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Biotechnology and conservation of plant biodiversity: A review

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Abstract
With deforestation, environmental pollution, un-restricted collection, genetic modification and all other kinds of human related measures together with climatic change, considerable amount of plants are under threat of eradicating from the world. Though in-situ conservation measures can be applied, ex-situ measures are more practical and efficient for the conservation of plant species. With continuously increasing technology, biotechnological approaches deliver some most promising methods for long term conservation of germplasms. *In vitro* conservation, cryopreservation and DNA storage techniques help to conserve plant genomes for a considerably long time. Objective of this review is to discuss the potential of conserving plant species through biotechnology.

Keywords: biotechnology, biodiversity conservation, invitro, plant tissue culture, synthetic seeds

1. Introduction

The conservation of plant biodiversity is a vital issue concerning the human population worldwide. The challenge of sustainable agriculture development to address increasing food demand is one of the single most demanding concerns which challenge humans today and diversity of biological resources provides the basis for that. Plant biodiversity is a natural source of products to the medical and food industries. It provides different basic raw materials and contributes to supply new genetic information useful for breeding programs and for developing more productive crops and more resistant plants to biological and environmental stresses [1]. Biological diversity or biodiversity refers to genetic materials and the ecosystems of which they are a part. Diversity rich ecosystems possess higher resilience and are able to recover and adapt more readily from natural tragedies and/or anthropogenic activities and habitat degradations. Plant genetic resources (PGR), as a vital segment of biodiversity in general and agrobiodiversity in particular, constitute the genetic material of plants having value as a resource for present and future generation of human being [2]. As genetic resource, the PGR may be of reproductive or vegetative propagule such as seeds, shoots, tissues, cells, pollen, DNA molecule etc, containing the functional unit of heredity in addition to corresponding information and knowledge about their use that can be applied in crop improvement programme and other product development [3]. The anthropogenic pressure, the introduction of alien species, as well as domesticated species have dramatic effects on plant diversity, which is reflected in rise in the number of threatened species [4].

The types of PGR vary from landraces and farmers' varieties, absolute cultivars, modern cultivars, breeding lines and genetic stocks, wild relatives, weedy races and potential domesticate species, exotic and indigenous [5, 6, 7]. There are approximately 400,000 species of higher plants, of which 250,000 have been identified or described [5]. Among these, 30,000 species are edible, but over the course of human Civilization just about 7,000 of them have been cultivated and or used by humans for food. Table 1 provides more facts about diversity of world plant species.

Table 1: Diversity of world plant species

Approximate number of plant species between	**13 - 14,000,000**
Number of described plant species	1,750,000
Number of higher plant species between	300,000 to 500,000
Approximate number of edible plant species	75,000
Number of plant species used for food	7,000
Commercially important plant species	150
Plant species producing 90% of calories in human diet	30
Crop species producing 60% of global food requirement (rice, wheat, maize).	3

Sources [2, 6]

Modern and sustainable conservation system requires complementary strategies involving both *in situ* and *ex situ* conservation. Emphasis needs to be given to the collection and conservation of the endemic wild relatives, rare and endangered species that are rapidly vanishing from their ecological niches [8].

The preservation of any plant species in their natural habitat, as well as the conservation of domesticated and cultivated species on the farm lands or in the surroundings where they have developed their distinctive characteristics denotes the *in situ* strategies [9]. However, there is a heavy loss or decline of species, populations and ecosystem composition, which can lead to a loss of biodiversity, mainly due to habitat destruction and the alterations of these natural environments; therefore, *in situ* methods alone are inadequate to save endangered plant species [4].Additional approaches, like storage in seed banks, field gene collections, *in vitro* collections and botanical gardens, complement the preservation programs for plant biodiversity. They are categorized as *ex situ* plant

conservation strategies, which mean to maintain the biological material outside their natural habitats. *Ex situ* conservation is a feasible way for saving plants from extinction, and in some cases, it is the only possible strategy to conserve certain species [10].

