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Guayulin content in guayule (*Parthenium argentatum* Gray) along the growth cycle

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ABSTRACT

The commercial development of guayulins might help spur industrial-scale applications of guayule as an alternative source of natural rubber, making necessary to build knowledge on their seasonal response and accumulation in plants. In the present study, the seasonal content of the four known guayulins (A–D) was profiled in 14 different guayule accessions, including hybrids, from age 13 months to age 23 months. Analysis revealed that the accessions could be categorized into four groups based on guayulin content and potentially reflective of their genetic origin and hybridization: one rich in guayulin A, one rich in guayulin C, one rich in guayulin D, and one with intermediate values. Despite the evident differences in guayulin profiles, all four groups shared the same general response. The content of guayulins A and B increased between April and September, followed by a drastic fall in content in November likely triggered by low temperatures, and then a gradual recovery. By contrast, the content of guayulins C and D was much more stable, especially for those accessions showing the highest production. The relationship between the guayulins was useful to characterize their evolution during the growth cycle, with some groups halting production of guayulins during the summer (group rich in guayulin C) and others initiating production earlier, after the winter period (groups rich in guayulin A and those with intermediate values). The higher producers of guayulins were the accessions 11591(CL-1) and AZ-6, and the best harvest time was between September and November, depending on climatic conditions.

1. Introduction

Since its discovery in 1852, guayule (*Parthenium argentatum* Gray) has been studied used as an alternative source of natural rubber (Mooibroek and Cornish, 2000; Rodriguez et al., 1971), attaining a degree of industrial importance in Mexico before 1930 and because of a shortage in raw materials during World War II and the oil crisis of the 1970s (Nakayama, 2005). Currently, however, guayule is not commercially produced in any great quantities despite the appealing properties of its products, which are equal or superior to those of *Hevea brasiliensis* (rubber tree), the major source of natural rubber production. For example, guayule latex is hypoallergenic and has attracted interest as a substitute for *Hevea* natural rubber (Cornish et al., 2009; Siler and Cornish, 1994), which can provoke allergic reactions (Nucera et al., 2020; Reshetnikova et al., 2019). Other studies have sought to investigate the commercial applicability of guayule byproducts from resin (Dehghanizadeh et al., 2021), including fatty acids (Banigan and Meeks, 1953), essential oils (Haagen-Smit and Siu, 1944; Nik et al., 2008) argentatin triterpenes (Romo et al., 1970; Schloman et al., 1986) and guayulin sesquiterpenes (Jara et al., 2019; Rozalén et al., 2021c).

Guayulin sesquiterpenes are only present in high concentrations in the guayule plant, which does not produce any of the pseudoguaianolide sesquiterpene lactones found in other *Parthenium* species (Rodriguez et al., 1971). Guayulins are a four-member family of cinnamic esters (Schloman et al., 1983; Spano et al., 2018), although new members might exist (Rozalén et al., 2021c). While the chemical structures of

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guayulins A, B, C and D were described many years ago (Martínez et al., 1986; Watkins et al., 1985), their specific role in the plant remains enigmatic (Rodriguez et al., 1976). It has been postulated that guayulins may be responsible for plant defence against microbes and termites (Durán-Peña et al., 2015; Gutiérrez et al., 1999). Guayulin A is the most abundant of the guayulins, followed by guayulin B, and guayulins C and D have been suggested to be oxidation products of A and B, respectively (Schloman et al., 1983). The total guayulin content account for 3.5 to 13.3 g kg^{-1} of guayule stems (Rozalén et al., 2021b).

Latex production is known to be activated in the autumn, when the temperature drops (Benedict et al., 2008; Sundar and Reddy, 2001). Several studies have reported that guayulin content also changes along the growth cycle of the plant (Coffelt et al., 2009, 2005; Rozalén et al., 2021c; Schloman et al., 1986), but the reason for this is unknown. In this line, we recently described a relationship between the autumn growth season of guayule and guayulin accumulation (Rozalén et al., 2021a). Specifically, we found that the content of guayulin A and B decreases significantly in the stem during this time, while the ratio of guayulins A–D in the leaves is maintained (Rozalén et al., 2021b). The disappearance of guayulins A and B did not result in an increase in the content of guayulins C and D generated by oxidation of the former, as occurs with isolated compounds and during chemical extraction at high temperature (Martínez et al., 1986; Schloman et al., 1983). A limitation of our previous study was that we only examined the autumn events, and a more exhaustive analysis is warranted to study the growth cycle response of guayulin.

Against this background, the present study sought to: 1) establish the profile of guayulin accumulation along the growth cycle of the plant; 2) determine whether the response profile is linked to the genetic origin of the accession or hybridization; and 3) assess whether a relationship exists between the four major guayulins along their growth cycle, to test for the possibility of interconversion.

2. Materials and methods

2.1. Guayule

The following 14 guayule and hybrid accessions were used in the present study: 11591 (CL-1), AZ-6, CFS17-2005, CFS18-2005, 593, 11604, 11693, A48118, CAL-2, AZ-2, R1092, R1100, R1101 and R1103. All seeds were obtained from the USDA-ARS National Plant Germplasm System (NPGS; www.ars-grin.gov/npgs) and were germinated and transplanted at three months of age, except accession 11591 (CL-1), which was received from CIRAD (France) as four months of age plants. The experimental field was conducted in *Santa Cruz de la Zarza* (Toledo, Spain). The site was chosen as representative of areas in Castilla-La Mancha where guayule is expected to be grown. The crop was transplanted in May 2017, and the accessions were sampled five times until they were 2 years of age: harvest (H1-June 2018, H2-July 2018, H3-September 2018, H4-November 2018 and H5-April 2019). A randomized complete block design with three replications was used with two border rows. Each line consisted of two blocks, 1.5 m apart and 8 m long, 20 cm between plants with a density of 33,333 plants/hectare and 3 m between blocks. In each sampling date (H1 to H5), a sample of four adjacent plants was taken for each of the three repetitions, that is, from each accession, twelve plants were taken per harvest.

