STOMATA: THE PHYSIOLOGY AND BIOCHEMISTRY OF THEIR REGULATION IN LEAVES

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THERE has been a phenomenal expansion of our knowledge on the physiology and biochemistry of stomata in the past decade. We would focus our attention primarily on the work carried out from this laboratory in the light of the recent advancements in this field. The readers are particularly referred to a number of publications that have appeared recently[1-9] to provide an overview of the developments in stomatal functioning.

STIMULATION BY LIGHT - ROLE OF CYCLIC PHOTOPHOSPHORYLATION

The stomatal opening is invariably enhanced on illumination and the conspicuous presence of chloroplasts in guard cells makes light one of the important factors to regulate stomatal movements[10]. Light is not absolutely essential for stomatal opening since the lowering of ambient CO_2 can cause widening of stomata even in the dark. This observation has led Raschke[1] to believe that the wide stomatal opening in light is due only to an indirect effect through modulation of CO_2 levels. However, even in CO_2 free air, illumination accelerates stomatal opening[11,12]. The light-induced enhacement is due to the availability of energy from cyclic photophosphorylation mediated through photosystem (PS) I[13-16]. Evidence for such a conclusion was based on the fact that the stomatal opening in light was sensitive to 2,4-dinitrophenol (DNP) and salicylaldoxime, inhibitors of cyclic photophosphorylation while 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and *o*-phenanthroline, inhibitors of noncyclic electron flow did not cause significant stomatal closure. The sensitivity of stomatal opening to inhibitors of cyclic photophosphorylation is maintained under varied experimental conditions such as low pH, presence of ADP or induction of opening by the removal of bicarbonate[17].

The guard cell chloroplasts are photochemically active and exhibited an active PS I *in vivo* as evidenced by the reduction of nitroblue tetrazolium chloride[17]. The isolated guard cell chloroplasts showed greater activities of PS I mediated NADP reduction and cyclic photophosphorylation than the PS II mediated ferricyanide reduction or noncyclic photophosphorylation. Although PS I could more readily be demonstrated in guard cell chloroplasts[17,18], appreciable activity of PS II in guard cell chloroplasts has been detected recently[19-21]. The photochemical capacities of guard cell chloroplasts are therefore well established although structurally they lack fully developed grana and resemble more like the agranal bundle sheath chloroplasts of C_4 plants[22,23].

Since the stomatal movements do occur in darkness, oxidative phosphorylation appeared to be a source of energy[24]. On illumination, photophosphorylation becomes an additional source and becomes more critical under conditions where respiration is blocked. There may be a common pool of ATP derived from both oxidative—and photophosphorylation in guard cells, which mediates energy dependent H^+/OH^- efflux facilitating their exchange with cations or anions[25]. Guard cell chloroplasts shrink during illumination indicating light dependent ion movements[26]. The epidermal tissues contain remarkable levels of possibly membrane bound ATPase[27-29] which can mediate ATP-dependent H^+ efflux.

The delaying of leaf senescence in light has recently been discovered to be closely related to the maintenance of stomatal aperture[30,31]. The operation of cyclic photophosphorylation in light is presumably responsible for the maintenance of wide stomatal aperture and retarded the senescence process that the illumination did not control senescence through photosynthetic carbon assimilation was clear from the fact that

