Seed Dormancy Breaking

of Temperate Region Deciduous Tree Species.

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by

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Declaration

I hereby declare that this thesis has been composed by myself, and has not been accepted in full or part, in any previous application for a higher degree. The work of this thesis is a record of my own work, any collaborative work has been specifically acknowledged as have all sources of information.

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Abstract

Tree seeds often exhibit dormancy and require several months of moist chilling (cold stratification) before germination can proceed. Seed dormancy is poorly understood with no clear identified mechanism as to why cold stratification works. Endogenous plant hormones and chemicals have been implicated in this process.

The requirement for a lengthy cold stratification period (often 2-4 months) limits the popularity of growing trees from seed in the amateur gardening market and reduces productivity for professional tree growers. A treatment product or protocol which breaks dormancy would address these issues.

We have been able to define the seed dormancy status of a range of temperate region tree species. The deeply physiologically dormant seeds of *Acer tataricum* (tartar maple) were chosen as a test seed species. It was determined that water and oxygen permeability of the seed coats were not a major factor in maintaining dormancy in this seed. Nor was the oxygen concentration that the seed was incubated in. There was also no indication of inhibitory substances (to germination of lettuce seeds) within the seed. The testa of *A. tataricum* was found to weaken over time after imbibition in both cold and warm conditions and it's physical restriction of the embryo is likely to be a secondary factor in prolonging dormancy.

It was found that cytokinins can break the dormancy of *A. tataricum*. A treatment for breaking the dormancy in temperate region tree seeds is proposed using a 350 mg/l kinetin/20% dimethylsulphoxide solution. This treatment successfully removed the dormancy and substituted for up to 3 months of cold stratification in these species in *Acer platanoides* (Norway Maple 'Crimson King') and *Tilia cordata (*Small-leaved Lime).. It also increased the germination percentage of *Acer pseudoplatanus* (Sycamore). Possible mechanisms of how cold stratification breaks dormancy in relation to this finding are discussed. Other species and varieties of dormant seed respond to other plant hormones such as *A. platanoides* to jasmonic acid. Future areas of study in order to understand

the mechanism of dormancy release are suggested along with further ideas to develop a dormancy breaking seed treatment.

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Chapter 1

General Introduction

Temperate area trees are typically defined as those species which naturally occur between the polar and tropical regions between 25 and 50 degrees latitude. This mid latitude location results in exposure to both hot and cold air masses at different times of year. This causes the region to experience the distinctive four seasons of spring, summer, autumn and winter. Winters can be cold with often freezing temperatures. Average annual temperatures across world temperate regions are 10ºC with typical annual precipitation being between 750 mm and 1500 mm. Temperate area forests are predominantly found in North America, Western and Central Europe and East Asia however are also present in South America and Oceania (Currie and Bergen, 2008).

The majority of temperate area tree species are deciduous which is characterised by leaf loss and a resting or dormant period in winter with most active growth in the spring and summer seasons. Often these deciduous trees are called broadleaved to differentiate them from the predominantly evergreen or non deciduous conifer species which are characterised by thin needle like leaves or scales.

Temperate area deciduous trees are extremely diverse and include such extensive species as Oak, Maple, Beech, Ash and Chestnut. A feature of many of these species is production of large quantities of seed (Harper, 1977). Many species exhibit a form of seed dormancy. Seed dormancy is defined by the absence of germination in seeds which are subjected to environmental conditions which are conducive to germination. In other words if the seed's requirements for warmth, oxygen, moisture and absence or presence of light are optimal germination still will not occur. The dormancy of the seed prevents germination. It is only when the dormancy is removed that germination can proceed. Dormancy can also be described as a block to germination (Finch-Savage and Leubner-Metzger, 2006). It is probable that many plant species have evolved seed dormancy to

prevent germination in conditions which would be unfavourable to seedling survival. A prime example is that of temperate deciduous trees. Seeds are dispersed in autumn where environmental conditions may be favourable to germination (warmth) however the soon to arrive winter season with potential freezing conditions would result in severe seedling mortality. A dormancy period of several months holds holds back germination until the more favourable spring and summer seasons. The seedling will then have the whole growing season to develop and harden up for the subsequent winter. This improved survival of individual seedlings gives a strong evolutionary pathway for the development of seed dormancy in species which experience harsh winter environments.

Seed dormancy presents challenges to the forestry industry. The lag time between sowing of the seed and germination directly affects profit margins as the time, energy and resources required to maintain a favourable germination environment for large quantities of seed can be considerable (Kermode, 2011).

What is germination?

It is pertinent to define what germination is. There are many different interpretations of what germination is and no single standard exists. However in basic seed science, germination can be defined most simply as “germination begins when a dry seeds takes up water and is completed with protrusion of the radicle”. A more economic and practical definition for a tree grower might be “the emergence and establishment of the seedling” (Basra 2007).

Frequently, researchers declare germination to have occurred when the radicle just emerges from the seed coat. However, there are some species of plant where other organs such as the shoot or cotyledons protrude first. In excised embryos, where there is no seed coat, often germination is recorded when the embryonic axis (usually the radicle) starts to elongate or curl away from it's start position. Some researchers declare germination when biochemical processes start within the seed

before any outside visible evidence is present (Nonogaki, 2010).

Germination can be said to proceed in 3 phases. Phase one is where dry seed (although not all seed starts as completely 'dry') quickly absorbs water until its tissues and cells are fully hydrated. This is called imbibition. This is then followed by phase two where the seed does not take up any more water. This phase is associated with DNA repair and translation of stored as well as newly synthesised mRNA. The seed can remain in this state for a variable amount of time. In dormant seeds this is such a time until dormancy is broken. In many physiologically dormant tree seeds this time corresponds with when they would naturally be in the moist soil after being dispersed. They cannot proceed to phase three until the dormancy is overcome. Phase three of germination is characterised by significant DNA replication, embryonic axis elongation, mobilisation of storage reserves and a second period of water uptake as the seed enlarges. Once this phase is complete the seed will show radicle protrusion and the seed can be said to have 'germinated' (Bewley, 1997).

Seeds are made up of several basic components. All seeds have an embryo from which a radicle emerges during germination. The radicle forms into the roots of the young plant. Seeds can be endospermic or non-endospermic. An endospermic seed has its embryo surrounded by a mass of storage tissue which protects and nourishes the embryo. The endosperm and embryo are thought to signal to each other and this signalling between the two is a major mechanism in the regulation of germination (Yan, 2014). Common ash (*Fraxinus excelsior)* is an endospermic seed. Tatar maple (*Acer tataricum)* is a non-endospermic seed (as are most of the Maples). There is no endosperm tissue. The embryo consists of two cotyledons with an attached radicle. The cotyledons contain the seed's energy storage, mostly in the form of lipids (in particular linolenic acid). This is in contrast to endospermic seeds which store most of their energy in the form of starch.

Both *F. excelsior* and *A. tataricum* have an outer seed coat called the pericarp. When the pericarp is

present it can be described as the fruit of the seed. The pericarp in both species forms a wing shape and is sometimes called a samara. The wing shape allows the seed to more easily be dispersed by the wind. *Acer* seeds typically come as a pair of joined samaras/pericarps.

In both seeds the samaras/pericarps can physically be removed by hand exposing the inner seed. This exposed seed can be described as the true seed rather than the fruit. In *A. tataricum* the true seed coat is called the testa and is thin, sticky and clings to the surface of the embryo like a skin. It too can be removed revealing the embryo underneath.

Figure 1. Anatomy of *Acer tataricum* and *Fraxinus excelsior* seeds.

Seed dormancy is not specific to trees and is a feature of many plant species. There is considerable variation in how seed dormancy is manifested. Various researchers over the years have attempted to classify seed dormancy into different categories. The soviet researcher Nickolaeva proposed a dormancy classification system based on the morphological and physiological characteristics of the seed (Nikolaeva, 1967). Decades later Baskin & Baskin updated this system and split seed dormancy into five main types (Baskin and Baskin, 2004).

1.Physiological Dormancy (PD)

This is the most prevalent form of seed dormancy in all plants and features in the majority of temperate zone tree species. Physiological dormancy is an intrinsic state where the seed will not germinate until either chemical or hormonal signals within the seed are activated. The exact process is not completely understood. Such signals are initiated by exposure to environmental factors such as temperature, light exposure or gas concentration among others. Each species has specific environmental requirements to break physiological dormancy.

PD can be divided further into three subcategories, that of deep PD, intermediate PD and non-deep PD. The depth of PD dormancy can be classified by the seed's response to embryo excision and treatment with the plant hormone gibberellic acid. Gibberellic acid is linked to promoting germination.

When removed from the rest of the seed, deep PD seed embryos fail to grow, or produce non-viable, abnormal or stunted seedlings. Gibberellic acid has no effect on germination in deep PD seeds. Dormancy can only be overcome by a period of cold stratification (typically 3-4 months) and may also need a period of warm stratification in order for germination to proceed successfully. Examples of PD temperate zone tree seeds are Tatar Maple (*Acer tataricum*).

Intermediate PD seed embryos when excised will produce viable seedlings. There is also some response to gibberellic acid. These seeds require a shorter period of stratification than deep PD seeds in order to germinate successfully (typically 2-3 months). An example is the common Sycamore (*Acer pseudoplatanus*).

Non Deep PD is the most common seed dormancy across all plants. Excised embryos grow into viable plants and gibberellic acid can promote germination. Dependent on species, non deep seeds can also have their dormancy removed by processes including scarification (scratching the seed coat), cold or warm stratification and after ripening in dry conditions.

2. Morphological Dormancy (MD). These seeds have underdeveloped embryos. They are not physiologically dormant but need time to grow and develop before they can germinate.

3. Morphophysiological Dormancy (MPD). These seeds also contain underdeveloped embryos however the embryos also have a degree of physiological dormancy. They require time to grow and mature as well as a dormancy breaking treatment such as cold stratification. An example of a temperate zone tree which exhibits MPD is Common Ash (*Fraxinus excelsior*).

4. Physical Dormancy (PY). These seeds are characterised by a water impermeable seed or fruit coat. This coat prevents water penetrating inside the seed and as water is essential to germination this acts as a form of dormancy. The seeds will remain dormant until some factor renders the coat permeable to water. Depending on the species this may be high temperatures, freeze/thawing, fire or passage through the digestive tracts of animals. These seeds can be artificially released from dormancy by mechanical or chemical scarification of the seed/fruit coat. In trees this type of dormancy is more common among tropical species.

5. Combinational Dormancy (PY + PD). These seeds exhibit physiological dormancy however are also covered in a water impermeable coat. These two forms of dormancy appear to act independently of each other.

Evolution of dormancy

Seed dormancy is thought to be an ancient process dating back to the very beginning of seed-bearing plants. Willis et al. (2014) suggest that the first plants to set seeds most likely exhibited morphophysiological dormancy. At some point in history the plants adapted to produce mature embryos at the time of seed dispersal. Thus, morphological and physiological dormancy characteristics became uncoupled. The resulting physiological dormancy is thought to be an evolutionary hub from which the remaining forms of dormancy have evolved. The diversity of physiological dormancy, in only allowing germination in response to specific environmental factors, suggests it allows plants to time germination in new or difficult environments. This would aid colonisation of new areas. In particular the spread of trees into temperate areas where the principal trigger to releasing dormancy is exposure to cold temperatures (Willis et al. 2014).

Cold stratification

Moist chilling is a widely used technique for breaking seed dormancy in many tree species (Schopmeyer, 1974). It involves subjecting the seed prior to sowing with moisture and cool temperatures in the range of 0-5 degrees centigrade. The seed is often maintained in this state for several months before being exposed to higher temperatures. The process simulates a natural cold season cycle where the seed may spend several months overwintering in cool soil only to break dormancy after a set period of time. After treatment the seed can be exposed to higher temperatures to allow germination. Tree growers in the forestry and horticulture industries frequently bury tree

seeds in the ground in several layers of soil and sand over the winter months to break dormancy to promote germination the following spring. This method of layering the seed in the ground is called stratification. Cold treatment is also carried out in refrigerators with the seed mixed in bags of moistened moss, soil and/or sand although the process is still commonly called stratification.

It is the cold state whilst being fully moistened that breaks the dormancy. Cold treatment of dry seeds (moisture content less than 15%) does not break dormancy. Unfortunately the biochemical mechanism as to why this works is poorly understand. Cold stratification is the most reliable method of breaking seed dormancy for physiologically dormant seeds. The disadvantage of stratification to the tree grower is primarily the time it takes for the treatment to work. Most tree species seeds require cold treatment for 1-3 months and some in species it takes significantly longer. Whilst undergoing this lengthy process, seeds are susceptible to mould, insects and bacterial pests which can reduce their viability and the resulting successful germination rate. For many deciduous temperate region trees propagation from seed is the only economically viable method of growing new trees. Vegetative methods such as cuttings, grafting and tissue culture have proven expensive on a large scale. If a method were available to speed up seed dormancy breaking this would increase seedling production significantly by freeing up propagation, glasshouse and nursery space. There is a rising demand for young trees to supply many national reforestation commitments worldwide (UN Press Release, 2019)

The rather onerous stratification requirements also limit the sales of tree seeds to the amateur gardening market. If a method or treatment were available to either pre-break dormancy or hasten the breakage a new market for tree seed may be available.

Other methods of breaking seed dormancy

It is currently thought that plant hormones are significantly involved in breaking physiological seed

dormancy. The exact biochemical mechanism that hormones play in dormancy is not well understood. It is possible that hormonal actions are different across species and even across different depths of Physiological Dormancy (Staszak et al. 2019).

Abscisic acid (ABA) is an important plant hormone present in almost all plants and some fungi and bacteria. First characterised in the 1960's it was thought to cause abscission (the annual process of leaves falling off trees in autumn) and acquired the name abscisic acid. ABA has since only been found to affect leaf fall in a few cases. We now understand it to have many important regulatory roles within the plant, of which the most documented effect is the action of ABA in slowing the shoot growth of plants in preparation for winter. Some of the roles of ABA are related to external stresses on the plant. An example of one of these actions is where ABA signals for the stomata in leaves to close in response to water drought thus lowering transpiration and therefore preserving water within the plant (Li et al. 2017; Graeber et al. 2012).

