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New technologies to improve the ex situ conservation of plant genetic resources

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1 Introduction

Plant genetic resources, which include traditional and modern varieties, crop wild relatives, genetic stocks, breeding lines and weedy species, form the genetic basis for the improvement and selection of crops through breeding (IPGRI, 1994). While the ex situ conservation of crop genetic resources will contribute towards addressing the challenge of food insecurity, as set out in the United Nations Sustainable Development Goals (www.sustainable development.un.org/), the ex situ conservation of wild species can facilitate strategies (e.g. habitat restoration and species reintroduction) that improve ecosystem resilience under various climate change scenarios (Frankel, 1990; McFerson, 1998; Abbas and Qaiser, 2011). Biotechnology, that is, the use of living organisms or parts thereof to manufacture or modify a product, develop microorganisms for specific uses, or improve plants or animals (Uyoh et al., 2003), has also made plant genetic resource conservation an industry priority in many developed parts of the world.

In this regard, seed storage is the most efficient and cost-effective means of ex situ plant germplasm conservation, and has been used to conserve a sizeable amount of plant biodiversity worldwide for decades (IBPGR, 1976; Engels and Engelmann, 1998). The science of seed genebanking – of storing orthodox seeds under dry, cold conditions (orthodox seeds being those that tolerate such conditions) – is still relatively basic and, given the lengths of time that the seeds

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are expected to remain alive compared with the age of many well-documented collections, could still be considered somewhat experimental. Nonetheless, over recent years, we have gained greater insight into the effectiveness of the basic processes that most genebanks follow in managing their collections of orthodox seeds and, since budgets are often limited, advanced technologies are being introduced, with the tag line of improving efficiency.

However, seed storage cannot be used to conserve genetic variation of all species, due to fundamental differences in innate reproductive characteristics (specifically the ability of seeds to survive long periods of dry storage) or the need to fix and multiply favourable genetic combinations. In such cases, seed-derived embryonic tissues, somatic embryos or vegetative tissues (e.g. shoot tips) are the materials of choice for storage. Collections housed in both the developed and developing world (Thailand, Australia, United States of America, Italy, Belgium, India and China, among others) include germplasm of vegetatively propagated fruit, commercial and speciality crops, ornamentals, nuts, vegetables and wild relatives, from temperate, subtropical and tropical zones (Jenderek and Reed, 2017). Where these are clonal collections the plant material is maintained in an actively growing state using a variety of techniques for one or more of the following purposes: (1) to preserve the selected genotypes; (2) to maintain their sterile nature; (3) to produce seed unamenable to storage. While the development of cryopreservation techniques for clonal crops began as far back as the 1970s, involving controlled-rate cooling and subsequent plunging in liquid nitrogen (LN) of shoot tips (Grout et al., 1978) and buds (Sakai and Nishiyama, 1978), the last two decades has seen the development of a range of cryopreservation techniques that have been applied across numerous genera and species (Benson, 1999; Reed, 2008; González-Arnao et al., 2014), including both orthodox-seeded (desiccation tolerant) and recalcitrant-seeded (desiccation sensitive) species (Berjak et al., 2011a; Jenderek and Reed, 2017).

In this chapter, we describe some of the recent applications of science and technology to improve the management of genebank collections of orthodox seeds, not least through the introduction of automation. In addition, we look at how routine cryopreservation procedures are now possible for many species, and how storage procedures for recalcitrant-seeded and vegetatively propagated species continue to evolve, in light of the challenges they present in terms of long-term ex situ germplasm conservation.

2 Improving the management of orthodox seeds

2.1 Routine operations

Differences in seed post-harvest physiology are reflected in the categorisation of seeds as orthodox, recalcitrant (Roberts, 1973) and intermediate (Ellis et al.,

1990), based on their responses to loss of moisture and to storage at different temperatures. Orthodox seeds can tolerate drying without damage to low-moisture contents (2–6%; Roberts, 1973; Chin and Roberts, 1980; Hong and Ellis, 1996) and their longevity, spanning perhaps many decades, is increased as the moisture content and temperature at which they are stored (within limits) are reduced (Ellis and Roberts, 1981). The core operations of a genebank with the mandate to conserve seeds of orthodox species comprise seed production, drying, cleaning, viability and health testing, packing, storage and distribution; all of these activities are documented and driven by the genebank information system (Fig. 1).

Most genebanks store seeds of every accession in both an active collection and a base collection. The active collection is generally stored at 2–4°C (medium-term storage; MTS) and it is from this collection that samples are taken for distribution. This is therefore where the bulk of the seeds are stored. The base collection is stored at a lower temperature, typically −20°C (long-term storage; LTS). Samples in the base collection are usually much smaller than the samples in the active collection, and the purpose is long-term conservation. The point of having all the accessions in two environments is to reduce costs. In theory, the cost of storing a sufficient quantity of seeds for distribution at the lower temperature is too high. This is particularly true for agricultural genebanks with high rates of distribution. In contrast, at, for example, the Millennium Seed Bank (MSB) of the Royal Botanic Gardens Kew, whose main purpose is long-term conservation of species and which has relatively few sample requests, all seeds are under LTS conditions. Other genebanks may also maintain only one storage environment, based on operational needs and costs. The United States Department of Agriculture has a network of genebanks holding the active collections for different crops, with one main base collection serving all the regional genebanks (National Research Council, 1991). Some genebanks additionally cryopreserve samples of orthodox seeds, for example, of orthodox seeds which are expected to be very short-lived, even under 'conventional' genebank storage conditions of −20°C (Davies et al., 2018; Ballesteros et al., 2021).

Testing the viability of seeds before they are placed into storage, and at regular intervals during storage, is a key 'quality control' activity that genebanks are expected to do as a matter of routine (FAO, 2014). For genebanks with accessions in both an active and a base collection, if the sample in both comes from the same seed lot, it is not necessary to check the viability of the seeds in both collections. Since the longevity of orthodox seeds improves in a predictable manner as the temperature is reduced, the seeds in the base collection should always have higher viability than the seeds from the same seed lot stored in the active collection after the same period of time. In other words, the viability of the seeds in the active collection

