

Chapter 21

Assessing Desiccation Sensitivity:

from diagnosis to prognosis



Olivier Leprince

Institut National d'Horticulture, UMR Physiologie Moléculaire des Semences (INH/INRA/Université d'Angers), 16 bd Lavoisier, F-49045 Angers, France

Summary

To optimise *ex situ* preservation methods, it is imperative to understand the nature of desiccation sensitivity in seeds in order to quantify and predict it. Desiccation tolerance has been regarded as two components: survival during drying and survival in the dried state. Orthodox seeds tolerate complete water loss. Non-orthodox seeds exhibit a wide range of desiccation sensitivity, the quantification of which is still a matter of debate. Desiccation sensitivity can be assessed by determining the water contents corresponding to the onset of, and half of the loss of, viability during drying. Statistic models to determine these water contents are presented. Five factors affecting the response to drying are identified: the “population effect”, the seed characteristics, the drying conditions, the hydration level to which the seed is dried, and the rehydration method. Designing diagnosis tools to test desiccation sensitivity will not be possible without a better understanding of the biochemical and physical processes that occur during drying. Several of these processes are reviewed. There is strong interest in developing molecular and biochemical markers to avoid using assays that only give a retrospective indication of the effects of drying and ageing. Because the presence or the level of protective substances cannot so far predict the level of desiccation sensitivity, an alternative strategy is to use desiccation-induced processes leading either to tolerance of complete or partial water loss (drought tolerance), as a prognostic tool to assess desiccation sensitivity. An assessment is made of whether the emerging techniques, such as functional genomics and quantitative genetics, can help identify global markers as prognostic tools in a wide variety of species.

Introduction

Quantifying and predicting desiccation tolerance and desiccation sensitivity is a prerequisite to optimise *ex situ* long-term preservation methods. Currently, the terms “orthodox” and “recalcitrant” are used to distinguish whether or not a particular seed is able to be stored dry for long periods of time. Experimentally, it is rather simple to identify a seed with an orthodox behaviour because it survives complete drying if it is rehydrated properly. Thus the orthodox behaviour appears as a qualitative trait (“all or nothing” feature) even if it is recognised that desiccation tolerance requires a variety of protective mechanisms. Mechanisms of desiccation tolerance have been studied extensively and received recent in-depth coverage in books (Vertucci and Farrant, 1995, Buitink *et al.*, 2002) and reviews (Leprince *et al.*, 1993; Bewley, 1995; Ingram and Bartels, 1996; Pammenter and Berjak, 1999; Hoekstra *et al.*, 2001). In contrast to orthodox seeds, we are still facing difficulties in categorising recalcitrant seeds because they characteristically exhibit a wide range of desiccation sensitivity (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999). This is illustrated by the late introduction of

the term “intermediate” to characterise seed behaviour between orthodox and recalcitrant and by the occasional use of inappropriate terms such as “sub-orthodox” or “semi-recalcitrant” in conferences. Thus, desiccation sensitivity appears to be a quantitative trait, although the quantification is still a matter of debate. Various factors affect the seeds’ response to drying and there is a lack of consensus on methods to assess survival. This lack of consensus has constrained our efforts to understand desiccation sensitivity. Recent studies have attempted to resolve the contradiction between the qualitative aspect implied in the definition of “orthodox” and the quantitative aspect implied by the term “recalcitrant”.

Compared to the wealth of information on protective mechanisms involved in desiccation tolerance, our understanding of the nature of damage experienced by drying tissues is limited. Furthermore, we are still unable to predict whether a seed of a poorly or non-characterised species is desiccation tolerant before testing its viability after drying, thereby destroying it if it did not tolerate the loss of water (Box 21.1). A new strategy to find markers at the physiological and molecular level that could predict the level of desiccation sensitivity of seeds of a particular species is presented here.

Box 21.1 Distinguishing the dead from the living or the Heisenberg Uncertainty Principle applied to seed storage. It is also relevant to testing desiccation tolerance. [Walters (1998) *Understanding the mechanisms and kinetics of seed aging (reproduced with permission of CABI Publishing)*]

The nature of the seed system confounds our ability to assay and quantify the progress of seed deterioration upon drying and in the dried state, and hence to make predictions about susceptibility. It is difficult to distinguish living from non-living. At present, determining whether a seed is alive or dead has similar limitations as pinpointing the location of an electron. According to the Heisenberg Uncertainty Principle, the position of an electron can only be inferred after it has been moved. Analogously, the question of a seed’s viability is only answered after the seed has germinated. Because sampling for viability necessarily affects the experimental outcome, we cannot evaluate the potential longevity of an individual seed. Once it has been germinated, its longevity can no longer be tested. And once the seed dies, it cannot be retrieved for future studies. Because the health of an individual seed cannot be directly assayed, inferences are made based on the overall quality of the population. This involves sampling the population to determine the proportion of germinable versus non-germinable seeds. Correlative studies of physical and chemical changes that occur as the percentage germination declines must be viewed with caution as it is unclear whether the measured changes occur in all the individuals of the population, the ones that are dying most rapidly, or the ones that are already non-viable.

The Diagnosis:

Testing and Quantifying Desiccation Tolerance/Sensitivity in Seeds

1. Desiccation Sensitivity Based on a Critical Water Content

It has been proposed since 1994 that recalcitrance is not an “all or nothing” feature (Berjak and Pammenter, 1994). This suggestion evolved into the notion that desiccation tolerance is a quantitative feature (Berjak and Pammenter, 1994; Dussert *et al.*, 1999; Pammenter and Berjak, 1999; Walters, 1999). This review abides by the original assertion that it is the sensitivity to drying (i.e., recalcitrance) rather the ability to survive drying (i.e., desiccation tolerance) which is a quantitative feature. The nuance is of importance because it is the responses of non-orthodox seeds to drying that are complex and cause problems to laboratories around the world.

