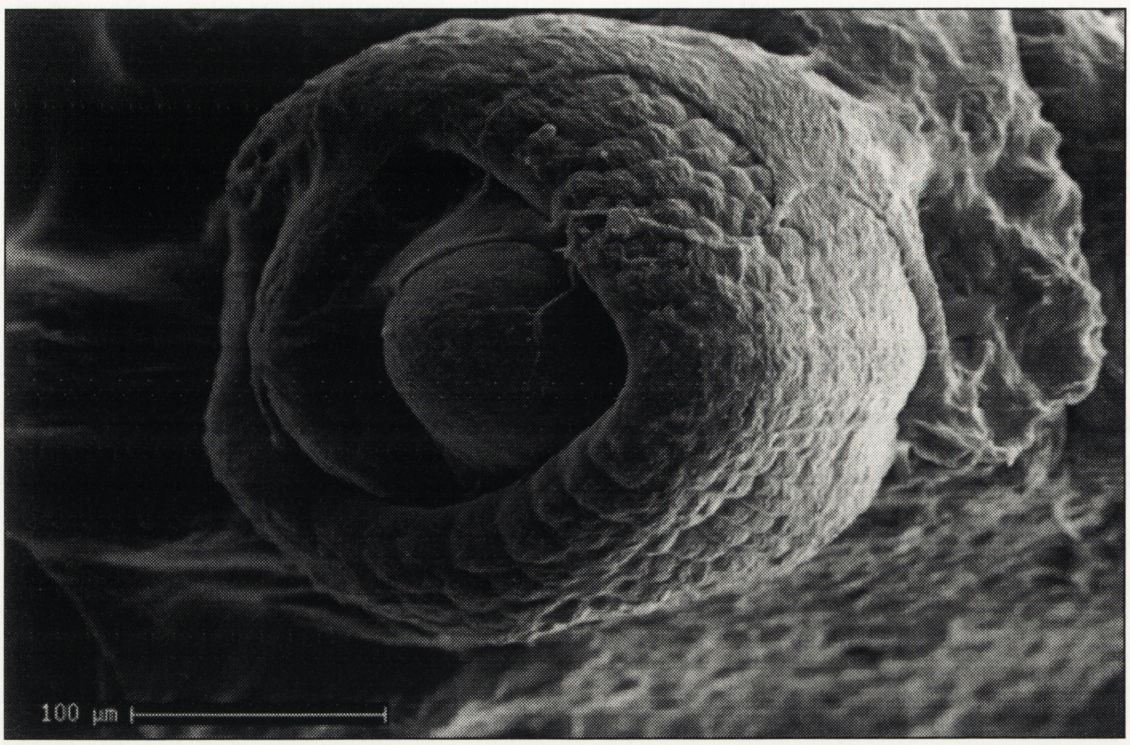
leaves. This is an attractive hypothesis, especially for grass leaves, which early in their development form a hood that completely encircles the axis (Fig. 1 and Williams, 1974). Does it apply to environmentally induced variation in leaf width? Results such as those of Mitchell and Soper (1958) in *Lolium* and *Paspalum* show that, not surprisingly, environmental variations can cause marked variations in leaf width in the absence of effects on the size of the apical meristem *per se*. Interestingly, however, in that study variation in leaf width with light intensity seemed to correlate with variation in the circumference of the sub-apical region (defined as in Fig. 2). In view of these observations, one aim of the present study was to specifically address the following question: Are the reduced number of formative divisions (cellular files in the leaf intercalary meristem) and leaf width at high R_s due to limitations of apex enlargement during the early stages of leaf formation?

Materials and methods.

Growth conditions

Wheat plants (*Triticum aestivum* L. cv. Egret) were grown in pots in a controlled growth chamber set at 18/15 °C day/night temperature, 600 µmol quanta m^{-2} s^{-1} irradiance, 11 h photo- and thermo-period and a relative humidity of 80%, day and night. Calibrated seeds (42 to 44 mg) were sown 15 mm deep in soils with contrasted mechanical resistance to root penetration. The soil used in this study (a silty loam with 5% coarse sand, 40% fine sand, 31% silt, 19% clay and 4.5% organic matter) was the same as in earlier experiments (Chapters 3&4). After thorough mixing of 2.2 g finely ground superphosphate and 357 mg NH_4NO_3 per kg dry soil, the soil was homogeneously packed into cylindrical PVC pots (200 mm high and 87 mm diameter), so as to achieve a penetrometer resistance of 0.58 ± 0.03 MPa and 6.98 ± 0.09 MPa. These contrasted soil resistances were obtained through two different combinations of soil bulk density and soil water content (1.12 g cm^{-3} and 0.24 g H_2O g^{-1} dry soil at low

a)



b)

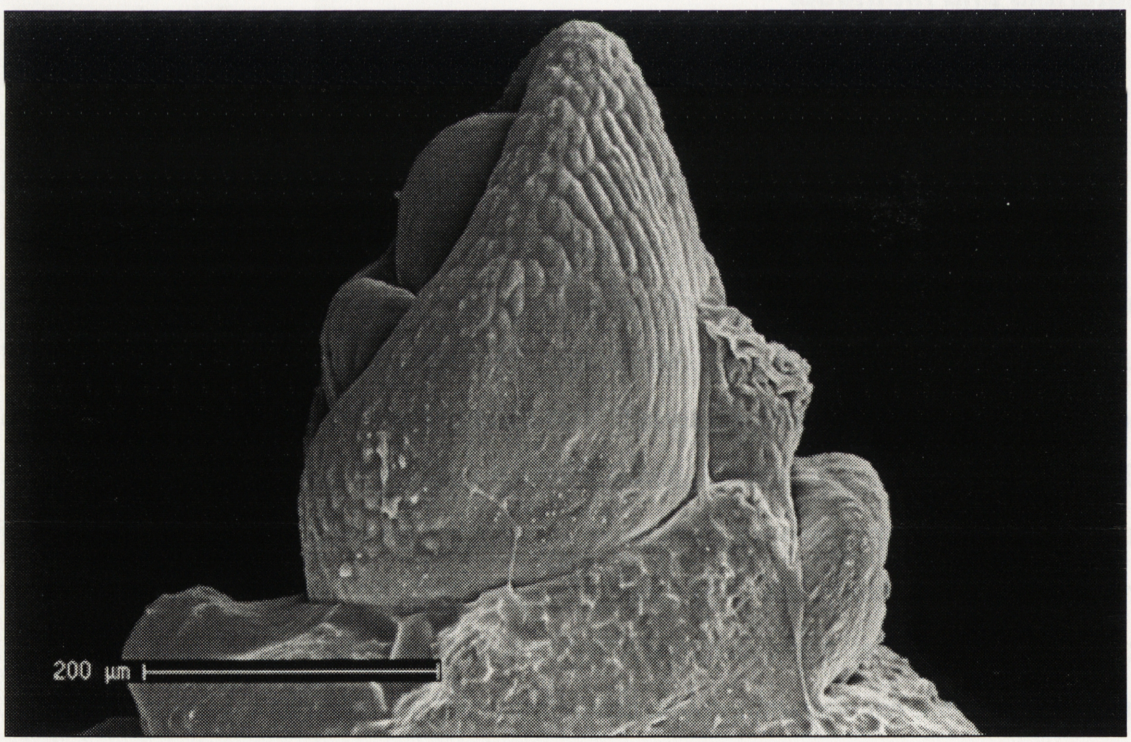


Figure 1. Scanning electron microscopy image of a replica (see Materials and Methods) of an apex at *PI* = 6.75. a) Top view. b) Side view.

R_s and 1.42 g cm^{-3} and 0.22 g H_2O g^{-1} dry soil at high R_s). Pots were watered at least daily, so as to maintain soil water content as constant as possible around its initial value.

Plant harvest

Five randomly selected plants were harvested daily from d7 through d14 at low R_s, and d8 through d16 at high Rs. This allowed to cover a similar range of developmental stages at the two R_s, from the initiation of leaf 5 to that of leaf 7. These plants were dissected for microscopic examination of the apical and subapical meristem (defined as in Fig. 2) and of the young leaf primordia, leaf 5 especially (see below). The technique used was a slightly modified version of that described by Green and Linstead (1990), which enables scanning electron microscopy and light microscopy on the same sample. Five other plants were harvested at each R_s at the emergence of leaf 7, from which mature leaf 5 was sampled.

Dissection and sample preparation for scanning electron microscopy (SEM)

In order to prevent excess evaporation from the specimen the dissection microscope was placed in a tray layed with wet filter paper and enclosed in a small plastic tent. The oldest leaves were removed until the apical meristem and leaf 4 (on d7-10) or 5 (later harvests), were exposed. The apex with this leaf and usually two younger leaf primordia still attached (see Fig. 1) was then quickly placed upside down in a gelatine capsule (size 0, Alltech, Australia) filled with dental impression material (ESPE Permagum, low viscosity). After ca 3 minutes, the material had hardened enough so that the specimen could be removed leaving a mould with a clear impression. Replicas of these impressions were obtained by filling the moulds with resin (Araldite "M", Ciba-Geigy, Australia) which was let to polymerise overnight at 60 °C. The replicas were sputter-coated with 20 nm gold using an SEM coating unit (type: E5000, Polaron Equipment Ltd.), and mounted in a vertical position on the stage of a scanning electron microscope (Jeol, JSM 6400). Several photographs were taken for each sample (Fig 1); the sample was rotated so as to select views which would expose the insertion site of leaf 4 and 5 or 5 and 6 and allow reproducible measurements of the length of these two leaves and of the height of

