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## Innovative approaches to the preservation of forest trees

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### ABSTRACT

The recent acceleration of actions to conserve plant species using *ex situ* and *in situ* strategies has revealed the need to understand how these two approaches might be better developed and integrated in their application to tree species. Here we review some of the recent successes relating mainly to tree seed biology that have resulted in the development and application of innovative actions across five areas: (1) the expansion of living collections to conserve threatened tree species in sufficient numbers to ensure a broad genetic diversity in their progeny; (2) the generation of viability constants to enable estimates to be made of storage longevity of tree seeds in the dry state; (3) improvement in the diagnosis of tree seed storage behaviour through the development of predictive models, reliable prognoses of desiccation tolerance and use of botanical information systems, such as GIS, to correlate information on species distribution and their physiological characteristics; (4) advances in storage preservation biotechnology to enhance the future application of cryopreservation procedures to recalcitrant species in biodiversity hotspots where many are under threat of extinction; (5) integration of *ex situ* and *in situ* conservation approaches to ensure that best practice in horticultural and forestry are combined to maintain or enhance genetic diversity, especially in high value species and those with small and vulnerable populations. These actions can lead to greater impact if supported by greater efforts to create seed banks and to collate databases world-wide so that data, knowledge and collections are more available to the scientific, forestry and NGO communities. Throughout this review we have used examples from the mega-biodiversity countries of Brazil and China, as a way of illustrating wider principles that can be applied in many countries. Future development of current research approaches, the adherence to conservation policy and the expanding needs for education are also considered briefly.

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### Introduction

The Convention on Biological Diversity (CBD, 1992) provides a legal framework for accessing, conserving and using biodiversity in a fair and equitable manner. Within its instructions there are clear obligations on each contracting party to identify components of biological diversity important for its conservation and sustainable use having regard to: (1) ecosystems and habitats; (2) species and communities; and (3) described genomes and genes of social, scientific or economic importance. Responding to these ambitions, the botanic gardens' community launched a Global Strategy for Plant Conservation (GSPC, 2002) with 16 targets for concerted action. This strategy was revised in 2010 and broadly fits under the Aichi Biodiversity Targets (2010–2020), which articulate five

strategic goals of which strategic Goal C seeks to '...improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity.' (ABT, 2014). Three associated targets address the need to preserve tree germplasm and the role that Botanic gardens can play in this through: conserving areas of particular importance for biodiversity and ecosystem services' (Target 11); preventing the extinction of known threatened species and improve their conservation status (Target 12); safeguarding the genetic diversity of cultivated plants, wild relatives and other socio-economically as well as culturally valuable species (Target 13).

Of all life forms, trees in particular require special attention for conservation; for their myriad of ecosystem services, their high level of extinction threat and their socio-economic and cultural value (Oldfield et al., 1998; Dawson et al., this special issue). However, for the conservation of trees to be successful there are a considerable number of challenges to overcome. Many of these

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are being addressed by the activities of the botanic gardens community (Oldfield, 2009). The purpose of this article is to present recent *in situ* and *ex situ* innovative approaches to conserving species in their natural habitat, living collections, or germplasm banks. These will be described through the major methods being used, developed and applied to: (1) the use of living collections, often held by botanic gardens, to conserve threatened species; (2) the development of the technology and understanding of the longevity of dry tree seeds held in seed banks; (3) the diagnosis of tree seed storage behaviour; (4) the use of biotechnological approaches to improve the preservation of recalcitrant (desiccation intolerant) species; and (5) provide integrated *in situ* and *ex situ* conservation strategies, especially in their development to conserve 'exceptional' species that produce low seed numbers.

## 1. Living collections and threatened trees

For decades botanic gardens have offered a service to conservation through targeted programmes of collecting seeds and other tissues (e.g. cuttings) of threatened species, as a means of providing safe haven to the germplasm and to complement *in situ* conservation efforts. An excellent summary of such work across 700 botanic gardens in 118 countries can be found on the Botanic Gardens Conservation International web site (BGCI, 2014). Recent acceleration of these conservation efforts have resulted from the availability of country-based Red Lists of threatened species and online access to the International Union for Conservation of Nature (IUCN) Red List that provides a global assessment of threatened plants. An example of a concerted effort to provide shelter to Red List trees is Xishuangbanna Tropical Botanical Garden, Yunnan, PR China (XTBG, 2014). As one of the main botanic gardens within the Chinese Academy of Sciences (CAS), XTBG has developed its arboretum, opened in 1959 and comprising an area of 3.2 ha, into an important haven for 750 tropical plant species. The main function of the arboretum is to collect and preserve endangered and rare plants, including important commercial and economic trees, such as *Cananga odorata* (ylang ylang), *Podocarpus nagi* (Javan podocarpus) and state protected species such as *Shorea wangi* (the towering tree). *Shorea* is one of five genera in the Dipterocarpaceae within which 13 species have been given national protection in China due to their rare or endangered status. The arboretum has now been expanded to cover seven ha, and contains a total of 34 species from seven dipterocarp genera, including all species distributed in China and Southeast Asia, thus fulfilling a regional conservation role as well as a national one. The region is rich in tree species of many families, including Fagaceae, Lauraceae, Theaceae and Magnoliaceae. A second example in China is the South China Botanic Garden, CAS, which holds the world's largest collection of Magnoliaceae with more than 130 species, and palms with 382 species (c. 17% of the species in the family). Overall, the 160 botanic gardens in China play a key role in implementing China's Strategy for Plant Conservation (Huang, 2010). Botanic gardens in other countries fulfil a similar role.

A concern relevant to this approach to *ex situ* conservation is the limited genetic capture and the limited extent of duplication against unpredicted losses. However, the need for duplication of *ex situ* conserved plants across different gardens is now better recognised, with 8216 (33%) of the 24,667 plant species cultivated *ex situ* in the 10 main botanic gardens in China duplicated in at least one other botanic garden, and nearly 5% in four gardens (Huang, 2010). One study on optimal sampling for plants for *ex situ* conservation in living collections, based on *Leucothrinax morrisii* (thatch palm), has recommended sample sizes of at least 15 individuals at each site to capture an acceptable level of genetic variation (Nammoff et al., 2010). Across 13 populations of *Chamaedorea ernesti-augusti* (fishtail palm; averaging 25 individuals), allelic

capture was estimated at 5–58% (Cibrian-Jaramillo et al., 2013). This level of genetic representation (i.e., 15–25 individuals) is impossible to achieve in most individual botanic gardens, hence current efforts by BGCI to co-ordinate the species' conservation through more intensive species planting within gardens is critical to success (BGCI, 2014). If species are represented by individual trees at botanic gardens the progeny may be severely inbred, if they produce seed at all. Representation by only a few individuals may also lead to inbreeding problems in subsequent generations, a situation somewhat analogous to that observed with 'pasture' trees following forest clearance (Lowe et al., 2005). However, in assigning a management priority for garden collections, consideration should be given to species information available on a basis of their imperilment and operational costs to maintain diversity (Cibrian-Jaramillo et al., 2013).

