Plant Physiology, 2024, pp. 1–12

doi: DOI HERE
Advance Access Publication Date: Day Month Year
Paper

PAPER

How mucilage helps seeds from being washed away? A mechanical interpretation of myxodiaspory using rheology

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FOR PUBLISHER ONLY Received on Date Month Year; revised on Date Month Year; accepted on Date Month Year

Abstract

Myxodiaspory is an adaptation by seeds/achenes of certain angiosperms, where a gelatinous polysaccharide (mucilage) is exuded when hydrated. The polysaccharide composition of seed mucilage includes pectin, cellulose, hemicellulose and starch. The mucilage plays multiple roles in seed physiology including seed hydration, protection and anchorage. In nature, seeds encounter various forces due to different environmental factors. Studies have shown that the mucilage envelope found around certain seeds supports in withstanding these forces by anchoring them to the substrate. What could be the mechanistic basis that triggers the resistive behaviour of mucilage against dislodgement still remains unclear. Rheology, a widely used technique to understand the mechanical response of soft viscoelastic materials is used in the present investigation. The characteristic large amplitude oscillatory shear (LAOS) response of biopolymers can be associated with the corresponding changes in the microstructure during deformation. Herein, we investigate the *in vitro* mechanical response of two model seed mucilages: freshly collected mucilage of sweet basil (*Ocimum basilicum*) and chia (*Salvia hispanica*) seeds under shear deformations using LAOS. Tack experiments conducted on mucilage along with rheology suggest that the strain stiffening response of seed mucilage is crucial in resisting the forces during water flow leading to seed anchorage. Unlike pectin gels, mucilages show resistance to large scale deformations through strain stiffening at different length scales. This arises from the hierarchical microstructure of mucilage consisting of hemicellulose, cellulose fibrils and fibrillary bundles (CF and CFB) in addition to the crosslinked pectin matrix. Present investigation also paves way to a novel methodology using rheological techniques to study plant mucilage physiology.

Key words: seed mucilage, strain stiffening, oscillatory shear rheology, seed anchorage, hierarchical microstructure

Introduction

Plants adapt various unique strategies for their survival in diverse environments. Myxodiaspory is one of those strategies shown by seeds/achenes of angiosperms (Ryding, 2001). Myxodiaspory refers to the exudation of mucilage, a gelatinous complex polysaccharide upon imbibition of water by the seeds. Generally, polysaccharides present in the seed/achene mucilage contain pectin, cellulose, hemicellulose and starch, varying in combinations and compositions across different species (Phan and Burton, 2018; Western, 2012; Naran et al., 2008). The mucilage is a highly functional trait in seed/achene physiology such as seed hydration, protection, anchorage, germination and also in early seedling development (Western, 2012; Tsai et al., 2021; Yang et al., 2012). Mucilage also helps in protecting the structural integrity of the seed by acting as a protective sheath surrounding the seed, (Pan et al., 2021) in addition to regulating the seed germination (Western, 2012; Yang et al., 2012). Interestingly, mucilage is also shown to play a central role in the anchorage of seeds to the soil during events, such as, rain and heavy wind (Pan et al., 2022; Engelbrecht et al., 2014). Seed dislodgement experiments show that the forces are in the range of 0.0043 - 2 N for water flow velocities ranging from 6.7 x 10^{-5} to 1.4 x 10^{-2} l/s cm (Pan et al., 2022). When the flow velocities of the water are converted to strain experienced by the seed, it varies from 0.2 - 200 %. Even though it is reported that the composition of mucilage plays an important role in withstanding the flow velocities experienced by the seed (Pan et al., 2022), the mechanism in detail is not understood in the context of mucilage microstructure. A quantitative analysis of the structure-function relationship of the mucilage can help to understand the role of different components of the mucilage and their hierarchical structural organization in its mechanical response (Amsbury, 2020; Kreitschitz et al., 2021).

Rheology is a widely used approach to investigate various material systems to understand their structural response to mechanical deformation (linear and non-linear) (Jacob et al., 2019; Kurniawan et al.,

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2012; Hashemnejad and Kundu, 2016; Piechocka et al., 2016). When subjected to large strains/deformations, one of the key characteristic response of certain soft biological materials is 'strain stiffening' (Erk et al., 2010). Some of the classic examples of such biological materials exhibiting strain stiffening include F-actin (act; Scheff et al., 2021; Wen and Janmey, 2013), fibrin (Piechocka et al., 2016), collagen (Kurniawan et al., 2012; Motte and Kaufman, 2013), neurofilaments (Wagner et al., 2007) which are of animal origin and alginate (Hashemnejad and Kundu, 2016; Zhang et al., 2007), agarose (Bertula et al., 2019) and pectin gels (Jacob et al., 2019; Schuster et al., 2012) of plant origin. The strain stiffening response of these systems is also associated with their physiological functions. Alginate and low-methoxy pectin get ionically crosslinked in the presence of divalent cations such as calcium (Ca^{2+}) resulting in hydrogel network by forming egg-box dimers and bundles (Jacob et al., 2019; Grant et al., 1973; Braccini and P´erez, 2001). Low-methoxy pectin, which is also a major constituent of seed mucilages, is shown to exhibit strain stiffening response under large amplitude oscillatory shear experiments. The strain stiffening response of the pectin gels is shown to originate from the calcium-crosslinked polygalacturonan chains which form rodlike egg-box bundles connected through semi-flexible polymer chains (Jacob et al., 2019). However, seed mucilage being a hierarchical complex system where different components may have different roles in its overall mechanical response. It will be interesting to investigate what roles these different components play in its mechanical response. Does mucilage also show strain stiffening response like pectin and other biomolecular networks? If so, what are the possible microstructural features and their changes that contribute to such responses? And, what physiological roles necessitates strain stiffening, if it is present in the seed mucilage?

Rheological behaviour of seed mucilages reconstituted from dried powders of certain species has been studied earlier (Malick et al., 2018; Bru¨tsch et al., 2019; Troshchynska et al., 2022; Garcia e Silva et al., 2022; Capitani et al., 2015). However, in all these studies, the structure of the native mucilage was altered by one or more of the processes followed: use of other chemicals, centrifugation and freeze-drying (Liliana and Alfredo, 2015). And the linear rheological response of the reconstituted mucilage was studied with food applications in mind. The main objective of the present study is to understand the role of different components of mucilage, such as, pectin, cellulose, hemicellulose and starch in the mechanical response of mucilage and the seed physiology. Hence, in the present study, linear and non-linear rheological response of freshly collected native mucilage from two widely studied myxodiasporic model species, sweet basil (*Ocimum basilicum*) and chia (*Salvia hispanica*) were explored along with low-methoxy pectin gels (comparison purpose).

Materials and Methods

Sweet basil (*Ocimum basilicum*) and chia (*Salvia hispanica*) seeds were purchased from Essence Organics (Aurangabad, Maharashtra, India.) and Anveshan Farm Technologies Pvt.
Ltd. (Gurugram, Haryana, India.) respectively. The seeds were certified organic to avoid chemical contamination. Low methoxy pectin (LMP) and high methoxy pectin (HMP) of citrus origin with a galacturonic acid content of 87% and 81% and degree of esterification 39% and 67% respectively were purchased from Krishna Pectins, Jalgaon, Maharashtra, India. Calcium chloride dihydrate ($CaCl_2.2H_2O$) used for cross-linking low methoxy pectin was procured from Merck Specialities Private Limited, Mumbai, Maharashtra, India.