Importantly ABA is also implicated in seed dormancy. It is thought that elevated ABA levels within the seed suppress the processes which lead to germination. This inhibitory activity maintains dormancy. In many species of plant and in particular the well studied *Arabidopsis thaliana,* germination is associated with decreasing levels of ABA. There is considerable evidence that ABA works antagonistically with gibberellic acid (GA), another majorly important plant hormone. Simply put, an increase in gibberellic acid reduces the level of ABA and therefore promotes germination hence dormancy is broken. The balance between ABA and GA is influenced by factors such as temperature and light (Skubacz and Daszkowska-Golec, 2017).

Treatment of seeds with GA has been reported as accelerating germination or breaking dormancy altogether in some species of plant whose seeds normally require a period of cold stratification. This is especially the case where seeds exhibit intermediate to non deep physiological dormancy. Deeply dormant seeds have shown little response to GA (Frankland, 1961).

Other hormones

Jasmonates are lipid derived plant hormones. They are involved in regulating a wide range of functions in plants including photosynthesis and seed formation. These hormones are particularly linked to eliciting plant defence mechanisms in response to outside stressors. For example when tomato leaves are attacked by herbivores such as red spider mite, jasmonates (among other molecules) signal for the plant to produce deterrent chemicals to protect from further damage (Howe and Jander, 2008).

Jasmonates are oxylipins, in that they are derived from oxygenated fatty acids. Jasmonates begin their synthesis in the chloroplasts of the plant cell. Linolenic acid, a fatty acid common in plant cells is converted to 12-oxo-phytodienoic acid (OPDA) (Roberts, 2016). The OPDA is then transported to the peroxisomes of the cell where is undergoes further oxidation reactions to form jasmonic acid (JA). Jasmonic acid can also be converted to slightly different forms still within the jasmonate family of molecules. Adding a methyl group results in methyl jasmonate (MeJA) and jasmonic acid can also form conjugates with amino acids of which the most prevalent conjugate is JA-Ile (JA with isoleucine). The amino acid conjugated forms such as Ja-Ile were originally thought to be a storage and transport form of jasmonate. It is now well established that JA-Ile is biologically active in it's own right. There is evidence that the different forms of jasmonate hormone have varying signalling roles within the plant (Schuman 2018; Babenko et al. 2015).

Jasmonic acid has been linked to seed germination in dormant seeds where in some species it displays an inhibitory effect while in others it may promote dormancy release (Linkies and Leubner-Metzger 2012). Berestetzky et al., (1991) found that a 48 hour treatment with jasmonic acid could induce germination in dormant *A. tataricum* seeds. *A. tataricum* (Tatar Maple) normally exhibits a deep physiological dormancy in its seeds, requiring approximately 90 to 120 days cold

stratification before germination can proceed. The researchers found that a 2 day treatment of seeds could completely substitute for the cold stratification. However, the treated seeds would only germinate if their testae (seed coats) were removed. This effect upon *A. tataricum* seeds was also found by Babaenko et al., (1996).

In other species commonly thought to have deep physiological dormancy in their seeds, Yildiz et al. (2008) found that a jasmonic acid treatment of pear (*Pyrus communis*) seed embryos promoted germination. The percentage germination rates were lower than that of cold stratified seeds but significantly more than that of untreated and unstratified seeds. Ranjan and Lewak, (1992) reported similar results. They also later demonstrated successful germination after JA treatment, and reported germination occurred after GA treatment (Ranjan and Lewak, 1994).

In dormant varieties of wheat, Xu et al. (2016) suggests that jasmonates have a key role in the cold stratification mechanism of dormancy breaking. Jacobsen et al. (2013) demonstrated that methyl jasmonate (a naturally occurring methylated form of jasmonic acid) stimulated germination in dormant wheat seeds. In Arabidopsis it was found that herbivore damage stimulated an increase in jasmonate iso-leucine (a jasmonic acid/amino acid conjugate) which was associated with reducing dormancy in subsequently produced seeds (Singh et al. 2017).

This makes jasmonic acid and its derivatives an interesting area of study in potentially developing a dormancy breaking tree seed treatment.

Hypoxia and seed coat inhibition

Côme et al. (1985) found that apple seed embryos exposed to low oxygen concentrations (hypoxia) increased the rate of germination. Apple (*Pyrus Malus L.*) is normally thought to exhibit a deep physiological dormancy in its seeds (Lewak, 1981).

The experiments were conducted on seeds without their seed coats. Raising the oxygen concentration higher than normal air levels (>21%) suppressed germination.

However, in dormant barley seeds, hypoxia inhibited germination and was associated with an

increase in ABA within the seed (Benech-Arnold et al. 2006). The authors also speculated that the seed glumellae (the phenolic rich seed coat coating) received significant oxidation effectively lowering the oxygen concentration within the seed. This they state may cause the hypoxia and therefore maintain dormancy.

Acer pseudoplatanus can be induced to germinate without a period of cold stratification by removing it's outer and inner seed coats. Pinfield and Dungey, (1985 & 1980) suggest that there may be a relationship between the testa of *A. pseudoplatanus* (common sycamore) seeds and a restriction of oxygen to the embryo. Successful germination depends on sufficient oxygen concentrations at the seed embryo. The permeability of the seed coats may be a factor in dormancy maintenance, however the above studies suggest that there may not be a consistent effects across different tree species.

Nitric oxide and cyanide

Nitric oxide (NO) is thought to be a trigger of dormancy release in wheat (Jacobsen et al. 2013) and also in apple seeds (Andryka-Dudek et al. 2019).

Nitric oxide as well as cyanide (HCN) is often present in seeds during germination and treatment with either of these substances can accelerate germination in apple seeds (Renata and Agnieszka, 2006; Ranjan and Lewak, 1994).

Gniazdowska et al. (2010) exposed apple embryos to gaseous NO and HCN for 6 hours and found it broke dormancy compared to untreated embryos. They also found that treatment with a solution of sodium nitroprusside (which breaks down into NO and HCN) also promoted germination.

Like in much of this area of study, the mechanism as to why this breaks dormancy is poorly understood. It is theorised that reactive nitrogen species substances (such as NO and HCN) cause an

increase in reactive oxygen species (free oxygen and hydrogen peroxide) within the seed embryo. These reactive oxygen species are important signalling molecules within the embryo and it is possible that their mode of action is to modify proteins seed proteins which either promote or inhibit germination (Oracz et al. 2007).

Aims and objectives

Many species of economically important temperate region trees exhibit seed dormancy. They will not germinate until this dormancy is broken. This is traditionally achieved with a period of cold stratification and may take up to 120 days to achieve. A shortened time to germination would be economically advantageous to tree growers. It is the overall aim of this study to develop a method or treatment to bypass the cold stratification period. An additional aim is to further understand the mechanism of dormancy with tree species exhibiting seed dormancy.

In order to work toward these aims the following objectives are proposed:

- Define the seed dormancy characteristics of various temperate tree seed species.
- Test the physical characteristics of the seed and it's coverings that may relate to dormancy maintenance.
- Discuss the possible mechanism of cold stratification.
- Identify and test chemical treatments for breaking seed dormancy.
- Identify and test plant hormone treatments for breaking seed dormancy.

Chapter 2

General Materials and Methods

Seeds

Seeds were obtained mostly from United Kingdom based tree seed suppliers. All seeds unless otherwise mentioned were of UK provenance.

Some seeds were collected at various sites in the UK including Lancaster, Morecambe, Kendal and Anglesey specifically for the purposes of these investigations. They collected in September 2019 and dried to a moisture content of approximately 10% and kept in airtight containers in darkness.

Chemicals and hormones

Chemicals, reagents and plant hormones were obtained from Sigma Aldrich, APC Pure (UK), Dr Ehrenstorfer GmbH (Germany) and Phygenera (Germany).

Germination tests

In order to determine germination, seeds and embryos were laid out in 90 mm Petri dishes with a single sheet of Whatman #1 filter paper. The filter paper was moistened with distilled water (dH_20). Where seeds were surface sterilised this was achieved by a 5 minute immersion in 6% H_20_2 with frequent stirring followed by rinsing in dH_20. Seed embryos were also set out this way too. Large seeds such as walnut were placed on moistened cotton pads in 300 ml polypropylene containers with lids.

Petri dishes were then placed in a growth area with at a temperature of 19ºC ± 2ºC. They were either exposed to light or in the dark. Darkness was achieved by wrapping the dishes in two sheets of aluminium foil. Petri dishes were set out with 20 seeds or embryos each. Initial experiments to

define dormancy in various tree species were tested once (Table 1). Subsequent germination experiments were repeated 2 times (to give a total of 3 germination tests). Germination was scored over time and expressed as a % germination or mean % of germination.

For cold stratification studies, seeds were placed upon moistened paper towels and enclosed in plastic food containers. They were then placed in a refrigerated cold room (approx. 4ºC) in darkness. Stratification studies were also conducted using fresh sphagnum moss instead of paper towels.

For germination studies seeds were either placed out whole or with their embryos excised. In order to remove the seed coats to excise the embryo, the seeds were soaked first for several days until fully imbibed (where they would take up no more water) and the pericarp (outer seed covering) was removed by hand. Then using forceps and a scalpel (under a magnifying glass where needed), the seed coat was carefully removed. In *Acer* species seeds this involved scraping the seed coat away while being careful not to damage the delicate radicle. In *Fraxinus* seeds embryo removal was achieved by slicing partway along the side of the seed and prising the resulting slit apart. The embryo could then be removed with forceps.

Germination was recorded as successful when the radicle emerged from the seed coat. This means the radicle could be seen through the testa and if present for that experiment the pericarp too (the covering layers). This is called visible germination. In excised embryos germination was noted when the radicle elongated significantly from it's start position. Some seedlings were grown on to check for abnormal characteristics and morphology. For this, germinated seeds were transferred to general purpose compost and placed in an unheated greenhouse with natural lighting.

Statistics

The standard error of the mean was calculated for seed germination tests. Where appropriate a one way repeated measures ANOVA (analysis of variance) calculation was performed to the 0.05 confidence level to indicate statistical significance.

Chapter 3

Defining seed dormancy in a range of tree species and investigating the physical characteristics of a model tree seed species in relation to seed dormancy

Materials and methods

Defining seed dormancy for various tree species

The following seeds were obtained and subjected to germination tests as described in the general methods above. *Acer campestre* (Field Maple), *Acer ginnala* (Amur Maple), *Acer palmatum* (Japanese Maple), *Acer pseudoplatanus* (Sycamore), *Acer tataricum* (Tatar Maple), *Betula papyrifera* (Paper Birch), *Betula pendula* (Silver Birch), *Fraxinus excelsior* (Common Ash), *Humulus lupulus* (Hops), *Juglans nigra* (Black Walnut), *Juglans regia* (English Walnut), *Malus domestica* (Apple 'Cevaal'), *Malus domestica* (Apple 'Cox's Orange pippin'), *Malus domestica* (Apple 'Kidds Orange Red'), *Pyrus communis* (Pear 'Conference'), *Quercus robur* (English Oak), *Quercus rubra* (Red Oak), *Robinia pseudoacacia* (Black Locust), *Sorbus aucuparia* (Rowan), *Tilia cordata* (Small leaved Lime) and 3 seperate samples of *Acer platanoides* (Norway Maple) (Lot A collected from Anglesey, North Wales, Lot L collected from Lancaster, Lancashire and Lot S collected from Kendal, Cumbria).

A. tataricum was chosen as the main (but not only) seed species to conduct further experiments on. *A. tataricum* exhibits deep physiological dormancy and is reported to respond to treatments of jasmonates to break this dormancy. This is an interesting avenue of research and may shed light on the mechanisms of dormancy. It was decided to attempt to attempt replicate this finding as described in chapter 4. Also due to the numerous experiments anticipated and the many repeats needed it was important to choose a variety of seed that was available in sufficient quantities which was the case for *A. tataricum*.

Testing seed component extracts for inhibitory properties

20 Seeds of *A. tataricum* and *F. excelsior* were de-pericarped and allowed to imbibe in distilled water for 3 days. Using forceps and a scalpel, the testa was removed from the embryo in *A. tataricum*. The embryo from *F. excelsior* was removed and the leftover seed housing (which contains the endosperm) was retained. Figure 1 above shows the difference between the seed components.

The isolated *A. tataricum* pericarp, testa, and embryos were ground separately with a pestle and mortar and each added to 20 ml distilled water. The same was done for the *F. excelsior* pericarps and endosperm. These extracts were shaken and allowed to stand for 3 hours before being used.

3 ml of each extract were added to Petri dishes containing 20 seeds of Lettuce (*Lactuca sativa* 'Little Gem'). Seeds were placed upon Whatman #1 filter paper. Dishes were placed in daylight at 19ºC ±2ºC. Distilled water was used as the control. Germination was scored as when the radicle emerged from the seed coat. Each experiment was repeated a further two times.

Another *F. excelsior* pericarp extract was made up however this time it was used to treat excised *F. excelsior* embryos instead of lettuce seeds. Distilled water was used as a control. Germination was scored when the embryos enlarged and started to show a green colour. Some embryos were grown on to check viability and morphology.

Water uptake of dry *A. tataricum* seeds

10 dry seeds of *A. tataricum* had their pericarps removed and were weighed on a set of micro scales. The seeds were then added to 20 ml distilled water and left for 24 hours at 15ºC. They were then removed carefully from the water and placed on blotting paper until all surface water was absorbed by the paper and the seeds were dry to the touch. The seeds were weighed again. They were then added again to another 20 ml distilled water and the procedure repeated for several periods of 24

hours. The experiment was conducted 3 times and the average weight calculated.

Chemical permeability of the testa of *A. tataricum*

A. tataricum in various states of seed coat removal (with pericarp, without pericarp but with intact testa, partially removed testa and fully removed testa) were immersed in an indigo carmine solution (10 mg/ml in distilled water) for 2 days. Indigo carmine stains the embryo, testae and pericarp of *A. tataricum* seeds a dark blue colour. Seeds were removed and blotted dry. Where present, pericarps and testae were removed and the embryos examined for staining. In order to test if the solvent used to carry the stain had an effect on permeability of the seed coats this experiment was repeated with a dimethylsulphoxide solution (10 mg indigo carmine in 20% DMSO).

