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Growth Regulator Effects on Rubber and Resin Production in Guayule Callus and Proliferating Shoot Cultures

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Parthenium argentatum *Gray callus and shoot tissue cultures established from germinated seeds were treated with various growth regulators, growth retardants and sodium acetate to determine their effects on resin and rubber production. Callus (one variety) and shoot cultures (two varieties) were grown in a 16-h light/8-h dark regime in Murashige and Skoog's revised tobacco medium supplemented with the various growth regulators or sodium acetate. Other cultures were grown under short-day conditions (8-h light) or with low (4°C) night temperature. On a dry weight basis both callus and liquid shoot cultures accumulated 0.20% to 0.34% and 0.12% to 0.37% rubber, respectively, for all treatments inclusive of the control. Resin content in callus and liquid shoot cultures ranged from 2.80% to 6.89% and 2.89% to 6.30% respectively.*

Guayule, *Parthenium argentatum* Gray (Compositae), is a rubber-producing shrub native to the deserts of northern Mexico and the southern United States. Rubber production in cultured stem segments of guayule was first reported in 1950 by Arreguin and Bonner[1]. Today, considerable work is directed towards the improvement of plant growth and rubber production[2,3] and to the development of tissue culture systems for hybridisation, propagation and regulation of rubber biosynthesis[4].

The substance TEA [2(3,4-dichlorophenoxy)-triethylamine] increased rubber production in field-grown guayule plants[5] and at lower concentrations increased guayule tissue culture growth[6]. Also reported are guayule callus induction and organogenesis[7], rubber and hydrocarbon production in callus tissue[8], the partial purification of an enzyme catalysing the incorporation of isopentenyl-PP into rubber from guayule leaves[9] and conditions for the multiplication of guayule shoots[10].

The guayule cultures were maintained for approximately one year prior to this study. Rubber and resin production occurred in both callus and shoot tissue cultures. This paper summarises the general trends evident in both resin and rubber production for selected growth regulators and for two conditions of light and temperature.

MATERIALS AND METHODS

Plants and Tissue Culture

Milled guayule plant tissue (Cultivar N396, Dr William Schlomann, Jr., Goodyear Tire and Rubber Company, Akron, Ohio 44 316) and guayule plants maintained in a University of Minnesota greenhouse (*ca.* eighteen-month-old plants, commercial nursery, Pasadena, California) were assayed for resin and rubber content. The analytical data reported for plant tissues were based on eight replicate extractions of 0.5 g lyophilised tissue.

Guayule cultures were established from two strains of guayule seed (Mesa 593 and Mesa 11 591) as previously described[10]. Callus tissue

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(seventeenth subculture since culture was established) was grown in 30-ml square vials for thirty days on 18-ml solid (1% agar) Murashige and Skoog's revised tobacco medium (MS) containing 20 p.p.m. benzyladenine (BA) *(Figure 1)*. For the experimental studies, the callus tissue was subcultured in 125-ml Erlenmeyer flasks containing various liquid media and grown in defined light and temperature for either seven or fourteen days *(Table 1)*, then harvested and analysed. In cultures harvested at Day 14, the medium was decanted and replenished on Day 7.

Shoot tissues of Mesa 593 or Mesa 11 591 (twenty-fourth subculture since culture was established) were transferred from MS solid medium containing 3 p.p.m. BA into 250-ml Erlenmeyer flasks that contained MS liquid medium with 1.25% sucrose and 0.1 p.p.m. BA. The shoots were not subcultured; however, the growth medium was decanted and replenished every ten days *(Figure 2)*. After fifty-eight days, the medium was decanted and replenished with 10 ml of MS medium containing various experimental growth regulators. The liquid shoot cultures were then grown for either fifteen or thirty days, harvested and analysed. In thirty-day-old cultures, the experimental medium was decanted and replenished on Day 15.

