

Chapter **35**

## **Predicting Seed Longevity:**

*the use and abuse of seed viability equations*



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### Summary

The development of the viability equations from the 1960s onwards has underpinned the *ex situ* conservation of plant genetic resources as seeds, i.e., seed banking. The equations include constants that explain the empirical effects of both moisture content and temperature on longevity. The temperature constants appear to be universal, over the range c.  $-13^{\circ}\text{C}$  to  $90^{\circ}\text{C}$ , whilst the moisture content-related terms are species-dependent. The generation of the full viability constants requires considerable experimentation and germplasm, but there are short cuts to a rapid estimation of longevity. Early studies focussed on crop species, however seed viability constants are now available for at least 66 species from 26 families. Empirical data reveal low-moisture-content limits to the response of seed longevity to hydration, generally at moisture contents in equilibrium with c. 15% RH at  $15^{\circ}\text{C}$ . Below this level of hydration, longevity may increase slightly, remain the same or decrease slightly, depending on the seed lot. This point appears to be below the moisture content at which seeds are thought to enter the 'glassy' state, indicating that removal of water from the glass may not destabilise the seeds, at least over shorter experiments. In addition, there is some evidence that this low moisture content limit varies with both seed chemical composition and storage temperature. A similar limitation applies to the effects of low temperatures; although there is little long-term data available, the sub-zero temperature response appears not to fit the quadratic term appropriate for higher temperatures. These qualifications to the seed viability equations have contributed to the recent debate about the validity of predicting, by extrapolation, longevity in the region of millennia for dry, cold-stored seeds. By comparison, practical evidence on buried seeds supports the notion of seed germination after storage for centuries.

### Introduction

*"The purpose of models is not to fit the data but to sharpen the questions"*

*Samuel Karlin, 1983 (cited by Mackay, 1991)*

The initial driving force for the development of seed conservation science and technology was the need to provide seedsmen and seed producers with advice on how best to store agricultural seeds between growing seasons. To meet this demand, and the needs of the seed conservationist, seed viability models have been developed, over the last 40 years, to characterise how environmental conditions impact on seed survival during storage.

As the seed viability models can be applied only to seeds with certain physiological properties, it is first appropriate to explain the well-known and variable seed storage responses. Thereafter, the mathematics behind the model development is considered, followed by an explanation of the basic assumptions needed to use the various models. Practical evidence provides verification of their potential value, but also raises issues about their validity when predicting the likelihood of encountering ancient seeds.

## Seed Storage Responses

Seed storage behaviour can be assigned to two main classes based on the seed population response to dehydration, *viz.* desiccation tolerant and long-lived ('orthodox'), desiccation sensitive and short-lived ('recalcitrant') (Roberts, 1973). A third class of seed response has been proposed for seeds that tolerate moderate desiccation, down to around 8–12% moisture (c. -100 MPa), and this has attracted various epithets: 'orthodox with limited desiccation ability' (OLDA) (Tompsett, 1984a, b; Tompsett and Kemp, 1996), 'sub-orthodox' (Bonner, 1990) and 'intermediate' (Ellis *et al.*, 1990c; 1991a). The intermediate descriptor also included a sensitivity to dry storage at low temperatures of 0°C and -20°C.

Recent evidence on the seed storage responses of a range of species has clearly shown that the critical moisture contents and temperatures delimiting the three classes is dependent on a number of factors, including the developmental age of the material, the method of processing (particularly the temperature and rate of drying) and post-storage methodology. Nonetheless, it is possible to redefine the storage classes described above in relation to the physiological response of seeds to critical ranges of moisture content, which appear to relate to zones of water sorption (Pritchard, 2004). Although these zones are not a direct measure of water binding, they can be used to infer approximate levels of occupancy of sorption sites based on the D'Arcy-Watt model (Sun, 2002). Sorption zone I of the isotherm extends up to c. 15% RH at which point > 90% of 'strongly bound' water sorption sites are occupied. In sorption zone II, c. 15–80% RH, all 'strongly bound' water sorption sites are occupied, > 80% of 'weakly bound' hydration sites are occupied, and only about 5% of multilayer sorption sites are occupied. In sorption zone III (> 80% RH) the occupancy of multilayer hydration sites continues to increase (Sun, 2002)<sup>1</sup>. In an attempt to simplify terminology and to emphasise the biophysical basis of seed storage responses articulated earlier by Walters (1998a), Pritchard (2004) has proposed a seed storage classification system in relation to the zones of the water sorption isotherm in which a change of response (essentially desiccation sensitivity or longevity) is observed:

- Type I seeds tolerate removal of both 'free, multilayer' water (sorption zone III) and the vast majority of 'weakly bound' water (zone II). As the latter is reduced longevity is increased, at least down to moisture contents in equilibrium with around 15% RH (see Roberts and Ellis, 1989). This type includes orthodox seeds;
- Type II seeds may lose viability as mainly loosely bound water (about < 80% RH) is being removed, e.g., some species of *Araucaria* Juss. (Tompsett, 1984a) and coffee (Dussert *et al.* 1999), and they tend to be relatively short-lived when stored at moisture contents within sorption zone II (see Bonner, 1990; Ellis *et al.*,

<sup>1</sup> For a typical sorption isotherm for yellow maize seed see Probert (2003) – Chapter 19.

1990c). Low temperature stress and reduced longevity may occur at specific sub-zero temperatures, not necessarily  $-20^{\circ}\text{C}$ , e.g., some orchids (Pritchard *et al.*, 1999). This type includes seeds previously described as ‘orthodox with limited desiccation ability’ (OLDA) (Tompsett, 1984a,b; Tompsett and Kemp, 1996), ‘sub-orthodox’ (Bonner, 1990) and ‘intermediate’ (Ellis *et al.*, 1990c; 1991a).

- Type III seeds display signs of desiccation sensitivity when mainly multilayer water (zone III) is being removed, i.e., down to about 15–25% moisture content (80–90% RH). The loss of this water can only be tolerated following rapid desiccation of seed parts (see Pritchard and Prendergast, 1986; Pence, 1995; Vertucci and Farrant, 1995), thereafter, the removal of water from weak binding sites tends not to be tolerated (Pammenter *et al.*, 1991; Pritchard, 1991). Cryopreservation is the only long-term storage option. The Type includes all ‘recalcitrant’ seeds.

Thus at the seed lot and, in many cases, species level, desiccation tolerance and longevity tends to partition into three ‘types’: desiccation tolerant and inherently long-lived seeds in the dry state (Type I); partially desiccation tolerant seeds that are relatively short-lived unless cryopreserved at c.  $-80^{\circ}\text{C}$  or lower (Type II); and essentially desiccation sensitive seeds that may be cryopreserved by careful manipulation (Type III) (Pritchard, 2004).

## Seed Viability Equations

Reductions in seed moisture content and storage temperature within broad limits are known to enhance longevity in orthodox, Type I seeds (see Roberts and Ellis, 1989; Pritchard, 2003). The seed viability equations, which quantify such relationships, have influenced the design of seed storage facilities, and this has culminated in the adoption of international gene bank standards (Genebank Standards, 1994). To appreciate fully why the seed conservation/banking community has arrived at this position, it is important to reflect on how work on the modelling of seed longevity has developed over more than 40 years (see Roberts, 1973; Ellis and Roberts, 1980a; Roberts and Ellis, 1989; Dickie *et al.*, 1990). The details reveal gradual improvements in the mathematical description of the variable rates at which populations of seeds die in relation to environment, initial quality of the seed lot and some inherent characteristic of the species. More recently, considerations of the molecular mobility of viscous cytoplasm (Sun and Leopold, 1994; Sun, 1997; Walters, 1998; Buitink, 2000), have opened up new opportunities to use different ‘constants’ in viability equations that reflect the biophysical basis of longevity rather than an empirical description of the pattern of population decline.

A brief history of the development of seed viability equations is given in Box 35.1, whilst a more detailed description of the science is provided thereafter.

#### Box 35.1 Seed viability equations: a brief history

- The seed viability equations were developed as a means of predicting the longevity of crop seeds in store, as a support to seedsmen and seed producers.
- Much of the ground work was done in the 1960s, and this culminated by the early 1970s in the development of the 'basic viability equation,' incorporating three constants to explain the dependence of seed survival on moisture content, temperature and an inherent property of the species (see Roberts, 1973).
- Within 10 years, subtle changes had been made to the equation to take into account: 1) the added complexity of the temperature dependence of seed longevity (i.e., a second temperature term was added); 2) the importance of the initial quality (viability) of a seed lot on potential longevity (Ellis and Roberts, 1980a). This resulted in four viability constants (two for temperature, one for moisture content and one related to moisture that defined the species response), which when combined with the initial viability of the seed lot, were able to explain variability in longevity between seed lots of the same and different species (Ellis and Roberts, 1980a).
- By the late 1980s, it was clear that consideration of seed relative humidity, in addition to seed moisture content, would provide further insight to inter-species variability in longevity by effectively removing the effect of seed chemical composition on the moisture content term (Roberts and Ellis, 1989).
- At about the same time, it was proposed that the temperature constants were 'universal' across species (Dickie *et al.*, 1990). This development paved the way for the characterisation of species seed longevity on the basis of experiments at one temperature, assumptions then being made about responses at other temperatures.
- Limits to such extrapolations using the improved viability equation (Ellis and Roberts, 1980a) were suggested when longevity was related to the glass transition temperature of the seeds (Sun and Leopold, 1994). A glass is a fluid that is so viscous that it acquires the mechanical properties (strength) of a solid, which in the context of seeds is what happens when they are dried to low humidity. As the glass transition temperature is dependent on the moisture content of the seeds, it was possible to relate longevity to where seeds were, in terms of storage environment, above the glass transition temperature (Sun and Leopold, 1994; Sun, 1997).
- A driving force for viscosity-dependent longevity in seeds is the molecular mobility of viscous cytoplasm (Walters, 1998; Buitink, 2000), leading to further opportunities to use different 'constants' in viability equations that reflect the biophysical basis of longevity rather than an empirical description of the pattern of population decline.

### 1. Basic Viability Equations

Roberts (1973) noted that the “relationships between temperature, moisture content and period of viability has now been examined in detail in five orthodox species (*Hordeum vulgare* L., *Triticum aestivum* L., *Oryza sativa* L., *Vicia faba* L. and *Pisum sativum* L.). In all five species percentage of germination after any given period under any combination of temperature and moisture content may be defined by three basic viability equations which contain a total of four constants, the values of which differ between species.” The first equation (not shown here) described the normal distribution of viability periods of the individual seeds in a population (Figure 35.1a). Such a pattern of seed deaths gives rise to the typical negative sigmoidal seed survival curve (Figure 35.1b). The second equation, showed that the standard deviation (a measure of the spread) of the distribution of deaths in time ( $\sigma$ ) is proportional to the mean viability period ( $\bar{p}$ ), thus:

$$\sigma = K_{\sigma} \bar{p} \quad [1]$$

where  $K_{\sigma}$  is a constant. The third equation then related temperature, moisture content and mean viability period, as follows:

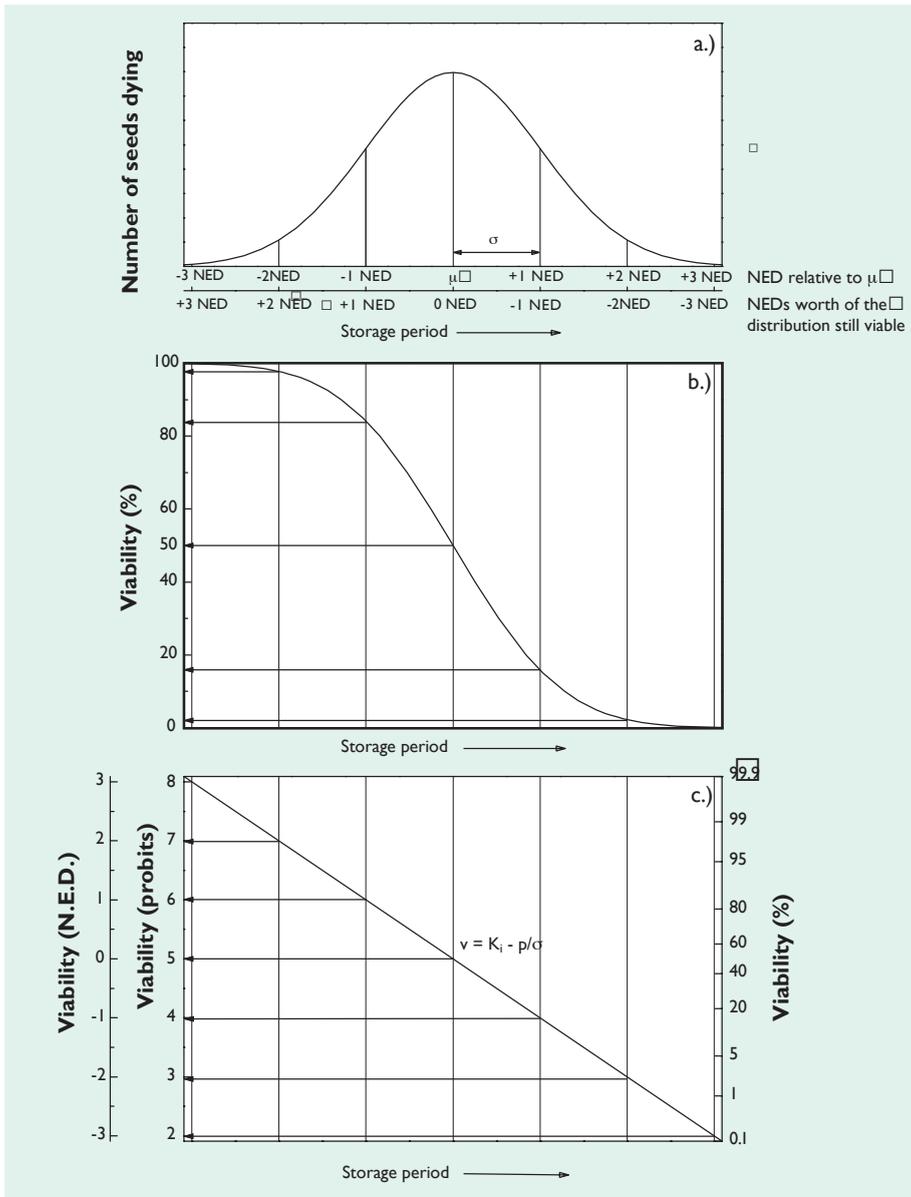
$$\log \bar{p} = K_v - C_1 m - C_2 t \quad [2]$$

where  $m$  is % moisture content (fresh weight basis),  $t$  is temperature ( $^{\circ}\text{C}$ ) and  $K_v$ ,  $C_1$  and  $C_2$  are constants. For broad beans (*Vicia faba* L.) the values of the constants determined were:  $K_v = 5.766$ ,  $C_1 = 0.139$  and  $C_2 = 0.056$ .

Once the value of the constants for a species have been determined the pattern of loss of viability can be presented in the form of a nomograph, showing the relationship between scales for mean longevity and the required viability at the end of storage under the chosen conditions (moisture content and temperature). This makes predicting seed survival easier than when using the three equations (Roberts, 1973). Nowadays, the wide availability of statistical software packages has reduced the importance of nomographs, their main use being as a teaching aid. In particular, they strongly emphasise that the effects of time, temperature and moisture content on seed longevity should be considered simultaneously as their inter-relationships over a range of conditions are smooth. Thus, the notion of a ‘safe moisture content’ for storage is rather misleading; longevity will always depend on the combination of conditions used (Roberts, 1973).

Nomographs based on these basic viability equations are only rough guides to seed longevity, their value being limited by concerns about a number of impacting features (Roberts, 1973):

- intra-specific genotypic, i.e., seed lot, differences in longevity under the same storage conditions;



**Figure 35.1** Modelling seed longevity using the probit transformation of survival curves. (a) The frequency of seed deaths over time is normally distributed, such that the time for the population to reduce in viability by one normal equivalent deviate (NED = standard deviation) provides the value of  $\sigma$  for use in the seed viability equation of Ellis and Roberts (1980a). (b) The negative sigmoidal seed population survival curve over storage time. (c) The probit transformation of a survival curve which can be described by the equation shown (= Equation [6] in text). (Note that the probit value = NEDs + 5). Adapted from Probert and Hay (2000), with permission of Blackwell Publishing.

- effects of pre-storage conditions, such as high temperature post-harvest drying and mechanical damage; these may affect the subsequent loss of viability;
- changing oxygen partial pressure during hermetic storage<sup>2</sup>;
- inaccuracies in determining seed moisture content.

Genotypic differences and pre-treatment effects could be expressed as differences in the initial quality of a seed lot, and thus their potential longevity. The lower the initial quality, the quicker the seed lot will expire under the same storage conditions. As a consequence, it has always been emphasised that the equations, not just the nomographs, should be used as a 'rough guide to the expected behaviour of high-quality seeds lots' (Ellis and Roberts, 1980a). In earlier work, 'high quality' could be achieved for all seed lots by applying the correction factor,  $100/x$ , where  $x$  is the initial percentage viability of the seed lot, thus ignoring the dead seed fraction before the experiments started (e.g., Roberts, 1961). Even so, substantial differences in longevity can occur in seed lots > 99% viability (Ellis, 1976). So, this correction is not justified and modifications to the basic viability equation sought to take into account such variations in potential longevity. This was achieved by introducing a term to account for initial differences in seed quality,  $K_i$  (Ellis and Roberts, 1980a).

## 2. The Improved Viability Equation and $K_i$

In addition to accommodating initial differences in seed quality,  $K_i$ , the improved viability equation included modifications to enable its application over a far wider range of environmental conditions (Ellis and Roberts, 1980a; Ellis *et al.*, 1986). In Equation [1] survival in storage is quantified as the mean viability period,  $\log \bar{p}$ , which is related to the standard distribution of deaths in time,  $\sigma$ , by the constant  $K_\sigma$ . It is therefore possible to derive a new equation describing the relationship between the standard deviation of the normal distribution of seed deaths in time with environment, thus:

$$\log \sigma = \log K_\sigma + K_v - C_1 m - C_2 t \quad [3]$$

Equation [3] may be re-written as

$$\log \sigma = K_L - C_1 m - C_2 t \quad [4]$$

where

$$K_L = \log K_\sigma + K_v \quad [5]$$

<sup>2</sup> It is worth noting that longevity for lettuce seeds stored at  $\leq 10\%$  moisture content and 40–50°C nearly doubles when nitrogen replaces air as the storage environment (Ibrahim and Roberts, 1983), indicating that gaseous environment will affect viability modelling, and this has not yet been taken into account in developing the viability constants.

When seed survival curves are plotted as probit percentage viability against time, straight lines of negative slope are produced which can be described by  $1/\sigma$  (Finney, 1977). Therefore, survival (viability remaining) can be predicted, thus:

$$v = K_i - (1/\sigma) p \quad [6]$$

where  $v$  is viability (probit %),  $p$  is storage period (d) and  $K_i$  is viability (probit %) before storage (see Figure 35.1c).

The relative differences in longevity between seed lots is maintained in all environments and thus is not dependent on genotype. In addition, the constants  $C_1$  and  $C_2$  (see Equation [2]) are not affected by genotype either, nor by seed quality. Finally, genotype and seed quality do not affect the slope of the survival curves. Therefore, only the intercept of the survival curve,  $K_i$ , is affected by genotype and pre-storage treatments (Ellis and Roberts, 1980a). It follows from Equation [4] that  $K_L$  too is independent of these factors. As a consequence, it has been possible to propose a single viability equation to account for the performance of seeds in storage by substituting  $\sigma$  in Equation [4] in Equation [6], thus:

$$v = K_i \frac{p}{10} K_L - C_1 m - C_2 t \quad [7]$$

where viability,  $v$  (probit %), at any time in storage,  $p$ , is related to a combination of moisture content,  $m$ , and temperature,  $t$ . Remember that  $K_L - C_1 m - C_2 t$  are species-specific. For example, the constants  $K_L$ ,  $C_1$  and  $C_2$  are estimated to be 4.606, 0.135 and 0.034 respectively for finger millet and 6.713, 0.267 and 0.033 respectively for amaranth (Mutegi *et al.*, 2001). Note here the similarity of the temperature term ( $C_2$ ) of the species.

