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Author for correspondence:
Chris Holland
e-mail: christopher.holland@sheffield.ac.uk

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Investigating the rheological properties of native plant latex

Georg Bauer[1], Christian Friedrich[2], Carina Gillig[2], Fritz Vollrath[3], Thomas Speck[1,4] and Chris Holland[3,5]

[1]Plant Biomechanics Group, Botanic Garden, Faculty of Biology, University of Freiburg, Schänzlestraße 1, Freiburg 79104, Germany
[2]Service Group 'Rheology and Processing', Freiburg Materials Research Centre, Stefan-Meier-Straße 21, Freiburg 79104, Germany
[3]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK
[4]Freiburg Materials Research Centre (FMF) and Freiburg Institute for Interactive Materials and Bioinspired Technologies (FIT), Stefan-Meier-Straße 21, Freiburg 79104, Germany
[5]Department of Materials Science and Engineering, University of Sheffield, Sir Robert Hadfield Building, Mappin Street, Sheffield S1 3JD, UK

Plant latex, the source of natural rubber, has been of interest to mankind for millennia, with much of the research on its rheological (flow) properties focused towards industrial application. However, little is known regarding the rheology of the native material as produced by the plant, a key factor in determining latex's biological functions. In this study, we outline a method for rheological comparison between native latices that requires a minimum of preparatory steps. Our approach provides quantitative insights into the coagulation mechanisms of *Euphorbia* and *Ficus* latex allowing interpretation within a comparative evolutionary framework. Our findings reveal that in laboratory conditions both latices behave like non-Newtonian materials with the coagulation of *Euphorbia* latex being mediated by a slow evaporative process (more than 60 min), whereas *Ficus* appears to use additional biochemical components to increase the rate of coagulation (more than 30 min). Based on these results, we propose two different primary defensive roles for latex in these plants: the delivery of anti-herbivory compounds (*Euphorbia*) and rapid wound healing (*Ficus*).

1. Introduction

Natural latex is a material of significant scientific and commercial interest. It is a milky white, yellow, red or colourless fluid stored in specialized cells throughout the plant known as laticifers [1] and can be found in more than 20 000 species from some 40 families [2,3]. The ubiquity of this material attests to its biological significance; however, its diversity and function in nature are yet to be fully understood. Currently, there is no evidence that latex performs a metabolic role. Instead, it is most likely used by the plants in defence against herbivory deploying two separate defensive mechanisms: biochemical and mechanical. Several studies have investigated latex biochemistry, finding it to be a complex aqueous mix of alkaloids, phenolics, proteases and chitinases to name but a few (see [4] and references therein). In terms of mechanical defence, a component of latex that has received particular attention over the past 100 years is *cis*-1,4-polyisoprene, more commonly known as rubber. Rubber can be found in the latex of some 300 genera and eight plant families [5,6] often in high concentrations (e.g. up to 40% dry weight in *Hevea brasiliensis* [7]). Upon coagulation latex is able to seal wounds, preventing infection and further fluid loss or act as an adhesive by snaring insects or blocking their mouthparts [8,9]. Therefore, by probing the biochemical and mechanical properties of latex from a range of different families and species, it should be possible to gain greater insights into the range of selective pressures these materials are under and deduce their primary biological functions.

We propose that rheology, the study of deformation and flow, can offer new insights into latex mechanics, and thus into the evolutionary processes acting upon this material. From a rheological perspective, latex behaves like a complex colloidal suspension of a polymeric substance in an aqueous medium. Rheological studies on latex have been undertaken since the 1950s; however, the focus has been primarily on industrial latex, and as such these systems are difficult to draw relevant biological conclusions regarding latex's natural function.

Non-synthetic industrial latex is produced by concentrating natural latex through centrifugation, evaporation or creaming and subsequent dilution and preservation using additives (e.g. ammonia) [10]. These studies have demonstrated that many factors influence the viscosity and stability of industrial latex, including concentration, particle size and distribution, biochemical components, chemical environment and storage time (for a review see [11]). However, in addition to significant processing of these materials prior to testing, they have been limited to a few key agricultural species, namely *H. brasiliensis* (Euphorbiaceae), *Parthenium argentatum* (Asteraceae) and *Ficus elastica* (Moraceae) [12].

