Project title:	Application of post-harvest treatments to extend storability of pedunculate acorns (*Quercus robur* L.) without loss of viability or germinability.
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The results and conclusions in this report are based on an investigation conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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SCIENCE SECTION

Introduction

The genus *Quercus*, commonly known as oak, comprises about 450-600 deciduous and evergreen species that occur naturally mainly in the northern hemisphere (Kaul, 1985; Nixon, 1993; 2002; Deng et al., 2006). The classification system, however, is complicated and disputed due to the large number of species that often hybridize. In Nixon's classification system, there are two subgenera, *Cyclobalanopsis* (cycle-cup oaks) and *Quercus* (true oaks) (Nixon, 1993) (Table 1). The subgenus *Quercus* is further subdivided into three sections.

Table 1. Classification of the genus *Quercus* (Nixon, 1993, 2002 and others).

Subgenus	Section	Common name	Examples
Quercus	*Quercus*	White oaks	*Q. alba, Q. ilex, Q. macrocarpa, Q. robur,Q. petraea, Q. serrata*
	Protobalanus	Intermediate oaks	*Q. cedrosensis, Q. palmeri*
	Lobatae	Red oaks	*Q. hintonii, Q. nigra, Q. pagoda, Q. rubra*
Cyclobalanopsis		Cycle-cup oaks	*Q. fleuryi, Q. lamellosa, Q. schottkyana*

The section *Quercus* (white oaks) is widely distributed in Europe, Asia and America, while the section *Lobatae* (red oaks) is restricted to North and South America (Nixon, 1993). These groups include several important species that have multiple uses for timber, firewood, charcoal, cork, tannin and animal feed (Olson, 1974; Oldfield & Eastwood, 2007). Under natural conditions, regeneration is often poor as acorns do not persist due to desiccation, predation, insect infestation and fungal infection. So silviculturists often use artificial means to establish forests (Korstian, 1927).

In general oak fruiting is irregular, resulting in poor seed crops during inter-mast years (Brookes & Wigston, 1979; Sork & Bramble, 1993; Dey, 1995). This is largely due to the abortion of flowers or premature abscission of immature fruits during unfavourable weather conditions such as spring frosts or summer droughts (Olson & Boyce, 1971; Suszka et al., 1996; Bonner, 2003). Some individual trees produce no acorns while others contribute heavily to the annual seed crop. Thus, mast seed crops vary not only from tree-to-tree within populations but also from year-to-year among regions (Koenig et al., 1994; Pons & Pausas, 2012; Pérez-Ramos et al., 2014).

Oaks produce large-seeded fruits (acorns), which are classed as 'recalcitrant'. Unlike 'orthodox' seeds, recalcitrant seeds are shed with high moisture content and lose viability rapidly below a species-specific critical threshold (Suszka & Tylkowski, 1980; Bonner & Vozzo, 1987). Recalcitrant seeds also remain metabolically active, which leads to germination without the need for additional water. Thus, these seeds can only be stored until

germination is initiated, which can occur within a few days or several months after shedding, depending on species (Berjak & Pammenter, 1994; 2008; 2013).

Factors influencing shelf-life of acorns

At present, recalcitrant seeds are usually stored under 'wet-storage' conditions that reduce desiccation and respiration, thereby increasing their shelf-life. This is a delicate balancing act as these responses are influenced by temperature, relative humidity and gas atmosphere during storage.

1. Desiccation

Acorns are shed with high moisture content, typically ranging from 50-55% (fw) for white oaks and 30-40% (fw) for red oaks (Bonner, 1974, 1976, 2008; Gosling, 2002). Unlike orthodox seeds, recalcitrant seeds such as acorns do not undergo maturation drying, which is the final stage of seed development before shedding. Thus, there is no clear end-point to fruit development and fruits are shed at different moisture content in different years on the same tree (Finch-Savage & Blake, 1994). This reflects fruit developmental status (i.e. maturity), which in turn is influenced by weather conditions and natural habitats (Koenig et al., 1994; Daws et al., 2004a; Diaz-Pontones & Reyes-Jaramillo, 2012).

Critically, acorns are desiccation-sensitive and lose viability rapidly below a critical moisture content. However, there are different degrees of desiccation-sensitivity between species, which are largely linked to the chemical composition of the storage reserves in the cotyledons. The red oak fruits typically have higher lipid contents (8-31% fw) (Bonner & Vozzo, 1986) than white oaks fruits (1-12% fw) (Xia et al., 2010). In general, red oak fruits are more desiccation-tolerant than white oak fruits. This is reflected in the critical water content thresholds, which typically range from about 25-40% for white oaks and 15-20% for red oaks (Bonner, 2008; Xia et al., 2012). In *Q. robur*, acorn viability decreases when moisture content drops below *circa* 38% (Gosling, 1989; Doody & O Reilly, 2008).

The critical water content, however, can be masked by seed size (Daws et al., 2004b). Thus, the level of deviation from the mean water content of a seed lot can vary significantly for acorns within a population (Suszka & Tylkowski, 1980). Small acorns desiccate more rapidly than larger ones (Morgan & Brubaker, 1986). Some acorns, therefore, can germinate even when seed lot moisture content is below the critical threshold.

2. Respiration and fermentation

Acorns remain metabolically active, producing carbon dioxide, water and heat as by-products of respiration (Brown, 1939; Suszka & Tylkowski, 1980). The fruits, therefore, require adequate ventilation to prevent oxygen depletion (Szczotka, 1978), which can lead to the production of fermentation by-products including ethanol and acetaldehyde (Akimito et al.,

2004; Pasquini et al., 2012). In *Q. serrata*, the accumulation of acetaldehyde rapidly reduced the shelf-life of acorns in closed systems (Akimito et al., 2004).

Under laboratory conditions, white oak fruits have been stored successfully for short periods (12 weeks) in modified gas atmospheres. In *Q. alba*, the respiration rate of acorns is lower in nitrous oxide atmospheres or 98:2% N_2O:O_2 than ambient air (Lakovoglou et al., 2010). In *Q. macrocarpa*, however, the respiration rate is similar under both conditions. This reflects differences in the metabolic activity of the species. Thus, *Q. alba* produces non-dormant acorns that germinate rapidly while *Q. macrocarpa* often produces dormant acorns that require some stratification depending on seed source.

3. Premature germination

In white oaks, acorns are typically not dormant when shed and often germinate rapidly in autumn sometimes while still on the tree (Bonner, 2008). These fruits are difficult to store for longer than a few months even at near freezing temperatures due to premature germination. Premature germination often leads to damaged radicles, which may be susceptible to fungal infection (Suszka & Tylkowski, 1980; Bonner, 2003). Premature germination though is not usually fatal as acorns have large storage reserves and can recover from damage by producing secondary radicles.

In red oaks, however, acorns are frequently dormant when shed in autumn (Wirges & Yeiser, 1984). This may be due to their high lipid content that requires stratification to convert lipids to soluble carbohydrates (Korstian, 1927). At low storage temperatures, these dormant acorns effectively undergo stratification due to their high moisture contents. The degree of dormancy differs amongst species and is also influenced by the weather before seed shed (Tylkowski & Wrzeéniewski, 1986).

In general, dormant acorns require at least 8-12 weeks' stratification. The relationship between storability and dormancy suggests that species with dormant acorns (largely red oaks) are easier to store than those with non-dormant acorns (usually white oaks) (Bonner & Vozzo, 1986). Dormancy is also influenced by geographic distribution. In the white oak, *Q. macrocarpa*, for example, acorns from northerly populations are dormant and require stratification while those from southerly populations are non-dormant at shedding (Gucker, 2011; Row et al., 2012). The red oak, *Q. emoyri*, however, produces non-dormant acorns that do not require stratification (Nyandiga & McPherson, 1992).

4. Fungal infection

Fungal infection can cause significant losses during storage. In Europe, the most serious acorn pathogen is *Ciboria batschiana* (Zopf) N.F. Buchw., which belongs to the Ascomycetes (Schröder, 2002). This fungus can spread slowly at sub-zero temperatures (-3°C). Thus,

freshly harvested acorns are often subjected to a hot-water treatment (41°C for 2 hrs), which usually reduces infection by *C. batchiana* during subsequent storage (Knudsen et al., 2004). Hot-water treatments, however, can affect other saprophytic fungi, decreasing the frequency of *Cladosporium* and *Papulaspora* species, but promoting *Alternaria* and *Penicillium* species amongst others (Knudsen et al., 2004).

Past and present storage practices

In the late 1750s, John Ellis conducted several experiments to preserve acorns during long transatlantic voyages (Murphy, 2014). He coated acorns with various substances including bees wax, brewer's loam and gum arabic. The acorns were packed in boxes containing dry sand. These boxes were then stowed in the upper regions of the ship's hold. In most cases, acorns shrivelled or decayed. Some acorns in bees wax, however, remained fresh for a full season and were successfully used by William Aiton, the Botanic Gardener at Kew, to raise seedlings (Elllis, 1759/60, 1770).

