http://journal.unoeste.br/index.php/ca/index
DOI: 10.5747/ca.2023.v19.h535
ISSN on-line 1809-8215

Colloquium Agrariae

Submetido: 05/06/2023 Revisado: 26/07/2023 Aceito: 01/08/2023

CHALLENGES FOR STORING RECALCITRANT SEEDS

Cristina Rossetti, Cariane Pedroso da Rosa, Guilherme de Oliveira Pagel, Tiago Zanatta Aumonde, Lilian Vanussa Madruga de Tunes

Universidade Federal de Pelotas – UFPel, RS. E-mail: cristinarossetti@yahoo.com.br

Abstract

The perception that seeds had differences in their water content when disconnected from the mother plant, and consequently, variations in the period of maintenance of viability goes back to the beginning of agriculture, when people around the world started having to produce food as a result of population growth, ceasing to be exclusively extractive. Recalcitrant seeds do not tolerate desiccation and temperatures close to zero degrees, due to the lack of antioxidative mechanisms and maintenance of cellular compartments. Thus, this study aims to review the main concepts and techniques of drying and storage, usually used in so-called recalcitrant seeds.

Keywords: packaging; seed drying; seed moisture; seed conservation; deterioration.

DESAFIOS PARA O ARMAZENAMENTO DE SEMENTES RECALCITRANTES

Resumo

A percepção de que as sementes apresentavam diferenças em seus teores de água quando desligadas da planta-mãe, e consequentemente, variações no período de manutenção da viabilidade retoma ao início da agricultura, quando povos ao redor do mundo passaram a ter que produzir alimento em decorrência do crescimento populacional, deixando de ser exclusivamente extrativistas. Sementes recalcitrantes não toleram dessecação e temperaturas próximas a zero grau, devido à inexistência de mecanismos antioxidativos e de manutenção dos compartimentos celulares. Dessa forma, este estudo tem por objetivo revisar os principais conceitos e as técnicas de secagem e armazenamento, usualmente utilizadas em sementes ditas recalcitrantes.

Palavras-chave: embalagens; secagem de sementes; umidade de sementes; conservação de sementes; deterioração.

Introduction

Recalcitrant seeds are more difficult to store when compared to other seeds. This fact is due to the high susceptibility to water loss, which makes storage with high moisture content necessary. This internal humidity favors the attack of microorganisms and germination during storage. The application of low temperatures is also limited, as recalcitrant seeds are damaged by temperatures close to or below zero (King; Roberts, 2019). According to Chin and Roberts (2021), recalcitrant seeds are produced by plants that grow in aquatic environments, where natural drying of the seeds is not expected to occur, as well as by perennial plants that have adopted in their evolution a reproduction strategy in which the seeds, usually large in size, are placed at regular intervals in relatively humid environments. being the survival of the species, over time, more dependent on the perennial growth habit of the adult plant than on the life span of the propagation units.

The viability of seeds in general results from several factors such as the genetic characteristics of the species or cultivar, vigor of the parent plants, prevailing climatic conditions during seed maturation, degree of mechanical damage and environmental storage conditions (Carvalho; Nakagawa, 2012).

However, the biodiversity conservation strategy involves both *in situ* and *ex situ* methods. *In situ* conservation refers to the maintenance of species in their habitat through conservation units, such as national parks (Brazil, 2010). The *ex situ* conservation method consists of the conservation of species outside their habitat and must be carried out in a complementary way to *in situ* conservation (Brazil, 2010). *Ex situ* conservation can be carried out through seed storage (FAO, 2022). However, the success of seed storage depends on knowledge about their behavior during this process, which enables the use of appropriate conditions to maintain viability (Hong; Ellis, 2016).

Therefore, this study aims to review the main concepts and techniques of drying and storage, usually used in so-called recalcitrant seeds.

1. What are recalcitrant seeds?

Seeds show atypical behavior for living beings: they manage to survive even with a small amount of water in their tissues. This behavior is that of the so-called orthodox seeds, which support desiccation up to levels of 5% of water content in their seeds, which allows their storage for long periods of time (Barbedo, 2021). They are called orthodox, because they present behavior considered as the standard for seeds.

However, it is observed that there is a category of seeds that are intolerant to desiccation, losing their viability when they are dehydrated and that do not allow long periods of storage at low temperatures (Barbedo, 2018). They are called recalcitrant seeds, because they have a “stubborn” behavior, which does not suit the known behavior of seeds (Barbedo, 2021), with examples of this category being the seeds of the rubber tree *Theobroma cacao, Araucaria angustifólia, Carapa guianensis, Persea americana, Mangifera indica end Citrus* sp. (Fonseca; Freire, 2022). One of the theories for the emergence of recalcitrant seeds is that their occurrence is due to evolutionary adaptation, and they have a metabolic "shortcut", in which their desiccation does not occur, due to being in a more humid environment (Subbiah *et al.*, 2019).

