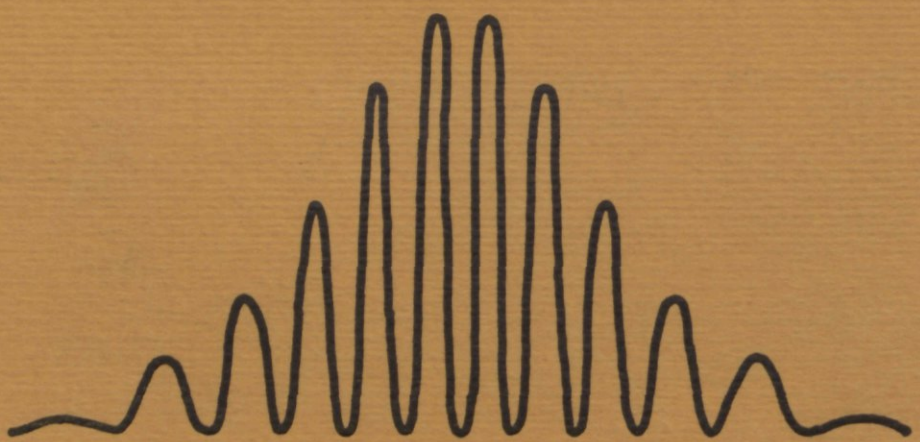
RHYTHMS IN STOMATAL OPENING OF BEAN LEAVES

Ritmen in de opening van de huidmondjes bij de boon



P. A. M. HOPMANS

Dit proefschrift met stellingen van

PETRUS ADRIANUS MARIA HOPMANS,

landbouwkundig ingenieur, geboren te Fijnaart, 28 maart 1939, is goedgekeurd door de promotor, Dr. Ir. S. J. WELLENSIEK, emeritus-hoogleraar in de Tuinbouwplantenteelt.

De Rector Magnificus van de Landbouwhogeschool
J. M. POLAK

Wageningen, 26 februari 1971

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RHYTHMS IN STOMATAL OPENING OF BEAN LEAVES

Ritmen in de opening van de huidmondjes bij de boon

(MET EEN SAMENVATTING IN HET NEDERLANDS)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS, MR. J. M. POLAK,
HOOGLERAAR IN DE RECHTS- EN STAATSWETENSCHAPPEN
VAN DE WESTERSE GEBIEDEN,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP WOENSDAG 12 MEI 1971 TE 16 UUR

DOOR

P. A. M. HOPMANS

H. VEENMAN & ZONEN N.V.-WAGENINGEN-1971

VII

Dat de overheidshulp aan fruitteeltbedrijven tijdens de huidige crisisperiode sinds enige tijd daarin resulteert, dat niet-draagkrachtige bedrijven in stand worden gehouden, terwijl aan het slechter worden van de nog goede vermogenspositie van efficiënt producerende bedrijven vrijwel niets wordt gedaan, is bedrijfseconomisch onjuist en wordt als sociaal onrechtvaardig ervaren.

VIII

Ontharden van gietwater met ionenwisselaars ter voorkoming van vlekken op de bladeren van potplanten is voor Nederlandse potplantenbedrijven een veelbelovende methode.

IX

Veel wetenschappelijke arbeid is al verloren gegaan door pogingen om ze in de vorm van stellingen te publiceren.

Proefschrift P. A. M. HOPMANS
Wageningen, 12 mei 1971

Aan Ineke
Aan mijn Ouders

VOORWOORD

Bij het gereed komen van dit proefschrift wil ik mijn erkentelijkheid uitspreken aan allen, die op enigerlei wijze eraan hebben bijgedragen.

Hen, die mij op de HBS en aan de Landbouwhogeschool natuurwetenschappen hebben bijgebracht, noem ik hierbij speciaal.

Mijn promotor, Professor Wellensiek, ben ik dankbaar voor zijn stimulering een promotieonderzoek aan te vangen en voor de grote mate van vrijheid, die hij mij gegeven heeft tijdens dit onderzoek. De presentatie van de dissertatie is dank zij zijn kritiek ingrijpend verbeterd.

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Aan Dr. Kuiper dank ik talloze suggesties, die de leesbaarheid hebben verhoogd.

Mijn dank gaat ook uit naar mijn collega's van het Laboratorium voor Tuinbouwplantenteelt voor hun kameraadschap en naar de medewerkers van dit laboratorium, van het IBS en van het ITAL, die aan het onderzoek direkt en indirekt hebben bijgedragen. Met vaardigheid hebben Anke Visser en Truus Hoving het manuscript getypt en Henk van Lent en Reyer Jansen het teken- en fotowerk verzorgd.

CONTENTS

GLOSSARY

active ion transport transport of ions requiring metabolic energy

active stomatal movement stomatal movement originating from a change of turgor of the guard cells

air boundary layer of the leaf the zone of air surrounding a leaf exposed to a certain wind speed in which air movement is laminair and transport of water vapour is by diffusion

amplitude difference between maximum (or minimum) value and mean value in oscillation

ATP adenosine triphosphate; an important energy donor in biological processes

β-gauge instrument to measure the mass of a part of the leaf placed between a β radiation source and a β radiation detector, short periodic fluctuations in leaf mass being attributed to changes in water content

boundary layer resistance resistance to diffusion of the air boundary layer of the leaf

build-up period for stomatal opening see 'Spannungsphase' of stomatal opening

carbon dioxide compensation point carbon dioxide concentration at which the absorption of carbon dioxide by photosynthesis exactly balances carbon dioxide output by respiration

circadian rhythm circa = about, diem = day; rhythm with periods approximating but different from 24 hours

cycling of stomata rhythm in stomatal opening with periods smaller than 120 minutes

damping of cycling the gradual decrease of the amplitude of cycling with time

day time interval of 24 hours

diffusion conductance reciprocal of diffusion resistance, expressed as cm sec^{-1} (see leaf diffusion resistance)

diurnal or daily rhythm rhythm with periods of exactly or approximately one day

endogenous rhythm rhythm continuing in constant environment

entrained stomatal cycling stomatal cycling forced to match exactly that of some oscillation in the environment or another part of the same plant

forcing rhythm rhythm capable of synchronizing or entraining another rhythm

free-running cycling self-sustained cycling under constant conditions

full turgor state of turgor when a cell or tissue is in equilibrium with pure water (water potential is zero)

half-time the time for completion of half a process

hydroactive stomatal closure active stomatal closure caused by the reaction of the guard cells turgor to a decrease of plant water potential

ion pump active ion transport mechanism or process

latent heat heat of evaporation

eaf diffusion resistance mean diffusion resistance to gas of both surfaces of the leaf expressed on single side leaf area in sec cm^{-1} and composed of resistances in parallel of the stomatal and the cuticular pathway

leaf 'thickness' mass per unit area (mg cm^{-2}) measured by the β-gauge

leaf water content absolute quantity of water in the leaf

light compensation point light intensity at which the absorption of carbon dioxide by photosynthesis exactly balances the production of carbon dioxide by respiration

negative feedback control system a system in which the value of some factor is controlled by feeding back and using its value in such a manner as to bring the value of the controlled factor closer to a desired (reference) value (see figure 1 on p. 7)

osmosis the transport of a solvent across a differentially permeable membrane

osmotic pressure an evaluation of the potential maximum turgor pressure which will develop in a solution if it is permitted to come to equilibrium with pure water in an ideal osmotic system

overshoot of stomatal movement stomatal movement shooting beyond the degree of stomatal opening that is aimed at

oxidative phosphorylation ATP generation by the transport of electrons from the substrate to oxygen in respiration

passive stomatal movement stomatal movement caused by variation of the mechanical pressure of the surrounding cells on the stomata

peak-to-trough difference difference between maximum and minimum value of oscillation

period time required for an oscillation to make a complete cycle

phase the stage of the cycle; instantaneous state of an oscillation within a period

phase shift a single displacement of the whole cycle along the time axis without changing the period

photoactive stomatal opening active stomatal opening caused by the reaction of the guard cell turgor to light

photoperiod part of the day during which the plants are illuminated

photophosphorylation ATP generation using chemical energy produced by the photoreactions of photosynthesis

plant water potential thermodynamic expression of the difference in chemical potential of the plant water and pure free water, the difference being divided by the partial molal volume of water; it is expressed as energy per volume and its dimensions are equivalent to pressure; water potential of a cell is turgor pressure minus osmotic pressure

positive feedback control system a system working as a negative feedback control system, but bringing the value of the controlled factor further away from the reference value

Q_{10} see temperature coefficient

relative water content ratio of actual water content and water content at water saturation (= at full turgor)

root resistance the resistance to water transport of the root system

self-sustained cycling cycling being able to continue without an influence of a periodic source of energy

short wave radiation visible and infrared radiation up to wavelength 3000 nm

'Spannungsphase' of stomatal opening primary stage of the stomatal opening process during which the pore is not yet opened and preparatory processes in the guard cells gradually build up their turgor pressure

stable control system control system capable of bringing the controlled factor to a constant value near the reference value without overshooting or with successively decreasing overshoots (see figure 2)

stomatal diffusion resistance resistance to gas diffusion through the stomatal opening

sub-period a part of a period of cycling

synchronization state in which two or more oscillations have the same period due to mutual or unilateral influences

temperature coefficient the ratio of the rate of progress in any reaction or process in a plant at a given temperature to the rate or the linearly interpolated rate at a temperature 10 °C lower

time constant the time required for a physical quantity to change its initial magnitude by a factor $1-1/e$ ($e = 2.71828$), being 0.632

turgor pressure the excess pressure exhibited by solution inside plant cells or an osmometer above the external pressure; the equal opposite pressure is exerted upon the cell contents by the walls of the cell

unstable control system control system not capable of bringing the controlled factor to steadiness near the reference level, but cycling continuously and causing the controlled factor to cycle (see figure 2 on p. 8)

vapour pressure deficit the difference in vapour pressure between the actual vapour pressure and saturation vapour pressure of the air at the same temperature

water potential see plant water potential

water stress water shortage

1. INTRODUCTION

1.1. PURPOSES

Cycling stomatal behaviour was encountered during investigations on deviating plant behaviour due to the use of artificially controlled environments. The preliminary investigations concerned the behaviour of the stomatal opening in *Phaseolus vulgaris* plants. At the time, that a vigorous cyclic stomatal movement with short periods (35–50 minutes) and a diurnal rhythm in stomatal opening were found (1967), only a few papers had been issued on these phenomena.

The interpretations were rather inconvincing and far from uniform. During continuous measurement of photosynthesis and transpiration, cycling had been found also by GAASTRA and KUIPER (personal communications) as a seriously disturbing factor.

On first sight as well as during extended analyses, the cycling stomatal behaviour seemed to contradict some ideas concerning the relation between stomatal opening and environmental factors. For these reasons the rhythms in stomatal opening found were challenging.

An analytic study of the stomatal cyclic behaviour was made in order to explore the cyclic behaviour, to explain it, and to utilize the cycling of transpiration rate to obtain information on different aspects of general plant water relations and of properties of the action mechanism of the stomata.

1.2. LITERATURE

In the present condensed literature treatment, publications on stomatal cycling are listed. Literature on diurnal rhythms in stomatal opening will be reviewed in the introduction of chapter 5, whereas several chapters and paragraphs will be introduced with the literature on the subjects concerned. For comprehensive up-to-date treatments of the physiology of stomata reference is made to MEIDNER and MANSFIELD (1968) and ZELITCH (1969). The publications on stomatal cycling can be arranged in 7 groups in relation to the present study.

1. Observations of rhythms, difficult to interpret at present due to the methods used, by MAXIMOW und KRASNOSSELSKY-MAXIMOW (1928) and BORESCH (1933).
2. Stomatal cycling, synchronous for the different plants of a crop in the field with periods of about 2 hours, found by BROWN and ROSENBERG (1970) in *Beta vulgaris*.
3. Microscopic observations. NIKOLIC (1925) found an oscillation in stomatal opening during the adaptation to a changed light intensity and WENT (1944) found it on intact plants. STÅLFELT (1929) found rhythms with periods of 15 minutes in pieces of leaves immersed in water under the microscope. KUIPER

(1961) described separate rhythmic changes with very short periods of individual stomata.

4. Oscillations of stomata with short periods. RASCHKE (1965, 1967) and APEL (1967) found oscillation in maize leaf segments with periods of 3 to 4 minutes. They suggested that this rhythm would originate from the function of the stomata to control the concentration of carbon dioxide in the leaf.
5. Cycling in stomatal opening measured indirectly as rate of transpiration, photosynthesis or leaf temperature or directly with viscous and diffusive flow porometers, with periods ranging from approximately 20 to 100 minutes. Description of damping or sustained rhythms with or without suggestions for an explanation have been published by GREGORY and PEARSE (1937), SCARTH et al. (1948), ANDERSSON et al. (1954), FLORELL and RUFELT (1960), HOWE (1964), SKIDMORE and STONE (1964), KARMANOV and SAVIN (1964), RASCHKE (1965, 1967), KUIPER (1965), KARVE and MISHAL (1966), APEL (1967), COX (1968), EHRLER (1968), GRAU (1968), KRIEDEMANN (1968), UNGER (1968), KANEMASU and TANNER (1969), TAYLOR and GATES (1970).
6. Experimental analyses of the water relations of plants with cycling as indicated under 5 were made by KARMANOV et al. (1965, 1966), EHRLER et al. (1965), HARRIS (1968), BARRS and KLEPPER (1968), HOPMANS (1968, 1969a), LANG et al. (1969) and by RASCHKE and KÜHL (1969). In order to explain the stomatal cycling as an oscillation of the control mechanism of the water relations of the plant, MELESHCHENKO and KARMANOV (1966), MEISTER und APEL (1968), HOPMANS (1969b), LANG et al. (1969) proposed models with a negative feedback control system. HOPMANS (1969b) also indicated a positive feedback loop, which might amplify the cycling, because the stomata seem to be subject to passive opening movements during cycling (COX, 1968; BARRS and KLEPPER, 1968).
7. BARSS (1968), TROUGHTON and COWAN (1968), and TROUGHTON (1969) utilized stomatal cycling to test and estimate the importance of the mesophyll resistance to carbon dioxide transport.

1.3. CYBERNETICS AND RHYTHMICITY OF STOMATA

The stomata control the water status and the carbon dioxide concentration in the leaves by varying the vapour and the carbon dioxide fluxes through the stomatal pores. When the water potential in the leaf falls beneath a certain value, the rate of water loss through the stomatal opening is reduced by partial stomatal closure and the water balance is re-established. When the carbon dioxide concentration in the leaf falls due to an increase in the assimilation rate at a certain stomatal opening, the guard cells increase the stomatal opening until a certain concentration is regained. The interaction of both control functions is only partly known. From what is known about the responses of the stomata to changes in the water status in the plant (STÅLFELT, 1956), combined with the types of oscillatory behaviour published, evidence is available and has

been presented that the stomata behave just as controllers with negative feedback for the water status in the plant (MAERCKER, 1965; KARMANOV et al., 1966; HOPMANS, 1969a, b; LANG et al., 1969; RASCHKE and KÜHL, 1969) and of the carbon dioxide concentration in the intercellular spaces of maize by RASCHKE (1965).

For such a control the stomata have to execute the functions of sensors, comparators, amplifiers and actuators as in figure 1.

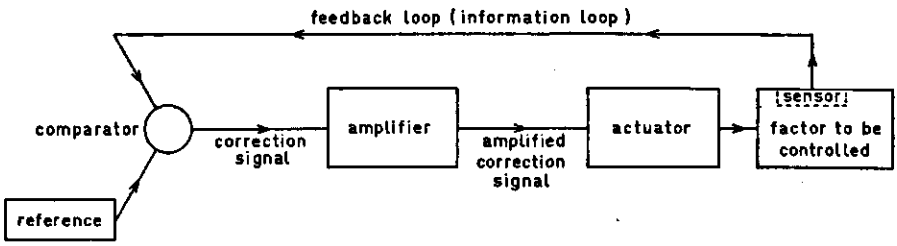

FIG. 1. Diagram of a controller with negative feedback. The comparator supplies the correction signal by comparing the sensor signal with the reference signal.

These components in the control circuit are far from physiologically identified in the stomata. In stomatal control as described above, the information loop to and within the stomata (water potential in the leaf) is different from the control step (changing vapour diffusion resistance). The reference values for both controlled factors, water and CO_2, are dependent on some environmental and internal factors. RASCHKE (1966) indicated an influence of light on the carbon dioxide reference value in his experiments with maize leaves. Also daily rhythms in stomatal opening, independent of photosynthesis, vary the reference value. The reference for the water status is dependent on the species. See for example the ranges of water saturation deficits at which different species close their stomata (STÅLFELT, 1956).

In negative feedback controllers the time delay in the feedback loop and the amplification can be combined with a damping on the actuator in such a way, that a correction of a deviation from the level of the reference overshoots. When these overshoots are repeated, but with decreasing amplitudes, proceeding to steadiness, a damped rhythm occurs. Both in the latter case and when no overshoots occur in the correction, the control system is called stable (SOLLBERGER, 1965). When the overshoots are repeated and reach a maximum level, a sustained rhythm results; the control system is then called unstable. Figure 2 illustrates both cases.

In this theoretical case factor x could be stomatal opening and the control system the stomatal apparatus.

For definitions and explanations of most terms, used in rhythm research, reference is made to ASCHOFF et al. (1965), SWEENEY (1969) and WILKINS (1969). Some terms, much used in the present work will be clarified with the following examples. The stomatal system of many bean plants with 2 primary leaves

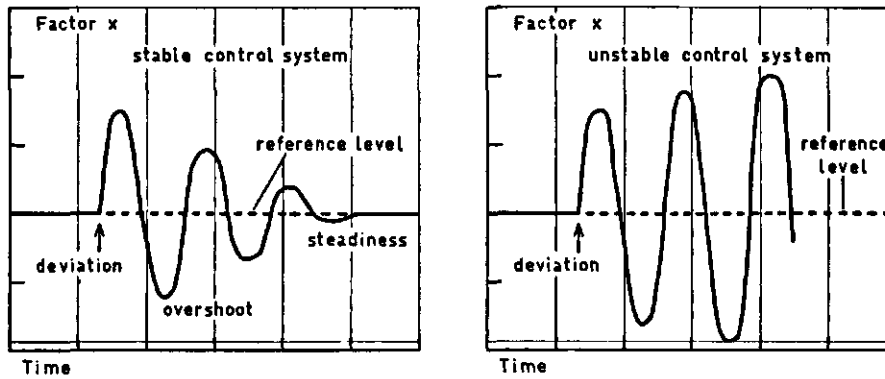

FIG. 2. Course of factor α after deviation from the reference level (arrows), when controlled by a stable control system (left figure) and by an unstable control system (right figure).

exhibited a continuous cycling in a constant environment. This cycling will be called self-sustained; its period is the free-running period. The cycling might be called endogenous in order to stress that there is no external oscillator which causes this cycling. If an external factor e.g. light intensity or humidity, affecting stomatal opening, would be cycling, and stomatal cycling would get the same period, this exogenous agent is called a forcing oscillator, which entrains the cycling of the stomata. A self-sustained oscillation can be entrained only to a range of periods near its own free-running period (KLOTTER, 1960).

1.4. PRESENTATION OF RESULTS

After having dealt with materials and methods in chapter 2, the conditions required for stomatal cycling to be induced and sustained, and the properties of cyling are presented in a mainly descriptive manner in chapter 3. The interaction of the plant water potential with stomatal movement and the way the leaf water content cycled due to resistances and water potential gradients in the transpiration flow pathway will be analysed in chapter 4. Also from this analysis it will become clear that cycling originated from and was synchronized by the interaction of the stomatal apparatus with the water potential in the transpiration pathway, as will be illucidated by a diagrammatic conceptual model. Stomatal cycling has been used as an extraordinary sensitive way to show the effects of factors on stomatal action. The influence of an endogenous circadian rhythm on stomatal opening tendency in light and darkness, as amplified by the cycling, is presented in chapter 5. It is necessary to be aware of this rhythm and its influence on cycling, when interpreting the influence of other factors on stomatal cycling. The influence of light intensity, carbon dioxide concentration and temperature on cycling, separated from the components of the water relations proper, involved in the reactions, will be used to reveal information on the physiology of the stomatal mechanism (chapter 6).

2. GENERAL MATERIALS AND METHODS

2.1. Plants

Beans, *Phaseolus vulgaris* 'Vroege Wagenaar', were sown in a greenhouse at 20 °C and natural daylight. Supplementary irradiation from fluorescent tubes was given from 600 to 2200 with an intensity of approximately 20,000 erg $cm^{-2}sec^{-1}$. The plants were either cultivated in the soil or in water cultures. In the case of water cultures, sowing was performed in river sand; thereafter, the seedlings were placed on a non-aerated nutrient solution with the following composition:

5.5 mM $Ca(NO_3)_2$	0.9 mM $FeSO_4$ (EDTA)
5.5 mM KNO_3	5.1×10^{-2} mM H_3BoO_3
2.5 mM $MgSO_4$	1.1×10^{-2} mM $MnSO_4$
1.2 mM KH_2PO_4	1.5×10^{-3} mM $ZnSO_4$
	4.3×10^{-4} mM H_2MoO_4
	3.5×10^{-4} mM $CuSO_4$

The other plants were grown in peat-perlite in 0.3 l pots and remained in the greenhouse until the primary leaves were fully developed. After approximately 18 days all plants were put in a climate room at an air temperature of 24 °C, a relative air humidity of 75% and an irradiation intensity of 35,000 erg cm^{-2} sec^{-1}. Irradiation was obtained from Daylight type fluorescent tubes (Philips TL 55) for a period of 16 hours per day. If not stated otherwise, all trifoliates had been removed from the plants. The daily light–dark periods were not altered from cultivations to experiments.

2.2. Environment and plant position

Most experiments were carried out in a large climate room with ventilation, but without an operating air temperature or humidity control during the course of the experiments. This procedure prevented short periodic fluctuations of temperature and relative humidity. Each morning after the lights were switched on, the air temperature increased to a fairly constant level within a few hours. The wind speed varied from 10 to 40 cm sec^{-1} depending on the site of the measurement. At the leaf surface the short wave radiation, supplied by 10 40W Daylight type fluorescent tubes (Philips TL 55), was 35,000 erg cm^{-2} sec^{-1}.

The environmental factors, existing during the experiments and shown in the figures, will be presented in an abridged form. For example an air temperature of 25 °C, a vapour pressure of 10 mm Hg, a short wave radiation of 35,000

erg $cm^{-2}sec^{-1}$, and a wind speed of 10 to 15 cm sec^{-1}, will be presented as 25 °C/ 10 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–15 cm sec^{-1}.

Experiments were also carried out in a plexiglass plant chamber of 25 l. Cold air of 5 °C with a vapour pressure of 5–6 mm Hg was forced into the chamber by 2 inlets with a rate of about 800 l h^{-1}. Inside the chamber the air was circulated by 2 ventilators placed on opposite sides and giving a wind speed of 50–60 cm sec^{-1}. The primary leaves and the epicotyledonary part of the stem were only inserted in the chamber. In all experiments the leaves were kept in position between fine nylon wires.

Measurements of transpiration and CO_2 exchange were performed with facilities to be described furtheron.

Measurements on 6 plants in the large room or on 2 plants in the plant chamber were recorded simultaneously.

2.3. Measurement of environmental factors

Air temperature was measured with a copper-constantan thermocouple, built in an injection needle, which was shielded from direct radiation of the light source. The relative humidity was measured with Pernix hair elements. The mV-output of both measurements was registrated with a millivoltrecorder. Wind speed was measured with a hot wire anemometer. Short wave radiation was measured with a Kipp-solarimeter or a selenium photocell, calibrated against the solarimeter.

2.4. Leaf temperature measurement and interpretation

The temperature difference between leaf and air was measured with a double set of copper-constantan thermocouples of 0.1 mm threads, wired in series. Two juntions were pressed against the lower epidermis by their own spring and the 2 other junctions, which were built in an injection needle, were placed in the air underneath the leaf. In combination with air temperature and relative air humidity, the leaf temperature was recorded by a mV Philips recorder. As practically always the relative changes in leaf temperature in a constant environment were to be investigated, measuring the leaf temperatures with thermocouples pressed to the leaf surface was estimated sufficiently accurate. Changes in leaf temperature T_l (°C) at a constant wind speed, air temperature, air vapour pressure and absorbed short and long wave radiation can be ascribed to changes in transpiration rate. Transpiration rate is dependent on the vapour pressure gradient from leaf to surrounding air and diffusion resistance according to the well known relation (see e.g. Slatyer, 1967):

$$E = \frac{c_1 (e_s - e_a)}{r_l + r_a} \qquad (1)$$

E transpiration rate in mg cm^{-2} h^{-1}
c_1 conversion factor
e_s vapour pressure within the leaf in mm Hg
e_a actual vapour pressure of surrounding air in mm Hg
r_l and r_a leaf and boundary air layer resistance to water vapour diffusion in sec cm^{-1}

It is assumed that e_s is the saturated vapour pressure at the leaf temperature. Furthermore, e_s is taken to be linearly related to the leaf temperature T_l for the leaf temperature variations under investigation.

$$e_s = c_2 T_l \tag{2}$$

Equation (1) becomes

$$E = \frac{c_1 (c_2 T_l - e_a)}{r_l + r_a} \tag{3}$$

From theoretical and experimental analyses by e.g. WOLPERT (1962) and GATES (1968) a linear relation may be assumed to exist between T_l and E at constant environmental factors:

$$T_l = c_3 E \tag{4}$$

Equation (3) becomes

$$T_l = \frac{c_3 c_1 (c_2 T_l - e_a)}{r_l + r_a} \tag{5}$$

This equation can be converted into

$$T_l = \frac{c_1 c_3 e_a}{c_1 c_2 c_3 - r_l - r_a} \tag{6}$$

Because the air vapour pressure e_a and the boundary layer resistance r_a in the present experiments were constant, constants are introduced for it into equation (6).

$$T_l = \frac{c_1 c_3 c_4}{c_1 c_2 c_3 - r_l - c_5} \tag{7}$$

The constants in equation (7) are grouped to new constants.

$$T_l = \frac{c_6}{c_7 - r_l} \tag{8}$$

The relation between T_l and r_l is illustrated in figure 3 for different radiation intensities.