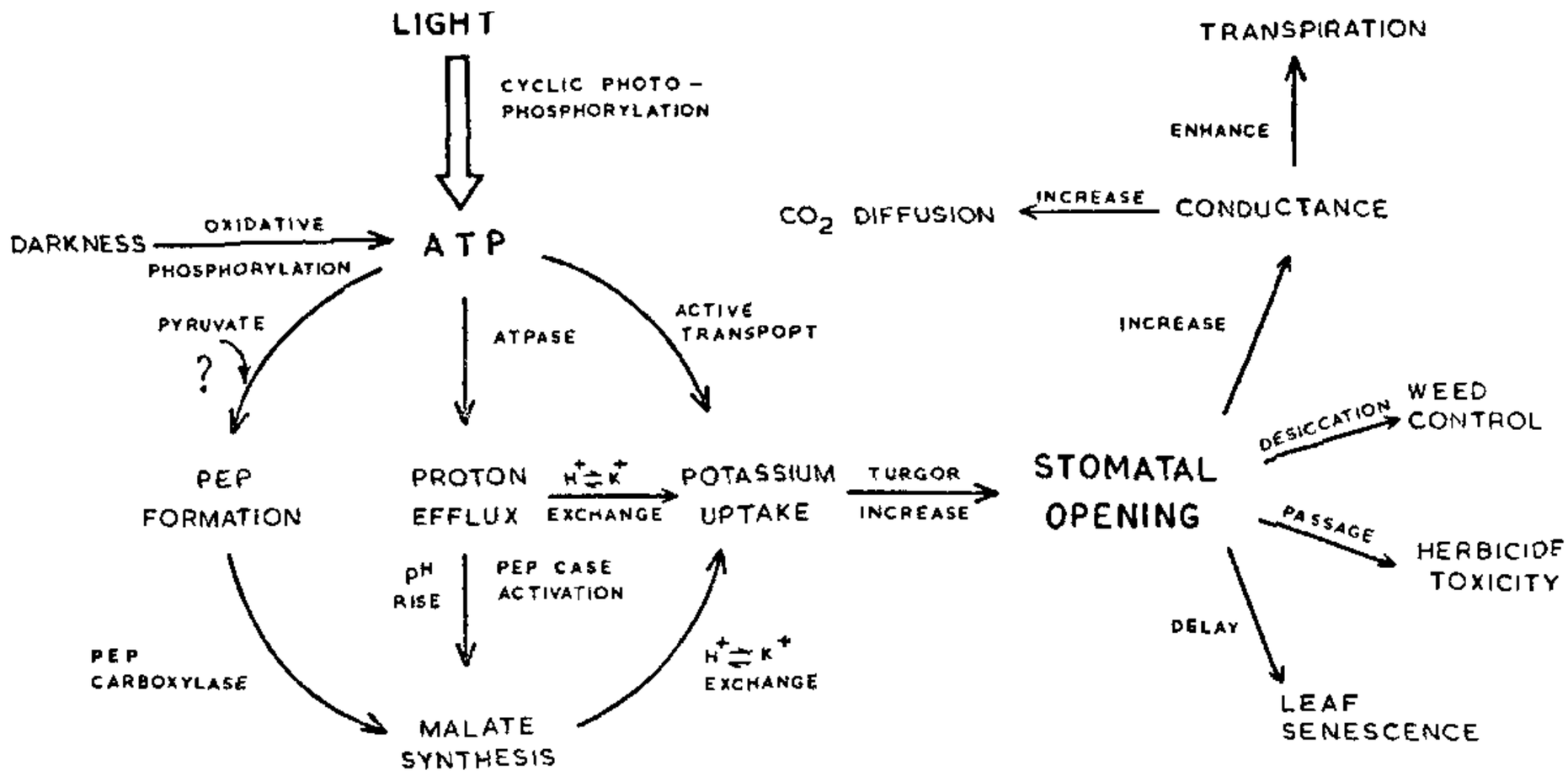

Figure 1. A summary of the contemporary outlook of events leading to stomatal opening and the regulation of other physiological processes by stomatal movements.

DCMU, a classic inhibitor of photosynthesis, did not affect the progress of senescence. Treatments or chemicals which cause stomatal closure accelerated leaf senescence while the compounds which bring out wide stomatal opening delayed senescence. A contemporary understanding of the control of stomatal aperture by light through direct and indirect effects is presented in figure 1.

IONIC RELATIONS

That the monovalent cations stimulate stomatal opening while divalent cations suppress the opening has long been known. The specific role of potassium in stomatal movements was demonstrated independently by Fujino[32] and Fischer[33]. The stimulation by light of stomatal opening also exhibited a specific requirement for potassium ions[34]. The width of stomatal aperture reached an optimal level at low concentration of potassium salts, *e.g.* 10 mM KCl in epidermal strips of *Vicia faba*. If the concentration of cation is increased, say upto 100 mM, other cations such as Na^+, Li^+ or NH_4^+ also supported the stomatal movements.

The movement of potassium into and out of guard cells during stomatal opening or closure is an universal phenomenon occurring in plants ranging from pteridophytes to most advanced higher plants[35,36]. A positive correlation exists between the degree of stomatal opening and potassium content of the guard cells as revealed by histochemical techniques[33,37], flame photometry[38] and electron microprobe analysis[39,40].

