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RECALCITRANT SEEDS
A STATUS REPORT

by

H.F. CHIN

including a

BIBLIOGRAPHY 1979-87

by

H.W. PRITCHARD

INTERNATIONAL BOARD FOR PLANT GENETIC RESOURCES

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The International Board for Plant Genetic Resources (IBPGR) is an autonomous international scientific centre under the aegis of the Consultative Group on International Agricultural Research (CGIAR). IBPGR was established by the CGIAR in 1974. The basic function of IBPGR is to promote and coordinate an international network of genetic resources centres to further the collecting, conservation, documentation, evaluation and use of plant germplasm and thereby contribute to raising the standard of living and welfare of people throughout the world. Financial support for the core programme is provided by the Governments of Australia, Austria, Belgium, Canada, China, Denmark, France, FRG, India, Italy, Japan, the Netherlands, Norway, Spain, Sweden, Switzerland, the UK and the USA, as well as the World Bank.

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RECALCITRANT SEEDS
A STATUS REPORT

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Introduction

Most of the world's agriculture depends on seeds, and so the maintenance of seed viability in storage is of particular importance. The life spans of seeds may vary from a few days to a few hundred years, and there have been claims that some seeds may remain viable for up to 3000 years.

Most seeds remain viable for longer periods than normal if they are first dried and then stored at low temperature in sealed containers. Roberts (1973) introduced the terms 'orthodox' and 'recalcitrant' to describe the storage behaviour of seeds. Orthodox seeds can tolerate desiccation and freezing temperatures while recalcitrant seeds are killed if their moisture contents are reduced below some relatively high critical value (12-31%). If they are maintained in a moist condition they retain their ability to germinate for a short time, which typically varies from a few weeks to a few months.

Many of the major plantation crops, fruit trees and timber species have recalcitrant seeds. Little attention has been given to the storage of the seeds of tropical tree fruit species (Hanson, 1984) and there are many storage problems yet to be solved in tropical plantation crops (Chin, 1978). Williams (1984) stressed that research work on clonal crops, particularly for major ones, should be accelerated. At the 21st International Seed Testing Association (ISTA) Congress in Brisbane, Australia in 1986, there was active discussion on the problems and importance of recalcitrant seeds. A working group was formed at this Congress to investigate methods for the assessment of moisture content and the storage of recalcitrant seeds. At present there are only about a dozen laboratories actively pursuing research on recalcitrant seeds.

King and Roberts (1979) produced a comprehensive literature review for IBPGR on recalcitrant seeds. This was the first review of this subject which had been undertaken and it was very useful for researchers. Subsequently, a book on recalcitrant crop seeds was published (Chin and Roberts, 1980). Farrant et al. (1988) gave a current assessment of the problem at the 21st ISTA Congress. The aim of this status report is to fill in some of the gaps with recent developments, and to make research proposals for the future.

Characteristics and identification

The major characteristic difference between orthodox and recalcitrant seeds lies in the physiology of their response to desiccation. Hanson (1984) suggested that it was more accurate and meaningful to call orthodox seeds desiccation-tolerant and recalcitrant seeds desiccation-sensitive. Furthermore, most recalcitrant seeds are sensitive to chilling injury at low temperatures (King and Roberts, 1979). Many recalcitrant seeds do not tolerate freezing temperatures, e.g. Hevea brasiliensis and Nephelium lappaceum (Chin, 1975; Chin et al., 1981). Some, such as Theobroma cacao, do not even tolerate temperatures of 15°C (Hor et al., 1984), or 4°C for Shorea talura (Sasaki, 1976).

Roberts et al. (1984) observed that species that produce recalcitrant seeds were of 2 main types: (a) species from aquatic habitats; and (b) some of the large-seeded trees. The latter include many important tropical plantation crops such as rubber, cocoa and coconut; tropical fruit crops such as mango, mangosteen, durian, rambutan and jackfruit; and tropical timber species belonging to the families Dipterocarpaceae and Araucariaceae. Temperate representatives include oak, chestnut and horse chestnut. The average weights and volumes of recalcitrant seeds usually far exceed those of orthodox seeds.