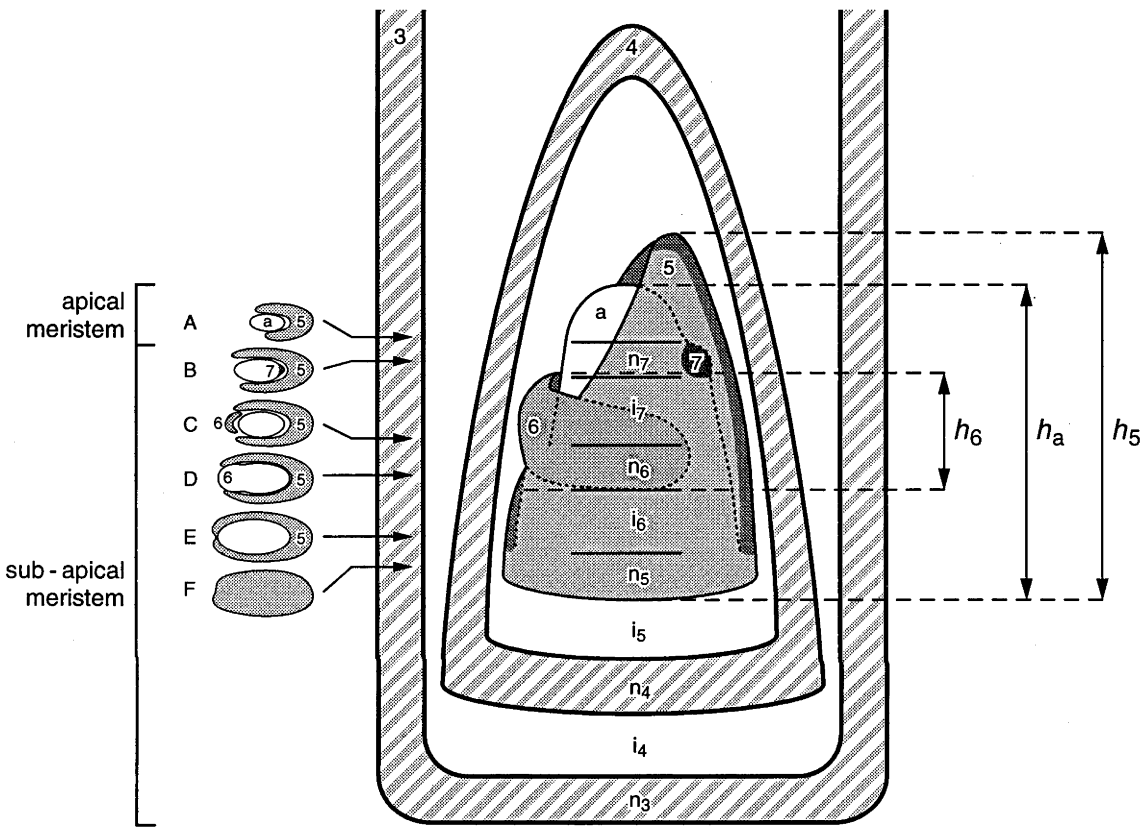


Figure 2. Schematic representation of an apex with identification of the apical meristem (a), leaves 3 to 7 (numbered in bold), and the corresponding nodes and internodes (n_3 to n_7 and i_4 to i_7, respectively) defined as in Lyndon (1990). h_a denotes the height of the apex measured from the base of leaf 5 and h_5 and h_6 are the heights of leaf 5 and 6, respectively. The positions of the six cross sections which were analysed by light microscopy (see Materials and Methods and Fig. 3) are indicated by horizontal arrows labelled A to F.

the apical dome (lengths labelled h_5, h_6, and h_a in Fig. 2). At the latest harvests where leaf 5 was completely encircling the apex with its margins overlapping on each other, only h_5 could be accurately measured.

Preparation of transverse sections for light microscopy examination

Immediately after being removed from the impression material the plant samples were fixed overnight in 0.25% glutaraldehyde and 0.38% formaldehyde in 0.05 M phosphate buffer (pH =6.7), and after a quick wash with 0.05 M phosphate buffer, in 1% osmium tretroxide for 2.5h. All samples were then stored in 70% ethanol. At the end of the experiment two sets of fixed apices, which represented the most extreme stages of development common to the two R_s treatments, were selected for sectioning. For the first set, leaf 5 had just been initiated; the average plastochron index (*PI*, see later) was 5.17 ± 0.15 and 5.30 ± 0.07 at low and high R_s, respectively. On the set of older apices, leaf 7 was the youngest initiated primordium, with a plastochron index of 6.69 ± 0.11 and 6.68 ± 0.14 at low and high R_s, respectively.

These two sets of apices were dehydrated in ethanol in three 1h steps (90, 95 and 100% ethanol and infiltrated overnight with 50% LR-white resin (London Resin Company Ltd.) in ethanol, followed by 4h in pure LR-white resin. They were then transferred in gelatine capsules filled with pure resin, care being taken to position the apex with its axis parallel to the capsule wall. The resin was let to polymerise at 60 °C for 24h.

For the set of most advanced embedded apices, several series of 5 transverse sections, alternatively 1 and 2 µm thick, were taken through the apical and subapical meristem (Fig. 2). The sequence of these sections was recorded. All 1 µm sections were mounted on slides and stained with 1% toluidine blue in 1% sodium borate buffer and examined under the light microscope. Six sections (labelled A to F in Fig. 2) were selected for morphometric analysis, from six similar positions along the axis of the sample: at the base of the apical dome, just above the youngest initiated leaf primordium (leaf 7, section A); through the site of initiation of that primordium (B); through the disc of insertion of

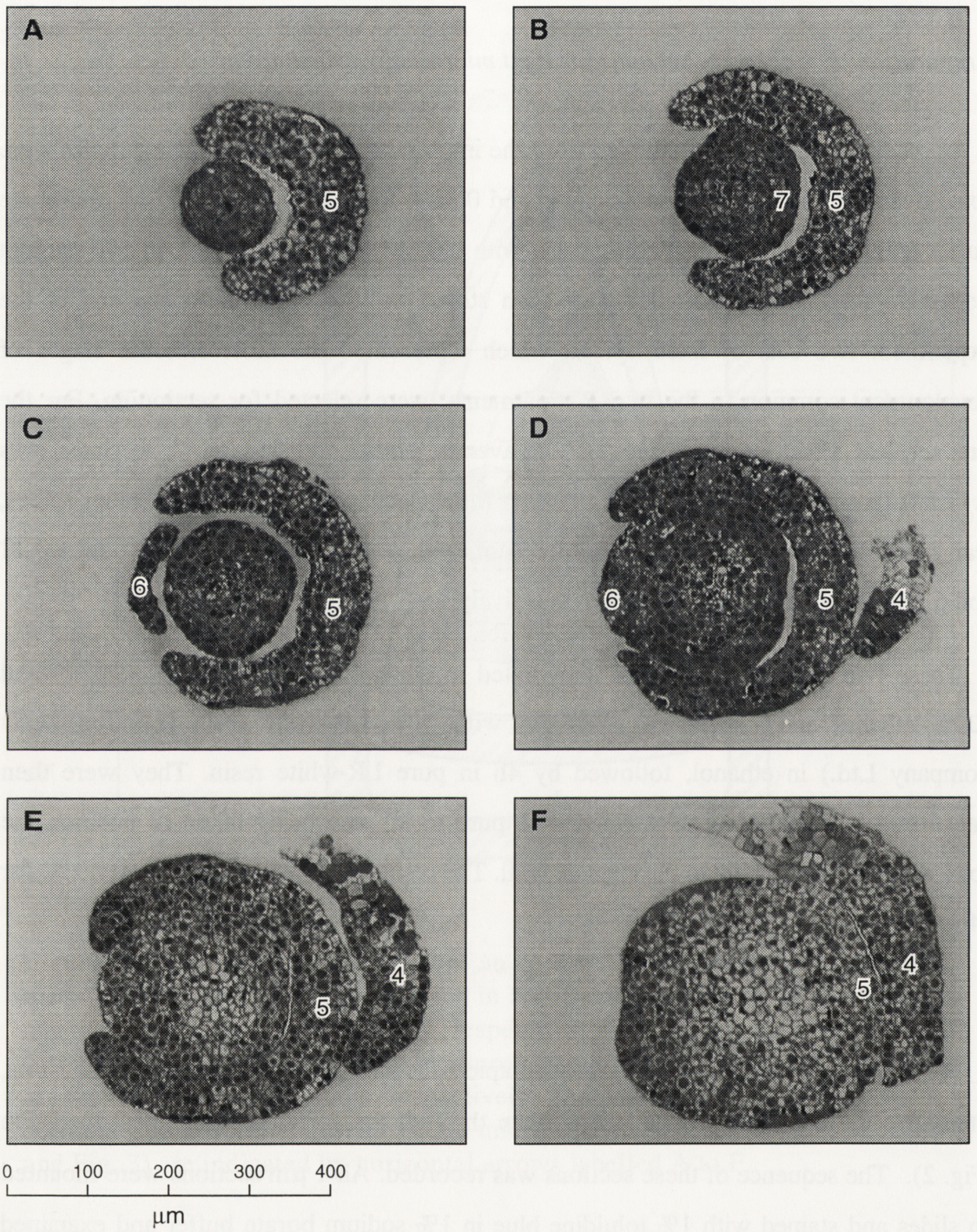


Figure 3. Example of light microscopy images of transverse sections taken at 6 positions along the apex as indicated in Fig. 2.

the two oldest leaves (leaf 6 and 5, sections D and F), which will be referred to as "nodes" (Lyndon, 1990) and through the meristematic regions separating these discs ("internodes", sections C and E). For the set of young apices, only one section (section A, just above the site of initiation of leaf 5) was selected for analysis. Light microscopy images from the sections (Fig. 3) were obtained with a video camera mounted on a light microscope. On these sections, the following determinations were made: circumference of the central axis (sections A-C-E) or of the node (sections B-D-F) and number of cells on the outer most layer; width of leaf 5, measured as the distance between margins on the abaxial side of the primordium, and number of epidermal cells along that distance. Average cell width was then calculated from leaf width divided by that number of epidermal cells.

Description of developmental stages (developmental scale)

The number of visible leaves on the main stem (n) and the emerged lengths of the two youngest blades (l_{n-1} and l_n) were recorded on all plants at each harvest. These measurements were used to determine the foliar stage of each plant according to Haun (1973) as: $(n-1)+\frac{l_n}{l_{n-1}}$ (later referred to as "Haun index", *HI*), where $\frac{l_n}{l_{n-1}} \leq 1$. The Haun index provides a continuous scale for the description of foliar stages, which furthermore is linear with temperature ie in our conditions, linear with time. The constant time interval taken for the Haun index to increase by 1 unity defines the phyllochron.

The total number of initiated leaves was also counted. In order to analyse the effects of R_s on meristem and leaf characteristics independently of possible age differences, all measurements were referred to the apex, or to the leaf, plastochrone age. Plastochronic age was measured by the value of the plastochron index (*PI*) as defined by Erickson and Michelini (1957):

$$PI = n + \frac{\ln(l_n) - \ln(l_r)}{\ln(l_n) - \ln(l_{n+1})} \quad \text{(Eq. 1)},$$

Table I. Effects of soil resistance on the characteristics of mature leaf 5 blades. Data in the first two columns are means and standard errors (into brackets, n = 5). The third column gives the difference between R_s treatments as a percentage of the value at low R_s and the statistical probability level of the difference determined from ANOVA analysis of variance (n.s. denotes not significant).

	R_s (MPa)		Difference (%)
	0.6	7.0	
Length (mm)	254.2 (4.8)	235.0 (6.1)	-7.6 (0.038)
Width (mm)	8.6 (0.2)	7.3 (0.3)	-15 (0.006)
Area (mm^2)	15.8 (0.9)	12.5 (0.8)	-21 (0.026)
# files across	338 (5)	288 (9)	-15 (0.001)
Cell width (µm)	25.4 (0.4)	25.3 (0.5)	n.s.

where n is the number of leaves longer than a reference length (l_r), l_n and l_{n+1} are the lengths of leaves n and $n+1$, respectively with, by definition $l_n \geq l_r$ and $l_{n+1} < l_r$. The reference length was chosen as 50 μm which was the smallest leaf primordium length that could be measured with precision on the SEM images. The assumptions embedded in the derivation of that index are that leaves are initiated at regular interval and expand exponentially, according to the same curve (Erickson and Michelini, 1957). This implies constancy of the denominator in Eq. 1, sometimes referred to as plastochron ratio (PR, see for example Silk (1980)).

Results

An unexpectedly low percentage of seed germination (about 60% at both R_s) prevented us from thinning at seedling emergence for greater uniformity between plants within each R_s treatment. This caused a greater variability in the data than in the companion studies conducted under similar conditions (Chapter 3&4).