Testing testa tensile strength of *A. tataricum*

Based on the ideas outlined by Steinbrecher and Leubner-Metzger (2017) a simple experiment to assay the tensile strength of the testa of *A. tataricum* was conducted. Professional laboratory equipment was not available at the time however a setup to test the breaking force of the testa using common materials was devised.

The testae from individual *A. tataricum* seeds were carefully removed with scalpel and forceps. In many cases it was possible to remove relatively large sections of the testa intact. These were cut to pieces 2 mm by 4 mm in size with a scalpel. This was done for freshly imbibed seeds and also for seeds which had 2 and 3 month cold stratification treatments. It was also done for seeds which had a 3 month 'warm' stratification period (kept at 19ºC).

The cut testa pieces were kept moist before testing. An apparatus as illustrated in Figure 2 was constructed. A micro bulldog clamp was used to suspend the pieces from a hook and another

clamp was connected to the bottom of the testa piece. From this a weight basket made from a filter paper disc was hung. Micro weights made from lead in 0.5 g increments were added to the weight basket one by one. When the testa broke this was recorded as the breaking force. The experiment was repeated 10 times for each seed treatment.

Figure 2. Apparatus set up for measuring the tensile strength of the testa in *A. tataricum.*

Note the 2 mm strip of testa clamped between the bulldog clamps. The paper disc below was folded in half and attached to the lower clamp to act as a holder for the weights. 0.5 g weights were gently added one by one until the testa broke.

Permeability of *A. tataricum* testa to oxygen

Due to a closure of laboratory facilities and a lack of acces to dedicated measuring instruments a simple method of testing the passage of oxygen through the testa of *A. tataricum* was devised. A common High School experiment as described by Engerer, (1999) which is normally conducted to demonstrate reduction and oxidation was adapted to test for the presence of oxygen.

Testa pieces from freshly imbibed *A. tataricum* seeds were removed as intact as possible. The larger pieces were set aside and kept moist.

An oxygen indicator solution was prepared as follows: 0.3 g of glucose and 0.175 g of KOH was added to 11 ml of distilled water and mixed. 0.003 g of indigo carmine was dissolved in 1 ml of distilled water and added to the glucose and KOH solution. The resulting oxygen indicator solution was sealed in an air-tight vial. The solution turned green then red/brown in colour and after several hours stabilised at a yellow colour. The resulting liquid was quickly transferred to a 2.5 ml syringe. The syringe was closed to the 1 ml mark. An intact piece of testa, large enough to cover the hole at the top of the syringe was sealed in place with a piece of sticky moulding putty. This procedure was carried out several times. A sealed and unsealed syringe were used as controls. The indicator solution turns orange/red when exposed to oxygen and eventually purple when fully oxygenated. Any change in colour in the solution within the syringe was noted as oxygen penetrating the testa.

Antimicrobial activity of seed coat of *A. tataricum*

10 *A. tataricum* seeds were de-pericarped and their testas removed. The testa material was ground in a pestle and mortar and added to 10 ml of distilled water and mixed. 8 mm discs punched from Whatman #1 filter paper were soaked in this extract for 2 hours.

Petri dishes were prepared containing either type 1 agar, more suited to growing fungi, (1% agar + 2% malt extract) or Type 2, more suited to bacterial growth (1% agar, 2% yeast extract). Each plate was inoculated with 1ml of soil extract (10 g garden soil mixed in 100 ml water and allowed to settle). A glass spreader was used to evenly distribute the soil extract across the agar.

6 testa extract soaked filter paper discs were placed on each agar plate and then incubated at 27ºC in the dark. Plates were inspected daily for fungal and bacterial growth.

Investigating the effect of oxygen concentration on seed dormancy

Seeds of apple (*M. domestica* 'Cox's Orange pippin'), *A. tataricum* and pear (*P. communis* 'Conference') were imbibed in distilled water for 4 days and their embryos excised. Whole seeds and embryos were placed on wetted filter paper on Petri dishes. The dishes were then transferred to the gas box. The box was kept in the light at 19ºC for 21 days and observed for germination. After this time the box was opened and exposed to normal atmospheric conditions and observed for a further 14 days for germination.

The gas box was made from a sealable airtight polyethylene box with a volume of 2 L. An inlet valve and an outlet valve were fitted. A Crowcon gasman oxygen meter was calibrated and placed within the box. Once sealed the box could be flushed with Argon to reduce the oxygen concentration to 5% or flushed with pure oxygen to increase it to 50%.

Results

There are a vast number of temperate tree species and each may have a different classification of dormancy. To confirm the dormancy status of several commonly available tree seeds and to select a test species with known baseline dormancy characteristics for further experimentation, germination tests were conducted on whole seeds and excised embryos from a range of species (Table 1)

Table 1. Germination characteristics of common temperate tree species.

20 seeds or embryos per germination test. Results expressed as percentage germination.

Species	**% Germination after 14 days Whole seeds [1]**	**% Germination after 14 days Excised embryos [1]**
Acer campestre (Field Maple)	0	0
Acer ginnala (Amur Maple)	0	0
Acer palmatum (Japanese Maple)	0	50

Acer platanoides (Norway Maple) [2] Lot L Lot S Lot A	 0 0 0	 80 90 0
Acer pseudoplatanus (Sycamore) [3]	0	60
Acer tataricum (Tatar Maple)	0	0
Betula papyrifera (Paper Birch)	90	N/A
Betula pendula (Silver Birch)	95	N/A
Fraxinus excelsior (Common Ash)	0	95
Humulus lupulus (Hops)	0	80
Juglans nigra (Black Walnut)	0	0
Juglans regia (English Walnut)	0	95
Malus domestica (Apple 'Cevaal')	0	50
Malus domestica (Apple 'Cox's Orange pippin')	0	50
Malus domestica (Apple 'Kidds Orange Red')	0	55
Pyrus communis (Pear 'Conference')	0	35
Quercus robur (English Oak)	80	85
Quercus rubra (Red Oak)	100	100
Robinia pseudoacacia (Black Locust)	40	85
Sorbus aucuparia (Rowan)	0	10
Tilia cordata (Small leaved Lime)	0	0

Notes: [1] Seeds were unstratified. [2] Lot S was collected from Staveley, Cumbria. Lot L was collected from Lancaster, Lancashire. Lot A from Angelsey, North Wales. [3] Collected fresh and set out to germinate without drying. N/A Birch seeds were too small to easily excise the embryos.

The results from Table 1. above show that the birch, oak and black locust seeds germinated in their intact state (without having their embryos excised) readily within 14 days at 19ºC. Japanese maple,

pear, apple, rowan, sycamore, hops, ash, English walnut and Norway maple (Lots L&C) did not show any germination while intact. However, all of these species showed some germination once they were removed from their seed coats. Seeds of *A. tataricum, A. campestre, J. nigra, T. cordata, A. ginnala* and *A. platanoides* (Lot A) did not germinate either whole or with embryos excised within the 14 day time frame.

In addition, the effects of different periods of cold stratification were tested on *A. tataricum*, *F. excelsior*, *S. aucuparia, T. cordata, A. platanoides Lot L, A. campestre* and *A. ginnala (Table 2).*

Table 2. Effects of cold stratification on selected temperate region tree seeds[1].

20 seeds per germination test, for 3 replicates. Mean percentage germination shown. Standard errors are reported in parentheses.

Species	**% germination after 1 month stratification**	**% germination after 2 months stratification**	**% germination after 3 months stratification**
A. campestre	0 (0)	0 (0)	0 (0)
A. ginnala	0 (0)	0 (0)	70 (2.89)
A. platanoides Lot A	0 (0)	0 (0)	55 (10.41)
A. tataricum[2]	0 (0)	0 (0)	30 (5)
*A. tataricum (*in sphagnum*)*	0 (0)	0 (0)	30 (0)
F. excelsior	0 (0)	0 (0)	0 (0)
T. cordata	0 (0)	0 (0)	30 (7.64)
S. aucuparia	0 (0)	20 (5)	90 (2.89)

[1]Stratification was in darkness at 4-6ºC. [2]This experiment was repeated for *A. tataricum* but with sphagnum moss instead of paper as the medium surrounding the seeds.

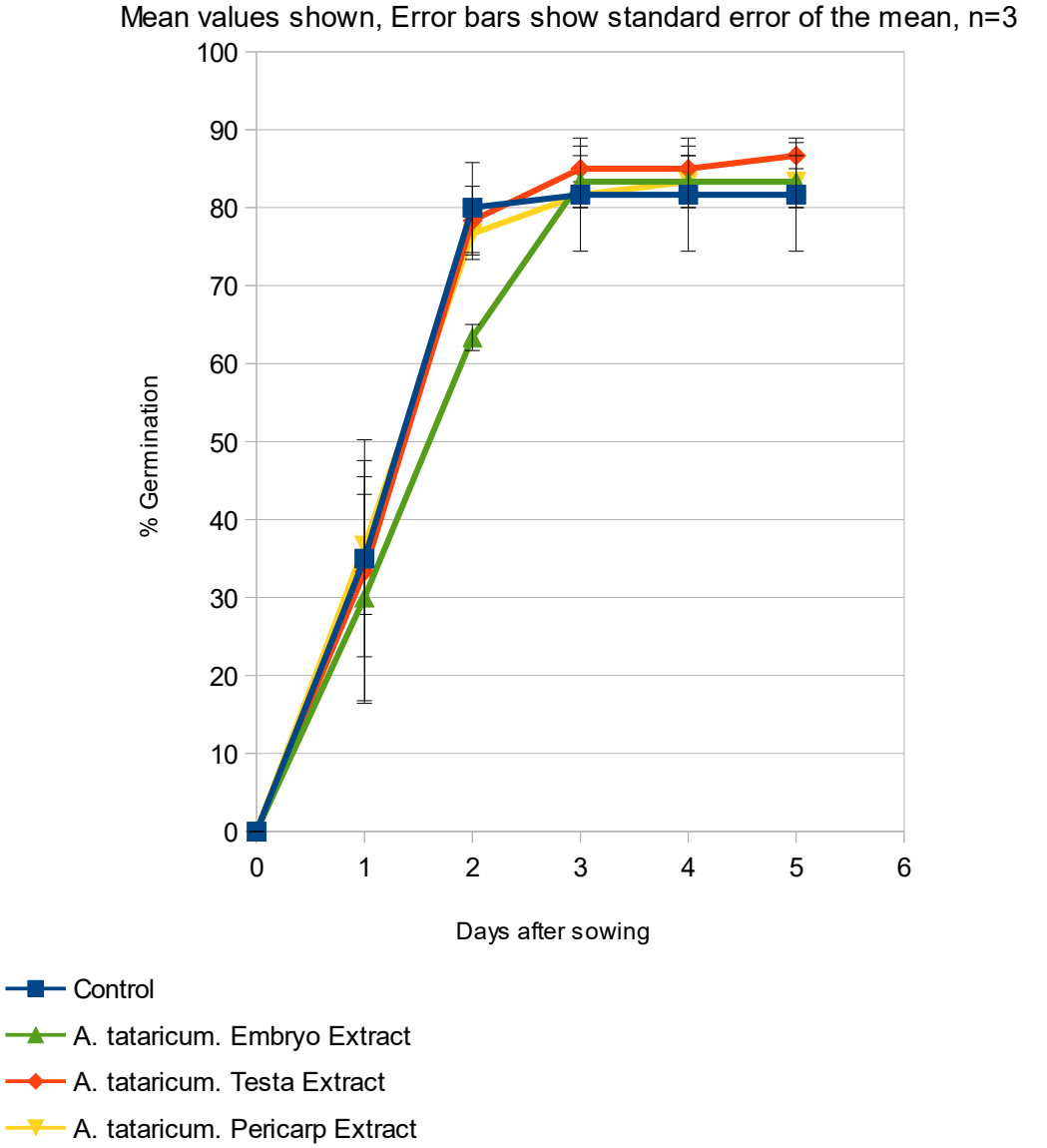
A. campestre, and *F. excelsior* failed to germinate after any of the cold stratification periods tested. The other seeds tested germinated following 3 months of cold treatment. None of the seeds were capable of germination after 1 month cold treatment and only *S. aucuparia* germinated after 2

months of treatment.

It was decided next to investigate if the seeds of *A. tataricum* and *F. excelsior* contained substances that may inhibit germination. Presence of these substances could be a mechanism in maintaining dormancy.

Figure 3 below shows the effect of pericarp, testa and embryo extracts from *A. tataricum* upon the germination of lettuce seeds. Figure 4 shows the effect of *F. excelsior* pericarp and endosperm extracts upon the germination of lettuce seeds.