Sodium can replace to some extent the cation requirement of stomatal opening but the degree of stomatal opening is usually lower in the presence of sodium than potassium[41,42]. It is therefore concluded that a preferential effect of potassium for the stomatal opening is established.

Anions also participate in stomatal movements. Chloride or nitrate stimulate stomatal opening. An active uptake of chloride into the guard cells occurs in leaf epidermis[43]. Chloride ions can balance 30–50% of potassium in stomata[12,44,45]. Chloride accompanies potassium during influx and efflux into/out of guard cells from/into the subsidiary cells of maize[46].

If inorganic anions are not available, stomata balance all the potassium by organic acids, mainly malate. Malate synthesis in epidermal tissue is remarkably reduced in presence of chloride or nitrate[44,47]. Since malate balances 2 equivalents of potassium while chloride or nitrate ions need only one potassium, not only malate synthesis but also the potassium requirement is reduced in the presence of chloride or nitrate[47].

Sulphate ion is a poor promoter of stomatal opening[34,47]. It therefore appears that only monovalent anions or cations are involved in stomatal movements. There is also a good difference in anionic requirements of starch containing stomata (*e.g. Vicia faba*) and starch lacking ones,

such as of onion (*Allium cepa*). The former synthesises large quantities of malate, as the principal anion to balance potassium entering the guard cells. Stomata of *Allium cepa* cannot derive malate by the breakdown of starch molecules and therefore use chloride to equilibrate potassium[48,49]. They may however use malate as a proton primer.

BIOCHEMISTRY

Apart from previous classic findings, on the presence of chlorophyll in guard cell chloroplasts, their ability of $^{14}CO_2$ fixation and histochemical demonstration of certain enzymes[10], a lot of new information is now available on chracteristics of chloroplasts and biochemistry of carbon metabolism in guard cells.

As already described in this article, the guard cell chloroplasts, not only possess chlorophyll but also are functional photochemically[17-21]. There is only one instance where chlorophyll could not be detected in guard cells although they have responded to light[50]. The involvement of phytochrome in stomatal movements is suggested[51,52] but such mechanism is yet to be convincingly demonstrated. The ratio of chloroplast-to-cell volume is far higher in guard cell than in the other mesophyll cells and makes the guard cell chloroplasts highly efficient organelles, if they choose to produce ATP[53]. Pallas and Dilley[54] calculated that guard cell chloroplats could produce sufficient ATP through photophosphorylation to drive active cation transport.

The ability of guard cells to fix CO_2 is well known[10]. The quantity of carbon fixed by guard cells was too small to generate enough soluble carbohydrates to increase the turgor and initiate stomatal opening. It is now established that the epidermal tissues/guard cells fix carbon primarily into malate and aspartate[53,55,56]. The carbon assimilated initially into organic acids moves later into starch *via* gluconeogenesis[57]. The stomata are unable to produce Calvin cycle intermediates and seem to lack reductive pentose phosphate pathway for carbon fixation[58,59].

The epidermal tissues possess high activities of enzymes involved in β-carboxylation of phosphoenolpyruvate (PEP) leading to malate or aspartate formation. Remarkable activities of PEP carboxylase, NAD-, NADP malate dehydrogenase aspartate- and alanine aminotransferases were demonstrated in epidermal tissues as well as in individual guard cells[60-62]. The PEP carboxylase from epidermal tissues has optimal pH of 8.0; the activity increasing sharply as the pH rises from 7.0 to 8.0; low Km for PEP, is sensitive to malate or oxalacetate and appears to be similar to the PEP carboxylase of C_4 plants[47,63-65]. We have also noted that PEP carboxylase might also be acting as the CO_2 sensor in the guard cells. Low concentrations of externally added bicarbonate stimulated stomatal opening stimulation being enhanced by PEP and suppressed by malate or oxalacetate, the inhibitors of PEP carboxylase[66]. At higher concentrations of bicarbonate, a feed back inhibition of PEP carboxylase in guard cells by malate can occur.

Ribulose 1,5-bisphosphate (RuBP) carboxylase was demonstrated in extracts of leaf epidermal tissues[60,61,67] but guard cells have recently been shown to lack RuBP carboxylase by immunoprecipitation technique[58]. The absence of RuBP carboxylase in guard cells and the inability of epidermal tissues to generate Calvin cycle intermediates suggest the noninvolvement of reductive pentose phosphate pathway in stomatal movements.

