TRANSTONOPLAST pH CHANGES AS A SIGNAL FOR ACTIVATION OF THE CYTOSOLIC METABOLISM IN HEVEA LATEX CELLS IN RESPONSE TO TREATMENTS WITH ETHREL (AN ETHYLENE RELEASER). IMPLICATIONS FOR THE PRODUCTION OF A SECONDARY METABOLITE : NATURAL RUBBER

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The latex of Hevea brasiliensis is a specialized true fluid cytoplasm which is expelled from wounded latex vessels (D'Auzac et al., 1982). It is collected industrially for its high content of a secondary metabolite of high economic interest : Natural rubber.

The application of Ethrel (an ethylene releaser) or auxin-like substances to the bark of rubber trees induces a sharp, though transient, increase in latex latex production (D'Auzac and Ribaillier, 1969). The main known effect of ethylene was shown to consist of a significant alkalinization of latex cytosol. This increase in the cytosolic pH occurs some 24 hours after treatment and is parallel with increase in the production of latex and hence of rubber (Fig. 1) (Tupy, 1973; Coupé et al., 1976; Coupé, 1977; Brzozowska-Hanover et al., 1979).

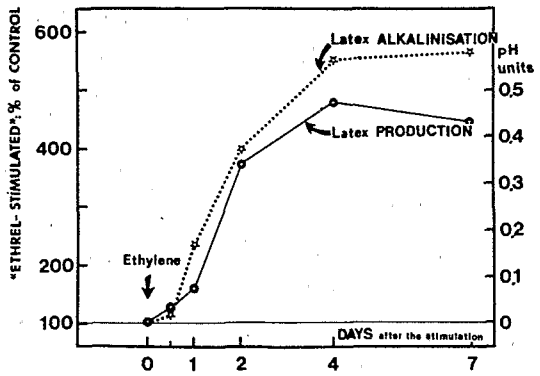

Figure 1

Kinetics of effect of treatment of Hevea bark with Ethrel
on the production of natural rubber
and on the pH changes (alkalinization) of the latex cytosol

Laticiferous cells contain a vacuolar compartment : the so-called lutoids which can easily so isolated intact and purified by simple centrifugation (D'Auzac et al., 1982).

We reported (see Chrestin et al., 1987) that highly significant inverse relationships have been demonstrated between the pH of the cytosol and of the vacuolar compartment, as well as between the vacuolar pH and the production of rubber. Accordingly direct correlation was demonstrated linking the transtonoplastic pH gradient and the production of rubber. Moreover, significant acidification of the intravacuolar fluid was shown to be a response to treatment of Hevea with Ethrel (Coupé, 1977; Coupé and Lambert, 1977; Brzozowska-Hanower et al., 1979; Chrestin, 1985).

These findings and correlations could be fully explained by demonstration of the functioning of two opposing H^+ pumps located on the lutoidic tonoplast :

- one is an Mg-dependent ATPase which catalyses the electrogenic influx of protons in the vacuolar compartment (vacuolar acidification) and the alkalinization of the cytosol (Marin et al., 1981; Marin, 1982; Crétin, 1982; Chréstin, 1985; Marin et al., 1986; Chréstin et al., this issue).

- the second is a tonoplast redox system which consumes NADH and cytochrome c (used as artificial acceptor). When assayed with very freshly isolated intact lutoids, its functioning induced an electrogenic efflux of protons from the vacuolar space into the cytosol, causing vacuolar alkalinization and concomitant acidification of the cytosol (Crétin, 1983; Chréstin, 1985; Chréstin et al., 1987).

The present paper demonstrates the functioning of the two opposing proton-pumping systems as a true tonoplastic biophysical pH-stat, and shows some conditions of the maintenance of a high transtonoplastic $\Delta\overline{\mu}_{H^+}$ in quasi in vivo conditions.
The pyrophosphatase activity found in Hevea latex was demonstrated to be soluble and exclusively located in the cytoplasmic compartment (Jacob and Marin, unpublished data). This activity do not participate at all in any transfer of protons across the tonoplast. Such results contrast with those described in the literature concerning the tonoplast of the different higher plants studied until today.