The thin bean leaves (approximately 1 mg fresh weight cm^{-2}) have a low heat capacity and therefore the time constant for leaf temperature variation is small. If, for example, a latent heat flux of 0.1 cal cm^{-2} min^{-1} (equal to 1 mg cm^{-2}h^{-1} transpiration) represents the net loss of energy from a leaf of 1 mg fresh weight cm^{-2} for a time interval, the temperature will fall 1 °C per 8

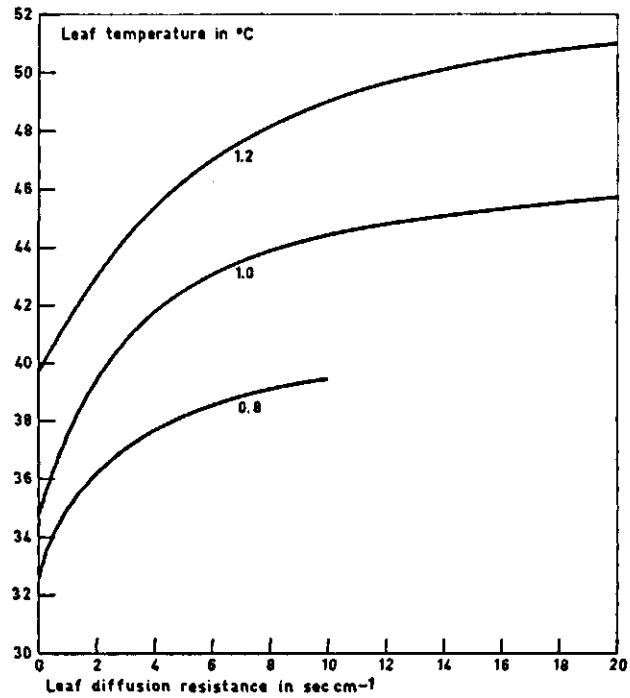

FIG. 3. Relation of leaf temperature to leaf diffusion resistance for radiation intensities 0.8, 1.0 and 1.2 cal $cm^{-2}min^{-1}$ absorbed by a leaf of 5×5 cm. Derived from computed data of GATES (1968). 40°C/27.7 mm Hg/ see figure/10 cm sec^{-1}.

seconds. The time lag of leaf temperature behind transpiration rate may be neglected, even if rapid changes in stomatal opening occur as described in the following.

3. INTRODUCTORY OBSERVATIONS ON CYCLING

3.1. INTRODUCTION

In this chapter conditions favouring the susceptibility of the plants of stomatal cycling will be discussed. The way how cycling is induced will be described. Together with a description of the properties of cycling it may serve as an introduction to the reader to the complicated cyclic behaviour of the stomata.

3.2. PRACTICAL REQUISITES

3.2.1. *Plant cultivation*

In spite of the fact that *Phaseolus* plants have been widely used in studies on transpiration and photosynthesis, cycling stomatal openings have rarely been mentioned (HOWE, 1964; KARMANOV and SAVIN, 1964; KARMANOV et al., 1965; KUIPER, 1965; HOPMANS, 1969a). In the present study, however, cycling was readily induced in the cultivar 'Vroege Wagenaar' and in 'Berna' either grown in peat-perlite or in nutrient solution. In order to test the importance of previous treatments on the plants susceptibility of cycling, an experiment was done in which the effect of various growth factors, described on p. 9, was compared with those in use at the Institute for Biological and Chemical Research on Field Crops and Herbage, IBS (Brouwer, 1960). At that Institute stomatal cycling was rare, considering the continuous recordings of transpiration and photosynthesis available.

In one climate room at 25–26 °C air temperature and 75% relative humidity, plants were grown in aerated or non-aerated nutrient solution in fluorescent light of 31,000 erg $cm^{-2}sec^{-1}$ (low) and in HPLR light of 80,000 erg cm^{-2} sec^{-1} (high). All seedlings were treated as plants for water culture from sowing until the stage that the primary leaves were 3–4 cm long. At high light the plants with aerated roots showed more and longer roots and larger leaf areas than the non-aerated plants, on which adventitious roots were present on the hypocotyl. At low light no morphological differences were observed between aerated and non-aerated plants. The test on susceptibility of cycling consisted of an effort to induce cycling by a dark-light transition shortly after removal of the plants from the growing-environment to the measurement room. Previous aeration was continued. Tabel I shows the results.

Comparing low with high irradiation it is evident, that sustained cycling was induced at once in plants grown in low irradiation intensity, whereas no sustained cycling nor an overshoot occurred in a large part of the plants grown in high irradiation. The effect of aeration was important for plants from high irradiation.

Without aeration cycling was induced in the majority of the plants at once,

TABLE 1. The effect of the growth conditions light and aeration on stomatal reaction on a dark-light transition shortly after removal from the growth conditions.

growth conditions		absolute and relative (in brackets) numbers of plants			
irradiation	root aeration	plants investigated	sustained rhythm	damped rhythm	no overshoot
low	—	7 (100)	7 (100)	0 (0)	0 (0)
	+	10 (100)	9 (90)	1 (10)	0 (0)
high	—	6 (100)	4 (66)	1 (17)	1 (17)
	+	12 (100)	2 (17)	2 (17)	8 (66)

whereas with aeration cycling was induced in only a small number of the plants, the majority not even showing an overshoot. In conclusion, plants from low irradiation intensity showed a higher tendency to sustained cycling, irrespective of aeration, than those from high irradiation intensity. In high irradiation intensity non-aeration increased the tendency to cyling with a more than additive effect (interaction). It should be added, however, that these clear differences diminished considerably when the plants were in the experimental setup in 35,000 erg $cm^{-2}sec^{-1}$. The results suggest that high irradiation and aeration of the root environment make bean plants unsusceptible of cycling.

BROUWER (1960) observed that non-aerated nutrient solutions inhibited leaf and root growth and he found evidence that leaf growth was reduced because of an unfavourable water balance of the plants due to limited root growth. Also permeability of the roots to water in non-aerated nutrient solution was reduced (BROUWER, 1954, 1965). This in turn may explain the higher tendency to cycling as presented by table 1.

Only after a period of water stress the bean plants of KARMANOV and SAVIN (1964) could be induced to cycling. The region of the main water uptake via the roots decreases during water stress, because root growth is limited whereas suberization proceeds (BROUWER, 1965), so that the root resistance to water transport increases. The same explanation might be applied to the observations of KARMANOV and SAVIN (1964).

3.2.2. *Position of the leaves*

Plants, susceptible of cycling of the stomatal apparatus, showed different instability of the stomatal system. The position of the leaves under the light source turned out to be an important factor. In case the primary leaves of one plant overlapped each other, the rhythm was often damped, whereas it turned to a self-sustained rhythm by simply removing the overlapping. The rhythm could be made self-sustained from damped also by taking away young shoots or by bending a trifoliate from its natural place into the same horizontal plane as the primary leaves without causing overlapping of leaf surfaces. These findings suggest that all sites of the total leaf area with active stomata should

be in an equal environment in order to approach the conditions, that the stomatal behaviour over the whole plant without entrainment would be rather similar. It may be concluded that the synchronized cycling over the whole plant, as described earlier (EHRLER et al., 1965; COX, 1968; BARRS and KLEPPER, 1968; HOPMANS, 1968; LANG et al., 1969) and as found here, was frequently disturbed by bringing a part of the active stomata out of phase.

3.3. INDUCTION

3.3.1. *Sharp changes in environmental factors*

Cycling was induced when factors, which affect stomatal opening, were changed abruptly, e.g.:

- Switching on the light after a long dark period.
- A light break by 1 to 10 minutes darkness.
- Increasing the carbon dioxide content of the air from 300 to 900 or more ppm for some minutes.
- A sudden fall of air humidity.
- Applying water to the roots of water stressed plants in peat-perlite.

3.3.2. *Entrainment*

In this experiment (figure 4) the vapour pressure in the plant chamber was cycling with variations of about 3 mm Hg, caused by the cycling stomatal opening in one plant (curve 1). This induced stomatal cycling in another plant (curve 2) in the same chamber. The amplitude in curve 2 gradually increased and cycling turned from entrained into free-running with a period deviating from that of the originally forcing rhythm.

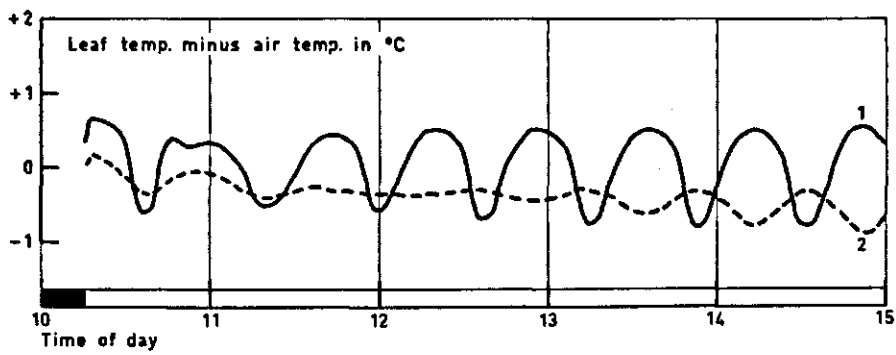

FIG. 4. Self-sustained stomatal cycling in one plant (curve 1), inducing cycling by entrainment in another plant (curve 2) in the same plant chamber. Air temperature and air humidity cycled. 24.8–25.2 °C/9.9–10.2 mm Hg/50,000 erg $cm^{-2}sec^{-1}$/50 cm sec^{-1}.

Figure 5 shows an example of how stomatal cycling in one leaf was mediated to other leaves on the same plant through induction by entrainment. The plant

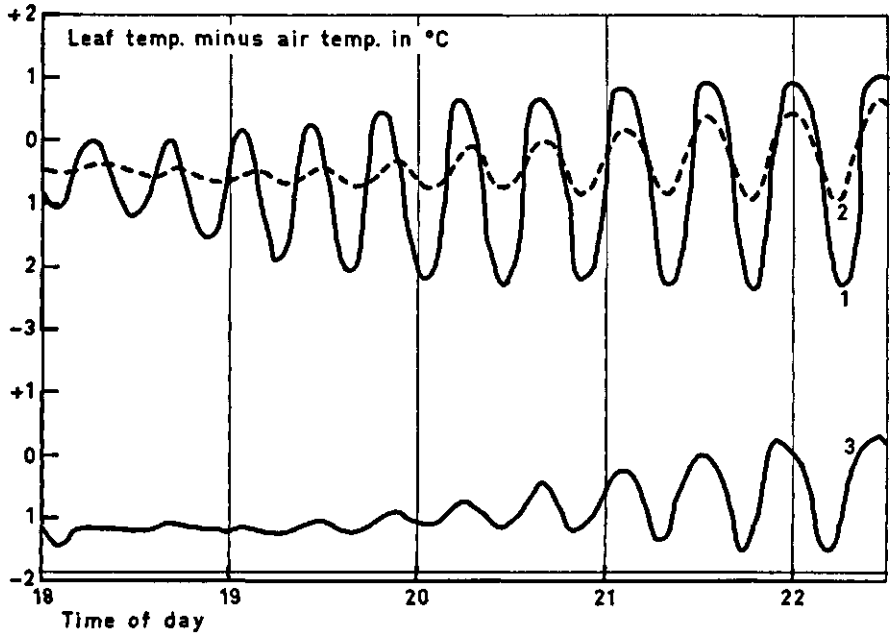

FIG. 5. Cycling in an old trifoliate (curve 1), inducing by entrainment cycling in the primary leaf (curve 2) and in a young trifoliate (curve 3). 25 °C/9 mm Hg/30,000 erg $cm^{-2}sec^{-1}$/10–20 cm sec^{-1}.

under investigation was on nutrient solution and possessed 2 primary and 2 trifoliate leaves, all bent in one horizontal plane, without overlapping leaf parts.

In the older trifoliates (curve 1), stomata had been induced by putting the plant in the experimental setup at 1700. The phase delay in the beginning of overt entrainment in the curves 2 and 3 in relation to curve 1 is in line with the explanation, that a cycling water potential as a result of cycling transpiration rate from the older trifoliate is the forcing rhythm. When the delay disappeared, induction may be considered completed. According to MELESHCHENKO and KARMANOV (1966), COX (1968) and LANG et al. (1969) the water potential in the plant works as a synchronizer of the rhythm in the leaves on one plant.

3.3.3. *Spontaneous*

When a plant is capable of self-sustained stomatal cycling, the stomatal system is unstable and the steady state is labile as long as cyling has not been induced. Figure 6 illustrates how in such a situation an extremely small change in the stomatal opening was followed by gradually increasing overshoots.

The rhythm had damped after some hours of self-sustained cycling in the same environment some hours before this spontaneous beginning of cycling. Hence, an unknown internal factor had brought the stomatal system from instability to stability and again to instability.

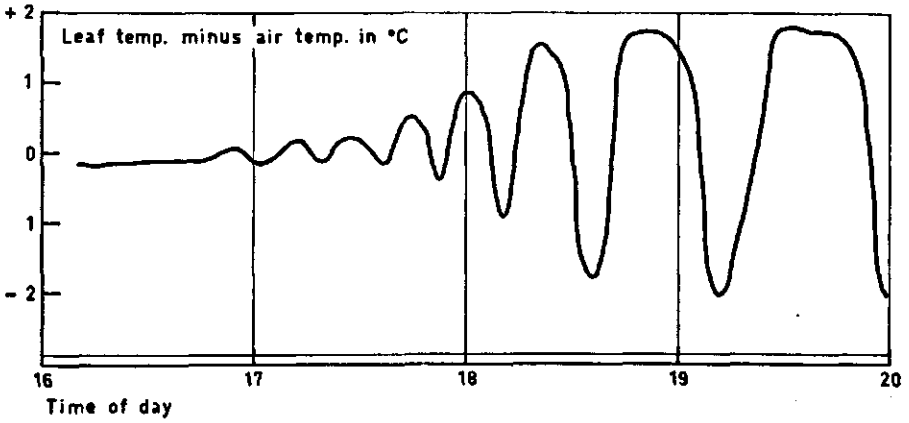

FIG. 6. Example of seemingly spontaneous start of cycling in a constant environment. 26 °C/ 13–14 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–20 cm sec^{-1}.

3.4. PROPERTIES

3.4.1. *Amplitudes*

In the course of the investigations a very wide range of amplitudes of leaf temperature has been recorded. The largest peak-to-trough difference found was 6.2 °C. Between this maximum and the still measurable variation in leaf temperature of 0.1 to 0.2 °C all intermediate values were found. The most common cycling showed peak-to-trough variations in leaf temperature of 2 to 3 °C. The amplitudes of stomatal cycling could be constant or gradually increase in course of time in self-sustained rhythms or gradually decrease in a damped rhythm. The damping of the rhythm could be either rapid, completed after a few periods, or slow, or result in a long damped rhythm of up to 7 hours duration.

3.4.2. *Free-running and entrained periods*

The periods of cycling lasted between 15 and 90 minutes with a frequently occurring range between 40 to 50 minutes. When stomata in primary and trifoliate leaves were cycling synchronously in the intact plant, the younger the leaves participating in the synchronized cycling, the shorter the periods were. An experiment was performed to find how the period was affected by the different leaves on one plant. The immediate response of the periods in the primary leaf was investigated upon removal of leaves, beginning with the youngest, and proceeding to the older trifoliates and to one primary leaf. The period increased each time when a trifoliate was removed. When a primary leaf was detached, the period in the other primary leaf did not alter.

It did not alter either, when one third of the lamina at the top side was cut from the remaining leaf.

Furthermore, the period in a young trifoliate leaf was recorded, while successive leaves were removed proceeding from the oldest to the younger leaves of a plant. No change of the period was observed.

Cycling in the younger leaves showed shorter periods and it appeared to entrain cycling in the older ones. The free-running period of the primary leaves could only be studied, when the trifoliates were detached.

3.4.3. *Changing from one type to another*

Sometimes different types of cycling from one moment to the other were observed in a plant. The difference in cycling consisted mainly of the amplitudes and to a smaller extent of the periods. Figure 7 shows 2 types of cycling, which were induced by subsequent dark-light transitions.

The occurrence of either of the types had no relation to the time of the day.

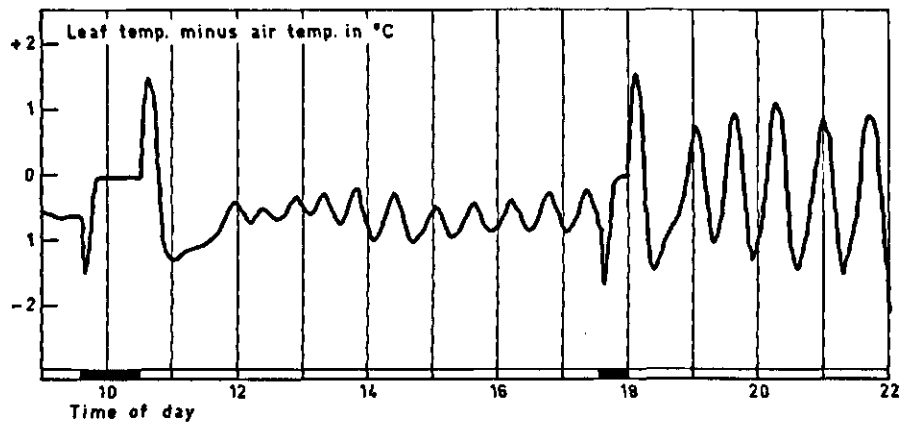

FIG. 7. Two types of rhythm in the same plant differing mainly in amplitude, both induced after small dark periods indicated by black bars. 25 °C/14 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/ 10–20 cm sec^{-1}.

3.4.4. *Irregularity in form*

One type of irregularity from the form of the smoothly running curves was rather frequent. It can be indicated as a delay in stomatal closure during cycling, though it appeared in different ways. Figure 8 is a representation of different ways performed by one plant.

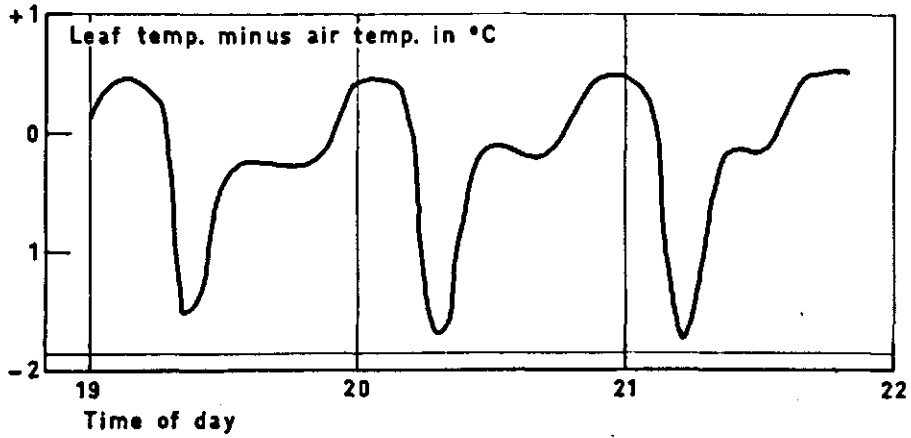

FIG. 8. A delay in the closure during stomatal cycling, presenting itself in different ways. 26°C/8 mm Hg/50,000 erg $cm^{-2}sec^{-1}$/20–25 cm sec^{-1}.

4. WATER RELATIONS OF CYCLING

4.1. INTRODUCTION

Several authors on cyclic stomatal openings payed attention to the water relations. HOWE (1964) proposed that photoactive opening and hydroactive closure (STÅLFELT, 1956) were alternating without indicating a reason. Several analyses and interpretations of aspects of the water relations in relation with cyclic stomatal opening showed, that the type of cycling in the present study originated from the control function of the stomata on the water status of the leaf (EHRLER et al., 1965; RASCHKE, 1965; APEL, 1967; HARRIS, 1968; COX, 1968; BARRS and KLEPPER, 1968; HOPMANS, 1969a; LANG et al., 1969; RASCHKE and KÜHL, 1969). BARRS and KLEPPER (1968) mentioned as essential factors for cycling the resistance to water transport in the roots, whereas HOPMANS (1969a) and LANG et al. (1969) pointed to the delay in the adjustment of the turgor of the guard cells to the water potential in the leaf cell walls. COX (1968), BARRS and KLEPPER (1968) and HOPMANS (1969a) suggested, that the stomata might open passively during cycling.

In the present chapter these factors will be analysed by different methods in rather uniform environments. A clear understanding of the different mechanisms in the water relations of the whole plant, integrated in the cyclic behaviour of the stomata, is aimed, including their interaction and phase relations.

4.2. MATERIALS AND METHODS

Transpiration measurement

The transpiration rate was determined psychrometrically by measurement of the water vapour pressure of air, flowing in and out of the plant chamber in which one plant was placed. Both 35.5 liter chambers and 1.5 liter 'sandwich' type chambers were used. Leaf temperature and radiation intensity were measured inside the plant chamber. The transpiration rate and the diffusion resistance for water vapour of the leaf were calculated by a computer from the data obtained. The facilities used have been described by LOUWERSE and VAN OORSCHOT (1969). When stomata were cycling, rapid changes in transpiration rate were followed by changes in vapour pressure of the outflowing air with a time delay and with a reduced amplitude, because of the amount of water vapour needed to balance the vapour concentration in the chamber at the existing air flow rate and transpiration rate. The real transpiration rate was found by correcting the computed transpiration rate with the quantityof vapour, lost or accumulated in the chamber for the time interval under consideration. For this computation the computer program was extended as described by MILLENDORFER and BORGHORST (1963). Sometimes processing was extended by plotting the computed data.

β-gauging of water content

A β-gauge (NAKAYAMA and EHRLER, 1964) was used to measure changes in water content of the leaf. A suitable β-source for these leaves proved to be a source of 10 μc of Ca-45 mounted on top of a small perspex rod (Institute of Atomic Sciences in Agriculture). The source was placed over the leaf.

A GM-tube, type GM18505, with a window of 1.5 mg cm^{-2} was placed at a distance of 8 cm from the isotope. The leaf, attached to the plant, was positioned half way between source and detector. A continuous recording of the radiation transmitted through the leaf was made with a set-up of voltage supply amplifier, count rate meter (40 sec time constant), and time recorder. A calibration curve was made for each geometry of the β-gauge, using aluminum foils as absorbers. The calibration curves were adapted to a decrease of the amount of radiation emitted by the β-source. Before relating variations in leaf 'thickness' (mass per unit area) to variations in leaf water content, the extent of leaf area shrinkage had to be known, because area shrinkage increases thickness. This relation was determined for leaves of 3 different ages by measurement of the distance of thin marks in length and width direction on detached leaves and measurement of the fall in weight. Measurements are given in figure 9. The plants had not

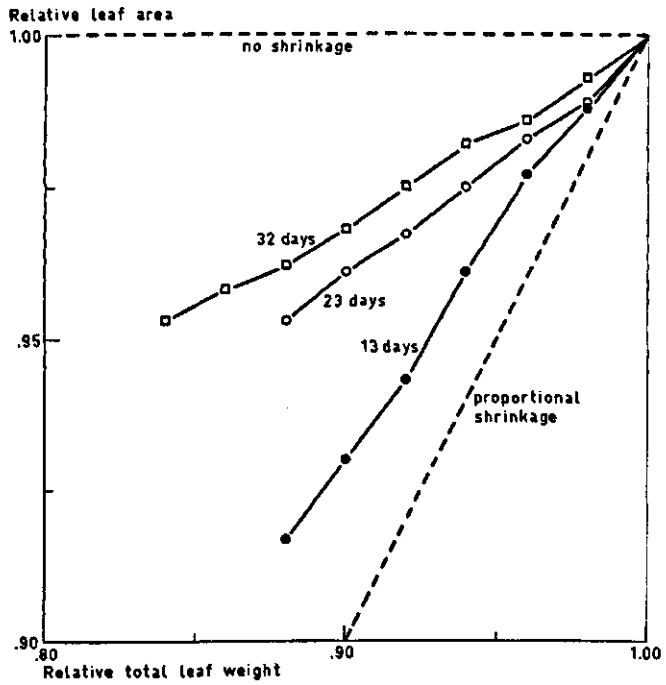

FIG. 9. Relation of relative leaf area and relative total leaf weight during falling leaf weight due to water loss of primary leaves from plants of 13 days, 23 days and 32 days old. The values 1.00 on abscissa and ordinate represent the relative magnitudes of total leaf weight and leaf area some seconds after detaching the leaves during the photoperiod. The theoretical relations for leaves with no shrinkage and with proportional shrinkage of leaf area are indicated by broken lines. The curve for each age is the average of 4 leaves from separate plants.

been defoliated previously, had been cultivated on nutrient solution and at low light intensity.

Leaf area shrinked considerably at all ages investigated, but young leaves were more sensitive. Relative leaf 'thickness' was calculated by dividing relative total leaf weight by relative leaf area. This calculated relative 'thickness' was plotted against relative total leaf weight in figure 10 in order to show how to correlate total leaf weight values with leaf 'thickness' data measured by the β-gauge.

