Title: Optimizing Seed Water Content: Relevance to Storage Stability and Molecular Mobility

Running title: Optimum Seed Moisture Content

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Abstract

This research was conducted to determine the optimum MC (moisture content) that gave maximum longevity to seeds. Three species were used to represent seeds with different dry matter reserves which gives them different sorption properties: maize (Zea mays L.), elm (Ulmus pumila L.) and safflower (Carthamus tinctorius L.). The seeds of elm, safflower, and maize embryos with MC ranging from 0-0.15 g H₂O /g DW (Dry Weight) were stored at 35°C for different time. The results showed the optimum MC for seeds and embryos storage varied between species (0.057 g H₂O/g DW for maize embryos, 0.045 g H₂O/g DW for elm, and 0.02 g H₂O/g DW for safflower). Drying below this optimum MC increased the aging rate and there were detrimental effects of drying. The relative humidity corresponding to optimum MC in embryos of maize, elms and safflower was about 15%, 12% and 7% respectively, according to the lipid composition of the embryos. The data provided confirmatory evidence that molecular mobility (ΔAzz) in elms, maize and safflower embryos was compatible with the optimum moisture content.

Key words: seed storage; Molecular mobility; Optimum water content; relative humidity.
Seed moisture content (MC) and storage temperature are the most important factors affecting seed longevity and vigor during storage (Vertucci and Roos 1991, 1993; Vertucci et al. 1994). FAO/IPGRI (1994) recommended drying seeds to water content between 3 and 7%, depending on the lipid content of the seeds, for seed conservation in genebanks. However, studies have consistently indicated that the longevity of seeds of many crops, ornamentals and trees containing different chemical components could be greatly increased by drying them below the recommended moisture range (Ellis et al. 1988, 1989, 1990ab, 1995; Ellis and Hong 1996, 2006, 2007ab; Vertucci and Roos 1990, 1993, 1994; Wang et al. 2003; Zheng et al. 1998). Tests also clearly showed that seed life spans could not be prolonged indefinitely by progressively drying seeds (Ellis et al. 1988, 1989, 1990ab). These data indicate that seed life span at a certain temperature is finite, and the MC below which longevity could not be improved was considered as a critical MC. The value of the critical MC varies among species (Ellis et al. 1988, 1989, 1990ab; Vertucci and Leopold 1987; Vertucci and Roos 1990). There is evidence that the value of the critical MC varies among species in an inverse relationship with the lipid content of the seed (Ellis et al. 1989, 1990ab; Vertucci and Roos 1990).

The possibility that drying below a critical MC might result in faster seed aging sparked a debate on the benefits and risks of a newly introduced technology to ultradry seeds for seed conservation. Vertucci and colleagues (Vertucci and Roos, 1990, 1991; 1993; Vertucci et al., 1994; Walters, 1998a, b; Walters et al., 2005) introduced the concept of optimum MC or relative humidity for seed storage because they found that drying below a certain relative humidity increased seed aging rates. They demonstrated that the relative humidity at the optimum was similar among diverse species and storage temperatures, and consequently, the MC at the optimum varied widely. However, Ellis and colleagues (1989, 1990a,b) did not observe detrimental effects of drying, and so concluded that aging rates MC for seeds dried to less than a critical MC. Recently, Ellis and colleagues demonstrated a change in critical MC with storage temperature that was similar to Vertuccis earlier observations (Ellis and Hong, 2006). The reason for the controversy was the incorrect assumption of the Viability Equations that MC and temperature are independent factors regulating seed aging rates.
Differences in the temperature at which storage experiments were conducted between these two labs account for differences in the ability to detect detrimental effects of extreme drying on seed aging. Ellis and colleagues performed storage experiments at 65°C and observed a critical MC but no detrimental effects of drying. Assuming that MC and temperature were independent factors regulating seed aging rate according to empirically-derived Viability Equations, these authors extrapolated observations made at high temperatures to lower temperatures more typically used for seed storage or rapid aging experiments. In contrast, Vertucci and colleagues used thermodynamic considerations to deduce water content-temperature interactions and supported their conclusions with experiments conducted at a range of temperatures less than 65°C, in which they were able to detect deleterious effects of overdrying.

