Ethylene-Induced Increase in Glutamine Synthetase Activity and mRNA Levels in *Hevea brasiliensis* Latex Cells

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Ethylene, used as a stimulant of latex production in *Hevea brasiliensis*, significantly activates the regenerating metabolism within the laticiferous cells. In this context, attention was focused on glutamine synthetase (GS; EC 6.3.1.2), a key enzyme in nitrogen metabolism. A specific and significant activation of the cytosolic glutamine synthetase (GS$_{cyt}$) in the laticiferous cells after ethylene treatment parallels the increase of latex yield. A marked accumulation of the corresponding mRNA was found, but in contrast, a slight and variable increase of the polypeptide level is at the limit of detection by western blotting. The GS response to ethylene might be mediated by ammonia that increases in latex cytosol following ethylene treatment. The physiological significance for such a regulation by ethylene of the GS$_{cyt}$ is discussed in terms of the nitrogen requirement for protein synthesis associated with latex regeneration.

Hevea brasiliensis latex is a rubber-producing cytoplasm from specialized cells called laticifers (d'Auzac et al., 1989). These anastomosed cells constitute an articulated system from which latex is expelled upon tapping, until coagulation of the rubber particles blocks the latex flow. In situ regeneration mechanisms allow reconstitution of the exported latex before the next tapping. In quantitative terms, 100 mL of latex, exported by a moderately producing tree during one tapping, are completely regenerated within 3 d. This corresponds to the net synthesis of about 50 g of dry rubber and 1.2 g of protein. Thus, a very intense metabolic activity is required, in particular energy-generating catabolic pathways like glycolysis (Jacob, 1970), as well as anabolic processes allowing reconstitution of the intracellular components. In this context, nitrogen metabolism involved in protein and nucleic acid synthesis takes a prominent part.

One way to enhance rubber production is to provide the trees with exogenous ethylene using the ethylene generator, ethephon (d'Auzac and Ribailler, 1969). Treatment with ethephon both increases the volume of exported latex and stimulates latex regeneration between tappings (Coupé and Chrestin, 1989). A 1.5- to 2-fold increase of latex production can be obtained by this method.

The mechanisms of ethylene action are not completely

elucidated. Physiological and biochemical evidence shows that ethylene acts on membrane permeability, leading to prolonged latex flow, as well as on general regenerative metabolism (Coupé and Chrestin, 1989). Glycolysis acceleration (Tupy, 1973) and increased adenylic pool, polysomes, and rRNA contents (Coupé and Chrestin, 1989; Amalou et al., 1992) are obvious indications of such metabolic activation. Furthermore, several enzymic activities have been shown to be specifically modulated by ethylene in the rubber tree (Coupé and Chrestin, 1989) and other plants (Lieberman, 1979).

Working on the assumption that the regenerative metabolism is intensely activated after ethephon treatment, we focused our attention on one key enzyme of nitrogen metabolism, GS (EC 6.3.1.2), which allows NH$_4^+$ integration into organic compounds. This enzyme, in particular, supplies the cells with the amino acids needed for protein synthesis via the pathway involving glutamate synthase (EC 1.4.1.13). Recently, a GS$_{cyt}$ has been identified in rubber tree latex (J.L. Jacob, A. Clement, and J. C. Prevôt, unpublished data), where no glutamate dehydrogenase, another plant enzyme involved in nitrogen assimilation, could be detected (Jacob et al., 1978), suggesting that the GS-glutamate synthase cycle might be the major pathway for the amino acid and protein synthesis required for latex regeneration.

Our objective in this study was to investigate the putative influence of ethylene on the GS$_{cyt}$ in latex. We show here that GS$_{cyt}$ in *H. brasiliensis* latex is indeed regulated by ethylene, probably through de novo protein synthesis, as revealed by changes in GS$_{cyt}$ activity, protein, and mRNA levels.