Collection of basil and chia seed mucilage

For collecting the mucilage, seeds were soaked in double distilled water at a seed-to-water ratio of 1:40 for 30 min at room temperature (25-30°C). Seeds upon uptake of water secrete the mucilage. Excess water was filtered off using a nylon mesh and the swollen seeds were squeezed gently between two meshes to separate the mucilage from the seed surface. The separated mucilage was collected from the mesh surface. The collected mucilage was kept for degassing in a desiccator for 15 min to remove entrapped air. The mucilage was allowed to rest for 30 min before the rheological studies.

Determination of solid fraction

The solid fraction of the mucilage gives an estimate of the total polysaccharide present in it. The solid fraction of the freshly collected seed mucilage was determined by drying the mucilage. Mucilage was spread on glass slides and placed in a vacuum oven at 27°C and at a vacuum pressure of 50 mbar. The sample was weighed every hour until the weight remained constant. The solid content of the mucilage was calculated using the following equation,

$$S_w = \frac{w_d}{w_w} \tag{1}$$

where S_w is the solid fraction in the mucilage, w_w and w_d are the initial (as collected) and final (as dried) weight of the mucilage. The polysaccharide content in freshly collected basil and chia seed mucilage is 0.8 wt% and 0.35 wt% respectively.

Preparation of pectin gels

Pectin gels with different amounts of pectin and crosslinker concentrations were prepared for comparison with the mucilage. Low methoxy pectin gels were prepared according to the procedure reported in the literature (Jacob et al., 2019). Low methoxy pectin solutions of the required concentration were prepared in de-ionised water and stirred for 16-18 h at 60°C for complete dissolution. Calcium chloride ($CaCl_2.2H_2O$) is used as a crosslinking agent. The stoichiometric ratio (R) relates the concentration of calcium ions to the non-methoxylated galacturonic acid content in the pectin. R is expressed as,

$$R = \frac{2[Ca^{2+}]}{[COO^-]} \tag{2}$$

The extent of crosslinking was varied by varying the R value. Pectin-Ca gels were prepared for different R values by adding the prepared calcium chloride solution to the pectin solution drop by drop with stirring at 25-30°C. The mixture was stirred for 10 min and left undisturbed for 24 h at room temperature (25-30°C) for gel formation.

Rheological studies

Rheological studies help in understanding viscoelastic response of the material under mechanical shearing. The resultant storage modulus ($G'(\omega)$) and the loss modulus ($G''(\omega)$) (hereafter referred to as G' and G'' respectively) describe the elastic and viscous response of the material and can be obtained from the oscillatory shear experiments carried out on the mucilage. Rheological studies were carried out on the freshly collected mucilage to understand its mechanical behaviour on a stress-controlled rheometer, Anton Paar Physica MCR 301. A cone and plate geometry (CP 50-2/TG-SN5712) with a diameter of 50 mm, a true gap of 47 μm and a cone angle of 1.995° was used. Oscillatory shear experiments were carried out to study both the linear and non-linear viscoelastic behaviour of the mucilage. To study the linear deformation behaviour, small amplitude oscillatory shear (SAOS) experiments were performed at a strain amplitude (γ_0) of 1% and angular frequency (ω) ranging from 0.1 to 100 rad/s. Large amplitude oscillatory shear (LAOS) experiments were carried out in the strain amplitude range 0.01 to 1000% at a constant angular frequency of 1 rad/s to study the non-linear deformation behaviour. All the measurements were carried out at a temperature of 25 °C unless

otherwise specified and were repeated thrice on separate mucilage samples for reproducibility. A silicone oil solvent trap was used to prevent the evaporation of water from the sample during the experiments.

Analysis of non-linear rheological response and strain stiffening

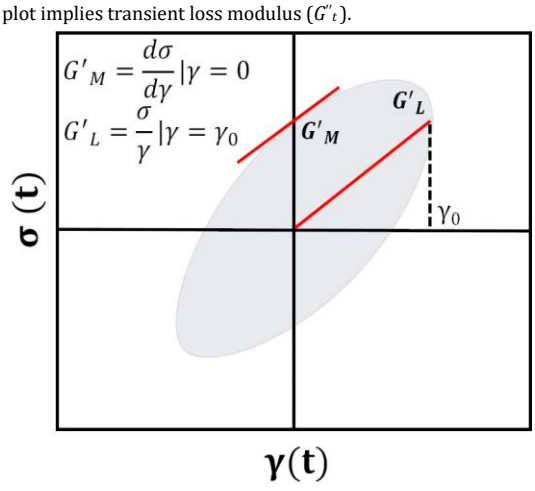
In the non-linear regime during a LAOS measurement, the stress response becomes a function of higher harmonics of the sinusoidal strain input. The overall moduli obtained from strain amplitude sweep contains the first harmonic information without reflecting the influence of higher harmonics. Analysis of intra-cycle stress-strain raw data using Lissajous-Bowditch (LB) plots helps to understand the influence of higher harmonics on the material response (Ewoldt et al., 2008, 2007a). One approach for this analysis is based on strain stiffening ratio that can be calculated from modulus at minimum and maximum strain values (Ewoldt et al., 2008), while another approach known as Sequence of Physical Process (SPP) is based on calculation of transient moduli at every point on the L-B plots (Rogers, 2017).

Non-linear behavior observed in L-B plots is quantified by defining the strain stiffening index S, as a function of the minimum-strain elastic modulus G'_M and the maximum strain elastic modulus G'_L using equation (Ewoldt et al., 2008),

$$S = \frac{G'_L - G'_M}{G'_L} \tag{3}$$

where G'_M - tangent modulus at minimum strain ($\gamma = 0$) and G'_L - secant modulus at maximum strain ($\gamma = \gamma_0$) during one cycle at a given strain amplitude (Figure 1(a)). S = 0 implies the linear response where the material functions do not depend on the strain. 0 < S < 1 indicates strain stiffening and S < 0 indicates strain softening behaviour of the material system (Ewoldt et al., 2008).

Sequence of Physical Process (SPP) analysis estimates transient moduli from the L-B plots by calculating the slope of the L-B curve at each point in the cycle for a given strain amplitude. The transient or instantaneous elastic modulus (G'_t) is obtained from the slope of stress-strain L-B plot and the slope values obtained from stress-strain rate L-B plot implies transient loss modulus (G''_t).



