

# Assessment of phenotypic diversity in the USDA, National Plant Germplasm System (NPGS) guayule germplasm collection

V.M.V. Cruz<sup>\*</sup>, D.A. Dierig, A. Lynch, K. Hunnicutt<sup>1</sup>, T.R. Sullivan, G. Wang, J. Zhu<sup>2</sup>

Bridgestone Americas, Inc., Eloy, AZ 85131, USA

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## ABSTRACT

Phenotypic characterization on historical breeding lines and wild genetic resources from the 1950's to 1990's of the rubber-bearing plant guayule (*Parthenium argentatum* A. Gray) and hybrids was conducted. Variation among germplasm on seed weight and germination was determined along with leaf traits and trichomes using image analyses. Flow cytometry was performed to estimate diversity in ploidy levels. Tetraploid guayule was the predominant type in the germplasm collection (64%), followed by triploids (34%), pentaploids (1.5%), and hexaploid (1%). No plants with octaploid or higher ploidy level were observed. Guayule 100-seed weight averaged 57.5 mg and was determined to have significant positive correlation to 7 and 14-day seed germination and seed size for this seed lot. Phenotypic similarity among germplasm was determined from quantitative analysis of leaf traits and trichomes. Measurements of other traits on young leaves were highly correlated to those on mature leaves. Most leaf traits within each development stage were found to be positively correlated. Guayule leaves averaged 6.85 cm long and 2.06 cm wide, with 1–6 serrations. Leaf serration height ranged from 2.9 to 6.4 mm. Germplasm lines AZ-2, AZ-3, and N566 had the greatest number of serrations, while R1093 and W6-429 with the least number. Trichome density was consistent between young and mature leaves with average density of 17 trichomes per 0.04 mm<sup>2</sup>. Accessions R1100, AZ-3, and CAL-4 had the highest trichome density (>21 trichomes per 0.04 mm<sup>2</sup>). The results from analyzing the relatively high number of plants for seed parameters, leaf traits and ploidy are useful for optimizing germplasm collection and to better aid agronomic management practices and utilization of guayule germplasm for crop improvement activities.

## 1. Introduction

Guayule (*Parthenium argentatum* A Gray) is a perennial species in the Asteraceae family and has a long ethnobotanical history that dates to the Aztecs in the 1500 s (Lloyd, 1911). However, like other new crops, it has a sporadic research interest historically (Coffelt et al., 2015). Native guayule populations in Mexico have been harvested and utilized as source of natural rubber since the 1800's. During World War II, when the U.S. rubber supply from the Asia-Pacific region was cut-off, the federal government embarked on mass planting guayule and increased research activities to ensure a stable domestic supply. Though there was a significant amount of acreage successfully established in California, Arizona, and Texas during the 1940's, large scale guayule research and development activities were abandoned when the war ended (USDA, 1946). Bridgestone Corp. started its research on guayule in 2012 to

pursue commercialization of guayule in the U.S. Southwest as part of its sustainability initiatives. Other private companies in Europe and Australia, such as Versalis, Guatecs, Nokian Tyres, and Guayule Australia Pty. Ltd., currently also have ongoing research activities (Jara et al., 2019; Rousset et al., 2021).

Germplasm characterization efforts in guayule and long-term commitment to breeding are important to allow development of improved varieties for commercial deployment. In plant breeding, information derived from the assessment of diversity at the morphological, physiological, genetic levels and their interactions to different growing environments can be integrated into downstream research to tailor crop development activities to specific breeding targets (Swarup et al., 2021).

This study analyzed trait variability in seed, leaf, and ploidy of guayule. Seed size traits are often positively correlated to vegetative

<sup>\*</sup> Corresponding author.

E-mail address: [cruzvonmark@bfusa.com](mailto:cruzvonmark@bfusa.com) (V.M.V. Cruz).

<sup>1</sup> Present address: University of Nebraska, Lincoln, NE 68583.

<sup>2</sup> Present address: Northeastern University, Boston, MA 02115.

growth and biomass (Aparicio et al., 2002; Herron et al., 2020). Leaf traits are important indicators of plant growth and photosynthetic capability. Leaves were also reported as a suitable indicator of genetic diversity and selection efficiency in some breeding programs with traits that were under strong genetic control (Ren et al., 2020). In guayule, trichome morphology and leaf shape were proposed to have potential uses for identifying high rubber plants in wild populations as well as verifying accession identity and hybridity (Mehta et al., 1979; Hashemi et al., 1988a; Abdel-Haleem et al., 2019). Thompson and Ray (1989) indicated that the formation of leaf lobing is also an indication of guayule plant maturity. Until now, there has been limited quantitative morphological characterization of leaf traits in guayule germplasm collections.

The ploidy level of a subset of plants from several guayule germplasm accessions have been analyzed and reported in the literature. Even though there have been investigations on ploidy of different accessions through cytology and flow cytometry, the number of plants analyzed to determine intra- and inter- accession variability was quite limited (Stebbins and Kodani, 1944; Gore et al., 2011; Sanchez et al., 2014). In this study, we included a greater number of representative plants for each germplasm accessions to have an updated assessment of inter and intra-accession guayule ploidy diversity.

## 2. Materials and Methods

### 2.1. Germplasm and seed treatment

Seeds of 70 guayule accessions were obtained from the U.S. National Arid Land Plant Genetic Resources Unit (NALPGRU), Parlier, CA (Supplemental Table 1). The seeds were cleaned for debris and were soaked in gibberellic acid (GA<sub>3</sub>) for 6 h to remove dormancy using a modified procedure from Naqvi and Hanson (1980). Seed viability data were obtained by conducting germination tests at 25/15 °C in a growth chamber (Conviron, USA) for 14 days with light, 12 h/day at 17,688 lux, 19.2 DLI (mol/m<sup>2</sup>/day), and 221.8 PPFD (μmol/m<sup>2</sup>/s). Observations were collected seven days after planting (7DAP) and 14 days after planting (14DAP).

### 2.2. Planting, field trial design and maintenance

Gibberellic acid (GA<sub>3</sub>) treated seeds were mixed with potting soil (Pro-Mix PGX Soil, PT Horticulture, USA) and direct seeded on raised beds. Planting was conducted in Eloy, AZ on fields at the Bridgestone Americas, Guayule Agro-Operations Farm (32.679057, -111.625182). Seedlings were established using sprinkler irrigation. Subsequent irrigations were done using the furrows until harvest. Each germplasm was planted in four row plots (3 m long x 4 m wide) that were replicated four times following a randomized complete block design. Weeds were controlled by pre-emergence herbicide Prowl H<sub>2</sub>O (pendimethalin) at 3 qt/acre (7 L/ha), post-emergence herbicide Aim® EC (carfentrazone-ethyl) at 1.6 oz/acre (117 ml/ha), and Fusilade DX (fluazifop-P-butyl) at 20 oz/acre (47 L/ha). Mustang® (S-Cyano (3-henoxyphenyl)methyl (±) cis/trans 3-(2,2-dichloroethenyl)- 2,2 dimethylcyclopropane carboxylate) was applied at 4.3 oz/acre (10 L/ha) for flea beetle control one week after emergence.