It especially focuses on the activation of the ATPase moiety of this tonoplastic pH-stat through increase of the ATP content and of potential tonoplastic ATPase activity in the latex after treatment of rubber trees with Ethrel (an ethylene releaser).

THE TWO TONOPLASTIC OPPOSING H^+-PUMPS WORK AS A BIOPHYSICAL pH-STAT IN QUASI IN VIVO CONDITIONS

Plotting the activity of the two H^+-pumps as a function of the pH of the medium (buffered deproteinized latex cytosol obtained by ultrafiltration on PM-10 membrane) clearly shows that the ATPase remains at its maximal potential activity over the physiological pH range (6.5 to 7.3: Brzozowska-Hanower et al., 1979) while the tonoplast e^- transport system, being more pH sensitive in the same pH range, becomes more efficient at a slightly alkaline pH (Fig. 2). This suggests that an excess alkalinization of latex cell cytosol, possibly caused by excessive working of the H^+-pumping ATPase, will be efficiently counteracted by the activation of the redox chain-dependent efflux of protons from the lutoids. Cytosolic pH will then theoretically tend to stabilize itself in the pH range comprised between the optimum pH of each proton pump, insofar as their respective substrate availability is not limiting in the latex (cf. different papers referenced in Marin, 1985; Chrestin, 1985).

We therefore conclude that this system consists of a typical biophysical (bio-osmotic) pH-stat, based on controlled (energy-dependent) transtonoplastic fluxes of free H^+. Its operation will actively participate in the fine regulation of cytosolic pH, and be closely involved in the control of the cytosolic metabolism of latex (cf. Marin, 1985).

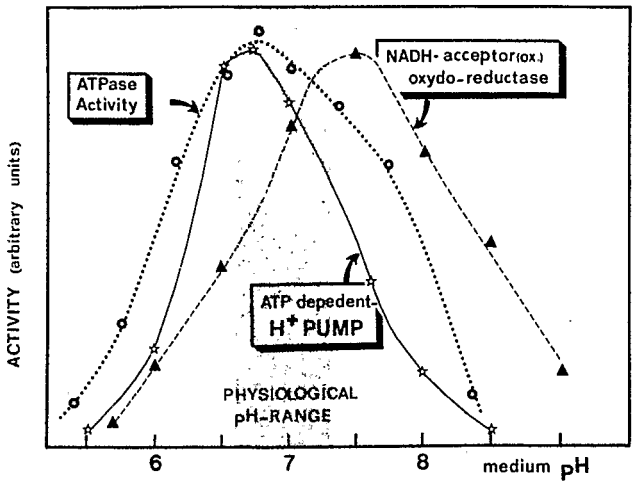

Figure 2

Dependence on pH of tonoplastic ATPase
and NADH-cytochrome c oxido-reductase activities,
and of their proton pumping efficiency,
measured in buffered ultrafiltered cytosol from Hevea latex

OBLIGATORY CONTINUOUS SUPPLY OF ATP TO MAINTAIN HIGH TRANSTONOPLASTIC ELECTROCHEMICAL PROTON GRADIENT

When fresh lutoids were preincubated with ^{14}C-methylamine in the presence of glucose (10 mM) they also accumulated the pH probe (as in the absence of glucose Crétin, 1982), according to their initial transtonoplastic proton gradient (Fig. 3-A). The addition of MgATP (2.5 mM) also induced rapid further influx of methylamine indicating a marked vacuolar acidification caused by the well-demonstrated functioning of the tonoplastic H^+-pumping ATPase. When, after 30 min incubation, the same lutoid suspension was provided once more with an extra but limited (1.5 mM) amount of MgATP (without hexogenous hexokinase : circles and dotted lines in Fig. 3-A), it could be seen that the lutoids maintained, and even slightly amplified, their ATP-dependent transtonoplastic pH gradient. Fig. 3-A (triangles and dotted line) reports a similar experiment, where the second addition of MgATP was accompanied by a addition of a saturating amount of exogenous hexokinase (in presence of glucose 10 mM) in order to use and then eliminate as rapidly as possible all the ATP present in the medium.