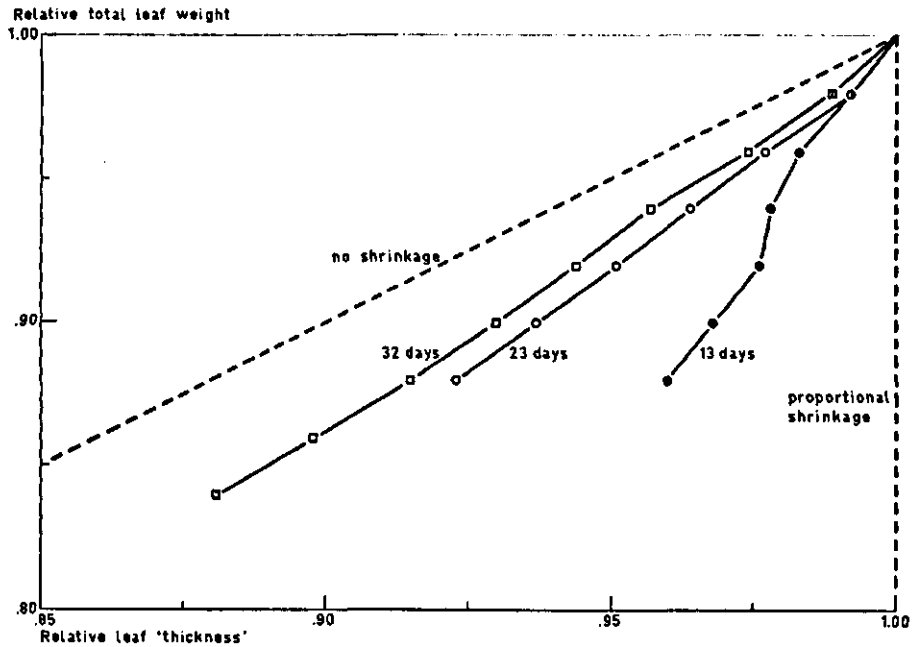

FIG. 10. Relation of relative total leaf weight during water loss to relative leaf 'thickness' (the parameter which a β-gauge would measure) of primary leaves from plants being 13 days, 23 days and 32 days old. The values 1.00 on abscissa and ordinate represent the relative magnitudes some seconds after detaching the leaves during the photoperiod. The theoretical relations for leaves with no shrinkage and with proportional shrinkage of leaf area are indicated by broken lines. The curve for each age is the average of 4 leaves from separate plants.

The difference in the ordinate direction between the curves for actual behaviour of plants of different ages and the theoretical line for no leaf area shrinkage shows the errors made, if leaf 'thickness' values are interpreted as leaf weight without correction for shrinkage.

If for example the β-gauge showed a decrease of 4% in leaf 'thickness' from 1.00 to 0.96, this would mean a decrease of total leaf weight due to water loss in the 13 days old plants of 12% and in the 23 days plants of 6.6%. If shrinkage had not been taken into account, however, only a 4% decrease would have

been measured. Earlier data on leaf shrinkage (HOPMANS, 1969a) proved to be not reliable. Variation in total leaf weight is indicated in the text also as variation in water content. In the experimental data presented no plants of 13 to 15 days have been used, but corrections for shrinkage as indicated by the curve for 23 days old plants had to be considered.

Root temperature control

For root temperature control a small pump transported water from a 100 l container of controlled temperature to the 1 l plant pot, equipped with an overflow back to the reservoir. Root temperature was changed by moving the water pump and tubing to a container of the desired temperature. The half-time of the root temperature transition produced was about 2 minutes. Root temperatures were sensed by thermocouples and recorded.

4.3. TIME COURSE OF SOME FEATURES OF THE LEAF WATER RELATIONS

4.3.1. *Transpiration rate, leaf temperature and diffusion resistance*

As shown in the preceding chapter a wide diversity in amplitudes of leaf temperatures, hence in transpiration rates and diffusion resistances was found. Figure 11 presents an illustration of cycling of leaf temperature, transpiration rate, and leaf diffusive conductance and resistance in a young plant with 2 fully developed primary leaves in a 'sandwhich'-type plant chamber.

The resistance to vapour diffusion in the boundary air layer, at a wind speed of 100 cm sec^{-1} was approximately 0.7 sec cm^{-1} (compare SLÅTYER and BIERHUIZEN, 1964).

4.3.2. *Variation in water content*

Cyclic variations in leaf weight of 15–20 % due to water loss were found during stomatal cycling with the β-gauge using the correction for leaf area shrinkage. A time lag between minimal leaf 'thickness' and minimal leaf temperature was observed. As has been discussed earlier (HOPMANS, 1969a), this time lag originates from the lag of change in water absorption rate behind the change in transpiration rate.

Variations in leaf water content determined by the β-gauge may be related to variations in water potential in the leaf in a semi-quantitative way, due to the fact that an almost linear relation between relative water content and water potential may be assumed (ALTMAN and DITTMER, 1966; WEATHERLEY, 1965; KNIPLING, 1967).

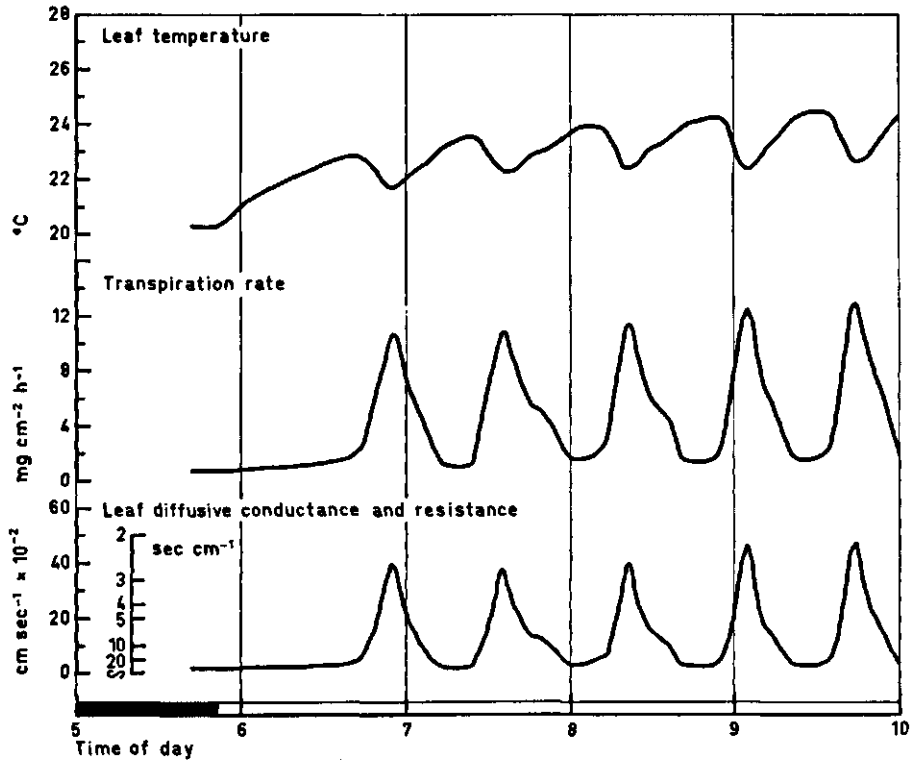

FIG. 11. Time course of leaf temperature, transpiration rate, and calculated diffusive conductance in cm sec^{-1} and resistance in sec cm^{-1} for water vapour (stomatal and cuticular). Transpiration rate and diffusion conductance and resistance are expressed on single side leaf area. Air temperature cycling with amplitudes approximately 0.7 × leaf temperature amplitudes, with the same mean and 1.8 to 2.4 min phase delay/mean vapour pressure 8.7 mm Hg/ 39,000 erg $cm^{-2}sec^{-1}$/100 cm sec^{-1}.

4.4. REACTIONS OF CYCLING UPON INDUCING A CHANGE IN PLANT WATER POTENTIAL IN DIFFERENT WAYS

4.4.1. *Changing air humidity*

At a constant stomatal diffusion resistance to water vapour the vapour pressure gradient from the intercellular spaces in the leaf to the leaf exterior is the only controlling factor for transpiration rate. Leaf temperature and vapour pressure of the air affect this gradient. Stomata are assumed to close at increased transpiration rate only if the rate of water loss gives a water deficit in the leaf (MEIDNER and MANSFIELD, 1968; RASCHKE and KÜHL, 1969). The effect of a rapid change of air humidity and the effect of air humidity on the properties of sustained cycling were studied by affecting transpiration rate by a change in the vapour pressure of the air.

Figure 12 shows an example of a frequently occurring response of cycling to an increase in air humidity. As soon as the vapour pressure deficit decreased, the amplitudes of cycling decreased and the rhythm damped. The vapour pressure gradients from leaf to air at b and d were 12.1 and 9.4 mm Hg respectively and the differences of the troughs b and d with their preceding peaks a and c were 1.5° and 0.8 °C respectively. The ratio of the peak-to-trough differences was larger than the ratio of the corresponding vapour pressure gradients. Therefore, the peak-to-trough difference in stomatal opening d–c was lower than b–a, and it was assumed that the minimal stomatal openings at a and c were equal.

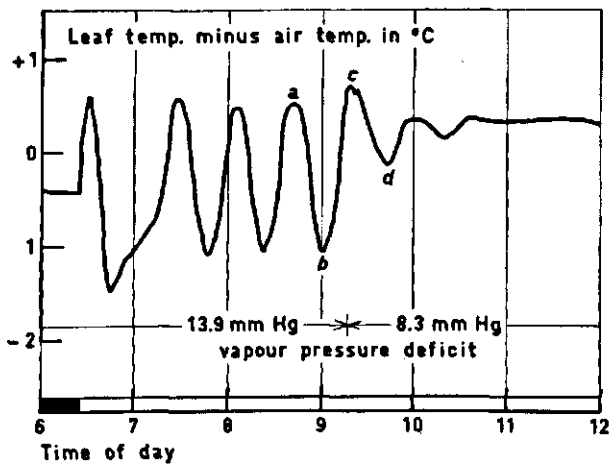

FIG. 12. Influence of decreasing the vapour pressure deficit of the air on stomatal cycling. 26 °C/see figure/35,000 erg $cm^{-2}sec^{-1}$/10–15 cm sec^{-1}.

Although at high air humidity cycling often damped completely (figure 12), it was also frequently found to be sustained. Thus properties of self-sustained cycling could be compared at different air humidities. Figure 13 illustrates that amplitudes of the cycling transpiration were reduced by decreasing the vapour pressure deficit. This response was obtained, when the time between the different vapour pressures was small.

The amplitude of leaf temperature and transpiration rate would double when the vapour pressure deficit increased from 6.4 to 13.3 mm Hg, if the amplitude in the cycling of stomatal opening was not affected. The measured ratio was, however, approximately 3, indicating that the stomatal cycling was larger when the vapour pressure deficit was large. This reasoning assumes that the mean leaf temperature at different air humidities was equal. The period turned out to be hardly affected by vapour pressure deficit.

Table 2 shows that different vapour pressure deficits (v.p.d.) at equal or at practically equal air temperatures affected peak-to-trough difference in leaf

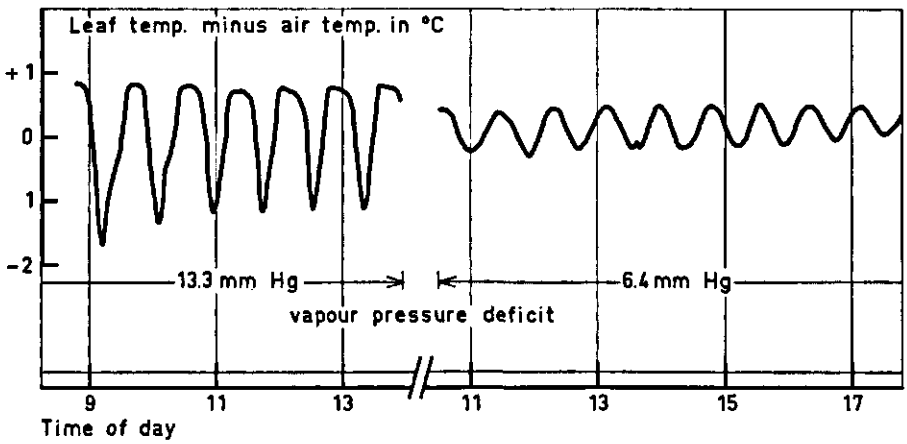

FIG. 13. Influence of air humidity, indicated as vapour pressure deficit of the air, on period and amplitude of leaf temperature cycling in one plant. The different air humidities were given on 2 consecutive days. 28 °C/see figure/35,000 erg $cm^{-2}sec^{-1}$/10–15 cm sec^{-1}.

TABLE 2. Influence of air humidity expressed as vapour pressure deficit (v.p.d.) of the air on peak-to-trough difference and periods of leaf temperature cycling on 3 days in succession for one plant. Short wave radiation 35,000 erg $cm^{-2}sec^{-1}$, wind speed 10–15 cm sec^{-1}.

day	time of day	air temperature	v.p.d. air in mm Hg	peak-to-trough difference in °C	periods in min
1	7–9	28.0	13.3	2.1	52
	11–13	28.0	13.3	1.8	47
2	11–13	28.0	6.4	0.4	51
	14–16	28.0	6.4	0.4	47
3	13–15	27.5	11.5	1.7	47
	21–24	27.6	5.9	0.4	48

temperature as was demonstrated in figure 13. Also here the peak-to-trough difference of stomatal cycling was larger in dry air, without a considerable change in the period.

4.4.2. *Enhancing the water potential in the root medium*

In this section the immediate response of the stomata to an increase of the water potential in the roots will be studied and the effect of increased soil water potential on cycling.

If peat-perlite in pots with gradually decreasing soil water potential is wetted, the water potential near the root surface rapidly increases. Figure 14 shows the effect of such a rapid increase in water potential on cycling and steady stomatal opening. In both plants (curve 1 and 2) cycling damped during gradual drying of the soil in the pot prior to watering. The immediate response was

stomatal closure, during cycling as well as when the stomatal opening was constant.

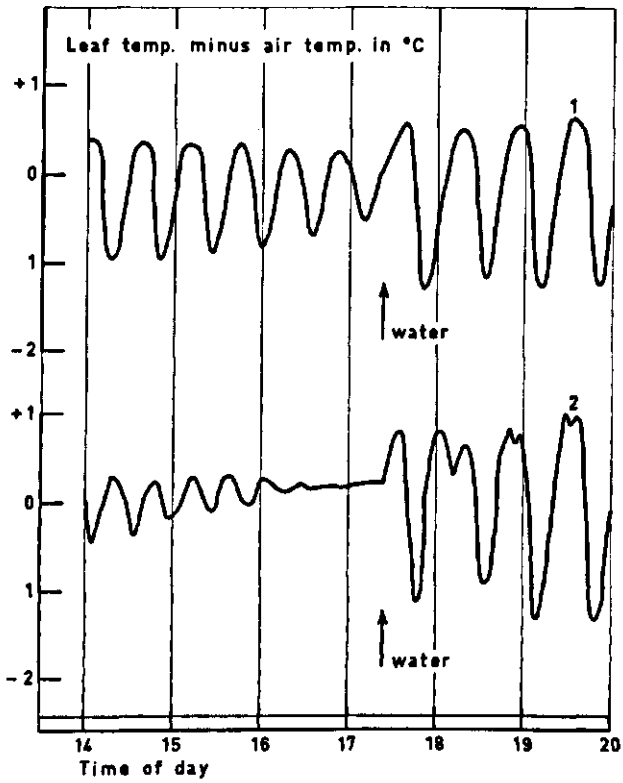

FIG. 14. Effect of watering plants on the roots (arrows) on the course of leaf temperature when stomata were cycling (curve 1) and when they were steady (curve 2). Plants in small pots with peat-perlite, which was dry before watering. 25 °C/10.5 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/ 15–20 cm sec^{-1}.

The mean increase in leaf temperature directly after watering of 15 randomized cases in approximately similar environmental conditions was 0.6 °C. When water stress was extreme, leaf temperature did not rise further after watering, but began to decrease slowly after some hours. The response of the leaf temperature of 4 plants is presented in table 3, indicating the relative intensity and duration of the temporary stomatal closure. The increased leaf 'thickness' shown in the table developed slowly during the time of increased leaf temperature.

No increase in leaf 'thickness' was observed during the closing movement itself (figure 15).

TABLE 3. Examples from 4 plants of leaf temperature increase, increase in leaf 'thickness' and duration of the period of temporarily increased leaf temperature after watering plants in peat-perlite.

leaf temperature increase in °C	leaf 'thickness' increase in mg cm^{-2}	duration of increased temperature in min
0.60	0.40	12.5
0.47	0.25	16.7
0.30	0.00	12.4
0.90	0.10	14.5

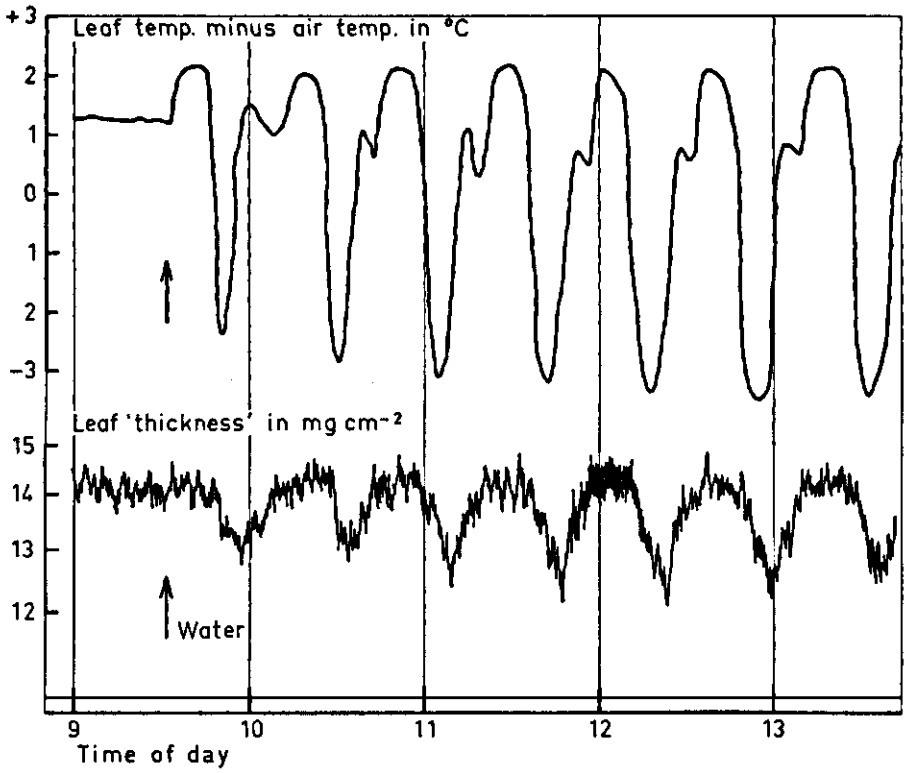

FIG. 15. Effect of water supply to the roots (arrow) of a plant in peat-perlite on the course of leaf temperature and leaf 'thickness'. Before watering, the plant had a slight water stress. 26.5 °C/9.6 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10 cm sec^{-2}. (After HOPMANS, 1969a).

4.4.3. *Reducing the water potential in the root medium*

The effect on cycling of reduction of the plant water potential by decreasing the water potential in the root medium was studied. The latter may be decreased by a gradual decrease of the soil moisture content or by an increase in the osmotic pressure of the nutrient solution. When the water potential in the root medium is reduced, the water potential in the plant will follow as assumed by SLATYER (1967), COWAN and MILTHORPE (1968) and JANES (1970). When the soil moisture content decreases, part of the plant water stress may be caused by increased resistance to water transport in the soil adhering the roots (GARDNER, 1960; MACKLON and WEATHERLEY, 1965; WEATHERLEY, 1965). Experimental data to evaluate the impact of this resistance will be presented.

When the peat-perlite in small pots gradually became dryer, cycling that had been sustained for a long time, at a certain moment began to damp more or less slowly. During damping the leaf temperature of the minima increased more than the temperature of the maxima decreased. Some time after steadiness was reached, a gradual stomatal closure movement set in. Figure 16 shows an example.

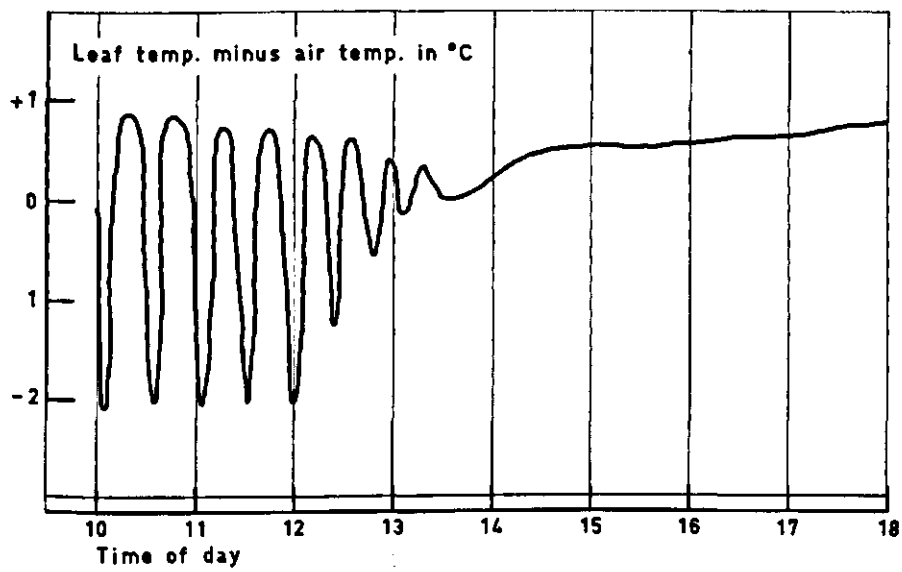

FIG. 16. Damping stomatal cycling caused by gradual drying of peat-perlite in a small pot. 27–28 °C/10.5–11.0 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/15 cm sec^{-1}.

The water potential of the nutrient solution was decreased with glucose, mannitol or sodium chloride as osmotically active substances. The osmotic pressure of 0.8 atm of the nutrient solution was increased with 1 atm by replacing the pot by another one, filled with identical nutrient solution in which the osmoticum had been dissolved. A control changing with unchanged nutrient

solution did not affect cycling. Glucose and mannitol were used 12 times and the cycling stopped 10 times, whereas 2 times it continued. In such an experiment stomatal opening of a cycle was unchanged but subsequent closure began at the normal phase or in advance of it. The extent of closure was less than normal and it was followed by a slight and slow reopening to a steady state. Cycling could continue after decreasing the water potential with 1 atm by glucose (1 experiment), by mannitol (1 experiment) and by sodium chloride (4 experiments), as illustrated by figure 17. Immediately after the water potential of the root surface was decreased (arrow), the stomata closed less during sub-period b, coinciding with a decreased leaf 'thickness' at that sub-period b', whereas the preceding minimum in leaf 'thickness' a' was at least as low as the previous ones. The further amplitudes in leaf temperature remained smaller, the maxima remaining lower and the minima higher than before the osmoticum was applied.

The importance of a possible resistance to water transport in the soil around the roots was tested as follows. No differences in cycling were found between plants in water culture and in soil. Also no clear difference in the rate of recovery from the periodic leaf water deficits were observed between plants in the different root media. These observations suggest that the resistance to transport was situated in the roots and not in zones of soil adhering the roots.

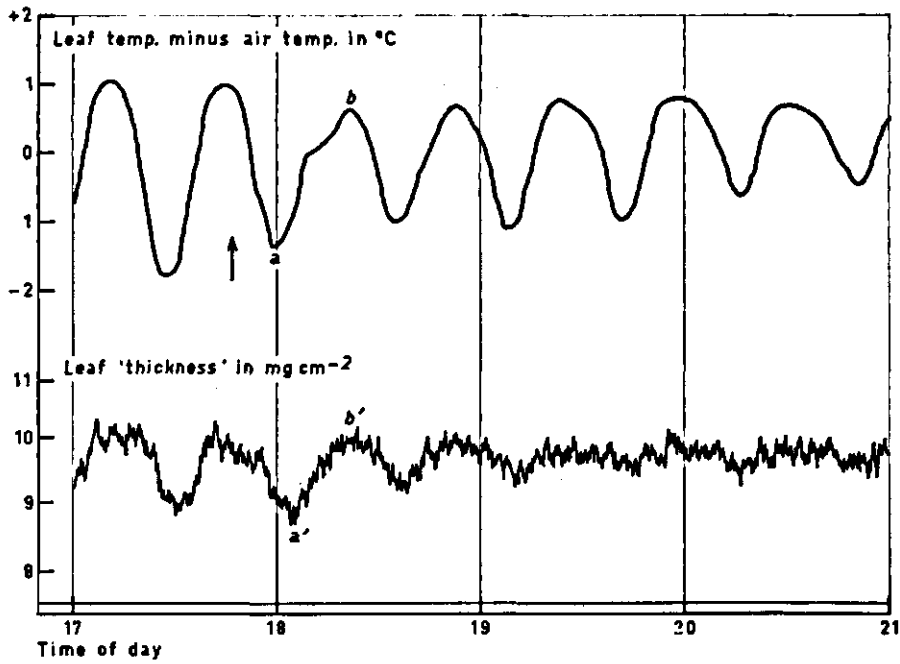

FIG. 17. Effect on sustained cycling in leaf temperature and leaf 'thickness' of decreasing the water potential in the root medium by 1 atm (arrow) with sodium chloride. 27°C/15 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10 cm sec^{-1}.

