Effect of drought stress on the cytological status in *Ricinus communis*

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Abstract

Growing leaves of dicots are characterized by the simultaneous development of cytological structure and physiological function. Cytological development of growing leaves of castor bean (*Ricinus communis* L.) and the impact of drought on this process was studied. Cell division was observed when the middle lobe of the leaf was below 8 cm length. Cell densities dropped when the middle leaflet had reached 4 cm. Identical relationships between leaf size (length of the middle lobe) and (i) exposed surface area of epidermal cells, (ii) height of palisade cells, (iii) cell density and stomatal density were observed. During drought, areal growth decreased, but the relationships between the cytological parameters and leaf size did not change. The impact of drought on the cellular growth processes depended on the stage of cytological development at the onset of the drought. These results are the basis for an analysis of physiological and biochemical parameters in forthcoming studies.

Key words: Drought, leaf growth, cell division, cell density, stomatal density, cytological status, *Ricinus communis*.

Introduction

Growth zones are regions with a high demand for substances and energy. When plants are subjected to unfavourable conditions these may not be sufficiently supplied. It has long been known (Hsiao, 1973) that growth rate rapidly declines in response to stress often prior to a decline in activity of the supplying tissues ('sources'). Growing parts of a plant are highly sensitive to environmental constraints and often determine the behaviour of the whole plant via feedback regulation (Krapp *et al*., 1993). However, the response of cell division to drought is still a matter of debate (Clough and Milthorpe, 1975; Yegappan and Patton, 1982).

In contrast to monocots, growing dicot leaves are characterized by a complex spatial and temporal pattern of cell division and cell elongation (Maksymowych, 1963). The entire leaf blade is involved in growth, which makes analysis of the response of leaf growth in dicots much more complicated than in monocot leaves, roots or other primarily linear-organized growth zones (Silk, 1992).

In parallel, the physiological status of a growing leaf changes dramatically both during its development (Turgeon, 1989; Maksymowych, 1963) and in response to environmental factors. On the other hand, environmental factors also influence the rate of development of the plant. Prior to experiments on the impact of drought on the physiology and biochemistry of growing leaves of *Ricinus communis*, a thorough analysis was conducted of the influence of drought on basic cytological parameters including cell division and cell expansion. This study aimed to analyse the cytological background for physiological processes studied in experiments characterizing the response of growing leaves to drought and their contribution to drought tolerance in *Ricinus*.

Materials and methods

Seedlings of *Ricinus communis* L. were germinated in vermiculite and transplanted to special planting pots (4470 cm³) topped by an aluminium cap and fitted into a root pressure chamber (Schurr and Schulze, 1995). This was because the root pressure chamber was to be used in follow-up experiments. The root of a seedling was introduced into the moist sandy loam (bulk soil density: 4.1 g dry soil cm⁻³) through the hole in the aluminium cap (Gollan *et al*., 1992). The plants were cultivated for 3 weeks in a growth cabinet at a day/night cycle of 12/12 h (300 μmol photons m⁻²s⁻¹ during the photoperiod), a constant air temperature of 25 °C and a relative humidity of 60/45% during the light and dark periods, respectively. Plants used in...
the experiments had developed one pair of green cotyledons, one pair of primary leaves and one fully developed main leaf (Fig. 3). Until the start of the drought experiments, the gravimetric soil water content was kept above 0.20 g g\(^{-1}\) by repeated watering with modified Hoagland solution (in mol m\(^{-3}\): K\(^+\) 3.0, NO\(_3^-\) 4, H\(_2\)PO\(_4^-\) 1, Ca\(^{2+}\) 1, SO\(_4^{2-}\) 2, Mg\(^{2+}\) 2; in mmol m\(^{-3}\): NH\(_4^+\) 0.12, Fe\(^{3+}\) 18, BO\(_3^{3-}\) 1, Mn\(^{2+}\) 0.09, Zn\(^{2+}\) 0.035, Mo\(_{5}\)O\(_{2}\) 0.02).

**Experimental design**

The experiment was designed to study the course of changes occurring in the growing leaf No. 3 during drought stress. One half of the plants was subjected to drought stress from day 18 after germination on, while control plants were kept well watered. Samples from the leaf base and the leaf tip of leaf 3 were taken from water-stressed and control plants at day 24 (no apparent decline in growth rate), day 26 (significant decline in growth rate), day 29 (cessation of areal growth), and day 35 (growth had stopped for several days) after germination. Control plants were also sampled on day 32.