Plants were manually cut 5-cm above the ground and stored in kraft bags. Samples were dried in an oven for 48 h at 60 °C until attaining a moisture content of 12 % and the leaves and flowers were then removed. Moisture content was determined with a halogen lamp moisture analyzer (model XM-120 T; Cobos, Barcelona, Spain) at 105 °C. Samples were considered to have reached constant mass when moisture loss was <0.1 % in 180 s. Subsequently, the dry biomass of branches was measured. Branches were cut into pellets of ~1-cm length with a manual cutter, and were ground in a two-step process using a 2-mm hammer grinder followed by a 0.5-mm centrifuge grinder. Dried ground samples were stored in screw-cap glass vessels at room temperature.

Field temperature was recorded in a nearby meteorological station (Ocaña, Toledo) from the Spanish State Meteorological Agency (in Spanish, *Agencia Estatal de Meteorología*).

2.2. Resin extraction

Sequential resin extraction was performed in an ASE E-914 Speed Extractor (BUCHI, Postfach, Switzerland), as described (Rozalén et al., 2021a). Briefly, a sample of 1.5 ± 0.005 g of guayule (0.51-mm-sized particle) was weighed and homogenized with approximately 32 g of sand as a dispersing agent and packed with 37 g of sand above and below to fill the stainless steel 80-cm^{-3} extraction cell. The resin extraction conditions using acetone as solvent were as follows: temperature 40 °C, pressure 100 bar, hold 3 cycles 10/20/30 min, 1-min heat-up, 3-min discharge, 2-min flush with solvent, and 5-min flush with N_2. Resin extract was collected in a 240 mL flask and transferred to a pre-weighed flask equilibrated for 30 min in a desiccator. Solvent evaporation was carried out in a Multivapor BUCHI P-6 (Postfach, Switzerland) parallel system at 50 °C and 150 mbar. After evaporation, the pre-weighed flasks were stored for 60 min in a desiccator before final weighing. The resin percentage was then determined gravimetrically considering dry weight. Each sample was extracted twice.

2.3. Guayulin quantification by high-performance liquid chromatography-diode array detection

Twenty microliters of resin dissolved in ethanol (Panreac, Barcelona, Spain) at 10 mg mL^{-1} was filtered (0.22 μm) and then injected into an Agilent 1200 HPLC chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD) (Agilent G1315D). A reverse-phase ACE Excel 3 C18-PentaFluorPhenyl (PFP) column (150 × 4.6 mm, 3 μm particle size) protected with an ACE Excel HPLC Pre-column Filter (0.5-μm particle size) (both from Advanced Chromatography Technologies Ltd., Reading, Berkshire, UK) was used for separation at 30 °C. Acetonitrile (Sigma-Aldrich, St. Louis, MO) (solvent B) and milli Q-grade water (solvent A) were the solvents. The elution gradient for solvent B was as follows: 0 min, 60 %; 10 min, 60 %; 20 min, 80 % and hold 5 min; 35 min, 100 % and hold 2 min. Agilent ChemStation software (version B.03.01) was used for quantification of the four guayulins (A–D) using a guayulin A standard calibration curve (0.1–250 mg L^{-1}, $r^2 = 0.995$). The limit of detection and quantitation was 31.24 μg L^{-1} and 112.82 μg L^{-1}, respectively. The guayulin A standard (98 % purity) was isolated in our laboratory due to a lack of commercial standards.

To prepare the guayulin A standard, low molecular weight guayule rubber was removed from the resin extract as described (Zoeller et al., 1994). Rubber-free resin was then fractionated using flash chromatography on a VersaFlash Station System I equipped with a Versaflash cartridge (23 mm × 110 mm) containing Spherical C18 bonded silica (30 g, 20–45 μm) with acetonitrile:water (80:20) as solvent and with a flow rate of 20 mL min^{-1}. The fractionation procedure was followed using HPLC-DAD. The guayulin A fraction was evaporated under vacuum and crystalized from hexane/chloroform, as described (Martínez et al., 1986). The molecular weight and mass fragmentation pattern was confirmed by liquid chromatography-mass spectrometry (LC–MS) (Rozalén et al., 2021c). Guayulins B, C and D were tentatively identified by their characteristic UV absorption spectra (maximum at 256 nm for guayulins B and D, maximum at 276–278 nm for guayulin C) and retention time (Rozalén et al., 2021c).