Each experimental condition and control was determined in quadruplicate. The MS medium contained 1.25% sucrose and either 0.1 p.p.m. BA; 10 p.p.m. naphthaleneacetic acid (NAA); 2 p.p.m. and 20 p.p.m. 2(3,4-dichlorophenoxy)-triethylamine (TEA, Dr Henry Yokoyama, USDA, San Diego, California 92 135); 50 p.p.m. N-[2,4-dimethyl-5-[[(trifluoromethyl)sulfonyl]amino]phenyl] acetamide (Embark, Dr Kestutis Tautvydas, 3M Company, St. Paul, Minnesota 55 414); 2 p.p.m. and 20 p.p.m. α-cyclopropyl-α-(p-methoxyphenyl)-5-pyrimidine methanol (A-rest, Dr Edward Tschabold, Eli Lilly Company, Indianapolis, Indiana 46 285); 100 p.p.m.

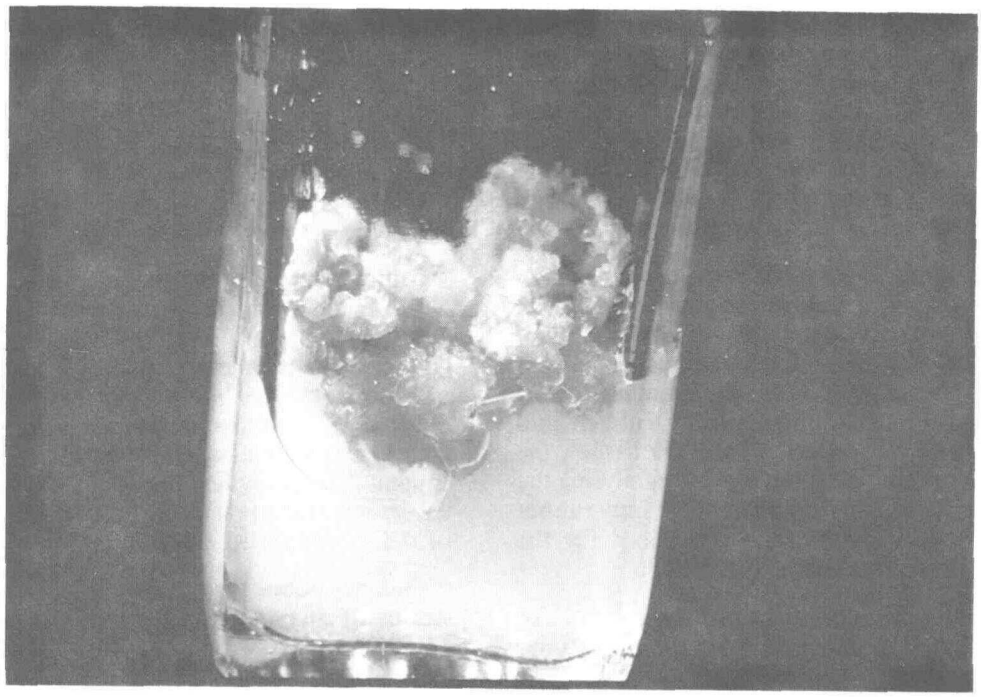


Figure 1. Parthenium argentatum *Gray (var. 593 Mesa) thirty-day-old callus growing on Murashige and Skoog's agar medium containing 20 p.p.m. benzyladenine in a 30-ml vial.*

TABLE 1. GUAYULE CALLUS (MESA 593) GROWN IN LIQUID MEDIUM: GROWTH CHANGES, RUBBER AND RESIN ANALYSES

Experimental condition	Analyses[c]		
	Growth changes[b]	Rubber (mg %)	Resin (g %)
2 p.p.m. TEA	+118	I	I
Control–45 days	+103	H	I
2 p.p.m. A-rest	+101	H	H
Control–37 days	+ 97	—	—
10 p.p.m. NAA	+ 94	L	L
20 p.p.m. TEA	+ 88	L	L
25 p.p.m. GA	+ 85	H	I
100 p.p.m. Sodium acetate	+ 82	I	L
50 p.p.m. Embark	+ 81	I	L
20 p.p.m. A-rest	+ 81	L	L
Temp./Light[a]	+ 66	H	I
Temp./Light[a]–45 days	+ 60	I	I