## 2. Tree seed longevity in the dry state

There are numerous challenges in developing strategies for tree seed regeneration, including long-term space requirements to accommodate mature growth, the time taken to create a seed orchard and their maintenance costs, as times to first flowering and maximum seed output from trees vary considerably between species. Consequently, for seed storage to be more widely accepted and adopted as an effective long term *ex situ* conservation strategy it is necessary to demonstrate that tree seeds can be stored for periods well in excess of the time to reach reproductive age and preferably the tree's lifespan.

Long-term storage is not the main purpose of the many 'active' seed collections maintained by tree seed centres, scientific institutions and private commercial suppliers. Based on the World Agroforestry Centre's Tree Seed Suppliers Directory (TSSD), such collections contain 2846 woody perennial species of known natural source (Dawson et al., 2013). Clearly, this is an important *ex situ* reservoir of biodiversity. However, the environmental conditions for storage are less stringent than those applied in long-term seed banks, such that these 'active' seed collections only maintain a high viability for short-term use in afforestation programmes and require regularly replenishment.

What then is the prospect of storing tree seeds for longer than the time to reproductive age or species lifespan? There are records of thousands of years' lifespan, e.g. bristlecone pine; and some large emergent trees from the Amazon rainforest have been carbon dated at more than 1400 years old (Chambers et al., 1998). However, the average lifespan of trees is probably closer to 150 years. For species grown in Britain, for example, expected lifespans are: 50–70 years for *Alnus glutinosa* (alder), *Betula* (birches) and *Populus* (poplars); 70–100 years for *Fraxinus excelsior* (ash) and *Picea abies* (Norway spruce); 150–200 years for *Acer platanoides* (Norway maple); 200–300 years for *Tilia* (limes), *Quercus robur* (English oak) and *Pinus sylvestris* (Scots pine) and 300+ years for *Picea sitchensis* (Sitka spruce) (British Hardwood Tree Nursery, 2014).

Detailed estimates of the storage biology of desiccation tolerant tree seeds are uncommon. On the Seed Information Database (SID) there are viability constants (used to describe the sensitivity of seed lifespan to temperature and moisture) for only 16 tree species (RBC, Kew, 2014b). Accounting for the variation in desiccation tolerance, it is possible to compare estimated seed lifespans under a storage temperature that may be accessible to foresters, e.g. 10 °C, and the lowest safe moisture content (MC) for the seed.

Table 1 shows that the estimated seed half-lives (time for viability to fall from 97.9% to 50%) varies from 0.95 and 1.36 years for *Dipterocarpus alatus* (Gurjum tree) and *Araucaria columnaris* (Cook pine), respectively, at the short end of the scale to 324 and 342 years for *Acer platanoides* (Norway maple) and *Liquidambar*

**Table 1**

Predicted times for tree seeds to lose half their viability when stored at the MCs shown and at 10 °C, to match the temperature achievable in most forest tree seed stores, based on published viability constants reported in the Seed Information Database (Royal Botanic Gardens, Kew, 2014b). Oil contents are from SID or other, specified sources.

Species	Oil content (% mass)	Moisture content (% fresh mass) <sup>#</sup>	Seed half-life (years)
<i>Acer plantanoides</i>	c. 22 <sup>a</sup>	3.1	324
<i>Agathis australis</i>	64.6	5.3	12
<i>Araucaria columnaris</i>	40.7	12.0	1.4
<i>Araucaria cunninghamii</i>	24.8	2.0	14.9
<i>Dipterocarpus alatus</i>	7.0	17.0	0.95
<i>Dipterocarpus intricatus</i>	15.9	10.0	6.0
<i>Entandrophragma angolense</i>	68.1	5.0	2.65
<i>Khaya senegalensis</i>	52.5 <sup>b</sup>	6.0	2.85
<i>Liquidambar styraciflua</i>	27 <sup>c</sup>	3.2	342
<i>Malus pumila</i>	25.7 <sup>d</sup>	4.5	85
<i>Pinus elliottii</i>	19 <sup>e</sup>	5.5	68
<i>Pinus taeda</i>	17.5	6.1	27
<i>Platanus occidentalis</i>	6.3	5.0	19
<i>Swietenia humilis</i>	70.6	4.0	21
<i>Terminalia brassii</i>	4.8	4.6	9
<i>Ulmus carpinifolia</i>	18–32 <sup>f</sup>	3.0	56

<sup>a</sup> Grodziński and Sawicka-Kapusta (1970).

<sup>b</sup> Okieimen and Eromosele (1999).

<sup>c</sup> Bonner (1972).

<sup>d</sup> Kamel et al. (1982).

<sup>e</sup> Wolff et al. (1998).

<sup>f</sup> Sorensen and Soltoft (1958).

<sup>#</sup> Lowest safe MC.

*styraciflua* (sweet gum), respectively, at the other end of the scale. The shortest life-spans are driven by the need to store the seeds partially hydrated at 10–17% MC. In contrast, the longest lived seeds can be stored much drier at around 3% MC. It should be noted that this comparison is used to illustrate a point and that to compare precisely inter-species performance would require accounting for the effects of oil on the equilibrium moisture status of the seed; and as Table 1 shows, ‘seed’ (not always morphologically a seed) oil content varies enormously between species. Nonetheless, it is clear that seed life-span beyond the life-span of the tree is possible. This will be more the case when internationally-agreed long-term seed bank (3–7% MC and –20 °C) conditions can be used, rather than the 10 °C used in this analysis.

Interestingly, there are well documented examples of long-term ‘tree’ seed survival of hundreds of years, validated by carbon dating. The longest lived ‘tree’ species seed that germinated is that of *Phoenix dactylifera* (date palm), recovered from an archaeological excavation at Masada, near the Dead Sea, and dated at 2000 years old (Sallon et al., 2008). In addition, some seeds of three woody species of the Cape Flora of South Africa were germinated after about 200 years museum storage under non-ideal conditions. The species, still requiring species verification, belong to the genera *Acacia*, *Lipparia* (both in the Fabaceae) and *Leucospermum* (Proteaceae) (Daws et al., 2007).

