International Journal of Biology Research
www.biotechjournals.com
Volume 1; Issue 1; March 2016; Page No. 10-15

In vitro Germplasm collection and storage: A review

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Abstract
The efficient use of plant genetic resources requires the careful collection of germplasm, its conservation, evaluation, documentation and exchange. Over the last few decades, plant tissue culture has been used to propagate hundreds of plant species. The use of this technique has been particularly important for conservation and multiplication of plants that produce seeds, propagated vegetatively, recalcitrant or have unpredictable seed germination. *In vitro* collection is a natural outgrowth of this work. Tissue culture being a basic component of plant biotechnology; can expand the possibilities for obtaining plant germplasm irrespective of the nature of the material. It can supplement seed collecting, providing an alternative source of material for propagation and preservation when seeds are not available. Even so, as an *in vitro* operation with a certain risk of genetic instability is preferable to losing the material completely. Similarly, taking more than one type of explants can provide more opportunities for recovering plants. *In vitro* collection is an extension of laboratory activities and once the tissue is brought into the laboratory, further measures can be taken to acquire or maintain sterility. It is technically relatively easy; success depends entirely on the availability of methods for growing and multiplying the original explants *in vitro* in order to produce complete plants. In several cases (e.g. cacao, cotton), although the *in vitro* collection protocols successfully provided living material to the laboratory, the lack of techniques for maintaining and propagating plants from that tissue precluded the widespread application of the technique. Similarly, a better understanding of the basic biology of regeneration would likely improve the ability to regenerate plants from *in vitro* collected explants, thereby increasing the possibilities for collection. Currently, the number of species that have been the object of *in vitro* collection is limited, but as this number grows, improvements in the technique will naturally follow. The paper considers *in vitro* collection of germplasm as an alternative sustainable means of capturing plant genetic diversity during collection expedition.

Keywords: germplasm, *in vitro*, totipotency, conservation, tissue culture protocol.

Introduction

A wide genetic base helps to contain biotic and abiotic stresses that destroy entire crop stands. Steady and progressive genetic erosion has resulted in a marked decrease in plant genetic diversity. Developing countries, particularly those in the tropics, are still the repositories of vast biodiversity in their agro-ecosystem and act directly or indirectly as reservoirs and suppliers of germplasm. These sites are regarded as genetic "hot spot" for plant genetic collection and conservation. Germplasm collections can range from collections of wild species to elite, domesticated breeding lines that have undergone extensive human selection. Plant genetic diversity must be preserved and to achieve this, a well-planned exploration and collection expedition is needed. This is important in order to collect enough plant germplasm that will represent as much diversity needed for conservation purpose.

Germplasm is the living genetic resources such as seeds or tissue that is maintained for the purpose of plant breeding, preservation, and other research uses. however, the loss of biodiversity due to exploitation of natural populations, harvest without permits from the wild, natural hazards, cultural, political and economic issues, pose a great threat to plant genetic resources (Valarie *et al*, 2002) [18, 13, 10]. Wild and cultivated species stand to gain immensely from *in vitro* techniques of conservation in genebanks. Although many genebanks exist worldwide today, only about 30 countries provide secure long-term storage because there is little provision for long-term sustainable management of genebanks. The world's 7.5 million genebank accessions are largely of the crops on which humans and livestock most rely for food and feed, including important wild relatives and landraces, but others are of crops of local importance and underutilized species (FAO, 2014) [7]. As a result of these militating factors there is need to collect and store plant genetic resource for future sustainable usage.

Method of germplasm storage depends on the nature of germplasm material: seed or non-seed (clonal material). Methods for the storage of seeds are a factor of storage tolerance of the seed to low temperature and dry environment, seeds are further divided into storable (orthodox) and non-storable (recalcitrant) types. The orthodox types include small-seeded grains crops and vegetables. They can be stored for a long time under low-temperature and low-humidity environment. This storage could be done on a long, medium or short-term periods in accordance to the purpose of storage and the viability of the germplam are important. The recalcitrant

type are classified as non-storable and therefore cannot be stored under low temperature and humidity conditions, examples are certain vegetables, cocoa, mango and so on. Such seeds may be stored in low temperature ranges (0-10 ^{0}C) for short periods such as 1-5 years. While storage of vegetatively propagated materials is attained by a number of procedures which includes: tissue culture, cold storage, cryopreservation in liquid nitrogen and field maintenance.