Leaf dimensions at maturity. As expected from earlier experiments (Chapter 3) high R_s reduced mature leaf size. Effects on the dimensions of leaf 5 are shown in Table I. The blade was shorter and narrower. Reduction in width was due to a decrease in the number of cell files constituting the blade while cell width was unaffected (Table I).

Leaf elongation rate at the primordial stage and plastochron index. Figure 4 shows the elongation curve of leaf primordium 5 during the 2 to 3 plastochrons following initiation. Elongation was approximately exponential at the two R_s but proceeded at a much reduced rate at high than low R_s (p=0.006). Figure 4 gives indication of a slight curvature of the relationship between ln (l_5) vs time towards the end of the observation period. However, this deviation from exponentiality was not significant and was similar for the two treatments. The plastochron ratios calculated from measurements on leaf 4 and 5, or 5 and 6 over the period shown in Fig. 4, were stable in time, and similar at the two R_s and for the two pairs of leaf primordia, with an average value of 1.13 (s.e.=0.04).

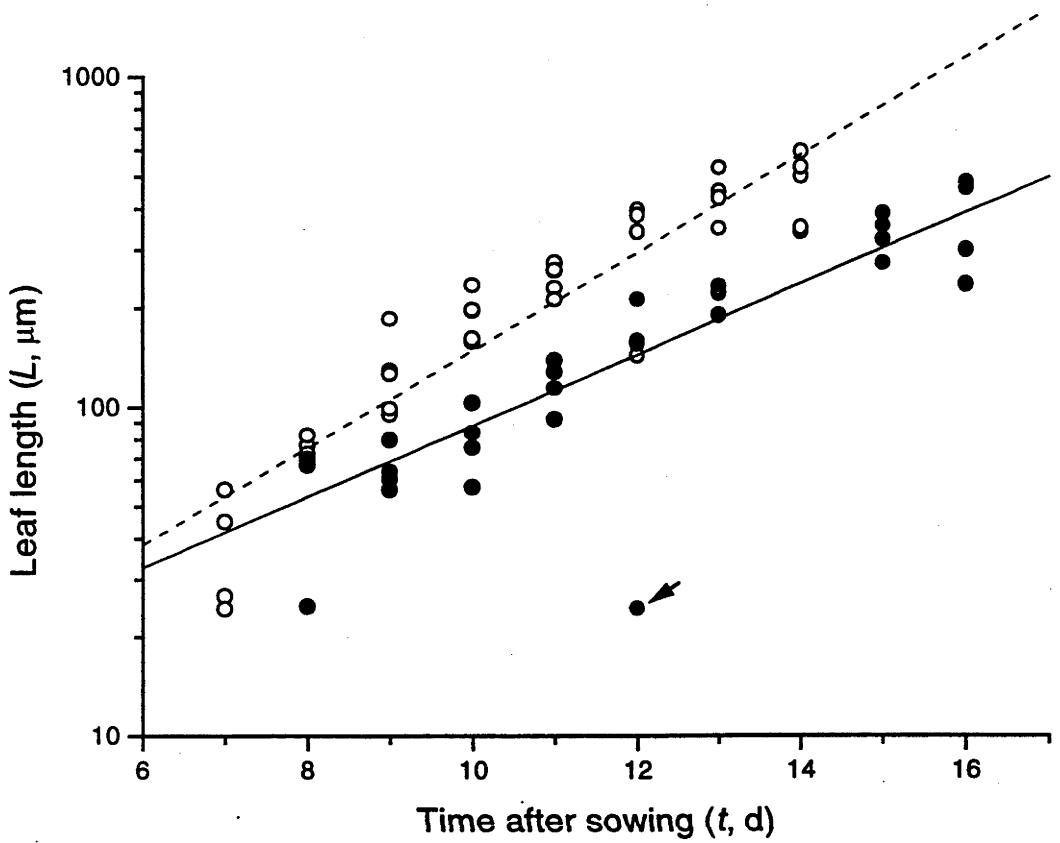


Figure 4. Length of leaf 5 (L) as a function of time (t) at low and high R_s (open and solid circles, respectively). Equations of the regression lines (excluding the data point marked with an arrow): $L = e^{(1.60 + 0.34t)}$ at low R_s (dashed line) and $L = e^{(1.94 + 0.25t)}$ at high R_s (solid line). The slopes of the two lines were statistically different ($p = 0.006$).

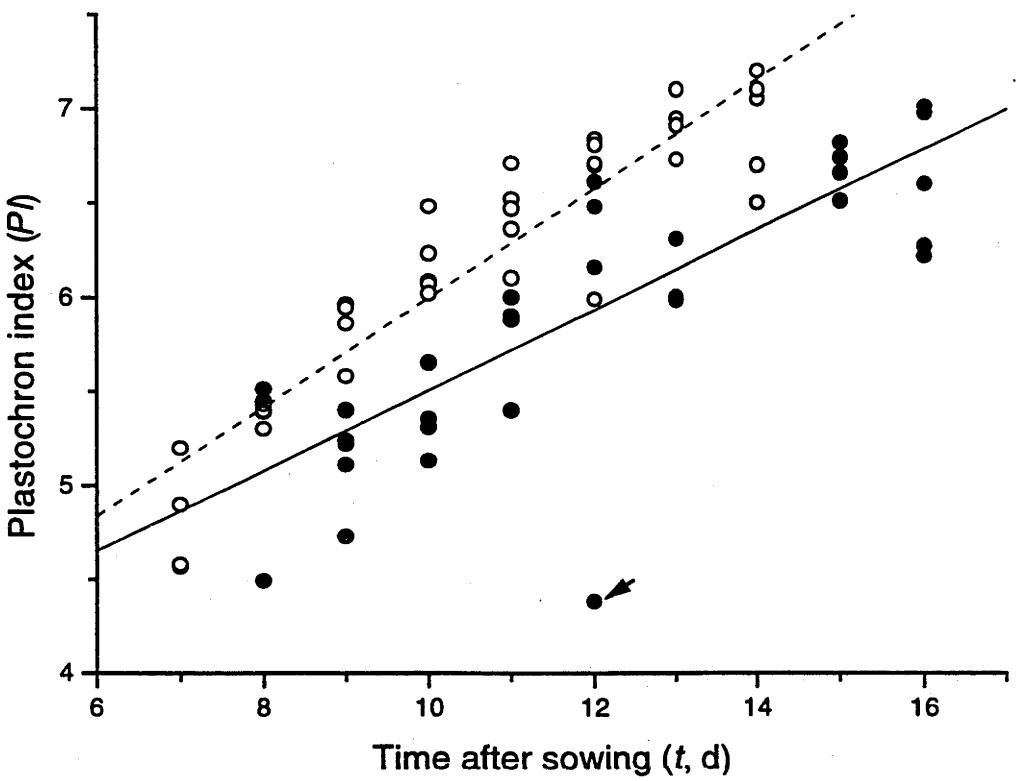


Figure 5. Plastochron index (*PI*) as a function of time after sowing (*t*, *d*) at low and high R_s (symbols as in Fig. 4). *PI* was calculated with a reference length of 50 µm (see Materials and Methods). Equations of the regression lines (excluding the data point marked with an arrow): $PI = 3.10 + 0.29\ t$ for low R_s (dashed line) and $PI = 3.37 + 0.21\ t$ at high R_s (solid line). The slopes of the two lines were statistically different ($p = 0.010$).

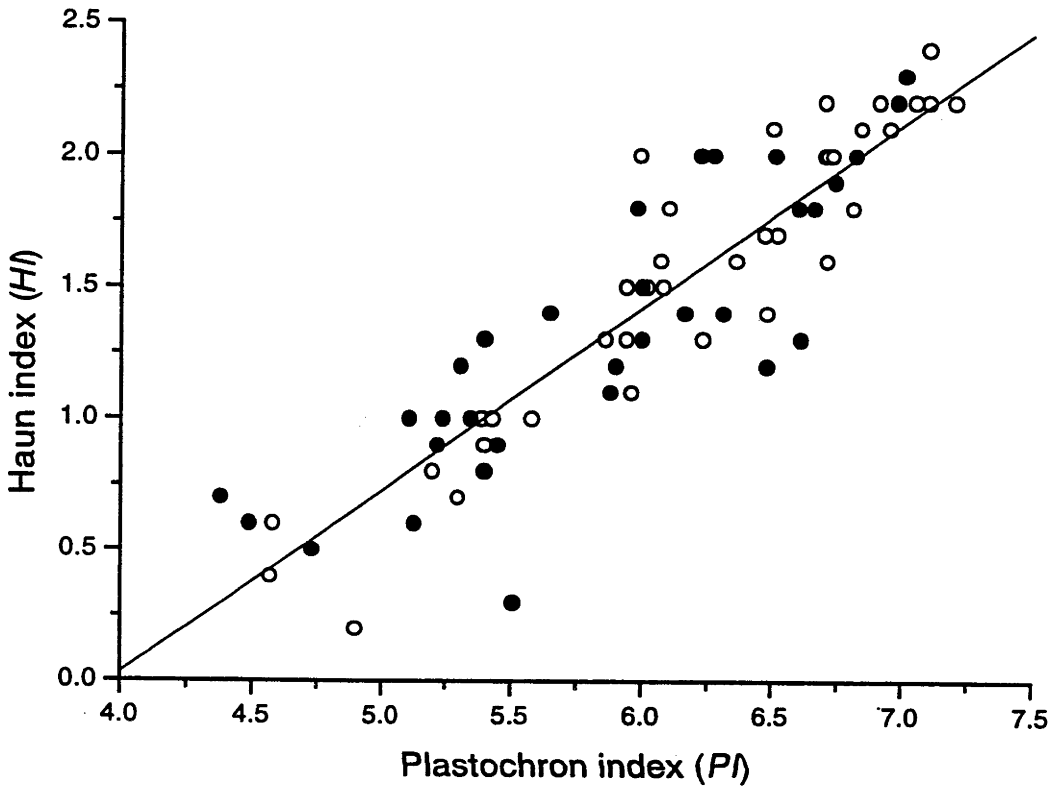


Figure 6. Relationship between Haun index (*HI*) and plastochron index (*PI*) for the main stem at low and high R_s (open and solid symbols, respectively). Equation of the regression calculated on all data points: $HI = 2.75 + 0.7\ PI$.

In these conditions, the plastochron index calculated using Eq. 1 gives a way of accurately measuring developmental age of both leaf primordia and apex.

Figure 5 shows a plot of the plastochron index *vs* time at low and high R_s. Because the reference length used to calculate *PI* (of 50 μm) corresponds to a very early stage of primordium development, this plot gives a good representation of the influence of R_s on the rate of leaf initiation. The average plastochron duration between d6 and 16 as estimated from the slope of the linear fittings of the data in Fig. 5 was significantly increased at high compared to low R_s (4.7 d and 3.4 d, respectively ($p=0.01$)). Given these effects of R_s on development rate, effects on patterns of development will be analysed using plastochronic rather than chronological time.