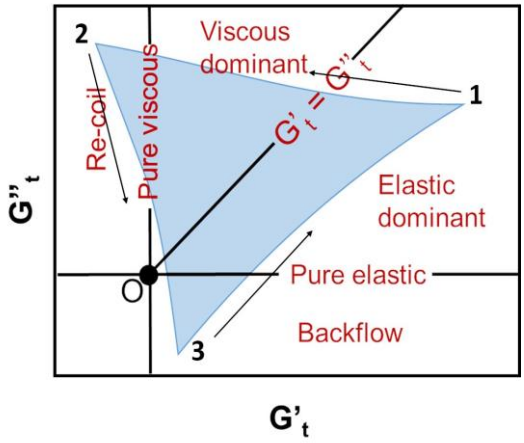


Fig. 1. (a) Model Lissajous-Bowditch (L-B) plot - stress-strain cycle data with tangent and secant moduli at minimum and maximum strain (Ewoldt et al., 2008) (b) SPP analysis - Position of different regions in the Cole-Cole plot of transient moduli(Rogers, 2017)

The transient moduli can be given as,

$$G'_t = \frac{d\sigma}{d\gamma}; \tag{4a}$$

$$G''_t = \frac{d\sigma}{d(\frac{\dot{\gamma}}{\omega})} \tag{4b}$$

The earlier mentioned moduli G'_M and G'_L imply the response only at limiting conditions of minimum ($\gamma = 0$) and maximum strain ($\dot{\gamma} = 0$), whereas, the transient moduli encompasses the deformation response continuously along the L-B curve. This allows detailed transient information related to non-linear features such as, strain stiffening and strain softening (Erturk et al., 2022).

The Cole-Cole plot of the estimated transient moduli (G'_t, G''_t) is analysed by classifying it into different regions as shown in Figure 1(b). The horizontal line and vertical line passing through the origin indicate purely elastic and purely viscous behaviour respectively. The 45° line through the origin represents the flow condition where the transient storage modulus (G'_t) is equal to the transient loss modulus (G''_t). The positive region above and below the flow condition represents viscous and elastic dominant regions respectively. The region between positive G''_t and negative G'_t represents viscous recoil and the region between negative G''_t and positive G'_t represents backflow (Rogers, 2017; Erturk et al., 2022; Donley et al., 2019).

Tack response of seed mucilage and pectin

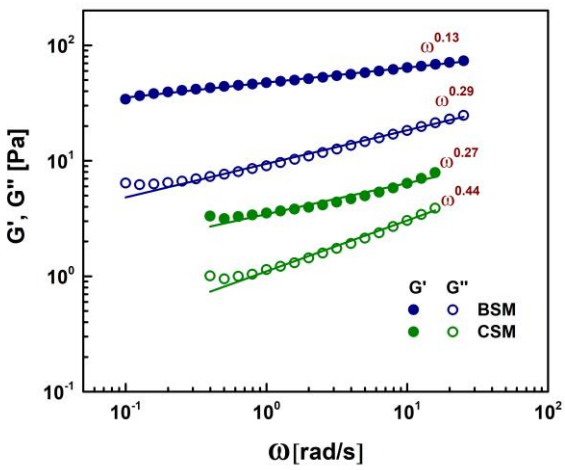
Tack experiments were performed on the rheometer using whole swollen seeds and freshly collected basil and chia seed mucilage to understand the wet tack behaviour of the seed mucilage. Three different experiments were conducted for this: 1) on swollen basil and chia seeds with mucilage envelope, 2) on freshly collected mucilage without seed and 3) on pectin gels. On the lower platen of the rheometer basil and chia seeds with mucilage envelope were loaded at 2.5 mm and 2 mm gap respectively and parallel plate of 25 mm dia. is used. The gap was chosen based on the size of the swollen seeds so that the top plate comes in contact with the gel on the seed surface without force application. This was followed by compressing the swollen seeds by 1 mm and then tack test was performed by lifting the top plate at a velocity of 10 μm/s. Similar experiments were also performed on the collected seed mucilage and model pectin gels. The

normal force was measured and the wet tack strength of the mucilage was calculated from the area of normal force vs gap plot (Baron et al., 2009).

Results and Discussion

Basil and chia seed mucilage - weak hydrogels ?

The linear rheological analysis and the variation of storage ($G'(\omega)$) and loss modulus ($G''(\omega)$) with angular frequency (ω) is used to understand the physical nature of the gel. Small amplitude oscillatory shear (SAOS) response of freshly collected basil seed mucilage (BSM) and chia seed mucilage (CSM) is shown in Figure 2. For both the mucilages, the storage modulus is higher than the loss modulus throughout the frequency range studied indicating viscoelastic solid-like behaviour. BSM shows higher storage and loss moduli than CSM at all the frequencies. The storage and loss moduli of both the mucilages show a frequency dependence, $G' \propto (\omega)^a$ and $G'' \propto (\omega)^b$. A power law fitting of the data gives a and b in the range of 0.1 0.4 indicating the weak frequency dependent nature of moduli exhibited by weak gels (Ross-Murphy, 1993). In comparison to reconstituted mucilages from dry powders, freshly collected mucilages



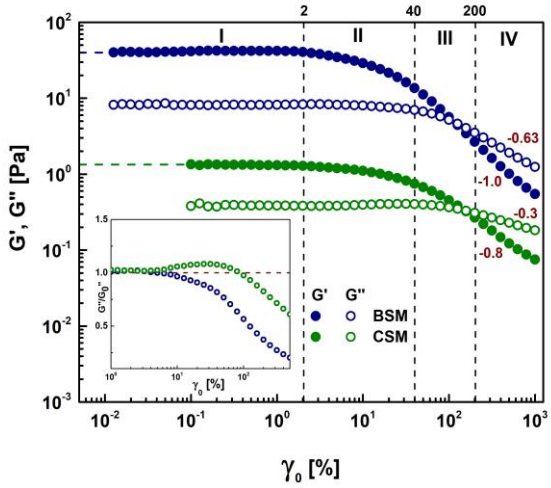
show lower storage modulus. This could be due to the higher solid content in the reconstituted mucilage and also the structural changes (Capitani et al., 2015).

The small amplitude oscillatory shear (SAOS) analysis is limited to studying the general nature of these biopolymers since the response is linear in this range. To understand the contributions of microstructure and the structural changes in the mucilage during the mechanical deformation, large amplitude oscillatory shear (LAOS) experiments were carried out. The variation of storage ($G'(\gamma_0)$) and loss ($G''(\gamma_0)$) moduli with strain amplitude for both basil and chia mucilages is shown in Figure 3. The overall response can be classified into four different strain regions. Region I: $\gamma_0 \leqq 2\%$, linear response region where both G' and G'' are independent of strain. Region II: $2\% < \gamma_0 \leqq 40\%$, the region where G' tends to decrease with increasing strain amplitude indicating non-linear response. Region III: $40\% < \gamma_0 \leqq 200\%$, the cross-over region where

(G') and (G'') crossover. For BSM, this occurs over γ_0 of 100125 % and for CSM, it is at 150%. Region IV: transition to viscoelastic fluid-like behaviour occurs beyond the crossover at $\gamma_0 > 200\%$, (where $G'' > G'$). The zero shear modulus (G'_0) for BSM is one order of magnitude higher than that of

Fig. 2. Small amplitude oscillatory shear response of BSM and CSM at 1% strain amplitude

Fig. 3. LAOS response of BSM and CSM at angular frequency of 1 rad/s. The vertical lines indicate the strain amplitude limits for the four rheological regions. The horizontal lines indicate the G'_0 values of the mucilages. The inset in the figure shows the overshoot in G'' observed for chia seed mucilage.