**Determination of the length of the middle lobe of the leaf**

The length of the middle lobe of the leaf was used as a measure of leaf size, since it was linearly related to leaf area and measurable with a ruler when the leaf was still covered by the bud scales. Individual analysis of the growth rates of all lobes of the leaves revealed that the middle lobe was representative for the others in relation to the relative growth rates (data not shown).

**Cytological parameters**

For determination of cell density, leaf discs (4 mm diameter) were incubated in 500 μl of digestion mix (1U Pectinase, 50 mM Na-acetate pH 4.0, 2 mM EDTA) for 7 h in 37°C with intermittent shaking. Thereafter samples were forced through a syringe at least twice to shear any remaining tissue fragments. This procedure was repeated, if microscopic analysis still showed intact tissue residues. The density of the cell suspension was estimated in a Neubauer chamber.

Cell numbers per leaf were estimated to determine the endpoint of mitotic activity. Since all other parameters were determined in samples from the leaf tip and the leaf base, tests were made to see if the number of cells could readily be calculated from samples taken from these regions of the leaf only. Two methods were compared (i) cell densities were determined from samples taken from 13 regions of the leaf covering all interveinal areas and regions from the inside of the leaf to the margin. Cell numbers were calculated from

\[
N = \frac{\sum_i \{\text{cell density}_i \times \text{area}_i\}}{\sum_i \text{area}_i} \times \text{leaf area}
\]

(ii) Alternatively, cell numbers were calculated from the samples taken at the leaf tip and the leaf base by

\[
N = \frac{\text{cell density}_{\text{tip}} + \text{cell density}_{\text{base}}}{2} \times \text{leaf area}
\]

The number of cells per leaf obtained by either of these methods was almost identical (Fig. 1).

For the determination of cell density and mitotic indices in individual cell layers, leaf discs sampled at the leaf base and the leaf tip of leaf 3 were fixed in fixation buffer (100 mM PIPES, 10 mM EGTA, 5 mM MgSO\(_4\), 5% DMSO, 2% paraformaldehyde; pH 6.8) for 45 min. Thereafter the fixation buffer was removed and leaf discs were washed once in PBS buffer (140 mM mM NaCl, 2.6 mM KCl, 8 mM Na\(_2\)HPO\(_4\), 1.4 mM K\(_2\)HPO\(_4\), pH 7.2). Samples were placed in 90% water-saturated phenol for 30–45 min (depending on the age of the tissue) and were then washed 2–3 times with 4 M KOH. Then leaf discs were washed again 3 times for 5 min in PBS buffer. The cuticle was made permeable by a short application of 5% H\(_2\)SO\(_4\) in diethylther, followed by washing three times in PBS for 5 min. Leaf discs were vacuum-infiltrated with DAPI-solution (4.6'-diamidino-2-phenylindol 0.2 HCl, 1:100 000 in PBS). To prevent photobleaching of the stained tissue, the samples were mounted on glass slides with a mounting solution (100 mg ml\(^{-1}\) 1,4 diazobicyclo-[2,2,2]-octane in 90% phosphate buffered glycine). Nuclei per area were counted under a Zeiss ICM 405 with a mercury vapour pressure lamp and a filter set Zeiss G 365. Anaphases and telophases were counted separately to calculate the mitotic indices (nuclei in mitosis per stained nucleus).

The height of the palisade cells was determined with a microscope from the cell suspension obtained from the pectinase digestion. This suspension could be kept at 4°C overnight without changes in the cellular parameters. Statistical analysis (SAS Wilcoxon) showed that the measurement of 10 palisade cells per sample gave mean palisade cell heights, which were not significantly different from measurements of a larger number of palisade cells.