In 2013, the UK launched its first native tree seed bank at the RBG, Kew to protect against species and genetic diversity loss due to the interrelated impacts of climate change and increasing threat of pests and diseases (RBG, Kew, 2014a). The aim is to increase the species range and genetic diversity of the collection of UK tree seeds already in long-term storage in the Millennium Seed Bank (at c. 5% MC and –20 °C). These seeds will be available to research organisations and for restoring trees to the UK countryside and increasing tree cover. The Forestry Commission is a key partner, providing advice on target species and help with collection. A priority list of 50 trees and shrubs has been targeted for collection, including: *Juniperus communis* (common juniper), and the subspecies *Juniperus ssp. hemisphaerica*, designated as critically endangered on the IUCN Red List; *Cotoneaster cambricus* (wild cotoneaster), a rare species with just a handful of wild trees; and

*Pyrus cordata* (Plymouth pear), designated as vulnerable on the IUCN Red list and one of Britain’s rarest trees.

### 3. Diagnosis of tree seed storage behaviour

Information on the seed biology (storage and germination) of tree species is sparse beyond the main species of interest to commercial forestry (Young and Young, 1992; Vozzo, 2002). Consequently, the prospects for *ex situ* seed banking is unclear for the vast majority of the estimated 60,000–100,000 tree species in the world (Oldfield et al., 1998). Such shortcomings can be addressed, in time, through screening seeds of representative tree species for storage physiology (i.e., desiccation tolerance and longevity) and through the development of predictive models for physiological responses.

#### 3.1. Screening for seed storage

In all countries it is the case that not all tree species produce seeds that can be stored in conventional seed banks (dry and cold) due to sensitivity of the seeds to desiccation; the so-called recalcitrant response. For example, in Central Amazonia (Brazil) indications are that c. 60% of tree species produce recalcitrant seeds. Over the last 20 years considerable progress has been made in: (1) understanding the mechanisms of desiccation-induced viability loss on drying; (2) estimating the proportion of the world’s flora that produce such seeds (i.e., diagnosis); and (3) developing methods that can help conserve such species in *ex situ* cryo-banks (i.e., storage biotechnology).

In contrast to desiccation tolerant (orthodox) seed, recalcitrant seeds lack the ability to “switch-off” metabolically late in development or to undergo intracellular dedifferentiation (Berjak and Pammenter, 2013), such that these seeds must be stored under moist conditions, where longevity is often curtailed by germination and the proliferation of seed-associated fungi. Reactive oxygen species and programmed cell death have been implicated in desiccation stress in recalcitrant seeds, e.g., *Q. robur* (English oak) (Kranter et al., 2006), and during accelerated ageing of orthodox

seeds, e.g., *Ulmus pumila* (Siberian elm) (Hu et al., 2012), which has led to increased interest in manipulating the gaseous environment during seed storage, including through the use of nitrous oxide (Iakovoglou et al., 2010). Studies on the mechanistic basis of seed desiccation (in)tolerance are ongoing.

Characterisation of tree seed storage responses received attention during the mid-1990s through a global initiative led by Danida and Bioversity International (formerly International Plant Genetic Resources Institute; IPGRI). Scientists in c. 20 countries screened seeds of 52 tropical forest trees, belonging to 27 families, for recalcitrant and intermediate (partial desiccation tolerance but with sensitivity to storage at  $-20^{\circ}\text{C}$  and  $0^{\circ}\text{C}$ ) responses. The project employed a storage protocol (Fig. 1) that assessed seed responses to multiple desiccation states and subsequent storage at a range

of temperatures (Hong and Ellis, 1996). The approach is a reliable way to resolve seed storage behaviour. For a summary of the findings of the project see Sacandé et al. (2005). One limitation of relying upon the full protocol is that it uses thousands of seeds, which may not be easily available for rare tree species or reliably with trees with supra-annual seed production.

An alternative screening approach deals with only aspects of the effects of drying and short term storage at the initial MC of the seed sample (at receipt or harvest), called the 100-seed test, to reflect the target number of seeds to use (Pritchard et al., 2004b). The approach, which was developed for palm seeds, has been adopted by tree seed experts at INPA in Brazil, the University of KwaZulu Natal, Durban, Republic of South Africa and at the University of Queensland, Australia (e.g., Hamilton et al., 2013).

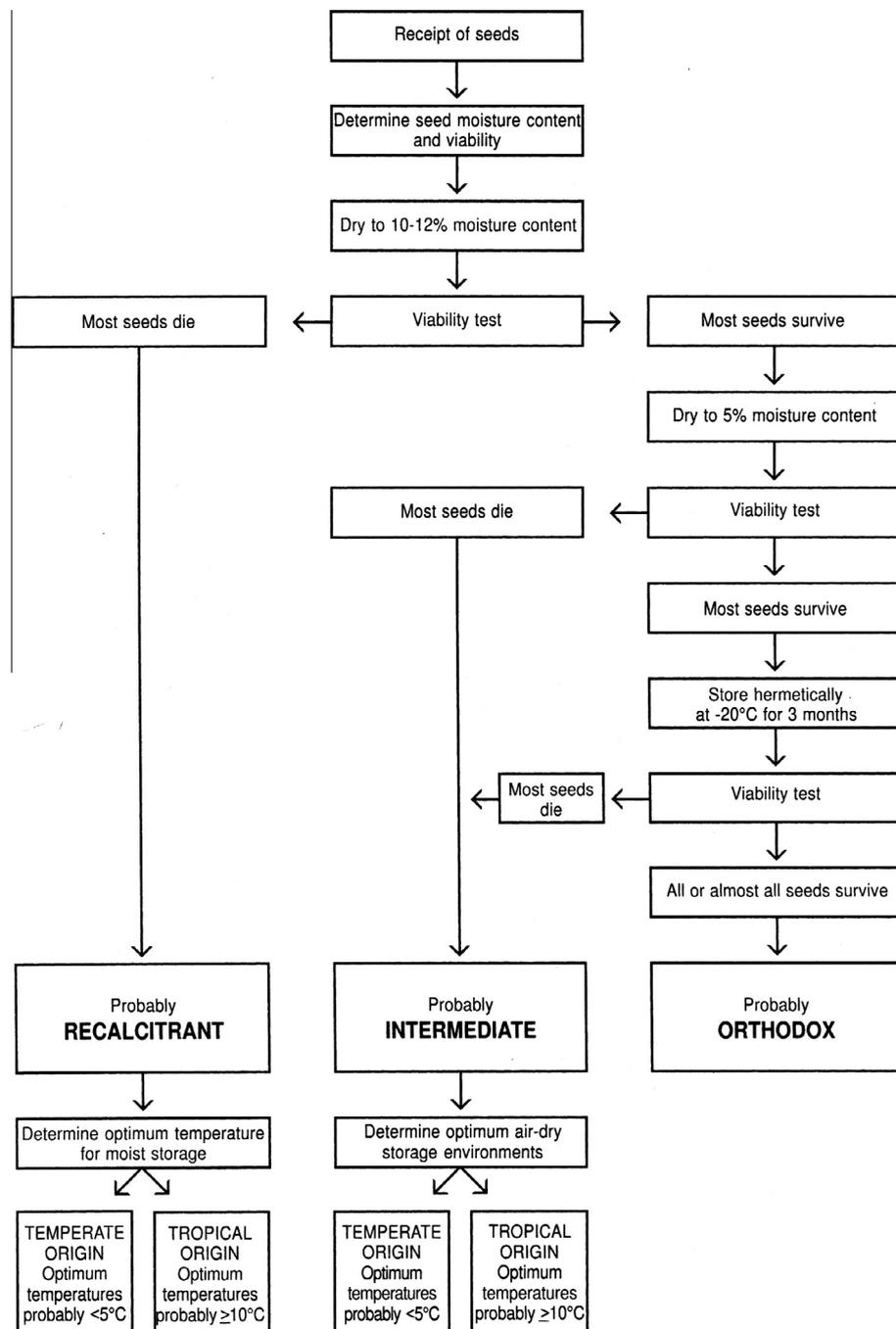
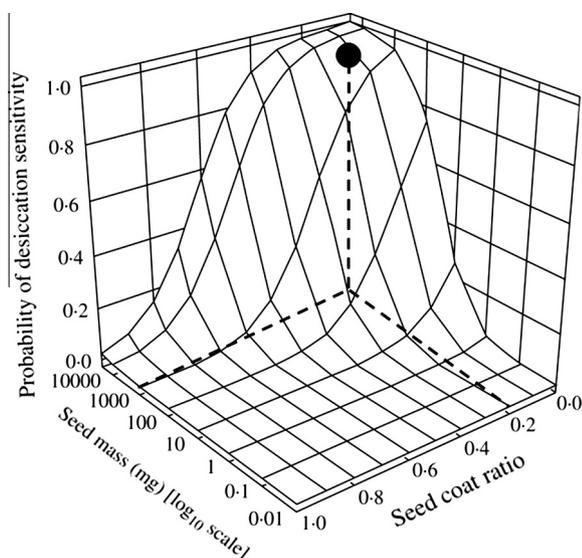