This study examines the rheological properties of microlitre samples of latex from a range of species within minutes of extraction. We have selected species that are not cultivated for their latex to ensure biological relevance, although the species chosen are closely related to industrial cultivars for commercial relevance (i.e. the Euphorbiaceae *Euphorbia characias*, *Euphorbia myrsinities* and *Euphorbia amygdaloides*, and the Moraceae *Ficus benjamina*). We demonstrate distinctive differences in the rheological properties of the different latices and interpret these findings from a mechanical and biochemical perspective in order to unveil potential selective pressures acting upon these materials and their respective roles in plant defence.

2. Material and methods

2.1. Plant material

Materials from mature, free-rooted, *F. benjamina* L. (grown in a temperate glass house) and *E. characias* L. (grown outside) were collected from Oxford University Botanic Gardens. *Euphorbia amygdaloides* L. and *E. myrsinites* L. plants were purchased from a commercial nursery in Freiburg and in the same growing season, they were potted, stored outside and taken to the laboratory for testing. All plants were well watered.

Given it was impractical to transport whole plants into the laboratory for testing, we adopted an approach to ensure that sufficient material was removed from the plant to be comparable with that of an intact plant. For sample preparation, whole stems (more than 60 cm) were cut from the base of the plant, with severed ends instantly placed into water filled 15 ml centrifuge tubes in water and sealed with Parafilm. The whole severed stems were then returned to the laboratory for immediate latex extraction and testing.

Latex extraction involved using a razor blade to make a shallow lateral incision across the stem until latex began to ooze out. Exuded latex was quickly removed via pipette and collected onto Parafilm. In scenarios where one stem was used several times to collect latex, great care was taken to ensure that cuts were sufficiently far apart to ensure consistency in sample preparation (i.e. that latex exudate amount and laticifer pressure was comparable and samples were not becoming depleted or diluted). Once a sufficient amount of latex was collected, aliquots were taken for further analysis.

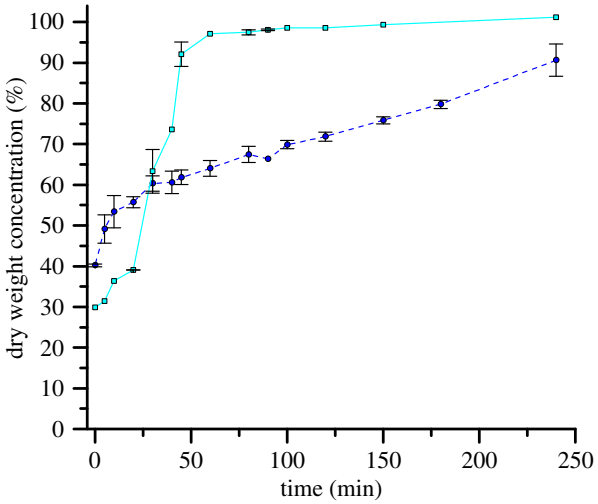

Figure 1. Average latex concentration versus time for *Euphorbia characias* ($n = 8$, circles and dotted line) and *Ficus benjamina* ($n = 3$, squares and solid line) dried side-by-side with rheology samples in standard laboratory conditions (25°C, 40% relative humidity). Error bars are standard error, although the number of samples differs per time-point for *F. benjamina*, and thus should be used as an indicator of data quality only. (Online version in colour.)

2.2. Thermogravimetric analysis

Single aliquots of 10 µl of fresh latex from *E. characias* and *F. benjamina* (with type II water as a control) were spread onto 8 mm diameter platinum pans for thermogravimetric analysis (TGA) and then tested in a Q50 thermogravimetric analyser (TA Instruments, USA) under isothermal conditions for 120 min with an N_2 purge gas at 50 ml min^{-1}.

2.3. Rheological testing and parallel concentration measurements

For rheological testing, 10 µl aliquots of fresh latex were transferred onto the bottom plate of the rheometer and spread out into a droplet of 8 mm in diameter. Owing to differences in the drying environment in the TGA instrument compared with the rheometer, in order to determine sample concentration as a percentage dry weight at the time of rheological testing, 10 µl aliquots were placed onto a piece of aluminium foil, spread to the same size (8 mm diameter), massed for initial weight and then placed next to a rheological testing sample. For samples from Freiburg, concentration was measured directly from parallel concentration sample. For samples from Oxford, these parallel concentration samples were massed at specific time intervals and pooled to construct a generic drying curve for each species and used to determine rheological sample concentration (figure 1 with each datapoint representing an average of eight repeats for *E. characias* and owing to sample limitations up to three repeats for *F. benjamina*). To determine absolute dry weight, samples were air dried for at least 3 days then freeze dried for a further 24 h and massed.