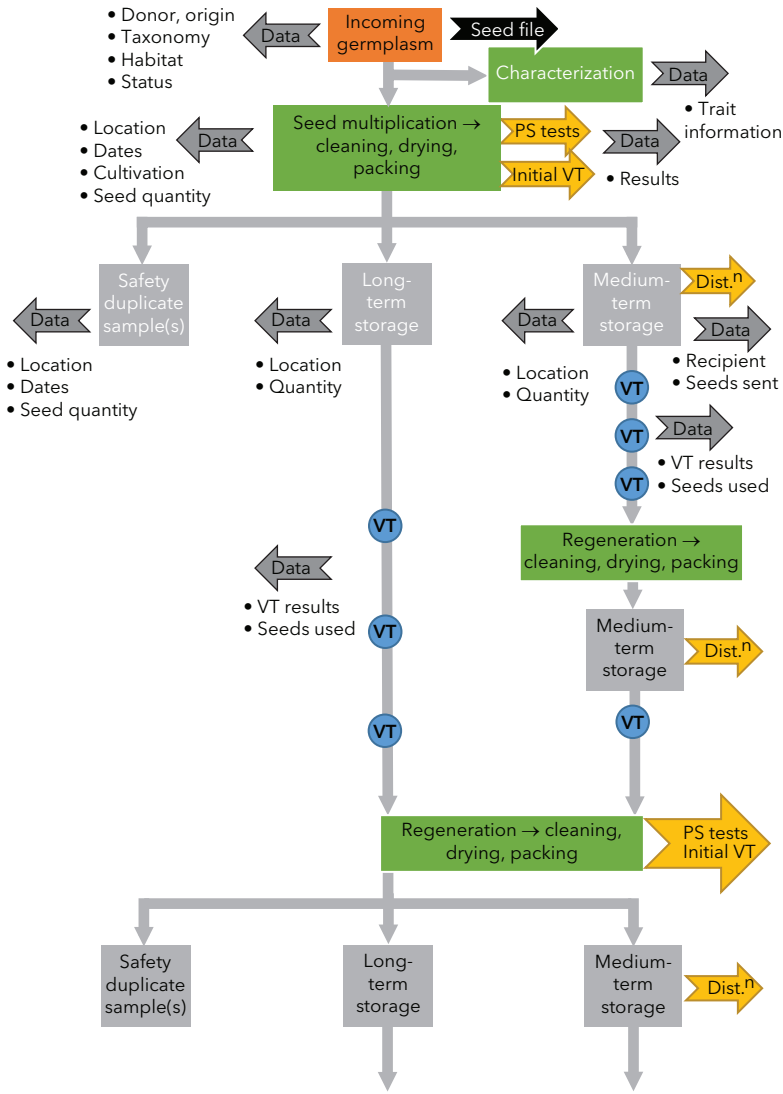

Figure 1 Overview of operations in a typical seed genebank. When a sample first arrives at the genebank, it will be assessed for uniqueness and initially grown under quarantine conditions to ensure there is no disease. If there are enough seeds, a small sample (e.g. 20–30 seeds) of the incoming seeds will also be added to the seed file, for future reference and variety verification. The accession will then be multiplied to produce sufficient seeds for storage. Characterisation of the accession for morpho-agronomic traits according to defined crop descriptors may be carried out on plants used for seed multiplication, or may be an independent operation. Characterisation also includes collection of data on features of the seeds. After harvest, seeds will be cleaned and dried. They may also undergo a pest control treatment. Samples will be taken for phytosanitary (PS) testing and for an initial viability test (VT). After final equilibrium drying, seeds will

Figure 1 (*Continued*)

be packed for storage in the long-term (base) and medium-term (active) collections (if appropriate). Some genebanks may additionally cryopreserve a sample of seeds. Safety duplicate samples will be sent for long-term conservation, without any management interventions, at another genebank and/or in the Svalbard Global Seed Vault. Seeds in the active collection are used for distribution (Dist.[n]). They are also regularly tested for viability (viability test; VT). Once the viability falls below the threshold level or the quantity of seeds remaining gets low, the accession will be regenerated and the seeds produced are processed for storage in the active collection again. A few cycles of regeneration using seeds from the active collection may occur, before seeds from the base collection should be used. After replacing the seeds in the active collection, meaning that the seeds in the two collections are not from the same seed lot, viability testing of seeds in the base collection should commence. When the viability of the seeds in the base collection falls below the threshold level (or, but theoretically unusually, the quantity of seeds remaining gets low), a new sample should be produced for the base collection. A sample of seeds from this seed lot should also be sent as a safety duplicate sample, because it is expected that the samples in the base collection and in long-term storage in another location would lose viability at a similar rate. Seeds from this same seed lot may also be used to replenish the active collection and the 'cycle' begins again. Across all the operations, data is collected and entered into the genebank information system. This data includes information on the processes themselves, such as number of seeds sown, number of plants harvested, viability test date and so on. The data examples shown represent only a fraction of all the data that would, in reality, be recorded.

will fall faster than the seeds stored in the base collection – unless there has been any divergence in the handling that compromises the initial quality or moisture content of the seeds intended for storage in the base collection. It is only necessary to start testing the viability of seeds in the base collection when the seeds in the active collection are replaced by a new seed lot (Whitehouse et al., 2020). Similarly, if seed samples are taken from the same seed lot and prepared in the same way for storage in the base collection and for sending to another genebank as a safety duplicate, it is not necessary to monitor the viability of that safety duplicate sample. Safety duplicate samples are only intended to be returned to the originating genebank, and only if that genebank cannot recover the accessions from their own samples. When the viability of the seeds in the base collection declines, the safety duplicate sample may be replaced Until now, this has been happening at low rates, although it is likely to increase, particularly for species with relatively short-lived seeds, as the viability of more samples in the base collection falls and the samples are replaced. For many genebanks, in addition to having every accession in the two collections, a very small sample of the 'most original seeds' of each accession is also placed in the seed file. The seed file is the reference sample against which comparisons can be made, for example, of regenerated material or of new material that is submitted to the genebank. Ideally, the most original sample would comprise some of the seeds originally

donated or arriving at the genebank, but in some cases it will be seeds from the first round of multiplication.

Given the interdependency of operations, a robust information system is crucial to record all the data collected for each process and to ensure timely and efficient sequencing of operations. Unfortunately, data management may be overlooked as key 'infrastructure', particularly in national genebanks, where resources may be limited. Genebank information systems also store passport data and, increasingly, characterisation and evaluation data, and links to genomic data, where available. More genebanks are genotyping accessions or even, whole collections, and/or are handling genetic stocks such as mapping populations, mutant populations and recombinant inbred lines. Genebank information systems such as GRIN-Global (www.grin-global.org) also allow users to search collections and order accessions online.

