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MISCELLANEOUS TOPICS

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Rain events at maturity severely impact the seed quality of psyllium (*Plantago ovata* Forssk.)

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Abstract

Plantago ovata Forssk. is an emerging crop yielding psyllium husk, a material comprised of hydrophilic polysaccharides that form mucilage upon wetting. Psyllium husk has important industrial uses including as a dietary fibre supplement and a textural alternative in gluten-free bread production. Industrial applications require high-quality and purity psyllium husk, but consistent supply of uniform quality material is often limited by climatic constraints, especially unseasonable rainfall at crop maturity. Here we compared the seed quality of four *P. ovata* varieties harvested before and after 26 mm of rain and validated our key findings in the following season. Colourimetry showed that the rain event caused the seeds to be darker and greener, possibly from pigment oxidation and microbial growth. Sugar profiling, water absorption assays and microscopy showed that premature hydration of the husk in rain-damaged samples caused loss of the most soluble mucilage components and an increase in non-mucilage contaminants, leading to a reduction in seed water absorption capacity, which is a key indicator of psyllium husk functionality. Germination was also diminished in rain-affected seeds. In this study we show for the first time the extent that unseasonable rain at maturity has on *P. ovata* seed quality. We suggest that rain-damaged seeds are unsuitable for husk production and resowing and outline potential screening methods to identify rain-damaged seeds before purchase. Additionally, the extensive quality impacts described here may make *P. ovata* a suitable model or indicator species for studying acute climate effects on seed quality, especially from rain.

KEYWORDS
mucilage, *Plantago ovata*, psyllium husk, rain damage, seed quality, weathering

1 | INTRODUCTION

Plantago ovata Forssk. is an important emerging herbaceous crop native to central Asia and commonly cultivated in India, Pakistan and Iran. *P. ovata* is the source of psyllium husk – a papery material milled from the seed surface that is comprised almost entirely (c. 86%) of non-fermentable polysaccharides that, when hydrated, swells into a gel called mucilage (Cowley & Burton, 2021). Psyllium husk is a widely consumed dietary fibre supplement that assists with laxation thereby relieving constipation (Marlett et al., 2000; McRorie et al., 1998) and aids in alleviating metabolic disorders like hypercholesterolaemia (Anderson et al., 2000). Psyllium husk is also an important component of gluten-free food production, as it replicates some of the textural and structural

properties of gluten (Cappa et al., 2013; Fratelli et al., 2018; Haque & Morris, 1994; Mancebo et al., 2015; Ren, Linter, et al., 2020). Its many uses lead to high demand for high-quality psyllium husk, which current global supply is not always able to match, so alternative growing regions are needed. The majority of the world's *P. ovata* production is from North Gujarat province, India, where summers are hot, winters are cool, and rainfall is low, particularly in winter after the initiation of flowering (Kumar, 2015). Few alternative growing regions have the requisite hot summers and dry late season/winter climate for high-quality psyllium production, but for over 35 years, *P. ovata* has been undergoing development as a crop in the Ord River Irrigation Area in Northern Australia (McNeil, 1989a, 1989b; McNeil, 1991a, 1991b; McNeil, 2017; McNeil & Duran, 1992). The outlook from these trials is promising, but climatic constraints and variability remain a poorly understood and significant hurdle to *P. ovata* crop success, widespread adoption and the production of the highest quality psyllium husk.

Climate change is causing extreme weather events with rainfall particularly anticipated to become more intense but less frequent, and the seasonality of rainfall is also expected to change (Dey et al., 2019). Variation in rainfall affects crops in many ways from drought to flooding, but an effect not often considered is the impact unseasonable rain can have on seed quality. Rain damage has been reported to diminish seed quality, physiologically and via harvest quality, in a number of crops including wheat (*Triticum aestivum* L.; Ellis & Yadav, 2016), common bean (*Phaseolus vulgaris* L.; Greven et al., 2004), mung bean (*Vigna radiata* L. and *Vigna mungo* L.; Williams et al., 1995) and soybean (*Glycine max* L.; Castro et al., 2016). Reductions in seed quality have been linked to transient rewetting after the maturation drying phase of seed development had initiated and perhaps also physical weathering of the seed coat. Where non-mucilage-producing seeds could allow evaporation or droplet roll-off before significant moisture damage occurs, the impact of even a brief rain event on seed quality of mucilage-producing species (e.g. canola, flax, chia and psyllium) is likely to be more profound as the hydrophilic mucilage polysaccharides could trap water at the seed surface. Despite being somewhat protected by the capsule at maturity, dehiscent zones still allow moisture entry. High levels of humidity have been shown to diminish mucilage quality in flax (*Linum usitatissimum* L.; Dorrell, 1973; Dorrell & Daun, 1980; Gubbels & Kenaschuk, 1993) although these authors found that the main oil quality indicators, given it is used mainly as an oilseed, were unaffected and thus mucilage impacts were not considered an important determinant of flax seed quality. By contrast, the mucilage-forming psyllium husk is the primary product obtained from *P. ovata* seeds, and thus any impacts on its quality are considered critical. This is exacerbated by the morphology of *P. ovata* seeds and the exposed nature of its mucilage polysaccharides. In species like flax and *Arabidopsis thaliana* L. Heynh., mucilage polysaccharides are synthesised and stored within specialised cells on the outer surface of the seed coat called mucilage secretory cells (MSCs) (Cowley & Burton, 2021). In contrast, we recently reported that *P. ovata* seeds are covered by a cell-free layer of dry laminated polysaccharides (the MSCs disintegrate during development and are absent at maturity on the seed's surface), forming the basis of the 'psyllium husk' obtained when the seeds are milled (Phan et al., 2020). Unlike flax or Arabidopsis where MSCs must reach a certain level of hydration before rupturing and releasing their mucilage (Miart et al., 2019; Windsor et al., 2000), the cell-free mucilage polysaccharides of *P. ovata* make mucilage release independent of a hydration threshold and cell rupture and thus it is likely that *P. ovata* seeds are inherently more susceptible to damage from high humidity/rain. The economic implications of what are likely to be rapid and profound impacts on psyllium husk quality caused by rain and other abiotic factors are significant but remain poorly understood or characterised. Furthermore, the hallmarks of *P. ovata* mucilage quality, mucilage purity and seed quality in general are poorly defined and these would aid in improving post-harvest quality testing of *P. ovata* seed for industrial and pharmaceutical end users of psyllium husk, and even for other mucilage-producing crops.

In this study we have performed the first investigation of the impact that damage from adverse rain events has on the physiological and harvest quality of *P. ovata* seed using field-grown material from Kununurra, Western Australia harvested before and after 26 mm of unseasonable late season rainfall at crop maturity.

Key Points

- Psyllium husk from *Plantago ovata* seeds has industrial and medicinal use due to its ability to swell greatly in water.
- Psyllium husk quality varies, often due to late season rain when seed is mature, but this process is poorly understood.
- In field-grown *P. ovata*, rain events at maturity caused yield loss and reduced seed quality hallmarks.
- Rain affected seeds yielded unwanted pigments and absorb less water making them unsuitable for most food uses.
- Rewetting after maturation reduced germinability making them also unsuitable for resowing.

2 | MATERIALS AND METHODS

2.1 | Plant materials

In 2020 and 2021, four *P. ovata* varieties, V1, V9, V11 and V12, were grown at the Frank Wise Institute for Tropical Agriculture, Department of Primary Industries and Regional Development in Kununurra, Western Australia (S 15° 39′ 18″, E 128° 43′ 1″) as part of replicated variety trials. The trials were planted in mid-May in both years dependent on prevailing weather conditions. The crops were sown and managed according to recommendations in McNeil (2017). Plant stands of 300–500 plants per square meter per replicate were

obtained from sowing rates of 14–15 kg/ha based on pre-sowing seed germination rates. The crops were sown 0–5 mm deep in Kununurra clay irrigation bays consisting of 70–100 m long, 1.2 m wide bed tops separated by 0.6 m furrows used for irrigation. Weeds and pests were controlled using approved chemicals as needed and in line with pesticide registrations. In the 2020 trials pre- (BR) and post-rain (AR) samples were obtained from all four lines, while in the 2021 trial, V1, V9 and V11 were not available in the ongoing variety trials and so samples were obtained from V12 only. Fertiliser was applied as recommended in both years consisting of a basal application of N (51 kg/ha), P (21 kg/ha), Zn (5 kg/ha), S (12 kg/ha) and a urea top dressing was applied (46 kg/ha N) at early flowering. The plants were irrigated as required throughout the season with no in-season rainfall occurring except as indicated near harvest. In both years, top growth was good, averaging 9–10 t/ha in 2020 though somewhat less in 2021 due to the presence of foliar disease around flowering. Harvest indices were relatively low in both seasons but not inconsistent with other seasons.