It is assumed that the high viscosity of intracellular glass decreases molecular mobility and impedes diffusion, thus slowing down degradable processes during aging (Vertucci 1990; Sun and Leopold 1993; Walters 2004). A relationship between longevity and the mobility of molecules in the glassy cytoplasm has been found in Typha latifolia pollen and pea seeds (Buitink et al. 1998a), and a marked increase in cytomatrical viscosity has been described for pea (Pisum sativum) seeds at tissue water concentrations below 0.3gH2O/DW, typical of a glass formation system (Buitink and Leprince 2004). However, the relation between molecular mobility and critical MC has received little attention (Walters, 1998; Walters et al., 2005).

This experiment was designed to elucidate the optimum MC of three types of seeds embryos with different chemical components according to our previous work (Wang et al. 2001) such as: protein rich seeds of elms (Ulmus pumila L.), starch rich seeds of maize (Zea mays L.) and oil rich seeds of safflower (Carthamus tinctorius L.), which were stored at 35°C. Moreover, we identified water properties in seeds and investigated the possible relationship between molecular mobility and optimum MC for seeds storage.

Results

Effect of Low-Moisture Storage on Seed Germination and Vigor

The influence of MC on longevity of seeds stored at 35°C was studied using three seed species with different chemical components. All seed species had initial germination
percentages greater than 90%.

The elm seed is a typical short-lived seed that survives only about one month at 35°C temperature. It loses its vigor rapidly during the high temperature and humidity of the summer season. The results showed loss of germination percentage of elm seeds was progressive with time after one month of storage at 35°C. The decline in germination percentage with MC was faster for seeds stored with higher water content (ranging from 0.053-0.105 g H₂O/g DW) and lower water content (from 0.020 to 0.030 g H₂O/g DW) (Figure 1A and B). There was no significant loss of germination potential at 0.045 g H₂O/g DW, implying that this was the optimum water content for storage at 35°C. Aging rates are greater if seeds are store at MC above or below the optimum MC.

The change in germination percentage of maize embryos at 35°C for different MC followed the same pattern as that of the elm seeds. An optimum MC for maize embryos stored at 35°C was observed at 0.057 g H₂O/g DW, a value that is considerably higher than that observed for elms and safflowers. The viability of maize embryos decreased sharply if grains were dried to MC values less than about 0.057 g H₂O/g DW. Sensitivity to drying to very low MC was clear in maize and elms seeds (Figure 2A and B).

The oil-rich safflower seeds maintained stronger vigor at the very low MC. Figure 3 shows that the germination percentage of safflower seeds with high level of MC decreased greatly when stored at 35°C and the seeds went bad. Seeds with a low MC still remained at a high level of germination percentage (Figure 3A and B). The germination percentage of safflower seeds began to decrease when the water content fell below the optimum MC of 0.024 g H₂O/g DW.

**Analysis of water sorption isotherms**

The RH (relative humidity) corresponding to this optimum MC can be determined by water sorption isotherm analysis. The isotherms produced for the three kinds of seeds used in this experiment have the same reverse-sigmoidal shape reported earlier for other seeds (Waters and Hill 1998) (Figure 4), which can be divided into three regions: a convex region at RHs below 20%, a linear region at RHs between 20 to 65%, and a concave region at RHs above 75%. The amounts of lipids are 5%, 18% and 52% for the of maize embryos, the seeds
of elms and safflowers, respectively. The relationship between RH and MC appeared to depend on the different lipid content of the three species, with maize and safflower giving the highest and lowest water content for a given RH, respectively. The RH corresponding to optimum MC of 0.057 g H₂O/g DW in maize embryos at 35°C, 0.045g H₂O/g DW in elms and 0.024g H₂O/g DW in safflower is about 15%, 12% and 7% respectively (Figure 4).