$K_i$  can be estimated in one of two ways. First, via a large (i.e., hundreds of seeds) initial germination test, just as the storage experiment is to start. Second, by back extrapolation by probit analysis, based on subsequent germination tests carried out at regular intervals during a few accelerated seed ageing experiments. For example, seeds can be adjusted to around 5 to 15% moisture content and then stored at warm temperature (c. 40°C). Irrespective of the method used, it is important to have a reasonably high measure of confidence in this initial viability value (determined or deduced) as small differences in  $K_i$  can have a large effect on predictions of longevity. Of course, a consequence of a higher  $K_i$  is that the absolute longevity (50% viability period) will be enhanced, e.g., soybean (Zanakis *et al.*, 1993).

To determine the species constants, seeds should be aged under several combinations of temperature and moisture content. Then from the survival curves (Figure 35.1b, c), the constants can be estimated by fitting Equation [4] using multiple regression analysis. In this context, it is conventional to constrain the survival curves for a single seed lot to a common origin, a procedure that often reveals significant differences in  $K_i$  among seed lots but not among ageing environments, e.g., in barley and wheat (Pieta Filho and Ellis, 1992). Then this deduced  $K_i$  for the seed lot is inserted in the viability equation (see next section).

Another important change in the development of the seed viability equations at this time was the logarithmic transformation of moisture content. Hutchinson (1944) had suggested such a transformation when dealing with seed viability data, and Ellis and Roberts (1980a) adopted this in an attempt to extend the viability equations to a wider range of conditions.

### 3. Temperature and Seed Ageing ( $C_H$ , $C_Q$ )

Whilst the overall range of temperatures used in seed ageing work is large, each study is often limited to a range of about 25°C. Over this range, there is generally no evidence of abrupt limitations to the log-linear relationship between longevity and temperature described by the equation. This implies that a single value of the term  $C_2$  is appropriate, as shown in Equations [2, 3, 7]. However, for seeds stored at one moisture content and a wide range of temperatures it became apparent that  $C_2$  became progressively greater at the higher temperatures, i.e., the relationship was slightly convex (Ellis and Roberts, 1980a). To account for this it was proposed that the relationship was better described by including a negative quadratic term in Equation [4]. When combined with the change in moisture content term  $C_1m$  to log moisture content (mentioned above), and accepting a slight change in convention, the ‘improved viability equation’ was described thus

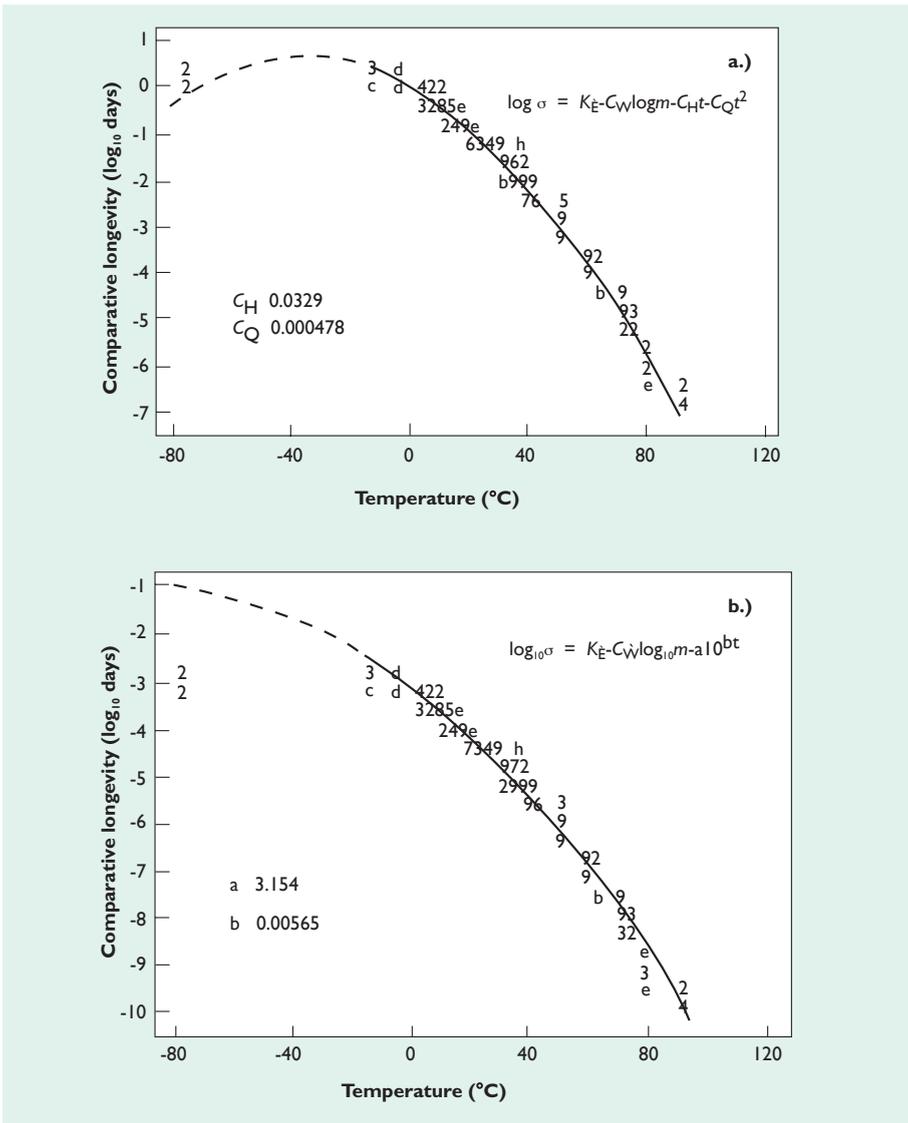
$$\log \sigma = K_E - C_W \log m - C_H t - C_Q t^2 \quad [8]$$

In addition, Equation [7] can now be modified to

$$v = K_i \hat{p} / 10^{K_E - C_W \log m - C_H t - C_Q t^2} \quad [9]$$

The values of the constants  $C_H$  and  $C_Q$  of the improved viability equation for barley are 0.04 and 0.000428 respectively (Ellis and Roberts, 1980a). Subsequently, it has been shown that this semi-logarithmic quadratic relationship adequately describes the relative effect of temperature on seed longevity in more than 20 species of crop and flower seeds (Ellis and Roberts, 1981a). In a further more detailed comparison of seed longevity data for eight diverse species, Dickie *et al.* (1990) concluded that the temperature constants are universal, being 0.0329 and 0.000478 for  $C_H$  and  $C_Q$  respectively. In other words, the relative sensitivity of seed longevity to changing temperature is identical between species (Figure 35.2a). These constants fitted the data well for temperatures between -13 and 90°C. General support for the ‘universal’ values comes from a long-term study on seeds of four trees stored at -18°C to 10°C for up to 10 years; average  $C_H$  and  $C_Q$  values were 0.0375 and 0.000676, respectively (Bonner, 1994).

However, the universal values do not explain all empirical data sets, for various reasons. Contrary to the early estimates for  $C_Q$  for barley and other species (e.g., see Ellis and Roberts, 1980a, b),  $C_Q$  for apple and lupin seed was estimated to be a negative term (Dickie *et al.*, 1985, Dickie and Bowyer, 1985).



**Figure 35.2**

Relations between comparative seed longevity in eight species and temperature, using the quadratic (a) or exponential (b) terms. Data sets shown are for lettuce, mahogany, elm, terb, barley, cowpea, soybean and chickpea. The numbers 2 to 8 indicate two to eight coincident observations on several species, while 9 indicates nine or more coincident observations. The letters indicate single observations for the species listed in order above. In (a) longevity is described by Equation [8], shown inset, and comparative longevity =  $\log_{10} \sigma - K_E + C_W \log_{10} m$ . In (b) longevity is described by Equation [11], shown inset, and comparative longevity =  $\log_{10} \sigma - K_E + C_W \log m$ . The continuous lines show the fitted relationships, whilst the broken curves are extrapolations to  $-75^\circ\text{C}$ . Figures re-presented from Dickie *et al.* (1990) with permission of Elsevier.

This implies a lowering of the  $Q_{10}$  (temperature coefficient)<sup>3</sup> for the rate of loss of viability with increasing temperature. However, the standard error on the determination for apple was high, casting doubt on the validity of the  $C_Q$  estimate. This may have been a consequence of using a limited range of temperatures for the ageing experiments (Dickie and Bowyer, 1985). Moreover, constraining the viability constants to the ‘universal’ values can have implications for the other constants. For example in two varieties of both delphinium and salvia stored at 20°C and 30°C, the application of the universal temperature constants lowered the value of  $K_E$  by about one; however,  $C_W$  remained unchanged (Kwong *et al.*, 2001). Similarly, using the ‘universal’ constants increased the variance of fitting the viability constants in two *Arabidopsis thaliana* (L.) Heynh. ecotypes; whilst a quadratic relationship between temperature (6°C to 55°C) and seed longevity was confirmed, the magnitude of the non-linearity was smaller than that indicated by the ‘universal’ value of  $C_Q$ . (Hay *et al.*, 2003).

The universal temperature constants are not recommended for use at temperatures below -20°C, mainly because they could not fully account for the response of *Ulmus carpinifolia* Gleditsch seeds when held at -75°C (Dickie *et al.*, 1990). At this low temperature, *Ulmus* seeds appeared to store equally as well as at -13°C (Tompsett, 1986). This would not be predicted from the quadratic temperature term, as it imposes a reversal in the relation between longevity and temperature at the point  $t_L$  defined by

$$t_L = -C_H / (2C_Q) \quad [10]$$

If we accept the universal temperature constants apply, then  $t_L$  is -34.4°C (Dickie *et al.*, 1990). The inference is that at equal temperatures above and below this point, longevity will be the same. So cryopreserved seeds at -150°C (i.e., 115°C below  $t_L$ ) should have the same longevity as seeds stored at 80°C. There is no evidence to support this perspective.

The possibility that an exponential term might account for the effects of temperature on seed longevity for eight diverse species was also explored by Dickie *et al.* (1990), revealing that

$$\log_{10} \sigma = K_E - C_W \log m - a10^{bt} \quad [11]$$

where  $K_E$  and  $C_W$  have modified values after fitting the new temperature term and two new coefficients  $a$  and  $b$  are associated with this term. Estimates of the coefficients are  $a = 3.154$  and  $b = 0.00565$  (Figure 35.2b). Because the quadratic and exponential models fitted the temperature data equally well, it appears that the quantitative relations between temperature and seed longevity in diverse species is remarkably similar (Dickie *et al.*, 1990).

<sup>3</sup> The ratio of the rate of a process at one temperature to that at 10°C lower.

Predictions of longevity at very low temperatures is not a precise science because of the need to extrapolate considerably. In elm seed, inclusion of the quadratic temperature term appears to underestimate longevity at  $-75^{\circ}\text{C}$ , whilst the exponential term tends to overestimate observed longevity (Tompsett, 1986; Dickie *et al.*, 1990). However, if we accept that the exponential term describes seed responses at ultra-low temperatures more closely, it is possible to theorise considerable benefits (175-fold) of storing seeds in or just above liquid nitrogen (i.e.,  $< -150^{\circ}\text{C}$ ) compared with conventional storage (i.e.,  $-20^{\circ}\text{C}$ ) (Pritchard, 1995). But is there any evidence that this is the case? The average seed germination level of onion (*Allium cepa* L.) cultivars did not decline after 10 years storage at  $-18^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$  and there was no significant difference in survival (92%) between the two storage temperatures (Stanwood and Sowa, 1995). However, seedling growth rate (root length) and oxygen uptake were significantly higher for the cryopreserved seeds, suggesting greater preservation of seed vigour. The mean time to germinate, which is a measure of seed vigour, is negatively related to the total germination level, e.g., in onion, and subtle changes in this characteristic of a seed lot are easier to detect than statistically significant alterations in total germination during the first part of any seed survival curve (Ellis and Roberts, 1981a). This suggests that the lower vigour of onion seeds stored at  $-18^{\circ}\text{C}$  compared to  $-196^{\circ}\text{C}$  (Stanwood and Sowa, 1995) is a harbinger of a faster rate of seed viability loss under conventional storage conditions. Moreover survival at conventional seed bank temperature ( $-20^{\circ}\text{C}$ ) might be less than predicted, as ageing studies on *Hordeum distichum* cv. Proctor [*Hordeum vulgare* L. ssp. *distichum* (L.) Thell. cv. Proctor] at 15.4% moisture content revealed an ageing rate equivalent to that predicted at  $-6.3^{\circ}\text{C}$  (Roberts and Ellis, 1977). Nonetheless, regeneration intervals (in this instance, the time for viability to fall to 95% of its original) of decades to a millennium were suggested for seeds of seven crops held in cold store at 5% moisture (Roberts and Ellis, 1977). Thus, banking at c.  $-18^{\circ}\text{C}$  still offers considerable opportunity for long-term seed storage.

The exception to the rule is the accelerated rate of viability loss (in as little as 3 months) at  $-20^{\circ}\text{C}$  for 'intermediate' seeds of coffee (Ellis *et al.*, 1990c, 1991a), papaya (Ellis *et al.*, 1991b) and oil palm (Ellis *et al.*, 1991d). Clearly, such a response is not predicted by the universal temperature constants. However, this specific behaviour now appears to be relatively uncommon in seeds. Firstly, seeds of other species can show different kinetics for low temperature stress, often with a biphasic pattern (faster immediately and slower longer-term), e.g., *Agathis* Salisb. species (Dickie and Smith, 1995), three palms (Davies and Pritchard, 1998) and three orchids (Pritchard *et al.*, 1999). Moreover, cold-stress in dry seeds is not necessarily limited to  $-20^{\circ}\text{C}$ , e.g., orchids (Pritchard *et al.*, 1999) or in coffee (Hong and Ellis, 2002). As a consequence, it has been suggested that a much broader group of (Type II) seeds be established (see 'Introduction to seed storage responses') for which a further understanding of the water relations and temperature control of longevity is needed (Pritchard, 2004).

#### 4. Moisture and Seed Ageing ( $K_E$ , $C_W$ and $C_W r$ )

At a single, constant temperature, there is a negative logarithmic relationship between  $\sigma$  and moisture content (Figure 35.3, central zone)<sup>4</sup>.  $C_W$  is the slope of this relationship and  $K$  the intercept, if extrapolated back to  $\log m = 0$  (i.e.,  $m = 1\%$ ), so that

$$\log_{10}\sigma = K - C_W \log_{10}m \quad [12]$$

where,

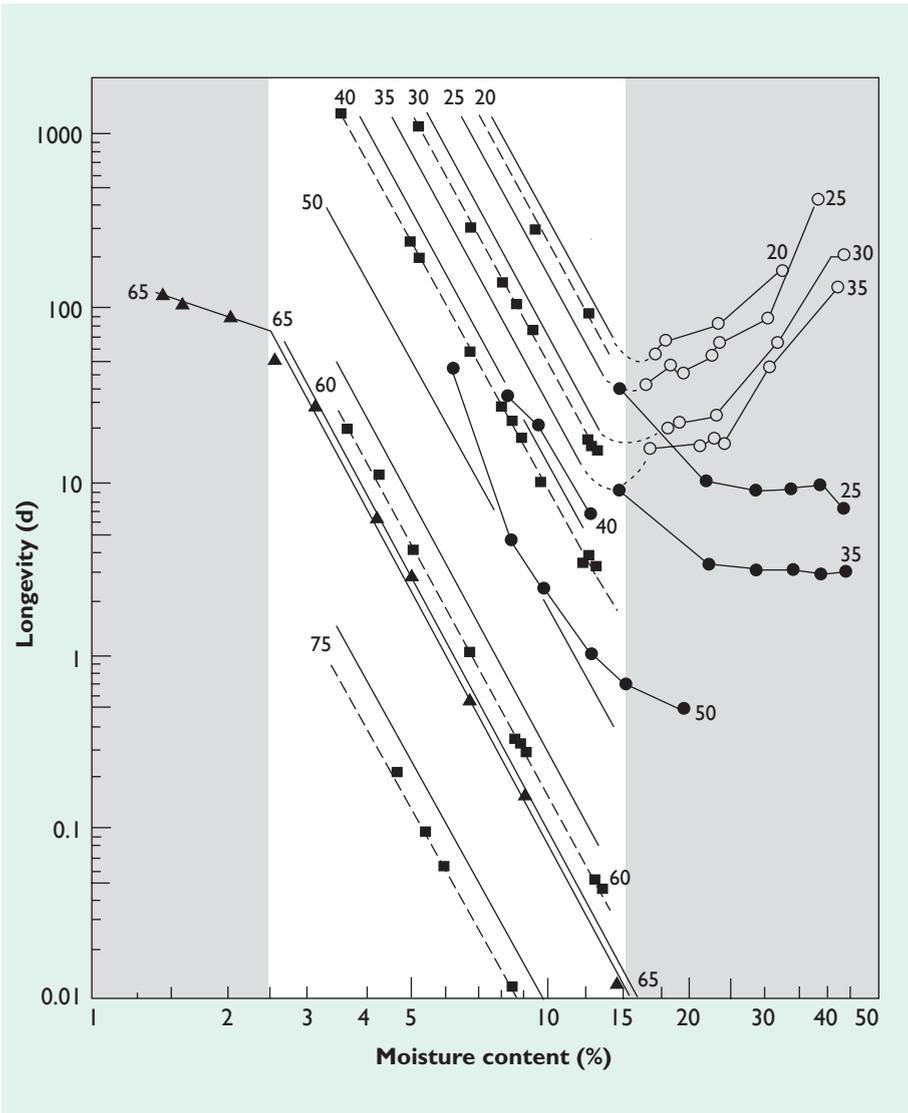
$$K = K_E - C_H t - C_Q t^2 \quad [13]$$

$K$  is a means of simplifying Equation [8], when only one temperature is being considered. In this way, and assuming that the barley temperature constants could be applied to other species, Ellis *et al.* (1986) estimated that  $K_E = 7.19$  and  $C_W = 4.02$  for sesame seeds. Subsequently, there have been many estimates of  $K_E$  and  $C_W$  and these have been shown to differ significantly between species, mainly as a result of the dependency of moisture content on seed chemical composition (e.g., Ellis *et al.*, 1986; 1988; 1989; 1990b; and later discussion).

$K_E$  is the theoretical value of  $\log\sigma$  at 1% moisture content ( $\log_{10} 1 = 0$ , the intercept of the slope) and 0°C determined by a multiple regression-fitting process. Whilst the term is widely used in longevity models, its contribution to an understanding of longevity under ultra-dry conditions is unclear. First, such ultra-dry storage of seeds has been rarely attempted, as it is exceptionally difficult to dry seeds to that extent. Indeed, the best opportunity to reach such low moisture contents would be with oily seeds, e.g., 1.6 to 2.1% moisture content for seeds of *Araucaria columnaris* Hook. (Tompsett, 1984a), *Sesamum indicum* L. (Ellis *et al.*, 1986) and *Nothofagus obliqua* (Mirb.) Oerst. (Leon-Lobos and Ellis, 2003 – Chapter 40). For non-oily seeds, reaching such low moisture is probably unattainable within suitable experimental time frames. Consequently, no biological or physiological significance has been assigned to  $K_E$ .

The practical determination of seed moisture content is easily achieved by gravimetry, before and after drying at a temperature of about 103°C. However, plant-water relations tend to be considered in the context of water potential (MPa), which can be related to the relative vapour pressure (RH) of a system via the partial molar volume of water (see Equation [15] and Sun, 2002). It is possible to determine seed water potential indirectly as RH (Cromarty *et al.*, 1982). Moreover, although a complex relationship, moisture content can be related to RH and temperature. Pixton and Warburton (1971a, b) noted a linear relationship between the logarithm of RH for linseed and the reciprocal of absolute (Kelvin) temperature at any given moisture content. Using this information, Roberts and Ellis (1989) modelled the ageing of lettuce seed in relation to RH. Whilst the moisture content term

<sup>4</sup> Note that  $\log m$  values are used to determine  $C_W$ , but  $m$  is shown in Figure 35.3 on a log scale.