To adequately quantify the level of desiccation sensitivity, many authors determine the so-called critical water content (CWC). It is defined as the water content below which loss of viability (Finch-Savage, 1992; Walters, 1998; Reisdorph and Koster, 1999) or apparent membrane damage (Berjak *et al.*, 1993; Sun *et al.*, 1994; Leprince *et al.*, 1995b, 1999; Reisdorph and Koster, 1999) is observed during drying. The CWC is determined from curves where percentages of survival (usually germination, Figure 21.1A) or electrolyte leakage are plotted against the seed/embryo water content or thermodynamic properties of water (water activity or water potential) obtained at different intervals. The CWC is defined either as the water content corresponding to the discernible loss of viability (see Vertucci and Farrant, 1995) or to 50% of viability loss (Dussert *et al.*, 1999; Walters, 1999). To differentiate between both, Reisdorph and Koster (1999) introduced the term ‘threshold water content’ (TWC) to describe the moisture content corresponding to the onset of loss of viability or membrane damage and instead used CWC to describe the water content at which drying was lethal for a proportion of the seed population (i.e., <50% survival). Thus, CWC and TWC allow a simple assessment of the level of desiccation sensitivity in seeds within a species (e.g., different maturity stages, different origins) and between species. For example, viability curves determined for intact seeds (Figure 21.1A) allow various species to be ranked according to their desiccation sensitivities (Table 21.1).

Using CWC or TWC to characterise the responses to drying and compare the level desiccation sensitivity with different species is however fraught with difficulties that may lead to misinterpretation or inaccurate diagnosis of the level of desiccation sensitivity. The CWC and TWC are dependent on 5 factors that should be taken into account and preferably specified in published articles and databases. These factors are: a) the “population effect” – the viability of a seed cannot be easily and directly assayed against its moisture

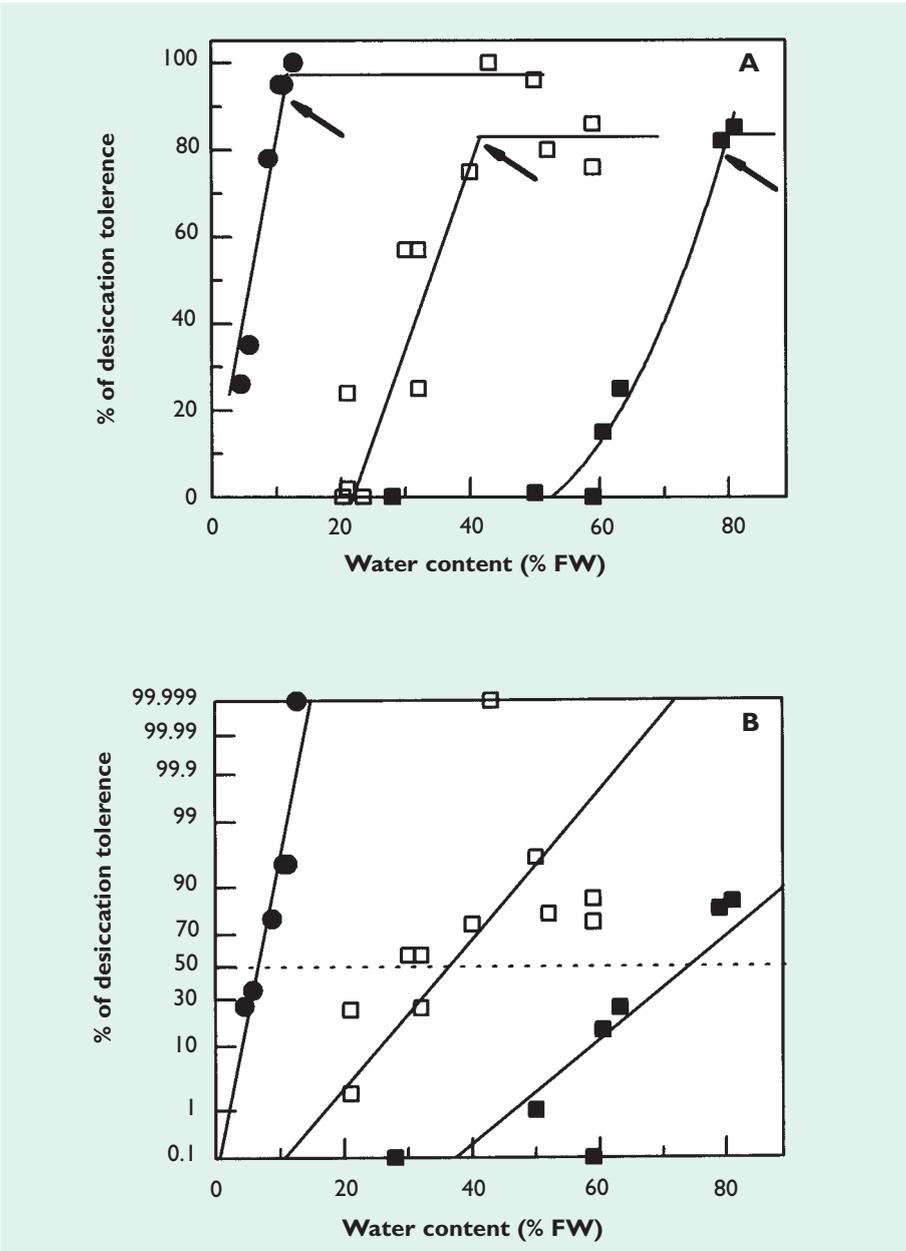


Figure 21.1

The relationship between viability and water content during drying of seeds of cocoa (*Theobroma cacao*, ■), red oak (*Quercus rubra*, □) and coffee (*Coffea arabica*, ●). Viability is expressed as % germination after drying on a linear scale (A) and probit scale (B). Water contents are expressed on a fresh weight basis. Germination data represent the mean of 2 batches of 20–25 seeds. Arrows indicate the threshold water content. (after Leprince et al., 1998).

