IPGRI

In vitro collecting techniques
for germplasm conservation

Valerie C. Pence, Jorge A. Sandoval, Victor M. Villalobos A. and Florent Engelmann, editors

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J.M.M. Engels, volume editor

Introduction to the Series

The Technical Bulletin series is targeted at scientists and technicians managing genetic resources collections. Each title will aim to provide guidance on choices while implementing conservation techniques and procedures and in the experimentation required to adapt these to local operating conditions and target species. Techniques are discussed and, where relevant, options presented and suggestions made for experiments. The Technical Bulletins are authored by scientists working in the genetic resources area. IPGRI welcomes suggestions of topics for future volumes. In addition, IPGRI, would encourage, and is prepared to support, the exchange of research findings obtained at the various genebanks and laboratories.

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Cover image: Collecting a shoot tip of *Gonzalagunia hirsuta* by *in vitro collecting*. Valerie C. Pence, Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden.

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List of authors

The authors of this publication include the four teachers[†] and 15 students[‡] of an international course on 'In vitro Techniques for Germplasm Collection', jointly sponsored by IPGRI/IBPGR, FAO and CATIE at Turrialba, Costa Rica, in 1990 and several other contributors.

[†]**Alvarenga, V. Silvana**
Departamento de Química
Instituto Tecnológico de Costa
Rica, Apartado 129, Cartago,
Costa Rica

[†]**de Bem Bianchetti, Luciano**
Sain Parque rural s/nº
Centro Nacional de Recursos
Genéticos de Biotecnología
(CENARGEN) de EMBRAPA,
Brazil

[†]**Borbor Ponce, Miryam M.**
Programa de Maíz
Universidad Nacional Agraria
'La Molina', Avenida
Universitaria s/n
La Molina, Peru

[†]**Brenes Hine**, Abdenago
Coordinador Programa de
Recursos Fitogenéticos
Escuela de Ciencias Agrarias
Universidad Nacional,
Apartado 86, 3000
Heredia,
Costa Rica

[†]**Calderón Díaz, José H.**
Faculdad de Agronomía
Area Tecnológica
Subárea Manejo y
Mejoramiento de Plantas
Universidad de San Carlos
de Guatemala

Ciudad Universitaria
Zona 12 Guatemala

[†]**Clark, John R.**
Center for Research of
Endangered Wildlife,
Cincinnati Zoo and Botanical
Garden
3400 Vine St., Cincinnati, OH
45220, USA

[†]**Engelmann, Florent**
Institut de recherche pour le
développement (IRD)
BP 5045, 34032 Montpellier
Cedex 1 France

[†]**Espadas Y Gil**, **Francisco L.**
Centro de Investigación
Científica de Yucatán
A.C. Km 7 antigua carretera a
Progreso Ex. Hda. Xcumpich,
AP 87,Cordemex, Yucatán,
Mexico

[†]**García Tapia**, **Vildama**
Instituto de Investigación
Agropecuaria de Panamá,
Panamá

[†]**González, Luis G.**
Consultant on
PGR/Agrobiodiversity
Research Management
PO Box 8686-1000, San José
Costa Rica

[†]**Jayanthi, N.**
Forest Research Institute
Malaysia (FRIM), Kepong
52109, Kuala Lumpur
Malaysia

[†]**Krishnapillay, B.**
Forest Research Institute
Malaysia (FRIM), Kepong
52109, Kuala Lumpur
Malaysia

[†]**López González, Patricia E.**
CIFAP Morelos, Apartado
Postal #12, Zacatepec, Morelos,
Mexico

[†]**Lozoya Saldaña**, Héctor
Departamento de Fitotecnia,
Universidad Autónoma
Chapingo
Mex. CP 56230, Mexico

[†]**Montoya Henao, Luz M.**
Universidad Nacional de
Colombia, Departamento
Agronomía
Apartado Aéreo 568,
MedellínColombia

[†]**Oicatá López, Mabilia**
Instituto Colombiano
Agropecuario ICA Apartado
aéreo 151123, El Dorado
Bogotá, Colombia

[†]**Pence, Valerie C.**
Center for Research of
Endangered Wildlife,
Cincinnati Zoo
and Botanical Garden, 3400
Vine St., Cincinnati
OH 45220,
USA

[†]**Plair, Bernadette L.**
Center for Research of
Endangered Wildlife, Cincinnati
Zoo and Botanical Garden, 3400
Vine St., Cincinnati
OH 45220, USA

[‡]**Sandoval, Jorge A.**
Tissue Culture Laboratory,
Corporación Bananera
Nacional (CORBANA)
Apartado 390-7210 Guapiles,
Limon Costa Rica

[†]**Sandoval, Olga E.**
Encargada de Lab. Cultivo de
Tejidos Centro Nacional de
Tecnologia Agropecuaria
(CENTA), Km 33 $^{1}/_{2}$
carretera a Santa Ana,
San Andrés Departamento
La Libertad,
El Salvador

[†]**Tapia B., César**
Instituto Nacional de
Investigaciones Agropecuarias
(INIAP)
Departamento Nacional
de Recursos Fitogenéticos
(DENAREF)
Estación Experimental
Santa Catalina
Panamericana Sur
Km 14
casilla 17-01-340430, Quito,
Ecuador

[†]**Taylor, Mary**
Secretariat of the Pacific
Community, Regional
Germplasm Centre
Private Mail Bag, Suva,
Fiji

[†]**Velasco Urquizo, Eyla**
Instituto Nacional de
Investigación Agraria (INIA)
Estación Experimental
Agropecuaria La Molina,
Programa Nacional de
Investigación de Recursos
Genéticos y Biotecnología
(PRONARGEB) Avenida
Universidad s/n, Lima, Peru

[‡]**Villalobos A., Víctor M.**
Secretario Ejecutivo
Comisión Intersecretarial de
Bioseguridad y Organismos
Genéticamente Modificados
Insurg. Sur #489, Col
Hipodromo Condesa, 06100
México DF, México

[‡]**Withers, Lyndsey A.**
International Plant
Genetic Resources Institute
(IPGRI), Via dei Tre Denari
472/a, 00057 Maccarese, Rome,
Italy

[†]**Zacher de Martínez,
Marta B.**
Instituto Agronómico Nacional
(IAN), Ministerio
de Agricultura y
GanaderÌa Ruta II Km 48.5,
Caacupe,
Paraguay

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Preface

Food security is fundamental to sustaining life for the peoples of any given country and plant genetic resources form the foundation on which the achievement of that goal depends. Today, however, germplasm is increasingly being lost, due to habitat loss and other factors. This loss, whether of cultivated or of wild species, or genetic diversity within species, diminishes the resources available for the future and thus, irreversibly erodes the genetic base on which crop improvement depends. The cost of conserving these genetic resources can be high; even so, the cost of not confronting the problem might become even much higher.

Over the past three decades, plant biotechnology has developed tools which impact on work with genetic resources in a variety of ways. These include notably methods for the *in vitro* propagation and the long-term storage (cryopreservation) of germplasm; detecting and eliminating diseases in germplasm collections, thereby improving the speed and safety of germplasm exchange; producing synthetic seeds; and identifying useful genes.

During the early 1980s, while searching for ways to more fully utilize the techniques of plant biotechnology, IPGRI (the International Plant Genetic Resources Institute, then the International Board of Plant Genetic Resources, IBPGR) suggested that germplasm be collected by means of *in vitro* methods. The use of tissue culture to develop innovative and practical protocols for the *in vitro* collecting of plant germplasm was an attractive alternative, particularly for problematic species (e.g. those with recalcitrant seeds or with propagules that are difficult to collect and transport). In 1984, IPGRI recommended that this method be used for several tropical species of particular interest and initiated several research projects in various countries. This eventually led to the proposal of an international course, to be held in Latin America, on the theory and practice of the principles of *in vitro* collecting, using concrete examples. With the collaboration of the Biotechnology Unit at CATIE (Centro Agronómico Tropical de Investigación y Enseñanza, Turrialba, Costa Rica), IPGRI coordinated and implemented the course between 22 April and 5 May 1990. At the same time, an attempt was made to demonstrate the advantages of analysing and using this promising methodology in a coordinated, inter-institutional fashion involving both, national and regional/international research institutes. Research was carried out with collections of *Citrus* spp., *Musa* spp., *Theobroma cacao*, *Coffea arabica* and *Persea americana*.

Between 1990 and today, research in this area has been performed by different groups, leading to the development and/or optimization of *in vitro* collecting techniques for various additional species including coconut, taro, tropical rainforest tree species and wild and endangered species.

Recognizing the very high potential and broad applicability of *in vitro* collecting techniques for improving the efficiency of germplasm collecting for problem species, FAO and IPGRI decided to disseminate within the plant genetic resources community worldwide the information on these techniques through the publication of a Technical Bulletin on this topic. The initial report produced after the CATIE international course, which forms the backbone of the present publication, was therefore translated into English, edited and complemented with chapters authored by researchers working on *in vitro* collecting of the additional species mentioned above.

This Technical Bulletin comprises three separate parts. The first part (theoretical background to *in vitro* collecting), consists of three chapters. These chapters present the rationale behind the development of *in vitro* collecting and its potential for the conservation of crops and wild or endangered species (Chapters 1 and 2). Chapter 3 deals with the control of contamination, a critically important step which conditions the successful development of any *in vitro* collecting protocol. The second part (case studies) comprises nine chapters, each describing the work performed for the development of *in vitro* protocols for a particular species or group of species. The protocols described can be applied directly for collecting germplasm of any of the species concerned. However, circumstances will differ from one collecting mission to the next and it can be expected that these protocols will have to be adapted to these circumstances. Therefore, the aim of these chapters is to illustrate the range of protocols, from the simplest to the most sophisticated, which can be developed for *in vitro* collecting germplasm of a given species and to highlight the critical steps of such protocols. Such information should be used by the readers as a guide for the development of protocols for the species of their own interest. The last part (prospects) consists of a single chapter which analyses the future of *in vitro* collecting for improving the conservation and use of plant genetic resources. This Technical Bulletin includes references up to 2001.

Within their framework of collaborative activities, IPGRI, FAO and CATIE aim to continue to promote *in vitro* collecting activities in the future and as a means of disseminating specific information,

present this Technical Bulletin, which includes both theoretical and practical aspects. This publication aims to provide a resource for those wishing to understand the basic concepts of adapting plant tissue culture methods to field collecting. As such, it will hopefully stimulate research directed at improving *in vitro* collecting and will spark the imagination of those who will adapt the technique to new species and new applications.

Jan Engels   **Florent Engelmann**
Director   IPGRI Honorary Research Fellow
Genetic Resources   *In Vitro* Conservation
Science and Technology   and Cryopreservation

Part I.
Theoretical background
to *in vitro* collecting

Chapter 1. *In vitro* collecting—concept and background

Lyndsey A. Withers

The need for new collecting techniques

Experienced germplasm collectors will base their collecting expeditions on available information and on their knowledge of the problems that may limit the effectiveness of their work. There may exist only one limited opportunity to carry out an expedition because of problems of availability of personnel, permission from the country hosting the expedition and time of year that is appropriate for collecting (e.g. because of climate and the plant's cycle). Economic and common sense indicates that the most advantage must be taken of an expedition to collect available germplasm, in that resources are limited and each opportunity is unique. This concept of 'unique opportunity' becomes highlighted even more if the germplasm to be collected is threatened through a change in land use or other factors that could affect its future availability.

Despite careful planning, the collector's considerable experience and the collaboration of the host country and its administrative authorities, a collecting expedition may still encounter difficulties, such as those related to the quantity and quality of the propagules collected. Perhaps not enough material was available because of bad harvests, long-term scarcity of the plant in the collecting zone, animals that ate the available populations of the plant (particularly in the case of forage plants), a high incidence of pests and/or diseases, or immaturity of available plants.

In seeds and propagules, these problems usually relate to a seasonal pattern of development. Plant parts that are not strictly organs of propagation or perpetuity, such as shoots of trees, are more flexible in that they are likely to be available for collecting at any time. However, because these are vegetative tissues, they have less likelihood of surviving a long trip to the genebank. The problem of deterioration, caused by natural processes and by attack from microorganisms, also occurs for recalcitrant seeds, which may germinate and/or suffer mortal damage in transit. A last problem, shared to a certain extent by some seeds and their fruits, is excessive volume and weight. The costs and inconveniences of transporting large quantities of plant material can seriously limit a collecting expedition.

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.

Searching for a solution

Because of the problems just described, the germplasm collector must take advantage of as many alternatives as possible to increase the efficiency and effectiveness of an expedition. One such alternative is to use an *in vitro* method of collecting. In 1982, the IBPGR (now IPGRI) inaugurated the Advisory Committee for *In Vitro* Storage, with the aim of examining the current situation of *in vitro* conservation of germplasm and highlighting the opportunities for developing and applying new approaches to solving problems of conserving plant genetic resources.

The difficulties associated with collecting germplasm of the two principal categories of problem crops—those having either recalcitrant seeds or vegetative planting materials—were considered as 'bottlenecks'. Even more, the Committee emphasized that any plant type could benefit from the use of improved techniques of collection. With the goal of investigating this approach in detail, a subcommittee of the Advisory Committee was established in 1983. Members of this subcommittee included experts in germplasm collecting, in genetic resources of problem species and in the application of *in vitro* techniques to the propagation and conservation of plants.

For the conservation of germplasm that cannot be collected by other means, the technology of *in vitro* propagation had been already confirmed as being adaptable to the collecting of such germplasm (Withers 1980; 1982). The basic premise studied by the subcommittee, therefore, comprised some fundamental aspects of *in vitro* culture techniques, such as inoculation and regeneration of plants, that could be adapted to the collecting of 'problem' material. This would be, however, a support operation, not a large-scale exercise in *in vitro* propagation.

With the help of preliminary experiments carried out at the institution hosting the meeting (School of Agriculture, University of Nottingham, UK), the subcommittee came to the proper conclusion that *in vitro* collecting had high potential. One outcome of the meeting was a report with recommendations (IBPGR 1984). In the following years, two models of collecting methods were developed, one based on the woody shoots of cacao (*Theobroma cacao* L.) and the other on the embryos of coconut (*Cocos nucifera* L.) and these became the basis of future research projects. These are studied in more detail in the section below on 'Cases of *in vitro* collecting'. Now, however, we will analyse more deeply the use of *in vitro* procedures and their adaptation for germplasm collecting.

Adapting the basic in vitro *procedures*

The establishment of *in vitro* cultures in the laboratory involves:

1. Selecting the appropriate tissue for inoculation.
2. Cutting the tissue to an appropriate size.
3. Eliminating soil residues and visible diseases and pests by a preliminary wash.
4. Surface sterilizing the plant tissue.
5. Washing to remove the disinfectant.
6. Cutting off unnecessary or damaged tissue.
7. Inoculating into culture vessels containing a nutrient medium and closing the vessels.
8. Transferring the germplasm to incubation chambers.

To protect the explant from a possible reintroduction of contaminants, surface sterilization and inoculation are carried out in a laminar-flow chamber. Incubation is conducted under controlled conditions where temperature, quality and intensity of light and day length are regulated to promote optimal growth and development. The culture medium also contains nutrients needed by the tissue to reach maximum growth and development.

The inoculated tissue will develop according to the culture medium used. Under certain conditions, an explant may be induced to produce many lateral shoots that can be separated and which, in their turn, can be multiplied. Under other conditions, only one independent shoot may be grown from an explant, which is then induced to produce roots for later transplanting to soil.

This model applies to non-adventitious systems, for example, those that can produce a plant from a pre-existing shoot, as in the case of nodal explants or a zygotic embryo. Alternatively, it is possible to induce *de novo* apices in an adventitious form from almost any tissue, raising the possibility of mass clonal propagation over a shorter time. For genetic conservation, non-adventitious propagation systems are favoured, which are more likely to avoid genetic instability through somaclonal variation. Although non-adventitious systems are more appropriate, it should be emphasized that any material is better than none. As such, any tissue that regenerates adventitiously should not be rejected if it is the only material available. Researchers must be imaginative and take advantage of the great flexibility that *in vitro* culturing offers. For example, a leaf segment can produce plants through somatic embryogenesis, as can an infertile ovary through *in vitro* culturing and pollinating.

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. To illustrate this, each step of the inoculation procedure is analysed below:

Selecting the appropriate tissue for inoculation
Observations: where possible, a strong tissue that can withstand surface sterilizing is preferred. Young, growing tissues are generally most suitable for further growth *in vitro*.