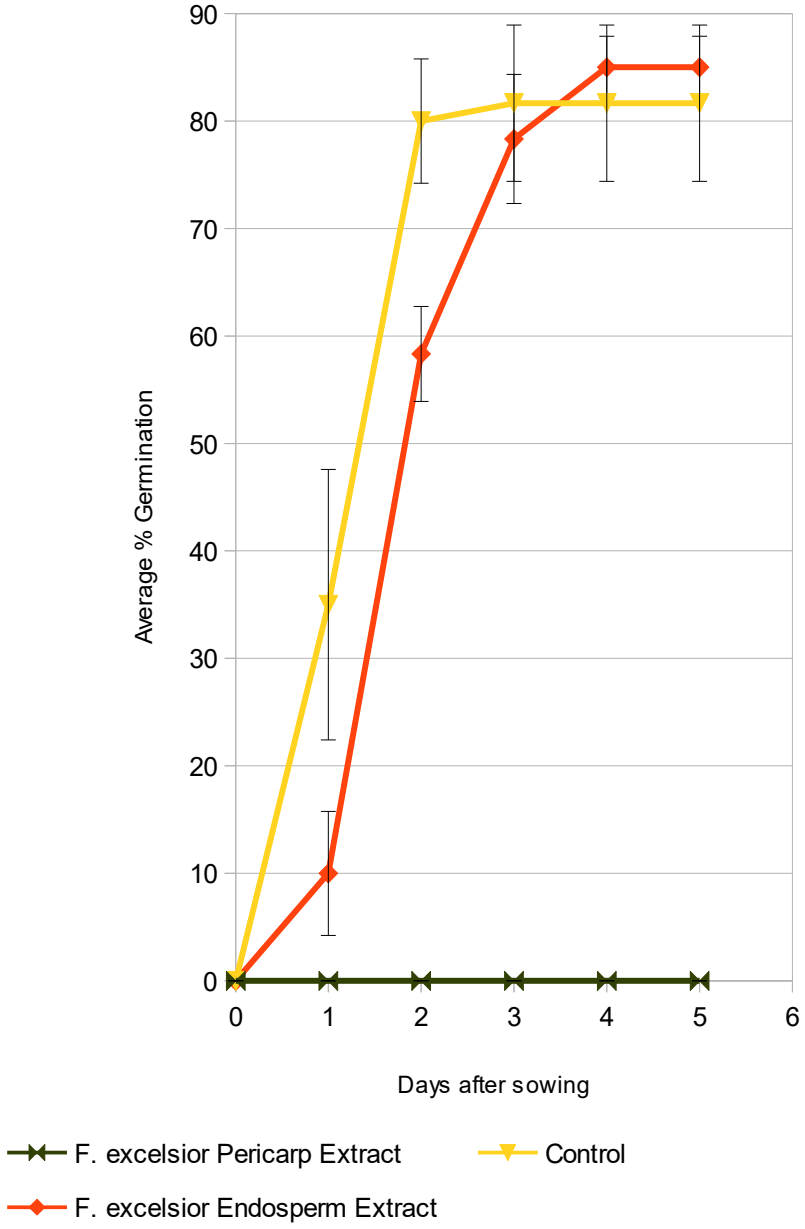
Figure 3. Effect of A. tataricum seed component extracts on lettuce germination



A one way repeated measures ANOVA calculation produced a *P* value of 0.21. This indicates the null hypothesis (that the means of each sample show no statistical difference from each other to the 0.05 confidence level) cannot be rejected and therefore the extracts of *A. tataricum* show no or very little inhibitory effects on lettuce germination.

Figure 4. Effect of F. excelsior seed component extracts on lettuce germination

Mean values shown, Error bars show standard error of the mean, n=3



This indicates that there was no significant inhibitory effect exerted by the *F. excelsior* endosperm extract compared to the control ($P = 0.23$). There was however a dramatic effect exerted by the pericarp extract which completely prevented germination in all lettuce seeds over the 5 days tested.

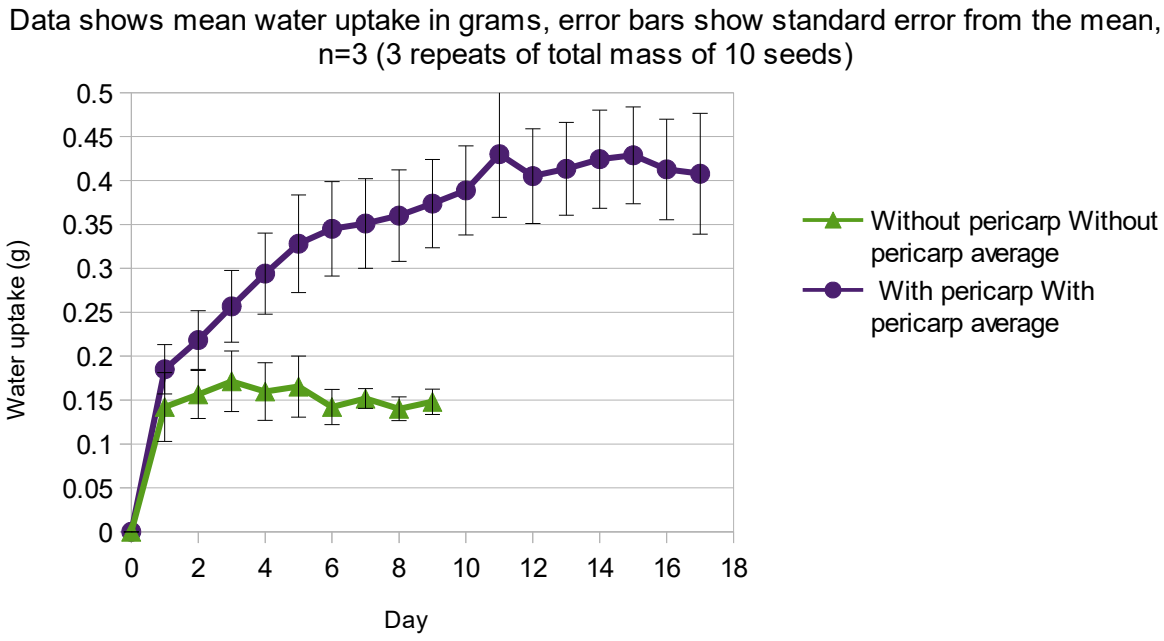
The *F. excelsior* pericarp extract was further tested on excised unstratified *F. excelsior* embryos. Distilled water was used as the control. The control embryos showed 80% germination after 14 days while those subjected to the pericarp extract showed a germination rate of 50%.

The physical properties of the seed coat of *A. tataricum* were next investigated. It was decided to conduct some investigations into the basic properties of the testa of *A. tataricum* to better understand this important seed component and it's links to seed dormancy.

Water uptake

Dry seeds need to first take up water (imbibition) before they can break dormancy and germinate. The seed coat may restrict water uptake prolonging the time requirement to get to this stage (and therefore influencing dormancy periods). An experiment charting the water uptake of seeds of *A. tataricum* with and without testa was conducted. The results are shown in figure 5 below.

Figure 5. Water uptake of dry A. tataricum seeds over time



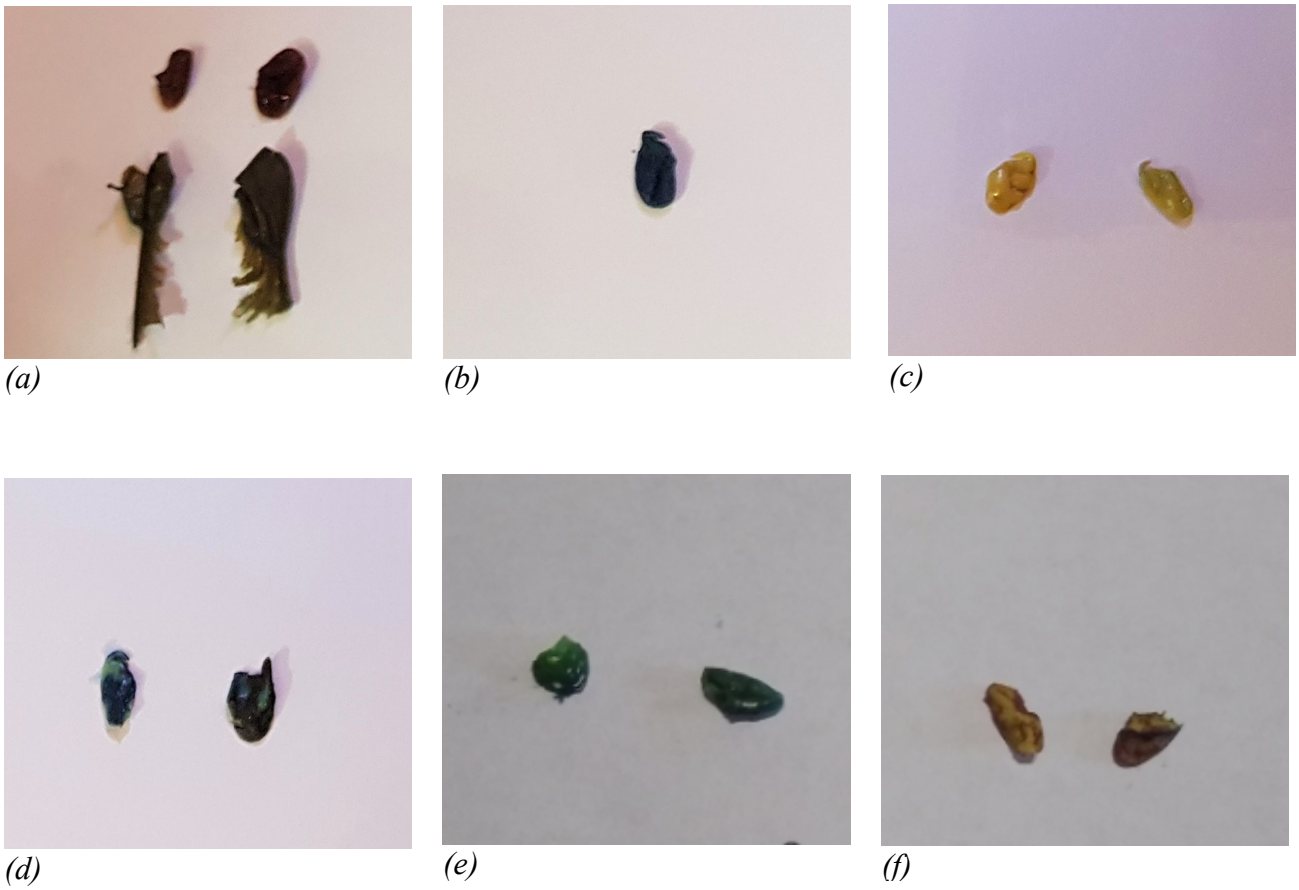
Seeds with pericarps removed showed rapid water uptake in the first day of imbibition. Maximum imbibition was observed after 3 days on average. After this, seeds appeared to reduce in weight. It

was also observed that mould growth started to appear alongside this. For seeds that had an intact pericarp initial uptake of water was similar, but continued to increase over 12 days to a maximum value that was much higher than in the pericarp-free seeds.

Chemical permeability of the testa

The permeability of the testa of *A. tataricum* is thought to be a factor in the passage of chemicals or hormones in or out of the seed. Figure 6 shows the results of treating *A. tataricum* seeds with and without its seed coats with the stain indigo carmine.

Figure 6. Measuring permeability of *A. tataricum* testa with indigo carmine staining.



(a) *(b)* *(c)*

(d) *(e)* *(f)*

Treatment with indigo carmine in water solution (*a*) full seed with pericarp treated and pericarp removed after treatment – indigo carmine did not penetrate through pericarp and did not stain the testa inside. (*b*) naked embryo without testa treated – indigo carmine stained the embryo dark blue. (*c*) fully intact testa treated with testa removed after – picture shows stain did not penetrate the testa. (*d*) partially intact testa treated and remaining testa removed afterwards – stain coloured the embryo less where testa was intact.

Treatment with indigo carmine in 20% DMSO (*e*) seed with testa intact and removed after treatment – stain penetrated through the testa to colour the embryo. (*f*) treatment of whole seed with pericarp intact and pericarp and testa removed afterwards – indigo carmine did not penetrate the pericarp nor the testa.

When the indigo carmine was dissolved in water only the exposed embryo (consisting mainly of the cotyledons) readily took up the indigo carmine stain. In seeds which had an intact testa the testa completely prevented the dye penetrating into the underlying embryo. The pericarp also prevented the stain penetrating to the seed below. Where a seed had it's pericarp removed during staining but

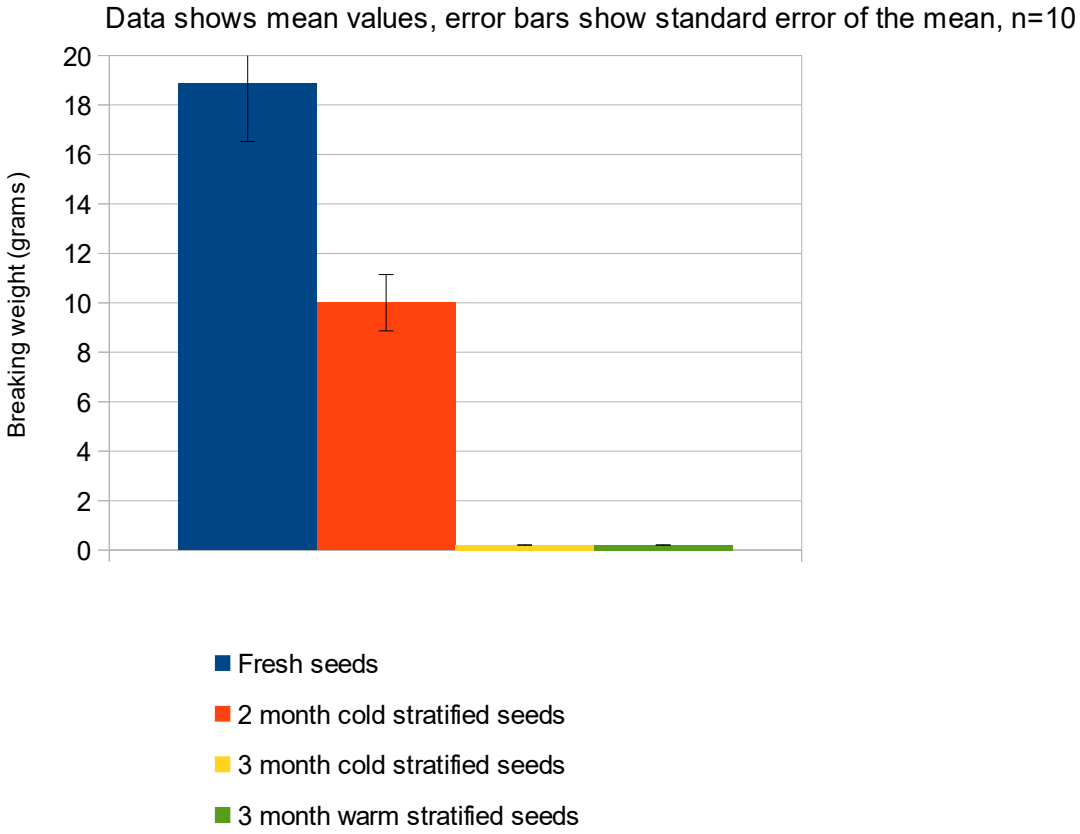
only part of it's testa, it showed a mottling effect. The naked areas on the embryo would be stained but much less so where there was a covering of testa.

When the indigo carmine dye was dissolved in 20% DMSO, exposed embryos again readily took the stain. In addition, the stain penetrated the testa, which it did not do when water was the solvent. The pericarp completely prevented penetration of the dye for both solvents tested.