[a]Cultures maintained under 27°C–8-h day/4°C–16-h night

[b]Percentage difference in growth index (GI = final wet weight/initial inoculum wet weight) at Day 37. Day 30, GI = 3.6; Day 37, GI = 8.3; Day 45, GI = 12.3

[c]Concentrations determined from dried tissues at Day 37 or Day 45 compared to the control concentration at Day 37:

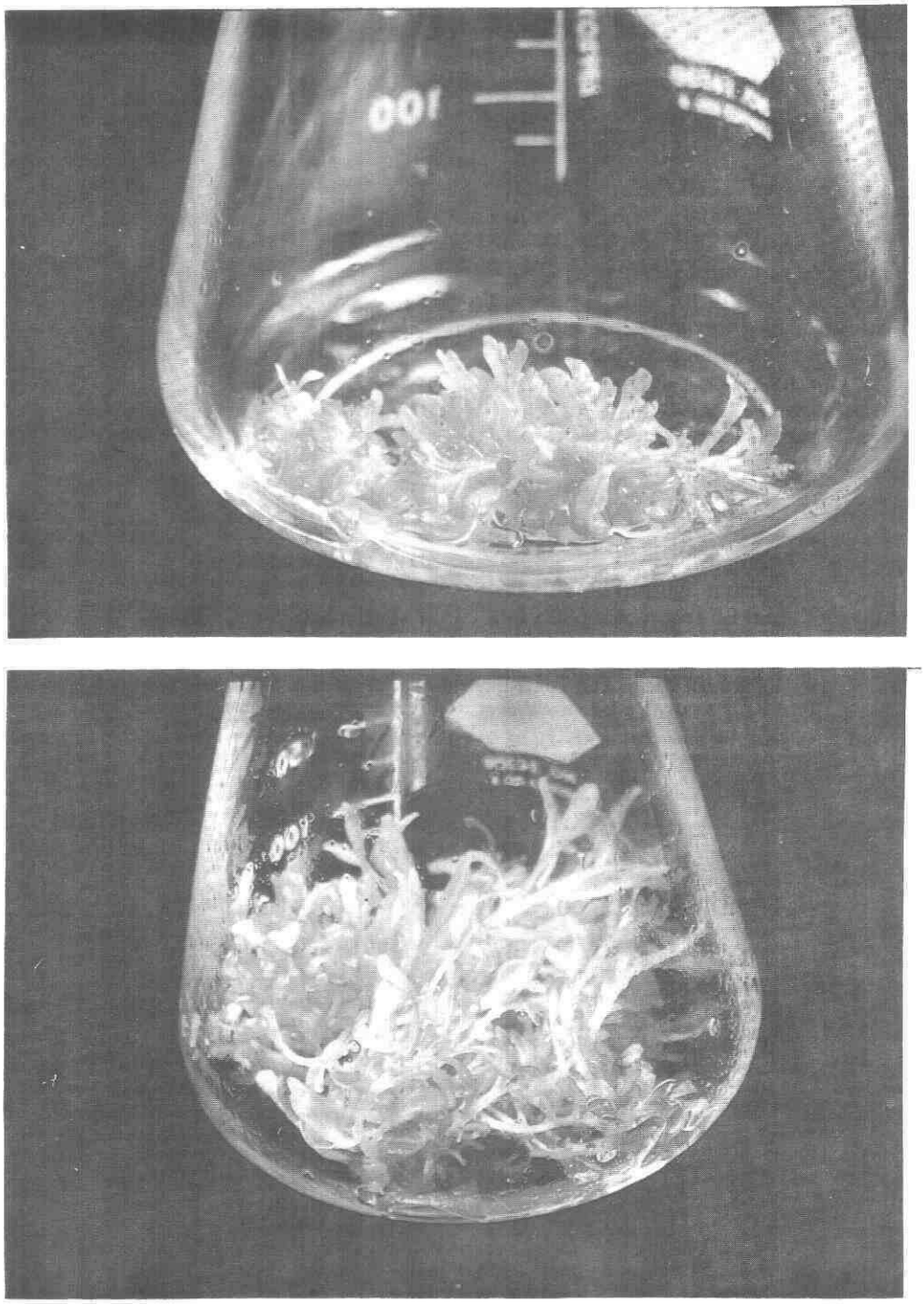
	Rubber (mg %)	Resin (g %)
High (H)	0.32 ± 0.003 to 0.34 ± 0.002	5.00 to 6.89
Intermediate (I)	0.21 ± 0.002 to 0.23 ± 0.003	4.65 to 5.00
Low (L)	0.19 ± 0.006 to 0.20 ± 0.002	2.80 to 3.41

