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Ex situ conservation of rare and valuable forest tree species through seed-gene bank

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There is growing concern throughout the world about the uncontrolled exploitation and depletion of the earth's natural resources, especially affecting the plant biodiversity of tropical forests. The extinction potential of a species is related to the degree of its biological vulnerability and the degree of threat by biotic and abiotic factors. Therefore, the need for conservation is exceptionally high and of paramount importance to preserve this plant heritage for posterity. One of the most effective biological techniques to conserve this biodiversity is the establishment of gene banks, i.e. *ex situ* conservation. Conventional seed storage is believed to be a safe, effective and inexpensive method of *ex situ* conservation of plant genetic resources, which not only maintains its viability but also its vigour without hampering the genetic makeup. The elucidation of various factors that regulate seed viability and vigour in storage is essential. An ideal condition to prolong the seed longevity is mainly dependent on seed moisture content, temperature and type of container used during storage. The optimum stage of seed maturity, seed-lot quality, their processing and harvesting techniques, germination eco-physiology and degree of dormancy too play a crucial role in maintaining seed longevity that need to be considered before large-scale seed storage is initiated. The present review is an attempt to discuss the importance of the aforementioned aspects of forest tree seeds in detail, to conserve their germplasm for *ex situ* conservation through seed-gene bank.

FORESTS, the biological diversity they contain and the ecological function they maintain, are our heritage. In tropical and subtropical regions, complex and species-rich ecosystems are being rapidly destroyed or altered, and in arid and semi-arid regions fragile environments are threatened by the increasing stress from human populations, domestic animals and fluctuating climates[1]. At

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the current rate of deforestation, mass extinction of species seems imminent. Furthermore, a considerable amount of genetic diversity within species that might survive is likely to be lost. Deforestation and fragmentation of habitats adversely affect the fate of rare species. Rare species in fragmented habitats may be reduced to such low numbers that they may not constitute viable populations. In such populations, genetic drift and inbreeding may result in inbreeding depression, with deleterious effects on reproductive output. Thus, a combination of demographic and genetic factors may hasten the extinction of rare species in small, isolated fragments[2]. There has been a general recognition in the recent years that the genetic variation present in a species is a valuable biological resource in this era of genetic engineering. Species preservation is not just the preservation of the Latin binomials/species; rather, it is the preservation of an evolutionary lineage consisting of genetically diverse individuals[3]. The loss of species genetic diversity represents a type of partial extinction[4] that often presages its total extinction. Thus, it is necessary to understand the biology of such species to find out the causative factors which lead to reproductive and regeneration failure. Presently, there is a great awareness regarding the need to conserve natural plant resources worldwide. Studies have shown that many plant species are in danger of extinction, while some have already become extinct. On a global basis, the IUCN has estimated that about 12.5% of the world's vascular plants, totalling about 34,000 species, are under varying degrees of threat. Among these, a staggeringly high 91% are limited in their geographical distribution to a single country – which links their potential for extinction to national, economic and social conditions[5]. Thus, priorities need to be determined as conservation strategies in order to build a relatively small amount of relevant work on rare and threatened species[6]. The present rates of habitat loss, landscape alteration and extinction at the species, community and even at the ecosystem levels, have sent conservation biologists scrambling to devise methods and tools for species protection and preservation. One of the goals of many conservation programmes, in addition to habitat preservation, is to maintain existing level of genetic variation in species that are rare or threatened[7,8]. The two commonly used strategies for conserving plant genetic resources are – *in situ* conservation, which allows evolution to continue within the area of natural occurrence, and *ex situ* conservation, providing a higher degree of protection to germplasm compared to *in situ* conservation[9]. *In situ* conservation refers to the conservation of a species or population on-site where they occur naturally whereas *ex situ* conservation involves the conservation of a species off-site, in seed banks or botanical gardens.

