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Visualization of the Malleability of the Rubber Core of Rubber Particles from *Parthenium argentatum* Gray and Other Rubber-Producing Species under Extremely Cold Temperatures

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Rubber particles from *Parthenium argentatum* Gray (guayule) were frozen in liquid nitrogen (−196°C), fractured, and visualized using cryo-scanning electron microscopy. We observed that the rubber polymer core of the rubber particles was still malleable at this extremely cold temperature, and the core stretched substantially during separation of the fracture planes. This malleability was observed *in situ* in tissue sections, as well as in purified rubber particles, and was found to be independent of purification procedure, guayule line, tissue age, or season. The malleability or stretching phenomenon suggests that *P. argentatum* rubber has some unique properties because rubber particles from *Hevea brasiliensis* Müll. Arg. and *Ficus elastica* Roxb. were brittle at this temperature, fractured cleanly, or showed only tiny threads of material pulling out of the core.

KEY WORDS: Guayule; natural rubber; polyisoprene; rubber particles; cryo-scanning electron microscopy; field emission scanning electron microscopy.

INTRODUCTION

Parthenium argentatum (guayule) is currently being introduced into the southwestern United States as a commercially viable source of high-quality rubber latex that can be used to manufacture products safe for use by people suffering from Type I latex allergy [1–10]. This life-threatening allergy is caused by proteins in rubber products made from latex of *Hevea brasiliensis* (the Brazilian or para rubber tree), which is currently the sole commercial source [11] of rubber.

P. argentatum latex is similar to *H. brasiliensis* latex in that both are formed in membrane-bound rubber particles [12, 13] and both make high-quality, high molecular weight rubber [14], which in turn can be used to manufacture high-quality products [7]. However, some distinct differences also exist. For example, the protein and lipid complements are quite different between the two species [15], the particle size distribution is different [14, 16], and *P. argentatum* latex forms a softer and more elastic film than *H. brasiliensis* latex when compounded with a simple *H. brasiliensis* formulation [K. Cornish and H. Bader, unpublished results]. It has recently been shown that the *P. argentatum* latex consistently is more viscous than *H. brasiliensis* latex when compared over a wide rubber particle concentration range [16]. These results suggest that other properties also may be different in *P. argentatum* latex, compared to *H. brasiliensis* latex. The discovery and characterization of novel properties may lead to new value-added products and markets.

In this paper, we describe an unforeseen property of rubber particles: malleability of the rubber particle core at

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−196°C. We have extended our initial, very unexpected, single observation of this property [13] to a wide range of *P. argentatum* samples, including rubber particles *in situ*, rubber particles purified using different procedures, and rubber particles from different guayule lines, tissue ages, and seasons. We also compare our results with cryo-scanning electron microscope (SEM)-fractured rubber particles of *H. brasiliensis* and *Ficus elastica* (which, unlike *P. argentatum* and *H. brasiliensis* is known to produce low molecular weight, poor-quality rubber [14,17–19]).

EXPERIMENTAL

Cryo-SEM

P. argentatum isolated rubber particles and tissue pieces, as well as *H. brasiliensis* and *F. elastica* rubber particles, were prepared using an Alto 2500 Cryo Preparation System (Gatan, Pleasanton, CA; previously Oxford, Inc., UK) and viewed in a Hitachi S-4700 field emission SEM (Hitachi, Japan). All preparations were viewed and photographed at 1.5–5 kV. Details are described below.

In Situ Rubber Particles

Tissue Excision

Pieces of tissue (approximately 3 × 5 mm) were excised from stems of three different diameters (<5, 5–10, and >10 mm) from three different lines (O-16, N9-5, and AZ101) of *P. argentatum* plants, that were greenhouse-grown in Albany, CA. The stem is still part of the living plant, thus the method of sampling is relatively nondestructive. All branches were still living 1 year after this procedure. The excised pieces were cut lengthwise into smaller pieces ≤0.5 mm in diameter and immediately frozen in liquid nitrogen [−196°C, N_2(l)]. The tissue lengths were transferred to chilled cryo-vials and stored under N_2(l) until they were prepared for microscopy.

Cryo-SEM Preparation

A cryo-vial was lifted from the N_2(l) storage container and a single tissue length was quickly removed, using chilled forceps, and placed into a small insulated bath of N_2(l) containing a brass specimen holder previously equilibrated to N_2(l) temperature. While immersed in N_2(l), the tissue length was placed on end in the adjustable slot of the brass specimen holder such that the tissue protruded above the top of the holder. The slot was tightly closed around the sample and the entire unit was placed into a N_2(l)-filled styrofoam cup that fits into the slushing chamber of the cryo-preparation system. The rod of the vacuum transfer device was screwed onto the specimen holder and fitted to the top of the slushing chamber. The slushing chamber was evacuated until the N_2(l) was semisolid and the specimen was pulled up into the vacuum transfer device. The small chamber of the vacuum transfer device was then closed with the specimen inside (thus, the specimen remained frozen and under vacuum during the subsequent transfer), the vacuum was released, and the specimen was transferred to the cryo-preparation chamber. Once inside the cryo preparation chamber, the tissue length was fractured crosswise using the internal fracturing knife [alternatively, the tissue length was fractured during the initial transfer of the tissue length to the specimen holder under N_2(l)]. The specimen holder was then heated to −85°C to sublimate water, which is opaque to electrons, from the surface of the sample for about 10 min, brought back to −130°C or below, and coated with gold–palladium under an argon atmosphere. Finally, the specimen holder was transferred to the cryo-stage in a field emission SEM, where the rubber-containing cells in the fractured tissue length were observed and photographed.