An exception is the seeds of Anthurium. The 1000 seed weights of recalcitrant seeds often exceed 500 g (Chin et al., 1984). This is both due to their high moisture contents, which range from 30% to 70% (on a fresh weight basis), and also to their large sizes. For example, the 1000 seed weight of durian exceeds 14 000 g and the average volume of each seed is 8 ml. If 3000 seeds is taken to be the minimum for each accession required for storage in a genebank, a much larger storage capacity is clearly required than with orthodox seeds. An extreme case is that of coconut, in which each seed weighs about 1 kg and occupies about 1000 ml.

Most recalcitrant seeds are spherical or oval in shape, and dicotyledonous, and some are also endospermic. There is a wide range of morphological and structural variation amongst recalcitrant seeds. The seeds or nuts are often surrounded by thick endocarps, in which case the structures are botanically true fruits. Many tropical fruits are covered with a fleshy or juicy arilloid structure. The fleshy arils of rambutan and jackfruit are covered by a thin papery membrane, which, in the case of jackfruit, becomes impermeable to water on drying.

Recalcitrant seeds show some other peculiarities. For example, the tiny embryos of rambutan seeds are attached at different positions between the 2 unevenly sized cotyledons (Chin, 1975). The seeds of a number of species, such as mango, mangosteen and langsat, are polyembryonic. It is important to understand the basic structures of the seeds if the results of studies on their physiology are to be interpreted properly.

The main characteristics of recalcitrant seeds, such as size, short life span and sensitivity to freezing temperatures, are typical, but not diagnostic. Roberts et al. (1984) warned that the diagnosis of whether or not seeds are recalcitrant may not always be straightforward, and there have been a number of cases of seeds which had originally been classified as recalcitrant but are now known to be orthodox, for example, Citrus limon (Mumford and Grout, 1979), cassava (Ellis et al., 1981; Mumford and Grout, 1978) and oil palm (Grout et al., 1983). Correct diagnosis is of prime importance, because it determines whether methods of conservation which are applied are likely to succeed or not. Roberts et al. (1984) listed 9 ways in which information concerning germination behaviour might be misinterpreted which would result in the incorrect classification of orthodox seeds as recalcitrant.

King and Roberts (1979) listed 75 species from 29 families as provisionally having recalcitrant seeds; the definitive identification of some of them is, however, difficult. In 1981, and on the advice of the IBPGR Advisory Committee on Seed

Storage, it was decided that a through study should be made of this problem and IBPGR provided a grant to the Universiti Pertanian Malaysia for this purpose. The main objective was to develop a screening method for distinguishing between orthodox and recalcitrant seeds involving desiccation, which would ensure that the cause of seed death was not due to the method and temperature of drying. A screening technique using a drying temperature of around 20°C, which is a lower temperature than is normally employed in drying seeds, with a fan or desiccants, was used (Chin et al., 1984). Farrant et al. (1988) suggested that freshly harvested seeds are tested and recommended rapid drying because seeds become increasingly sensitive to desiccation on storage.

Moisture content of recalcitrant seeds

Recalcitrant seeds are shed from the parent plant at high moisture contents usually after a limited amount of maturation. However, unlike orthodox seeds, they are highly intolerant of further desiccation. Because of their size, these seeds may lose water at a slower rate than do orthodox seeds. This may effectively reduce the rate at which the viability of such seeds declines and Tamari (1976) has shown that the viability of seeds belonging to the family Dipterocarpaceae after storage is positively correlated with seed size. It is possible, however, that large seed size may actually contribute to recalcitrant behaviour (King and Roberts, 1979) as there are greater problems of internal transport of water than with smaller seeds. Water is more difficult to remove from large seeds, especially from those with impermeable testas, than from small seeds. We have found that the moisture contents of individual seeds within a sample of recalcitrant seeds may vary considerably, and so the coefficient of variation is large as compared with that of a similar sample of orthodox seeds (Table 1).

The 1985 ISTA rules do not cover the testing of recalcitrant seeds with regard to sampling, and moisture and germination testing. Samples may not be representative because the seeds are large, and relatively few of them may be available

Table 1. The coefficient of variation in moisture content of recalcitrant and orthodox seeds

Type	Species	Coefficient of variation
Recalcitrant	*Hevea brasiliensis*	13.26
	Nephelium lappaceum	13.38
	Artocarpus heterophyllus	5.29
Orthodox	*Zea mays*	2.36
	Hibiscus esculentus	2.86
	Vigna sesquipedalis	2.68

for testing, and because of their high and variable moisture contents. There is validity in measuring moisture content of individual seed parts based on the findings of a variation in moisture content between components (e.g. oil palm; Grout et al., 1983: Avicenia marina; Berjak et al., 1984: Araucaria hunstecinii; Pritchard and Prendergast, 1986). Hence, at the 1986 ISTA Congress, a working group was constituted for the assessment of the moisture content of recalcitrant seeds. At present we are using 20 seeds for moisture determination. The seeds are sliced and dried in an oven at 105°C for 16 hours and the moisture content calculated.