These seeds come from species that generally inhabit environments with a humid tropical climate, arboreal, with seed production concentrated in a few months of the year, in which there is a high rate of precipitation (Barbedo, 2018; 2021). Thus, some strategies are used by recalcitrant seeds in order to achieve the perpetuation of the species, such as: availability of propagules distributed throughout the year; the seed development cycle is prolonged and it acquires the ability to germinate before completing maturation; have the ability to survive when submerged in water (Barbedo, 2018).

Despite being grouped under the same name, recalcitrant seeds present different behaviors regarding the degree of recalcitrance, since they have variable tolerance to desiccation and storage time, among different species and even within the same species (Guardia *et al.*, 2020).

The difference in behavior within the category of recalcitrant seeds can be explained when studying seed formation and which formation stage is prioritized, given that the characteristics are acquired progressively during seed maturation (Barbedo; Centeno; Figueiredo-Ribeiro, 2020; Barbedo, 2018).

Some seeds follow route number 1 indicated in Figure 1: they accumulate dry matter, acquire the ability to germinate, tolerance to desiccation and long-term storage, and finally are dispersed. However, in environments where rapid seed germination is required, investment in training goes directly so that the seed acquires the germination potential, to the detriment of the accumulation of reserves, and quickly disperses, without obtaining the characteristics of tolerance to desiccation and long-term storage, exemplified by route 2.

However, within these two extremes there may be several other behaviors, such as those represented by routes 3, 4, 5 and 6 in Figure 1, which exemplify how the gradient between orthodox (1) and recalcitrant (2) behaviors emerges. The sacrifice of some stages in the formation of the seed is linked to the environment in which it is found, and the further the seed is from orthodox behavior, the more the environment influences the characteristics that are prioritized by the seed (Barbedo, 2018).

2. Desiccation tolerance mechanisms

It is believed that recalcitrant seeds do not have some of the apparatus that allow their dehydration as well as orthodox seeds. Establishing the glassy state of the cytoplasm is an important step in seed dehydration, as it is in this state that the molecules contained in the cytoplasm have reduced movement (Barbedo, 2021). The glassy state is reached by the accumulation of sucrose, which increases the viscosity of the cytoplasm, consistent with a decrease in seed metabolism, a situation that is not observed in recalcitrant seeds (Barbedo; Centeno; Figueiredo-Ribeiro, 2020).

When the glassy state does not occur during desiccation, the movement of molecules continues to occur as if seed germination were happening, which causes an increase in free radicals that lead to lipid peroxidation of membranes of organelles and cells, the which leads to the death of the seed (Barbedo, 2021). An excessive generation of reactive oxygen species is perceived in recalcitrant seeds during their dehydration, combined with a lower antioxidant apparatus, which leads to the unfeasibility of these seeds in this situation (Chandra *et al.*, 2021).

Another mechanism that is adopted by orthodox seeds to tolerate desiccation is the folding of the cell wall to avoid rupture of the plasma membrane (Caccere *et al.*, 2022), a behavior not seen in recalcitrant seeds. The latter have lower levels of wall plasticizers, such as polysaccharides with arabinose in their composition, making it impossible for this folding to occur in the cell wall without breaking the structure during desiccation (Barbedo; Centeno; Figueiredo-Ribeiro, 2020).

The LEA “late embryonic abundant proteins” proteins are abundant in orthodox seeds, preventing damage caused by dehydration resulting from the maturation process, with a specific group, the dehydrins, being the main ones accumulated in response to any stress that causes a lack of water in the plant (Azarkovich, 2020). However, depending on the degree of recalcitrance, dehydrins are synthesized at different times of seed development. “Typical” recalcitrant seeds do not synthesize dehydrins either at the time of embryogenesis or during storage; the “atypical” recalcitrant ones, which undergo a little dehydration, present dehydrins, allowing these seeds to be stored for short periods; and intermediate seeds synthesize dehydrins during storage, showing greater tolerance to desiccation than the others (Azarkovich, 2020).

3. Determination of seed sensitivity to desiccation

Although the determination of sensitivity to desiccation of seeds is often carried out empirically, in the form of trial and error, Barbedo (2021) and the Royal Botanic Gardens (Gold; Hay, 2014) establish some steps to be followed in order to determine the desiccation tolerance.