Limitations in Conventional Germplasm Storage Methods
Over the last 30 years, there has been significant increase in the number of plant collections and in accessions in *ex situ* storage centres throughout the world. Most of these collections are seed based. However, seeds of a number of species, predominantly tropical or subtropical, belong to the recalcitrant or intermediate categories; have a limited longevity which renders their long-term preservation impossible. For example, *Cocos nucifera* (coconut), *Theobroma cacao* (cacao), and many tree and shrub species have seeds which are physiologically immature when shed, have high moisture content, cannot withstand much desiccation and are sensitive to chilling, therefore cannot be stored in low temperatures. Others, such as *Coffea* spp. and oil palm (*Elaieis guineensis*) can be stored only for a short period of time and long term conservation remains unattainable (Ellis *et al*., 1990, 1991) [5, 6]. Whatever the cause, the final result of these difficulties may be the loss of valuable germplasm, loss of invested funds and an expedition that was deficient in terms of geographic coverage and sampling of the genetic diversity of the targeted population or area.

More so, plant parts that are not strictly organs of propagation or perpetuity, such as ex-plants from shoot of trees, are more flexible and are likely to be available for collection at any time. Considering the problems involved in germpasm exploration, one must take advantage of as many alternatives as possible to increase the efficiency and effectiveness of an expedition. The above listed limitations associated with conventional germplasm collection and storage, have necessitated the development of alternative, the use of *in vitro* technique.

***In vitro* Germplasm Collection and Storage: A Tissue Culture Approach**
Tissue culture is a process that involves exposing plant tissue to a specific regimen of nutrients, hormones, and lights under sterile, *in vitro* conditions to produce many new plants, each a clone of the original mother plant, over a very short period of time. The technique essentially involves separation of a cell/tissue/organ from the donor plant under aseptic conditions and growing it on a synthetic medium in a suitable container under controlled environment (John and Lorin, 1987) [9]. The concept that the individual cells of an organism are totipotent is implicit in the statement of the cell theory. Schwann (1839) expressed the view that each living cell of a multicellular organism should be capable of independent development if provided with the proper external conditions (John and Lorin, 1987) [9].

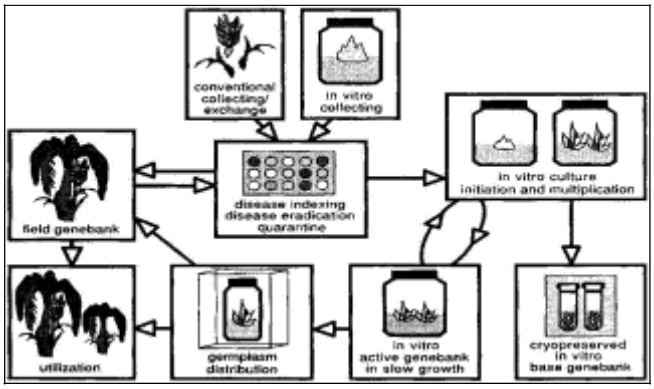
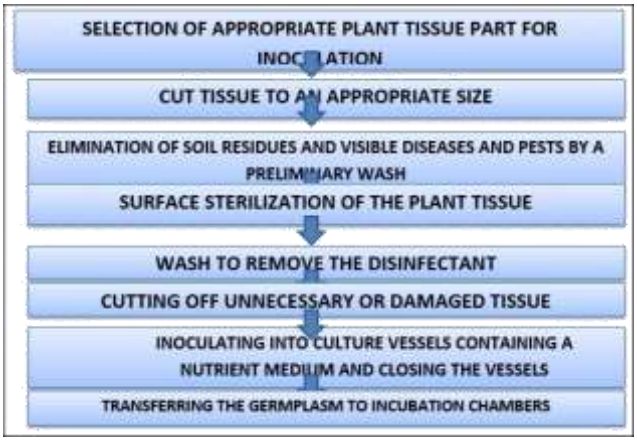