The exposed surface area of epidermal cells and stomatal density were analysed from replicas of the upper surface of leaf discs (diameter 4 mm) made with Formvar (dissolved in 3% methanol). After 1 min the replica could be peeled from the leaf disc and mounted on to a glass slide for microscopic analysis. Images of the replica were captured with a frame grabber (Truevision Targa 32+) from a CCD-camera (Sony XC75) mounted on a microscope (Olympus BX 40). The exposed surface area of 20 individual epidermis cells was determined with a image processing programme (Sigma Scan, Jandel Scientific). No significant differences of the measure were obtained when larger numbers of cells were evaluated (SAS Wilcoxon). The density of fully differentiated stomatal complexes with visible central stomatal pores was also determined with the Formvar replicas.

**DNA content per leaf**

Whole leaves were sampled and stored at −80°C until they were extracted following the procedure by Doyle and Doyle.
Ground leaves were extracted in centrifuge tubes with 15 ml extraction buffer at 60 °C (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM TRIS, 1% PVP 40, 0.2% mercapto-ethanol, pH 8.0). After 45 min 10 ml chloroform/isoamylalcohol was added. The mixture was centrifuged for 5 min at 4000 rpm at 25 °C in a Beckman J2–21 centrifuge. The supernatant was removed and the remaining solution was discarded. The same procedure was repeated three times. Afterwards the supernatant was mixed with 10 ml cold (4 °C) isopropanol. After centrifugation (5 min, 14 000 rpm, 4 °C) the pellet was washed three times with 70% ethanol for 5 min. Thereafter the residual ethanol evaporated and the dried pellet was dissolved in 2 ml bidistilled H₂O overnight. RNA in the solution was digested by incubation with 100 μg ml⁻¹ RNase A (Sigma) for 30 min at 37 °C. The extraction procedure with chloroform/isoamylalcohol was repeated. After the last centrifugation the pellet was dried and taken up in 500 ml bidistilled H₂O and the optical density at 260 nm was determined at the photometer (Pharmacia). Significant impurities with proteins and RNA were excluded by simultaneous determination of OD at 230 and 280 nm.

Results

Relationship between cell division, cell elongation and the leaf length

The total number of cells per leaf and the DNA content per leaf were analysed in order to determine the leaf size, at which cell division is terminated. The number of leaf cells (Fig. 2a) and the amount of DNA per leaf (Fig. 2b) increased almost exponentially up to a length of the middle lobe of 6–8 cm and remained constant thereafter. The fact that cell number and DNA amount plateau at the same leaf size indicates that no significant polyploidy is present in *Ricinus communis*: at most a doubling of DNA content per cell could be present.

Leaf development of *Ricinus communis* can therefore be separated into three phases: up to length of 4 cm cell division activity is found throughout the entire leaf blade; on reaching 4 cm a significant increase of leaf area was recorded, while cell division became progressively limited to the leaf base and eventually ceased when the middle lobe reached a size of 8 cm.

Leaf growth during drought

When a plant is subjected to a stress treatment it possesses leaves in very different developmental stages. Old leaves do not grow any more, while younger leaves are still undergoing cell expansion or even cell division. During drought stress rapidly expanding leaves reduce areal growth first (Fig. 3B, leaf 3). However, even leaves that show high cell division activity stop growth in response to drought (Fig. 3b, leaf 4).

Leaf conductance declines very rapidly below that of control plants due to the closing of stomata in large leaves. In small leaves the difference between water-stressed and well-watered plants is also caused by the absence of the developmental increase of leaf conductance in water-stressed plants (Fig. 3c).

Exposed surface area of epidermal cells

In leaves of well-watered plants with a length of the middle lobe of 2.5 cm (day 24), where cell division was

![Fig. 2.](https://example.com/figure2.png)  
**Fig. 2.** (a) The number of cells per leaf, (b) leaf DNA content in relation to the length of the middle leaflet of leaf 3 of *Ricinus communis*.

![Fig. 3.](https://example.com/figure3.png)  
**Fig. 3.** (a) Gravimetric soil water content, (b) length of the middle lobe of leaves 2, 3 and 4 and (c) leaf conductance of leaves 3 and 4 during a drought stress treatment. Open symbols: well-watered plants, closed symbols: water-stressed plants. Watering stopped at day 18 after germination.
still occurring, no significant tip-to-base gradients of the exposed surface area of epidermal cells were present (Fig. 4a). However, 2 d later, the area of epidermal cells at the leaf tip had increased approximately 7-fold, while it had only doubled at the leaf base. During the following 9 d the exposed surface area of epidermal cells increased linearly being always 400 μm² smaller at the leaf base than at the leaf tip. Even fully differentiated leaves showed the tip-to-base gradients of exposed surface area of epidermal cells. During drought stress an identical difference of cell size between tip and base of the leaf developed, however, the rate of increase of projected cell area was halved compared to control plants (Fig. 4a).