It was observed that under these conditions (lack of ATP), the lutoids progressively lost the pool of free protons they had previously accumulated during the functioning of the tonoplastic H_+-pumping ATPase when ATP was still available in the medium (i.e. before addition of hexokinase).

As addition of the hexokinase reaction product, glucose-6-P, did not lead to efflux of protons (not shown), the pool of free electrogenic protons accumulated

in the vacuolar compartment (against the thermodynamic equilibrium) during the functioning of the H^+-pumping ATPase, clearly tends to dissipate with time if insufficient ATP is available.

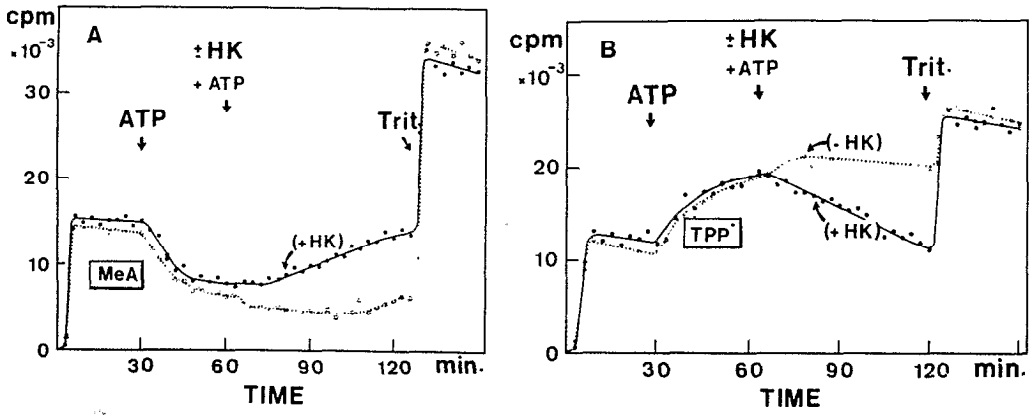

Figure 3

Efflux of protons and tonoplast depolarization of intact lutoids
in a medium without ATP
after its complete consumption by hexokinase plus glucose

The assays were performed with a fresh lutoid suspension using flow dialysis (Crétin, 1982) at pH 7.1, in the presence of glucose (10 mM). In A: Fluxes of ^{14}C-methylamine (using as pH probe) in the presence of MgATP alone (2.5 mM), then MgATP (1.5 mM) in the presence (full symboles and lines) or not (open symboles and dotted lines) of saturating amounts of hexokinase. In B : as in A but fluxes of ^{14}C-TPP^+ were measured to monitor the transmembrane potential changes.

Fig. 3-B demonstrates that the dissipation of the transtonoplastic gradient of free protons in the absence of ATP is accompanied by polarization of the membrane (interior more negative), as shown by the concomitant influx of the potential probe TPP^+ in the vacuoles when hexokinase was added.

We conclude that high transtonoplastic electrochemical proton gradient, then intravacuolar acidity and hence cytosolic (external medium) pH are actively controlled by a continuous, energy-consuming H^+-pump. This leads to proposing "kinetic control" of the H^+-pumping ATPase, essentially by the availability of its own substrate.

INCREASE OF THE TRANSTONOPLASTIC ΔpH AND ACTIVATION OF THE LUTOIDIC H^+-PUMPING ATPase IN HEVEA BY TREATMENT OF BARK WITH ETHREL

Compartmental pH Changes Induced by Treatment With Ethrel

The "stimulation" of rubber production by treatment of Hevea bark with Ethrel (ethylene releaser), is known to induce a rapid alkalinization of the latex cytosol, and a more or less delayed acidification of the vacuolar compartment (Coupé, 1977; Coupé and Lambert, 1977; Brzozowska-Hanower et al., 1979; Chréstin, 1985). We attempted to see if the functioning of the tonoplastic proton pumps were involved in these compartmental pH changes.