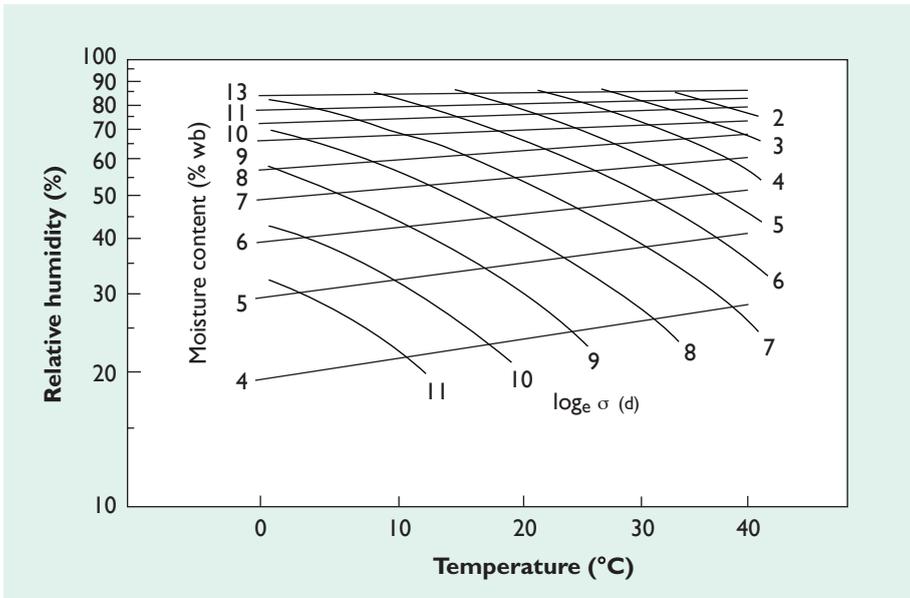


**Figure 35.3** Relations between the logarithm of longevity (d), log moisture content and temperature (°C, labelled) for lettuce seed. Equation [8] applies in the central unshaded region. The affects of ultra-drying and repair in the presence of air (open symbols) are shown to the left and right hand side of the figure, respectively. Longevity is presented as  $\sigma$  (dashed lines) and  $2\sigma$  (solid lines). Re-presented from Roberts and Ellis (1989) with permission of Elsevier.

of the viability equations has been shown to be unaffected by temperature, this is not the case when RH is considered (Figure 35.4). Now we observe that at a given moisture content, the RH of the seeds is predicted to decrease by about 10% as the temperature falls by 40°C. Thus, during seed storage there is an indirect effect of temperature on RH and thus on the water potential of the seeds. Roberts and Ellis (1989) investigated this effect by plotting interpolated  $\log\sigma$  values for lettuce seeds against equilibrium relative humidity (eRH) at various constant temperatures, and observed linear relations at each temperature. From the slopes, and on the basis of a similar analysis for barley seeds, it was proposed that

$$\log \sigma = K_E - C_W r - C_H t - C_Q t^2 \tag{14}$$

where  $r$  is relative humidity (%). For barley and lettuce seeds,  $C_W$  of Equation [14] had a common value of 0.0376, meaning that longevity increased 2.4-fold for each 10% fall in eRH. It is worthy of note that whilst Equation [14] is similar to Equation [8], the viability constants have different values. Moreover, the estimated temperature terms ( $C_H$  and  $C_Q$ ) also varied between barley and lettuce seeds, unlike the universal temperature constants (Roberts and Ellis, 1989).



**Figure 35.4** Relations between RH, temperature and seed moisture content (% wet basis or fresh weight) in linseed and longevity ( $\log_e\sigma$ , d) data for lettuce (curved lines), calculated from published constants. The straight lines reveal the dependency of moisture content on RH as it changes with temperature. Re-presented from Roberts and Ellis (1989) with permission of Elsevier.

As stated earlier, seed RH can be related to seed water potential ( $\psi$ , MPa), thus

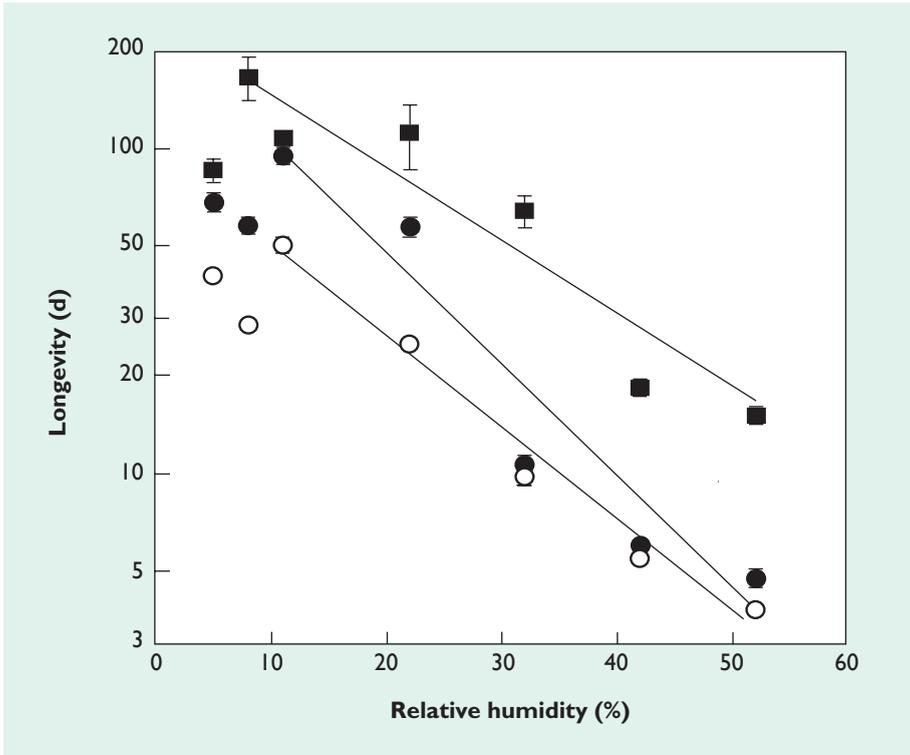
$$\psi = (RT/\bar{V}_W) \ln (e/e^\circ) \quad [15]$$

where  $\bar{V}_W$  ( $\text{cm}^3 \text{mol}^{-1}$ ) is the partial molar volume of water,  $R$  is the universal gas constant ( $8.314 \times 10^7 \text{ ergs mol}^{-1} \text{ deg}^{-1}$ ),  $T$  the absolute temperature and  $e/e^\circ$  is seed RH (i.e., vapour pressure of the seed/vapour pressure of water, and inserted in the equation as a proportion rather than a percentage). As a consequence, it is possible to modify Equation [14] as follows

$$\log \sigma = K_E - C_W 4.6052 + (\psi \bar{V}_W / RT) - C_H t - C_Q t^2 \quad [16]$$

Now this viability equation encapsulates the full effects of moisture on seed longevity, taking into account the dependency of seed RH or water potential on temperature. The importance of this is that it predicts that the water potential of the seed will alter when a sample is moved from one temperature to another. This matters because two key activities in seed conservation involve such a temperature shift from ambient (c.  $15^\circ\text{C}$  to  $25^\circ\text{C}$ ), where the seeds are routinely handled, to: 1) lower temperatures, for seed banking; and 2) higher temperatures, for accelerated ageing studies. We will return to the seed banking issue later under 'Complementary models,' and concentrate first on seed storage studies.

Generally, in seed ageing work an isotherm (the relationship between seed moisture content and RH) is determined at one temperature, close to ambient, and the ageing work carried out at an elevated temperature, for practical reasons (Ellis *et al.*, 1989, 1990b). Given the temperature dependency of isotherms, there are clearly limitations to this approach. However, when pre-storage humidity at one temperature ( $20^\circ\text{C}$ ) has been used to interpret longevity data at a different temperature ( $65^\circ\text{C}$ ), the longevity-RH relationship has been observed to be similar to that for longevity-eRH; for example, seeds of 12 and 8 crops had a  $C_W$  values of 0.0358 (Ellis *et al.*, 1989) and 0.0346 (Ellis *et al.*, 1990b) respectively. These values are equivalent to 2.2 to 2.3-fold improvements in longevity for each 10% fall in RH, very similar to those estimated for barley and lettuce using eRH (Roberts and Ellis, 1989). Moreover, seeds of other species that had been pre-equilibrated at one temperature and aged at about  $20^\circ\text{C}$  higher had similar  $C_W$  values: 0.034 for *Dactylorhiza fuchsii* (Druce) Soó (Pritchard *et al.*, 1999) (Figure 35.5); an average of 0.0293 and 0.0362 for two varieties of each of delphinium and salvia, respectively (Kwong *et al.*, 2001); and 0.031 for seeds of a *Salix* L. hybrid (Wood *et al.*, 2003). By comparison, lower  $C_W$  values have been reported for *Astronium urundeuva* (Allemão) Engl. seeds pre-equilibrated at  $6^\circ\text{C}$  and then aged at  $60^\circ\text{C}$  ( $C_W = 0.021$ ; Medeiros *et al.*, 1998) and two orchids, pre-equilibrated at  $16^\circ\text{C}$  and then aged at  $40^\circ\text{C}$  ( $C_W = 0.022$  and  $0.027$ ). For these three species, seed longevity would increase only 1.6- to 2.2-fold for each 10% fall in RH.



**Figure 35.5** Relations between seed longevity ( $\log\sigma$ , d) at 40°C and pre-storage RH at 16°C for three orchids: *Dactylorhiza fuchsii* (●), *Dendrobium anosmum* (○) and *Eulophia gonychila* (■). The effects of ultra-drying below c. 10 to 15% RH on reduced longevity are evident, these data not being including in the linear regressions. Fitted line parameters are: (●)  $\log\sigma = 2.37 - 0.034RH$ ; (○)  $\log\sigma = 1.98 - 0.027RH$ ; (■)  $\log\sigma = 2.39 - 0.022RH$ . Re-presented from Pritchard *et al.* (1999) with permission of the American Orchid Society, Inc.

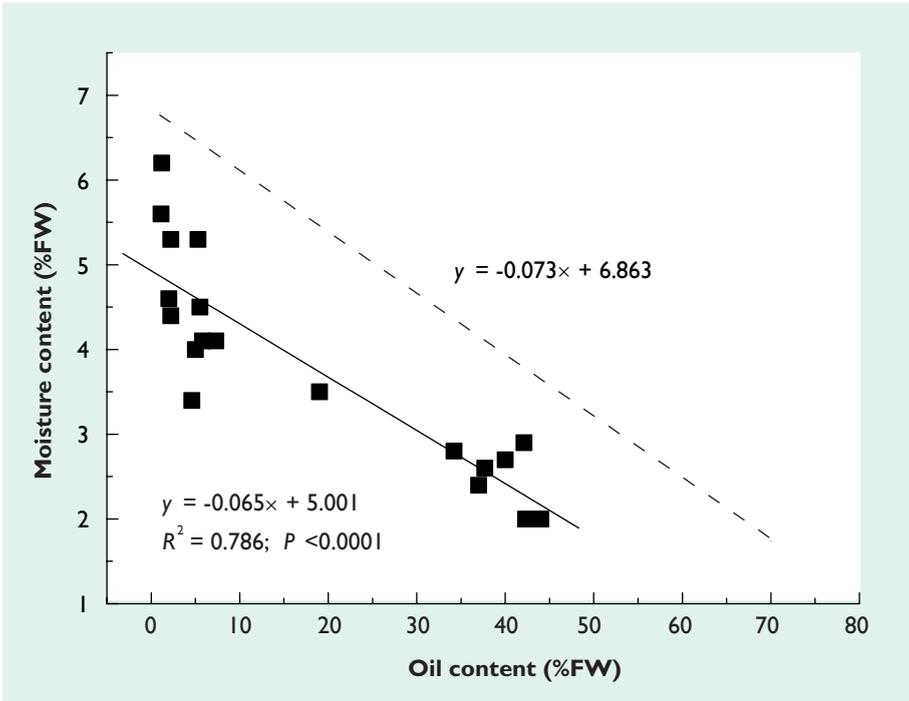
Thus, the general impression is that a 20°C to 40°C shift in temperature may not have a very large effect on the interpretation of the general relationship between longevity and RH. In support of this notion, Fang *et al.* (1998), who modelled isotherms, noted that orthodox seeds respond similarly to temperature and RH. Nonetheless, the apparently subtle overall differences in the sensitivity of seed longevity to reducing RH between species could be cumulatively large after dehydration to low RHs (e.g., Figure 35.5).

#### 4.1. Lower and upper moisture limits

Several investigations have shown that there is a low-moisture-content limit ( $m_c$ ) to the linear relations between the logarithms of longevity and moisture content. For example in Figure 35.3, this limit appears to be about 2.5% moisture content for lettuce seeds (Roberts and Ellis, 1989). Across 25 species, this limit has been estimated to vary between 2% and 6.2% moisture content for seeds stored at 65°C, corresponding to 10–15% RH at c. 20°C (Ellis *et al.*, 1988; 1989; 1990a; 1990b; Leon-Lobos and Ellis, 2003 – Chapter 40). Similarly, *Taxus brevifolia* Nutt. seeds stored best at 5–35°C when at 14% RH (Walters-Vertucci *et al.*, 1996) and, as Figure 35.5 reveals, the seeds of three orchids had maximal longevity at 40°C when pre-treated to c. 11% RH at 16°C (Pritchard *et al.*, 1999). When much of the data on these diverse species is combined, a negative linear relationship [ $y = 5.00 - 0.065x$  ( $R^2 = 0.786$ )] is observed between the value of  $m_c$  (% wet mass) and oil content (% dry mass) (Figure 35.6, 20 species).

In contrast, it has been proposed that maximum longevity at 35°C for seeds of a range of species (see Vertucci and Roos, 1990; Walters-Vertucci *et al.*, 1996) corresponds to moisture contents in equilibrium with 22% RH at this temperature (Walters *et al.*, 1998). Moreover, this optimum moisture content for storage is negatively related to oil content ( $y = 6.86 - 0.073x$ ) (recalculated for 6 species from Walters *et al.*, 1998). When compared with the  $m_c$  – oil content relationship, it is noticeable that both relationships have very similar slope parameters (-0.065 cf. -0.073), with the plotted lines separated by < 2% moisture content (Figure 35.6). What might happen to seeds within, and below, this moisture content divide is considered in a later section (Interaction between temperature and moisture).

An upper limit to the relationship between longevity and moisture content has also been identified, above which longevity increases when oxygen is present (see Figure 35.3). In sesame, seed longevity at 50°C is greater than predicted by extrapolation from lower moisture contents when above 15% (data point at 20.3%). Other species where an upper moisture content limit has been identified include: onion, *Allium cepa* (18%; Ellis and Roberts, 1977); barley, *Hordeum vulgare* (24%; Ellis and Roberts, 1980b); lettuce, *Lactuca sativa* L. (15%; Ibrahim *et al.*, 1983); niger, *Guizotia abyssinica* (L.f.) Cass. (22%; Zewdie and Ellis, 1991a); tef, *Eragrostis tef* (Zucc.) Trotter (24 to 28%; Zewdie and Ellis, 1991a); and durum wheat (26%; Petruzzelli, 1986). This upper limit approximates to about 90–93% RH, water potentials of about -10 to -20 MPa (Roberts and Ellis, 1989; Zewdie and Ellis, 1991a) and the onset of sorption zone III of the water sorption isotherm (see Roberts and Ellis, 1989; Sun, 2002; Probert, 2003 – Chapter 19).



**Figure 35.6** Dependence of critical moisture contents (% fresh weight) for seed survival on oil content (% dry weight). The critical moisture contents are defined as: the low-moisture-content limit to the logarithmic relations between  $\sigma$  at 65°C and moisture content, for 20 species (■, solid line; see Ellis *et al.*, 1988, 1989, 1990a, b); and the moisture content optimal for longevity at 35°C, based on vigour after storage for 6 species (dashed line, see Walters *et al.*, 1998b). Fitted lines are shown on the graph. Oil content data are taken from Tweddle *et al.* (2003) and M. Sacandé (Royal Botanic Gardens, Kew, pers. comm.).

## 5. Complementary Longevity Models

### 5.1. Potential storability index and storage environment coefficient

Tang and co-workers (TeKrony *et al.*, 1999; Tang *et al.*, 2000) developed a model to better predict the decline in hybrid corn seed, based on the potential storability index (PG, the time for germination to decline to a level G) and the storage environment coefficient (SEC, the factor by which seed longevity alters by a change in temperature and moisture content). The model maintains a quantitative relationship between seed longevity, initial seed quality and the storage environment via four variables derived from the ratio of seed longevity of the same seed lot in two environments, thus:

$$P_{1,50} = P_{2,50} 10^{[-C_1\Delta m - C_3t - C_5\Delta(tm) - C_6\Delta(t \log m)]} \quad [17]$$

where  $P_{1,50}$  and  $P_{2,50}$  are the half viability periods (days) in environments 1 and 2,  $\Delta m$  is the difference in seed moisture,  $\Delta t$  is the difference in temperature,  $\Delta(tm)$  is the difference of the product of moisture and temperature,  $\Delta(t \log m)$  is the difference of the product of log moisture and temperature, and  $C_{1,3;3,5;6}$  are constants. The benefit of this approach is that it avoids the need to determine  $K_E$  of Equation [9] (Tang *et al.*, 2000). Good agreement was observed between predicted [Equation (17)] and observed (by probit analysis) SEC values for three medium-to-high vigour lots of hybrid corn (TeKrony *et al.*, 1999). Predicted [Equation (17)] and observed (probit analysis)  $P_{50s}$  were also in close agreement. However, Equation [9] (Ellis and Roberts, 1980a) predicted a much shorter longevity at 12% moisture content and 30°C than observed with three seed lots; although Equation [9] worked well for seeds aged at 10°C higher (TeKrony *et al.*, 1999). The suggestion was made that the constants from one seed lot are applicable to other seed lots of similar initial quality (TeKrony *et al.*, 1999). In a follow-up study on inbred corn, however, TeKrony *et al.* (2000) noted that as  $P_{50}$  increased with improved storage conditions (cooler and/or drier), the predicted values were much larger than those observed. Such overestimation in seed longevity limits the usefulness of the alternative model. So for corn it appears that neither the Ellis and Roberts (1980a) nor the Tang *et al.* (2000) models accurately predict longevity at the lower temperatures commonly used by the seed industry to store seed (TeKrony *et al.*, 2000).

## 5.2. Longevity and the glass transition (T<sub>g</sub>)

Seed ageing in the dry state may involve many physical and chemical changes, including free radical activity, lipid peroxidation and non-enzymatic reactions (Priestley, 1986; Walters, 1998). The rates of at least some ageing reactions are thought to depend largely on the mobilities (diffusion) of molecules and this will be related to the viscosity of the aqueous phase of the seeds; the greater the viscosity, the slower the reactions. Indeed, seed viscosity under air-dry conditions is so high that seeds are considered to be in the glassy state (Williams and Leopold, 1989; Walters, 1998), i.e., a fluid with the mechanical properties (strength) of a solid. As with longevity, the glass transition temperature (T<sub>g</sub>) in seeds (and other biological materials) is dependent on moisture content, which suggests that there may be a direct link between seed viscosity and longevity. As a consequence, new models for seed longevity have been proposed in the last 10 years that are complementary to the original seed viability equations (Sun and Leopold, 1994; Sun, 1997; Walters, 1998; Buitink, 2000). These models rely on the use of a different set of 'constants' in viability equations that reflect the biophysical basis of longevity rather than an empirical description of seed population decline.

The glass transition temperature (T<sub>g</sub>) in seeds varies with moisture content, e.g., in soybean ranging from about -20°C at 15% MC to around 50°C at 8% MC (Sun and Leopold, 1994). It follows that air-dry soybean will be in the glassy state at room temperature, thus explaining (at least in part) their considerable longevity (years) under such conditions.