Nowadays, acorns from temperate regions are stored at low temperatures (-3 to +5°C) while those from tropical regions at held at higher temperatures (+7 to +17°C) (Willan, 1987; Hong et al., 1996). In white oaks, such as *Q. robur*, acorns have been stored between -1 to -3°C (Suszka & Tylkowski, 1980). These acorns often germinate prematurely at higher temperatures (+1 to 5°C) but are killed at lower temperatures (-5°C). In red oaks, such as *Q. rubra*, acorns have been stored under similar conditions with little loss of viablilty over 18 months (Noland et al., 2013; Suszka & Tylkowski, 1982). However, it is not recommended to store red oak acorns for longer than two years due to significant deterioration.

Under laboratory conditions, acorns are typically stored in bags with varying degrees of success depending on bag composition, wall thickness, and bag seal (Rink & Williams, 1984; Devine et al., 2010). The bags are often made from polyethylene, which is sometimes perforated to improve gas exchange. In general, polyethylene bags with a wall thickness between 75-100 microns prevent excessive water loss but permit sufficient ventilation for respiration (Bonner, 1990). In contrast, polyethylene bags thinner than 75 microns are permeable to water while those thicker than 250 microns are impermeable to oxygen and carbon dioxide.

In *Q. ilex*, acorns have been successfully stored for up to 12 months in sealed polyethylene bags (30 microns [1.2 mils]) at 3°C (Pasquini et al., 2011, 2012). The acorns respired at low levels, reaching a steady-state in the oxygen-limiting conditions of the bags. There was, however, little or no premature germination, which was attributed to the accumulation of ethylene, a potential germination inhibitor, in the bags. After 12 months, stored acorns still had a germination capacity of *circa.* 60% (Pasquini et al., 2012).

In *Q. alba*, acorns have been successfully stored for 6 months in sealed polyethylene bags (106 microns [2.7mils]) at 1.6°C (Devine et al., 2010). After 6-12 month's storage, premature germination occurred but the percentage varied for different seed sources. In general, the radicles were less than 30mm long. These radicles were susceptible to breakage during handling and sowing but this did not prevent a successful seedling crop. Similar results were reported for acorns stored in specialised gas-permeable polyethylene bags (Star-Pac™).

In commercial operations, however, acorns are usually stored in containers often with a substrate such as charcoal, peat, sand or sawdust, which allows for diffusion of sufficient oxygen but maintains the high moisture content of seeds and prevents the spread of fungal pathogens (Hong & Ellis, 1996, Hong et al., 1996; Suszka & Tylkowski, 1982). In *Q. robur*, acorns have been stored for up to three years (-1°C) although there was significant year-on-year deterioration (Suszka & Tylkowski, 1980). Similar results have been reported for acorns of *Q. rubra* (Suszka & Tylkowski, 1982).

At present, however, there is no single solution for the successful short-to mid-term storage of acorns of different species despite research and experience spanning several centuries.

APPLICATION OF POST-HARVEST TREATMENTS TO EXTEND STORABILITY OF PEDUNCULATE OAK ACORNS (*Quercus robur* L.).

This project focuses on two approaches to extend the shelf-life of pedunculate oak acorns (*Quercus robur* L.) [hereafter 'oak']. The first approach aims to reduce water loss by coating acorns with either a commercial anti-transpirant (Wiltpruf®) or different types of waxes viz. bees, soya and microcrystalline wax. The second approach aims to reduce respiration by storing acorns in different types of bag viz. biopolymer, polyethylene or polyester, which interact with the acorns to passively modify the storage atmosphere. Both approaches have been exploited in the food industry, which suggests potential for use when storing acorns.

Aims and objectives

The aim of this proof-of-concept project is to examine methods for extending storage duration of acorns for up to 18 months without significant loss of seed quality. If this was possible, it may help overcome the supply and demand problems for seed traders and nursery managers, resulting from irregular fruiting of oak.

The objectives are as follows:

1. To use two approaches to extend storage duration for acorns:
    1. wax coatings/anti-transpirants to reduce water loss;
    2. permeable/barrier bags to reduce respiration of acorns.
2. To determine which treatments are effective by tracking changes in seed quality using a range of seed tests:
    1. to track water loss by measuring moisture content.
    2. to detect changes in seed occupancy (shrinkage) by taking x-rays.
    3. to detect membrane damage by measuring electrolyte conductivity/solute leakage.
    4. to determine loss of viability/germinability of acorns.
3. To determine the seedling performance/vigour of treated acorns under nursery conditions.

Methods - General

Acorns (*circa*. 100 kg) were purchased from Forestart, Shropshire, in November 2015. The seed lot contained many damaged and germinating acorns, which were excluded from the experiments where possible. On arrival, moisture content, x-rays, electrical conductivity, and germination tests were carried out on the bulk seed lot to determine baseline seed quality. These tests were then repeated periodically through the project to monitor changes in seed quality.

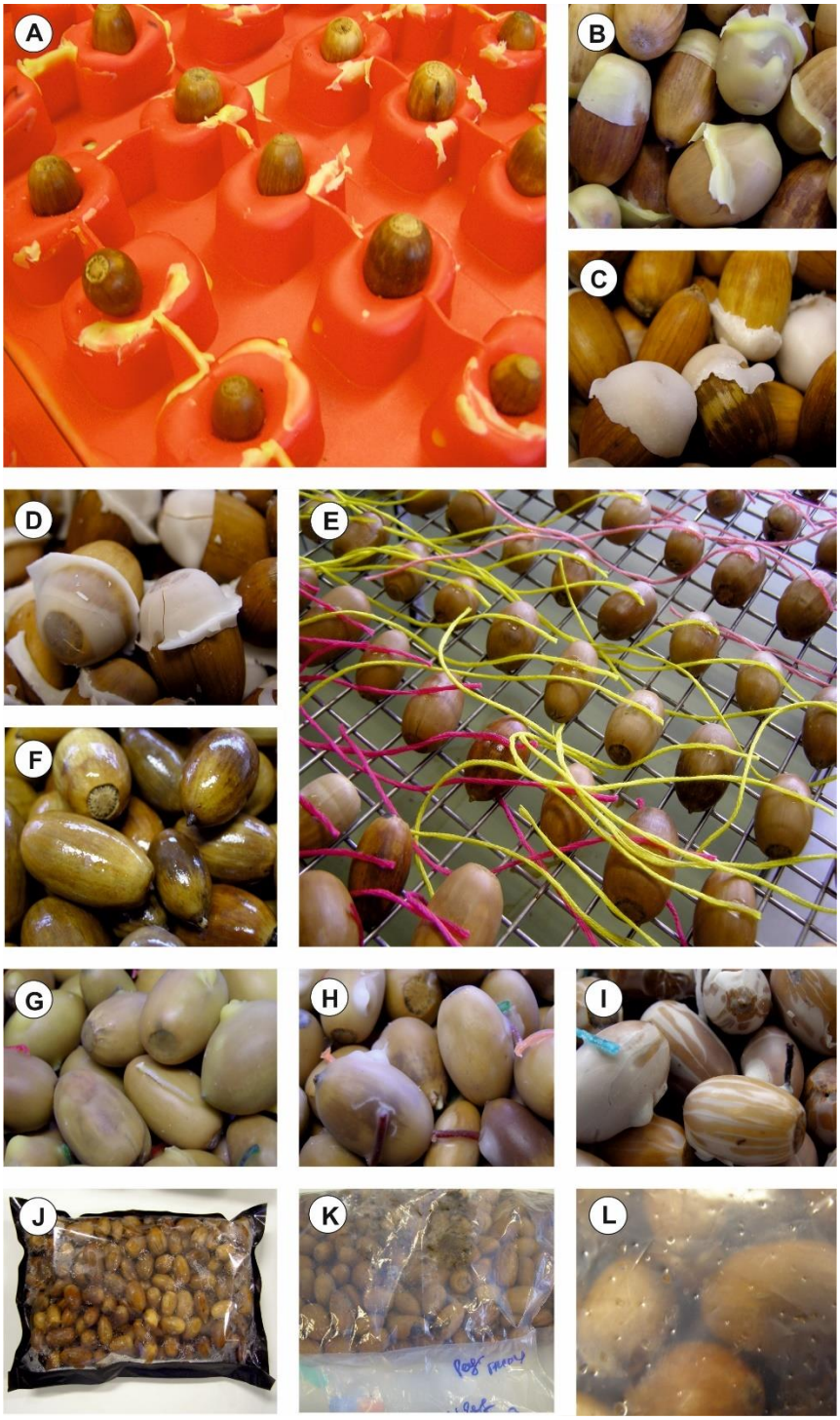

Figure 1. A - Ice-cube jig used to partially coat cup-scar end of acorns with wax; Acorns partially coated with B - bees-, C - microcrystalline-, or D - soya wax, E - Threads were glued to acorns, which were then dipped entirely into molten wax; Acorns coated entirely with F - Wiltpruf ®, G - bees-, H - microcrystalline-, or I - soya wax, J – Acorns stored in polyester bags, K – peforated biopolymer bags with L- 25 microperforations per square inch.