Leaf emergence. Figure 6 shows that there was a good correlation between Haun index and plastochron index, which furthermore was independent of R_s. In other words the date of emergence of a leaf from the whorl of older enclosing sheaths was directly related to its date of initiation ie developmental age, and this relationship was unaffected by R_s. The slope of the relationship (0.7 ± 0.04) in Fig. 6 indicates that the rate of primordium initiation was about 30% greater than the rate of leaf emergence. As a result of this, non-emerged leaf primordia gradually accumulate during vegetative development and the time between initiation and rapid linear growth increases for successive leaves

Elongation of the apex. Figure 7 shows that apex height was also directly related to plastochron age. On that scale, elongation of the apex was approximately exponential and was unaffected by R_s. The slope of the fitted line in Fig. 7, of 0.7, is lower than that relating the logarithm of leaf length to *PI* which, by definition of *PI*, is equal to *PR* (see Eq. 1) ie 1.13. This is why the tip of the young leaf covered the top of the apex in less than two plastochrons (see Fig. 1).

Lateral expansion of leaf primordium and growth in girth of the apical and sub-apical meristems. The width of leaf 5 was measured just after initiation of leaf 7 on 5 on successive transverse sections taken across the disc of insertion of leaf 5 (section F in

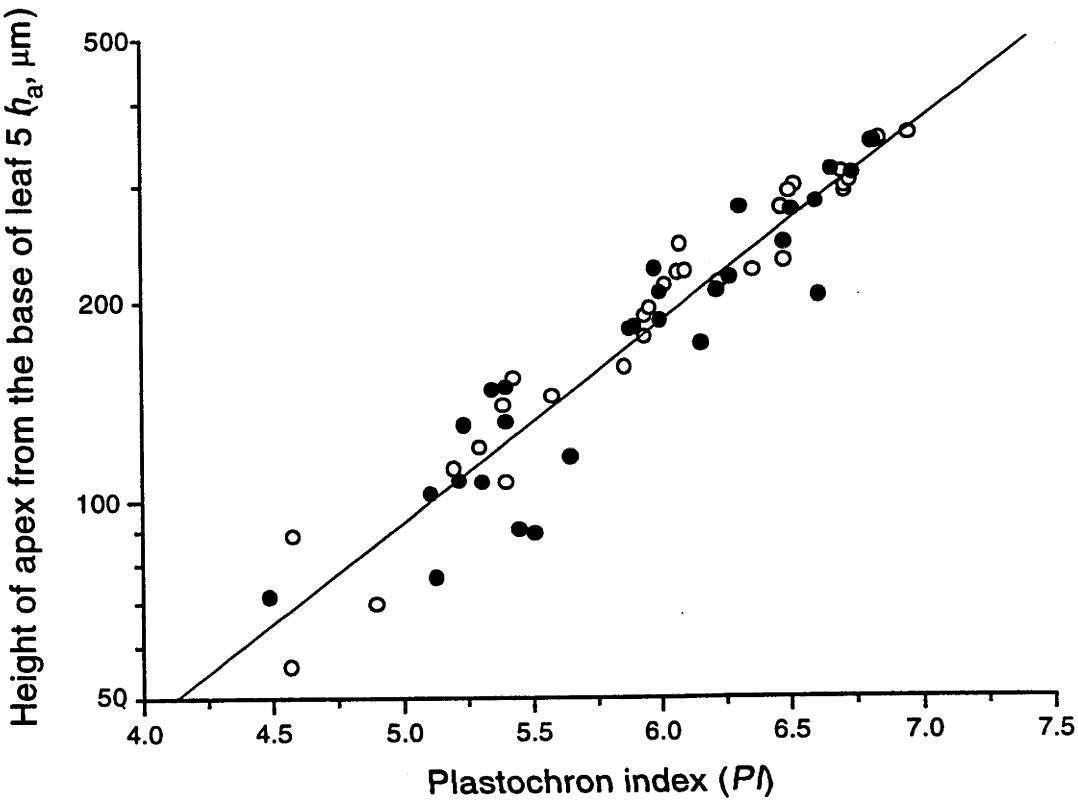


Figure 7. Height of the apex relative to the base of leaf 5 (distance h_a in Fig. 2) as a function of plastochron index (*PI*) at the two R_s (same symbols as in Fig 4). Equation of the regression on all data points $h_a = e^{(1.01 + 0.70\,PI)}$.

Figs 1 and 2) and across the younger nodes and internodes above it (sections A-E). The data are shown in Fig. 8a, plotted *vs* nodal or internodal position along the axis ie, in effect, *vs* plastochron age. The width of the young leaf increased progressively towards more basal locations, especially at low R_s There was no consistent R_s effect in the distal part of the leaf, while the two most basal sections showed reduced lateral expansion of the primordium at high R_s. Due to large plant to plant variation in that parameter, this reduction was statistically non significant (p=0.028). However, given the significant reduction in diameter of the sub-apical meristem axis with R_s measured on the same sections (p=0.019 at node 5, Fig. 8a), it is most likely revealing a real effect of R_s on the primordium lateral growth rate, starting during the second plastochron after initiation. The circumference of the apical meristem at the level of the newly initiated primordium was unaffected by variation in R_s (Fig. 8a and Table II for plants sampled early at *PI*=5.2). This observation too is consistent with the fact that the distal part of leaf 5, which was formed the closest to initiation, had similar width at the two R_s.

Both the reduced growth in girth of the subapical meristem and reduced lateral leaf expansion in the same region at high R_s were due to reduction in cell number (Fig. 8c). Cell widths across the leaf epidermis and in the outer layer of the subapical meristem in sections E and F were indeed similar at the two R_s (Fig. 8b). There was also little variation in cell width along the young leaf ie with developmental age. The increasing leaf width towards node 5 was mostly due to an increase in the number of cell files. In contrast, cells were much wider on the perimeter of the apical meristem and meristematic axis at the site of insertion of leaf primordium 7 than at lower positions in the sub-apical meristem. But because the number of cells was comparatively more reduced (Fig. 8c), variation in meristematic axis circumference was correlated to variation in cell number rather than in cell width, like in the young leaf.

Discussion.

In this study, we examined the effects of high soil resistance on leaf early development and on its relationship to apex growth. The analysis was focussed on leaf 5 which, in the conditions of this experiment and of companion studies (Chapters 3&4) where

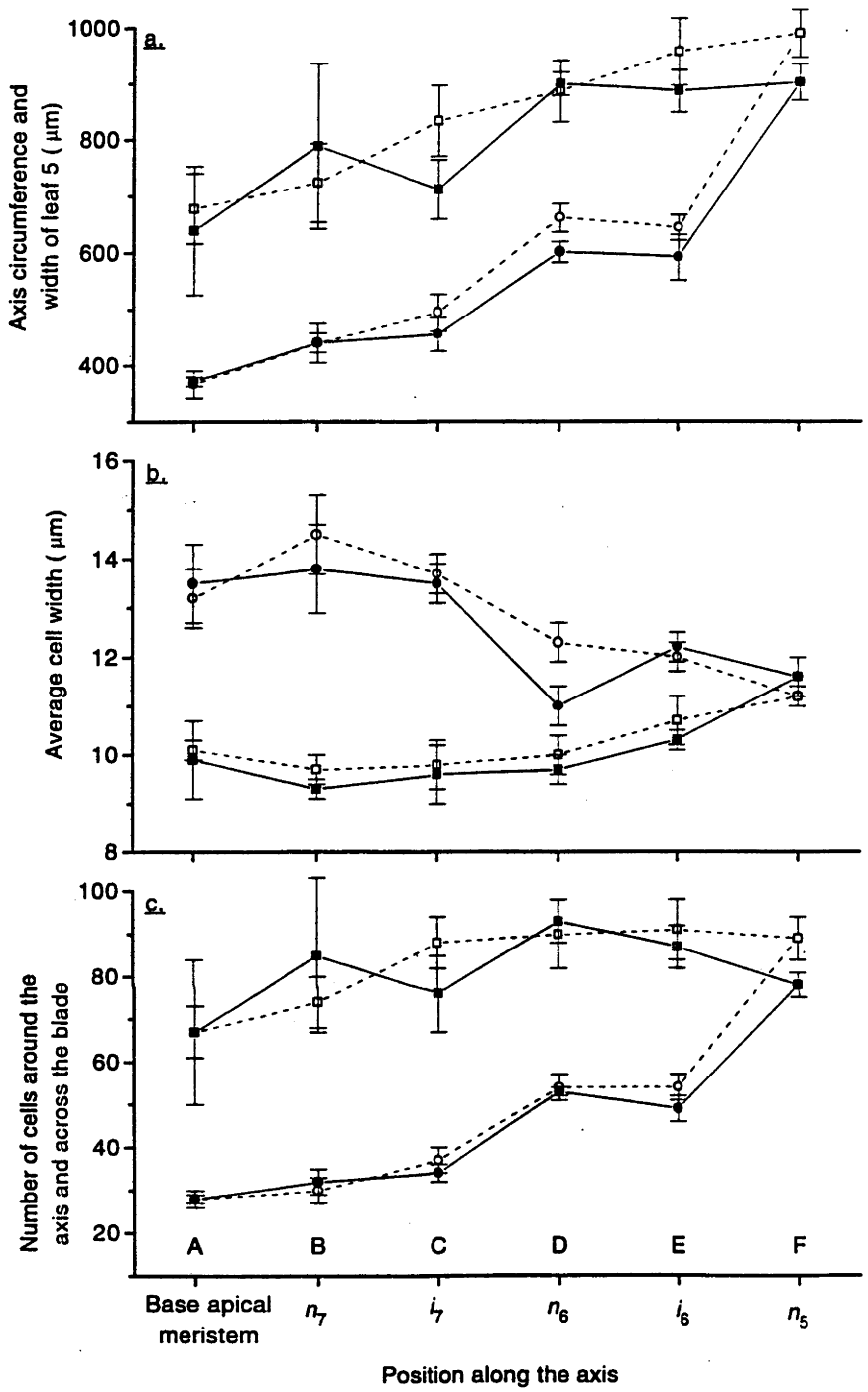


Figure 8. Longitudinal variation in the characteristics of leaf 5 and of the apical and sub-apical meristem axis at low and high R_s (open and soild symbols, respectively): (a) Axis circumference (○ , ●) and leaf width (□ , ■); (b) number of cells in outer layer; (c) average width of these cells, on 6 successive transverse sections (see example in Fig. 3) positioned as indicated in Fig. 2 at the base of the apical dome and in the 2 or 3 youngest internodes and nodes below it (sections C and E, and B, D, F, respectively).

differences in R_s were imposed from germination, fully developed under contrasted soil conditions.

The activity of the apical meristem (label *a* in Fig. 2) is discrete. At regular interval (defining the plastochron) a new group of cells are recruited on the side of the apical dome which, through a change of growth polarity and a number of periclinal and anticlinal divisions, will give rise to a new vegetative phytomer made of a leaf, a node corresponding to the site of insertion of the leaf and eventually an internode below it (Sharman, 1942; Poethig and Szymkowiak, 1995). These phytomers pile up and define morphological units characterised by a given position in the sub-apical meristem. Under constant environmental conditions, the interval between the initiations of successive units is constant (eg Friend et al., 1962; Miglietta, 1989; Hay and Kemp, 1990 and Fig. 6) ie there is a strict correspondence between position number and plastochronic age. This implies that the characteristics of successive nodes and internodes at a given time provide a record of apical development as a function of age. During the two plastochrons following its initiation leaf 5 elongated approximately exponentially with respect to time (Fig. 4), at a similar rate to that of leaf 4 and 6, at the two R_s. In these conditions the length of the youngest leaf can be used as a measure of developmental time within a plastochron (Eq. 1). As a whole these features enabled us to convert chronological time or position along the leaf or apex axis into developmental time (see methods) and thereby to separate the effects of R_s on rates *vs* pattern of development.