**Molecular Mobility in Different MC of Seeds**

Molecular mobility can be characterized by the rotational correlation time (τᵣ) of the rotating molecule (Buitink et al. 1998a). The τᵣ values of 10⁻¹²~10⁻⁹ s can be assessed from EPR spectra and calculated by the parameters h₊₁, h₀ and h₋₁ shown in Figure 5a. For low MC seeds, where the formation of glasses in dehydrating biological tissues had been established, the τᵣ was difficult to detect. The distance between the outer extreme of the electron paramagnetic resonance spectrum, with 2Azz to mark the distance (Figure 6), was used as a measure of molecular mobility in our study. An increase in 2Azz denoted a decrease in molecular mobility (Van et al. 1974; Dzuba 1996).

Figure 5 shows representative EPR spectra of CP (3-Carboxy-Proxyl) in the embryos of elm, maize and safflower at different MC. Curves showed representative EPR spectra of CP in the seeds recorded at 35°C. At a MC below 0.1 g water g⁻¹ dry weight, a powder spectrum (characterized by the two broad peaks at the extremes) overlapped the mobile spectrum in three kinds species, indicative of slow molecular mobility of the spin probe. The distance between the two broad peaks at the extremes is referred to as 2Azz, which can indicates molecular mobility. In addition, we measured the 2Azz value at -150°C as the maximum values of molecular mobility, which can be assumed that motion of the probe was almost completely immobilized. The value of 2Azz gives information about the polarity of the spin probe’s environment in the tissue at this low temperature (Knowles et al. 1976). In elm seed the maximum 2Azz decreased with decreasing MC from 70.5 to 72.2 G, whereas in maize embryos, it changed from 70.5 to 71.8 G, and 70.6 to 71.6 G in safflower seeds (Figure 6).

To determine the change in molecular mobility as a function of MC, it is necessary to correct for the polarity change of the environment in which CP is present for each MC (Figure 6). The mobility at a certain MC was expressed as the difference between the maximum 2Azz
(at -150°C, where the spin probe is assumed to be immobilized) and the 2Azz measured at the
35°C. We refer to this parameter as ΔAzz. An increase in Δuzz represents a relative increase in
molecular mobility compared with the completely immobilized situation at -150°C. Figures 7
show the dependence of the molecular mobility (ΔAzz) on MC in the seeds of elm, maize
embryos and safflower, respectively.

As shown in Figure 7, between approximately 0.105 to 0.045 g H₂O/g DW in elm seed
(Figure 7A), 0.133 to 0.057 g H₂O/g DW in maize embryos (Figure 7B) and 0.116-0.024 g
H₂O/g DW in safflower seeds (Figure 7C), molecular mobility decreased with decreasing MC.
The MC corresponding to the minimum mobility (lowest ΔAzz) varied with different seeds,
when the tissues reached approximately 0.045 g H₂O/g DW for elm, 0.057 g H₂O/g DW for
maize and 0.024 g H₂O/g DW for safflower seeds, the 2Azz reached its largest value,
indicating that the molecular mobility was lowest under the optimum MC. When MCs of
seeds were decreased further, molecular mobility slightly increased again.

Discussion

Maize embryos, elm and safflower seeds exhibited sensitivity to drying to very low MC.
The MC at which sensitivity was observed varied among the species, presumably as a result
of differences in chemical composition of the dry matter reserves. Above and below the
optimum MC, seeds deteriorate faster (Figure 1, 2 and 3). The results from this experiment
give a clear indication of optimum MC for three kinds of seeds storage, and support the idea
that seeds deteriorated faster if dried to very low water content (Vertucci and Roos

Water sorption isotherms describe the process of hydration in terms of physical sorption
of water on molecular surfaces (Vertucci and Leopold 1984; Walters and Hill 1998). Our
species have different dry matter reserves which gives them different sorption properties. The
lower equilibrium MC of the safflower seeds compared with those of the maize embryos and
elms can be explained by differences in lipid content (Walters and Hill 1998).

The RH range from our results (15% in maize, 12% in elms and 7% in safflower
respectively) (Figure 4) were consistent with those determined for Typha latifolia pollen and
lettuce seeds (Buitink et al. 1998a; Walters et al., 2005b). Higher RH ranges of 19-27%
(Vertucci and Roos, 1990) and approximately 30-40% (obtained when seeds dried at 20oC
and 10% RH are heated to 65°C (Vertucci and Roos, 1993) were once proposed; but accumulated evidence suggests seed storage optimum may occur at less than 20% RH at the storage temperature.