Fig 1: germplasm flow

In vitro collection of plants employs the basics of plant tissue culture. Collection of plant germplasm is in process in many countries especially in centres of diversity of the particular crop plant. Tissue culture systems allow for axenic propagation of plant material with high multiplication rates. Virus-free plants can be obtained through meristem culture in combination with thermotherapy. The miniaturization of explants reduces space requirements and consequently labour costs for maintenance of germplasm collections and recently, help to exchange certain crop germplasm across countries using embryo cultures because of the obvious advantages (figure 1).

Developing a Tissue Culture Protocol
Before embarking on an *in vitro* collecting of a particular species of plant, it is mandatory to first develop an optimum tissue culture protocol for the plant. Previous case studies carried out in cocoa, cotton and some forages illustrated the flexibility of this method (Valarie *et al.*, 2002) [18, 13, 10]. The major constraints to this procedure were lack of a good method for *in vitro* propagation of the collected material. The general basic protocol is that the operations to be carried out in the field should be those that are truly necessary. Such operation is defined by the condition of the plant material, the type of environment found at the collecting site (s) and the length of the trip back to the receiving laboratory. Once a culture has been established, the collected germplasm is safe (at least for the short term). In designing a tissue culture protocol for a plant it is recommended that the taxonomy of the plant be considered. Plants in the same taxa tend to share close affinity in phylogenetic relationship as a consequence of similar physiological, biochemical and molecular characters. Therefore to produce a tissue culture protocol for a test plant starts with a search for an already established protocol of a plant in the same taxa with your test plant or has close taxonomic affinity with the chosen test plant. After this is done, one can then attempt to optimize this protocol by varying parameters used in previous work.

***In vitro* Germplasm Establishment Procedure**
Establishing of germplasm using tissue culture method employs the basic *in vitro* cultures as used in the laboratories.



Methods of *In vitro* Germplasm Conservation/Storage

Prolonged maintenance of *in vitro* material provides an effective system for establishing both active and base germplasm collections particularly for the conservation and maintenance of those plant species that cannot be stored as true seed and are amenable to micro propagation technology. The properties required of a successful storage system are the ability to:

1. Reduce the growth rate of *in vitro* plants, resulting in extension of intervening sub-culture frequencies.
2. Retention of vitroplant viability during storage at maximum simultaneously maintaining genetic stability at minimum.
3. Maintain the full developmental and functional potential of stored material when it is returned to physiological temperature.
4. Make significant savings in labour input, materials and commitment to specialized growing facilities.

Collecting Germplasm *in vitro*

The laboratory facilities required for *in vitro* culture normally include a sterilizer, a laminar flow hood (to provide a clean, sterile work surface), incubators, growth chamber and greenhouse in addition to a pure water source, chemicals, glass and plastic labware, and other standard items of equipment. Some success has been claimed for collecting material in the field directly *in vitro*. The level of sophistication ranges from using a fully equipped local laboratory, for transfer of collected material as soon as possible, to working in the field in a portable glove box or on a clean table with a simple box-like cover to exclude contamination. Following surface sterilization, explants are removed and either inoculated to sterile culture media in the field or held in sterile buffer for later inoculation in the laboratory (Sossou *et al*., 1987) [17].

A cruder alternative is to sterilize tissue explants with nontoxic agents and inoculate them to media containing antibiotics and fungicides. Although there is some potential for using *in vitro* collection for vegetatively propagated crops and those with short- lived seeds, there are serious implications for plant quarantine since the collected explants may carry pests or pathogens that might well be detected or excluded in material cultured by more rigorous methods.