### 2.3. Seed and plant data

Comparison of seed weights was made using clean seeds of each germplasm prior to initial germination testing and field planting. Seed sizes were obtained using Image-J v. 1.52 (Schneider et al., 2012) on AZ-2 to see if seed weight can be a proxy for size. After plant establishment in the field, sampling for leaf trichome density, leaf area and morphology was done using five random plants from plot replicates selecting five leaves that were in the early growth and five in mature growth stages. Forty-five accessions were analyzed for leaf area and

morphology, while 42 for trichome density and morphology. Only healthy leaves were sampled from the plant canopy to represent each development stage.

For whole leaf morphology, measurements on 19 traits were obtained from 45 *P. argentatum* accessions by scanning leaves using a Canon LiDE 220 (Canon, USA) at 600 dpi and extracting contours using MorphoLeaf software (Biot et al., 2016). The leaf area estimates from MorphoLeaf was compared with measurements from LI-3100 C leaf area meter (LI-COR, USA), and looking at the measurement correlation as well as doing a paired *t*-test analysis. For trichome analysis, a clear tape was pressed on the leaves and peeled off following Siebert (2004). The lifted trichomes were observed for length and width, where the latter was obtained from the widest section of the trichome, using a Nikon Eclipse Ci microscope system equipped with an NIS-Elements D v. 4.5 image analysis software (Nikon, USA). A 200 × 200 μm (0.04 mm<sup>2</sup>) viewing area was used to count leaf trichomes to estimate density. Observations on ten germplasm (N565, CAL-7, 11635, 11646, R1101, R1093, AZ-5, A48118, AZ-3, and N566) were analyzed first to compare estimates from looking at different leaf sections and sides on both young and mature leaves before proceeding to get observations on the remainder of the accessions.

Plant biomass data were collected on one and two-year old shrubs after cutting whole plants at ground level. Rubber and resin data were collected by air drying the shrubs under the shade to approximately 15% moisture. Plants were chipped and ground for laboratory analysis. Rubber and resin content estimates were obtained using near infrared spectroscopy (NIR) that was calibrated to the Soxhlet solvent extraction method (Black et al., 1985; Suchat et al., 2013; Placido et al., 2020).

Flow cytometry analysis was done on 56 accessions (*P. argentatum* and hybrids) to determine ploidy content using leaf samples according to the method described in Cruz et al. (2017).

### 2.4. Data analysis

Comparison among entries was carried out using JMP v. 16 (SAS, USA). All data were checked for normality. The biomass data was log-transformed due to non-normality. The Shannon's index (Shannon and Weaver, 1949) was calculated using ploidy data according to the formula  $H = - \sum_{i=1}^n p_i \ln p_i$ , where  $I$  was the relative proportion of the ploidy type to the total types per germplasm ( $p_i$ ).  $H$  accounts for both abundance and evenness of the ploidy types that are represented by plants in each germplasm.

## 3. Results

### 3.1. Variation in seed weight and germination

Seed weight. The guayule germplasm set had an average 100-seed weight (HSW) of 57.5 ± 13.8 mg, with CV of 24.08%. Seed weight was found to have high correlation to seed size (seed shape area, perimeter, and diameter estimates) using observations from AZ-2 ( $r = 0.80$ – $0.85$ ). The average seed diameter (Feret diameter) of AZ-2 was 3.4 ± 0.3 mm.

Heavier seed weights were observed on PARL 914 (83.3 mg), PARL 919 (80.5 mg), AZ-6 (79.1 mg), PARL 915 (75.9 mg), and 11591 (74.4 mg), while it was the opposite on PARL 934 (23.4 mg), CAL-1 (32.1 mg), CAL-2 (35.5 mg), AZ-2 (38.7 mg), and R1109 (39.1 mg). Since these seeds may have been from different seed lots and harvest times from the NALPGRU genebank and guayule seed filling was reported to be influenced by several factors (such as genetics, pollinator activity, and climatic factors) as reviewed by Jorge (2005), fair comparison among germplasm was difficult.

In general, guayule seed weight was found to have significant positive correlation to 7- and 14-day seed germination percentages,  $r = 0.82$  and  $0.81$ , respectively (Fig. 1). It was also observed to have weak

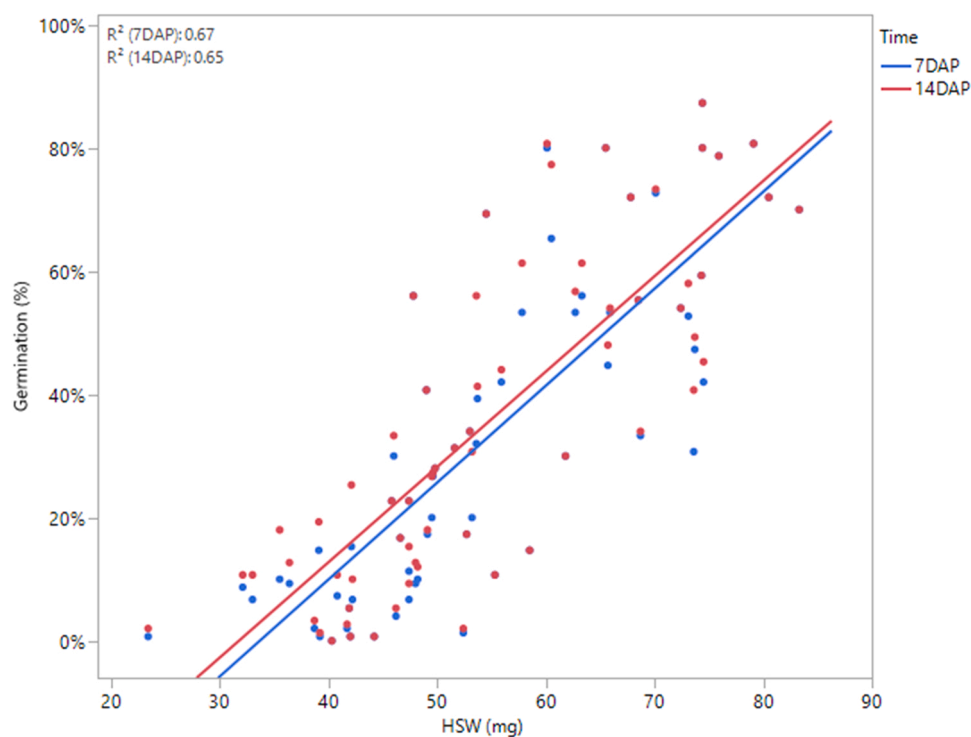


Fig. 1. Correlation between 100-seed weight (HSW) and germination percentage at 7 and 14 days after planting (DAP) on 67 guayule germplasm.