In agriculture, most crop species are conserved by *ex situ* means using seed-gene banks, field-seedling banks and in certain cases, tissue culture and cryopreserve gene bank. In contrast, in forestry, because of the long regeneration time required by trees, the preferred conservation approach is to incorporate *in situ* conservation principles into sustainable forest management[10]. Increasing the area of managed forest reserves and strictly protected areas, complementing these with *ex situ* gene banks and conservation stands[11] is also recommended for conservation. In reality, both *ex situ* and *in situ* conservation are complementary and should not be viewed as alternatives[12,13]. The best defence against the erosion of forest genetic diversity is a combination of *in situ* conservation, *ex situ* storage and the use of living collection of plants (known as *ex situ* field gene banks)[14]; this complementary conservation technique is described as *inter situ* conservation[15]. However, *in situ* conservation in tropical ecosystem, particularly like those of the Indian subcontinent, is difficult, not only owing to environmental disasters, landslides, unpredictable rainfall, flood, etc. but also due to man-made disasters and pressures like forest fire, illicit felling and overexploitation of wild gene resources for commercial purpose. Thus, in the tropics, *ex situ* conservation of forest genetic resources has become a common practice due to the alarming rate of deforestation, and the loss of species and genetic diversity. A major challenge of *ex situ* conservation is to ensure that sexually propagated samples of plants do not become museum specimens, incapable of surviving under natural conditions[16]. In 1975, FAO/UNEP conducted a pilot study on the methodology for long-term conservation of forest genetic resources within the global context[17]. The report of the study recommended the complementary role of *ex situ* seed banks in genetic resource conservation and identified the need to establish research programmes especially in the testing, storage and regeneration of tropical-forest tree seeds. The greatly increased interest in tree seeds during the past few decades may be attributed not only to the large-scale practices of artificial regeneration, but also to the growth of the agroforestry, social forestry, commercial nursery operation, watershed management and restoration of degraded areas. This interest has caused high demands for seedlings of different tree species for plantation work, both in good and poor seed years. To fulfil this requirement, we need basic knowledge about seed biology and technology such as seed maturation index, seed harvesting, processing, germination, dormancy, viability, vigour and storage physiology for various tropical species. Much of the work to date has been done mainly on temperate species, and research on tropical and subtropical species is lagging behind; thus our knowledge about tropical tree seed physiology is still inadequate. This article focuses on all the above aspects of seeds of both temperate and tropical species.

Seed-storage behaviour

Seed storage by both conventional and cryopreservation technologies offers a relatively cheap method of conserving a broad range of germplasm. According to Harrington[18,19], among all the *ex situ* conservation strategies the easiest and least expensive method of preserving the world's existing plants genotypes would appear to be conventional seed storage. Seed storage plays a complementary role in germplasm conservation, as temporary means until *ex situ* stands are established as a safety measure against disastrous losses for limited number of seed-lots. To fulfil the conservation roles, seed-storage life must exceed the natural interval between germination and seed production for the next generation. Successful long-term preservation is dependent on continuous viability monitoring with the re-collection or regeneration, whenever viability drops below a minimum level. However, development and use of optimum storage conditions will decrease the frequency of viability tests and re-collection or regeneration[20]. The ideal storage conditions are always those which reduce the growth processes of respiration and transpiration to the lowest possible degree, without impairing in any way the inherent vitality and strength of the seed embryo[21]. The longevity of seeds varies from species to species, even if they are provided with identical storage conditions. In fact, longevity of seeds in storage depends on their sensitivity and tolerance to desiccation and low temperature. Accordingly, during 1970s, seeds were classified into two major categories, i.e. orthodox and recalcitrant based on their inherent nature[22]. Thereafter, in the early 1990s, seeds of woody species were again classified into four categories based on the length of their viability and tolerance to freezing temperature as true orthodox, sub-orthodox, temperate recalcitrant and tropical recalcitrant[23]. The temperature at which recalcitrant seed can be stored may also provide valuable insight into the adaptive significance of recalcitrance. Seeds which are shed in a highly hydrated state and endure a chilling spell during their maturation are adapted to low temperatures in storage in comparison to those which have no such opportunity as in warm tropical environments. These responses have further served as the basis for the identification of recalcitrant types, as temperate-recalcitrant and tropical-recalcitrant seeds[23]. Temperate-recalcitrant seeds of species from genera such as *Quercus* and *Aesculus* cannot be dried at all, but they can be stored for several years at near-freezing temperatures. By maintaining high seed moisture content (35–40%) and a certain amount of gas exchange, seeds of some *Quercus* species can be stored for 3–5 years at near-freezing temperature with only moderate loss of viability[24,25]. Seeds of tropical-recalcitrant species have the same moisture and gas exchange requirements as the temperate-recalcitrant species, but they are sensitive to low temperature. Even short periods below 10–15°C will cause loss of viability; species included in this group are from genus *Shorea*[26], *Hopea*[27] and several tropical fruit trees[28].