Water in seeds is found as free water and bound water. The free water is necessary for the movement of molecules from one centre of metabolism to another. On drying, the free water is removed, and the loss in weight is expressed as the moisture content of the seeds. The moisture contents of seeds may be reported on a fresh weight or a dry weight basis. Measurements of seed moisture content on the dry weight basis may exceed 100%, which may seem puzzling when reported in the literature. It is more realistic to express moisture content on the fresh weight basis, as has been done in this report, but it is essential always to state the basis of moisture content assessment.

Free water is easily removed from seeds on drying while bound water, which is strongly held, is not. It has been suggested that the bound or subcellular water is closely associated with macro-molecular surfaces and has a structure imposed upon it (Drost-Hansen and Clegg, 1979). The structured water may possibly ensure the stability of macromolecules and subcellular surfaces and may stabilize membranes in desiccated orthodox seeds (Berjak et al., 1984). Clegg (1979) suggested that structured water is involved in ensuring the precise functioning of multi-enzyme systems. All metabolism can, and probably does, take place in structured water. Loss of structured water results in disruption of metabolism; in the case of orthodox seeds this presumably does not occur because of their tolerance to desiccation. In recalcitrant seeds, however, this does not appear to be the case (Berjak et al., 1984).

Desiccation and chilling sensitivity

The degree of sensitivity of recalcitrant seeds to drying varies between species. For example, seeds of Borneo camphor (Dryobalanops aromatica) are damaged at moisture contents below about 35% (Tamari, 1976); cocoa (Theobroma cacao) seeds are damaged at below 27% (Hor et al., 1984); whereas rambutan (Nephelium lappaceum) seeds are damaged at moisture contents less than 20% (Chin, 1975). The critical moisture content which kills all seeds varies from species to species, but it is a relatively high value which is usually within the range from 12% to 31% (Roberts, 1973). Critical moisture content referred to here is probably best described as the "lowest safe moisture content" (LSMC) of Tomsett (1987b). The variation in susceptibility to desiccation is found not only between species but also within the same species or seed lot. The high coefficient of variation of

moisture content in recalcitrant seeds may possibly account for the variation in their susceptibility to drying injury.

The reason why dehydration causes the death of recalcitrant seeds is still not clear. King and Roberts (1979) suggested the following 2 ways: either death occurs rapidly at or below some critical moisture content (critical moisture content hypothesis); or loss of viability occurs at a rate which is negatively related to moisture content over a wide range of moisture contents (noncritical moisture content hypothesis). It is also possible that a reduction in moisture content causes loss of membrane integrity and nuclear disintegration as has been shown with rubber seeds on sun drying (Chin et al., 1981). The seeds of many tropical plants contain high concentrations of phenolic compounds and of phenolic oxidases. These compounds are normally compartmentalized within cells; on desiccation the cell membranes are damaged and the phenolic compounds are released. They are then oxidized and protein/phenol complexes are formed with a consequent loss of enzyme activity (Loomis and Battaile, 1966).

Farrant et al. (1988) recently proposed a new hypothesis based on work with the recalcitrant seeds of Avicennia marina. These seeds behave as imbibed orthodox seeds when they are first shed, and can withstand the loss of at least 18% of their initial content of water and still remain viable. The seeds normally start to germinate on shedding. They become more sensitive to desiccation with the onset of cell division and vacuolation and the early stages of germination (Bewley, 1979). Dehydration of seeds stored for more than 4 days caused a marked decline in germination. As germination proceeds further, the lower limit below which seeds died also increased.