CSM during LAOS. BSM shows no overshoot in G'' while a weak overshoot is observed for CSM as shown in the inset in Figure 3. BSM with no overshoot represents type I material system in which polymer chains/microstructural entities align in the direction of flow (Hyun et al., 2011). On the other hand, CSM with weak overshoot represents type III materials with additional rearrangement and formation of temporal networks in the microstructure during deformation (Sim et al., 2003).

How close are seed mucilages to pectin gels ?

The presence of crosslinked pectin in seed mucilage is well established in the literature (da Silveira Ramos et al., 2021; Kurd et al., 2017; Thessrimuang and Prachayawarakorn, 2019). The FTIR spectra for BSM and CSM along with low and high

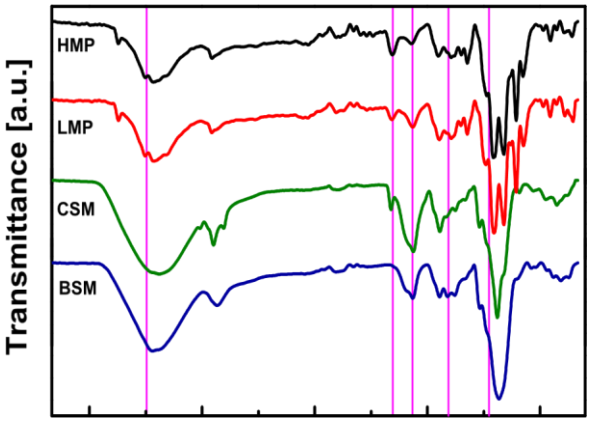


Fig. 4. Fourier Transform Infrared spectra of BSM, CSM, LMP and HMP. The straight line at 1740 cm^{-1} indicates the presence of peak corresponding to esterified groups

methoxy pectin for comparison are shown in Figure 4. The peaks at 1630 and 1740 cm^{-1} correspond to the carboxylic acid and the esterified carboxylic acid groups present in the pectin. Except for the BSM, all other materials have a peak at 1740 cm^{-1} which indicates the presence of methyl-esterified pectin. Homogalacturonan chains (HG1) present in pectin can crosslink in the presence of divalent ions to form gels. Additionally, Energy Dispersive Spectroscopy (EDS) analysis conducted along with SEM studies on the dried mucilages showed the presence of different divalent ions such as calcium and magnesium in both the mucilages (Supplemental Information Fig.S1). Our rheological studies on freshly collected mucilages clearly indicate the weak gel-like nature of both basil and chia seed mucilages (Figure 2 and Figure 3). In order to compare the mucilage gel behaviour with that of pectin gels, LAOS rheology experiments are carried out on calcium cross-linked pectin gels and the results are shown in Figure 5.

Among the different low methoxy pectin-Ca gels, the gels with 0.5 and 0.35 wt% pectin and R = 0.5 showed comparable zero shear storage modulus (G'_0) values to that of BSM and CSM respectively (Figure 5). The linear viscoelastic limit for pectin gels is one order of magnitude higher than that of the seed mucilages. The overshoot observed in G'' in region II is prominent in the case of pectin gels, weak in the case of CSM and absent in BSM. The limit of the linear viscoelastic region and the presence of overshoot are shown to be related to the presence of egg box bundles in the HG1 region of pectin chains in the presence of Ca (Jacob et al., 2019). It is interesting to observe that the non-linear region in seed mucilages occurs at much lower strain limits indicating possible differences in the microstructure of the mucilage or type of pectin and its crosslinking in the mucilage. In region IV, $G' \propto (\gamma_0)^a$ and $G'' \propto (\gamma_0)^b$. The values of a and b for pectin gels (Table 1) are higher compared to seed mucilages. Weak/no overshoot in G'' and gradual decay of moduli at higher deformations in seed mucilages imply the differences

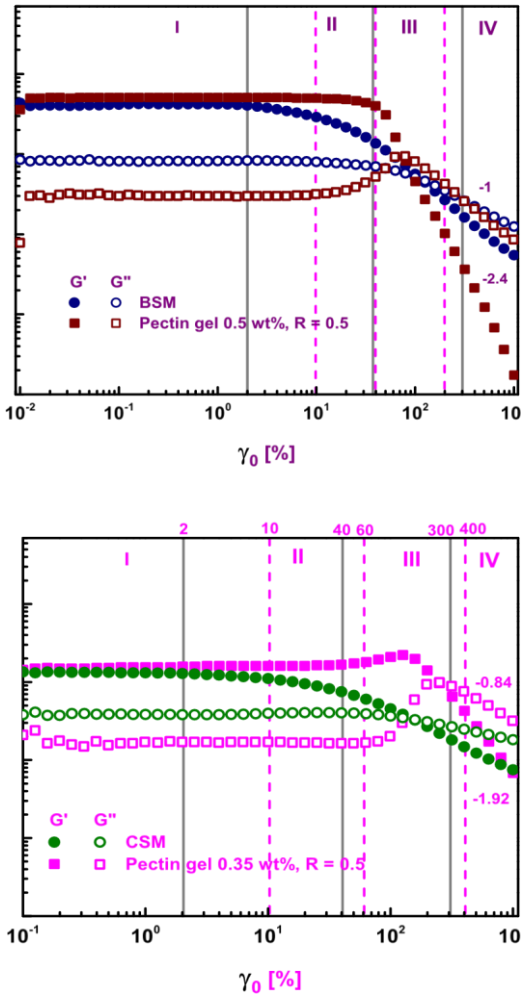


Fig. 5. Comparative LAOS response of pectin gels along with seed mucilages (a) response of pectin gel of 0.5 wt% and R = 0.5 along with the response of BSM and (b) response of pectin gel of 0.35 wt% and R = 0.5 along with the response of CSM. The vertical lines represent different LAOS regions. The onset and extent of LAOS regions are different for seed mucilages (gray lines) and pectin gels (magenta dashed lines).

involved in their composition and microstructure which might be the reason for the differences in observed response. However, at higher strain amplitudes, due to the presence of higher harmonics, G' and G'' data needs to be interpreted using other methods such as, intracycle (within a cycle of oscillation) stress-strain analysis using Lissajous- Bowditch (L-B) plots.

Strain stiffening in seed mucilage?

The L-B plots give better insights into the microstructural contributions of the mucilage to the observed LAOS behaviour. The L-B plots for (a) BSM (b) CSM (c) pectin gel with 0.5 wt% pectin and R=0.5 (d) pectin gel with 0.35 wt% pectin and R=0.5, at four strain amplitudes are shown in Figure 6

and summarised in Table 1. These strain amplitudes represent the four regions of the LAOS shown in Figure 5. The shape of these plots is elliptical within the linear viscoelastic region.