The acorns were treated as described in Objectives 1.1 and 1.2 below (Figure 1). The treatments were then randomly assigned to four fridges (2.2±1.4°C and 60.8±13.0% RH). After each storage duration (12-weeks), acorns were withdrawn from treatments to track changes in seed quality as follows: 20 acorns for moisture content, 20 acorns for electrical conductivity, and 120 acorns (30 x 4 replicates) for germination tests in the laboratory; see Appendix 1 for more detail.

Statistical analysis

Data from the experiments were analysed using statistical models from the R package (R Core Team, 2016); more detail on this can be found in Appendix 2. Factors were considered statistically significant if P≤0.05.

OBJECTIVE 1.1. COATINGS TO REDUCE WATER LOSS OF ACORNS

Methods – Coatings

The control acorns were stored loosely in 16 trays to provide baseline quality data while the remaining acorns were treated with four products (1:10 dilution Wilt-pruf S600®, bees-, soya- or microcrystalline wax (Table 2), which were applied as a partial (cup scar end = half) (Figures 1a-d) or entire coating (whole) (Figures 1e-i). There were 1000 acorns per treatment.

Table 2. Characteristics of different products used in the experiment.

Coating	Supplier	Congealing point (°C)
Wilt-pruf S600® (1:10 dilution)	Vitax Grower, Leicester	n/a
Beeswax (WHC059)	Willam Hodgson & Co, Cheshire	-
Soyawax (WHC690)		36-40
Microcrystalline wax (Techniwax 9356)		70-77

Results – Coatings

1. Moisture content

Figure 2 shows that moisture content of control and treated acorns decreased with increasing storage duration. However, there were significant differences between treatments. Most treatments, including all the half coated acorns, lost moisture at a similar rate to the controls except for two (bees- and microcrystalline waxes whole), which had significantly higher moisture contents during storage (i.e. the interaction of storage duration and treatment was significant at P<0.001).

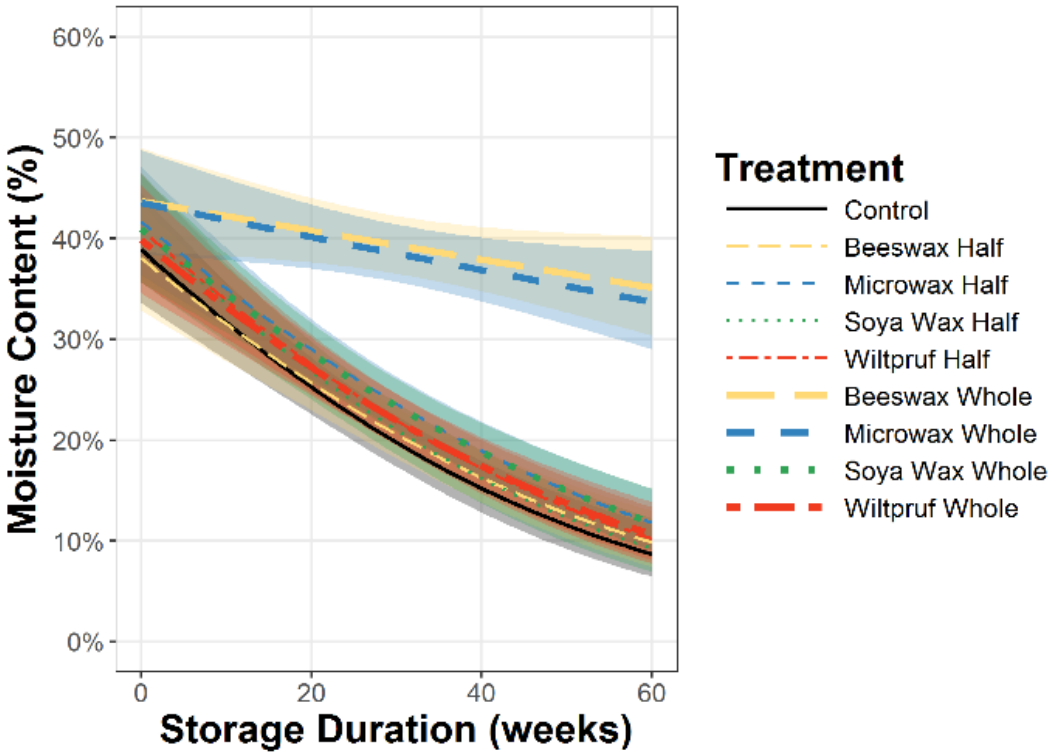

Figure 2. Wax data best fit model for moisture content, predicted by storage duration and treatment. Coloured ribbons indicate 95% confidence intervals. Microwax = microcrystalline wax

2. X-rays

Figure 3 shows that seed occupancy of control acorns decreased from 80% to 62% during storage. The partially coated acorns followed a similar trend. However, acorns entirely coated with bees- and microcrystalline waxes had significantly higher seed occupancy after 48 and 60 weeks compared with the other treatments (i.e the interaction of storage duration and treatment was significant at P<0.001).

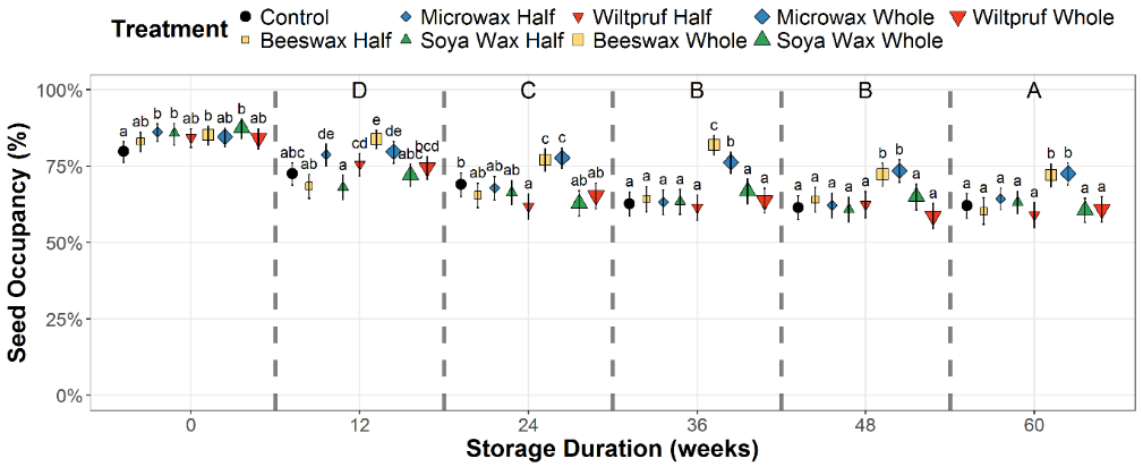

Figure 3. Coating data plot of seed occupancy (%) by storage duration and treatment. Lettering (lowercase) refers to significant differences within storage durations; lettering (uppercase) refers to significant differences across storage durations. Error bars = 95% confidence intervals. Microwax = microcrystalline wax

3. Electrical conductivity

Figure 4 shows the effect of storage duration on the probability of solute leakage across all treatments. Between 12 and 60 weeks storage there was no significant difference indicating that this measure of seed quality is perhaps not a good measure of viability.

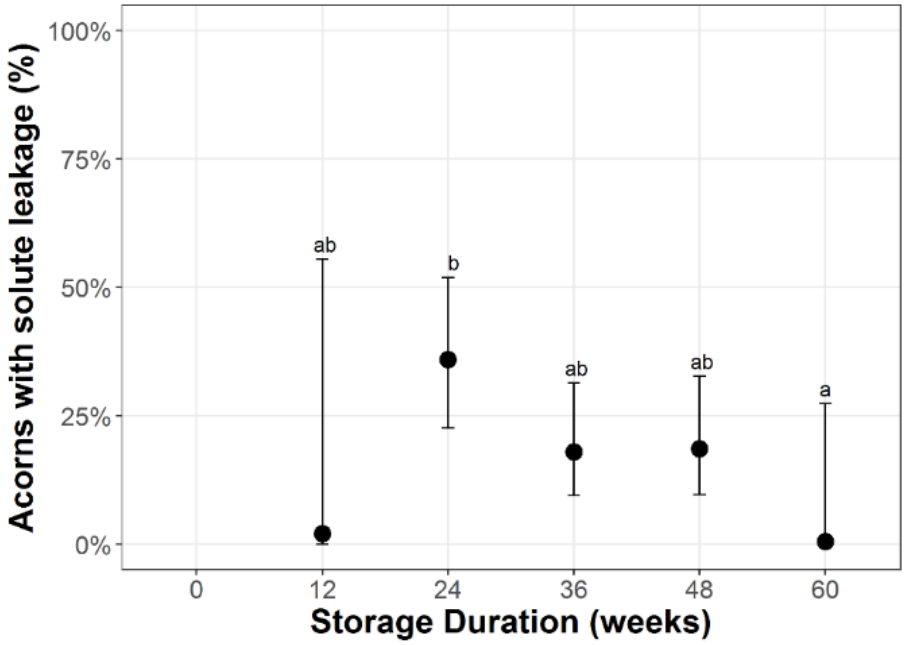

Figure 4. Coating data plot of percentage of acorns with solute leakage by storage duration. Lettering (lowercase) refers to significant differences across storage durations. Error bars = 95% confidence intervals.