sodium acetate; or 25 p.p.m. filter-sterilised gibberellic acid (GA). The cultures were grown on a gyratory shaker (78 r.p.m.) at 24°C under a 16-h light/8-h dark cycle (5500 lx, fluorescent plant-grow bulbs, 40 W cool-light, Sears Roebuck Company). Two different culture conditions were studied for effects on growth rate and rubber and resin production. These conditions were light — 16-h light or 16-h dark cycle, and temperature — 37°C in light and 4°C in the dark.

Rubber and Resin Analyses

Replicates for each experimental condition were combined for assay, lyophilised and ground in a mortar. Resin content was determined gravimetrically and the rubber content by a turbidimetric method based upon the methods of Traub[11] and Perry[12].

Material was weighed (0.5 g) into a centrifuge tube containing 20 ml of acetone and homogenised by a Polytron (Brinkman Willems Model PT-10-20, 3500, Des Plaines, Illinois 60 016) for 30 s at a setting of 7. The blades were rinsed with additional acetone into the centrifuge tube. The acetone-guayule homogenate was then centrifuged at 1870 r.p.m. for 15 min. The supernatant was filtered through Whatman #1 filter paper into a tared round-bottom flask and the filter paper rinsed. The acetone extraction was repeated twice and the combined extracts were flash evaporated to dryness at room temperature to obtain the resin weight. The acetone-insoluble pellet was



After ten days of growth

Figure 2. Parthenium argentatum *Gray (var. 593 Mesa) inoculum in Murashige and Skoog's liquid medium containing 3 p.p.m. benzyladenine and grown in a 250-ml Erlenmeyer flask maintained on a gyratory shaker (78 r.p.m.).*

immediately used for rubber analysis. About 5 ml of diisobutylketone (DBK; Aldrich, Milwaukee, Wisconsin 53 233) was added to the pellet in the centrifuge tube and ground as for resin for 30 s at a setting of 7. The blades were rinsed with an additional 5 ml of DBK and the homogenate centrifuged at 1870 r.p.m. for 30 min. The supernatant was filtered through Whatman #1 filter paper into a 25-ml volumetric flask. The procedure was repeated once, the filter paper rinsed with DBK, and the final volume adjusted to 25 ml.

A guayule natural rubber standard (CIQA, Saltillo MX) was obtained from Dr William Schlomann, Jr., for the preparation of a turbidimetric standard curve. Diethylene glycol stearate for the assay was obtained from Glyco Chemicals, Inc., Greenwich, Connecticut 06 830, and morpholine was obtained from Aldrich. Spectrophotometric readings were made on a Hitachi Model 100-10 Spectrophotometer (Beckman Instruments, Fullerton, California 92 634).