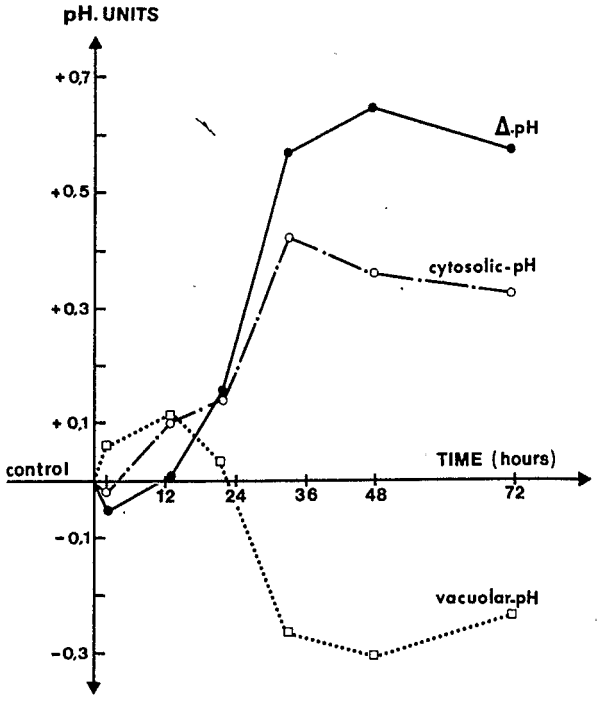

Figure 4

Kinetics of the effects of Ethrel
on the cytosolic and vacuolar pH,
and on the resulting transtonoplastic ΔpH in Hevea latex

The results are expressed as mean differences in latex characteristics between Ethrel-treated trees and control (basal line).

Fig. 4 shows some kinetic aspects of the effects of Ethrel on the cytosolic and lutoidic (vacuolar) pH changes in the latex vessels. It can be seen that the response to Ehtrel treatment could be subdivided into two distinct phases :

- an initial stage (lasting about 21 hours), characterized by slow, slight alkalinization of the cytosol and a transient, slight rise in the intravacuolar pH (maximum after 13 hours). During this early stage, responses of the pH of the two compartments varied in the same way and with similar amplitude. The transtonoplastic pH gradient (ΔpH) then remained constant for at least 13 hours.

- a delayed wide response with simultaneous alkalinization of the cytosol and acidification of the lutoids (vacuoles). The pH of the cytosol increased by 0.42 unit compared with control (maximum after 33 hours) and then remained 0.3 unit higher for more than 2 days. At the same time, the intravacuolar pH decreased to 0.2 to 0.3 unit below control (Fig. 4 and 5).

The resulting transtonoplastic ΔpH increased by 0.57 pH unit (33 h) and then by 0.55 pH unit against the control. Although the pH changes of the two cellular compartments moved in symmetrically opposite ways, the cytosolic pH was shown to be more affected than the intravacuolar pH. This could be explained by the greater buffering capacity of the intravacuolar fluid (Chrestin, 1985).

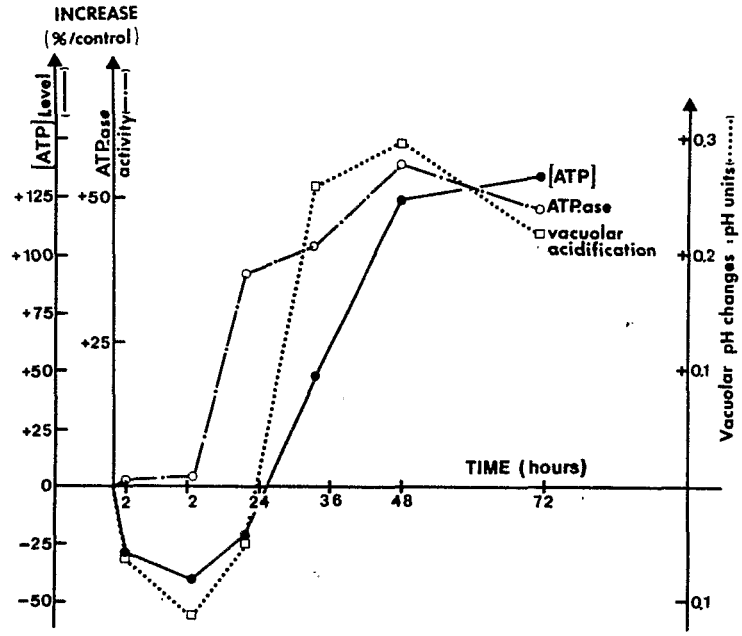

Figure 5

Kinetics of the effect of Ethrel
on the potential tonoplastic ATPase activity
and cytosolic ATP level, expressed in % variation compared with control
and on vacuolar acidification in latex from Ethrel-treated trees