MATERIALS AND METHODS

Plant Material

Three different *Hevea brasiliensis* genotypes, GT1, PB217, and RRIM600, from plantations in Ivory Coast and Malaysia, were used in the present work. These genotypes are among the most commonly cultivated in Africa and Asia. Each

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genotype was represented by two groups of trees, one as a control (C) and the other (T) treated with ethephon. Groups consisted of 25 trees each for protein quantification, enzyme assays, and immunoanalysis, 20 trees each for latex yield measurements, 8 trees each for northern blotting, and 6 trees each for NH_4^+ measurements. Both control and treated trees were tapped simultaneously, and the collected latex was pooled in each group and for each tapping day. Ethephon treatment was performed on group T by spreading 2.5% ethephon in palm oil over the tapping cut and a 1-cm stripe on the bark just underneath the tapping cut. In the case of the northern blot experiments, the bark area to be treated was gently scraped before application of ethephon and palm oil mixture. Control trees of the genotype RRIM600 were scraped and treated with palm oil without ethephon.

Assays

The cytosol was isolated from the latex collected on ice by a 40,000g centrifugation for 60 min at 4°C and freeze-dried for storage before analysis. Total cytosolic proteins were quantified using Bradford's method (Bradford, 1976). NH_4^+ concentrations were measured in the cytosol following the method described by Brzozowska et al. (1974).

The plant GS catalyzes two types of reaction: a biosynthetic reaction (L-glutamate + ATP + NH_4^+ → L-glutamine + ADP + Pi) or a transferase reaction (L-glutamine + NH_2OH → γ-glutamyl hydroxamate + NH_3). Both activities of the cytosol were assayed at 30°C, according to Shapiro et al. (1970). The GS_{cyt} biosynthetic assay measured the formation of ADP or Pi released in 0.1 M Tris-HCl buffer, pH 7.7, containing 20 mM glutamate, 2 mM ATP, 10 mM $MgCl_2$, 2 mM $(NH_4)_2SO_4$, and 100 μM ammonium molybdate to inhibit latex phosphatase activity (Jacob and Sontag, 1974). Assays without glutamate and without enzyme solution were run as controls. The γ-glutamyltransferase assay measured spectrophotometrically (at 500 nm) the formation of glutamyl hydroxamate in 40 mM imidazol buffer, pH 7.15, containing 20 mM glutamine, 20 mM arsenate, 0.3 mM $MnCl_2$, 0.2 mM ADP, and 20 mM hydroxylamine.

Glutamate dehydrogenase (NAD-GDH: EC 1.4.1.2; or NADP-GDH: EC 1.4.1.3) was assayed by incubating 400 μg mL^{-1} of freeze-dried latex cytosol in 0.1 M Tris-HCl, pH 7.5, containing 10 mM 2-oxoglutarate, 1 mM $(NH_4)_2SO_4$, and 0.2 mM NADH (or NADPH) at 30°C. The NAD (or NADP) formation was followed spectrophotometrically at 340 nm for 20 min. Exogenous GDH (EC 1.4.1.3, Boehringer, 10–20 units mL^{-1}) was then added as a positive control in the presence of the latex cytosol.

Immunoanalysis

Polypeptides from freeze-dried cytosol were solubilized directly in Laemmli buffer (Laemmli, 1970). Forty micrograms of each sample were separated following SDS-PAGE in 15% acrylamide and transferred to nitrocellulose membrane (Nitro Plus, Micro Separation, Inc.; Westboro, MA) for immunoblotting analysis. The membrane was first incubated for 1 h in TBST buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Tween 20) containing 10% (w/v) powdered milk, then rinsed

briefly and incubated with a rabbit antiserum directed against tobacco GS_{cyt}, kindly provided by Dr. B. Hirel (Hirel et al., 1984). After washing in the same buffer, the membrane was incubated for 45 min with an anti-rabbit IgG conjugated with alkaline phosphatase. The activity of this enzyme was revealed by using the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. A duplicate gel was stained with Coomassie blue to check whether the protein quantification and deposits were reliable. The relative intensities of the signals were quantified using the Millipore Bio-Image system.

