I. Introduction

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Coffee is one of the most important beverages in the world and is consumed by more than a third of the world's population. It is also a very important commodity crop for many developing countries, once contributing over US$ 10–11 billion annually (Bolvenkel et al. 1993) and providing a source of income for thousands of small-scale farmers, as well as being a significant source of employment. However, during the crisis years that began in 2000 and continued to the end of 2004, earnings slumped to just over US$ 5.5 billion annually, while the value of retail sales in industrialized countries continued to remain healthy and to increase steadily, increasing to exceed US$ 70 billion (Osorio 2005).

The commercial coffee comes from two main species, *Coffea arabica* L. and *C. canephora* Pierre ex Froehner, and many varieties of coffee have been developed in response to widespread prevalence of pests and diseases, such as Coffee berry borer, Coffee berry disease, Coffee leaf rust, and, more recently, *Fusarium* wilt and others, all of which undermine coffee production and quality. It is recognized that the cultivated varieties, in particular *C. arabica*, have a very narrow genetic base (Anthony et al. 2002) and their improvement depends on the availability of adequate amounts of genetic diversity. The genus *Coffea* is endemic to the Old World tropics of Africa, particularly Madagascar, and over 100 wild species are found in the Afrotropical-Madagascar region, including the Comoros and the Mascarene Islands (Chevalier 1947; Bridson and Verdcourt 1988; Stoffelen 1998). This region, together with farmers' fields growing old and traditional coffee varieties, represents the ultimate source of coffee genetic diversity, on which the future of coffee improvement depends. However, deforestation and encroachment by agricultural activities, population pressures and economic hardships threaten all these reservoirs of genetic diversity, and with these threats comes the danger of significant erosion of the *Coffea* genepool. Chapter 2 of this publication provides a detailed account of the coffee genetic resources and the threats they are facing. The conservation of coffee genetic resources has not received much attention recently, but efforts to collect and conserve coffee genetic resources were initiated in the 1960s and 1970s by ORSTOM (now IRD), FAO and IBPGR (now Bioversity), and several options for their conservations have been developed.

Conservation options

Two basic conservation strategies, each comprising various techniques, are employed to conserve genetic diversity, namely *in situ* and *ex situ* conservation (Engelmann and Engels 2002). Article 2 of the Convention on Biological Diversity provides the following definitions for these categories (UNCED 1992):

- *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitat.

- *In situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.

There is an obvious fundamental difference between these two strategies: *ex situ* conservation involves the sampling, transfer and storage of target taxa from the collecting area, whereas *in situ* conservation involves the designation, management and monitoring of target taxa where they are encountered (Maxted et al. 1997). Another difference lies with the more dynamic nature of *in situ* conservation compared with the more static *ex situ* conservation. These two basic conservation strategies are further subdivided into specific techniques, including seed storage, *in vitro* storage, DNA storage, pollen storage, field genebank and botanic garden conservation for *ex situ*, and protected area, on-farm and home garden conservation for *in situ*, each technique presenting its own advantages and limitations (Engels and Wood 1999). *Ex situ* conservation techniques are in particular appropriate for the conservation of crops and their wild relatives, while *in situ* conservation is especially appropriate for wild species and for landrace material on-farm.

In situ conservation

In situ conservation offers the possibility of conserving a greater diversity of species and genepools at the same time. It is a dynamic conservation process, as plants continue to evolve with changes in their environment, most importantly pests and diseases (Maxted et al. 1997; Hodgkin and Ramanatha Rao 2002). It is suitable for crop evolution and genetic studies, and represents a viable alternative for conservation of non-orthodox-seed species. However, *in situ* conservation leaves the plant material vulnerable to natural and human-induced disasters, and the plant material is not readily accessible for use. The appropriate management regimes are poorly understood and high levels of supervision and monitoring are required to implement *in situ* conservation. Finally, the amount of genetic diversity that can be conserved in any one reserve is not easily measurable.