At harvest, a 0.5 m by 1 bed wide sample plot was hand cut from the middle of the central bed of a sample. Any obviously disease affected, low population, trampled or otherwise abnormal areas were avoided. The entire sample was placed in large paper or cloth bags at the point of harvest and then air-dried at approximately 50°C. The seed was then threshed using a Kimseed (Wangara, Australia) laboratory plant thresher including any that had shattered into the bag. The seed was subsequently cleaned of very small debris (including early aborted seeds) and large trash using air flow and vibrating oval shaped slotted screens on a Kimseed (Wangara, Australia) laboratory seed cleaner. Threshed and cleaned seeds were stored at room temperature (22–25°C) in sealed polypropylene containers until analysis.

Climate data were obtained from the Government of Western Australia Department of Primary Industries and Regional Development weather station located at the DPIRD Station in Kununurra approximately 500 m from the trial site.

2.2 | Hundred seed weight

For each plot replicate, 100 seeds were manually counted and weighed on a microbalance.

2.3 | Seed size

For each plot replicate, 50 seeds were randomly selected, backlit with a LED light box and imaged with a macro digital camera from a fixed height (Galaxy S10+, Samsung, South Korea). Seed size was determined by image analysis using the 'Analyse Particles' function of Fiji ImageJ. The image scale was set to a steel drafting ruler (also imaged at the same fixed height) and the average seed area was calculated for each plot replicate.

2.4 | Seed colour analysis

Seed colour was determined using digital image colourimetry. For each replicate, cleaned seed was loaded into triplicate wells in a 12-well plate (Corning, United States). The plates were tapped several times to settle seeds onto the bottom of the well before scanning the plate on a flat-bed scanner (C7270i, Canon, Japan) at 600 dpi. Images were calibrated to an internal pure white reference before determining the L^*, a^* and b^* colour space values with Fiji ImageJ (Woolf et al., 2021). Colour space values of technical triplicates were averaged for each plot replicate. Total colourimetric difference of AR compared to BR (ΔE 2000) was determined using the ColorTools add-in for Microsoft Excel (rbcmyk.com.ar, 2021).

2.5 | Pigment extraction and spectrophotometry

To estimate the ability of pigment to leach from internal seed tissues and, simultaneously, the heat-responsive colour change capacity, a spectrophotometric method was used based on Abdel-Aal and Hucl (1999) with modifications. For each sample, 150 mg of cleaned seeds were weighed into 2 ml microcentrifuge tubes. To each tube, 1.5 ml of acidified ethanol (15% v/v 1 M HCl and 85% v/v denatured ethanol) was added and samples were incubated in a shaking incubator (Thermomixer Comfort, Eppendorf, Germany) at 60°C for 1 h. Samples were centrifuged to pellet debris and 1 ml of supernatant was transferred to a clean cuvette and samples read at 600 nm on a spectrophotometer (Cary 50 Scan, Varian, United States). The absorbance of a blank of acidified ethanol was subtracted from the absorbance of each sample. The average solution colour was obtained by the digital image colourimetry of images taken through the side of the cuvette, backlit by an LED-light box. Total colourimetric difference (ΔE 2000) compared to the acidified ethanol blank (solvent) was determined using the ColorTools add-in for Microsoft Excel (rbcmyk.com.ar, 2021). The mean colour of BR and AR samples were obtained using the colour average function of Photoshop CC 2018 (Adobe, United States).

2.6 | Mucilage staining and microscopy

Mucilage architecture was observed by staining with ruthenium red based on Arsovski et al. (2010) and Cowley et al. (2020) with modifications.

Ruthenium red (0.01% w/v) (C075, ProSciTech, Australia) was dissolved in water containing 0.001% (v/v) Tween-20. The ruthenium red solution was degassed by sonication under vacuum for 2 min to prevent bubble formation during imaging.

Fifteen to twenty seeds per sample were randomly selected and placed into wells of a flat-bottomed 24-well microplate (Linbro, ICN Biomedicals, USA) before adding 500 µl of ruthenium

red solution. The seeds were imbibed undisturbed for 30 min before being backlit with a LED lightbox and imaged with a macro digital camera from a fixed height (Galaxy S10+, Samsung, South Korea).

Soluble mucilage was stained and quantified using MuSeeQ, a supervised image analysis tool for phenotyping soluble mucilage parameters (Miart et al., 2018). Twenty seeds from each sample were placed onto a 10 × 10 cm square petri dish (Sarstedt, Germany) filled with 0.6% (w/v) agarose (25 ml) impregnated with 0.4% toluidine blue (C078, ProSciTech, Australia), 0.1% sodium tetraborate (BDH Chemicals, Australia) and 0.001% Tween 20 (Sigma, USA). Mucilage was allowed to release and stain for 24 h before imaging as above for the ruthenium red assay. The ratio of mucilage to seed area was calculated using the *Camelina* MuSeeQ macro for Fiji ImageJ (Miart et al., 2018). Severely affected seeds released minimal mucilage, so were poorly segmented by the macro, necessitating confirmatory manual measurement of some seeds. Mucilage to seed area ratios were averaged for each sample.

2.7 | Water absorption capacity

The water absorption capacity of seed samples was determined following Cowley et al. (2020). After weighing 30 mg of cleaned seed into a 2 ml microcentrifuge tube, 1 g of water was added, and mucilage was allowed to expand undisturbed for 45 min at 25°C. Using a 1 ml micropipette, unabsorbed water was removed and weighed. The water absorption capacity was determined using the following equation:

$$\text{Water absorption capacity (mg / mg)} = \frac{\text{Initial mass of water added (mg)} - \text{mass of excess water removed after 45 mins (mg)}}{\text{Initial mass of seeds (mg)}}$$

The water absorption capacity was performed in technical duplicate and averaged for each plot replicate.

2.8 | Total mucilage extraction

Crude total mucilage content was determined following Cowley et al. (2020) with modifications. Briefly, 30 mg of whole, unmilled seed was extracted for 1.5 h with 1.5 ml of deionised water at 65°C and 1300 rpm agitation using a ThermoMixer® Comfort (Eppendorf, Germany). The samples were centrifuged for 2 min at 15900 *g* and the supernatant removed to a clean tube. Solution volume was made up to approximately 1.5 ml with water based on tube markings and the samples agitated at 30 Hz for 10 min on a MM400 Mixer Mill (Retsch, Germany) with a 2 ml tube adapter. Tubes were centrifuged at 15900 *g* for 2 min and the supernatant removed. The two supernatants were homogenised in a water bath at 85°C for 3 h, then freeze-dried to a constant weight using a benchtop vacuum freeze-drier (Dynavac FD-1-50, Australia).

Freeze-dried total mucilage extracts were dispersed at 1 mg/ml in ultra-pure water in a water bath at 85°C for 3 h with intermittent shaking for further analysis.

2.9 | Spectrophotometry of mucilage dispersions

Pigment carry-over during mucilage extraction was determined by spectrophotometry. One volume of the same 1 mg/ml mucilage dispersions were acidified with one volume of 2 M HCl (0.5 mg/ml mucilage in 1 M HCl) before incubating in an oven at 60°C for 1 h. One millilitre of incubated mixture was placed into a cuvette and read at 600 nm on a spectrophotometer (Cary 50 Scan, Varian, United States).

2.10 | Monosaccharide analysis

Monosaccharide profiles of crude mucilage extracts were determined as per Cowley et al. (2021).

2.11 | Microbial growth assay

As a preliminary investigation of seed infestation, seeds from each sample (5 per plot replicate) were plated on sterile potato dextrose agar before sealing and incubating at 37°C. After 1 and 7 days plates were placed on an LED light box and imaged with a commercial camera.

2.12 | Germination assay

In a laminar flow chamber, 20 seeds per plot replicate were counted and placed into a 1.5 ml microcentrifuge tube. To the tube, 1 ml of sterilisation agent (50% commercial bleach, 25% ethanol, 25% water) was added, before sealing and inverting repeatedly for a minute. After a minute, the sterilisation agent was then discarded. This process was repeated for a total of 5 times to ensure maximum sterility. To wash away residual sterilisation agent, 1 ml of autoclaved deionised water was added, and the tube sealed and inverted repeatedly for 1 minute. This process was repeated twice. From each sterilised batch of seeds, 15 seeds were evenly distributed into a sterile 90 mm petri dish lined with autoclaved glass fibre paper (Whatman, United Kingdom). The glass fibre paper was wetted with 5 ml of autoclaved deionised water and the plate sealed with cling film and placed in an incubator at 25°C with a 16 h/8 h light/dark photoperiod.