RNA Isolation

The procedure for total RNA isolation from latex was derived from the method described by Kush et al. (1990). Latex was collected in an alkaline buffer consisting of 50 mM Tris-HCl, pH 9, containing 150 mM LiCl, 5 mM EDTA, and 5% SDS, then immediately frozen in liquid nitrogen for storage. After thawing, most of the rubber was discarded by a 10,000g centrifugation for 30 min at 4°C. The "white fraction" recovered was deproteinized through at least two phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and one chloroform:isoamyl alcohol (24:1, v/v) extractions. RNA precipitation was performed overnight in 2 M LiCl at 4°C and followed by a 10,000g centrifugation for 30 min at 4°C. Additional purification with chloroform:isoamyl alcohol was done before ethanolic precipitation in the presence of 300 mM potassium acetate, pH 5.5.

Northern Blot Analysis

Poly(A)$^+$ RNA isolated from total RNA by chromatography on oligo(dT)-cellulose was subjected to agarose/formaldehyde gel electrophoresis (Sambrook et al., 1989) then transferred onto a nylon membrane (Hybond N, Amersham) according to the manufacturer's recommendations. The probe used to detect GS mRNA from rubber tree latex was a radiolabeled insert corresponding to a full-length cDNA clone of soybean GS_{cyt} (Miao et al., 1991) obtained from Dr. B. Hirel. Hybridization was performed overnight at 45°C in 5× SSC solution (from a 20× SSC stock solution: 3 M NaCl, 300 mM trisodium-citrate, pH 7), 10× Denhardt's reagent (from a 50× stock solution: 1% Ficoll, 1% PVP, 1% BSA), 7% SDS, 20 mM sodium phosphate buffer, pH 7.2, and 100 μg/mL sonicated and heat-denatured salmon sperm DNA. Final washes were carried out at 50°C in 1× SSC and 0.5% SDS. After autoradiography, the relative intensities of the signals were quantified using the Millipore Bio-Image system.

RESULTS

Analysis of GS Activity

The GS_{cyt} activity was measured for two H. brasiliensis genotypes (GT1 and PB217) in the soluble fraction of latex freed from plastids and other organelles. Measurements were made before and after ethephon treatment (Fig. 1). A significant increase in GS_{cyt} biosynthetic and transferase activities could be detected 48 h after ethylene treatment (Fig. 1). The highest increases in activity were 1.5- and 2-fold for the
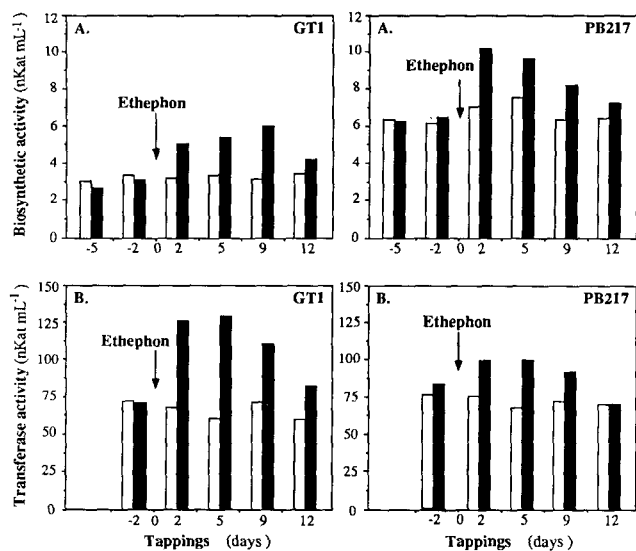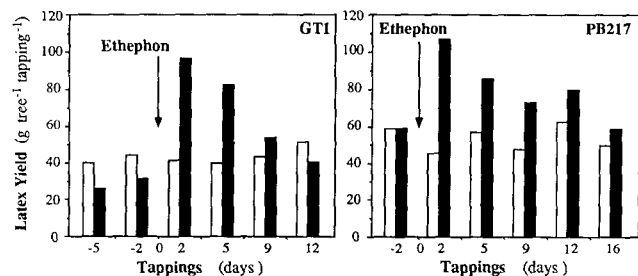
Figure 1. Influence of ethylene on GS_{cyt} activities in latex from the *H. brasiliensis* genotypes GT1 and PB217. GS_{cyt} activities were measured in latex cytosol pooled from 25 control trees (light bars) and 25 ethephon-treated trees (black bars). The measurements were repeated six times on each pool of latex. Both groups of trees were tapped simultaneously, twice a week, before and after ethephon treatment (indicated by the arrow, on d 0). A, GS_{cyt} biosynthetic activity. Variability over six repeats, on the same pool of latex: 4 to 7%. B, GS_{cyt} transferase activity. Variability over six repeats, on the same pool of latex: 4 to 7%.