4.4.4. *Discussion*

The following factors, causing a rapid fall in the water potential in the plant were found to induce a temporary increase in the opening of the stomata in bean plants by HOPMANS (1969a): an abruptly enhancing of the osmotic pressure of the nutrient solution by 5 atm with sodium chloride, a severing of the petiole in air. This effect was attributed to passive opening of the stomata. Explanations of this type of temporary opening are very old. VON MOHL (1856) indicated that guard cells regulate the pore opening actively by their turgor pressure and that opening also may occur passively: 'Es ist also deutlich, dass bei *Amaryllus* die Oeffnung und Schliessung der Spaltöffnungen nicht von der Thätigkeit der Porenzellen allein abhängt sondern dass zwischen diesen und den Epidermiszellen ein Antagonismus existiert'. VON MOHL also already used the word 'passiv' for movement of stomata caused by the pressure of the epidermis cells. Evidence for the passive nature has been increasing up to recent data (LEITGEB, 1886; DARWIN, 1898; DARWIN and PERTZ, 1911; WILLIS et al., 1963; MEIDNER and HEATH, 1963; FALK, 1966; LOUGUET, 1968; RASCHKE and KÜHL, 1969; RASCHKE, 1970a).

Temporarily overshooting opening movements have been induced by enhancement of the transpiration rate by wind (MILTHORPE and SPENCER, 1957), by sharply decreasing the vapour pressure of the air around the leaf or of the air blown through the leaf (MACKLON and WEATHERLEY, 1965; RASCHKE and KÜHL, 1969). These opening movements were identified as passive.

In the present experiments a rapid increase of the water potential in the root medium induced stomata to close temporarily. HOPMANS (1969a) discussed this response, keeping in mind the explanation in the literature and the theory for water transport (STRUGGER, 1943; WEATHERLEY, 1963, 1965). He concluded that a passive stomatal closure was involved.

On page 25 it was described that the stomatal opening during cycling was reduced when in the course of the opening movement the acceleration of transpiration rate was diminished by increasing air humidity. The increasing air humidity possibly decreased a passive component in stomatal opening movement. Hence during the cycling in dry air the mechanism of passive stomatal opening contributed to the opening movement.

The immediate reaction on the decrease in water potential in the root medium during cycling (figure 17) is explained as follows. During stomatal closure, the water content and therefore the turgor of the surrounding epidermal cells increased less at a lower water potential at the roots, thus indicating a decrease in the contribution of passive stomatal closure, present before the water potential at the root was decreased.

In figure 14, curve 1, the increased stomatal closure during cycling immediately after watering the plant represents an example of passive closure during cycling, caused by a rapid increase of the soil water potential.

The susceptibility of the stomatal apparatus of passive opening made BARRS and KLEPPER (1968), COX (1968) and HOPMANS (1969a) suggest that passive stomatal opening movements contributed to stomatal cycling.

In conclusion, the present results indicate that during cycling in dry air and at a high water potential at the roots both passive opening and passive closure contributed to the stomatal openings and closures.

WILLIS et al. (1963) found that with opened stomata, the smaller the initial water deficit in the leaves, the more intense the stomatal opening movement was in response to excision of the petiole in air. This effect would mean that, the higher the water content reached during the sub-period with minimal stomatal opening, the more the contribution of passive opening to the following opening movement.

HOPMANS (1969a) suggested that the important factor underlying the cycling was the time delay in the adjustment of the turgor of the guard cells to the water potential of the epidermis, being the same time delay as involved in passive stomatal movements. With the indication of the contribution of both passive opening and passive closure during cycling, the evidence for this delay factor has increased.

In the present findings the amplitude of cycling was increased by a decrease of the air humidity and an increase of the water potential in the root medium. The cycling was found to appear only in dry air by several authors. These effects on cycling are interpreted according to the following picture.

A delay in the reaction of the guard cells to increasing water deficit during stomatal opening causes excessive water loss in dry air. The increasing water deficit in the course of the lag period, during which stomatal opening continues, induces passive stomatal opening which amplifies the overshoot. This effect would according to WILLIS et al. (1963) be stronger, the smaller the water deficit was during the preceding sub-period with minimal stomatal opening. The delay factor again causes overshoot in the active movement in the subsequent stomatal closure. The extent of the contribution of passive closure is affected by the water potential of the water supplied by the root. The higher the water potential in the root medium, the more the turgor of the surrounding cells will increase and the more the contribution of passive movement to the stomatal closure.

These effects lend support to the importance of the delay in the adjustment of the turgor of the guard cells to the plant water potential as the factor causing overshooting of the active stomatal movement and as the factor amplifying the importance of passive stomatal movement.

4.5. THE ROLE OF THE RESISTANCE TO WATER TRANSPORT IN THE ROOTS

From the previous sections it is clear that a cycling of the water deficit of the leaves accompanied cycling of transpiration rate. A water deficit will arise, however, only when the supply is limited. BARRS and KLEPPER (1968) identified the roots as the sites of major resistance to water flow associated with cycling.

The objective of the following investigations was to study the importance of the root resistance and the effect of root temperature in relation to cycling.

4.5.1. *Influence of severing the roots*

Tips of 2–3 cm were cut under water with a sharp edged cutter from the roots while approximately 20 cm remained on the plant. Cutting was done during a sub-period with minimum transpiration and maximum and constant leaf water content. As an example: the peak-to-trough differences of 2.3 °C and periods of leaf temperature cycling were slightly affected, but the peak-to-trough differences in leaf 'thickness' fell from 2.5 to 0.5 mg cm^{-2}, immediately after cutting the roots.

The maxima in leaf 'thickness' did not change and were maintained longer. The fact that the maxima in water content continued 10–15 minutes during each cycle both before and after root cutting, suggests that during that sub-period a high water potential was reached and maintained throughout the whole plant. Because in the next sub-period the decrease in water content was affected by the decrease of the resistance to water flow into the roots, it is concluded that the water potential in the roots cycled down to a resistance in the roots. This resistance apparently was not located within the xylem vessels of the remaining part (80%) of the roots.

Some hours after cutting the roots, the amplitudes of leaf 'thickness' gradually began to rise again, presumably because of gradual blocking of the xylem vessels in the cut ends of the roots.

Frequently the amplitudes of cycling in stomatal opening were also reduced after severing the roots.

When a large part of the roots was cut off, stomatal opening was set in at the normal phase, but remained smaller and the water content fell hardly any more. This indicates that an increasing transpiration rate no longer induced a passive part in the opening movement, when the water supply was improved.

4.5.2. *Influence of cycling on the root resistance*

It was mentioned in the previous section that the xylem vessels in the cut ends of the roots gradually blocked. The blocking was removed by cutting a few more millimeters from the root end. The cycling transpiration, as indicated in figure 18, did not change by this decrease of the resistance to water flow. The time curve of leaf 'thickness' shows that the fall in water content during sub-period d' was considerably less than in b', indicating that the water supply was improved. The rate of change of leaf 'thickness' is represented by the steepness of the curve. It indicates that after reopening the xylem vessels in the roots, both the fall (c'–d') and the rise (d'–e') of water content slowed down after cutting, (a'–b') and (b'–c') respectively. Hence, during the increasing transpiration rate (c–d) and synchronously falling water content (c'–d') the rate of water supply was increased by the cutting.

This response was explained above as a decreased resistance of the xylem vessels. The slower rise of water content over identical ranges of leaf 'thickness' indicates a relatively lower rate of water uptake during this part of the cycle in spite of the reduced viscous flow resistance. The resistance to water uptake in the roots before cutting must have fallen considerably during the sub-period

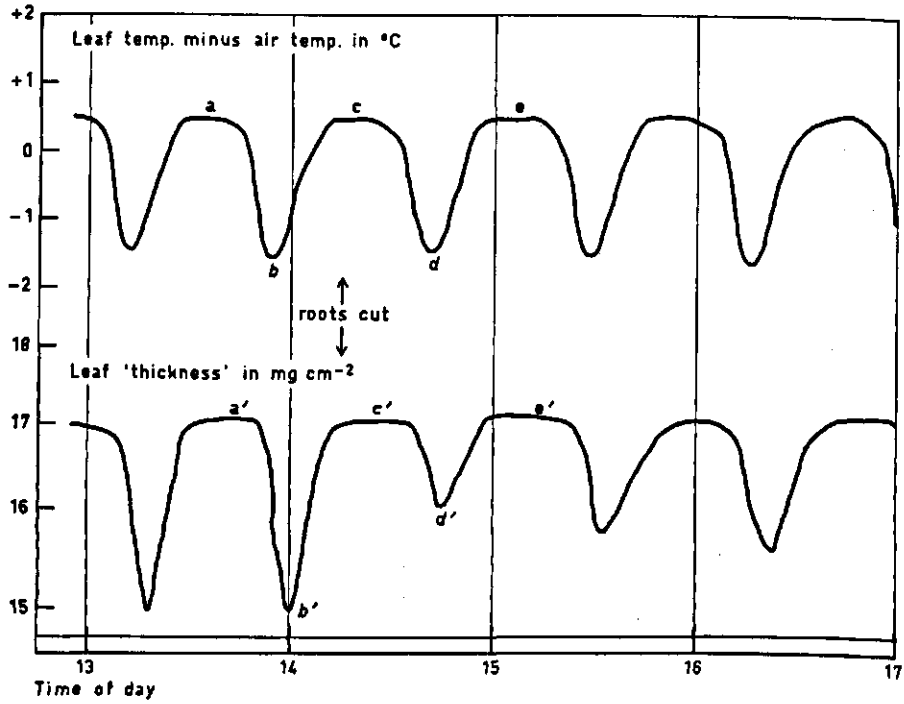

FIG. 18. Reaction of stomatal cycling and variation in leaf 'thickness' on reopening blocked vessels in the roots of a plant on nutrient solution by cutting some millimeters from the ends. 28 °C/19 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/30 cm sec^{-1}.

with reduced water content (b'). During each cycle this course of events was repeated. During the sub-periods with low transpiration, constant high water content, and low water uptake rate (e.g. a and a'), the resistance to water uptake in the root was again established at the previous high level.

Leaves detached from the plant with the petiole submerged in water seldom showed cycling. If present, this cycling vanished by cutting off under water a few millimeters from the tips of the petioles, suggesting that a resistance to water flow had developed at the initial cut. Figure 19 shows an example of cycling in a detached leaf. The rate of decrease of leaf 'thickness' during each cycle was comparable to the corresponding rate in leaves on intact plants. The subsequent increase was much slower, indicating that the resistance to water uptake in the petiole limited the water uptake more than intact root systems did during that sub-period.

The fall in leaf 'thickness' was often twice as large as the falls in the next cycles with approximately equal amplitudes in leaf temperature, when an overshooting stomatal opening was preceded by a long period of low transpiration rate, like a daily dark period. Obviously the resistance to water flow was larger when preceded by a longer period of low rate of water uptake.

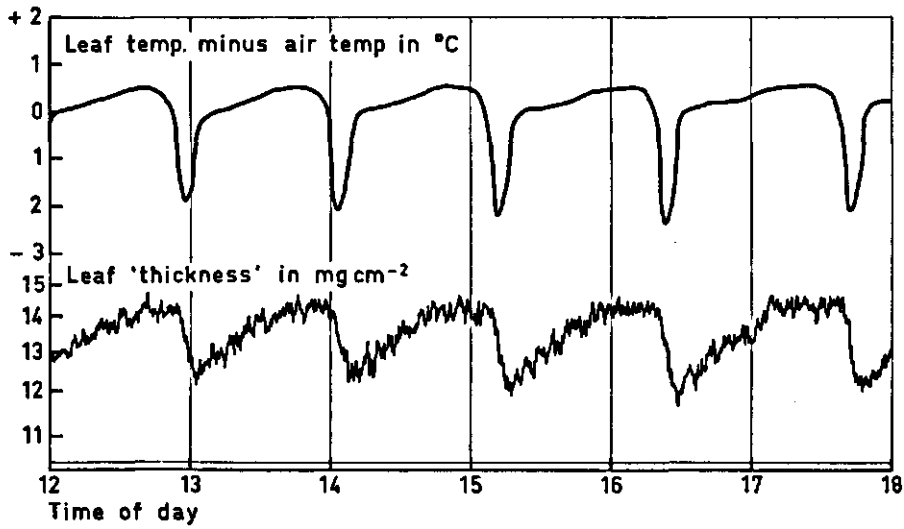

FIG. 19. Cycling in leaf temperature and in leaf 'thickness' in a detached leaf with the petiole in water. 27.5 °C/11 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/30 cm sec^{-1}.

4.5.3. *Interaction of root resistance and cycling in different leaves*

It was concluded in chapter 3 that younger leaves generally have shorter free-running periods than older leaves on the same plant and that they entrain cycling in the older leaves.

Figure 20, upper left part, once more demonstrates, that cycling in the primary leaf showed the same period as, but lagged behind, cycling in a trifoliate on the same plant. Removal of the trifoliate changed the variation in the 'thickness' of the primary leaf. The water content decreased more during the sub-periods of high transpiration. This indicates that after the detachment the resistance to water transport was higher when the stomata opened. There was a time of approximately 10 to 15 minutes during each cycle, during which the rate of water loss was very low and leaf water content was constant, hence a period of very low water uptake by the roots.

Removal of the primary leaves from a plant did hardly produce any change of the amplitude and period of stomatal cycling in the remaining trifoliate, but the amplitude of the variation in leaf 'thickness' increased considerably. This conduct is in complete harmony with the first case.

4.5.4. *Influence of root temperature*

It is generally found that an increase of the root temperature induces a decrease of the root resistance to water uptake and vice versa (GAVRILOFF, 1926; DÖRING, 1935; KRAMER, 1942; 1956; JENSEN and TAYLOR, 1961; KUIPER, 1964; BROUWER, 1965).

In the present experiments an abrupt decrease of root temperature of plants with steady stomatal openings induced a temporary stomatal opening move-

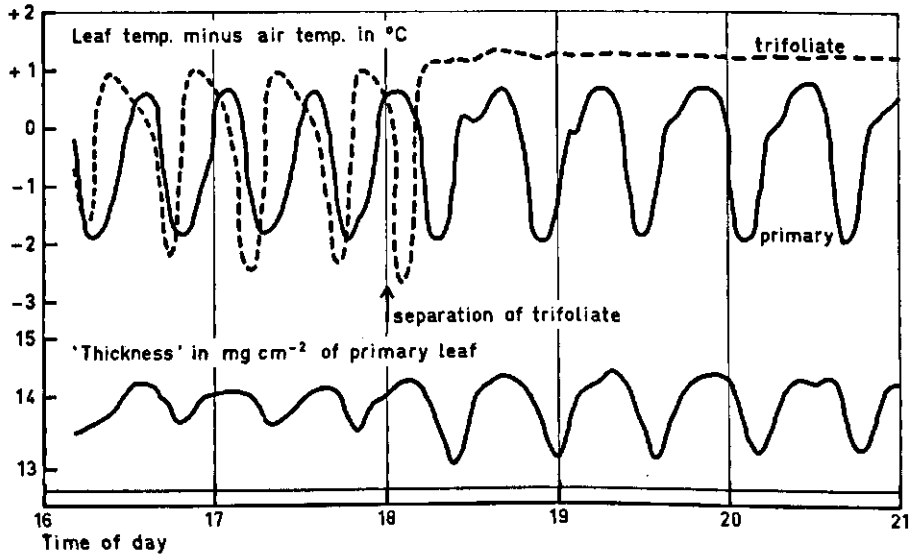

FIG. 20. Stomatal cycling in a primary leaf and in a trifoliate on one plant and variation in 'thickness' of the primary leaf before and after removal of the trifoliate. Roots in nutrient solution. 26°/10 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–15 cm sec^{-1}.

ment, probably of a passive nature, which was caused by impaired water supply.

The reaction of stomatal opening and the change in water content during cycling upon abruptly decreasing or increasing root temperatures have been investigated in the usual way.

Initial and stationary effects of lowering the root temperature on cycling like in figure 21 were found in 6 out of 16 cases. In the other cases stomatal opening remained steady after the water content had regained a new constant level.

A deep fall of leaf 'thickness' (b') indicated, that the water uptake was seriously impaired soon after the root temperature had decreased.

Stomatal closure during prolonged water deficit (c) was not complete, but closure did not continue earlier than after a certain leaf water content had been reached. This final closure probably was merely passive. The leaf 'thickness' at c', e', g', coinciding with the sub-periods of low transpiration rate, remained lower at lower root temperature than at higher root temperature. The transpiration rate at c, e and g did not reach the same minimum either. Effects of an increase of root temperature like in figure 21 were found in 14 out of 18 cases. The increase of leaf 'thickness' (from h' to i') was more rapid, and the extent of stomatal closure (at i) increased immediately after the increase of the root temperature. The amplitude remained larger.

The gradual increase of the amplitude at the lower root temperature was a more frequently observed symptom of adaptation to low temperature. The same reactions as shown in figure 21 appeared after changing the root temperature

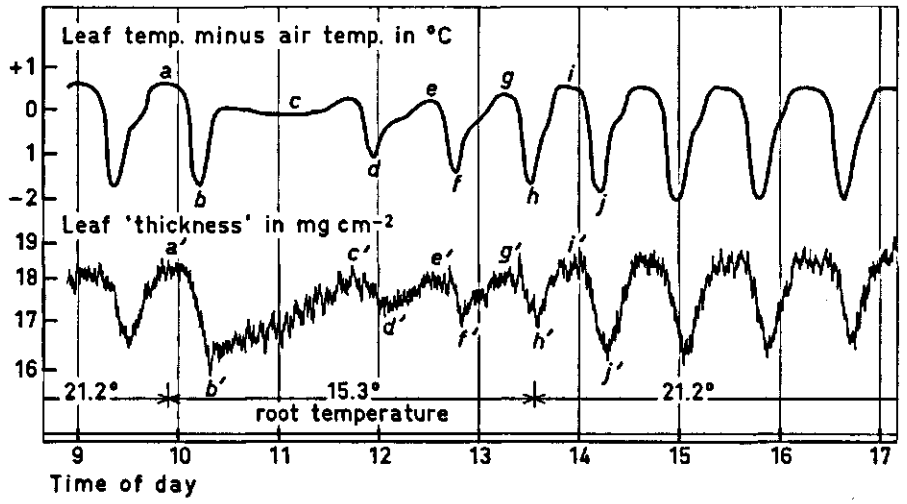

FIG. 21. Reactions of cycling leaf temperature and variations in leaf 'thickness' on root temperature decrease (21.2°→15.3°C) and increase (15.3°→21.2°C). 25°C/7 mm Hg/50,000 erg $cm^{-2}sec^{-1}$/40–50 cm sec^{-1}.

from 15°C to 12°C and back to 15°C after an adaptation time of approximately 20 hours in 15°C.

In general, the effect of an increase of the root temperature consisted of a decreased root resistance during the sub-periods of stomatal closure and increased water content. The rapid and high rise of the water content probably fortified passive closure.

4.5.5. *Discussion*

Cycling water potential as the entraining agent

LANG et al. (1969) concluded that the water potential in the xylem cycled and proposed with MELESHCHENKO and KARMANOV (1966) and COX (1968) that cycling of the water potential was a necessary condition for the observed synchronization of stomatal cycling on different leaves of one plant.

The present results indicate that the water potential cycled down to the site of resistance to water uptake into the roots. The location of the main resistance in the roots is still being discussed (DAINTY, 1969).

The mechanism of entrainment of cycling in the older leaves to periods shorter than their free-running periods may be explained by the cycling water potential. It was common that the onset of stomatal opening of the entrained cycling in the primary leaves lagged behind the opening in the trifoliate. This was probably caused by a delayed fall in water potential in the trioliates, which was propagated to the primary leaves and accelerated the point where the active stomatal opening force exceeded the pressure of the surrounding cells.

Cycling of the resistance to water flow into the roots

During stomatal cycling the root resistance was found to be high during sub-periods with increasing and high plant water deficit that followed sub-periods with low rate of water uptake, and to be considerably decreased in the subsequent sub-period of decreasing and low water deficit. Hence, the root resistance decreased and increased during each cycle. It is proposed, that the decrease of the root resistance was induced by the temporary low water potential in the roots during the sub-period with high water deficit. Similar reactions have been described by JOST (1916), KÖHNLEIN (1930), BREWIG (1937), BROUWER (1953, 1965), MEES and WEATHERLEY (1957a, b) and WEATHERLEY (1965). MEES and WEATHERLEY (1957a, b) and BROUWER (1965) indicated a time delay in this response of the root resistance.

KUIPER (1963) reported that the root resistance decreased with increasing water potential gradient and remained at the low level when the gradient was decreased subsequently. Also during the present cycling the root resistance apparently remained at the low level during gradual decrease of the gradient. It returned to the prior high level, however, during the subsequent sub-period with very low water potential gradient.

Influence of root temperature on cycling of the root resistance

The decrease of root resistance, induced by the high water potential gradient between root interior and exterior, was fortified by an increase of the root temperature. As a result the leaf water content increased more in the following sub-period and passive stomatal closure was increased, thus lowering the rate of water uptake. The rise in root resistance will have been stimulated by the decreased flow rate as concluded above. The steeper fall of the water content during cycling with an increased root temperature supports this view. The increased root resistance at that particular sub-period stimulated passive opening.

In conclusion: Due to the fact that the fall of root resistance was enhanced by increasing temperature, both cycling of stomatal opening and cycling of root resistance were intensified.

4.6. SYNTHESIS

Models for the mechanism of water transport through the plant becoming more refined and comprehensive from author to author, were presented by VAN DEN HONERT (1948), BROUWER (1961), COWAN (1965), and WOO et al. (1966a, b).

KARMANOV et al. (1966), MELESHCHENKO and KARMANOV (1966), CLAUS (1968), MEISTER und APEL (1968), HOPMANS (1969b), LANG et al. (1969) and RASCHKE (1970a) proposed models, simulating stomatal cycling. The salient component in these models is a control system with negative feedback for the control of the water balance in the leaf by the stomata. In this control circuit the water balance is controlled by the stomatal opening (actuator, see p. 7).

The information on the status of the water balance in the leaf reaches the guard cells as a water potential value through the information loop. HOPMANS (1969b) and LANG et al. (1969) proposed a time delay in this information loop to be caused by a resistance for water transport to the guard cells. This delay causes a time lag in the correcting action of the stomata upon a deviation of the water balance.

The present study provides evidence in support of the action of the negative feedback circuit as indicated earlier. Furthermore, evidence was found for the existance of a positive feedback circuit, which controlled the water balance of the leaf by the stomata as proposed by HOPMANS (1969b) and RASCHKE (1970a). In addition, a variable root resistance as a component involved in cycling is introduced. In a conceptual model the action of the new components will be illustrated. Figure 22 presents this model.

How the model works will be illustrated by the following course ot events, based on the preceding part of this chapter. The guard cells are assumed to be triggered by other factors to keep or bring their turgor to a high level, when not a low water potential induces active stomatal closure.

When stomata are opened actively during cycling, the water vapour diffuses through r_l, r_s and r_a. Evaporation from the cell walls of the substomatal cavities rises and the water potential decreases in each segment of the pathway from the leaf cell walls to the xylem vessels in the roots. The water potential of the cell walls continues to fall, accompanied by a falling water content in the mesophyll (c_m) and epidermal cells (c_e), which is mainly due to the root resistance r_r.The intercellular pressure in the epidermis, caused by the turgor in the epidermal cells, induces already passive stomatal movement by a small alteration. The passive opening will be larger, the higher the turgor in the guard cells when the opening movement is started and the higher the vapour pressure gradient leaf-air, when the water supply to the leaf is impaired. This passive stomatal opening is proceeding due to the delayed reaction of the guard cells by active closure (delayed negative feedback correction,–). A relatively high resistance to water transport through the guard cell membranes, r_{mg}, might be the cause of this delay. In the course of this lag time the passive opening tendency increases, induced by the continuing decrease in turgor of the epidermal cells (positive feedback,+).

During the subsequent active stomatal closure the water content rises rapidly due to a delayed decline of r_r in response to the high water potential gradient from root exterior to interior. The turgor of the epidermal cells increases, causing passive stomatal closure by the above mentioned positive feedback circuit. This passive closure is more pronounced, the lower the resistance in the root during this part of the cycle and the higher the water potential in the root medium.

Summarizing, the effects of delay factor(s) in the negative feedback of stomatal control become apparent if combined with a resistance in the water supply pathway to the leaf. Overshooting of stomatal movement, as a consequence of this combination, is amplified by positive feedback actions as passive stomatal movement. Cyclic variations in the resistance to water transport into the roots

fortify the overshooting effect on stomatal movement of both feedback systems. The variation in the water potential synchronizes stomatal cycling within one leaf and within the leaf system of one plant.

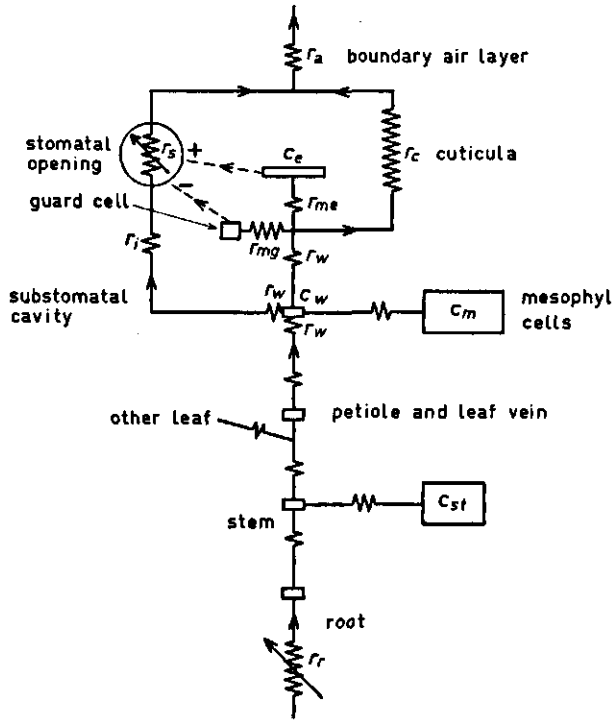

FIG. 22. Diagrammatic conceptual model of the pathways of the transpiration stream, of the transfer of water within the leaf, and of the stomatal control system of the water balance of the leaf. The relative sizes of the symbols for water storage (rectangulars) and resistance indicate in a semi-quantitative sense the relative magnitudes of the entities they represent.
Denominated resistances, subsequently met by the transpiration stream from the root to the surrounding air, are in the liquid phase:
r_r root,
r_w cell walls of the leaf,
r_c cuticula,
and in the vapour phase;
r_i substomatal cavity,
r_s stomatal opening,
r_a boundary air layer.
Water storages are:
c_{st} stem tissue,
c_m mesophyll cells,
c_w cell walls inside the leaf,
c_e epidermal cells.
r_{mg} guard cell membranes,
r_{me} epidermal cell membranes.
The stomatal control system is indicated to contain a negative (–) and a positive (+) feedback loop.