Fig. 1. Simplified scheme for screening seed storage behaviour (Hong and Ellis, 1996), with permission of the publisher (Bioversity International, formerly IPGRI).

### 3.2. Predictive models

With an estimated 223,000–353,000 species of higher plants (Scotland and Wortley, 2003; Chapman, 2009) and the physiological screening of all species unlikely in the short- to medium-term, it is important to develop predictive biological models that help indicate risks associated with handling seeds with particular features. One of the earliest efforts in this direction revealed broad associations between heavier seed weights in the Araucariaceae (Tompsett, 1984) and Dipterocarpaceae with seed desiccation sensitivity. More complex multiple criteria keys for seed storage have been developed for a few more plant families, including Meliaceae, drawing on seed weight, MC at the time of seed shedding, seed shape data and general habitat (Hong and Ellis, 1998). Further developments in this direction have found associations between specific habitat conditions and desiccation intolerance across a broad range of vegetation types; with low levels of frequency (c. 10% or less) in the driest regions of the world, and high frequency (close to 50%) for tropical moist evergreen forests (Tweddle et al., 2003). Taking the ecological concept further, it can be hypothesised that as recalcitrant seeds must maintain water status or else they die, such seeds will be dispersed in the rainy season in seasonally dry environments. This is indeed the case with c. 70 African tree species (Pritchard et al., 2004a). However, in environments that are wet throughout the year, such an association between time of seed dispersal and desiccation sensitivity may not be evident, as indeed is found in an analysis of 16 plant species from a seasonally wet tropical forest in Queensland, Australia (Hill et al., 2012). Similarly, relatively quick germination in recalcitrant seeds would enable the sourcing of an external water supply in the soil. The relevance of this is that recalcitrant seeds do not need proportionally as great a defence mechanism against predation, e.g., thick seed coats, as germination is relatively quick (Pritchard et al., 2004a). This possibility was assessed for 104 Panamanian tree species (Daws et al., 2006). By plotting seed mass and the seed coat ratio (dry weight of the covering layers compared to the internal tissues: the embryo in a non-endospermic seed; and the embryo and endosperm in an endospermic seed) and the associated seed storage physiology, it was possible to develop a predictive model for the probability of a seed being recalcitrant (Fig. 2).



**Fig. 2.** The topography of seed desiccation sensitivity. Known seed (in)tolerance to drying was correlated with seed mass and seed coat ratio (SCR) to generate a predictive model of probable desiccation sensitivity in 104 Panamanian tree species (Daws et al., 2006). Dashed line and closed circle: a seed of 1 g and a SCR of 0.2 has >0.95 probability of being desiccation sensitive (recalcitrant).

The best logistical model describing the topography of the recalcitrant seed response, i.e., for predicting the likelihood of desiccation sensitivity ( $P$ ) is:

$$P(\text{Desiccation sensitivity}) = \frac{e^{3.269 - 9.974a + 2.156b}}{1 + e^{3.269 - 9.974a + 2.156b}}$$

where  $a$  is Seed Coat Ratio and  $b$  is  $\log_{10}$  seed mass (grams). It is important to note that 14 (13%) of the Panamanian species appear to have been misclassified by the model but that validation of the model was accurate for 38 African and European woody species for which published literature was available. A practical implication of this is that detailed characterization of the response to desiccation should be conducted on these 14 species to determine additional elements to add to the model to improve accuracy. An additional consideration is the variation in seed mass that can occur within and between seed lots, where smaller seeds will dry more rapidly to below their critical water content, while larger seeds retain proportionally more water and so maintain viability (Daws et al., 2004).

In a comparative study of seed morphology in relation to desiccation tolerance and other physiological responses in 71 Eastern Australian rainforest species, representing 30 families, sensitivity to desiccation to low MC (<10%) occurred in 42% of species. Taken with earlier findings, 49% of Eastern Australian rainforest species have non-orthodox seeds. In broad agreement with other studies, across the 71 species the desiccation sensitive seeds were found to be larger than desiccation tolerant seeds (1663 mg vs 202 mg) and had less investment in seed coats (0.19 vs 0.48 seed coat ratio) (Hamilton et al., 2013). Similar studies are underway at the Germplasm Bank of Wild Species, Kunming Institute of Botany, CAS in SW China and at INPA, Manaus on species in the Amazon. Across 60 economically-valuable timber species from 18 families found in non-flooded forest near Manaus, 62% had seeds that were difficult to store and these seeds probably have non-orthodox behaviour (Ferraz et al., 2004). Another analysis, for 60 tree species growing in Vietnam (51 native and 9 introduced), found 43% to be non-orthodox in storage behaviour (Ellis et al., 2007).