On-farm conservation

On-farm conservation is also a dynamic process in which plants continue to evolve (Jarvis et al. 2000; Watson and Eyzaguirre 2002). It ensures the conservation of valuable genetic diversity in traditional landraces, weedy crops and ancestral forms. The material is easily accessible for use by farmers and local communities. However, it is vulnerable to changes in management practices and the appropriate management regimes are poorly understood. On-farm conservation requires the maintenance of traditional cultivation systems. The amount of genetic diversity that can be conserved on farm remains to be evaluated.

Conservation in field genebanks

Conservation in field genebanks is suitable for species with non-orthodox storage behaviour. In some ways, this method offers a satisfactory approach to conservation (Engelmann and Engels 2002). The genetic resources under conservation can be readily accessed and observed, thus permitting detailed evaluation. However, there are certain drawbacks that limit its efficiency and threaten its security (Engelmann 1997a; Withers and Engels 1990). The plants are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism. In addition, they are not in a condition that is readily conducive to germplasm exchange because of the great risks of disease transfer through the exchange of vegetative material. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very

survival in times of economic stringency (Dulloo et al. 2001). Even under the best circumstances, field genebanks require considerable inputs in the form of land (often needing multiple sites to allow for rotation), labour, management and materials, and, in addition, their capacity to ensure the maintenance of much diversity is limited (Engelmann and Engels 2002).

In vitro conservation

In vitro conservation methods represent a relatively easy alternative for medium- to long-term conservation of a large number of non-orthodox, sterile or clonal species (Withers and Engelmann 1998). Cryopreservation (in liquid nitrogen at -196°C) provides long-term safety of the stored material, with limited maintenance and monitoring once the material is in storage. Germplasm exchange is facilitated by *in vitro* methods as they permit the production of virus-free material through meristem culture and their rapid multiplication (Engelmann 1997b). However, there are risks of somaclonal variation in some species when maintained under *in vitro* slow growth. *In vitro* storage is relatively high-tech and maintenance costs of the material are high. Individual slow growth and cryopreservation protocols need to be developed or adapted for most species. Difficulties are encountered in storing non-orthodox-seed species, and only a limited number of accessions can be conserved, especially when using slow growth storage.

Pollen storage

Pollen conservation is another viable alternative for conserving species with non-orthodox seeds. Pollen storage is a relatively easy and low-cost procedure (Towill and Walters 2000). Using pollen facilitates germplasm exchange, as a relatively small quantity of material is required for a single sample. Most importantly, pollen is generally less likely to be infected by pathogens than other propagules. The disadvantages of pollen conservation are that only paternal material is conserved, i.e. less than half of the total genetic make-up of an organism; individual regeneration protocols need to be developed to produce haploid plants; and further research is needed to produce diploid plants.

DNA storage

DNA storage in DNA libraries is yet another alternative for conserving species with non-orthodox seeds (Adams and Adams 1992). It is also a relatively easy and low-cost procedure. It is particularly useful for conserving specific genes responsible for heritable characteristics of particular value (e.g. disease resistance). DNA is easily accessible and is especially convenient for exchange among plant breeders. However, procedures for regenerating entire plants from conserved DNA are not available at present, and numerous problems exist with gene isolation, cloning and transfer.

Seed storage

Seeds are regarded as the most convenient material for *ex situ* conservation, and they make secure medium- to long-term conservation feasible (FAO 1996). Seed storage is both efficient and reproducible, allowing the conservation of a wide range of genetic diversity. Seeds are also a convenient form for germplasm use and exchange. Moreover, they require only limited maintenance and monitoring once the material is placed in storage. However, seed storage does not allow for the conservation of useful genotypes. There are risks of losing genetic diversity with each regeneration cycle and it is a static conservation process, as it 'freezes' the evolutionary development of useful characteristics, especially related to resistance to pests and diseases. Most importantly, the usability of seed for long-term storage depends on its storage behaviour.