Base line = Control

Increase in the Tonoplastic ATPase Activity

It can be seen in Fig. 5 that Ethrel induced a sudden increase in the tonoplastic ATPase activity in vitro 21 hours after treatment. The specific activity of the H^+-pumping ATPase measured on intact lutoids after 21 hours was shown to increase by 37% compared with the control, and then by 55 to 60% after 48 hours.

The specific ATPase activity measured on purified tonoplast, and in optimal conditions of pH and substrates (Fig. 6-A) was shown to be four times higher in membranes from Ethrel-treated trees than in membranes of the control.

Kinetic studies (Fig. 6-B) indicate that the K_m of the enzyme for MgATP remained unchanged whereas increase in V_{max} could be interpreted as an increase in the number of catalytic sites on the tonoplast, probably owing to activation of the de novo synthesis of the enzyme (Gidrol, 1984; Chréstin, 1985).

Increase in ATP and Total Adenine Nucleotides Pools
in the Latex Cytosol from Ethrel-treated Trees

Fig. 5 shows that as early as 2 hours after treatment with Ethrel, the ATP content of latex cytosol from "stimulated" trees fell by 30% below control, then fell again by 47% after 13 hours. Following this transient decrease, the ATP content increased rapidly and exceeded control level about 24 hours after treatment. Later, the ATP content in the latex from "stimulated" trees reached values 225% (after 33 h) and then 240% (after 71 h), of that of control.

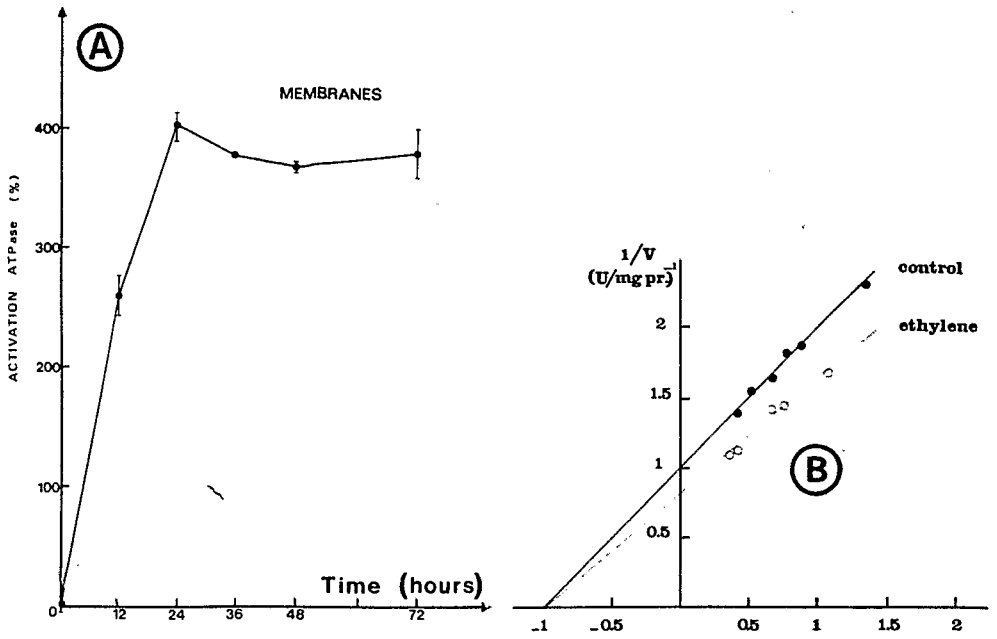

Figure 6

Effects of Ethrel treatment
on the potential activity and on the kinetic parameters
of the tonoplastic ATPase as measured on the purified tonoplast

In A: Time-course of the Ethrel treatment on the potential ATPase activity measured on purified tonoplast; in B : Effect of the Ethrel treatment on the K_m and V_{max} of tonoplastic ATPase (Ethrel : circles; control : black dots).