The biophysical state of desiccated cells is very important for survival of seed (Walters and Engels 1998; Waters 1998; Waters and Hill 1998; Waters et al. 2005ab; Hoekstra 2005). The optimum MC for *Typha latifolia* L. pollen storage corresponds closely to the MC at which minimum mobility was observed (Buitink et al. 1998a). Our results showed that the molecular mobility ($\Delta Azz$) in elms, maize and safflower were related to optimum MC for storage longevity (Figure 7), which is in agreement with Buitink et al report. Due to the differences in composition and content of samples, the spectra of EPR for dry seeds are dissimilar to those given for *Typha latifolia* L. pollen and there are a lot of subtle differences among seed spectra (Buitink et al. 1998a). The data in this paper provide very nice confirmatory evidence that molecular mobility interpreted from rotational spin of a probe decreases and then increases with seed drying in a similar pattern to seed aging kinetics.

We observed that mobility slightly increased again when the water content of seeds was far lower than optimum MC (Figure 7). A similar observation has been made in cattail (*Typha latifolia* L.) and Pea (*Pisum sativum* cv Karina) seeds (Buitink et al. 1998a, 2000). Withdrawal of water below the critical level to maintain glass integrity could result in increased porosity, free radical or other toxic species trapped in a relatively stable glassy matrik above the critical hydration level could become considerably more mobile, implying the spin probe partitions into a more mobile environment (Berjak 2006).

In conclusion, the maximum seed viability during storage of elm, maize and safflower can be achieved only by strict control of seed. The optimum MC varied among the species and depended on seed lipid content, drying below this water content can increase the aging rate and there may be detrimental effects of drying. Using the MC and temperature at which molecular mobility is at a minimum as a measure is more accurate for predicting optimum storage conditions.

**Materials and methods**

**Plant material**
Seeds of elm (*Ulmus pumila* L.), maize (*Zea mays* L.) and safflower (*Carthamus tinctorius* L.) were used in this study. The seeds of elm and safflower were provided by Institute of Botany, Chinese Academy of Sciences, and those of maize were provided by the Institute of Germplasm Resource, Chinese Academy of Agricultural Sciences. All seed samples were freshly harvested in 2006. The initial germination percentage of embryos in elm, maize and safflower were 97.33%, 91.33% and 91.33%, and their MCs (Moisture content) were 0.105 H$_2$O/g DW, 0.132 H$_2$O/g DW and 0.115 H$_2$O/g DW respectively.

Seeds of elm, safflower and maize embryos isolated from the seed were adjusted to different water contents using regularly regenerated silica gel. In detail, they were packed in nylon bags and were embedded into silica gel in a desiccator at ambient temperature (the ratio of seeds to silica gel was 1:10 (w/w). Silica gel in the desiccator was changed with fully dried silica gel everyday and the seeds and maize embryos were regularly weighed. After dehydration, the seeds and maize embryos were kept at several levels of MC, ranging from 0.105 g H$_2$O/g DW to 0.020 g H$_2$O/g DW for elm, 0.132 g H$_2$O/g DW to 0.034 g H$_2$O/g DW for maize embryos, and 0.115 g H$_2$O/g DW to 0.003g H$_2$O/g DW for safflower. MC was determined according to the rules of International Seed Testing Association using the oven-dry method under constant high temperature. After that, they were then sealed in aluminum foil and stored at -20°C for further use.

Lipids were quantitatively extracted from seeds and maize embryos by soaking ground embryos in chloroform: methanol (2:1) solution for 10 min (Vertucci and Roos 1990). The procedure was repeated twice and all soaking solutions combined. The extract was washed with 0.9% NaCl aqueous solution, followed by 2 washes of a 1:1 mixture of 0.9% NaCl in water: methanol solution. The mass of the extracted lipid was measured after evaporation of the solvent using a rotary evaporator apparatus. The amount of lipid was expressed as a percentage of the dry weight.