The main results of this study are:

- High R_s reduced the rate of apex and leaf development. At a given time, the apex was smaller, in length and girth, and had initiated fewer leaves. Leaf primordia were, at any given position, less developed. Fewer leaves had emerged.

- Most of these differences disappeared when apex and leaf characteristics were compared on a developmental scale. The size of the apical dome and the longitudinal growth of a new vegetative phytomer during the two first plastochrons following

Table II. Characteristics of a transverse section of the apical meristem just above the site of initiation of the youngest leaf primordium (5 and 7 at *PI* = 5.2 and 6.7, respectively): circumference, number of cells in the outer layer, average width of these cells. Data are averages and standard error (n = 5 to 6) of two extreme plastochron stages (*PI*, see methods). R_s had no significant effect on any of these characteristics. The right column describes variations with *PI* (difference expressed as a percentage of the value at *PI* = 5.2) and the statistical probability level of the difference as in Table I.

	PI = 5.2		*PI* = 6.7		Difference
R_s (MPa):	0.6	7.0	0.6	7.0	(%)
Circumference (μm)	332 (14)	341 (21)	374 (23)	373 (7)	+ 11% (0.045)
Number of cells	24 (2)	27 (2)	28 (2)	28 (1)	+ 10% (n.s.)
Cell width (μm)	14.0 (1.0)	12.8 (0.2)	14.1 (0.6)	13.4 (0.7)	+ 1% (n.s.)

initiation, were directly related to plastochronic age, according to a similar relationship at low and high R_s (Figs 7 and 8). The elongation rate of the young leaf remained little affected by R_s until about the time it emerged from older sheaths (Fig. 6).

- However, within one plastochron (node axis) to two plastochrons (attached leaf primordium) following initiation, some effect of R_s on the ontogenetic pattern of phytomer development became apparent. Increase in axis diameter and primordium lateral expansion were slower at high R_s (Fig. 8).

From these observations we conclude that:

The reduced leaf width observed at high R_s at later stages, on expanding and mature blades (see previous chapters) is not due to the apical meristem being smaller at the time of initiation nor to reductions in the number or size of cells initially recruited at the margins of the primordium initiation site. Soil resistance had no detectable effect on either of these parameters. However, limitations on the number of cells contributing to the primordium lateral expansion did take place soon after initiation. Our data indicate that these limitations were related to slower radial expansion of the subapical meristem and reduced cell production rate in the external cell layer from which a large part of the leaf tissue is initially derived (Roesler, 1928; Sharman, 1945). Meristematic cell width remained unaffected; this is consistent with the similarity in meristematic cell length observed later at the base of the intercalary meristem (Chapter 4).

There is little precise quantitative data on the temporal pattern of formative divisions which give rise to the number of cellular files and cells layers in the leaf lamina. However, detailed anatomical studies on several cereals and grasses (Sharman, 1942&1945 (rice and various graminae); Kaufman, 1959 (rice) and Skinnner and Nelson, 1994 (*Festuca*)), all indicate that most epidermal files and cell layers are formed within 4 to 5 plastochrons after primordium initiation. Extrapolating the relationship between *PI* and *HI* (Fig. 6) and in agreement with Malvoisin (1984), we estimate that this approximately coincided with the time of leaf emergence. Assuming that the

increase in apical meristem diameter with plastochron age is exponential (Williams, 1966) data in Table II yield an average radial relative growth rate between *PI*=5.2 and 6.7 of 0.07 per plastochron. If following Abbe et al. (1941) it is further assumed that the radial expansion rates of the apical dome and of the nodes in the subapical region are proportional, and using the circumferences measured for node 6 and 7 at *PI*=6.7, the estimated values for the circumference of node 5 at *PI*=9.0 (ie at emergence of leaf 5) are 2.97 mm and 2.39 mm at low and high R_s, respectively. Taking an average cell width in the node external layer of 11 µm at the two R_s (Fig. 8), the corresponding cell numbers are 270 and 217, respectively. Since by the time of emergence the leaf margins have been overlapping for some time (Sharman, 1945; Williams, 1960; Silvy, 1982) these numbers are already an underestimate of the number files which were actually contributing to the lamina at that stage, and a fortiori later (see Table I and earlier chapters). However, their relative difference (-21% taking leaves grown on loose soil as a reference) is of the same order as, or very close to, the difference observed in this experiment (14%) or earlier experiments (22%, see Chapters 3&4) between final file numbers. Some of the parameters involved in the above calculations were extrapolated from the two plastochrons following initiation to the following 2-3 plastochrons preceding blade emergence. This may be inaccurate and needs to be examined by further more exhaustive studies of leaf development from the primordial stage to emergence. However these calculations show that when compounded over time, the apparently small differences in cell production rates which were detected in the subapical meristem during plastochron 2, may quantitatively account for substantial decrease in the final number of cellular files in mature blades.

The leaf lengths measurements taken from several hours to 1 d after emergence in this experiment and many others (Chapters 3&4; Masle and Passioura, 1987; Masle, 1990) consistently showed that high R_s significantly reduces the elongation rate of emerged leaves. These effects persisted even when leaf length was plotted against time from emergence which from this study, is equivalent to plastochronic age. Given that the lag between leaf initiation and emergence was similar at the two R_s these effects seem to be mostly generated around the time of leaf emergence ie within plastochron 4-6 after

initiation. Data in Chapter 4 showed that high *R*s significantly reduced the number of proliferative cells per file in the basal meristem of fast expanding emerged blades. It is indeed well established that during plastochrons 1 and 2 the bulk of the primordium is meristematic (Sharman, 1942&1945; Freeling, 1992). Cell enlargement correlated with loss of the ability to divide starts during plastochron 3, progressing basipetally from the tip of the blade. The length, and presumably also the number of cells per file, of the short basal meristem characteristic of fast expanding emerged blades are therefore determined between then and emergence from the whorl of older sheaths after which stage they appear to remain stable (Bernstein et al., 1993; Skinnner and Nelson, 1994). We therefore conclude that the longitudinal pattern of leaf development becomes sensitive to variation in R_s before blade emergence, at the post primordial stage during plastochrons 3 to 5.

General conclusion

High R_s slows down the rate of apex and leaf development but does not appear to have immediate effects on the pattern of development of the newly initiated phytomers. Such effects were first detected on radial phytomer expansion during plastochron 2. The ontogenetic pattern of longitudinal leaf development appear to be affected later, at the post-primordial stage. Differences in the number of formative divisions and in the number of proliferative cells along the intercalary meristem that were reported earlier are generated at the primordial and post-primordial stage. They do not appear to be related to the size of the apical dome at leaf initiation nor to the size and number of meristematic cells initially recruited to the leaf primordium. Further studies towards the understanding of the effects of *R*s on the kinematics of leaf growth and mature leaf anatomy should now concentrate on the development of the young leaf from plastochrone 1 to 5.

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General discussion.

Main conclusions.

The results described in this thesis, lead to a number of conclusions about the nature of the effect of soil resistance to root penetration (R_s) on leaf growth and development and of the cellular basis of leaf development in general. These two aspects will be discussed separately in the following discussion.

The effects of R_s.

Whole plant scale.

Whole plant and leaf growth response to variations in R_s in wheat have been described in the literature (Masle and Passioura 1987; Masle and Farquhar 1988; Masle 1990; Masle et al., 1990; Masle 1992). The present results confirm earlier observations that high R_s causes a substantial reduction in growth of young wheat plants. Relative rates of leaf area expansion (r_l) and dry weight accumulation (r_w) were mainly affected during the first days after emergence, except at R_s = 6.6 MPa where r_l and r_w were still reduced after 12 days of growth (Chapter 2; Figure 4). Because of the early differences in r, absolute growth rates remained generally inversely related to R_s. Leaf growth rate was reduced due to lower rates of leaf appearance, reduced tillering, lower leaf expansion rates and reduced final leaf sizes.

Cellular basis of reduced size of the mature leaf.

Both length and width of mature blades were reduced by high R_s. These reductions were associated with shorter and narrower mature epidermal cells; a decrease in the number of cellular files across the blade and a shift in relative proportions of epidermal cell types. There was a marked difference in the degree to which these effects were expressed between different leaf positions, suggesting differences in the underlying developmental processes. In leaf 1, smaller leaf sizes were mainly due to cell size being reduced, while the number of cell files and number of cells per file were hardly affected. In leaf 3 cell sizes were less affected than in leaf 1 but the overall effect of R_s on leaf

dimensions was of similar magnitude as the number of files was significantly reduced. In leaf 5 both size and number of cells were decreased.

Kinematics of leaf expansion.

Leaf expansion in grass leaves is generated by the production and subsequent expansion of new cells in the growth zone located at the base of each cell file (see Introduction, Figure 1). Individual cells within each file are gradually displaced into more distal locations, due to the production of new cells in more basal positions. While being displaced, cells expand and divide at rates that greatly depend on their position along the file. Cells in the most basal part of the blade both divide and expand, whereas cell expansion in the absence of division occurs in the elongation only zone, resulting in an increase in the length of cells as they are moving through this region. At the end of the elongation zone cells stop expanding and become part of the longer mature section of the blade.

The mechanisms underlying variations in leaf expansion rates with differences in R_s are also markedly different between leaf positions: In leaf 1, leaf elongation rate was reduced because of slower relative cell expansion rates in the whole of the growth zone and slower cell partitioning rates in the division zone. In contrast, in leaf 3 and 5 leaf elongation rates were reduced by a reduction of the number of proliferative cells and by a smaller length of cells entering the elongation only zone. The smaller length of cells at the end of the division zone was due to increased rates of cell partitioning, while relative elongation rates in the division zone were unaffected by R_s.

<u>The steady-state assumption in the kinematic analysis of leaf elongation.</u>

Steady-state growth is a fundamental assumption on which the kinematic analysis of local cell partitioning and expansion rates in the growth zone during the phase of rapid leaf growth is based (Chapter 4). In steady-state conditions, the rate of leaf elongation, the distribution of cell lengths along the growth zone and of recent cell divisions are constant. The assumption of steady-state is often taken for granted (Volenec and Nelson 1981; MacAdam et al., 1989; Meiri et al., 1991; Bernstein et al., 1993; Walker and Hsiao 1993), although diurnal variations (Schnyder and Nelson 1988) as well as variations in

daily expansion rates with developmental stage have been reported (Schnyder *et. al.* 1990).

In this study, steady-state was not strictly the case: there were significant differences between day and night rates of leaf elongation (See Chapter 2, Figures 7 and 8). However, because migration of cells from the base of the growth zone to the mature region takes a number of days (2 to 3 days are required for cells to move from the end to the division zone to the end of the elongation only zone, See Chapter 4; Figure 2). An implicit assumption that is made when analysing the data according to average elongation rates over 24 hour, as in this study (Chapter 4) is that the daily fluctuations of cell expansion and partitioning rates throughout the growth zone are of similar magnitude. No data is currently available to ascertain the validity of this assumption. Leaf elongation rates are approximately linear with respect to time for the greater part of the expansion of the blade (See Chapter 2, Figure 7). To improve the comparability between R_s treatments, care was taken to harvest leaves at a similar developmental stage.