Rubber confirmation. ^{1}H-NMR spectra (JEOL FX 90Q) were obtained for guayule natural rubber (standard), extracted guayule stem tissue and shoot tissue cultures. The natural rubber standard was dissolved directly in $CDCl_3$, and for extracted tissue samples the DBK was removed by rotovap under vacuum at 40°C before dissolving in $CDCl_3$. The presence of rubber in the tissue culture extracts was confirmed at the University of Minnesota by nuclear magnetic resonance (NMR). Further confirmation was obtained by IR (Dr Anthony Verbiscar, Anver Bioscience Design, Inc., Sierra Madre, California 91 204) and NMR and ^{13}C-NMR (Dr James Visintainer, Goodyear Tire and Rubber Company). Biosynthetic tracer studies in our laboratory have also confirmed rubber in both callus and shoot tissue cultures of guayule[13].

RESULTS AND DISCUSSION

Guayule is the only species of *Parthenium* known to produce commercially important amounts of rubber[14]. Mesa 593 is a low-rubber-producing variety of guayule, and Mesa 11 591 a higher rubber producer. A two-year-old plant normally contains 10% rubber by dry weight, although its range of concentration may be considerably higher depending on the environmental and genetic factors controlling rubber production. The rubber values for callus and shoot tissue cultures for Mesa 593 were generally lower than for Mesa 11 591 *(Tables 1, 2* and *3)*. The range of values for growth index, rubber and resin levels are given in *Table 4*.

Tissue Cultures

Callus of Mesa 593 grown in liquid medium (callus liquid) grew as large (50 mm), light-green, compact cell aggregates with no browning of tissues or growth medium during the seven-day or fourteen-day experimental periods *(Table 1)*. Over the experimental period, the greatest increase in growth was noted with 2 p.p.m. TEA and 2 p.p.m. A-rest while a smaller growth increase was evident under the conditions which varied in temperature and photoperiod.

Liquid cultures of proliferating shoots of Mesa 593 and Mesa 11 591 grew best in a small volume (10ml/250-ml flask) of MS[15] containing 1.25% sucrose *(Tables 2* and *3)*. Such cultures grew very well if the medium was replaced every ten days over the extended experimental period of seventy-three days or eighty-eight days. Leaf and stem tissues of Mesa 593 control tissues were smaller and morphologically more uniform than Mesa 11 591 tissues. Stem tissues of Mesa 11 591 were larger than those of Mesa 593, and the silvery sheen noted in the intact plant was evident in leaf and stem tissues. Over the experimental period, growth of Mesa 593 was greatest with 25 p.p.m. GA and 2 p.p.m. A-rest, and the greatest growth in Mesa 11 591 was noted with 25 p.p.m. GA and 20 p.p.m. TEA. The effects of photoperiod and temperature were consistent in both Mesa 593 and Mesa 11 591 cultures in that they resulted in negligible growth or decreased growth over the experimental period.

Rubber and Resin Analyses

Plant tissue. The rubber and resin contents of combined guayule stem and leaf tissues from approximately eighteen-month-old plants grown

TABLE 2. GUAYULE PROLIFERATING SHOOTS (MESA 593) GROWN IN LIQUID MEDIUM: GROWTH CHANGES, RUBBER AND RESIN ANALYSES

Experimental condition	Analyses[c]		
	Growth effect[b]	Rubber (mg %)	Resin (g %)
25 p.p.m. GA	+33	L	I
2 p.p.m. A-rest	+25	L	H
Control-73 days	+21	—	—
2 p.p.m. TEA	+18	H	H
20 p.p.m. TEA	+16	I	H
100 p.p.m. Sodium acetate	+15	L	I
50 p.p.m. Embark	+14	I	L
10 p.p.m. NAA	+11	I	L
Temp./Light-88 days[a]	+ 9	L	I
Light/Dark[a]	+ 2	H	H
20 p.p.m. A-rest	− 1	L	H
Temp./Light[a]	− 9	H	H

[a]Cultures maintained under 27°C-8-h day/4°C-16-h night. Light/dark culture received four days of dark treatment followed by three days of light treatment immediately before harvest at Day 73.