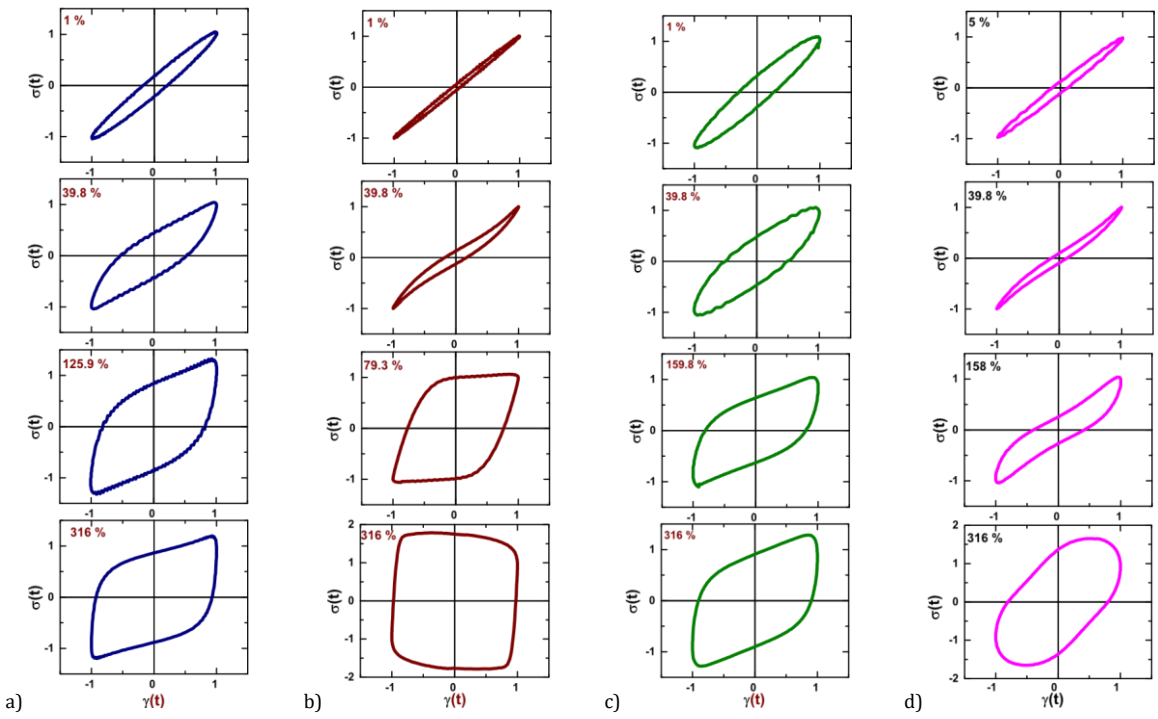


Fig. 6. Lissajous plots for (a) BSM (b)Pectin gel 0.5 wt%, R=0.5 (c) CSM and (d) pectin gel 0.3.5wt%, R=0.5 based on regions indicated in Figure 5

However, beyond the linear limit, the L-B plots become nonelliptical indicating non-linear response of the material system. The shape and slope of the forward curve of the L-B plot (positive-half cycle) is indicative of material behaviour, such as, strain stiffening and strain softening. The strain stiffening ratio, S for the mucilage and pectin gels (Equation 3) was calculated using MITlaos software (Ewoldt et al., 2007b) and is shown as a function of strain amplitude in Figure 7. 0<S<1 implies strain stiffening and S<0 implies strain softening in the material system. The S values and the strain stiffening/softening regions for pectin gels are comparable to that of the results reported previously (Jacob et al., 2019). The differences in the galacturonic acid content, degree of esterification and the source of low methoxy pectin do not change the qualitative nature of the strain stiffening response in a significant way.

The characteristic behaviour of both the mucilages and pectin gels, across their respective LAOS regions is interpreted using L-B plots. In region I (linear), the L-B curves are elliptic for all the systems (Figure 6) and S~0 (Figure 7) for both the mucilages and pectin gels implying linear response. In region II, for all the systems, L-B curves show concave upward nature and 0<S<1 indicates the strain stiffening behaviour. Even though the L-B curves appear steeper for pectin gels in this region, the S values for the seed mucilages are comparable with those for the pectin gels. At the end of region III and in region IV, in the case of pectin gels, the shape of the L-B curve changes and the S values are either <0 or >1. Interestingly, in the case of seed mucilages, the shape of L-B curve remains nearly the same with 0<S<1. It is important to note that S values should always be less than 1, i.e. S>1 has no physical meaning and cannot be interpreted as either stiffening or softening. This shows that beyond the cross-over strain amplitude, pectin gels do not show strain stiffening response, whereas, seed mucilages continue to exhibit strain stiffening even at larger strains.

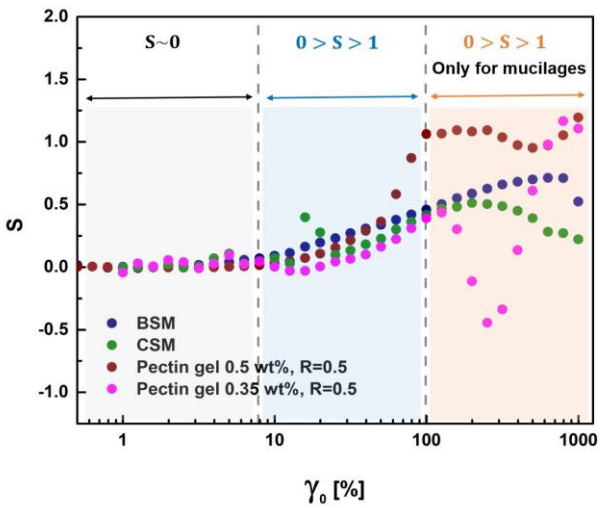


Fig. 7. Strain stiffening ratio, S during large amplitude oscillatory shear for BSM, CSM and pectin gels. The dashed lines represent the different regions of strain stiffening of these gels.

The area of the L-B plot indicates the energy dissipated in the material during deformation. It can be observed that for all the systems studied, the area of the L-B plots increases with increasing strain amplitude indicating increasing strain energy dissipation. In region IV (beyond the crossover), the energy dissipation is higher in the case of pectin gels. Similarly, the slope of the decay (a) of $G'(\gamma_0)$ in this region is also higher for pectin gels than seed mucilages as discussed earlier. This also points to the differences in the microstructure of the mucilage