Purified Rubber Particles

Rubber particles from *P. argentatum* were purified in a Tris/HCl pH 7.5 buffer [14], or in 0.05% ammonium alginate, adjusted to pH 10 with NH_4OH [20]. Rubber particles from *H. brasiliensis* and *F. elastica* were purified in a Tris/HCl pH 7.5 buffer [14, 19] only. Until the samples were prepared for microscopy, particles purified in Tris/HCl were stored in buffered 10% or 30% glycerol at −20°C, whereas particles purified in alginate were stored at 4°C. *H. brasiliensis* and *F. elastica* rubber particles were thawed then washed three times in alkaline water (adjusted to pH 10 with NH_4OH, to avoid coagulation), centrifuged in a microcentrifuge between washings to remove glycerol, and then prepared for SEM as the other suspensions.

Cryo-SEM Preparation

A drop of rubber particle suspension was placed on a roughened, brass specimen holder. A thin sheet of roughened copper (approximately 5 × 10 mm) was placed on the surface of the suspension. The holder was plunged into liquid nitrogen, allowed to equilibrate, and the copper sheet was forced off the sample with forceps, thus fracturing the sample. Samples were transferred under vacuum (using a vacuum transfer device, as described above) to a cryo-preparation chamber, sublimated at −85°C for 10