Finally three main categories of seed-storage behaviour were recognized (although each may be further sub-divided); the third category 'intermediate', has been identified between the orthodox and recalcitrant categories[29]. Seeds of species that can be dried and kept under favourable conditions (low temperature and low moisture content) in a viable state, satisfactorily *ex situ* over a long term in an appropriate environment are called 'orthodox' seed[22]. However, many forest and fruit tree species from temperate and especially tropical regions produce seeds that are damaged by desiccation and are often sensitive to low temperatures and are called 'recalcitrant' seeds which have a short storage life[22]. Seeds of some species show 'intermediate' storage behaviour, surviving desiccation to fairly low moisture content, but suffering injury due to low temperature[30]. The maintenance of viability of seeds of species with intermediate or recalcitrant storage behaviour is problematic. However, in general, medium-term storage is feasible for seeds of species with intermediate storage behaviour, provided the storage environment is well defined (and well controlled), but short-term storage is usually the best that can be achieved with seeds which show recalcitrant storage behaviour (and again, only under well-defined and well-controlled environment)[31]. Therefore, if an accession of seeds of a particular species is to be conserved, then it is essential to know whether the species shows orthodox, intermediate or recalcitrant seed-storage behaviour, in order to determine the most suitable storage environment and the likely duration of successful storage[32]. At present, however, information on seed-storage behaviour is available only for about 3% of higher plants. A problem in seed research nowadays is that laboratories around the world have achieved variable success in drying the same species of seeds. Generally many tropical forest tree species have been labelled as recalcitrant just because they lost viability quickly when, in fact, this loss may have been due to other causes such as poor seed-handling practices, our knowledge of which is incomplete[33]. Studies have shown that several species considered to be recalcitrant are actually orthodox. These include the temperate *Fagus sylvatica*[34] and two tropical species *Citrus limon*[35] and *Elaeis guineensis*[36]. Further work on *E. guineensis* has indicated that this species should be classified as intermediate in storage behaviour[37]. Later research on individual species describes seeds as minimally, moderately or highly recalcitrant depending on their degree of desiccation sensitivity, hydrated storage lifespan and sometimes, chilling sensitivity[38]. There are several genera within which some species show orthodox and some others recalcitrant behaviour. *Acer* spp. as a whole shows contrasting seed-storage behaviour; for example, *A. pseudoplatanus*, *A. saccharinum* are recalcitrant, *A. marcophyllum* is intermediate, and *A. platanoides*, *A. saccharum*, *A. circinatum*, *A. campestre*, *A. japonicum*, *A. mono*, *A. palmatum*, *A. pectinatum*, *A. rubrum*, *A. spicatum* and *A. caesium* are orthodox in nature[31,32,39–42]. On the other hand, *Azadirachta indica* is perhaps the only example in which intraspecific differences in the post-harvest storage physiology have been reported. While the seeds of Asian origin appear to be 'more or less recalcitrant', those of the African provenance could be described as orthodox[43]. Recent research on seed storage physiology of *A. indica* classified it as intermediate on the basis of its capacity to withstand desiccation to 6–7% moisture content[44].

The use of many rare and high-value indigenous tropical forest species in planting and conservation programmes is still hindered by problems associated with seed-handling and storage. Generally, knowledge about seed physiology of most forest species is scarce or non-existent. In order to improve the sustainable forest management and conservation of biodiversity through the conservation and use of tropical forest species, Danida Forest Seed Centre and the International Plant Genetic Resources Institute (IPGRI) initiated a project in 1996, funded by DANIDA, on handling and storage of such seeds, involving about 30 national institutes in the tropics. In the first phase of the project, a protocol for determining the minimum moisture content and optimal storage conditions was developed and tested on about 30 species. A second phase of the project is now well under way, participants will be gathering more information on additional species, and the practical applicability of the results will be tested in large-scale trials[45]. The Royal Botanic Garden, Kew, UK is also working in this field, their Millennium Seed Bank Project is one of the largest international conservation projects ever undertaken. It aims to safeguard plant species worldwide against extinction through seed storage. It is believed that seed banking is a relatively new and under-exploited tool in combating the loss of global plant diversity, and has the unique feature as a conservation technique of making plants rapidly and easily available, under the 'Convention on Biological Diversity', for investigation and evaluation. The project intends to collect and conserve at least 10% of the world's flora, i.e. over 24,000 species by 2010, concentrating on the drier areas of the tropics and subtropics. The project hopes that this huge task of collecting the seed will be based on international collaboration and information sharing[46]. Under this project a unique seed database has also been developed for information on seed-storage behaviour[41]. The database classifies seeds in different storage categories, taking full precaution for some tropical forest species due to lack of knowledge about their proper seed handling and storage physiology. On the other hand, the National Seed Storage Laboratory, USA is working extensively on the perspective of ultra-drying of orthodox seeds for their cost-effective long-term conservation, hermetically, at ambient room temperature where freezing conditions cannot be provided, especially in developing country[47].