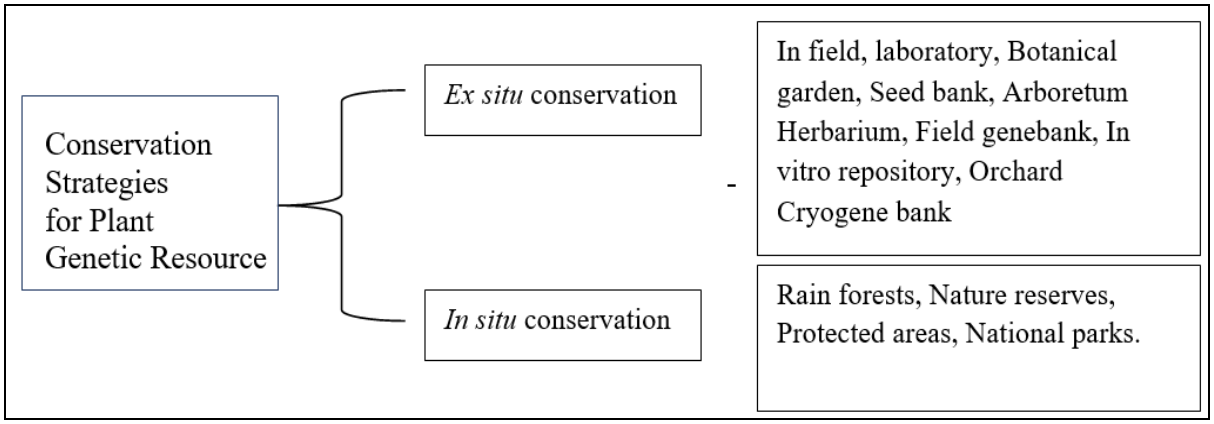

Fig 1: Different conservation strategies used in conserving plant genetic diversity resources

In situ and *ex situ* methods are complementary and are not exclusive. Advances in plant biotechnology, especially those associated to *in vitro* culture and molecular biology, have also provided powerful tools to support and improve conservation and management of plant diversity [11].

This review is mainly targeted on explaining the plethora of the biotechnology conservation strategies including *in vitro* conservation and cryopreservation techniques, which can be efficiently used to improve the conservation of plant biodiversity.

Biotechnological strategy of plant diversity conservation

In vitro conservation and cryopreservation techniques

Efficient and reliable conservation of genetic resources in case of vegetatively propagated plants and recalcitrant (non-orthodox) seed species has been hampered due to problems faced during application of their conventional method of *ex situ* conservation in field gene banks. To handle these challenges, *in vitro* techniques have been widely used for conservation of these plant species, which produce little or no seeds and its related activities like collecting and exchange of germplasm of these species. *In vitro* slow or normal growth techniques offer up to medium-term storage option, avoiding the risk of losses of germplasm on field gene banks due to pests or disease attacks and natural disasters. Advances in plant biotechnology, especially *invitro* culture and molecular biology, have also provided powerful tools to support and improve conservation and management of plant diversity[11]. At present, biotechnological methods have been used to conserve endangered, rare, crop, ornamental, medicinal and forest species, allowing the conservation of pathogen-free material, elite plants and genetic diversity for short, medium and long term. Furthermore, *in vitro* techniques offer a safe mean to internationally exchange plant material, enable the establishment of extensive collections using minimum space, allow supply of valuable material for wild population recovery and facilitate molecular investigations and ecological studies [12]. While cryopreservation at ultra-low temperature, usually in liquid nitrogen (-196°C), is the only option currently available for the long-term conservation of these PGR avoiding exogenous contamination, requiring small space and least maintenance. The main target of using this very low temperature is to cease all metabolic activities of cell and theoretically the cell or tissue can be stored for an indefinitely period. Both *in vitro* conservation and cryopreservation techniques use tissue culture principles for conservation [13].