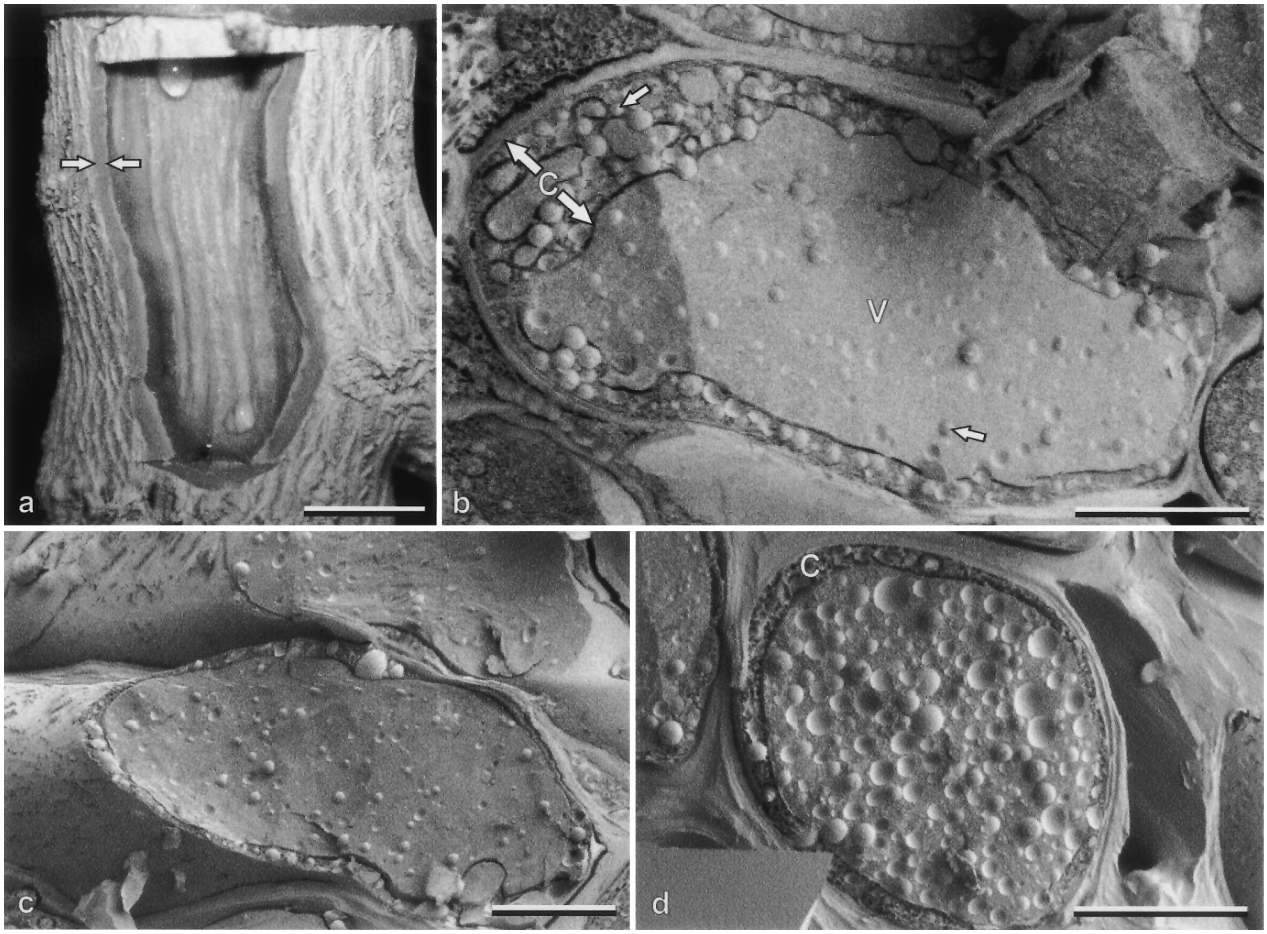

Fig. 1. *Parthenium argentatum* (a) stem segment (line O16-1, large, older stem) showing the region where the excised tissue piece for SEM study was taken. The cells containing the rubber particles are located in the tissue indicated between the two arrows, just beneath the bark. (b–d) Cryo-scanning electron micrographs of *P. argentatum* rubber-containing cells showing cells from (b) young tissue containing rubber particles in the cytosol as well as in the vacuoles; (c) maturing cells from medium-sized stems showing an increasing accumulation of rubber particles in the large central vacuole and rubber particles compressed into a single layer in the much diminished, surrounding cytosol; and (d) mature cells from large-sized stems showing a central vacuole filled with rubber particles, and a single layer of rubber particles in the remaining cytosol. Cells are from the following lines: O16-1 (b); AZ101 (c); N9-5 (d). C, cytosol; cw, cell wall(s); V, vacuole. Magnification bars: (a) 5 mm; (b–d) 10 μm.

min, chilled to −130°C, sputter-coated with gold–palladium, and transferred to the cold stage in the SEM.

RESULTS

Tissue Sections from *P. argentatum*

In *P. argentatum*, rubber is synthesized in rubber particles localized in the bark parenchyma cells of the stems (Fig. 1a, between arrows) and the roots. When tissue samples were examined, it was clear that the number of rubber particles in the stem bark parenchyma cells increased with stem size (Fig. 1b–d), which agrees with yield data previously reported [21, 22]. The smallest stems (<0.5 cm in diameter) contained very little woody xylem and represented the current year's growth. The medium-sized stems (0.5–1.0 cm in diameter) possessed a woody xylem, were more than 1 year old, and had experienced one winter season. The largest stems (>1.0 cm in diameter) were at least 2 years old, contained a woody xylem with two or more growth rings, and had experienced at least two winter seasons.

To avoid confusion with previously used terminology to describe fracturing methods while specimens are frozen [23–25], we've chosen to use a new term, cryo-SEM fracture, for our method. The behavior of the rubber particle cores was examined after cryo-SEM fracture, in tissue sections excised from three different *P. argentatum* lines grown as greenhouse plants.

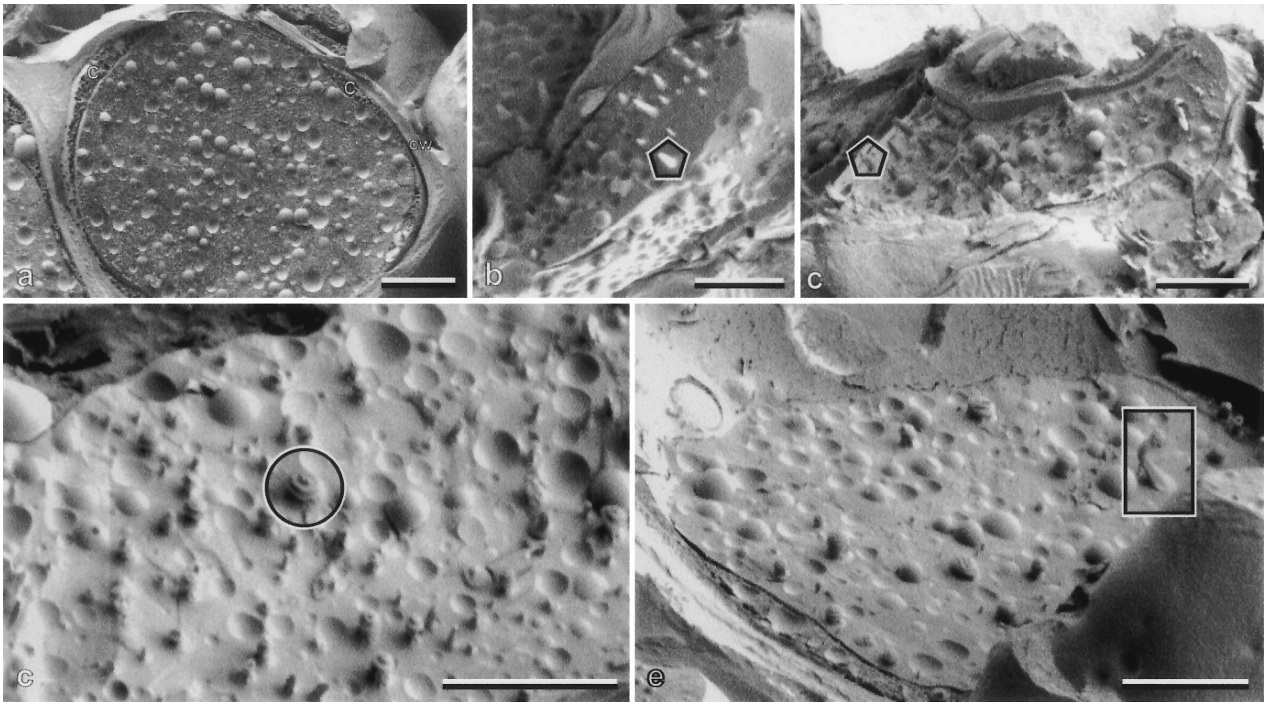

Fig. 2. Scanning electron micrographs of mature, rubber-containing cells from *Parthenium argentatum* showing stretching of the rubber particle core at −196°C *in situ* that was independent of the age of the tissue or of the line. The stretchiness was also independent of whether the rubber particles were located in the cytosol (a) or had passed into the vacuole (b–e). Different types and degrees of stretching occurred in the rubber particles. The plastic type of stretching (b, c pentagon) seemed to be the result of the rubber core (particle interior) stretching to its limit and then breaking. The elastic type of stretching (d, circle) occurred where the rubber particle snapped back on itself after being stretched and broken. A third type of stretching could be described as both plastic and elastic (e, rectangle) where the particle stretched quite a lot and then the tip rebounded. Lines and stem sizes: (a) O16-1, large stem; (b) AZ101, small stem; (c) N9-5, small stem; (d) AZ101, large stem; (e) N9-5, medium stem. Magnification bars: 5 μm.