Cutting the tissue to an appropriate size
Observations: superficial damage should be minimized as far as possible, but the opportunity should also be taken to eliminate external tissues that are very dirty, infected, or damaged.

Eliminating soil residues and visible diseases and pests
Observations: in the field, there may not be sufficient quantities of water to carry out this activity.

Surface sterilizing plant tissue
Observations: in those places where the quantity of sterile water is inadequate for later washings, disinfectants should be portable and of a relatively low toxicity to plant tissue. Non-conventional disinfectants can be used, such as water purification tablets, agricultural fungicides and combinations of products at low concentrations. Surface sterilization and the following steps will most probably be carried out under conditions that are not aseptic (see below), but a sterilizing vessel with a lid is more appropriate than a beaker. Surface sterilization can be repeated once the material reaches the laboratory, which means that simple, effective, short-term treatments that maintain adequate cleanliness are more suitable than more severe treatments that may damage the tissue. One fact that should be kept in mind is that many meristematic plant tissues are free of microbial contamination by being protected by many leaves, bracts, seed testa, etc. Advantage can be taken of this situation by selecting an explant that can be surface sterilized to remove most of the contamination, then dissected to remove the external tissues. Quick work and a frequent change of dissection instruments can prevent the internal tissues from becoming contaminated.

Washing to remove the disinfectant
Observations: the number of washes needed will depend on the concentration and toxicity of the product used. If a post-

sterilization step is included, then sterile water will need to be used, together with an adequate technique for preventing the re-introduction of contaminating microorganisms. Even so, any residual effect of the disinfectant, such as of a fungicide, will continue to be lost with each wash.

Cutting off unnecessary or damaged tissue
Observations: this is another step where contaminants can be re-introduced and, as such, should be avoided where possible.

Inoculation into culture vessels containing a nutrient medium and closing the vessels
Observations: several factors must be considered when selecting the vessel: its type, the quantity of inoculum and the culture medium to be used. The container should be portable, thus strong, but not too heavy. Synthetic materials are more suitable than glass and, under some circumstances, something as simple as a plastic bag may be enough (see next section under 'Coconut').

Although placing more inoculum in the culture vessel means that, overall, more germplasm is transported, the risk of cross contamination increases. The decision on how much inoculum to place in each receptacle is influenced by the efficiency of the surface sterilization method, the time the tissue will be in transit and the susceptibility of the tissue to damage from contaminants.

The culture medium must be so designed that it can be adapted to the purpose in hand. If the tissue's development must be encouraged (e.g. to stimulate embryo germination or the growth of axillary buds), then appropriate growth regulators must be included in the medium. If, on the contrary, development must be suspended, then a minimal medium or a medium containing growth retardants must be used. A minimal medium is less likely to support the growth of residual contaminating microorganisms than a complete medium. A medium may also contain antimicrobial additives to retard the growth and destructive effects of bacteria and fungi. The potential side effects on the explant, however, must also be considered. The advantages of a liquid versus solid medium must also be weighed. A liquid medium is more accessible for the inoculum, but it is less effective in retarding the growth of contaminating microorganisms. Moreover, containers with liquid medium must not leak.

Transferring the germplasm to incubation chambers
Observations: This stage is much longer and much more risky than the simple transfer from one room to another in the laboratory. The

maximum effort must be made to protect the inoculated material while in transit. This involves both the receptacles used for the medium and their container. Special attention must be given to the likely conditions of transport, such as fluctuating temperatures and unexpected movements (e.g. blows, shaking). The possibility of using refrigeration to delay deterioration of cold-tolerant material must also be considered.

Limitations imposed by the expedition's structure
The above procedures have been analysed in terms of the requirements of inoculation under field conditions. A second group of factors to be considered are the constraints imposed by the nature, scale and duration of the collecting expedition, the degree to which *in vitro* collecting is central to the expedition and the experience of the expedition's personnel. Some examples will be given as explanation.

For an expedition collecting various species, a very general approach must be used 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 principal method, then this will influence the levels of re-application and the amount of resources and time available for this activity. If the collectors have only basic experience in *in vitro* techniques, then the field operations must be designed according to their level of experience, leaving, where possible, all other work for the laboratory. 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 collecting expeditions.

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 collecting 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'. The examples of successful *in vitro* collecting highlighted in the next section 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* collecting in the field is regarded as a 'support operation', then the collector must be able to rely on there being a suitable laboratory available as soon the collection is made. Some activities, such as preparing the culture medium, labelling 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. It should be emphasized once more, however, that the great advantage of *in vitro* collecting is its adaptability and flexibility. For this reason, there are no quick and fixed rules or recipes, only general guidelines to help adapt this concept to situations with new species.

Cases of *in vitro* collecting

A number of case studies are presented and analysed in details in Part II of this Bulletin. This section briefly reviews the results obtained with several species, with the aim of demonstrating the reach of this method and its use for germplasm collecting expeditions.

Cacao

One of the first species explored as a target for *in vitro* collecting was cacao. Recent experiments have included both buds and embryos, but from the beginning, the goal was to find an alternative for transporting segments of woody shoots from the collecting site to the greenhouse. Experiments by Yidana and coworkers (Yidana *et al.* 1987; Yidana 1988, see also Box 1, Chapter 5) showed that surface sterilizing nodal segments from the stem, using water purification tablets and an agricultural fungicide (e.g. FBC protective fungicide at 0.05%) were effective, without further washings. (The tablets contained the active ingredient 'Halozone' [carboxybenzene-sulphodichloroamide], at 0.4 g/L, prepared as tablets of 10 ± 4 mg and added to 100 mL of boiling water.)

Inoculation was on a semi-solid medium containing a fungicide (e.g. Tilt® MBC at 0.1%) and sometimes antibiotics (e.g. rifamycin + trimethoprim, each at 15 mg/L) to maintain the tissues under relatively clean conditions, although not necessarily free of contamination, for as long as 6 weeks. Occasionally, there was bud growth and rooting. (Optimum levels of disinfectants and antimicrobial compounds were reached through a system of trials with leaf discs.) The field equipment was simple, consisting of racks of plastic tubes containing the culture medium, jars of boiled water, disinfectants, plastic tweezers and pruning scissors. Inoculation was

carried out in the open air. The major constraint to this procedure was the lack of a good method for *in vitro* propagating the collected material. Nevertheless, this demonstrated the extent to which the norms of *in vitro* culture can be complied with. This basic approach has also been applied successfully to buds from trunks of fruit trees in temperate latitudes (K. Elías, unpublished data).

Cotton

The collecting of cotton (*Gossypium hirsutum* L.) germplasm and its wild relatives was hampered by the unpredictable availability of viable seed. As a result, Altman and coworkers developed an *in vitro* technique to use in the field in Mexico (Altman *et al.* 1990). They surface sterilized nodal stem cuttings by dipping in a 20% solution of commercial sodium hypochlorite mixed with 30% ethanol, for 45 s. The cuttings were then inoculated directly onto a medium with half-strength salts; 1% glucose; the antibiotics rifamycin and trimethoprim, each at 15 mg/L; the fungicide Tilt® MBC at 1 g/L; naphthaleneacetic acid at 1 mg/L; hydrolysed casein at 0.5 g/L; and agar at 9 g/L. The inoculations were carried out in the open air. The cuttings were in transit for almost 3 weeks, after which they were resterilized with a 4% solution of commercial sodium hypochlorite, then treated with rooting hormones and planted in a mixture of sand, soil and a sterilized mix of vermiculite, supplemented with calcium and slow-release nutrients.

Although the actual step of collecting was in itself successful, it was difficult to stimulate rooting or further growth in the cuttings. This experience emphasized the importance of having a technique that can support the continued growth of the collected material.

Forages

Collecting of forage germplasm involves many of the same problems as collecting cacao and cotton and also includes the risk of grazing animals. Forages, however, have very different structures. For example, the available explants for *Digitaria* Haller and *Cynodon* Rich. are herbaceous. Despite the less robust nature of this material and the different necessities for its *in vitro* culture, it could be managed in the same way as woody material. Ruredzo (1989) used a simple technique that was similar to Yidana's, described above. In this case, the collecting site was very close to the hotel where the collector was staying, so he carried out the inoculation there without having the complications of field inoculation.

Coconut

The last example in this short review is the coconut, which is included for two reasons. First and primarily, the coconut is voluminous and heavy, which makes it difficult to transport. Second, coconut seeds are recalcitrant and several researchers have adapted techniques for the *in vitro* culture of coconut embryos to the field collecting of coconut germplasm.

In all cases of collecting, the basic sequence has been as follows: to strip and open the nut; to extract, with a perforator, a portion of the endosperm containing the embryo; and to preserve and inoculate it onto the culture medium. The differences in approach among researchers has been based on the extent to which they reproduce laboratory conditions in the field, the number of cultures they make in the field versus the number made in the receiving laboratory and, consequently, the level of sterilization that is used. Using the simplest technique—that used by Rillo and coworkers in the Philippines (Rillo 1991)—portions of endosperm were transported from the field, under refrigeration, in plastic bags filled with coconut water. Sterilization and inoculation were carried out in the receiving laboratory (Rillo and Paloma 1990).

Assy-Bah and coworkers (1987, 1989, see also Box 2, Chapter 10) carried out direct inoculation in the field. Their method involved surface sterilization of endosperm plugs with calcium hypochlorite, extraction of embryos and their inoculation *in vitro*. This procedure required a higher level of skill to carry out the inoculation adequately, more care in handling explants and a more protected environment, such as an inverted box to keep out aerial contaminants.

The approaches just described recognized the limitations of working in the field and used relatively simple methodologies to overcome them. The approach used by Sossou *et al.* (1987), also with coconut, followed a very different logic. They tried, where possible, to overcome the deficiencies of the field environment and to provide facilities for inoculation that were almost as sophisticated as those of the laboratory. They used an alcohol-sterilized inflatable glove box and followed exactly the same procedures as those used in the laboratory and requiring the same degree of skill. The glove box, inflator, instruments, lamp, solutions and culture vessels had to be transported with care, both to and from the collecting sites.

Conclusions

This chapter aimed to illustrate that some of the problems in collecting germplasm can be overcome by an imaginative application

of *in vitro* procedures. While certainly this new approach to collecting is not sophisticated, it does require adequate knowledge, preparation and planning.

The previous case studies illustrate the flexibility of *in vitro* collecting, involving several levels of complexity in the field and a range of explants appropriate for different species. The spectrum of examples is offered as a stimulus to imagination and not for literal adoption. In fact, it may not have been necessary for the last example (Sossou *et al.* 1987) to be so complicated. Before going into the field, the need to use complex procedures must be carefully reviewed, bearing in mind the case of the coconut, where embryos located in portions of endosperm were easy to collect and safely transport. Ingenious complex procedures need to be compatible with the other tasks that must be carried out during a collecting expedition and be appropriate in terms of weight for transport, training of the collectors and technical support.

The basic message, therefore, is that the operations to be carried out in the field should be those that are truly necessary. Such operations are defined by the conditions 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. Any other activity should be left until the germplasm reaches that laboratory.

Once a culture has been successfully established, the collected germplasm is safe, at least for the short term. However, as in any conservation exercise, there must be a plan for the safe preservation of the germplasm in sufficient quantities to be available for use. Even more important, collectors must be fully aware of the fact that *in vitro* collecting is not a means of avoiding either quarantine or the procedures for indexing diseases. It may reduce the risks of introducing diseases and pests (IBPGR 1988), but it does not reduce the need to be alert and to comply with phytosanitary procedures and regulations. As such, *in vitro* collecting should be seen as part of a broad scheme for conservation.

Chapter 2. *In vitro* collecting—a tool for wild or endangered species conservation

Valerie C. Pence

Introduction

Plant conservation efforts have traditionally fallen into two complementary and sometimes overlapping spheres of activities: conservation of germplasm of economically important species; and conservation of wild germplasm of rare or endangered species, regardless of their present usefulness to humankind. While the former efforts are driven by strong economic and humanitarian forces, the latter are driven by the understanding that biodiversity is the cornerstone on which economic benefits from plants is derived. Perhaps only 5% of all plant species have been tested for any beneficial use (Farnsworth 1988; ten Kate and Laird 1999) and it is likely that among the thousands of species yet unexamined, there are many which will provide benefits to humans as food, fiber, oil, pharmaceuticals and other uses. It is sobering, however, that of the 300 000 known species of vascular plant, over 30 000 are of immediate conservation concern (Walters and Gillett 1998).

While preserving species in their natural habitat is of primary importance, there are many places where habitats are under threat, from logging, agriculture, urbanization, pollution, etc. Species which are rare or endemic are of particular concern, since even the loss of relatively small areas of habitat may prove to move these species irreversibly toward extinction. *Ex situ* conservation methods are required to back up the germplasm of these species, in the event they be lost in the wild.

Traditional methods for *ex situ* conservation of endangered or rare species have included the growth of plants in botanical gardens and arboreta and, more recently, the establishment of seed banks for wild species preservation (Falk 1987; Laliberté 1997; Linington 1997). Biotechnology has advanced plant conservation further, by making available the techniques of tissue culture propagation and tissue and DNA cryopreservation (see Benson 1999 and Bowes 1999 and articles therein). These methods have considerably broadened the ability to propagate wild species as well as the ability to preserve non-seed tissue, as well as seeds, in long-term liquid nitrogen storage.

The more recent adaptation of tissue culture to field collecting, in the technique of *in vitro* collecting, provides researchers with the ability of expanding collecting opportunities to those species which do not have available seed or easily transportable propagules. While

the basic techniques of collecting are similar regardless of the species, there are particular issues which arise when dealing with wild and particularly endangered, taxa.

In vitro collecting for wild or endangered species

The approaches used for collecting wild species will vary depending on whether a particular species is targeted, or whether collections will be made opportunistically from species available within the area of a particular expedition. It may be possible to tailor media and procedures for species of interest. Alternatively, protocols may be chosen which are adaptable to a wide range of species.

The number of plants available for collecting wild species will often be more limited than those of crop species and the plants may be scattered within a given area. This can be particularly true in the tropics, where the number of individuals of a given species may be very low. Little may be known about the growth and phenology of the species and the plants may be difficult to access, growing in remote areas, on cliff faces, etc. Because such plants cannot be monitored easily, even the best plans may not successfully coordinate a collecting trip with the production of seed or of young growth for *in vitro* collecting. In the wild, even young tissues may suffer predation by insects or infection by microorganisms, further limiting chances for successful establishment of cultures.

In vitro collecting can be adapted to a variety of situations for collecting endangered species germplasm. Although many endangered species propagate readily from seed and suffer primarily from habitat loss, some rare species do not produce seed, or the seed they produce is of low viability. If seeds are produced, they may have unusual dormancy requirements which can be difficult to meet. And, finally, as with other plants, seeds may not be available or may be immature at the time of a collecting trip.

Dealing with endangered species, by definition, means that plant material will be limited in supply and extreme care must be taken not to harm the *in situ* population. Although voucher specimens are recommended for any collection, collecting such specimens from endangered species, which may be few in number, can harm the population and may be prohibited legally. In such cases, photographs can be substituted for pressed specimens. *In vitro* collecting, however, may, at times, be allowed from plants when even seed collection is restricted. Carefully removing small amounts of appropriate tissues from the plant should not harm the individual and can allow replication of the germplasm *ex situ* while maintaining the germplasm *in situ*.

Endangered species are also often legally protected and proper permits and permissions must be obtained before any seeds or tissues are collected. In some cases, collecting may be prohibited entirely because of the rarity of the species. Transport and importation of certain species is also restricted by countries adhering to international conventions such as CITES (Convention on International Trade in Endangered Species) or the CBD (Convention on Biological Diversity) and additional permits or material transfer agreements may be required (Wyse Jackson and Sutherland 2000).

Little may be known about the *in vitro* culture of a wild species and thus, developing appropriate media and protocols for *in vitro* collecting can be difficult. Guidance must be taken from literature reports or experience with related or similar species. It is also unlikely that enough tissue will be available for experimentation and so educated judgements must be used in developing procedures for the limited amount of material that will be available.

Laboratory researchers requiring tissue from specific endangered plants can organize trips for collecting the desired material. Alternatively, *in vitro* collecting kits can be sent to field collaborators, who, by following simple instructions, can make the collections and send the tissues back to the laboratory. In addition, an *in vitro* collecting kit can be taken to the field as a back-up on seed collecting trips. If seeds have not been produced or have already been shed, it may be possible to collect tissues by IVC. If seeds are available but immature, placing them into culture can maintain viability even when they are not developed enough to survive normal seed handling procedures. Examples of the application of these techniques to wild endangered species are given in Chapter 12 of this volume.