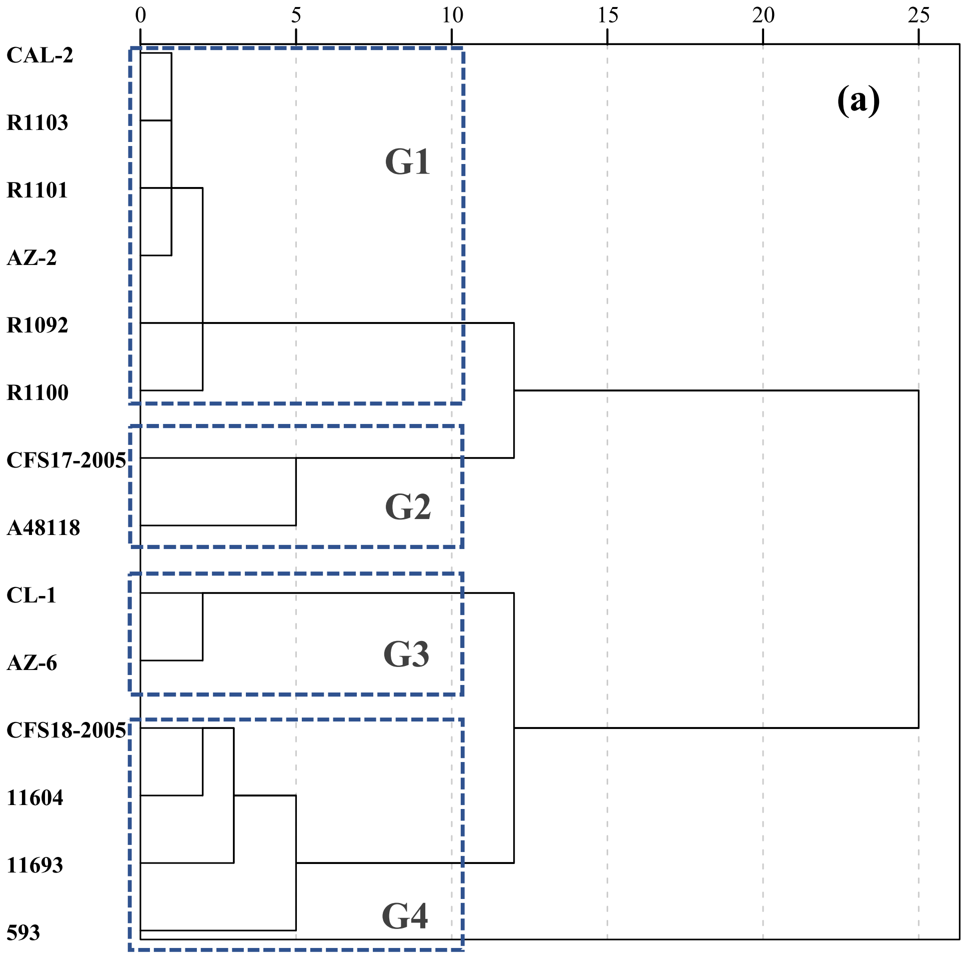
2.4. Data analysis

An analysis of guayule based on the content of guayulins A, B, C and D (mg g^{-1} resin) was performed in the 14 accessions across the five harvest dates of the study. This was based on a hierachical cluster analysis using the nearest neighbor method, obtaining a dendrogram (tree diagram) showing an estimation of four likely clusters. Statistical analysis was performed with IBM SPSS Statistics v25 (Templeton, 2011). The clusters were compared by discriminant analysis to determine whether the accessions were correctly classified, to establish "models of response". Two-factor multivariate analysis was then used to compare the four clusters as established "models" of guayulin response (Models 1–4) and the five harvest samplings (H1–H5). The interaction between the two factors (Model × Harvest) and their individual effects were examined by comparing means and establishing homogeneous subgroups using Tukey's honest significant difference test.

Discriminant analysis was repeated to examine whether the ratios among guayulins (A/B, A/C, A/D, B/C, B/D and C/D) allowed the correct classification within the established models of response. Two-factor multivariate analysis was again performed to compare the ratios among guayulins attending to the established models (M1–M4) and harvest samplings (H1–H5). As before, the interaction among factors (Model × Harvest) and their individual effects were examined by comparing means, analyzed with Tukey's test, to study guayulin inter-relationships.

3. Results

The seasonal variation of guayulin content in 14 guayule accessions (including hybrids) cultivated in the same field was evaluated to build knowledge on their seasonal response and accumulation in plants, which may ultimately aid in optimizing crop management and establishing the best harvesting periods.



(b)

	Guayulins (mg g^{-1} resin)				
	A	B	C	D	Total
Model	***	***	*	***	***
Harvest	***	***	***	ns	***
Model x Harvest	***	**	*	*	***