The sampling of families used in the analyses described above is extensive and reveals that desiccation sensitivity is also wide spread in non-woody species. For example, desiccation sensitivity is quite widespread in palms and particular attention should be given to phenotypic plasticity in the seed storage response as a result of differences in seed developmental age at the time of natural seed dispersal. Changes in relative desiccation tolerance can also be found in seeds from trees across their native range, and other aspects of seed quality, such as seed germination are seen to vary; for example, *Acer pseudoplatanus* (Daws and Pritchard, 2008).

### 3.3. Botanical information – species inventories and distributions

Whilst the botanical inventory for the developed world is comprehensive, that of the world's tropics is not. For example, vast areas of the Brazilian Amazon await exploration and it is estimated that when fully recorded the number of species of angiosperms for the Brazilian flora will minimally double to 44,000–50,000 (Shepperd, 2003). Limited knowledge of plant diversity reflects the complexity of the tropical moist forest biome, difficult access, lack of systematic collections and the small number of botanists and other specialists in such regions. However, the need for conservation in tropical moist forests is greatest (World Commission on Forests and Sustainable Development, 1999). Even with stricter controls on deforestation, the Amazonian rainforest contracted by about 6000 km<sup>2</sup> per year between 2005 and 2009 (Nepstad et al., 2009). At stake is the conservation of tree diversity, e.g. more

than 1400 tree species in just two reserves close to Manaus, Brazil. In Brazil protected areas have been extended, reaching 16.6% of the continental area of the country by 2011 (Ministerio do Meio Ambiente, 2014). Nonetheless, deleterious human influence in these areas can still be a problem for the adequate preservation of forest species.

Whilst current species lists can be limited to investigations carried out close to major cities, along roads and rivers (Nelson et al., 1990), they remain a key tool in conservation planning. Although most attention is often given to species of economic priority (FAO, 2014), from a global conservation perspective more consideration of endemic and endangered species is required to ensure their survival. Conservation status can be determined through field work, the analysis of historic herbarium specimen data, and by drawing on institutional and global resources, e.g. the Global Biodiversity Information Facility (GBIF, 2014). However, there is the matter of how many herbarium specimens are needed to detect whether a species is threatened. In a study on 661 endemic species of legumes and orchids from Madagascar, it was concluded that capturing 15 geo-referenced specimens per species was sufficient to produce robust preliminary conservation assessments based on range estimates (Rivers et al., 2011).

To support the integration of *in situ* and *ex situ* conservation approaches, both vegetation maps and assessments of individual species distributions are needed. At the habitat level, priority has been given to assessing the level and rate of destruction of the world's biodiversity hotspots through monitoring, so as to understand threats to habitat and species loss and demonstrating the potential value of Geographic Information Systems (GIS) for the management of sites. An example of the role of GIS in contributing to conservation activity is the monitoring of vegetation cover changes in the area of Mount Oku and the adjoining Ijim ridge in Cameroon, a tropical montane rainforest, using satellite and aerial sensor detection (Baena et al., 2010). Following strong spatial patterns of deforestation between 1958 and 1988, regeneration was observed following the first Conservation Project (started in 1987) which resulted in 7.8% of the 1988 montane forest extent being recovered by 2001. Whilst there were differences in forest vegetation boundaries across the study area, regeneration was observed from the commencement of the project.

Deforestation increases fragmentation and edge effects and large trees have a higher probability of dying due to physical damage (Laurance et al., 2000) or physiological constraints from microclimatic changes (Camargo and Kapos, 1995). In addition, forest fragmentation seems to lessen the number of reproductive events (Lowe et al., 2005) and may also cause an asynchronism in the reproduction cycle between trees located in fragments and in adjacent continuous forests. Consequently, over time, less trees will fruit, which will reduce seed rain and may affect the natural regeneration of certain tree species in forest remnants (Benitez-Malvido, 1998). This is a particular concern when considering recalcitrant seeded species, as they can be more frequent in families of large trees (e.g. oaks, dipterocarps).

As the example from Cameroon illustrates, focused attention can support forest tree conservation in biodiversity hotspots. However, the conservation of evolutionary process should also be a priority in the face of global change to ecosystems. Phylogenetic diversity (PD) is a biodiversity index that measures the length of evolutionary pathways linking taxa. Although taxon richness is a good surrogate for PD, the two have been found to be decoupled in a study in South Africa, based on an assessment using GIS of genus absence/presence per quarter degree square using data from the Pretoria National Herbarium database – PRECIS (Forest et al., 2007). Thus providing the ability to develop PD biodiversity indices matching the local geography and supporting specific conservation planning.

GIS is also a valuable tool in deciding which sites would not be good for *in situ* conservation or have the potential to be lost quickly based on climate change and habitat loss, including sea level rise, and thus providing a rationale for intervention through *ex situ* conservation. Such intervention is supported by phenological information from herbarium specimens and up-to-date seasonal climate data (e.g. rainfall, which affects flowering and seeding), thus enabling conservationists to target collecting sites and times for (also pollen) seed harvesting. The MSBP, RBG, Kew applied such an approach in producing seed collecting guides for seasonally-dry habitats in more than 10 countries. It is worth noting however that seed production in moist forests can be hindered by low species density and less predictability in fruiting to a specific month, plus supra-annual fruiting. Seed collecting in tall trees located in remote areas may be a further challenge. In undisturbed forest, the high predation of fruits and seeds by the local fauna may reduce even more seed availability for storage or propagation. Future developments in online and in-field mapping, better integration of multiple datasets (climate, species, weather, satellite imagery, etc.), and access to high resolution and hyper special imagery will improve the capability of these innovative conservation tools and are areas of major activity by botanic gardens.

There are numerous efforts to collate information on species biology of relevance to *ex situ* conservation, at the national: (NatureServe Explorer for North America) regional (EUFGIS for the EU) and global (FAO's REFORGEN) level. In some cases, these information sources collate a broad range of information (e.g., on the seed trade, seed handling, plant pests, relevant institutes, education programmes) and provide recommendations for *ex situ* conservation activities (e.g. EUFORGEN, 2014). However, there are few publicly accessible databases or knowledge management systems that are seed specific. Examples include GRIN (2014) and the SID (RBG, Kew, 2014b). Although not exclusively dedicated to trees, there is much information that can be gleaned from searching by the name of the species. Searches on SID are possible for seed chemistry (oils and protein), germination, mass (thousand seed weight), longevity and morphology. Even when there is no data on the species of interest, there may be information on a con-generic species or a perspective that can be gleaned from information across species in the same family. An example of such an analysis is shown in Table 2 for the trees listed in the Global Trees Campaign, a partnership between Fauna and Flora International and BCGI. This is the only international campaign dedicated to saving threatened trees. The situation is critical as over 8000 tree species, 10% of the world's total, are threatened with extinction.