In vitro collecting for botanical research

In some cases, *in vitro* collecting is used for a single taxon, but it can also be used for more broad based collecting by researchers on botanical collecting trips for herbaria or botanical gardens. In these cases, using general procedures to accommodate a wide range of species can be useful, since the species that will be collected may not be identified prior to the trip.

In vitro collecting for biodiversity conservation education

In vitro collecting is a 'low-tech' approach requiring relatively simple tools and procedures and as such, it is also well adapted as an educational tool (Plair and Pence 2000a). When full tissue

culture facilities are not available, teachers can use *in vitro* collecting to introduce students to *in vitro* methods. Topics related to sterility requirements and the effects of plant growth regulators on the growth and development of plant tissues can be discussed. *In vitro* collecting can also be used as a platform for introducing concepts involving the value of biodiversity and collecting germplasm for *ex situ* conservation. Students can participate in the implementation of a technique which is being used by researchers for preserving biodiversity worldwide.

Future research and applications

Because *in vitro* collecting is based on tissue culture techniques, its limitations are the same as those of tissue culture in general. The recalcitrance of some species to regenerate or even to grow *in vitro* is well known and the growth of tissues collected by *in vitro* collecting will be under the same restraints as any tissues grown *in vitro*. Continued research to help broaden the ability of species to grow and propagate *in vitro* will benefit *in vitro* collecting as well as other areas of tissue culture work.

Thus, the goal of developing an *in vitro* collecting procedure which can be used successfully for every plant species is likely unattainable. It is, nevertheless, a useful goal around which to fashion research which can expand the applicability of *in vitro* collecting. As more species are collected, more information will become available on the factors affecting contamination rates and growth of collected tissues. Just as the methods of the technique are flexible and adaptable, so too, the uses to which the technique can be put cross a wide range of interests and disciplines. *In vitro* collecting can facilitate basic research studies as readily as it can be used to collect useful germplasm for long-term storage and its value will be determined, ultimately, by the imagination and needs of those who use it.

Chapter 3. Controlling contamination during
in vitro collecting

Valerie C. Pence and Jorge A. Sandoval

Introduction

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. *In vitro* culture is conducted under aseptic conditions; however the routine aseptic procedures practised during *in vitro* culture operations seldom control systemic microorganisms present in the plant (endogenous microorganisms), even under good laboratory conditions. The technique of *in vitro* collecting, however, poses challenges beyond those of normal tissue culture. Work is done in the field, often in the open air, exposing cultures to air-borne contaminants. In addition, field-grown plants generally have a high rate of endogenous contaminants and dealing with these is often the most challenging step in establishing a viable culture from *in vitro* collected tissues (Leifert and Waites 1990; Leifert *et al*. 1994; Norman and Alvarez 1994).

Surface sterilization is the first step in establishing aseptic cultures and this is often done at the collecting site. However, tissues may be placed on a transport medium and sterilized or resterilized in the laboratory (Rillo and Paloma 1991; Predieri, pers. comm.).

Although surface sterilization is necessary, bacteria and fungi that are found beneath the epidermis of the plant and even in intercellular spaces, will not be affected by surface treatments. Antimicrobial agents must be added to the culture to systemically kill such contaminants, which may not appear for days or even weeks after the culture is initiated. This chapter will focus primarily on these contaminants and methods for their control.

Factors affecting contamination of *in vitro* collected cultures

A number of observations have suggested factors which may be important in determining rates of contamination by endogenous microorganisms from field collected materials. Older plant tissues taken later in the growing season are often more infected than younger plant tissues (Bernstein and Carroll 1977), while certain endophytes may have preferences for particular host tissues (Carroll and Petrini 1983). Underground tissues, such as roots, rhizomes and corms, generally have high levels of endogenous contaminants and can be extremely difficult to clean (Roy and

Saha 1997; Rabe and van Staden 1999; Smith *et al.* 1999). Vegetative and floral buds often harbor contaminants in complex tissues which can protect even external microorganisms from surface sterilants (Merkle *et al.* 1997).

Contamination may also be affected by environment. Explants taken from plants in a moist tropical site had a 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 disinfested than tissues from moister areas (McKay 1999). A seasonal effect on contamination rates has also been observed in some collections (Thomas and Ravindra 1997), although others have reported no seasonal differences (Vianna *et al.* 1997; Pence 1999).

Contaminating agents in cultures initiated by IVC

It is likely that most, if not all, plant species harbour endophytes (Boullard 1979; Dreyfuss and Petrini 1984; Zenkteler 1995; Hallmann *et al.* 1997; Carroll 2000) and many apparently benefit from a mutualistic relationship in which fungi or bacteria inhibit predation of the plant (e.g. Bacon *et al.* 1975; Carroll 1986; Chanway 1998). Studies have indicated that species of *Pseudomonas*, *Enterobacter*, *Agrobacterium*, *Xanthomonas*, *Flavobacterium* and *Bacillus*, among others, are common endogenous contaminants of plants and thus of tissue culture explants (Cornu and Michel 1987; Duhem *et al.* 1988; Enjalric *et al.* 1988; Gunson and Spencer-Phillips 1994; Norman and Alvarez 1994; Cassells and Tahmatsidou, 1996; Tanprasert and Reed 1997; Kamoun *et al.* 1998; Mantell 1998; Moutia and Dookun 1999). In the case of fungi, some groups of species are ubiquitous as endophytes, such as species of *Cladosporium*, *Geniculosporium* and *Nodulisporium*, while others show a high affinity for certain plant families (Petrini 1986). Fungi which have been identified from original cultures of field collected tropical plants include species of *Colletotrichum*, *Glomerella*, *Pestolotia*, *Penicillium*, *Fusarium*, *Phomopsis*, *Alternaria* and *Xylaria* (Gochenauer and Pence, unpublished).

Bacteria and fungi develop rapidly as saprophytes in culture media. Their requirements for nutrients are essentially the same as those of plants in that they need macro-elements, micro-elements and vitamins to grow and reproduce and consequently, they compete with the explants for nutrients. In culture, they may produce phytotoxic metabolites that can inhibit growth or even kill the explants (Falkiner 1990).

On standard plant tissue culture media many bacteria or fungi will be visible within three or four days and some may be visible 24 h after culturing. Others can be very slow-growing, however

and may not appear for several weeks or even months. In some cases, cultures with no visible signs of fungi or bacteria have revealed contamination when transferred to richer media (Reed *et al.* 1995; Seyring 1998). Placing tissues from field grown plants directly onto a rich detection medium allows for an early determination of contamination and early elimination of contaminated cultures (Poonawala *et al.* 1999).

Conditions in culture may not favor the growth of all the microorganisms present in an explant. Fungus may physically obscure bacterial growth or may chemically inhibit it by producing antibiotic compounds (Di Menna *et al.* 1996; Rodrigues *et al.* 2000). As a result, when fungal contamination was inhibited by the addition of benlate to cultures of leaf discs initiated by *in vitro* collecting, apparent bacterial contamination rates increased (Pence 1996). Similarly, some bacteria are known to produce compounds which can inhibit the growth of fungi (Ganova-Raeva *et al.* 1998).

In most cases it may be sufficient to determine whether contaminants are bacterial or fungal in order to choose an appropriate antimicrobial agent. In other cases, it may be useful to undertake microscopic and biochemical identification of the contaminant to genus or species. More sophisticated techniques are also available, such as electron microscopy, ELISA tests for serological determination, or tests for the cDNA of specific contaminants (Leifert and Waites 1990). The expense of such tests, however, usually limits their use to particular, economically important situations.

Using antimicrobial agents

A variety of antimicrobial compounds have been used to control contamination in tissue cultures initiated in the laboratory from field collected material, as well as in *in vitro* collecting procedures (Tables 1 and 2). Depending on the target tissues, these can provide direction in designing new protocols for *in vitro* collecting. These chemicals differ considerably in their characteristics (Falkiner 1990). Their solubility, stability in light, interactions with other media components, thermolability, pH requirements and allerginicity or toxicity to humans should all be considered when they are used. If the primary contaminants of a targeted species are known and isolated, the effects of various antimicrobial compounds can be examined to select those which are most effective (e.g. Silva *et al.* 1988).

A combination of two or more antimicrobial compounds can increase the range of microorganisms that can be targeted (Young *et al.* 1984; Haldeman 1987; Alvarenga 1990; Jiménez 1990; Reed *et*

Table 1. Some examples of plant species for which various antibiotics have been used in plant tissue culture media

In vitro plant species	Antibiotic	Concentration	Reference
Allium longicuspis Regel	Ciprofloxacin	5–100 mg/L	Fellner 1995
Camellia japonica L.	Rifampicin	10–50 gm/L	Haldeman *et al.* 1987
Camellia sinensis (L.) Kuntz	Rifampicin	10–50 gm/L	Haldeman *et al.* 1987
Carica papaya	Rifampicin	25–100 mg/L	Vianna *et al.* 1997
Cattleya aurantiaca (Batem.) P. Don.	Gentamycin	50 mg/L	Thurston *et al.* 1979
	Penicillin G	100 mg/L	Thurston *et al.* 1979
	Vancomycin	50 mg/L	Thurston *et al.* 1979
Citrus sinensis L.	Ampicillin	100 mg/L	Brenes Hines *et al.* Ch. 8, this vol.
	Chloramphenicol	2.5 mg/L	Brenes Hines *et al.* Ch. 8, this vol.
Coffea arabica L.	Gentamycin	100 mg/L	Lozoya Saldaña *et al.* Ch. 5, this vol.
Coffea canephora Pierre var. *robusta*	Cefotaxime	500 mg/L	Duhem *et al.* 1988
	Rifampicin	15–50 mg/L	Duhem *et al.* 1988
	Trimethoprim	15 mg/L	Duhem *et al.* 1988
Corylus avellana	Gentamycin	6.2–50 mg/L	Reed *et al.* 1998
	Streptomycin	62.5–1000 mg/L	Reed *et al.* 1995
	Timentin	62.5–1000 mg/L	Reed *et al.*1998
	Combinations	various	Reed *et al.* 1998
Corylus contorta	Gentamycin	6.2–50 mg/L	Reed *et al.* 1998
	Streptomycin	62.5–1000 mg/L	Reed *et al.* 1995
	Timentin	62.5–1000 mg/L	Reed *et al.*1998
	Combinations	various	Reed *et al.* 1998
Gossypium hirsutum L.	Cefotaxime	100–1000 mg/L	Agrawal *et al.* 1998
Hevea brasiliensis Muell. Arg.	Tetracycline		Mendanha *et al.* 1998
Malus sp.	Rifampicin	25–100 mg/L	Waldenmaier *et al.* 1986
	Rifamycin	25–100 mg/L	Waldenmaier *et al.* 1986
	Tetracycline	25–100 mg/L	Waldenmaier *et al.* 1986
	Combinations	various	Young *et al.* 1984
Mentha L. spp.	Gentamycin	50 mg/L	Reed *et al.* 1995
	Rifampicin	30 mg/L	Reed *et al.* 1995
	Streptomycin	1 mg/L	Reed *et al.* 1995
Musa spp.	Ampicillin	100 mg/L	Montoya Henao *et al.* Ch. 7, this vol.
	Chloramphenicol	100 mg/L	Montoya Henao *et al.* Ch. 7, this vol.
	Gentamycin	100 mg/L	Montoya Henao *et al.* Ch. 7, this vol.

In vitro plant species	Antibiotic	Concentration	Reference
	Rifampicin	100 mg/L	Van den Houwe and Swennen, in press.
Pelargonium x domesticum L.H. Bailey cv. 'Grand Slam'	Cefotaxime	100–500 mg/L	Barrett and Cassells 1994
Persea americana Miller	Ampicillin	400 mg/L	Sandoval and Villalobos, Ch. 9, this vol.
	Chloramphenicol	12.5–200 mg/L	Biasi 1995
	Nalidixic acid	12.5–200 mg/L	Biasi 1995
	Streptomycin	12.5–200 mg/L	Biasi 1995
Pinus pinea L.	Cefotaxime	250 mg/L	Humara and Ordas 1999
	Kanamycin		Humara and Ordas 1999
	Ticarcillin	300 mg/L	Humara and Ordas 1999
	Vancomycin	250 mg/L	Humara and Ordas 1999
Primula vulgaris Hudson	Aureomycin	40 mg/L	Seyring 1999
	Gentamycin	40–80 mg/L	Seyring 1999
	Oxytetracyclin	60–120 mg/L	Seyring, 1999
	Vancomycin	10–80 mg/L	Seyring 1999
	Rifampicin	25-100 mg/L	Waldenmaier *et al.* 1986
	Rifamycin	25–100 mg/L	Waldenmaier *et al.* 1986
Prunus avium	Tetracycline	25–100 mg/L	Waldenmaier *et al.* 1986
Pseudotsuga menziesii	Combinations	various	Young *et al.* 1984
Rhododendron L.	Combinations	various	Young *et al.* 1984
Stanhopea oculata (Lodd.) Lindl.	Gentamycin		Thurston *et al.* 1979
	Penicillin G	100 mg/L	Thurston *et al.* 1979
	Vancomycin	50 mg/L	Thurston *et al.* 1979
Tanacetum vulgare L.	Cefotaxime	250 mg/L	Keskitalo *et al.* 1998
	Gentamycin	40–80 mg/L	Keskitalo *et al.* 1998
	Rifampicin	10–50 gm/L	Keskitalo *et al.* 1998
Theobroma cacao L.	Cefotaxime	500 mg/L	Duhem *et al.* 1988
	Gentamycin	40 mg/L	Alvarenga *et al.* Ch. 6, this vol.
	Rifampicin	15–50 mg/L	Duhem *et al.* 1988
	Trimethoprim	15 mg/L	Duhem *et al.* 1988
Ulmus procera Salisbury	Cefotaxime	100 mg/L	Fenning *et al.* 1993
	Kanamycin		Fenning *et al.* 1993
Various	Cefotaxime	100 mg/L	Pence *et al.* Ch. 13, this vol.
	Vancomycin		Pence *et al.* Ch. 13, this vol.
Vitis vinifera	Kanamycin	5 mg/L	Peros *et al.* 1998

Table 2. Some examples of fungicides used in plant tissue culture media

Fungicide	Other names	Chemical name	Concentration	*In vitro* plant species	Reference
Amphotericin B	Amphozone, Fungizone, etc.	C47H73NO17, polyene	10 mg/L	*Cattleya aurantiaca* (Batem.) P.Don., *Stanhopea oculata* (Lodd.) Lindl.	Thurston *et al.* 1979
			2.5 mg/L	*Eucalyptus grandis* W. Hill ex Maiden	Watt *et al.* 1996
Benomyl	Benlate	[1-[(Butylamino)carbonyl]-1*H*-benzimidazol-2-yl] carbamic acid methyl ester	100 mg/L	*Sechium edule* Jacq. Sw.	Alvarenga 1990
			1–2 g/L	*Erythrina* L. spp.	Jiménez 1990
			1–1.5 g/L	*Coffea arabica* L.	Cerón 1987
			10–50 mg/L	*Nicotiana tabacum* L.	Pollock *et al.* 1983
			100 mg/L	variety of tropical	Pence 1996
			1 g/L	*Dendrocalamus giganteus* Munro	Ramanayake and Yakandawala 1997
					Thurston *et al.* 1979
			50 mg/L	*Cattleya aurantiaca*, *Stanhopea oculata*	Watt *et al.* 1996
				Eucalyptus grandis	
			1 g/L	*Camellia sinensis* (L.) Kuntze,	Haldeman *et al.* 1987
			1–4 g/L	*Camellia japonica* L.	Lozoya Saldaña *et al.* Ch. 5, this vol.
			100 mg/L	*Coffea arabica* L.	Alvarenga *et al.*, Ch. 6, this vol.
			100 mg/L	*Theobroma cacao* L.	Montoya Henao *et al.*, Ch.7, this vol.
			100 mg/L	*Musa* L. spp.	Brenes Hines *et al.*, Ch. 8, this vol.
			50 mg/L	*Citrus sinensis* L.	
Bavistin	Carbendazim, carbendazole; BMC, MBC, etc.	1*H*-Benzimidazol-2-ylcarbamic acid methyl ester (degradation product of benomyl)	10–50 mg/L	*Nicotiana tabacum* L.	Pollock *et al.* 1983
			50 mg/L	*Litchi chinensis* Sonn.	Das *et al.* 1999
Chlorothalonil	Daconil, Bravo, Termil	2,4,5,6-Tetrachloro-1,3-benzenedicarbonitrile	250–500 mg/L	*Eucalyptus grandis*	Watt *et al.* 1996
Clotrimazole	FB-5097, Lotrimin, Mycosporin, Tibatin, Trimysten, etc.	1-[(2-Chlorophenyl)diphenylmethyl]-1*H*-imidazole	10-50 mg/L	*Nicotiana tabacum*	Pollock *et al.* 1983