For *F. benjamina* and *E. characias*, rheological testing was performed on a Bohlin Gemini 200 HR nanorheometer (Malvern Instruments, UK) located in Oxford and for *E. amygdaloides* and *E. myrsinites* testing was performed on a Physcia MCR 301 (Anton Paar, Germany) located in Freiburg. Both rheometers were regularly serviced, fully calibrated and performed exactly the same test methods using a parallel plate geometry (8 mm diameter) at 25°C. A plate–plate set-up was chosen to ensure that the samples completely filled the gap upon testing, and to ensure consistent loading a constant compressive force of 0.5 N was applied. Two rheological tests were conducted: (i) an initial oscillation test within the material linear viscoelastic region (between 155 and

0.623 rad s^{-1} (25–0.1 Hz) at 0.02 strains) followed by (ii) a stepped shear viscosity test (between 0.01 and 100 s^{-1}). For certain samples, low-viscosity, high-frequency datapoints that fall outside the sensitivity/accuracy limit of the rheometers are not reported (as defined by the instrument software).

2.4. Data analysis

Data were recorded and processed using instrumentation software (for TGA, Thermal Advantage Q Series, TA Instruments; for rheology, Bohlin, Malvern Instruments, UK and Rheoplus, Anton Paar, Germany) or in the case of weight measurements, data were entered directly into Excel (Microsoft, USA). Further analysis was performed in Excel, and linear regression and figures created in Origin v. 7 (Origin Labs, USA).

3. Results and discussion

3.1. Drying kinetics

The dry weight concentrations of fresh latex saps not only vary within species but also within individuals (G Bauer, C Friedrich, C Gillig, F Vollrath, T Speck, C Holland 2012, unpublished data) and perhaps even by time of day and/or locality. In order to relate sample concentration to rheological properties, the drying kinetics of the latices studied was investigated (see Material and methods). TGA was initially used to determine sample drying kinetics and dry mass (see electronic supplementary material, figure S1). Upon comparison with water, chosen as an evaporative control as it is the main solvent of latex, we observed that *Ficus* dries rapidly, adopting the same drying profile until nearly completely dry. This suggests a standard mechanism of evaporation with little interaction between the water and the solid contents/particles of the *Ficus* latex. This is in direct contrast to the *Euphorbia* latex, which dries more slowly, suggesting that the water is better retained in this material (perhaps owing to interactions with specific components of this latex).

The observed difference may be attributed to the size distribution of latex particles in these species. G Bauer *et al.* (2012, unpublished data) revealed large differences between the latices of *F. benjamina* and that of *Euphorbia* spp.: the latex of *F. benjamina* exhibits a widespread bimodal latex particle distribution (with peaks at about 0.9 and 3.6 µm and a range from 0.3 to 9.4 µm), whereas *Euphorbia* species used in this study have been observed to display a narrower distribution of much smaller particles resulting in a higher packing density (with a peak at approx. 0.2 µm and a range from 0.1 to 0.5 µm—depending on species). It is not unreasonable to suspect that the slower evaporation rate observed in *E. characia*'s latex may be attributed to an increased surface area between the smaller more densely packed particles and water, which served to either increase hydrophilic interactions or exposed surface area and as a result lower the evaporation rate.

Figure 1 further highlights the differences in average drying kinetics for two species tested, *F. benjamina* and *E. characias*. Initial latex concentrations differed by 10% dry weight, with *F. benjamina* having the lowest (approx. 30% versus *E. characias* 40%). However, *F. benjamina* was approximately five times quicker to dry to a constant mass, doing so in 50 min compared with *E. characias* which failed to dry completely during the time tested (more than 240 min).