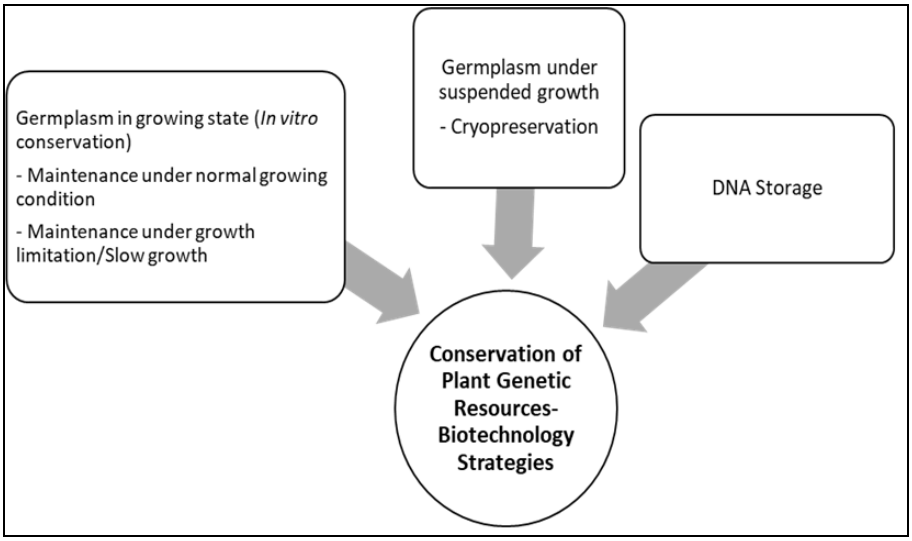

Fig 2: Biotechnological approaches in Conservation of Plant Genetic Resources.

Germplasm in growing state (In vitro) conservation

In vitro conservation is centered on tissue culture techniques, and represents a complementary strategy for the conventional conservation. Tissue culture techniques have the advantages of producing pathogen-free plants with high multiplication rates, under controlled and aseptic environmental conditions. Tissue culture systems or *in vitro techniques* present advantages which are listed below:

1. very high multiplication rates
2. aseptic system:- production of pathogen-free stocks
3. reduction of space requirements
4. genetic erosion reduced to zero under optimal storage conditions
5. reduction of the expenses in labor costs

Tissue culture is an essential tool for the production of transgenic plants and high-value phytochemicals [14, 15, 16]. The short and medium term storage is mainly achieved by reducing the growth and increasing the intervals between sub cultures[16]. This is gained by modifying the culture conditions, mainly lowering the culture temperature.

Maintenance under normal growing condition

In vitro techniques are mainly used to achieve medium-term conservation allowing the storage of biological material from several months to 2-3 years without sub culturing, depending on the technique used and accordance with the type of plant material. The basic of *in vitro* conservation depends by implying growth reduction which is generally attained by modifying the culture medium and/or the environmental conditions [4]. The principal factors that affect short and medium term conservation are temperature, culture medium, physiological stage of *ex-plant*, size and type of culture vessels, modifications of gaseous environment, encapsulation, desiccation and stability of stored plant material [17].

Modifications of the culture medium can include dilution of mineral elements, reduction of sugar concentration, changes in the nature and/or concentration of growth regulators and addition of osmotically active compounds [18]. The culture environment, it can be changed by maintaining lower temperature levels, combined or not, with a decrease in light intensity or by placing cultures in complete darkness. The most frequently used combination of physical and chemical factors involves decrease of temperature, reduction of mineral elements and carbon source concentration in the medium and the use of low light intensity [19]. The temperatures reported for medium-term conservation are usually from 4 °C to room temperature [20]. However, when regards the tropical plant species they have to be stored in the temperature range of 15-20°C or even higher, depending on their cold sensitivity [21]. Therefore, the procedure to enable extending subculture periods will mainly focus on modifying the chemical composition of culture medium.

Maintenance under slow growth condition

Other additional parameters may also influence the efficiency of slow growth storage, such as the type of explants, their physiological state, as well as the type, volume and the kind of closure of culture vessels [17]. Standard *in vitro* culture conditions can also be used for medium-term storage when dealing with species that have a natural slow growing habit. Alternatively, the explants may be covered with paraffin, mineral oil or with liquid medium to reduce the growth rate. Modifications in gaseous environment, desiccation and/or encapsulation are other possible options [18]. Artificial seeds, which are produced by encapsulating plant propagules (shoot buds or somatic embryos) in a synthetic media, enable medium-term conservation of various plant species, like orchids, through encapsulation of protocorms [22].

Short- and medium-term conservation is regularly used in many laboratories in order to increase the intervals between subcultures required under the multiplication procedure. At the end of a storage period, cultures are transferred onto fresh medium and usually placed for a short period in optimal conditions to stimulate regrowth before entering the next storage cycle [18]. Slow growth has been successfully used for plant species of both temperate and tropical origin, including crops, forest trees, endangered species and medicinal plants [23]. As an example, rare wild species, like *Gladiolus imbricatus*, which is an important resistance-gene pool in this genus by having resistance to abiotic and biotic stress, was stored up to one year [24] on Murashige and Skoog (MS) medium [25] at low temperature and kept in the dark. After one year of storage, 25%of the plants could be successfully recovered.