Seed maturation, harvesting and processing

It has been suggested that larger seeds with high moisture content be characterized by shorter span of viability compared to highly desiccated seeds, with hard, impermeable seed-coats. Fully ripened seeds with high initial viability retain their viability longer than immature seeds[18,48]. Knowledge of the right stage and time of maturity is essential for collection of an abundant quantity of healthy and vigorous seeds at an economic cost, especially in case of rare and endangered species. It is useful to know the physical and biochemical changes that occur during the maturation of seeds, as such information may determine the proper time of seed collection, storage and testing methods essential for the species. A colour change in fruit or cone provides a simple, and for some species, reliable criterion for judging seed maturity[49]. Physical characteristics have a definite advantage as indices of seed maturity. Moisture content and colour are the best criteria for maturity in sugar maple seeds, with germination reaching 95% when seed moisture dropped below 14.5% (dry weight basis)[50]. Edwards[51] has reviewed the work done on the maturity and seed quality of forest tree species. More recent work on tree species has been done on *Ulmus wallichiana*[52], *A. indica*[53], *Michelia compressa*[54], *M. kusanoi*[55], *M. thunburgii*, *Podocarpus macrophylla*[56], *Aesculus indica*[57] and *Shorea robusta*[58]. Recently, work on electrical conductivity (EC) of seed leachates during maturation has gained some importance as an operational test. Low level of vigour (high EC) for immature seeds and high level of vigour (low EC) for physiologically mature seeds has been reported in seven crop species[59] the same trend observed in tree seeds, e.g. *Pinus sylvestris*[60], *U. wallichiana*[52] and *S. robusta*[58]. Literature on seed development is dominated by studies on annual crops which mature in a specific time, but in forest species the duration of seed development can range from a few days in ephemeral species to over two years in conifers[61]. The period between seed maturation and seed dispersal is often short, whereas the effect of climate in a given year may displace the dates of seeding by several weeks from the average. Therefore, an effective schedule of seed collection requires prior knowledge of the length of time between anthesis (flowering/pollination) and fruit maturity. The interval between flowering and maturation of seeds and fruits varies greatly with the species, even within the same genus; for example, in *Eucalyptus* it varies from one month in *E. brachyandra* to 10–16 months in *E. diversicolor*[62].

Response of seeds to storage conditions depends on the quality of seed collected. Other than seed maturity, emptiness also affects the quality of seeds. Intermittent seeding patterns and high incidence of empty (aborted) seeds are regular features in forest tree seeds. Intermittent seeding is widespread in tree species, the reasons for which are not quite understood. Seed emptiness could possibly result from low pollen availability, loss of effective size of population resulting in inbreeding or unnatural factors such as frequent lopping. After extraction from the fruit, seeds must be processed properly before they are fit to go into storage. Sound and filled seeds must be separated from empty and non-viable seeds and from inert fragments of fruits, before the seeds are stored. For research purposes, when basic knowledge on germination or other seed characteristic is sought, repeated cleaning to a high standard is necessary. For the effective separation of empty, dead and viable (sound) seeds, their specific physiological properties need to be used. The ease with which sound seeds can be differentiated depends on the degree of difference which exists between the seeds and the matter to be separated from them, and the degree of uniformity among the seeds themselves[63]. The physical properties used in the cleaning process are: floating velocity, thickness, width and length of the seed, friction coefficient, specific gravity, elasticity, surface structure and colour[64]. Separation of seed by float has been used effectively for a number of forest tree species including *A. caesium* and *U. wallichiana*[65]. The two principles on which such separations are based are density and absorption. Simancik[66] made use of the differences in the rate of imbibitions of empty, dead and viable seeds for grading of several coniferous species. These weight and density differences were used for an effective separation of dead and viable seeds of *Pinus contorata*[67], *P. roxburghii*[68,69] and *P. caribaea*[70]. This separation method is called the IDS-method, i.e. incubation (I), drying (D) and separation (S). Modern cleaning machines often combine more than one method, so that the cleaning process is both effective and quick. However, the species in concern and the amount of seeds to be handled for storage will determine whether cleaning is best carried out by hand, by improvized equipment or by special machinery. All the above information may aid in devising a strategy for the preservation and conservation of germplasm.