However, despite the TGA being highly accurate and comparable between samples tested, the flow of the purge gas in the TGA furnace resulted in samples drying faster than those used for rheological testing, rendering concentration predictions invalid if based on TGA residence time. Therefore, we adopted side-by-side parallel concentration measurements for the latex samples (see Material and methods; figure 1; and electronic supplementary material, figure S2).

From looking at figure 1, we can infer that the rapid drying time of *F. benjamina* latex (less than 60 min) may have undergone selection for first and foremost mechanical defence (i.e. wound healing; see [13]) and the relatively slower drying time of *E. characias* (more than 240 min) being a result of optimization for biochemical protection, ensuring that the latex remains liquid in order to more effectively transfer anti-herbivory compounds to a predator [4,8], yet still ultimately drying and providing mechanical protection. However, it is unlikely that any of these protection mechanisms is a sole selective pressure on plant latex function, especially when anti-herbivore compounds, such as proteases and chitinases, can be found in both genera (*Ficus* and *Euphorbia*) [14–16] (overview in [4]).

Additionally, we must remember that these drying rates were obtained under controlled laboratory conditions. In the field, *F. benjamina* is native to regions with high humidity and precipitation, whereas especially *E. characias* and *E. myrsinites* prefer dry and warm habitats [2]. Thus, when compared with coagulation rates in the laboratory, *Euphorbia* latices may in fact coagulate faster in the field owing to being found in conditions which promote faster evaporation. Such an area is worthy of further study in order to determine environmental influences on the biological function of plant latices in the field.

Finally, it is also worth considering the effect of interspecific differences in quantity and drop sizes of the exuding latex on the drying rates in the field. However, while our experimental design negated this factor by assessing latex droplets of the same size (10 µl in volume, 8 mm diameter, see Material and methods) an examination of typical latex drop sizes and their influence on the latex drying rate might be of interest in the future.

3.2. Rheological properties

For each species, three factors were investigated: (i) the mechanical properties of fresh latex at the point of extraction, (ii) its coagulation mechanism, and finally (iii) a comparison between species. For all samples, two tests were performed. The first was an oscillation test that measures modulus, essentially testing how the latex stores or dissipates energy over different time scales. The second was a viscosity test, which determines the latex's flow properties at different shear rates [17]. Overall, all samples tested exhibited non-Newtonian flow behaviour (where viscosity is dependent on shear rate, unlike water or oil [17]), which is also seen in high concentrations of industrial processed latex [11].

Figure 2 compares latices from two species at the same time post-extraction (figure 2*a*,*b*) and at similar concentrations (figure 2*c*,*d*). Comparing samples just after extraction allows us to investigate the properties of fresh latex. Owing to *F. benjamina* undergoing a rapid change in mechanical properties within the first few minutes, a time-point of 10 min post-extraction is shown (figure 2*a*,*b*). Our results showed clear time-