The advantage of slow growth techniques is that they use the same basic facilities used in plant tissue culture with little or less modifications. Micro propagation and that the storage regimes are based on modifying the conditions previously established for rapid multiplication. However, they do not alleviate the main problem associated with the high costs of labor and space requirements of any micro propagation system, in addition to the potential risks of *somaclonal* variation for some species [26].

Cryopreservation techniques

Cryopreservation refers to storage of biological samples at ultra-low temperature in liquid nitrogen at -196 ^{0}C. Cryopreservation has been identified as the most practical and efficient tool for long-term storage of germplasm. At ultra-low temperature basically all cellular divisions and metabolic processes cease providing opportunity to store plant tissues for a long time without any change [17]. Cryopreservation requires a small volume, demands very limited maintenance and relatively cheap when compared with other long-term conservation methods [27]. Classical cryopreservation techniques, which are based on freeze-induced dehydration, are mainly employed for freezing undifferentiated cultures and apices of cold-tolerant species. New cryopreservation techniques, which are based on vitrification of internal solutes, are successfully employed with all explant types, including cell suspensions and calluses, apices, and somatic and zygotic embryos of temperate and tropical species [28]. Vitrification is the freeze-avoidance mechanism that enables hydrated cells, tissues and organs to withstand exposure to the temperature of liquid nitrogen [29]. Seven different vitrification-based procedures can be identified as: (i) pre-growth (ii) dehydration; (iii) pre-growth-dehydration; (iv) encapsulation-dehydration; (v) vitrification; (vi) encapsulation-vitrification and (vii) droplet-vitrification [30]. They have allowed improvements in survival and recovery after cryopreservation compared with conventional crystallization-based protocols, proving their effectiveness for large scale application with different plants.

DNA Storage

Genomic DNA refers to the complete set of DNA within an organism. By preserving genomic DNA, all the information related to the particular organism can preserved, hence can be identified as an advanced conservation method. DNA extraction is the first step of DNA storage. The literature contains large number of DNA extraction protocols with are slightly different from one another [31]. In recent years, commercial manufacturers have developed kits that allow rapid and efficient isolation of high-quality DNA from a wide variety of plant species [32]. Regardless of how DNA material is collected, proper storage and handling is essential to the maintenance of high-quality DNA that can be used in the future. Extracted pure DNA free from the risk of biological contamination can be stored for a considerable period under room temperature though low temperature storage is recommended [33]. DNA material used within a short period may be stored at -20 ^{0}C, while DNA stored for a long period should be kept in ultra-low freezers, at or below-80 ^{0}C. DNA can be stored as a precipitation in ethanol at -164 ^{0}C in cases where storing at -80 ^{0}C is not working [34].

***In vitro* collecting techniques for plant germplasm conservation**

Plant material collection is the first step to acquire plant germplasm either in any of the main conservation techniques. But *in vitro* techniques can significantly increase collecting efficiency through the use of *in vitro* collecting, which is the process to initiate tissue cultures in the field [35]. The same author has stated that *in situ* collection techniques come in handy for some species, with sterile seeds or without seeds, or they have short longevity or viability. In some cases, only few individuals of a selected species still restricted to a certain area; therefore, *in vitro* collecting of tissues would be less invasive than removing whole plants and will result in a more efficient method for sampling a large number of plants when seeds are not available [36].

Also, there is impossibility in collecting some species by traditional means, due to a seasonal pattern of development. Some more issues in collecting germ plasm like some organs that are not strictly used for propagation, like shoots of trees, are more easily available for collecting at any time, the deterioration of plant material, due to natural processes and microorganism attack which affect material integrity and the excessive volume and weight of certain fruits (Ex: Coconut) [11]. Due to various limiting factors mentioned above, *in vitro* collecting broadens the possibilities for collecting living tissues. The material to be collected depends on each species, in theory, almost any part of the plant is sufficient to regenerate a whole organism under the appropriate growth conditions because of the cell totipotency.