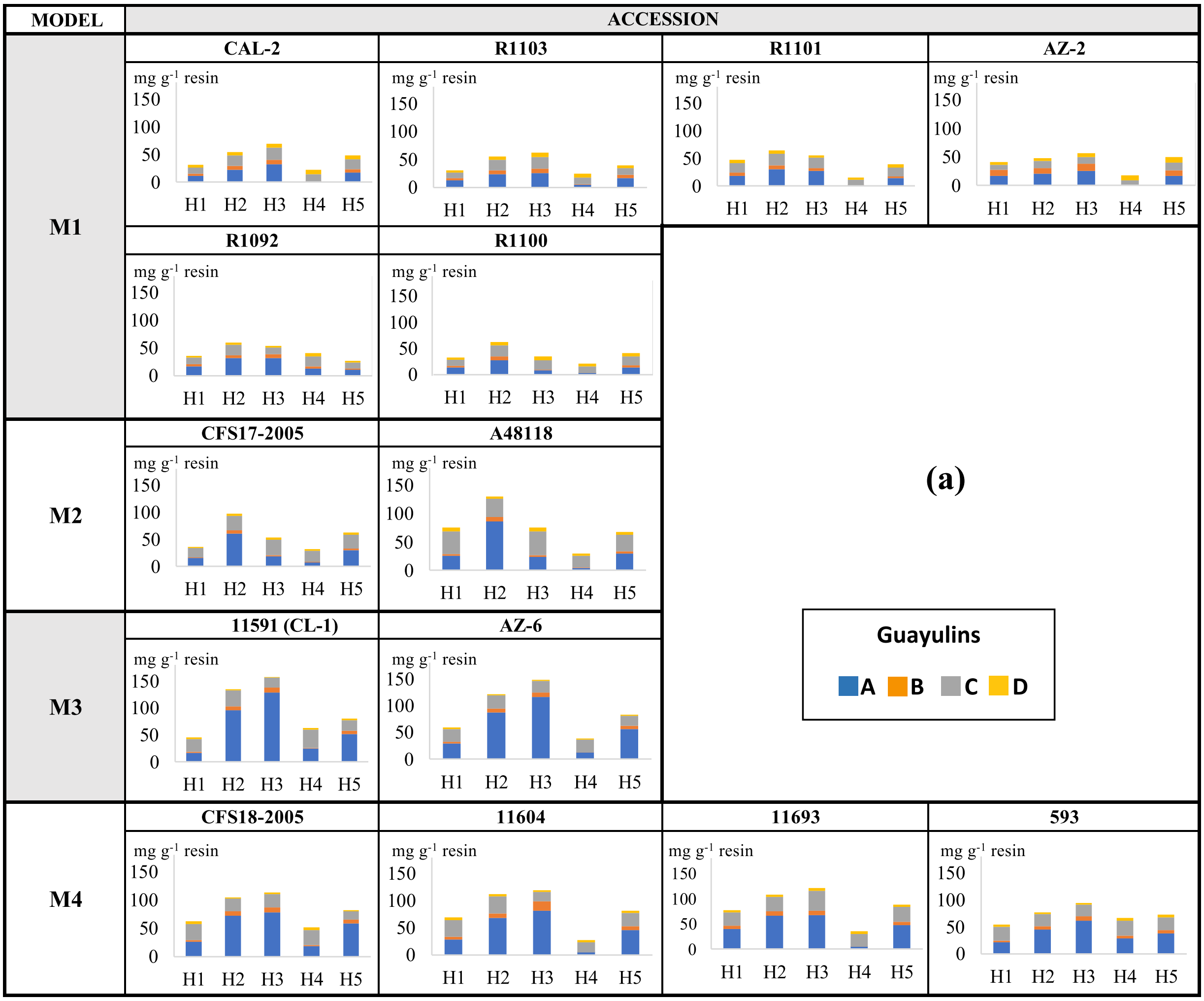
Fig. 1. (a) Neighbor joining trees clustering the content of guayulins A, B, C and D and total guayulins in every accession and harvest period; (b) multivariate analysis considering Model (the four groups identified in the cluster, Fig.1a) and Harvest factors (five harvesting dates) and their interaction.
**P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant*

3.1. Guayulin response

The four known guayulins (A–D) were quantified across five sequential harvests (H1-June, H2-July, H3-September, H4-November 2018, and H5-April 2019), including the cold weather stress conditions that are associated with rubber formation, and possible interrelationships were examined. The 14 accessions clustered into four well-defined groups based on absolute guayulin content calculated (Fig. 1a): *Group 1* contained CAL-2, R1103, R1101, AZ-2, R1092, R1100, with three of the six accession as Mariola hybrids (R1103, R1101, R1100), CAL-2 is a *P. argentatum x P. fruticosum hybrid* and AZ-2 is a *P. argentatum x P.* (unknown Parthenium species) hybrid, and R1092 is pure guayule (Ilut et al., 2017). *Group 2* contained CFS17−2005 and A48118, which are both wild collection accessions from different periods (2005 and 1948, respectively), according to Ilut et al. (2017).

Group 3 was represented by two accessions, 11591(CL-1), AZ-6. Accession 11591 (CL-1) has been given this denomination to clarify that it is seed from 11591 plants grown at CIRAD in a mixed field along with other accessions (Taurines et al., 2019). *Group 4* contained CFS18-2005, 11604, 11693, 593, including the oldest accession (593) and the 11604 and 11693 accessions from the 1950's. The CFS18-2005 accession originated from a bulk collection in an abandoned experimental guayule field and is a mixture of older genotyped accessions (prior to 1978), which precludes its classification into a single genotypic group (Ilut et al., 2017).

Canonical discriminant analysis correctly classified 84.3 % of the four groups, and significant differences between all four guayulins were revealed in multivariate analysis (Model, Fig. 1b). Analysis of the second



(b)

		Guayulins (mg g^{-1} resin)				
		A	B	C	D	Total
Model	**M1**	16.45 a	5.20 b	14.84 a	5.73 c	42.22 a
	M2	29.72 b	3.30 a	28.18 c	4.44 b	65.64 b
	M3	61.62 d	5.24 b	23.50 b	2.58 a	92.93 d
	M4	44.85 c	6.29 b	25.32 bc	4.26 b	80.73 c

(c)

		Guayulins (mg g^{-1} resin)				
		A	B	C	D	Total
Harvest	**H1**	21.57 b	3.64 b	22.72 ab	4.35 a	52.30 b
	H2	63.03 d	7.46 c	25.19 b	3.83 a	99.51 d
	H3	59.82 d	7.38 c	24.35 ab	4.15 a	95.69 d
	H4	10.27 a	1.26 a	21.19 a	4.57 a	37.29 a
	H5	36.09 c	5.29 b	21.36 a	4.37 a	67.11 b

Fig. 2. Total and individual guayulin content (A, B, C and D) by Model and Harvest: (a) profile for every model along the growth cycle; (b) mean values considering Model factor (M1–M4); (c) mean values considering Harvest factor (H1–H5).
Different letters within columns denote significant differences between models (b) or harvest time (c) for every guayulin and total content ($p < 0.05$).

factor (Harvest) revealed significant differences in the content of all guayulins except for guayulin D, although this became highly significant when total guayulin content was considered. An interaction factor (Model × Harvest) will need to be considered for the significant differences in guayulin content (Fig. 1b).