5. CIRCADIAN RHYTHM IN STEADY AND CYCLING STOMATAL OPENINGS

5.1. INTRODUCTION

The periodicity in stomatal opening with periods of exactly or approximately one day is expressed as daily or diurnal rhythm. When periods explicitly approximate 24 hours, the term circadian rhythm is used. Only the short period rhythm will be indicated as cycling. A diurnal rhythm in the stomatal opening of *Prunus laurocerasus* leaves in continuous light was found by MASKELL (1928) and it was the reason for an observed diurnal rhythm in the rate of photosynthesis. GREGORY and PEARSE (1937), HEATH and RUSSELL (1954), MEIDNER and MANSFIELD (1965) found this rhythm in periods of 24 to 30 hours continuous light in different plants. KANEMASU and TANNER (1969) also observed it in *Phaseolus vulgaris*. GRAU (1968) described a daily rhythm modulating the cycling of stomata in a piece of lettuce leaf in continuous light.

Diurnal rhythms in darkness have been studied more extensively. Stomatal opening was observed in continuous darkness by KUYPER (1915), SAYRE (1926), SCHWABE (1952), MONTERMOSO and DAVIS (1942), DALE (1961), STÅLFELT (1963, 1965, 1967) and PALLAS (1969). STÅLFELT (1963) observed stomatal opening in carbon dioxide free air in darkness. In all these reports stomatal opening in continuous darkness was found during the first day and in a few cases during the second day. It was damped on subsequent days in darkness.

BRUN (1962) concluded that the delay in the onset of stomatal opening in banana leaves after turning on the light was dependent on the length of the preceding dark period in accordance with a daily rhythm. The rate and the degree of opening in soybean and *Xanthium* was found to depend on the length of the dark period as indicated above for banana leaves (MANSFIELD, 1963, 1965). In the above mentioned examples of opening in darkness the phase of the rhythm in darkness turned out to be set by the termination of the light period.

Daily rhythms in stomatal openings in constant environments were found by WILLIAMS (1952), PALLAS (1969) and SEIDMAN and RIGGAN (1969) during the normal daily photoperiods. The latter authors, working with bean plants, demonstrated a daily course of the stomatal opening and carbon dioxide concentration in the climate room, which did not correspond.

SKIDMORE and STONE (1964), BARRS and KLEPPER (1968), HARRIS (1968) and HOPMANS (1968, 1969a) found daily patterns in the stomatal opening, coinciding with a daily course in the tendency to cycling during the photoperiod. Evidence was presented that the water balance of the leaf did not cause this daily pattern in stomatal opening. MEIDNER and MANSFIELD (1965) suggested that the rhythm in light and darkness might proceed by the same mechanism, because the tendency to open in continuous darkness coincided with the rhythm in the ability to open as a response to illumination.

MEIDNER and MANSFIELD (1968) stated that no conclusive evidence was available yet for the endogenous nature of the diurnal rhythm in light.

5.2. IN CONTINUOUS LIGHT

The diurnal periodicity of stomatal opening in continuous light was studied using the experimental setup described in the general materials and methods, p. 9. Plants in each experiment were of the same age and grown in equal conditions in nutrient solution. The primary leaves of the bean plants, if not held in a fixed position, performed the often described diurnal movement. For leaf temperature measurements a diurnal change in the contact of the thermocouples with the leaf surface was prevented by keeping the laminae fixed between nylon wires.

A rhythm with mean period of 25.7 hours was found in the leaf temperature of the primary leaves without stomatal cycling, hence the term circadian rhythm was applied here. Within one group of synchronously recorded plants the periods differed only slightly between the plants, but differences between the observed phases varied between 2 and 5 hours. The leaf temperature increased during the time which corresponded with the last part of the light period and the dark period of the light-dark cycle of the pretreatment. The latter was composed of 16 hours light and 8 hours darkness.

In figure 23 a phase shift of a circadian stomatal rhythm is shown, brought about by 12 hours darkness. The rhythms in both plants during day 4 and 5 were irregular and synchronization was lost. The dark period induced a shift of the phases and put both plants in approximately the same phase.

Often stomatal openings were cycling during continuous light. Cycling some-

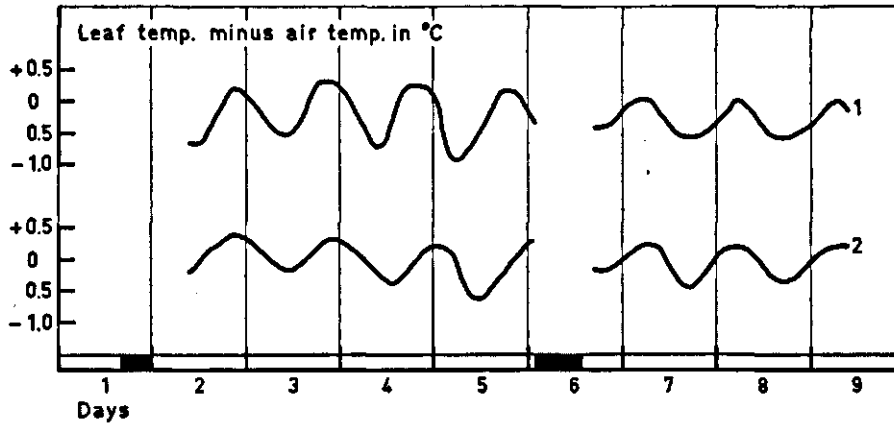

FIG. 23. Circadian rhythm in leaf temperature of 2 plants, that had been grown in daily dark-light alternation until day 2 as indicated on day 1. On day 6 a light break of 12 hours darkness was given. 27.2–27.4 °C/9.2–9.6 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/15–20 cm sec^{-1}.

times sustained for several days, in one case even for 6.5 days without interruption, but it frequently occurred that time intervals with cycling and with non-cycling opening alternated as shown in figure 24. Air temperature and humidity did not vary with a daily periodicity. Curve 1 is representative for 3 plants, curves 2 and 2′ for 2 plants and curves 3 and 3′ for one plant.

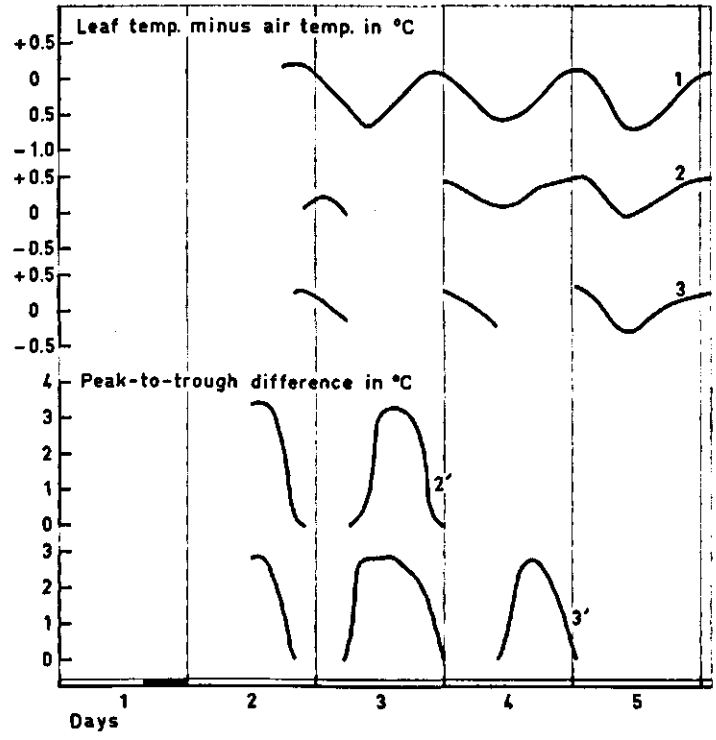

FIG. 24. Leaf temperature in continuous light in curve 1, indicating circadian rhythm in persistantly non-cycling stomatal opening and in curves 2 and 3 in non-cycling stomatal opening, interrupted by time intervals of cycling (interruptions of the curves). For the time that stomata of 2 and 3 were cycling the peak-to-trough differences of leaf temperatures are indicated in curve 2′ and 3′ respectively. Up to day 2 the daily light-dark alternation was as on day 1. 23.4–24.6 °C/7.4–9.0 mm Hg/20,000 erg $cm^{-2}sec^{-1}$/15–25 cm sec^{-1}.

Stomatal cycling started at the phase of the circadian rhythm, where stomatal opening approached the daily maximum. At other times the amplitude of cycling gradually decreased and cycling damped.

As an example of a circadian rhythm, modulating sustained cycling, figure 25 is shown. From sowing until day 1 in the figure the daily dark periods were given during the last 7 hours of the day. The dashed curve is representative for 5 plants which behaved similarly with slight differences of phases and amplitudes. The relation of the peak-to-trough differences of cycling to the phases of the circadian rhythm in non cycling stomatal opening was approximately that

presented in figure 24. However, the circadian rhythm in the peak-to-trough differences in the present figure fully matched the circadian rhythm in steady stomatal openings. The periods of sustained cycling were modulated by the circadian rhythm in such a way that they increased during the circadian subperiod, in which overall stomatal opening tended to decrease. The opposite combination applied to the other half of the circadian period. The phases of circadian rhythm in the peak-to-trough differences and in the periods of cycling were not exactly equal. In figure 25 the maxima in the periods lag some hours behind the minima of the amplitudes, but the opposite was found in other plants.

The above results showed, that in continuous light the phase of circadian

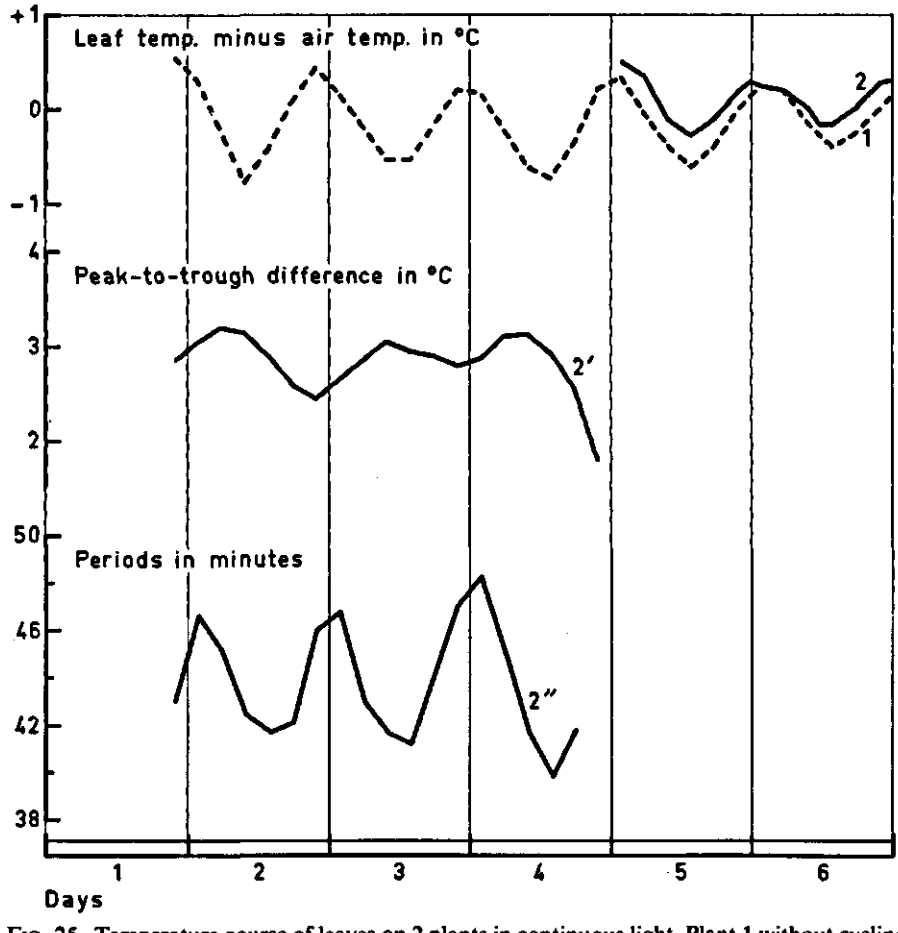

FIG. 25. Temperature course of leaves on 2 plants in continuous light. Plant 1 without cycling at all (dashed line), plant 2 without cycling on days 5 and 6 (drawn line). The peak-to-trough differences and the periods of plant 2 during sustained cycling are curves 2' and 2'' respectively. Air temperature and humidity without a diurnal periodicity. 25.5–26.5 °C/9.6–10.8 mm Hg/ 20,000 erg $cm^{-2}sec^{-1}$/15–25 cm sec^{-1}.

rhythm in non-cycling stomatal opening could be caused to shift. Cycling started spontaneously and the amplitude of sustained cycling was largest approximately at the time that in similarly treated plants maxima were attained in the circadian rhythm in the opening of non-cycling stomata. The amplitude was smallest and the period largest and cycling stopped temporarily half a circadian period later.

5.3. In continuous darkness

Two typical examples of diurnal rhythm in darkness are shown in figure 26. The large amplitude in curve 1 shows that stomatal opening during cycling in darkness may be of the same magnitude as stomatal opening during cycling in light. The amplitude sometimes showed a clearly daily pattern (curve 1), but a less regular pattern was frequently found (curve 2).

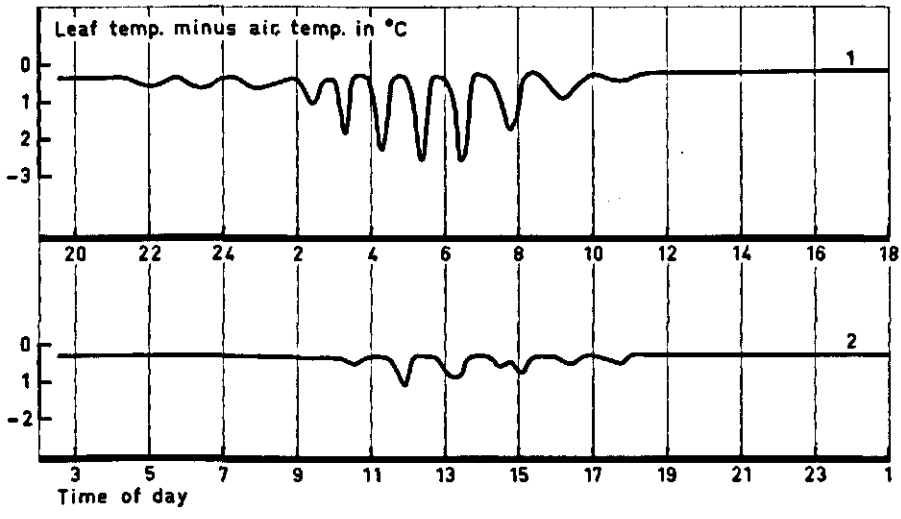

Fig. 26. Temperature course of leaves on different plants during the first day in continuous darkness. Curve 1: 22.1 °C/10.5 mm Hg/ –/20–30 cm sec^{-1}. Curve 2: 23.3–23.8 °C/ 12.5 mm Hg/ –/20–30 cm sec^{-1}.

Stomatal opening only occurred during the first day of darkness, and during that part of the day when the plant used to be in the light, viz. from 2300 to 1100 (curve 1) and from 600 to 2300 (curve 2).

Groups of 5 or 6 plants were investigated during periods of 31 hours darkness for a quantitative description of the diurnal rhythmic stomatal opening in darkness (table 4).

The maximum of stomatal opening was gradually attained at 12–14, 6 to 8 hours after the time that on the previous days the light period started. Later on

TABLE 4. Stomatal cycling in continuous darkness per time interval of the day. Analyses derived from 18 plants. Daily light on the previous days from 600 to 2300.

	time intervals in hours of the day						
	6–8	8–10	10–12	12–14	14–16	16–18	18–20
relative leaf temperature decrease due to stomatal opening	16.2	55.8	92.5	100.0	95.4	54.5	20.6
cycles with peak-to-trough difference larger than 1.0°C	2	7	11	10	8	3	1
smaller than 1.0°C	4	4	8	7	13	8	4
total	6	11	19	17	21	11	5
mean period in minutes	79.0	79.6	80.0	101.0	116.0	138.0	
coefficient of variability	9.7	12.8	34.0	25.2	23.8	33.6	

stomatal opening gradually decreased. Also the total number of cycles was centered around this time interval. The cycles with peak-to-trough differences larger than 1.0°C appeared earlier than the smaller ones. The mean period was almost constant throughout the first 3 time intervals (600 to 1200), and it considerably increased during the following ones.

5.4. DURING THE DIURNAL PHOTOPERIOD

The influence of a diurnal rhythm in the opening of the stomata was almost always found during days with common light-dark alternation with photoperiods of 16 or 17 hours. This tendency interacted with the susceptibility of the stomata of cycling and it varied from case to case. The main types of interaction are presented in figure 27. In curve 1 the stomatal system was stable throughout the whole photoperiod; only at the beginning some overshoots occurred. Cycling damped in curve 1 and 2, indicating that the stomatal system became stable when the opening tendency of a daily rhythm reached a certain level. Interaction of a daily rhythm with cycling specially occurred as shown in curve 2 in younger leaves, in which stomata cycled with short periods. Curve 3 represents the main type of interaction, where continuous instability of the stomatal apparatus led to sustained cycling. By superposition of a diurnal rhythm on the amplitude (and the periods) of cycling, the amplitudes reached a maximum (and the periods a minimum) near the middle of the daily photoperiod.

The rhythmic behaviour shown in curves 2 and 3 has been found synchronously in the same plant in a few cases viz. type 2 in the primary leaf and type 3 in a trifoliate leaf of the same plant. This suggests that the frequency of cycling was increased by the increase of some form of activity. The activity was increased to a level, where stomata became stable. The level of this activity depended on leaf age and on the phase of the daily rhythm.

According to the influence of temperature on cycling, to be described in

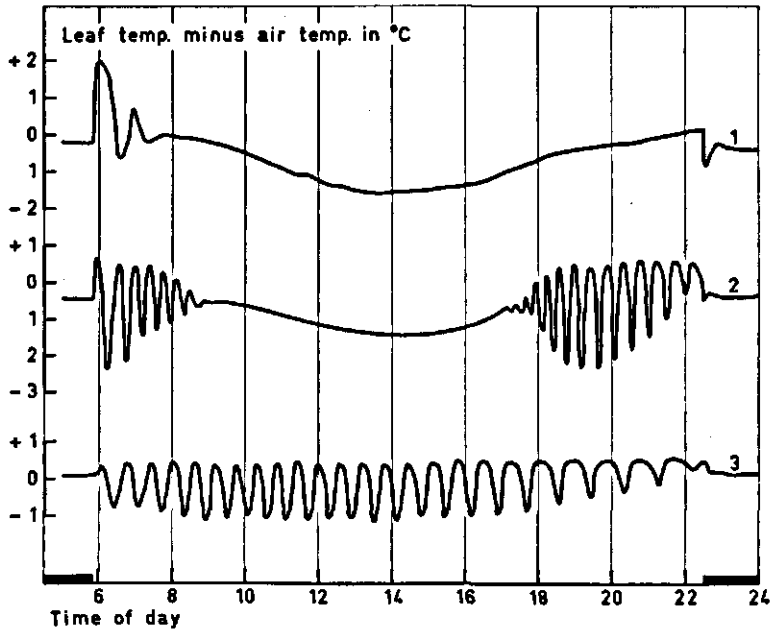

FIG. 27. Leaf temperature minus air temperature in the course of the daily photoperiod of 17 hours to which the plants were adapted. Three different plants from different days. Air temperatures, gradually increasing until about 1800, especially during the first half of the light period, are for 800 and 1800; curve 1: 24.3 °C, 26.6 °C/15 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–20 cm sec^{-1}; curve 2: 25.1 °C, 27.0 °C/9 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–20 cm sec^{-1}; curve 3: 23.7 °C, 25.3 °C/10 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–20 cm sec^{-1}.

section 6.6.1, the increase of the amplitude and the decrease of the period in the first part of the photoperiod could be assigned to the increase of temperature in the course of the photoperiod as indicated in the legend of figure 27. However, the development of both the amplitude and the period in the second half of the photoperiod could not be explained this way at all. Furthermore, daily patterns during the photoperiods similar to curve 3 also were obtained from plants in a plant chamber with constant air temperature, constant vapour pressure and constant carbon dioxide concentration of the incoming air.

The mean periods during approximately 2 hours at 4 time intervals in the course of the daily light periods were determined from 25 plants (figure 27, curve 3). These mean periods were corrected for the deviation from the mean daily temperature and expressed on a relative basis per day. For the separate time points the mean of the relative values of the 25 day-plant combinations were multiplied by the overall mean period. The following data were obtained.

Hours since start of light period:	1.5	6.0	10.5	15.0
Period in minutes:	41.1	39.6	42.6	46.4

The periods at the beginning and at the end of the daily light periods were longer than the periods at both medium times (probability level exceding 0.5%).

The period of cycling changed during the whole photoperiod possibly due to an internal factor.

A group of uniform plants on nutrient solution was observed during consecutive days, either intact, or after removal of the root system, in order to ascertain whether the daily rhythm in the light period was governed by factors, situated in the leaves or in the roots. Precautions were taken that the xylem vessels would not be blocked. Cutting was done under water, a sharp-edged cutter was used, and the hypocotyl was desinfected and was washed with distilled water. The stem was placed in distilled water with 10^{-7}M $CuSO_4$. Figure 28 shows that the stomatal openings completed the daily rhythm during the light periods after root excision in the same way as before.

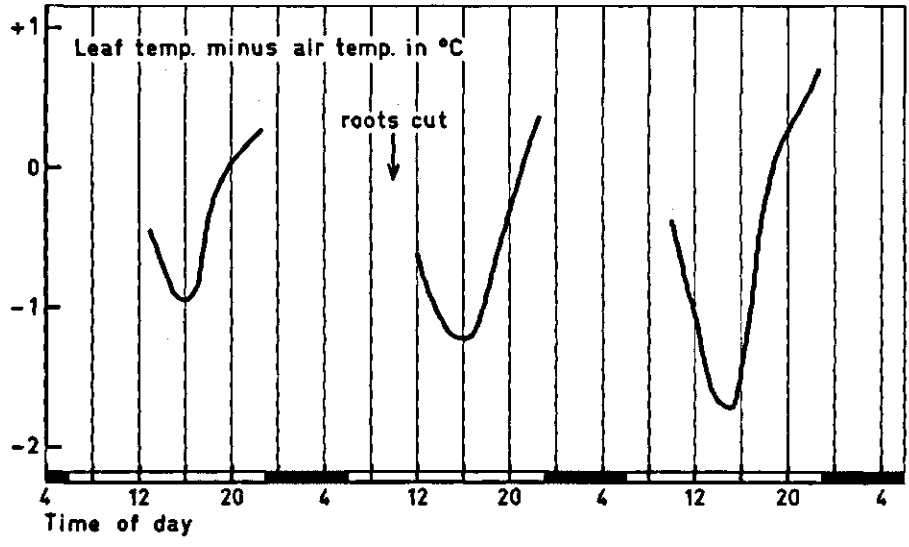

FIG. 28. Leaf temperature minus air temperature, representing stomatal behaviour in the course of the light periods of 3 consecutive days of plants on nutrient solution, before and after the root system had been cut off under water. The curves are means of 4, 2 and 2 plants respectively for the consecutive days. Air temperatures gradually increased during each photoperiod as indicated. 26 °C–27 °C/16.5 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–25 cm sec^{-1}.

Also in isolated leaves the same daily rhythm was performed by the stomatal opening.

It is concluded that factors governing the daily rhythm in stomatal opening during the light period are situated in the leaves.

The ability of the stomata to open after closure by short dark periods of 40 minutes in the course of the daily light period was tested (figure 29).

A daily rhythm existed in the opening ability of the stomata after the light was switched on.

The variation in the opening ability consisted fully of a variation in the time

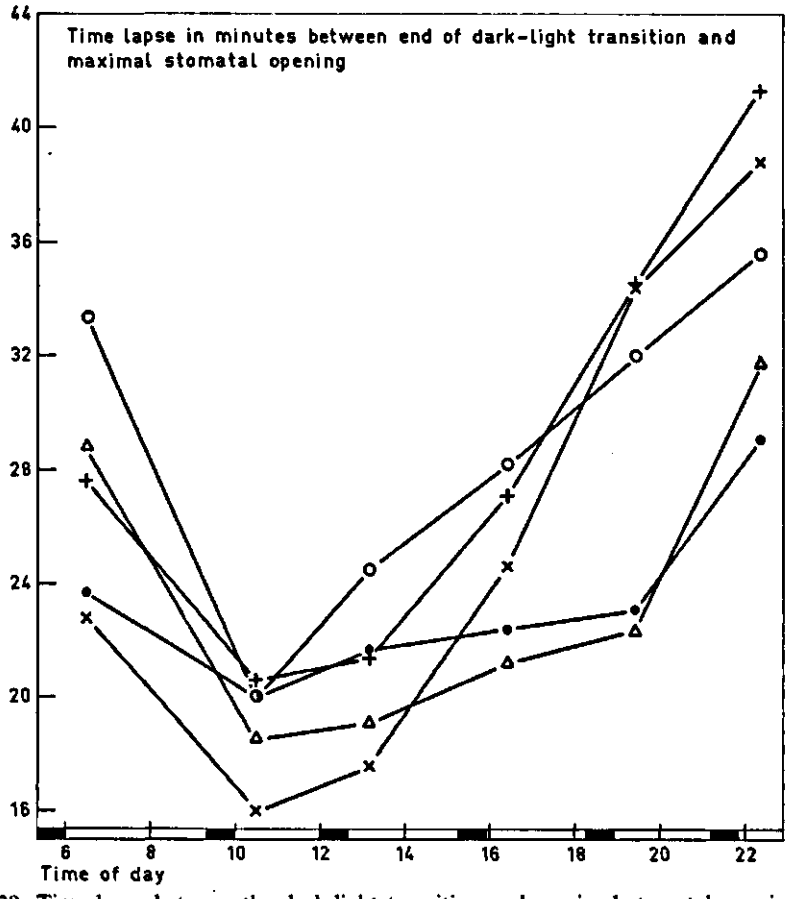

FIG. 29. Time lapse between the dark-light transition and maximal stomatal opening of 5 plants on the same day after short dark periods, distributed over the daily photoperiod, to which the plants were adapted. Air temperature gradually increasing. 27–29 °C/10 mm Hg/ 30,000 erg $cm^{-2}sec^{-1}$/10–30 cm sec^{-1}.

between the light switching on and the beginning of stomatal opening. Once the opening movement had started, the rates of opening were equal throughout the light period.