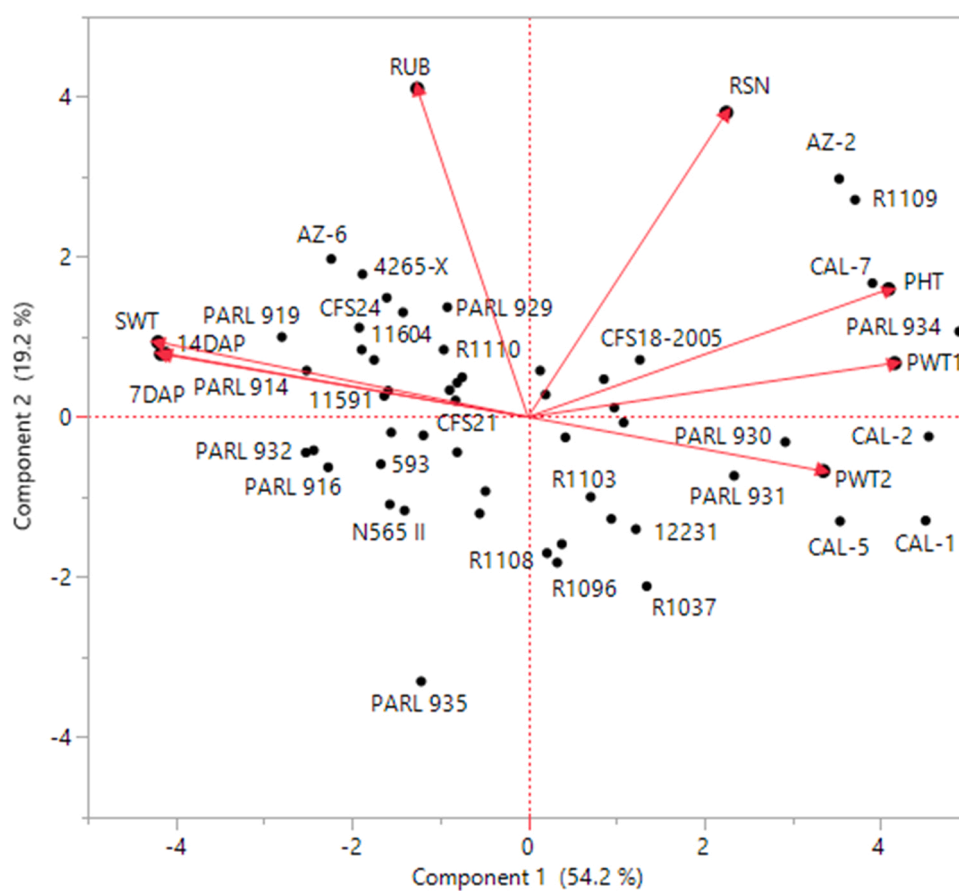


Fig. 2. Biplot of seed weight and other plant traits (SW = seed weight, 7DAP and 14DAP = seed germination, PWT1 = plant biomass after one year, PWT2 = plant biomass after two years, PHT = plant height, RUB = rubber content, RSN = resin content).

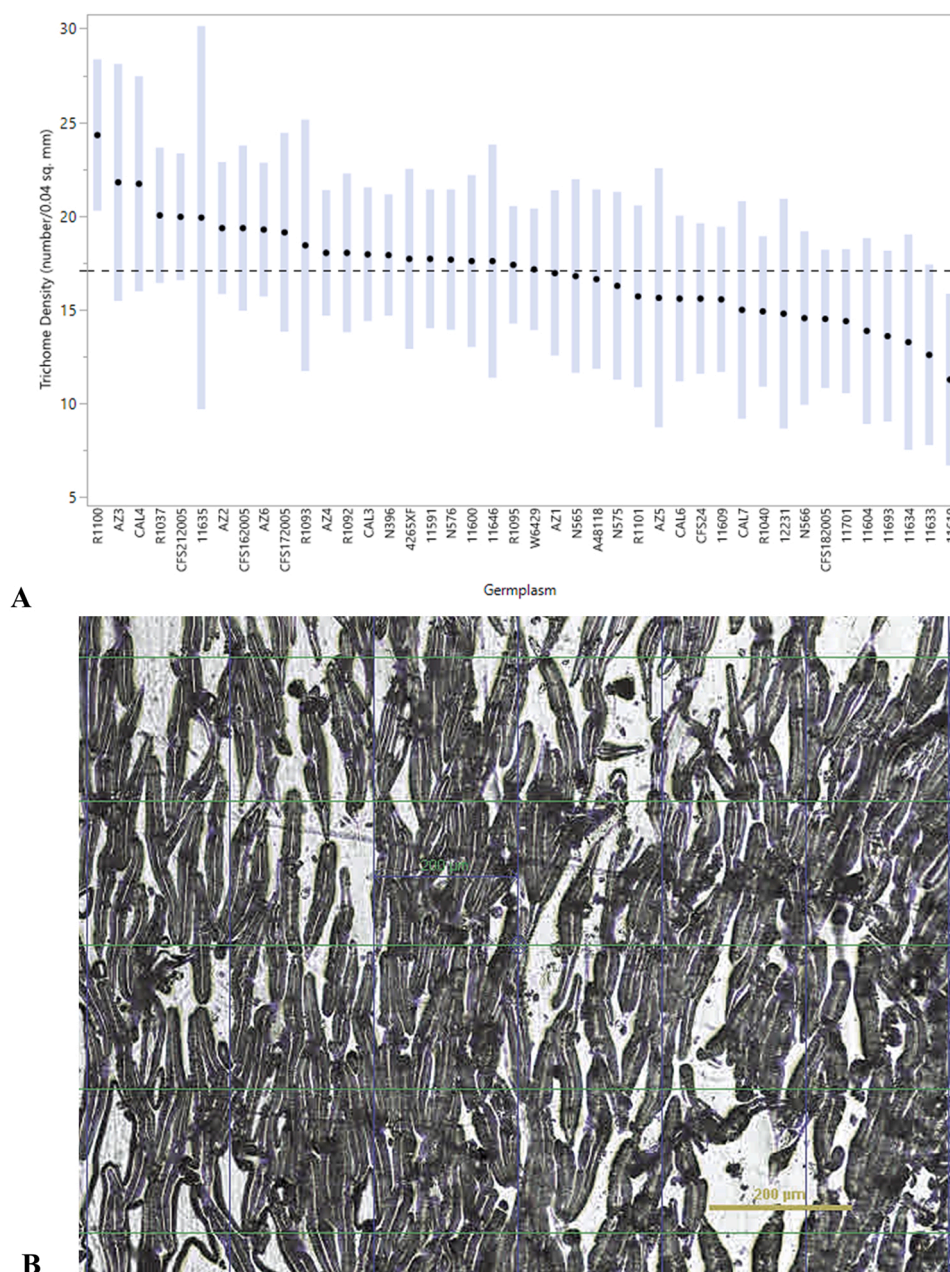
positive correlation to rubber content (RUB) ( $r = 0.34$ ,  $p = 0.0132$ ). Seed weight had significant negative correlation to plant height (PHT) ( $r = -0.55$ ,  $p = <0.0001$ ) and biomass (PWT1) ( $r = -0.60$ ,  $p = <0.0001$ ), suggesting a possible trade-off between sexual reproduction and vegetative growth. For these specific seed lots, accessions with low germination and lighter seeds were those with large plants such as R1109, CAL-2, AZ-2 compared with PARL 932, PARL 914, and 11604 having the opposite. The biplot summarizing the relationship among these variables is shown on Fig. 2.

