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Review article

A REVIEW ON *IN SITU* AND *EX SITU* CONSERVATION STRATEGIES FOR CROP GERMPLASM

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INTRODUCTION

Plant Genetic Resources (PGR) is the basic material for launching a crop improvement programme. PGR, also known to be germplasm is a vital segment of biodiversity in general and agro-biodiversity in particular, constitute the genetic material of plants having value as a resource for present and future generation of human being (Dhillon and Saxena, 2003; LEISA, 2004). 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 (Ogbu *et al.,* 2010). The components of PGR include landraces and farmers' varieties, obsolete cultivars, modern cultivars, breeding lines and genetic stocks, wild relatives, weedy races and potential domesticate species, exotic and indigenous species (FAO, 1996; Engels and Visser, 2006; Sharma, 2007). Conservation refers to protection of genetic diversity of crop plants from genetic erosion. Conservation of plant genetic resources for food and agriculture as one sector of biodiversity, is considered to be a major element of any strategy to achieve sustainable agricultural development (Withers and Engelmann, 1998). The major threats for food security in long term and loss of diversity are replacement of highly diverse local cultivars and land races of traditional agro-ecosystems by genetically uniform modern varieties, deforestation, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species, climate change, changing life styles, globalization, market economies, over-grazing and changes in land-use pattern (Kaviani, 2011). Therefore, essential measures must be taken for the conservation of the genetic variability of natural populations. There are two distinct methods of plant germplasm conservation viz., *in situ* and *ex situ*. *In situ* conservation involves conservation in the natural habitats i.e., wilderness areas, reserves, protected areas and within traditional farming systems (so called, on-farm conservation). *Ex situ* conservation involves removal of the plant genetic resources from their natural habitat and placing them under artificial storage conditions (Withers and Engelmann, 1998). *Ex situ* conservation provides the opportunity to study the biology of and to understand the threats to endangered species, in order to eventually consider successful species recovery programs, which would involve restoration and reintroduction. It also has the advantage of preserving plant material and making it available for research purposes, without damaging the natural populations (Temitope, 2013).

Approaches of *In situ* conservation:

In this approach, plant species are promoted to grow in their natural habitats where evolutionary processes continue to operate; thus making it a dynamic system (Ogbu *et al*., 2010). This method had number of advantages that includes conserving a large range of potentially interesting alleles, enhance and ensure sustainable use of genetic variation for present and future human needs, assures protection of associated species and facilitates research on species in their natural habitats, thus allow a better evaluation and utilization. In particular, this method of conservation is of significance to the wild relatives of crop plants and a number of other crops, especially tree crops and forest species where there are limitations on the effectiveness of *ex situ* methods of conservation. *In situ* conservation, in addition to natural habitats in protected areas and national reserves, also need to be carried out on-farm in the areas where landraces and locally adapted farmers varieties are cultivated. This requires active farmers participation to conserve landraces and traditional farmers varieties. The novel genetic resources may be conserved even in home gardens (Rathore *et al*., 2005; Tao, 2003).

With *in situ* approach, however, there are many disadvantages like cost, size and maintenance aspects (over-exploitation, competition from invasive alien species, genetic drift and inbreeding and human disturbances), cover only very small portion of total diversity of a crop species, political and social issues, and the danger of genetic wipe out as a result of natural disasters, fire etc. In such situations, the alternative way to conserve diversity is to maintain it *ex situ*.

Approaches of *Ex situ* conservation:

An approach to *Ex situ* conservation includes classical methods like seed storage in seed banks, field gene banks and botanical gardens. When the biological material (organs, seeds) cannot be stored in a traditional/classical manner, biotechnological methods like *in vitro* storage and cryopreservation are alternative approaches for *ex situ* conservation of such plant material.

Classical approaches:

Seed banks: Seed storage is the most convenient method of long-term conservation for plant genetic resources (Kaviani, 2011). Seeds are classified mainly on the basis of their storability, into two major groups *viz*., Orthodox and Recalcitrant seeds (Roberts, 1973).

Orthodox seeds	Recalcitrant seeds
Amenable to dehydration to 5% or less (dry weight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible.	Cannot withstand much desiccation, relatively high critical water content value (10-12% or 20% of fresh weight). They remain viable only for a short time (weeks or months), even if kept in the required moisture conditions.
Examples: most of the agricultural crop seeds	Examples: mostly seeds from tropical and sub tropical species; oil palm, coconut, cacao, rubber, mango, jack fruit and coffee etc.