2.2 Changing procedures

Recommendations on how to handle seeds in genebanks were first published in 1975 (Cromarty et al., 1990) and, while there has been some evolution, the essence of those recommendations has barely changed. For example, Cromarty et al. (1990) recommended drying seeds in a drying room at 'about 15°C and 10–15% relative humidity with good air circulation'; in the IPGRI/FAO genebank standards published in 1994, the recommendations were to dry at 10–25°C and 10–15% relative humidity (IPGRI/FAO, 1994); and in the latest version of the standards, the recommended drying environment is 5–20°C and 10–25% relative humidity (FAO, 2014). Similarly, storage temperatures and monitoring intervals remain largely unchanged, even after decades of storing seeds in genebanks. Unfortunately, because some of those original recommendations are now relatively old, it may be difficult to know how they came about; old data sets might not be archived and knowledge of the hows and wherefores not passed on to younger genebank managers/scientists. Nonetheless, we should still be considering whether genebank standards are optimal and in particular, given that the standards are intended to cover such a broad range of species, whether they are optimal for particular species.

2.2.1 Seed drying

The conditions under which orthodox seeds are dried are intended to result in seeds being at a moisture content where seed longevity is optimal (regardless of whether the seeds are subsequently stored at 2–4°C or −20°C). Various combinations of temperature and humidity would allow seeds to reach the same moisture content, however, the drying treatment itself should not be

detrimental to the quality of the seeds. In theory, if very wet seeds are placed at a high temperature there could be a high rate of seed ageing. Thus, the genebank standards recommend drying at a relatively cool temperature, in contrast with the higher temperatures used for drying grain. Many genebanks have a drying room that runs at 15°C and 15% relative humidity. In the case of rice, however, it has been found that drying at 15°C is not optimal for the subsequent longevity of the seeds (Crisostomo et al., 2011; Whitehouse et al., 2015, 2018a). If the rice seeds, due to the typically humid environment of rice fields, are not able to dry in situ and have a high moisture content at the time of harvest (> 16.5%), initial drying at 40–45°C followed by final equilibrium drying at 15°C and 15% relative humidity improves the subsequent longevity of the seeds significantly compared with only drying at 15°C and 15% relative humidity (Whitehouse et al., 2018a). This response is perhaps not surprising for rice: in the tropics, ambient temperature is, of course, relatively high, and rice (and other grains) is often dried on roads or flat cement areas, where temperatures in the middle of the day under bright sunlight can get quite high. A similar response was also seen for seeds of four wild rice species (Timple and Hay, 2018). As a consequence of this research, the rice genebank at the International Rice Research Institute now routinely uses two-stage drying for all the collection. Freshly harvested seeds are initially dried for 3 days in a drying room at 40°C and 30% relative humidity, after which, they are transferred for final equilibrium drying in the drying room at 15°C and 15% relative humidity. There is some evidence that drying seeds of some other species at a higher temperature than 15°C may similarly be better for the subsequent longevity of the seeds (Whitehouse et al., 2018b).

2.2.2 Revising monitoring intervals

As indicated above, many genebanks still adopt the default viability testing intervals of 5 or 10 years, depending on the expected longevity in storage (FAO, 2014). Otherwise, '*Viability monitoring test intervals should be set at one-third of the time predicted for viability to fall to 85% of initial viability or lower depending on the species or specific accessions, but no longer than 40 years*' (FAO, 2014). The recommended way to predict the viability period is to use the Ellis and Roberts (1980) viability equation, using parameters determined from experiments in which seeds are stored at a range of temperatures and moisture contents (e.g. Hay et al., 2003). This can be done using the seed viability constants menu in the Seed Information Database (SID; Royal Botanic Gardens Kew, 2020), but is only possible for a small number of species. Furthermore, as discussed by Hay and Whitehouse (2017), the time for viability to fall to 85% of the initial viability is perhaps not an ideal standard, since it depends on an estimate of initial viability, and because it means that the absolute threshold

value could potentially vary among seed lots (i.e. of seed lots of the same or different accessions). For example, if the initial viability ranges between 85% and 100%, the monitoring intervals would be calculated based on the time for viability to fall anywhere between 72% and 85%. Further, it is known, based on the typical shape of the seed survival curve (the curve describing the loss of viability during storage), that once the viability of a seed lot has fallen to 72%, the rate of percentage viability loss is nearing its peak. In practice, most genebanks still use set-standard monitoring intervals, at the species level at least, and have a fixed viability threshold (e.g. 85%), rather than setting seed lot-specific test intervals and thresholds. Nonetheless, in future, there could be a more dynamic approach for setting viability-monitoring intervals (Whitehouse et al., 2020).

A more dynamic, flexible approach will probably only emerge as we understand more about the real performance of seed lots in genebanks. Some genebanks have already analysed their historical viability monitoring data, using a variety of approaches to model the longevity of seed lots, within species or species groups and in some cases, to formulate more efficient monitoring intervals (e.g. Walters et al., 2005; van Treuren et al., 2013; Hay et al., 2015; Ellis et al., 2018, 2019; Yamasaki et al., 2020). In some cases, the conclusions drawn from such studies, while contributing to scientific knowledge, are only really relevant to the genebank where the data was collected, due to crop focus or more likely, regeneration and processing protocols, and storage conditions that are perhaps unique to that genebank, even if they are more-or-less consistent with the Genebank Standards (which cannot be species-prescriptive because they are meant to cover so many species). In future, there may be more meta-analyses, using viability data for the same crop from different genebanks. In particular, this could help verify the applicability of the Ellis-Roberts viability equations, particularly at low temperatures for which data is still lacking (Pritchard and Dickie, 2003).

There have also been some advances in terms of understanding the molecular basis of seed longevity for genebank accessions, which raises the possibility of using genotype data to predict the relative longevity of seeds in genebank storage and hence set appropriate monitoring intervals (Lee et al., 2019). Screening seed lots for longevity under experimental conditions to get an initial measure of relative longevity could also help identify which seed lots to test first (Davies et al., 2016; Hay and Whitehouse, 2017), perhaps in particular if used in conjunction with genotype data. Taking this idea further, it has been suggested that a more efficient strategy for genebanks would be to preclude the need to do viability monitoring tests, by taking other measurements (ideally simple, cheap and non-destructive tests) that are predictive of the extent to which a seed lot has aged (Fu et al., 2015). However, this still seems to be somewhat futuristic in practice, and may only ever be feasible for large agricultural genebanks with many accessions of a single or few crops.

Figure 2 Barcodes are used to track samples through genebank operations: seed drying (a) and transplanting (b) at the International Center for Tropical Agriculture (CIAT) in Colombia; and germination testing and scoring (c, d) at the International Institute of Tropical Agriculture (IITA) in Nigeria. All photos taken by the first author (Fiona R. Hay).

2.2.3 Electronic data collection

The use of barcodes to label the packets of seeds going into genebank storage has been a common practice for some years now (Rao et al., 2006). Indeed, barcodes are now used to track samples through many, if not all, genebank operations, from the field to storage, including during characterisation/evaluation activities and distribution (Fig. 2). This has also made it possible to collect data electronically, even in the field: the barcode can be scanned and then the relevant data entered. To further minimise the risk of wrong data entry, genebank staff may use a portable tablet or similar device with software that is customised to show, for example, value options with pictures for a particular characterisation trait. In theory, not only is this more accurate, but also improves efficiency since staff do not have to manually enter data from paper score sheets after data collection.