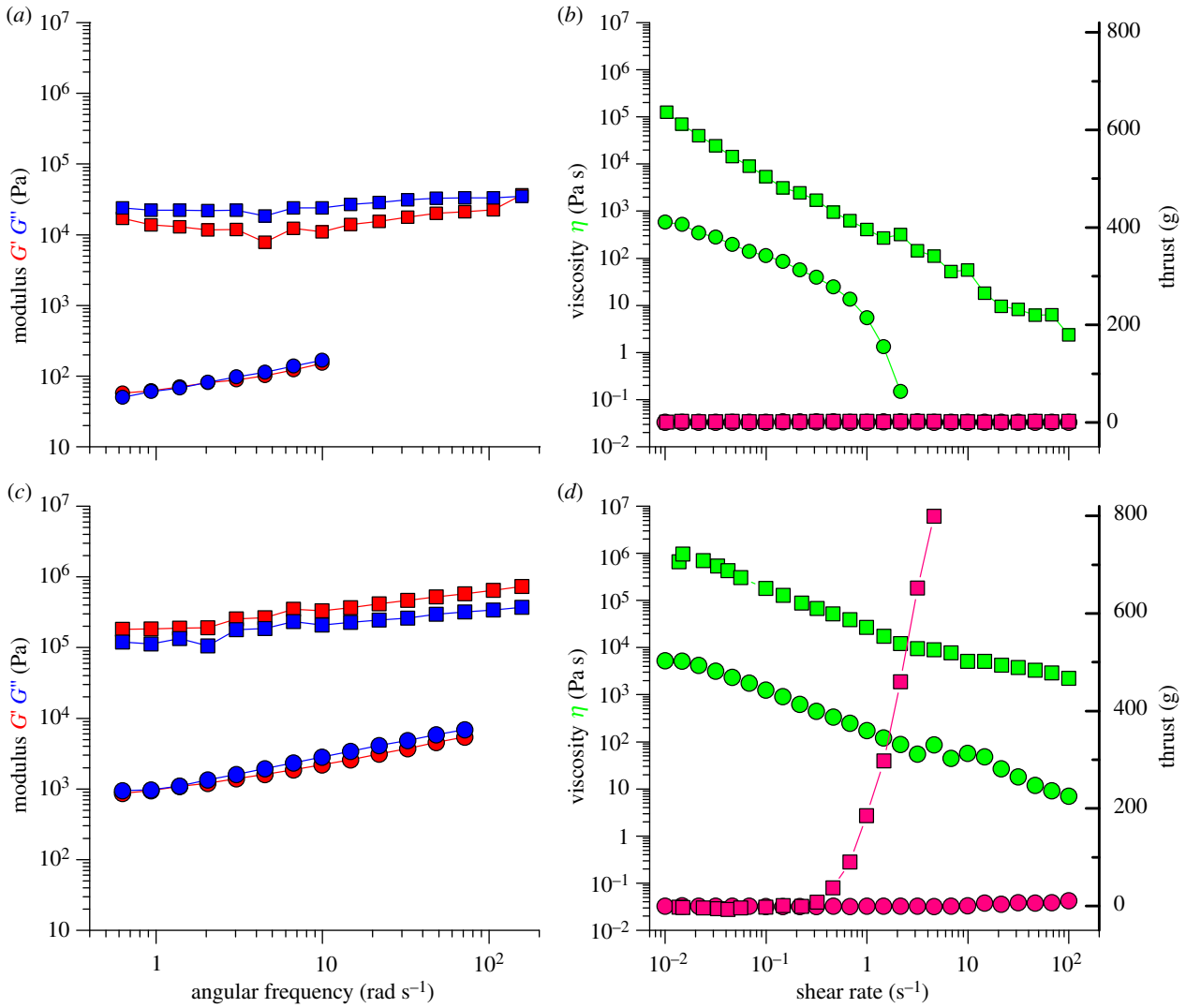

Figure 2. Rheological tests performed on exuded latex of *Euphorbia characias* (circles) and *Ficus benjamina* (squares), at comparable times since extraction ((*a*,*b*) 10 min post-extraction) and concentrations ((*c*,*d*) 66% *E. characias* and 63% *F. benjamina*). Oscillation tests ((*a*,*c*) with G', light grey; G'', dark grey (red and blue respectively in online colour version) after 10 min of drying. Viscosity tests ((*b*,*d*) with viscosity, light grey; normal force, dark grey (green and pink respectively in online colour version). Each figure represents an individual sample from each species. (Online version in colour.)

independent differences in the rheological properties of the latices between species. From the oscillatory tests shown in figure 2*a*, both plant latices appeared to behave like liquids with G'' (loss modulus) dominating G' (elastic modulus); however, *E. characias* was two orders of magnitude lower in moduli compared with *F. benjamina*. For viscosity measurements, both latices displayed a yield stress and shear thinning behaviour, with *F. benjamina* having the highest viscosity. For *E. characias*, the shear thinning was more pronounced and the sample became unstable in the shear test post-0.5 s^{-1} (figure 2*b*). This we attribute to a combination of sample modulus and viscosity at these rates that created an unstable flow profile [18]. For both species, normal force (thrust) readings did not rise during measurements, supporting the yield stress measurements and suggesting the shear thinning phenomenon was most likely the result of a breakage of a weakly bonded network of latex particles within all samples.