Seed germination. Guayule seeds from NALPGRU collection showed a wide variation in germinability (7DAP, CV=65.0%; 14DAP, CV=60.6%). The average germination at 7DAP was 39.3% that increased at 14DAP to 41.8%, a 2.5% overall increase. There was significant positive correlation between the 7DAP and 14DAP germination with  $r^2$  of 0.99. Fig. 2 shows how seed germination was related to other phenotypic traits.

For these batch of seeds, the highest germination was observed on 11604 (87.3%), AZ-6 (80.7%), CFS24 (80.0%), PARL 932 (80.0%), and PARL 915 (78.7%) while poor germination was recorded on PARL 934 (1.0%), PARL 931 (2.0%), AZ-2 (2.0%), PARL 930 (4.0%), and CAL-7 (7.3%). CFS21 had the largest increase in germinability of 24% between 7DAP and 14DAP, followed by PARL 923 with 12.0%, then CAL5 and N576, both exhibited a 10% increase. Twenty-three germplasm did not display any difference in germination percentage between 7DAP and 14DAP.

### 3.2. Variability in leaf morphology

Nineteen leaf traits were analyzed on 42 accessions. All guayule leaves were oblanceolate to lanceolate with the leaf blade length on young leaves ranging from 3.19 to 7.65 cm, with an average of  $5.40 \pm 1.1$  cm., while on mature leaves 3.72–9.33 cm, with an average



**Fig. 3.** A. Mean and standard deviation of trichome density. Analysis of Means ( $\alpha=0.05$ ) showed that R1100, AZ3, and CAL4 have significantly higher density while 11604, 11619, 11433 and 11634 have significantly lower density. B. Trichomes of 11619 on leaf adaxial side (bar = 200  $\mu$ m).



of  $6.86 \pm 1.1$  cm. Accessions with large leaves according to leaf area, included AZ-2, R1040, 11600, and 12229, while those with the smallest were W6-429, R1100, R1093, R1101, and 11633. The accessions with most leaf tooth (serrations) on mature leaves were AZ-3, R1037, AZ-2, R1101, and CFS21, while those with the least were W6 429, R1093, 593, N575, and 12229 (Supplemental Table 3).

There was a strong positive correlation between blade leaf length from the two sampled leaf developmental stages ( $r = 0.80$ ). Among all traits, leaf blade perimeter had the highest correlation to blade length ( $r = 0.98$  on young (new) and mature (old) leaves). The leaf area estimates from the MorphoLeaf software were found to have strong correlation ( $r = 0.97$ ) to values from the LI-COR leaf area meter (Fig. 5a). However, the average estimate from the LI-COR leaf area meter was significantly lower by  $0.55 \text{ cm}^2$  compared with the average estimate from the MorphoLeaf software (paired  $t$ -test results not shown). The

small discrepancy between the leaf area estimates from image analysis and LI-COR leaf area meter in this study might be from fresh leaves curling when fed through the machine's conveyor system. The correlations among the other leaf variables are shown in Fig. 5b, indicating that more than half of all variable combinations have moderate to strong positive correlation. Between the wild and cultivated germplasm set, it was determined that the former had significantly shorter leaf blade length (average of 5.9 vs. 7.2 cm) and less width (1.8 vs. 2.1 cm).

The petiole width of young leaves ranged from 0.82 mm to 2.27 mm, while mature leaves had petiole that were 0.90 (W6 429) to 2.63 mm (11600) wide. The accessions with the greatest number of teeth or leaf serrations were AZ-2 (6.1), AZ-3 (5.6), N566 (5.4), CAL-4 (5.2), and R1037 (5.0), while those with lowest average number of serrations were R1093 (1.5), W6 429 (1.7), 11693 (1.8), R1092 (1.8), and 12231 (1.9). There was a strong positive correlation between the total number of