In addition, it was found that the total adenine nucleotides pool (ATP + ADP + AMP) in fact closely followed the kinetic variations of the ATP content alone, resulting in scarcely detectable changes in the energy charge and ATP:ADP ratio (Chrestin, 1985) in the latex cytosol. This led us to conclude that de novo synthesis of adenine nucleotides was induced by ethylene (at least 24 h after the treatment), with rebalancing of the energy charge, possibly through adenylate kinase and metabolic activities.

It can be seen from Fig. 5 that the intravacuolar pH changes closely followed the variations in ATP content in the latex. Lower concentrations of ATP were associated with a less acid vacuolar pH, and higher ATP concentrations were associated with considerable acidification of the vacuolar compartment. These data clearly show that the early, marked activation of the tonoplastic proton-pumping ATPase (between 13 and 21 h) did not immediately result in any expected vacuolar acidification. This might be attributed to the transient relative lack in availability of the substrate for the H^+-pumping ATPase (i.e. : ATP) as an early "side-effect" of treatment with ethylene releaser.

DISCUSSION AND CONCLUSIONS

The variations in the total adenine nucleotide pool, and especially the decrease in the ATP content are the earliest known biochemical characteristics of latex to be affected after treatment with Ethrel. Early disturbance in the adenine nucleotide pool might result from the enhancement of the utilization of triphosphate-

nucleotides in the synthesis of nucleic acids and proteins. In support of such an hypothesis, Coupé et al. (1976) and Coupé (1977) reported that treatment of Hevea bark with Ethrel (or with auxin-like substances) induced a increase in the total RNA and polyribosome content in latex during the first 12 hours following treatment. It is then possible that ethylene induces a true de novo adenine nucleotide synthesis within less than 24 hours, and that the total adenine nucleotide pool will be continuously rebalanced.

The striking biochemical event which follows treatment with Ethrel is the marked sharp increase in tonoplastic ATPase activity after a time-lag of 13 to 21 hours. This observed in vitro in intact washed lutoids and also in purified lutoidic tonoplast (Crétin et al., 1983; Gidrol, 1984), hence in the absence of any possible soluble cytosolic activator. This increase in specific ATPase activity was shown elsewhere to be concomitant with significant stimulation of protein synthesis at the lutoidic tonoplast (Gidrol, 1984). Thus the lutoids from "Stimulated" trees keep - at tonoplast level - an indelible print of ethylene treatment ! The fact that only V_{max} was affected, and not K_m for MgATP, supports the hypothesis that there is an increase in the number of ATPase catalytic sites on the tonoplast, ascribable to de novo ATPase synthesis, as an early biochemical event induced by ethylene.

The simultaneous opposite changes in cytosolic and vacuolar pH, together with the satisfactory stoicheiometry of the estimated transmembrane H^+ fluxes (according to the respective buffer capacity of each compartment : Chrestin, 1985), suggest some stimulation of tonoplastic proton pumps by ethylene.

The fact that intravacuolar acidification was shown to run parallel with the increase in the cytosolic ATP content leads to concluding that transtonoplastic H^+ fluxes might depend on the lutoidic ATPase activity, as controlled by the availability of ATP. The K_m of ATPase for MgATP was shown to be about 0.5 to 0.7 mM measured in physiological conditions (with ultrafiltered cytosol at pH 6.8 to 7.2) (Gidrol et al., 1985; Gidrol, 1984) while the mean cytosolic ATP content was shown to remain less than 0.25 mM in the control (i.e. : not Ethrel treated) (Chrestin, 1985).

Thus, the tonoplastic H^+-pumping ATPase always operates at far less than its maximum potential in vivo, and its real activity depends in a linear manner on the ATP content of the cytosol. The initial decrease in the ATP content in the latex (30 to 40% less than control) is then assumed to result in a decrease of the ATPase activity and cause some vacuolar alkalinization. The data showing the progressive discharge of the transtonoplastic ΔpH under ATP depletion in vitro (Fig. 3) agree with such an hypothesis.