A more detailed analysis of how longevity, as predicted from viability constants, might be linked to Tg was undertaken by Sun and Leopold (1994). They showed similarities between the phase diagram (i.e., the relationship between Tg and moisture content) and the temperature at which pea, soybean and maize seeds had a mean viability period ( $\bar{p}$ ) of 50 years, based on a modification of the seed viability Equation [8], thus:

$$m = 10^{(\log_{10}K_I - \log_{10}\bar{p} + K_E - C_H t - C_Q t^2) / C_W} \quad [18]$$

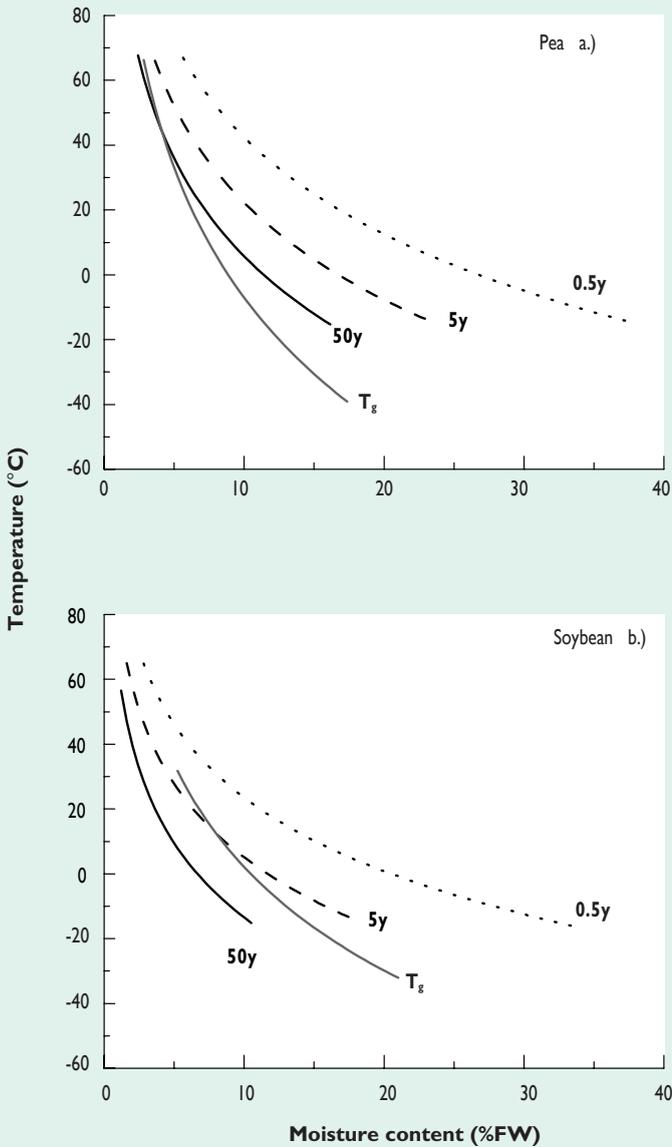
where  $\bar{p}$  is the mean viability period in days,  $m$  is moisture content on a fresh mass basis and  $K_I$ ,  $K_E$ ,  $C_W$ ,  $C_H$  and  $C_Q$  the viability constants as defined in equations [6] and [8].

In Figure 35.7 we show recalculated data for pea and soybean and include additional analyses for mean viability periods of 5 years and 0.5 years based on published viability constants. There is a semi-logarithmic relationship between temperature for mean viability period and moisture. A similar relationship was observed for Tg and moisture content, based on interpolated Tg values from Sun and Leopold (1994). Some species-dependent differences are evident, with the Tg line closest to the 5-year and 50-year mean viability periods for soybean and pea seeds, respectively (Figure 35.7). Moreover, Tg appears to be more sensitive to changing moisture content in pea than in soybean. Thus, the relationship between the glass transition and longevity in pea (Figure 35.7a) and soybean (Figure 35.7b) is different, perhaps as a consequence of subtle differences in the nature of their intracellular glasses (Sun, 1997). Nonetheless, these analyses appear to be consistent with the known dependency of longevity improvement on moisture content, such that  $C_W$  tends to be smaller in oily (e.g., soybean) compared to non-oily (e.g., pea) seeds.

Another feature of the data in Figure 35.7 is that the Tg line is steeper than the mean viability period lines, for both pea and soybean. This means that Tg might not be a perfect predictor of longevity at both low and higher moisture contents. Several suggestions have been offered as to why this might be the case, including the non-equilibrium nature of glasses and the complexity of the intracellular structures involved in the glassy matrix, such that water may not act solely as a solvent of the glass or induce uniform changes in viscosity (Leprince and Walters-Vertucci, 1995; Sun, 1997).

By interpolation from the information in Sun and Leopold (1994), and the information in Figure 35.7, we estimate that the mean viability period in pea and soybean would be about 200 years when seeds are stored c. 20°C to 40°C below Tg. From a practical point of view, such longevity is probably acceptable to most gene bank managers.

The observation has been made that the maximum storage stability (measured as radicle length  $\times$  % germination after storage) for pea (Vertucci and Roos, 1990) corresponds to the Tg/moisture content relationship in bean, e.g., Tg is c. 35°C when seed moisture content c. 7% (Leprince and Walters-Vertucci, 1995). In addition, though, it has been noted that the maximum heat capacity



**Figure 35.7** Relations between moisture content (% fresh weight) and both the glass transition ( $T_g$ , °C) and mean viability period (years) for pea (a) and soybean (b).  $T_g$  data are interpolated from Sun and Leopold (1994) and shown as solid grey lines. Mean viability periods are shown for 50 (solid lines), 5 (dashed lines) and 0.5 years (dotted lines) based on published viability constants: pea,  $K_E = 9.858$ ,  $C_W = 5.39$ ,  $C_H = 0.0329$  and  $C_Q = 0.000478$  (Ellis *et al.*, 1989); and soybean,  $K_E = 7.525$ ,  $C_W = 4.086$ ,  $C_H = 0.0329$  and  $C_Q = 0.000478$  (Dickie *et al.*, 1990).

of the glass in dry bean seeds corresponds to c. 5.5% moisture content, which means that in ageing studies at 35°C, the seeds would be about 30°C below T<sub>g</sub> (i.e., T<sub>g</sub>-30; see Leprince and Walters-Vertucci, 1995). Similarly, it has been suggested that optimum storability in *Typha latifolia* L. pollen is around T<sub>g</sub>-40, when there is an upward shift in heat capacity of the glass (Buitink *et al.*, 1996; Walters, 1998; Buitink, 2000).

Under what conditions then are seeds held in the gene bank? If we accept that soybeans at c. 18% (dry mass) oil content would equilibrate to about 5% MC at 15% RH and 20°C (Vertucci and Roos, 1993), then conventionally stored (-20°C) soybeans would be at approximately T<sub>g</sub>-65. The use of such storage conditions may be important to reduce the likelihood of crystallisation of the aqueous glass, which would lead to an accelerated rate of deterioration (Bernal-Lugo and Leopold, 1998). In model solutions, including sucrose, it has been determined that it is necessary to cool to at least T<sub>g</sub>-50 before the molecular motions could be considered to be negligible over the lifetime (multiple year) of a typical pharmaceutical product (Hancock *et al.*, 1995; Hancock and Shamblin, 2001). Thus, it is conceivable that the thermodynamics of seed stability are similar to pharmaceuticals. But is there practical evidence to support the theory that seed storage at just less than T<sub>g</sub>-50 is appropriate?

It is estimated that T<sub>g</sub> is c. -60°C in seeds dried to c. 20% moisture content (Sun and Leopold, 1994) and thus T<sub>g</sub>-50 would therefore be ≈ -110°C. In such relatively moist material, there would be considerable risk of crystallisation during storage unless temperatures were a minimum of those in the vapour phase above liquid nitrogen, i.e., ≤ 130°C. In other words, storage at T<sub>g</sub>-70. For very dry seeds also, T<sub>g</sub>-70 appears to provide excellent conditions for storage. Assuming that ultra-dry (about 3.1% moisture) seeds of barley and oat seeds, which survived 110 years at 10°C to 15°C (Steiner and Ruckenbauer, 1995), had a phase diagram similar to those published so far for seeds, then this exceptional longevity was achieved at around T<sub>g</sub>-70 (Pritchard, 2004). Finally, and as mentioned above, T<sub>g</sub>-70 does appear to provide storage conditions very close to the current international standards in gene banks, i.e., when seeds are at 3-7% seed moisture content and -18°C (Genebank Standards, 1994).

### 5.3. WLF kinetics

A second analysis of the relationship between longevity and the glassy state was provided by Sun (1997), who related seed ageing to the temperature dependence of mechanical relaxation processes in amorphous systems above T<sub>g</sub> as described by the Williams-Landel-Ferry (1955) equation. Accepting that ageing is a diffusion-limited reaction, longevity can be modelled using a modified WLF equation, thus:

$$\ln(K_g/K) = -C_1(T-T_g)/(C_2+T-T_g) \quad [19]$$

where  $K$  and  $K_g$  are the reaction rate constants at  $T$  and at  $T_g$ , respectively, and  $C_1$  and  $C_2$  are derived constants (Sapru and Labuza, 1993). With respect to storage stability,  $K$  was equal to  $1/\sigma$  in Equation [6] (Sun, 1997). The rates of seed viability loss for bean, pea and soybean were functions of  $T-T_g$  (in the region  $20^\circ\text{C}$  to  $90^\circ\text{C}>T_g$ ) and fitted the WLF kinetics well, such that  $\log\sigma$  was negatively related to temperature above  $T_g$  in a concave, curvilinear fashion (Sun, 1997). The analysis revealed that pea and bean were almost identical in their storage responses, but that soybean was both shorter-lived and more sensitive to  $T>T_g$ , i.e., increasing temperature above  $T_g$ . This latter effect is, however, temperature independent, as the analysis was performed on seeds at variable moisture content while storage temperature was kept constant. The conclusion is that above  $T_g$ , temperature and moisture content may have independent effects on longevity, as suggested by the Ellis and Roberts (1980a) seed viability equation.

The derived constants of the WLF equation (Sun, 1997) suggest that seed intracellular glasses are readily crystallisable, based on the classification of Slade and Levine (1991). Crystallisation of intracellular glasses, i.e., devitrification, in seeds would presumably contribute to accelerated ageing, as proposed by Bernal-Lugo and Leopold (1998). Moreover, Pritchard (2004) has hypothesised that devitrification in Type II seeds during storage at some sub-zero temperatures could be a contributory factor to rapid viability loss. A contrary view is that this is unlikely as degradative reactions in a glass might be faster than the relaxation processes, including crystallisation (Leprince and Walters-Vertucci, 1995).

#### 5.4. Molecular mobility

It is thought that the high viscosity of intracellular glasses decreases molecular mobility and slows deleterious reactions, thus enhancing longevity. Molecular mobility can be determined using saturation transfer electron paramagnetic resonance (ST-EPR) spectroscopy to measure the rotational motion of a spin probe which is taken up by imbibing seeds (Buitink *et al.*, 2000b). Buitink *et al.* (2000b) investigated the relationship between ageing rates and rotational motion in the cytoplasm of pea and *Impatiens walleriana* Hook.f. seeds and also in the Type I-behaved pollen of *Typha latifolia*. For pea axes at 6.5% moisture content, there was a systematic increase in rotational correlation time for the probe as temperature decreased until  $T_g$ ; further cooling resulted in a continued lengthening in correlation time, but the temperature dependency was smaller. The rate of vigour loss (taken from Vertucci *et al.*, 1994) followed the same biphasic pattern, with improvements in longevity being evident as temperature reduced below  $T_g$  (Buitink *et al.*, 2000b). Similarly, there was a linear relationship between  $\log\sigma$  for pea seeds at 7 to 14% moisture content and the logarithm of rotational motion for pea axes. Extrapolations then enabled predictions of pea seed longevity at different moisture contents and temperatures (Buitink *et al.*, 2000b). These revealed that for all storage temperatures ( $35^\circ\text{C}$  to  $-18^\circ\text{C}$ ) the viability equation and its constants

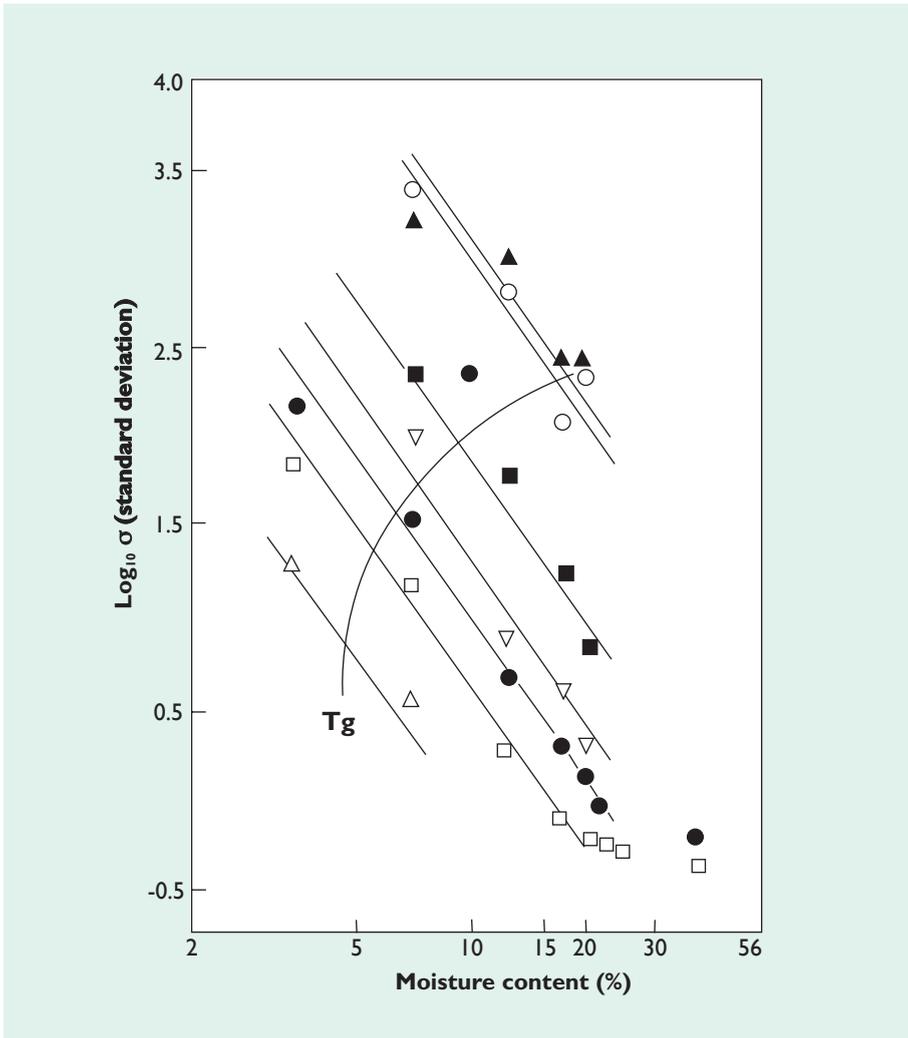
overestimated longevity ( $\sigma$ , years) for dry seeds (c. 7% moisture content), but underestimated longevity for wetter seeds (c. 14% moisture content) compared with projections based on the rotational mobility of the spin probe. The cause of this systematic difference in extrapolations is not clear, but appears to relate to being below and above  $T_g$  respectively.

It was also shown that the slopes of the relationships (plotted on a double log scale) between ageing rate (i.e., the reciprocal of  $\sigma$ ) and rotational correlation time were similar (i.e., -2.4 to -3.1) for *Typha latifolia* pollen, pea and *Impatiens* L. seeds (Buitink *et al.*, 2000b). This suggests a similar relative effect of changing viscosity/molecular mobility between species (Sun, 1997; Buitink *et al.*, 2000b). Interestingly though, longevity of *Typha* pollen < pea < *Impatiens* at the same rotational correlation time, suggesting inherent differences between species even when mobility is similar. Given that reduced longevity of osmo-primed impatiens (*Impatiens walleriana*) and bell pepper (*Capsicum annuum* L.) seeds is not associated with the increased molecular mobility of the cytoplasm, as determined by the polar spin probe 3-carboxy-proxyl (Buitink *et al.*, 2000a), there is clearly much to be learned of the molecules responsible for the detrimental reactions impacting on the kinetics of longevity (Walters, 1998).

### 5.5. A glass ceiling?

Considerable evidence now points to the value of considering seed ageing studies in relation to  $T_g$  (Sun and Leopold, 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997; Walters, 1998; Buitink, 2000). Thus, correlations can be observed between  $T_g$ ,  $\sigma$  and the mean viability period for seed longevity, and these suggest that viscosity is one of the main drivers of seed survival. It might be expected then that enhanced seed viscosity on entering the glassy state should have a positive impact on seed longevity and this might be observable in co-plots of  $\log \sigma$  against  $\log$  moisture content. However, when  $T_g$  for neem seed axes from Sacandé (2000) is superimposed on longevity data for *Ulmus carpinifolia* (Tompsett, 1986), no clear deviation in the longevity/moisture relationship is suggested as moisture is removed from the glass (Figure 35.8); longevity continues to increase. Of course, a major limitation of this analysis is that the  $T_g$  and longevity data relate to different species. However, this approximation would hardly alter if the phase diagrams for bean axes (Leprince and Waters-Vertucci, 1995) or *Typha latifolia* pollen (Buitink *et al.*, 1998a, b) were used instead.

It has been suggested that there is a 'glass ceiling' to pollen longevity and the inference has been made that this corresponds to the low-moisture-content limit to the logarithmic relations between  $\sigma$  and moisture content (Buitink *et al.*, 1998a,b; Hong *et al.*, 1999a). Moreover, it has been suggested that removal of water from intracellular glasses might increase ageing rates and/or decrease storage stability (Leprince and Walters-Vertucci, 1995; Buitink *et al.*, 1998a, b; Walters, 1998). We will return to the subject of ultra-dry seed storage later (see 'Beyond the limits').



**Figure 35.8** Linear relations between  $\sigma$  (log d) and moisture content (% fresh weight, log scale) for *Ulmus carpinifolia* seeds when stored at 52°C ( $\Delta$ ), 42°C ( $\square$ ), 36°C ( $\bullet$ ), 31°C ( $\nabla$ ), 21°C ( $\blacksquare$ ), -13°C ( $\blacktriangle$ ) and -75°C ( $\circ$ ). The curvilinear line for the glass transition (Tg) in neem axes is also shown, as interpolated from Sacandé (2000). Longevity data are from Tompsett (1986) with permission of Elsevier.

Perhaps some of the contradiction in general viewpoints about critical moisture contents for longevity and  $T_g$  stems from the complex nature of glasses; the water contained therein does not behave as an ideal solvent, unlike in model (sugar solution) systems. This makes the interpretation of glassy behaviour difficult, particularly when comparing studies in which various methods of determination and interpretation have been used, e.g., mid-point and onset-point of  $T_g$  (see Leprince and Waters-Vertucci, 1995; Sun, 1997; Buitink *et al.*, 1998a, b). Moreover, the glassy state is a non-equilibrium state with an effectively infinite response time, so its determination is strongly influenced by rate (experimental) effects. And these experimental effects are determined on a much shorter time scale (orders of magnitude different) than seed ageing studies. Thus, it is actually difficult to be unequivocal about the precise relationship between the determined  $T_g$  and longevity. Nonetheless, considering seed ageing in relation to the glassy state has been shown to be a very valuable innovation in seed science in the last fifteen years (Williams and Leopold, 1989; Sun and Leopold, 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997; Buitink *et al.*, 1998a,b; Walters, 1998; Buitink, 2000).

### 5.6. Thermodynamics

Discrete changes in water properties in seeds coincide with changes in physiological activity possibly as a result of the properties of water at macromolecular surfaces affecting metabolism. In other words, the thermodynamic status of water controls seed ageing reactions, and these happen if they are thermodynamically favoured (Walters, 1998). By comparison, in glassy models, reactions are slowed because of a physical barrier (the structure of the glass) to the movement of molecules, the reactants for ageing. However, both of these mechanisms, glass behaviour (discussed above) and thermodynamics (discussed here), appear to be relevant to our perception of seed ageing.

The easiest way to determine the thermodynamic status of water in seeds is via the water sorption isotherm, that relates moisture content to RH [e.g., Probert (2003) – Chapter 19]. As noted earlier (see ‘Moisture and seed ageing’), RH can be related to water potential ( $\Psi_w$ ) via Equation [15], providing an opportunity to relate moisture content to the thermodynamically more relevant parameters of water activity,  $a_w$  ( $RH/100 = a_w$ ), and  $\Psi_w$ . Both of these parameters are informative about the availability of water for chemical reactions (Roberts and Ellis, 1989; Vertucci and Roos, 1990; Walters, 1998). Based on vigour determinations following ageing, mainly at 35°C, Walters (previously Vertucci) and co-workers have proposed that the optimum conditions for seed storage coincide with a water activity of 0.14 to 0.22 (equivalent to RH of 14 to 22%) (see Walters, 1998; Walters, *et al.*, 1998b). In contrast, Ellis and co-workers have linked the low-moisture-content limit to the relations between longevity ( $\log\sigma$ ) and  $\log$  moisture content to a slightly lower water activity of about 0.10 (equivalent to RH of 10%) (Ellis *et al.*, 1988, *et seq*). These humidities approximate to the saturation of strong water binding sites, and are presumed to relate indirectly to the interface between water sorption zone I and II (see Roberts and Ellis, 1989;

Walters, 1998; Sun, 2002). These subtle differences in critical RHs suggest differences in optimum or maximum moisture contents for longevity (see 'Ultra-dry storage'). In addition, though, thermodynamic considerations dictate that these critical moisture contents change with temperature.