Table 21.1 The assessment of desiccation sensitivity of seeds of various species representing various storage behaviours

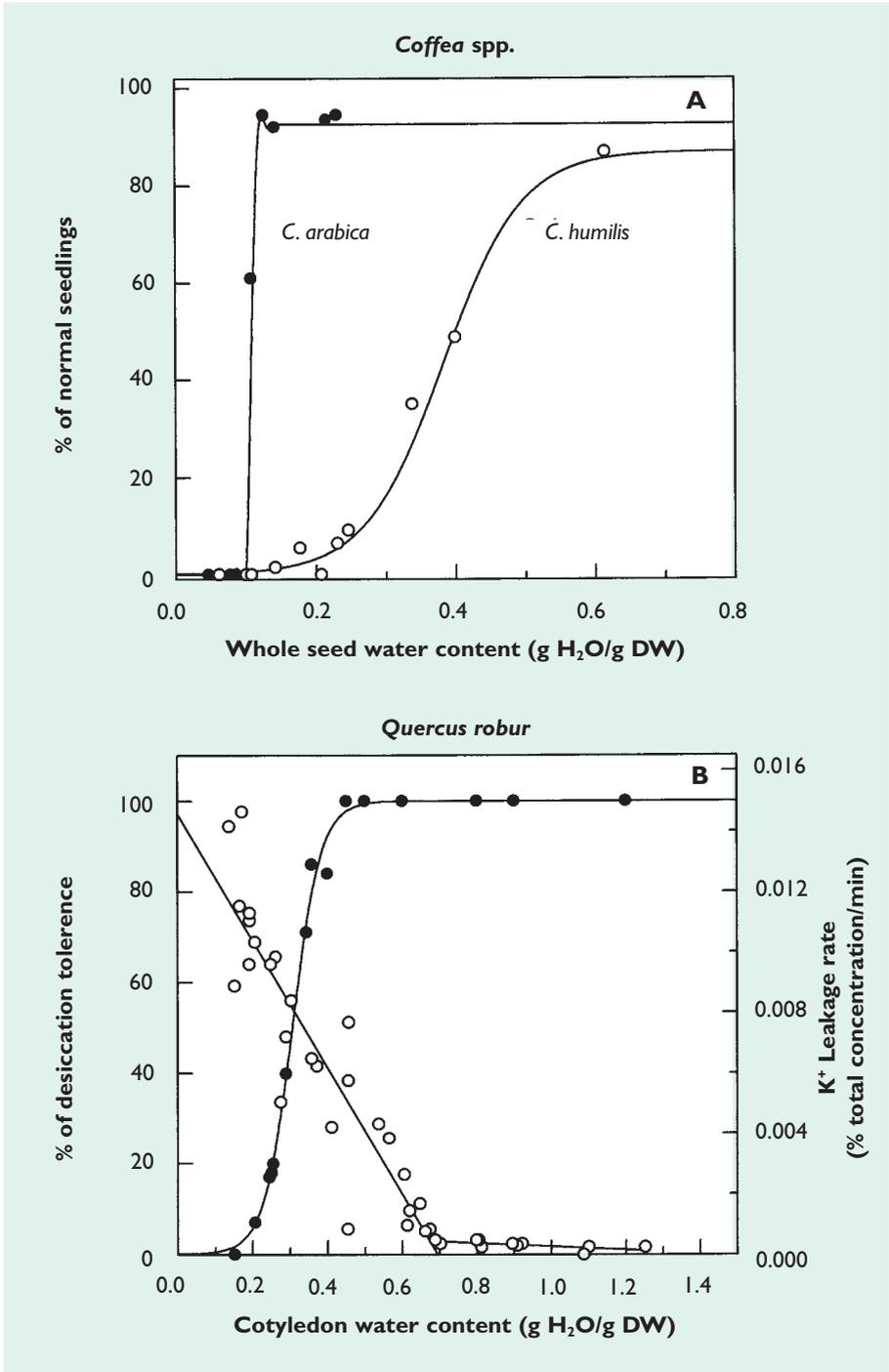
Species	Storage behaviour	Desiccation sensitivity		References
		TWC	CWC	
<i>Brassica campestris</i>	Orthodox	NA	NA	Leprince et al., 1998
<i>Vigna unguiculata</i>	Orthodox	NA	NA	Leprince et al., 1998
<i>Coffea arabica</i>	Intermediate	12	7	Leprince et al., 1998
	Intermediate	ND	11	Dussert et al., 1999
<i>Azadirachta indica</i>	Intermediate/	10	5	Sacandé et al., 1998
	Recalcitrant	13	6	Leprince et al., 1998
<i>Quercus rubra</i>	Recalcitrant	40	15	Leprince et al., 1998
		20	16	Pritchard, 1991
<i>Theobroma cacao</i>	Recalcitrant	80	72	Leprince et al., 1998

TWC and CWC refer to as threshold and critical water content, respectively, and are expressed on a fresh basis without correction for the seed oil content. TWC corresponds to the onset of loss of viability during drying and CWC to the water content at which drying was lethal to 50% of the seed lot. NA, not applicable because orthodox seeds survive complete water loss. ND, not determined. For comparison, data are taken from studies in which drying conditions were similar.

content and inferences are made based on the overall desiccation sensitivity of the population; b) the seed characteristics (anatomy and physiology); c) the drying conditions (rates, temperature); d) the hydration level to which the seed is dried together with the time spent at that level, and e) the rehydration method. These factors are discussed in the following sections.

2. Distribution of Desiccation Tolerance: Continuous or Discrete?

Various levels of desiccation sensitivity within non-orthodox species have been described and quantified by a CWC (as illustrated by Figure 21.1A). However, the determination of CWC can be more easily determined by converting the percentage values into probits (i.e., probability units, Figure 21.1B). When plotted on a probit scale, the relation between percentages of survival and water content fits significantly to a linear regression (Probert and Longley, 1989; Pritchard, 1991; Finch-Savage, 1992; Kovach and Bradford, 1992). Such an approach allows for a statistical estimation of the response of the seed population to drying and infers that desiccation sensitivity is continuously and



normally distributed within the population. This approach has been used to model and quantify the loss of viability during storage (viability equation; Ellis and Roberts, 1980), the temperature/water potential dependence of germination (Pritchard and Manger, 1990; Bradford, 1995) and dormancy loss (Pritchard *et al.*, 1996). Despite the goodness of fit (Figure 21.1B), it is not always possible to conclude whether the linear relationship reflects a continuous distribution of desiccation tolerance or a high variation of seed water contents within each population upon drying. In other words, a linear relationship between percentage of survival and water content during drying may be due to the wide range of desiccation sensitivities among the seed batch or to large experimental variation in individual seed water contents within the sample taken during drying (Probert and Longley, 1989; Pritchard, 1991; Finch-Savage, 1992).