Fungicide	Other names	Chemical name	Concentration	*In vitro* plant species	Reference
Diniconazole	Spotless, Sumi-8	(*E*)-(±)-β-[(2,4-Dichlorophenyl)-methylene]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol	125 μM	*Trigonella foenum-graecum* L. cv. Gouka	Cerdon *et al.* 1995
Ethirimol	Milstem, Milgo, Micurb Super	5-Butyl-2-(ethylamino)-6-methyl-4(1*H*)-pyrimidinone	50 mg/L	*Cattleya aurantiaca, Stanhopea oculata*	Thurston *et al.* 1979
Fosetyl-Al	Aliette, Efosite Al, etc.	Phosphonic acid monoethyl ester aluminium salt	1.5 mM (MW 354)	*Vitis vinifera* L.	Lopez-Serrano *et al.* 1997
Metalaxyl	Ridomil, Subdue, Apron	*N*-(2,6-Dimethylphenyl)-*N*-(methoxyacetyl)-DL-alaninate	0.5-100 mg/L	*Sorghum bicolor* (L.) Moench	Gowda and Bhat 1988
Miconazole	Monostat, etc.	1-[2-(2,4-Dichlorophenyl)-2-[(2,4-dichlorophenyl) methoxy]ethyl]-1*H*-imidazole	10-50 mg/L	*Nicotiana tabacum*	Pollock *et al.* 1983
			20 mg/L	15 species	Tynan *et al.* 1993
Imazalil	Imaverol, Clinafarm, Enilconazole	1-[2-(2,4-Dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole		*Spathiphyllum floribundum* Schott	Werbrouck and Debergh 1995
Omadine	Pyrithione, PTO	1-Hydroxy-2(1*H*)-pyridinethione	5 mg/L	*Cattleya aurantiaca, Stanhopea oculata*	Thurston *et al.* 1979
PCNB	Botrilex, Terraclor, Qunitozene, etc.	Pentachloronitrobenzene	100 mg/L	*Cattleya aurantiaca, Stanhopea oculata*	Thurston *et al.* 1979
Tilt	Banner, Desmel, Orbit, Radar, Propiconazole	1-[[2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole	1 g/L	*Theobroma cacao*	Yidana *et al.* 1987; Yidana 1988
Thiabendazole	MK-360, Omnizole, Thiaben, TBZ, etc.	2-(4-Thiazolyl)-1*H*-benzimidazole	10–50 mg/L	*Nicotiana tabacum*	Pollock *et al.* 1983

al. 1995). An antibiotic and a fungicide may be combined, or a combination of antibiotics may be used to target more than one type of organism. Care should be taken, however, so that no incompatibility exists between the antimicrobial agents and components of the medium. For example, antagonism has been found between magnesium and gentamycin and between magnesium and calcium and polymyxins E and B. Moreover, pH can affect the activity of some antibiotics. Streptomycin, for example, is 500 times more active at pH 8.5 than at pH 5.5 (Falkiner 1990). In contrast, inorganic salts are more available to plant tissues *in vitro* when the pH ranges between 5 and 6 (George and Sherrington 1984).

The effects of antimicrobial compounds on the plant tissue must also be considered, although this will depend on the species and may even vary between tissues of the same plant (Mathews 1988). Antibiotics have been shown to inhibit the initiation of callus, shoot growth, or embryogenesis from explants of various species (Dodds and Roberts 1981; Loschiavo *et al.* 1986; Waldenmaier *et al.* 1986; Biasi 1995; Teng and Nicholson 1997; Seyring 1999). The antibiotic chlortetracycline can disrupt pollen tube elongation by binding calcium (Reiss and Herth 1982), while penicillins can alter the activities of enzymes involved in nitrogen metabolism (Santos and Salema 1989). On the other hand, there are a number of reports where no negative effects have been observed from the use of antibiotics in tissue culture and in some cases antibiotics have stimulated regeneration and/or callus growth (Owens 1979; Phillips *et al.* 1981; George and Sherrington 1984; Mathias and Boyd 1986; Borrelli *et al.* 1992; Pius *et al.* 1993; Yepes and Aldwinckle 1994).

Similar results have been reported with fungicides *in vitro*. One of the most widely used fungicides in plant tissue culture is benomyl (benlate), although a number of others have also been used (Table 2). In some cases growth inhibition has been reported (Tynan *et al.* 1993; Watt *et al.* 1996), but in other cases, fungicides have shown growth regulator activity and stimulated growth (Skene 1972; Thomas 1974; Werbrouck and Debergh 1995).

More recently, a non-traditional antimicrobial compound, Plant Preservative Mixture™ (PPM) (Plant Cell Technology, Inc., Washington, DC) has also been investigated for *in vitro* collecting (Renfroe *et al.* 1999; 2000; Pence *et al.* 2000). As with antibiotics and fungicides, it can be used for surface sterilization as well as in the medium to eliminate endogenous contamination. PPM has been used in plant tissue culture to prevent air-borne contamination in situations where a laminar flow hood was not available (Cumbee

1998; Neidz 1998), suggesting its potential usefulness in *in vitro* collecting. It has been tested on a number of species at concentrations between 0.05 and 0.2 % (v/v) (Compton and Koch 1999; George and Tripepi 1999; Renfroe *et al.* 1999; Babaoglu and Yorgancilar 2000; Coloue *et al.* 2000; Geerts *et al.* 2000; Guri *et al.* 2000; Pence *et al.* 2000; Reed 2000; Pereira, pers. comm) and, as with many other antimicrobial compounds, it is generally of low toxicity, but may inhibit growth depending on the species and the concentration of PPM used. Because of its stability compared with many antibiotics, it may find use in *in vitro* collecting, either alone or in combination with traditional antibiotics or fungicides.

In addition to incorporating antimicrobial compounds directly into the tissue culture medium, a number of protocols have used antibiotics, fungicides or PPM to treat tissues before culturing (Haldeman *et al.* 1987; Suri *et al.* 1999; Guri *et al.* 2000). Such treatments are easily adapted to *in vitro* collecting situations (see Chapters 5, 9 and 12).

Some chemicals, particularly certain antibiotics, are not stable to autoclaving and must be filter sterilized before use, while in other cases activity is short-lived once they are in solution. These compounds may need to be taken into the field as powders, dissolved and filter sterilized at the time of collection and added to the medium in the field (Pence *et al.* this vol.). Other, non-chemical, treatments may also be useful in reducing contamination. For example, a cold treatment after surface sterilization on temperate woody species decreased endogenous contamination with *Bacillus subtilis* (Kowalski and Van Staden 1998). This approach might be adaptable to *in vitro* collecting cold tolerant species in cases where ice or refrigeration is available.

For an antimicrobial compound to be most useful, contaminants should be identified in advance in order to select the most effective chemicals. The toxicity of these to the plant should then be tested in order to minimize damage to the tissues in vitro. If this is not possible, broad spectrum chemicals of low toxicity should be used to increase the chances of producing clean, healthy cultures. The collector should be alert to the possibility that the pathogen may not remain sensitive to the antimicrobial agent for long, but is likely to develop a resistance to the product. The alternation of different chemical treatments is one strategy for handling this problem. Many authors consider antimicrobial treatment as preventive, not curative (Falkiner 1990; Leifert and Waites 1990; Barret and Cassells 1994). Nevertheless, when dealing with valuable material (e.g. the only introduction of a given species), the use of such compounds is amply justified to rescue

the explants until aseptic cultures are obtained. In the case of Musa, this was a successful procedure: the contaminated material was rescued, then placed in the greenhouse until new growth was achieved and later it was re-introduced in vitro (J.A. Sandoval, unpublished data).

Salvaging cultures

If an explant becomes contaminated, attempts can be made to salvage the culture, if the value of the tissue warrants the investment of time and materials. At times, a fungus may be slow growing and confined to only a portion of an explant. In some such cases, it has been possible to cut away the infected area, resterilize the surface of the tissue and then reculture it with a fungicide. This method was effective in salvaging tissue from half a leaf disc of *Hippobroma longiflora* (L.) G. Don. f. which then went on to establish healthy shoot cultures (Plair and Pence 1999). On the other hand, bacterial contamination is generally more difficult to eradicate and it is often more difficult to isolate uninfected tissues than with a slow growing fungus. For very valuable germplasm, however, application of antibiotics may be worth attempting. Important lines should not be discarded until the tissue fails to grow. Some tissues may grow in the presence of bacteria and in very rare cases, the growth of healthy plant tissue may eventually suppress bacterial growth (Pence 1999).

It is important to note that any salvaging operation is more likely to be successful the earlier it is attempted. Thus, cultures should be monitored frequently for signs of contamination and appropriate steps taken as soon as any contamination is observed.

Conclusions

In vitro collecting can be used to collect germplasm for propagation in order to provide material for field collections or to store species that are difficult to conserve using traditional methods. Obtaining contaminant free cultures is the first and likely most challenging, step in initiating tissue cultures using this technique, because procedures are often performed in the open air and involve field grown plants.

Surface sterilization is generally effective in removing epiphytic contaminants, but much of the contamination resulting from *in vitro* collecting originates from endophytic bacteria and fungi. The use of antimicrobial agents either in combination with surface sterilization or incorporated into the culture medium is required to eliminate these contaminants. The response of tissue to antimicrobial chemicals will differ according to species and care

must be taken to balance effectiveness with toxicity to the host tissue. Nevertheless, many such agents have been used either individually or in combination, to successfully recover aseptic cultures. The added time, labor and expense involved in using these compounds in *in vitro* collecting protocols is amply justified by the need to obtain clean tissues for germplasm conservation.

Part II.
Case studies

Chapter 4. Coffee

Héctor Lozoya Saldaña, Mabilia Oicatá L., Miriam M. Borbor Ponce and José H. Calderón Díaz

Introduction

Coffee (*Coffea arabica* L.) is a crop of major importance to the tropics. Genetic improvement centres on increasing its yield, adaptability and disease resistance, but this requires a functional genebank that is accessible and where germplasm is safely conserved. Such banks have been established with international expeditions which collected small plants, stem segments and seeds (Anthony *et al.* 1987). In some cases, however, a collecting trip is made and no seeds are found. Similarly, depending on the location of the species, the duration of the expedition, time of year, etc., seeds or vegetative materials may deteriorate and lose viability in transport. *In vitro* collecting is an alternative solution to such problems; it is a means of extending the viability of the plant material gathered in the field (Withers 1987).

Since the beginning of the 1980s, *in vitro* culture of coffee has been described using a variety of explants: somatic embryos, vegetative apices, axillary buds from orthotropic and plagiotropic branches, microcuttings and *in vitro* germinated seeds (Staritsky and Van Hasselt 1980; Sondhal *et al.* 1981; Guzman and Berthouly 1987; Schöpke *et al.* 1987; Bertrand-Desbrunais 1991). The main problems in establishing the explants are tissue browning and contamination of explants by fungi and bacteria, for which antioxidants, fungicides and antibiotics have been used (Guzman and Berthouly 1987; Duhem *et al.* 1988). The fungi and bacteria that are present are usually saprophytes, which contaminate the medium, but not the tissue. Nevertheless, they either acidify the medium or cause its decomposition, in addition to invading the small space available for plant growth.

A number of procedures are available for dealing with the problem of contamination in plant tissue cultures, which have been described in details in the previous chapter. This study attempts to adapt these laboratory methodologies, with a special emphasis on the control of contamination and browning, to their utilization in the field in order to increase the efficiency and effectiveness of collecting of coffee germplasm.

Materials and methods

Explants were dissected and cultures inoculated on a stainless steel tray placed over a permanent flame. If collecting was carried

out outdoors, the tray was placed as far away from the ground as possible, on stumps or stones. Explants were inoculated on two culture media: Medium A comprising the salts of Murashige and Skoog (1962), with 1 mg/L thiamine, 100 mg/L myo-inositol, 30 g/L sucrose and 6 g/L agar; and Medium B, consisting of Medium A supplemented with 100 mg/L benomyl, 100 mg/L gentamycin and 1 g/L activated charcoal.

A series of experiments tested different surface sterilization methods. Chemicals were used at the following concentrations: alcohol, 70%; commercial sodium hypochlorite, 10% dilution; ascorbic acid, 100 mg/L; Tween, as Tween-80®, 0.05%; benomyl, 100 mg/L; gentamycin, 100 mg/L. Samples were immersed for 10 min in the various sterilization solutions tested. All treatments were followed by rinsing in sterile water, unless indicated otherwise.

Two groups of surface sterilization treatments were tested, using single nodes with axillary buds from orthotropic stems collected at or near CATIE.

CATIE field genebank inoculations
Samples of cv. Catuai were collected in CATIE's field genebank, 600 m above sea level. Surface sterilization treatments included immersion in: (a) alcohol; (b) commercial sodium hypochlorite; (c) BGA, a mixture of benomyl, gentamycin and ascorbic acid, without rinsing; or (d) sterile water (control).

Laboratory inoculations
Corresponding laboratory inoculations were performed in CATIE's Biotechnology Unit on the same day as the inoculation in CATIE's field genebank, using the same procedures and materials. Small twigs were transported to the laboratory in an ascorbic acid solution.

'La Lola' Experiment Station inoculations
Explants of cv. Catimor were collected in 'La Lola' Experiment Station, 60 m above sea level and 70 km from the CATIE field genebank. Surface sterilization treatments included immersion in: (a) alcohol plus Tween; (b) commercial sodium hypochlorite plus Tween; (c) BGA plus Tween (BGAT), without rinsing; or (d) ascorbic acid plus Tween, without rinsing.

Second field genebank inoculations
A second set of explants from cv. Catuai were collected, with modifications in the technique based on the previous experiments. Segments of orthotropic stems, each with two nodes starting from

the apex, were selected as explants. They were surface sterilized by immersion in Tween under constant shaking, then immersion in the BGAT solution and finally washing again in the Tween solution.

Four days after inoculation, measurements were made of contamination, degree of tissue browning, presence of fungi and bacteria and tissue's general vigour, which indicated viability and potential for recovery through further sterilization and culture. Each treatment included 5–7 replicate samples.

Five days after inoculation, the cultures were re-sterilized under laboratory conditions, by immersion in a solution of sodium hypochlorite and Tween, followed by immersion in the BGAT solution, then washing in an ascorbic acid solution. Tissues were then transferred to Medium B without activated charcoal.

Results

CATIE field genebank inoculations

Contamination: Control tissues, rinsed only with water before inoculation, had 100% contamination by bacteria or fungi on both Media A and B. None of the other surface sterilization treatments showed consistency in the control of fungi and bacteria, although tissues treated with sodium hypochlorite and cultured on Medium B were free of contamination.

Browning: On medium A, browning ranged from 8 to 31%, while on Medium B, browning ranged from 11 to 73%. On both media, browning was greatest on tissues which had been surface sterilized with alcohol.

Vigour. The potential for tissue recovery was greatest when tissues were surface sterilized with sodium hypochlorite or with the BGA mixture, being 85% for both treatments on Medium A and 71 and 100%, respectively, on the more complex Medium B.

Laboratory inoculations

These tissues differed from the CATIE field genebank inoculations in that they were soaked in ascorbic acid for several hours during transport and were inoculated in the laboratory.

Contamination: There was a high rate of bacterial contamination on both media. In contrast, fungi were eliminated by treatments with alcohol or sodium hypochlorite in Medium A, while they were reduced to below 30% in Medium B.

Browning: Here, browning rates were similar, ranging from 8% to 36% on Medium A and from 11 to 31% on Medium B. Browning was greatest on tissues which had been surface sterilized with sodium hypochlorite, on Medium A, or alcohol, on Medium B.

Vigour: When tissues were washed with BGA and inoculated onto the simpler Medium A, tissue vigour was conserved in 85% of the explants when they were subsequently washed with sodium hypochlorite and inoculated on the more complex Medium B.