[b]Percentage difference in growth index (GI = final wet weight/initial inoculum wet weight) at Day 73. Day 58, GI = 31.8; Day 73, GI = 41.5; Day 88, GI = 41.9

[c]Concentrations determined from dried tissues at Day 73 or Day 88 compared to the control concentration at Day 73:

	Rubber (mg %)	Resin (g %)
High (H)	0.28 ± 0.002 to 0.32 ± 0.002	4.53 to 6.21
Intermediate (I)	0.21 ± 0.002 to 0.25 ± 0.002	3.66 to 4.44
Low (L)	0.12 ± 0.002 to 0.19 ± 0.003	2.89 to 3.01

in the University of Minnesota greenhouse were 1.39% and 8.50% respectively. Rubber and resin contents given in the analysis of milled guayule stem tissue (N396 material obtained from the Goodyear Tire and Rubber Company) were 7.53% and 8.30%, respectively.

Tissue cultures. Tables 1, 2 and *3* report the rubber and resin contents of the cultured tissues. It is evident that cultures contain a lower concentration of rubber (0.12% to 0.37%) than the plant leaves (*ca.* 10%). Secondary metabolism is often dependent on morphological complexity and some undifferentiated tissue cultures may produce only a portion of the secondary metabolites present in the intact plant[16]. In this study, shoot cultures as well as callus cultures produced rubber and resins.

Growth regulator effects on rubber and resin production were noted *(Tables 1, 2* and *3).* A-rest is a growth retardant that inhibits diterpene synthesis, an effect that may be reversed by GA[17]. The high resin content in all cultures treated with 2 p.p.m. A-rest may be due to an accumulation of diterpene precursors. A higher concentration of A-rest (20 p.p.m.) inhibited growth and appeared toxic. Embark is a growth retardant that may decrease the chlorophyll-a content of leaf tissue[18]. Although none of the shoot cultures appeared chlorotic after treatment with 50 p.p.m. Embark, both growth inhibition and a dramatic decrease in resin content were evident. NAA may induce elongation in shoot cells and stimulate root formation[19]. The use of NAA did not increase growth, resin or rubber pro-

TABLE 3. GUAYULE PROLIFERATING SHOOTS (MESA 11591) GROWN IN LIQUID MEDIUM: GROWTH CHANGES, RUBBER AND RESIN ANALYSES

Experimental condition	Analyses[c]		
	Growth effect[b]	Rubber (mg %)	Resin (g %)
20 p.p.m. TEA	+17	I	I
Control–73 days	+16	—	—
25 p.p.m. GA	+16	L	I
10 p.p.m. NAA	+13	I	I
100 p.p.m. Sodium acetate	+ 1	I	L
2 p.p.m. TEA	+ 0	H	H
50 p.p.m. Embark	− 4	I	I
Temp./Light[a]	− 5	H	H
2 p.p.m. A-rest	− 6	I	H
20 p.p.m. A-rest	−18	L	H
Temp./Light–88 days[a]	−19	L	L
Light/Dark[a]	−24	H	L

[a]Cultures maintained under 27°C–8-h day/4°C–16-h night. Light/dark culture received four days of dark treatment followed by three days of light treatment immediately before harvest at Day 73.

[b]Percentage difference in growth index (GI = final wet weight/initial inoculum wet weight) at Day 73. Day 58, GI = 21.6; Day 73, GI = 31.2; Day 88, GI = 38.7

[c]Concentrations determined from dried tissues at Day 73 or Day 88 compared to the control concentration at Day 73:

	Rubber (mg %)	Resin (g %)
High (H)	*0.31 ± 0.001 to 0.37 ± 0.002*	*4.86 to 6.30*
Intermediate (I)	0.24 ± 0.004 to 0.29 ± 0.006	3.73 to 4.73
Low (L)	0.17 ± 0.008 to 0.22 ± 0.004	3.25 to 3.34