***In vitro* Storage**

There are two approaches to which have proved successful in *in vitro* culture, namely the slow growth and cryopreservation methods. The slow growth approach involves applying retardant chemicals or reducing the culture temperature. Subculture intervals can be extended up to 1 or 2 years, thereby greatly reducing the time, labor, and materials required to maintain the cultures. Slower growth reduces the frequency of cell division and consequently the number of times a random mutation is multiplied in the culture. Such genetic changes that occur in tissue cultures are called somaclonal variations. Stress is an intrinsic factor in slow growth, and little is known about its effect on somaclonal variation. What began as a clonal culture may change into a population of cells consisting of the original genotype plus variant genotypes. Also, stress factors may act differently on such a population of genotypes, favoring some somaclonal variants. This could result in a changed population of cells and the failure to conserve the genetic integrity of the original clonal material. Undifferentiated callus cultures are more susceptible to somaclonal variation than organized tissue systems, such as shoot cultures. Only organized cultures are recommended for slow-growth storage. This technique of long-term root, tuber, or shoot tissue culture storage is well developed for some crops such as banana (*Musa*) (National Research Council, 1993) [14].

Cryopreservation involves suspending growth by keeping cultures at an ultralow temperature, typically that of liquid nitrogen (-196 °C). It offers the prospect of storage for indefinite periods with minimal risk. However, certain cultures can suffer damage during freezing and thawing. Until recently, routine cryopreservation methods were available or under development only for cell suspension cultures. Larger, organized structures frequently suffered serious structural injury and loss of viability.

However, two new approaches to cryopreservation may lead to more widespread applications for genetic conservation. They focus on reducing cell damage from ice crystal formation. One approach is through vitrification of cellular water by a cryoprotectant mixture and the other involves encapsulation of specimens within an alginate gel that is then dehydrated. For vitrification the specimen is infused with a cryoprotectant mixture that promotes the conversion of much of the cellular water into a noncrystalline, vitreous solid when rapidly cooled (Sakai *et al.*, 1990) [15]. For encapsulation the specimen, such as a shoot tip or somatic embryo, is encased in an alginate gel to form an artificial seed. This artificial seed is then dehydrated in her air before cooling (Dereuddre *et al.*, 1990) [3]. The enveloping gel appears to minimize deleterious effect from dehydration and also protects the specimen from physical damage, being larger and more robust than an isolated shoot tip or embryo.

Many technical barriers remain that prevent the routine use of cryopreservation for plant meristems, pollen, and plant cell cultures. No conservation collection or germplasm banks are yet using cryopreservation for non-seed germplasm storage, although several are involved in cryopreservation research.

Adapting *In vitro* Procedures to Field Collections of Germplasm

In adapting *in vitro* procedures to field collecting, several aspects should be remembered: one is that *in vitro* collecting comprises a support activity and is not merely for propagation. The second is that field work implies certain limitations, which is why only essential stages are carried out, whereas

others are left until later when conditions are more sophisticated. Furthermore, additional or alternative steps may be needed to overcome the limitations of fieldwork.

A collecting expedition that various species should require a general approach to accommodate the range of facilities and procedures that must be adapted to the different needs of the materials to be collected and the diverse laboratory treatments following collection. If *in vitro* collecting is to be a supportive technique, rather than the sole method, then this will influence the levels of re-application and the amount of resources and time available for this activity.

Most germplasm collectors have only basic experience in *in vitro* techniques and as such field operations must be designed according to their level of experience, leaving, where possible, all other work for the laboratory for specialist to handle. This is, in fact, the most likely scenario, because it is more logical and easier to train collecting experts in the principles of *in vitro* inoculation than to train them in *in vitro* techniques for specialized collection expeditions (Valarie *et al*., 2002) [18,13,10].

As with the inoculation procedures, the equipment must also be adapted to the field, taking into account the essential requisites of the operation and the need to carry all the instruments into the field, because of limited services. Questions arise, such as 'What is the maximum load that the expedition's vehicles and personnel can take?' 'What is the collection site like?' 'How close is it to the nearest services for electricity and potable water?' Equipment must be strong, easy to operate, require minimal maintenance and, if possible, be multipurpose. For example, the box carrying the instruments and culture vessels should also be able to serve as a working table and/or inoculation 'chamber'. Examples of successful *in vitro* collection highlighted in this review illustrate the great flexibility of this technique in terms of equipment that can be used and the degree of sophistication of the procedures tried. If *in vitro* collection in the field is to be regarded as a 'support operation', then the collector must be able to rely on a suitable laboratory available as soon as the collection is made. Some activities, such as preparing the culture medium, labeling the culture vessels and first sterilizing the instruments, must be done beforehand.