Presumably, one and the same factor caused the daily rhythm in the onset of stomatal opening in the light after dark periods, the daily rhythm in the steady stomatal opening in attached and isolated leaves, as well as the daily rhythm in period and amplitude of cyclic stomatal opening.

5.5. Discussion

In continuous light the period of the daily rhythm in stomatal opening differed from 24 hours. The phases were not the same in different plants with the same treatment and interruption of continuous light by a dark period caused a phase shift. These findings supply sufficient evidence that the circadian rhythm in light is endogenous in the bean plant.

It is assumed that the maintenance of the daily rhythm during only one day in continuous darkness originates from the lack of energy required for stomatal opening on the second day.

Similar phase relations to the preceding dark-light alternation were found for the daily rhythm in stomatal opening during daily photoperiods for the circadian rhythm in stomatal opening in continuous light and for the daily rhythm in continuous darkness. This corroborates the assumptions made by Stålfelt (1965, 1967) and by Meidner and Mansfield (1965) that the rhythm in light and darkness might proceed by the same mechanism.

Evidence was presented in the preceding section that the daily rhythm in stomatal opening during normal daily light periods originated from the leaf itself. This confirms the data of Grau (1968), who described a daily rhythm in light in the cycling stomata of pieces of lettuce leaves. By bringing different leaves on one *Xanthium* plant in different phases Meidner and Mansfield (1965) demonstrated that the source of the rhythm in darkness was in the leaf. These facts lead to the hypothesis that one general endogenous oscillator behind the circadian rhythms in continuous darkness, in continuous light and in the normal daily photoperiod is situated in the leaf.

The amplitude and period of stomatal cycling in continuous light and darkness and during the daily light periods were all modulated by the circadian rhythm in a way that cycling was most intensified at approximately the phase where the non-cycling stomatal openings reached their circadian maximum. This suggests that the same endogenous circadian oscillator mentioned above, caused the modulations. Stålfelt (1967) found that in darkness the diurnal rhythm in the width of the pore and in the width of the stomatal apparatus of *Vicia faba* was accompanied by a rhythm in the same phase of the surplus of the osmotic value of the cell sap in the guard cells over that of the epidermal cells. This surplus was mainly caused by a variation in the osmotic value in the guard cells.

From chapter 4 it is clear, that the amplitude of cycling depends on the turgor of the guard cells, the active contribution to the combined active and passive opening movement. This active component being subject to a diurnal rhythm, will have caused the amplitude to assume a daily pattern.

In all cases of high instability of the stomatal apparatus the daily variation in the period of cycling was made up mainly by the variations of the sub-period between stomatal closure and the onset of stomatal opening. As will be further clarified in the next chapter, the length of that sub-period reflects the time required to increase the turgor of guard cells enough to open the stomata. A circadian

rhythm in the osmotic pressure of the guard cells might very well partly have modulated these sub-periods to daily patterns.

The hypothesis of an endogenous oscillator in the leaf, causing circadian rhythm in non-cycling stomatal openings in light and darkness, is extended by hypothesizing that this same endogenous oscillator modulates stomatal cycling to the daily patterns.

JONES and MANSFIELD (1970) found an endogenous circadian rhythm in the carbon dioxide compensation point in detached leaves of *Bryophyllum* and *Coffea*. A similar rhythm in bean might play an important part during the circadian rhythm in the stomata in light. Probably in the present study a circadian rhythm in the compensation point was not the primary source for the circadian rhythm in stomatal opening, because also in darkness the primary oscillator affected the stomata.

6. ENVIRONMENTAL EFFECTS ON CYCLING STOMATA

6.1. Introduction

The cyclic opening and closing of the stomatal apparatus in a constant environment might be affected by changed environmental factors in a special way. The responses of cycling stomata to different factors have been studied, because they might provide useful data for the further analysis of the cyclic behaviour as well as for the still unrevealed action mechanism of the guard cells.

6.2. Materials and methods

Light intensities in the large climate room were partly varied by switching on different numbers of fluorescent tubes and partly by using grey cinemoid filters. In the assembly for the measurement of carbon dioxide exchange, described by Louwerse and van Oorschot (1969), the light in each plant chamber was obtained from 4 Philips HPLR-lamps of 400W, with a layer of running water of 5 cm underneath. Light intensity was varied here using perforated metal screens.

Carbon dioxide concentration was varied in the plant chamber described in section 2.2 (p. 10). Short time increases of the carbon dioxide concentration in the plant chamber were realised by adding a roughly controllable, measured flow of carbon dioxide from a cylinder to the normal air stream into the plant chamber. In the plant chambers of the assembly for measurement of the rate of carbon dioxide exchange the concentration was controlled by mixing continuously carbon dioxide to carbon dioxide free air (Louwerse and van Oorschot, 1969), hence a carbon dioxide free atmosphere could be obtained as well.

Variation of leaf temperature. Leaf temperature was varied in 2 different ways. The most frequently used method was by varying the air temperature. In some experiments a specially built device was used, consisting of a clear panel of glass with an electrical conducting metal coating, which could be heated up to 100 °C by an electric current passing through the coating. The temperature of the panel was controlled by a thermostate. The panel was placed between the light source and the leaves. By placing the sensor of the thermostate (a thermocouple) on the leaf, the leaf temperature could be controlled in a wide range above the air temperature independently of air temperature and short wave radiation.

Measurements of the carbon dioxide exchange rates. The carbon

dioxide contents of the in- and outgoing air of the plant chambers were measured with an infra-red gas analyser. Both 35.5 l chambers and 1.5 l 'sandwich'-type chambers were used, Together with the environmental factors, leaf temperature and transpiration measurement were processed by a computer (LOUWERSE and VAN OORSCHOT, 1969). In addition, the computer program was extended as indicated in section 4.2 (p. 20) to correct for delays due to the volume of the plant chambers at rapid variations of CO_2 exchange.

6.3. PRECONDITIONING FACTORS

6.3.1. *Introduction*

Responses of stomata to external factors can only be compared if important preconditioning factors were equal for the different plants. The importance of 2 preconditioning factors will be dealt with in this section.

6.3.2. *Duration of the preceding dark period*

The rate of stomatal opening in the light, as well as the degree of stomatal opening are affected by the length of the preceding dark period. BRUN (1962), MANSFIELD and HEATH (1963) and MANSFIELD (1963) found that the reactivity of the stomata on the onset of light followed a daily rhythm after increasing dark periods. The response time of stomatal opening in banana leaves, investigated by BRUN (1962), tended to increase as the length of the previous dark period was extended. The higher the temperature during the dark period, the more pronounced the increase was. The combination of a decreasing opening activity of the stomata in prolonged darkness with a diurnal rhythm was found by most authors who studied diurnal rhythm in darkness (e.g. STÅLFELT, 1963). It was also mentioned in section 5.3 (p. 45). ALVIM (1949) indicated, that in bean plants the rate and the degree of stomatal opening in light decreased with the length of the previous dark period.

The effect of the length of the preceding dark period on stomatal opening in light was investigated at equal phases of the diurnal cycle of 16 hours light and 8 hours darkness of the pretreatment. At the onset of light after 8 hours dark periods and after 8 + 24 hours dark periods the time lapses between the dark-light transitions and maximal stomatal openings were compared. At the beginning of 3 consecutive photoperiods the mean time lapses after the indicated dark periods were:

preceding dark period	32 hr	8 hr	32 hr
time lapse in minutes	73.7	37.0	78.2

The environmental conditions during the lapse periods were: 25 °C/14 mm Hg/35,000 erg $cm^{-2}sec^{-1}$/10–30 cm sec^{-1}. The time between dark-light transition and maximal stomatal opening was about doubled by the longer dark periods. No difference was found in the rate of the opening movement, and the effect was confined to the period from the onset of light until the beginning of stomatal opening.

The onset of light induced stomata to cycling in all the treatments presented above. In order to ascertain, whether the influence of the length of the preceding dark period on the rate of stomatal opening remained in the course of the light period, the mean lengths of the second periods of cycling after the first stomatal opening were compared. This mean length was 43 minutes after the 32 hours dark periods and 38 minutes after the 8 hours dark period, indicating that the effect of a dark period, which was 24 hours longer than normal, had almost disappeared after 1 hour in the light.

6.3.3. *Competition between leaves with relation to the period*

The free-running period of the primary leaves of plants, grown while the trifoliates remained on the plant, was found by determining the period immediately after detachment of these trifoliates. The period increased when the plants grew older. The increase of the period consisted mainly of an increase in the sub-period with relatively closed stomata. It was about 25% in 2 weeks, following the stage of just completed extension of the primary leaves. At the same time the primary leaves gradually yellowed.

The yellowing did not appear when the trifoliates had been removed, and it was reversed when removal of the trifoliates was done at a later stage. One or 2 days after removal of the trifoliates, the period in the primary leaves was always seen to return to shorter periods, approximately of the length as when the trifoliates were not yet developed.

6.3.4. *Discussion*

The effects of prolonged dark periods and of the presence of younger leaves on the same plants have in common a reversible after-effect, consisting of increased sub-periods with small stomatal openings. For the interpretation of the effect of detaching the younger leaves the following literature data for *Phaseolus vulgaris* are relevant. WAREING et al. (1968) showed an increased rate of photosynthesis in the remaining leaves of partially defoliated plants. They suggested that partial defoliation leads to increased rate of photosynthesis due to increased supply of cytokinins, produced by the roots, to the remaining leaves. This, in turn, leads to an increased enzyme synthesis including carboxylating enzymes. MEIDNER (1969, 1970) found increased photosynthesis and stomatal opening upon defoliation of bean plants like WAREING et al. did. The response of the stomata appeared to coincide with the increase in the levels of carboxylation enzymes.

In the present stomatal cycling the smaller periods – or rather the shorter sub-periods of relatively closed stomata – could have been brought about by a reduced competition for minerals, enzymes, and pro-enzymes from the roots.

According to ALVIM (1949) and BRUN (1963) the declining stomatal reactivity to light after long dark periods might be caused by starvation. This explanation is preferred for the declined reactivity found in the present experiments after longer dark periods. Starvation might consist of a deficiency of a product of photosynthesis, since the reduced reactivity disappeared rapidly in the light.

6.4. LIGHT INTENSITY

6.4.1. *Literature*

KUIPER (1961) found that the stomatal opening in bean increased with increasing light intensity until the maximum was reached at 50,000 erg cm^{-2} sec^{-1}. KANEMASU and TANNER (1969) found the stomatal diffusion resistance of the lower leaf surface of bean to attain its maximum at approximately 30,000 erg $cm^{-2}sec^{-1}$, when the leaf was irradiated from above and at 7000 erg cm^{-2} sec^{-1} when it was irradiated from below.

Some publications reported sensitivity of the stomata to extremely low or moderately low light intensities as 30–40 erg $cm^{-2}sec^{-1}$ (VIRGIN 1956) or 1290 erg $cm^{-2}sec^{-1}$ (MANSFIELD and MEIDNER, 1966). The data of KANEMASU and TANNER (1969) indicate an appreciable stomatal opening in the lower epidermis upon irradiation from 400 to 800 erg $cm^{-2}sec^{-1}$.

6.4.2. *General influence*

Cycling continued or damped, when light intensity was increased in the course of self-sustained stomatal cycling. When cycling damped, it often damped just as well after a new induction to cycling by a short dark period. In figure 30 an example of a damping cycling with increase in light intensity is presented. The irradiation of a plant in a plant chamber was abruptly increased from 38,000 to 115,000 erg $cm^{-2}sec^{-1}$ during sustained cycling. The other environmental factors remained unchanged.

The minimum of leaf diffusive conductance increased from a to c and was followed by a decrease of the maximum from b to d, hence, primarily the stomata closed less and subsequently opened less than at the lower light intensity. The overshooting stomatal closure movement was counteracted at the higher light intensity by an intensified opening trigger and/or by the lower carbon dioxide concentration in the leaf. The subsequent decrease in stomatal opening (d) might result from a change in the course of events in the water relations, suggested as follows. Since the stomata closed less in sub-period c, the water stream into the root did not stagnate and the root resistance did not increase as in the preceding periods according to section 4.5.2 (p. 34). Consequently a less serious water deficit developed in the leaf during the sub-period c to d and the passive component in stomatal opening was diminished.

The figure shows that the rate of CO_2 uptake increased markedly with the increased light intensity. This confirmed other experimental data on the influence of light intensity on CO_2 uptake during cycling. For instance in 5 cases the change in the mean rate of CO_2 uptake per unit change of irradiation was 70%, 57%, 120%, 108% and 90% of the mean change in the figure.

6.4.3. *Influence on sustained cycling*

The influence of light intensity on the period of sustained cycling was studied. The mean value of 2 or 3 periods in each light intensity immediately before and after the change of the light intensity was measured and corrected for the

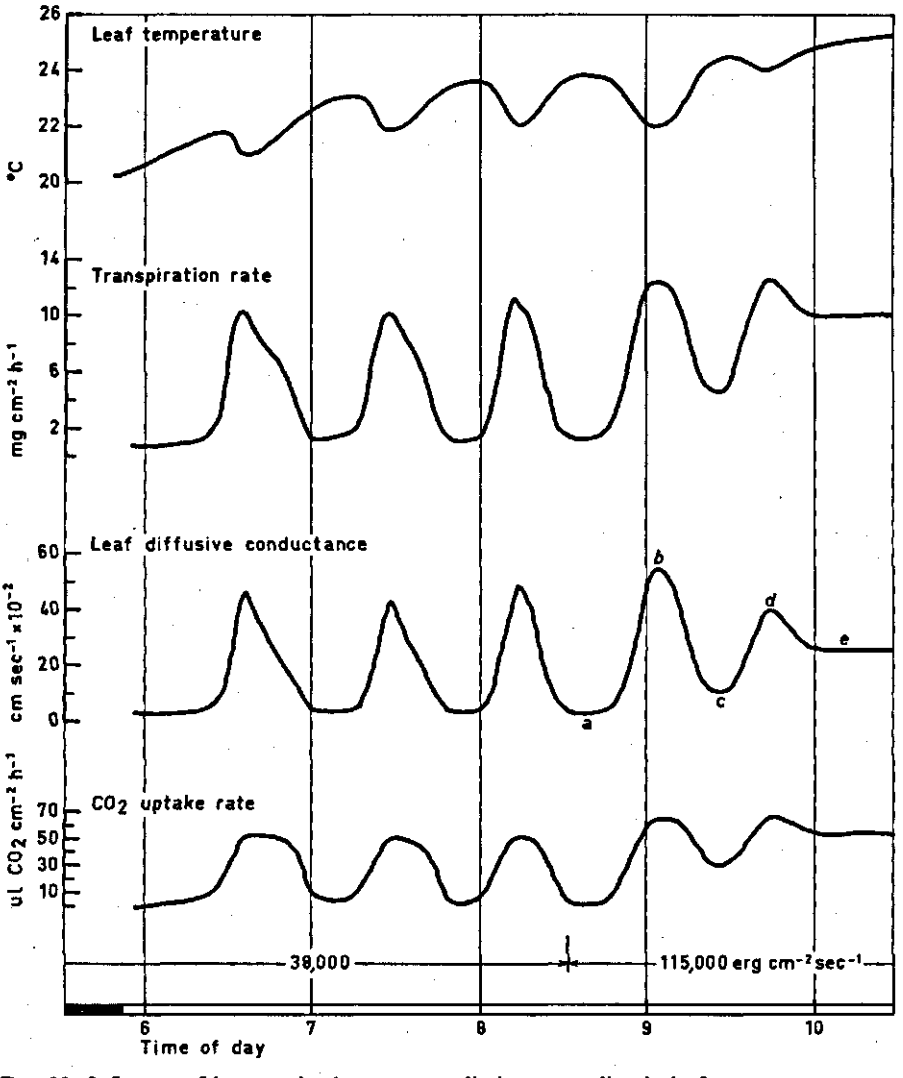

FIG. 30. Influence of increase in short wave radiation on cycling in leaf temperature, transpiration rate, calculated leaf diffusive conductance to water vapour, and rate of carbon dioxide uptake of a plant with 2 primary leaves. Plant chamber 1.5 l. Air temperature until 830 cycling with an amplitude of 0.7 times leaf temperature and with the same mean, after 830 cycling in average 1.0°C below leaf temperature; vapour pressure between 9.3 and 13.7 averaging 11.5 mm Hg; wind speed approximately 100 cm sec^{-1}.

estimated change of the period due to the diurnal rhythm. At irradiation intensities higher than 16,000 erg $cm^{-2}sec^{-1}$ the correction was derived from the daily pattern in normal photoperiods as described in section 5.4 (p. 47), whereas at the intensities lower than 2100 erg $cm^{-2}sec^{-1}$ the much larger correction coefficients, derived from the daily pattern of cycling in darkness, were used. A correction was applied for the influence of temperature on the period (to be dealt with in section 6.6.2 on p. 65), when the mean leaf temperature was changed by the changed light intensity. The experiments on the effect of light intensity on sustained cycling are divided into 3 groups; viz. short wave radiation above 35,000 erg $cm^{-2}sec^{-1}$ on the upper side of the leaves, short wave radiation from 34,000 to 16,000 erg $cm^{-2}sec^{-1}$ on the upper side of the leaves, and short wave radiation lower than 2,100 erg $cm^{-2}sec^{-1}$ on the lower side of the leaves.

Short wave radiation above 35,000 erg $cm^{-2}sec^{-1}$ on the upper side of the leaves

The changes of irradiation intensity mainly consisted of 20,000 or 40,000 erg $cm^{-2}sec^{-1}$ between 75,000 and 35,000 erg $cm^{-2}sec^{-1}$ (17 replications with different plants), whereas a few increases up to 100,000 erg $cm^{-2}sec^{-1}$ (3 replications) and up to 200,000 erg $cm^{-2}sec^{-1}$ (2 replications) were performed. Statistical analysis with the t-test did not yield a significant influence of irradiation intensity on the period of cycling.

Short wave radiation from 34,000 to 16,000 erg $cm^{-2}sec^{-1}$ on the upper side of the leaves

The experiments consisted of 6 replications with different plants, each replication comprising 3 to 5 plants. The mean period at 16,000 erg $cm^{-2}sec^{-1}$ and 34,000 erg $cm^{-2}sec^{-1}$ was 51.8 min and 47.6 min respectively at an air temperature 21–23 °C. The t-test indicated the influence to be more than probably significant, t exceeding the 2.5% probability level.

The above results indicate that between 16,000 and 34,000 erg $cm^{-2}sec^{-1}$ a level in the effect of irradiation intensity on the period was reached above which irradiation intensity had no effect and under which the period increased with decreasing irradiation intensity. At the lower irradiation intensities the compensation point was approached. At 7,000 erg $cm^{-2}sec^{-1}$ the rate of CO_2 uptake was 2–3 $\mu l\ cm^{-2}h^{-1}$ and could no longer be distinguished from zero in some cases. The respiration rate in darkness was 1–2 $\mu l\ cm^{-2}h^{-1}$ at 22 °C.

Short wave radiation lower than 2,100 erg $cm^{-2}sec^{-1}$ irradiated on the lower side of the leaves

KANEMASU and TANNER (1969) found that irradiation from below the leaf was more effective than irradiation from above. The effect of low light intensities was studied by starting at 2,100 erg $cm^{-2}sec^{-1}$ at about 1100 and progressively decreasing the irradiation during 5 hours with the steps as in figure 31. The periods and peak-to-trough differences have been corrected for changes of the influence of the circadian rhythm using the course of the periods and

peak-to-trough differences in continuous darkness (table 4, p. 46). The effect of irradiation intensity has presumably been attenuated by this correction, because the increase of the period and the decrease of the peak-to-trough difference in darkness were larger than in light. The effects of low light intensities on the period and peak-to-trough differences of sustained cycling are indicated in figure 31.

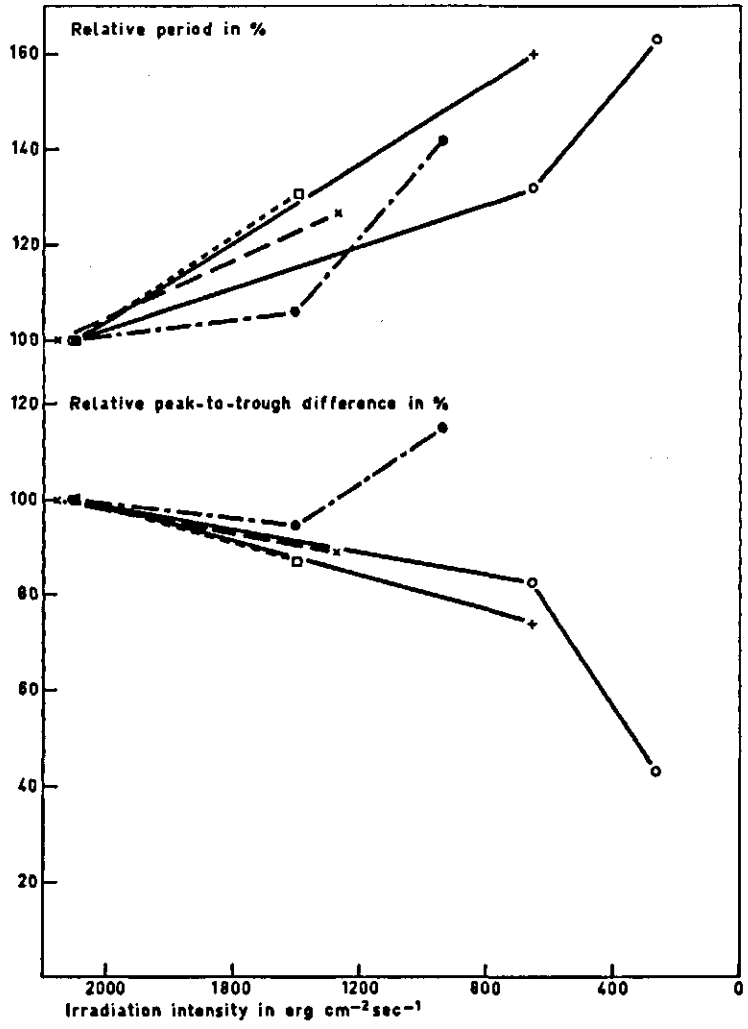

FIG. 31. Periods and peak-to-trough differences of cycling leaf temperatures in different irradiation intensities, expressed as percentages of the values at 2100–2110 erg cm^{-2} sec^{-1} and corrected for the circadian rhythm. Each curve represents the mean of one uniformly treated group of 6 plants studied on one day. Temperature varied 0.1–0.2 °C per day and from 25.3 to 29.0 °C between days. Vapour pressure 9–12 mm Hg, wind speed 10–30 cm sec^{-1}.

The periods consistently increased with decreasing irradiation intensity, which was due to the increase of the sub-period with minimal stomatal opening (not to be seen in the figure). At decreasing irradiation intensities the differences between peaks and troughs of cycling became smaller with one exception.

Hence, when the irradiation intensity decreased from 2,200 to 200 erg $cm^{-2}sec^{-1}$, the rate of cycling decreased. Obviously, the lower the radiation absorbed by the leaf, the longer the accumulation time to induce stomata to open. Furthermore the peak-to-trough difference indicates that, once the stomata are open, the level of the turgor of the guard cells, low enough to close the stomata, was sooner reached at low light intensity. According to chapter 5, the opening tendency by the endogenous circadian factor was at a high level during the circadian sub-period when the investigations were made. Hence an important part of the turgor of the guard cells was due to this factor.

6.4.4. *Influence of very low light intensity*

Sensitivity of the stomata to very low light intensities during the period of high effect of the circadian rhythm on stomatal opening was tested at 150 erg $cm^{-2}sec^{-1}$ on the upper side of the leaves and 50 erg $cm^{-2}sec^{-1}$ on the lower side of the leaves. Irradiation was performed by distant fluorescent tubes during 2 periods of approximately 2 hours at daytime. The 3 curves in figure 32 demonstrate different degrees of reaction of the stomatal opening to light.

Table 5 indicates the frequency of these types of reaction.