Tensile strength assays

Some seeds show dormancy when they are intact but are able to germinate when their embryos are excised. Berestetzky et al. (1991) reports *A. tataricum* could only germinate after a dormancy breaking treatment once released from its seed coat. The testa may physically hold back the embryo from germinating. An experiment was conducted in order to test if the action of cold stratification influenced the biomechanical properties of the testa by using the tensile strength assay. Results are shown in Figure 7.

Figure 7. Breaking force of A. tataricum testa after different stratification treatments



A one way repeated measures ANOVA calculation gives a *P* value of 0.00001. This indicates that freshly imbibed seeds showed significantly stronger testae than those seeds that had a period of stratification. Seeds which had been stratified either in warm or cold conditions for over 3 months had such weak and brittle testae that it was impossible to obtain a reading. They would break under the weight of the clamps alone.

Oxygen permeability of the testa

As oxygen availability to the embryo has been thought to influence dormancy in seeds an experiment to indicate the oxygen permeability of the *A. tataricum* testa was developed. Results are shown in Figure 8.

Figure 8. Measuring oxygen permeability of *A. tataricum* testa

The syringe on the left hand side has it's tip sealed with the testa of *A. tataricum* seed. Note the orange plume descending into the indicator solution. This indicates the presence of oxygen permeating through the testa. The syringe in the middle is open to the atmosphere, again note an orange plume showing oxygen ingress. The syringe on the right hand side is fully sealed, note there is no orange colour change indicating no oxygen has penetrated.

After 1 day plumes of orange could be seen developing in the oxygen indicating solutions of the open (positive control) and testa capped syringes. The fully sealed syringe developed no orange colour indicating it was not being exposed to oxygen. Several repeat experiments with different samples of testa were run, and in all cases appeared permeable to oxygen.

Effect of oxygen concentration on seed germination

Côme et al., (1985) and Thévenot et al., (1987) both report that apple embryos exposed to low

oxygen concentrations (hypoxia) germinate more readily than in normal air or at elevated oxygen levels. If low oxygen concentrations can break the dormancy of apple seeds then perhaps there may be a similar effect on *A. tataricum*. The results of an experiment subjecting the apple and *A. tataricum* seeds to hypoxia and hyperoxia are shown in table 3 below.

Table 3. Germination of tree seeds in hypoxia and hyperoxia at 19ºC

20 seeds per germination test repeated twice for a total of 3 tests per species/lot. Mean percentage germination shown. Standard errors are reported in parentheses.

Species	**% germination in hypoxia (5% Oxygen)**	**% germination in normal air (21% Oxygen)**	**% germination in hyperoxia (50% Oxygen)**
A. tataricum - no pericarp	0 (0)	0 (0)	0 (0)
A. tataricum - embryo	0 (0)	0 (0)	0 (0)
Apple - whole seed	0 (0)	0 (0)	0 (0)
Apple - embryo	20 (0)	50 (2.89)	0 (0)
Pear - whole seed	0 (0)	0 (0)	0 (0)
Pear - embryo	10 (0)	35 (5)	0 (0)

Neither a reduction in oxygen concentration or increase promoted germination in *A. tataricum*. Germination did occur in apple and pear embryos in 5% oxygen, although germination in normal air was over twice the level. Only isolated embryos germinated. No embryos or whole seeds germinated during the experiment at 50% oxygen levels.

Antimicrobial activity of the testa

The testa of A. tataricum is thought to protect the embryo beneath from microbial damage. It was of interest to determine if this protection is provided by chemicals exuded by the testa. It may help to know when developing a potential dormancy breaking treatment if the embryo normally requires an

antimicrobial additive.

Incubated agar plates were observed for any zones of inhibition around the testa extract soaked discs. An even spread of mainly fungal species was observed across the type 1 agar plates and the type 2 plates showed again an even spread of bacterial species. No zones of inhibition were observed and in both types of plate microorganisms grew up to and over the discs.

Discussion

When initially tested for germination some tree species tested, such as the Oaks and Birches, showed no seed dormancy. They readily germinated in their intact state without requiring either seed coat removal or a period of cold treatment. These species showed very good germination percentages and grew on to be vigorous seedlings so could be described a being non dormant. Black Locust seeds have a hard shell for a seed coat which physically holds back the radicle from emerging. Once the shell is broken or removed germination percentages increase markedly. These particular seeds could therefore be described as having physical dormancy only.

Both the non dormant and physical only dormant seeds pose little problems for tree growers so were ruled out for further study.

Many of the other seeds tested (Japanese maple, Norway maple, sycamore, hops, rowan, ash, apple, pear and English walnut) showed no germination in their intact state but once their embryos were isolated germination occurred. This would suggest that of these seeds tested their dormancy status is that of either intermediate or non deep physiological dormancy. Interestingly these species are commonly classed as having physiological dormancy which normally requires a period of cold stratification first. Much research has been conducted into tree seed dormancy by Russian researchers from the 1950's to 1980's. Rosbakh et al. (2020) have compiled this research into perhaps the most comprehensive list of temperate tree species seed dormancy studies available.

Listed are the suggested dormancy classifications for thousands of species. However there does appear to be some doubt and variability in the data, especially when differentiating between deep, intermediate and non-deep dormancy traits within a species. This is reflected in the data in Table 1. For example, Nikolaeva suggests English and black walnut (*Juglans regia* and *J. nigra*) both show deep physiological dormancy. Results from Table 1 would indicate that of the seeds tested, English walnut show non-deep or intermediate physiological dormancy due to it's embryos germinating when excised, while black walnut is deep due to it's embryos failing to do so.

The results from Table 1 indicate that *Acer campestre, A. ginnala, A. tataricum, J. nigra* and *T. cordata* could be classed as exhibiting deep physiological dormancy due to their excised embryos being unable to germinate. These seeds require a period of moist chilling to break their innate dormancy and therefore should be able to germinate after a period of cold stratification. In order to confirm this classification the seeds were subjected to cold treatment (Table 2).
These seeds tested are described as having deep dormancy. It was found from the results in Table 1 that *S. aucuparia* (Rowan) could be better described as having intermediate dormancy due to it's ability to germinate without cold stratification (at a low percentage) when excised form it's seed coat. Cold treatment enhanced it's germination percentage (Table 2). Apart from *F. excelsior* (described below) the other seeds tested displayed characteristics of deep physiological dormancy. *A. campestre* (field maple) showed no signs of germination after 3 or even 4 months cold treatment. Either the seeds tested have an extremely deep dormancy or it is possible that they were unviable. The Norway maple seeds (Lot A) successfully germinated after 3 months cold treatment which confirms their viability. It also suggests that dormancy characteristics can vary across the same species as the other types of Norway maple seeds tested from Table 1 above showed shallower dormancy as they readily germinated after seed coat removal.
The seeds of *A. tataricum* showed low germination percentages. When paper towels were used as

the stratification medium, considerable fungal mould formed on the surface of the seeds. Since this may be have reduced germination, and as no mould was present on the other seed species, it was decided to repeat the stratification experiment but with sphagnum moss as the medium instead. Sphagnum moss contains natural anti-fungal chemicals and it is in common use in the tree growing industry for refrigerated stratification of seeds. Little sign of mould was present using the moss, but the germination results were similar to those where paper towels were used.
Interestingly, of the un-germinated *A. tataricum* seeds, germination could be induced by removing the pericarp and the inner seed coat. It wasn't necessary to remove the whole seed coat; just the area covering the radicle. This suggests that physical restraint of the embryo by the seed coat in this species may be a factor in dormancy regulation and was investigated further in relation to the breaking strength of the testa after stratification.

After reference to the data in Rosbakh et al., (2020), it is possible to speculate that the relatively poor germination of the *T. cordata* and *A. tataricum* seeds was due to inadequate chilling. The cold room used for these experiments varied in temperature from 4-6ºC. It is suggested that 0-3ºC is optimal for stratification and that only a few degrees higher can take significantly longer to break dormancy. It can be speculated that the longer time spent at suboptimal temperature may decrease viability in these seeds.

Interestingly, none of the seeds of *F. excelsior* germinated, even after 4 months of cold treatment. The excised embryos were clearly shown to be viable from the earlier germination experiments. The cold treatment was not enough to break the dormancy of the whole seed. Subsequent removal of the embryo after 4 months stratification showed healthy embryos that quickly germinated. This suggests that the endosperm surrounding the embryo restricts the embryo from germinating. *F. excelsior* seeds may require a period of moist warmth before cold stratification in order to weaken

the endosperm. In these experiments no period of initial warmth was allowed for the seeds. This may explain the results obtained.

Seed dormancy can vary according to the local and seasonal conditions that the parent plant was subjected to prior to and during pollination. This finding is consistent with previous research. In *Arabidopsis,* the dormancy of the seed can be affected by nitrate levels fed to the mother plant (Aloresi et al, 2005). It is even possible that the seed itself may regulate it's depth of dormancy according to the season. Again, in *Arabidopsis* seeds, depth of dormancy varied according to soil temperature (Footitt et al. 2011). However, it can't be assumed that findings in *Arabidopsis* apply to tree species tested here. It was also found that herbivore damage in the mother plant could remove the dormancy of the first generation of seeds formed but not in subsequent generations (Singh et al. 2017).

This situation of variation within a species due to differences in the maternal environment may be occurring with the Norway maple samples shown in Tables 1 and 2. Three different samples were collected from different areas of the country. Two of the samples showed germination of isolated embryos. These grew on to produce viable seedlings, suggesting that dormancy is non-deep or intermediate. One sample (labelled Lot A) collected from Anglesey, North Wales, did not germinate with seed coat removal suggesting it has deep dormancy. Norway maple is commonly thought to have deep dormancy requiring around 3 months of cold stratification These results suggest that dormancy within seeds of these species can vary. It is possible that this variation occurs in other tree species too. One explanation is the effect of the maternal environment. Another explanation may be there are a wide spectrum of different varieties within the species, each being similar enough to be classified as the same species but showing minor variation in such characteristics as seed dormancy. Conklin and Sellmer, (2009) found that there is a large variation in germination percentages shown by different varieties of Norway maple under identical growing conditions. This may of course be

due to the location of the parent plants of each of the varieties. Either way, it can be concluded that seed dormancy is not consistent across a species. One sample of seeds may not necessarily exhibit the the same dormancy as another.

Most of the other species tested above showed germination results consistent with what would be expected. Common ash (*F. excelsior*) germinated easily once freed from their endosperm and seed coats. The resulting seedlings grew slowly but eventually formed normal looking saplings. It is suggested that *F. excelsior* seeds show a deep morphophysiological dormancy. This is where the seed has an undeveloped embryo which needs to mature before germination but also needs a period of cold stratification to break an innate dormancy. The results above broadly agree with this classification and suggest that the maturation of the embryo can occur outside of the seed coat and endosperm, and also that the level of physiological dormancy is non deep or intermediate on account of being able to germinate once excised.
As with Norway maple there is evidence that *F. excelsior* seeds originating from different geographical areas show different depths of dormancy. Seeds from colder areas require a longer period of stratification than those from warmer climates (Fărcaş, 2000).

Once the basic germination and dormancy status for several species of seed had been established, *A. tataricum* was chosen as a test seed (and to a lesser extent *F. excelsior*) for further investigation. A range of experiments were designed to explore the physical and chemical properties of the seed.

Inhibitory properties of seed component extracts from *A. tataricum* and *F. excelsior*
One of the possible factors in dormancy regulation in seeds is the idea of inhibitory substances being present which may suppress germination. The most studied of inhibitory plant substance is abscisic acid. It is known that ABA can be present in different quantities in different tissues of the seed (Sondheimer, 1968).

Extracts of *A. tataricum* appeared to show no significant inhibitory effect on lettuce seed germination. All lettuce seeds tested reached 80%+ germination after a few days. This would suggest that (upon lettuce at least) there are no or few/weak inhibitory substances within the recently imbibed *A. tataricum* seed.

An endosperm extract from *F. excelsior* also showed insignificant inhibition of lettuce germination. However, the pericarp extract showed an extremely strong inhibitory effect. No seeds would germinate in its presence. This would suggest that there are substances inhibitory to germination within the pericarp tissues of Common ash seeds. One obvious candidate would be ABA, although this was not tested for specifically.

As ash seeds in their natural form include the pericarp and do not normally separate before germination, it was decided to test the inhibitory effect of the pericarp extract on excised ash embryos. The pericarp extract showed a clear inhibitory effect on the embryos, albeit much less severe than the total halt to germination it caused in lettuce seeds. It is possible that the pericarp contains substances such as abscisic acid (Sondheimer, 1968).

The inhibitory properties exhibited by the pericarp may be a factor in the regulation of dormancy in the seed. It is possible the inhibitory substances may slowly leach out during winter exposure to soil and rain. The seed would then have a lower level of inhibition to germination in springtime when it is safer for it to develop into a seedling. It is also a possibility that this observed effect of suppressing other plant's germination is an evolutionary survival method. The pericarp may leach out inhibitory substances into the surrounding soil suppressing the germination of competing trees species. This would be a classic case of alleopathy, where an organism produces biochemicals

which influences either in a positive or negative way other organisms in it's surroundings (Friedman and Waller, 1983).

Physical properties of the seed coat of *A. tataricum*

A. tataricum seeds are covered with an external pericarp. Removal of the pericarp exposes the seed. The seed is covered in a thin seed coat called the testa. After imbibition of the seed the testa can be scraped free from the underlying embryo, sometime coming off in large pieces. It has been observed in many species of seed that removal of the seed coat of a seed can allow germination to proceed particularly species showing non deep and intermediate physiological dormancy. It was therefore hypothesised that the seed coat is a factor in dormancy.

The range of experiments in this chapter allow us to understand more fully the properties of the *A. tataricum* seed coats. Before the seed can proceed to germination it must first imbibe, by taking up water. This corresponds with phase one of germination where seeds reach a plateau of hydration. It appears that both testa and pericarp readily permit water passage through them however the pericarp is less permeable to water than the testa alone (Figure 6). Imbibition tests show that a seed with intact pericarp reaches it's maximum water uptake after 11 days. A seed without pericarp (but with testa) reaches a maximum water uptake after only 3 days. Unfortunately it was physically impossible to remove the testa from a dry seed in order measure water uptake of a naked embryo so there is no data showing how much water a naked embryo might take up from a dry state. It can be seen from the data that the pericarp intact seeds take up more water in total suggesting the pericarp absorbs significant amounts of water as well as the seed. It is possible that the seed within the pericarp becomes fully imbibed after 3 days also and that further water absorption is simply due to the pericarp. A further experiment to test this would be to remove and weigh the pericarps separately to the seed each day and plot the results.