For species producing orthodox seeds, the most common way to acquire plant material is through seed collection; nonetheless, different circumstances, such as seed absence or inadequate seed development, may hinder seed collection and for these cases, zygotic embryos or vegetative tissues, like bud woods, shoots, apices or leaves, can be collected. For vegetatively propagated species, it is necessary to collect stakes, pieces of bud wood, tubers or corms [17].

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Mst suitable tissue for *in vitro* collecting size of the tissue, soil residues and presence of diseased tissue sterilization of plant tissue and procedure to remove the disinfectant nutrient medium and the conditions of storage

Furthermore, *in vitro* collecting may pose more challenges beyond those of normal tissue culture, as work is done in the field and culture exposure to air-borne contaminants maybe unavoidable [35]. Microorganism removal is a critical factor that must be strictly controlled during *in vitro* collecting of plant material. The first *in vitro* collecting systems were developed for *Theobroma cacao L.* (Cocoa) and *Cocos nucifera L.* (coconut), generating two *in vitro* collecting methods that were used as a model to develop other protocols [37]. *In vitro* collecting represents an alternative for rare and endangered species, since usually thismaterial is limited in supply and seed collection may be restricted. The removal of small amounts of appropriate tissue from the plant should not harm *in situ* populations [38].

***In vitro* conservation of horticultural crops**

Slow growth technique has been successfully applied for plant species of both temperate and tropical origin,

including diverse types of crops, forest trees, endangered species and medicinal plants [39]. The first report of successful *in-vitro* storage was of shoot tips of *Vitisrupestris* where at 9° C, cultures could be stored up to 290 days. In several cases, the cultures are maintained at standard temperature [17]. Yet, satisfactory storage durations are obtained only with slow growing species. Almost all tropical species are cold sensitive. Therefore, when these stored at low temperatures physiological damages likechanges in the metabolism, protein content, composition and functioning of the membranes caused by chilling injuries are induced. These disorders increase with the degree of chilling, but they are generally reversible following short-term exposure to low temperature. The storage temperature depends on the cold sensitivity of the species.

In vitro Conservation of Ornamental Plants

In this section, the application of slow growth storage and cryopreservation techniques for the medium- and long-term conservation of ornamental plants is discussed. Up to now, most of the *in vitro* conservations of ornamental plants are based on the cold storage approach. Under this, ornamental plants are generally stored just a few degrees above the freezing (mainly, at 4–5°C) and most of the researchers prefer storage in total dark conditions to slow down cell metabolic activities.

However, storage under low light intensity (from 3 to 8 mmol/m^2/s) showed to be effective for shoot cultures of *Humulus* spp. [40]. In general, maximum time of conservation of shoot cultures in cold conditions ranges from some months to 1 year, and ornamental species are not exception. As for synthetic seeds of ornamentals, have a shorter time of conservability, ranging from 1.5 to 9 months. But, in *Hibiscus moscheutos* (almost 20 months of storage [41] and in *Splachnum ampullaceum* (30 months [42] showed an exception.

In vitro seed germination has been widely employed for multiplication of a large number of orchid species and it could be a fast mean for multiplying rare and endangered orchids. *In vitro* seed germination, micro propagation, somatic embryogenesis, zygotic embryo culture and callus culture systems have been developed successfully for a considerable number of native endangered species in Sri Lanka [43].

In vitro multiplication of spontaneous flora species of *Arnica montana* L, *Dianthus spiculifolius* Schur, *Drosera rotundifolia*, *Leontopodium alpinum* Cass, *Syringa josikaea* and *Sequoia sempervirens* in order to conserve and repopulate their natural areas, as the technique ensures the obtainment of a large number of individuals in a relatively short time-span, identical from a phenotypical and genotypical stand point with the parent-plant from which the tissue is harvested originally[44]. *Vriesea inflate*, an ornamental bromeliad has been successfully preserved using slow growth technique at 15°C and can be transferred to growth at *ex vitro* condition to achieve 100% survival rate [45].