The leaf epidermal tissues use malate as the principal organic anion to balance K^+ and to increase the guard cell turgor during stomatal opening. A positive correlation has been established between the width of stomatal aperture and malate content of epidermal strips, individual guard cells and guard cell protoplasts (cf. Ref. 47).

The stomata are able not only to synthesise malate but also metabolise it fast. The epidermal tissues can use externally supplied malate to produce starch through gluconeogenesis[57]. During stomatal closure the malate content of stomata decreases. It is however not clear whether malate is extruded out or decarboxylated by malic enzyme during closure of stomata. High levels of NAD malic enzyme do occur in leaf epidermal

TABLE 1

The biochemical characteristics and enzymic composition of the leaf epidermal tissue compared to the underlying mesophyll tissue of Commelina species

Observation	*Guard cell/ Epidermal tissue*	*Mesophyll Cell/tissue*	*Reference No.*
Morphology[a]			53
Chloroplast number per cell	10	28	
Cell volume occupied by chloroplast (as %)[c]	5.2	3.7	
Photochemical characteristics of chloroplasts[b]			17
Chlorophyll a/b ratio	5.2	3.2	
Ferricyanide reduction[d]	72	347	
Cyclic photophosphorylation[d]	576	426	
Noncyclic photophosphorylation[d]	27	243	
Carbon fixation rates[a]			53
Per chloroplast (pg CO_2 min^{-1})	0.41	0.26	
Per unit volume of chloroplast (fg CO_2 μ m^{-3} min^{-1})	24.7	4.0	
Primary carbon fixation products[a]	Malate and aspartate	PGA and sugar phosphates	53, 55
Enzyme levels[b,d]			61
PEP carboxylase	1125	37	
Pyrophosphatase	2250	168	
Malic enzyme	1152	153	
NAD malate dehydrogenase	1968	658	

[a] *C. cyanea.*
[b] *C. benghalensis.*
[c] when stomata were closed.
[d] μ mol mg^{-1} (chlorophyll) hr^{-1}.