'La Lola' Experiment Station inoculations

Contamination: Contamination was notably lower in these experiments in which a surfactant (Tween 80®) and ascorbic acid were incorporated in the surface sterilizing treatments. Explants on Medium A showed no fungal contamination and only those which had been prewashed with alcohol or ascorbic acid had bacterial contamination. On Medium B, fungi were detected only on cultures prewashed with ascorbic acid and bacteria appeared only on explants washed with sodium hypochlorite.

Browning: Tissues on Medium A showed the most browning when treated with sodium hypochlorite or alcohol, while on Medium B browning was greatest on those pretreated with sodium hypochlorite or ascorbic acid. Browning was lowest on both media (14 to 18%) when tissues were surface sterilized with the BGAT solution.

Vigour: Tissue vigour was high on Medium A, unrelated to the degree of tissue browning, contamination, or surface sterilization method used. In contrast, on Medium B, maximum vigour (80%) coincided with the least browning of tissue and the total absence of microorganisms after surface sterilization with alcohol. Rinses with sodium hypochlorite or ascorbic acid reduced the tissue's overall vigour and only 20% of explants could be recovered.

Thus, in this collecting trial, the best results were obtained using Medium A and the BGAT mixture for surface sterilization. This favoured the least browning (14%), the absence of microorganisms and 80% of the plant material could be recovered.

Second set of field genebank inoculations

With the modifications in the explants selected and the sterilization process, contamination by microorganisms was nil, tissue browning minor and the two nodes conserved the vigour of the explant.

Discussion and conclusion

The culture media used in these experiments did not contain growth regulators because the objective was to use *in vitro* collecting as a temporary measure for transporting tissues to the laboratory. The option of transfer at a later stage to other media would depend on vigour, contamination, tissue browning and degree of development of the buds.

In our study, the high rate and inconsistent control of contamination during both the collecting in CATIE field genebank and laboratory inoculation appeared to be a result of the absence of surfactants in the surface sterilizing solutions. This was corrected in further collecting experiments. Methods of surface sterilization which included biocides were the most successful, resulting in about 80% clean cultures, followed by methods involving sodium hypochlorite. Sterilizing with alcohol induced tissue decomposition and browning, whereas washing with water alone or with ascorbic acid resulted in unacceptably high contamination. The growth of microorganisms in these cultures indicated that the fungicide and antibiotic that were incorporated into the culture medium were not effective in controlling this type of contamination.

Recommendations for future work include:

1. Prewashing the twigs with detergents as the first step of surface sterilization;
2. Using more complex mixtures of selective biocides and antioxidants;
3. Specifically for coffee, using 4–5 cm long explants that include two nodes with two buds on each.

Considering that coffee explants inoculated *in vitro* in the field are kept on the inoculation medium for a limited time only, the *in vitro* collecting procedures described here provide a highly workable technique for collecting coffee germplasm. Overall, this method minimizes the risk of rapid deterioration of the collected samples and extends the collecting period. It also confines pathogens endemic to the plants *in situ* to the test tube and, except for obligate parasites, the remaining pathogens can be determined visually by colony growth in the culture medium. These additional advantages make *in vitro* collecting a powerful alternative to traditional methods used in expeditions for collecting coffee germplasm.

Chapter 5. Cacao

V. Silvanna Alvarenga, Luciano de Bem Bianchetti, Patricia E. López González, Olga E. Sandoval and Marta B. Zacher de Martínez

Introduction

Collecting germplasm of cacao (*Theobroma cacao* L.) for *ex situ* conservation is a particular challenge, because seeds of this species are highly recalcitrant. Cuttings and mature seeds are generally used for propagation, but, because they lose viability rapidly, they must be transferred quickly to the field genebank. As a result, it can be difficult to maintain planting material alive over long distances between the collecting and final planting sites. The utilization of tissue culture techniques, however, provides an alternative for collecting and conserving genetic resources. Microcuttings and embryos can be collected and maintained in genebanks that conserve germplasm and support breeding programmes.

Cacao (Order Malvales; Family Sterculiaceae) originated in Colombia, Ecuador, Peru and Brazil, according to Cheesman (1935) and Pound (1935), although other important centres of diversity, such as Mesoamerica, also exist (Enríquez 1985). More than a thousand million cacao trees are cultivated throughout the world, occupying an area of about 7.36 million hectares. According to FAO (2001), world production of dried seed was about 7.36 million tons in 2000, but every year millions of these trees become diseased and must be replaced.

Cacao seeds are recalcitrant and lose viability in less than 30 days, making conservation of the seeds very difficult (Enríquez 1985). In 1983, the IBPGR (now IPGRI) Subcommittee in charge of *in vitro* storage decided to examine tissue culture techniques and their potential application for the conservation of species with recalcitrant seeds (IBPGR 1984). In the case of cacao, *in vitro* collecting of microcuttings and mature embryos were identified as alternative methods to test in the field.

A simple *in vitro* collecting technique for cacao was developed at the University of Nottingham by the IBPGR and in Ghana by the Cacao Research Association (Yidana *et al*. 1987; Yidana 1988). Cuttings with nodes were surface sterilized by immersion in a mixture of fungicides and water purification tablets dissolved in boiled water. The cuttings were then transferred with clean tweezers to tubes with semi-solid medium containing fungicides and antibiotics. Five weeks later, no fungal contamination was observed and bacterial contamination was about 10% (Withers 1987).

Box 1. *In vitro* collecting protocol for cacao as developed by Yidana (1988)

Equipment
The basic equipment required for *in vitro* collecting and field inoculation of cacao cuttings includes the following items:
   1 pair of secateurs for taking cuttings; 1 pair of forceps for handling explants; 10 mL sterile plastic tubes containing semi-solid medium supplemented with fungicide (Tilt MBC 0.1%) and antibiotics (rifamycin + trimethroprim, 15 mg/L each); tube racks for transporting the test tubes; water sterilizing tablets; fungicide (FBC Protectant Fungicide); boiled tap water; screw cap containers.

Protocol
- Take 2–4 cm long stem nodal cuttings with a small portion of leaf (ca 2 cm x 2 cm).
- Place explants in a glass jar and a known volume of boiled water, sufficient to completely cover the explants.
- Add the water sterilizing tablets (10/100 mL) and the fungicide (0.05 %).
- Close the lid of the container, shake it frequently for 10 min and leave it to stand for an additional 20 min.
- Open the lid, take the cuttings with forceps and insert them into the collecting tubes.
   All explants are handled with forceps sterilized in the same manner as explants.

In addition to collecting vegetative material, establishing adequate *in vitro* collecting methods for embryos would increase the potential for conserving cacao germplasm and could serve as a model for other species with recalcitrant seeds. The specific objectives of the research described below were to compare embryos and vegetative explants with regard to methods for surface sterilization and to selecting the best culture medium for these explants.

Materials and methods

Collecting was carried out in CATIE field genebank and in its 'La Lola' Experiment Station in the Province of Limón. Materials were handled in a simple protective chamber made of thick cardboard.

Both microcuttings and mature embryos were collected. Microcuttings were taken from young basal orthotropic branches, consisting of about 1 cm of petiole and 4 cm of the main shaft, with the leaves removed. For embryos, mature fruits, about 180 day-old, were selected. The seeds were extracted from the fruits and dissected to obtain embryonic axes with a piece of cotyledon attached.

Several surface sterilization treatments were tested with microcuttings (Table 3). In the case of embryos, the entire cacao fruit was surface sterilized by flaming after immersion in 95% alcohol. The fruit was then cut longitudinally, taking care not to damage the seeds and the funiculus scar was located to identify the position of the embryo axis. A longitudinal cut was then made into the cotyledons on about two-thirds of their length, in the region opposite the embryo axis. Using a scalpel and tweezers, the cotyledons were separated, exposing the embryo axis, which was then carefully removed and placed on the culture medium. Tests were then made to examine the effect of media components on contamination and survival of microcuttings and embryos.

Table 3. Surface sterilization treatments used for *in vitro* collecting of cacao microcuttings at CATIE, Costa Rica

Sterilization method		Treatment	
Code	Treatment	A	B
1	Sterile water + soap	Wash	Wash
2	Sterile water	Rinse	Rinse
3	Fungicide	Ridomil® (1 g/L) for 15 min	Benomyl (80 mg/L) for 15 min
4	Antibiotic	None used	Gentamycin (80 mg/L) for 15 min
5	Fungicide + antibiotic	Benomyl (1 g/L) + ampicillin (100 mg/L) for 15 min	Benomyl (1 g/L) + gentamycin (80 mg/L) for 15 min
6	Calcium hypochlorite + Tween	Commercial calcium hypochlorite at 10% dilution + Tween-20® for 15 min	Commercial calcium hypochlorite at 10% dilution + Tween-20® for 15 min

All media contained half-strength Murashige and Skoog (1962) (MS) salts. For microcuttings, all media contained 10 g/L sucrose and 2 g/L Gelrite, except for the control medium which contained 30 g/L sucrose. Additions to the media included, as indicated, polyvinylpyrrolidone (PVP), 10 g/L; benomyl, 100 mg/L; and gentamycin, 40 mg/L. For embryos, all media included gentamycin, 40 mg/L; activated charcoal, 2 g/L; and agar, 8 g/L; with 0, 1 or 3% sucrose. All media were adjusted to pH 5.7 and then autoclaved for 21 min at 121°C (1.1 kg/cm^2). After transport to the laboratory, the cultures were placed in a controlled environment, with a temperature of 27±2°C, a photoperiod of 16 h light/8 h dark and a light intensity of 2000 lux.

Microcuttings were evaluated for the presence and type of contamination (bacteria or fungi), the degree of mortality, the amount of mucilage and tissue browning and the capacity for recovery. Embryos were evaluated for the presence of contamination, tissue browning, elongation and growth of the radicle and plumule.

Results

Microcuttings were collected from both the CATIE field genebank and the 'La Lola' Experiment Station and used to evaluate the different surface sterilization methods before transfer to control medium. All but two of the surface sterilization treatments described in Table 3 resulted in 100% contamination and loss of the shoots. Treatment 3A, containing Ridomil® at 1 g/L, produced a high degree of tissue browning and secretion of mucilage from the explants. All 12 explants on this medium were contaminated, 11 with fungi and 1 with bacteria, although this produced only 33% mortality among the explants. Treatment 5B, which included

benomyl and gentamycin, showed the least contamination (57%), although all of this was fungal. Twelve of the 14 explants treated survived, however and there was less browning than on medium 3A. On the basis of these results, only treatment 5B was used in the next experiment.

A second batch of microcuttings were collected in CATIE's field genebank and in the 'La Lola' Experiment Station. Some bacterial contamination was observed on all media tested, although the presence of antibiotics and/or fungicides helped reduce contamination compared with the control (Table 4). The PBG medium, which contained an antioxidant as well as an antibiotic and a fungicide, showed the highest explant recovery rate and the lowest degree of tissue browning and mortality.

In the case of embryos, 9 days after collecting, there was very little contamination on any of the media tested (Table 5). A number of them had started to germinate, particularly on 1% sucrose and there was little browning of the tissues.

Table 4. The effect of culture medium on contamination of cacao microcuttings after *in vitro* collecting. Ten explants were tested on each medium

Culture medium[†]	Contamination (%)	Type of contamination		Recovery (%)	Mortality (%)	Tissue browning (%)
		Bacteria	Fungi			
—	90	+	–	80	20	50
PG	50	+	–	70	30	40
PBG	40	+	–	100	0	20
PB	30	+	–	90	10	20

†P = PVP (10 g/L); B = benomyl (100 mg/L); G = gentamycin (40 mg/L).

Table 5. Observations made on mature cacao embryo axes after *in vitro* collecting. Twelve embryos were tested for each medium

Sucrose (%)	Contamination (%)	Type of contamination	Embryos with plumule (%)	Embryos with radicle (%)	Tissue browning
0	0	—	0	33	+
1	8	Fungi	8	83	+
3	0	—	0	45	+

+ = Low.

Discussion and conclusion

The control of contamination in microcuttings of cacao initiated by *in vitro* collecting was improved by surface sterilizing in a solution containing the fungicide benomyl and the antibiotic gentamycin.

The use of Ridomil® as a surface sterilant increased the rate of tissue browning, although some browning was seen in all treatments. Browning in explants is related to their production of polyphenoloxidases and tyrosinases, enzymes that act as regulators of IAA, which are toxic when concentrated (Forrest 1969). Browning is also related to the specific contents of tannins and polyphenols, to metabolic activity in the area from which the explant was taken and to the time of year in which the collection was made. However, polyvinylpyrrolidone (PVP), a polyamine that absorbs phenols (George and Sherrington 1984), was an effective antioxidant when incorporated into the medium.

Good recovery of embryos was obtained by flaming the cacao fruits to eliminate surface contamination. Embryos grew more quickly when they were transferred on a medium containing some sucrose, as was also reported by Esan (1977). Tissue browning was minimal, possibly as a result of the presence of activated charcoal. Compton and Preece (1986) suggested that activated charcoal absorbs toxic metabolites released into the medium, thus reducing browning. In general, embryos were more easily manipulated than microcuttings, making *in vitro* collecting of cacao embryos a viable option for rescuing the genetic variability of this species.

Further research with cacao is needed to determine whether the age of the explant, hour of collecting, presence of pathogens, genotype and time of year influence mucilage secretion and production of polyphenols in microcutting explants initiated by *in vitro* collecting. Work should also continue on the use of fungicides and antibiotics in the culture medium, with the goal of reducing contamination to the lowest degree possible, while still permitting the survival of the explants.

Trials should also be carried out to increase viability during transit to the laboratory and to optimize the physiological conditions required by the explants to guarantee their capacity for morphogenesis. Because of their proven capacity for embryogenesis (Esan 1977; Pence *et al.* 1979; Lopez-Baez *et al.* 1993), *in vitro* collecting methods should also be attempted using floral structures and immature embryos.

Chapter 6. *Musa*

Luz M. Montoya Henao, César Tapia B., Francisco L. Espadas y Gil and Jorge A. Sandoval

Introduction

Conservation of plant genetic resources is a priority for maintaining genetic variability–the basis of all plant breeding programmes. *Musa* L. is among many groups of species that are suffering from genetic erosion. Jaramillo (1983) and De Langhe (1984) pointed out that, little by little, cultivars and wild types of particular interest (e.g. *Musa* AA, BB, ABB, AAB, AAA, AAAB, etc.) are disappearing from the centre of genetic diversity of this crop and from other areas as a result of natural disasters, nomadic agriculture and deforestation.

For this reason and because of its importance as a food crop, IBPGR (the predecessor of IPGRI) considered collecting *Musa* germplasm a Type I priority activity, in order to establish *ex situ* collections (IBPGR 1983). Propagation in most *Musa* species is vegetative, however, because their fruits are parthenocarpic (Stover and Simmonds 1987). Corms are used for propagation, but their large size makes transport from collecting sites to research centres or genebanks very expensive. Hence, collectors will benefit from methods that improve the management of these propagules. Tissue culture is one such method and its use for *in vitro* collecting of explants in the field is a real possibility. The goal of the following studies was to define the minimal conditions needed for the *in vitro* collecting of *Musa* explants, such that viable explants could be recovered under standardized conditions.

Materials and methods

Work was carried out with the cv. Valery (*Musa* AAA), found in the Musaceae collection held at CATIE. Sword shoots, 30–50 cm high, were selected. The external parts of the corm were removed, together with foliar sheaths, until sections about 8 cm long and 5 cm in diameter were obtained, which enclosed vegetative apices. This material was surface sterilized by immersion for 20 min in undiluted commercial bleach (Ajax® chlorine; 5.25% sodium hypochlorite), followed by two sterile water washes, 5 min each. In some cases, the explants were also washed with soap or immersed for 10 min in an antioxidant solution (ascorbic acid at 100 mg/L). The material was then reduced to a manageable size (4–5 cm) for *in vitro* inoculation, using either forceps and a scalpel or by hand (after previous disinfection with 70% alcohol). The resulting segments were cultured on a Murashige and Skoog (1962) medium with or

without vitamins, supplemented, as indicated (Table 6), with activated charcoal at 0.15%, or wide-spectrum fungicides and bactericides (benomyl, 100 mg/L; gentamycin, 100 mg/L; ampicillin, 100 mg/L; chloramphenicol, 100 mg/L), but in concentrations of low toxicity for the explants (Pollock *et al.* 1983; Shields *et al.* 1984). Media also contained sucrose, 30 g/L; and either Difco agar, 7 g/L, or Gelrite, 2 g/L.