Comparing samples at different times post-extraction but with similar concentrations (63% *F. benjamina* time = 30 min and 66% *E. characias* time = 90 min) suggests that differences between these species cannot be attributed to drying time or concentration alone (figure 2*c*,*d*). In general, the trends are as before, with *F. benjamina* latex having higher modulus and viscosity readings than *E. characias* latex. At this concentration, *F. benjamina* has coagulated into a stiff gel indicated by G' dominating G'' during the frequency sweep, unlike *E. characias* which is yet to fully coagulate. Taken together, these observations explain the apparently unusual increase in normal force observed when *E. characias* is sheared (figure 2*d*). Here, the combination of lower modulus and viscosity enables the sample to store recoverable elastic energy as it is being deformed, leading to an increase in normal force that may be attributed to either the Weissenburg effect [18] or flow instability [19]. However, while this is unlikely to have any direct biological relevance, this effect is of particular note for industrial processing of these materials.

In summary, figure 2 demonstrates that the differences observed in species can be attributed to the molecular constituents of the samples and not merely to concentration or drying time, a finding which is consistent with observations and explanations provided by [12] for industrial latex.

3.3. Coagulation mechanism

To deconvolute the coagulation mechanisms present in the different latices, two generic rheological parameters were

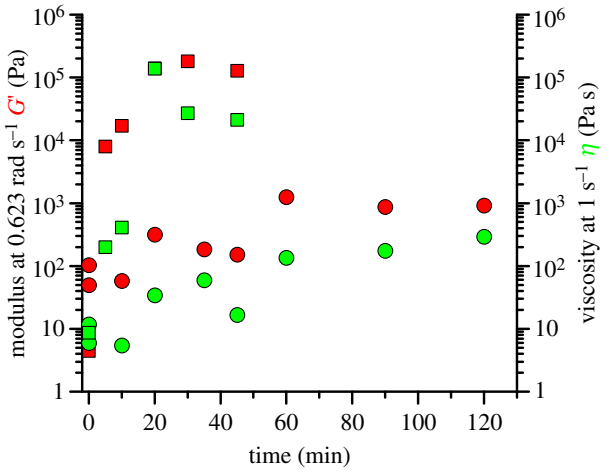

Figure 3. Property–time plot of exuded latex of *Euphorbia characias* (circles) and *Ficus benjamina* (squares) (elastic modulus at 0.623 rad s^{-1} (0.1 Hz), dark grey; viscosity at 1 s^{-1}, light grey (red and green respectively in online colour version)). Datapoints represent individual samples from each species. (Online version in colour.)

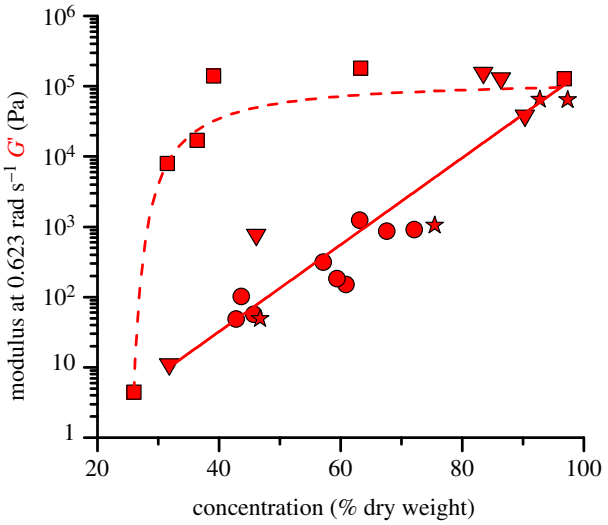

Figure 4. Modulus–concentration plot of exuded latex of *Euphorbia characias* (circles), *E. amygdaloides* (stars), *E. myrsinites* (triangles) and *Ficus benjamina* (squares). *E. characias and F. benjamina*: concentration derived from generic drying curve; *E. myrsinites* and *E. amygdaloides*: concentration measured directly from parallel concentration sample. Datapoints represent individual samples from each species. Solid line represents a linear regression fitted to *Euphorbia* data ($y = -0.96 + 0.06x$, $R^2 = 0.85$). Dashed line represents an exponential fit to *F. benjamina* data; however, owing to the limited number of samples this is intended purely as a guide to the eye only. (Online version in colour.)

chosen to aid comparison. First is the elastic modulus reading at low frequency (G' at 0.623 rad s^{-1}, $G'_{0.6}$), which represents the inherent strength/stiffness of the latex network, and second is the basal shear viscosity (η at 1 s^{-1}, η_1), which represents the dissipative losses (e.g. friction) between latex particles as they flow past one another (figure 3) [17].