4. Germination

Figure 5 shows the cumulative germination of control and treated acorns after different storage durations (0-60 weeks). In general, germination was similar for control and seven treatments at time 0 with the exception of acorns entirely coated with bees- or microcrystalline waxes. In all other treatments, there was some germination after 12-weeks storage but little or no germination after longer storage durations. In contrast, acorns entirely coated with bees- or microcrystalline waxes had 8.3% and 6.7% germination respectively after 60-weeks storage.

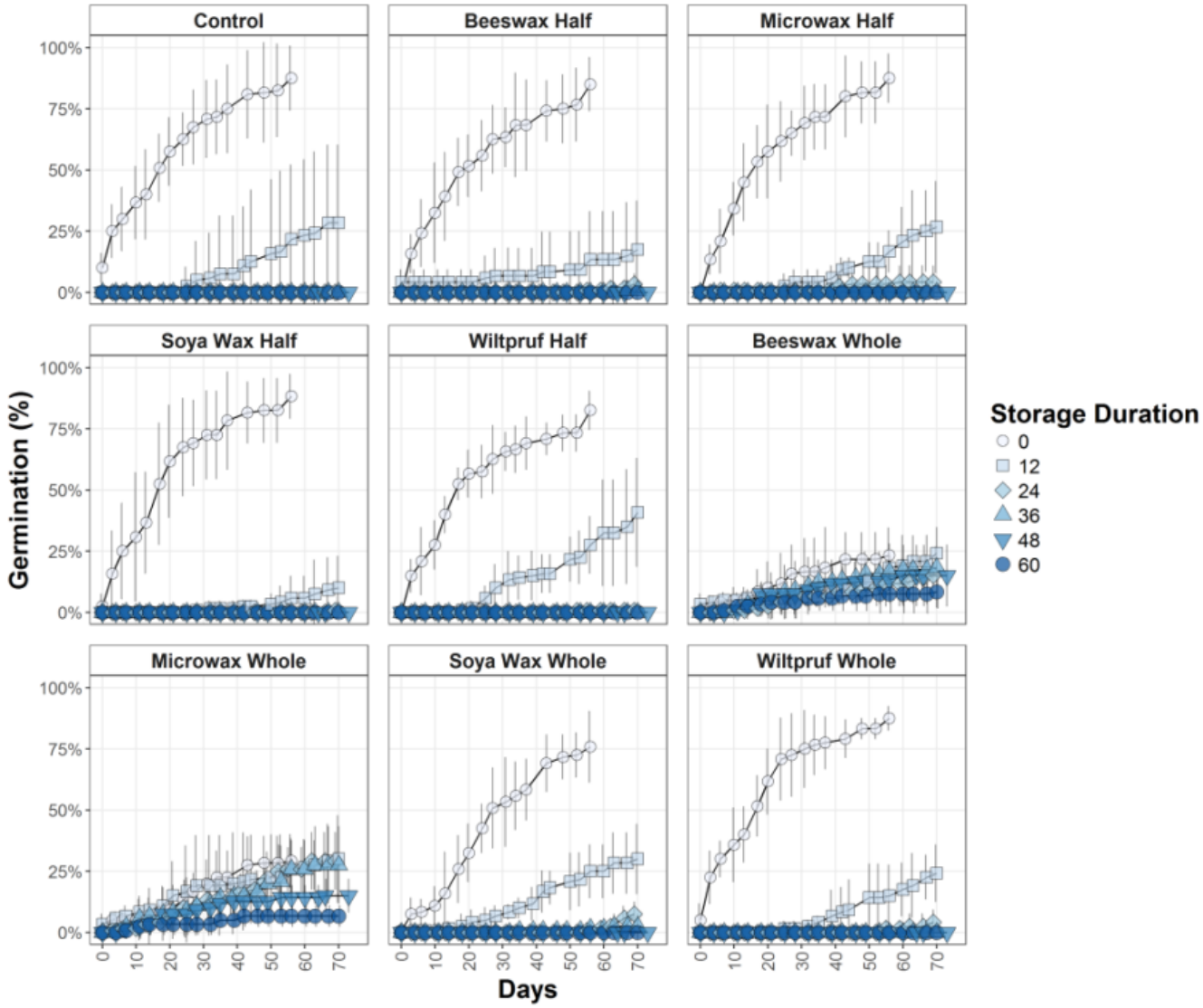

Figure 5. Cumulative germination (mean values of four replicates) for each of the acorn covering treatments over 60 weeks storage. Error bars = 95% confidence intervals. Microwax = microcrystalline wax

Figure 6 shows that there was a clear (sigmoidal) relationship between germination and moisture content. Percent germination decreased rapidly between 35-45%, and therefore, 38% was used as the critical threshold below which there was less than 50% germination. The bees wax whole and microcrystalline wax whole treatments were excluded from this analysis because the acorns had trapped radicles.

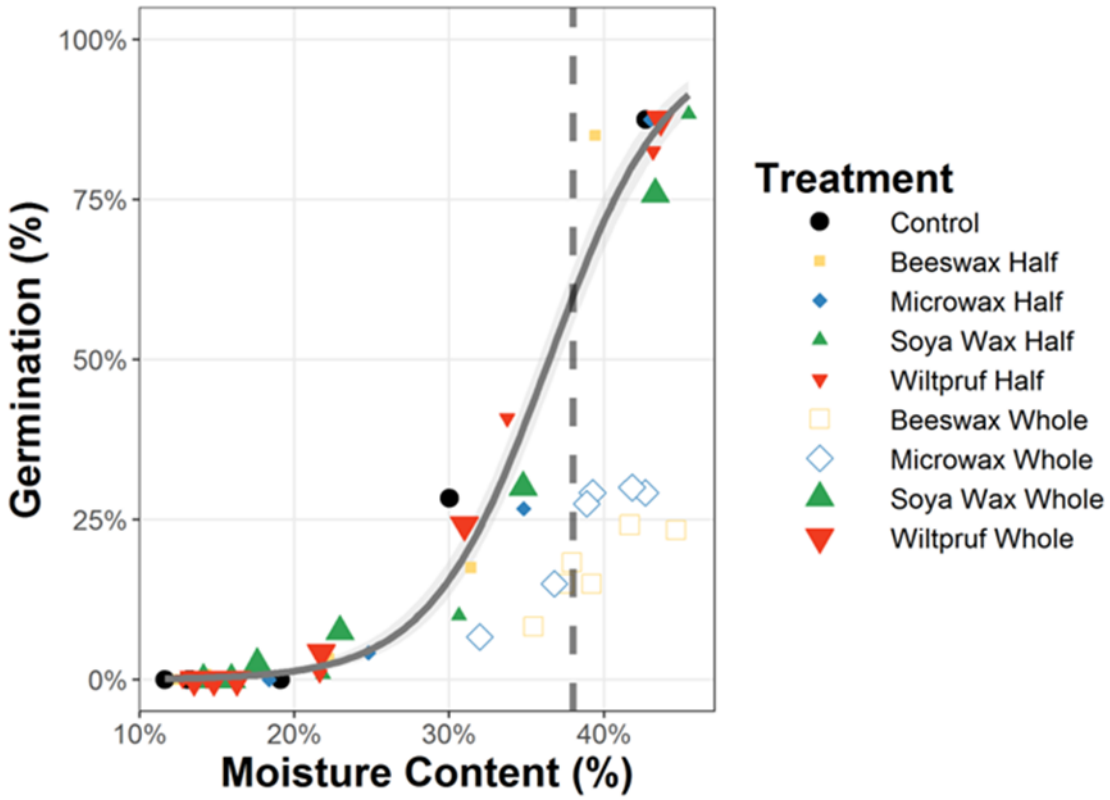

Figure 6. Coating data best fit model for germination capacity, predicted by moisture content. Grey ribbon indicates 95% confidence intervals. Fill for data points indicates inclusion/exclusion from the best fit model (filled = included, unfilled = excluded, see text).

Discussion – Coatings

There is a relationship between temperature and relative humidity, which in turn influences moisture content of recalcitrant seeds during storage. The control acorns had 43% moisture content and 88% germination at time 0. After 12-weeks storage, the acorns had lost water rapidly, dropping below the critical threshold (*circa.* 38%), which resulted in corresponding losses in germination. During the following weeks, moisture content decreased even further, and thus, most control acorns failed to germinate during subsequent tests. In some cases, control acorns split, exposing the seeds to dry air (Figurcoae 7a). A few acorns, however, germinated despite these low moisture contents.

The acorns coated partially with bees-, microcrystalline-, or soya waxes desiccated at similar rates to control acorns regardless of wax type. This indicates that water loss occurs over the entire pericarp not just through the cup scar. In *Quercus* species, the cup scar is often the main route for water movement and influences rates of water uptake and loss (Xia et al., 2012b). In *Q. suber*, for instance, the cup scar not only lacks a thick protective cuticle but also contains large xylem vessels, which potentially provides a pathway for water loss (Sobrino-Vesperinas & Viviani, 2000).