Unique relationships between the exposed surface area of epidermis cells and the leaf size (length of the middle lobe) were obtained for control and stressed plants. Tip-to-base gradients were identical in leaves of a given size (Fig. 4b).

**Height of palisade cells**

In the smallest leaves studied, the height of the palisade cells tended to be larger in the leaf tip than in the leaf base (Fig. 5a). These gradients disappeared during the further development of the leaves. Fully-developed leaves did not exhibit significant differences in the height of their palisade layers between the leaf tip and the leaf base. The same was observed in drought-stressed plants, but the tip-to-base gradients were present for a longer time, when plants were drought-stressed.

Unique relationships between the height of the palisade cells and the leaf size (length of middle lobe) were obtained for control and stressed plants (Fig. 5b). The relationships support the observation that the leaf tip of *Ricinus* has slightly higher palisade cells.

**Cell density**

Cell density is the result of cell division, cell elongation and the formation of intercellular spaces. In well-watered plants cell density dropped within 2 d by a factor of 4 after the leaf had reached the size at which the relative rates of cell division and cell elongation started to deviate (Fig. 6a). At that developmental stage the leaf base still had twice the cell density as the leaf tip. These gradients had disappeared, when the leaf reached its final size. Cell density of drought-stressed plants were slightly lower than in controls at day 24 even though no significant decline in areal growth rate was observed in leaf 3 at that stage. This would be consistent with an early impact of drought on cell division in leaves at low rates of expansion, as found in some other recently characterized plant systems (see Discussion). However, cell density of drought-stressed plants decreased at a much lower rate than in control plants and tip-to-base gradients were still present 11 d after the onset of the stress treatment, when they had already disappeared in the corresponding leaves of the control plants.

Unique relationships were found between the cell densities and the leaf size (Fig. 6b). No difference was detected between plants grown under stress and well-
declined due to a relative increase of cell expansion/formation of intercellular spaces over cell division. In the palisade parenchyma, cell density initially increased (Fig. 7c) indicating higher relative rates of cell division than cell expansion during that stage of development. In this tissue layer cell density remained constant due to the synchronized activities of cell division and cell expansion until a length of the middle lobe of the leaf of 10 cm was reached. Cell density in the middle part of the leaf lobe studied was always intermediate between tip and base (data not shown). The relative contribution of the individual tissue layers to the overall cell density varied due to the differences in the decline of cell density with leaf size in three different tissue layers. The ratio between epidermal cells and spongy mesophyll cells was almost constant at 1.5 spongy mesophyll cells per cell of the upper or lower epidermis throughout leaf development. However, the ratio between palisade and epidermal cells increased during leaf development transiently.

No significant differences in the cell density gradients between tip and base as well as within the different tissues were found in response to drought stress.

Mitotic indices in different tissue layers

Mitotic indices in the epidermis and spongy parenchyma of the leaf tips declined throughout the studies of leaf sizes (Fig. 8a, b, d). In contrast, mitotic indices in epidermis and spongy parenchyma remained high up to a length of 4–6 cm at the leaf base, corresponding to the leaf size when cell densities started to decline. Mitotic indices were high up to a length of the middle lobe of 8 cm in the palisade parenchyma at the leaf base (Fig. 8c) supporting the interpretation of a maintenance of cell division activity in this tissue layer in larger leaves. The decline in mitotic indices is correlated to the decline of cell densities in the individual tissue layers.

Mitotic activity ceased in all tissues at a middle lobe length of 10 cm. Mitotic activity in the middle part of the lobe studied was always intermediate between tip and base values (data not shown). No significant deviations in the relation between mitotic activity and leaf size were present between well-watered and water-stressed plants.