In nature, many trees and shrubs can survive freezing temperatures, but some are prone to injury. Most of the recalcitrant seeds belonging to timber, plantation crop and fruit species grow in, and are adapted to, a moist and warm tropical forest habitat. Thus, it is not surprising that they do not tolerate freezing temperatures, although the failure of the seeds of some species to survive at 15°C is hard to understand. In contrast, many orthodox seeds can survive even at -196°C. Among the recalcitrant seeds there are varying degrees of tolerance to low temperature. The temperate species are more tolerant to low temperature. Seeds of Quercus spp., for example, can germinate at 2°C after 8 months in cold storage, whereas the seeds of many tropical species are killed at subambient temperatures or suffer chilling injury. Examples are Theobroma cacao (Hor et al., 1984), Nephelium lappaceum (Chin, 1975), Dryobalanops aromatica (Jensen, 1971), Hopea odorata (Tang and Tamari, 1973), Shorea ovalis (Sasaki, 1976), Garcinia mangostana (Winters, 1953) and Elaeis guineensis (Mok and Hor, 1977).

The literature on chilling injury in seeds covers both orthodox and recalcitrant seeds. It was already known 50 to 60 years ago that orthodox seeds, if not dried to a sufficiently low moisture content, are killed by freezing temperatures (Becquerel, 1925; Lipman and Lewis, 1934). Stanwood (1983) stated that there is a high moisture freezing limit (HMFL) which is the threshold that if exceeded will result in a decrease in viability of a seed sample during liquid nitrogen cooling.

This threshold is normally a relatively narrow range of seed moisture content within a species, but it can vary between species. The cause of seed death in these cases is similar to that of recalcitrant seeds with high moisture contents when frozen. Freezing damage in moist seeds is presumably associated with the formation of ice crystals, and usually occurs at moisture contents above 14-20% (Roberts, 1972).

The reasons why recalcitrant seeds may be killed at subambient temperatures are not known. Some of the results on the deleterious effects of subambient temperatures on orthodox seeds may be applicable to recalcitrant seeds. Simon et al. (1976) attributed the low temperature susceptibility of the orthodox seeds of cucumber (Cucumis sativus cv. Long Green Improved) and mungbean (Vigna radiata) to protein denaturation. Wolfe (1978) suggested that the declining fluidity of membranal lipids which occurs during chilling can result in changes in membrane thickness and permeability which can affect the membrane-bound enzymes.

Studies on cocoa seeds have shown that the fall in viability with declining temperature can be extremely abrupt. Possible reasons given by Boroughs and Hunter (1963) were: (i) the presence of some temperature-dependent, rate-limiting reaction, the cessation of which causes lethal metabolic disruption; (ii) the absence of some protective substance present in seeds which are not susceptible to chilling; and (iii) the liberation of some toxic material owing to cold induced changes in membrane permeability.

Hor (1984) showed there was a very sharp reduction in storability of cocoa seeds at 15°C compared to 17°C, indicating that they are very sensitive to slight temperature changes around a critical value. The temperature difference of 2°C means that seeds die in less than 2 weeks and suggests that only a few inter-related reactions may be involved in causing seed death. A number of physiological, biochemical and ultrastructural changes were detected. There was a 3-fold increase in leachate conductivity and lower [^{14}C]leucine incorporation, and major ultrastructural changes were observed in the cell membrane system.

Storage of recalcitrant seeds

In the past half century many different methods of storage have been proposed for the long-term storage of recalcitrant seeds, but without exception their use has been unsuccessful. At best, and with the latest developments, truly recalcitrant seeds can hardly be stored for a year so their conservation in genebanks is not yet practical. As recalcitrant seeds are desiccation-sensitive, the obvious answer would seem to be to store them in moist media, but this has only been successful for short-term storage. Moist or imbibed storage has been used for a number or crops, including rubber (Ang, 1976), rambutan (Chin, 1975) and cocoa (Evans, 1953; King and Roberts, 1982).

The major problems to overcome in moist storage are to prevent germination and to control fungal growth. Attempts have been made to store seeds within

the fruit or in fruit juice, but these have been generally unsuccessful as has been shown with cocoa (Pyke et al., 1934) and rambutan (Chin, 1975). Now a new method has been tried in our laboratory. This is partial drying with the application of fungicide. The method has been fairly successful in prolonging the storage life of rubber seeds from the normal 3 months in moist storage to 1 year. Freshly collected rubber seeds were soaked in 0.3% 'Benlate' and then drained and surface dried in air. Seed were stored in perforated polyethylene bags at ambient temperatures. Over 50% of the seeds were still viable after a 1-year storage period. Similarly, Hor (1984) treated cocoa seeds with 0.2% 'Benlate'/'Thiram' mixture and stored them in a polyethylene bag for 24 weeks; after this time over 50% of the seeds germinated.