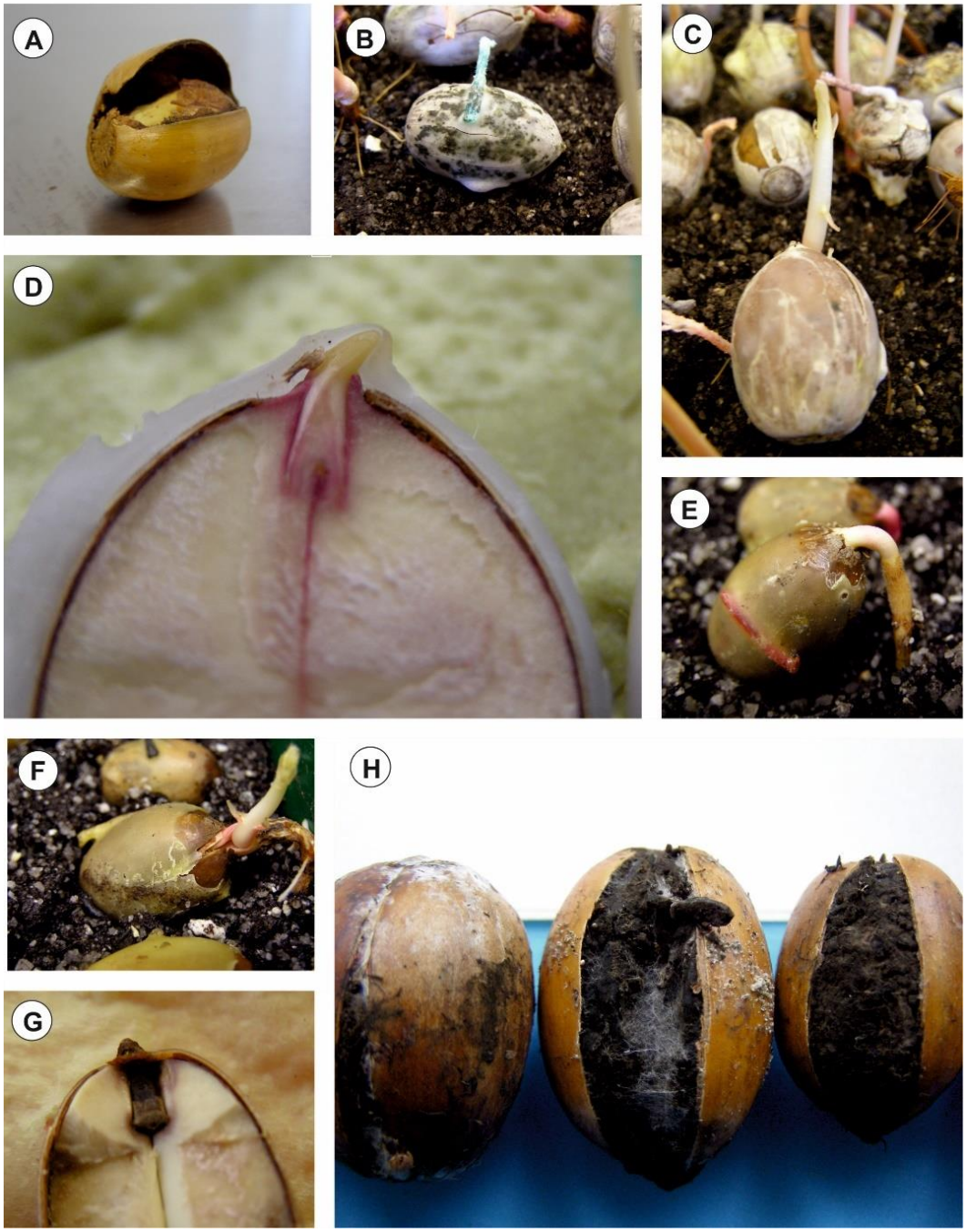

Figure 7. A - Control acorn that has split, exposing seed, B - Acorn coated entirely with soya wax that is covered with superficial mould, C – Seedling from acorn entirely coated with soya wax, D – Acorn entirely coated with microcrystalline wax with trapped radicle, E – De-capped acorn coated entirely with bees wax germinating; and F – producing shoots, G – Acorn with necrotic embryo due to fermentation, H – Acorns succumbed to *Ciboria batschiana.*

The acorns coated entirely with waxes had moisture content of 43-45% at time 0. During storage, however, the acorns responded differently depending on wax type. This reflected the chemical and physical properties, which in turn influenced the thickness and pliability of the coatings. Soya wax formed thin, brittle coatings, which flaked off during storage. At time 0, therefore, the acorns coated entirely with soya wax germinated as readily as the control acorns (Figure 7c). During storage, however, soya wax proved ineffective at reducing desiccation, resulting in a corresponding loss in germination capacity within 12 weeks. In addition, soya wax appeared to be susceptible to superficial fungal infection during germination tests (Figure 7b).

In contrast, bees- and microcrystalline waxes formed thicker coatings, which reduced desiccation in entirely coated acorns. The moisture contents, therefore, were close to the critical threshold even after 60-weeks storage. However, these acorns often struggled to germinate as the wax prevented the radicles from emerging (Figure 7d). This was largely due to mechanical resistance as some 'de-capped' acorns subsequently germinated although very slowly (Figures 7e and f). During longer storage durations, however, these acorns gradually lost viability due to fermentation. After 60-weeks storage, cut-tests showed that only 25-35% acorns were viable while the remaining non-viable acorns had necrotic embryos.

The anti-transpirant, Wilt-pruf S600®, resulted in very glossy, slightly sticky acorns. This natural product forms a soft, flexible film when applied in water-based solutions (Wiltpruf – Plant Protector [2017]). On application, the film starts to polymerize into a longer chain, higher weight molecule. This does not occur at the same rate with the surface layer undergoing rapid polymerization in contrast to the contact layer. This anti-transpirant was not harmful to acorns, which germinated successfully at time 0. During storage, however, the acorns coated entirely or partially with Wilt-pruf S600® lost water and viability at similar rates to the control acorns.

Overall, there was a sigmoidal relationship between moisture content and germination for the control and six treatments. For two treatments, however, there was no clear relationship between moisture content and germination. After 60-weeks storage, the acorns coated entirely with bees- or microcrystalline wax still had moisture contents above the critical threshold. These wax coatings, however, not only hampered germination but later promoted fermentation due to oxygen depletion.

In general, desiccation was the major cause of acorn death. The x-ray images provided a snapshot of desiccation during storage. At time 0, control acorns had a seed occupancy of 80%, dropping to 62%. During storage, however, percentage seed occupancy decreased at different rates depending on treatment. Thus, acorns coated entirely with bees- or

microcrystalline waxes had higher seed occupancies than other treatments, which reflects the higher moisture contents particularly at longer storage durations. In terms of solute leakage there was no significant relationship between treatments and storage duration. For acorns coated entirely with bees- and microcrystalline waxes, this may have been due to solutes being trapped inside the wax coatings.

OBJECTIVE 1.2. – BAGS TO REDUCE RESPIRATION OF ACORNS

Methods – Bags

Control acorns were stored loosely in trays while the remaining acorns were packed into four types of bags (200 acorns per bag) (Table 3). There were 6 bags per treatment.

Table 3: Characteristics of different bags used in the experiment.

Trade name (* = samples only)	Polymer	Thickness (µm)	Perforations (psi)
-	Polyethylene	125	0
Sira-Flex™Resolve®	Biopolymer	25	0
Sira-Flex™Resolve®	Biopolymer	25	25
Sirane Self Seal Cook Bag*	Polyester with aluminum oxide	~32	0

Results – Bags

1. Moisture content

Figure 8 shows that the moisture content of control acorns decreased with increasing storage duration. Generally the acorns stored in perforated and non-perforated biopolymer bags followed a similar trend although moisture content was higher than the corresponding control acorns. In contrast, the moisture content of acorns stored in polyethylene or polyester bags increased significantly with increasing storage duration (i.e. the interaction of storage duration and treatment was significant at P=0.05).

2. X-rays

Figure 9 shows that seed occupancy of control acorns decreased significantly from 80 to 62% during storage. The acorns stored in perforated and non-perforated biopolymer bags followed a similar trend. In contrast, seed occupancy of acorns stored in polyethylene or polyester bags remained constant during storage.