Other sources of information on tree species to draw upon include various species-based information leaflets or booklets that summarise, *inter alia*, taxonomy, distribution, uses and seed biology. For example, the 'Seed Leaflet' series (København Universitet, 2014) for about 150 species, e.g., *Borassus aethiopicum* (African fan palm; Sanon and Sacandé, 2007). A similar series on Amazonian species is emerging from Manaus, Brazil (INPA, 2014). However, it would be valuable to consolidate these and other resources on one web site under a general theme of 'Tree seeds of the world' so that *ex situ* conservation actions can be supported. Sources of information should include compendia of national and regional forest seed programmes.

#### 4. Storage preservation biotechnology

Target 8 of the GSPC directs that approximately 75% of threatened plant species be present in *ex situ* collections by 2020. This target can present particular challenges for mega-diverse countries, in terms of the scale of the task, the range of species for protection, and in the application of the most appropriate techniques

**Table 2**

An analysis of seed storage options for the trees listed by the Global Trees Campaign (FFIBCGI, 2014) based on the Seed Information Database (RBG, Kew, 2014b). The details were presented at the Second meeting of the IUCN/SSC (2012).

Group A	Species
Group A – Data deficient at the species and genus level	<i>Ardisia</i> ; <i>Baillonella</i> ; <i>Chirantodendron</i> ; <i>Fitzroya</i> ; <i>Kokia</i> ; <i>Lansan</i> ; <i>Microberlinia</i> ; <i>Tambourissa</i> ; <i>Tectiphiala</i> ; <i>Tieghemella</i> ; <i>Wollemia</i> ; <i>Xanthocyparis</i>
Group B – Species evidently orthodox	<i>Acacia anegadensis</i> ; <i>Commidendron robustum</i> ; <i>Dalbergia melanoxylo</i> ; <i>Dracaena draco</i> ; <i>Larix decidua</i> ssp <i>carpatica</i> (species level) <i>Pericopsis elata</i> ; <i>Pilgerodendron uviferum</i> ; <i>Taiwania cryptomeroides</i> ; <i>Widdringtonia cedarbergensis</i>
Group C – Species with congeners that are evidently orthodox	<i>Adansonia grandidieri</i> & <i>A. suarezensis</i> (data on 3 sp.)  <i>Caesalpinia echinata</i> (data on 22 sp) <i>Cypripedium dupreziana</i> (data on 12 sp) <i>Dalbergia nigra</i> & <i>D. stevensonii</i> (data on 29 sp.) <i>Dracaena ombet</i> (data on 1 sp.) <i>Magnolia</i> × 4 sp (data on 6 sp. = O; 1 uncertain; 1 Recalcitrant) <i>Malus niedzwetskyana</i> (data on 12 sp.) <i>Pinus longaeva</i> (vast majority of species in <i>Pinus</i> = O) <i>Rhododendron protistum</i> var <i>giganteum</i> (vast majority of species in <i>Rhododendron</i> = O; 2 sp. = uncertain) <i>Tectona philippinensis</i> (data on 2 sp.) <i>Ulmus mexicana</i> (data on 15 sp.)
Group D – Species, or congeners (number in brackets) are often recalcitrant	<i>Aquilaria crassara</i> – recalcitrant (+1 sp. = R)  <i>Araucaria araucana</i> – recalcitrant (+3 sp. = R, 5 sp. = I, 2 sp. = O) <i>Carapa megistocarpa</i> – DD (+2 sp. = R) <i>Cinnamomum cebuense</i> – DD (+7 sp. = R; 1 sp. = U; 1 sp. = I; 1 sp. = O) <i>Dyopsis pambana</i> – DD (+1 sp. = R) <i>Elaeocarpus bojeri</i> – DD (+3 sp. = R) <i>Quercus hintonii</i> – DD (vast majority sp. = R; + 1 sp. = I, <i>Q. emoryi</i> ) <i>Sideroxylon marginata</i> (+2 sp. = R; 1 sp. = O) <i>Zelkova secula</i> (+1 sp. = R)
Group E – Species with some seed desiccation tolerance?	<i>Acer leipoense</i> & <i>A. yambianse</i> – DD (but 2 sp. = R; 3 sp. = U; 1 sp. = I; 40 sp. = O) <i>Attalea crassispata</i> – DD (but 2 sp. = I) <i>Medemia argun</i> – Uncertain <i>Pritchardia limahuliensis</i> – DD (but 1 sp. = O; 4 sp. = U) <i>Swietenia macrophylla</i> – Intermediate (but 2 sp. = O)

Key: DD = data deficient regarding seed storage behaviour; R = desiccation sensitive; I = partially desiccation sensitive; O = tolerant of drying and dry storage; uncertain = data is limited to make a certain diagnosis.

and innovations, particularly for recalcitrant seeds (Harding et al., 2013; Walters et al., 2013). The lifespan of fully hydrated recalcitrant seeds is limited mainly by the extent to which they can be safely cooled. For both temperate and tropical species cooling is limited by the risk of ice formation and chilling stress, respectively. Thus in practice, storage can be close to 0 °C for temperate recalcitrant seeds and 15 °C for tropical representatives on this functional trait. Generally under such conditions, lifespan is limited to a few months to rarely more than one year. Consequently, alternative conservation solutions are needed if the seeds of these species are to be conserved longer-term *ex situ*. Cryopreservation (usually storage below c. –130 °C, often in the vapour phase above liquid nitrogen) is the method of choice (Li and Pritchard, 2009). However, as whole recalcitrant seeds tend to be large, the development of innovative approaches have mainly related to shoot tip and embryo (and embryonic axis) tissue preservation, followed by recovery *in vitro*. By reducing tissue mass, it is possible to control better the target MC for cryopreservation, and the cooling and warming phases of the process.