Longer storage periods have been reported with seeds of Coffea and Citrus spp., although seeds of these 2 genera have been found to be not truly recalcitrant; their storage lives have been extended to over 2 years. The technique used with coffee was to store moist seeds in polyethylene bags at 15°C (Vossen, van der, 1979). Citrus seeds can also be maintained for several years if given heat and a fungicide treatment. They are then stored imbibed in a thin closed polythene bag at 0°C to 4°C (King and Roberts, 1979).

Other methods, besides imbibed storage, have also been tried but so far none of them have been successful. Examples include the storage of durian in air-tight containers (Soepadmo and Eow, 1976), Shorea spp. in sealed inflated polyethylene bags, cocoa in a sealed CO_2 atmosphere (Villa, 1962) and rubber in brine and polyethylene glycol (Sakhibun, 1981). Tompsett (1983) stressed the importance of oxygen availability in imbibed storage. In spite of the short periods that recalcitrant seeds can presently be stored, existing techniques are useful to ensure the survival of genetic material collected in the field and also for planting the next season's crop. For example, in 1876, rubber seeds were first collected by Wickham in Brazil. When they arrived in Kew Gardens only 4% of the 70 000 seeds germinated. Ong et al. (1983) reported a similar expedition to collect wild Hevea germplasm in 1981 which gave better results with over 65% of the 64,736 seeds germinating on reaching their destination in Malaysia. As many of the recalcitrant seeds belong to perennial plantation crop or timber species, which may not produce seeds every year, there is still the need for short-term storage.

Therefore, new techniques, such as cryogenic storage of the embryos of recalcitrant seeds, need to be developed. The most promising method according to Roberts et al. (1984) is storage in liquid nitrogen, but so far there has been no success with truly recalcitrant seeds and the technical problems are formidable. If seeds are to survive, they must be dried prior to freezing (Becquerel, 1925; Lipman and Lewis, 1934; Fedosenko, 1974; Sakai and Noshiro, 1975) and at low moisture contents seeds can survive at -196°C (Stanwood and Bass, 1978). Stanwood (1984) provided a preliminary guide to the practical preservation of seed germplasm in liquid nitrogen. However, even some species with orthodox seeds have a higher than normal threshold of moisture content; if their moisture content is above this level freezing temperatures are damaging to seeds.

The threshold of seed moisture above which a decrease in viability of a seed sample occurs during cooling to the temperature of liquid nitrogen is defined as the high moisture freezing limit (HMFL). This threshold is normally within a relatively narrow seed moisture content range within a species but can vary between species. For example, Stanwood (1983) recorded a range from 9.6% for sesame to 28.5% for bean. This indicates the possibility that the embryos of some recalcitrant seeds can be dried to below their HMFL and subsequently cooled and stored in liquid nitrogen. The other alternative is by using the method of "colligative cryoprotection" (Merryman and Williams, 1981). In this technique, cell water is replaced with a cryoprotectant such as glycerol, DMSO and l-proline. Seeds of Anthurium spp., an ornamental plant with desiccation-sensitive seeds, with l-proline infiltration at 23 °C gave a consistently higher germination percentage than with other cryoprotectants (Stanwood, 1985, pers. comm.). Another study simulating recalcitrant seeds was that by Grout (1979), who used hydrated tomato seeds infiltrated with 15% DMSO, as a model. These showed no loss of viability when exposed to liquid nitrogen.

Work on the cryopreservation of plant systems is fairly recent, but cases of the survival of embryos in liquid nitrogen have been reported in a number of crop species and their subsequent regeneration into plants shows that it may be possible to conserve recalcitrant seeds in this way. Bajaj (1985) noted that the germplasm of plants with short-lived seeds could possibly be conserved through the cryopreservation of excised embryos. In addition, for clonally reproduced species with a long life cycle it may be possible to use asexual embryos which can be produced in large numbers. In fact, somatic embryos are now produced to be marketed as 'artificial seeds' in the USA. Such somatic embryos, especially those of species with recalcitrant seeds, can be further exploited by freeze storage of somatic embryos.