In tissue sections many particles did not fracture, and instead the fracture plane passed either over or under the intact particles (Figs. 1b–d and 2). However, when the *in situ* rubber particles did fracture, we observed that the rubber cores of almost all fractured particles were stretched out (Fig. 2). The stretching occurred independent of the age of the tissue or of the line (Fig. 2a–e). The stretchiness at −196°C also was independent of whether the rubber particles were located in the cytosol (Figs. 1d and 2a) or had passed into the vacuole (Fig. 2b–e). Different types and degrees of stretching occurred in the rubber particles. The plastic type of stretching (Fig. 2b, c) appears to be the result of the rubber core (particle interior) stretching to its limit and then breaking. The elastic type of stretching (Fig. 2d) occurred where the rubber particle snapped back on itself after being stretched and broken. A third type of stretching involved both the plastic and elastic type of stretching (Fig. 2e) where the particle core stretched out, broke, and then the tip rebounded.

Latex from *P. argentatum*

Two forms of aqueous rubber particle suspensions (latex) were examined: one was a buffer and glycerol-stabilized suspension (pH 7.5) of enzymatically active rubber particles stored under N_2(l) [26], and the other was an inert suspension purified in ammonium alginate (pH 10) and stored at 4°C [20]. Five different samples of glycerol-stabilized rubber particle suspensions were examined that had been purified at different times during a year from mature plants of line 11591, grown in the field in Phoenix, AZ (Fig. 3a–f). The core of many of the rubber particles stretched before breakage occurred as the cryo-SEM fracture planes were pulled apart. All five samples showed this property, demonstrating that it is not seasonally dependent. Cleanly fractured particles were not observed.

Similarly, the cores of purified rubber particles stored in ammonium alginate also stretched during fracture, and to a more extreme degree in most cases (Fig.