In *Lolium perenne*, a grass species, the cell length distributions along the growth zone were very similar at 4 different stages of leaf development, despite variation in leaf elongation rates with developmental stage of the leaf, similar to that shown in Chapter 2 (Figure 7; Schnyder *et. al.*, 1990). It was therefore assumed that the same would apply to wheat leaves, *i.e.* steady state for the cell length distribution through time. However, no direct evidence for this is available from our own data or from the literature.

Although convenient for kinematic calculations, the steady-state assumption precludes the application of the kinematic approach (as described in Chapter 4) for the analysis of varying leaf elongation rates. Analyses of the cellular basis of diurnal variations in leaf expansion rates and rapid changes due to changing environmental conditions therefore require adaptations of the method. If variations in leaf expansion rates are not accompanied by changes in the cell length distribution along the growth zone, the approach outlined by Gandar and Rasmussen (1991) can be utilised. This method allows for variations of the velocity field with time. It may be impossible to verify that cell length distribution is stable when these changes in leaf elongation rate in fact do occur. There are two reasons for this: 1. The variability in cell length at any location along the growth zone could easily obscure relatively small changes in the distribution of the

average cell length. 2. Changes in cell length that originate in the division zone, take at least three days in our experimental conditions to move through the division zone into the mature blade (See Figure 2, Chapter 4). This duration effectively determines the temporal resolution for this method and make it unusable for the analysis of constantly changing leaf elongation rates.

Primordia development at the apex.

High R_s slows down the rate of apical development: leaf primordia are initiated at a lower rate and grow slower with respect to time after their initiation. Many developmental characteristics of the apex at a given time are strictly correlated with its developmental stage measured in terms of plastochron age: the circumference of the apical meristem, its height relative to the base a given leaf primordium and the height of the primordia were all correlated to plastochron age according to a relationship independent of R_s. Growth in girth of nodes in the sub-apical meristem as a function of plastochron age, however, is reduced by R_s. This was due to similar reductions of meristematic cell expansion and partitioning, because cell size at any given position in the apex at a given developmental stage was not significantly affected by R_s.

Relating the results between experiments.

In order to facilitate comparison of successive experiments and test reproducibility of the response to R_s, all experiments were conducted in controlled climate growth chambers providing the same environmental conditions. However, it was impossible to perfectly reproduce soil conditions from one experiment to the other. Even with the combination of soil bulk density and water content constant, there were small variations of R_s between experiments (see Table III, Chapter 2). This is unavoidable when working with large batches of soil, in which the soil sub-structure was deliberately kept as undisturbed as possible, *i.e.* close to those *in situ*. Differences between the two runs conducted to sample mature blades or between these two runs and experiment 2 in which the kinematics of cell expansion was analysed were small and had negligible influence on plant response to R_s (Chapter 2; Table 1). However, in experiment 3, where the apical development was analysed, an apparently similar range of R_s led to smaller differences in dimensions of mature leaves and cells (Chapter 2; Table I). It is suspected that the

sequential harvesting (from d7 to d16) of plants from pots where more than one plant was growing may have loosened the soil and decreased the effect of R_s so that the plants harvested between day 24 and 31 had similar mature leaves. The fact that the results on apical development obtained on the first harvested plants are comparable with the other experiments is consistent with this interpretation. However, to avoid any ambiguity in future experiments of this nature, it would be wise to leave the soil undisturbed before harvesting plants from a particular pot.

Relationship between effects of R_s on cell division and expansion and anatomical characteristics of the mature blade.

Despite small variations in the magnitude of the growth response to R_s between experiments, the similarity in the nature of the response allows us to identify at what stage during the developmental process differences in anatomical characteristics observed in the mature blade are determined:

1. The reduction of mature cell length (Chapter 3, Figure 7) is a consequence of differences in the kinematics of cell division and expansion during the phase of rapid leaf expansion. As discussed when explaining differences in leaf expansion rates, smaller mature cell length in leaf 1 growing at high R_s were mainly due to differences in elongation rates in the elongation only zone. In leaf 3 and 5 they were a consequence of increased rates of cell partitioning in the zone of cell division, whereas cell expansion was not significantly affected.
2. The number of cells per file along the blade is greatly dependent on the cell production rates during the phase of linear leaf expansion, and on the duration of this phase. Ong and Baker (1985) have shown that the linear phase of leaf expansion accounts for the formation of 90% of the leaf. It is remarkable that the total number of cells in a file was not or only little affected by R_s (10 % at the most) while the kinetics of cell production rates by the leaf intercalary meristem and/or their rate of elongation showed a large and variable response to R_s.
3. Given that the number of cell files constituting the blade is approximately constant along most of its length (Chapter 3; Figure 5; and Silvy, 1982), formative divisions must have stopped before, or shortly after the onset of rapid leaf expansion. Our results showed that the reduction of the number of files constituting leaf 5 at high R_s were not related to effects of R_s on the size of the apical meristem at initiation, but

from a reduction in the size of the sub-apical meristem during the 2 plastochrons following initiation. Since apical development in the embryo in wheat is arrested after initiation of leaf 3 (Williams, 1960), leaf 1 had accomplished this early part of its development in the embryo. The fact that the number of formative divisions was unaffected by R_s in that leaf, for which reduction in the rate of elongation were first seen 1 day after emergence (*i.e.* 5 days after germination), indicates that in, at the most, 4 first plastochrons after leaf initiation formative divisions are completed.

4. Variations in R_s also caused a shift in proportions of different epidermal cell files (Chapter 3). Two lines of evidence suggest that the increase in the fraction of files with ground tissue is also determined at the start or shortly before the onset of rapid leaf expansion. Firstly, no differentiation between adjacent epidermal cell files could be observed during the first two plastochrons of development of leaf 5 (Chapter 5, Figure 2). Secondly, the fact that similar changes in the relative proportions of different types of cell files are observed in leaf 1, 3 and 5 (Chapter 3) suggests that the identity of individual files (*i.e.* cell type formed within each file) is determined just prior to leaf emergence. c) Differences in length between cells of different types in adjacent cell files are associated with differences between files in the relationship between r and p and in the size of the zone where divisions occur (Chapter 4; Figure 6). The fact that the length of elongated cells is more reduced by high R_s than any of the other cell types, indicates a differential response of cell division to high R_s (rates or extent of division zone) between files producing different types of cells. d) The increased trichome density at high R_s can be explained by higher rates of asymmetrical divisions (Chapter 4, Figure 5) and possibly also in increased residence times in the asymmetrical divisions zone (Chapter 4, Figure 2).

Variation between successive leaves.

The difference of the response of successive leaves to variation in R_s, is interesting and deserves attention. Under the experimental conditions used in the experiments described in this thesis, the level of stress applied to the growing seedlings varied little during seedling life. We were therefore able to conclude that the differential response to R_s of successive leaves was due to the timing and duration of stress imposition with respect to the progression of the leaf through its developmental program. Unfortunately, differences

in timing of stress imposition with respect to developmental stage of the leaves was confounded with ontogenetic variations between the same leaves.

In order to investigate in more detail the interaction between leaf ontogeny and time of stress perception, one could establish plants on soils made of layers of different R_s. Masle (1990) showed that leaf elongation rates of plants growing in a layer of high R_s soil on top of a low R_s layer increased compared to plants in pots with high R_s soil throughout. Moreover, this response could be observed shortly after the first root tips entered the loose soil. Interestingly, the first detectable difference in leaf elongation was seen for in the leaf that had just started to elongate when the root tips first entered the loose soil. Leaves that were already expanding, kept growing at the same rates, again suggesting differences in sensitivity to R_s related to the leaf developmental stage. It would be interesting to use a similar system to investigate the effects of releasing the stress at different stages of development of leaves that have started their development under root impedance. In response to such a treatment one would expect differences in the characteristics that are determined during development in the sub-apical meristem (number of files constituting the blade) to persist, while differences in later determined characteristics (cell size) would not be seen. The fact that in experiment 3, where the soil was loosened after leaf 5 had partly developed to the hood stage, resulted in a reduced number of files but not cell width (Chapter 5; Table I) supports this assumption. Also initiation of the stress just before emergence of leaf 5 should result in a response similar to that observed in the current investigation in leaf 1 (*i.e.* similar number of files at low and high R_s and a great reduction in cell size at high R_s).

Adaptive function of response to high R_s

The anatomical and morphological characteristics in response to high R_s which were described in Chapters 2 and 3, closely match the description of xeromorphism (Maximov 1929; Oppenheimer 1960). The observation that high R_s results in more anatomical features characteristic of drought resistance, strengthens the argument put forward by Masle and Farquhar (1988) that the response to high R_s may be part of an early warning system to prepaire for imminent water stress.

From an agronomical perspective one could wonder about the desirability of the growth responses to high R_s that are described in this thesis and by other authors. It has been shown that the growth response to drying soil and high R_s occurs before root functions such as water and nutrient uptake limits growth (Masle and Passioura 1987; Passioura 1988; Passioura and Gardner 1990). Even in irrigated crops, dry soil conditions, resulting in increased R_s (see Introduction) are encountered locally, especially in the top layer of soil. Similarly, a layer of soil with a high R_s can be encountered by only part of the root system. Both of these conditions result in reduced leaf growth and transpiration rates (Blackman and Davies 1985; Saab and Sharp 1989; Neales *et. al.* 1989; Zhang and Davies 1989). This conservative response could be judged as undesirable when the bulk of the roots still have access to plentiful water. In dryland conditions, however, the situation is more complicated. It has been argued that fast early seedling growth is desirable to enable rapid coverage of the soil, thus preventing evaporative losses from bare soil surface (López-Castañeda and Richards 1994). However, by inducing higher stomatal resistances, adverse soil conditions result in improved efficiency of carbon assimilation in relation to transpirational water losses (drought (Farquhar and Richards 1984; Guinta et al., 1995); root impedance (Masle and Farquhar, 1988)). It may be possible to develop wheat genotypes for growth under dryland conditions which would not show the undesirable aspects of the growth response to high R_s (slower early growth), but retain beneficial aspects (higher water use efficiency). The partial independence between the response of stomatal conductance and leaf growth rates to high R_s seen among genotypes of wheat and barley (Masle 1992) indicates that there are opportunities for such an approach.

General aspects of leaf development.

Cell size and number in mature leaves and assessment of cell division and expansion rates.

Many investigations of the relationship between variations in mature leaf size and changes in number or size of cells constituting the leaf under various environmental conditions have been published. Often the authors try to infer from these measurements the effect of the particular environmental factor investigated on cell division and expansion rates (*e.g.* Mitchell and Soper, 1958; Friend and Pomeroy, 1970; McCree and

Davis, 1974; Randall and Sinclair, 1988; Körner et al., 1989). Our results clearly show that the conclusions reached by such an approach must be assessed with care, because there are a number of different ways in which the same result can be obtained: In all leaves investigated we find the number of cells along a file was little affected by high R_s (Chapter 3, Table II) *i.e.* that differences in leaf length of the leaf were mainly associated with differences in cell length. From these observations one could infer that the mechanism by which leaf length is decreased is the same in all leaves; that cell division is not affected, whereas cell expansion is. Taking into account the duration of leaf expansion (much increased in leaf 1, very similar in leaf 5; Chapter 4), it becomes obvious that the mechanisms by which final length was reduced is varied between leaves. One could conclude that cell division rate is not affected in leaf 5, whereas it is lower at high R_s in leaf 1. Our results show that in fact cell *division* rates in leaf 5 are increased by high R_s, but that due to a decrease in the population of proliferating cells the overall rate of cell *production* was similar. These observations therefore illustrate the need for caution in interpreting differences in mature organs and show the power of the method used for the kinematic analysis.