2.2.4 Improving viability testing

Modifying and improving germination procedures used in viability monitoring should be a constant process in genebanks, particularly those conserving wild species diversity. Such species, compared with major crop species, are likely to have some sort of dormancy mechanism which prevents germination when moisture, temperature and light requirements are met. It is also more likely that dormancy-breaking procedures have not been documented and/or widely

validated for such species. Despite the need for reliability in viability testing and to provide germplasm users with seed germination advice, research in this area has been under-resourced, particularly at crop genebanks where wild species have historically perhaps received less focus in the context of managing germplasm. Seed banks entirely focussed on conserving wild species, in contrast, have invested more resources in determining optimum germination protocols, as documented, for example, in the Seed Information Database (Royal Botanic Gardens Kew, 2020). There are other comprehensive resources that would be helpful to any genebank needing to improve their germination protocols (e.g. Baskin and Baskin, 2014; ISTA, 2020).

The preferred germination-test sample size in official testing is 400 seeds, sown as four replicates of 100 (ISTA, 2020). This is far too many seeds than most genebanks would like to use in a viability monitoring test, though the actual number tested varies among genebanks depending on species, quantity of seeds available, facilities and other logistical constraints. As also discussed elsewhere (Hay and Whitehouse, 2017), there are other ways in which the number of seeds used overall for viability monitoring might be reduced, not just by having more knowledge of longevity in storage, but also, for example, by following a sequential monitoring scheme (Ellis et al., 1985). A recent study across three international genebanks, involving 111 accessions from 11 species, confirmed that sequential sampling is an efficient alternative to the fixed size sampling method for making decisions based on viability for tropical forage species, although for three *Leucaena* species, there was no advantage in using sequential sampling to save seeds in viability monitoring (A. Sartie, L. Santos and Z. Kinyanjui, *pers. comm*.).

While 'viability' is most often assessed using a germination test, there has long been an interest in developing a non-destructive test to predict the viability of a seed lot. Research in this area has considered, for example, the nature and quantity of volatiles released by seeds during storage (Colville et al., 2012) and molecular markers (Boniecka et al., 2019; Fleming et al., 2019). However, while these studies certainly cast light on the process of seed deterioration in storage, there is still a lot of research needed before we get close to a practical non-destructive predictive test of seed viability during genebank storage (reviewed by Fu et al., 2015).

2.3 Automating processes

Some genebanks, notably those of the international genebanks of the CGIAR and a few national genebanks, have very large collections. For example, the genebank at the International Rice Research Institute (IRRI) in the Philippines is the largest single-crop collection with more than 132 000 accessions (https://www.genesys-pgr.org/). Staff at such genebanks may work as if they were

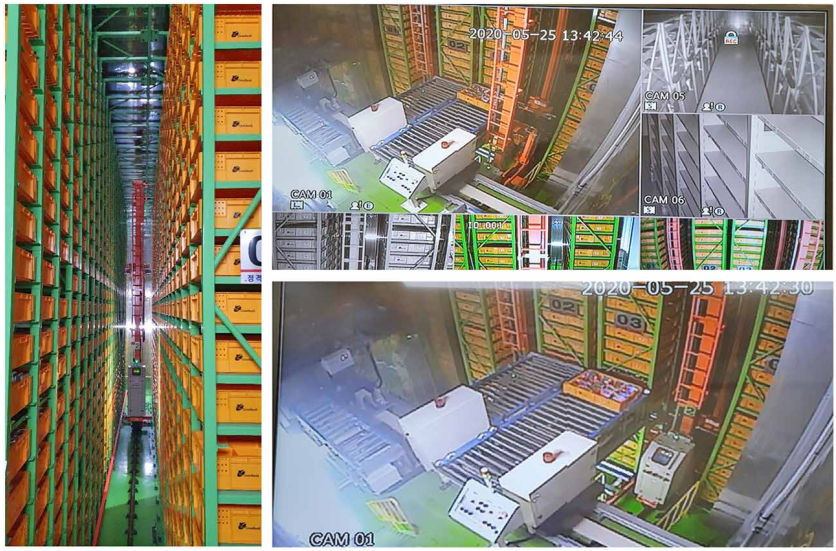

Figure 3 Images of the robotic storage and retrieval system in the long-term storage room of the National Agrobiodiversity Center, Rural Development Administration, Republic of Korea. Images provided by the National Agrobiodiversity Center.

working in a factory line, with groups of staff focussing on one particular activity at any one time and often for quite long periods of time. In such genebanks, that process thousands of seed samples of one or just a few crops each year, automating some of those processes is more likely to be feasible, compared with a genebank that is perhaps processing one species for one week, and a completely unrelated species, the next. The genebank storage rooms can thence be considered the warehouses for the factory and also be managed in a similar way.

2.3.1 Robotic storage and retrieval

A few genebanks around the world, for example, the Genetic Resources Center of Japan's National Agriculture and Food Research Organization and the National Agrobiodiversity Center in the Republic of Korea (Fig. 3), have incorporated warehouse management-type technology to manage their collections. All the placing and retrieval of samples is handled by a robot, so staff rarely enter the stores themselves. There are a number of reasons why this could be considered advantageous. The long-term storage environment, at −20°C, is not a pleasant working environment for staff and could even present a health hazard to some people, even if they wear insulated clothing. Therefore, restricting access and limiting the length of time anyone is in that room, might reduce any risk associated with working at such a low temperature. On the other hand, it should

not be necessary to enter this storage room very often, as its intended use is long-term conservation. The active collection is stored at a higher temperature (2–4°C), which is a more tolerable temperature, nonetheless, a robotic system will increase security and ensure correct placement of samples. It may also mean that there is less exchange of air so it is easier to maintain the correct temperature and energy is not being wasted. This is perhaps particularly relevant in hot and humid environments. Of course, genebanks that do not install a robot system, will still make sure that the location of accessions in both stores (e.g. shelf number, tray number) is recorded in the genebank information system.