Table 6. Media used for *in vitro* collecting of explants from *Musa* AAA cv. Valery, at CATIE, Costa Rica

Medium no.	Components[†]
1 (control)	MS, vitamins, sucrose, agar
2	MS, activated charcoal, sucrose, agar
3	MS, vitamins, activated charcoal, benomyl, gentamycin, sucrose, agar
4	MS, vitamins, ampicillin, sucrose, gelrite
5	MS, vitamins, chloramphenicol, sucrose, gelrite

[†]See text for concentrations

Dissection (i.e. the reduction of explant to a size of 4–5 cm) was carried out in a 'rustic chamber', with a thin wood base and a double thickness of filter paper for its sides and top. This chamber was placed with its opening away from the prevailing wind, sprayed with 70% ethanol and an alcohol burner was introduced to sterilize instruments (e.g. forceps and scalpels) by flaming.

Once the field work was completed, cultures were packed in a box previously sprayed with 70% alcohol, transported to the laboratory and placed in a growth chamber at 26±1°C, 80% relative humidity, a photoperiod of 16 h light/8 h dark and a light intensity of 50 µE m^{-2} s^{-1}. The explants were carefully evaluated for contamination, tissue browning and survival rate at 5 or 7 days after inoculation. The degree of tissue browning was estimated on a scale of 0–3, where 0=no browning, 1=some browning, 2=intermediate browning and 3=considerable browning.

Results

Five days after inoculation, the only contamination observed was on medium 3 (20% with fungi). Seven days after inoculation, medium 2 also showed contamination, with 20% of explants infected with bacteria, suggesting that, with increasing time, more contamination would be seen.

Large differences were seen with tissue browning in that explants handled with forceps and scalpel had much more

browning than those handled only with a scalpel or by hand. This type of browning, however, appeared only on the surface of the explant. When an ascorbic acid solution was used, browning was considerably reduced.

Explant survival was 100%. As with contamination, this percentage was independent of the handling or surface sterilization method. No significant differences were seen among the different media used. In the control medium, which received no components to counteract possible contaminants, fungi and bacteria were also not detected.

Discussion and conclusion

The effectiveness of sodium hypochlorite for surface sterilizing explants in this study corroborated the successful findings of Vuylsteke (1989) and Sandoval *et al.* (1991) under laboratory conditions. We did not observe fungal contamination, most probably because sodium hypochlorite was very effective. Moreover, the morphology of *Musa* is such that the apical meristem is located in a closed depression between the foliar bases surrounding the corm (Simmonds 1973) and is thus well protected from external infection.

In contrast, contamination by bacteria was about 40% in all treatments. Some endogenous bacteria in *Musa* are known to cause problems for *in vitro* culture (Bakry 1984). Media supplemented with antibiotics provide an alternative (Leifert and Waites 1990), although, in this study, gentamycin was not fully effective. Nevertheless, in general, contamination was not a serious problem and the methodology used to manage it was efficient. Contaminated explants could be surface sterilized again under laboratory conditions.

Tissue browning in explants was greater when they were dissected with forceps and a scalpel than when they were excised by hand or with the scalpel alone. Tissues were more likely to be injured by the first method and thus more likely to release compounds that can be oxidized by air, such as peroxidases or polyphenoloxidases. These cause tissue browning and/or the release of phenols to the environment (Compton and Preece 1986). Nevertheless, ascorbic acid was very efficient in preventing browning, a finding that was in line with those of Compton and Preece (1986), Vuylsteke (1989) and Sandoval *et al.* (1991) for *Musa*. In this latter study and in our work with the 'Valery' clone, tissue browning was relatively minor (a value of 1). However, depending on the cultivar or species, browning can be very severe, as in the 'Curraré' plantain (AAB), *Musa balbisiana* Colla. and *Musa textilis* Née (J.A. Sandoval 1995, pers. comm.).

Vegetative apices in *Musa* are suitable explants for *in vitro* germplasm collecting in the field, requiring only a temporary, relatively simple medium. Once in the laboratory, they can be surface sterilized again and established on a standardized medium.

Trimming of the material can be done by hand, provided operators have disinfected their hands in alcohol. This type of handling prevents possible injury from forceps and scalpel, thus minimizing tissue browning, although a scalpel may help in the final stages. In addition, the use of ascorbic acid is advisable to prevent browning in explants of *Musa* AAA cv. 'Valery'.

Future *in vitro* collecting trials should include other parts of the *Musa* plant, for example, the floral apex. This apex, because of its location on the plant, is usually cleaner than vegetative apices (J.A. Sandoval, pers. comm.). Methods for the *in vitro* culture of floral apices of *Musa* have been published (Srinivasa Rao *et al.* 1982; Cronauer and Krikorian 1985). The high percentage of culture survival (100%) in this study, however, confirms the efficiency of the method used and the likely support it will offer to collecting *Musa* germplasm.

Chapter 7. *Citrus*

Abdenago Brenes Hines, Vidalma García Tapia and Eyla Velasco Urquizo

Introduction

Orthodox seeds represent the simplest and most economical type of material for collecting and conserving plant germplasm. In the case of vegetatively propagated species or species with recalcitrant seeds, however, the use of alternative techniques, such as *in vitro* collecting, is required (Withers 1987).

In the case of *Citrus* L., grafting of vegetative cuttings is the most widely used method for propagating valuable genotypes (Platt and Opitz 1973). Even when seeds are used, Marte (1987) showed that the germinability of *Citrus* seeds falls drastically if their moisture content drops below 70%, a situation that can occur rapidly when seeds are exposed to hot tropical conditions. This may be due to the induction of dormancy, rather than true recalcitrance (Soetisna *et al.* 1985), but in either case, a decrease in moisture makes germination of *Citrus* seeds difficult. These factors justify studying the possibility of using *in vitro* collecting of microcuttings and seeds as a means for collecting and preserving valuable *Citrus* germplasm.

Navarro (1984), when studying traditional tissue culture techniques for the genus *Citrus*, found the following to be the most important applications:
1. Culture of the nucellus to obtain plants from the maternal genotype in mono-embryonic varieties;
2. Culture of the ovules to obtain nucellar plants from poly-embryonic parthenocarps;
3. Micrografting to obtain virus-free plants.

When refrigeration cannot be used, Navarro (1988) recommended that collecting be carried out during a period of dormancy, i.e. during winter in sub-tropical areas and the dry season in tropical areas. Young tissues are the best source of buds for micrografting (Skirvin 1981; Marte 1987).

Although some experiments on *in vitro* techniques of germplasm collecting have been carried out, research on this theme is still preliminary (IBPGR 1984). Withers (1987 and Chapter 1) has discussed the main principles of *in vitro* collecting in terms of adapting the aseptic conditions of the laboratory. Building on this idea, the objective of this study was to determine the applicability of *in vitro* collecting to *Citrus* spp. with the goal of preserving explants for the minimum time needed for transport from the field to the laboratory.

Materials and methods

Germplasm collections of *Citrus sinensis* L. were made from the Cabiria collection held by CATIE. Straight twigs, about 8 cm long, were collected from the distal part of young, orthotropic branches. These segments were divided into 3–4 cm long microcuttings, which constituted the vegetative explants. Seeds were also extracted from mature healthy fruits and their testa were removed before surface sterilization and *in vitro* inoculation.

Surface sterilization of the microcuttings consisted of immersion in a 3% or 5% dilution of commercial sodium hypochlorite for 5 minutes, followed by two rinses with sterile water, 1 min per rinse. In the case of seeds, the entire fruit was surface sterilized by flaming, the seeds were extracted and the testa was removed. The seeds were then immersed for 3 min in either sterile distilled water or in a 3% solution of commercial sodium hypochlorite+Tween-20® and this was followed by two rinses (1 min each) in sterile distilled water. The seeds were then held in sterile water to await inoculation.

The media used for both microcuttings and seeds were:

1. MS (Murashige and Skoog 1962) (control)
2. MS+ampicillin (100 mg/L) + benomyl (50 mg/L) (MS-AB medium)
3. MS+chloramphenicol (2.5 mg/L) + benomyl (50 mg/L) (MS-CB medium)

The media were autoclaved at 121°C and 15 p.s.i. for 20 min. Antibiotics were filter sterilized and added to the corresponding media after autoclaving. Each culture flask contained 8 mL of medium.

Surface sterilization and explant inoculation were carried out in the open air. Aseptic measures were limited to sterilizing the working surface (a metal tray, 53 x 30 cm) and forceps by wiping them with 70% ethanol and then exposing them to the flame of a portable alcohol burner. After inoculation, the explants were taken to the laboratory, where they were placed in a controlled environment room (27±2°C; light intensity 2500 lux; photoperiod 16 h light/8 h dark).

The following variables were evaluated at 2 and 4 days after field inoculation: (1) percentage of contaminated explants; (2) percentage of recoverable explants; (3) percentage of microcuttings with tissue browning; and (4) temporary half recovery rate.

In this study, a *recoverable explant* was defined as an explant in one of the following states which could provide at least one healthy node:

1. Healthy, with no contamination and no necrotic lesions.

2. Partially contaminated, with sufficient tissue free of contamination to be used as a source of new explants which could be surface sterilized, dissected and inoculated *in vitro* under laboratory conditions.
3. Having some necrosis, but with sufficient healthy tissue to be used as a source of new explants, which could be dissected, surface sterilized and inoculated *in vitro* under laboratory conditions.

Results

The overall recovery rate of microcuttings was high (87.7%), both 2 and 4 days after inoculation (Table 7). Contamination after 2 days was low, averaging only 3.3% and caused by bacteria. After 4 days, however, contamination levels rose to 13.3% as fungi appeared. Surface sterilization method 2 resulted in more microcuttings recovered at 2 and 4 days, even though the treatment showed a higher percentage of contamination. The percentage of recoverable microcuttings on MS and MS-AB media was similar, while 100% recovery, was obtained on MS-CB medium. No tissue browning was seen among the microcuttings.

Table 7. The effect of surface sterilization treatments and culture media on contamination of microcuttings of *Citrus* collected *in vitro* in the field

Six treatments were tested, two sterilization treatments combined with each of three media, 10 microcuttings/treatment. Results are summarized as averages across treatments.

Treatments[†]	Average recoverable explants at day 2 (%)	Average contamination at day 2 (%)		Average recoverable explants at day 4 (%)	Average contamination at day 4 (%)	
		Fungi	Bacteria		Fungi	Bacteria
All treatments	87.7	0	3.3	87.7	13.3	3.3
Surface sterilization						
3% NaClO	80	0	0	80	6.7	0
5% NaClO	93.3	0	6.7	93.3	20	6.7
Medium						
MS	80	0	10	80	20	10
MS-AB	80	0	0	80	10	0
MS-CB	100	0	0	100	10	0

[†]NaClO=surface sterilant for 5 min followed by 2 rinses of sterile water; AB = ampicillin, 100 mg/L, plus benomyl, 50 mg/L; CB = chloramphenicol, 100 mg/L, plus benomyl, 50 mg/L.

In the case of seeds, recovery was also high, while contamination was relatively low (Table 8). A simple immersion in sterile water

reduced fungal contamination to a low level (16.7%, on average), probably resulting from the removal of the testa. No bacterial contamination was observed. Within four days, seeds exhibited a high level of germination (83.3%). MS-CB medium was the most promising, although by four days, the seeds had begun germinating on all three media.

Table 8. Effect of three media on contamination and germination of *Citrus* seeds without testa, 2 and 4 days after *in vitro* inoculation in the field (10 seeds/treatment)

Medium[†]	Recoverable explants (%)	Contamination (%)		Germinated seeds (%)
		Fungi	**Bacteria**	
Evaluation at day 2				
MS	100	0	0	0
MS-AB	75	25	0	0
MS-CB	100	0	0	0
Average	91.7	8.3	0	0
Evaluation at day 4				
MS	75	25	0	75
MS-AB	75	25	0	75
MS-CB	100	0	0	100
Average	83.3	16.7	0	83.3

[†]AB = ampicillin, 100 mg/L, plus benomyl, 50 mg/L; CB = chloramphenicol, 100 mg/L, plus benomyl, 50 mg/L.

Discussion and conclusion

In this study, the overall contamination of microcuttings was relatively low, although surface sterilization with 5% sodium hypochlorite gave more recoverable explants and slightly more contamination than with 3% sodium hypochlorite. These apparently contradictory findings may have resulted from the fact that inoculations were performed by several different workers and the number of replicates was low.

Fungi were more harmful to the explants than bacteria and, as expected, the level of contamination detected at four days was higher than that observed at two days. This means that the problem of contamination should be evaluated by taking into account the time between *in vitro* inoculation and arrival at the laboratory.

The recovery of an appreciable number of explants (87.7% for microcuttings and 83.3% for seeds, on average) was independent of the sterilization treatment and culture medium used. The highest rate of recoverable explants (100%), however, was obtained

with medium containing the antibiotic chloramphenicol. Pollock *et al.* (1983) did not recommend the use of this antibiotic because of its high toxicity for plant cell culture. They found that the survival rate of protoplasts from *Nicotiana plumbaginifolia* Viv. dropped from 50 to 20% with concentrations of this antibiotic of 2 and 5 mg/L, respectively. Our findings may be explained by the relatively low concentration of the antibiotic that was used (2.5 mg/L) and the use of shoots instead of protoplasts in our experiments. Very little browning was observed, resulting in a high percentage of apparently healthy and normal microcuttings.

Contamination of seeds was also relatively low, but was also higher at the second evaluation. As with the microcuttings, the most promising treatment for seeds was MS + chloramphenicol + benomyl.

From these experiments with *in vitro* collecting of *Citrus,* the following recommendations are made:

- Evaluate, through preliminary experiments, the capacity of collected explants to grow *in vitro* from the moment they are inoculated onto the medium.
- Study the use of temporary, portable chambers that will improve conditions for inoculation in the field.
- Study the effect of other antibiotics, such as cefoxitin and cefotaxime that are wide spectrum, have low toxicity for plant tissues and resist betalactamases (Pollock *et al.* 1983).
- Divide the tasks among the members of the collecting team to refine the procedure for each step, thus reducing contamination produced as a result of variability in skill and inadequate handling by different collectors. The division of labour in the field is an important aspect of planning and executing *in vitro* germplasm collecting experiments.

Chapter 8. Avocado

Jorge A. Sandoval and Victor M. Villalobos A.

Introduction

Despite continuing efforts to provide as many desirable genotypes as possible for *ex situ* conservation, collecting germplasm in remote areas is particularly difficult (Withers 1984). At least three factors impact these collecting efforts:

1. The type and size of the propagule (e.g. stem, tuber, stolon, or cutting)
2. The longevity of the propagule (e.g. affected by pretreatment, packing conditions and deterioration)
3. The distance from the collecting site to the final destination (e.g. *in vitro* culture laboratory, greenhouse, or field genebank)

Large propagules, short-lived tissues and long distances each present hurdles which can decrease the success of a collecting mission. In the search for ways to overcome these problems, *in vitro* collecting has been proposed as a technique with high potential for the efficient collecting of plant material.

Few aspects of *in vitro* collecting in the field have been examined, but the elimination of microorganisms and the survival and later development of the explant are worth studying. Likewise, understanding the factors that interact in the field is essential, as was shown in the successful *in vitro* collecting of coconut (Assy-Bah *et al.* 1987; Sossou *et al.* 1987).

This project aimed to define the minimal conditions for *in vitro* collecting of avocado (*Persea americana* Miller) germplasm. The overall objectives were: (1) to collect germplasm from the field and maintain it *in vitro* until it reaches the laboratory, where it can be handled under aseptic conditions; and (2) to use a convenient culture medium for its conservation and later distribution. The specific objectives of these experiments were: (1) to develop an adequate system for surface sterilizing explants; (2) to determine the most suitable type and size of explants; and (3) to determine the most suitable culture medium for *in vitro* collecting.

Materials and methods

Twigs from trees were collected in the field and inoculated onto a half-strength Murashige and Skoog (1962) medium with 3% sucrose; 400 mg/L filter sterilized ampicillin; 1 g/L activated charcoal; and agar at 1 g/L for semi-liquid medium or 7 g/L for semi-solid medium; pH 5.7.

Seven trials examined a variety of surface sterilization protocols, all of which were conducted in the field. Commercial sodium hypochlorite was used at a dilution of 5%, while ethanol was used at 70%. In some cases the fungicide benomyl and the antibiotic streptomycin were tested, at the concentrations indicated. Unless otherwise indicated, treatments were for 5 min.

Trial 1
The following treatments were tested on 2.5 cm long twigs, followed by washing in sterile water: (1) detergent; (2) sodium hypochlorite; (3) ethanol, for 4 min; (4) detergent + sodium hypochlorite; (5) sodium hypochlorite, followed by ethanol for 1 min.