Where possible, work such as sterilizing the inoculated tissue or transferring the inoculum to a complex medium should be left for the laboratory receiving the material. One unique advantage of *in vitro* collecting is its adaptability and flexibility. For this reason, there are no quick and fixed rules, only general guidelines to help adapt this concept to situations with new species.

Applications of *In vitro* Germplam Collections

Plant conservation efforts have traditionally fallen under two complementary and overlapping spheres of activities: Conservation of germplasm of economically important species (driven by strong economic and humanitarian forces) and Conservation of wild germplasm of rare and endangered species (driven by understanding that biodiversity is the cornerstone on which economic benefit from plants is derived). *In vitro* conservation techniques were developed previously to protect the genetic resources of food and other economic species. However, there is also an increasing recognition of their usefulness in other aspects of biodiversity conservation. It will be so surprising to know that only 5% of all plants species have been tested for any beneficial use (Farnswarth, 1998). Therefore considering an alternative collection possibility will further be used to capture a wild range of other wild plant species that have not be studied.

In vitro collection of germplasm finds useful applications in the following areas:

***In vitro* collection of wild or endangered species** Endangered species are generally rare. Little may be known about the growth and phenology of the species and plant may be difficult to access, growing in remote areas, on cliff faces and so on. Because such plants cannot be monitored easily, even the best plans may not successfully coordinate a collecting trip with the production of seed or young growth for *in vitro* collecting. Therefore dealing with endangered species, by definition, means that plant materials will be limited in supply and extreme care must be taken not to harm the in situ population.

***In vitro* collection for botanical research**

In vitro collection can be used for a single taxon and also for broader based collecting by researchers on botanical collecting trips for herbaria or botanical gardens.

***In vitro* collection for biodiversity conservation education** *In vitro* collecting is a "low tech" approach requiring simple tool and procedures for collecting species. It can be adapted as an education tool for learning more about *in vitro* methods especially when the full tissue culture facilities are not available.

***In vitro* collection for germplasm exchange**

The importance of national and international germplasm exchange *in vitro* can hardly be over-emphasized considering the tremendous advantages it offers over conventional methods. Besides occupying far less cargo space, it also affords exchange of axenic plant material free of undesirable contaminants. This is of special significance in plant movement, quarantine and to breeders who can import a large number of clones of choice, multiply them and distribute them among user groups.

In vitro exchange is, therefore catching on fast. The principles are applicable to perennial fruits. It is important to use suitable, impact resistant and well-sealed culture containers. The packaging should provide adequate thermal insulation and protection against rough handling. The culture medium should be of a formation that includes a higher than usual concentration of gelling agent. The most rapid available shipping method should be preferred.

Contamination during *In vitro* Germplasm Collection

The problem of contamination is fundamental to the technique of *in vitro* collection and it can only benefit from the application of new approaches to controlling the growth of microorganisms *in vitro*. It should also be remembered that protocols for dealing with contamination will not necessarily be done entirely in the field.

Generally *in vitro* culture is conducted under aseptic conditions; however the routine aseptic procedures practiced during *in vitro* culture operations seldom control systematic microorganisms present in the plant (endogenous microorganism), even under good laboratory conditions.

Asepsis is a requisite for *in vitro* culture and is perhaps the greatest constraint in developing *in vitro* collection protocols. Different tissues and different species may show differing sensitivities to surface sterilants, which must be considered. Explants that are covered by bracts, foliar sheaths, or are enclosed in capsules or other types of structure, may be surface sterilized by flaming with alcohol, thereby reducing the probability of contamination. With many species, however, the morphology of the plant does not permit this approach and the integrity of the surface tissue must be preserved for further growth *in vitro*.

Most field collected tissues usually harbour some contaminating microorganisms even after surface sterilization and these must be overcome with antimicrobial agents, including fungicides and antibiotics. A variety of choices are available, as evidenced by the variety of compounds outlined in this review. The use of non-traditional compounds, such as PPM, is increasing, as well.