From the data obtained, other than the 9 day difference in achieving maximum hydration between intact and de-pericarped seeds, these tests suggest that water restriction by the seed coats is not a major factor in maintaining seed dormancy. The seed coats appear to be freely permeable to water. When the seed reaches a plateau of hydration it enters phase two of germination. The seed will remain in the phase until such a time as dormancy is broken. This is useful when considering potential dormancy breaking treatments. It may be the case that dormancy can only be broken when the seed has achieved initial hydration and that the time allowed for imbibition before treatment may be important.

Chemical permeability of the seed coat

The seed coats of *A. tataricum* may be permeable to water, but it is important to know how permeable they may be to other substances. This may help us understand if substances can pass out of the seed (such as endogenous inhibitory substances) or if we are interested in passing substances into the seed (such as chemical or hormone treatments to break dormancy).

Indigo carmine is a chemical dye that readily stains *A. tataricum* embryos a blue/violet colour. The seed coats (pericarp and testa) prevented its passage to the embryo when the dye was dissolved in water. This is an interesting result. Indigo carmine is a relatively small molecule with a molecular weight of 466.4 g/mol. For comparison abscisic acid is 264.3 g/mol. This barrier to substances presumably works the same way for substances wishing to exit the seed. If inhibitory substances need to be leached from the seed before germination it could be speculated that they need to be of a small size. There is also the need to consider factors such as solubility and polarity. This barrier to external substances by the seed coat may be a consideration when treating seeds with potential chemical or hormone treatments for future experiments.

Perhaps more interesting and relevant to introducing exogenous chemicals and plant hormones into

the seed for potential dormancy breaking treatments is the use of DMSO. The indigo carmine dye when dissolved in 20% DMSO readily penetrated the testa of *A. tataricum* where it would not when dissolved in water only. DMSO is well known as a membrane permeability enhancer although it's exact mechanism is unknown. It is thought that it may displace the water molecules at the surface of the membrane therefore changing the equilibrium across the membrane and thus enhancing diffusion into the seed/cell (Cheng, 2015). This ability for DMSO to shuttle other chemicals through the seed coat is utilised later in chapter 4.

Oxygen permeability of the testa of *A. tataricum*

The oxygen permeability experiment illustrated in Fig. 7 suggests that the testa of *A. tataricum* is freely permeable to gaseous oxygen. It was initially a consideration that the seed coats may restrict diffusion of oxygen to the embryo and that this lack of oxygen maintained dormancy. The presumption being that oxygen is necessary for germination to occur. Due to the simple nature of the experiment, a quantitative measure could not be obtained. Nevertheless, it does show that despite the testa regulating germination to some extent, it does not limit oxygen diffusion to the embryo. It is therefore unlikely that the oxygen permeability of the testa is a significant factor in dormancy maintenance in *A. tataricum*.

It was observed during germination tests that *A. tataricum* seeds that had been stratified and had failed to germinate could be induced to germinate if they had their testas removed. Only the area of the testa that covered the radicle needed to be removed. In many seeds, it was observed that after 2 months stratification, seeds with intact testae (but without pericarps) did not germinate. However, when these seeds had all or part of their testa removed the radicle quickly elongated and germination proceeded. The experiments conducted here (Fig. 6) show that over time the strength of the testa diminishes and that this is not dependent on the temperature the seed has been kept at. A weak testa allows the radicle to emerge. This suggests that some form of physical restraint is present

in seed dormancy. However, it cannot be the main mechanism of dormancy, as seeds stratified in warm conditions over 3 months, despite having weakened testae, do not germinate. A period of cold is still required for germination. It can be supposed that the physical restraint is a minor additional form of dormancy which extends the time required for germination. Perhaps the period of cold required is much less than commonly thought, and the physical restraint (time taken for the testa to fully degrade) contributes to the dormancy breaking period. An experiment where naked embryos were cold stratified alongside intact seeds may show this more clearly (if the naked embryos did not degrade before germination). Another explanation could have been that removal of the testa allows more oxygen to the embryo which is a driver for germination. However, as the testa appears to be permeable to oxygen, the physical restraint theory is the more likely explanation.

The testa of *A. tataricum* showed little to no antimicrobial activity. It was observed that intact seeds often when placed out for germination would be susceptible to a covering of fungal contamination. However, where the seeds had an intact testa, the underlying embryo would remain firm with no signs of mould in the embryo. Exposed embryos were very susceptible to fungal damage and would decompose quickly if contaminated. This would suggest that the testa of *A. tataricum* acts as a physical barrier to microbial contamination but doesn't contain any obvious chemical antimicrobial properties. This is an important consideration when developing a dormancy breaking treatment. If the treatment requires a naked embryo (perhaps because it cannot penetrate the seed coat) the incidence of fungal damage will be higher without an intact testa, reducing the effectiveness of the treatment. If the testa could be retained during a treatment, this may protect the seeds long enough to improve germination percentage.

Acer ginnala (Amur Maple) is closely related to *A. tataricum*. Seeds appear identical to *A. tataricum* except that they are smaller. The resulting trees are similar also except for leaf formation.

A. ginnala has glossy lobed leaves while *A. tataricum* has matt and mostly unlobed leaves. Many botanists class *A. ginnala* as a subspecies of *A. tataricum.* Dumbroff and Webb, (1970) found that in *A. ginnala* removal of the pericarp halved the time taken for stratification. They added an extract from the pericarp to the stratifying seeds and found it to have no effect on the stratification time. They also suggested that oxygen was not a limiting factor in germination. These results correlate with the findings here for *A. tataricum*. Dumbroff also reports similar conclusions for *A. saccharum* (Webb and Dumbroff, 1969). However it should be noted that *A. saccharum* exhibits a less deep dormancy and responds well to gibberellic acid treatment.

Vordtriede et al. (2012) studied some of the properties of *A. ginnala*. They conducted inhibition studies of a testa extract and as in the above studies on *A. ginnala* and *A. saccharum*, found no significant inhibitory properties from the testa. This is in line with the experimental results for *A. tataricum* presented here.

They also found that removal of the testa allowed rapid germination without stratification. This is at odds with the results from the germination experiments here which showed *A. ginnala* (along with *A. tataricum*) to require a period of cold stratification before germination, and testa removal alone did not break dormancy in either species. This may be, as described above, that dormancy can vary within a species possibly influenced by environmental conditions affecting the mother plant.

Oxygen concentration and germination

Varying the oxygen concentration had no effect on *A. tataricum* with no germination shown in any of the experiments (Table 3). This suggests that oxygen levels are not the main driver in breaking dormancy in this species. Neither apple nor pear, which were also tested, showed any germination at elevated oxygen conditions suggesting that either this concentration of oxygen is toxic, inhibitory to germination or promotes dormancy.

In apple and pear, conditions of hypoxia lowered the percentage of germination in exposed embryos compared to normal air. This is at odds with the results of Côme et al. (1985) and Thévenot et al. (1987) who found that 5% oxygen increased germination percentages. They used a different variety of apple (Golden delicious) collected from a different geographic area (northern France as opposed to northern England) which may be a factor in the varying results. The experimental procedure is also different. In this study argon (an inert gas) was used to flush out the air in the gas box to reduce the oxygen levels. Côme and Thévenot used a system of mixing nitrogen and oxygen to fill their germination chambers. This meant that they had a germination environment containing 5% oxygen and 95% nitrogen. In this study the conditions would have also been 5% oxygen but with the remaining gas being mostly argon. It could be speculated that their method by way of contamination in the nitrogen source or reaction within the chamber may have increased levels of reactive forms of nitrogen. Reactive nitrogen species (in particular nitric oxide) have been implicated in breaking dormancy in apple seeds (Gniazdowska et al. 2010) . It is possible that rather than the lowered oxygen concentration being the factor in breaking the dormancy of apple seeds in the above studies it is actually the increased level of reactive nitrogen that may be the active factor. The effect of nitric oxide on the dormancy of *A. tataricum* along with a range of other chemicals are investigated in chapter 4.

Chapter 4

Identifying and investigating chemical and hormone treatments to break seed dormancy in temperate region tree species.

Various treatments for breaking dormancy have been reported over the years in many different species of plant. It was decided to investigate the effectiveness of some of the reports of successful chemical treatments on *A. tataricum* with the aim of narrowing down a mechanism of dormancy regulation.

A search of the literature brings up many chemicals reported to promote germination in full or partially in dormant seeds. Calcium hydroxide (Jennings and Tulloch, 1965). Calcium hypochlorite (Okonkwo and Nwoke, 1975; Miyoshi and Mii, 1998). Hydrogen peroxide in apple seeds (Bogatek et al. 2003). Nitric oxide (both in gaseous form and in solution via sodiumnitroprusside) and cyanide again in apple seeds (Gniazdowska et al. 2010). Ethanol (Taylorson and Hendricks, 1979) and Thiourea (Çetinbaş and Koyuncu, 2006). All are potential candidates for dormancy breaking treatments.

Ranjan and Lewak, (1992) suggest that in some cases light may have an effect upon seed germination in apple seeds.

Based on the fact that *A. tataricum* is a non-endospermic seed and stores it's metabolic energy as lipids it could be speculated that a treatment which replicates or stimulates the lipid breakdown may assist with germination. Linolenic acid is the main seed lipid in *A. tataricum* (Codreanu et al. 2007). A treatment with this acid or a lipase enzyme may break dormancy.

It was found in chapter 3 that *A. tataricum* had little or no inhibitory properties on the germination of lettuce seeds however a simple leaching experiment may remove any inhibitory substances present which may be specific to *A. tataricum* itself. The phenolic content of the testa may have an effect on dormancy and germination. Polyvinylpyrrolidone (PVP) absorbs polyphenols

(Gray, 1978), and a treatment with this may indicate if the phenolic content of the seed testa in *A. tataricum* has an influence on dormancy.

Plant hormones have important signalling roles on seeds. It has already been described in the previous chapters that seeds exhibiting anything less than deep physiological dormancy may respond positively to gibberellic acid treatment. *A. tataricum* seeds are classed as having deep physiological dormancy and it would not be expected that they would respond to GA alone.
As outlined in the introduction it was previously reported that a 2 day treatment of 500 mg/l jasmonic acid could completely substitute for cold stratification in these seeds (Berestetzky, 1991; Babenko, 1996). It was decided to try to recreate these results in order to provide a base from which to fully understand how seed dormancy may be regulated in *A. tataricum* and by extension, other deeply dormant tree seeds.
It was also decided to test the effects of fluridone. Although not a hormone, fluridone is a chemical that has been reported to break dormancy in certain tree seeds which otherwise need a period of cold stratification (Feurtado et al. 2007; Worarad et al. 2017).
Fluridone (1-methyl-3-phenyl-5-(3-tri-fluoromethyl-(phenyl))-4-(1*H*)-pyridinone), blocks the phytoene desaturase enzyme during synthesis of carotenoids in plant cells. Since ABA is synthesised from carotenoids, fluridone inhibits ABA biosynthesis within plant tissues (Bartels and Watson, 1978). As ABA is thought to be a major factor in the maintenance of seed dormancy, fluridone may have an effect on *A. tataricum*.

Materials and methods

Seeds were imbibed before treatment by soaking them in distilled water for 12 days. Pericarps if present were then removed. For most treatments, seeds were soaked in the relevant chemical or hormone solution for a specific amount of time as show below in Tables 4 and 5. Once treated,

seeds were either retained intact or had their embryos excised. They were then placed on moistened filter paper in 90 mm Petri dishes and kept in natural light at 19ºC. For the darkness experiment, the dishes were wrapped in two layers of aluminium foil.

One experiment tested involved leaching of the seeds. This involved frequent (every 3 hours during daylight hours) changes of the distilled water the seeds were soaking in.

Experiments involving nitric oxide were conducted by placing the seeds in a small wire basket within a sealable test tube. The basket could be positioned halfway up the test tube. 1 g of copper filings were placed in the base of the tube and 1 ml of 0.1 M nitric acid was added. The test tube was sealed and the seeds were exposed to the resulting nitric oxide for 3 hours before removal.

For the lipase treatment, seeds were exposed to a commercial lipase enzyme dissolved in distilled water (0.825 mg/ml). For the experiment with ethanol, 0.5 mM ethanol was used as the solution to moisten the filter paper during incubation.

Seeds were checked for germination daily for 20 days.

Results

A range of chemical treatments identified as exhibiting potential seed dormancy breaking properties as described in the introduction to chapter 4 (but also including darkness and a leaching treatment) were conducted on various tree seed species. The results are shown below in table 4.

Table 4. Effect of various chemical treatments on seed dormancy.

20 seeds per germination test repeated twice for a total of 3 tests per species/lot. Mean percentage germination shown. Standard errors are reported in parentheses.

Species tested	Treatment	% Germination whole seed	% Germination excised embryos
A. tataricum	2 d 0.8% sodium nitroprusside 1 d 0.8% sodium nitroprusside 3 hr 0.15% sodium nitroprusside	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)
M. domestica 'Cox's Orange pippin'	2 d 0.8% sodium nitroprusside	0 (0)	10 (5)
A. tataricum	2 d linolenic acid (50%)	0 (0)	0 (0)
A. tataricum	1% polyvinylpyrrolidone	0 (0)	0 (0)
A. platanoides Lot L	Darkness	0 (0)	0 (0)
A. platanoides Lot L	Light	0 (0)	80 (4.41)
A. platanoides Lot A	Darkness	0 (0)	0 (0)
A. platanoides Lot A	Light	0 (0)	0 (0)
A. tataricum	Darkness	0 (0)	0 (0)
A. tataricum	Light	0 (0)	0 (0)
A. tataricum	0.5 mM ethanol	0 (0)	0 (0)
A. tataricum	2 d 1% calcium hypochlorite	0 (0)	0 (0)
F. excelsior	2 d 1% calcium hypochlorite	0 (0)	0 (0)
A. tataricum	2 d calcium hydroxide (80mg/l)	0 (0)	0 (0)
A. tataricum	2 d 1% hydrogen peroxide	0 (0)	0 (0)
A. tataricum	10 d leaching	0 (0)	0 (0)
A. tataricum	3 hrs nitric oxide exposure	0 (0)	0 (0)
A. ginnala	3 hrs nitric oxide exposure	0 (0)	0 (0)
A. tataricum	2 d potassium hydroxide (250mg/l)	0 (0)	0 (0)
A. tataricum	2 d 1% polyethylene glycol	0 (0)	0 (0)
A. tataricum	2 d Lipase solution	0 (0)	0 (0)
A. tataricum	1 d 1% thiourea	0 (0)	0 (0)
A. tataricum	2 d 1% thiourea	0 (0)	0 (0)

Most of the chemical treatments showed no effect upon the seeds tested. Thiourea or nitric oxide exposure at the concentrations tested here showed no effect on seed germination. There was a low level of germination observed in apple seed embryos exposed to sodium nitroprusside. There was a strong response to light in exposed *A. platanoides* embryos which showed 80% germination as opposed no germination in those seeds of the same type incubated in darkness.