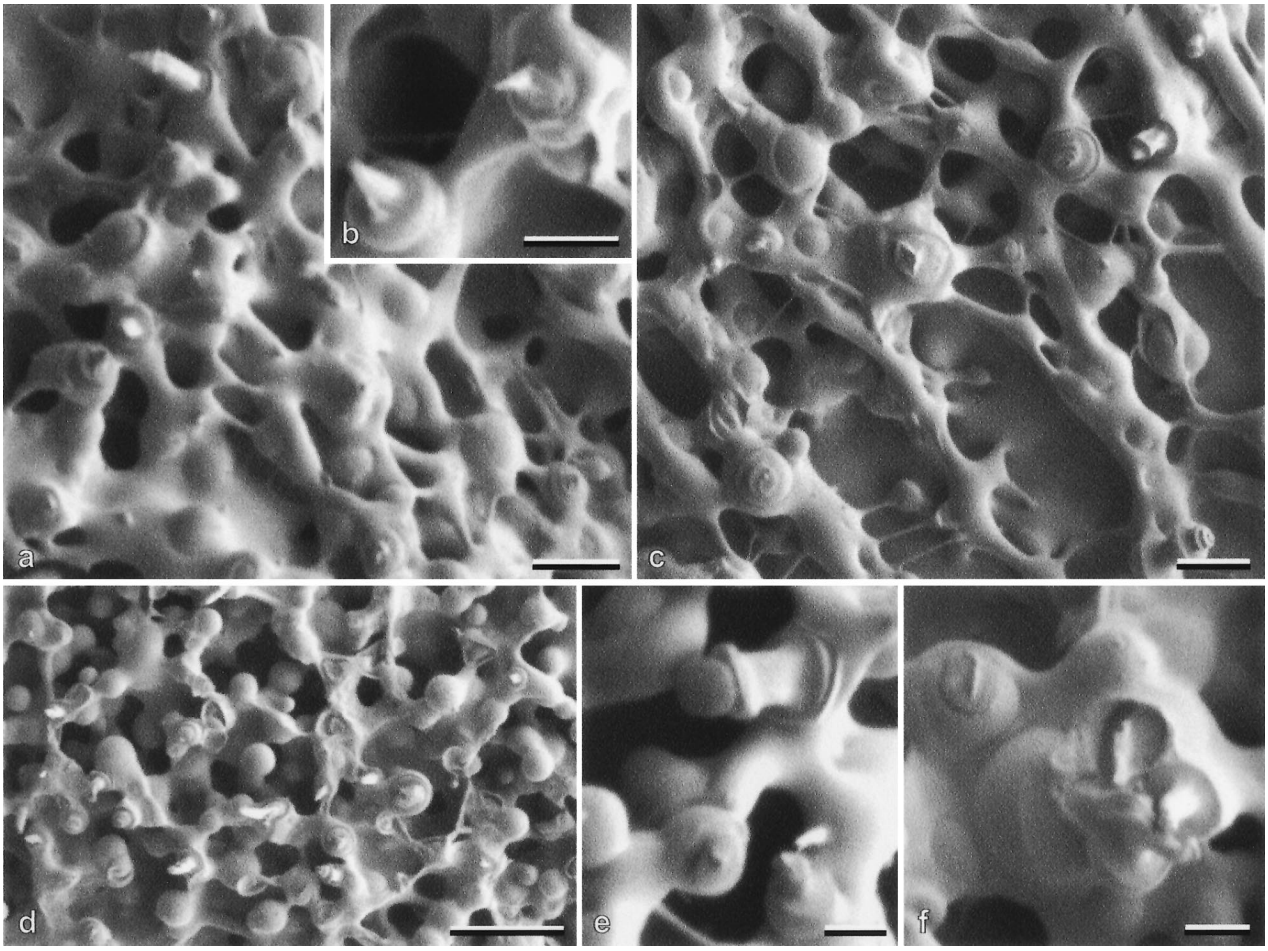

Fig. 3. *Parthenium argentatum* rubber particles purified from mature, field-grown plants of line 11591 harvested at five different times over a year. Purified, enzymatically active particles suspended in 10% glycerol and Tris/HCl buffer were stored in N_2(l) until used. Harvest dates: (a, b) 01/08/97, (c) 02/06/97, (d) 03/12/97, (e) 10/29/97, (f) 11/18/97. Both elastic and plastic fracture types can be seen in the micrographs. The matrix material in which the particles are interspersed is glycerol does not appear to have any effect on the fracture plane of the rubber particles. Magnification bars: (a,c) 2 μm; (b) 0.5 μm; (d) 5 μm; (e, f) 1 μm.

4a–g) than observed in the buffered samples (Fig. 3). Cleanly fractured rubber particles were not observed.

Latex from *H. brasiliensis* and *F. elastica*

Buffered and glycerol-stabilized suspensions (pH 7.5) of enzymatically active rubber particles stored under N_2(l) [26] purified from *H. brasiliensis* (Fig. 5a–c) and *F. elastica* (Fig. 5d, e) were cryo-SEM-fractured under the same conditions as the *P. argentatum* samples and examined. Particles of both species generally fractured quite cleanly (Fig. 5a, d), but *F. elastica* particles sometimes revealed multiple tiny threads of core material pulled out during fracture (Fig. 5e). The fracture plane of *H. brasiliensis* rubber particles showed some unevenness, which may be evidence of threads of core material that broke and fell back to the surface as the two halves of the particles pulled away from each other (Fig. 5a–c).

DISCUSSION

Stretching of the rubber particle core was unique to rubber particles of *P. argentatum* and was not observed in *H. brasiliensis* or *F. elastica* particles, which at most exhibited tiny thread-like extensions of their cores. Freeze-fracturing and freeze-cleaving techniques, combined with transmission electron microscopy, have shown a stretching (or deformation) phenomenon thought to be an artifact caused by heat produced by friction during the fracturing process [23]. Although artifactual in nature, the stretching occurred in specific types of materials such as latex spheres