In recent comparative studies on desiccation sensitivity, Dussert *et al.* (1999) and Reisdorph and Koster (1999) introduced a mathematical model that allows a straightforward estimation of both CWC and intraspecific variation of desiccation tolerance, without resorting to probit calculations. These authors argued that the typical S-shape of the viability curves (illustrated in Figure 21.2) corresponds to a quantal response which can be modelled using the equation:

$$V = V_i / [1 + \exp(\beta (WC - WC_{50}))]$$

where V is the % of survival obtained after drying a seed lot to water content WC ; V_i is the initial viability of the seed lot before drying; β is an experimental constant and WC_{50} is the CWC at which 50% viability loss is observed (Dussert *et al.*, 1999). From the equation, the intraspecific variation can be estimated by calculating the interval between WC_{90} - WC_{10} , corresponding to the water

Figure 21.2

A. Quantification and comparison of desiccation tolerance in two species of *Coffea* [*C. arabica* (●) and *C. humilis* (○)]. Survival represents the percentage of normal seedlings, which are plotted as a function of seed water content at different intervals of slow drying. Data are from Dussert *et al.* (1999) and reproduced with permission (CABI Publishing). B. The relationship between cotyledon seed water content and both viability (●) and membrane integrity of axis (○) of *Quercus robur* during fast drying. Viability is assessed on the embryonic axis and expressed as % of radicle emergence. Membrane integrity is assessed as the rate of K^+ leakage during rehydration following drying (Leprince, Wutz and Hoekstra, unpublished observations). *Coffea* spp. and *Q. robur* viability data were fitted with the desiccation sensitivity model developed by Dussert *et al.* (1999) and computed by the Quasi-Newton method. Leakage data were fitted with linear regressions and computed by the method of Berjak *et al.* (1993).

content at which 90% and 10% of viability is reached during drying, respectively. This interval can be calculated as:

$$\Delta WC = WC_{90} - WC_{10} = 2\ln(9)/\beta$$

This model is very attractive because it does not involve the mathematical complexity of a normal distribution and probit analysis. Furthermore, two statistical descriptors, WC_{50} and ΔWC (or β), can adequately assess the level of desiccation tolerance and the seed-to-seed variation for desiccation sensitivity, respectively (N.B. Similar parameters can be estimated using the probit approach). Therefore, both parameters are considered to be extremely helpful in assessing the level of desiccation sensitivity from seed lots for which it is not always possible to control the origin of the lot, its maturation stage at harvest, and its genomic purity. For example, this model was used in a comparative study to show that desiccation sensitivity in seeds of various species of *Coffea* spp. cannot be categorised in discrete levels and may be considered as an adaptive feature (Figure 21.2; Dussert *et al.*, 1999, 2000). These authors also argued that from the interval, it can be inferred whether one or more damaging factors can be held responsible for the level of desiccation tolerance obtained from a seed lot. The smaller the value, the lesser number of putative factors are likely to be involved (Figure 21.2). The model also allows the comparison of the seed-to-seed variation of desiccation sensitivity with the seed-to-seed variation of water content or any factor that is potentially linked with the desiccation sensitivity (for example, the lack of a particular protective substance). The use of the seed-to-seed responses to compare desiccation sensitivity in recalcitrant seeds is further illustrated in Table 21.2.

3. Seed Characteristics

Both seed anatomy and physiology may exert an influence on the two parameters that determine the CWC (i.e., water content and physiological criteria assessing survival after drying). Seed size, the presence of impermeable testa and/or reserve tissues surrounding the embryo (endosperm or perisperm), and the nature and amount of the storage, reserves all influence the hydraulic conductance and water properties. Thus, the seed characteristics can affect the kinetics of drying (reviewed in Probert and Hay, 2000), the water distribution and properties within the different tissues during and after drying (for example, Pritchard, 1991; Grange and Finch-Savage 1992; Eira *et al.*, 1999), and the kinetics of rehydration after drying (reviewed in Osborne *et al.*, 2002). The complexity of the relationship between seed characteristics and the response to drying is illustrated by studies on coffee seeds that are considered to have storage behaviour intermediate between orthodox and recalcitrant (Ellis *et al.*, 1991). When the water sorption properties of seeds of several *Coffea* spp. were assessed on a whole seed basis, they were found to be typical of orthodox seeds (Eira *et al.*, 1999). However, when these properties were assessed on excised embryos,

they appeared to be intermediate between those of desiccation-tolerant and -sensitive embryos. Thus, expressing a CWC based on a whole seed when the viability assay does not integrate the whole seed physiology (for example, germination which involves mainly the embryonic axis) may be misleading. This point is illustrated in Table 21.2 with seeds of *Quercus rubra*.

It should be emphasised that for oily seeds, the amount of oil may lead to an underestimate of the actual water content present in the tissues. For instance, the water content of neem seeds held at 60% relative humidity and 20°C is about 6% (fresh weight basis) when it is calculated on the whole seeds. However neem seeds may contain up to 50% of storage lipids, a component that does not interact with water. Therefore, the amount of water actually present in the cytoplasm is much higher than 6%. Taking into account the oil fraction, the hydration level of the non-lipidic components is about 13% (Sacandé *et al.*, 1998). For oily seeds, the interaction between water and seed is then more easily assessed by a water sorption isotherm which can be obtained by measuring equilibrium moisture content as a function of relative humidity at constant temperature. Typical water sorption isotherms and their determination have been published elsewhere (Walters, 1998; Dussert *et al.*, 1999; Eira *et al.*, 1999; Probert and Hay, 2000). Thus, in order to compare the responses to drying of seeds from different species, the respective water content should be expressed on the basis of the non-lipidic seed material rather total seed weight.