Quantification of guayulins in all 14 accessions are shown in Supplementary Table S1 and in Fig. 2a. When the individual and total mean guayulin content were compared between the response Models independently of the vegetative cycle (Fig. 2b), the content of guayulin A and total guayulins could differentiate between the four models, with Model 3 containing the highest values. Model 2 was the only model showing differences in guayulin B, with the lowest content, and with the highest content of guayulin C. Model 4 presented with intermediate concentrations of guayulins, although it had the second most abundant overall content. Finally, Model 1 showed the lowest total content, with the lowest values for guayulins A and C but the highest value for D (summarized as $M3 > M4 > M2 > M1$).

In a similar manner, when the individual and total mean guayulin content was compared between harvests independently of the response (Fig. 2c) all guayulins with the exception of guayulin D showed significant differences in content between the periods of the growth cycle. The most evident differences were that H2 and H3 generally showed a higher content of A, B and C, and therefore for total guayulins, whereas H4 shows the lower content of all guayulins, which was recovered for guayulin A and B in H5

As an interaction effect was found between the model and harvest (Fig. 1b), the response of the guayulins attending to this interaction was also analyzed (Table 1). Comparing the accumulation of guayulin A along the growth cycle within every model (Table 1, capital letters), in addition to the described evolution (decrease in H4 and recovery in H5), three different responses were observed for H2 and H3 depending on the Model: 1) Model 2 peaked in H2 and then declined in H3; 2) Models 1 and 4 reached their maximum in H2, which they also maintained in H3; and 3) Model 3 peaked in H3. Comparisons of the Models for guayulin A content within every harvest (Table 1, regular letters) extended the previous findings ($M3 > M4 > M2 > M1$; Fig. 2b). The superiority of Model 3 was evident only in H2 and H3, and shared the highest values with Model 4 in H5, which was second on the scale in H3. Model 2 was superior to Model 1 in H2 and H5, with the latter Model always inferior.

Table 1
Two-factor multivariate analysis (Model × Harvest) comparing the accumulation of each guayulin (A, B, C and D) and total guayulin content.

Guayulin A	H1	H2	H3	H4	H5
M1	a, B	a, C	a, BC	a, A	a, B
M2	ab, AB	b, C	a, AB	ab, A	b, B
M3	ab, A	c, C	c, D	b, A	c, B
M4	b, B	b, D	b, D	ab, A	c, C
Guayulin B	H1	H2	H3	H4	H5
M1	a, B	a, B	b, B	a, A	a, B
M2	a, A	a, B	a, A	a, A	a, AB
M3	a, AB	a, C	bc, C	a, A	a, BC
M4	a, AB	a, CD	c, D	a, A	a, BC
Guayulin C	H1	H2	H3	H4	H5
M1	a, A	a, B	a, AB	a, A	a, AB
M2	b, AB	b, AB	c, B	b, A	b, AB
M3	b, A	b, A	ab, A	b, A	ab, A
M4	b, A	b, A	b, A	b, A	b, A
Guayulin D	H1	H2	H3	H4	H5
M1	a, A	b, AB	c, AB	b, B	b, B
M2	a, A	bc, A	bc, A	a, A	ab, A
M3	a, A	a, A	a, A	a, A	a, A
M4	a, A	a, A	b, A	ab, A	a, A
Total G	H1	H2	H3	H4	H5
M1	a, AB	a, D	a, CD	a, A	a, BC
M2	ab, B	bc, C	a, B	ab, A	b, B
M3	ab, A	c,C	c, D	b, A	b, B
M4	b, B	b, C	b, C	b, A	b, B

Different letters between rows (small case letters) denote significant differences between models within each harvest time ($P < 0.05$). Different letters between columns (capital letters) denote significant differences between harvest times within each model ($P < 0.05$). M1, M2, M3 and M4 are the four different response models for guayulin production identified in Figs. 1 and 2. H1-H5 correspond to the five harvesting dates (June, July, September and November 2018, and April 2019 respectively).

In the case of guayulin B, comparing the accumulation within every model (Table 1, capital letters), a very similar evolution to that of guayulin A was observed. Model 2 reached a maximum in H2 and then decreased in H3, where the remaining models maintained the level in H3, before all decreasing in H4. In the Tukey test, guayulin B showed the lowest values for Model 2 while the remaining models showed higher values (Fig. 2b). Reviewing the interaction and comparison within each harvest (Table 1, regular letters), guayulin B showed differences for Model 1 only in H3.

Concerning guayulin C, Models 3 and 4 did not show significant changes between harvests (Table 1, capital letters). Model 1 showed the significantly lowest content of guayulin C in H1 and H4 in relation to H2. In Model 2, guayulin C content was significantly higher in H3 than in H4. When the models were compared between every harvest (Table 1, regular letters), H3 was the sampling time where Model 2 presented with significantly higher values than the other models, which would explain the results obtained previously (Fig. 2b, guayulin C M2 = 28.18c).

Guayulin D seemed to be the most stable compound along the growth cycle as significant changes were observed only in the case of Model 1 (Table 1, capital letters), in which guayulin D was significantly lower in H1 than in H4 and H5. When the models were compared within every harvest (Table 1, regular letters), some differences were found in all with the exception of H1. The pattern shown in Fig. 2b. ($M1 > M2=M4 > M3$) could be explained by the fact that Model 1 had significantly higher values in H3 and Model 2 and 4 only differ in H2 (higher in M2).

When considering total guayulin content along the growth cycle within every model (Table 1, capital letters), the predominance of guayulin A was evident, as the same response patterns were observed: M2 peaked in H2 and decreased in H3; M1 and M4 remained constant in H3 from H2, M3 rose until H3. Comparison of the models within every harvest (Table 1, regular letters) served to explain the individual pattern (Fig. 2b, $M3 > M4 > M2 > M1$): Model 3 showed the highest values in H2 (same level as M2) and H3, and Model 4 in H3 had significantly higher values than Model 1 and Model 2.