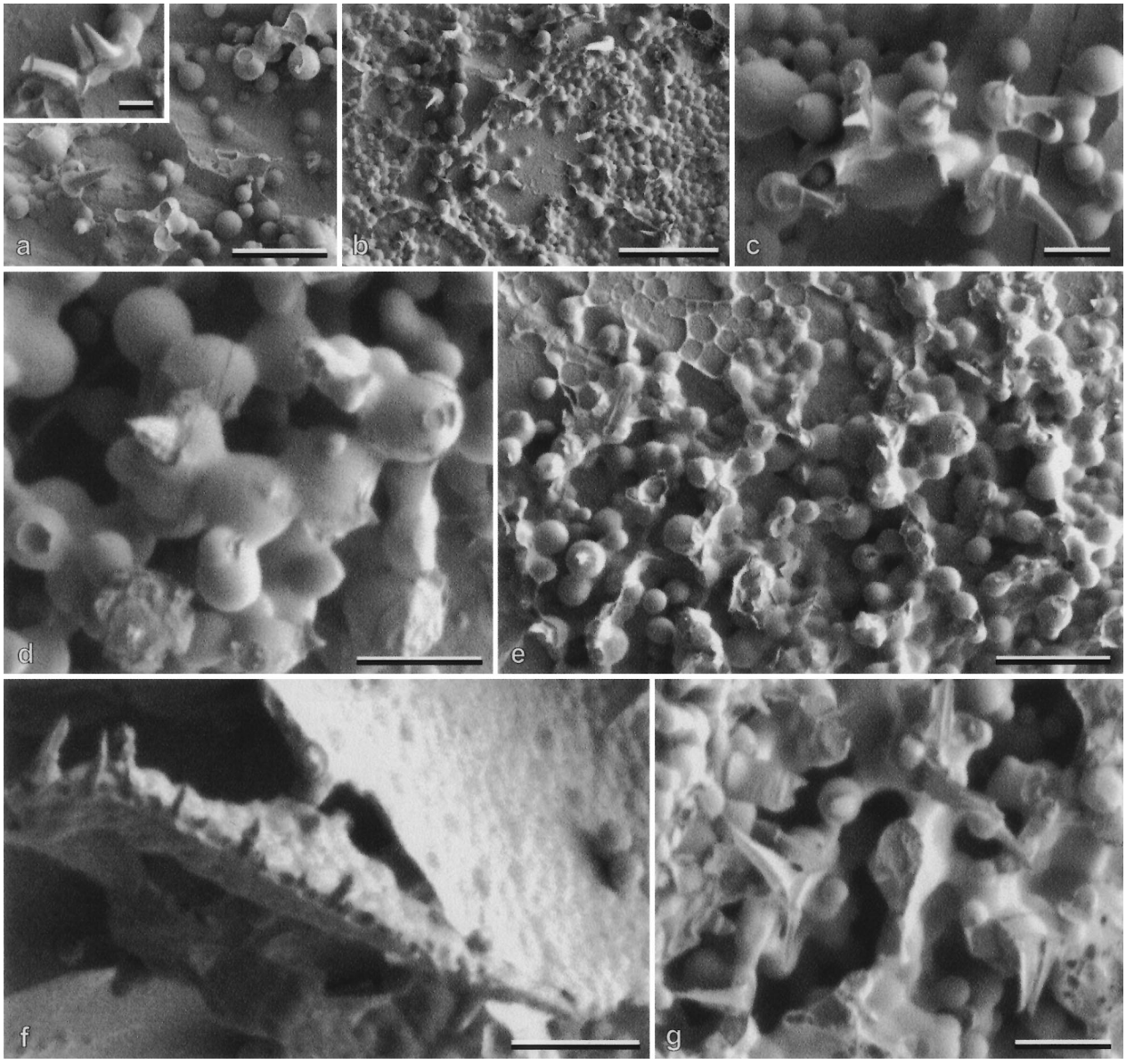

Fig. 4. (a–g) *Parthenium argentatum* ammonium alginate-purified rubber particles from different lines and harvest dates. Lines and harvest dates: (a, inset) 11591, 11/99; (b, c) G7-15, 1/00; (d) 11591, 3/00; (e) 11591, 5/00; (f) G7-11TC, 7/00; (g) 11591, 9/00. Partially coagulated rubber particles (which formed a sheet in f) exhibited stretching while coagulated (f, g). Magnification bars: (a, e, f) 5 μm; (inset) 1 μm; (b) 10 μm; (c,d,g) 2 μm.

(made from polystyrene or polyacrylate) and biopolymers such as poly-β-hydroxybutyrate [24]. Stretching was also reported to occur in other complex biological molecules such as membranes and protein macromolecules [23], although to a much lesser degree, visible in transmission electron microscopy as fibrillar extensions rather than the extreme deformation obvious in these studies. Nonetheless, in the SEM studies reported in this article, the different behavior of the cores of the particles from the three species suggests that the core stretching is a genuine property of the *P. argentatum*. If the stretching was an artifact, it would seem likely that either all three particle types would exhibit the phenomenon or that at least *H. brasiliensis* and *P. argentatum* would behave similarly. The molecular weight of the rubber in *F. elastica* particles is much lower than in either *H. brasiliensis* or *P. argentatum* [14, 17] and particle core is more fluid [13]. However, *H. brasiliensis* and *F. elastica* fracture similarly in cryo-SEM, whereas *P. argentatum*, which has similar molecular weight rubber to *H. brasiliensis*, behaves differently.