Stomatal density

The density of differentiated stomata is the result of the formation of stomatal complexes by cell division and differentiation as well as the dilution of these complexes by cell elongation, mainly of the surrounding epidermal cells. Even very small leaves had a high number of undifferentiated stomata (data not shown), but differentiation of stomatal complexes started later in the development. The density of differentiated stomata
increased until the relative rate of formation of stomatal complexes dropped below the relative rate of cell expansion (Fig. 9b). The fact that stomata differentiate at a smaller size at the tip of the leaf compared to the base caused a large increase in stomatal density at the leaf tip even before significant development of stomatal complexes was observed at the leaf base. The fast expansion of epidermal cells at the leaf tip caused an inversion of the stomatal density during the development with higher stomatal densities at the leaf base. Highest stomatal density at leaf tip and leaf base coincided with the highest palisade cell-to-epidermis cell ratios.

This complex pattern between stomatal density and leaf size was again unique for well-watered and water-stressed plants and caused a complex variation of stomatal densities, while leaf growth was changed by drought. At
the leaf base the increase of stomatal density was delayed in water-stressed plants compared to well-watered plants (Fig. 9a), whereas it was significantly higher at the leaf tip in water-stressed plants. Higher stomatal densities than in well-watered plants were reached several days later at the leaf base. Thereafter stomatal density declined in well-watered and water-stressed plants, but water-stressed plants always exhibited a higher stomatal density than well-watered plants. This higher density of sites of potential water loss in water-stressed plants was due to the developmental pattern of the formation of stomatal complexes. Despite this, leaf conductance strongly decreased (see Fig. 3c) in response to drought.

Discussion

Stress causes a systemic response of the plant on all levels of organization. Therefore, analysis of drought stress has to take into account stress effects from the molecular up to the macroscopic level. This is relevant not only because stress effects are present at all these levels, but also because changes on one level strongly interact with those on another level. In growing leaves cytological and physiological differentiation occurs in parallel. Therefore, differences between drought-stressed and well-watered plants could be due either to specific effects of drought or to variation of the speed of the development. The aim of this study was to set the base for further studies on the physiological and biochemical level by analysis of the effect of drought on the cytological status of growing leaves.

Effects on cell division and cell elongation

It has long been debated whether the decline of growth rate in drought-stressed plants involves changes in cell division rate. In dicot leaves cell division occurs during a large fraction of the growth period of the leaf (Figs 2, 8; Maksymowych, 1963). Thus effects on the individual processes of division and elongation cannot be inferred on the basis of cell size changes alone. The analysis of leaf elongation shows that leaves, which are growing without further cell division decline in and stop growth during drought stress. Therefore cell elongation itself is influenced by drought (Fig. 3).

Leaves subjected to the stress, when cell division occurs, also slow down and eventually stop growth (Fig. 3). There are several lines of evidence to indicate that cell division was affected in these leaves. (i) If the rate of cell expansion is diminished without a similar decline of cell division activity, cell densities would increase in leaves in which cell division is going on. However, cell density in leaves from drought-stressed plants declined with leaf size in an identical manner (Fig. 6). This was even true when very small leaves were included (Fig. 7). It might even be deduced from the lower cell density of stressed plants in the leaf base that increase of stomatal density was delayed very small leaves during the initial phases of the drought stress cycle. The density of data during that phase is low, but it would nicely fit data from root tips growing at different water potentials (Sacks et al., 1997).

This interpretation is supported by the decline of mitotic indices (Fig. 8). It can be argued that cells can remain in a mitotic phase for some while without continuation of cell division activity. However, this is contrary to observations noted here. Firstly, mitotic indices are rapidly declining in leaves within 1–2 d after a certain leaf size has been reached. Secondly, the diurnal courses of mitotic indices obtained during conditions identical to those reported here show distinct changes with mitotic indices varying within 1–2 h (unpublished results).

Dicot leaves are characterized by a complex pattern of distribution of cell division and cell expansion activity between the leaf tip and the leaf base. During that differentiation cell division ceases first at the leaf tip and last at the leaf base. If only one of these processes would be affected by the stress treatment this would cause a change in tip-to-base gradients of cell density as well as in cell size parameters. The same argument applies during the early phases of leaf development, when the mitotic activities within the tissues layers differ from each other. If drought would affect only cell division or cell expansion,
the relationships between the various tissue layers would change. Since, however, unique relationships between cytological parameters and leaf size were observed under our experimental conditions, cell division and cell elongation were equally affected.