An opening reaction in the light was most frequent. This indicates, that this irradiation intensity affected stomatal opening. Often the stomata opened rhyth-

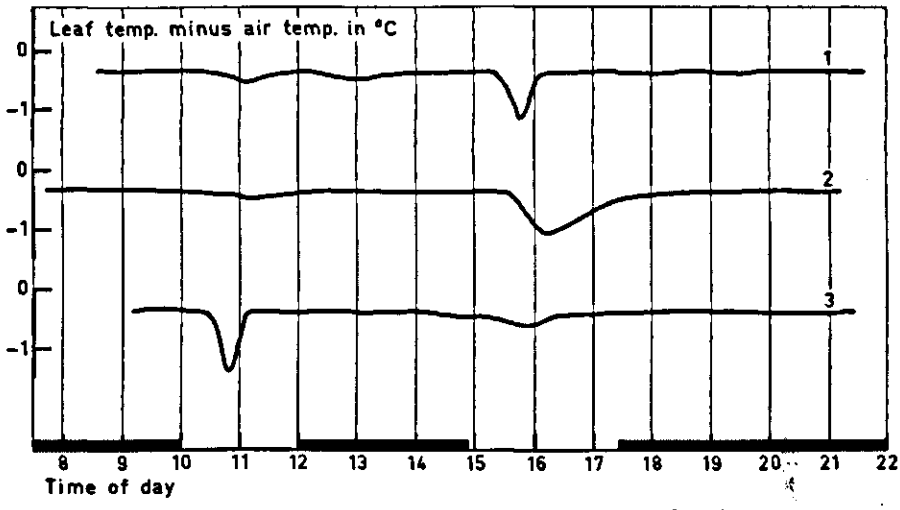

FIG. 32. Some examples of the influence of 2 periods of 150 erg $cm^{-2}sec^{-1}$ on the upper and 50 erg $cm^{-2}sec^{-1}$ on the lower side of the leaves on stomatal opening during the time of the day that normally the light period with 35,000 erg $cm^{-2}sec^{-1}$ was given (600 to 2300). Curve 1: 18 °C/5.7 mm Hg/–/15–20 cm sec^{-1}. Curves 2 and 3: 24 °C/ 11 mm Hg/ –/15–20 cm sec^{-1}.

TABLE 5. Numbers of plants per type of reaction of stomatal opening on irradiation with 150 erg $cm^{-2}sec^{-1}$ on the upper and 50 erg $cm^{-2}sec^{-1}$ on the lower side of the leaf during 2 hours, beginning at 1000 and at 1500. 22–24 °C/9–12 mm Hg/10–30 cm sec^{-1}.

	time		totals
	1000–1200	1500–1700	
number of plants	24	29	53
opening at 1500	4	12	16
opening at 1000	5	9	14
sum of opening by light	9	21	30
no reaction	6	7	13
opening indistinguishable from daily rhythm	9	1	10

mically in the dark as a consequence of the daily rhythm, so that an effect of weak light could not be distinguished. This suggests that a large part of the turgor increase of the stomata was due to the endogenous daily rhythm, even so, the stomata clearly reacted on the light.

6.4.5. *Influence of low light intensity on entrained cycling*

In section 4.5.3 (p. 35) it has been described how cycling with short free-running periods was able to entrain the cycling in older leaves from larger free-running periods to shorter ones by the water potential.

Entrainment was studied in the case, when a decreased light intensity brought the free-running period of the entrained cycling further away from the period of the forcing cycling. During self-sustained cycling one of the 2 primary leaves was kept at a constant irradiation intensity (figure 33, curve 1), whereas irra-

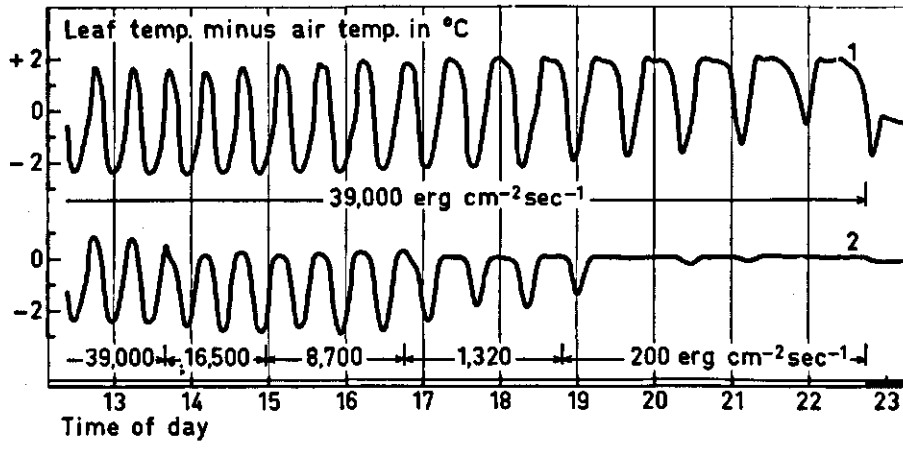

FIG. 33. Stomatal behaviour of 2 primary leaves on one plant. One leaf (curve 1) was irradiated with constant intensity, whereas the intensities on the other leaf (curve 2) were gradually decreased as indicated. 27 °C/13.5 mm Hg/see figure/ 10–15 cm sec^{-1}.

diation on the twin leaf was stepwise decreased from the original 39,000 erg $cm^{-2}sec^{-1}$ to 200 erg $cm^{-2}sec^{-1}$ on the upper leaf surface (figure 33, curve 2). The period of the leaf during constant irradiation was increased due to the daily rhythm as dealt with in 5.4 (p. 46). The period of the other leaf remained equal to that of the fully irradiated leaf, in spite of the fact that the free-running period may be assumed to have increased due to the decreased light. The amplitude, however, decreased. A whole cycle was sometimes omitted at 200 erg $cm^{-2}sec^{-1}$, hence the period was doubled, together with a strong further decrease of the amplitude.

The time lag between the onset of stomatal opening in the primary leaf at 39,000 erg cm^{-2} sec^{-1} and the onset in the other primary leaf at low light intensity was measured. The lag time, averaged over 4 periods for 2 plants, was 4.1 min at 1,320 erg $cm^{-2}sec^{-1}$ and 8.3 min at 200 erg $cm^{-2}sec^{-1}$, indicating that the lower the light intensity, the larger the delay of the onset of opening of the shaded leaf compared with the onset of opening of the fully irradiated leaf.

The response of the shaded leaf is interpreted as follows. As discussed in section 4.3.2 (p. 23) and section 4.5.3 (p. 35) the cycling water potential of the leaves lagged behind stomatal cycling, and changes in the water potential were rapidly propagated from one leaf to the other. A decrease of the water potential in the fully irradiated leaf, when propagated to the shaded leaf, decreased the turgor pressure in the epidermal cells of that leaf, and thus reduced the level of the turgor pressure of the guard cells, necessary to open the stomata. The lower the light intensity on the shaded leaf, the lower the turgor pressure in its guard cells and therefore the more the water potential in the leaf had to fall before the stomata could be opened. This explains why at decreasing irradiation the onset of stomatal opening lagged gradually more behind the onset of stomatal opening of the fully irradiated leaf. The turgor of the guard cells increased so slowly at 200 erg $cm^{-2}sec^{-1}$ that the minimal pressure exerted by the surrounding cells was sometimes not exceeded and the cycle was doubled.

The sub-periods with minimal stomatal openings increased with decreasing light intensity due to an increase of the time required for the guard cells to overcome the pressure from the surrounding epidermis cells.

6.4.6. *Discussion*

The influence of low light intensity on the period and more specifically on the sub-period of minimal stomatal opening suggests that the guard cells accumulate the effect of a small impulse over a long time, building up the turgor pressure gradually to a level where the stomata open. This build-up period is identical to the 'Spannungsphase' of STÅLFELT (1927, 1956), who claimed that a product rule could be applied to this build-up period: $k = (i+x)(t+y)$, in which i is light intensity, t the duration of irradiation required for the onset of the opening movement, and k, x and y are constants. STÅLFELT's experimental data did not conform to this rule, but he ascribed this to the variable initial condition of the leaves in his experiments. The effect of the low light intensity on the sub-periods with minimal stomatal apertures or on the

'Spannungsphase' obviously conformed an approximately similar rule. For 2 reasons the product rule is believed to be manifest in the present stomatal behaviour contrary to STÅLFELT's observations: (a) the same leaves returned to and started each cycle at the same situation of water balance and stomatal position, so an identical initial situation was reached at each cycle; (b) the build-up period ('Spannungsphase') proved to be only sensitive to unusually low light intensities.

6.5. CARBON DIOXIDE CONCENTRATION

6.5.1. *Literature*

Many investigators found that the stomatal opening in light was larger, the lower the CO_2-concentration of the air (FREUDENBERGER, 1940; HEATH and RUSSELL 1954; GAASTRA, 1959; KUIPER, 1961; PALLAS, 1965; HARRIS, 1968). According to MEIDNER and MANSFIELD (1968) the stomata of most plants appeared to be insensitive to reductions in CO_2-concentration below the 100 ppm level.

In darkness stomata were generally found to be closed at 300 ppm. Stomata were open at low concentrations, provided however, that they had not closed prior to the decrease in CO_2-concentration (LLOYD, 1908; MEIDNER and MANSFIELD, 1968).

Stomata were reported to open at extremely high CO_2-concentrations in light (PALLAS, 1965) and in darkness (LOUGUET, 1965). The rates of response of stomatal opening to changes of the CO_2-concentration and to changes of the light intensity were of the same order of magnitude (GAASTRA, 1959; KUIPER, 1961). Stomata of *Xanthium pennsylvanicum* immediately closed when the CO_2-concentration was increased from 400 ppm to 1050 ppm for 5 minutes (MANSFIELD, 1965a). In CO_2-free air a transfer from light to darkness induced a decrease in stomatal aperture initiall as rapid as in 300 ppm, but gradually slowing down (KUIPER, 1961; MEIDNER and MANSFIELD, 1968). A transfer from darkness to light in CO_2-free air increased stomatal aperture (KUIPER, 1961), as did an increase of the light intensity (HEATH and RUSSELL, 1954).

APEL (1967) concluded that CO_2-concentrations from 200 to 500 ppm did not affect sustained stomatal cycling in young seedlings of barley in light.

6.5.2. *General influence*

My experiments showed that a rapid increase of the CO_2-concentration from 300 to 1000 ppm after some minutes induced a rapid decrease of stomatal opening in light. When stomatal opening was cycling, however, cycling was not altered significantly by a similar increase of the CO_2-concentration. Cycling stopped with small stomatal opening only when still higher concentrations were applied. A rapid decrease of the CO_2-concentration from 300 to 0 ppm caused an increase of stomatal opening, when initially the stomatal opening was constant.

When in the course of cycling the CO_2-concentration was decreased, cycling

damped in some exceptional cases with approximately doubled periods; in most cases, however, cycling continued.

In CO_2-free air in light as well as in darkness the CO_2-output ranged from plant to plant from 4 to 20 μl $cm^{-2}h^{-1}$. During stomatal cycling in CO_2-free air the rate of CO_2-output did not cycle measurably.

6.5.3. *Influence on the period of sustained cycling*

The effect of decreasing CO_2 on sustained cycling is presented in figure 34. The increase in the period was due to the extension of the sub-period with minimal opening. The amplitude was not affected. The period also increased to a relatively small extent in time by the daily rhythm. This effect, however, was always much smaller than the increase of the period upon a change of the CO_2-concentration.

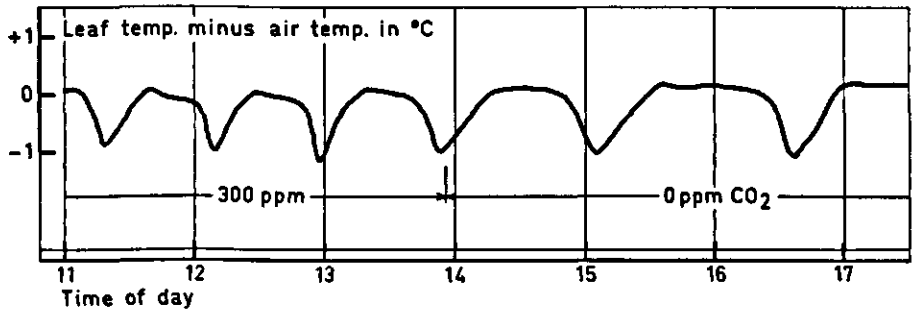

FIG. 34. Influence of decreasing the carbon dioxide concentration of the air from 300 to 0 ppm on stomatal cycling. 27.2–27.4 °C/10.0–10.4 mm Hg/70,000 erg $cm^{-2}sec^{-1}$/50 cm sec^{-1}.

In figure 35 the influence of variation of the CO_2-concentration from 300 to 200 and 0 ppm during sustained cycling on the period is presented. The periods were smaller at 300 ppm than at 200 ppm and smaller at 200 ppm than at 0 ppm. Opposite and therefore consistent results were obtained, when the CO_2-concentration was increased from 0 to 300 ppm.

6.5.4. *Discussion*

In literature stomatal opening has been reported to be stimulated by decreasing CO_2-concentration and the present results seem to contradict these earlier findings. My experimental results, however, supply the new information, that the build-up period ('Spannungsphase') is increased by decreasing CO_2-concentration. Therefore it is not justified to accept without reservations the effect of decreasing CO_2-concentration to stimulate stomatal opening.

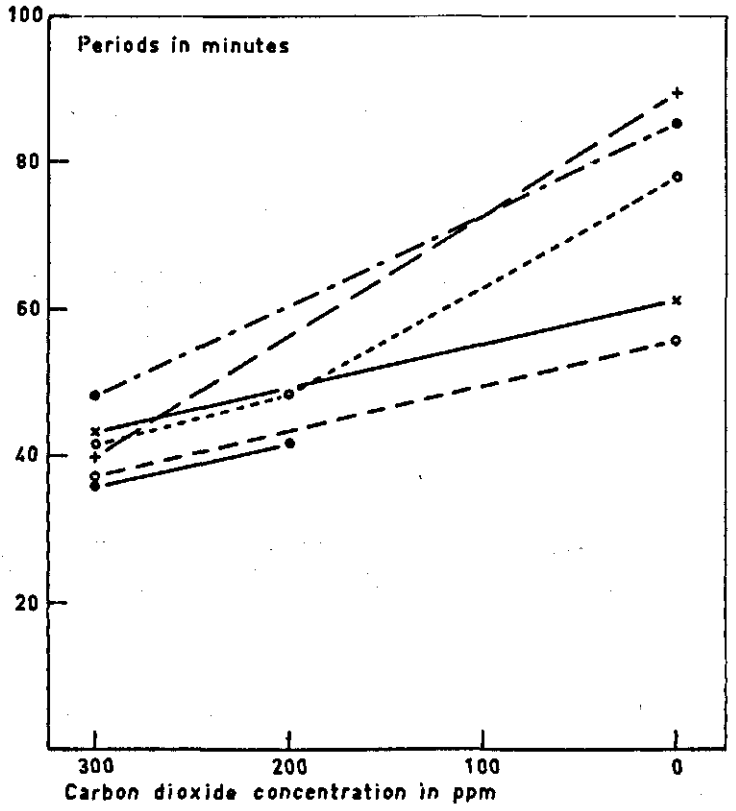

FIG. 35. Influence of carbon dioxide concentration on the periods of stomatal cycling of 6 plants, represented by 6 different symbols. Environmental factors constant during each experiment with one plant (equal symbols), but different for different plants: 23–27 °C/6–10 mm Hg/33,000–90,000 erg $cm^{-2}sec^{-1}$/60–100 cm sec^{-1}.

6.6. TEMPERATURE

6.6.1. *Literature*

Reports on the effect of temperature on stomatal opening have been contradictory. Several authors found no significant effect of temperature. It has been demonstrated that the reaction of the stomatal mechanism to temperature changes is obscured by accompanying active and passive reactions of the stomata to changes in the water balance of the leaf (STÅLFELT, 1962), and reactions of the stomata to changes in the internal CO_2-concentration, called forth by this temperature change (MEIDNER and MANSFIELD, 1968). The effect of temperature may be distinguished in effects on the rate of opening and on the final steady opening. MEIDNER and HEATH (1959) found a Q_{10}-value of 2.2 for the rate of stomatal opening in onion. When the accumulation of CO_2 in

the leaf was prevented by sweeping the leaf cavity with normal air, the rate of opening and the final opening increased with increasing temperature. STÅLFELT (1962) found in *Vicia faba* leaves, well supplied with water, that the degreeof stomatal opening was practically proportional to temperature in light as well as in CO_2-free air in darkness. HOFSTRA and HESKETH (1969) concluded that the temperature response of the stomata of several species appeared to be correlated with the response of photosynthesis to temperature. RASCHKE (1970b) found that between 15° and 35°C the stomatal conductance in maize leaves was proportional to the net rate of photosynthesis. He concluded that this was an indication that the stomatal opening was controlled by the CO_2-concentration. Above 35°C stomatal opening in maize increased with temperature, irrespective of the high internal CO_2-concentration. The increase of stomatal opening at very high temperatures was also found by EL-SHARKAWY and HESKETH (1964), while net photosynthesis had fallen to zero. A temperature increase from 27°C to 36°C without removing CO_2 from the leaf induced stomatal opening in darkness in *Xanthium pennsylvanicum* (MANSFIELD, 1965b). This reaction only occurred during the phase of the diurnal rhythm, at which the stomata tended to open. In this experiment the stomata still responded to CO_2-concentration. DARWIN (1898) and MANSFIELD (1965b) reported that high temperature delayed the rate of nocturnal closure.

6.6.2. *In light*

The leaf temperature in light was varied by increasing the air temperature from 17°C to 29°C in one experiment and from 15.5°C to 31.3°C in another in 5 intermediate steps. The vapour pressure, which was not controlled, increased from 8.4 mm Hg at the minimum temperature to 10.7 mm Hg at the maximum temperature. The vapour pressure gradient between the leaf interior and the external air, determined from leaf temperature and air humidity measurements, increased from 6.5 to 18.3 mm Hg. During the temperature transitions light was switched off for half an hour. At each new temperature level the stomata were induced to cycling by the onset of light. This was necessary because cycling in several plants was interrupted and stopped by the rapid air temperature increase. The mean period of cycling was determined over the whole time of 2–4 hours, during which the temperature period lasted. The temperatures 17°C up to 22.5°C were given from 1200 to 2300 on one day and 25°C up to 29°C from 1100 to 1800 on the next day. In the data presented no correction has been made for the effect of the daily rhythm, which especially from 1900 to 2300 maximally increased the periods. In figure 36 the mean period per plant as affected by temperature is presented.

From 17°C to 20°C the period decreased strongly and a temperature coefficient (Q_{10}) of 3 to 4 was measured. As temperature increased further, the Q_{10}-value in general decreased. Figure 37 indicates how sub-periods with minimal stomatal openings and sub-periods with open stomata during the rest of the period were influenced by temperature.

Sub-periods with closed stomata were more sensitive to temperature than

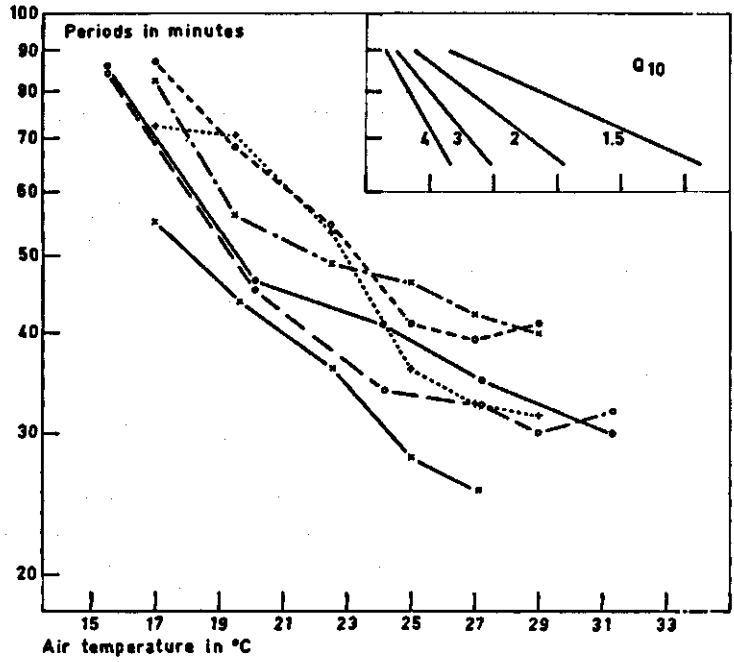

FIG. 36. Influence of air temperature on the periods (ordinate in log scale) of stomatal cycling of 6 plants, designated by different symbols. In the insertion the steepnesses of the lines indicate Q_{10}-values, applicable to the curves of the present figure. Air vapour pressure increased from 8.4 to 10.7 with increasing temperature. Short wave radiation 35,000 erg $cm^{-2}sec^{-1}$. Wind speed 10–30 cm sec^{-1}.

sub-periods with open stomata. The irregularity in the closed stomata curve at 22.5 °C is ascribed to the daily rhythm, because its influence is mainly apparent during the sub-periods with closed stomata (chapter 5, p. 47).

The first stage of the opening movement and the second half of the closing movement were decreased by increasing temperature during the sub-period with open stomata. The time of maximal opening and the first half of the closing movement were only influenced to a small degree.

Obviously, the difference from peak to trough in leaf temperature during cycling was greatly affected by the combined increase of air temperature and vapour pressure deficit. The mean difference amounted from 0.9 °C in 17 °C to 2.5 °C in 25 °C. The peak-to-trough differences in leaf temperature were divided by the mean vapour pressure difference between leaf and air during the sub-periods with open stomata. These data indicated that from 17° to 22.5 °C the amplitudes in stomatal opening had increased, but no further increase was observed at higher temperatures.

According to section 4.4.1 (p. 25), no effect of vapour pressure on the period was found, but only an effect on the amplitudes of stomatal opening. This

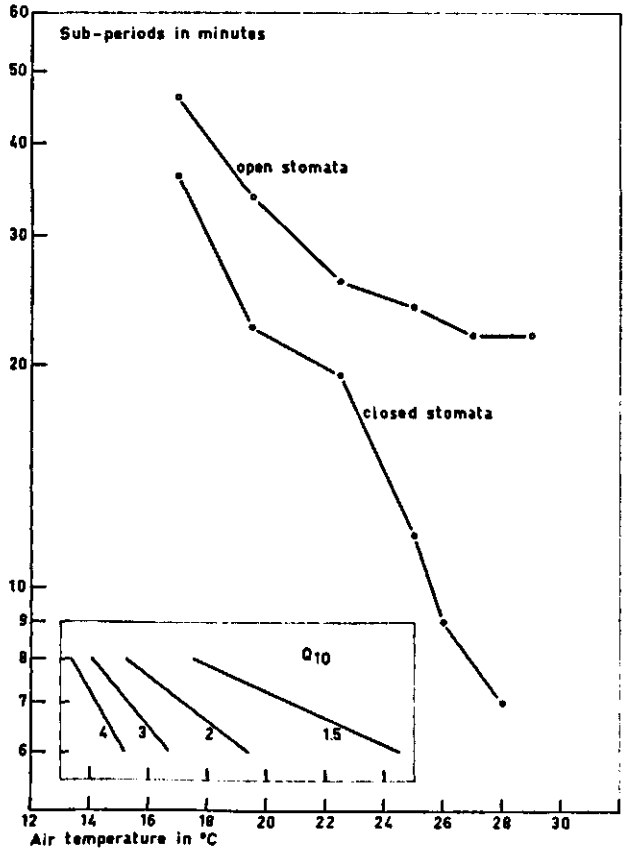

FIG. 37. Influence of air temperature on the sub-periods with open and closed stomata, presented as the mean of 4 equally treated plants for which figure 36 illustrates the total period-temperature relation. In the insertion the steepnesses of the lines indicate Q_{10}-values, applicable to the curves of the present figure. Air vapour pressure increased from 8.4 to 10.7 mm Hg with increasing temperature. Short wave radiation 35,000 erg $cm^{-2}sec^{-1}$. Wind speed 10–30 cm sec^{-1}.

suggests that only temperature and not vapour pressure deficit had affected the periods.

In section 4.5.4 (p. 36) the effect of an increase of the root temperature on the intensity of cycling was described. Apart from a temporary shock effect of a temperature decrease, the amplitudes and not the periods appeared to be affected by changing the root temperature. The influence of temperature on cycling in isolated leaves was studied to exclude possible changes in the water balance of the leaf, caused by a changed root resistance. The averaged period of 3 leaves in 35,000 erg $cm^{-2}sec^{-1}$ at 17.5°C and 26.0°C were 83.4 and 45.6 min respectively at air vapour pressures of 8 and 9 mm Hg respectively. Hence, an increase of temperature also strongly decreased the period in isolated leaves. The sensitivity

of the root resistance did not seem to play an important role in the reaction of the period to temperature.

The period was equally affected by temperature in normal air as in CO_2-free air in light when the temperature was raised from 22° to 29°C. No increase in the rate of CO_2-output could be detected during cycling in CO_2-free air as a response to this increase of temperature.

In conclusion, the period of stomatal cycling and specially the sub-period with minimal stomatal opening was strongly affected by temperature in the light. The water balance of the leaf did not appear to be primarily involved.

6.6.3. *High temperature in darkness*

When the light was turned off at a high leaf temperature, the stomata started closing, which movement soon reversed into an opening movement and was followed by cycling (figure 38). Attention is drawn to the relation between the course of the transpiration rate and of the CO_2-exchange rate in figure 38.