3.2. *Guayulin relationship*

We previously demonstrated that the ratios between the individual guayulins changed in autumn (Rozalén et al., 2021b). For this reason, we attempted to establish relationships between guayulins considering the ratios A/B, A/C, A/D, B/C, B/D, and C/D. Discriminant analysis showed that these ratios improved the classification of the four previously established Models (Fig. 1a) achieving 87.1 % of correctly classified accessions in the assigned model cases.

Multivariate analysis (Table 2) revealed significant differences for all the guayulin ratios when the average for every model was considered, independently of the growth cycle. However, when the Harvest factor was considered, all of the ratios showed significant differences, except the A/B ratio. When the two-factor interaction (Model × Harvest) was

Table 2
Multivariate analysis considering the factors Model and Harvest and their interaction for guayulin ratios A/B, A/C, A/D, B/C, B/D and C/D.

	Ratios among guayulins					
	A/B	A/C	A/D	B/C	B/D	C/D
Model	***	***	***	***	***	***
Harvest	ns	***	***	***	***	***
Model × Harvest	ns	***	***	ns	***	***

***$P < 0.001$; ns: not significant.

considered, all of the ratios except the A/B and B/C ratios showed significant differences.

The statistical significance of mean test considering the model factor (Fig. 3) was in accord with the response of the models when total content was considered, as shown in Table 1. In terms of ratios, Model 1 showed the lowest values for all ratios, except for B/C (Fig. 3), which was reasonable because this model showed the lowest values for guayulins A and C (Fig. 2a and Supplementary Table S1). Model 2 showed the lowest A/C and B/C ratios, as the absolute values were the lower for guayulin B and higher for guayulin C (Fig. 2a and Supplementary Table S1). Thus, the ratios with guayulin C as the denominator were the lowest. By contrast, Model 3, which clusters 11591(CL-1) and AZ-6, showed the highest ratio values, particularly A/C, A/D and B/D (Fig. 3), because of its high guayulin A and low guayulin D content, which emphasized those ratios in comparison with the other Models. Finally, Model 4 showed intermediate ratios, in accord with findings for the absolute values.

When the harvest factor was considered, although the ratio A/B did not change significantly along all the growth cycle (Table 2), the results showed that the concentration of both guayulins decreased notably at H4, reaching the lowest values along the growth cycle (Table 1). The decrease in H4 resulted in all of the involved ratios (A/C, A/D, B/C and B/D) showing the lowest values at this time (Table 2). By contrast, H3 showed the maximum values for A/C and A/D ratios, as guayulin A reached its maximum value at that time (Fig. 2). The concentration of guayulins A and B recovered significantly at the end of the winter (H5), (Fig. 2), explaining why all of the ratios that included A and B increased significantly. The ratio A/B was an exception, which decreased (Fig. 3).

As interactions between the two factors considered in the multivariate analysis (Model × Harvest) were observed in four of the six ratios among the guayulins (Table 2), this was also analyzed in detail (Table 3). The response of each ratio and their significant differences during the growth cycle was represented for each model (Table 3, regular letters). Furthermore, the models were compared within every harvesting time along the growth cycle (Table 3, capital letters). Adding to the previous results, Models 3 and 4 recovered the ratios A/C and A/D in H5 significantly better than Models 1 and 2, likely due to the greater

Table 3

Two-factor multivariate analysis (Model × Harvest) comparing the guayulin ratios A/B, A/C, A/D, B/C, B/D and C/D.

Ratio A/B	H1	H2	H3	H4	H5
M1	a, A	a, A	a, A	a, B	a, A
M2	a, A	ab, A	ab, A	a, A	a, A
M3	a, A	b, A	b, A	a, A	a, A
M4	a, A	ab, A	ab, A	a, A	a, A
Ratio A/C	H1	H2	H3	H4	H5
M1	a, B	a, B	b, B	a, A	a, B
M2	a, A	ab, B	a, A	a, A	a, A
M3	a, A	c, B	d, C	a, A	b, B
M4	a, B	b, B	c, C	a, A	b, B
Ratio A/D	H1	H2	H3	H4	H5
M1	a, A	a, A	a, A	a, A	a, A
M2	a, A	b, B	a, A	a, A	a, A
M3	a, A	c, C	c, D	a, A	b, B
M4	a, A	b, BC	b, C	a, A	b, B
Ratio B/C	H1	H2	H3	H4	H5
M1	b, B	a, B	b, B	a, A	a, B
M2	a, A	a, A	a, A	a, A	a, A
M3	a, AB	a, AB	ab, B	a, A	a, AB
M4	a, A	a, AB	b, B	a, A	a, AB
Ratio B/D	H1	H2	H3	H4	H5
M1	a, BC	a, BC	b, C	a, A	a, B
M2	a, A	ab, B	a, A	a, A	a, A
M3	a, A	c, C	d, D	a, A	b, B
M4	a, A	b, BC	c, C	a, A	b, B
Ratio C/D	H1	H2	H3	H4	H5
M1	a, AB	a, B	a, B	a, A	a, AB
M2	bc, AB	b, B	b, AB	b, A	b, AB
M3	c, A	c, C	c, C	c, B	b, A
M4	b, AB	b, C	b, BC	b, A	b, BC

Different letters between rows (small case letters) denote significant differences between models within each harvest time ($P < 0.05$). Different letters between columns (capital letters) denote significant differences between harvest times within each model ($P < 0.05$). M1, M2, M3 and M4 are the four different response models for guayulin production identified in Figs. 1 and 2. H1-H5 correspond to the five harvesting dates (June, July, September and November 2018, and April 2019 respectively).