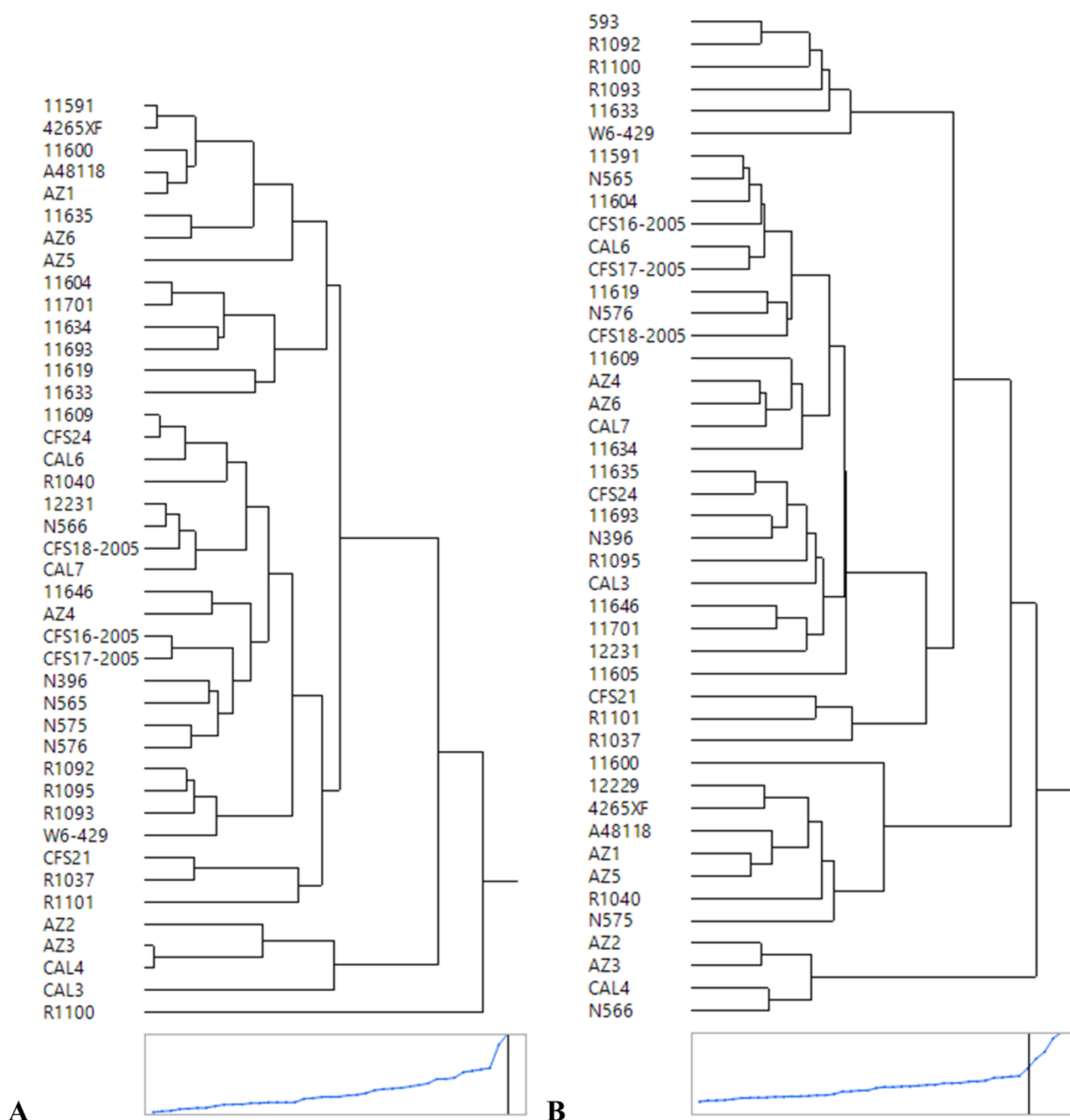
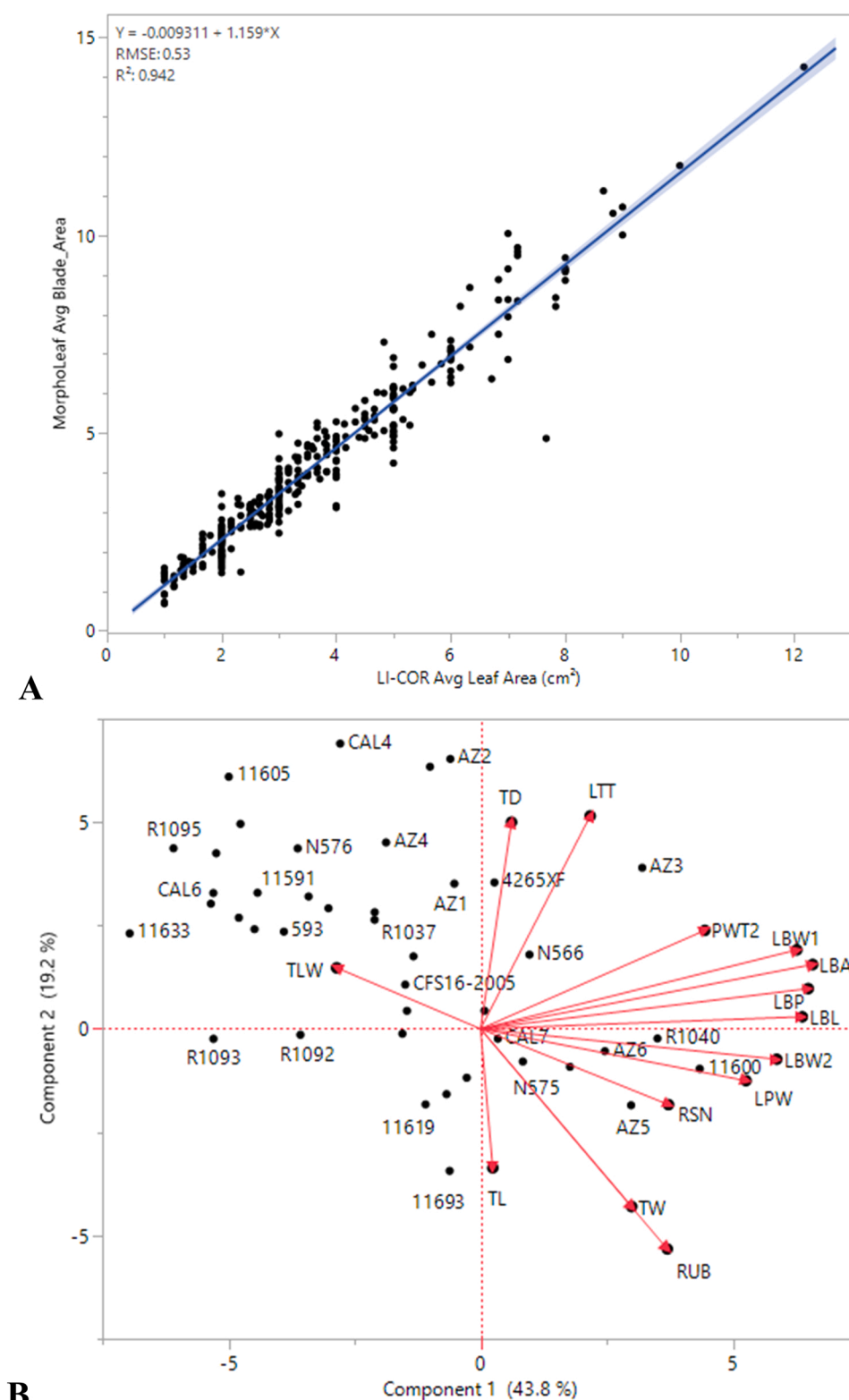


Fig. 4. Clustering of accessions based on leaf trichome morphology and density (A) and overall leaf morphology, size, and serration parameters (B).



**Fig. 5.** Correlation between mature guayule leaf area measurements of LI-COR and MorphoLeaf (A) and relationships among guayule leaf, trichome and plant phenotypes (B). Abbreviations denote the following: RUB = rubber content, RSN = resin content, PWT2 = dry weight of 2 year old plants, TL = trichome length, TW = trichome width, TLW = trichome length:width ratio, LBL = blade length, LBW1 and LBW2 = MorphoLeaf blade widths BB and IS, respectively, LPW = leaf blade perimeter.

serrations in young and mature leaves of guayule ( $r = 0.80$ ).

The accessions with the deepest serrations (tallest teeth) on mature leaves were 11600 (5.52 mm), AZ-5 (4.96 mm), 11605 (4.78 mm), 4265XF (4.72 mm), and A48118 (4.38 mm), while the shallowest serrations were on W6 429 (1.05 mm), R1100 (2.02 mm), R1093 (2.03 mm), R1092 (2.12 mm), and 593 (2.18 mm). The average leaf tooth height/width ratio was 0.45. The accessions 11605, A48118, and

CFS24 showed 1.0, 0.82, and 0.72 height/width, respectively indicating their leaves have serrations that were tall as they were wide. Fig. 4b shows the grouping of germplasm based on these leaf trait measurements.

Plant dry biomass had significant positive correlation with leaf measurements, except the number of teeth (serrations) (Fig. 5b). Leaf width (BB) and blade area showed high correlations with plant dry

biomass ( $r = 0.82$  and  $0.78$ , respectively). All leaf trait measurements had weak correlation to rubber content. Only leaf petiole width showed a slightly higher value at  $r = 0.30$ . Resin content had moderate correlation to most leaf traits with leaf blade area, leaf and petiole width being significant ( $r = 0.66$ – $0.79$ ). Compared with rubber, guayule resin content was described to be more constant throughout the growing season and plant development (Rozalén et al., 2021).

### 3.3. Characterization of leaf trichomes

The trichome densities of young and mature guayule leaves from the first ten accessions were found to be highly correlated ( $r = 0.97$ ), thus the full analysis was subsequently performed on the rest of the accessions using older leaves only. The density estimates from random observation points on the adaxial, abaxial, and midrib epidermal surface sections of the leaves were all found to be highly correlated with  $r > 0.98$ , indicating uniformity across the leaf sections.