Proliferative and formative divisions are partly independently controlled.

In the growth zone of root tips, two types of divisions have been distinguished: Formative divisions result in an increased number of cell files, whereas proliferative divisions increase the number of cells within each file (Gunning et al., 1978). In the growth zone of *Azolla* roots, these two types of divisions occur simultaneously and in a strictly organised pattern.

In leaf meristems the number of files across the blade increases during development in the sub-apical meristem. The constant increase in the length of the leaf primordium during this phase (Silvy, 1982), suggests that the number of cells along a file increases at the same time. Both formative and proliferative divisions occur therefore during at least the first 2 plastochrons after leaf initiation. However, as mentioned earlier, there is no further increase of the number of files across the blade after emergence of its tip from the whorl of surrounding leaves. This implies that after emergence only formative divisions occur in the meristem. Therefore, in contrast to the situation in the root meristem, formative and proliferative divisions are separated temporally.

An important question in relation to the cellular basis of leaf development is how the number of formative and proliferative divisions (*i.e.* the number of files across the blade and the number of cells per file) are determined. Given that proliferative divisions cease just prior to leaf emergence from the whorl of surrounding leaves, the number of formative divisions is determined by the rate of those divisions and the duration between initiation of the primordium on the apex and the onset of rapid leaf expansion.

The results in chapter 3 show that the number of proliferative divisions varies between cell type and leaf position, but is rather insensitive to R_s. The number of cells along the blade for each cell type is determined by the rate of cell production by the population of dividing cells as a whole and the duration of cell division activity in the blade (*i.e.* distal to the ligule, which gradually moves through the division zone; see Schnyder *et. al.*, 1990). The rate of cell production in each file depends on the number of dividing cells in that file and the rates at which those cells divide. Currently not much is known about the functional relationships between cell expansion, the volume at which cells divide and partitioning rates. Not much more is known about how the number of dividing cells within a file is determined. In *Lolium perenne,* cell length distribution along the growth zone was very similar between different stages of development of the leaf (Schnyder *et. al.*, 1990), suggesting constancy of both the length and number of proliferative cells. For root meristems, two alternative points of view have been put forward: 1. the number of divisions per founder cell is determined at its inception at the base of the growth zone (Silk, 1992), and 2. the presence of a spatial gradient of one or more morphogens controls spatial distribution of proliferation (Barlow, 1976). The fact that asymmetrical divisions occurred at the same location in files of most cell types (Chapter 4; Figure 6 and Schnyder *et. al.*, 1990), supports the spatial control mechanism. However, the variation in length of the division zone between adjacent epidermal files (Chapter 4; Figure 6) and between epidermal and mesophyll cells (MacAdam et al., 1989), is not easy to explain on the basis of a spatial control mechanism. How the number of divisions per founder cell can be determined at inception and how this number can be so different between cells in neighbouring files is also not easily understood from a mechanistic point of view. Hopefully research into the molecular mechanism underlying the regulation of the cell cycle will provide us with new insights into these problems.

Preserved parameters.

One interesting outcome of the present work is the identification of a number of parameters that are unaffected by R_s in all leaves. Although minor differences between the number of cells per file in any given leaf in response to variations in R_s were found, these differences were surprisingly small. Similar results were obtained in response to light intensity and ambient CO_2 concentrations by Masle (unpublished data). Other researchers however found that in other grasses the number of cells along the blade was greatly affected by light intensity (Mitchell and Soper, 1958; Friend, 1966; Friend and Pomeroy, 1970) and photoperiod (Ryle, 1966).

The constancy of the number of cells constituting the elongation only zone, not only between R_s treatments, but even between leaves (Chapter 4; Table III) is even more striking. Only few kinematic investigations into the cellular basis of leaf expansion rate have determined the number of cells in the elongation only zone or even in the growth zone as a whole. Volenec and Nelson (1983) found that the number of cells in the whole of the growth zone of tall fescue increased from 300 to 487 with increasing nitrogen fertilisation which resulted in 30 and 89 % increase in leaf elongation rate, respectively. Unfortunately no distinction between cells in the zones of cell division and expansion was made. Because growth in the elongation only region accounts for 85 to 90% of the overall leaf elongation rate, the mechanism that determines the size of this zone is of great importance in the determination of the growth of individual leaves and potentially of crop growth in general. Large differences in the number of cells in the growth zone as a whole were shown to account for a great part of the differences in genotypically determined differences in leaf elongation rates of tall fescue (Volenec and Nelson, 1981; Volenec and Nelson, 1983).

Relationship between cell volume and partitioning.

The results of Chapter 4 (Figure 1) show that there is no set threshold length at which cells divide. This suggests that cell division is somewhat independent of cell size. This is at odds with the assumption that attainment of a given critical cell size determines when a cell divides (Körner et al., 1989). It seems unlikely that variations in cell width and thickness would compensate for the differences we observed in the length at which cells

divide at different locations in the division zone, in different leaves or between R_s treatments. However, determination of cell volume at which cells partition will have to be made before a direct relationship between timing of cell partitioning and cell size *per se* can be ruled out.

Spatial gradients of cell partitioning and expansion rates.

As a consequence of working at the cellular level, we were able to obtain data with a very high spatial resolution which was particularly useful in the kinematic analysis. These data show that, similar to cell expansion rates, cell partitioning rates vary spatially within the division zone (Chapter 4; Figure 5). This implies that average cell cycle values conceal useful information, and that for understanding of the process of cell division and its role in relation to whole leaf growth, determinations of local partitioning rates are necessary.

Putative cell compounds involved in cell cycle regulation.

In Chapter 4 it was shown that the processes of cell expansion and partitioning in the division zone are not tightly linked, as is sometimes assumed. The question arising from this is how progress through the cell cycle is regulated and what is the nature of the relationships between cell expansion and partitioning.

The cell cycle is controlled at specific points, notably at the transition between G1 and S, and G2 and M phase. At these control points, the activity of certain key enzymes is required to trigger progression through the cycle. Cyclin dependent kinases (CDK's) determine the competence of a cell to divide, *i.e.* to progress past the control points. The activity of CDK's depends on activation by cyclins. Each cyclin specifically affects only one of the two control points. The concentration of these cyclins oscillates during the cell cycle, and cyclin concentrations are therefore thought to be determining cell cycling rates. Moreover, in animals and yeasts, G1 cyclins have been shown to be involved in signalling pathways that control cell division. In plants however, many functions of putative cell cycle regulators have not yet been examined, although many enzymes homologous to those identified in yeasts and animals have been found (for a recent review of cell cycle regulation in plants, see Doerner, 1994).

Aspects of interest with respect to cell expansion are: the minimal and maximal size at which cells can divide and what determines these sizes; the nature of the interactions between cell volume expansion and the accumulation of cyclins and CDK's; and how this relates to the observed cell division rates and volume at which cells divide. Kinematic analysis of cell partitioning rates as a function of location in the division zone, as done in chapter 4, is a necessary basis for further investigations into regulation of the cell cycle in growing plant organs.

Asymmetrical divisions.

Although the formation (Stebbins and Jain, 1960; Stebbins and Shah, 1960; Tomlinson, 1974) and functioning (Zeiger et al., 1987) of stomata have been the subject of a great body of research, surprisingly little is known about stomatal initiation. Why are stomata confined to a limited number of well defined files in grass leaves, and why do they seldom occur in two adjacent files? It is striking that both trichomes and stomata originate from an (initial) asymmetrical cell division, and that these divisions occur in all files at a similar location in the growth zone (Chapter 4, Figure 6; see also Stebbins and Shah, 1960) even when symmetrical divisions in these files stop at different distances from the base of the leaf (Chapter 4; Figure 6). It is also interesting that bulliform cells are apparently incapable of trichome formation. Grass leaves, in which the spatial positions of cells along files are a measure for their developmental stage, form an ideal system for investigating the physiological basis of these differentiation processes.

Modelling leaf expansion.

Modelling is often used as a method to enhance our understanding of biological processes. Only one model of cell division and expansion in relation to leaf growth has been published to date (Arkebauer and Norman 1995a; Arkebauer and Norman 1995b; Arkebauer et al., 1995). In addition to this, a number of models describing steady-state root growth based on the dynamics of cell division and cell expansion have been published (López-Sáezet al., 1975; Baake and Buff, 1986; Bertaud *et al.*, 1986). In contrast to the model proposed by Arkebauer and Norman, which incorporates temperature and water relations, none of the latter models includes the effects of environmental factors on cell division and expansion rates.

There are major differences between these models in the methods by which cell expansion and division in the division zone are simulated, which is illustrative of our current lack of understanding of the relationship between these two processes.

Cell division

Baake and Buff (1986) avoid modelling cell division and expansion in the division zone by introducing an injection function, which adds new cells to a cell file at the base of the elongation only zone. López Sáez *et al.* (1975) assume constancy of the number of cells in the division zone and the duration of the cell cycle and assume proliferating cells to be of constant length, regardless of their progression through the cell cycle. Arkebauer and Norman (1995a) propose a simple model in which the size of proliferating cells increases from 600 to 1200 μm. Cell division occurs by definition upon reaching the upper limit. The duration of the cell cycle (growth from 600 to 1200 μm^3) of dividing cells (and thereby indirectly also cell expansion rates) is determined by an empirical dependence of cell cycle duration on temperature. The number of proliferative cells in their model varies throughout leaf development, as function of daughter ratio (the proportion of cells that will remain proliferative (capable of dividing again) after each division) vs time. When this daughter ratio becomes 0, leaf expansion ceases after all cells have completed their expansion. Bertaud *et. al.* (1986) also modelled cell expansion during progression through the cell cycle. Their model allows for random variation in the length at which cells divide. According to this model cell division occurs at a random length between $2*L_{min}(x)$ and $L_{max}(x)$, with $L_{min}(x)$ and $L_{max}(x)$ the minimal and maximal lengths observed in experiments with roots at location x. The length and number of cells in the division zone in this model are not input parameters; they are derived from cell division rules and empirically determined distributions of spatial velocity and cell length.

The data presented in this thesis shows that:

1. There is no single threshold size at which cells divide. Cell division occurs at different lengths, depending on the position of a cell along the growth zone. The relationship between position and length at which cells divide is sensitive to R_s and varies with leaf position (Chapter 4; Figure 1).
2. Cell partitioning rates vary with position along a file in the division zone between different leaves and in response to environmental conditions. Cell cycle duration was

found to increase in successive leaves and to be variously affected by R_s (Chapter 4; Table IV).

3. Meristematic cells do expand. In fact, expansion in the division zone accounts for 10 - 15% of the overall leaf elongation rate. Moreover, cell elongation rates vary spatially and are somewhat independent of cell division rates (Chapter 4; Figures 2 and 5).