Seed bank collections classification:

Storage	Base collections	Active collections	Working collections
Period	Long term (~50 or more)	Medium term (8-10 years)	Short term (3-5 years)
Temperature	-18 or -20^{0}C	0^{0}C	5-10^{0}C
Moisture	5±1 %	8 %	8-10 %
Use	Disturbed only for regeneration	Breeding programmes	Crop improvement programmes

The main drawback of seed storage is that its wide application is confined to orthodox seeds only. Moreover, there are practical problems in applying long term seed storage to most long live forest trees, including gymnosperms and angiosperms, since their juvenile period is very long and they do not produce seeds for several years (Engelmann, 1991). Different categories of crops that present problems for seed storage (Withers and Engelmann, 1998) are mentioned as: 1) Crop species that do not produce seed at all and are vegetatively propagated, for example: banana and plantain (Musa spp.). 2) Vegetatively propagated species such as cassava, potato, yam, sweet potato and sugar cane etc. 3) Recalcitrant and intermediate seeds 4) Some seeds remain viable only for a limited duration and 5) Some seeds deteriorate rapidly due to seed borne pathogens. These problem materials require field gene bank for their conservation.

Field gene banks/plant bank:

Field or plant bank is an orchard or a field in which accessions of fruit trees or vegetatively propagated crops are grown and maintained. The genetic resources under consideration are readily accessed and observed, permitting detailed evaluation (Withers and Engelmann, 1998). Conservation in field gene bank is very difficult to carry out due to the following limitations: an adequate sample has to be taken for the conservation of genetic diversity, more space requirement, expensive, requires trained personnel, cumbersome to manage and are vulnerable to natural vagaries (genetic erosion).

New or biotechnological approaches:

New or biotechnological approaches *viz., in vitro* slow growth storage and cryopreservation are developed for conservation of the recalcitrant and vegetatively propagated species where conventional methods are not applicable. Both the techniques use tissue culture principles for conservation (Roca *et al*., 1989; Reed, 1993; Mandal, 2003).

The advantages associated with tissue culture systems include clonal propagation, production of insect and disease free material, zero genetic erosion, requires short time for obtaining new plants, less space and less labour intensive, allows safe exchange of material and lead to genetic modifications like somaclonal variations (stress factor resistance, production of useful compounds etc.). The *in vitro* storage of large quantities of material induces various problems such as reduction in the populations genetic base since very small size samples are taken for conservation, management of plant material is difficult, which needs to be subcultured and risks of genetic variation, which increase with in vitro storage duration and can lead to loss of trueness to type (Kaviani, 2011; Engelmann, 1991 and Vasile *et al.,* 2011).

In vitro conservation

In vitro slow/normal growth techniques offer up to medium-term storage option, avoiding risk of losses of germplasm on field gene bank due to insects, nematodes, disease attacks and natural disasters. It is commonly used for vegetatively propagated species, non-orthodox seeded species and wild species which produce little or no seeds (Ogbu *et al.,* 2010).

The art and science of plant tissue culture is based on devising media for each genotype that would elicit the optimal response in terms of growth rate of the explants. However, when tissue techniques are employed for conservation, the aim is to devise a medium that would decrease the growth rate of explants to the minimum, thereby increasing the subculture intervals. Slow growth techniques have been developed for medium-term conservation of crop species (Engelmann and Drew, 1998; Sarkar and Naik, 1998 and Ogbu *et al.,* 2010).

The various methods used to achieve this includes use of growth retardants, use of minimal growth media, use of osmotic regulators, reduction in oxygen concentration, size and type of culture vessels, type of enclosures, maintenance under reduced temperature and light intensity and combination of more than one treatment. Explants used for in vitro conservation must be of right type as well as physiological stage. The apical and auxiliary meristems of very small size are the preferred explants for in vitro storage. In fact, organized explants have proved better than unorganized tissues, in terms of genetic stability of the germplasm (Mandal, 2003; Reed *et al.,* 2004; Chaudhury and Vasil, 1993; Kameswara Rao, 2004 and Ogbu *et al*., 2010).

Cryopreservation

Cryopreservation refers to the non-lethal storage of biological tissues at ultra-low temperature, usually that of liquid nitrogen (LN) which is -196°C. Currently, it is the only option available for the long-term conservation of germplasm of vegetatively propagated and recalcitrant seed species. Due to storage at the temperature of the vapor phase (-150 to -180° C) or liquid phase (-196° C) of LN, cell divisions and metabolic activities are arrested and thus, plant material can be stored for unlimited periods of time. Conservation of germplasm using cryogenic approach required very limited space; the plant material stored is protected from exogenous contamination and needs very limited maintenance. It causes no change in viability, vigor and genetic makeup of the conserved materials. It also eliminates the need to test stored materials frequently, thus making storage cost-effective (Kameswara Rao, 2004 and Ogbu *et al*. 2010).