In vitro collecting poses contamination challenges beyond those of normal tissue culture. Work is done in the field, often in the open air thereby increasing the chances of contamination.

Factors Affecting Contamination of *In vitro* Collected Cultures

1. **Age**: Older plants tissue taken later in the growing season are often more infected than younger plants tissue (Bernstein and Carool, 1977) [2]
2. **Position**: Underground tissues, such as roots, rhizomes and corms, generally have high levels of endogenous contaminants and can be extremely difficult to clean (Smith *et al*, 1999) [16].
3. **Complex tissue:** Vegetatitive and floral buds often habour contaminats in complex tissue which can protect even external microorganism from surface sterillant (Merkle *et al*., 1997) [11].
4. **Environment:** Contamination may also be affected by environment. Explants taken from plants in a moist tropical site has higher rate of contamination than those from a temperate site (Pence 1999). On the other hand desert species appear to have less surface contamination by bacteria and fungi and are more easily disinfected than tissue from moister areas (McKay, 1999) [12].

Table 1: Some Examples of Plant Species for Which Various Antibiotics Have Been Used In Plant Tissue Culture

***In vitro* Plant species**	**Antibiotics**	**Concentration**	**Reference**
Coffea arabica L.	Gentamycin	100 mg/l	Lozoya saldana *et al*., 2002
Hevea brasiliensis Muell. Arg.	Tetracycline	1000 mg/l	Medanha *et al*., 1998
Musa spp	Ampicillin	100 mg/l	Montoya henao, 2002
	chloramphenicol	100 mg/l	Montoya henao, 2002
	Gentamycin	100 mg/l	Montoya henao, 2002
Theobroma cacao L.	Rifampicin	15-50 mg/l	Dohem *et al* 1988
	Trimethoprim	15 mg/l	Dohem *et al* 1988
	Cefotaxine	100 mg/l	Fenning *et al* 1993
Gossypium hirsitum L.	Cetotaxine	100-1000 mg/l	Agrawal *et al* 1998 [1]

Table 2: Some Examples of Fungicides Used In Plant Tissue Culture Media

Fungicide	**Chemical name**	**concentration**	***In vitro* plant species**
Amphotericin		10 mg/l	Cattlega aurantiaca (Batem)
$C_{47}H_{73}NO_{17}$		2.5 mg/l	Eucalyptus grandis (w. Hill ex Maiden)
Benomyl	Carbomic acid-methyl ester	100 mg/l	Sechium edule (Jacq.)
		1-2 g/l	Erythrina L.
		1-1.5 g/l	Coffea Arabica
		10-50 mg/l	Nicotiana tabacum L.
		100 mg/l	Variety of tropical
		1 g/l	Dendrocalamus giganteus (Munro.)
		100 mg/l	Theobroma cacao L.
		100 mg/l	Musa spp. L.
		50 mg/l	Citrus sinensis L.
Bavistin	1-H-Benzimidazol-2-ylcarbamic acid methylester	10-50 mg/l	Niotiana tabacum L.

Source: Adapted from Valarie *et al.*, 2002 [18, 13, 10]

Conclusion

The paper has highlighted and demonstrated the importance of an alternative germplam collection method and storage. *In vitro* collection can be an important tool for solving a number of the problems associated with germplasm collection. *In vitro* collection is one way of increasing this number the number of Germplasm collections in gene banks worldwide. A major priority is, therefore, to prepare proposals for analyzing this possibility in plant species that produce recalcitrant seeds or propagate asexually. Another theoretical-practical alternative should be to refine principles and methods, to update existing knowledge and to integrate *in vitro* collection into the overall goals for specific crops and geographic and political areas.

In vitro collecting demonstrates the applicability and flexibility of tissue culture, a technique that was originally developed for research and then for propagation. Further research will not only broaden the application of *in vitro* collecting to areas such as botanical collecting, basic research and education, but will provide the area of plant tissue culture with an increasing body of information on the growth and

response of yet untested species and plant varieties *in vitro*. The conservation and utilization of plant genetic diversity, on which we all depend, can only benefit from these activities.

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