The methodology proposed by Barbedo (2021) consists of the following eight steps:

1. The seed sample must be separated into three groups, groups A, B and C. The seeds from group A must be sown immediately;

2. Seeds in groups B and C must be dried. After drying, those from group B should also be sown in the same type of substrate as those from group A.

3. If group B seeds germinate, group C seeds can be stored. If the seeds of group B do not germinate, check whether there is physical dormancy, comparing them with those of group C;

4. If the group B seeds are close in size to the group C seeds, the group B seeds must be scarified. They must be returned to the substrate in order to carry out a new germination test. If there is germination, the seeds of group C can be stored;

5. If the seeds in group B are larger than those in group C, indicating that there has been water absorption, these seeds must be placed, still in the humid substrate, inside the refrigerator, remaining there for two to three months;

6. The presence of physical dormancy observed in item 4 implies the need for scarification of group A seeds. the storage;

7. If the seeds that went through item 4 do not germinate, they must be taken to the refrigerator like those in item 5;

8. The seeds from item 5 must be placed to germinate after leaving the refrigerator. If germination occurs, these seeds exhibit physiological dormancy and can also be stored.

At the end of these steps, it can be concluded that: 1. Seeds from group A germinated, but other groups did not germinate: seeds intolerant to desiccation; 2. If group A seeds have not germinated, the seeds may be dead from the start.

The Royal Botanic Gardens (Gold; Hay, 2014) suggests the “100 seed test for desiccation tolerance”, which consists of the following steps:

1. Determine the initial relative humidity balance (eUR) of the seed lot with a hygrometer;
2. Separate 10 seeds and individually determine the water content using the official methodology;
3. The germination test must be carried out with two samples of 13 seeds each;
4. Sixty-four (64) seeds must be separated into two samples of 32 seeds each, in which: 32 seeds will be mixed with silica gel in the same weight as the 32 seeds for drying to occur; another two 32 seeds will be mixed with moistened vermiculite to serve as moisture control. Both samples must be taken to an oven set at 15 °C (for temperate species) or 25 °C (for tropical species);
5. The silica gel of the desiccated sample should be changed every 1-3 days, considering the seed size, when the seeds should be weighed. When they reach constant weight, the initial relative humidity balance should be measured again. When 15% of eUR is reached, 6 seeds must be separated for a new moisture determination;

6. The place where the control seeds with the vermiculite are stored should also be opened every 1-3 days. It should also be determined, in the same period as the sample with UR silica gel and remove 6 seeds to determine the moisture;

7. Two germination tests are performed: one for the desiccated sample and another for the control sample;

8. The results of the initial germination, of the dried seeds and of the control seeds must be plotted in a graph that relates % of germination x incubation period.

Both methodologies indicate the extremes: orthodox seeds and recalcitrant seeds. This is a more modern way of indicating the behavior of seeds, since, due to the great variability of behavior between these two extremes, each species must be analyzed case by case.

4. Challenges for Storing Recalcitrant Seeds

The moisture content of recalcitrant seeds of most tropical tree species, at the time of dispersal, is quite variable, ranging from 23-25% to 46-53%. In addition to sensitivity to desiccation, many recalcitrant seeds of tropical species are sensitive to cold, not tolerating storage at temperatures below 15 °C. This imposes serious limitations and challenges to the storage of these seeds in the long term, since the procedures traditionally used for the storage of orthodox seeds, which generally involve reducing their water content and storing them in a refrigerated environment, could cause damage to them. irreversible, leading to loss of viability. On the other hand, the maintenance of high-water contents during the storage of recalcitrant seeds can favor the development of microorganisms harmful to the seeds or culminate in their germination (Vieira *et al.*, 2018).

In this sense, any procedure developed for the storage of recalcitrant seeds must avoid water loss and maintain an adequate supply of oxygen to the seeds, while at the same time preventing the proliferation of microorganisms and germination during the storage period. Considering the difficulty of storing recalcitrant seeds in the long term, in situ conservation strategies for these species should also be considered, as a way to guarantee the preservation and conservation of the genetic heritage (Cruz, 2016).

5. Cryopreservation technique

Cryopreservation can be defined as the conservation of plant material in liquid nitrogen at -196 °C, or in the vapor phase at -150 °C, keeping all metabolic processes essentially paralyzed and in a latent state, making it possible to preserve the material at long term (Molina *et al.*, 2016).