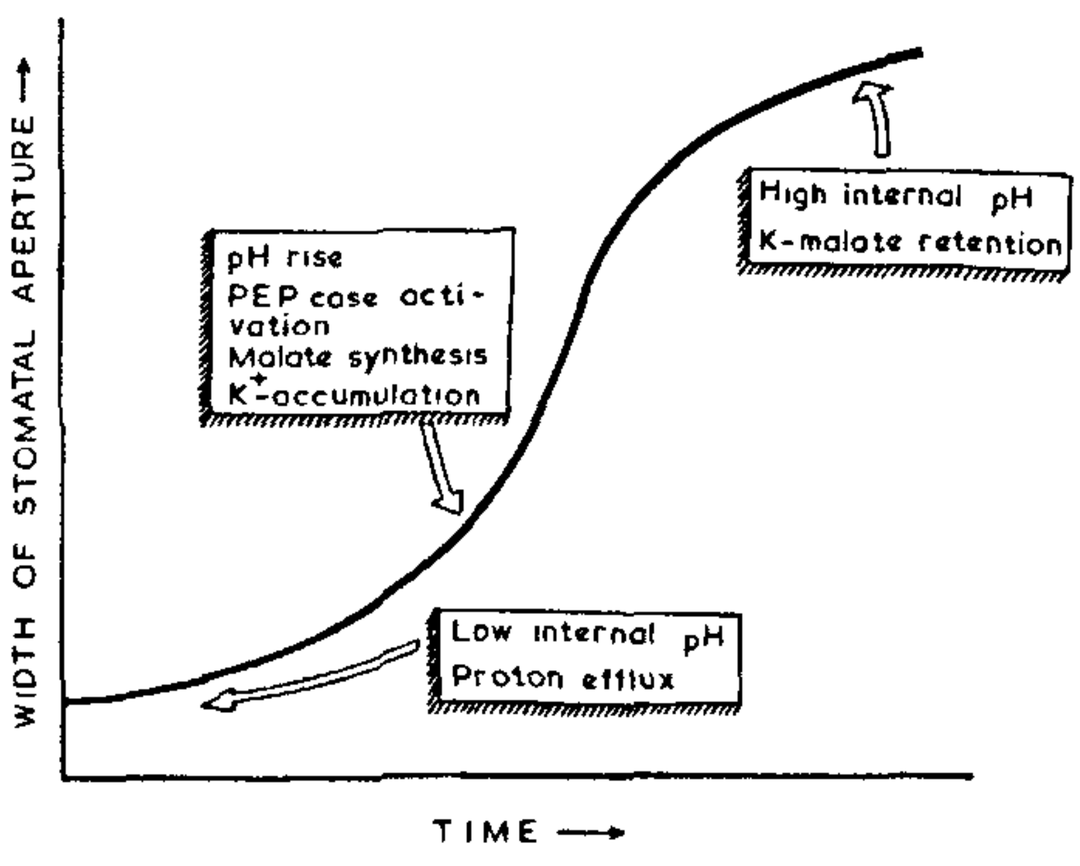

Figure 2. The sequence of events during stomatal opening in epidermal strips. The width of stomatal aperture exhibits by typical sigmoid curve. Proton efflux is the primary event while malate synthesis starts later. The increase in guard cell pH due to proton efflux activates PEP carboxylase resulting in malate production.

tisues[60,61,68]. Malate efflux during stomatal closure is demonstrated in epidermal strips of *Commelina communis*[69].

It is thus evident that the guard cells are highly active metabolically and are geared to carry out selected biochemical events (table 1). Proton efflux is the primary event during stomatal opening[70]. Such proton extrusion can be carried out by membrane bound ATPase already demonstrated in epidermal tissues. Further experiments are needed to reveal how exactly the ATPase is triggered. The proton efflux raises the guard cell pH activating PEP carboxylase which leads to malate synthesis (figure 2). The protons from malate facilitate potassium uptake, increase guard cell turgor and cause stomata to open.

HORMONAL REGULATION

Abscisic acid (ABA), one of the naturally occurring plant growth regulators, directly influences stomatal movements. It inhibits K^+ uptake by guard cells, restricts starch breakdown and suppresses stomatal opening[71–73]. It may also interfere with organic acid metabolism of guard cells but it is atleast certain that ABA restricts the process of stomatal opening rather than the stomatal closure[73]. The response of stomatal opening to the concentration of ABA in the incubation medium is so precise and reproducible that it is being used as a bioassay for ABA[74].

ABA is believed to be involved in the control of plant water balance because, under water stress ABA levels in the leaves increase rapidly resulting in stomatal closure thereby conserving further water loss[75]. The drought resistance of maize cultivars was correlated with their capacity to accumulate ABA under water stress[76]. Although the exact mechanism of action of ABA on stomata is still to be established, ABA is known to restrict ion flux into roots and coleoptiles by decreasing the proton efflux from them[77]. It therefore appears that ABA limits the proton efflux from guard cells and suppresses stomatal opening.

Cytokinins, on the other hand, promote stomatal opening and enhance transpiration[78]. However, the increase in stomatal opening by cytokinins has till now been observed in monocot species[79,80] and the effect of cytokinins on dicot stomata is under question. We observed that benzyl adenine (BA) could stimulate stomatal opening over a narrow range of concentration while kinetin was ineffective[79]. Livne and Vaadia[78] suggested that the balance between cytokinins and ABA in plants could modulate their water balance—an increase towards cytokinins causing more water loss while under the water stress increased ABA levels and reduced transpiration. Studies on a wilty mutant of tomato confirmed this phenomenon. The wilty mutant had more cytokinins which caused rapid wilting through excess transpiration and it could be prevented from wilting by exogenous application of ABA[81].

Besides cytokinins and ABA certain phenolic acids such as ferulic acid could suppress stomatal opening. However these compounds were effective only at very high concentrations and their role *in vivo* is yet to be established. Fusicoccin, a fungal toxin, is a potential plant growth regulator[82]. It promotes K^+ influx and causes a wide stomatal opening[82]. The suppression of stomatal opening by ABA is relieved to a considerable extent by FC. Another group of synthetic plant growth regulators, morphactins—including chloroflurenol and EMD 7301 W suppressed stomatal opening in epidermal strips at low concentrations[83].

STOMATA AND WATER BALANCE IN WATER

Stomatal control of water loss is one of the powerful physiological mechanisms which enables the plants to adapt to water stress. Similarly the water status of the plant in turn controls the stomatal movements.