Trichome density averaged from the three leaf sections (number of trichomes/ $0.04 \text{ mm}^2$ ) ranged from a high of 24 (R1100) to 11 (11619), with an overall average of 17 (Fig. 3). Three germplasm (R1100, AZ-3, and CAL-4) were found to have significantly higher average density, while four (11604, 11619, 11433 and 11634) have significantly lower density and were more glabrous. The AZ-2 germplasm had an average density of 19. W6 429 and CAL-3 have 17 which was comparable to the

polyploids N396, 4265XF, 11591, N576, 11600, 11646, and R1095 (Fig. 3). Between the wild and cultivated germplasm groups specified in the GRIN database (refer to Supplemental Table 1), the former was observed to have significantly greater trichome density ( $18.1$  vs  $16.6$ ,  $p = 0.04$ ).

Trichome length ranged from an average of  $65.86 \mu\text{m}$  (CAL-3) to  $114.91 \mu\text{m}$  (AZ-5), with an overall average of  $99.09 \pm 25.95 \mu\text{m}$ . AZ-2 and AZ-3 showed relatively shorter trichomes than 38 other germplasm, having an average of  $83.71 \mu\text{m}$  and  $81.23 \mu\text{m}$ , respectively. Trichome length had a CV of 27.9%. R1040, 11633, R1092, 11619, and CAL4 were the top five germplasm with high variability in length while 11591, N565, R1037, CFS21–2005, and R1095 had the least and were most uniform.

Trichome width ranged from an average of  $10.53 \mu\text{m}$  (R1100) to  $17.48 \mu\text{m}$  (11635). The average trichome width on all accessions was  $14.26 \pm 3.81 \mu\text{m}$ . The accessions with highest variability in width were CFS21–2005, CAL-4, CAL-6, 11619, and CAL-7 while those with relative uniform width were AZ-6, N396, R1095, A48118, and R1040. The trichome width and length have a very weak negative associations with density ( $r < -0.12$  and  $-0.14$ , respectively). The trichome length: width (L:W) ratio ranged from 11.03 (R1100) to 5.90 (CAL-3) (Table 1). Trichome width showed strong positive correlation to rubber content ( $r = 0.71$ ), while trichome length with weak correlation ( $r = 0.40$ ). Ten accessions had trichomes that were significantly longer than wide

**Table 1**  
Summary statistics of guayule leaf trichome parameters from microscopy measurements.

ID	Length (L)		Width (W)		L:W ratio		Density	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
11591	107.52	20.70	15.98	4.31	7.21	2.46	17.72	3.71
11600	103.23	26.33	16.78	4.66	6.67	2.65	17.60	4.59
11604	106.02	29.85	15.58	4.19	7.36	3.08	13.88	4.96
11609	98.99	24.53	14.84	4.28	7.26	2.95	15.56	3.87
11619	101.96	30.92	14.10	4.31	7.93	3.65	11.28	4.58
11633	92.20	27.82	15.20	4.36	6.57	2.76	12.60	4.83
11634	99.58	28.12	15.68	3.93	6.74	2.53	13.28	5.75
11635	96.78	24.87	17.48	4.92	6.00	2.44	19.92	10.22
11646	97.38	25.09	15.46	4.00	6.76	2.71	17.60	6.22
11693	102.55	28.00	17.11	4.44	6.47	2.80	13.60	4.56
11701	108.23	28.85	15.72	4.05	7.40	2.95	14.40	3.84
12231	103.99	28.74	13.02	3.47	8.51	3.62	14.80	6.13
4265XF	106.20	28.85	15.96	4.42	7.31	3.61	17.72	4.80
A48118	101.76	27.92	16.08	3.76	6.64	2.32	16.64	4.79
AZ-1	103.89	29.48	16.23	4.17	6.80	2.57	16.96	4.41
AZ-2	83.71	21.09	14.38	4.05	6.34	2.59	19.36	3.53
AZ-3	81.23	19.87	12.66	3.54	7.03	3.40	21.80	6.32
AZ-4	90.90	21.34	14.85	3.55	6.54	2.25	18.04	3.35
AZ-5	114.91	31.19	17.35	4.31	7.02	2.57	15.64	6.92
AZ-6	100.68	28.70	16.99	3.89	6.27	2.44	19.28	3.58
CAL-3	65.86	15.60	12.18	3.50	5.91	2.40	17.96	3.58
CAL-4	80.88	26.04	12.50	3.70	7.01	3.09	21.72	5.74
CAL-6	103.01	28.48	15.17	4.53	7.44	3.23	15.60	4.43
CAL-7	107.81	29.09	13.03	4.49	9.40	4.67	15.00	5.80
CFS16–2005	100.26	24.44	14.07	3.80	7.77	3.45	19.36	4.41
CFS17–2005	101.09	26.48	14.59	3.78	7.52	3.14	19.13	5.30
CFS18–2005	100.89	24.64	13.51	3.87	8.10	3.12	14.52	3.69
CFS21–2005	110.41	24.97	12.04	3.54	10.03	4.05	19.96	3.38
CFS24	98.44	25.64	15.12	3.72	6.87	2.41	15.60	4.02
N396	101.00	28.55	12.62	2.90	8.40	3.05	17.92	3.24
N565	96.32	21.46	13.32	3.17	7.66	2.77	16.80	5.17
N566	104.26	26.87	13.44	3.79	8.37	3.11	14.56	4.64
N575	101.89	29.20	13.92	3.46	7.80	3.31	16.28	5.01
N576	102.37	25.38	14.17	3.41	7.68	2.87	17.68	3.75
R1037	108.97	24.49	13.04	3.66	9.04	3.41	20.04	3.62
R1040	94.43	27.64	13.87	3.28	7.21	2.83	14.92	4.02
R1092	95.95	28.98	12.53	3.61	8.32	3.87	18.04	4.25
R1093	91.49	22.51	11.93	3.26	8.26	3.28	18.44	6.71
R1095	92.06	21.28	12.66	2.95	7.66	2.56	17.40	3.14
R1100	107.67	24.91	10.53	2.86	11.03	4.13	24.32	4.04
R1101	108.10	27.58	11.18	3.18	10.51	4.34	15.72	4.86
W6 429	86.79	23.56	11.91	3.13	7.83	3.16	17.16	3.25
Average	99.09	25.95	14.26	3.81	7.59	3.06	17.04	4.69

compared to the average ( $L:W > 7.61$ ). This included R1100, R1101, R1093, R1092, R1037, N566, N396, CFS21-2005, CAL-7, and 12231. Cluster analysis using trichome traits resulted in groups of germplasm that have long and wide trichomes occurring with intermediate density (represented by 11591, 11600, 4265XF, AZ-6), long and wide trichome occurring with low density (AZ-5, 11604, 11619, 11633), short and thin trichomes with high density (AZ-2, AZ-3, CAL-4), and long and thin trichomes with very high density (R1100) (Fig. 4a).