PB217 and GT1 genotypes, respectively, for both biosynthetic and transferase activities. The stimulation lasted two to three tappings after the ethylene treatment and then decreased.

Latex Yield and Total Protein Content

Latex yield of the ethylene-treated trees increased significantly (2.4-fold) in both genotypes studied (GT1 and PB217). It was maximum 48 h after treatment and then decreased over the three to four subsequent tappings (Fig. 2). The

Figure 2. Influence of ethylene on latex yield from the *H. brasiliensis* genotypes GT1 and PB217. Twenty control trees (light bars) and 20 ethephon-treated trees (black bars) were tapped simultaneously, twice a week, before and after ethephon treatment (indicated by the arrow, on d 0). In each group, latex yield was measured on two separate pools of latex from 10 trees each. The mean values are given in this figure. Variability over the two measurements: 0 to 7%.
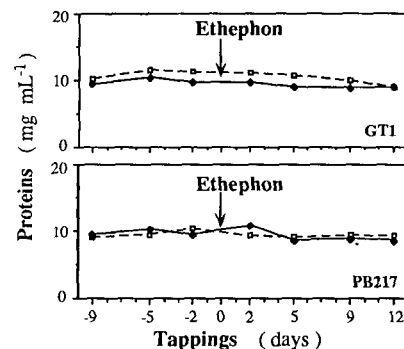
Figure 3. Influence of ethylene on total protein content in latex cytosol from the *H brasiliensis* genotypes GT1 and PB217. Total protein content was measured in latex cytosol pooled from 25 control trees (□) and 25 ethephon-treated trees (♦). For each pool, six measurements were performed. Both groups of trees were tapped simultaneously, three times before and four times after ethephon treatment (indicated by the arrow, on d 0). Variability over six repeats, on the same pool of latex: 4 to 7%.

increase in latex yield is partly due to an increased volume of exported latex. However, the concentrations of total cytosolic proteins were not modified in the ethylene treatment or by the successive tappings in either genotype (Fig. 3).

Northern Blot Analysis

We tested first the hypothesis that ethylene could regulate the expression of the GS gene. Northern blots demonstrated a clear accumulation of the GS transcripts in the latex of ethylene-treated trees from three different genotypes (Fig. 4, A and B). A 4- to 20-fold increase in GS mRNA levels could be measured, depending on the genotype, 48 h after ethylene treatment. This stimulatory effect was specific for ethephon, since the basal levels of GS transcripts remained very low in control trees submitted to bark scraping and application of palm oil alone (Fig. 4B). The ethylene-induced accumulation of GS transcripts appeared to be transitory: in genotype RRIM600 (Fig. 4B) the stimulatory effect was maximal 2 d after treatment, notably decreased after 5 d, and disappeared after 7 d.