The main development of the last 25 years in plant cryopreservation has been the improvement in vitrification methodologies, particularly encapsulation-dehydration (Fabre and Dereuddre, 1990) and the use of complex solutions of cryoprotectants that reduce the risk of ice formation of partially hydrated tissues during cooling and rewarming. These ‘plant vitrification solutions’ (PVS) combine cryoprotectants that vary in permeability, enable removal of cellular water, increase cell viscosity and alter the properties of any remaining water (Volk and Walters, 2006). PVS2 is the most commonly used cryoprotectant, consisting of 30% glycerol, 15% dimethyl sulfoxide and 15% ethylene glycol in Murashige and

Skoog medium with 0.4 M sucrose (Sakai et al., 1990). Across the various compositions of vitrification solutions and methodological variations (i.e., vitrification, encapsulation-vitrification and droplet vitrification), these technologies have been successfully applied to shoot tips, somatic embryos, nodal explants, etc., of 110 species (>80 genera) (Sakai and Engelmann, 2007). Such techniques are now being applied on a large scale; for example, to protect the potato collection at the International Potato Center, Peru and the banana and plantain collection in the Laboratory of Tropical Crop Improvement, Catholic University of Leuven, Belgium. Some examples of the tree species cryopreserved by vitrification methods are given in Table 3. Most of the species are of interest to commercial forestry or are valuable fruit trees. Vitrification of the intracellular constituents during cooling can be achieved by partial drying of the sample in air; for example, the embryos or embryonic axes of five species of citrus cryopreserved after desiccation to c. 12% MC (Malik et al., 2012). In this example, longevity of the partially-dried embryos at –20 °C was limited to a few months only, whilst the cryopreserved samples were reported to retain high levels of viability after 6–8 years.

There remain species-specific and specimen-specific subtleties in successful embryo cryopreservation, as a result of differing physiological states, intraspecific genetic variation, morphological variation from the shoot to root poles, and large differences in chemical composition and visco-elastic properties. Necessary adjustment to the methods relate to the pre-culture phase, exposure to loading solution prior to PVS treatment, and the unloading phase. Consequently, there has been a determination to devise and apply generic methods. Two such methods are cathodic amelioration and vacuum-infiltration vitrification (VIV) cryopreservation.

**Table 3**

Some tree and shrub species cryopreserved by various vitrification methods. Information is summarised from Sakai and Engelmann (2007), unless otherwise stated.

Species	Tissue	Method
<i>Actinidia chinensis</i>	Shoot tip	Vitrification
<i>Actinidia deliciosa</i>	Shoot tip	Vitrification
<i>Aesculus hippocastanum</i>	Embryogenic culture	Vitrification
<i>Artocarpus heterophyllus</i>	Embryonic axis	Vitrification
<i>Camellia sinensis</i>	Shoot tip	Vitrification
<i>Castanea sativa</i>	Somatic embryo; shoot tip	Vitrification
<i>Citrus aurantium</i>	Shoot tip	Vitrification; encapsulation-vitrification
<i>Citrus junios</i> × <i>C. grandis</i>	Somatic embryo	Vitrification
<i>Citrus madurensis</i>	Embryonic axis	Vitrification
<i>Citrus sinensis</i>	Nucellar cells; callus	Vitrification
<i>Diospyros kaki</i>	Shoot tip	Vitrification; encapsulation-vitrification
<i>Diospyros lotus</i>	Shoot tip	Vitrification
<i>Eriobotrya japonica</i>	Shoot tip	Vitrification
<i>Malus domestica</i>	Shoot tip	Encapsulation-vitrification
<i>Olea europea</i>	Somatic embryo	Encapsulation-vitrification
<i>Parkia speciosa</i>	Shoot tip	Encapsulation-vitrification <sup>a</sup>
<i>Picea mariana</i>	Embryogenic culture	Vitrification
<i>Poncirus trifoliata</i> × <i>Citrus sinensis</i>	Shoot tip	Vitrification; encapsulation-vitrification
<i>Populus alba</i>	Shoot tip	Vitrification
<i>Populus canescens</i>	Shoot tip	Vitrification
<i>Populus nigra</i>	Shoot tip	Vitrification
<i>Populus tremula</i> × <i>P. tremuloides</i>	Shoot tip	Vitrification
<i>Prunus</i> (4 species)	Shoot tip	Vitrification
<i>Prunus domestica</i>	Shoot tip	Encapsulation-vitrification
<i>Pyrus communis</i>	Shoot tip	Vitrification
<i>Quercus robur</i>	Embryogenic culture	Vitrification
<i>Quercus suber</i>	Somatic embryo	Vitrification
<i>Robinia pseudoacacia</i>	Shoot tip	Vitrification

<sup>a</sup> Nadarajan et al. (2008).

Innovations around *in vitro* storage technology are covered elsewhere in the literature, (FAO, 2013).

Cathodic amelioration aims to counteract the reactive oxygen species produced during cryopreservative procedures; for example, both excision and dehydration of recalcitrant seed axes of *Castanea sativa* (sweet chestnut) are known to trigger the production of superoxide radicals (Roach et al., 2008). Improved cryopreservation success (promoting shoot development) has been achieved through the immersion of axes of *Strychnos gerrardii* (coast monkey-orange) in cathodic water after cryopreservation (Berjak et al., 2011). The strongly reducing, high pH cathodic water is produced by electrolysis of a solution containing calcium and magnesium chloride.

Vacuum infiltration vitrification (VIV) cryopreservation seeks to increase the uniformity of PVS penetration into plant embryos that vary in permeability due to differences in tissue mass, morphology, hydrophobicity and visco-elasticity (Nadarajan and Pritchard, 2014). Such variability in tissue properties has previously demanded empirical determinations of PVS exposure times, to balance the benefits of tissue dehydration whilst avoiding excessive chemical toxicity. By applying a gentle vacuum during PVS2 treatment at 0 °C and 25 °C it has been possible to reduce optimal exposure times by about 10-fold compared with conventional vitrification procedures and improve survival. The method has been applied to the embryos or axes of three oilseed species (from the Mediterranean to the tropics) with lipid melting properties that span –45 °C to 35 °C (Nadarajan and Pritchard, 2014).

## 5. Integrated conservation

As eluded to above, further integration of *ex situ* and *in situ* conservation strategies is an increasing necessity (Volis and Blecher, 2010), as is consideration of *circa situ* conservation approaches, e.g. in smallholders' agroforests (Dawson et al., 2013). The trees planted in such agroforests can act as reservoirs of biodiversity,

provide alternative sources of product to wild harvesting and may stimulate species inclusion in seed collections or field gene banks.