The changes in rheological properties for both *Euphorbia* and *Ficus* follow the same trends as their respective drying kinetics (figure 1). This confirms that drying is associated with an overall increase in latex viscosity and modulus, which we define as coagulation. However, while it takes just over 50 min for *Ficus* to dry to a constant mass it only takes 20 min to reach the maximum of its rheological properties (*Euphorbia* does not appear to plateau within the time tested). This insight alone highlights the value of direct mechanical property measurements of latex samples; it appears that coagulation of latex in *Ficus* occurs 30 min *before* drying is complete.

Moving beyond drying times and investigating coagulation as a function of concentration, it is possible to incorporate data from latex samples that have not been subjected to precisely the same drying environments as well as determine whether coagulation is due to simple evaporation or if there are additional biochemical drivers (figure 4 and viscosity measurements in the electronic supplementary material, figure S3). Taken together, our data suggest that for any given concentration, *Ficus* latex has a much higher value of rheological properties than *Euphorbia* latex. However, both latices appear to be convergent at low and high concentrations, suggesting that the material is generally constrained in mechanical properties within the plant and when fully dry outside the plant. Therefore, differences between the species tested are most likely owing to the coagulation process itself.

Within the *Euphorbia* the latices of all three species tested appear to adopt the same linear trend (on log-scale plot, $\eta_1 = -2.67 + 0.08C$, $R^2 = 0.87$, and $G_{0.6} = -0.96 + 0.06C$, $R^2 = 0.85$, where C is dry weight concentration). This implies that the coagulation mechanisms of latices produced by these species are similar and (we believe) most likely to be mediated by evaporation rate which is consistent with observations in industry [11,12]. This agrees well with our hypothesis that the primary defensive role of *Euphorbia* latex is delivery of anti-herbivory compounds as selection would favour slow drying (see electronic supplementary material, figure S1) and coagulation times (figure 4) to ensure that predators are exposed to these chemicals for as long as possible (a matter of hours). While this exposure may not be of significance to large organisms, for example grazing mammals, whose residence time on a plant is a matter of seconds, a prolonged exposure to a free-flowing, uncoagulated latex would be of particular use in deterring parasitic predators, for example insects (e.g. aphids).

This strategy appears to be in direct contrast with observations for *Ficus* latex which coagulates at 40% concentration. Two possible explanations exist for this phenomenon: passive particle packing or active biochemical processes. Particle packing may account for these results, as *Ficus*' larger latex particles with their wider distribution may pack more efficiently than *Euphorbia*'s smaller and narrowly distributed particles. This would enable an interconnected coagulated gel with a higher water content in *Ficus* [12,20] (G Bauer *et al*. 2012, unpublished data). Alternatively, an active biochemical explanation may act to increase a latex's mechanical properties (through a curing/cross-linking reaction) that occurs in the latex upon damage.

Recent microscopic observations appear to support a biochemical explanation; in addition to latex particles, even larger, but collapsed structures have been reported (G Bauer *et al*. 2012, unpublished data), similar in size and concentration to lutoids in *H. brasiliensis* ([20] and citations therein, [21,22]). In *Hevea brasiliensis*, latex coagulation is mediated by the protein hevein, which is released upon rupture of lutoids after injury, which acts to cross-link any latex particles [23,24]. Thus, a stable coagulated network could be created even in the presence of quite high amounts of water, whereas a further evaporation would not increase the

modulus or the viscosity significantly. Regardless of the specific coagulation mechanism, our observations suggest that the primary defensive role of latex in *Ficus* may be wound healing, as selection would favour mechanisms that achieve a rapid increase in mechanical properties over the shortest period of time.

4. Conclusion

Our rheological measurements have demonstrated that it is possible to perform detailed quantitative studies on the flow characteristics of microlitre samples of native latex. The data provide a new window onto the mechanical properties of latex from which we can begin to infer the biological function and evolution. Specifically, we have investigated latices from two plant genera, *Euphorbia* and *Ficus*, and suggest that their coagulation mechanisms rely on either evaporative or biochemical processes which are finely tuned towards adaptation for anti-herbivory or wound healing, respectively. In the future, we envisage the application of microrheological techniques to continue to help elucidate the true nature of these multi-functional materials, leading us forward in both fundamental understanding and industrial applications.

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