However, the success of this technique depends on the combination and efficiency of its steps, which are pre-cooling, cooling and reheating (Pieruzzi, 2013). The water content is the most critical point for the survival of the material to be cryopreserved, with the reduction of the degree of humidity being essential to avoid the formation of ice during freezing (Stegani *et al.*, 2017). This reduction must occur quickly, in order to reduce the stress caused during the cooling process, avoiding damage, normally fatal, to recalcitrant seeds (Pieruzzi, 2013).

Cryoprotection is a methodology that consists of treating samples in a concentrated medium of cryoprotective solutes, which act in the dehydration of plant material by osmosis, followed by direct immersion in liquid nitrogen. After dehydration, the material changes from a liquid state to a glassy state. In this transition, the interior of the cells becomes viscous enough that all chemical reactions that require molecular diffusion stop occurring, leaving the cells in dormancy and stability over time (Benson *et al.*, 2022).

Cryoprotectants can be classified as penetrating and non-penetrating. Penetrating cryoprotectants are glycerol, ethylene glycol and dimethylsulfoxide (DMSO), low molecular weight molecules capable of crossing the plasmatic membrane and acting in the adjustment of the osmotic balance in substitution of the water removed from the intracellular environment, allowing the cell to reach the glassy state during the freezing (Benson *et al.*, 2022). Non-penetrating cryoprotectants are sugars such as sorbitol, trehalose, and sucrose, which act as external osmotic agents, removing excess intracellular water by osmotic flow (O’Brien *et al.*, 2021).

6. Ultra-rapid dehydration of embryos

The technique called flash-drying or ultra-rapid dehydration of embryos shows to be promising to solve or alleviate the problem of conservation of Brazilian species with recalcitrant seeds (Pammenter *et al.*, 2002). Berjak and Pammmenter pioneered study with ultra-rapid dehydration. This method allows the withdrawal of water from the embryonic axis to occur very quickly, without enough time for the lethal degenerative processes to take place (Berjak *et al.*, 2020).

7. Packaging used to store these seeds

The type of packaging to be used for storage is another factor that can prolong the conservation of seeds, by protecting them from contact with the environment and against attack by insects and animals, in addition to facilitating handling and optimizing the use of space in storage (Freitas, 2019).

Recalcitrant species generally need to maintain the moisture at which they were harvested, not supporting losses greater than 5% of the initial moisture to remain viable. The suitable environment for conservation can be obtained by burying them in damp coal, wet sawdust, or wet sand; but there are species that need good aeration and cannot be buried, they must be packed in paper bags or open boxes to allow good oxygen diffusion, and they must be placed in an environment with high relative humidity to avoid dehydration (Floriano, 2014).

Any method to be developed must prevent water loss and maintain an adequate supply of oxygen to the seeds, while also preventing the proliferation of microorganisms and germination during storage. A proposed alternative has been the partial dehydration of the seeds before storage and packaging in packages resistant to the exchange of water vapor between the seeds and the atmosphere, such as polyethylene packages (Ferreira; Gentil, 2023). These packages allow the maintenance of high-water content by the seeds throughout storage without blocking the gaseous exchanges between them and the atmosphere which, due to their high respiratory activity, are intense (Bonner, 2018). The proliferation of microorganisms can be inhibited by treating the seeds with sodium hypochlorite solution and fungicides, in addition to frequent inspection to eliminate visibly contaminated seeds (Berjak; Pammenter, 2022).

Another technique used consists of stratifying the seeds in a moistened hygroscopic substrate, such as sand, sawdust, vermiculite and charcoal powder. The main disadvantages of this method are the excessive volume of material to be stored; the possibility of seed germination; and the proliferation of microorganisms. To circumvent seed germination, the use of inhibitors such as abscisic acid, osmotic solutions and superficial drying of seeds have been recommended for the conservation of seeds of some species (Andrade *et al.*, 2013).

8. Germoplasm Banks

Seed storage constitutes a set of procedures aimed at preserving its quality, acting as an instrument for the formation of regulatory stocks and the maintenance of genetic resources through germplasm banks (Aguiar *et al.*, 2021).

However, currently, the so-called *in vitro* technologies represent important strategies for the conservation of recalcitrant seeds: cultures of zygotic embryos and isolated embryonic axes, derived from mature seeds, normally survive partial desiccation and freezing, in the same way as shoot apexes and buds. can be cryopreserved (Towill, 2020).

Conclusion

The loss of water in recalcitrant seeds triggers some deteriorating processes, such as protein denaturation, changes in the activity of peroxidase enzymes and damage to the membrane system, resulting in the complete loss of viability. Thus, it is still necessary to improve scientific knowledge about its physiological mechanisms, related to sensitivity, desiccation and low temperatures, to determine efficient methods of seed storage.

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