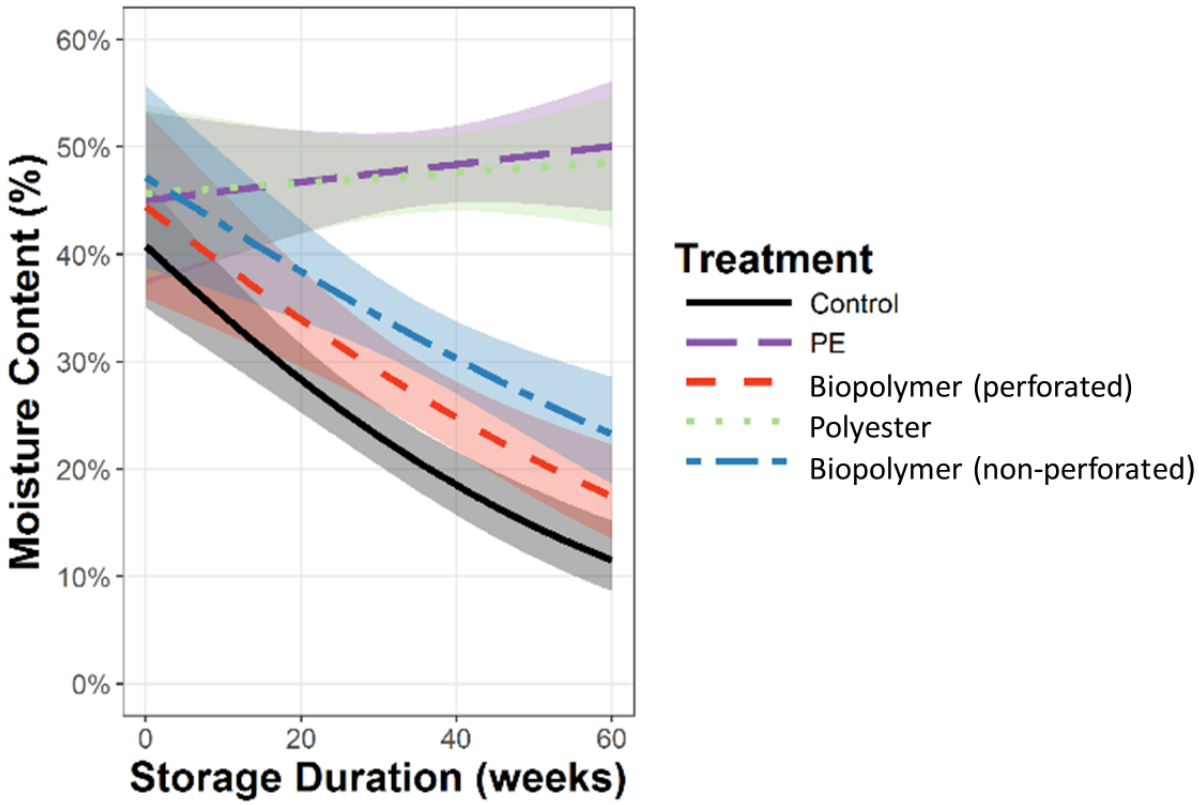

Figure 8. Bag data best fit model for moisture content, predicted by storage duration and treatment. Coloured ribbons indicate 95% confidence intervals.

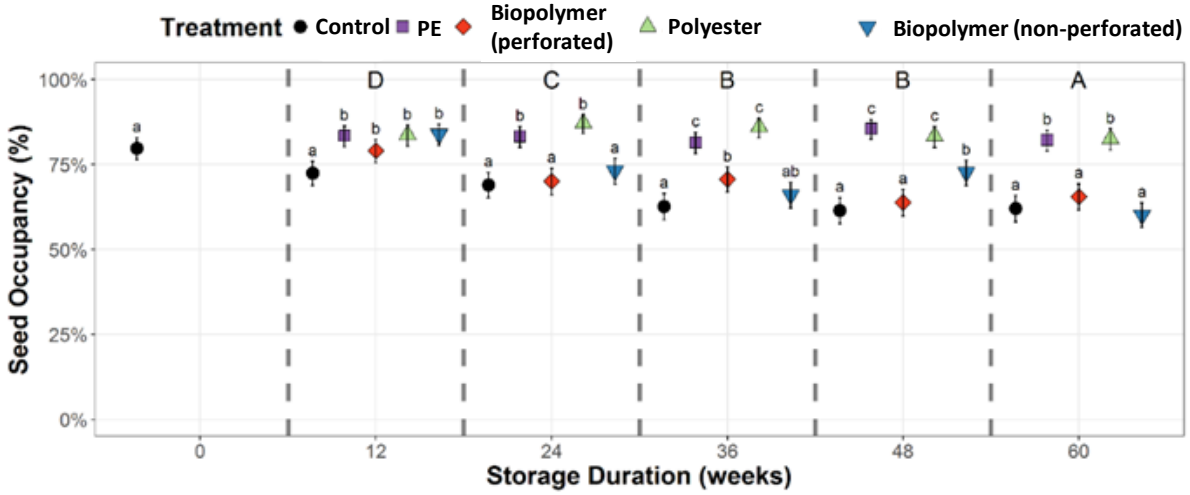

Figure 9. Bag data plot of seed occupancy (%) by storage duration and treatment. Lettering (lowercase) refers to significant differences within storage durations; lettering (uppercase) refers to significant differences across storage durations. Error bars = 95% confidence intervals.

3. Electrical conductivity

Figure 10 shows that there was no significant relationship between storage duration and solute leakage. However, there was a great deal of variation between treatments and notably soluate leakage of acorns stored in polyester bags increased with storage duration.

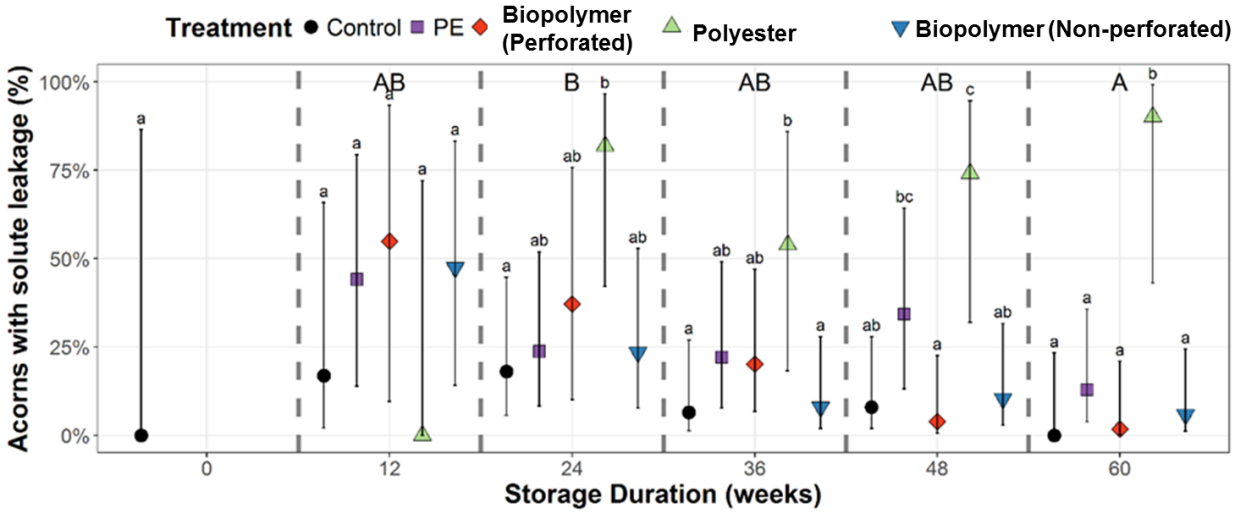

Figure 10. Bag data plot of proportion of acorns with solute leakage by storage duration and treatment. Lettering (lowercase) refers to significant differences within storage durations; lettering (uppercase) refers to significant differences across storage durations. Error bars = 95% confidence intervals.

4. Germination

Figure 11 shows the cumulative germination of control acorns after different storage durations. In general, germination decreased rapidly with increasing storage duration. The acorns stored in biopolymer (perforated and non-perforated) and polyethylene bags followed similar trends. In contrast, germination in polyester bags remained constant during storage. In this particular treatment, fungal infection was a problem. Infected acorns (*circa*. 16-18%) were not sown, and therefore, germination percent was adjusted accordingly.

Figure 12 shows that there was a clear (sigmoidal) relationship between moisture content and germination for the acorns stored in different bags. Data for the Polyethylene bags were excluded due to acorn fermentation.