**Aging Treatment and Germination Assays**

Foil packets were incubated at 35°C and seeds deteriorated rapidly over the 140 (maize embryos) or 30 days (elm, safflower) storage period. To avoid injury of seeds and embryos by the imbibing treatment, they were put into individual nylon bags and imbibed gradually in three different desiccators with relative humidity of 36%, 45%, and 100% before germination.
The seeds and embryos were treated in each desiccator for 24 hours. The germination tests were conducted according to the Rules of International Seed Testing Association. Four replications with 50 seeds in each were germinated at 25°C. The germination percentage was estimated after 7 days.

**Sorption Isotherms**

A 100 g subsample of the seeds or maize embryos was placed in desiccators above a range of saturated salt solutions at a constant temperature of 35°C to determine their sorption isotherms. Saturated salt solutions comprised H2SO4 (1%), ZnCl2 (5.5%), NaOH (7.5%), LiCl (13%), CaBr2 (15%), CaCl2 (22%), MgCl2 (32.8%), NaBr (54.5%), NaNO2 (62%), NH4Cl (75.5%), KCl (84%), BaCl2 (87%) and KNO3 (90%) (Vertucci and Roos, 1993). Seeds and embryos were removed weekly, reweighed and returned to the desiccators. When they reached constant weight (i.e. in equilibrium with the saturated salt solution), their moisture content was determined.

**Determination of Molecular Mobility**

According to the method described by Buitink (1998a), the molecular mobility was determined by EPR spectroscopy with CP (3-Carboxy-Proxyl) as a spin probe. For labeling of the tissues with CP (Sigma), 0.5g of the seeds or embryos were incubated in a 5ml solution containing 1 mM CP at 35°C in the dark for 45 min. Potassium ferricyanide was then added to a final concentration of 200 mM, and the seeds and embryos were incubated for another 15 min. Ferricyanide broadens spin probe signals in the solution surrounding the cells to invisibility. Subsequently, the samples were surface-dried with filter paper and auto-blown over silica gel in a sealed desiccator to obtain the various MCs. Then 0.1g of dehydrated seeds or embryos was sealed in a quartz tube of diameter 5mm to carry out the molecular mobility determination at -150°C and 35°C. EPR spectroscopy (Model 300E, Bruker Analytik, Rheinstetten, Germany) was used to characterize the molecular motion of the polar nitroxide spin probe CP and the values of 2Azz obtained were considered as an estimate of molecular mobility. Measurement conditions were as follows: microwave power was 200 μW-2 mW and modulation amplitude was 0.4 G.
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Appendix

Abbreviations:

**Figure legends**

Figure 1 Rate of deterioration of *U. pumila* seeds stored at 35°C with different MC (ranging from 0.020-0.105 gH₂O/gDW). Higher water content as A, and low water content as B. Data are the means ±SE.

Figure 2 Rate of deterioration of *Z. mays* seeds stored at 35°C with different water contents (ranging from 0.034-0.132 gH₂O/gDW). Higher water content as A, and low water content as B. Data are the means ±SE.

Figure 3 Rate of deterioration of *C. tinctorius* seeds stored at 35°C with different water contents (ranging from 0.115-0.003 gH₂O/gDW). Higher water content as A, and low water content as B. Data are the means ±SE.

Figure 4 Moisture sorption isotherms of three seed species. Data were collected at 35°C. elm, closed circles; maize, closed squares; safflower, closed diamonds. Data points represent the mean of 3 replicates.

Figure 5 EPR spectra of CP in *T. latifolia* pollen, *U. pumila*, *Z. mays*, *C. tinctorius* seed at various MCs. Spectra were recorded at ambient temperature.a: *T. latifolia* pollen (Buitink et al, 1998); b: *U. pumila* seed; c: *Z. mays* seed; d: *C. tinctorius* seed.

Figure 6 Change in the distance between the outer extrema of the EPR spectra (2Azz) of CP in the seeds of elm, maize and safflower at -150°C as a function of water content. dw, dry weight.

Figure 7 Change in ΔAzz at different MC. ΔAzz was calculated as the difference between the maximum 2Azz (-150°C) and 2Azz at 35°C, with MC for spectra of CP in elm(A), maize(B) and safflower seeds(C). Curves were fitted with a third-order polynomial. dw, dry weight.