The mechanism, by which this apparently synchronized behaviour of cell expansion and cell division occurs, is not clear. Small deviations from this co-ordinated behaviour in very small leaves with lower cell densities in drought-stressed compared to well-watered plants seem to be readily overcome resulting in the unique relationships between leaf size and cytological parameters. Several alternative mechanisms might be considered.

On the one hand, common signals including plant hormones (Munns and Cramer, 1996) could affect both cell division and cell elongation. On the other hand, interaction between cell division and cell elongation might occur, if the same processes limiting cell wall expansion in elongating cells (Kutschera and Schopfer, 1986) are active during the phase of plasma doubling in mitotically active cells. It is not clear in plants if, as a regulatory step, a certain size has to be reached prior to the next cell division, as has been suggested by Barlow and Pilet (1984), and if the same processes control cell expansion in dividing cells and non-dividing cells.

Alternatively, the unique relationships of cell parameters to leaf size could be due to the leading role of one process over the other. This might be the case in drought stress cycle experiments, in which stress progressively increases. Therefore the rate of stress development might have an effect on the relationships between cell division and cell elongation.

The constancy of the relations of the different tissue layers to each other during drought stress and well-watered conditions is remarkable in this context. Drought stress did not lead to an unbalanced development of the different tissue layers in relation to leaf size. This was also true for local gradients on the leaf. Tip-to-base gradients for the size of epidermal cells were most pronounced in middle-sized leaves, but were present even in fully-developed leaves.

Co-ordinated development of different tissue layers might also be due to common signals including hormones. However, due to the variation of concentration in and influxes to the tissues, varying sensitivities of the tissues would then have to be the reason for the co-ordinated response. Alternatively, one tissue could trigger the processes in the others as suggested in cylindrical organs (Kutschera and Schopfer, 1995). The epidermis is also suited to play such a role in leaves because it is (i) the tissue where signals transported in the transpiration stream accumulate (Zhang et al., 1987), (ii) its differentiation runs in front of the other tissues, and (iii) its role in the formation of intercellular spaces in the palisade parenchyma is obvious (Esau, 1953).

Interaction between cytological and physiological status of the leaf

This study aimed to set the base for further work on the physiological responses to drought in growing leaves. The interaction between cytological status and physiological processes is already apparent in the data presented in this paper. During differentiation of the stomatal complexes tip-to-base gradients of stomatal density occurred. Due to drought effects on leaf growth stomatal density was higher at a given time after the onset of stress compared to leaves of well-watered plants. Nevertheless, leaf conductance (on an areal basis) not only did not increase as in the developing leaves of well-watered plants, but even declined.

Therefore the physiological process of stomatal closure had to override the higher stomatal density in drought-stressed plants and one part of the lower stomatal conductance in response to drought could be explained by the low status of differentiation of the stomata in small leaves.

Analysis of growing leaves in physiological experiments

Effects of stress on growing parts are very important to the performance of the plant in its present and future environment. Growing tissues determine the immediate behaviour of a plant because of their high demand on energy and substances. In the long-term, changes of leaf growth inevitably influence the structure of the plant by which it has to gain, for example, carbon and light. It is therefore obvious that understanding the stress physiology of plants requires studies on growing parts of the plants during unfavourable growth conditions. Usually in experiments in stress physiology, samples are taken after a defined time of stress impact. This approach causes problems in studies on growing leaves. If the stress treatment causes the sampled leaves to grow at a lower rate or to stop, leaves from control plants and treated plants have a different cytological status. Differences in physiological, biochemical or molecular parameters between the samples could then be biased by the varying overall cell density or the relative contribution of the various tissue layers to the sample. The importance of the second aspect can be seen from the varying ratio between palisade and epidermis cells with leaf size. Palisade cells obviously have different biochemical equipment compared with epidermal cells. If after a defined duration of stress treatment medium-sized leaves of stressed plants are compared to fully developed leaves of controls the ratio between these tissue layers with different biochemical stocks is very different and biases the results on changes in the biochemical composition.

It is obvious from these results that leaves have to be compared on the basis of the same cytological status, which is closely linked to leaf size—at least in Ricinus.
R. communis under the reported drought conditions. This is not only relevant for studies in stress physiology, but equally in any other area where plants exhibiting a growth phenotype are studied (e.g. transgenic plants).

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References