TABLE 4. SUMMARY OF COMPARISON OF GROWTH INDEX[a], RUBBER AND RESIN LEVELS[b] IN UNORGANISED CALLUS (MESA 593) AND PROLIFERATING SHOOT CULTURES (MESA 593 AND MESA 11591)

Culture conditions	Growth index		Rubber (mg %)		Resin (g %)	
	Control	For all experimental conditions	Control	For all experimental conditions	Control	For all experimental conditions
Callus in liquid (Mesa 593) Table 1	8.32 ± 0.88	4.70 ± 0.24 to 10.46 ± 0.76	0.23 ± 0.003	0.20 to 0.34	5.00	2.80 to 6.80
Shoots in liquid (Mesa 593) Table 2	41.55 ± 10.68	37.04 ± 5.35 to 51.61 ± 8.60	0.24 ± 0.002	0.19 to 0.29	3.57	2.89 to 6.30
Shoots in liquid (Mesa 11591) Table 3	31.24 ± 5.22	21.74 ± 4.79 to 45.32 ± 4.56	0.28 ± 0.003	0.17 to 0.37	4.14	3.25 to 6.30

[a]Growth index = final wet weight/initial inoculum wet weight

[b]The lowest and highest values obtained at the end of the experimental period. Control values were determined at thirty-seven days for callus grown in liquid and at seventy-three days for proliferating shoot cultures grown in liquid.

duction in shoot cultures, and caused their decrease in callus-liquid cultures. Rubber may accumulate in guayule root bark[20] but no roots were observed in this study.

Yokoyama reported that TEA stimulated rubber accumulation in both stem and root tissues of guayule[5]. The effects of two concentrations of the TEA derivative were studied; the lower concentration (2 p.p.m.) gave higher rubber and resin contents. Bioregulators similar to the TEA used have been observed to stimulate carotenoid formation in citrus fruits and may act by depressing genes that regulate enzymes affecting carotenoid biosynthesis. The early precursors for cartenoids are similar to those for isoprenoid and terpenoid syntheses, which may explain the accumulation of rubber and resin in the TEA-treated cultures.

A precursor of rubber synthesis, sodium acetate, was also administered to guayule cultures. The concentration of sodium acetate used was previously found to be optimal for promoting rubber formation in seedling plants[21] and stem explants in aseptic cultures[1]. However, the rubber content, resin accumulation and growth of all cultures treated with sodium acetate were low. The explanation for this observation is unknown.

Rapid growth and significant rubber production are mutually exclusive phenomena in guayule plants. Guayule plants with vigorous growth contain more xylem tissue and proportionally less rubber[21]. It is also reported that the greatest level of rubber formation appears late in the year when vegetative growth is negligible[22]. This phenomenon apparently occurred in GA-treated shoot cultures where increased growth was associated with low rubber content; however, the reverse effect was observed for GA in callus-liquid cultures *(Table 1)*. The late seasonal requirement for rubber production in plants[23] may have been evident in cultures grown under 27°C-8-h day/4°C-16-h night cycles. These cultures grew slowly but produced high rubber contents in all cultures and high resin contents in liquid shoot cultures. Cultures maintained under short 8-h-light/long 16-h-dark conditions also showed increased rubber accumulation with little or no increase in growth. It is surprising that rubber content is high in these cultures as rubber accumulation is said to be dependent on active leaf metabolism[24], and photosynthesis may be necessary for the synthesis of rubber precursors[25].

In summary, the best overall conditions for growth and yield of rubber and resin in callus grown in liquid were obtained with 2 p.p.m. A-rest. In both shoot cultures those treated with 2 p.p.m. TEA produced high rubber and resin contents although their growth was moderate (Mesa 593) or unchanged (Mesa 11 591). In both callus and shoot cultures, a short light photoperiod (8-h day/16-h night) and low temperature (27°C day/4°C night) reduced the growth rate and increased the rubber concentration. Under these same light and temperature conditions, the resin concentrations were high in shoot cultures but lower in callus grown in liquid.

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