Trial 2
The following treatments were tested on 5 cm long twigs: (1) sodium hypochlorite; (2) alcohol; and (3) sodium hypochlorite + alcohol.

Trial 3
Twigs were immersed in: (1) sodium hypochlorite, followed by the fungicide benomyl, 500 mg/L, for 20 min; or (2) alcohol, followed by benomyl.

Trial 4
Twigs were treated with: (1) detergent + 2 g/L benomyl + sodium hypochlorite + ethanol; (2) detergent + ethanol, followed by benomyl; or (3) detergent + benomyl.

Trial 5
Twigs were exposed to two series of treatments: (1) sodium hypochlorite, then ethanol, then sodium hypochlorite; or (2) ethanol, then sodium hypochlorite, then ethanol. Explants were then immersed for 15 min in either (1) 500 mg/L streptomycin; (2) 3 g/L benomyl; or (3) streptomycin + benomyl.

Trial 6
A final treatment was tested: alcohol + sodium hypochlorite, followed by immersion in a mixture of 500 mg/L streptomycin + 4 g/L benomyl for 15 min.

Results
Trial 1
The lowest contamination was noted with the treatment including sodium hypochlorite followed by alcohol. Explants did not show contamination for the first 12 days of culture.

Trial 2
The most promising treatment from Trial 1 was examined further. Explants did not begin to show contamination until day 10. Sodium hypochlorite + alcohol was the most effective treatment, in that the explants remained alive and free of contamination for 43 days after culturing.

Trial 3
Evaluation at 8 days after inoculation showed that the combinations of sodium hypochlorite + benomyl and alcohol + benomyl were not toxic to the explants, but contamination occurred within a few days in all treatments.

Trial 4
These treatments were ineffective in controlling contamination.

Trial 5
The best results were obtained with the sodium hypochlorite–alcohol–sodium hypochlorite series followed by treatment with benomyl–streptomycin. The explants were free of contamination for 25 days. In most cases new bud growth was observed.

Trial 6
This treatment proved to be very effective. After 15 days, developing buds were removed from contaminated explants. The buds were surface sterilized again with the same compounds, to which they were exposed for 3 minutes only. Contamination was successfully eliminated, establishing the explants under the standardized conditions of laboratory *in vitro* culture. This procedure was used later, with satisfactory results, for *in vitro* collecting of the coral or flame tree (*Erythrina* L. sp.), vanilla (*Vanilla planifolia* Jackson) and sapodilla (*Pouteria* Aublet sp.).

A number of other observations were made in the course of these experiments. Young, green and durable explants were better able to withstand the surface sterilization treatments, which injured weaker explants and the best size for a microcutting was 1.5–2 cm. The semi-liquid medium is not recommended because it stimulated exudation from explants. Benomyl was beneficial in that it controlled the presence of fungi, but it tended to induce callus formation.

Discussion and conclusion
Sodium hypochlorite and ethanol were not toxic to the explants and their use in combination with the benomyl–streptomycin

mixture was highly satisfactory. This protocol provided a method for controlling contamination in the original explant long enough to allow new outgrowth to occur from lateral buds. These were sufficiently clean so that they could be successfully isolated and used to establish aseptic shoot cultures.

There is potential to improve the quality of the initial material by studying the effects of other fungicides and the characteristics of other disinfecting compounds and procedures. The effects of other factors, such as plant growth regulators, further sterilizations and the use of antioxidants in the medium could also be explored. The use of this combination of sterilants and antimicrobial compounds, however, has been effective in isolating clean, new growth from *P. americana*, as well as three other species and should allow for the efficient collecting of germplasm from a variety of woody species.

Chapter 9. Taro

Mary Taylor

Introduction

Colocasia esculenta L. var. *esculenta*, or taro, is an important traditional root crop in the Pacific Island region. It is a valuable source of food, produced for home consumption, domestic markets and also, in some countries, for export. The importance of this crop, however, is far greater than its contribution to nutrition and revenue: it is very much a part of people's custom and so assumes a high cultural status.

Over the years, taro genetic resources have been collected in several Pacific Island countries. These collections have been maintained as field genebanks. Few have been duplicated or supported by complementary methods of conservation. As a consequence, losses of accessions and, in some cases, of whole collections, have occurred.

The use of *in vitro* culture for taro was first introduced in the Pacific in the mid-1980s, when the opportunity to collect, document and utilize taro genetic resources came with assistance from three UNDP/FAO root crop projects. A regional, pathogen-tested taro collection was established and maintained in the tissue culture laboratory of, what was then, the South Pacific Commission (SPC). This collection was later duplicated at the EU-funded tissue culture laboratory at the University of the South Pacific, Samoa, as part of the Pacific Regional Agricultural Programme. The sustainability of this *in vitro* collection has demonstrated the security of this method of conservation and has also shown the ease with which taro adapts to *in vitro* methods.

The need to use *in vitro* methodology for collecting arose when the taro leaf blight disease, caused by the fungus *Phyophthora colocasiae*, spread to American Samoa and Samoa in mid-1993. One high-yielding cultivar, favoured on domestic and overseas markets, dominated production in both countries. This cultivar proved to be susceptible to the disease and consequently, infection was severe. No local cultivars were found with any tolerance to the disease. Therefore, there was a need to look elsewhere for disease tolerant/resistant germplasm. Cultivars showing some resistance were available in the Federated States of Micronesia. However, no tissue culture facilities existed in that country. Because of this, it was decided that attempts would be made to tissue culture the taro cultivars without the usual sterile facilities.

In vitro collecting has been used more recently within the AusAID funded Taro Genetic Resources project (TaroGen). Collecting has been carried out in several Pacific Island countries for this project, but with many of these countries, maintenance of the collection either in the field or *in vitro* is not feasible because of limited resources. Consequently, *in vitro* collecting has been used to collect the germplasm and to transfer it to the Regional

Germplasm Centre (RGC) in Suva, Fiji. This RGC was established as part of the TaroGen project and is based at SPC, (now the Secretariat of the Pacific Community).

Materials and methods

When initiating tissue cultures directly from plants in the field, the steps which are used in the laboratory must be adapted for use in the field (see Chapter 1). Prior to any collecting mission, equipment that is not obtainable in the collecting country must be organized. This usually includes: labelled culture containers with sterilized media; dissection tools (forceps, scalpels and scalpel blades); a small container of wetting agent (Tween); sterile Petri dishes and sterile paper for trimming and cutting the explants; a small knife; a few small plastic containers for sterilizing and for holding dissection tools; and a methylated spirits burner for flaming instruments. It is assumed that in any country where taro is to be collected, at the very least, domestic bleach is obtainable, if not also methylated spirits.

Suckers are detached from the mother plant and all outer leaves removed using a bush knife. The sucker is then trimmed with a small, sharp knife so that the final explant is approximately 4 cm long, with the basal corm tissue approximately 1 cm^2. Rinsing with water is not necessary. In fact, it has been found that if there is any delay in the processing of the suckers into tissue cultureexplants, any previous contact with water can exacerbate problems with endogenous contaminants. Any soil can be removed during the trimming process and/or with kitchen towels.

From this stage, all manipulations are carried out inside a cardboard box with the front side open, which has been swabbed with a bleach solution prior to use. The trimmed explant is then sterilized with a solution of 2% sodium hypochlorite plus a wetting agent (Tween) for 15 min. During sterilization, the container is shaken as much as possible. After sterilization, the outer tissue is removed with instruments that have been sterilized using full strength domestic bleach. If possible, these instruments should also be flamed using a methylated spirits burner. The explants are then inoculated onto a basic Murashige

and Skoog (1962) culture medium. Ideally, the culture containers are then sealed with some form of plastic wrap. If plastic wrap is not available in the country, then parafilm can be included in the list of supplies that must be taken to the collecting site.

Once these cultures have reached their final destination, further sterilization is only considered if there is contamination. The cultures are usually placed in the growth room for a period of two to four weeks and are closely monitored. After this recovery stage, the 'explants' are further reduced so that the size of the final explant is 1 cm in length with corm tissue approximately 0.2 cm^2. At this stage they are also transferred to fresh medium.

Results and discussion

This technique has been used successfully many times and allows for the efficient collecting and transfer of taro cultivars from the field to the laboratory. The maximum time in transit occurred when germplasm was collected in the Federated States of Micronesia and plants were in transit for ten days from collection to arrival in the laboratory. No cultures were lost as a result of contamination. The key to success is the speed with which the manipulations are carried out after the explant has been sterilized. This has been demonstrated in workshops when shoot-tips have been excised from plants in an ordinary room with no air conditioning and no sterile cabinet. Provided the speed of manipulation is optimized, the degree of contamination is minimized.

Chapter 10. Coconut

Florent Engelmann

Introduction

In vitro collecting offers the plant collector an additional option for solving various problems which can be encountered during collecting expeditions (Withers 1995). In the case of coconut, seeds are bulky and heavy, making them costly to transport. They are also highly recalcitrant. These characteristics limit the amount of material that can be collected and restrict the geographic range of collecting missions. These limitations may have serious consequences for genetic resources conservation, since it is recognized that a large amount of the untapped genetic diversity in coconut is located in remote areas, such as atoll islands. The key to solving these problems, however, lies in recognizing that only the embryo is needed to propagate a coconut palm.

Research on the development of *in vitro* culture methods for coconut began in the Philippines in the early 1960s and was then advanced by research teams in Asia, Africa, India, the Americas, Europe and, more recently, Australia. As a result, various protocols for culturing coconut embryos *in vitro* have been published, all ensuring the production of plantlets, but still requiring optimization. In 1997, a global project coordinated by COGENT (International Coconut Genetic Resources Network), involving 18 coconut research institutes worldwide, was initiated to develop an improved and standardized protocol for *in vitro* embryo culture (Batugal and Engelmann 1998). Significant improvements were achieved during the first phase of the project and an improved protocol was presented during the second meeting of the project, held in Mexico in 2000 (Engelmann and Batugal 2001).

Research on the adaptation of *in vitro* culture techniques to collecting coconut embryos was initiated 15 years ago under the aegis of the IBPGR (International Board for Plant Genetic Resources, the predecessor of IPGRI), with the aim of facilitating not only the collecting but also the international exchange of coconut germplasm. In addition to the advantages offered by this technique for collecting genetic resources, *in vitro* collecting would also avoid the transmission of important coconut diseases, which do not pass through the embryo. This is particularly important with the expected increase in international exchange of coconut germplasm linked with the establishment of regional collections of coconut germplasm currently underway (Ramanatha Rao and Batugal 1998). Various *in vitro* collecting techniques have been

developed by different teams, thereby demonstrating not only the feasibility of collecting isolated embryos, but also the great flexibility that can be exercised within the basic concept.

In vitro collecting protocols

The *in vitro* culture of coconut embryos has been adapted by several researchers to the field collecting of coconut germplasm. All techniques include the following sequence of operations: (1) dehusking and cracking open the nut; (2) using a cork borer to extract a plug of endosperm containing the embryo; (3) dissecting the embryo from the endosperm; and (4) inoculating the embryo into culture. The methods developed differ in the degree to which attempts are made to reproduce laboratory conditions in the field, the amount of *in vitro* work actually performed in the field and, therefore, the point at which sterilization is carried out. Their utilization requires varying levels of technical expertise and the method selected will depend on the circumstances of the collecting mission and on the tissue culture expertise available among the collecting team.

The simplest method, which does not require specific expertise at the collecting site, is the one developed in the Philippines (for details, see Rillo and Paloma 1991; Rillo 1995). Plugs of endosperm containing the embryos are extracted in the field, brought to a simple isolation room close to the collecting site, disinfected with alcohol and commercial bleach, placed in sterile plastic bags with sterile, moist cotton and transported in cold storage. Upon arrival in the laboratory, subsequent manipulations are carried out aseptically, under the laminar flow hood. The cylinders of endosperm are resterilized with commercial bleach and the embryos are extracted and inoculated *in vitro* for germination and growth. This protocol is used routinely in the Philippines in the framework of programmes for mass production of Makapuno embryos (Rillo 1999).

Another protocol, which has been established by Australian researchers, requires some technical expertise but allows transport times of up to 6 weeks (for details, see Ashburner *et al.* 1995, 1996; Samosir *et al.* 1999). Plugs of endosperm are collected in the field and transported to an improvised laboratory close to the collecting site, where the embryos are extracted from the albumen, sterilized with commercial bleach and inoculated into 2 ml sterile plastic cryotubes containing sterile water. As previously, manipulations after arrival in the laboratory are performed aseptically under the laminar flow hood. The embryos are resterilized and inoculated *in vitro* for germination and growth.

Box 2. Example of coconut embryo *in vitro* collecting protocol
(adapted from Assy-Bah *et al.* 1987)

Equipment
The basic equipment required for in vitro collecting and field inoculation of 100 embryos includes the following items:
- 1 machete or nut dehusker; 1 sponge; 1 portable gas burner; 1 hammer; 2 forceps (30 cm long); 2–4 corkborers (20 mm); 4 jars, 500 mL; 1 folding table; 1 wooden box; 4 liters of commercial bleach; 2 scalpels; 20 sterile Petri dishes; 1 roll of plastic cling film; 100 culture tubes each containing 20 mL semi-solid medium; 100 flasks, 30 mL, each containing 15 mL sterile water.

Protocol
- Preliminary operations are performed in the open air, on the folding table which has been washed and disinfected with bleach.
- Select and dehusk mature nuts (11–12 months after pollination).
- Break nuts open with a clean hammer.
- Use a corkborer to remove a cylinder of solid endosperm containing the embryo and use forceps to transfer the cylinder to a jar containing 500 ml of commercial bleach. Disinfect all instruments with commercial bleach and sterilize in the flame of the gas burner.
- Immerse batches of 25 cylinders in bleach for 20 min.
- The following operations are performed inside the wooden box, which provides some protection from external contaminants. The inside walls of the box are disinfected with bleach.
- Place one cylinder in a sterile Petri dish and dissect out the embryo using forceps and a scalpel. Flame dissecting tools before manipulating a new embryo to reduce the risk of cross-contamination.
- Rinse the embryo once in sterile water (using one flask per embryo to reduce the risk of cross-contamination) and transfer it to solid medium in a culture tube.
- Seal the tube with cling film and place it on a rack for transport to the laboratory.

In the protocols developed by other researchers (Assy-Bah *et al.* 1987; Sossou *et al.* 1987; Karun *et al.* 1993), *in vitro* inoculation of the embryos is performed directly at the collecting site, thus requiring the relevant expertise to be available within the collecting team. The field equipment requirements are greater than in the protocols described above, but even these methods range in complexity. The technique of Sossou *et al.* (1987) attempts to reproduce laboratory facilities and methods as much as possible in the field, using an inflatable glove box. The protocols established by Assy-Bah *et al.* (1987) and Karun *et al.* (1993), however, accept the limitations of working in the field and represent a lower-technology approach. Endosperm plugs are extracted from the nuts and disinfected with commercial bleach; the dissection and inoculation of embryos into sterile culture tubes is then performed inside a wooden or plexiglas box to provide protection from airborne contaminants. Using the protocol developed by Assy-Bah *et al.* (1987) which is detailed in Box 2, embryos inoculated on semi-solid growth medium could be kept under non-controlled environmental conditions for 2 months before being grown in the culture room of a laboratory (Engelmann and Assy-Bah 1992). With the protocol developed by the Indian research team, embryos are either directly

inoculated on growth medium or kept for 2–4 months in sterile water (Karun *et al.* 1996). This protocol has been used successfully by Indian researchers to collect several thousand embryos from remote Indian Ocean islands (Karun *et al.* 1998; 2001).

Conclusion

The various examples of *in vitro* collecting protocols developed for coconut embryos range from extreme simplicity to a relatively high level of sophistication and illustrate the flexibility and adaptability of the basic concepts of the procedure. It is with coconut that the largest amount of research has been directed towards the establishment of *in vitro* collecting protocols, because of the particular difficulties encountered with germplasm collecting and exchange for this species. *In vitro* collecting is currently used on a routine basis for coconut more than with any other species. The utilization of this technique is expected to increase with the establishment of regional coconut germplasm collections, thus making coconut one of the best examples of the application of *in vitro* collecting.