Immunoblot Analysis

The relationship between the increase in mRNA levels and the amount of GS protein was explored by immunoblot analysis performed on latex cytosol from the GT1 genotype (Fig. 5). Two polypeptides of about 39 and 40 kD were recognized by the antibodies directed toward the tobacco GS_{cyt} (Hirel et al., 1984). Quantification of the immunoblot revealed a slight intensification (1.3-fold) of the band corresponding to the major polypeptide, suggesting that ethylene might increase the amount of GS polypeptide. This increase in the protein level, although consistent with that measured for the GS activity (1.5- or 2-fold, as shown in Fig. 1), displayed a significant variability, likely due to the fact that the western blot method was not sensitive enough to detect
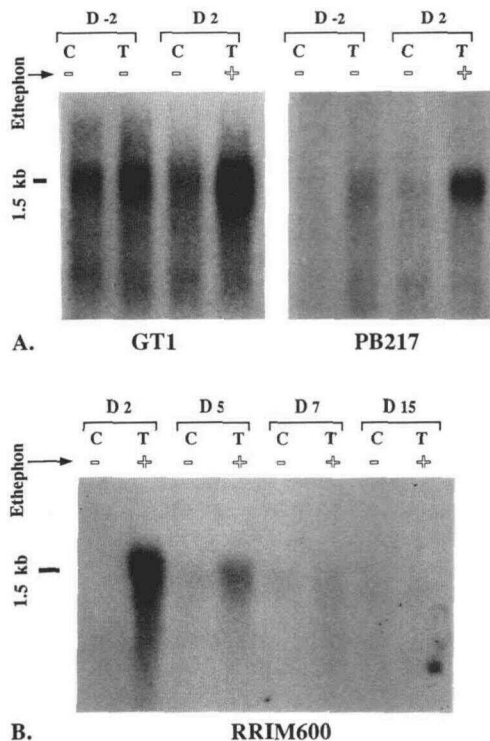
Figure 4. Changes in GS mRNA levels in *H. brasiliensis* latex cells in response to ethylene. Three rubber tree genotypes (GT1, PB217, and RRIM600) were tested by northern blot analysis. Poly(A)⁺ RNA (3 μg/lane) were separated by agarose/formaldehyde gel electrophoresis and probed with a soybean GS cDNA clone. C, Control group of trees; T, ethephon-treated group of trees. A, Genotypes GT1 and PB217; both control and treated trees were tapped once 2 d before treatment (D$_{-2}$), and then 2 d after treatment (D$_2$). B, Genotype RRIM600. Ethylene treatment was performed by applying 2.5% ethephon in palm oil to scraped bark; control trees were treated with palm oil without ethephon, applied to scraped bark. Both control (C) and treated trees (T) were tapped repeatedly 2, 5, 7, and 15 d after treatment (D$_2$, D$_5$, D$_7$, and D$_{15}$, respectively).

changes of small amplitude, such as those that can be expected from the measured increases in GS activity.

Analysis of NH₄⁺ Content

Ethylene treatment induced NH₄⁺ accumulation in the cytosol (Fig. 6). An 18% increase was measurable 24 h after ethylene treatment and reached 40% on d 10. This stimulation was specific for ethephon and independent of bark scraping or palm oil application.

Analysis of GDH Activity

The search for GDH activity in latex cytosol was performed by testing NAD(P) formation from NAD(P)H. When freeze-dried latex cytosol was added to the assay medium, the OD measured (OD$_{340}$) remained constant over 20 min. To demonstrate that the assay medium containing the freeze-dried latex cytosol was suitable to measure GDH activity, a positive control was performed by adding exogenous GDH (10–20

units mL⁻¹), which induced a 1.25-OD unit decrease in 1 min. Under the conditions used, the sensitivity of the assay was such that a GDH activity higher than 0.1 μmol h⁻¹ mL⁻¹ of latex cytosol (i.e. about 1% of the GS activity) would have been detected.