Seed dormancy can also interfere with accurate assessment of germination after drying and complicate the diagnosis of desiccation sensitivity (Tompsett and Pritchard, 1993, 1998; Pritchard *et al.*, 1996; Wood *et al.*, 2000).

The level of desiccation sensitivity in some species varies among the different organs of the mature seeds and depends on whether these organs are dried intact in the seed or after excision. Cotyledons of *Castanea sativa* (Leprince *et al.*, 1999) and tea (Kuranuki and Yoshida, 1996) appear to be more sensitive to drying than axes. In orthodox seeds, differential tissue sensitivity to drying was also noted during the loss of desiccation tolerance of germinating seeds of pea (Reisdorph and Koster, 1999). Thus, depending on how survival is assessed, seed tissues may need to be examined separately.

The stage of maturity also has a strong influence on the level of desiccation sensitivity (Table 21.2; Berjak *et al.*, 1993; Finch-Savage and Blake, 1994; Pammenter and Berjak, 1999). For seeds of wild species, this factor cannot always be controlled when the seeds are collected in the field. Therefore, efforts should be made at the seed cleaning step in the laboratory to homogenise the seed batch to a single developmental stage.

In conclusion, it is advised that for species that are poorly characterised for their seed storage behaviour, basic information on the seed characteristics should be obtained and taken into account when determining the level of desiccation sensitivity (Box 21.2).

Box 21.2 Check-list to assess seed characteristics of a seed batch collected in the field prior to testing desiccation sensitivity

- 1) Obtain information on seed anatomy and physiology through data base searching or experimentation.
- 2) Identify the nature of seed envelopes. Are they permeable to water and vital stains?
- 3) Dormancy status: Yes/No? If yes, is it coat-imposed or embryonic?
- 4) Determine the nature and amount of storage reserves (protein/starch/lipids).
- 5) Assess water content of the embryonic axis, cotyledons, and endosperm, if present.
This determination will allow the calculation of the intra-tissue variation (and)
- 6) Check if the seed is sensitive to chilling temperature (before and after drying)

4. Drying Conditions

Seeds of both orthodox and recalcitrant types are sensitive to the rate of drying and temperature (Hong and Ellis, 1990; Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Walters *et al.*, 2001). For instance, in imbibed embryos of pea, an orthodox seed, drying rate can be manipulated so that a CWC can be observed as high as that of tea seeds, a recalcitrant species (Walters *et al.*, 2001). Table 21.2 further illustrates the impact of drying conditions on desiccation sensitivity and our interpretation of it. Table 21.2 shows that the rate of drying influences the seed-to-seed variation of desiccation sensitivity in tea seeds. Alternatively, this variation could be interpreted as an indication of a continuum rather than discrete levels of desiccation sensitivity. However, such an interpretation warrants validation.

Despite its importance, the rate of drying is poorly quantified in the literature and often has different meanings in different laboratories (for example slow drying can refer to hours, days or weeks). Additional confusion is also brought about by the fact that the rate of water loss depends both on the drying method and the hydraulic conductance of the seed, which is a complex factor of size, shape and anatomy. Regardless of the precise rate of water loss, both positive and negative effects of rate on survival have been observed, suggesting that there may be no universal drying rate to characterise the level of desiccation sensitivity. Therefore, in any comparative study, reference to the rate of drying should be based on the rate of seed/organ water loss rather than the drying time *per se*. Recently, Liang and Sun (2000) proposed a method to estimate the rate of water loss by calculating the slope of the linear regression between time of drying and seed water loss expressed on a dry weight basis and plotted on a semi-logarithmic scale.

Table 21.2 Examples of factors affecting the assessment of the desiccation sensitivity in three recalcitrant species, *Quercus rubra* (red oak), *Camellia sinensis* (tea) and *Quercus robur* (pedunculate oak)

Desiccation sensitivity parameter	Water content (%)	
	whole fruit	embryonic axis
<i>Quercus rubra</i>		
Threshold water content	20	32
Critical water content	14.6 ± 0.2	21.6 ± 1.6
Seed-to-seed variation (DWC)	8	16
<i>Camellia sinensis</i>	Fast drying	Slow drying
Threshold water content	27	56
Critical water content	21.1 ± 0.7	46.9 ± 0.8
Seed-to-seed variation (DWC)	17	41
<i>Quercus robur</i>	early harvest	late harvest
Threshold water content	36	29
Critical water content	27.5 ± 0.6	25.5 ± 0.2
Seed-to-seed variation (DWC)	17	8

Desiccation sensitivity was established by measuring the percentage of germination at different intervals of drying and assessed using the model of Dussert *et al.* (1999) with the data of Pritchard (1991) for red oak, of Walters *et al.* (2001) for tea and of Finch-Savage and Blake (1994) for pedunculate oak. The threshold water content corresponds to the onset of loss of viability during drying and the critical water content corresponds to water content at which drying was lethal to 50% of the seed lot. The seed-to-seed variation reflects the distribution of desiccation sensitivity within the seed population and is calculated as the interval of water content corresponding to the interval between 90 and 10% viability. For the critical water content, the fitting procedure allowed calculation of the standard error. Water contents are expressed on a fresh weight basis. In red oak, it was found that when water contents are expressed on the whole seed basis, the sensitivity to drying appears to be higher than when water contents are expressed on the embryo axis basis. However, this differential sensitivity is an artefact. For tea, the drying rate was found to influence the three parameters characterising desiccation sensitivity. For pedunculate oak, the level of desiccation sensitivity depends of the stage of the fruit maturity. Maturity stage was characterised as two harvests: early and late after shedding. It is noteworthy that the decrease in seed-to-seed variation with increasing maturity reinforces the suggestion that *Q. robur* seeds exhibit an indeterminate development.