2.3.2 Seed phenotyping

Analysis of images captured by different types of cameras has many potential applications in seed testing (Dell'Aquila, 2009; Boelt et al., 2018). One way in

Figure 4 (a) Samples from the genebank's physical seed file at the International Rice Research Institute (IRRI); (b) selecting the training-set seeds from the bulk prior to automated sorting (image used with permission of N. Ruaraidh Sackville Hamilton); (c) the custom seed sorter at IRRI. (a) and (c) were taken by the first author (Fiona R. Hay).

which image analysis could be applied to improve genebank operations is by using it as a virtual seed file (Edberg Hansen et al., 2016). Most agricultural genebanks keep a small sample of seeds of perhaps the material originally received at the genebank, or otherwise, of the first cycle of seed multiplication, for use as a reference against which new harvests can be compared. This is called the seed file (Fig. 4A). This seed file does not have a physical backup, and if any seed file samples are lost, they would have to be replaced by the next available 'most original sample' (i.e. from the first regeneration cycle, if available, rather than the second or third). The seed file is not just used to verify that harvested seeds appear to be of the same accession, but they are also used, for example, to check incoming material to see whether the sample is a potential varietal mixture with different seed phenotypes and/or to determine whether it might be a variety that is already represented in the genebank. However, use of the seed file relies on having trained personnel who are able to detect subtle differences between seeds. Some genebanks already capture images of the seeds of different accessions (as well as other parts of the plants), to use internally and/or to make available to germplasm browsers on their online ordering portal, and there is a lot of potential to analyse such images and make the validation/checking process more objective. Edberg Hansen et al. (2016) proposed the use of multispectral imaging since it offers the possibility of identifying and measuring some of the more subtle characteristics of seeds, which might not be apparent in images from an ordinary digital RGB camera.

2.3.3 Seed sorting

Most seed lots intended for genebank storage will go through a cleaning process, though the extent and method of cleaning may vary. There are various types of seed cleaning equipment developed for the seed industry, including seed blowers, sieves, brushes and colour sorters, which can be used to remove non-seed material such as plant stems or dispersal structures. The choice of method will very much depend on the seeds being cleaned and the quantity of seeds to be cleaned. For very small seed lots, the amount of time it takes to clean the equipment between seed lots may mean that it is not practical to use such machines. Some genebanks do a considerable amount of hand sorting. For many years, all the seeds intended for storage in the rice genebank at the International Rice Research Institute (IRRI) were entirely sorted by hand, removing off-types, immature (green), damaged or diseased seeds. This used to be a year-round activity and as a consequence, for some seed lots, the length of time between harvest and final storage was many months. Although the seeds were kept in the drying room until they could be cleaned, this could nonetheless impact the initial viability and storage potential of the seeds when put into the genebank stores. The IRRI genebank now has a unique,

purpose-built seed sorter that selects seeds by individual-seed image analysis, making sure the colour and shape parameters of each seed matches that of a training set (Fig. 4B, C). This automated seed sorting is considered a first-step in the cleaning process, and the 'selected' seeds are still manually sorted, but introducing this automation has increased the throughput of the seed cleaning process.

2.3.4 Germination scoring

Automated scoring of the germination process has attracted quite a lot of interest in the seed testing community over the last decade or so, to increase the number of samples that can be tested for germination in a short period of time and/or to get more information on the rate of germination as a measure of seed lot vigour (Joosen et al., 2010; Harper and Long, 2011; Demilly et al., 2015). Some genebanks test the germination of thousands of samples every year, as part of the routine viability monitoring. For genebanks handling seeds of many different species, germination protocols (pretreatment, germination method and germination temperature) are likely to vary from one species to the next, and there is little opportunity to automate the scoring process. However, for large crop genebanks, testing one or a few species following the same protocols for each accession within a crop, automated scoring of germination may be feasible. Various systems are available, from fully automated robotic systems in a controlled-environment room with the capacity for hundreds of tests at any one time, to semi-automated systems in which tests are manually moved from the germination environment to the camera. The software for these systems use image analysis to determine how many seeds have germinated at each observation time and, once the test is finished, calculates various parameters, for example, final germination percentage and mean germination time. From the genebank perspective, the parameter of most interest is the final germination percentage at the end of the germination test, but other parameters may provide insight into the seed-ageing process (since vigour is expected to decline before there is loss of viability) and/or may be of interest to breeders who are trying to enhance the seed vigour of varieties, so that they can, for example, still give good crop establishment in marginal environments.

3 Improving the management of recalcitrant-seeded and vegetatively propagated species

3.1 Routine operations: recalcitrant seeds and vegetatively propagated species

Recalcitrant seeds are sensitive to desiccation (Roberts, 1973), freezing (reviewed by Walters et al., 2008) and very often chilling, in some cases at temperatures

as high as 15°C (Berjak et al., 1995). This precludes their maintenance under conventional orthodox seed storage conditions (e.g. King and Roberts, 1980; Farrant et al., 1989). Short- to medium-term storage methods and long-term storage via cryopreservation of seed-derived explants represent some of the storage options employed for such species over the last few decades. Similarly, alternative germplasm conservation strategies, such as storage of actively growing cultures, minimal-growth storage and cryopreservation, have been adopted for species that do not produce seeds (e.g. banana), root and tuber vegetables, clonally propagated crops, and where the unique true-to-type genomic constitution of a cultivar needs to be maintained.

For recalcitrant seeds, short- to medium-term storage has involved maintenance at water contents as close to that at shedding, and at ambient or slightly lower temperatures, and this is defined as 'hydrated-storage' (e.g. Berjak et al., 1989). However, under these conditions, recalcitrant seeds still initiate germination-associated events, culminating in seed death (Berjak et al., 1989; Pammenter et al., 1994; Chandra et al., 2019). Storage at temperatures lower than ambient has been shown to postpone the onset of germination in recalcitrant seeds by slowing down the metabolic rate (e.g. Pritchard et al., 1995), provided the seeds are not chilling-sensitive. However, even then, storage longevity generally ranges from a few weeks to months e.g. for *Scadoxus membranaceus* and *Landolphia kirkii* (Farrant et al., 1989), various amaryllid species (Sershen et al., 2008) and *Madhuca latifolia* (Chandra et al., 2019). The lifespan of recalcitrant seeds in hydrated storage is often further curtailed by the proliferation of a spectrum of fungi (Mycock and Berjak, 1990; Sutherland et al., 2002), even when seeds are treated with fungicidal agents (Mycock and Berjak, 1995; Moothoo-Padayachie et al., 2018).