Cryopreserved explants (but pollen) should eventually regenerate whole plants to be used and therefore, regeneration protocols need to be clearly defined prior to embarking on crypopreservation. Regenerated plants should also maintain genetic integrity of the starting material (Ogbu *et al*. 2010).

Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing (Kaviani, 2011). The various techniques currently in use include classical and new cryopreservation techniques (Kameswara Rao, 2004; Withers and Engelmann 1998). Classical techniques involve freeze-induced dehydration, whereas new techniques are based on vitrification. Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice (Fahy *et al.,*1984).

Classical freezing procedures include the following successive steps: pre-growth of samples, cryoprotection, slow cooling (0.5–2.0°C/min) to a determined pre-freezing temperature (usually around −40°C), rapid immersion of samples in liquid nitrogen, storage, rapid thawing and recovery. Classical techniques are generally operationally complex since they require the use of sophisticated and expensive programmable freezers. In some cases, their use can be avoided by performing the slow-freezing step with a domestic or laboratory freezer (Kartha and Engelmann, 1994).

In vitrification based procedures, cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotective media and/or air desiccation. This is followed by rapid cooling. As a result, all factors that affect intracellular ice formation are avoided. Vitrification based procedures offer practical advantages in comparison to classical freezing techniques. Like ultra-rapid freezing, they are more appropriate for complex organs (shoot tips, embryos), which contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration.

By precluding ice formation in the system, vitrification based procedures are operationally less complex than classical ones (e.g. they do not require the use of controlled freezers) and have greater potential for broad applicability, requiring only minor modifications for different cell types (Engelmann, 1997). Engelmann (2000) described seven vitrification based procedures in use for cryopreservation, which include: (i) pre-growth (ii) dehydration (iii) pre-growth -dehydration (iv) encapsulation-dehydration (v) vitrification (vi) encapsulation-vitrification and (vii) droplet-vitrification.

i) Pregrowth:

The pregrowth technique consists of cultivating samples in the presence of cryoprotectants and then freezing them rapidly by direct immersion in liquid nitrogen. The pre-growth technique has been developed for Musa meristematic cultures (Panis *et al*., 2007).

ii) Dehydration:

Dehydration is the simplest vitrification-based procedure, since it consists of dehydrating explants and then freezing them rapidly by direct immersion in liquid nitrogen. This technique is mainly used with zygotic embryos or embryonic axes extracted from seeds. It has been applied to embryos of a large number of recalcitrant and intermediate species (Engelmann, 2000).

Desiccation is usually performed in the air current of a laminar airflow cabinet, but more precise and reproducible dehydration conditions are achieved by using a flow of sterile compressed air or silica gel. Ultra-rapid drying in a stream of compressed dry air (a process called "flash drying" developed by Prof. Berjak's group in South Africa) allows freezing samples with relatively higher water content, thus reducing desiccation injury (Berjak *et al.,* 1989). However, optimal survival is generally obtained when samples are frozen with water contents between 10 and 20% (fresh weight basis).

iii) Pre-growth -dehydration:

The pre-growth- dehydration procedure involves pregrowth of explants in the presence of cryoprotectants, dehydration under the laminar airflow cabinet or with silica gel and then rapid freezing. This method has been applied notably to asparagus stem segments, oil palm polyembryonic cultures and coconut zygotic embryos (Uragami et al., 1990; Assy-Bah and Engelmann, 1992; Dumet *et al.,* 1993).

iv) Encapsulation-dehydration:

Cryopreservation using the encapsulation-dehydration procedure has been very effective for freezing apices of different plant species from temperate and tropical origin (Gonzalez-Arnao and Engelmann, 2006).

The basic protocol comprises encapsulation, preculture of alginate coated samples in liquid medium with high sucrose concentration, desiccation, rapid cooling and slow rewarming. It is important to note that after such a drastic drying process (desiccation down to around 25% moisture content in alginate beads, fresh weight basis), survival of explants after thawing may become independent of the warming rate, as noted for example with carrot somatic embryos (Dereuddre *et al.,* 1991) and orchid seeds with fungal symbiont (Wood *et al.,* 2000). For recovery, encapsulated samples are generally placed on standard culture medium without having to extract the explants from their alginate coating (Gonzalez-Arnao and Engelmann, 2006).

v) Vitrification:

The freezing procedure referred to as vitrification comprises a pretreatment (loading treatment) at room temperature, followed by exposure to a vitrification solution at 25° or 0°C, rapid cooling and warming, and final removal of the vitrification solution by washing samples with an unloading solution consisting of liquid culture medium supplemented with 1.2 M sucrose (Withers and Engelmann, 1998).