There is a general acceptance that temperature does influence the equilibrium RH of seeds (Roberts and Ellis, 1989; Vertucci and Roos, 1990; Figure 35.3), and as articulated in Equation [16]. At constant water content, a rise in temperature will cause an increase in water activity. Alternatively, if the water activity is kept constant and temperature altered, the moisture content changes: moisture content increasing when temperature decreases, and vice versa [see Probert (2003) – Chapter 19]. Because of these effects, it has been proposed on thermodynamic grounds that seeds should be pre-treated to around 60% RH prior to ultra-low temperature storage (e.g.,  $-150^{\circ}\text{C}$ ), as such cooling will effectively lower the seed eRH to around the optimum (Vertucci and Roos, 1993). For pea seeds, this is equivalent to a moisture content of c. 14%, which is more than twice the optimum (c. 6%) moisture content predicted for storage at  $35^{\circ}\text{C}$ . By a similar argument, the optimum moisture content for the storage of pea seed at  $-20^{\circ}\text{C}$  can be interpolated from isotherms to be about 11%, the inference being that equilibration to c. 6% moisture content in gene bank drying rooms will be excessive with respect to subsequent cold storage in the bank. Such predicted changes in the critical moisture content for storage are supported by molecular mobility projections for pollen and seeds (Buitink, 2000; Buitink *et al.*, 2000b; Buitink and Hoekstra, 2003 – Chapter 37).

At a practical level, such large changes in critical moisture contents for longevity ( $\sigma$ ) are less apparent when ageing seeds in closed containers, e.g., lettuce at 65 and  $35^{\circ}\text{C}$  (Ellis *et al.*, 1988; 1989; 1995) and coffee, at 15 to  $-20^{\circ}\text{C}$  (Hong and Ellis, 2002). Such observations sparked a lively debate in the literature about how longevity theory and practice come together (see Ellis *et al.*, 1991c; Vertucci and Roos, 1991; Smith, 1992). A key issue here appears to be how much seed isotherms change with temperature, and our perception of this might depend on the methodology used. There appear to be two main methods used for isotherm presentation: after equilibration above saturated salt solutions, seed moisture content is plotted against tabulated RHs (Vertucci and Roos, 1990, *et seq*) or against eRH determined on the seeds at the appropriate temperature (Hay *et al.*, 2003). In the first case, seed moisture is projected to alter by 4 to 5% over  $50^{\circ}\text{C}$ , compared with about 1% by the latter method. Recently, Fang *et al.* (1998) developed a three-dimensional model to represent seed moisture content as a function of RH and temperature, showing that a temperature shift from  $50^{\circ}\text{C}$  to  $5^{\circ}\text{C}$  has only a slight negative effect on moisture content. On these grounds it is perhaps the case that the optimal moisture content for storage does not change over the usual gene bank operational temperatures (dry room to bank) as much as previously suggested by Walters and co-workers. As discussed above, and in Walters (1998), seeds do not have the characteristics of ideal solutions/model systems with respect to glassy properties. Some aspects of their sorption properties appear similarly to be non-ideal.

## Seed Viability Equations:

### *Assumptions and Improvements*

#### 1. Normal Distribution of Survival and the Probit Model

The probit model has been applied for more than 70 years to dose-response studies, initially testing for toxicity responses (Bliss, 1934). Its application to seed survival curves assumes that the distribution of seed deaths in time is normally distributed (Ellis and Roberts, 1980a). This assumption was tested by Tang *et al.* (1999). Eleven corn seed lots with a wide range of initial vigour were stored in various combinations of constant temperatures (20, 30, 40 and 50°C) and moisture contents (10, 12, 14 and 16% fresh weight basis), and the goodness-of-fit assessed on full or truncated (i.e., only including the data where germination is between 5 and 95%) data sets. When the data were truncated, the majority (79%) of the 187 survival curves analysed were classified as normal or near normal. In comparison, only 57% of the curves from the full data set followed a normal or near-normal distribution. It follows that survival of a low-vigour seed lot is more likely to be normally or near-normally distributed than a high-vigour seed lot. In contrast, there was good agreement between the predictions of the viability equation and practice when the initial viability of maize seed ( $K_i$ ) corresponded to 99.5 to 99.95% (Parkes *et al.*, 1990).

Probit transformed survival curves can deviate from linearity in lettuce, field bean and soybean, with the highest initial germination data points contributing to the significant deviation (Kraak and Vos, 1987; Wilson *et al.*, 1989; Fabrizius, 1998). This means that estimating the initial quality ( $K_i$ ) of a seed lot using the probit model has some inherent dangers. Nonetheless, in a study of seed longevity of pearl millet [*Pennisetum americanum* (L.) Leeke], comparing the use of probit analysis, Weibull (four-parameter), Richards and polynomial functions, Moore and Jolliffe (1987) found that all had some weaknesses in estimating initial seed quality ( $K_i$ ).

There are numerous potential contributory factors to inaccurate  $K_i$  determination, including the presence of empty or insect-infected seeds. These seeds are effectively non-respondents and these should be accounted for when analysing ageing data (see 'Control Mortality').

#### 2. Control Mortality

Probit analysis of field bean (*Phaseolus vulgaris* L.) seed ageing data without adjustment for initial or 'threshold response rate' (failure to germinate) yielded significant chi-square values (Wilson *et al.*, 1989). When the threshold response rate was fixed at 6% the fit of the ageing curves was improved and differences between the intercepts lost their significance as estimates of initial germination.

Similarly, Mead and Gray (1999) suggested a modification to the viability model based on the control mortality model developed for insecticide bioassays. Using Abbot's formula (Abbot, 1925), Finney (1977) corrected bioassay data to take into account the proportion of insects that would die irrespective of the insecticide dose being applied, i.e., the 'control mortality.' For seed ageing studies, control mortality can be incorporated into the Ellis and Roberts (1980a) viability equation thus:

$$\%v = 100 \times C_v \times \phi \left( \frac{K_i - p/10}{K_E - C_W \log m - C_H t - C_Q t^2} \right) \quad [20]$$

where  $\phi$  is the cumulative normal function,  $K_i$  is the initial viability of the responding part of the population in NEDs (see Figure 35.1),  $C_v$  is the proportion of responding seeds. Where the proportion of non-respondents is equal to zero,  $C_v = 1$  and the equation is equivalent to the original Ellis and Roberts (1980a) equation (Mead and Gray, 1999; Hay *et al.*, 2003). This new control viability model for seed ageing fitted well data from both carrot (Mead and Gray, 1999) and *Arabidopsis thaliana* seed storage experiments (Hay *et al.*, 2003).

### 3. One- and Two-step Fitting

The conventional approach to determining seed viability constants is to carry out probit analysis on the germination counts from each temperature-moisture content combination (environment), and then to regress the log of the inverse of the slope (i.e.,  $\sigma$ ) on percentage seed moisture content for each storage temperature (see Ellis and Roberts, 1980a). In other words, the analysis is performed in two-steps. One limitation of this approach is that all the environments are equally weighted in the regression of  $\log \sigma$  against  $\log$  moisture content, irrespective of the number of data points in the survival curves from which the  $\sigma$  values, and their standard errors, are derived. As a consequence the viability parameter estimates will tend to be less precise. A more equitable approach would be to perform probit analysis jointly for all environments (Wilson *et al.*, 1989; Hay *et al.*, 2003). Moreover, the probit transformation creates nonhomogeneity of variance and so Equation [9] cannot be used as a model for regression (Wilson *et al.*, 1989).

Wilson *et al.* (1989) implemented a nonlinear regression procedure (SAS Institute, 1982), which involved the weighting of binomial data points through the inclusion of angular transformation, thus

$$ga = \arcsin \sqrt{\phi \left( \frac{K_i - p/10}{K_E - C_W \log m - C_H t - C_Q t^2} \right)} \quad [21]$$

where  $ga$  = germination angle and  $\phi$  is a function that computes the probability that a random variable with a normal (0,1) distribution falls below the argument of the function. When studying field bean data, Wilson *et al.* (1989) initially took parameter estimates from earlier work on cowpea (Ellis *et*

*al.*, 1982), but ultimately determined the constants for field bean of  $K_E = 9.08$ ,  $C_W = 5.20$ ,  $C_H = 0.0057$  and  $C_Q = 0.00079$  with  $K_i = 6.68$ . The estimates were comparable to those determined using the two step method.

Hay *et al.* (2003) have adopted a similar one-step approach, to the analysis of *Arabidopsis thaliana* seed ageing data, using the general non-linear model fitting facilities of GenStat. Again, both the one- and two-step models appeared to provide reasonable fits to the data (Hay *et al.*, 2003). However, both  $K_E$  and  $C_W$  were found to differ significantly between two ecotypes of the species using the one-step analysis, whereas this was not the case for two-step fitting (Hay *et al.*, 2003). Thus, with the one-step approach it is more likely that it will not be acceptable to constrain parameters to a common value for all seed lots. As a consequence, it seems likely that the notion of viability constants being immutable at the species level will be increasingly challenged in time.

#### 4. Effective Mean Temperature

A common assumption of the improved viability equation (Ellis and Roberts, 1980a) is that there is no effect *per se* of temperature alternation on longevity. This is an important point as a feature of any seed bank operation is the periodic assessment of seed viability, a process that requires seed lots to be withdrawn from the bank and warmed up before sowing for germination.

To address this question, the effects of temperature on seed longevity have been estimated on the basis of storage under sets of constant temperature conditions. Of course, it would not be sufficient in dealing with fluctuating temperature to take the arithmetic mean of the temperature since viability is logarithmically (rather than linearly) related to temperature (Roberts, 1973). As a simple aid to applying Equation [2] to fluctuating conditions, Roberts (1963) suggested an equation for estimating the ‘effective temperature’ during storage, thus

$$T_E = \frac{\log \left\{ \frac{\sum [\text{antilog}(tC_2) \times w]}{100} \right\}}{C_2} \quad [22]$$

where  $T_E$  is the effective temperature (substituting  $t$  in Equation [2]),  $t$  is the recorded temperature ( $^{\circ}\text{C}$ ),  $w$  is the percentage time spent at each temperature  $t$ , and  $C_2$  is the value of the constant applied in Equation [2]. This equation was used by MacKay and Flood (1968) to assess the performance of wheat seeds held under fluctuating temperatures, finding that the actual and predicted mean viability periods were similar when the seeds were at 16–20% moisture content. At lower moisture contents, actual mean viability periods were greater than predicted.

Parkes *et al.* (1990) confirmed the value of using an expression that gives effective mean temperature for diurnal temperature fluctuations using winter

wheat (*Triticum aestivum*). Two more recent studies on tomato (*Lycopersicon esculentum* Mill.) seeds (Hung *et al.*, 2001) and conidia of *Metarhizium flavoviride* W. Gams & Rozsypal (Hong *et al.*, 1999b) also showed that the loss in probit viability is solely a function of the current storage environment, with no effect of the change in temperature *per se*. This purely additive effect of storage temperature suggests little risk to seed bank collections from repeated access (warming and cooling). This view is supported by the observation of only slight differences in germination in seed lots of four species subjected to 200 cycles of cooling-warming between 15°C and -10°C (Specht *et al.*, 2000).

## 5. Interaction Between Temperature and Moisture

The Ellis and Roberts (1980a, *et seq.*) seed viability model views moisture content and temperature as independent variables, whilst accepting that temperature has a relatively small effect through RH on moisture content over a c. 40°C range (Roberts and Ellis, 1989). Moreover, the theoretical model of longevity (Vertucci and Roos, 1990, *et seq.*) defines moisture in terms of a critical water activity (or RH), the corresponding moisture content for which must change with temperature. Thus, moisture content and temperature are inter-dependent variables, although the temperature effect is small (Fang *et al.*, 1998). As discussed under ‘Thermodynamics’ and ‘Ultra-dry storage’ these two longevity models can be brought closer together, if we accept the proposition that seeds do not ‘behave’ as model glass or sorption systems.

# Application of the Conventional Seed Viability Model

## 1. Seeds

### 1.1. How to generate species constants

The amount of work needed to determine a full set of viability constants ( $K_E$ ,  $C_W$ ,  $C_H$ ,  $C_Q$ ) is considerable and the quantity of seeds used may be large compared to the conservation collection size. However, only four germination tests are needed, theoretically, to determine the four viability constants in the original viability equation (see Roberts, 1973). Initially, the recommendation was that the tests should relate to three sets of conditions, including at least two moisture contents and two temperatures. In the first environment, germination after two storage times allows the determination of the slope of the survival curve and from this the values of the mean viability period ( $\bar{p}$ ) and standard deviation ( $\sigma$ ) can also be calculated. By substituting these values in Equation [1], the value of  $K_o$  is determined. Then for two more storage environments, single germination tests are used to estimate survival curves

using a common intercept (zero time of ageing) for all three storage treatments. The constants  $K_v$ ,  $C_1$  and  $C_2$  in Equation [2] can then be determined using the three values for the mean viability periods.

In practice, rather more temperature/moisture content combinations are now used, although this varies with study. For Iceland poppy (*Papaver nudicale* L.), seeds were kept at 16 combinations of temperature and moisture content over a period of 48 months (Belletti *et al.*, 1991). For pepper (*Capsicum annuum*), 9 combinations of temperature and seed moisture were used in ageing tests over a 16 month period (Lotito and Quagliotti, 1993). In contrast, Hay *et al.* (2003) used 37 temperature/moisture content combinations in a longevity study on *Arabidopsis*. However, a different approach can be adopted if the universal temperature constants are used; longevity can be investigated at a range of moisture contents (usually about 10) and only one temperature (Dickie *et al.*, 1990). Indeed of the 51 species for which there are constants listed in Hong *et al.*, (1998b), 24 were derived from seed ageing studies at one temperature only. Prior to the availability of the universal temperature constants, the barley  $C_H$  and  $C_Q$  values were commonly (20 species) used, i.e., 0.04 and 0.000428, respectively (Ellis and Roberts, 1980a; Ellis and Roberts, 1981a).

As a first step to ageing studies, seed moisture content is adjusted; however, the method of achieving this depends on the study. Often seeds are equilibrated at a range of RHs (or moisture contents) above water (Ellis *et al.*, 1988, *et seq*) or saturated salt solutions in sealable jars (see Vertucci and Roos, 1990; Sun, 2002). Usually the seeds are then: 1) aged at a different temperature in the presence of salts; 2) transferred to another container (without salts) and hermetically sealed in prior to a change in temperature.

Finally, to quantify accurately the pattern of seed viability decrease with time under each storage environment, multiple samples need to be withdrawn and germination assessed using methods appropriate to the species under investigation. The subsequent analysis of seed survival curves has been introduced earlier (see 'Seed Viability Equations'). A general guideline on how to determine seed viability constants is given in Box 35.2.

Hong *et al.* (1998b) have tabulated viability constants for 51 species, plus some subspecies. By 2003, the number of species for which constants were available had extended to 66 species across 26 families (Seed Information Database – SID, Tweddle *et al.*, 2003). Whilst nearly one third of the data comes from two families, the *Leguminosae* Adans (*Fabaceae* Lindl.) and *Poaceae* Barnhart (Table 35.1), it is clear that the quantification of seed ageing has been applied to a considerable range of plant diversity. This diversity is apparently not limited by the variation in seed attributes, such as oil content, morphology, etc. Indeed, the wide applicability and acceptance of the Ellis and Roberts (1980a) seed viability model has spawned the development of a computer programme to predict seed storage behaviour (Kraak, 1992).

**Box 35.2 General guidelines on how to determine seed viability constants**

- Adjust seed moisture content over water, saturated salt solutions or silica gel, depending on the required moisture (hydration) status. Monitor changes in weight of the seed sample to determine when equilibrium has been reached.
- Transfer equilibrated seeds to higher and/or lower temperatures for storage. Seeds can be placed above saturated salt solutions at the ageing temperature or can be moved to a clean container, sealed in and then moved to the storage temperature of choice. In the latter case, try to ensure that the seeds fill the container (i.e., minimise the air volume). Note also that some salts can have a detrimental effect on seed quality, i.e., accelerate their ageing.
- Sample the experiment at regular intervals. Determine seed moisture content gravimetrically before and after drying overnight at a temperature of about 100°C. Also determine seed germination level (i.e. the viability test) for the sample. Aim to create survival curves (i.e., viability against time) with at least 6 data points between 95% and 5% viability, for each combination of temperature and moisture content.
- If planning to use the universal temperature constants ( $C_H$  and  $C_Q$ ), adjust seeds to about 10 moisture contents equivalent to c. 5% to 95% RH and age at one temperature – this should enable the lower and upper moisture limits to the longevity-moisture content relationship to be determined. Alternatively, use about 5 different temperatures over at least a 40°C range so that specific estimates of  $C_H$  and  $C_Q$  can be made.
- Subject the survival data to probit analysis (a weighted regression), thus generating values for the standard deviation of the frequency of seed deaths in time ( $\sigma$ ). Then plot  $\log \sigma$  against  $\log$  moisture content (% fresh weight basis), in order to determine where the moisture limits occur; fit linear regression within these limits to determine  $C_W$  (slope) and  $K$  (intercept).  $K$  can then be used to provide an estimate of  $K_E$  using the universal temperature constants and Equatron [13].
- Note that to generate full viability constants will require using thousands of seeds.

**1.2. Factors that affect constants**

It is well established that the initial seed quality of a seed lot ( $K_i$ ) will affect longevity. What is perhaps less apparent is that the viability constants ( $K_E$ ,  $C_W$ ,  $C_H$ ,  $C_Q$ ), which are meant not to vary within species, do appear to vary within or between studies on the same species. Such variation in the constants and in  $K_i$  has a profound effect on both our perception and understanding of seed longevity. The main factors that appear to influence the constants and/or  $K_i$  are: genotype, seed development and the environment, seed mass, seed chemical composition, seed dormancy, seed treatment and method of describing germination.

**Table 35.1 Families and number of species for which seed viability constants are available. Adapted from Hong *et al.* (1998b); Yang, 1999; Hay *et al.*, 2003; and F.R. Hay, Royal Botanic Gardens, Kew, pers. comm.)**

Family	Number of species	Family	Number of species
<i>Alliaceae</i> Batsch	1	<i>Leguminosae</i> Adans. ( <i>Fabaceae</i> Lindl.)	10
<i>Amaranthaceae</i> Adans. (incl. <i>Chenopodiaceae</i> Vent.)	3	<i>Linaceae</i> DC. ex Perleb	1
<i>Anacardiaceae</i> Lindl.	1	<i>Meliaceae</i> Juss.	4
<i>Araucariaceae</i> Henkel & W. Hochst.	2	<i>Papaveraceae</i> Adans.	1
<i>Asteraceae</i> Martynov (i.e., <i>Compositae</i> Giseke)	3	<i>Pedaliaceae</i> R.Br.	1
<i>Brassicaceae</i> Burnett (i.e., <i>Cruciferae</i> Adans.)	3	<i>Pinaceae</i> Adans.	2
<i>Cactaceae</i> Durande	4	<i>Platanaceae</i> T. Lestib.	1
<i>Caryophyllaceae</i> Durande	1	<i>Poaceae</i> Barnhart (i.e., <i>Gramineae</i> Adans.)	11
<i>Combretaceae</i> R.Br.	2	<i>Ranunculaceae</i> Adans.	2
<i>Cucurbitaceae</i> Durande	2	<i>Rosaceae</i> Adans.	1
<i>Dipterocarpaceae</i> Blume	2	<i>Sapindaceae</i> Juss. (incl. <i>Aceraceae</i> Durande)	1
<i>Euphorbiaceae</i> J.F.Gmel.	4	<i>Scrophulariaceae</i> Durande	1
<i>Hamamelidaceae</i> R.Br.	1	<i>Ulmaceae</i> Mirb.	1

### *Genotype*

Generally, there is remarkable similarity between the seed storage performance of seed lots of the same species when held under identical conditions, once consideration has been given to differences in the initial quality of the lots. For example, seed longevity in cashew (*Anacardium occidentale* L.) at 40°C did not vary between clones, having the same  $C_W$  of 2.877 (Mwasha *et al.*, 1997). Moreover, there were no systematic errors when predicting the longevity of barley cultivar ‘Doublet’ using the viability constants derived for cultivar ‘Proctor’ (Pieta Filho and Ellis, 1992). In addition, there was no significant difference among genotypes of Indonesian and American soybean (*Glycine max*) in the semi-logarithmic relation between  $\sigma$  and seed equilibrium humidity, with the regression slope equivalent to a doubling of longevity for each 8% reduction in RH (Zanakis *et al.*, 1993). Similarly, the effects of moisture content and temperature on longevity (i.e., the values of  $C_W$ ,  $C_H$  and  $C_Q$ ) did not differ amongst four cultivars of tef (*Eragrostis tef* (Zucc.) Trotter), although  $K_E$  was significantly different for one cultivar (Zewdie and Ellis, 1991b).