In vitro storage, that is, the use of tissue culture, by which cells, tissues or organs are excised from parent plants, decontaminated and then transferred to artificial growth media in vitro (Krøgstrup et al., 1992; George, 1993; Mandal et al., 2000), represents the major short- to medium-term germplasm storage option for vegetatively propagated species. A wide range of explants have been used (e.g. buds, cuttings, seeds, shoot apices and leaves) based on the fact that media can be manipulated to produce different cultures such as unorganised, undifferentiated callus or organised tissues and organs that can be converted into plantlets in the regeneration phase. Variations to impose minimal growth include reduction in nutrient (e.g. Schnapp and Preece, 1986) and/or sucrose concentration (e.g. Kartha and Engelmann, 1994) in the growth medium, alterations to culture medium osmotic potential (using osmotica such as sucrose, sorbitol, ribose and mannitol (e.g. Zandvoort et al., 1994)), the addition of growth retardants such as abscisic acid (ABA) (e.g. Jarret and Gawel, 1991; Taylor et al., 1996), and placing explants in a controlled atmosphere or beneath a liquid medium or mineral oil (Paunescu, 2009). Lowering the partial pressure

of oxygen with temperature (between 0°C and 10°C (e.g. Blakesley et al., 1996)) or light (e.g. Grout, 1995) below optimum in the culture environment can also limit in vitro growth. However, irrespective of whether germplasm is stored in vitro as actively growing cultures (e.g. Krøgstrup et al., 1992) or as minimal-growth cultures (e.g. Schnapp and Preece, 1986), such storage will at some stage require transfer of material onto new media (Krøgstrup et al., 1992; Mandal et al., 2000; Mycock et al., 2004), introducing the risk of contamination. Surviving, uncontaminated material can potentially be rapidly micropropagated to bulk up reserves (Mandal et al., 2000) but such clonal propagation, apart from being labour intensive and expensive, limits biological diversity within the collection and can impose selection pressures and environmental stresses, resulting in plants with genetic modifications (Staritsky, 1997). This variation arises from somaclonal variation during culture (Panis and Lambardi, 2006), but the use of organised systems such as embryos, meristems and shoot tips can reduce this risk (Engelmann, 1997; Mandal et al., 2000).

Long-term germplasm storage of recalcitrant-seeded and vegetatively propagated species is achieved via cryopreservation which involves the cooling of biological material to, and subsequent storage at, cryogenic temperatures, typically in liquid nitrogen (LN; −196°C), or its vapour (at approximately -150°C). Less ideally, material can be stored at some other temperature below −80°C (Finkel and Ulrich, 1983; Berjak et al., 1999a). Cryopreservation is regarded as the ultimate long-term storage approach since it is believed to arrest metabolic activity and deterioration, thus minimising, if not precluding, genetic changes (Krøgstrup et al., 1992; reviewed by Harding, 2004). With the exception of mature orthodox seeds (Pritchard and Nadarajan, 2008) and certain varieties of pollen (Ganeshan et al., 2008) and spores (Ingram and Bartels, 1996), biological tissues almost always contain considerable cellular water. This implies that successful cryopreservation of hydrated explants from recalcitrant seeds and vegetatively propagated species is best achieved when lethal intracellular ice-crystal formation is mitigated, as this can cause irreversible intracellular damage (Wesley-Smith et al., 1992). While cryopreservation is generally considered in terms of water's liquid and solid (ice) phases, it is also possible to cryopreserve plant material by inducing the process of vitrification, that is, the transition from liquid to glass phase without ice-crystal formation (Sakai, 2004). The phenomenon of vitrification has allowed for the development of 'ice-free' cryopreservation, which has been extensively applied to plant tissues (Sakai et al., 1992) of a number of vegetatively propagated species. However, freezing of plant tissues that inherently have high water content will inevitably involve the conversion of at least some of the water to ice and this is what precludes this approach in recalcitrant seed-derived explants of a number of species (Wesley-Smith et al., 1992).

Even though cooling and dehydration are the greatest sources of failure, and under other circumstances, the greatest contributors to post-cryo preservation survival, the success or failure of any plant cryopreservation protocol is a consequence of optimisation of all the manipulations involved in the preparation of the tissue for cooling, and all the steps involved in the recovery of that tissue after cooling (Berjak et al., 1999b; Pammenter and Berjak, 2014). These manipulations generally include variable combinations of the following (Funnekotter et al., 2017; Kaczmarczyk et al., 2012):

1 excision of explants such as seed embryos/embryonic axes or shoot tips to obtain sufficiently small samples;
2 partial physical desiccation or pre-growth on osmotic media to reduce water content;
3 cryoprotection through exposure to penetrating or non-penetrating cryoprotective agents to promote vitrification;
4 cryopreservation in LN;
5 re-warming which can be accompanied/followed by unloading of the cryoprotectant(s); and
6 washing (unloading of CPA solutions), recovery and regeneration of germplasm into plantlets (usually in vitro).

Samples that have been used for cryopreservation over the last three decades or so include buds, shoots, meristems, cell cultures, protoplast cultures, anthers, pollen, somatic and zygotic embryos, embryonic axes, callus, and whole seeds, if they are sufficiently small (Benson, 2008a). Success has been achieved in cryopreserving whole seeds (e.g. Hor et al., 1990; Kioko et al., 2003), shoot tips (Varghese et al., 2009), embryonic axes and zygotic embryos (Berjak et al., 2011b; Sershen et al., 2012) and somatic embryos of recalcitrant-seeded species (e.g. Mycock and Berjak, 1993). However, the number of successful protocols developed for embryonic axes and zygotic embryos from recalcitrant seeds is extremely low in the context of the number of species within this seed category (Subbiah et al., 2019).

A number of protocols for vegetative explants incorporate cold acclimation to pre-condition samples for exposure to cryogenic temperatures (reviewed by Reed, 1996, 2008; Benson, 2008b). Cold acclimation is generally induced in the laboratory by exposing explants to low in vitro growth temperatures (1–6°C) generally in combination with shortened day-length and/or high-sugar pre-treatments (Reed, 1996, 2008). Most of these protocols have also been based on what are termed classical methods (reviewed by Engelmann, 1997) which generally involve explant chemical cryoprotection, followed by slow cooling (0.5–2.0°C min^{-1}) down to −30°C to −40°C, or even −60°C (Krøgstrup et al., 1992). This controlled slow cooling (step 1) is said to encourage the formation

of extracellular ice, progressively dehydrating the cells, as intracellular water is lost to exterior ice nucleation sites. This step is usually followed by immersion in LN (step 2). When freeze-induced dehydration during step 1 is too intense, various damaging events associated with the concentration of intracellular salts and changes in cellular membranes are possible (Mazur, 1990; Pritchard et al., 1995a). Also, some of the cells may fail to reach the optimum intracellular concentration and upon super-cooling undergo lethal intracellular ice-crystal formation (Mazur, 1990). Thus, while slow cooling may retain the integrity of individual cells, it has been shown to be less efficient at maintaining the tissue integrity required for survival of complex tissues, that is, meristems and embryos (Panis and Lambardi, 2006). Nevertheless, this 'two-step' cooling method, regarded as the first standard protocol developed for hydrated plant tissue (e.g. Withers and King, 1980), is still used for the cooling of undifferentiated culture systems (e.g. cell suspensions and calli (Withers and Engelmann, 1997)), and even differentiated structures such as the shoot apices of cold-tolerant species (e.g. Reed and Chang, 1997) and in isolated cases, excised embryonic axes/zygotic embryos from recalcitrant seeds (Mroginski et al., 2008).