(a)

	Ratio A/B	Ratio A/C	Ratio A/D	Ratio B/C	Ratio B/D	Ratio C/D
M1	4.81 a	1.00 a	3.16 a	0.37 b	0.96 a	2.71 a
M2	8.72 bc	1.03 a	7.29 b	0.11 a	0.80 a	6.47 b
M3	11.48 c	2.81 c	29.54 d	0.24 ab	2.43 c	9.57 c
M4	736 ab	1.87 b	12.40 c	0.27 b	1.69 b	6.17 b

	Ratio A/B	Ratio A/C	Ratio A/D	Ratio B/C	Ratio B/D	Ratio C/D
H1	6.83 a	1.02 b	5.44 a	0.21 b	0.85 b	5.50 a
H2	8.46 ab	2.42 d	20.62 c	0.31 b	2.19 c	7.59 b
H3	8.23 ab	2.89 e	26.03 d	0.38 b	2.59 c	7.15 b
H4	10.05 b	0.42 a	2.74 a	0.06 a	0.30 a	5.44 a
H5	6.90 a	1.76 c	10.66 b	0.27 b	1.41 b	5.48 a

(b)

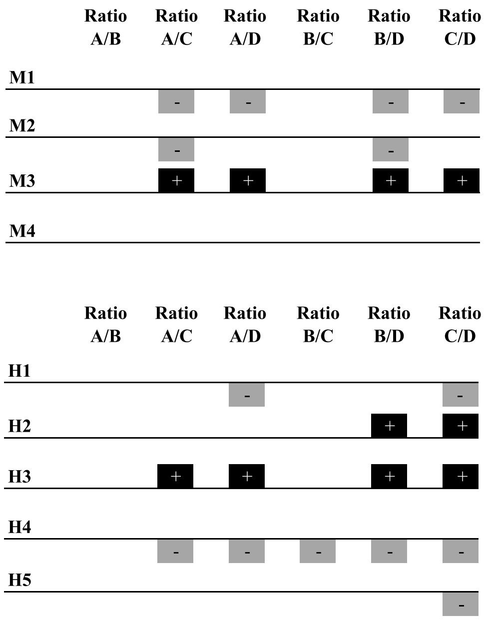


Fig. 3. (a) Mean ratios between guayulins for the four models established (M1–M4) and the five-harvest dates (H1–H5); (b) Graphical representation of significant differences between models or harvest dates.

Different letters within the same column indicate significant differences ($p < 0.05$) within models or harvest dates. Black bars indicate the significant superior values and shaded bars denote the lowest values.

production of guayulin A. This could indicate that the accessions in Models 1 and 2 are accessions with a later cycle, likely meaning that they need more time to recover the normal levels of guayulin A and B after the winter.

4. Discussion

Our results confirm our previous findings in CAL-1 and 11619 (Rozalén et al., 2021b), showing that the profile of guayulins changes during the autumn season. As validated here using 14 guayule accessions, there is a marked decrease in guayulin A and B content at the same time of the year. Analysis of the entire plant growth cycle revealed the existence of heterogeneity between the accessions. Most previous studies note that guayulin A is the major guayulin (Sidhu et al., 1995; Teetor et al., 2009), although guayulin C content can also be very high in some accessions (Spano et al., 2018). In total, 14 accessions were categorized into 4 groups or clusters based on their guayulin accumulation profile, which correspond to four response models. The findings suggest that guayulins *per se* might be good biomarkers of guayule origin or hybridization. It is known that breeding efforts to increase the content of natural rubber of this plant in the 1980s and 1990s also resulted in an increase in the resin content of the newly developed accessions. It is possible that guayulins, which are constituents of the resin fraction, also increased in content with the selection and development of new accessions. Mariola hybrids (Model 1) had the lowest content of guayulins A and C, whereas Model 3, which comprises the more recently developed pure guayule accessions, had the highest content of guayulin A and C.

Of note, examination of the relationship between guayulins allowed for a better classification of the accessions than analysis of individual content (87.1 % vs 84.1 %). Model 1 showed the lowest values for all relations; Model 2 had low A/C and B/C values due to its remarkable guayulin C content; Model 3 had the highest values for A/C, A/D and B/D; and Model 4 showed intermediate values. In addition, there was a significant interaction between the established models and harvests when the guayulins were considered individually or as relationships, except for guayulin D and the A/B guayulin ratio. This response has been described previously in autumn (Rozalén et al., 2021b). Guayulin D proportionally changed the least between the harvests of September and November, and the A/B guayulin ratio was the most stable. While the content of both decreased significantly in November, they maintained the same relationship as in September (Rozalén et al., 2021b). This seasonal decrease in guayulins A and B had been previously described (Schloman et al., 1983), but the close inter-relationship between the two has not.

Considering the evolution of the guayulins or their relation throughout the year increased the complexity of the study. A global glance revealed low values of guayulins in H1 (June), which increased in H2 (July) and H3 (September), reaching maximum values, to later decrease to a minimum in H4 (November), and showing partial recovery in H5 (April), particularly the content of guayulins A and B. The low absolute values in H1, independently of the model, could be due to the young age of the plant, which might still be developing (2 years), while the minimum values in H4 are likely due to a yet unknown metabolic process. Perhaps, as was postulated in previous studies, there is a link with the plant's need for amino acids (Schloman et al., 1986) or the production of rubber (Rozalén et al., 2021c). It was shown that the disappearance in H4 was not due to a decrease in the content of resins, nor to a greater growth development and therefore a redistribution of the resin in the plant, but rather to a minimal presence of the guayulins in the resin (Rozalén et al., 2021c).