The petri dishes were removed every 24 h for 144 h to count the number of germinated seeds (determined by radicle emergence, visible without a microscope). The seed counting was performed in

the laminar flow cabinet to maintain sterility. The germination index, which emphasises both the percentage and velocity of germination, was calculated as per Tan et al. (2017) with the following equation: $\sum(Gt/Dt)$ where Gt denotes the number of seeds germinated on day t and Dt is the number of days since plating (germination time).

2.13 | Statistical analyses

All analyses were performed on seed from three plot replicates per variety, both before and after the rain event. To determine statistically significant interactions from 'rain' and 'variety' factors in the 2020 trial, a two-way analysis of variance (ANOVA) was used, and statistically significant differences between BR and AR samples within a variety were determined using a Bonferroni's multiple comparisons test ($p < .05$). From the validation data from 2021 (one variety), agronomic data were analysed by unbalanced regression in GenStat (21st Edition, VSN International, UK) while significant differences in seed quality traits between samples before and after rain (performed blind) were determined by Student's t-test ($p < .05$). Unless otherwise stated, statistical analyses and plotting were performed in Prism 8.4.2 (Graphpad, United States). Multivariate principal component analysis and PERMANOVA of monosaccharide profiling in Figure S2 was performed in PAST 4.03 (Hammer et al., 2001).

3 | RESULTS

3.1 | Rain effects on seed from the 2020 harvest

3.1.1 | Between harvests, 26 mm of rainfall was recorded

In the week of harvest, the average daily temperature at the field site ranged from 27.9°C to 31.9°C (Figure 1a) with a weekly minimum of 21.5°C and weekly maximum of 40.7°C. Average humidity ranged from 53% to 74.3% (Figure 1b) with a weekly minimum of 23.5% and a weekly maximum of 99.7%.

On the 22nd of September, *P. ovata* plants were harvested and seed from these plants constitute the Before Rain (BR) samples. On the 24th and 25th of September, 8.4 mm and 17.6 mm of rain fell, meaning that the After Rain (AR) samples had been exposed to 26 mm of cumulative rainfall before being harvested on the 26th of September (Figure 1c).

Prior to the week of harvest, no rain had fallen at the field site since the 22nd of May 2020.

3.1.2 | Rain did not cause an overrepresentation of smaller, shrivelled seeds

To assess if the rain events caused a shift in seed size populations, we compared the 100 seed weight of seeds harvested before and after then rain event, finding that while there were minor intervarietal differences ($p = .0043$), the rain event caused no global change in 100 seed weight ($p = .9450$; Figure 2a). Average seed size was similarly unaffected by the rain event ($p = .1075$) with no significant effect of variety ($p = .3594$; Figure 2b). Visual inspection of the seed samples showed that while BR samples had a typical appearance for high-quality *P. ovata* seeds (smooth, matte, pale husk with even colour between seeds), AR samples were more variable in individual seed colour but most noticeably, many seeds had a rough texture

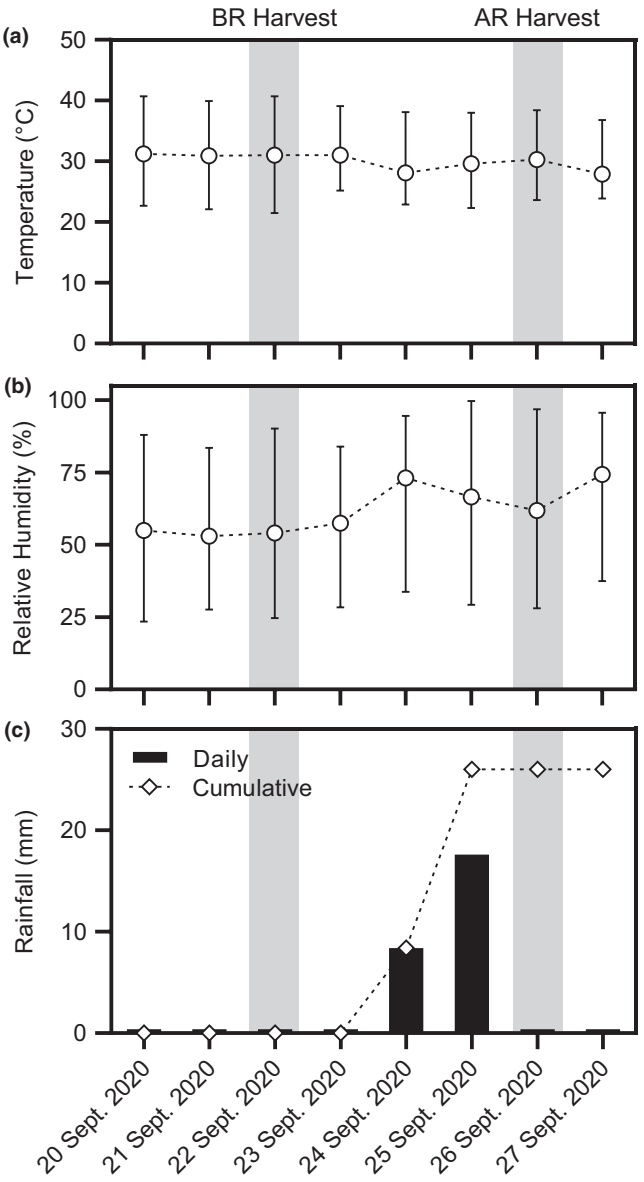


FIGURE 1 Climate data from the field trial site at the week of harvest (20–27 September 2020). (a) Daily temperature and (b) daily humidity. Open circles represent the daily mean while error bars represent the daily range where caps are the minimum and maximum. (c) Rainfall data. Black bars represent the daily rainfall while the open diamond symbols are cumulative rainfall. Grey boxes denote the two harvest dates: Seed harvested on the 22nd and 26th of September are the before rain (BR) and after rain (AR) samples, respectively

and 'scabbing', and at times, glossier and wrinkled surface similar to the appearance of medjool date (Figure 2c). In agreement with the 100 seed weight and seed size results, neither BR or AR samples had a noticeable presence of shrivelled or aborted seeds.

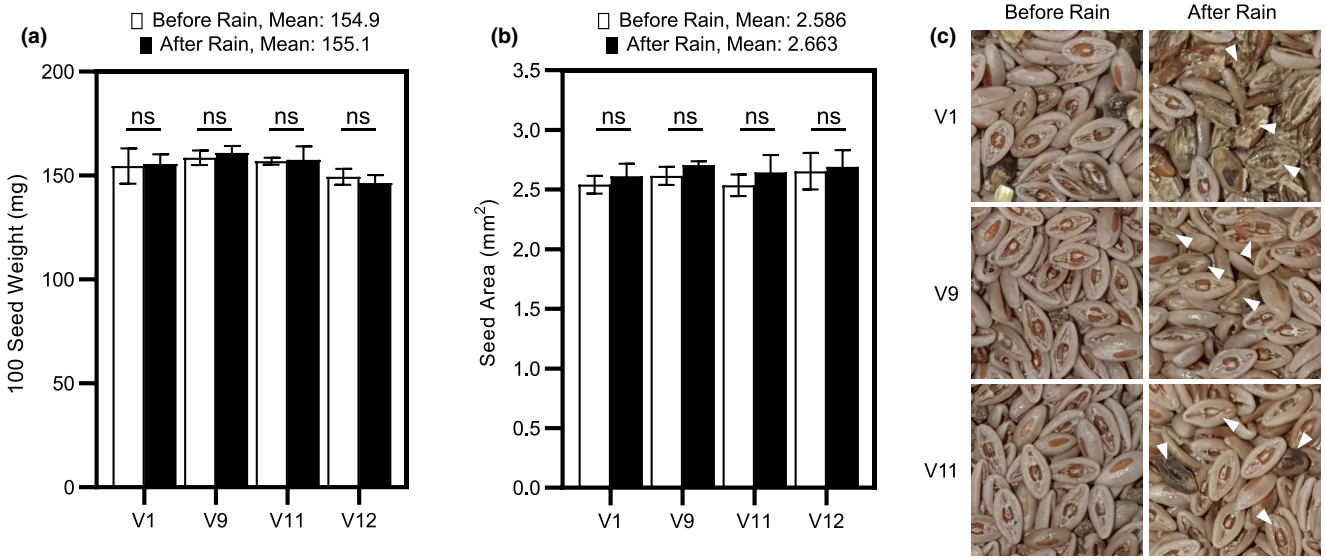
3.1.3 | Rain causes significant seed discolouration

Using colour analysis, we assessed the impact of rain damage on the chromaticity of psyllium seed samples. The three colour space values, L^* (representing lightness/darkness; lower values are darker, higher values are lighter), a^* (representing greenness/redness; lower values are greener, higher values are redder) and b^* (representing blueness/yellowness; lower values are bluer, higher values are yellower) were significantly affected by the rain event ($p < .05$; Figure 3). The rain event caused a significant global reduction in L^* ($p < .0001$), leading to significantly lower seed lightness in each variety (Figure 3a), and a significant global reduction in a^* ($p < .0001$), leading to significantly greener seeds in each variety (Figure 3b). While the two-way ANOVA found a significant global effect of the rain event on b^* ($p = .0367$), the variety contributed more b^* variation (32.13% vs 14.62%) and no significant difference in AR samples compared to BR was found between any of the lines ($p > .05$).