One question that remains unsolved is how the length of the division zone is determined. Two possibilities were addressed before: 1) Spatial control, *i.e.* length of the division zone itself is determined and 2) The number of cell cycles per initial cell is set at the inception of this cell at the base of the division zone, which indirectly determines the number of cells in the division zone at any stage.

Cell expansion

Another aspect of leaf development about which there appears to be no consensus is the relationship between mature cell length, the length of (and number of cells in) the elongation only zone and cell elongation rates.

Opposing assumptions were made in the different models mentioned above:

1. The length of the elongation zone is fixed; and it is determined by the position at which local velocity ($v(x)$, that is used as input for the model) becomes constant (Bertaud and Gandar, 1986; Bertaud *et. al.*, 1986).
2. The length of the elongation zone is a result of the number of elongating cells and of their growth kinetics (López-Sáez *et al.*, 1975).
3. The length of the elongation zone is the result of the duration of expansion of individual cells, which is determined by an empirical function $r(t)$, the number of cells entering the elongation zone per unit of time and their initial length (Baake and Buff, 1986).
4. The length of the elongation zone is indirectly determined by the constantly varying number of cells entering it and their expansion rate, which is a function of cell volume and decreases asymptotically to zero when the cell volume reaches a certain maximum length (Arkebauer and Norman, 1995a; Arkebauer and Norman, 1995b).

A central assumption in the kinematic analysis (Chapter 4) was a constant length of the growth and elongation zones throughout most of the linear phase of leaf expansion, an assumption that is supported by Schnyder *et. al.* (1990). Our data show a very constant number of cells in elongation zones of different lengths and that distributions of relative rates of cell expansion are very similar between low and high R_s when expressed on a basis of number of cells from x_{sd} (Chapter 4; Figure 2). This suggests that rather than a function of distance along the elongation zone or time since inception of an initial cell, local cell expansion rates could well be a function of cell rank within the file. Mature cell length is shown to vary extensively between different cell types, leaf positions and R_s treatments. It seems therefore more likely that cell size is determined by expansion rates rather than expansion rates being a function of cell size.

Summarising, modelling of expansion growth in intercalary meristems of roots and grass leaves is currently based on a number of assumptions, that are not supported by the experimental data presented in this thesis, or in the literature, or for which at present no supporting experimental data exist. These assumptions point to gaps in our current understanding of the regulation of leaf development that need further investigation, such as: the interrelationships between cell expansion and cell partitioning in the division zone; the determination of the number of proliferative cells; the nature of the relationship between mature cell size, relative cell expansion rates and size of the elongation only zone (both in terms of number of cells and overall length); the mechanism by which the duration of leaf elongation is determined and the cellular bases of differences in development caused by various environmental factors. Investigations like the one presented in this thesis extends our understanding of the leaf developmental process and should form the basis of further modelling efforts.

Variability

The results presented in this thesis show a considerable degree of variability in cell size data between different plants and spatially within the leaf. This variability remains often hidden in data presented the literature. However, it needs to be taken into account when sampling, and makes extrapolation from a sample taken at a single location to the whole leaf scale, which is often done in the literature (e.g. Mitchell and Soper, 1958; Guttridge and Thompson, 1963; Friend and Pomeroy, 1970), a highly questionable

approach. We largely overcame this problem by sampling at several locations (Chapter 3) and by working cell by cell (Chapter 4). To improve the analysis further, one could start with a more homogenous set of plants. Selection of a homogenous sub-sample needs to be such that the selected plants closely resemble the average for each treatment. Given the labor intensive analysis of the specimen and data processing in all of the work presented in this thesis, this approach is preferable to increasing the number of specimen per treatment.

Extrapolability of the results.

Cultivar Egret in comparison to other genotypes.

Masle (1992) compared the overall growth response to increased soil resistance across a range of genotypes of wheat and barley, including modern and land race lines. The growth response of cultivar Egret, which was used in the experiments described in this thesis, was representative of wheat and barley in general. However, of the modern wheat varieties tested, Egret appeared to be the least responsive variety to R_s. We would therefore expect similar results had we used another cultivar, in terms of directions and nature of the effects of R_s on leaf expansion. However, genotypic differences in the magnitude of the responses seen in Egret at the level of elemental growth processes are to be expected.

Dicotyledonous leaves.

In contrast to grass leaves, cell division and expansion in dicotyledonous leaves is not restricted to the base of the leaf during the phase of rapid leaf expansion. Although rates of cell division and cell expansion are highest near the base of the blade, both processes occur throughout the blade during a large portion of leaf expansion (Erickson, 1965; Maksymowych, 1973; Esau, 1977). Despite these differences in organisation of growth processes, responses to root stress (high R_s or drought) in dicotyledonous leaves appear to share many similarities with those observed in monocotyledonous species. Masle (1990) found a reduction of leaf area expansion and transpiration rates in tomato in response to high R_s similar to that observed in wheat and barley. Similarly, leaf elongation rate was reduced by low soil water potentials in sunflower (Sadras *et. al.,* 1993), *Phaseolus.* (Neuman and Smith, 1991), tobacco (Clough and Milthorpe, 1975) and

soybean (Randall and Sinclair, 1988). Similar to the results presented in this thesis, the response of soybean leaves was dependent on the developmental stage of the leaf when stress was first experienced: the final number of cells per leaf was affected most by a single eight day drought, occurring during the early stages of development (Randall and Sinclair, 1988). Morphological adaptations to drought in leaves similar, to those described in chapter 3, are generally referred to as xeromorphism and occur in a wide range of species, both dicotyledonous and monocotyledonous (Maximov, 1929; Oppenheimer 1960; Jones 1985). It may therefore be suggested that a number of results described in this thesis, may apply to responses to other root stresses related to R_s and to other species.

Experimental setup in comparison to natural conditions.

The use of controlled environment growth cabinets and of pots with homogenised soil prepared so as to achieve a standardised set of physical characteristics, enhanced the reproducibility of experiments and facilitated comparison between successive experiments. However, the ultimate aim of experimentation is to investigate the nature of processes that occur under natural conditions. The question therefore arises of how representative our data are in relation to more natural conditions.

In natural environments, plants are experiencing a much more complex and variable set of environmental conditions. Interactions between variations in several factors are likely to occur and affect the response of plant growth to any one particular environmental factor (see Introduction). Moreover, in the field, soils are not homogeneous. The presence of macro-pores, for example, greatly affects the magnitude of the plant growth response to otherwise hard soils (Passioura, 1991; Cornish, 1993). However, growth and yield reductions in response to high R_s under field conditions have been well documented (see references in Bowen (1981)). Therefore there is no doubt that qualitatively the responses studied in this thesis are relevant to agricultural conditions, and natural ecosystems.

Three dimensional growth aspects.

Length, width, thickness of leaf as a whole.

The investigations presented in this thesis have been restricted to two-dimensional growth of the epidermal cell layers only. However, the leaf is a three dimensional structure and comprises a number of different tissues. For a more complete understanding of the response of both leaf growth and functioning, further investigations into the relationships between growth in length, width and thickness of cells in various tissues and the origin of differences in the number of cell layers are needed.

The observed increase of leaf density (Chapter 2; Figure 5) with increasing R_s could reflect a reduction in average cell size, an increased number of mesophyll cell layers, a denser packing of cells (especially the mesophyll, *i.e.* smaller intercellular airspaces) and an increase in the concentration of solutes in the vacuoles and cytoplasm of cells. The results of this thesis only provide information on the first parameter: showing that cell density is increased. Data of MacAdam Volenec and Nelson (1989) show that the ratio of number epidermal cells to mesophyll cells in the adjacent layer is not fixed, suggesting that no direct inference on mesophyll cell density can be made from our observations on the epidermal cell layer. However, we observed similar variations in the number of mesophyll cell files constituting the blade as for epidermal cell files (data not shown). Currently we do not know of the effect of R_s on the number of mesophyll cell layers. Zagdanska and Kozdoy (1994) show that in wheat leaves the number of mesophyll cell layers is reduced by drought stress. Masle et al. (1990) showed that the concentration of soluble carbon in the cells is also a factor contributing to increased leaf density.

It would be very useful to obtain data on the effect of R_s on the development of the mesophyll. The mesophyll constitutes a great portion of the leaf volume (representing approximately 42% of the crossectional area in tall fescue (MacAdam et al., 1989) for example), and therefore mesophyll cells form a major sink for photosynthates in the leaf growth zone. The structure of the mesophyll is of course also of importance for CO_2 fixation (Björkman, 1981).

One aspect of mesophyll development of particular interest in the context of this work relates to its relationship to the development of the epidermal cell layer. MacAdam et al.

(1989) showed that high N nutrition increased cell division activity in the mesophyll of tall fescue leaves more than in the epidermal cell layer. As a result, a greater number of mesophyll cells was found adjacent to each epidermal cell in the mature part of the blade. The authors also showed that mesophyll cell divisions continue over a longer period than epidermal divisions (the length of the division zones in their study were 2 and 10 - 14 mm for epidermal and mesophyll cells, respectively) and that cessation of mesophyll division was associated with epidermal cells reaching a certain threshold size.

The vascular tissue is a prominent feature in cross-sections of the grass leaf. In mature blades, sclerenchyma cells appear in longitudinal plates extending from the larger vascular bundles (Esau, 1977). The relatively dense structure of the vascular tissue itself, and the sclerenchyma in particular, resulting from extensive secondary cell wall thickening, suggest that these tissues could possibly resist a great amount of tensile strength. My own observations and those published by MacAdam et al. (1989) showed the absence of sclerenchyma in the basal regions of the growth zone, whereas it is present in the mature region of the blade. Plants cells don't slide relative to one another; one tissue resisting extension affects the extension of the other tissues and thereby the leaf dimensions. Therefore it could be hypothesised that the secondary wall thickening involved in sclerenchyma formation may be involved in the termination of cell expansion at the distal end of the elongation only zone. To my knowledge, thorough comparative analysis between the processes of expansion growth and the development of this sclerenchymatic tissue has not been published.

Where from here?

Further research into R_s effects on leaf growth.

Most research into the effects of R_s, as well as other stresses, is usually focussed on either metabolic or growth processes. The work presented in this thesis may function as a basis for linking the two. A number of interrelationships between the metabolism of the plant and anatomical characteristics need further investigation: The relationship between carbohydrate levels and cell expansion and partitioning rates, *i.e.* how is the sensitivity of growth to carbohydrate supply of the growing tissues mediated (Masle et al., 1990); the

nature and development of anatomical differences in mesophyll structure and the consequences for photosynthetic characteristics of the leaf; the nature of the putative hormonal signal from the roots (Termaat et al., 1985; Masle and Passioura, 1987; Masle et al., 1990; Passioura and Gardner, 1990) and the mechanisms by which it affects leaf growth and carbohydrate partitioning between roots and shoots.

Further research into grass leaf development.

The work in this thesis shows that the development of grass leaves is a complex process, and many aspects of it remain to be investigated: Thorough analysis of the establishment and functioning of the growth zone during development in the sub-apical meristem would greatly increase our insight into the basis of structural differences of the growth zone as observed in this thesis in response to R_s and leaf position and in other studies in response to other environmental factors. This work identifies a number of processes in the developing leaf that are sensitive to environmental clues. Similar analyses of the response to other environmental factors will be necessary to demonstrate the generality of our observations and to enable investigations into the regulatory mechanisms involved.

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