### 3.4. Variation in ploidy

There was a total of 1410 plants with an average of 26 plants per germplasm characterized for ploidy. The highest number of plants analyzed was from R1109 (59) and the least on CAL5 (1) due to poor plant establishment on the latter. Approximately 64% of all plants were tetraploid (4X), 34% triploid (3X), 1.5% pentaploid (5X), and 1% hexaploid (6X) (Fig. 6a). Twenty-six accessions were uniform on ploidy or homogeneous, while 28 were mixtures. Among the accessions, 23 (42.6%) had plants that were all 4X and homogenous. This included AZ-2, AZ-5, and AZ-6. Unexpectedly, all CAL-3 plants in this study were determined to be 4X and was contrary to its breeding history and expectation of being a diploid (Tysdal et al., 1983; Gore et al., 2011).

Three accessions were homogeneous as 3X (0.6%). These accessions were PARL 935, R1093, and R1108. The remaining 28 accessions (51.8%) had mixed ploidy (Fig. 6b). There was an average of 5.2% occurrence of 6X plants within the 12 accessions where these were observed.

The Shannon diversity index on ploidy ranged from 0 to 1.1, with an average of 0.20. The most diverse accessions based on this index were 11619, CFS24, CAL-2, CAL-5, and N565-II. These were comprised of plants of different ploidy. 11619 and N565-II have plants that were 3X to 6X. These were the only two accessions that showed plants in all four ploidy classes.

## 4. Discussion

Characterization of germplasm collections is critical for plant genetic resources management and subsequent germplasm utilization. In guayule, there is only limited data on plant characterization and evaluation. A standard seed germination testing method on guayule has not been formulated according to Chirco and Turner (1986), but studies to optimize germination testing parameters and how to increase seed quality have been conducted. Wang et al. (2020) in particular, showed differential germination response of germplasm seed lots to various

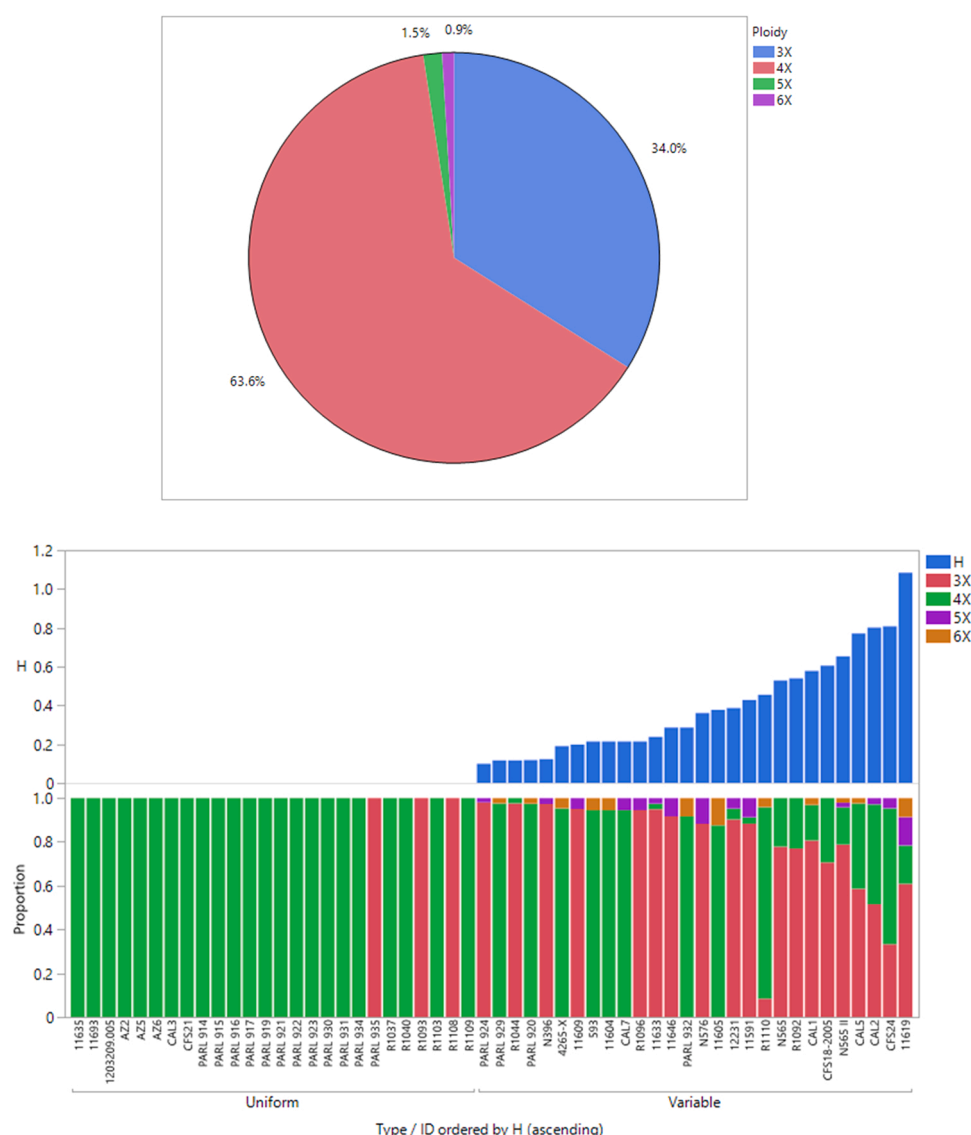


Fig. 6. Proportion of ploidy types in the total germplasm set (A) and occurrence per germplasm with the corresponding Shannon diversity indices (B).



temperature cycling treatments, indicating that optimal germination temperatures differ by germplasm. In relation to seed characterization, practices to look at seed color to assess seed filling has been done (Jorge, 2005). Only seed weight was among the descriptors set proposed for guayule where the focus was toward qualitative assessment of leaf shape, size, and serrations and extractable contents (Coffelt and Johnson, 2011). In this study, we assessed seed germination and conducted an alternate route of quantitative assessment of seed and leaf trait parameters that included analysis of trichome characteristics using imaging methods.

Determination of seed germination in guayule indicated that overall, only a small increase can be attained by extending the germination count to 14DAP. This suggests that estimates from 7DAP may enable shorter turnaround time when analyses of seed lots for planting decisions are quickly needed. There is still no official guideline for testing guayule seed germination and purity with some published methodologies doing initial germination count at either 7DAP or 10DAP and final count at 10DAP or 14DAP (Chirco and Turner, 1986). The results of this study reinforce that germination estimates at 7DAP are adequate when dormancy breaking treatments are successful. Overall, there was a wide range of germination percentage observed among the germplasm (1–87%) which indicated that seed lots of several accessions urgently need to be reviewed for regeneration and seed increase at NALPGRU. A comparison of seed germination from seed lots of freshly harvested seeds may provide additional information if there is differential response on dormancy breaking treatments among the various germplasm.

In general, guayule seed weight was found to be negatively correlated to plant height and biomass which was in line with observations on Asteraceae and other species where plants with lighter seeds exhibited higher growth rates (Fenner, 1983; Baraloto and Forget, 2007; Poorter et al., 2008). However, this trend does not seem to be universal (Benard and Toft, 2007; Turnbull et al., 2012). In another guayule study, Bedane et al. (2009) observed that taller AZ-2 plants exhibited larger and heavier seeds compared with smaller plants. If we combine this with our general observations, then a production cycle of small and large seeds will ensue.