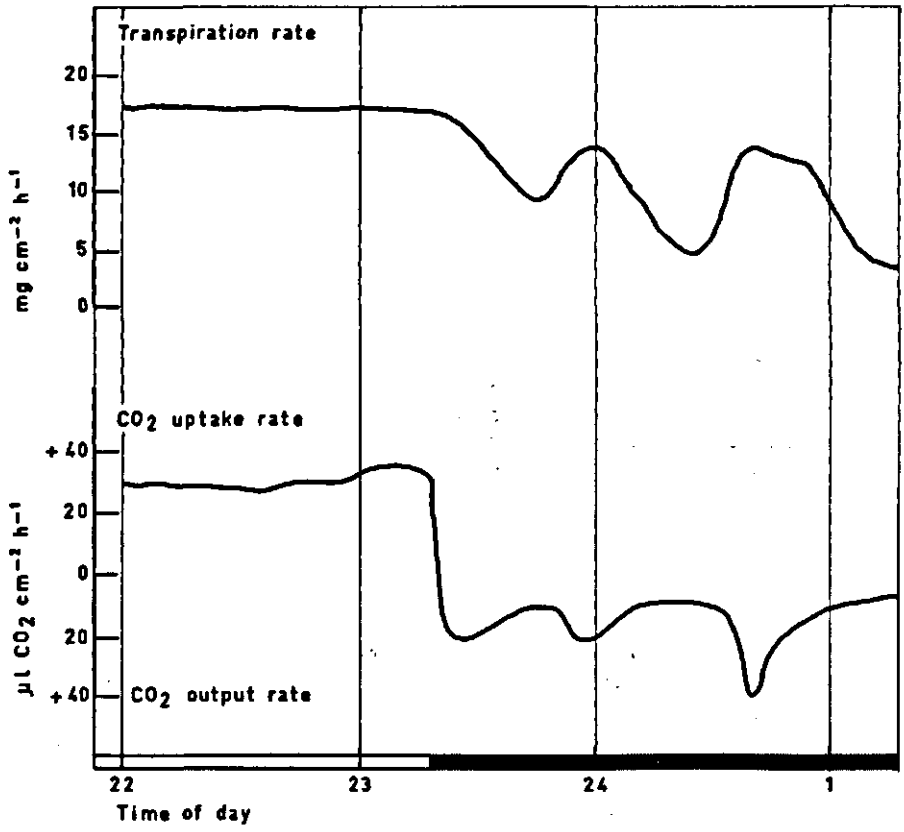

FIG. 38. Transpiration rate and carbon dioxide uptake and output rate in high air temperature in light and darkness, presenting stomatal cycling in high temperature in darkness. Leaf temperature 34-35°C.35.2-36.5°C/12-16 mm Hg/100,000 erg cm^{-2}sec^{-1}/50-60 cm sec^{-1}.

The rate of CO_2-output dropped in advance of a decrease in transpiration rate, especially during the last period in this figure. Hence, the rate of CO_2-output was controlled by another factor besides the stomatal diffusion resistance. The maximum rate of CO_2-output in 35° was 80 μl $cm^{-2}h^{-1}$.

Another experiment showed the sensitivity of the stomatal opening to increased temperature in darkness. The upper side of the leaf was irradiated with long wave infrared radiation, as described in section 6.2 (p. 52). During the daytime of the circadian rhythm at about 21 °C, leaf temperature was increased from 21 °C to 24 °C by the irradiation panel. Stomatal opening cycled in most plants with a mean period of 82 minutes and a mean peak-to-trough difference of 1.5 °C only during the time with increased leaf temperature. Stomatal cycling stopped and leaf temperature returned to the steady level of 0.1 to 0.2 °C below the air temperature, as soon as the heat source was turned off.

According to figure 38 it is supposed that CO_2-concentration in the substomatal cavities was very high when stomata opened. The enhanced temperature has stimulated the increase of turgor of the guard cells and it may have decreased the turgor of the adjacent epidermal cells by increasing the cuticular transpiration. The degree of turgor of the guard cells, required to open the stomata, might therefore have been decreased by temperature. It has been concluded, however, from the analysis of the water relations of cycling that especially those cells causing passive stomatal movements showed a low resistance contact with the roots. In figure 38 the reversal of the stomatal closing movement into an opening movement occurred at a time that the root resistance to water transport may be assumed not to have decreased yet by a lasting low water transport through the roots (p. 34). It is concluded therefore, that cycling at increased temperature in darkness was caused by enhancement of the passive as well as the active stomatal opening component.

6.6.4. *Discussion*

Apart from the influence on the water balance of the leaf, increasing leaf temperature decreased the sub-period with minimal stomatal opening, hence, the build-up period ('Spannungsphase'). Because increasing the CO_2-concentration in the light decreased these periods, the separate effects of CO_2 and temperature cannot be distinguished for several temperature light intensity combinations. From 17° to approximately 25 °C in light, however, net photosynthesis was stimulated by increasing the temperature, whereas at higher temperatures it was decreased. It is concluded that temperature exerts an influence independent of CO_2-concentration, because the effect of temperature increase on the build-up period was the same when CO_2-concentration increased or decreased.

Literature reports on the effect of temperature on stomatal opening (mentioned in 6.6.1 p. 64) cannot be directly related to the effects described in the present report, because the effect of temperature on the build-up period was not investigated earlier. The Q_{10}-values for the build-up period during cycling of 3 to 4 are much larger than the Q_{10}-values observed for stomatal opening of 2.2 (MEIDNER and HEATH, 1959), 1.5 (STÅLFELT, 1962) and 1.8 (RASCHKE, 1970b).

6.7. Discussion on the guard cell mechanism

The effects of environmental factors on stomata are so clearly demonstrated by the unstable stomata, because the onset of an opening movement is followed by synchronous overshoots. Mainly the sub-period with minimal stomatal opening was affected. This sub-period was called build-up period and according to Stålfelt (1956) 'Spannungsphase' (see p. 61). The length of the build-up period is determined by the time required for the guard cells to increase their turgor enough to open the pore. An external factor may influence the length of the build-up period by changing the turgor of the guard cells, but also by changing the turgor of the surrounding cells. The environmental factor may directly affect the osmotic pressure of the guard cells and/or that of the surrounding cells. The osmotic pressure in the guard cells may increase due to the production of osmotically active material inside them. Several authors admitted that starch-sugar conversion might contribute to variation of the turgor of the guard cells, but that it acted more or less as a stabilizer in addition to other mechanisms (Stålfelt, 1956; Fujino, 1967; Fischer, 1968a, b; Meidner and Mansfield, 1968). Solutes may be absorbed from the surrounding cells and water may follow passively. Evidence is rapidly growing that the osmotic pressure of the guard cells is enhanced by potassium ions being actively transported into the guard cells in amounts sufficient to raise the turgor so much that the stomata open (Imamura, 1943; Fujino, 1967; Fischer, 1968a, b; Fischer and Hsiao, 1968; Humble and Hsiao, 1969; Sawhney and Zelitch, 1969; Willmer and Mansfield, 1970). In the latter case the turgor of the guard cells might increase at the expense of the adjacent cells and accelerate the onset of stomatal opening.

Light appeared to limit the length of the sub-period with minimal stomatal opening at irradiation intensities lower than 2000 erg $cm^{-2}sec^{-1}$ on the lower side of the leaf. At the intensities in the range from 150 to 2000 erg $cm^{-2}sec^{-1}$ the CO_2-content of the stomata is not supposed to be affected by photosynthesis of the guard cells or the mesophyll. Light is reported to stimulate active potassium uptake by corn leaf tissue at intensities below 1100 lux (Rains, 1968). Apart from the potassium pump theory evidence has been accumulating that ATP, produced in the light by photophosphorylation of the guard cell chloroplasts, provides the energy needed for an increase of turgor (Zelitch, 1969; Kuiper, 1961, 1964; Fujino, 1967). The experimental results of Fujino (1967) suggested, that ATP is involved in the active uptake of potassium into guard cells in epidermal strips of *Commelina communis*, both in light and in darkness. Rains (1968) presented evidence that the transport mechanism for absorption of potassium is the same in the light as in the dak, the energy for uptake in the light coming partly from ATP produced by photophosphorylation and in darkness from oxidative phosphorylation.

My data on the sensitivity of stomatal opening to weak light in combination with these literature reports on the sensitivity to weak light of ion pumps, requiring ATP, support the hypothesis that weak light may increase the turgor

pressure of the guard cells by increasing ATP requiring active uptake of potassium ions.

The conclusion of RAINS (1968) that ATP from respiration would supply the energy for the potassium pump in corn leaves would enable such a pump to increase the turgor of the guard cells with respiratory energy, as proposed by FUJINO (1967).

The opening in darkness and the high Q_{10}-value in light and darkness of the build-up period of stomatal opening as presented, support the view of FUJINO (1967) that stomatal opening is largely dependent on ATP produced by respiration. A possibly important role of respiration has also been stressed by some of the investigators who noted numerous mitochondria in guard cells (PALLAS, 1966; THOMSON and DE JOURNETT, 1970).

The decrease of the build-up period with an increase of temperature was accompanied by decreased temperature coefficients. Several factors might have been involved. The permeability of the guard cell membranes to water might have been limiting in a part of or in the total temperature range investigated. In both cases the Q_{10}-value may change with temperature according to WARTIOVAARA (1956). Another possibility might be that the Q_{10}-value of the net result of active ion absorption and passive ion loss by the guard cells changed with temperature (WARTIOVAARA, 1956).

7. CONCLUSIVE REMARKS

Which factors determined the instability of the stomatal system?

It appeared that several factors played a part in causing instability of the stomatal apparatus:

a. Resistance to water transport in the water transport pathway. In intact plants the most important resistance was located in the roots.
b. Delay in effective adaptation of the turgor of the guard cells to changes of a factor they control.
c. Susceptibility of the stomata of passive movements.
d. Rapid propagation of water potential in the pathway from root to the epidermis and between different leaves.
e. Position and shape of the leaves such that environmental conditions, especially light, were uniform for the whole leaf area of one plant.
f. The level of stimulation to stomatal opening by e.g. light intensity, circadian rhythm.

When a large vapour pressure gradient existed between leaf and air and when the water potential in the root medium was high, the factors a, b and c caused and stimulated overshooting of the opening movement, whereas, the factors d and e caused synchronization over the whole plant. Increase of the level of stimulation of the opening movement (f) either increased or decreased instability.

Which part(s) of the circuit of the stomatal control system causes the delay in the adaptation of the turgor of the guard cells in response to changes of controlled factor(s)?

It is difficult to answer this obvious question satisfactorily as long as the action mechanism of the guard cells is unrevealed (MEIDNER and MANSFIELD, 1968; ZELITCH, 1969). The main controlled factor involved in the present cycling seems to be the water status of the plant as described in 4.6 (p. 39). The delay in the feedback circuit for the control of the plant water status may be caused by: (a) a delav of the informational water potential in entering the guard cell from the surrounding epidermal cells; (b) a delay in one or more of the steps of the active change of turgor of the guard cells in response to the information.

Which environmental conditions favour stomatal cycling?

Intact plants with flat leaves on petioles, the laminae not overlapping each other at short distances, may be expected to be suscetible of synchronized stomatal cycling in dry air. Cotton, *Helianthus* and *Phaseolus vulgaris* plants, of which the present type of cycling is most frequently described, conform to these features. Cycling could also be induced in *Xanthium* and *Vicia faba*. When the stomatal system is unstable, small rapid changes in the environment as in

wind and irradiation induce cycling, but also disturb it. A specially favourable situation for induction to cycling proved to be irrigation of the plants after a period of moderate water stress. It seems probable that stomatal cycling occurs in the field and in the glasshouse at low air humidity in the type of plants indicated above, standing isolated or in a crop without mutual shading between plants. Uniform plants may be expected to have approximately similar periods. The phases of cycling in different plants at short distance will be the same only in the course of a few periods after a common abrupt induction. In climate rooms the abrupt onset of irradiation induces optimally to cycling. When the above described conditions are fulfilled (as in the present study) in climate rooms stomatal cycling may not be rare.

What is the effect of stomatal cycling on long term plant behaviour?

It has to be borne in mind that the present stomatal cycling is a failure of the stomatal control system.

In case the amplitudes of stomatal cycling are large, cycling probably decreases mean net rate of photosynthesis. During the sub-periods with minimal stomatal opening the rate of CO_2 assimilation by the chloroplasts will decrease due to a decreased concentration gradient from chloroplasts to the substomatal cavity. During the sub-period with large stomatal opening, hence low stomatal diffusion resistance, the relative contribution of the boundary layer resistance and the mesophyll resistance for CO_2 transport to the total diffusion resistance increases.

At stomatal cycling in dry air the plant water content may cycle so severely that at each cycle wilting occurs. Not only for the photosynthetic capacity of the protoplast but also for other plant activities short periodic wilting may be disadvantageous. The question in which respect the effect of short periodic water deficiency on physiological activity differs from long term constant stress cannot be answered without further study.

SUMMARY

An analytical study of the cyclic stomatal behaviour in leaves of bean, *Phaseolus vulgaris* L. 'Vroege Wagenaar', was made in order to explore the cycling, to study different aspects of plant water relations and of the action mechanism of the stomata. A general method of investigation was recording of the difference of temperature of leaf and air with thermocouples in a constant environment.

Introductory observations

Plants grown at low light intensity showed a higher tendency to sustained cycling (higher instability of the stomatal apparatus) than those grown at higher light intensity. At the higher light intensity non-aeration of the nutrient solution increased the tendency to cycling with a more than additive effect. It is suggested that the lower capacity to take up water of non-aerated roots was involved in the higher tendency to cycling. Cycling over the whole plant was frequently disturbed by bringing cycling of a part of the active stomata out of phase. Cycling was induced by changing abruptly any factor that affected stomatal opening, by entrainment with cycling air vapour pressure, by entrainment with cycling plant water potential, and apparently spontaneously.

Younger leaves showed shorter free-running periods than older ones. When both were attached on the same plant, cycling in the younger leaves entrained cycling in the older ones.

Water relations of cycling

Amplitudes of cycling increased by decreasing air humidity and by increasing the water potential in the root medium. A time delay in the adjustment of the turgor of the guard cells to the plant water potential is assigned to be the cause of overshooting of the active stomatal movement and as to be the factor amplifying both passive opening and closing movement. Cycling only occurred when an important resistance to water uptake was present in the transpiration stream. In intact plants this resistance was located in the roots. The water potential cycled in the whole plant down to this resistance, synchronizing cycling in the whole plant. During each cycle the root resistance increased during the sub-period with low rates of water uptake and decreased delayed at subsequently decreasing plant water potential. Due to the fact that the fall of root resistance was enhanced by increasing root temperature, both cycling of stomatal opening and cycling of root resistance were intensified.

A diagrammatic conceptual model is presented, showing the pathway of the transpiration stream, of the transfer of water within the leaf, and of the stomatal control system of the water balance of the leaf. A negative feedback control circuit in it indicates how active stomatal movements function, while a positive feedback circuit shows how passive stomatal movements function during cycling.

Circadian rhythm in steady and cycling stomatal openings

In continuous light a circadian rhythm in non-cycling stomatal opening was found, which phase could be caused to shift. Cycling was most intense at the time that in similarly treated plants the maximum was attained in the circadian rhythm in the opening in non-cycling stomata and least intense half a circadian period later. In continuous darkness during the first day after the last photoperiod, stomata opened in a cyclic way with a daily pattern in the amplitudes as well as in the number of cycles. During photoperiods of 17 hours the non-cycling stomatal opening in leaves, attached to plants with and without root systems, as well as in isolated leaves followed a daily pattern. During these photoperiods the modulation of amplitudes and periods showed the same daily pattern as during the circadian rhythm in continuous light. During the daily dark periods stomata remained closed. A daily pattern was observed in the onset of stomatal opening after short dark periods in the course of the daily photoperiod. The data suggest that one endogenous circadian oscillator, situated in the leaves, caused the circadian rhythm in non-cycling stomatal opening and modulated stomatal cycling in continuous light, continuous darkness and during the daily photoperiods.

Environmental effects on cycling stomata

The time between the onset of light and the beginning of stomatal opening increased with increasing length of the preceding dark period at equal phases of the circadian rhythm. After 1 hour in the light the effect of the preceding dark period had almost disappeared. After removal of the trifoliates the periods in the primary leaves gradually shortened in the course of some days. It is suggested that this increased activity was caused by a reduced competition for minerals, enzymes and proenzymes from the root.

Cycling was frequently damped by increasing the irradiation intensity. Irradiation intensity beyond 34,000 erg $cm^{-2}sec^{-1}$ affected the rate of CO_2 exchange, but not the period or amplitude of sustained cycling. When the irradiation intensity on the lower surface of the leaf during the diurnal photoperiod was decreased from 2100 to 800 erg $cm^{-2}sec^{-1}$, cycling continued. The amplitude decreased and the period increased, mainly due to the increase of the sub-period with minimal stomatal opening. During the photoperiod 150 erg $cm^{-2}sec^{-1}$ on the upper surface of the leaf induced overshooting stomatal opening. The sub-period with minimal stomatal opening, being similar to STÅLFELT's 'Spannungsphase' (build-up period), increased at decreasing irradiation intensity due to an increase in time required for the guard cells to exceed the counterpressure from the surrounding cells, hence, the product rule of STÅLFELT applied here.

The build-up period of stomatal opening increased by decreasing the CO_2-concentration of the air from 300 to 200 to 0 ppm.

Enhancing the temperature in light decreased the period. This effect was clearly distinguished from the effect of increasing vapour pressure gradient. The Q_{10} of decrease fell gradually from approximately 4 between 17° and

20 °C to approximately 1.5 between 25° and 29 °C. The sub-period with minimal stomatal opening was mainly affected. In light the same effect of temperature on cycling was found in CO_2-free air as in normal air and the same on cycling in detached leaves as in attached leaves. A temperature increase in darkness induced stomatal cycling; this was assigned for the major part to the stimulation of the active opening component. Temperature is concluded to exert an influence independent of the effect of CO_2-concentration and of the water balance.

The effects of light and temperature on cycling support the existing hypothesis for an active ATP using potassium pump as a mechanism for stomatal opening.

Conclusive remarks

Several internal plant factors play a part in causing instability of the stomatal apparatus, but also the habit of the plant. Cycling must be expected to occur in the field and in the glasshouse, especially at low air humidity, when these factors favour instability. Severe stomatal cycling is suggested to decrease mean assimilation rate.

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SAMENVATTING

RITMEN IN DE OPENING VAN DE HUIDMONDJES BIJ DE BOON

Oscillaties van de stomata van bladeren van boon, *Phaseolus vulgaris* L. cv. 'Vroege Wagenaar', zijn onderzocht met de bedoeling waarnemingen te doen aan het oscillatiegedrag, het bestuderen van verschillende aspecten van de waterhuishouding in verband ermee en van het werkingsmechanisme van de huidmondjes. Als algemene methode van waarneming werd in constant milieu het temperatuurverschil tussen blad en lucht gemeten met thermokoppels en geregistreerd.

Inleidende waarnemingen

In lage lichtintensiteit opgekweekte planten vertoonden een sterke neiging tot continue oscillatie (sterkere instabiliteit van het stomata-apparaat) dan in hogere lichtintensiteit opgekweekte planten. Niet aëreren van de voedingsoplossing tijdens de opkweek in hoge lichtintensiteit verhoogde de neiging tot oscillatie meer dan additief. Waarschijnlijk komt de sterke neiging tot oscillatie voort uit het lagere vermogen van niet geaëreerde wortels om water op te nemen. De oscillatie over de hele plant werd dikwijls verstoord door de oscillatie van een deel van de actieve stomata uit de gemeenschappelijke fase te brengen. De oscillatie was te induceren door een factor, die de stomata-opening beïnvloedt, abrupt te wijzigen, door een meesleepeffect van een oscillerende waterdampspanning in de lucht, door een meesleepeffect van een oscillerende waterpotentiaal van de plant, en schijnbaar spontaan.

Jonge bladeren vertoonden kortere eigen perioden dan oudere. Als beide aan dezelfde plant zaten, werd de oscillatie in oudere bladeren meegesleept tot dezelfde periode (entrainment) door de oscillatie in de jongere bladeren.

Waterhuishouding van de oscillatie

De amplituden werden groter door de luchtvochtigheid te verlagen en door de waterpotentiaal van het wortelmedium te verhogen. Een tijdsvertraging in de aanpassing van de turgor van de sluitcellen aan de waterpotentiaal van de plant wordt geacht er de oorzaak van te zijn dat de actieve beweging van de huidmondjes aan zijn doel voorbijschoot (overshoot) en de factor te zijn, die zowel de passieve opening als de passieve sluitbeweging versterkte. De oscillatie trad alleen op als er in de transpiratiestroom een belangrijke weerstand bestond. In intacte planten lag deze in de wortels. De waterpotentiaal oscilleerde in de hele plant tot aan deze weerstand en synchroniseerde daarmee de oscillatie in de hele plant. In de loop van elke oscillatie steeg de wortelweerstand tijdens de subperiode met een lage snelheid van wateropname en daalde vertraagd bij de erop volgende lager wordende waterpotentiaal van de plant. Doordat de afname van de wortelweerstand versterkt werd door de worteltemperatuur te verhogen,

werden hierdoor zowel de oscillatie van de stomata-opening als die van de wortelweerstand geïntensiveerd.

Een schematisch principemodel is opgesteld voor de loop van de transpiratiestroom, voor het watertransport in het blad en voor het stomataire regelsysteem van de waterbalans van het blad. In het model geeft een regelcircuit met negatieve terugkoppeling de werking weer van actieve bewegingen en een regelcircuit met positieve terugkoppeling de werking van passieve bewegingen van de huidmondjes tijdens de oscillatie.

Dagritme in de niet en de wel oscillerende opening van de huidmondjes.

In continu licht bestond een dagritme met perioden niet geheel gelijk aan 24 uur (circadiaan ritme) in niet oscillerende openingen van stomata. De fase van dit ritme kon verschoven worden. De oscillatie was het hevigst gedurende de tijd, dat in identiek behandelde planten het maximum bereikt werd van het circadiane ritme in de opening van niet oscillerende huidmondjes. Een halve dag later was de oscillatie het zwakst. In de loop van de eerste dag in continu donker traden oscillerende stomata-openingen op, waarvan zowel de amplituden als het aantal optredende oscillaties een dagpatroon vertoonden. Tijdens fotoperioden van 17 uur verliep de niet-oscillerende opening van de huidmondjes van bladeren aan planten met en zonder wortel volgens een dagpatroon. Dit dagpatroon bestond uit modulatie van de amplituden en de perioden gelijk aan die tijdens het circadiane ritme in continu licht. Ook kwam een dagpatroon tot uiting in de inzet van de stomata-opening na herhaalde korte donkerperioden in de loop van een dagelijkse fotoperiode. Deze gegevens wijzen erop, dat een en dezelfde endogene circadiane oscillator in het blad het circadiane ritme veroorzaakte in de opening van niet oscillerende huidmondjes en de oscillaties volgens een dagpatroon moduleerde in continu licht, continu donker en tijdens de dagelijkse fotoperioden.

Invloed van milieufactoren op oscillerende huidmondjes

De tijd tussen het aangaan van het licht en het inzetten van de opening van huidmondjes bij dezelfde fase van het dagritme was langer na een langere eraan voorafgaande donkerperiode. Na een uur in licht was de invloed van de lengte van de voorafgaande donkerperiode vrijwel verdwenen.

Na het wegnemen van de drietallige bladeren werden in de loop van enkele dagen de perioden in de primaire bladeren geleidelijk korter. Deze stijging van de activiteit van de huidmondjes werd wellicht veroorzaakt door een vermindering van concurrentie om mineralen, enzymen en pro-enzymen vanuit de wortels.

De oscillatie dempte dikwijls door de stralingsintensiteit te vergroten. Bestralingsintensiteiten hoger dan 34.000 erg $cm^{-2}sec^{-1}$ beïnvloedden de snelheid van CO_2-uitwisseling, maar niet de periode of de amplitude van continue oscillatie. Bij verlaging van de bestralingsintensiteit op het onderoppervlak van het blad tijdens de dagelijkse fotoperioden van 2100 tot 800 erg $cm^{-2}sec^{-1}$ ging de oscillatie verder. De amplituden werden kleiner en de perioden groter.

Dit laatste hoofdzakelijk, omdat de subperiode met minimale stomata-opening langer werd. Een intensiteit van 150 erg $cm^{-2}sec^{-1}$ op het bovenoppervlak van het blad, toegediend tijdens de fotoperiode, induceerde opening met de gebruikelijke overshoot van de stomata. De subperioden met minimale stomata-opening, soortgelijk aan de 'Spannungsphase' van STÅLFELT (opbouwperiode), werden groter bij lager wordende lichtintensiteit als gevolg van verlenging van de tijd, die de sluitcellen nodig hadden om de tegendruk van de omringende cellen te overwinnen. Voor deze opbouwperiode ging dus de z.g. productregel van STÅLFELT op.

De opbouwperiode van de openingsactie van de stomata werd verlengd door de CO_2-concentratie van de lucht van 300 naar 200 en naar 0 dpm te verlagen.

Temperatuurverhoging in licht veroorzaakte verkleining van de periode. De invloed van de temperatuur was in dit opzicht duidelijk te onderscheiden van de invloed van verlaging van de dampspanning van de lucht. De temperatuur-coëfficient van de verkleining van de periode daalde van ongeveer 4 tussen 17° en 20°C tot ongeveer 1,5 tussen 25° en 29°C. Hoofdzakelijk de subperiode met minimale opening van de huidmondjes werd door de temperatuur beïnvloed. In licht had de temperatuur dezelfde invloed op de oscillatie in CO_2-vrije lucht als in normale lucht en dezelfde invloed op de oscillatie in afgesneden bladeren als in bladeren aan de plant. Een temperatuursstijging in donker induceerde oscillatie van de huidmondjes. Dit werd grotendeels toegeschreven aan de stimulering van de actieve openingscomponent. De temperatuur oefent een invloed uit onafhankelijk van de invloed van de CO_2-concentratie en van de waterbalans.

De invloeden van temperatuur en licht op de oscillatie bevestigen de bestaande hypothese, dat een actieve, ATP-energie verbruikende kaliumionenpomp het actiemechanisme is voor de openingsbeweging van de huidmondjes.

Opmerkingen ter conclusie

Verscheidene inwendige factoren zowel als de habitus van de plant beïnvloeden de instabiliteit van het huidmondjesapparaat. Als deze factoren de instabiliteit in de hand werken, is het voorkomen van oscillatie te verwachten in het veld en in kassen bij lage luchtvochtigheid. Het is waarschijnlijk, dat hevige oscillaties de gemiddelde assimilatiesnelheid verlagen.

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