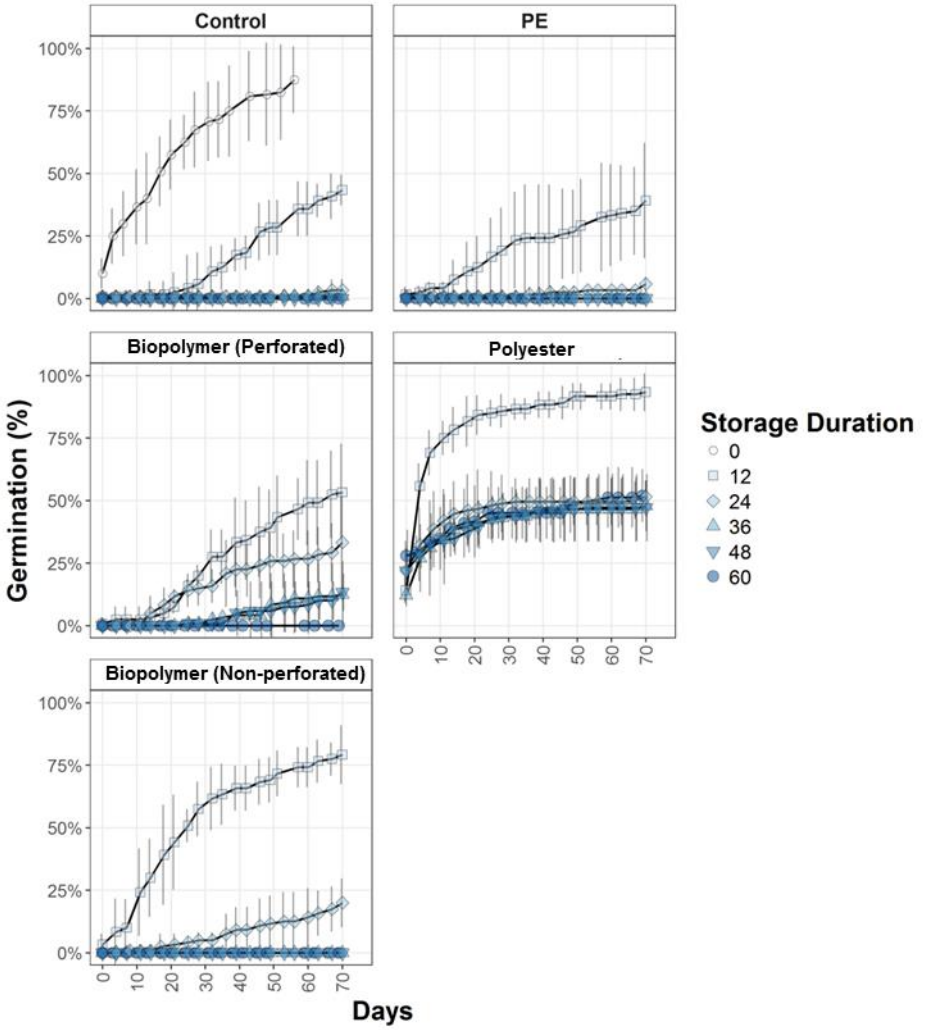

Figure 11. Bag data plot of cumulative germination (mean values per four replicates) by storage duration and treatment. Error bars = 95% confidence intervals.

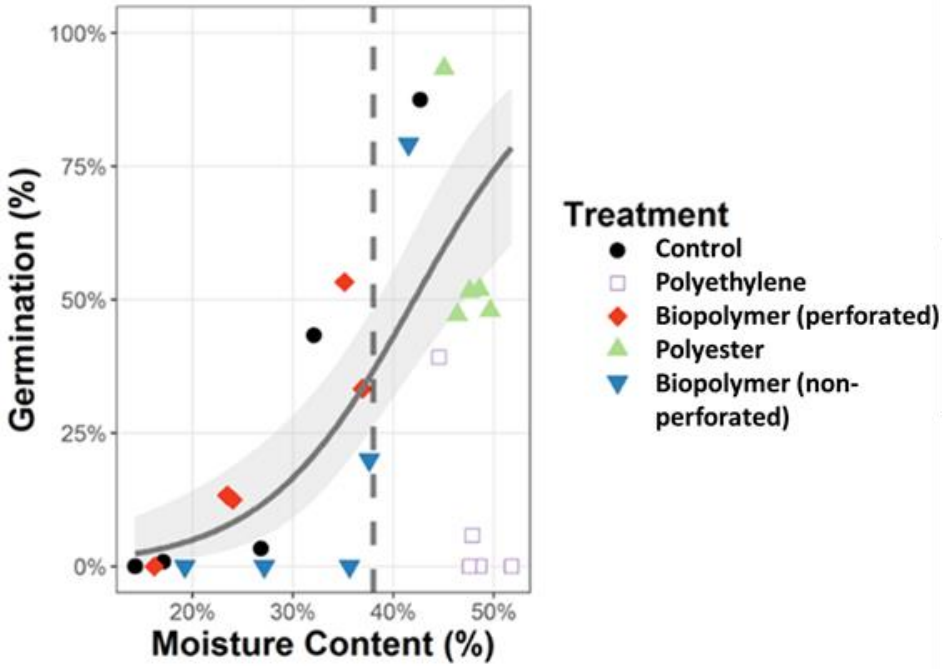

Figure 12. Bag data best fit model for germination capacity, predicted by moisture content. Grey ribbon indicates 95% confidence intervals. Fill for data points indicates inclusion/exclusion from the best fit model (filled = included, unfilled = excluded, see text).

Discussion – Bags

The shelf-life of acorns varied significantly depending on bag type. The bags had different properties, which interacted with the respiring acorns to passively modify the storage atmosphere. This in turn influenced not only desiccation but also respiration and fermentation.

The acorns stored in polyethylene bags lost viability rapidly even though moisture content remained above the critical threshold (*circa.* 38%). After 12-weeks storage, germination capacity was only 39%, dropping even further during the subsequent weeks. This was largely due to fermentation as the gas atmosphere was starved of oxygen in the sealed bags. These acorns were characterised by necrotic embryos (Figure 7g).

The acorns stored in biopolymer bags also lost viability rapidly, which was due to desiccation in both perforated and non-perforated biopolymer bags. In non-perforated bags, however, there was also some fermentation particularly at the longer storage durations. These bags are constructed from a plant-derived polymer, which is permeable to water, oxygen and carbon dioxide. This reduces potential problems with condensation and fungal infection (Shamash, 2012).

The acorns stored in polyester bags had high moisture content. After 12-weeks storage, acorns germinated rapidly and uniformly, which suggests that these were 'primed' for germination. The acorns often chitted but the radicles did not elongate further. This may be due to ethylene, which is a potential germination inhibitor. However, the acorns were prone to fungal infection (Figure 7b), which caused large losses (16-18%). Nonetheless, germination remained at similar levels (47- 52%) even after 60-weeks storage.

In general, desiccation was less of a problem when acorns were stored in polyethylene or polyester bags. This was reflected in the data for seed occupancy, which remained higher than the control acorns during storage. In contrast, seed occupancy decreased to similar levels to control acorns when acorns were stored in biopolymer bags (perforated or non-perforated). The probability of solute leakage though was higher for acorns in polyester bags possibly due to confounding by fungal infection. This confirms that solute leakage was not a good indicator of acorn viability.

OBJECTIVE 3. – TO DETERMINE THE SEEDLING PERFORMANCE OF TREATED ACORNS UNDER NURSERY CONDITIONS.

Methods – Nursery trial

Control and treated acorns were also tested in a nursery trial at Cheviot Trees, near Berwick-upon-Tweed in the Scottish Borders. In autumn, control acorns were sown in plug trays (25 acorns per tray), which were arranged as a block in the polytunnel. In spring (after 16-weeks storage), acorns from the storage treatments were sown as above (6 trays per treatment) in two randomized blocks. The first block had 9 treatments (4 coatings x 2 [partial and whole] plus control) and the second block had 5 treatments (4 bags plus control). The autumn-sown control acorns were then re-arranged as a buffer between and around the two blocks. In December 2016 the seedlings were counted and graded into three categories (> 40cm, 20-40cm and < 20cm) as per industry standards.

Results – Nursery trial

The control acorns sown in autumn were common to both parts of the study. Table 4 shows that the control acorns sown in autumn had a total seedling emergence of 54% but only 46% were classed as saleable (i.e.>20 cm height) . The majority of these saleable seedlings were between 20-40 cm tall. The control acorns sown in spring produced only 12% saleable seedlings, which was largely due to the high number of empty plugs (88%). The best coating treatment was microcrystalline wax whole, which produced 36% seedlings, the majority of which were graded as saleable (30%).

Table 4. Effects of coating treatment and 16-weeks storage on percentage seedlings in each category at December 2016.

	Percentage seedlings in each category					
	Saleable seedlings (%)			Failures/unsaleable seedlings		
Treatment	**>40 cm**	**20-40 cm**	**Total**	**Failures/Empty cells**	**<20 cm**	**Total**
Control (Autumn)*	19	27	46	46	8	54
Control (Spring)	1	11	12	87	1	88
Partially-coated with:						
Bees	1	7	8	89	3	92
Microcrystalline	2	16	18	81	1	82
Soya	3	14	17	81	2	83
Wiltpruf ®	3	17	20	75	5	80
Entirely coated with:						
Bees	2	14	16	77	7	84
Microcrystalline	9	21	30	64	6	70
Soya	2	15	17	81	2	83
Wiltpruf ®	1	7	8	90	2	92

* common control (autumn).

Table 5 shows the the control acorns sown in spring produced only 12% saleable seedlings. The best treatment was acorns stored in perforated biopolymer bags, which resulted in 35% saleable seedlings. The bulk of these saleable seedlings were between 20-40 cm tall.

Table 5. Effects of bag treatment and 16-weeks storage on percentage seedlings in each category at December 2016.