DISCUSSION

Our results have shown that ethylene induces a significant increase of the GS$_{cyt}$ activity in the latex of rubber trees in the various genotypes tested. The evolution of this stimulation is well correlated with the kinetics of latex yield (Fig. 2), as well as with other physiological parameters (pH, mineral ions, saccharose content, etc.) observed in rubber trees after ethylene treatment under similar conditions (Primot et al., 1979). The fact that ethylene treatment was without effect on the concentration of total cytosolic protein of the latex while increasing the amount of exported latex indicates that ethylene triggers a general activation of protein synthesis or protein turnover in the latex of the rubber tree. This is in agreement with the previously described increase of both ribosome polymerization and the incorporation of labeled amino acids into proteins following ethylene treatment (Coupé and Chrestin, 1989). From these observations, it can be concluded that the protein content of the latex is efficiently controlled, with full regeneration of the proteins lost through each tapping, even under conditions of ethylene-increased latex removal. The specific increase of GS activity over its initial level indicates that differential protein synthesis might
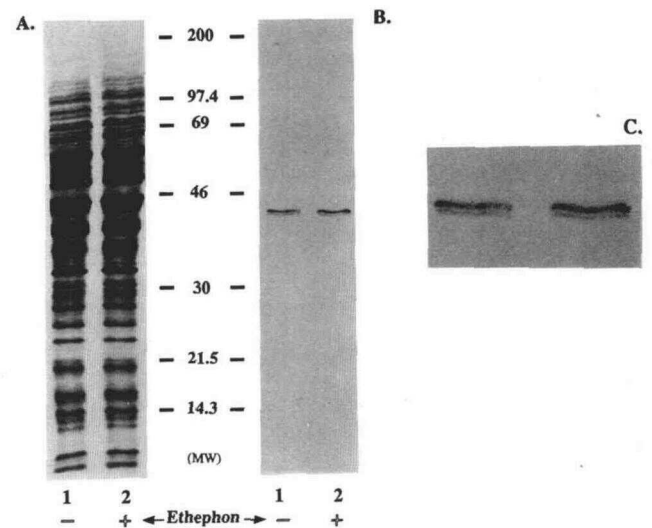
Figure 5. Steady-state levels of the GS polypeptides in latex cytosol from *H. brasiliensis* trees (genotype GT1) without treatment (1) or 2 d after ethylene treatment (2). Forty micrograms per lane of proteins were separated following SDS-PAGE. Molecular mass markers are indicated in kD. A, Polypeptides stained with Coomassie blue. B, Duplicate samples were transferred to a nitrocellulose membrane for western blotting. The GS$_{cyt}$ polypeptides were detected with an antiserum raised against a tobacco cytosolic GS. An alkaline-phosphatase-conjugated secondary antibody was used for antigen visualization. C, Enlargement from Figure 4B, in the area between the 30- and 46-kD molecular mass markers.
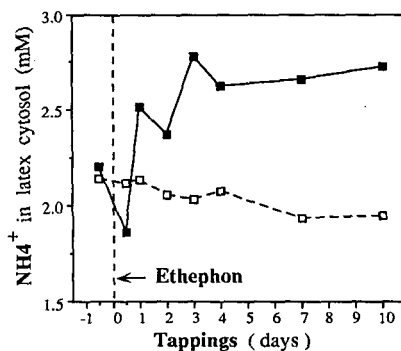
Figure 6. Influence of ethylene on the NH_4^+ content in latex cytosol from the *H. brasiliensis* genotype GT1. NH_4^+ contents were measured in latex cytosol pooled from six control trees (□) and six ethephon-treated trees (■). Treatment was performed by applying 2.5% ethephon in palm oil to scraped bark; control trees were treated with palm oil without ethephon. Both groups of trees were tapped once before treatment, then 12 h, 1, 2, 3, 4, 7, and 10 d after treatment (indicated by the dotted line, on d 0). Variability over two repeats, on the same pool of latex: 2 to 7%.

also be triggered by ethylene beyond the general maintenance of the protein level.

Northern and western blots were performed to test the hypothesis of an ethylene-induced synthesis of the GS protein. A strong regulation at the level of GS_{cyt} mRNA was indicated. However, the increase of GS_{cyt} transcripts appears notably higher than the increase of corresponding activity and polypeptide amount, suggesting that regulatory processes other than transcriptional regulation might also be involved. Such a discrepancy between GS enzyme and mRNA levels has been reported previously for other plant material. For example, in radish cotyledons submitted to dark-accelerated senescence, the relative content of GS_{cyt} mRNA increased 7-fold, whereas that of the corresponding polypeptide increased only 2-fold (Kawakami and Watanabe, 1988). Several other enzymes, as documented by Kamachi et al. (1992), exhibit the same behavior.

The immunoblotting experiments showed that at least two types of GS polypeptide, slightly different in size (about 1 kD), are present in the cytosol of *H. brasiliensis* latex (Fig. 4C). The possibility of contamination by a plastidial isoform is unlikely, because it has been shown by northern blot analysis that the chloroplastic form of GS in rubber tree is highly expressed in leaves but not in latex (Kush et al., 1990). The diversity in the structure of GS_{cyt} from various species is now well documented. In bean root nodules, for instance, among the eight GS_{cyt} isoforms identified, one is a homooctameric isozyme, whereas the others are made up by different ratios of two distinct polypeptide subunits (Cai and Wong, 1989). In *Hevea*, whether the two cytosolic polypeptides observed are different subunits of the same holoenzyme or two distinct isozymes is still to be determined.