Changes in seed colour of AR samples may reflect changes in the seed pigments. Using an acidified ethanol extraction on unmilled seeds, leachable pigments were extracted, and their absorbance measured by spectrophotometry (Figure 4a). The rain event had a significant influence on the absorbance of extracted pigments ($p < .0001$) increasing by 2.8-fold. Variety also had an influence ($p = .0081$) and within varieties absorbance of BR was correlated with the absorbance of the AR samples ($r = 0.84$, Figure S1). The increases in absorbance coincide with perceptible differences in extract colour (Figure 4b). When viewed in the cuvette, BR extracts are noticeably paler blue in colour, while AR samples are intense dark blue. Using the total colourimetric difference of the extracts compared to the solvent (ΔE; Figure 4c), rain had a significant discolouring effect on the extract ($p < .0001$) where BR samples displayed a discolouration of 16.15 and AR samples showed a discolouration of 40.14.

3.1.4 | Mucilage quality is reduced by rain damage

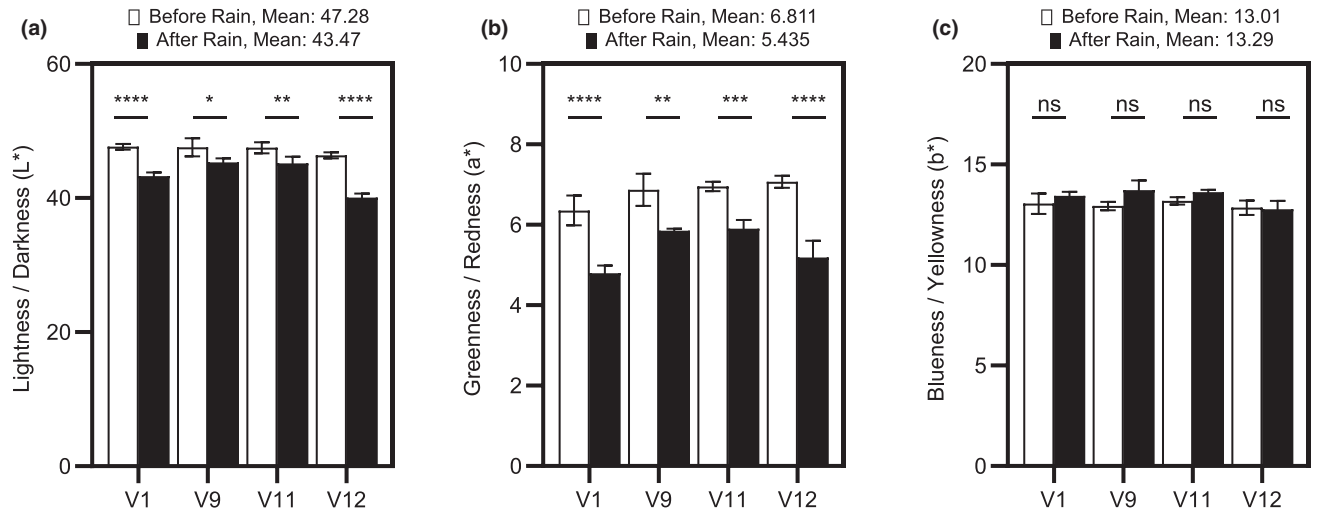
Disruption to mucilage release in rain-damaged seeds is evident by staining with ruthenium red (Figure 5a). Seeds of all BR samples produced mucilage envelopes with appearance typical of wild type *P. ovata*, while in AR samples some seeds did not release mucilage and often the amorphous, most soluble outer layer was significantly reduced. When quantified, the area of the soluble mucilage was smaller in AR samples and more variable (Figure 5b) and when quantified by image analysis was significantly reduced by rain damage ($p < .0001$; Figure 5c).



Source of Variation	Variation (%)	*p* value
Rain	0.01	0.9450 ns
Variety	57.44	0.0043**
Interaction	2.93	0.7871 ns

Source of Variation	Variation (%)	*p* value
Rain	12.81	0.1075 ns
Variety	15.20	0.3594 ns
Interaction	1.453	0.9531 ns

FIGURE 2 (a) Hundred grain weight and (b) seed size of four *P. ovata* varieties before (white) and after (black) 26 mm of rain in 2020. Data presented are means with standard deviation of three plot replicates. Below the plot are the outcomes of two-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$. (c) Macro appearance of seed samples harvested before and after 26 mm of rain. Arrowheads indicate seeds which are discoloured, textured and/or blackened. Scale bar = 2 mm and applies to all image panels

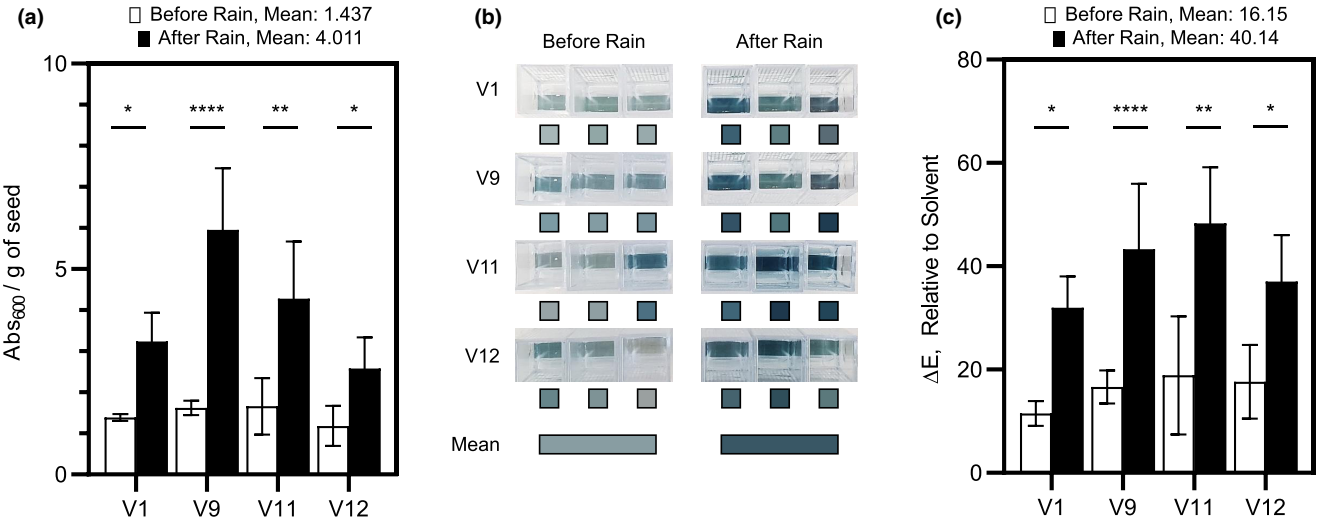


Source of Variation	Variation (%)	*p* value
Rain	56.54	<0.0001****
Variety	26.28	<0.0001****
Interaction	10.73	0.0011**

Source of Variation	Variation (%)	*p* value
Rain	70.87	<0.0001****
Variety	16.85	0.0002***
Interaction	4.92	0.058 ns

Source of Variation	Variation (%)	*p* value
Rain	14.62	0.0367*
Variety	32.13	0.0310*
Interaction	8.241	0.4283 ns