Tropical forests are typically diverse and comprise few abundant species and a large number of rare species which may be represented by less than one individual per hectare, as a consequence of differential survival of seedlings based on density-dependent (e.g. competition, vulnerability to herbivores) and other effects (Chave et al., 2006). Consequently, seed supply for *ex situ* programmes may be limited. Moreover, many of the species of interest produce difficult to handle, highly recalcitrant seeds. Developing the horticultural skills to handle seedlings can, therefore, provide additional opportunities to support conservation. One example is the use of seeds to produce seedlings to be planted out as framework species for the restoration of forests. The primary purpose of this group of species is rapid growth and accelerated regeneration at that site through the dispersal process, thus enhancing habitat heterogeneity (Tucker and Murphy, 1997). Out of 37 species native to northern Thailand trialled, nine were ranked as excellent framework species to accelerate the natural regeneration of the forest ecosystem and encourage biodiversity recovery on degraded sites (Elliot et al., 2003). Another example that demonstrates the scale of the horticultural task is that regulations governing re-afforestation programmes in degraded areas in South Brazil prescribe the use of a minimum of 80 species, distributed across pioneers and non-pioneers, and with these accounting for at least 40% of the total number of species; also any of the planted species cannot exceed 20% of this total (Camargo, J.L.C., pers comm.).

*In situ* conservation of seedling banks could be a means of maintaining a large number of young plants in a convenient reduced space. However, longer-term maintenance would be dependent upon restricting light levels to just above the compensation point, or limiting nutrition, to reduce plant growth, without stressing the plants. In the understorey, seedling banks of particular species can be usually observed soon after seed dispersal, although individuals in these populations are likely to experience depletion at different

rates. Few seedlings can withstand a long time in the shady understorey and advance to saplings, while seedling herbivory is a constant problem (Benitez-Malvido et al., 2005). Thus, whilst seedling bank management has appeal for the maintenance of recalcitrant species diversity, this innovative practice is quite uncommon in tropical forests and should be tested on some target species before any technical recommendation can be given.

In the absence of seed availability for planting purposes, collecting wildlings may sometimes be the most effective way to source germplasm for conservation purposes, as wildlings are available for a longer time than viable seeds can be collected. However, knowledge pertaining to seedling morphology is necessary as there can be significant differences between juvenile and adult growth phases. Enrichment planting of seedlings of species exhibiting poor seed storage could contribute to an integrated conservation approach, and enrichment planting in secondary growth forest may be an option; however, there are few long-term studies known. Finally, conservation stands near natural populations of species may be beset by problems of ‘monoculture’, unless the species naturally occurs in clusters of individuals, e.g. the Brazil nut, trees of which have survived 30 years after planting on c. 3500 hectares (Camargo, J.L.C., pers comm.).

Plantations of endangered species can be encouraged, e.g., essential oil producing trees of the Lauraceae family (Ferraz et al., 2009). A combination of CITES listing, prohibition of logging and the high price of the essential oil (linalol) distilled from its branches and leaves (about US\$180/kg) has generated interest in establishing new plantations of the *Aniba rosaeodora* (rosewood tree) in Amazonas State and raised the seedling prices (about 5 US\$/plant). However, there is, so far, no long-term experience of planting in close stands.

### 5.1. ‘Exceptional’ plant species, with extremely small populations

When the objective is to protect the world’s most threatened plant species that occur in small populations and produce few (or no) seeds, then approaches combining the conservation strategies outlined above are required. Signatory countries to the CBD are committed to developing and implementing biodiversity action plans for such nationally-threatened species. In 1994 the UK Government produced its first Biodiversity Action Plan (UK BAP) in response to the CBD, including detailed plans to aid recovery of the most threatened species and habitats. This work is overseen by the Joint Nature Conservation Committee (JNCC, 2014) which has collated information for all 1150 species included on the UK BAP priority species list (updated in 2007). Some of these protected species are in the genera *Pyrus*, *Salix* and *Sorbus*. In China, there is an acceleration of conservation work on ‘plant species with extremely small populations (PSESP)’ (Ma et al., 2013). The concept was first promulgated in Yunnan Province, SW China, and is now providing a focus for several national and regional-level conservation strategies. PSESP are recognised on the basis of low numbers of individuals due to serious human disturbance in recent times, a restricted habitat and an extremely high risk of extinction. The national list includes 120 species, of which about 80 are long-lived perennials (trees and cycads). For the top 20 PSESP for conservation priorities in Yunnan Province, 17 are long-lived perennials (15 tree species in 11 families and 2 species of cycad). One of the species, *Acer yangbiense* (yangbi maple), with only five individuals recorded, has been hand-pollinated and the resulting seeds used to produce more than 1000 saplings now growing in the Kunming Botanical Garden. Following the example of Malaysia in establishing *in situ* protection areas for *Rafflesia keithii* (monster flower), an *in situ* demonstration base for PSESP reintroductions has been created in SE Yunnan. As noted above (3.3 Botanical Information), very little information is available on the seed biology (and morphology) of these species;

although germination studies have started on *Manglietiastrum sinicum* (huagaimu; Zheng and Sun, 2009).

### Recommendations for future actions

One outcome of attempting to answer the questions raised at the start of this article, is that the following needs for scientific endeavour and policy intervention have become evident:

- (1) National programmes should continue to support the conservation work of botanic gardens and other institutes engaged in the introduction to living collections of forest species, particularly threatened trees; back-up conservation in seed banks should be accelerated and consideration given to a repository for international tree seed collections.
- (2) The innovative approach of generating viability constants for tree species needs to be properly assessed in a broader range of species, as numbers tested so far is restricted, so that predictions of seed storage for conservation and forestry purposes can be more comprehensive.
- (3) Screening a wider selection of the world’s forest species for seed storage behaviour and the further development of predictive models requires urgent acceleration, such as the incorporation of further morphological and biophysical characteristics (e.g. lipid content); to complement existing data for the Amazon and the Queensland forests, assessments should extend to species of West African, Indian and SE Asian forests, so that a better overall estimate of the likely frequency of seed desiccation tolerance can be made.
- (4) To better understand the underlying mechanisms of desiccation tolerance and intolerance, the physiological responses described require research at the metabolomic and transcriptomic levels. The possible relationship of different responses to phylogeny may further assist in developing taxa-specific strategies for *ex situ* and *in situ* conservation.
- (5) An online information facility dedicated to tree seed biology should be established to collate existing knowledge currently held in different databases and to assimilate new information as it is generated, so as to enable an acceleration of essential work by the scientific, forestry and NGO communities.
- (6) Cryopreservation research on plant species from the world’s biodiversity hotspots in the moist tropics should be accelerated, as these habitats contain the vast majority of recalcitrant species, many of which are threatened with extinction through habitat degradation.
- (7) It is crucial that baseline knowledge of rare species’ phenology, autecology, seedling bank management and fruit, seed (germination and storage), seedlings (and pollen behaviour) is improved; and supported by critical skills training in tropical plant and seed ecology and information technology, for undergraduates and post-graduates and through short-course provision, particularly in the tropics.

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