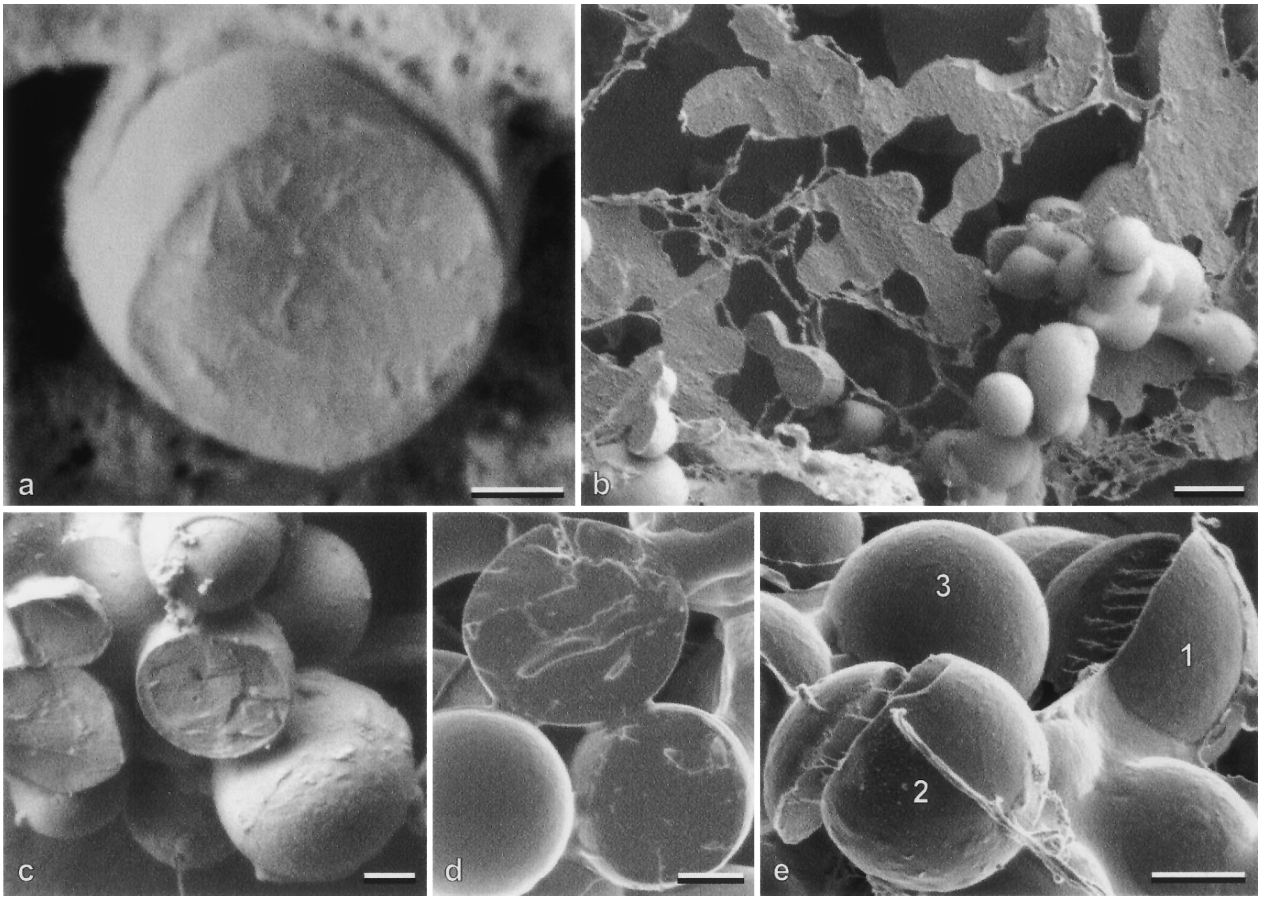

Fig. 5. *Hevea brasiliensis* (a, b) and *Ficus elastica* (c–e) rubber particles stabilized in glycerol and stored in N_2(l) until they were prepared for SEM. *H. brasiliensis* rubber particles which were thawed, frozen, and fractured while in glycerol (a) and those which had been washed (distilled water, adjusted to pH 10 with ammonia) prior to being fractured for SEM (b) exhibit clean fractures of the particles. *F. elastica* rubber particles, whether fractured from glycerol (not shown) or after washing (c, d), fractured cleanly. Both figures illustrate fractured and unfractured particles. *F. elastica* rubber particles purified in ammonium alginate (pH 10) and stored at 4°C until prepared for SEM fractured in two distinctly different ways. The fractured particle on the upper right (1) shows multiple tiny threads of core material pulled out during fracture. In contrast, the fractured particle on the lower left (2) shows threads of material from the particle biomembrane still connecting the two halves of the particle but the particle interior has fractured cleanly. The particle in the upper left (3) is unfractured. Magnification bars = (a) 0.5 μm; (b–e) 1 μm.

In earlier work using a different cryo-SEM (an Oxford CT1500-HF Cryo Preparation System [Oxford, Inc., UK] and viewed in a Hitachi S-4100 field emission SEM [Hitachi]), *H. brasiliensis* and *F. elastica rubber* particles fractured cleanly, with no stretched-out cores observed in either species, whereas stretched rubber cores of *P. argentatum* particles were again observed [13].

The underlying cause(s) of the core stretching during cryo-SEM are not yet understood and most interspecific differences in properties place *P. argentatum* rubber particle properties between those of *F. elastica* and *H. brasiliensis* particles, instead of in an outlying position: *P. argentatum* particles are larger than *H. brasiliensis* particles but smaller than those of *F. elastica* [14, 16]; *P. argentatum* latex viscosity is higher than *H. brasiliensis* but lower than *F. elastica* [16]; and *P. argentatum* rubber is more branched than *H. brasiliensis* rubber but less branched than *F. elastica* rubber [K. Cornish, unpublished results]. However, purified *P. argentatum* latex often contains a higher proportion of acetone-soluble resinous material (8.2%–9.9%) than purified *H. brasiliensis* latex (1.9%–2.3%) [10]. Because the latex tested was highly purified, the resin component is presumably contained inside the rubber particles mixed with the rubber polymers, and may be involved in the core stretching. In support of this argument, the cores of the alginate-purified latex rubber particles appear to stretch further (longer spikes) than the cores of the glycerol-buffer-purified particles. The alginate-purified particles were mainly purified in the summertime, whereas the glycerol-buffer-purified

particles were mainly purified during the wintertime: higher resin levels (11.6%) have been observed in July-harvested *P. argentatum* rubber than in March-harvested material (8.1%) [K. Cornish, D. K. Stumpf, and W. W. Schloman, unpublished results].

In conclusion, when rubber particles isolated from three species were examined, the core of the *P. argentatum* rubber particles stretched during fracture, indicating that the rubber was still malleable at −196°C, independent of purification procedure, guayule line, tissue age, or season. In contrast, at this temperature, only tiny threads of core material sometimes stretched from the rubber particle cores of *H. brasiliensis* and *F. elastica*. This cold-temperature-malleability property of the *P. argentatum* rubber particles may lead to new products and uses in cold-temperature applications.