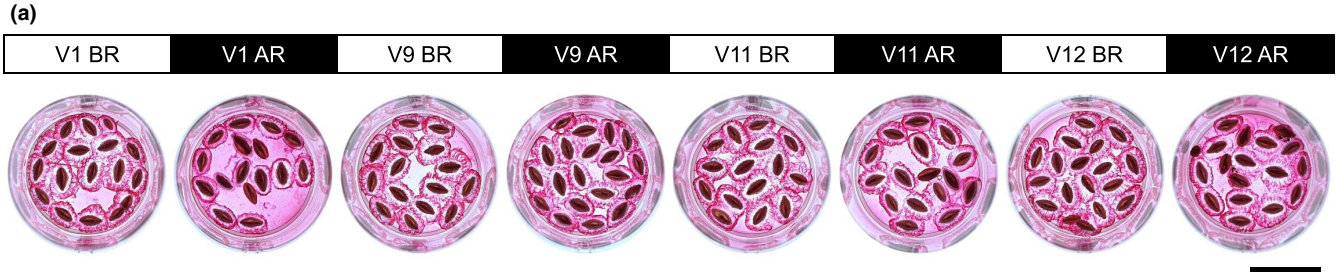
FIGURE 3 Colourimetric analysis of (a) L^*, (b) a^* and (c) b^* colour space values of four *P. ovata* varieties before (white) and after (black) 26 mm of rain in 2020. Data presented are means with standard deviation of three plot replicates. Below the plot are the outcomes of two-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$

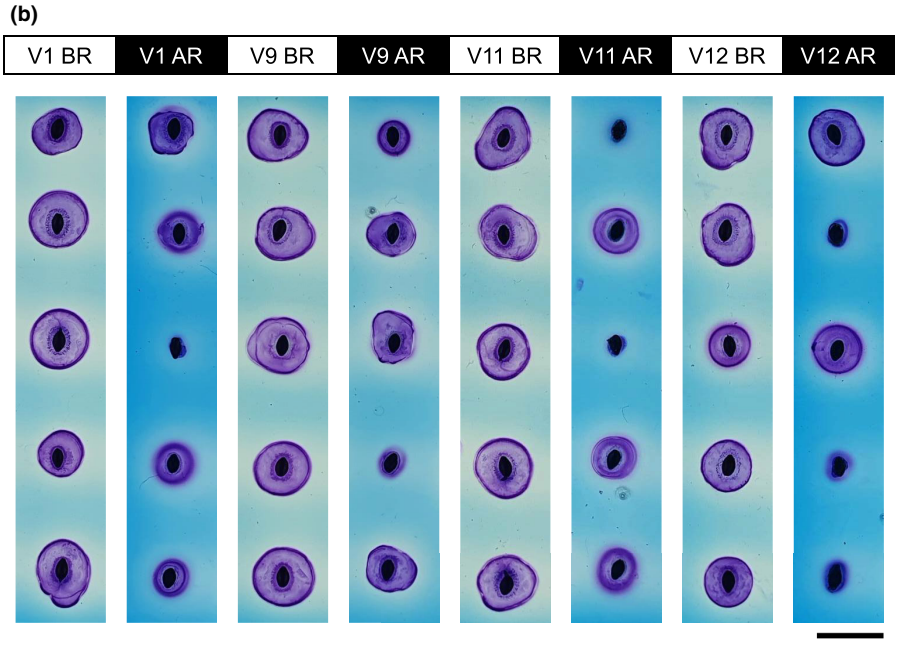


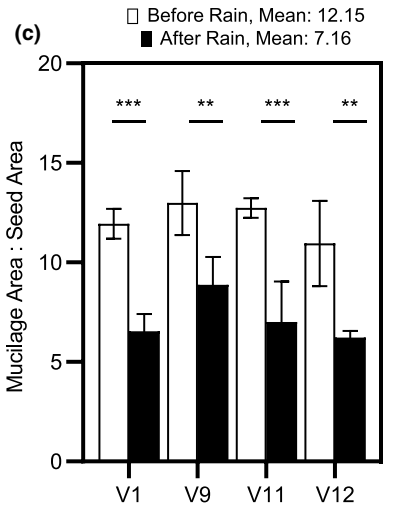
Source of Variation	Variation (%)	*p* value
Rain	55.31	<0.0001****
Variety	17.67	0.0081**
Interaction	10.14	0.0515 ns

Source of Variation	Variation (%)	*p* value
Rain	66.42	<0.0001****
Variety	8.64	0.1531
Interaction	2.03	0.7059

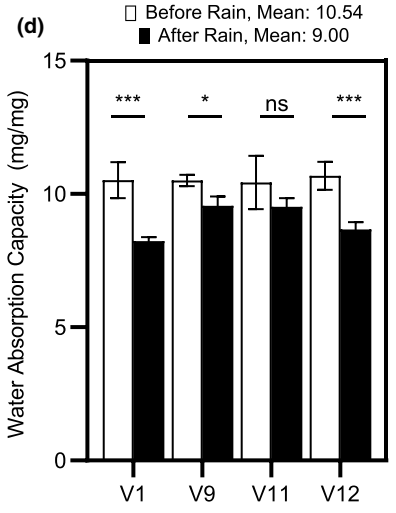
FIGURE 4 Analysis of pigments extracted with acidified ethanol from four *P. ovata* varieties before (white) and after (black) 26 mm of rain in 2020. (a) Absorbance at 600 nm of acidified ethanol extracts. Below the plot are the outcomes of two-way ANOVA. (b) Visual appearance of acidified ethanol extracts. Below each cuvette is the mean colour of each extract determined by colourimetry. Included below the images is the representative grand mean colour of extracts of BR and AR samples. (c) Total colour difference (ΔE) of extracts relative to the acidified ethanol solvent. Below the plot are the outcomes of two-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$



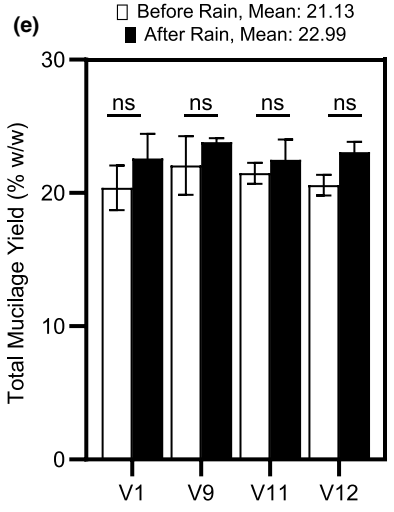




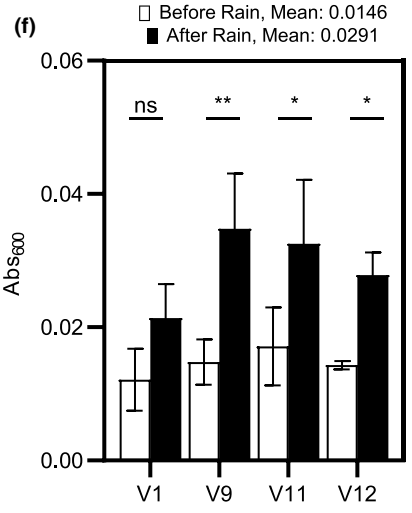
Source of Variation	Variation (%)	*p* value
Rain	74.91	<0.0001****
Variety	8.92	0.0529 ns
Interaction	1.16	0.7461 ns



Source of Variation	Variation (%)	*p* value
Rain	63.67	<0.0001****
Variety	7.436	0.1385 ns
Interaction	10.14	0.0681 ns



Source of Variation	Variation (%)	*p* value
Rain	34.52	0.0046**
Variety	11.51	0.339 ns
Interaction	3.086	0.808 ns



Source of Variation	Variation (%)	*p* value
Rain	58.77	<0.0001****
Variety	12.24	0.0857 ns
Interaction	4.160	0.4658 ns

FIGURE 5 Mucilage quality traits from four *P. ovata* varieties before (white) and after (black) 26 mm of rain in 2020. Mucilage stained by (a) ruthenium red shows finer architecture details while staining on toluidine blue-impregnated agarose (representative images of each sample) (b) allows quantification of released soluble mucilage area (c). The water absorption capacity (d), total mucilage yield (e) and absorbance at 600 nm of pigments in a 1 mg/mL dispersion of mucilage (f) were also tested. Data presented are means with standard deviation of three plot replicates. Below the plots are the outcomes of two-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$. Scales in (a) and (b) = 1 mm

The rain event caused a significant reduction in the water absorption capacity of *P. ovata* seeds ($p < .0001$; Figure 5d) from 10.54 to 9.00 mg/mg, representing a reduction of 15%. Rain did have a global effect on total mucilage yield ($p = .0046$) (Figure 5e), raising the grand mean yield from 21.13% to 22.99%, an increase of 9%. However, in post hoc tests, BR total mucilage yield was not significantly greater in any variety.