The most frequently used and efficient vitrification solution (Sakai *et al.,* 1990) so far is PVS2 (30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% (w/v) DMSO in culture medium with 0.4 M sucrose). However, direct exposure of samples to any vitrification solution often leads to detrimental effects due the toxicity caused by their high concentration (over 7 M for PVS2).

A pretreatment with cryoprotectants at a lower concentration has proved to significantly increase dehydration tolerance, and to mitigate the mechanical stress caused by the subsequent treatment with a highly concentrated PVS (Takagi, 2000; Thin and Takagi, 2000; Sakai, 2004). A mixture of 2 M glycerol + 0.4 M sucrose in liquid medium, termed "loading solution" (Nishizawa et al., 1993), applied for 20 min at room temperature is very effective to enhance osmotolerance (Sakai, 2004).

vi) Encapsulation-vitrification

Encapsulation-vitrification is a combination of the encapsulation-dehydration and vitrification procedures, where samples are encapsulated in alginate beads, and then subjected to freezing following the vitrification approach.

vii) Droplet-vitrification:

The droplet-vitrification technique is characterized by increased cooling and warming rates compared to other vitrification-based procedures, since samples are frozen in minute droplets of PVS placed on aluminium foil strips, which are plunged directly in liquid nitrogen. This protocol significantly increases the probability of obtaining a vitrified state during freezing, and of avoiding devitrification during warming (Panis et al., 2005).

Gene banks for various crops in India

Institutes	Crops
Central Institute for Cotton Research, Nagpur	Cotton
Central Plantation crops Research Institute, Kasargod	Plantation crop
Central Potato Research Institute, Simla	Potato
Central tobacco research Institute, Rajahmundry	Tobacco
Central tuber crops research Institute, Thiruvananthapuram	Tuber crops other than potato
Central Rice Research Institute, Cuttack	Rice
Directorate of Oilseeds research, Hyderabad	Oilseeds
Directorate of Wheat Research, Karnal	Wheat
Indian Agricultural Research Institute, New Delhi	Maize
Indian Grassland and Fodder Research Institute, Jhansi	Forge and fodder crops
National research centre for sorghum, Hyderabad	Sorghum
International Crops Research Institute for Semi-Arid Tropics	Groundnut, Pearl millet, Sorghum, Pigeon pea and Bengal gram

List of important International Institutes conserving germplasm

Name	Institute	Activity
IRRI	International Rice Research Institute, Los Banos, Philippines	Tropical rice
CIMMYT	Centre International de-Mejoramients de maize Trigo, El Baton, Mexico	Maize and wheat (Triticale, barely, sorghum)
CIAT	Center International de-agricultural Tropical Palmira, Columbia	Cassava and beans, (also maize and rice) in collobaration with CIMMYT and IRRI
IITA	International Institute of Tropical Agriculture, Ibadan, Nigeria.	Grain legumes, roots, and tubers, farming systems.
CIP	Centre International de-papa-Lima. Peru	Potato
ICRISAT	International Crops Research Institute, for Semi-Arid Tropics, Hyderabad, India	Sorghum, Groundnut, Pearl millet, Bengal gram, Redgram.
WARDA	West African Rice Development Association, Monrovia, Liberia	Regional Cooperative Rice Research in Collaboration with IITA and IRRI
IPGRI	International Plant Genetic Research Institute, Rome Italy	Genetic conservation.
AVRDC	The Asian Vegetable Research and Development Centre, Taiwan	Tomato, Onion, Peppers Chinese cabbage.

CONCLUSION

Biodiversity plays a great role in human existence and in healthy function of natural systems although it is on the way of depletion dominantly due to anthropogenic activities. This requires conservation of biodiversity either in *in situ* or *ex situ* or both methods in combination based on the conservation situation and its objective. Although in situ conservation is more encouraged to be used for biodiversity conservation, ex situ conservation is recommended as it complements through different techniques like zoo, captive breeding, aquarium, botanical garden and gene bank. *Ex-situ* conservation of crop diversity is a global concern, and the development of an efficient and sustainable conservation system is a historic priority recognized in international law and policy (Ola T. Westengen et al., 2013). The challenges for the future in the area of plant genetic resources conservation are technical and scientific, socio-economical, legal and political, including public awareness (Esquinas-Alcazar, 2005).

The field of germplasm conservation will have to address how to best integrate technological advances in the areas of molecular genetics, genomics, cryopreservation and other conservation techniques, and geographic information system to further facilitate conservation and utilization of these resources.

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