In contrast, when increase in potential ATPase activity was followed by increase in cytosolic ATP, efficient activation of the H^+-pumping ATPase occurred in vivo. It is suggested that this accounts for the rise in the transtonoplastic ΔpH and for the alkalinization of the latex cytosol which were demonstrated as favouring active metabolism in the latex.

As a conclusion, it is established that the two opposing proton pumps located on the lutoidic tonoplast can work as a true biophysical pH-stat under in vivo conditions. It is suggested that the differential functioning of the two moities is strictly controlled by the availability of their respective substrates. This was demonstrated in particular for the proton-pumping ATPase in vitro and in vivo. The ATPase moiety of the tonoplastic pH-stat was shown to be activated by exogenous ethylene (Ethrel) because of at least 2 early biochemical events :

- increase in the potential ATPase activity probably through de novo synthesis of the enzyme,

- and, increase in the real ATPase activity through a marked rise in the ATP content in the cytosol.

The combination of these two biochemical events induced by ethylene brings about efficient activation of the tonoplastic proton-pumping ATPase, and results in an increase in the transtonoplastic ΔpH (favouring detoxification of the cytosol) and in the concomitant alkalinization of the cytosol; both these phenomena favour the latex metabolism and hence rubber production (Chrestin et al., 1987).

These results should be regarded as an interesting model for the participation of vacuoles in the transduction of signals regulating the cytoplasmic metabolism and the production of secondary metabolites (natural rubber in this case) in higher plants.

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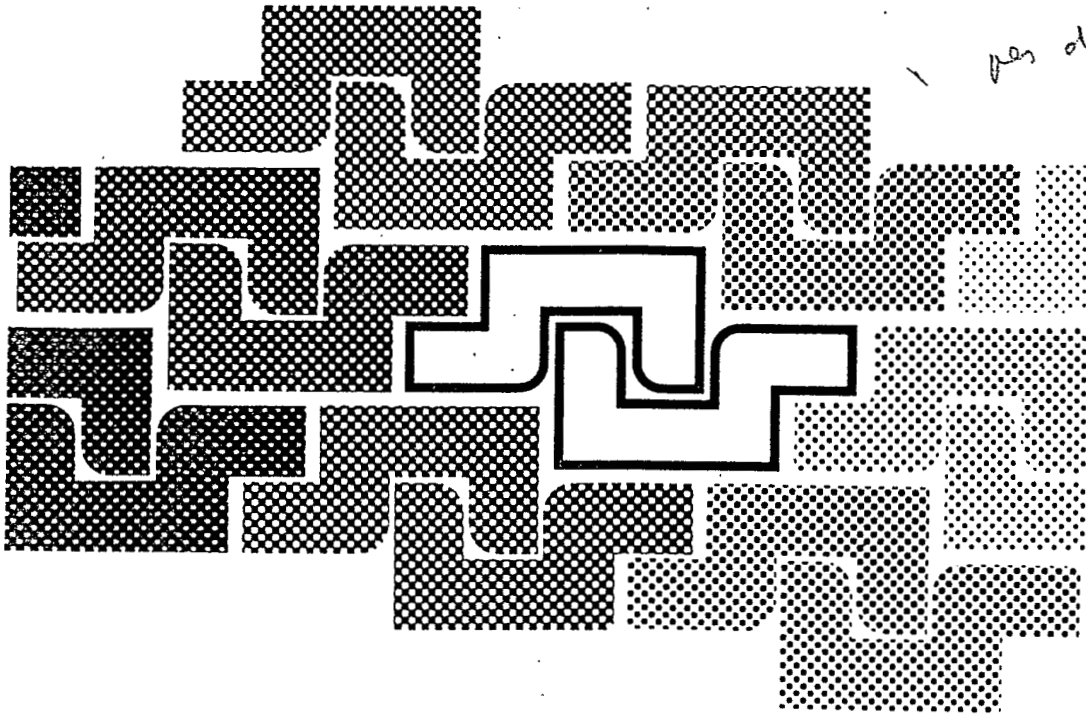

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