Seeds can be inherently sensitive to low and high temperatures. Seeds from tropical origin tend to be susceptible to chilling injury. Therefore, the drying temperature may have a direct impact on the level of desiccation sensitivity observed. The fast removal of water will also produce a temperature effect on desiccation sensitivity, although this is not always appreciated. Water evaporation is an endothermic process consuming 600 cal per g of water. Thus, when fast or flash drying methods using air convection are employed, the temperature within the seed will be significantly lower than that of the environment and could drop to the water freezing point. This may be of crucial importance for species of tropical origin which never experience chilling temperatures.

5. Sensitivity to Imbibitional Stress

Several species, particularly those of tropical and subtropical origin, are known to suffer when the seeds take up water at chilling temperatures. Injury may also occur at room temperature when the seeds are very dry before imbibition (Wolk *et al.*, 1989; Hoekstra *et al.*, 1999; Osborne *et al.*, 2002). Upon imbibitional stress, seeds leak solutes and macromolecules, leading to loss of cellular integrity and in turn, to the death of the tissues. Despite its widespread occurrence, the impact of imbibitional stress on the expression of desiccation sensitivity has not always been fully appreciated. For example, Kovach and Bradford (1992) showed that the loss of viability in wild rice was due to imbibitional damage that had previously been interpreted as desiccation intolerance. Imbibitional injury is also a risk in orthodox seeds, e.g., pea (Ellis *et al.*, 1990) and in neem, a tropical species, that only exhibits an orthodox-type behaviour when the stored seeds are rehydrated above 20–25°C (Sacandé *et al.*, 2001). The impact of imbibitional damage on seedling emergence is also illustrated in Table 21.3.

6. Membrane Permeability Assays

Most of the studies mentioned above have defined desiccation tolerance as the ability of a seed to germinate after drying. Next to survival assays, additional methods have been used to further quantify desiccation sensitivity and to adequately reflect the deleterious effect of drying on desiccation-sensitive seeds. Since membranes are considered a primary target of desiccation injury, plasma membrane integrity has been the method of choice and utilised as a criterion for desiccation tolerance. Two methods are currently used: leakage assays (Figure 21.2B; Poulsen and Eriksen, 1992; Berjak *et al.*, 1993; Sun *et al.*, 1994; Leprince *et al.*, 1995b; Reisdorph and Koster, 1999) and EPR spectroscopy of spin probes (Golovina *et al.*, 1998, 2001; Leprince *et al.*, 1999; Sacandé *et al.*, 2001). The advantages and disadvantages of both methods have

Table 21.3 Percentage of survival (% of radicles with a length of more than 5 cm) of *Vigna unguiculata* seeds following imbibitional stress together with the number of layers exhibiting dead cells in the cortex

Time of imbibition at 0°C (min)	% of survival	% dead cell layers
0	86 ± 12	0.1 ± 0.1
30	40 ± 6	36 ± 5
60	0	52 ± 11

Imbibitional stress was applied by soaking dry seeds at 0°C for 0, 30 and 60 min before placing them into optimal conditions of germination at 25°C for 5 d. The number of dead cells layers is expressed as % of total amount of layers within the cortex. Dead cells were assessed using vital staining and electron microscopy (Leprince *et al.*, unpublished observation). Data (mean ± SE) are the average of at least triplicate experiments

been described elsewhere (Hoekstra *et al.*, 1999; Golovina *et al.*, 1998, 2001). With both methods, a TWC can be detected, reinforcing the significance of this parameter to assess desiccation tolerance (Figure 21.2B). It should be pointed out that membrane permeability assays are also dependent on the seed characteristics, and drying and rehydration conditions.

Based on a study of membrane permeability in wheat embryogenesis, Golovina *et al.* (2001) argued that the inability to germinate after drying does not necessarily mean that the entire tissue is equally sensitive to drying. Reisdorph and Koster (1999) reached a similar conclusion using an argument based on the population effect. Obtaining 60% of survival at a particular water content does not necessarily imply that 60% of alive embryos did not suffer any damage. This is illustrated in Table 21.3 which shows the extent of imbibitional injury in a population of embryonic axis of *Vigna unguiculata*. To effectively prevent axes from germinating, only about 50% of the tissues need to be irreversibly damaged.

Strategies to Move From Diagnosis to Prognosis

The currently proposed mechanisms for desiccation tolerance are based on three criteria that seeds must meet to survive desiccation: 1) limitation of any desiccation-induced damage to a repairable level; 2) maintenance of physiological integrity in the dried state; and 3) mobilisation of repair mechanisms upon rehydration. The first two criteria are well documented in the literature (reviewed in Crowe *et al.*, 1992; Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Buitink *et al.*, 2002) whereas the latter has not been extensively studied in relation to desiccation tolerance (Osborne *et al.*, 2002). It has been inferred that different processes confer protection against the deleterious effects of water loss and the absence of one or more of these processes could determine the level of desiccation tolerance of an individual species (Vertucci and Farrant, 1995; Walters, 1999; Pammenter and Berjak, 1999). However, qualitative and quantitative data relating the level of desiccation sensitivity to damage are scant.

1. Correlation Between Levels of Protective Substances and Desiccation Tolerance

There is a large body of evidence that shows that both non-reducing sugars (Crowe *et al.*, 1992) and LEA proteins (Close, 1996; Cumming, 1999; Sales *et al.*, 2000) exert protective effects against water deficit and dehydration. Based on these premises, several attempts have been made to correlate the level of desiccation tolerance with the presence and amount of non-reducing sugars (sucrose and/or oligosaccharides) and dehydrins – late embryogenesis abundant (LEA) proteins that accumulate during drying. There is increasing evidence indicating that failure to express certain LEA proteins (Bradford and Chandler, 1992; Finch-Savage *et al.*, 1994; Wechsberg *et al.*, 1994; Black *et al.*, 1999) or to synthesize non-reducing sugar (Hoekstra *et al.*, 1994; Ooms *et al.*, 1994; Lin *et al.*, 1998; Black *et al.*, 1999; Bentsink *et al.*, 2000; Dussert *et al.*, 2000; Buitink *et al.*, 2000a; Hong *et al.*, 2000) cannot be taken as a prognosis tool to assess the level of desiccation sensitivity of seeds of a particular species or to categorize its storage behaviour. The above mentioned studies do not actually argue against the importance of these protective substances in desiccation sensitivity. Rather, they illustrate the potential pitfalls from reliance upon simple correlations. A similar caveat was made earlier by Hendry (1993) concerning studies aimed at establishing a link between the efficiency of free radical processing systems and desiccation sensitivity.