The results for a variety of hormone treatments are shown below in Table 5. Treatments of water only and 20% DMSO solution only served as controls. Hormone treatments included jasmonic acid, methyl jasmonate, kinetin, 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (6 BAP) and thidiazuron (TDZ). Non hormones thiourea and fluridone were also tested in conjunction with hormones.

Table 5. Germination of *Acer* seeds after various hormone treatments.

20 seeds per germination test repeated twice for a total of 3 tests per species/lot. Mean percentage germination shown. Standard errors are reported in parentheses.

Species Tested	**Treatment**	**% Germination Whole Seed treated (with subsequent testa removal)**	**% Germination Exposed Embryo treated**
A. tataricum	2 d distilled water	0 (0)	0 (0)
A. platanoides Lot A	2 d distilled water	0 (0)	0 (0)
A. tataricum	2 d 20% DMSO	0 (0)	0 (0)
A. tataricum	2 d jasmonic acid (50, 125, 250, 500 & 1000 mg/l)	0 (0)	0 (0)
A. tataricum	2 d jasmonic acid (125, 250, 500 mg/l) In darkness	0 (0)	0 (0)
A. tataricum	2 d methyl jasmonate (125, 250, 500 &1000	0 (0)	0 (0)

	mg/l)		
A. tataricum	2 d methyl jasmonate (125, 250, 500 mg/l) In darkness	0 (0)	0 (0)
A. tataricum	250 mg/l jasmonic acid	0 (0)	0 (0)
A. tataricum	2 d 250 mg/l jasmonic acid + 2 d 10 mg/l NAA	0 (0)	0 (0)
A. tataricum	2 d 250 mg/l methyl jasmonate + 2 d 10 mg/l NAA	0 (0)	0 (0)
A. tataricum	5 & 6 d 250 mg/l jasmonic acid	0 (0)	0 (0)
A. platanoides Lot A	2 d 250 mg/l jasmonic acid	0 (0)	30 (9.28)
A. tataricum	2 wks 250 mg/l jasmonic acid @4ºC	0 (0)	0 (0)
A. tataricum	2 d & 5 d 500 mg/l jasmonic acid @ pH5	0 (0)	0 (0)
A. tataricum	2 d kinetin (50, 100 & 200 mg/l)	0 (0)	0 (0)
A. tataricum	2 d gibberellic acid, GA3 (50, 250, 500 &1000 mg/l)	0 (0)	0 (0)
A. tataricum	2 d jasmonic acid (250 mg/l) + kinetin (50 mg/)	0 (0)	0 (0)
A. tataricum	2 d jasmonic acid 250 mg/l in 20% DMSO)	0 (0)	0 (0)
A. tataricum	2 d methyl jasmonate 250 mg/l in 20% DMSO)	0 (0)	0 (0)
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO)	30 (7.64)	0 (0)
A. tataricum	2 d kinetin (500 mg/l in 20% DMSO)	20 (2.89)	0 (0)
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO) followed by 90 mins 1% thiourea	0 (0)	NT
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO) followed by 1d 1% thiourea	0 (0)	NT
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO) followed by 20 mins 1% thiourea	25 (7.64)	NT
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO) + 100 uM Fluridone	0 (0)	NT
A. tataricum	2 d kinetin (100 mg/l in 20% DMSO) + 50 mg/l NAA (1-naphthaleneacetic acid)	0 (0)	NT
A. tataricum	2 d thidiazuron (100 mg/l in 20% DMSO)	0 (0)	NT
A. tataricum	2 d 6-benzylaminopurine (200 mg/l in 20% DMSO)	25 (0)	NT
A. tataricum	1 d 100 uM fluridone in 20% DMSO	0 (0)	0 (0)

NT= not tested.

Jasmonates either in the form of jasmonic acid and methyl jasmonate had no effect on the *A.*

tataricum seeds tested. This was surprising considering the positive results reported by previous researchers. Considerable time was spent investigating this result. Solubility of the jasmonates was investigated, treatments were independently made up with water, ethanol and DMSO as the initial stock solution solvent. Several different concentrations were tested on seeds both in the light and the dark. It was feared the jasmonate supplies being used were not biologically active, so tests were carried out on wheat seeds (where jasmonates are reported to suppress germination). The wheat tests showed that indeed both types of jasmonate suppressed germination percentage with increasing concentration as expected. It was concluded that the jasmonate was entering the seed however it just had no effect. Suspecting that seeds from different areas may have different responses other seeds of *A. tataricum* were obtained. Batches from Poland, Russia, Italy and USA were tested also, however these also showed no response to the jasmonate. Attempts to contact one of the still active original researchers listed above were unsuccessful. Speculation as to this discrepancy between the results here and those reported follow in the discussion below.

However jasmonic acid treatment did break the dormancy in *A. platanoides* (Lot A) compared with no germination when water only was used as the treatment. Unfortunately, due to limited seed numbers available this effect couldn't be investigated with further experiments such as treatment with kinetin.

Kinetin initially did not induce germination initially when the treatment solvent consisted of water. However, after learning in earlier in chapter 3 of this study that DMSO enhanced permeability of the seed coat (and presumably the seed tissues) kinetin treatment was tried again in a 20% DMSO solution. This did result in germination of 20-30% which is similar to that obtained after 3 months cold stratification as shown in chapter 3 Table 2. Of other cytokinins tested, 6 BAP also induced germination but TDZ did not. Germinated seeds were grown on where they appeared to form

healthy specimens in the time observed.

Thiourea (at a one day and 90 minute treatment times) and fluridone both appeared to suppress germination when used alongside or following kinetin treatment. Also addition of auxin in the form of NAA negated the dormancy breaking action of kinetin. One way ANOVA calculations give *P* values of 0.0001 compared to kinetin treatment alone (500 mg/l in 20% DMSO). This is below the standard scientific 0.05 confidence level and implies that fluridone, thiourea (at the treatment times tested) and NAA have a significant negative effect on the ability of kinetin to break dormancy in *A. tataricum* seeds. However thiourea at a 20 minute treatment time (post kinetin treatment) did not suppress germination. This suggests thiourea is time or/and dose dependent in it's inhibitory effects.

It became clear that when excised embryos were treated rather than when the testa was intact, that they were susceptible to degradation before they had time to germinate. The embryos were very prone to mould growth. Treatment with hydrogen peroxide before or after treatment showed no improvement. It was observed that the most successful method of successfully germinating the seeds of *A. tataricum* (with cytokinins) was to perform the treatment on the seed with an intact testa then to place it into the Petri dish for several days before removing the seed coat. This appeared to protect the embryo from decay presumably while it started it's biological change from dormancy to germination.

The results in Table 5 indicate that kinetin and 6BAP can break the dormancy of *A. tataricum* seed. The ultimate aim of this study is to develop a treatment that will break the dormancy in a range of temperate region tree species. A key question is whether this effect can be replicated in other tree species. Building on this, a 350 mg/l (for 2 days) kinetin treatment was tested as per the methods above on a selection of other available tree species seeds that are commonly thought to exhibit deep

physiological dormancy. These results are shown in table 6.

Table 6. Responses to seed dormancy breaking treatment (350 mg/l kinetin in 20% DMSO) in other tree species. 20 seeds per germination test repeated twice for a total of 3 tests per species/lot. (pericarps were removed before treatment and testa removed post treatment). Mean percentage germination shown. Standard errors are reported in parentheses.

Species	2d 350 mg/l kinetin	Control (water only)
Acer dissectum	0 (0)	0 (0)
Acer pseudoplatanus	90 (2.89)	70 (5.77)
Acer platanoides (crimson king)	50 (5)	0 (0)
Tilia cordata	40 (5)	0 (0)
Taxus baccata	0 (0)	0 (0)

The treatment did not break the dormancy of *A. dissectum* nor *T. baccata.* It did promote germination in *A. platanoides* (crimson king) and *T. cordata* as opposed to the control which showed no germination. The treatment also increased the germination percentage of *A. pseudoplatanus* from 70% to 90%.

Discussion

The results of various chemical treatments (Table 4) show that in these experiments they were not capable of breaking the seed dormancy in *A. tataricum* or (where tested) *A. ginnala*. This is despite reported success in the literature for other species. This may be due to differing depths of dormancy between species and/or each species responds differently to particular chemical treatments
Apple seeds treated with sodium nitroprusside showed a very low level of germination (10%) when they subsequently had their embryos exposed. This is broadly in line with what Gniazdowska et al. (2010) found. However the initial experiments in chapter 3 Table 1 show that simple embryo

excision alone results in 50% germination in these seeds. This would suggest that at the concentration tested here (0.8% sodium nitroprusside) it was inhibitory to germination, at least for the variety of apple seed used (Cox's orange pippin). It is possible that lower concentrations or a shorter treatment time may result in increased germination for this species.
Neither sodium nitroprusside nor gaseous nitric oxide treatments showed any effect on *A. tataricum* or *A. ginnala* despite reported success in apple seeds. This, alongside the fact that apple seeds appear to have significant germination with simple embryo excision alone, suggest that the two *Acer* species tested have significantly deeper levels of dormancy than apple.

It was interesting that light had a positive effect on germination of *A. platanoides*. Ranjan and Lewak, (1992), suggest that there is a light activated mechanism in apple embryos which contributes to dormancy release and that this mechanism is separate to that which regulates the jasmonic acid response found in these seeds.
The stimulation of *A. platanoides* found here may follow a similar pathway to that described by Ranjan and Lewak, (1992). However, it was only observed in one sample of seeds collected from one area (Lot L). Another sample *A. platanoides* seeds (Lot A) collected from different geographic areas didn't show this response to light. An explanation may be that there is great variation in the depth of dormancy within this species from area to area. The Lot L seeds may be more sensitive to light induced dormancy release than that of Lot A. However this light regulated pathway is not sufficient to break dormancy in seeds of *A. tataricum*, our deeply dormant test species.

One of the main reasons that *A. tataricum* was chosen as a test seed species was the previous suggestion that jasmonic acid could entirely replace the need for cold stratification in these seeds (Berestetzky, 1991; Babenko, 1996). This would have then been a starting point for development of a dormancy breaking treatment and perhaps provide some insight into the biochemical mechanism

of cold dormancy. Unfortunately the results of of these researchers could not be successfully replicated here. There are some possibilities of why this might be so. As suggested in chapter 3, seeds appear to vary in their dormancy depths depending on which geographic area they are gathered from. Perhaps levels of dormancy might even vary from year to year depending on environmental conditions the mother plant was subjected to. It is a possibility that previous researchers worked with seeds that exhibited a shallower dormancy than those tested here. The researchers may have used freshly gathered seed for their tests (it is not mentioned in the studies) and this could have had a bearing on the results (tests here were performed on dried and stored seed). Their germination percentages when broken by hormone treatment or cold stratification were very high compared to result here (80%+ as opposed to 30%) and this may be because their seed samples were fresher and more viable to start with. A fresher seed may be more sensitive to exogenous hormones such as jasmonic acid. An experiment testing a sample of seeds stored for different lengths of time would be necessary to investigate this hypothesis.

Jasmonic acid did, however, effect dormancy in the Lot A *A. platanoides* when tested. This would indicate that in this particular variety, jasmonic acid does have a positive effect upon breaking dormancy. While *A. platanoides* Lot A shows deep physiological dormancy other varieties within the species exhibit intermediate dormancy. *A. platanoides* Lot A variety while being classed as having deep physiological dormancy, may not be quite as deep as that of *A. tataricum* and *A. ginnala*. It may be possible that jasmonates at the levels tested here do have an effect on all but the deepest physiologically dormant seeds.

This raises the question whether jasmonic acid is a significant player in natural innate dormancy release after cold stratification or whether it's exogenous application triggers some other mechanism.

Cold stratification is the predominant technique for breaking seed dormancy. The act of moist

chilling at temperatures between 0-5ºC causes some (as yet unknown) physiological change(s) within the cellular machinery of the seed that does not occur at higher temperatures. The presence (or lack of) certain plant hormones almost certainly has an influence on breaking dormancy and we know that many hormones are involved in germination. The question is what is it about the cold that may activate/deactivate them?

Babenko et al. (1996) observed that translation and transcription activity increased steadily within the embryo of *A. tataricum* during cold stratification increasing almost linearly over 120 days. They found that the same level of genetic activity occurred with 2 days treatment of jasmonic acid. There was minimal increase in activity with stratified embryos in warm conditions.

They also state that microscopy showed that glyoxysomes were present in the cytoplasm of the *A. tataricum* seeds but that the process of cold stratification caused them to disappear. Glyoxysomes are micro bodies present within some plant cells which contain enzymes implicated in the breakdown of fatty acid stores. They are commonly observed in seeds during the initial phases of germination where their role is to convert lipid stores to carbohydrates to fuel germination (Dey et al. 1997).

Babenko et al. (1996) found that *A. tataricum* seeds contained endogenous jasmonic acid when dormant and that 90% of this jasmonic acid was in a conjugated form. Jasmonic acid is often stored within a cell as a protein conjugate such as jasmonic acid – isoleucine (JA-Ile). We know that JA-Ile is not just a storage molecule but biochemically active hormone in it's own right.