A large number of plant species have seeds that are termed 'orthodox', meaning that the seeds are desiccation tolerant and can be dehydrated down to low water contents, and that they are also

cold tolerant and can be stored at low temperature for extended periods (Roberts 1973). There are three main categories of plant species for which seed conservation presents a problem. Firstly, some crops, such as banana and plantain, do not produce seeds and are thus propagated vegetatively. Secondly, some species, such as potato or sugar-cane, include both sterile genotypes and genotypes producing orthodox seeds. However, these seeds are generally highly heterozygous and are thus of limited interest for the conservation of particular genotypes. These species are normally maintained as clones. Thirdly, numerous fruit and forest tree species, especially of tropical origin, produce recalcitrant seeds, i.e. seeds that cannot be dried to sufficiently low moisture level to allow their storage at low temperature (Roberts 1973; Chin and Roberts 1980). There is also a large number of species, termed intermediate (Ellis et al. 1990), for which the seeds can be dried to some extent, but their long-term conservation remains problematic.

Cryopreservation

The traditional *ex situ* conservation method for these difficult-to-store categories of plant species is in the form of field collections, which present both advantages and major drawbacks, as described previously. Cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196°C), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of the genetic resources of the problem species mentioned above. At this temperature, all cellular division and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, cultures are stored in a small volume, protected from contamination, and require very limited maintenance (Engelmann 2000).

Cryopreservation of vegetatively propagated species is becoming a reality and is used routinely for long-term conservation of an increasing number of germplasm collections (Engelmann 2004). As concerns non-orthodox-seed species, a number of review papers have been published in the last decade that present extensive lists of plant species whose embryos or embryonic axes have been successfully cryopreserved (Kartha and Engelmann 1994; Bajaj 1995; Pence 1995; Engelmann et al. 1995; Engelmann 1997a, b; Engelmann and Takagi 2000; Towill and Bajaj 2002). This might lead to the conclusion that freezing of embryos is a routine procedure applicable to numerous species, whatever their storage characteristics. However, careful examination of the species mentioned in these papers reveals that only a limited number of truly recalcitrant seed species are in fact included. This is because research in this area is recent and addressed by very few teams worldwide and because recalcitrance is a dynamic concept that evolves with research on the biology of species and improvement in storage procedures. Some species previously classified as recalcitrant have thus been moved to the intermediate or even sub-orthodox categories, and can now be stored using classical or new storage techniques (Engelmann 2000).

In comparison with the results obtained with vegetatively propagated species, cryopreserved storage of non-orthodox seeds is still at a very preliminary research stage. There are a number of reasons behind this situation, including the huge number of (mainly wild) species falling within this storage category, a lack or insufficient knowledge of their biology, the inexistence or non-operationality of *in vitro* culture protocols for most of these species, and the large heterogeneity in the physical, biochemical and physiological characteristics of their seeds (most importantly concerning their moisture content) within and between seed lots (Engelmann 2000). Fortunately, there are various options to consider for improving storage of non-orthodox seeds, including employing very precisely controlled desiccation and cooling conditions, using other cryopreservation techniques that have so far seldom been employed, and selecting seeds or embryos at the right developmental stage, which is a parameter of critical importance for the success of any cryopreservation experiment (Engelmann 1999).

Development of complementary conservation strategies

It is now well recognized that an appropriate conservation strategy for a particular plant genepool requires a holistic approach, combining in a complementary manner the different *ex situ* and *in situ* conservation techniques available (Engelmann and Engels 2002). *In situ* and *ex situ* methods, including a range of techniques for the latter (storage of germplasm as seeds, plants in the field, pollen, *in vitro* cultures under slow growth, cryopreserved explants, DNA sequences), are options available for the different genepool elements (cultivated species, including landraces and modern varieties; wild relatives; weedy types; etc.). Selection of the appropriate method should be based on a range of criteria, including the biological nature of the species in question; the practicality and feasibility of the particular method chosen (which depends on the availability of the necessary infrastructure); as well as the cost-effectiveness and security afforded by its application (Maxted et al. 1997). Considerations of complementarity with respect to the efficiency and cost-effectiveness of the various conservation methods chosen are also important. In many instances, the development of appropriate complementary conservation strategies requires further research to define the criteria, refine the method and test its application for a range of genepools and situations (Dulloo et al. 1998, 2005; Nissilä et al. 1999; Ramanatha Rao et al. 1999). An important area in this is the linkage between *in situ* and *ex situ* components of the strategy, especially with respect to the dynamic nature of the former and the static, but potentially more secure, approach of the latter (Engelmann and Engels 2002; Reed et al. 2004). Cryopreservation needs to be integrated as a key component in the development of complementary conservation strategies for non-orthodox-seed species since, as mentioned previously, it is the only technique currently available to ensure the safe and cost-efficient long-term conservation for species producing non-orthodox seeds.