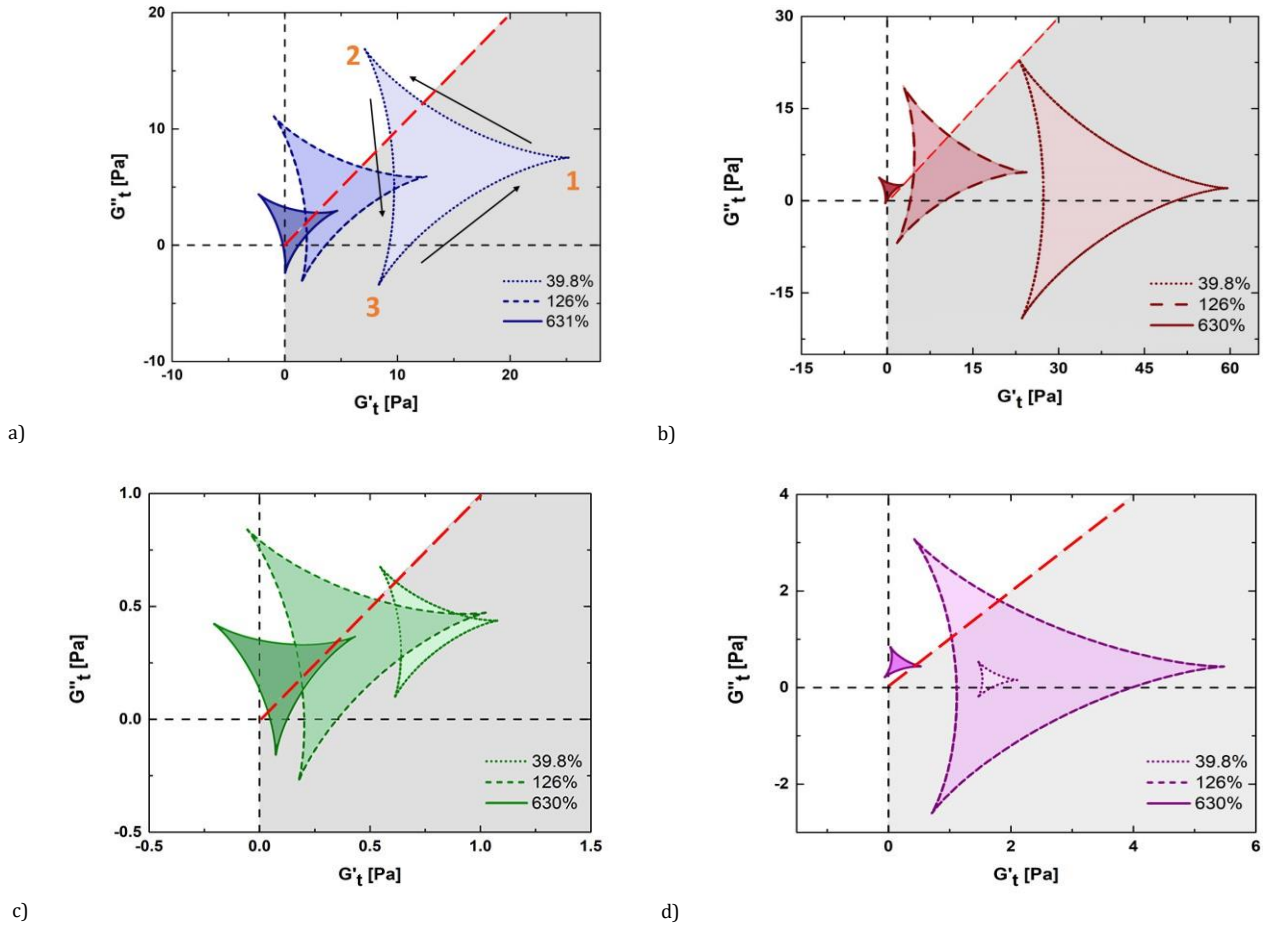


Fig. 8. Transient Cole-Cole plots for (a) BSM (b)Pectin gel 0.5 wt%, R=0.5 (c) CSM and (d) pectin gel 0.35 wt% R=0.5. The black dashed lines represent the X and Y axes passing through origin. The red dashed 45° line represents flow condition, $G'_t = G''_t$. The shaded region represents $G'_t > G''_t$ indicating elastic dominant region. The arrows indicate the path of deltoid and thus transient timescales such as yielding and recovery.

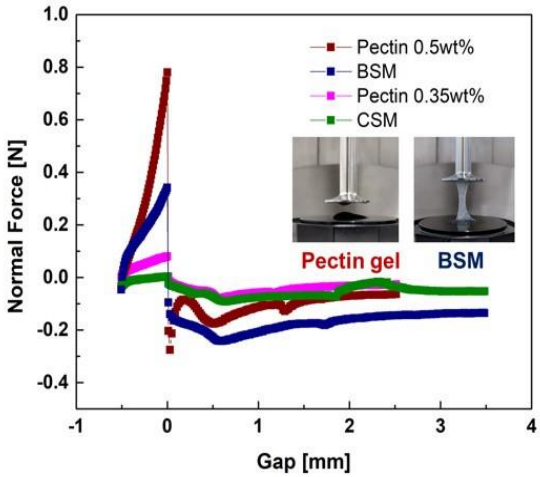
with that of the pectin gels and the processes contributing to the dissipation.

The transient Cole-Cole plots obtained from transient moduli are analysed using Sequence of Physical Processes (SPP) software (Luciano et al., 2021) and are shown in Figure 8. The deltoid plot is given for three different strain amplitudes each corresponding to the non-linear regions II, III and IV of the LAOS. Changes in the position and the area of the deltoid with increase in strain amplitude are quite informative about the structure of the seed mucilages.

At lower strain amplitudes (γ_0 = 39.8% in Figure 8), major area of the deltoid lies below the 45° line, where $G'_t > G''_t$ indicating more elastic nature of the gels. With increasing strain amplitude, the major portion of the deltoid shifts above the 45° line, where $G''_t > G'_t$ indicating a shift to fluidlike behaviour of the gels and also the yielding response. At higher strain amplitudes (region IV), the deltoid is completely located above the 45° line indicating more viscoelastic fluidlike response, especially in the case of pectin gels. However, in the case of seed mucilages, even at these higher amplitudes, some part of the deltoid still lies below the 45° line (in the shaded region) indicating the elasticity exhibited by the seed mucilages. This is also evident in the Lissajous curves, where the seed mucilages exhibit strain stiffening even at higher strain amplitudes, unlike the pectin gels. The significant observation from this analysis is that the seed mucilages have the capacity to maintain the elastic nature even at large strains.

Wet tack behaviour

The wet tack strength of the pectin gels and the seed mucilges is obtained from the normal force (F) versus gap (mm) plots and is shown in Figure 9(a) (Baron et al., 2009). The area under this curve gives the wet tack energy (J/m^2) of the material. For comparison, swollen whole seeds were also subjected to wet tack tests and the results are shown in Figure 9(b). Swollen whole seeds show higher wet tack strength when compared to freshly collected seed mucilages. Among these, swollen basil seeds and BSM show higher wet tack strength compared to swollen chia seeds and CSM. The force required to separate swollen basil seeds and chia seeds from the rheometer platen surface is approximately 22 and 12 N with wet tack energies of 18.34 and 2.05 J/m^2 respectively. The corresponding values of normal force for BSM and CSM are 0.4 N and 0.035 N and the wet tack energies are 0.143 and 0.0133 J/m^2. The pectin gels show normal force values between 0.1 and 0.8 N and wet tack energy between 0.051 and 0.28 J/m^2. In comparison to both BSM and CSM, pectin gels show higher wet tack energy.



a)

b)

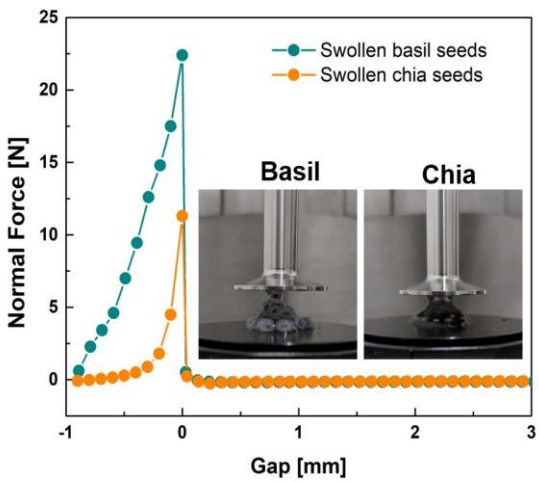


Fig. 9. (a) Tack response of the freshly collected mucilage and pectin gels. The inset shows that the basil seed mucilage still attaches to the platens unlike pectin (b) Tack response of the swollen basil and chia seeds. The inset shows the wet tack nature of swollen basil and chia seeds

However, the wet tack energy of seed mucilage when it is intact on seed surface is very high when compared to the pectin gels and extracted mucilages which is analysed later.