Stomatal closure decreases transpiration and increases the water use efficiency. The higher resistance to water loss in plants can therefore be expected by a low stomatal frequency or high stomatal resistance. Although the decreased stomatal frequency lowers transpiration per unit area, it is often accompanied by an increase in stomatal size or total leaf area[84]. Differences in stomatal resistance do occur even in cultivars of the same species but the measurements should be very critical and should be done under the same environmental variables; this is rather difficult[85].

As the plants are subjected to water stress, the stomata tend to close and this stomatal closure has been traced as a response to the leaf water potential or humidity in and around leaf. Stomata, of at least certain species respond directly to humidity in a feed forward manner; local water deficits develop in stomatal apparatus which induce stomatal closure before deficits occur in the remainder of the leaf[86]. This response acts as an early warning system that prevents excessive deficits by minimising further transpiration. The exact mechanism is still not identified[87] but the remarkable degree of isola-

tion between the stomatal apparatus and the rest of the leaf enables the water gradients to develop. The modulation of stomatal aperture in response to humidity again depends on the internal and external conditions of the leaf such as the degree of stress already prevailing, levels of growth regulators such as ABA and inherent variation among species[88].

Stomata show also a remarkable adjustment to leaf water potential. The general response of the stomata is to close, as the leaf water potential decreases but such sensitivity of stomata decreases, as the frequency of water deficits increases or as the evaporative demand increases, such as the one encountered in the field grown plants, compared to the glass house grown plants. The degree of stomatal adjustment in relation to the leaf water potential shows minimal variation among different species[88]. The decrease in stomatal aperture in response to water stress, is due to the lowering of the leaf water potentials, primarily through adjustment of osmotic potential[85], although it was earlier envisaged that the accumulation of ABA under water stress leads to stomatal closure.

CHEMICAL REGULATION OF STOMATAL MOVEMENTS

Based on the information obtained from studies on stomatal physiology, several chemicals were successfully employed to regulate the water requirements of plants. The suppression of stomatal opening reduces the water needs of plants while enhanced stomatal opening on the other hand leads to increased transpiration and desiccation[5]. Paraquat and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are some examples of herbicides which kill the plants by enhancing the transpirational water loss by forced opening of the stomata[89]. The stomatal behaviour also formed the basis of sensitivity of weeds to herbicides. Thiocarbamates (EPTC and molinate) inhibited transpiration in C_4 crop plants and resulted in their better water management. The transpiration in C_3 weeds was enhanced by thiocarbamates leading to their desiccation and death[90]. Thus under such crop-weed associations, thiocarbamate compounds are extremely useful in promoting the growth of crop plants alone.

The compounds which reduce transpiration and an improved water use efficiency of plants are known as 'antitranspirants'[7]. Antitranspirants are classified into three categories namely metabolic, film-forming and reflecting materials. Considerable work has been done in this laboratory on severally new metabolic antitranspirants which restrict stomatal opening by interfering with the metabolism of stomata and thereby reduce transpiration. Inhibitors of cyclic photophorylation like 2,4-dinitrophenol and chloromercuridinitrophenol and salicylaldoxime, when applied as foliar spray on test plants reduced transpiration and increased the transpiration ratio[91]. Since the discovery of suppression of stomatal opening[83], morphactins also were found to exhibit antitranspirant activity. The reduction in transpiration was more prominent under stress condition than that under irrigated conditions[92]. Several herbicides such as alachlor and butachlor reduced transpiration by restricting stomatal opening[90]. Alachlor even improved the yield of maize plants in spite of reduction in transpiration[93].

The herbicides can reduce transpiration also indirectly by enhancing the wax deposits on the leaf surface of several scrub species[94].

CONCLUSIONS

Current status of our knowledge of guard cell physiology and biochemistry leads us to believe that a reasonable interpretation is possible for the environmental and hormonal effects on stomatal movements in terms of ionic relations and specific metabolic events. Greatest lacunae however exist in the characterisation of proton extrusion and the precise role of carbohydrate constituents of guard cells in relation to stomatal aperture.

It is also evident that the stomata exert a critical role in plant water balance and that the behaviour of stomata is under the influence of water status of the plant. The basic science of stomatal physiology has wide range implications

for application in agricultural productivity through the possible reduction of water requirements of crop plants and thus soil water conservation at critical crop growth stages. Manipulation of stomatal behaviour may also be employed for the achievement of an eradication of weeds through desiccation under specific situation.

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