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REFERENCES

1. J. W. Whitworth and E. E. Whitehead Whitehead (eds.) (1991) Guayule natural rubber: a technical publication with emphasis on recent findings. Guayule Administrative Management Committee and USDA Cooperative State Research Service, Office of Arid Lands Studies, Univ. Arizona, Tucson.
2. D. T. Ray (1993) Guayule: a source of natural rubber. Pps. 338–346. *In:* Jules Janick and James E. Simon (eds.). New crops: exploration, research, and commercialization. John Wiley and Sons, Inc. New York.
3. A. B. Carey, K. Cornish, P. J. Schrank, B. Ward, and R. A. Simon (1995) Cross reactivity of alternate plant sources of latex in subjects with systemic lgE mediated sensitivity to *Hevea* brasiliensis latex. Annals of Allergy, Asthma and Immunology **74,** 317–320.
4. K. Cornish (1996) Hypoallergenic Natural Rubber Products from Parthenium argentatum (Gray) and other non-Hevea brasiliensis species, U.S. Patent No. 5580942.
5. K. Cornish (1998) Hypoallergenic Natural Rubber Products from *Parthenium argentatum* (Gray) and other non-*Hevea brasiliensis* species, U.S. Patent No. 5717050.
6. K. Cornish, J. L. Brichta, P. Yu, D. F. Wood, M. W. McGlothlin and J. A. Martin (2001) Guayule latex provides a solution for the critical demands of the non-allergenic medical products market. Agro-Food-Industry *hi-tech* **12**(6), 27–31.
7. D. J. Siler and K. Cornish (1994) Hypoallergenicity of guayule rubber particle proteins compared to *Hevea* latex proteins. *Industrial Crops and Products* **2,** 307–313.
8. D. J. Siler, K. Cornish, R. G. Hamilton (1996) Absence of cross-reactivity of lgE antibodies from *Hevea brasiliensis* latex allergic subjects with a new source of natural rubber latex from guayule (*Parthenium argentatum*). *J. Allergy Clin. Immunol.* **98,** 895–902.
9. K. Cornish and C. D. Lytle (1999) Viral impermeability of hypoallergenic, low protein, guayule latex films. *J. Biomed. Mater. Res.* **47,** 434–437.
10. W. W. Schloman Jr., F. Wyzgoski, D. McIntyre, K. Cornish, and D. J. Siler (1996) Characterization and performance testing of guayule latex. *Rubber Chem. Technol.* **69,** 215–222.
11. W. Davis (1997) The rubber industry's biological nightmare. *Fortune* **Aug. 4,** 86–95.
12. K. Cornish, D. F. Wood, and J. J. Windle (1999) Rubber particles from four different species, examined by transmission electron microscopy and electron paramagnetic resonance spin labeling, are found to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane. *Planta* **210,** 85–96.
13. D. F. Wood and K. Cornish (2000) Microstructure of purified rubber particles. *Int. J. Plant Sci.* **161,** 435–445.
14. K. Cornish, D. J. Siler, O. K. Grosjean, and N. Goodman (1993) Fundamental similarities in rubber particle architecture and function in three evolutionarily divergent plant species. *J. Nat. Rubber Res.* **8,** 275–285.
15. D. J. Siler, M. Goodrich-Tanrikulu, K. Cornish, A. E. Stafford, and T. A. McKeon (1997) Composition of rubber particles of *Hevea brasiliensis, Parthenium argentatum, Ficus elastica* and *Euphorbia lactiflua* indicates unconventional surface structure. *Plant Physiol. Biochem.* **35,** 281–290.
16. K. Cornish and J. L. Brichta, Rheological properties of latex from *Parthenium argentatum* Gray compared with latex from other rubber-producing species. *J. Polym. Environ.* **10,** 13–18.
17. K. Cornish (2001) Similarities and differences in rubber biochemistry among plant species. *Phytochemistry* **57,** 1123–1134.
18. C. L. Swanson, R. A. Buchanan, and F. H. Otey (1979) Molecular weights of natural rubbers from selected temperature zone plants. *J. Appl. Polym. Sci.* **23,** 743–748.
19. K. Cornish and D. J. Siler (1996) Characterisation of *cis*-prenyl transferase activity localised in a buoyant fraction of rubber particles from *Ficus elastica* latex. *Plant Physiol. Biochem.* **34,** 377–384.
20. K. Cornish and J. L. Brichta (2002) in J. Janick (Ed.), *Trends in New Crops and New Uses*, Proceedings of the 5th National Symposium on New Crops and New Uses: Strength in Diversity, November 10–13, 2001. Atlanta, GA, pp. 214–221.
21. K. Cornish, M. H. Chapman, F. S. Nakayama, S. H. Vinyard, and L. C. Whitehand (1999) Latex quantification in guayule shrub and homogenate. *Ind. Crops Prod.* **10,** 121–136.
22. K. Cornish, M. H. Chapman, J. L. Brichta, S. H. Vinyard, and F. S. Nakayama (2000) Post-harvest stability of latex in different sizes of guayule stems. *Ind. Crops Prod.* **12,** 25–32.
23. U. B. Sleytr and A. W. Robards (1977) Plastic deformation during freeze-cleavage: a review. *J. Microsc.* **110,** 1–25.
24. A. P. van Gool, R. Lambert, and H. Laudelout (1969) The fine structure of frozen etched *Nitrobacter* cells. *Arch. Mikrobiol.* **69,** 281–293.
25. W. J. Humphreys, B. O. Spurlock, and J. S. Johnson (1974) Critical point drying of ethanol-infiltrated cryofractured biological specimens for SEM. *Scanning Electron Microsc.* **1974,** 276–282.
26. K. Cornish and D. L. Bartlett (1997) Stabilisation of particle integrity and particle-bound *cis*-prenyl transferase activity in stored, purified rubber particles. *Phytochem. Anal.* **8,** 130–134.