2. Correlation Between Desiccation-induced Injury and Recalcitrance

Because the presence or the level of protective substances cannot so far adequately predict the level of desiccation sensitivity, an alternative strategy would be to use desiccation-induced processes leading either to tolerance of complete, or partial, desiccation (drought tolerance), or to death, as a prognostic tool to assess the level of desiccation sensitivity. Identification of various stresses that occur early upon drying has not received much attention in the literature and warrants further efforts for several reasons. If the damage occurs at high water content during drying, before the loss of viability is discernible, its early detection would make it possible still to resort to alternative methods of conservation without losing the seed batch. For example, in the field of post-harvest technology, a loss of chlorophyll content and a decreased photosynthetic activity is widely used as an early visible symptom of senescence (Buchanan *et al.*, 2000). Furthermore, a better understanding of the nature and causes of damage during water removal will also help to evaluate the role of various putative protective substances (Walters, 1999).

Increase in membrane permeability, loss of membrane function and disturbances in metabolism are characteristic features of desiccation-sensitive tissues. Currently, these defects are thought to originate from changes in the physical properties of the cytoplasm and macromolecular structures during drying. For example, cytoplasmic viscosity increases several-fold during drying. Consequently, metabolite diffusion, and O₂ solubility and diffusion are affected, which in turn may have an impact on metabolism (Leprince and Hoekstra, 1998). Such an impact has been observed in drying pea and cucumber axes that were sensitive to drying (Leprince *et al.*, 1998). In both species, an oxygen-dependent upsurge in acetaldehyde and ethanol was detected before the onset of membrane damage. Drying also induces a partitioning of amphiphilic molecules from the cytoplasm to the lipid phase, thereby delocalising key molecules such as antioxidants or fluidising phospholipid bilayers (Golovina *et al.*, 1998). Chromatin condensation/decondensation depends strongly on changes in ion concentrations and was found to be sensitive to the loss of water (Leprince *et al.*, 1995a). The loss of water may also concentrate solutes and ions present in the cytoplasm. The impact of the level of chromatin condensation on gene expression during drying and desiccation tolerance is unknown. The pattern of increase in cytoplasmic viscosity appeared to be different in desiccation-tolerant and -intolerant tissues and the difference was seen at high water content during drying (Leprince and Hoekstra, 1998; Golovina, *et al.*, 1998; Buitink *et al.*, 2000b).

There is compelling, albeit indirect, evidence that metabolism must be down-regulated in a coordinated way in order to achieve desiccation tolerance (Leprince *et al.*, 1999, 2000; Buitink *et al.*, 2002). In normal physiological conditions, regulation of metabolism is tight and controlled

both at the level of enzyme activity and gene expression (Buchanan *et al.*, 2000). If this control is lost because of stressful conditions, shifts in metabolism may occur. In drying tissues, they can be detected by rapid changes in concentrations of by-products of metabolism (adenylates, glycolytic products, organic acids, acetaldehyde and ethanol). Gas exchange measurements may be a rough indicator of unbalanced metabolism when abrupt changes in respiratory quotient (the ratio between CO₂ production and O₂ consumption rates) are observed.

Oxidative stress can also be categorised as unregulated or unbalanced metabolism. The rate of formation of reactive oxygen species is increased upon stressful conditions and cannot be matched by the activities of antioxidant systems, resulting in a net increase in free radicals. Symptoms of oxidative stress are usually the presence of peroxidized products or breakdown products of lipid peroxidation such as malonyldialdehyde and ethane. A rise in antioxidant levels during drying generally occurs as a change in the cellular redox balance and can also be interpreted as a response to a disturbance in free radical metabolism. For example, in the recalcitrant seeds of *Shorea robusta*, superoxide dismutase (SOD) activity increased upon drying before the loss of viability (Chaitanya and Naithani, 1994). In a comparative study on oxidative stress and antioxidants in seeds of *Acer* spp., an increase in γ -tocopherol concentration upon drying was the only difference found between desiccation-sensitive and -tolerant seeds (Greggains *et al.*, 2000). These above examples are counterintuitive because a rise in antioxidant defence was linked not to desiccation tolerance as expected by the assumption made in previous studies, but to desiccation sensitivity. In fact, these examples show that free radical metabolism in these recalcitrant seeds has been altered early during drying and that the system responded to it by an increased antioxidant activity. Symptoms of oxidative stress do not have to be interpreted necessarily as a cause of cell death, but rather an indication that the seed tissues are suffering from the water loss. Any disturbances in metabolism during drying should be set against a range of desiccation-induced damage and not only against percentages of survival after drying. This approach is necessary to ascertain that disturbances of metabolism are not due to the loss of membrane integrity during drying.

Another aspect of the role of metabolism in desiccation tolerance that has been largely ignored is the question as to how regulatory mechanisms of biochemical pathways are modified in relation to desiccation tolerance. This question can be appreciated by considering investigations on resurrection plants. Instead of accumulating starch as a photosynthetic carbon source, the hydrated leaves accumulate large amounts of 2-octulose, an unusual C8 sugar that is transported throughout the plant. Upon drying, octulose is rapidly converted into sucrose and stachyose (Bianchi *et al.*, 1991; Bartels and Salamini, 2001). There must be regulatory mechanisms that allow an accumulation of sucrose without it being used in the respiration. Despite the importance of sugars in desiccation tolerance, the biochemical basis for the switch in metabolism is poorly understood and its elucidation could help identifying markers for

desiccation sensitivity. It should be pointed out that biochemical markers will strongly depend on the nature of the chemical reserves (lipid *vs.* starch) in seeds and the overall composition of various organelles.