In 1986, IBPGR funded a project on techniques for the dehydration and conservation of recalcitrant seeds at Universiti Pertanian Malaysia. This project involved investigations on the possibility of drying embryos to below the HMFL and the use of colligative substitution of a cryoprotectant in the liquid nitrogen storage of embryos. Chin and Krishnapillay (1987) found that embryos of Artocarpus heterophyllus can be dried to 11% moisture content and survive in culture. Pritchard and Prendergast (1986) also found that tissue will survive from dried and cryopreserved Araucaria hunstecinii embryos. In the 6 months up to January 1987 preliminary studies on the screening of tree seeds and embryos dried to various levels of moisture content, and then cooled at different rates or plunged directly in liquid nitrogen were carried out. Treated seeds were planted in sterilized sand and embryos were cultured in enriched agar medium. The experiment was repeated with seeds which did not survive with the addition of either a single cryoprotectant, or a combination of them.

The possibility of partial desiccation of embryos prior to exposure to liquid nitrogen is also being pursued further. Recalcitrant seeds vary in their response to desiccation. In rubber seeds the critical moisture content was found to be between 15% and 20% (Chin et al., 1981). With this in mind, excised embryos of rubber seeds

were air-dried before cryopreservation. Normah et al. (1986), in our laboratory, found that at moisture contents between 14% and 20% (desiccation for 2-5 hours), 20-69% of the embryos survived cryopreservation for 24 hours and formed seedlings with normal roots and shoots when cultured in vitro. This is the first time that truly recalcitrant seeds have survived storage in liquid nitrogen.

The project on the cryopreservation of embryos of recalcitrant seeds with the colligative substitution techniques, is well underway, using a combination of cryoprotectants and various enriched media. The only species in which embryos have so far survived to produce roots is Nephelium lappaceum. All of the techniques used have to be refined and adapted with different species. A few major problems have been encountered which relate to the morphological structures of the seeds. There is much variation both between and within species. They differ in the location the embryo; some are tightly held between the cotyledons and are difficult or impossible to excise without damage. In order to overcome this problem with Nephelium lappaceum, a small block (2 mm x 4 mm) of cotyledonary tissue, together with the enclosed embryo axis, was used as the excised embryo for the various treatments. The second problem is the browning of tissues caused by the oxidation of phenolic compounds. Thirdly, it is sometimes difficult to achieve the infiltration of cryoprotectants into embryos, as some of the seeds are very large; hence, the use of excised embryos - in which case damage to cells caused by excision is accompanied by the release of phenolic compounds. In spite of the many problems yet to be solved, the in vitro culture of embryos after storage in liquid nitrogen is a potentially useful technique for long-term genetic conservation.

Future research

The following topics are proposed as research priorities:

(1) Sampling and moisture test
(2) Identification
(3) Causes of seed deterioration
(4) Storage of recalcitrant seeds
(5) In vitro culture - cryopreservation of embryos

(1) Sampling and moisture test

Because most recalcitrant seeds are very large and the numbers of seeds available for testing may be limited, it is not possible to follow the sampling rules recommended by ISTA for orthodox seeds. The sample size used for any test has to be both statistically significant and economical. Whereas a germination test for orthodox seeds involves 400 seeds and 4 to 16 replicates, it would be impractical to carry out this test, for instance, with coconuts. For a moisture test usually two

5 g samples are taken with 2 replicates, but a single recalcitrant seed may weigh from 10 g to over 1000 g. Besides, recalcitrant seeds have shown great variation in their moisture content with a high value of the coefficient variation of 12 to 13.

(2) Identification

Seeds of a number of species have in the past been classified as recalcitrant, but are now known to be orthodox. Even orthodox seeds are sensitive to desiccation if their moisture contents are sufficiently high. Dormancy, which can sometimes be induced by drying, is another important factor. Germination tests after desiccation may record little or no germination if seeds are dormant. Some seeds take a long time to germinate if not given the correct treatment, e.g. oil palm and Gnetum gnemon, which take 6 and 13 months respectively, to germinate. The seed coat can greatly affect assessment of whether or not seeds are orthodox or recalcitrant. The classic example is that of lemon seeds; without testas, they can be dried to a low moisture content and tolerate a temperature of -196 °C and yet retain their viability (Mumford and Grout, 1979).