By comparison, there appear to be subtle differences in longevity between some cultivars of soybean and rice.  $K_i$  (a measure of potential longevity)<sup>5</sup> was greatest for an Indonesian compared to an American soybean, with the cross being much closer to the American parent (Zanakis *et al.*, 1993). For sixteen cultivars of rice though,  $K_i$  did not appear to be determined by genotype (Kameswara Rao and Jackson, 1996). Nonetheless, two rice genotypes with purple pericarps had the highest longevity during ageing at 35°C and 15% moisture content;  $\sigma$  being 2.3 weeks compared to 1.5 weeks for the other cultivars (Kameswara Rao and Jackson, 1996). One cause of such a difference could be a different equilibrium RH for the moisture content used for ageing, reflecting genotype-dependent differences in seed chemical composition. In this case, the water relations of seed longevity (e.g.,  $C_w$ ) might vary with genotype. However, analyses of the seed storage performance of three subspecies of rice suggest that it is  $K_E$  rather than  $C_w$  that varies, i.e.,  $\sigma$  can be seen to vary any one moisture content (Ellis *et al.*, 1992). As a result, sub-species longevity of *indica* > *javanica* > *japonica*, and  $K_E$  follows the same sequence (Ellis *et al.*, 1992). Other indications of cultivar-dependent longevity of rice come from studies of ten cultivars, representing six isozyme groups (Rao and Jackson, 1997b). Seeds of cultivars belonging to isozyme Group II survived longer than other cultivars, and the floating rices of Group IV had shorter longevity.

As to the genetic basis of such differences in longevity within a species, it has been suggested, based on breeding studies on three generations, that storability (8 months under ambient conditions) in soybean (*Glycine max* cv. *Merrill*) is governed by one or two major genes (Dao *et al.*, 1999). In rice, recent quantitative trait loci (QTL) studies have elucidated further the inheritance and location of alleles involved in seed longevity. Using backcross inbred lines (BILs) derived from a cross between *japonica* cv. *Nipponbare* and *indica* cv. *Kasalath*, seed longevity (2 months at 30°C and 15% moisture content) was found to be linked to a QTL on chromosome 9 (Miura *et al.*, 2002). Consistent with earlier observations, *indica* alleles increased seed longevity at this QTL and two others (Miura *et al.*, 2002). The effect of this allele on longevity was verified by chromosome segment substitution.

#### ***Seed development, and the environment***

There is now considerable evidence that seed longevity increases after mass maturity (MM; maximum seed dry weight) has been achieved and during maturation drying (Hay and Smith, 2003 – Chapter 6). Most studies by Ellis and co-workers have recorded increases in the potential longevity in relation to  $K_i$ , i.e., the rate of ageing of seeds of different maturities was similar under identical moisture content and temperature conditions, but potential longevity was greater for more mature seeds as the initial viability ( $K_i$ ) was higher. For example, marrow seeds attain maximum longevity in air-dry storage about

<sup>5</sup> The greater the value of  $K_i$ , the longer the time for viability to fall to a given value, e.g., 50%, under a given set of conditions.

three to four weeks after MM (Demir and Ellis, 1993). Spring and winter cultivars of barley and wheat attain highest  $K_i$  values 3 to 21 d after MM, when the seeds have reduced to 16–28% moisture content (Pieta Filho and Ellis, 1992). Similarly,  $K_i$  in foxglove (*Digitalis purpurea* L.) seeds increased as development progressed from MM (36 DAF) to the point of natural dispersal (c. 50 DAF) when the seeds were c. 36% moisture content (Hay and Probert, 1995). Finally, *Ceiba pentandra* (L.) Gaertn. seeds reached maximum longevity 18 d after MM when the seeds were at 20% moisture content (Lima *et al.*, 2000).

Temperature during development and maturation drying can have a major impact on  $K_i$ . For example in wheat, the rate of increase in potential seed longevity (probits d<sup>-1</sup>) was positively related to temperatures between c. 14 to 19°C, as was predicted  $K_i$ ; interestingly, elevated CO<sub>2</sub> did not affect this relationship (Sanhewe *et al.*, 1996). Similarly, shading spring barley seeds such that growth was at a slightly reduced temperature resulted in seed lots with a lower  $K_i$ . In this species, maximum potential longevity occurred 18–27 d after MM, when seeds had maturation-dried to 15–19% moisture content (Pieta Filho and Ellis, 1991). In contrast, cooler conditions during development (27/21°C cf. 30/24°C) resulted in a higher  $K_i$  in *Phaseolus vulgaris* seeds, with maximum potential longevity occurring 34 d after MM and when the seeds had maturation-dried to about 16% (Sanhewe and Ellis, 1996). In comparison, *japonica* rice attained maximum potential longevity after maturation drying to 24–32% moisture content (Ellis and Hong, 1994). In *japonica* rice, the increase in  $K_i$  after MM was greater under cooler growing conditions (Ellis and Hong, 1994). Similarly, the mean potential longevity in four rice cultivars grown in the Philippines was higher when sown earliest in the season, as seed ripening then coincided with cooler and drier conditions (Rao and Jackson, 1997a).

Irrigation of the parent plant can also impact on potential longevity. Withholding water from rapid cycling brassica plants increased the rate of seed quality development and the maximum value for potential longevity;  $K_i$  peaked at 7 d compared to 10 d after MM in control plants, and was higher in droughted plants (Sinniah *et al.*, 1998a). The water status of the mother plant did not, however, alter  $\sigma$  throughout development, which remained at 4.7 d for seeds aged at 15% moisture content and 40°C (Sinniah *et al.*, 1998a).

Mature and immature seeds of barley, harvested at the same time, had similar  $\sigma$  values (Ellis and Roberts, 1981b). In foxglove (*Digitalis purpurea*) seeds, however,  $\sigma$  and potential longevity ( $K_i$ ) were greater the later the seeds were harvested; indeed, there was a positive relationship between these two parameters, such that  $\sigma = 2.46 - (0.71 \times K_i)$  (Hay *et al.*, 1997b). In foxglove, changes in  $\sigma$  resulted in variable estimates of  $C_W$  with developmental age, although these were not systematic unless  $C_W$  was constrained to a common value of 8.10. Then  $K_E$  was observed to increase from 9.1 to 10.1 during just less than two weeks further development (Hay *et al.*, 1997b). Although the cause of similar variation in  $K_E$  in rice was ascribed to genotypic effects (Ellis *et al.*, 1992), this is not likely to be the case for foxglove because the seeds were

harvested from the same group of plants within a single growing season (F.R. Hay, Royal Botanic Gardens, Kew, pers. comm.).

$K_E$  might also be influenced by environment during development. Conidia of *Beauveria bassiana* (Bals.-Criv.) Vuill. produced in the UK were about three times longer-lived ( $K_E = 6.7$ ) than those produced in Nairobi (Kenya) or Carolina (USA) ( $K_E = 6.2$ ); all other constants of the viability equation ( $C_W$ ,  $C_H$  and  $C_Q$ ) being the same between isolates (Hong *et al.*, 2001).

In addition to the time of harvest *per se*, the method of subsequent treatment (environment) can also impact on the longevity of seeds. For example, seeds of foxglove held briefly (i.e., a few days) at close to 100% RH exhibited increased  $K_i$  and  $\sigma$  (Hay and Probert, 1995). Moreover, foxglove seed longevity could develop when capsules were detached prematurely, but it did not reach the maximum value of seeds matured naturally on the parent plant (Hay *et al.*, 1997a). Similarly, immature seeds of cedro (*Cedrela odorata* L.) had increased longevity in air-dry storage when fruits were dried at a moderate rate in a single layer for 20 d (Lima *et al.*, 1998). These studies suggest the continuation of maturation events *ex planta* can improve seed longevity.

Clearly then, the time of harvest and subsequent treatment can have a profound impact on the *potential* and *actual* longevity of seeds in ageing studies. For further discussion of these topics see Hay and Smith (2003) – Chapter 6 – and Probert (2003) – Chapter 19.

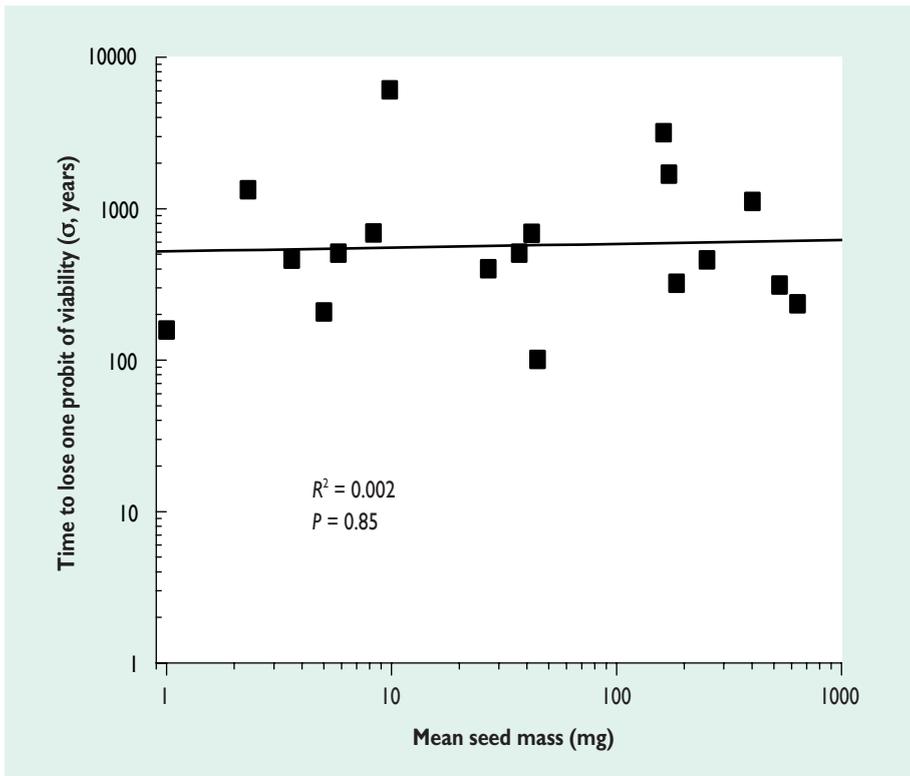
### ***Seed mass***

Small seeds are more likely than larger seeds to form persistent seed banks in the soil (Leck, 1989; Dalling *et al.*, 1998; Thompson, 2000). However, when the predicted seed longevity for 18 species at  $-20^\circ\text{C}$  after predrying to 15% RH at  $15^\circ\text{C}$  was analysed against seed mass (from 1 to  $> 600$  mg), no significant relationship was observed (Figure 35.9). Seed mass over this range appears to have no effect on longevity in dry storage and highlights the fact that at least some of the factors controlling the longevity of seeds *in situ* and *ex situ* are likely to be different.

### ***Seed chemical composition***

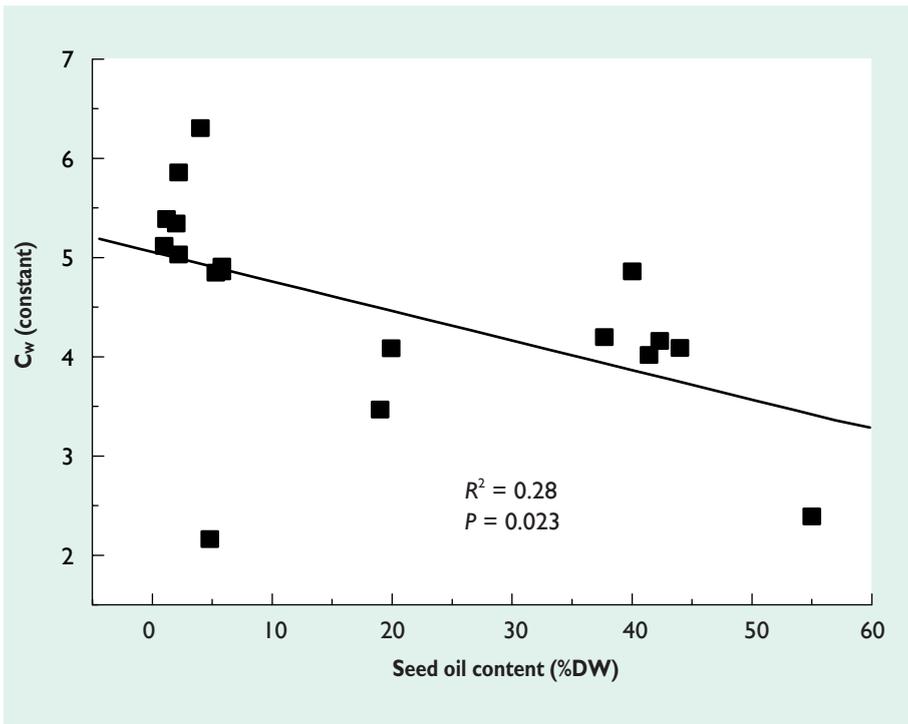
The chemical composition of seeds varies considerably between species and changes quite considerably as mass maturity approaches (see Bewley and Black, 1994). How might such differences in seed chemical composition influence seed longevity?

Oil content has a profound effect on the water relations in seeds, such that oily seeds have a lower equilibrium moisture content at a given RH compared to non-oily seeds (Cromarty *et al.*, 1982; Probert, 2003 – Chapter 19). In addition, the dependency of moisture content on RH in the central zone (II) of the isotherm appears to be smaller in oily compared with less oily seeds (Cromarty *et al.*, 1982; Vertucci and Leopold, 1987). As a consequence, it is conceivable that oil content could affect the moisture term of the viability equation,  $C_W$ .



**Figure 35.9** Relations between the predicted time to lose one probit of seed viability ( $\sigma$ , years) at  $-20^{\circ}\text{C}$ , following drying at 15% RH and  $15^{\circ}\text{C}$ , and mean seed mass for 18 species: *Allium cepa*, *Arachis hypogaea* L., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Helianthus annuus* L., *Hordeum vulgare* L., *Lactuca sativa* L., *Linum usitatissimum* L., *Oryza sativa* L., *Pennisetum glaucum* (L.) R.Br., *Pisum sativum* L., *Sesamum indicum* L., *Sorghum bicolor* (L.) Moench, *Swietenia humilis* Zucc., *Terminalia brassii* Exell, *Triticum aestivum* L., *Vigna unguiculata* (L.) Walp. and *Zea mays* L. Longevity data are taken from Hong *et al.* (1998b) and seed weights from Tweddle *et al.* (2003).

Figure 35.10 shows the relation between  $C_w$  and seed oil content for the 18 species indicated in Figure 35.9 and Table 35.2. The negative relation is significant, suggesting that low  $C_w$  values result from high seed oil contents. Nevertheless, the weakness of this association can be seen (Figure 35.10) from the large variation in  $C_w$  among species with similar oil contents. Indeed, a similar analysis of data presented by Medeiros *et al.* (1998), and that of an unpublished compilation of 48 species recently conducted in our laboratory, both failed to reach significance ( $P = 0.86$  and  $P = 0.07$ , respectively). In both these analyses, however, use of a mixture of whole seed and embryo-only oil contents could be an issue; and clarification of the relation between  $C_w$  and seed or embryo oil content deserves further effort.

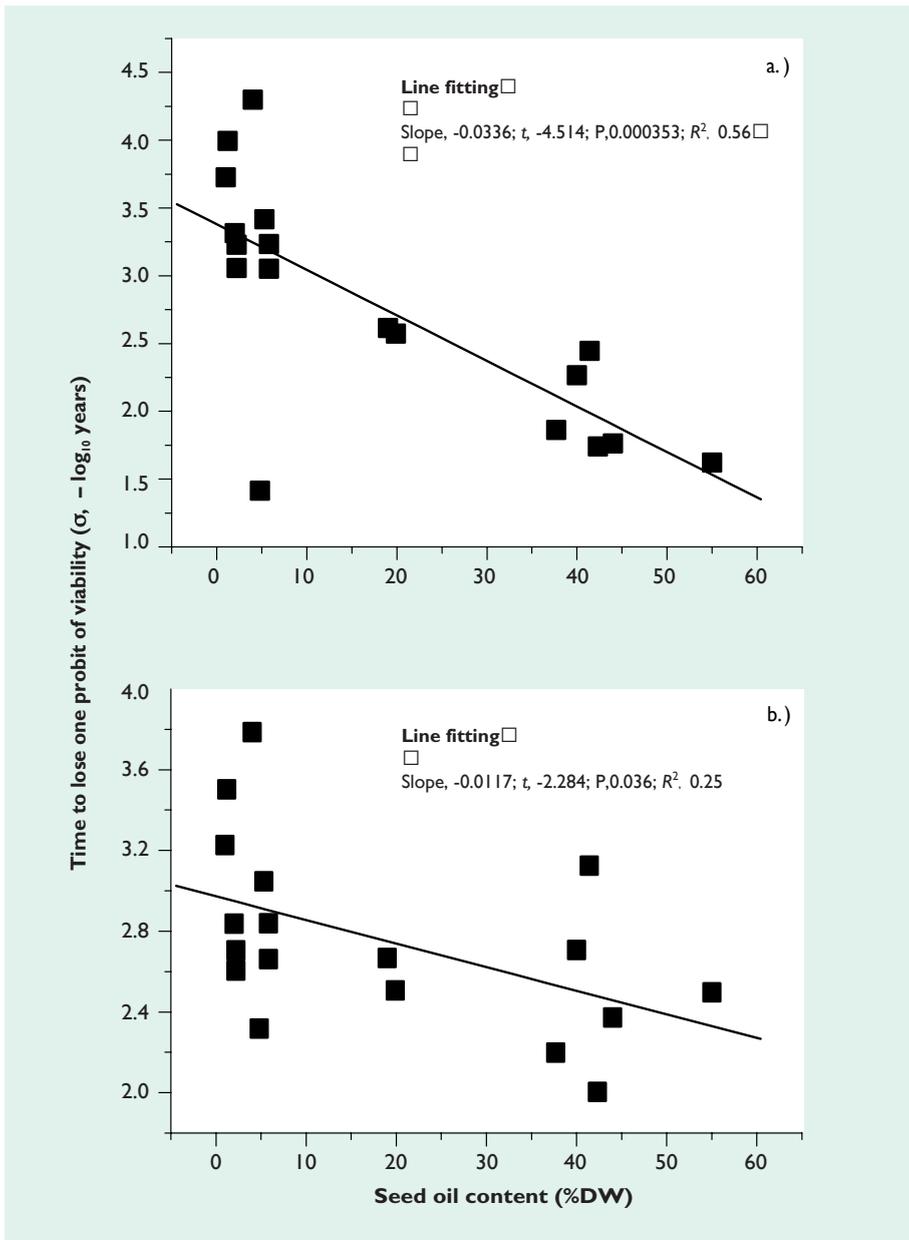


**Figure 35.10** Dependence of the viability constant  $C_w$  on seed oil content for 18 species (details as Figure 35.9). Longevity data are taken from Hong *et al.* (1998b) and oil contents from Tweddle *et al.* (2003), except for *Swietenia humilis* (M. Sacandé, Royal Botanic Gardens, Kew, pers. comm.).