	Percentage seedlings in each category					
	Saleable seedlings (%)			Failures/unsaleble seedlings		
Treatment	**>40 cm**	**20-40 cm**	**Total**	**Failures/ Empty cells**	**<20 cm**	**Total**
Control (Autumn)	19	27	46	46	8	54
Control (Spring)	1	11	12	87	1	88
Polyethylene	1	2	3	97	0	97
Polyester	2	9	11	88	1	89
Biopolmer Non-Perforated	0	0	0	100	0	100
Biopolymer Perforated	9	26	35	63	2	65

***** common control (autumn)

Discussion - Nursery trials

In commercial nurseries, nursery managers usually select germinating acorns for sowing to maximise crop production. Thus, it is expected that about 50-65% of an acorn seedlot will produce saleable seedlings depending on seed source and quality. As the initial seedlot in this project had 88% viable acorns, it would not be unreasonable for it to produce 44-57% saleable seedlings. The control acorns sown in autumn produced 46% saleable seedlings of which 19% were taller than 40cm. In contrast, acorns coated entirely in microcrystalline wax produced 30% saleable seedlings. In general, seedling emergence for acorns entirely coated with bees- or microcrystaline waxes was slow and as a result trays were often covered with thick moss plugs. The acorns stored in perforated biopolymer bags, produced 35% saleable seedlings while those in non-perforated biopolymer bags failed completely. Regardless of treatment, the quality of saleable seedlings was comparable with that of the control acorns sown in autumn. In all treatments, the proportion of seedlings below the saleable height threshold was low. In some cases, seedlings below this height threshold are retained for another season.

CONCLUSIONS

Oak fruiting is often irregular, resulting in poor seed crops during intermast years. In addition, acorns are difficult to store without significant loss of seed quality. As a result, seed traders

cannot easily stockpile acorns collected in mast years for use during inter-mast years, which results in supply and demand problems for nursery traders.

However, the shelf-life of acorns is strongly influenced by moisture content, which in turn is affected by storage conditions particularly temperature and relative humidity. In addition, acorns respire producing by-products that modify storage conditions. The type and intensity of respiration is also influenced by temperature and gas atmosphere. Thus the challenge is to provide 'wet storage' conditions, which have:

1. high humidity to reduce water loss from acorns but not free water that promotes fungal infection;
2. low temperature to reduce respiration and premature germination of acorns;
3. adequate gas exchange to prevent fermentation of acorns due to oxygen starvation.

This project confirmed that pedunculate oak acorns are difficult to store for longer than several months without significant crop losses due to desiccation, fermentation, fungal infection and premature germination. Desiccation below the critical threshold (*circa*. 38%), in particular, resulted in rapid decreases in germination of stored acorns regardless of treatment. However, the two approaches used in this study provided useful new information, indicating potential areas for further research and development.

Firstly, coating acorns entirely with bees- or microcrystalline-wax reduced desiccation during storage and maintained viability for up to 36-weeks. These coatings, however, hampered subsequent germination largely due to mechanical resistance, which trapped radicles. However, this could be overcome by partially removing the wax at the apex of acorns or developing methods that result in thinner/weaker coatings. With longer storage durations, acorns subsequently lost viability due to fermentation. It may be possible to solve both problems by modifying the wax formulations.

Secondly, storing acorns in polyester bags maintained high moisture content for 60-weeks. Critically, germination remained constant between 47-52% from 24-to 60 weeks storage. This suggests that acorns reached a steady-state in these polyester bags. It is possible that germination could be close to the target 70% if the fungal infection that caused 16-18% losses in this treatment was controlled more effectively during storage.

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APPENDIX 1 – SEED QUALITY TESTS

1. Moisture content

Twenty acorns (4 replicates of 5 acorns) were used to determine moisture content using the low constant oven method (17±2 hours at 103±2°C) (ISTA, 2003). Moisture content was calculated as a percentage fresh weight. Where applicable, wax was removed from acorns to accurately determine the fresh weight of acorns.

2. X-rays

Acorns (14-20) were x-rayed using a Faxitron MX20 (10s at 18-20kV). The resulting DICOM images were then manipulated to calculate percentage seed occupancy using image analysis software, ImageJ.

Calculating seed occupancy using ImageJ software.

Source data was taken in the form of the x-ray images used to assess seed quality. The images are in DICOM (*.dcm) file format (Digital Imaging & Communications in Medicine). Images saved in this format include all relevant data including patient/specimen, imaging equipment, hardware and image data in a single file.

The methodology used was image analysis software. Software used is 'ImageJ' 1.49v. This software is written in JAVA and runs on Linux, Windows and Mac OSX, 32 and 64 bit systems. ImageJ and its JAVA source code are freely available and in the public domain; no licence is required.

https://imagej.nih.gov/ij/download.html

To obtain cross sectional area measurement data the following method was employed:

1. IMAGE, DUPLICATE… Duplicate the image, so as not to alter the original image.
2. Use IMAGE, TYPE, 8-Bit (converts the 16 bit image to 8 bits as some functions only work on 8 bit black on white images)
3. Use the IMAGE, ADJUST, THRESHOLD function (to highlight the structure of the seed within the acorn) then APPLY this to the image after adjusting the slider accordingly on the default setting of the threshold. This will create an image with only the areas highlighted as a 'silhouette' using black and white pixels.
4. From this image the ANALYZE, ANALYZE PARTICLES… produced a count of the particles (acorns) and the associated area is calculated automatically in a table
5. Special attention is needed not to include unwanted structures, e.g. cup scars or wax cups that may appear in the seed image. These can be masked out using the software.
6. The analysis was set up to exclude and partial images on the edges of the frame
7. The data from the table can be exported to MS Excel

8. Each acorn in both image sets was checked to ensure they matched and the table data manually adjusted if the automatic particle (acorn) numbering was out of sequence.
9. This is repeated for the outer structure of the acorn and the data per acorn from each image compared, subtracted and calculated as percentage seed occupancy.

3. Electrical conductivity/Solute leakage

Twenty acorns were tested individually to detect solute leakage (Bonner, 1996). Acorns were soaked in 50ml of water for 24 hours at 27°C. Acorns were then removed and electrical conductivity (EC) of the water measured with a VWR CO30 conductivity meter. Results for solute leakage were expressed as microSiemens per gram (fw) ($\mu S\ g^{-1}$). Acorns were then cut longitudinally and scored as dead, alive or fungal infected. Where applicable, wax was not removed from acorns.

4. Germination

Acorns (30 x 4 replicates) were sown horizontally in trays containing moistened potting mix (1:1 peat: grit [v/v]). The acorns were not covered with potting mix to enable assesments. The trays were placed in loosely tied polyethylene bags and then incubated at 20°C for 8-10 weeks. Acorns were assessed twice weekly for germination. Germination was scored when acorns had chitted and radicles were 2mm or longer. At the end of germination tests, ungerminated acorns were cut and scored as dead or alive.

APPENDIX 2 - STATISTICAL ANALYSES

Data from the experiments were analysed using using the following R packages (R Core Team, 2016). Factors were considered statistically significant if P≤0.05.

Base R package (R Core Team, 2016)
Package “car” (Fox & Weisberg, 2011) – ANOVA
Package “ggplot2” (Wickham, H)
Package “lsmeans” (Lenth, 2015) – least-square means
Package “multcompView” (Graves et al., 2016) - least-square means lettering

For all statistical analysis, coatings and bag data were treated as two separate data sets.

For moisture and germination capacity data, there was a single replicate for each storage duration and treatment combination. As such, data could only be assessed using main effects, or by treating storage duration as a covariate within the model. Both model types were applied to the coatings and bag data, and the best fit model determined by assessing the residual deviance in the model. In each case, a generalised linear model (GLiM) with a logit-link function and binomial error, corrected for overdispersion (appropriate for proportional data) was applied to the data. The best fit model was determined using the F test statistic. The significance of the predictors for the best fit model was determined based on the likelihood ratio chi-square test statistics from the analysis of deviance, using the car package in R. Post hoc tests were conducted on the best fit model to estimate differences within significant factors, correcting for multiple comparisons using Bonferroni adjustments to the p value.

Germination capacity followed a sigmoidal function with respect to moisture content, therefore this relationship was also described a generalised linear model (GLiM) with a logit-link function and binomial error, corrected for overdispersion. The predict() function was used to plot the best fit model, with logit data being back-transformed to aid interpretation.

For the cumulative data set, maximum germination capacity was determined from each sample and analysed following similar methodology as above. As the data were replicated, the effect of storage duration could be analysed as a factor with treatment interactions. Model fit was compared between treating storage duration as a factor or continuous covariate, with the best fit model determined from analysis of deviance following the same methodology as above. The significance of the predictors was determined using analysis of variance. Post hoc tests were conducted on the best fit model to estimate differences within significant factors, correcting for multiple comparisons using Bonferroni adjustments to the p value.

Solute leakage from acorns was assessed for both coatings and bag data. Due to the high number of zero values within the data set, solute leakage was treated as a simple binary yes/no response and analysed using a generalised linear model (GLiM) with a logit-link function and binomial error, following a similar methodology as above, with the inclusion of acorns being live or dead as a predictor.

Seed occupancy was determined for both coatings and bag data. As these were also proportion data, the statistical analysis was the same as for the germination data, using a GLiM with a logit-link function and binomial error, corrected for overdispersion.