Detailed analysis of each guayulin revealed three different patterns for guayulin A: 1) Models 1 and 4 reached a maximum content in H2 (July) that was maintained in H3 (September) before decreasing; 2) Model 2 seemed to have an earlier evolution, peaking in H2 and beginning to decline in H3; 3) Model 3 continued to rise to H3 before declining at H4 (November). This indicates that some models exhibit a similar response depending on the time of harvest. For example, there were no differences between Models 2 and 4 in H2 (July). By contrast in H3 (September), Model 2, which was already decreasing from H2, further decreased to the lowest content found in Model 1 with no difference between the two, with Model 4 being higher and Model 3 higher still. For guayulin B, three patterns were also observed: 1) Model 2 reached its maximum in H2 and was already decreasing in H3; 2) Model 1 maintained a constant guayulin B content throughout the year, except for the marked decrease in H4; and 3) Model 3 and 4 reached maximum values in H2 that were maintained in H3 (the highest values corresponded to M4) to decrease in H4 and recover, as in the remaining models, in H5.

The response of guayulin C was much more stable than that of guayulins A and B over time, to the point that Model 3 and 4 did not change at all with the harvest date. Model 1 presented with the lowest values of all the models in four of the five sampling points (H1–H4) and did not show significant differences within the same model between H2 and H5. Model 2 presented differences only between H3 and H4. Finally, guayulin D was unquestionably the most stable of all, and changes were only observed throughout the year in the Model 1.

The different responses of the established models, which seem to be linked (at least in part) to the genetic origin and hybridization of the accessions, could be related to a different response of the plant to the abiotic stress created both by the increase in excessive summer temperatures (H2) and by the abrupt fall in temperature in autumn (H4).

The Model × Harvest interaction in the multivariate analysis suggested that the accessions grouped under Model 1 were unaffected by temperatures, as they maintained low values of all guayulins and their ratios, and they were able to maintain a high content of guayulin D in the autumn (H4). In addition, at the end of winter (H5), as occurred with the accessions of Model 2, the levels of guayulin A and B that decreased during the winter recovered more slowly. In addition to the late cycle, the Model 2 accessions seemed to be affected by the high temperatures of August and September, with maximums above 30 °C and minimums above 17 °C. In harvest H3, the Model 2 accessions showed a slowdown, and even a decrease, in the accumulation of some guayulins. Conversely, the guayulin content in the accessions for Models 3 and 4 continued to increase kept on growing from H2 to H3 and the contents of guayulins A and B recovered faster. Model 4 was unique in that it continued to show an increase in guayulin B content in H3, while, at the same time, the Model 3 accessions continued to accumulate guayulin A and total guayulins.

While the best time to harvest guayule for rubber production may be at the end of winter (Downes and Tonnet, 1985), this was not evident for guayulins, as the best time seemed to be between September and November. A more detailed study of this time period is warranted, because an earlier harvest, with high temperatures from extended summers that are common in this area, could reduce the number of plants that re-sprout. By contrast, a late harvest would mean losing the valuable content that plants store as guayulins.

With regards to industrial exploitation of guayulins, the accessions should be chosen depending on which guayulin is of the greatest interest, and based on the greatest activity in any type of bioassay. For example, if guayulin A is the goal, any accession of Model 3 could be chosen. Alternatively, an accession of Model 4 could be used to obtain guayulin B, linked to an early harvest in September; or a Model 2 accession in the case that guayulin C is the choice; or a Model 1 accession if guayulin D is chosen for exploitation.

Broadly, it can be stated that the most interesting accessions to produce significant amounts of guayulins are the 11591(CL-1) and AZ-6 accessions, both of them included in Model 3. They produce the highest concentration of guayulin A and, although they do not produce the highest amount of guayulin C, the purification of the guayulin C may be simpler, as the plants contains a lower amount of guayulin D, which is more structurally and chromatographically similar to guayulin C (Rozalén et al., 2021c). Whether one of these accessions is chosen for the global production of guayulins, guayulin B can be obtained by the semi-synthesis processes known from guayulin A (Querci et al., 2017).

5. Conclusion

A characteristic profile of guayulin accumulation has been established along the growth cycle of guayule. In general, all accessions increase their content of guayulins A and B between spring and autumn, after which the content of both is drastically reduced by the cold temperatures, and gradually recovers after winter. Guayulins A and B maintain a close correlation that does not change significantly throughout the cycle. By contrast, the amount of guayulins C and D is much more stable throughout the cycle. The accessions with the highest production of total guayulins do not show many changes in their profile, whereas seasonal variation is observed for plants that produce a lower quantity.

Four different response patterns were identified based on the accumulation of guayulins and these may be related to the genetic origin and the degree of hybridization of the accessions.

Models are affected differently along the year. In addition to the poorer accumulation of guayulins, Models 1 and 2 seem to respond slower to the arrival of the good weather, to recover the content of guayulins A and B that were lost during winter. By contrast, Models 3 and 4 are more resilient to high temperatures, as they continue accumulating guayulins during the hottest season (guayulin A in Model 3 and guayulin B in Model 4) and, in addition, increase the accumulation of guayulins A and B more quickly with the arrival of Spring. Finally, 11591(CL-1) and AZ-6 (Model 3 accessions) with 11.6 g of guayulins kg^{-1} DW and 13.2 g of guayulins kg^{-1} DW respectively, are the most interesting to produce guayulins in large quantities under the soil and climate conditions studied.

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CRediT authorship contribution statement

Juana Rozalén: Investigation. **M. Mercedes García-Martínez:** Investigation. **M. Engracia Carrión:** Data curation, Formal analysis, Visualization. **Amaya Zalacain:** Methodology, Supervision, Writing - review & editing. **Horacio López-Córcoles:** Methodology, Investigation. **Manuel Carmona:** Conceptualization, Methodology, Validation, Visualization, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113829.

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