The role of lipid stability in seed longevity has been the subject of numerous studies (e.g., see Priestley, 1986). Oxidations of unsaturated fatty acids are considered to be primary reactions in ageing, contributing to free radical production and subsequent attacks on other macromolecules (Benson, 1990). Oxidative processes are known to progress, albeit at a slow rate, in seeds at moisture contents coincidental with sorption zone II of the isotherm in which the viability equation is applicable (Vertucci and Leopold, 1987). Ponquett *et al.* (1992) proposed a model relating longevity ( $P_{50}$  or time to 50% of the original viability of the seed lot) in seven species to the levels of linolenic acid per unit of total tocopherols, higher values being associated with shorter longevity. There is ample evidence both in support of and against the lipid peroxidation model of seed deterioration (e.g., see Corbineau *et al.*, 2002), although comparisons between studies are hindered, even on the same species, by the use of different storage methods.

The primary site of lipid oxidation is thought to be membranes, thus leading to altered permeability and conductivity parameters (Priestley, 1986 *et seq.*). However, reserve lipids are also susceptible to attack, as observed in the diacylglycerol fraction of sunflower seeds subjected to accelerated ageing (Gidrol *et al.*, 1989). If oil-rich seeds have pools of lipid that can contribute to the initiation of free radical attack throughout the seed, then it follows that oily seeds may be generally shorter lived than non-oily seeds. To test this hypothesis comparisons were made between seed longevity and oil content for the 18 species in Table 35.2. Figure 35.11a shows that predicted longevity at  $-20^{\circ}\text{C}$  and 5% moisture content increases as oil content decreases from 55% to 1%. This result is not surprising as a seed with 50% oil will be at an equilibrium RH of about 45% when at 5% moisture content, compared with c. 10–15% RH for a non-oily seed. Accordingly, ageing will appear to progress more rapidly in the oily seed. A more meaningful comparison would be for seeds that had been equilibrated to the same RH before storage. The corresponding analysis is for the 18 species shown in Figure 35.11b, for seeds dried at 15% RH and  $15^{\circ}\text{C}$ . Whilst the dependence of predicted longevity at  $-20^{\circ}\text{C}$  on oil content is weak at equal RH (Figure 35.11b), when compared with equal moisture content (Figure 35.11a), the trend is just significant; oily seeds still appear to have shorter lifespans. In contrast, data for 22 species analysed by Medeiros *et al.* (1998) revealed no significant effect of oil content on predicted longevity ( $P > 0.05$ ), nor did a recent analysis in our laboratory of an unpublished compilation of data for 48 species ( $P = 0.75$ ). However, both these analyses are subject to the same caveats with regard to the mixing of whole seed and embryo only oil contents, mentioned above for the relation with  $C_w$ . Clearly, further investigations on the effects of seed oils (content and composition) on longevity are desirable, especially with respect to antioxidant systems.

Chemical composition is determined by both the phenotype (environmental effects) and genotype. The genetics of seed chemical composition is well established for *pea* (*Pisum sativum* L.), making this a good model species to investigate the effects of genetic background on longevity. In a recent study on mutant, near-isogenic lines (RRrbrb, rrRbRb, rrrbrb) and the wild type (RRRbRb) of pea, Lyall *et al.* (2003) showed that the effect of alleles at the (rugosus) r and rb loci on longevity was largely indirect; the lines had lower starch and higher lipid contents, and this reduced the impact of lowering moisture content on seed longevity (i.e.,  $C_w$  was lower), as a direct consequence of altered sorption properties of the seeds. Longevity was the same, however, when the lines and the wild type were compared on the basis of equilibrium RH rather than moisture content. Interestingly, below the low-moisture-content-limit to relations between longevity and moisture content the alleles directly affected seed longevity (Lyall *et al.*, 2003).



**Figure 35.11** Relations between the predicted time to lose one probit of seed viability ( $\sigma$ , log years) at -20°C and seed oil content for 18 species (details as Figure 35.9). Calculations are based on seeds being at the following hydration levels: 5% moisture content (a); equilibrium at 15% RH and 15°C prior to storage (b). Longevity data are taken from Hong *et al.* (1998b) and oil contents from Tweddle *et al.* (2003), except for *Swietenia humilis* (M. Sacandé, Royal Botanic Gardens, Kew, pers. comm.).

Soluble carbohydrates may protect seeds in the dry state as long as they are available on hydrophilic sites of cellular membranes and macromolecules. Carbohydrate metabolism, in relation to the relative quantity of oligosaccharides in a wide range of seeds and component tissues, has been used as a general marker for seed storage category (Steadman *et al.*, 1996). Moreover, seed longevity ( $P_{50}$ , years) in 16 species has been correlated with carbohydrate composition (Sun and Leopold, 1997). Greatest longevity occurred when the oligosaccharide fraction was c. 60%, seed longevity being lower when both higher and lower fractions were present, possibly because the tendency for crystallisation of carbohydrate mixtures is least when the ratio of oligosaccharide/sucrose is 0.33 to 1 (Sun and Leopold, 1997). Similarly, developing seeds of rapid-cycling brassica [*Brassica rapa* L. emend. Metzg. (includes *B. campestris*)] accumulate oligosaccharides, and stachyose content correlated positively with potential longevity ( $K_i$ ) (Sinniah *et al.*, 1998b).  $K_i$  also correlated with selected heat-stable proteins (Sinniah *et al.*, 1998b).

Overall, it appears that the main effects of seed chemical composition on longevity relate to: 1) the sorption properties of the seed; 2) potential sites for free radical attack; and 3) the presence of protective compounds and their action.

### ***Seed dormancy***

Environments used to investigate seed ageing, especially in cereals, are often in the region of 30°C to 40°C and c. 15% moisture content, as this will ensure that the kinetics of ageing is relatively fast. However, such conditions are also conducive to the removal of seed dormancy via after-ripening (see Baskin and Baskin, 1998; Pritchard and Toorop, 2003). Potentially then, these two physiological processes could be occurring concurrently during ageing studies.

Roberts (1963) showed this to be the case for seed of six cultivars of rice aged under exactly the same conditions of 13.5% moisture content and 27°C. For three of the cultivars, seed germination increased substantially during the early part of the experiment, indicative of loss of dormancy. However, the pattern of loss in viability ( $\sigma$ ) was identical in all cases (Roberts, 1963). Similarly, seed lots of the cactus *Cereus peruvianus* (L.) J.S.Muell. with considerable differences in red light sensitivity for germination (and likely reflecting differing underlying dormancy levels) had very similar moisture content/longevity relations (Yang, 1999). Thus, the evidence points to seed longevity and dormancy traits not being directly related. Recent quantitative trait loci studies on rice support this view. Using backcross inbred lines (BILs) derived from a cross between *japonica* cv. *Nipponbare* and *indica* cv. *Kasalath*, seed longevity and dormancy QTLs were found to be located on different chromosomes: chromosomes 2, 4 and 9 for longevity, and 1, 3, 5, 7 and 11 for dormancy (Miura *et al.*, 2002). These traits are thus controlled by different genetic factors.

One proviso should be added, though, concerning the trait of hardseededness, which is found, *inter alia*, in numerous species in the *Leguminosae*, *Anacardiaceae* Lindl., *Cistaceae* Adans. and *Malvaceae* Adans. Because ‘hard’ seeds are resistant to water uptake unless chipped, they may appear to have improved longevity under ageing conditions as a result of remaining dry. Such sealing in of dryness probably contributed to the exceptional longevity reported for seeds of *Nelumbo nucifera* Gaertn. – sacred lotus (see Shen-Miller, 2002).

### **Seed treatments**

Careful rehydration of dry seeds before the start of the germination test can result in higher  $K_i$  (see Genebank Standards, 1994; Dickie and Stuppy, 2003 – Chapter 15). Generally, seeds are ‘conditioned’ above water, the temperature of which is particularly important when dealing with tropical seeds, e.g., neem (Sacandé, 2000). Moreover, it has been known for many years that the germination rate of lower quality seed lots can be enhanced with hydration (soaking, osmo-priming) treatments before seed sowing, and that the benefits of such treatment can be retained after drying back (see Bewley and Black, 1994). Longevity also tends to be affected by such treatments being either enhanced (e.g., Burgass and Powell, 1984; Thanos *et al.*, 1989; Powell *et al.*, 2000; Probert *et al.*, 1991) or reduced (e.g., Hong and Ellis, 1992; Tarquis and Bradford, 1992).

Major factors contributing to these differences in response seem to be the length of the hydration treatment and the quality of the seed lot under investigation. Relatively short pre-treatments (< 1 d), especially on seed lots of relatively low vigour, tend to enhance longevity. In low quality (c. 60% germination) cauliflower (*Brassica oleracea* L. var. *botrytis* L.) seed, this effect appears to be manifest through an increase in both  $K_i$  and  $\sigma$ , the former increasing by about 0.5 probits of viability and the latter by about 3- to 7-fold during ageing at 12% moisture content and either 10°C or 20°C (Powell *et al.*, 2000). In low quality (90–95% germination) seed lots of three wheat cultivars (*Triticum aestivum*), hydration treatment (c. 10 h) nearly doubled longevity ( $\sigma$ ) (Hofman and Steiner, 1994). In contrast, when high quality seeds (94–99% germination) of three wheat cultivars were pre-hydrated for c. 10 h, dried back to 14% moisture content and stored at 30°C, they had similar intercepts for their survival curves but reduced longevity ( $\sigma$ ) 77–105 d compared with 147–286 d for untreated seeds (Hofman and Steiner, 1994). Also, high vigour cauliflower seeds given a 12 h aerated-hydration treatment had a longevity ( $\sigma$ ) of 1.5 months at 12% moisture content and 20°C, compared with 10.4 months for untreated seeds; treated seeds also had a lower  $K_i$  (Powell *et al.*, 2000). Seed longevity was reduced further when pretreatment was c. 1 d. Similarly, *Vigna radiata* (L.) Wilczek and *Hordeum vulgare* seeds have reduced longevity in dry storage when pre-hydrated for too long (Hong and Ellis, 1992).

Reduced longevity could be associated with altered properties of the glassy state. In the early stages of imbibition, peas seeds have an unaltered  $T_g$ ; however, longer-term treatment resulted in a substantial (c. 50°C) lowering of

the glass transition temperature (Williams and Leopold, 1995). A consequence of this would be that seed longevity at a single temperature would be dramatically shorter after pretreatment, as a result of an altered chemical composition and lowered cellular viscosity. However, Buitink (2000) could find no simple relationship between cellular viscosity and seed performance after hydration (priming) treatment.

What is particularly exciting about these types of manipulations and responses is that they facilitate a more detailed understanding of seed conservation science and practical outcomes. Thus, it is of interest to note that reduced longevity in primed seeds of several species can be alleviated through the application of mild water stress and high temperature stress for a period of several hours to days (Bruggink *et al.*, 1999). One wonders if such a combination of treatments might be of value in improving lower quality seed bank collections?

#### *Method of describing 'germination'*

Germination can be assessed by various means, each giving a different value of seed quality. Use of radicle protrusion as a germinability criterion tends to raise initial germination compared to normal seedling production (Ellis and Roberts, 1981a), and facilitates the use of uncorrected probit analysis (Wilson *et al.*, 1989). This has led to the suggestion that the conventional probit analysis method is less relevant for seed technologists employing more stringent germinability criteria (Wilson *et al.*, 1989). From this general thesis, it follows that ageing data constructed using normal seedling production might result in relatively higher estimates of  $\sigma$  (flatter ageing curves), potentially leading to higher estimates of  $K$  and, accordingly,  $K_E$ . However in delphinium,  $K_E$  was observed to be about a value of 2 higher when radicle emergence rather than normal seedling production was used as the viability measure (Kwong *et al.*, 2001). In the same study, the constants for salvia were not dependent on germination assessment method (Kwong *et al.*, 2001). Thus, solid evidence to substantiate the concerns of Wilson *et al.* (1989) about seed technologists using the viability equation appears, at this stage, to be lacking.

## **2. Pollen and Fungal Spores**

Interest in the storage of 'propagules' for conservation is not restricted to the seeds of higher plants. For example, knowledge of the storage characteristics of pollen is particularly important to fruit tree breeding programmes. In addition, there is interest in spore longevity of pathogenic and other fungi, in the context of opportunities for successful dispersal in natural environments. Understanding spore longevity could, thus, have applications in fungal biodiversity and plant pathology studies. Given the wide applicability of longevity models to multi-celled seeds, it is pertinent to consider whether such models are of equal value in quantifying and predicting longevity in single-celled systems.

Ageing studies on *Typha latifolia* pollen revealed considerable similarities to seeds with respect to the modelling of longevity (Hong *et al.*, 1999a). Thus, there was a negative logarithmic relation between longevity and pollen moisture content and a curvilinear semilogarithmic relation between longevity and temperature (-5°C to 45°C). Based on an analysis of the time for viability to fall to 50% (rather than  $\sigma$ ), the viability constants for the pollen were found to be:  $K_E = 4.607$ ,  $C_W = 2.464$ ,  $C_H = 0.0304$  and  $C_Q = 0.00065$ .

The mean lower-RH limit for *Typha latifolia* pollen ageing was 11.9% RH (Hong *et al.*, 1999a), very close to that previously suggested for seeds (Roberts and Ellis, 1989). However, the estimate of  $C_W$  in relation to  $r$  i.e., which describes the dependence of longevity on storage RH, was 0.0236. This implies that longevity increased 1.7-fold for each 10% fall in RH, a value that is somewhat lower than that observed in crop seeds (2.2-fold; Roberts and Ellis, 1989), but similar to that for conidia of the fungus *Metarhizium flavoviride* (1.9-fold; Hong *et al.*, 1998a).

Overall, the viability model (using either log moisture content or equilibrium RH) has been applied to the survival of conidia of five entomopathogenic fungi [*Metarhizium anisopliae* (Metschn.) Sorokin, *M. flavoviride*, *Beauveria bassiana*, *B. brongniartii* (Sacc.) Petch and *Paecilomyces farinosus* (Holmsk.) A.H.S.Br. & G.Sm.] and ascospores, conidia and uredospores of four phytopathogenic fungi (*Alternaria porri* (Ellis) Cif., *Helminthosporium oryzae* Breda de Haan, *Uromyces appendiculatus* (Pers.) Link and *Sclerotinia sclerotiorum* (Lib.) de Bary] across a wide range of temperatures and relative humidities (Hong *et al.*, 1997). The sensitivity of spore longevity to both temperature and eRH, and the upper and lower RH limits to the application of the model, varied considerably between and sometimes within strains of species (Hong *et al.*, 1997). The upper limits appeared to be around 65% in *Metarhizium flavoviride*, from > 75% to c. 40% RH in *Metarhizium anisopliae* conidia, depending on strain, and up to c. 95% in 'spores' of seven other species (Hong *et al.*, 1997). Longevity improvements in *Metarhizium flavoviride* conidia ceased on drying to a moisture content in equilibrium with 11% RH (Hong *et al.*, 1998a).

Clearly, considerable parallels can be drawn between the quantification of ageing in dry seeds, pollen and spores, supporting the notion of an 'economy of nature in anhydrous biology' (Hong *et al.*, 1998a).

## Beyond the Limits

It is clear that there are limits to the linear relations between the logarithms of  $\sigma$  and moisture content for seeds in 'dry' storage (e.g., lettuce, see Figure 35.3). How then do seeds respond to environments that are deemed to be beyond these limits, i.e., are either ultra-dry or ultra-wet?

### 1. Ultra-dry Storage

The initial recommendation for the moisture content that seeds should be stored at for long-term storage was 5% (fresh mass basis) (IBPGR, 1976; Cromarty *et al.*, 1982). Subsequent research on a range of species, including sesame, revealed that for some there were considerable benefits to seed longevity when moisture contents were reduced from 5% to about 3%; whilst for other species drying below about 7% had little effect (Ellis *et al.*, 1988; 1989; 1990a; 1990b). As a consequence, a FAO working group recommended preferred conditions for seed storage in base collections of 3 to 7% moisture content and -18°C (Genebank Standards, 1994). This range of moisture contents is related to differences in seed composition such that equilibrium relative humidities are similar across species, at about 10 to 12% RH at 20°C (Genebank Standards, 1994).

The observation that moisture contents lower than 5% could improve seed longevity in some species led to the concept of ultra-dry storage, such that additional drying might replace the need for refrigeration (FAO/IBPGR, 1992; Genebank Standards, 1994). For example, storage conditions could be 2% moisture content and 20°C. Clearly, there is merit to this approach when attempting to safeguard seeds when electricity supply is uncertain. However, concern has been expressed that using drying conditions of c. 10% RH at 20°C would over-dry and under-dry seeds for storage at lower and higher temperatures respectively (Vertucci and Roos, 1990; 1991, 1993; Vertucci *et al.*, 1994; Walters, 1998; Walters *et al.*, 1998a,b). This view is based on theoretical grounds of an optimum RH for storage of 19 to 27% (Vertucci and Roos, 1990, *et seq*), 14% (Walters, 1998) or an average of 22% across species (see Walters *et al.*, 1998a,b). It follows that ultra-drying to below this RH value (and associated moisture content) could significantly compromise seed longevity, including at gene bank temperature.

In support of such concerns are data showing that ultra-drying slightly reduces longevity (i.e., lowers  $\sigma$ ); for example in mung bean (Ellis *et al.*, 1989), *Phaseolus vulgaris* (Ellis *et al.*, 1990a) and sugar beet (Ellis *et al.*, 1990b) aged at 65°C, and seeds of three orchids, aged at 40°C (Pritchard *et al.*, 1999). However for many more species, seed longevity ( $\sigma$ ) either remains the same ( $n = 19$  species) or increases ( $n = 2$  species) below this critical hydration point (Ellis *et al.*, 1988; 1989, 1990b, 1992). On balance, the evidence suggests that when  $\sigma$  is negatively

affected by moisture reduction below this critical hydration level, the impact is relatively small compared with the effect on  $\sigma$  of increasing moisture content above this critical point. However, is this likely to be the case when dealing with storage at colder rather than warm temperatures?

In a study of 17 accessions (17 species from 12 genera) of crucifer stored in sealed glass vials with silica gel (c. 7% RH at 20°C) for 24–25 years at -5 to -10°C, it was observed that in only one of the accessions was there a significant difference in germination ability; germination in all others either remained constant or slightly increased (presumably as a result of the slow loss of dormancy) (Ellis *et al.*, 1993). In a related study, the viability of dry (5.5–6.8% moisture content) and ultra-dry (in equilibrium with 10% RH at 20°C) seeds of carrot, groundnut, lettuce, oilseed rape and onion were monitored during 5 years storage at 20°C and -20°C (Ellis *et al.*, 1996). For all species, seed longevity was greater for the ultra-dry samples. Similarly, ultra-dry seeds of hybrid *Salix* stored better than seeds equilibrated to 65% RH prior to storage when subsequently held at 16°C, 2°C, -20°C and in liquid nitrogen (Wood *et al.*, 2003). The results provide support to the view that seed storage at low MC is an acceptable procedure for the long-term maintenance of seed accessions in gene banks. This does not preclude the possibility, however, that conditions other than those currently recommended for long-term storage might provide even better seed longevity.

Why are there such divergent views on the risks/benefits associated with ultra-drying? Firstly, there is some discussion of whether the critical hydration level constitutes (in general) the ‘maximum’ (Ellis *et al.*, 1988; 1989, 1990b, 1992) or ‘optimum’ (Vertucci and Roos, 1990; 1991, 1993; Vertucci *et al.*, 1994; Walters, 1998; Walters *et al.*, 1998a, b) longevity at a given temperature. The latter viewpoint is supported by thermodynamic considerations of an optimum RH for storage, which does not change with temperature (see Walters, 1998; Walters *et al.*, 1998a,b). Because isotherms do change with temperature, however, assumptions about storage at one temperature are not necessarily appropriate at another. For example, pea seeds stored at 65°C have a critical moisture content of 6.2% moisture content (Ellis *et al.*, 1989), which is equivalent c. 10% RH at 20°C but 50 to 60% RH at the storage temperature (Vertucci and Roos, 1990; Walters *et al.*, 1998a, b). What is perplexing, though, is that the theoretical model based on thermodynamics would suggest that drying pea seeds from 6% to 3% moisture content should result in improved longevity at 65°C, but this is not observed on the basis of  $\sigma$  (Ellis *et al.*, 1989).