3.2 Changing procedures: recalcitrant seeds and vegetatively propagated species

While minimal-growth storage has been successfully applied to explants from recalcitrant seeds (e.g. Chin, 1996; Sershen et al., 2008), recent reports of its use for the storage of such germplasm are scarce. Its continued use for the storage of somatic embryos (Hassan, 2017) and vegetative tissues is, however, still prevalent in the literature (Chauhan et al., 2019). In this regard, temperature reduction appears to be the most widely applied procedure in slow growth preservation, but it is now clear that temperature requirements vary from species to species, possibly based on the agroclimatic conditions in which a particular species occurs (Thakur et al., 2015). A more recent development in the design of minimal growth storage protocols involves the use of artificial seeds, produced by encapsulating propagules such as shoot buds or somatic embryos in a synthetic matrix, and this approach has been used successfully for the medium-term storage of a variety of plant species and propagule types (Cruz-Cruz et al., 2013).

There is also an increasing emphasis being placed on monitoring explants during storage, for example, changes in sugar content (total and reducing and non-reducing) due to sucrose-imposed stress in order to understand the effect of sucrose concentrations during slow growth conditions on survival and regeneration rate (El-Dawayati et al., 2018). Accommodating the interactive effects of light and ABA, and assessing relative nuclear DNA content in

regenerants to ensure retention of ploidy level have also become important considerations (El-Dawayati et al., 2018).

The short- to medium-term storage of recalcitrant seeds via hydrated storage is no longer a common practice, but there are recent reports (Moothoo-Padayachie et al., 2016; Chandra et al., 2019) that the exogenous application of reactive oxygen species blocking agents (e.g. diphenyleneiodonium (DPI) and dimethyl thiourea (DMTU)) can be used to extend the storage lifespan of recalcitrant seeds (by days to weeks). In terms of long-term storage of recalcitrant seeds, in recent years it has become increasingly apparent that the success of cryopreservation protocols for their zygotic germplasm depends on the optimisation of cooling rates in parallel with explant moisture content, to eliminate or at least minimise intracellular ice crystal formation (Wesley-Smith et al., 2004). Success in this regard has, however, been hampered by (a) lethal freezing damage occurring when hydrated seeds/embryos/axes are exposed to LN (Wesley-Smith et al., 1992; Berjak et al., 1999b); and (b) drying to water contents precluding ice formation to the extent of desiccation damage, which generally culminates in loss of viability (Walters et al., 2008). In contrast, pollen (reviewed by Ganeshan et al., 2008) and seeds and somatic embryos of most desiccation-tolerant species (reviewed by Pritchard and Nadarajan, 2008) appear to be highly amenable to cryopreservation, shifting the focus for successful cryopreservation of recalcitrant seed germplasm from freezing tolerance to dehydration tolerance (Panis and Lambardi, 2006). The large-scale, routine application of cryopreservation to recalcitrant-seeded species is very limited globally, with many protocols remaining unpublished, but a recent study has validated the use of embryonic axes for cryopreservation of this group of species by showing that viability can be retained for decades (Ballesteros and Pence, 2019). For some recalcitrant-seeded species, such as cocoa and *Avicennia marina*, however, seed-derived germplasm will never be suitable for cryopreservation based on high levels of microbial contamination and/or the absence of suitably small zygotic explants. In these cases storage of alternate explants (e.g. shoot tips and meristems) generated via tissue culture may be more suitable.

While classical cryopreservation protocols used freeze-induced dehydration, modern protocols are predominantly vitrification-based (e.g. Fu et al., 1990; reviewed by Engelmann, 1999). In the latter case, cell dehydration to increase cytoplasmic viscosity precedes cooling, promotes the formation of glasses and avoids the factors that encourage ice-crystal formation – before exposure to the cryogen (e.g. Stanwood, 1985; Wesley-Smith et al., 1992; reviewed by Walters et al., 2008). Cooling rates typically used to cool embryonic axes/zygotic embryos in these modern protocols, range from 10°C min^{-1} (e.g. Vertucci, 1989; Sershen et al., 2007) to hundreds of °C s^{-1} (e.g. Wesley-Smith et al., 2004; Walters et al., 2002). Additionally, most recent

plant cryopreservation studies involve one of the following vitrification-based procedures: pre-growth, dehydration, pre-growth-dehydration, encapsulation-dehydration, vitrification, encapsulation-vitrification, droplet-vitrification (González-Arnao et al., 2008) and, over the last decade or so, the novel cryo-plate procedure (Yamamoto et al., 2011).

Pre-growth involves culturing explants on cryoprotectants, followed by rapid, direct immersion in LN (Engelmann, 2004), while for dehydration explants are usually partially dehydrated in a laminar flow or above a bed of silica gel before immersion in LN. Pre-growth-dehydration involves the combination of both these procedures. These methods are now widely applied for cryopreserving meristems and polyembryonic cultures, small seeds and seed zygotic embryos or embryonic axes (González-Arnao et al., 2008). However, reports on their successful application to recalcitrant seed embryonic explants are rare. The encapsulation-dehydration technique is based on the methods used for artificial seed production, and involves encapsulating explants in alginate beads, followed by pre-growth in sucrose-enriched liquid medium and then partial desiccation in a laminar flow or using silica gel. The explants, often shoot apices (González-Arnao and Engelmann, 2006), are usually dried to a water content around 20% (fresh mass basis), the effects of which are minimised by being encapsulated, and they are then rapidly immersed in LN.

Vitrification techniques have remained relatively unchanged over the last few decades and involve the immersion of explants (most often somatic embryos, apices and cell suspensions) in cryoprotective solution (loading), chemical dehydration in highly concentrated plant vitrification solutions (PVS), followed by rapid cooling and rewarming, and finally removal (unloading) of cryoprotectants before in vitro recovery (Sakai and Engelmann, 2007; Sakai et al., 2008). Encapsulation-dehydration and vitrification procedures are sometimes combined (encapsulation-vitrification) and involve encapsulating explants before cryoprotection and cooling. The droplet vitrification technique (Kartha et al., 1982) is also based on the vitrification procedure and is still in use; samples are loaded, dehydrated with vitrification solutions and then placed within small droplets of these solutions on aluminium foil, which are then immersed with the samples in LN.