In vitro conservation of medicinal plants

The World Health Organization has estimated that 65-80% of the world population's primary health care needs are met through plant-based traditional medicines [46] and there are more than 34,000 plants used for medicinal purposes worldwide [47]. Hence, an unregulated trade in medicinal plants has been emerged mainly from the wild which continue to grow dramatically in the absence of serious policy attention with environmental planning. As a consequence, some medicinal plants have become endangered and the problem of scarcity is escalating alongside the needs of the exploding world population. Therefore, requirement of reliable and efficient conservation methods for medicinal plants are becoming more and more significant. The conservation of threatened germplasm can be accomplished by storage in seed banks, field preservation, *in vitro* culture, and cryopreservation [48]. For medicinal plants, long-term preservation of plant propagules in plant tissue culture repositories is a more practical option. *In vitro* conservation and propagation of endangered germplasm has three main components as establishment of sterile cultures of the maternal plant tissue through meristem culture or axillary node culture, development of protocols for large-scale propagation of the plants from the living germplasm bank and long-term storage of the genetic resources[48].Wild-harvested plants can be introduced into culture through collection and surface sterilization of meristems. Axenic shoot cultures of selected medicinal species can be maintained under conditions that permit minimal growth for preservation. Cultures remain viable for 1-2 years at low temperatures (4-10°C) under optimized growth conditions though long-term preservation requires cryopreservation. Shoot tips explants of *Aloe vera* reported to maintain their regeneration frequency with morphogenetic competence even after prolonged *in vitro* culture [49]. Synthetic seeds are another viable option for conservation of medicinal plants. Some important medicinal plants as *Stevia rebaudiana*, *Valeriana wallichii*, *Gentiana kurroo*and *Lavandula officinalis* have been successfully conserved as synthetic seeds [50]. Synthetic seed production as a tool for the conservation and domestication of *Celastrus paniculatus* one of the rare medicinal plant in Sri Lanka has also been attempted [51].

In vitro conservation of forest species

For long-lived plants that have to cope with high temporal and spatial environmental heterogeneity, genetic diversity is of prime importance for species persistence. With continues deforestation, environmental pollution, climatic change and collection for various purposes many forest species are under threat of eradicating from the world. The preservation of genetic resources faces a continuing challenge whenever the long-term survival of forest tree populations is at stake due to the reduction of their genetic potential for adaptability [52]. Therefore, conservation of threatened forest species plays an important role in preserving the existing biodiversity. Conservation strategies for forest tree species differ from those employed in agricultural or wild short-lived plants. Though maintaining *in vitro* cultures under slow growth conditions is a feasible option, it has shown more success in herbaceous species rather than woody species where seeds of some species could possibly be stored under *in vitro* conditions, which stimulate the under-canopy conditions that arrest development in the wild[26].Synthetic seeds were successfully produced from woody species include shoot meristems of *Eucalyptus gunnii* [53] and somatic embryos of walnut [54].*Cedrela fissilis*, an economically important tree of the Brazilian Atlantic Forest has been conserved through synthetic seeds

and cryopreservation [55].Whole seeds and embryonic axis of *Swietenia macrophylla* and whole seeds of *Melia azaderach* have been successfully cryopreserved [56]. A procedure for the micropropagation and *in vitro* conservation of *Vriese areitzii*a, a bromeliad threatened of extinction from the Brazilian Atlantic Forest has been described [57]. Isozymes and DNA markers are useful in forest resource conservation because they can be used to identify resource populations, since today's genetic diversity in forest trees is predominantly the result of plant history [58]. DNA banks, tissue and cell cultures and cryopreservation provide excellent avenues and opportunities for ex-situ conservation of forest genetic resources [59]. The *in vitro* conservation techniques allow material exchanges among germplasm banks, and the germplasm keeps its sanitary conditions and viability during the transport [60].

Conclusions

Successful conservation methods are a crucial factor for the sustenance of plant genetic resources as some of them are rapidly depleting due to number of reasons. This paper, has presented some possibilities offered by biotechnology for improving ex situ conservation of plant biodiversity, through *in vitro* conservation, cryopreservation and DNA storage. These methods are mainly focused on conserving non-orthodox seed and vegetatively propagated species, rare and endangered species, as well as species with some economic value. In recent years, progress has been especially important in the area of cryopreservation, with the development of vitrification-based protocols. Still in many cases, research may be required to optimize the methods and to validate them on a range of genetically diverse accessions. All together biotechnological approaches can be identified as the most suitable way for plant germplasm conservation for longer periods.

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