To recap, compared with the empirical model the theoretical model appears to overestimate the potential negative effects of ultra-drying below the critical hydration level, and to not fully account for the improvements in longevity as reducing moisture approaches this level. How much of this difference in perception relates to different methods of data handling?

One difference between the theoretical and empirical models is that the former is based on a single measure of the vigour (product of percentage

germination and mean root length) of seeds after storage at 35°C (Vertucci and Roos, 1990, *et seq.*) whilst the latter is based on longevity at 65°C (Ellis *et al.*, 1988, *et seq.*). To address some of these concerns Ellis *et al.* (1995) assessed lettuce and sunflower seed quality after storage at 35°C using four criteria of vigour. It was observed that lettuce and sunflower seeds stored at c. 2.6% and 2% respectively (i.e., the optima from ageing studies at 65°C) were no less vigorous than seeds stored at 1.3 to 7%. Thus, it appears that neither ultra-drying nor a 30°C lowering in storage temperature has a dramatic effect on the critical hydration level for storage on the basis of vigour.

Another difference between the models is how the ‘maximum’ or ‘optimum’ moisture content for seed storage is identified mathematically. The theoretical model depends on a quadratic function (Vertucci and Roos, 1990, *et seq.*), which as discussed above may overestimate the negative effects of ultra-drying. Alternatively, the empirical model (Ellis *et al.*, 1988, *et seq.*) relies on a ‘broken stick’ approach based on the intercept of two linear regressions to the log  $\sigma$ /log moisture content relationship (see Figure 35.3). What happens when the two approaches are compared directly? In two species of *Nothofagus* Blume, the broken stick, quadratic and cubic models describe the dependency of log  $\sigma$  on log moisture content well at low moisture contents (Leon-Lobos and Ellis, 2003 – Chapter 40). A consequence of fitting such curvilinear models is much lower estimates of an optimum moisture content for longevity (i.e., about 3% cf. 5–6% with the broken stick model) (Leon-Lobos and Ellis, 2003 – Chapter 40). This finding indicates that aspects of the empirical and theoretical models can be brought closer together, leading ultimately to the need to reconsider the  $C_w$  and  $K_E$  terms of the viability equation.

## 2. Ultra-wet Storage

Relatively short-term, high moisture content pretreatments can be used to enhance seed longevity in dry storage for some species, particularly in low vigour seed lots (see ‘Factors that affect constants’). Repair may be a normal occurrence in the first few hours of imbibition and thymidine incorporation into non-replicating DNA has been observed during the first 30 minutes of the start of imbibition in aged seeds (see Osborne *et al.*, 2002). However, the effects of seed hydration on longevity can be sustained for much longer times when the seeds are fully imbibed, either continually or periodically in field conditions (Villiers, 1974). Indeed, under such conditions, seed longevity can be greater than that of drier seeds of the same species. In *Lactuca sativa* and *Fraxinus americana* L. seeds, such extended viability in the hydrated state results from failure to accumulate chromosome damage presumably as a result of repair (Villiers, 1974). Such repair is dependent on oxidative respiration (Osborne *et al.*, 2002) and such metabolism probably contributes to improved  $\sigma$  values at high moisture contents as it is dependent on the presence of oxygen, e.g., in wheat (Petruzzelli, 1986) and lettuce seeds (Ibrahim *et al.*, 1983; see Figure 35.3).

When oxygen is present in air-filled vials, lifespans for high moisture content seeds (c. 15–45%) are skewed (with a shoulder on the survival curve) rather than normally distributed as is the case at lower moisture contents (Ibrahim and Roberts, 1983; Ibrahim *et al.*, 1983). This suggests that oxygen is consumed by seeds early during storage, thus limiting the repair process later on; hence the accelerated rate of loss at that time. In contrast, Moore and Jolliffe (1987) observed a positively skewed distribution of mortality for 18% moisture content seeds of *Pennisetum americanum*, indicative of a more rapid progression of ageing than predicted by a normal distribution function.

As to the hydration threshold for ultra-wet responses in seeds, the higher the moisture content between c. 20% and 30–40%, the greater the longevity during aerated storage for both lettuce (Ibrahim *et al.*, 1983) and wheat seeds (Petruzzelli, 1986). Similarly, longevity at 48 to 55°C for spores of *Metarhizium flavoviride* at 15 to 19% moisture content was much greater than extrapolations from cooler and drier conditions, perhaps as a result of repair in conditions outside the normal limits to the viability model (Hong *et al.*, 1998a). Whilst in seeds (Figure 35.3) the onset for improved longevity appears to be around 90% RH (-15 MPa), the onset may be more variable (lower and higher limits) in fungal spores (Hong *et al.*, 1997; 1998a).

## Longevity Prediction: Fact or Fiction?

Ellis and Roberts (1980a) advocated the application of the improved viability equation to predict longevity under conditions within those used to derive the constants. Nonetheless, extrapolation to drier and colder conditions has been routinely performed (see Hong *et al.*, 1998b and references contained therein).

### 1. Comparative Seed Longevity: Seed Banking Predictions

Many seed banks store seeds at about 5% moisture content and -20°C. Under such conditions the expectation is that species will live for differing periods of time. This variability can be assessed, by comparing the predicted time taken to lose one probit of viability (e.g., falling from 84% to 50% ). Table 35.2 shows species differences with respect to projected longevity at -20°C under two levels of hydration: 5% moisture content; and pre-dried to c. 15% RH at c. 15°C. The estimates shown range from c. 20 to 20,000 years for seeds at 5% moisture content and from c. 100 to 6,000 years for seeds pre-dried to 15% RH. Thus, considering longevity on a RH rather than a moisture content basis reduces the variation in projected longevity from about 3 to 1.8 orders of magnitude.

**Table 35.2 Predicted times to lose one probit of viability ( $\sigma$ ) at  $-20^{\circ}\text{C}$  for 18 species. Longevity comparison based on information in Hong *et al.* (1998b); seed oil contents from Tweddle *et al.* (2003), except for *Swietenia humilis* (#) from M. Sacandé (Royal Botanic Garden, Kew, pers. comm.)**

Species	Longevity at 5% MC (y)	Longevity after pre-drying to 15% RH at c. $15^{\circ}\text{C}$	Difference in longevity (y)	Oil content (%)
<i>Vigna unguiculata</i> (L.) Walp.	5342	1687	- 3655	1
<i>Pisum sativum</i> L.	9876	3180	- 6696	1.2
<i>Hordeum vulgare</i> L.	2061	688	- 1373	2
<i>Oryza sativa</i> L.	1138	402	- 736	2.2
<i>Triticum aestivum</i> L.	1693	508	- 1185	2.2
<i>Sorghum bicolor</i> (L.) Moench	19890	6106	-13784	4
<i>Terminalia brassii</i> Exell	26	208	+ 182	4.8
<i>Cicer arietinum</i> L.	2613	1115	- 1498	5.3
<i>Pennisetum glaucum</i> (L.) R.Br.	1718	691	- 1027	5.8
<i>Zea mays</i> L.	1125	460	- 665	5.8
<i>Allium cepa</i> L.	413	465	+ 52	19
<i>Glycine max</i> (L.) Merr.	374	321	- 53	19.9
<i>Lactuca sativa</i> L.	73	158	+ 85	37.7
<i>Linum usitatissimum</i> L.	185	509	+ 324	40
<i>Sesamum indicum</i> L.	279	1332	+ 1053	41.4
<i>Helianthus annuus</i> L.	55	101	+ 46	42.3
<i>Arachis hypogaea</i> L.	58	236	+ 178	44
<i>Swietenia humilis</i> Zucc.	42	314	+ 272	55#

In general, low oil content seeds are predicted to have greater longevity when dried to 5% moisture content than if equilibrated to 15% RH, whilst the reverse is true for high oil content seeds. This reflects the different sorption properties of oily and non-oily seeds. Thus, for a non-oily seed, drying to 5% moisture content could be achieved by equilibration with < 10% RH air, and so raising the seed RH to 15% gives a shorter estimate for longevity. Alternatively, high oil content seeds would have an eRH of around 60% when at 5% moisture content. Not surprisingly, pre-treating oily seeds with 15% RH enhances longevity compared with 5% moisture content. Only for seeds at around 20% oil do the estimates of longevity appear similar (c.  $\pm$  50 years) whether on a 5% moisture content or 15% RH basis.

Irrespective of how we look at the data, the projected lifespans are considerable (millennia) in some cases. But how accurate are these extrapolations likely to be? Perhaps of most concern in making such projections is the size of the errors on the viability constant estimates, and their impact on the calculations. For two ecotypes of *Arabidopsis thaliana*, the common estimates (including standard errors) of the constants, using the standard two-step analysis, are:  $K_E = 8.17$  (0.217),  $C_W = 5.12$  (0.174),  $C_H = 0.0559$  (0.00619) and  $C_Q = 0.0000801$  (0.0000926) (Hay *et al.*, 2003). Longevity ( $\sigma$ ) is, thus, predicted to be 1302 years for seeds at 5% moisture content and -20°C. However, when the corresponding standard errors are added to all the constants, longevity is predicted to be 1984 years. Alternatively, lowering all parameters by one standard error gives a longevity of 856 years. Thus, errors on the parameters could account for more than a two-fold difference in the longevity estimate. In addition, the one-step fitting model gives about an 8% lower estimate of longevity at 5% moisture content and -20°C than the conventional two-step fitting model (Hay *et al.*, 2003). Clearly, how we use the constants can influence substantially our interpretation of projected longevity.

The projections discussed above are founded on a considerable amount of seed longevity data for crop species. However, of particular interest to the plant/seed conservationist is how to generate quickly a perspective on longevity for a broad range of hitherto un-researched species, as this may influence decisions on the monitoring of diverse bank collections. Table 35.3 suggests one way of achieving this based on our studies in the last few years on orchids, cactus and willow, to which have been added other published data. By using moderate humidity treatment (50% RH) and temperature (40°C), ageing studies can be completed in a practical time scale (few months) and a provisional ranking of species established.

We observe for the 12 seed species shown that longevity varies from c. 4 to 100 d, i.e., about 1.4 orders of magnitude, which is similar to that shown in Table 35.2 for projections at seed bank temperature. We also note that

**Table 35.3 Comparative longevity for seeds of 12 species and the spores of a fungus at 40°C and 50% RH.**

Species	Longevity ( $\sigma$ , d)	Reference
<i>Orobanche crenata</i> Forssk.	103*	Kebreab and Murdoch (1999)
<i>Orobanche aegyptiaca</i> Pers.	100*	Kebreab and Murdoch (1999)
<i>Lactuca sativa</i> L.	90.9*	Roberts and Ellis (1989)
<i>Hordeum vulgare</i> L.	66.6*	Roberts and Ellis (1989)
<i>Orobanche minor</i> Sm.	49*	Kebreab and Murdoch (1999)
<i>Cereus peruvianus</i> (L.) J.S. Muell	31.2	Yang (1999)
<i>Salvia</i> L. (mean of two varieties)	26.2	Kwong et al. (2001)
<i>Delphinium</i> L. (mean of two varieties)	22.3	Kwong et al. (2001)
<i>Eulophia gonychila</i> Schltr.	19.5	Pritchard et al. (1999)
<i>Dactylorhiza fuchsii</i> (Druce) Soó	4.7	Pritchard et al. (1999)
<i>Salix</i> × <i>sericans</i> Tausch × <i>S.</i> <i>viminalis</i> L.	4.4	Wood et al. (2003)
<i>Dendrobium anosmum</i> Lindl.	4.3	Pritchard et al. (1999)
<i>Metarhizium flavoviride</i> W.Gams & Rozsypal	4.3	Hong et al. (1997)

\* based on eRH values, whereas the other studies involved a temperature shift of about 20°C from pre-treatment to storage. On the basis that this temperature shift might increase RH by about  $\leq 10\%$  (Roberts and Ellis, 1989; Vertucci and Roos, 1990; Hay et al., 2003) and that the possible impact of this is a doubling in longevity (Roberts and Ellis, 1989), adjustment of all the data to eRH would raise  $\sigma$  to around 8 to 62 d for the species not marked\*.

spores of the fungus *Metarhizium flavoviride* are about as long-lived as the shortest-lived seed species listed. Whilst this is a small data set, it is worthy of note that the crops and weedy species appear to generally have greater longevity than the horticultural species. There are some limitations to this analysis, though, such as the use of both eRH and pre-storage RH, depending on species/study. An alternative approach to comparative seed longevity on diverse species, using a single set of ageing conditions, is underway within the Millennium Seed Bank Project (H. Davies and R.J Probert, Royal Botanic Gardens, Kew, pers. comm.).

## 2. Comparative Seed Longevity: Half-lives

The production of viability constants allows an objective comparison of seed longevity data, assuming that account is taken of the inter-species dependency of seed moisture content on relative humidity. At a lower level of precision, however, but still of practical interest is the retrospective analysis of historical data.

Priestley *et al.* (1986) carried out probably the largest comparative longevity analysis from storage data for 92 species derived from one or more of 13 localities in Europe, North America and Australia. Half of the species came from one locality only and the others from between two and nine localities. All data were subject to probit analysis to determine the half-viability period ( $P_{50}$ ). Environmental conditions presumably varied between sites and the estimated location effect varied from 2.7 to 15.4% (Priestley *et al.*, 1986). The most valid comparisons are, therefore, for species stored at one location. Some of the data are shown in Table 35.4 for 10 species, mainly grasses and pasture legumes, stored in the UK and France.

A couple of features are evident from the data set: 1) longevity is sometimes greater in the UK than in France, but there is no systematic pattern; 2) longevity in either country does not necessarily reflect the overall country average. When a ranking was produced for the 92 species, the estimated longevity varied from just under 3 years for *Perilla frutescens* (L.) Britton to more than 32 years for *Lycopersicon esculentum* Mill., i.e., a 10-fold difference (Priestley *et al.*, 1986).

Similar  $P_{50}$  data are also available for a range of vegetable species following long-term storage; usually under varying conditions of cool (5°C) to cold (-18°C) temperature, and following moderate drying (40–50% RH) (Table 35.5).

The variation in estimated longevity ( $P_{50}$ ) across these species is approximately 5-fold, from 27 years to 130 years on the average for *Capsicum annuum* and *Pisum sativum* respectively. Thus, the evidence from predictions based on the viability equations (Table 35.2) and observations based on practical experience (Table 35.5) indicate the considerable longevity of dry seeds. But what of survival in the natural environment under unregulated storage conditions?

## 3. Buried and Ancient Seeds

The longevity of ancient seeds has been the source of controversy and discussion for a number of years. There are many examples of anecdotal evidence for longevity in the region of centuries but far fewer confirmed reports (see Priestley, 1986).

Seeds of approximately 40 species were recovered from bricks of adobe walls of historic buildings in California and Northern Mexico, built between 1769 and 1837, and then dry-stored in glass vials at room temperature for c. 50 years (Spira and Wagner, 1983). Of these species, seven had one or more viable

**Table 35.4 Comparative seed longevity (time to 50% viability) for 10 species held under uncontrolled conditions in the UK, France and other countries. Data modified from Priestley *et al.* (1996)**

Species	Longevity ( $P_{50}$ , years)		
	UK	France	Average across all tested countries
<i>Brassica napus</i> L.	8.4	11.9	13.9
<i>Avena sativa</i> L.	11.6	8.2	13.0
<i>Medicago sativa</i> L.	7.1	15.6	10.6
<i>Lolium multiflorum</i> Lam.	8.1	9.0	9.4
<i>Triticum aestivum</i> L.	7.9	6.1	7.6
<i>Hordeum vulgare</i> L.	6.8	8.6	7.2
<i>Dactylis glomerata</i> L.	5.8	6.6	6.6
<i>Phleum pratense</i> L.	7.8	5.5	5.7
<i>Trifolium pratense</i> L.	5.1	11.7	5.4
<i>Festuca elatior</i> L.	6.1	4.9	5.0

seeds: *Malva parviflora* L. (183–200 years old); *Hordeum leporinum* Link (200 years); *Trifolium* L. sp (193 years); *Melilotus indicus* (L.) All. (183 years), *Chenopodium murale* L. (183 years); *Chenopodium album* L. (143 years); and *Medicago polymorpha* L. (200 years). The latter species produced normal seedlings. A similar record exists for seed of *Canna compacta* Roscoe (*Cannaceae* Durande) found within a walnut shell rattle in Argentina. However, subsequent seedling growth was not completely normal (Priestley, 1986).

The most ancient, viable seeds appear to have come from a fruit of *Nelumbo nucifera* (*Nelumbonaceae* Bercht & J.Presl) – sacred lotus – which sprouted after 1288 years burial in a dry lake bed deposit in southern Manchuria (Shen-Miller *et al.*, 1995). Other seeds recovered from dry, hard fruits of this species have been radiocarbon dated to c. 600 years (Shen-Miller *et al.*, 1983). However, whilst seeds 192 to 466 years old were capable of germination/cultivation, the offspring had abnormalities similar to those observed in modern seedlings given high doses of irradiation (Shen-Miller, 2002).

A review of the species listed above, reveals that most belong to genera well known to display the hardseededness trait. In which case, the exceptional longevities recorded may have been associated with maintenance of the dry state. However, there is also evidence that species not expected to have hard seed can display considerable longevity in the region of a century or more; for example, the seed burial experiment of Dr W. J. Beal that was started in 1879. Of 23 different species of locally common plants that were mixed with moist sand in unstoppered glass bottles and buried in a sandy knoll, one species,

**Table 35.5 Estimates of seed longevity (time to 50% viability) for 14 vegetable species following long-term, reduced temperature storage. Data modified from Roos and Davidson (1992)**

Species	Longevity ( $P_{50}$ years)		
	Average	Range	Number of cultivars tested
<i>Pisum sativum</i> L.	130	46–232	3
<i>Abelmoschus esculentus</i> (L.) Moench	125	24–258	5
<i>Lycopersicon esculentum</i> Mill.	124	56–230	5
<i>Zea mays</i> L.	65	52–86	5
<i>Cucumis melo</i> L.	61	47–79	5
<i>Solanum melongena</i> L.	54	27–119	4
<i>Phaseolus vulgaris</i> L.	46	20–90	5
<i>Cucumis sativus</i> L.	45	32–53	5
<i>Beta vulgaris</i> L.	43	34–66	6
<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai	43	34–48	5
<i>Spinacia oleracea</i> L.	37	30–43	3
<i>Daucus carota</i> L.	35	19–68	5
<i>Allium cepa</i> L.	29	11–38	5
<i>Capsicum annum</i> L.	27	17–37	4

*Verbascum blattaria* L., had viable seed (20% germination) after 90 years (Kivilaan and Bandurski, 1973). Similarly, non-hard seeds of *Hordeum vulgare* L. (barley) and *Avena sativa* L. (wild oat) showed 90% and 81% germination respectively after 110 years of hermetic sealed storage in the Vienna herbarium at temperatures between 10°C and 15°C and at c. 3% moisture content (Steiner and Ruckenbauer, 1995). Other seed samples of the same two species survived (10–20% germination) storage at about 7% moisture content for 124 years in a Nuremberg building (Aufhammer and Simon, 1957).

In conclusion, seed viability equations predict seed longevity for some species in the region of many centuries (Hong *et al.*, 1998b), whilst in practice just a few seeds (i.e., a small proportion of the population) are thought to have survived for so long. Thus, whilst seed longevity is quite remarkable it may be the case that the seed viability equations overestimate the longevity potential of seeds. Nonetheless, the inherent stability of seeds in the dry state still offers tremendous hope to seed conservationists wishing to protect against the loss of plant species across the globe.

## Seed Viability Equations: On Reflection

James D. Watson (1988) opined that “No good model ever accounted for all the facts, since some data was bound to be misleading if not plain wrong” (see Mackay, 1991). And this is clearly the case for the seed viability equations. The early models have undoubtedly been proven to be robust tools in the planning of seed storage studies and facilities over many decades, and their limits are now far better understood. As we step outside those limits in search of a new comprehension of the biophysical, mechanistic basis of seed performance, it becomes increasingly likely that the conventional viability equations will, one day, become obsolete with respect to predicting seed longevity at low moisture contents and in cold stores (sub-zero temperature). In the meantime, the underlying principles of survival (or death) articulated in the equations still remain highly pertinent to comparative seed longevity studies and the practical handling of seeds under a wide range of temperature and moisture content conditions.

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