Seed germination and pre-treatment conditions

The standard for judging seed quality (viability) is always a germination test under optimum conditions. Temperature, media and light are the critical factors affecting seed germination. Optimum temperature varies with ecotype; at this temperature, seeds are biochemically active, and any fluctuation above and below it, retards the rate of biochemical activity, which in turn results in inhibition or slowing of the rate of germination. Similarly, the light and media requirement for optimum germination varies with species. During the last couple of decades, considerable progress has been made towards the quantification of germination responses to temperature and the development of productive models. Several researchers have shown that cardinal temperature and thermal time for the rate of germination depends on species and within species may vary significantly among ecotypes[71–76]. Determination of the cardinal temperature and thermal time for seed germination rate will facilitate conservationists or seed-gene bank managers to select a suitable sowing season and agro-climatic zone for introduction of species in field for regeneration and as an *in situ* conservation stand.

In some mature seeds of woody species, especially from tropical highlands and the temperate zone, seeds fail to germinate promptly even under the optimum germination conditions. The absence of germination of an intact, viable seed under favourable germination conditions within a specified time lapse is termed as dormancy[77]. Dormancy may be of several types and more than one type may occur in the same seed. Seed dormancy is classified as physiological, morphological, morpho-physiological, physical, physical plus physiological, chemical and mechanical[78]. The dormancy conditions vary even within a species, depending on the differences between individuals, location, climatic conditions, time of collection, as well as nature and duration of seed storage after collection. In many seeds dormancy is caused by the inhibitory influence of structures covering the embryo like testa and endocarp[79]. Species of the temperate and tropical highland zones possess varying degrees of dormancy and require specific moist stratification treatments to overcome the condition. Depending on the type of dormancy, the pre-treatment requirement differs. The most commonly used pre-treatments are warm or cold moist stratification, chemical or mechanical scarification, soaking of seeds in hot and cold water, etc. In addition, a biochemical change controlled by the interaction between the inhibitor and growth promoter does have a major role in actual breaking of dormancy. Thus, knowledge of optimum germination and pre-treatment conditions of a species under consideration is essential prior to routine viability test of seeds for reproducibility of uniform results during long-term storage.

Seed viability and vigour test

Under certain circumstances, it is not possible to estimate the viability of seeds by a standard laboratory germination test. Seed technologists have always been interested in indirect methods of assessing the viability of seeds without the necessity of a routine germination test, particularly when dealing with the deep dormant seeds or seeds requiring a long period for the completion of germination test. Indirect tests can be performed within a few hours and are thus a great help in cases where results of the tests are required as soon as possible. The other objective of the quick, indirect viability tests is to determine the viability of samples which, at the end of the germination test, reveal a high percentage of fresh ungerminated or hard seeds. The triphenyl tetrazolium chloride (TTZ), electrical conductivity of seed leachates, excised embryo test, X-ray and cutting test are some of the indirect, reliable, routine viability tests. Recently, two more sophisticated seed viability tests, i.e. electrical impedance spectroscopy[80] (EIS) and fluorescein diacetate[81] (FDA) have been developed. In the past, the results of storage research were evaluated primarily in terms of germination and/or viability percentage. Now, however, all well-planned storage works incorporate some type of vigour test as an integral part of the evaluation. The importance of vigour as a factor in seed quality is clearly indicated by the trends in recent seed storage research. Loss in vigour can be thought of as an intermediate stage in the life of the seed, occurring between the onset and termination of germination/viability. Storage work that does not consider vigour tells only half the story. Decline in vigour is extremely difficult to measure. No generally accepted and satisfactory method has yet been found to measure the seed vigour of a particular species, but several vigour test methods have been developed and used for specific purposes. The tests include germination value[82], accelerated ageing test[83], cool temperature test, germination speed[84], cold test[85], electrical conductivity of seed leachates, mean germination time[86], excised embryo test[87], and germination index[88], etc. These vigour tests are of great importance to assess the actual decline in physiological quality of seeds, even if their viability remains constant during long-term storage.

Conclusion

Tropical forests are one of the last resorts of rich plant biodiversity with rare, endangered or otherwise valuable medicinal and commercial tree species that need immediate conservation for posterity and as future scientific material in the era of genetic engineering. On the other hand, forest tree seeds remain the most popular and common source of reproductive propagules in large-scale afforestation and reforestation programmes. Despite large-scale use, seed technologies for many tree species remain to be developed or are still inadequate. Therefore, we feel that it is high time to explore the storage physiology of valuable tropical forest species and gather scientific knowledge for their cost-effective long-term conservation. Thus, we recommend that, as a first step towards this, efforts should be made to develop post-harvest technology for proper handling of tropical seeds and classify the seeds according to their storage behaviour. Once seeds of a particular species are classified, then it is essential to develop complementary strategies for their conservation according to their storage physiology. For example, cryopreservation of embryonic axis of non-orthodox seeds and ultra-drying of orthodox seeds offers an effective and economically viable alternative for long-term *ex situ* germplasm conservation.

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