To complement the whole seed pigment analysis, pigment carryover during a typical water-based mucilage extraction was determined by spectrophotometry (Figure 5f). Rain damage caused a significant increase ($p < .0001$) in pigment absorbance of the total mucilage extract (100% global increase).

The monosaccharide composition of extracted mucilage was significantly affected by rain damage, leading BR and AR samples to separate in multivariate analysis ($p = .0255$; Figure S2). Analysing the molar abundance of individual monosaccharides found in *P. ovata* mucilage, rhamnose, glucose and xylose contents were significantly affected by rain ($p < .05$; Table 1). Rhamnose and xylose abundance reduced by 7.4% and 1.6%, respectively, while glucose abundance increased by 106%. Due to the shift in xylose abundance, the ratio of arabinose to xylose monosaccharides was also significantly affected by the rain event ($p = .0187$), increasing by 1.5%, although the ratio was more strongly influenced by variety (49.56%; $p = .0004$). Interestingly, mannose, a key marker of damaged seeds (Cowley et al., 2020) was variably detected. While no BR samples had any detectable levels of mannose, in V1 and V9, one replicate contained trace (detectable but below the limit of quantitation) amounts of mannose, and in V11 and V12, two replicates contained trace mannose. As all samples were below the quantitation limits, mannose was not quantified in these samples.

3.1.5 | Rain events increase microbial infestation on *P. ovata* seeds

In a preliminary study, the rain event caused a noticeable increase in microbial infestation on the *P. ovata* seeds (Figure 6). At 1 day after sowing onto growth media, there were noticeable bacterial colonies growing on all sample types but no evidence of fungal contamination. Incubation for up to 7 days after plating led to the growth of a wider variety of microbes (Figure 6). While BR samples were predominantly growing bacteria with some fungal growth in V11 and V12, all AR samples were growing several different types of fungi on more than half of the plated seeds. Importantly some potential mycotoxin-producing fungi including *Aspergillus niger*, *Alternaria* sp. and *Fusarium* sp. were tentatively identified growing on the AR samples.

3.1.6 | Germination capacity is affected by rain damage

The germination profile of each variety was delayed by the rain effect (Figure 7a), where the onset of germination was clearly slowed in AR samples. There was a global effect of rain on the final rate of germination ($p = .0014$) although the reduction was only significant for V9 (Figure 7b). By contrast, the germination index, representing both the percentage and velocity of germination, was significantly reduced by the rain events in all four varieties and so there was a very strong significant global effect ($p < .0001$; Figure 7c), mirroring the non-linear modelling of germination progress (Figure 7a). There was also a significant effect of variety and rain by variety interaction on the germination rate ($p = .0231$) linked to the lower baseline germination (BR) of V1.

3.2 | Validation of key rain effects in seed from the 2021 harvest

Field trials performed in the following season in 2021 were also impacted by rain at maturity and were used to validate the findings from the 2020 trial (Figure 8). On the 8th of September 2021, *P. ovata* plants were harvested and used as the BR samples. AR samples were harvested on the 17th of September after 9 mm of rain fell on the 16th (Figure 8a). Agronomic factors were assessed in the 2021 season finding that potential seed yield (Figure 8b) was significantly reduced after the rain event (64% of BR yield, $p = .049$), along with harvest index (Figure 8c; 82% of BR harvest index), though non-significantly ($p = .205$). We tested two major seed quality indicators from this study, germination index and water absorption capacity, on seed from 2021. As with the 2020 season, we found a significant reduction in germination index (50.0% decrease, $p = .0391$; Figure 8d) and water absorption capacity (20.5% reduction, $p = .047$; Figure 8e).

4 | DISCUSSION

Ensuring the highest quality of mucilage in *P. ovata* seeds is paramount as the psyllium husk is currently the only commercial use of this species with residual seed material having only a low value by-product return. Excess moisture at maturity has been shown to reduce seed quality and longevity in many species (Castro et al., 2016; Ellis & Yadav, 2016; Greven et al., 2004; Williams et al., 1995) and affect mucilage quality in flaxseed (Dorrell, 1973; Dorrell & Daun, 1980; Gubbels & Kenaschuk, 1993), but here we present

TABLE 1 Outcomes of two-way ANOVA of monosaccharide profiles of mucilage extracted from *P. ovata* seeds harvested before (BR) and after (AR) 26 mm of rain in 2020

	BR	AR	Rain			Variety			Interaction		
	Grand Mean	Grand Mean	Variation (%)	*p* Value	Significance	Variation (%)	*p* value	Significance	Variation (%)	*p* Value	Significance
Rha (% mol/mol)	2.971	2.750	30.180	.0074	**	16.930	.1951	ns	1.610	.9171	ns
GalAc (% mol/mol)	2.418	2.387	0.140	.7888	ns	7.270	.3004	ns	63.380	.0003	***
Gluc (% mol/mol)	1.181	2.438	36.210	.0017	**	8.800	.3600	ns	14.110	.1804	ns
Gal (% mol/mol)	3.319	3.538	6.230	.1921	ns	21.360	.1380	ns	18.650	.1787	ns
Xyl (% mol/mol)	70.830	69.710	21.510	.0166	*	16.380	.1844	ns	14.060	.2377	ns
Ara (% mol/mol)	19.190	19.170	0.050	.8326	ns	55.520	<.0001	****	26.350	.0020	**
Ara:Xyl	0.271	0.275	10.270	.0187	*	49.560	.0004	***	16.190	.0368	*

Abbreviation: Rha, rhamnose; GalAc, galacturonic acid; Gluc, glucose; gal, galactose; Xyl, xylose; Ara, arabinose. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$.

the first formal study demonstrating significant quality loss in rain-damaged *P. ovata* seeds.

As the impact of falling raindrops is known to cause extensive seed shattering from the dehiscent seed capsules (Dhar et al., 2005), we considered it possible that seed harvested after the rain event may have an overrepresentation of lighter aborted or immature seeds that are less susceptible to shattering dispersal, making subsequent analyses reflect differences in seed developmental age rather than damage from the rain event itself. When measured in the 2021 trial, reduced seed yield after rain showed that extensive shattering occurred (Figure 8b) but we found that 100 seed weight and seed size were unaffected by the rain events (Figure 2a and b), consistent with previous studies in flax (Dorrell, 1973; Dorrell & Daun, 1980), suggesting that different seed size classes (if present) were shattered to a similar degree.

In previous *P. ovata* field trials, seed discolouration has been a significant effect of unseasonable rainfall (David McNeil, personal communication). Colourimetric analysis presented here and used to determine the colour differences before and after rain damage showed that the rain event caused a significant darkening (reduction in L^*) and a significant greening (reduction in a^*) of the seeds (Figure 3a and b). The changes in seed colour determined using colourimetry likely reflect changes in the extractability and/or oxidation of pigments in the seeds which may be concentrated in an intensely stained layer between the mucilage polysaccharide layer and the endosperm tissues in *P. ovata* (Phan et al., 2020) and other *Plantago* species (Cowley et al., 2021; Jones & Albers, 1955; Patel et al., 2020; Qadry, 1963). Another possible explanation for the significant colour change in AR samples is microbial contamination. Fungal pathogens like *Alternaria* sp. which was putatively identified on AR seeds (Figure 6), are known to cause seed discolouration in soybean (*Glycine max* L.; Clear et al., 1989) and grain amaranth (*Amaranthus caudatus* L.; Noelting et al., 2011) and is a common seed-borne fungus of *P. ovata* (Choudhary et al., 2017). Regardless of the mechanism(s) causing the husk discolouration, visible pigment change in the extracts is likely to translate to pigment transfer and colour change in downstream products. This particularly impacts their use in gluten-free bread production where colour change is an unacceptable quality parameter (Silvano Ciani, personal communication) and is a persistent problem when baking using husk from damaged seeds (Cowley et al. unpublished work).