Except from substances associated with oxidative stress and deranged metabolism, it may also be interesting to screen for substances that are associated with osmotic adjustments associated with water stress. Osmotic adjustments are activated in tissues coping with mild protoplasmic water deficits (Buchanan *et al.*, 2000). Increase in proline concentration is not likely to serve a mechanism of desiccation tolerance but rather as a mechanism to adjust cell water potential when vegetative tissues are coping with a water deficit (Buchanan *et al.*, 2000). A 10-fold increase in proline concentration has been measured early during drying before the loss of viability in two recalcitrant species, *Quercus robur* (Poulsen and Eriksen, 1992) and *Machilus thunbergii* (Lin and Chen, 1995). To our knowledge, there is no report of proline accumulation in drying orthodox seeds. Further research is needed to establish whether osmotic adjustments upon drying in desiccation tolerant and intolerant tissues are accomplished via different mechanisms. If osmotic adjustments were dependent on the protoplasmic water content (Hoekstra *et al.*, 2001) and differentially regulated according to the level of desiccation sensitivity, an accumulation of osmolytes early during drying could serve as a biochemical prognosis tool.

The Prognosis:

Towards Identifying Molecular and Biochemical Markers

There is a strong interest in developing molecular and biochemical markers to avoid using assays that only give a retrospective indication of the effects of drying and ageing (Box 21.1). Understanding gene regulation is particularly important in this respect. Since desiccation tolerance is a multigenic trait (Leprince *et al.*, 1993; Vertucci and Farrant, 1995), there are likely to be many regulatory pathways that determine the activation of genes involved in the biosynthesis of protective substances and the repression of genes involved in biosynthetic pathways that are incompatible with the loss of protoplasmic water. There are two main approaches that can lead to the identification of regulatory molecules implicated in the response to drying (Ingram and Bartels, 1996). The first strategy would be to identify putative gene promoters, transcription factors and signalling molecules using molecular biology and genetic approaches, such as screening for dominant mutants, gene trapping and promoter analysis (Bartels and Salamini, 2001, and references therein). In the resurrection plant *Craterostigma plantagineum*, several genes encoding desiccation-induced transcription factors have been identified (Bartels and Salamini, 2001). Their role in desiccation tolerance is however still unclear. In

order to obtain markers of desiccation-induced damage, it would also be relevant to look for putative transcription factors or signalling molecules linked to a stress response that is indicative of desiccation sensitivity.

The second strategy comes in the wake of recent developments in the functional elucidation of the genome. Emerging technologies, such as mass sequencing, mRNA expression profiling analysis (i.e., transcriptomics), and protein and metabolite profiling analysis (i.e., proteomics and metabolomics), may be helpful in finding global markers as prognostic tools to assess the level of desiccation sensitivity. Transcriptomics, proteomics and metabolomics refer to a “brutal force”: systematic analysis of mRNA, proteins and metabolite populations present in a plant extract [for reviews on proteomics see Abott (1999), Blackstock and Weir (1999) and van Wijk (2001); on metabolomics see Fiehn *et al.* (2000), and on transcriptome analysis see Baldwin *et al.*, (1999) and Girke *et al.* (2000)]. In the present context, the idea is to use these technologies to screen for genes, proteins or metabolites that are characteristically present/absent in desiccation-tolerant or sensitive tissues. In seed science, these techniques have already been applied to study germination (Gallardo *et al.*, 2001) and seed development (Girke *et al.*, 2000). The main advantage of these high-throughput techniques is that they generate a vast amount of new information that could not have been otherwise obtained in a reasonable time, thereby shedding new light on the gene expression and metabolic pathways involved in stress response and development.

The road to discover markers for desiccation tolerance/sensitivity that can be used in the field is still dauntingly long. The caveats that have been described in the previous sections (seed characteristics and drying/rehydration conditions) are still valid. In addition, these technologies are still at their infancy and many technical challenges and pitfalls remain. For example, a proteomic study of germinating seeds of *Arabidopsis thaliana* could only identify with certainty 5% of the polypeptides extracted from the entire seed (Gallardo *et al.*, 2001). In addition, researchers carrying out investigations in the plant conservation and seed desiccation area will face two major drawbacks. First, these techniques are not yet applicable for species for which the genome has not been sequenced because it is too large, too complex or of no commercial interest. At present, sequencing efforts and functional genomics are focused on a handful of agronomically important crops (rice, maize, soybean, and *Medicago truncatula*) and on *A. thaliana*. It is estimated that the odds of identifying a polypeptide from a 2D gel in December 2001 is about 70% for maize and pea, 95% in yeast, but 0% for fungi of the *Fusarium* genus. Also there are increasingly raised voices against the current adage that “what is true for *Arabidopsis* is true for all plants” (Fernie and Willmitzer, 2001; Mathesius *et al.*, 2001). For example, the microsynteny between *A. thaliana* (*Brassicaceae*) and *M. truncatula* (*Fabaceae*) is less than 10% (Mathesius *et al.*, 2001). The second factor precluding a sensible accessibility and wide usage of these techniques is the high operational cost and the need for a high level of expertise in a wide array of techniques and disciplines.

Conclusion

There is such a considerable variation in the responses to drying that as yet no emerging pattern can be related to desiccation sensitivity. This review argues that some of these variations are inherent to the species but others originate from the lack of consensus of methods to assess desiccation tolerance. Unfortunately, this has not always been appreciated in the past. Therefore, great caution should be taken when comparing data from the literature. This review revealed a gap between the current state of emergency to preserve germplasm and our lack of understanding of the phenomena leading to desiccation tolerance/sensitivity, which may appear worrisome. To end on a positive note, two promising studies on the most recalcitrant organism are worth mentioning. Puhlev *et al.* (2001) and Wolkers *et al.* (2001) recently described two methods to successfully dry human blood cells while maintaining a high percentage of viability, an achievement that was not imaginable ten years ago.

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