The cryo-plate procedure (Yamamoto et al., 2011) is one of the most revolutionary technical developments in plant cryopreservation over the last decade and combines the droplet-vitrification and encapsulation-dehydration techniques. The methods involve using a thin layer of calcium alginate to attach shoot tips to an aluminium cryo-plate. The explants are then loaded, dehydrated with PVS and then cooled in LN by direct immersion of the cryo-plates (Yamamoto et al., 2011, 2012). It is evident that the commonalities between both these relatively recent cryopreservation techniques, droplet-vitrification

and cryo-plate, are the rapid cooling and warming rates (achieved by immersion in a sucrose-enriched medium at ambient temperature) compared with other vitrification-based procedures. These rapid cooling and warming rates are a consequence of the fact that the aluminium contact surfaces have a very high thermal conductivity and this increases the probability of vitrification during cooling and the avoidance of devitrification during subsequent warming. This may explain why vitrification-based protocols have been shown to improve post-cryo preservation survival in complex organs (e.g. shoot-tips) of species that responded poorly to classical protocols (Panis, 1995; González-Arnao et al., 2003).

4 Conclusion and future trends

Over the last 50–60 years since many of the first national and international genebanks were established, the science of conventional seed banking has barely changed and it seems unlikely to change drastically in the near future, not least since many genebanks have, to a seemingly large extent, effectively conserved and distributed seed germplasm. Despite that apparent success, it is difficult to know the extent of genetic erosion that might have occurred since the original sample arrived at the genebank, through cycles of regeneration or loss of alleles (due to seed death) during storage (Fu, 2017). There has been concern that seeds of many species, even though desiccation tolerant, are perhaps 'minimally orthodox' in that their lifespan in conventional genebank storage may not extend to many decades. Colville and Pritchard (2019) recently published a meta-analysis of seed longevity data that suggested that many more species have relatively short seed lifespans, compared with the extreme longevity reported for few species.

The number of seed accessions in genebank storage continues to increase, perhaps in response to the identification of gaps in existing collections (as discussed elsewhere in this book), but also due to changing priorities and advances in plant science. Many genebanks now conserve genetic stocks such as recombinant inbred lines, MAGIC populations and similar, and provide direct or indirect access to genomic information. A number of genebanks have also started or plan to genotype all or subsets of their collection (as discussed by McCouch et al., 2012). Genetic markers may also be used to, for example, identify duplicates within and among collections or to track accessions through storage and cycles of regeneration, although the cost and additional logistics required for such tracking is probably unworkable for most genebanks currently (van Treuren and van Hintum, 2014).

Large genebanks, particularly those conserving only one or two crops, process, perhaps, many thousands of new seed lots every year. As such, by necessity, they often adopt a factory-like approach to manage operations, with

staff trained for a particular activity and the seed lot then moved to the next position/task. In contrast, smaller genebanks, or seed banks conserving diverse species, particularly wild species, may take a different approach and have the same member(s) of staff follow the whole cycle for a particular seed lot. Whichever strategy is in place, it is beneficial for the genebank and for the wider seed conservation community, for there to be regular evaluation of procedures and active innovation where improvements are needed or offer advantages, for example, in terms of accuracy, consistency and/or efficiency. Related to this, the documentation of standard operating procedures (SOPs) is expected to be revised as procedures are adapted, as part of the continuous improvement principle of a genebank quality management system (as discussed elsewhere in this book).

Some genebanks have a research team responsible for developing new procedures, technologies and understanding, although in general, this is not something that has been prioritised at most genebanks. Nonetheless, it is helpful for genebanks to follow the seed science/testing and plant phenotyping communities to see whether emerging technologies could be applied in the genebank context (Whitehouse et al., 2020). As with many industries, more genebanks may have robots in the future. However, there will always be a need for people with an understanding of the value and uniqueness of germplasm samples, not least since it is for that very uniqueness and the underlying genes, that we conserve our species and agrobiodiversity for future generations.

The last two to three decades have seen significant progress being made in terms of conserving non-orthodox seeded and vegetatively propagated plant species from temperate, tropical and subtropical zones. This has been aided by the development of in vitro seed germination, zygotic embryo and callus culture, somatic embryogenesis and micropropagation systems for many of these species (e.g. a collection of Brazilian species (Pilatti et al., 2011)). The development of these in vitro systems has enabled short- and medium-term conservation for a number of these species, but successful cryopreservation protocols are still elusive for the vast majority. Nevertheless, there are presently collections in cryobanks in close to 15 countries, based on a list published by Cruz-Cruz et al. (2013) and our consultation with various specialists in the field. These collections, which include callus, pollen, shoot tips, dormant buds, seeds and embryogenic cell lines for vegetatively propagated species (Cruz-Cruz et al., 2013), have achieved significant species coverage, with cryopreservation protocols being established for root and tubers, ornamentals, crops and fruit trees of temperate and tropical origin (Engelmann, 2000; Benelli et al., 2013). As these collections expand and the production of clones obtained from elite genotypes, unique/important cell lines and genetically transformed material increases, genotyping collections in cryobanks to avoid duplication is going to become increasingly important. A recent review by Wang et al. (2020),

for example, reports on the use of stem disc-bearing adventitious buds, small leaf square-bearing adventitious buds, rhizome buds and microtubers as novel propagule types and hence, possible new alternative explants for cryopreservation. Cryobanks of the future are therefore likely to house much more diverse germplasm than at present.

While the latest two cryopreservation techniques, namely, droplet-vitrification and cryo-plate, have proven to be more beneficial for cryopreserving complex organs (e.g. shoot-tips) than classical protocols, of tropical species in particular, successful cryopreservation of explants from recalcitrant seeds remains challenging for the vast majority of species of tropical and temperate origin (Ballesteros et al., 2021). Large-scale, routine application of cryopreservation is therefore still very limited in comparison with conventional low-temperature seed storage. However, the benefits of cryotherapy in eliminating viruses in explants from vegetatively propagated species (e.g. sugarcane (González-Arnao et al., 2020)) increased production of artificial seeds in breeding programmes for non-orthodox or non-seed producing plants (Ravi and Anand, 2012), and more examples of orthodox species with poor longevity in conventional seed/genebank storage (e.g. Ali et al., 2007; Mondoni et al., 2011; Davies et al., 2018) may encourage practitioners to integrate cryopreservation into existing plant biodiversity conservation procedures, whether or not these facilities focus on vegetatively propagated, orthodox- or recalcitrant-seeded species.

5 Where to look for further information

- CGIAR Genebank Platform (www.genebanks.org).
- Food and Agriculture Organization of the United Nations (FAO) (www.fao.org/genetic-resources/en/).
- International Society for Low Temperature Biology and Medicine (www.societyforcryobiology.org/).
- Millennium Seed Bank (www.kew.org/wakehurst/whats-at-wakehurst/millennium-seed-bank).
- United States Department of Agriculture Germplasm Resources Information Network (GRIN) (www.ars-grin.gov).

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