Discussion

Comparison of pectin gels with seed mucilages

From the comparative analysis of the rheological response of the basil and chia seed mucilages with pectin gels, the following similarities and differences are summarised and are given in Table 1 (also in supplemental information Table 1.)

- Although two pectin gels with comparable zero shear modulus (G'_0) to that of the seed mucilages were selected to reflect the mucilage behaviour, the linear strain limits for both the seed mucilages are lower compared to that of the pectin gels.
- Pectin gels show strong overshoot in G'' curves. However, chia seed mucilage shows weak overshoot and basil seed mucilage shows no overshoot.
- In the case of seed mucilages, strain stiffening starts at lower strain amplitudes and persists till larger strain amplitudes (10-1000%). However, pectin gels exhibit strain stiffening over a short range of strain amplitudes only (25-150%).
- Strain stiffening ratio (S) values are comparable for seed mucilages and pectin gels at low strain amplitudes. However, at strain amplitudes > 150%, seed mucilages continue to exhibit strain stiffening while the pectin gels show fluctuating S values indicating the differences in the mechanisms contributing to the strain stiffening characteristics. Energy dissipation is also higher for the seed mucilages especially in region I and region II of LAOS response.
- Lesser decay in the G' values with increase in strain amplitude and recovery of elasticity are observed in the case of seed mucilages even above the crossover strain amplitudes, unlike in the case of pectin gels.

Role of hierarchical structure in strain stiffening

By analysing the differences between the responses of the mucilages and the pectin gels, the mechanisms and processes involved in the strain stiffening behaviour of mucilages can be elucidated. Eventhough the G'_0 values of the mucilages and the corresponding pectin gels are comparable, the overshoot behaviour in G'' values and strain stiffening are entirely different. Chia seed mucilage shows weak overshoot in G'' and basil seed mucilage does not show any overshoot in G''. This overshoot in G'' in the case of pectin gels and alginate gels (Jacob et al., 2019; Hashemnejad and Kundu, 2016) is attributed to the unzipping and reformation of the egg boxbundles. Hindrance in egg-box bundle formation results in diminished overshoot or absence of overshoot in G'' (Jacob et al., 2019). In the case of mucilages, the diminished overshoot or the absence of overshoot in G'' could be due to several other factors. EDS analysis of mucilage shows the presence of divalent ions, such as, calcium (Ca^{2+}) and magnesium (Mg^{2+}) and monovalent ion, potassium (K^{+}) in both basil and chia mucilages (Supplemental information - Figure 1). The free uronic acids in the mucilage have the ability to crosslink in the presence of calcium and magnesium ions. However, pectin forms weak gels in the presence of Mg, compared to that of Ca (Zsivanovits et al., 2004). The moduli (G', G'') of the mucilage indicate that they are preferably Ca-crosslinked gels (Supplemental Information - Figure 2). Additionally, the presence of monovalent ions can cause a charge screening effect hindering the egg box formation (Jacob et al., 2019) in these gels. These suggest that diverse crosslinking mechanisms are plausible in mucilages when compared to Ca crosslinked pectin gels which could be leading to the observed differences in the rheological behaviour (LAOS -regions II and III, Figure 5).

The above descriptions are based on the microstructural features arising at the molecular scale, considering pectin alone. However, it is known that, the microstructure of the seed mucilages are constituted of hierarchial organization of different components (Kreitschitz A, 2018). For example, the tubules found in the basil seed mucilage have thicker cellulosic fibrillary bundles which are connected by crosslinked pectin molecules and hemicellulose. Additionally, starch granules are also present along the cellulose fibrils. Chia seed mucilage also has similar hierarchical structure, however, lacks the starch granules and the thick cellulosic fibrillary bundles. Chia seed mucilage do not form tubules. This hierarchical structure of mucilage with cellulose, pectin, hemicellulose and starch granules and the interactions among them also play important role in determining the mechanical response of the mucilage, especially at higher strain amplitudes. Rheological studies on pectin gels containing cellulose nanofibrils reported weak overshoot in G'' (Jacob, 2021). Pectin can bind with cellulose nanofibrils and alter the rheological response (Bidhendi and Geitmann, 2015; Mendu et al., 2011).

The strain stiffening behaviour of the mucilages could originate from the microstructural features at multiple length scales present in the hierarchical structure. The strain stiffening region of mucilage is observed from strain amplitudes of 10 to 1000 % for both basil and chia seed mucilages, whereas, pectin gels show strain stiffening over a range of 25 -

Table 1. Comparison of LAOS behaviour of seed mucilages with pectin gels from Figures 5, 6, 7 and 8

S.No	Property	BSM	CSM	Pectin gel 0.5wt%	Pectin gel 0.35wt%
1.	G'_0[Pa]	40	1.4	50	1.5
2.	Linear strain limit [%]	2	2	10	10
3.	Overshoot in G''	Absent	Minor	Significant	Significant
4.	Yield strain [%]	125	160	80	250
5.	a, b (decay coefficients)	-1.0, -0.63	-0.8, -0.3	-2.4, -1	-1.92, -0.84
6.	Stiffening region [%]	10-1000	30-1000	25-80	40-150
7.	Stiffening ratio in region IV	increases, > 0	decreases, > 0	< 0	< 0