Another key component in the development of conservation strategies is the construction of core collections, which can be used either for conservation projects or evaluation purposes. A core collection is a subset of a larger germplasm collection and contains the maximum possible genetic diversity of the species with the minimum of repetitiveness (Frankel 1984). Despite the simple formulation of the core collection concept, construction of core collections appears often to be difficult because of lack of evaluation data for the whole collection. In many cases, a pragmatic approach can be encouraged, with the objective of structuring the germplasm accessions using passport data (see Chapter 5).

Research on the development of complementary conservation strategies and of the relevant storage methods required for their implementation can be effectively carried out through collaborative studies, involving fundamental and applied research organizations within countries, as well as through close cooperation with international institutions concerned with conservation research. Part of the work presented in this publication originates from the occurrence of such a situation with coffee.

Conservation of coffee germplasm

The conservation of coffee germplasm is closely associated with *C. arabica* domestication and has predominantly involved conservation in field genebanks because of the non-orthodox nature of coffee seeds. It first began on farms in the centre of origin, Ethiopia. Subsequently, worldwide extension of coffee cultivation contributed to the establishment of field genebanks in producer countries. The size of the collections increased greatly during the second half of the last century, when coffee germplasm collections were made during explorations across Africa (see Chapter 2). Considering the lifespan of coffee trees (about 30 years) and the inherent problems associated with maintenance of field genebanks (as described above), there is now an urgent need for rejuvenating the ageing coffee trees (Dulloo et al. 2001) and for the development of complementary methods of conservation.

Numerous *in vitro* techniques have thus been developed for medium-term storage of coffee germplasm (Dussert et al. 1997c). The establishment of an *in vitro* coffee core collection was initiated in 1991 at IRD Montpellier (France) but, a few years later, the limits of this technique was recognized with the occurrence of some genotypic selection and intraspecific genetic drift (Dussert et al. 1997a).

Research on the conservation of coffee seeds has also been promoted by Bioversity and IRD. Though *C. arabica* seeds can withstand desiccation down to 0.08-0.10 g $H_2O.g^{-1}$ dw water content (fresh weight basis) (Becwar et al. 1983; Ellis et al. 1990), they cannot be considered orthodox because they remain cold-sensitive (van der Vossen 1977; Couturon 1980; Ellis et al. 1990) and desiccation does not increase their longevity (van der Vossen 1977; Ellis et al. 1990). Fully hydrated seeds stored at 19°C under 100% relative humidity remained viable for 36 months for *C. arabica* and 15 months for *C. canephora* and *C. stenophylla* (Couturon 1980). Because of their intermediate storage behaviour, coffee seeds cannot be used for long-term conservation and coffee genetic resources are conventionally conserved as trees in field genebanks.

This highlights the importance of developing cryopreservation protocols for long-term conservation of coffee germplasm (Dussert et al. 2002). Research for cryopreservation techniques was performed with different explants, including seeds (Normah and Vendagasalam 1992), zygotic embryos (Abdelnour-Esquivel et al. 1992; Normah and Vendagasalam 1992), apices (Mari et al. 1995) and somatic embryos (Bertrand-Desbrunais et al. 1988; Tessereau et al. 1994).

However, seeds were considered the most interesting material for long-term conservation of coffee genetic resources using cryopreservation. Indeed, they are the only explant type for which a cryopreservation protocol could be developed that would not include any *in vitro* step, thereby allowing its implementation under low-tech conditions. In addition, seeds represent the base propagation unit for an autogamous species such as *C. arabica* and can be efficiently used for gene pool conservation in the case of allogamous species. Research was thus actively pursued at IRD Montpellier, leading after several years to the establishment of a simple, robust and efficient cryopreservation protocol based on the determination of very precise conditions for desiccation and freezing of seeds, which was applicable to a range of coffee species (Dussert et al. 1997b, 1998, 1999, 2000, 2002).