The observed increase in seed surface 'scabbing' (Figure 2c) strongly suggested that the psyllium husk/*P. ovata* mucilage layer had been affected by the rain events, making it important to assess the potential effect on mucilage quality. Per milligram of seed sample, samples affected by the rain event had a lower water absorption capacity (Figure 5d) but a greater yield of mucilage (Figure 5e). It is likely that the excessive moisture from the rain caused premature swelling of the outermost layer of mucilage which may have subsequently been microbially catabolised, physically washed by water movement or adhered to the inside of the capsule wall. Altering the outermost mucilage polysaccharide layer, which we have already found to be critical in initiating mucilage swelling in *P. ovata* seeds

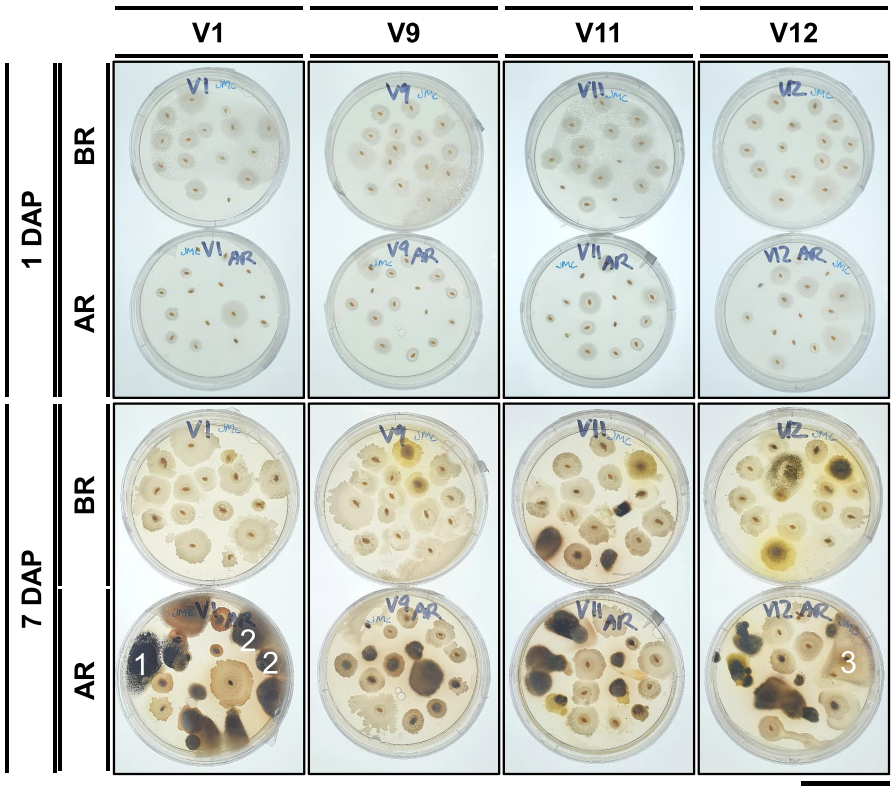
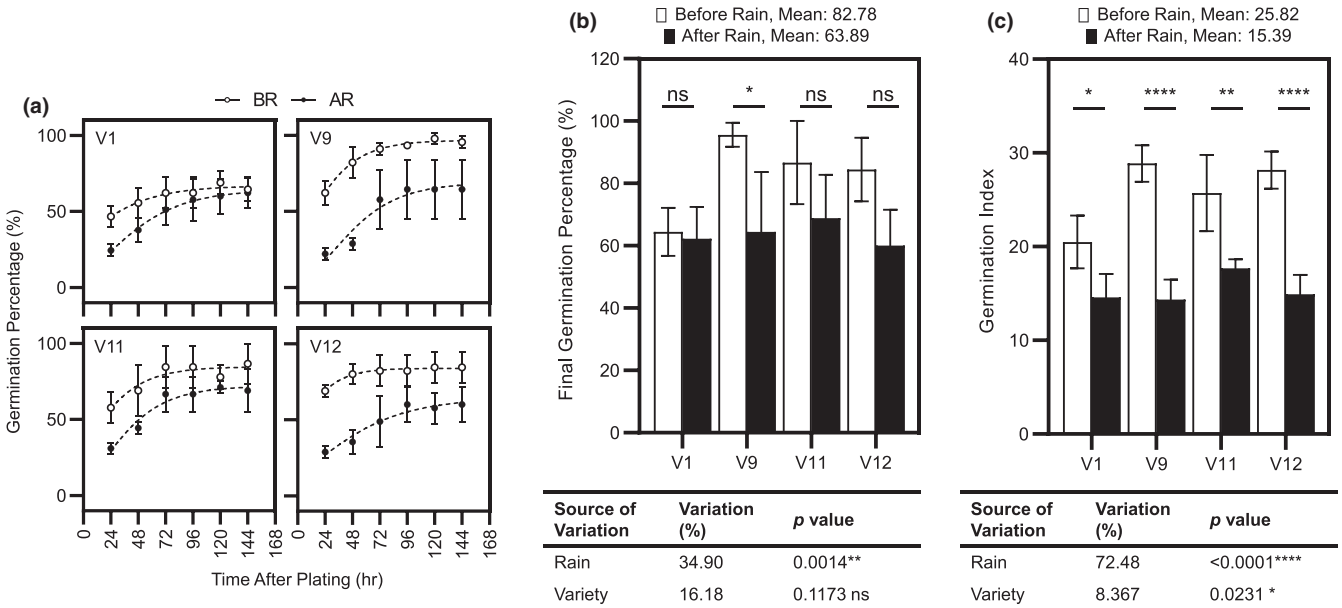


FIGURE 6 Microbial contamination on seeds of four *P. ovata* varieties before (BR) and after (AR) a 26 mm rain event. DAP = days after plating. Scale = 5 cm. 1 = *Aspergillus niger*; 2 = *Alternaria* sp.; 3 = *Fusarium* sp.



Source of Variation	Variation (%)	*p* value
Rain	34.90	0.0014**
Variety	16.18	0.1173 ns
Interaction	11.23	0.2310 ns

Source of Variation	Variation (%)	*p* value
Rain	72.48	<0.0001****
Variety	8.367	0.0231 *
Interaction	8.476	0.0221 *

FIGURE 7 (a) Germination profiles over 144 hours, (b) final germination percentage and (c) germination index of four *P. ovata* varieties before (white) and after (black) 26 mm of rain in 2020. Data presented are means with standard deviation of three plot replicates and in (a) germination progress is fitted by a Gompertz growth curve. Below (b) and (c) are the outcomes of two-way ANOVA. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$

(Phan et al., 2020), likely reduces the eventual water absorption capacity compared to unaffected seeds. A similar effect was seen in heavily rain-affected flax seeds, where the suspension value (related to swelling index) was linearly reduced in association with decreasing seed weathering grade (Dorrell & Daun, 1980). This may seem to contradict the findings of increased mucilage yield in AR samples but we have previously also found that poorer quality seeds had a higher mass yield of material obtained from mucilage extraction (Cowley et al., 2020). The yield of extracted material was higher due to an increase in the amounts of contamination from components originating

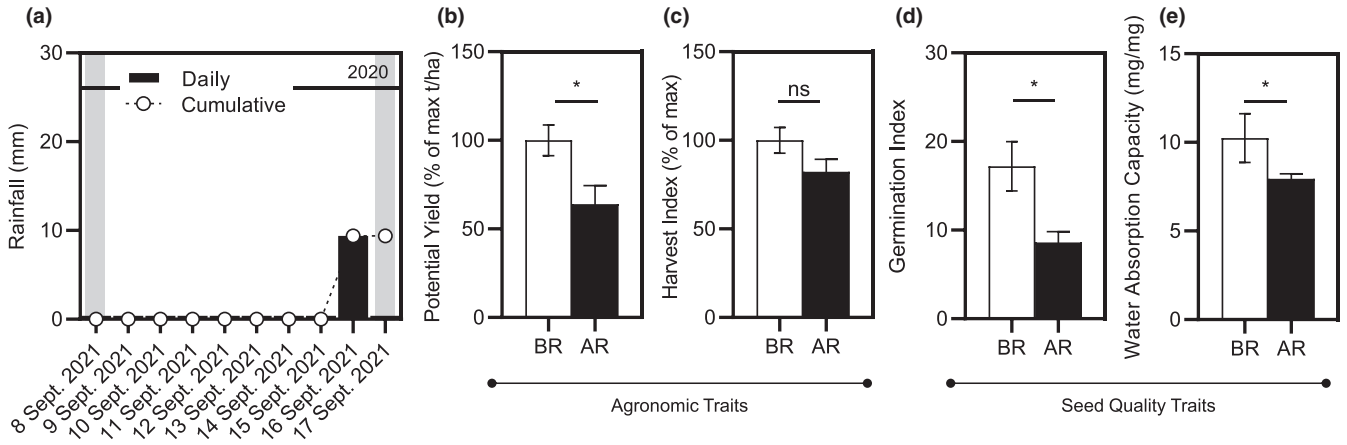


FIGURE 8 Validation of rain damage effects in V12 grown in 2021. (a) Rainfall data at the field trial site at the week of harvest (12–19 September 2021). Black bars represent the daily rainfall while the open diamond symbols are cumulative rainfall. Grey boxes denote the two harvest dates: Seed harvested on the 8th and 17th of September are the before rain (BR) and after rain (AR) samples, respectively. The black line represents the cumulative rainfall over harvest of the 2020 season (26 mm). (b) Reduction in potential seed yield. (c) Reduction in harvest index. (d) Germination index. (e) Water absorption capacity. ****$p < .0001$; ***$p < .001$; **$p < .01$; *$p < .05$; ns = not significant, $p > .05$