In the past, the use of drying techniques which have adverse effects on seed viability has resulted in the misclassification of seed types. It may be that at lower temperatures of around 20 °C and using desiccants the seeds will suffer less damage. Much of the work on recalcitrant seeds has been conducted in the tropics where the relative humidities of around 90% and temperatures around 30-35 °C are both very high. It is important to investigate whether recalcitrant seeds, particularly large seeds with impermeable seed coats, can be dried without loss of viability. Such seeds may be less sensitive to injury from desiccation in the first few days after harvest.

(3) Causes of seed deterioration

Basic work on the causes of seed deterioration is mainly being pursued with orthodox seeds, and much more needs to be done with recalcitrant seeds. Most of the current work deals with the observation of changes that take place when seeds deteriorate and lose their viability. Seed deterioration has been defined as the irreversible changes that reduce survival capacity and lead to loss of vigour and germinability (Anderson, 1973). It has been postulated that as the seed deteriorates various functions of the seed are impaired in a definite sequence (Delouche, 1969; Heydecker, 1972). Roberts (1979) cautioned against establishing which are the first causes for seed death unless they have been observed to occur under a range of conditions.

Basic studies on the causes of loss of viability should be encouraged. For example, research into membrane integrity, structure, damage and the biochemistry and physiology of membranes should be given priority. Delouche and Baskin (1973) suggested that among the first changes to occur are membrane degradation, impairment of energy and synthetic mechanisms and decreased respiration. A number of reasons have been suggested for the loss of membrane integrity in deteriorating seeds, which have already been mentioned (Wolfe, 1978).

(4) Storage of recalcitrant seeds

In spite of recent advances, it has hitherto not proved possible to conserve recalcitrant seeds successfully. There have been some reports on the lengthening of the storage life of rubber and cocoa by a few months based on partial drying and treatment with fungicides. However, long-term storage using the subimbibed technique has yet to be established. The observation by Farrant et al. (1988) that recalcitrant seeds may be more tolerant to desiccation soon after harvesting, but usually start to germinate rapidly, but may prove to be a useful basis for future research work. The new approach of harvesting seeds prior to seed fall and drying them partially may help to lengthen their storage life.

Past reports and experience indicate that it is unlikely that the technique of imbibed storage of recalcitrant seeds will be used successfully for the long-term conservation of genetic resources. There are too many problems with imbibed storage: under moist conditions seeds are liable to germinate; and the maintenance of constant relative humidity encourages the growth of fungus, both of which factors contribute to the rapid decline in viability with time. Although long-term storage may not be in sight, the development of short-term storage techniques is also important.

In the future we may have to work on entirely new approaches and techniques for the storage of recalcitrant seeds. In the last decade, progress has been achieved with orthodox seeds in the use of cryogenic techniques. Attempts with recalcitrant seeds have been initiated, and some success has been achieved with citrus and oil palm which had formerly been classified as recalcitrant. These examples indicate that it may be possible to store recalcitrant seeds, or their embryos, if the seeds are too large. Current work at Universiti Pertanian Malaysia has been partly successful with rubber seeds and research is being conducted on other species. The rates of chilling and thawing have to be worked out.

(5) In vitro *culture - cryopreservation of embryos*

There are 3 basic approaches to the storage of germplasm using in vitro methods: (a) maintenance under normal growth, (b) growth limitation, and (c) cryopreservation. Cryopreservation in plant systems includes protoplast, cell, callus, organ and embryo culture. To date more than 50 species have been successfully cryopreserved. Although it is feasible to cryopreserve various parts of plants, nonorganized cultures are not suitable for the conservation of genetic resources (Henshaw et al., 1980).

The cryopreservation of plant zygotic embryos involves the same basic principles as those used for cells or tissues, but the advantage is that embryos develop directly into seedlings. Zygotic, somatic, nucellar and pollen embryos have been successfully cryopreserved for a number of plant species. This technique may be particularly useful for the preservation of the excised embryos of recalcitrant seeds.

Two approaches are suggested for the preservation of embryos. The first involves using the partial drying method; for the less recalcitrant or partially

desiccation-sensitive species, pre-drying of embryos prior to cryopreservation should be attempted using embryos of different maturity and moisture content. For those partially desiccation-sensitive species the embryos can be preserved directly in liquid nitrogen without cryoprotectants. The second approach is to treat seeds or excised embryos with cryoprotectants prior to preservation in liquid nitrogen. The cooling and thawing rates have to be worked out. The optimum composition of the culture medium also has to be established as this can mean success or failure of the culture.

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RECALCITRANT SEEDS
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