Past research has also shown that pollen can also be effectively stored under vacuum at -18°C and remain viable for more than two years and fertile for at least six months. (Walyaro and van der Vossen 1977). Regrettably, to our knowledge, there been no further research on coffee pollen conservation, and this represents a major gap in coffee conservation research.

Research on the *in situ* conservation of coffee genetic resources has lagged behind the efforts made in developing *ex situ* conservation techniques. *In situ* conservation of coffee germplasm has often resulted passively from the establishment and management of protected areas in biodiversity hotspots (Dulloo et al. 1998). The natural habitats of coffee are principally forest ecosystems, and it is widely known that the biological diversity in these habitats is under threat from high rates of deforestation, land clearing and introduced invasive species. Efforts to conserve natural populations of coffee germplasm are very limited, and known examples come from work done in Ethiopia (Gole 2002) and in Mauritius (Dulloo 1998). There is still much to be done within the areas of coffee diversity hotspots in Madagascar and on mainland Africa, particularly in Tanzania, while major areas within the central Africa region, such as Gabon and the Central African Republic, still remain unexplored.

Aims of this publication

For many years, non-orthodox-seed research has been recognized by Bioversity and its predecessors as an area of critical importance for the conservation of plant genetic resources, and

numerous projects on this topic have been or are being implemented in collaboration with research institutions and genebanks worldwide (Engelmann and Engels 2002; Engelmann 2003). Recognizing the tremendous potential implications of the results achieved at IRD for long-term storage of coffee germplasm, and also for other non-orthodox-seed species, Bioversity decided to support a research programme aiming at transferring and testing on a large scale in a genebank located in a developing country the freezing protocol developed in France. This was implemented in 1998–2000 in the framework of two successive projects with IRD and CATIE, Costa Rica. CATIE was an ideal partner as it fulfilled the set of criteria required for participating in such a project. Indeed, CATIE holds one of the largest field collections of coffee worldwide, mainly of *C. arabica*, with 1852 accessions of this species (9760 trees), including wild plants and varieties from the diversity centre (Ethiopia), varieties from the dispersion centre (Yemen), varieties derived from two genetic populations spread worldwide in the 18th century (known as Typica and Bourbon), introgressed lines derived from interspecific hybrids, mutants and other selected genotypes (see Chapter 3). CATIE's fully equipped biotechnology laboratory includes all the facilities required for cryopreservation and molecular biology research, as well as highly skilled scientific and technical staff. Moreover, Bioversity and CATIE have a long and successful collaboration history in various areas, including cryopreservation of tropical plant germplasm (Abdelnour-Esquivel 2000; Engelmann 2003). At the time of the initiation of this programme, CATIE was also implementing collaborative research projects on coffee with IRD and another French research institute, CIRAD (*Centre de coopération internationale en recherche agronomique pour le développement*). Staff from these two institutes were working on a permanent basis in CATIE. These collaborative projects included research on the characterization and rationalization of the CATIE coffee germplasm collection (see Chapters 3 to 5); on the utilization of the material from the coffee collection for improvement purposes (Anthony et al. 1999; Bertrand et al. 1999, 2005); and on the use of biotechnology for large-scale propagation of improved material (Etienne et al. 1999, 2002; Etienne and Bertrand 2001).

The aim of this publication is to illustrate how new technologies (molecular biology and cryopreservation) can be efficiently employed to complement more classical ones for characterizing and rationalizing an *ex situ* germplasm collection, and to improve its conservation status. To our knowledge, the work described in this publication represents the first example of the application of these techniques in a genebank located in a developing country, in the framework of the development of an *ex situ* complementary conservation strategy for *C. arabica*, i.e. a crop of commercial importance at the global level. The approach that was applied to coffee genetic resources might be used with other perennial plants whose seeds also display non-orthodox storage behaviour.

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