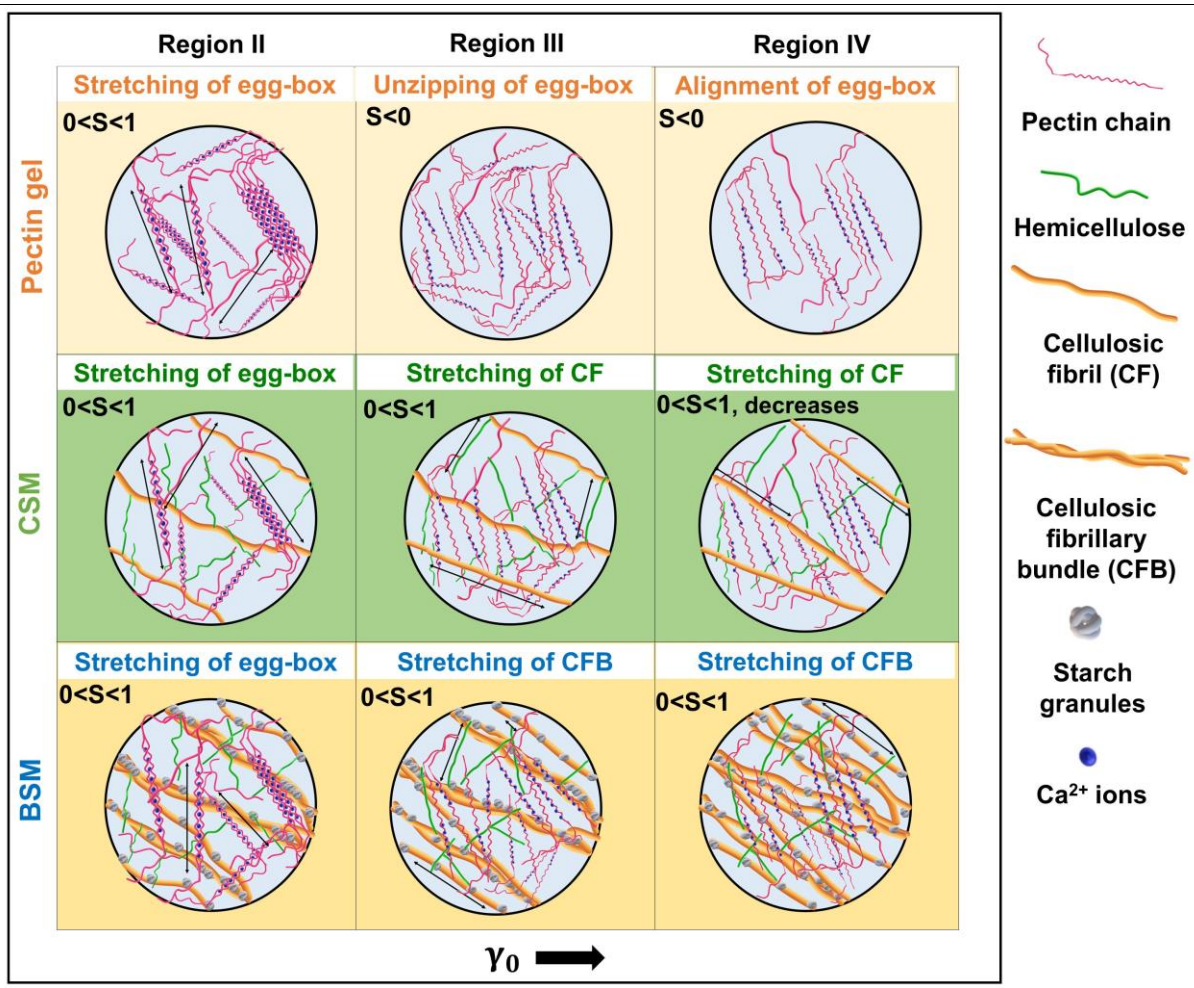


Fig. 10. Schematic representation of microstructural changes occurring in pectin gels and seed mucilage during deformation. The arrows indicate the cellulosic fibrils and fibrillary bundles.

150 % only. At lower strain amplitudes (< 100%), both the seed mucilages and the pectin gels show similar strain stiffening behaviour (Figure 7), indicating the role played by the Ca- crosslinked pectin gels. However, as the strain amplitude increases, only the seed mucilages show strain stiffening behaviour whereas,

the pectin gels cease to show strain stiffening. The dissociation of pectin network such as unzipping of egg box structures occurs and the role of pectin network in strain stiffening ceases. In fact, it has been shown that at higher strain amplitudes pectin gels show strain softening behaviour (Jacob et al., 2019). However, observation of strain stiffening of mucilage in this region highlights the fact that the load transfer happens from pectin to the cellulose and hemicellulose structures making them the load bearing components under large strains. The possible strain stiffening mechanism in region III could be originating from the stretching/bending of the cellulosic fibrils and their bundles. The schematic representation of the hierarchical structure and the microstructural changes during deformation are shown for both the seed mucilages alongGA with pectin gels in Figure 10.

The S values of CSM starts declining beyond $\gamma_0 > 250\%$ whereas, the S values of BSM continue to increase and declines only at 1000% strain amplitude (Figure 7). This could be arising from the microstructural differences between BSM and CSM as described earlier. BSM has thicker cellulosic fibrillary bundles, while CSM has thinner cellulosic fibrils. This emphasizes the fact that the hierarchical organization of components in seed mucilage and the systematic load transfer among different microstructural components helps the mucilage to withstand large shear deformations. This is also reflected in the wet tack strength and the role of strain stiffening in the seed anchorage which are discussed further.

Seed anchorage - role of strain stiffening and wet tack?

In the natural environment, swollen seeds of basil and chia are subjected to various mechanical forces in the form of shear, compression, tension etc. These different external conditions could arise from events, such as the flow of water during the rains or watering the seeds after sowing. The typical shear stress experienced in these events by the seeds is in the range of 1.8-10.6 Pa and shear strains of 0.2-200% (Engelbrecht et al., 2014; Poesen et al., 2003) . These conditions can be simulated in rheological experiments using LAOS mode. The role of the extraordinary strain stiffening response observed in the case of basil and chia seed mucilages in anchoring the seeds to the soil surface is further reinforced from the wet tack test results. The observed tack response also suggests that the mucilage layer helps anchor the seed strongly to the substrate and prevent its dislodgement by allowing large scale deformation of the mucilage without separating from the seed kernel. The tubular structures in the BSM may help in additional anchorage by penetrating into the porous soil media. The additional stretching during shearing of the mucilage enhances the area of contact with the soil. These inferences suggest that the strain stiffening of the mucilage has larger significance in seed physiology and this response even allows mucilage to act like a cytoskeleton to protect the seed from rupture and dislodgement during large external forces.

Conclusions

When exposed to external shear forces such as those originating from the flow of water, seed mucilage prevents seed dislodgement by anchoring the seed to the soil. To understand the possible mechanisms by which mucilage supports seed anchorage, the mechanical responses of the two model seed mucilages - basil and chia were analysed using both small and large amplitude oscillatory shear (SAOS and LAOS) rheology and wet tack studies. Similarities are observed in the qualitative mechanical response of both the mucilages exhibiting weak gel-like behaviour under SAOS and strain stiffening at large deformations. The response of seed mucilage to mechanical deformation is distinctly different from that of the model pectin-Ca gels studied. Especially, under large deformations, mucilage is capable of maintaining the elasticity and strain stiffening behaviour, unlike the pectin gels that undergo rupture and softening. Both the basil and chia seed mucilages show capability to recoil after larger deformations. This is also a unique feature of the seed mucilages when compared to the pectin gels. The presence of nano-scale structures such as cellulosic nanofibrils and their bundles interconneted by crosslinked pectin and hemicellulose in mucilage helps in withstanding large deformation through strain stiffening. The anchoring nature of the mucilage is established using wet tack experiments on a rheometer. Mucilage is known to support seed anchorage and the strain stiffening response of the mucilage is expected to maintain the anchorage strength during external forces. This is possible due to the contributions from the hierarchical structures present in seed mucilages resulting in more contact with the soil substrate. Our studies suggest that rheology can be a novel tool for understanding the physiological functioning of plant based soft gels *in vitro*.

Competing interests

No competing interest is declared.

Author contributions statement

K.B., P.S., A.P.D. and S.V. conceived the experiments, K.B. and P.S. conducted the experiments, K.B., P.S., A.P.D. and S.V. analysed the results. K.B., P.S., A.P.D. and S.V. wrote and reviewed the manuscript.

Acknowledgments

This work is supported in part by funds from the Science and Research Engineering Board (SERB), India.

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