Guayule seed weight was previously shown to be a good indicator of seed maturity, along with growing degree days and seed coat color (Jorge, 2005; Bedane et al., 2006). It should be noted that guayule produces flowers throughout the growing season resulting in seeds of different physiological maturity, thus variable seed weight. This primary factor causing the differences among seed weight among the germplasm analyzed in this study remain unclear because the seeds came from different seed lots at NALPGRU. However, careful hand seed collection of small seed lots is expected to be able to minimize this variability due to seed maturity since manual seed collection can target mature seeds visually. Future studies to compare seed weights using a common growing condition and harvest time may help provide information about this on the different guayule germplasm and validate our current results from using different seed lots.

The leaf sizes observed in the germplasm set was greater than what were reported in herbarium databases and formal taxonomic descriptions (SEInet, 2021; Flora of North America, 2021). The reported typical leaf length range in guayule was 1.5–2.5 cm versus 3.7–9.3 cm in this study. The larger leaves on guayule plants in this study could be from a more favorable growing environment because of soil, experimental plot location, and/or cultural management practices compared with plants in the natural habitat that depend only on natural precipitation. An inverse correlation between leaf size and moisture was reported to be typical in different plant communities (Jacobs, 1999).

The number of leaf serrations between young and mature leaves were found to be highly correlated ( $r = 0.83$ ). However, in terms of leaf serration there was a significantly greater number in mature compared with young leaves which supports the observations of Thompson and Ray (1989). The high correlation of resin to leaf size, specifically to blade area, blade perimeter, and petiole width would likely be due to the

leaves being the primary site for biosynthesis of polyisoprene and resin as reviewed by Rousset et al. (2021).

Leaf trichome characteristics in *Parthenium* species were variable and can indicate interspecific hybridization (Hashemi et al., 1988a and 1988b). The germplasm groups based on leaf and trichome-related traits indicated which accessions are phenotypically similar or unique in relation to the rest of the germplasm collection. R1100, is a wild germplasm and was found to have leaves with very thin trichomes occurring at a higher density compared with the other accessions in the collection. Genotyping data indicated that this is an interspecific hybrid, like R1037, R1101, AZ-2, and AZ-3 (Ilut et al., 2017). There were no leaf measurements from *P. incanum* and the other putative *Parthenium* parental species to compare these observations to in this study. The cluster analysis using MorphoLeaf trait parameters also indicated that the 'hybrid' accessions had large leaves with a greater number of serrations. This was consistent with observations by Rollins (1945) that interspecific hybrids, including those from tetraploid and triploid crosses, showed intermediate leaf serrations when compared with the parents. Several micro clusters of germplasm using the leaf measurements align with results of genetic similarity studies. For example, the morphological similarity among 593, R1092, and R1093 and among AZ-1, 4265XF, and 11600 were consistent to genetic similarity results in Ilut et al. (2017). Leaf similarity among AZ-4, 11634, and CAL-6 also agreed with observations on genetic similarity using SSR data by Cruz et al. (2015).

Healey et al. (1986) described trichomes of guayule as having cylindrical shapes compared with whiplike and conical trichomes of *P. fruticosum* and *P. schottii*. The high correlation in trichome density between the abaxial and adaxial leaf surfaces agreed with Healey et al. (1986) indicating that it was only the biserate trichomes in *Parthenium* that differ in density between the lower and upper leaf epidermis. The strong correlation of trichome width to rubber content that we observed necessitates further investigation if this trait can be used at a finer scale for identifying plants with higher rubber content.

In general, it is believed that trichomes play important roles in protecting plants from insect pests and in processes involving plant respiration and photosynthesis (Mofikoya et al., 2018; Amada et al., 2020; Karabourniotis et al., 2020). The higher trichome density on leaves of wild guayule germplasm suggest that these may have potential advantages. However, little information is known about leaf plasticity in guayule and the extent of direct environmental influence on leaf traits including that of trichomes. On other plants, trichome density varies with exposure to abiotic stresses. For example, in cotton and *Arabidopsis* there were greater trichome densities on plants that were subjected to drought stresses (Huttunen et al., 2010; Shahzad et al., 2021). There has been no study pertaining to this on guayule phenotypes. However, there was a related report on trichome abundance by Jayabalan et al. (2001). The authors reported trichomes that were  $> 180 \mu\text{m}$  long on one-year old guayule plants in India. Their estimates were about two times longer than those in this study on plants grown in Arizona after considering common germplasm (A48118, 11591, 11600, 11604, 11609, and N396). Jayabalan et al. (2001) did not find any correlation between trichome morphology and rubber content, contrary to Mehta et al. (1979) and results reported here.

Flow cytometry analyses indicated that 11635, 11693, AZ-2, AZ-5, and AZ-6 were 100% tetraploids supporting the findings of Gore et al. (2011). However, with relatively greater number of plants analyzed in this study, many accessions were determined to be comprised of plants with different ploidy. Among these were 11591, 11609, 11633, N565, and 12231 which were heterogenous and not uniformly triploids as previously described (Gore et al., 2011; Thompson and Ray, 1989). Another difference was on 11619, an accession observed to be predominantly composed of triploid plants, not tetraploids. Our results supported the observations in Sanchez et al. (2014) that 11591 and CFS21 were mixtures of different ploidy. On the other hand, CFS21 was uniform having all tetraploid plants, not a mixture of different ploidy.

The CAL-3 plants were determined to be all tetraploids, contrary to expectations that this is a diploid germplasm. Possible reasons for this include seed contamination or mislabeling in one of the seed handling process including but not exclusive to seed harvest during regeneration, seed cleaning and treatment, or planting.

## 5. Conclusion

Information about the diversity in leaf shape, trichome morphology, density and ploidy among guayule accessions was obtained. Accessions with contrasting traits and groups with general phenotypic similarity were identified which may help facilitate future studies where selection of diverse representatives is needed. Correlation among select plant traits were determined using a large representation of guayule germplasm accessions. Whether similar observations on the leaf and trichome traits can be obtained in a different characterization environment remain to be determined. The findings on ploidy level diversity in guayule germplasm collection supplements existing information on the collection and will be useful for formulating effective germplasm management strategies. We suggest that conservation methodologies tailored to germplasm with mixed ploidy should be framed to ensure collection integrity. In guayule breeding, the information about the ploidy of individual plants is useful for planning crossing schemes and anticipating results of crosses and seed increase activities.

## CRediT authorship contribution statement

**V.M.V. Cruz:** Conceptualization, Methodology, Data curation, Formal analysis, Writing -original draft, Writing - review and editing, Supervision, **D.A. Dierig:** Conceptualization, Writing - review and editing, Funding acquisition, **A. Lynch:** Methodology, Investigation, Resources, **K. Hunnicutt:** Investigation, Data curation, **T.R. Sullivan:** Methodology, Investigation, Writing - review and editing, **G. Wang:** Methodology, Data curation, Writing - review and editing, **J. Zhu:** Investigation, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indcrop.2021.114303](https://doi.org/10.1016/j.indcrop.2021.114303).

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