It was speculated that the conjugated jasmonic acid is attached to the glyoxysome membrane during seed dormancy and that cold stratification weakens the membrane releasing the endogenous JA which is then free to migrate to the cell nuclei and initiate transcription and start the process of germination. This would explain why exogenously added jasmonic acid was reported to stimulate germination in dormant seeds. Is it possible that other hormones are released the same way?

Glyoxysomes contain many enzymes including lipase enzymes which break down lipids. It is

possible these could breakdown the glyoxysome membrane lipids and therefore release bioactive molecules to stimulate germination.

In apple seeds, Żarska-Maciejewska, (1976) determined that the enzyme acid lipase had a temperature optimum of 0-4ºC and that this lipase was more active during cold stratification (Żarska-Maciejewska et al. 1980). This enzyme also was more active in acidic conditions.

It may be possible that enzymes present within the seed during dormancy are active at low temperatures and these enzymes breakdown the storage mechanisms of endogenous plant hormones. Once released the hormones are capable initiating germination. This would be a potential explanation of seed dormancy which would tie up the reported phenomena of cold response and exogenous hormone application in breaking dormancy.

Kinetin does break the dormancy of *A. tataricum*. Also the closely related cytokinin 6 BAP is effective. Although the percentage germination as a result of treatments with these hormones was low it was similar to that of 3 months of cold stratification. The resulting seedlings appeared to be morphologically correct so it can be concluded that a simple hormone treatment with certain cytokinins (when facilitated with dimethylsulphoxide) can substitute for cold stratification in *A. tataricum* seeds. Interestingly, the cytokinin thidiazuron (TDZ) showed no dormancy breaking effect upon *A. tataricum*. TDZ is a synthetic cytokinin that exhibits many of the same functions within the plant as other cytokinins. However, it also shows auxin like effects as well as its cytokinin roles (Murthy et al. 1998). The results here have shown that kinetin in combination with the auxin NAA prevents prevents the dormancy break that kinetin alone promotes. It is thought that auxin has a role in maintaining dormancy in *Arabidopsis* (Matilla, 2020). From this it is possible to speculate that the auxin effects of TDZ counteract or overrule its cytokinin role resulting in it being unable to break dormancy in *A. tataricum* seeds.

Auxin may have a role to play in the cross talk between hormones in maintaining seed dormancy. ABA is still the main hormone thought to influence dormancy and in this study fluridone (which suppresses its production) was tested as a potential dormancy breaking chemical. Worarad et al. (2017) report that fluridone had a minor effect (8% germination as opposed to 0% in the control) on breaking dormancy in ornamental peach seeds. When tested here on *A. tataricum* it had no effect on germination (at least in the seeds tested at the fluridone concentrations used). ABA is a direct antagonist to gibberellic acid (Liu X and Hou X, 2018). Seeds classed as having deep physiological dormancy do not respond to GA. It is perhaps the case that in less deeply dormant seeds, if ABA is reduced (such as with fluridone treatment) then the balance between GA and ABA moves in favour of GA. GA promotes germination whereas ABA suppresses it (Garciarrubio et al. 1997) so it is speculated that in non deeply dormant seeds (such as peach and *Arabidopsis*) it is the ratio of GA to ABA that determines dormancy. To break dormancy, either there needs to be an alteration in hormone biosynthesis and/or a change in the sensitivity in the embryo to ABA or GA.

It is likely that dormancy release in deeply dormant seeds is much less influenced by GA and alongside this ABA may be less important as a primary mechanism for maintaining dormancy. Another mechanism may dominate or another ratio of hormones may be more important. This would explain the lack of results from the fluridone treatment in *A. tataricum*. Furthermore, since fluridone inhibits the synthesis of ABA, it will have limited effect on pre-existing endogenous ABA. The fact that fluridone has some dormancy breaking effect in ornamental peach suggests there may be some ABA synthesis occurring in the dormant seeds. It is currently thought that ABA is synthesised when the seed is formed on the mother plant (Shu et al. 2016). Interestingly while exogenous ABA will suppress germination in Arabidopsis it does not induce dormancy. Only the ABA synthesised by the seed itself can establish dormancy. This is thought to be due to the location within the seed where the ABA is present or active (Kucera, 2005). Experiments, again in Arabidopsis, indicate that the testa in dormant seeds actively synthesises ABA and maintains

dormancy (Lee et al. 2010). It is unlikely that ABA is synthesised in the seed coats of *A. tataricum,* as previous experiments in chapter 3 have shown no inhibition from seed coat extracts.

The positive results shown for certain cytokinins (kinetin and 6BAP) in breaking dormancy in *A. tataricum* needed to be tested across a range of other deeply dormant tree seed species to see if the effect was a common one. The treatment with kinetin did not promote germination in *T. baccata* nor *A. dissectum*. One caveat is that it is possible these seeds were not viable to start with and no cold stratification tests were conducted on them in order to determine their viability. *T. cordata* which had (for this batch of seeds) been tested for viability in chapter 3 (Table 2) did germinate as a result of the treatment. When a water only treatment was used as the control no germination occurred. This suggests that kinetin does break dormancy in *T. cordata,* which has been defined here as possessing deep physiological dormancy. *A. platanoides* (crimson king) which is a different variety of Norway maple from those tested before (Lots A, S & L) is commonly defined as having deep physiological dormancy. The kinetin treatment broke the dormancy. Unfortunately, a lack of seed supplies and time prevented further testing of deeply dormant species. Kinetin treatment also increased the germination percentage of the intermediately dormant *A. pseudoplatanus* from 70% to 90%. This suggests that kinetin (and 6BAP) are ideal candidates for developing a dormancy breaking treatment.

Chapter 5

General Discussion

The results from Chapter 4, Table 6 show that the seeds of *A. tataricum* and *T. cordata* when subjected to a kinetin treatment can achieve germination percentages similar to that provided by cold stratification. It seems possible therefore that cold stratification either:

-causes the genes associated with germination to become more sensitive/less sensitive to endogenous hormones such as kinetin,

- counteracts the effect of ABA within the seed,

- releases stored cytokinin within the cell or

- promotes the biosynthesis of cytokinin.

We know that cold stratification has a cumulative effect with time. Up to a point the percentage germination increases with increasing time held at cold temperatures (bearing in mind some of the dormancy period may be extended due to physical restriction imposed by the seed coat). So this suggests a slow and gradual build up of signalling molecules and/or a slow reduction of inhibitory substances. This may take the form of a continually altering ratio of cytokinin to ABA within the seed or a least the genetic machinery's sensitivity to them. Currently the most popular theory of seed dormancy remains that ABA inhibits germination and that plant hormones such as gibberellins, cytokinins and possibly jasmonates counteract this with environmental factors controlling the ratio between the two (Feurtado and Kermode, 2018).

The genes responsible for germination may normally be inactive when the seed is dormant. This may be maintained by ABA. The effect of cold temperatures may be to make the genes less sensitive to ABA allowing them to start transcription/translation, therefore gradually starting the

process of germination. The cold may also at the same time make the genes more sensitive to endogenous plant hormones which promote growth.

We know that GA works agonistically against ABA. It has been found that kinetin also works opposite ABA (Chaloupkova and Smart, 1994). Recent study has highlighted the role of auxin in maintaining seed dormancy (Matilla, 2020). Auxin may be a secondary hormone acting alongside ABA. The roles of ABA and auxin in seed dormancy in *Arabidopsis* are linked and probably interdependent (Liu et al. 2013).

The results in Table 5 suggest that in *A. tataricum,* NAA (an auxin) prevents kinetin from breaking dormancy. Indeed Kurepa, (2019) shows that cytokinins and auxins are antagonistic to each other in shoot and root growth which suggests this may also be the case in the seed embryo. Auxins don't appear to be antagonistic to gibberellins (Weiss & Ori, 2007). There could be the case that in deeply dormant seeds such as *A. tataricum* auxin is a significant regulator of dormancy (alongside ABA) while less dormant seeds have less auxin (or sensitivity to it). This would explain why dormant seeds require cytokinin to break the dormancy and less dormant seeds are capable of germinating with gibberellic acid only.

Depending on species, added exogenous GA, cytokinin or jasmonate may antagonise the effect of ABA and or auxin therefore removing it's block to germination. The higher the concentration of added hormone would correspondingly antagonise the level of ABA/auxin and should result in increasing germination percentages with increased treatment concentrations. Indeed Berestetzky (1991) found this to be the case for jasmonic acid.

There is evidence that cytokinin levels increase rapidly due to cold stratification in *A. pseudoplatanus* and *A. sacharrum*. In both species during cold stratification cytokinin levels increased rapidly in the first 20 days of treatment and then fell. When subjected to warm

stratification (room temperature) instead cytokinin levels remained constant for the first 20 days and then fell with no increase (Julin-Tegelman and Pinfield, 1982; Walker et al. 1989). Unfortunately, no studies on cytokinin levels in *A. tataricum* could be found. However, jasmonic acid levels in *A. tataricum* followed a similar pattern during cold stratification where jasmonic acid naturally increased and then fell towards the end of stratification (Berestetzky, 1991). Babenko (1996) suggests that the jasmonic acid is already present in the seed and is released, rather than being synthesised during dormancy. This may be the case too for the cytokinins.

A simple explanation describing the mechanism of cold stratification may be that levels of germination promoting hormones are synthesised by cold sensitive genes. When these hormones accumulate to a certain level, dormancy is broken, perhaps by antagonising ABA or auxin. Addition of exogenous plant hormones can circumvent this process and either wholly or partly substitute for cold treatment.

It is clear that hormones have a key role in the regulation of seed dormancy. Plant hormones however have a wide range of actions upon the whole plant so there must be a specific regulation system that regulates dormancy and germination. The exact action of these hormones appears to influence the seed on a genetic level. Recent studies have shown the gene *DOG1* (*DELAY OF GERMINATION 1*) to be a key factor in dormancy maintenance. In *Arabidopsis DOG1* has to be present alongside ABA in order to sustain physiological dormancy in the seed. ABA alone can suppress germination but not maintain dormancy. The *DOG1* gene has to be present also. It is thought that *DOG1* and ABA act with independent signalling pathways but together maintain dormancy. *DOG1* codes for a protein which has been shown to block certain phosphatase enzymes. These enzymes belong to a family of phosphatases which influence ABA signalling and are thought to have a role in releasing dormancy (Carrillo-Barral et al. 2020; Soppe & Bentsink, 2020). *DOG1* has been linked to dormancy regulation in lettuce and wheat as well as *Arabidopsis,* but there have

been no studies in the more deeply dormant tree species. It may be possible that this particular gene is common to all physiologically dormant seeds.
How a plant hormone interacts with these genes is still not fully understood. They may suppress DOG1 (or similar dormancy genes in other species) or they may act more against ABA.

A protocol for treating deeply physiologically dormant seeds can be proposed based on the results for this study.

-Remove pericarps if present,
-Pre soak the seeds in water until imbibed 3-7 days,
-Soak in a solution of 100-500mg/l Kinetin dissolved in 20% DMSO for 2 days,
-Rinse the seeds and place in germination container in the light,
-After 3-7 days remove inner seed coats and replace in container until germination commences.
-Germinated embryos can be placed in potting media and transferred to a growing on area.

This treatment is subject to removal of both the pericarp and testa. It was found in chapter 3 that the testa imposes a sort of physical extra dormancy on the embryo and that some of the cold stratification period required for germination may be taken up with degrading of the seed coats. It was observed that the seed coats provide a degree of physical protection to the embryo form microbial attack. So although in nature the embryo may be ready to germinate, it cannot do so until the seed coat has sufficiently weakened, perhaps as a result of internal breakdown enzymes or external soil factors.
This protocol can eliminate the need for a cold stratification treatment of up to 3 months for some seeds. The time needed for this protocol before germination may commence is approximately 2 weeks, a significant saving in time over traditional cold stratification. However, it comes at the cost

of a significant increase in labour. Removal of seeds coats by hand is labour and time intensive. This may be acceptable for a research lab wishing to speed up its workflow, but it is unlikely to be viable for a large nursery grower who needs to plant thousands of seeds at a time. A process of eliminating the seed coat removal stage would majorly reduce the labour requirement. Enzymatic or chemical scarification could be investigated and this would likely be the next logical step for development of a dormancy breaking seed treatment. Chemical degradation of the seed coat with substances such as hypochlorite may damage the underlying embryo. Enzymatic scarification could be a more sympathetic treatment especially if the degrading action is specific to the seed coat and does not harm the embryo. Laccase which degrades lignin (which forms the structure of many seed coats) may be a promising candidate enzyme. (Pierce et al. 2019)

Unfortunately, even the simplest of protocol requirements would also negatively effect the viability of a seed treatment for the amateur gardening market. A simple one step (or one dip) product would be optimal.

In conclusion, we have been able to define the dormancy status of a range of temperate region trees. The deep physiologically dormant seeds of *A. tataricum* was chosen as a test seed species. It was determined that water and oxygen permeability of the seed coats were not a major factor in maintaining dormancy in this seed. Nor was the oxygen concentration that the seed was incubated in. There was also no indication of inhibitory substances (to germination of lettuce seeds) within the seed. The testa of *A. tataricum* was found to be an important part of the seed. It protects the seed from surrounding contaminants, restricts the passage of some chemicals yet is permeable to water and oxygen. The testa weakens over time after imbibition in both cold and warm conditions and is likely to be a secondary factor in prolonging dormancy. It has been found that cytokinins can break the dormancy of *A. tataricum*. A treatment for breaking the dormancy in temperate region tree seeds has been proposed using a 350 mg/l kinetin/20% dimethylsulphoxide solution. This treatment

successfully removed the dormancy and substituted for up to 3 months of cold stratification in *A. platanoides* (crimson king) and *T. cordata*. It also increased the germination percentage of *A. pseudoplatanus*. Possible mechanisms of how cold stratification breaks dormancy in relation to this finding have been discussed. Other species and varieties of dormant seed respond to other plant hormones such as *A. platanoides* to jasmonic acid. Future areas of study in order to understand the mechanism of dormancy release would include studies on cytokinin and other hormone levels in *A. tataricum* during cold stratification and studies on how cytokinins effect the levels of ABA and auxin within the seed during dormancy break. Future development of a dormancy breaking control would include treatments to weaken seed coats and further testing of effectiveness on other species of tree.

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