GS genes are subject to regulation by a large range of external or physiological stimuli, depending on plant organ, or GS isoform. Light regulation of GS expression has been described (Hirel et al., 1982; Tingey et al., 1988), as well as regulation by nitrate or ammonium (Kosaki et al., 1991; Miao

et al., 1991; Santos and Salema, 1992). Senescence also generates modifications of GS expression (Kawakami and Watanabe, 1988; Kamachi et al., 1992). We suggest that the modifications of the GS content and activity occurring during natural senescence could be mediated by a rise in endogenous ethylene, classically described as one of the major regulators of senescence in plants (Mattoo and Aharoni, 1988).

We do not know whether ethylene acts directly on the GS gene expression or indirectly through "secondary messages." For instance, the NH_4^+ level could well be an intermediate regulator of GS gene expression in latex, as it is in some other plants (Kosaki et al., 1991; Miao et al., 1991; Santos and Salema, 1992). This hypothesis is supported by the fact that ethylene treatment induces NH_4^+ accumulation in the cytosol of the laticifers (Fig. 6). The mechanism by which ethylene triggers this accumulation is not known; it might be through modifications of the membrane properties, which would trigger ammonia uptake, or through the activation of hydrolases able to release NH_4^+ from nitrogenous substrates. In latex, especially under ethylene stimulation, GS activation might be involved in preventing toxicity for the cell by limiting NH_4^+ accumulation. In this context, direct regulation of the GS gene by NH_4^+ would seem appropriate (Miao et al., 1991). However, the differences observed in the kinetics of changes in GS activity and NH_4^+ level (although the two types of data were obtained from separate experiments performed on different genotypes) suggest that the situation is more complex.

In most higher plants, the GS-glutamate synthase enzymic system is considered to be the main pathway for ammonia assimilation. Nevertheless, an alternative pathway based on GDH might exist in some plants showing high levels of this enzyme under certain environmental or nutritional conditions (Srivastava and Singh, 1987). When latex cytosol was incubated at 30°C in the presence of GDH substrate and cofactor (NADH or NADPH), no sign of cofactor oxidization could be detected spectrophotometrically, suggesting that neither NAD-GDH nor NADP-GDH was present in rubber tree latex cytosol, or that they were present in extremely low concentration. Therefore, GS-glutamate synthase should fulfill the requirements for protein synthesis associated with the regeneration of latex lost during tapping. We calculated that in the case of a moderately producing rubber tree genotype like GT1 or RRIM600, each tapping exported about 1.2 g of protein (14 mmol of nitrogen). The GS biosynthetic activity measured in vitro, under optimal conditions, would give about 26 mmol of nitrogen per day as Gln, providing the potential for complete regeneration of the removed proteins within 1 d. However, it has been observed that 3 d were necessary for the genotype GT1 to restore the integrity of the exported latex compounds in situ. This difference is due to the fact that in vivo conditions are clearly not optimal for GS activity, especially concerning pH, ATP, and glutamate (J.L. Jacob, A. Clement, and J.C. Prevôt, unpublished data). The requirements for protein synthesis become even more important in the case of ethylene-stimulated trees for which one tapping exports twice as much latex. In that case, 28 mmol of nitrogen have to be reincorporated into proteins, and the GS activity, already limited initially by the in vivo context, would become critical if kept at the initial level. The less than

2-fold activation of GS by ethylene measured in this work would thus provide the increased capacity for nitrogen incorporation necessary to compensate for the ethylene-increased protein removal in the same amount of time.

In conclusion, GS_{cyt} in *H. brasiliensis* latex is under close regulation, mediated by ethylene, directly or via secondary messages such as NH_4^+. Investigating the effect of ethylene on other important enzymes involved in nitrogen metabolism, such as glutamate synthase or nitrate and nitrite reductase, might also be of interest. Concomitant regulation of these enzymes is likely to occur, which would make it necessary to efficiently control nitrogenous compound regeneration and to prevent NH_4^+ toxicity in the very stressful context of latex exploitation.

ACKNOWLEDGMENTS

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