from the inner seed tissues, but which contribute minimally to the water absorption properties of the mucilage (Cowley et al., 2020). Therefore, mucilage content per seed was predicted to be relatively stable but more contaminants were extracted meaning that material extracted from damaged seeds will have a lower concentration of mucilage polysaccharides per gram, leading to the disconnect between mucilage yield loss and functionality loss. This observation has also been published previously for flax when comparing the least and most weathered seeds: while the suspension value of the most weathered seeds had reduced by over 43%, the mucilage yield had only reduced by 17.5% (Dorrell & Daun, 1980). In that study, the percentage of broken flax seeds was far greater in the weathered class and so it is likely that during the water-based mucilage extraction more inner seed components were extracted. Thus, the real abundance of mucilage polysaccharides in the extract is likely to have been much lower than reported, influencing the suspendability of seed. Similarly, in our study, the increased abundance of non-mucilage components is also apparent in the high levels of glucose (from the endosperm/embryo [Cowley et al., 2021]; Table 1) and the increased abundance of 600 nm-absorbing pigments (Figure 4 and 5f) that we linked to the total seed colour change (Figure 3). Interestingly we did not see consistently elevated levels of mannose (below quantitation limit; data not shown) as we reported previously for heavily damaged *P. ovata* seeds (Cowley et al., 2020). Samples from the 2017 field trial contained a high proportion of shrivelled, blackened and aborted seeds that were not seen in this study, and thus the mannan-rich endosperm cell walls (Cowley et al., 2021) of the shrivelled/blackened/aborted seeds may have been degraded or incompletely synthesised allowing mannose to more easily leach. The seeds in this study are likely to have been damaged more superficially than the 2017 field trial (Cowley et al., 2020) where the inner seed tissues were also degraded.

The layered nature of hydrated *Plantago* mucilage and the fine compositional differences between the layers have been shown to be a key determinant of mucilage functional properties (Cowley et al., 2021; Yu et al., 2017). Ruthenium red staining showed the typical layering of *P. ovata* mucilage was altered in AR samples (Figure 5a) and soluble mucilage quantification by MuSeeQ showed a strong reduction in the size of the released mucilage envelope (Figure 5b and c). Monosaccharide analysis on mucilage extracts found that the mucilage composition varied between BR and AR samples by multiple factors (Figure S2) but particularly in relation to subtle shifts in mucilage layer/fraction-specific monosaccharides (Table 1). This is evidenced in (1) the reduction in rhamnose and galacturonic acid and (2) the increase in arabinose to xylose ratio in the mucilage. Rhamnose and galacturonic acid monosaccharides are associated with pectin, a highly-hydrophilic mucilage component that is located at the mucilage periphery, which is the first component to be released upon hydration and is thought to 'prime' the mucilage hydration process (Phan et al., 2020; Yu et al., 2017). Arabinose and xylose are the main components of heteroxylan, the main component (c. 90%) of *P. ovata* mucilage and the ratio of the two monosaccharides (arabinose to xylose ratio) is known to increase proportionally from the mucilage periphery to the seed surface (Phan et al., 2020; Ren, Yakubov, et al., 2020; Yu et al., 2017). A reduction of heteroxylans with the lowest arabinose to xylose ratio (and incidentally, the greatest extractability [Yu et al., 2017; Ren, Yakubov, et al., 2020; Cowley et al., 2021]) would explain the slight overall increase in arabinose to xylose ratio. Altogether, it appears that moisture retention on the surface of *P. ovata* seeds during and after the rain events has caused premature mucilage hydration and limited release of the most soluble mucilage components. The loss of these 'priming' components may have impaired the hydration/swelling process and diminished the water absorption capacity of the seed and its husk, an essential property required for its functionality.

Rain has clearly affected the external surfaces/mucilage of the *P. ovata* seeds, and as mucilage acts as a reservoir for water uptake, its hydration, though potentially brief and non-extensive likely initiated seed

imbibition. Rewetting after the maturation drying phase has begun is known to reduce seed viability (Ellis et al., 1990; Greven et al., 2004), an effect we also found where the rain event significantly reduced the final germination rate and the germination index of AR samples (Figures 7 and 8d) potentially due to the premature onset of germination processes. It is important to consider that the germination test used here was conducted under sterile conditions (seeds were chemically disinfested and germinated in vitro) and thus does not take into account the increase in microbial load on AR seeds (Figure 6) which could additionally affect germination in the field. We have observed microbial overgrowth on non-disinfested seeds to significantly reduce seedling establishment under non-sterile conditions in pot experiments and in the field. Furthermore, mucilage has been implicated in successful establishment, especially under adverse conditions, in many mucilage-producing species (Pan et al., 2021; Pan et al., 2022; Tsai et al., 2021; Yang et al., 2012) including *Plantago* (Pan et al., 2021, 2022; Teixeira et al., 2020; Veiga-Barbosa & Pérez-García, 2014; Zhang et al., 2014). It is possible that changes to the mucilage in rain-damaged seeds will also contribute to reduced establishment in the field and thus trials should be conducted to determine if rain-damaged seeds are at all suitable for resowing. Sowing rates considering the reduction in germinability could be of particular importance. Furthermore, harvesting practices related to seed drying were found to reverse the negative effect on seed longevity caused by simulated rainfall in wheat (Ellis & Yadav, 2016). Drying practices will not reverse damage to the *P. ovata* seed mucilage but may improve the germinability/longevity and thus suitability for resowing rain-affected seeds. Management practices including drying processes could be the subject of continued research for *P. ovata*.

In conclusion, we have shown for the first time that rain damage significantly reduces the quality of *P. ovata* seeds directly related to deterioration of the mucilage/husk. Rain damage caused significant changes in seed colour which were likely due to oxidation and leaching of seed pigments located outside the mucilage layer and/or microbial growth, leading to significant colour changes in the purified mucilage. Premature hydration of the mucilage layer is the likely cause of seed surface 'scabbing' and resulted in the reduction/loss of the outer most layers of mucilage, affecting the water absorption properties, a key indicator of mucilage quality. Transient rewetting may have also caused reduced the germination potential of rain-damaged seeds due to premature initiation then stalling of germination. The potential for weathering and rain damage should be considered a critical determining factor when producing or purchasing *P. ovata* seed for husk production or resowing. Practically, we propose that the acidified ethanol assay used here may represent a diagnostic test for identifying rain-damaged seeds in the field or small laboratory (perhaps using a handheld spectrophotometer, mobile app or based on perceptible colour change) allowing simple identification/grading of low-quality *P. ovata* seeds and ultimately benefitting the consistent production of high-quality industrial and pharmaceutical products. Interestingly, the extensive quality impacts outlined here may also make *P. ovata* a suitable model or indicator species for studying acute climate effects on seed quality, especially from rain, particularly on crops with similar seed and capsule traits like canola or flax. Seed quality effects caused by other abiotic and biotic factors may be exacerbated by rain, either indirectly by shifting harvest times/conditions or directly as described here. Having an indicator species may aid in guiding the development of management strategies for responding to climate variability.

AUTHOR CONTRIBUTIONS

JMC conceived the study, performed experiments, analysed the data, prepared figures and wrote the manuscript. DLM and KYL conducted the field trials, contributed to experimental design and performed data analysis. JPB performed the germination experiment. SC and VC contributed to funding acquisition, project administration and experimental design, and supplied experimental materials. RAB conceived the study and contributed to funding acquisition, supervision, experimental design, data interpretation and writing the draft. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data underlying all figures is available from the corresponding author on reasonable request

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SUPPORTING INFORMATION

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