Chapter 11. Tropical rainforest trees

B. Krishnapillay, N. Jayanthi and Florent Engelmann

Introduction

The use of *in vitro* methods for collecting germplasm has many advantages and applications (IBPGR 1988). This technology is ideal when only vegetative explants are available, when clonal genotypes are required, or when collecting missions fail to coincide with seed production. They are even more useful for collecting short-lived recalcitrant seeds that do not survive transport from collecting to the seed centre, a period which may span a week or more.

About 70% of the tree species in the wet tropics produce seeds that are recalcitrant and not easily storable. Hence, storage of whole seeds in genebanks is not being undertaken, although liquid nitrogen storage of excised embryos is presently being researched. In this study, *in vitro* field collecting was attempted as the preliminary step in bringing healthy excised embryos to the laboratory. An economically important timber species in the seed production phase was selected for the study. Specifically, the compounds used to disinfect the embryos prior to culture were examined.

Shorea leprosula Miq., locally known as Meranti Tembaga, is a tropical lowland timber species belonging to the family Dipterocarpaceae. It produces recalcitrant seeds that are short lived and sensitive to desiccation and low temperatures. The seeds lack dormancy and lose viability if their moisture content is reduced below a relatively high value (12–31%) (Chin *et al.* 1984) and, thus, rapid germination is characteristic of these seeds (King and Roberts 1979). Their large fleshy cotyledons are also prone to attack by pests and diseases, which thrive in the high humidity (75±10%) and temperature (27±4°C) of the tropical rainforest. This species is also an erratic seed bearer which makes it impossible to obtain a consistent supply of sufficient planting materials. As a result, the use of regular seed collecting methods to collect *S. leprosula* germplasm has not yet proven to be successful. To overcome these potential problems and to provide material for long-term cryostorage, the possibility of using *in vitro* techniques for collecting embryos of *S. leprosula* was examined.

Materials and methods

Fresh seeds of *Shorea leprosula* were collected in August/ September, 1996, near the Forest Research Institute Malaysia (FRIM) compound (Kepong, Selangor, Malaysia). The whole fruit, after removal of the

wings, is about 1–1.5 cm in length and about 0.5–0.8 cm wide. Embryos, approximately 4–6 mm in length were excised from these in the field using a sharp scalpel and forceps. Excised embryos were placed on a wet paper towel in a covered Petri dish to avoid desiccation prior to culture.

The first stage of the experiment was carried out in the field. Excision and sterilization were carried out in a makeshift hood. A collapsible table and a box-shaped plastic hood with the front side open, which fitted onto the table, were used. These were swabbed with 70% alcohol before use. A small spirit lamp was used for sterilizing the scalpels and forceps.

Immediately after excision, the embryos were surface sterilized for 10 min in pre-sterilized, stoppered glass tubes, 25 mm x 10 cm. A freshly prepared solution of commercial bleach (Clorox®; active ingredient: sodium hypochlorite, 5.25%) was used at one of two dilutions (10% and 20%), 10–12 ml/tube, with one drop of wetting agent (teepol) added. The embryos were then disinfected by immersion for 10 min in a solution containing benlate, an agricultural fungicide (active ingredient: benomyl 50%), at 0.3 g/L and 0.5 g/L and the antibiotic penicillin, at 30 mg/L and 50 mg/L, either individually or in combination. Embryos were then rinsed in sterile distilled water for one minute. This was followed by inoculation in culture tubes containing an agar medium (8 g/L). The sealed tubes were brought back to the laboratory and the percentage of tubes with contamination was recorded after 10 days.

The second stage of the experiment was carried out in the laboratory under aseptic conditions and was initiated after the tenth day of culture. The non-contaminated embryos were sub-cultured onto MS medium (Murashige and Skoog, 1962) supplemented with 15 g/L sucrose, 1 mg/L benzylaminopurine (BAP) and 1 mg/L naphthaleneacetic acid (NAA). Embryo cultures were incubated in a controlled environment at 22–25°C with a light intensity of 30.4 µmol $m^{-2} s^{-1}$ and a 12 h light/12 h dark photoperiod.

Ten embryos were tested per treatment and experiments were replicated three times. A factorial experimental design with a CRD (Completely Randomised Design) was used. After analysis using ANOVA, the means were compared using the Duncan's new multiple range test (DNMRT).

Results

In the control treatment, 57% of the embryos were contaminated after 10 days of culture. However, treating the embryos with either surface sterilization, benlate or penicillin, either singly or in combination, significantly reduced contamination (Table 9).

Table 9. Contamination (%) of *S. leprosula* embryos after treatment with various compounds and culture for 10 days on agar medium. Values followed by the same letter are not significantly different at p=0.05

	Penicillin (mg/L)								
	0			0.03			0.05		
Benlate (mg/L)	0	0.3	0.5	0	0.3	0.5	0	0.3	0.5
Bleach 0%	57 [a]	7 [cdef]	0 [f]	10 [cdef]	17 [bc]	20 [bc]	20 [bc]	0 [f]	3 [ef]
Bleach 10%	0 [f]	7 [cdef]	17 [bcd]	3 [ef]	10 [cde]	17 [bcd]	20 [bc]	3 [ef]	13 [cde]
Bleach 20%	33 [b]	3 [ef]	17 [bcd]	7 [def]	3 [ef]	0 [f]	0 [f]	20 [bc]	13 [cde]

When 20% Clorox was used for surface sterilization, the contamination which appeared was delayed compared to the control treatment. Contamination appeared only after the sixth day and on the eighth day the embryos turned brown in colour, exuding phenolic compounds into the medium. The inclusion of benlate or penicillin generally reduced contamination further.

The second stage of the experiment was carried out to observe further development of the embryos. In most cases, treating embryos with Clorox, benlate or penicillin, either singly or in combination, increased the percentage of embryos which germinated (Table 10). All embryos showed normal development when treated with 10% Clorox alone, but many of the embryos treated with 10% Clorox and 0.5 g/L benlate were somewhat stunted. When 20% Clorox was used, the growth of most embryos was retarded and the presence of exudates was very high, particularly in the presence of benlate.

Table 10. Germination (%) of S. *leprosula* embryos after treatment with various compounds and culture on agar medium. Values followed by the same letter are not significantly different at p=0.05

	Penicillin (mg/L)								
	0			0.03			0.05		
Benlate (mg/L)	0	0.3	0.5	0	0.3	0.5	0	0.3	0.5
Bleach 0%	7 [h]	37 [gh]	77 [bcd]	80 [abcd]	67 [cde]	57 [defg]	47 [fgh]	100 [a]	63 [cdeff]
Bleach 10%	70 [bcd]	77 [bcd]	77 [bcd]	97 [ab]	73 [bcde]	87 [abc]	87 [abc]	90 [ab]	90 [ab]
Bleach 20%	60 [cdef]	77 [bcd]	77 [bcd]	57 [defg]	73 [bcde]	63 [cdef]	53 [efgh]	77 [bcd]	53 [efgh]

Discussion

Like with most Dipterocarps, *S. leprosula* seeds are infected by seed-borne fungi. One way to eliminate these fungi is to culture

excised embryos, which are relatively clean compared to the surrounding tissues.

In the first stage of the experiment, the embryos were cultured on an agar medium without nutrients, to reduce the growth rate. It was found that 10% or 20% Clorox and/or benlate or penicillin reduced contamination compared with controls. When 20% Clorox was used, however, the high concentration of the sterilant stressed the embryos, causing the production of phenolic compounds and eventual death. The same was generally observed when 0.5 g/L benlate was used.

The second stage of the experiment was carried out to detect the effects of the sterilizing compounds on development and growth of the excised embryos. Normal development was observed in all embryos when 10% Clorox was used as the surface sterilant. On the other hand, 20% Clorox was found to retard normal growth in most embryos. It was also found that 0.5 g/L Benlate inhibited the normal growth of the embryos, while 0.3 g/L did not. These results suggest that even a small difference in the concentration of the sterilizing compounds can affect growth of the embryos. Therefore, it is important to determine the sensitivity of the tissues to the antimicrobial chemicals used before selecting them for *in vitro* collecting procedures.

Conclusion

There are numerous compounds that can be used to eliminate contamination during *in vitro* collecting, but a few additional factors should be considered before selecting these disinfectants. These include cost, labour and toxicity to the explants. From this study, 10% Clorox employed alone was found to be the best and least expensive compound for controlling contamination in the *in vitro* collecting of *S. leprosula* germplasm. At this concentration, it was also not detrimental to the subsequent development of embryos in culture.

The *in vitro* collecting technique is a simple and unsophisticated method which can be used to collect and conserve pathogen free materials. This technique can also be used to rescue immature embryos and to conserve other plant parts. These studies with *S. leprosula* demonstrate the value of *in vitro* methods for embryos from large, recalcitrant seeds of rainforest trees. Used in combination with *in vitro* propagation and cryopreservation, *in vitro* collecting can provide an important tool for the *ex situ* conservation of valuable germplasm from rainforest tree species.

Chapter 12. Wild and endangered species

Valerie C. Pence, John R. Clark and Bernadette L. Plair

Introduction

The success of any *in vitro* collecting exercise depends on returning from the field with clean, viable tissue that is capable of resuming growth in the laboratory. Collecting a wild or endangered species presents particular challenges, since very little may be known about its contaminants or its requirements for growth *in vitro* and there will be little material available for experimentation (see Chapter 2).

Work in this laboratory has been directed at using tissue culture to propagate endangered species when other methods of propagation have proven to be inadequate. In collaboration with the Center for Plant Conservation (St. Louis, Missouri) and their network of botanical gardens throughout the United States, approximately thirty species have thus far been targeted for such work. In some cases, tissue cultures can be readily initiated from seeds or cuttings which are shipped to the laboratory. In other cases, however, seeds are not viable or they may experience dormancy which makes germination difficult. Cuttings from some species have proven to be fragile and not able to survive transport. In these cases, *in vitro* collecting has been used to initiate cultures from the plants *in situ* and these cultures have then been transported back to the laboratory for growth and propagation.

Research on developing techniques for use with endangered species has been performed using non-endangered, wild species from the Cincinnati area and from two locations in Trinidad and Costa Rica. Three techniques were investigated: leaf disc collecting, needle collecting of stem tissue and bud collecting. Information was obtained on controlling contamination and obtaining growth primarily from the first two of these methods, since material was more abundant for experimentation. The techniques were first tested with common species and the results were then applied to endangered US species. This work has been reported, in part, elsewhere, (Pence 1996; Clark and Pence 1999; Pence 1999; Plair and Pence 2000a; Pence *et al.* 2000; Pence 2001) but it will be summarized here to illustrate the techniques and potential of *in vitro* collecting for endangered species.

Materials and methods

Experiments with tissues from non-endangered species were made at several sites in the Cincinnati area and at the CRESTT (Centre

for the Rescue of Endangered Species of Trinidad and Tobago) Environmental Research Station, Morne Catherine, Chaguaramas, Trinidad and at La Selva Biological Research Station, Costa Rica. Endangered plant tissues were collected from the locations listed in Table 11.

Table 11. Endangered US species for which *in vitro* collecting has been used

Species	Collaborator	Location	Tissue	Results
Aconitum noveboracense Gray & Coville	The Holden Arboretum	Ohio	Bud	Shoot cultures established
Agalinis novasotensis Dubrule & Canne-Hilliker	Mercer Arboretum and Botanic Gardens, Texas A&M University	Texas	Bud	Callus lines established; browning
Astragalus cremnophylax Barneby var. *cremnophylax* Barneby	The Arboretum at Flagstaff	Arizona	Leaf and bud	Callus lines established
Clematis socialis Kral	North Carolina Botanical Garden, Alabama Nature Conservancy	Alabama	Bud	Shoot cultures established
Dicerandra frutescens Shinners *D. thinicola* H.A. Mill.	The Bok Tower Gardens	Florida	Leaf and bud	Callus lines established; browning; Browning and contamination
Hedeoma todsenii Irving	The Arboretum at Flagstaff	Arizona	Bud	Shoot cultures established
Houstonia purpurea (Small) Terrell var. *montana* (Small) Terrell	The North Carolina Arboretum	North Carolina	Bud	Contamination
Lobelia boykinii Torr. & Gray ex A.DC.	North Carolina Botanical Garden	North Carolina	Leaf and bud	Shoot cultures established
Mespilis canescens Phipps. *Rhexia aristosa* Britt.	Missouri Botanical Garden North Carolina Botanical Garden	Missouri North Carolina	Bud Leaf and bud	Callus lines established Shoot cultures established
Schoenocrambe suffrutescens (Rollins) Welsh & Chatterley	Red Butte Garden	Utah	Bud	Shoot cultures established

In most cases, the basic collecting medium consisted of the salts and minimal organics of Murashige and Skoog (Linsmaier and Skoog 1965) with 3% sucrose, 0.22% Phytagel and 0.5 mg/L each of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA). To control contamination in leaf disc and bud cultures, 100 mg/L active benlate was added to the medium before autoclaving. In addition, a drop of antibiotic solution, consisting of 5 mg/ml cefotaxime and 0.25 mg/ml vancomycin, was added on top of each tissue piece on the same day it was collected, for a final concentration of 100 mg/L

cefotaxime and 5 mg/L vancomycin, when diffused through the medium. The antibiotics were preweighed and taken into the field as dry powders. After all the collections for a day were completed, the antibiotics were dissolved in sterile water and filter sterilized through a 0.2 µm cellulose acetate membrane filter. Media for samples from needle collecting, however, did not contain fungicides or receive antibiotics. Borosilicate scintillation vials (7 ml) containing 2.5 mL of medium were used for leaf and bud tissues, while 1.5 mL microcentrifuge tubes containing 0.5 mL of medium were used for needle core samples.

In all cases, tissues were surface sterilized by swabbing with 70% ethanol just before excising the tissue. All instruments were also sterilized by soaking in 70% ethanol. No further rinsing of tissues or instruments was done and all activities were performed quickly, in the open air. The three methods used for experimentation and/or collecting endangered species included:

Leaf disc collecting
Leaves which were $1/4$ to $1/2$ expanded were chosen for collecting. After surface sterilizing the leaf and holding it by the petiole or edge, leaf discs were cut using a single-hole paper punch. Using forceps, discs were transferred from the punch to a vial of medium.

Needle collecting of stem tissue
After surface sterilizing a young stem, a small cross section of tissue was extracted using a no. 21 gauge hypodermic needle and a 3- or 5-ml glass syringe. The tissue was expelled onto medium in a microcentrifuge tube.

Bud collecting
A section of stem including a bud was swabbed with 70% ethanol. The bud was then excised from the plant with a scalpel or scissors and transferred to a vial of medium.

Vials and microcentrifuge tubes were grouped in plastic bags and these were carried in the field in cloth bags. Because of their large number, containers for experiments with non-endangered species were transported to the lab in a hard-sided trunk. Vials with endangered plant materials, which were fewer in number, were packed for transport or courier in small styrofoam boxes.

For experimental work with non-endangered species, tissues were examined 7–14 days after transfer to the laboratory, at which time most contamination was apparent. Endangered tissues were examined beginning 1–2 days after collection and appropriate re-sterilizations and other salvage procedures were undertaken,

when needed, in order to maximize the chances for survival and further growth.

Results

Leaf disc collecting

Early studies with leaf disc cultures indicated that contamination rates of 90–100% were common, particularly with tropical species, unless a fungicide and antibiotics were added. The addition of benlate and a solution of cefotaxime and vancomycin reduced average contamination rates from over 90% to approximately 30%. Even though no precautions were taken to shield the vial from the ambient air, less than 5% of fungi and bacteria were observed growing on the medium apart from the tissue, suggesting that more than 95% of the contamination originated from the explant itself.

Leaf discs have been collected from more than 200 species over the course of six years, many more than one time, with more than half of the species from the tropics (Trinidad and Costa Rica). A number of these have been regenerated into whole plants, including, *Hippobroma longiflora* (L.) G. Don f., *Spermacoce assurgens* Ruiz & Pavon, *Gonzalagunia hirsuta* (Jacq.) Schum., *Sida acuta* Burm. f., *Merremia glabra* Hallier, *Bryophyllum pinnatum* (Lam.) S. Kurz, *Prestonia quinquangularis* (Jacq.) Spreng., *Smilax cumanensis* Humb. & Bonpl. ex Willd., *Miconia virescens* (Vahl.) Triana, *M. lacera* (Bonpl.) Naud., *M. nervosa* (Sm.) Triana, *M. acinodendron* (L.) Sweet *and* several species of *Piper,* including *P. marginatum* Jacq., *P. aequale* Vahl., *P. hispidum* Sw. *and P. aduncum* L. Shoot tips or embryos from several of these species have also been cryopreserved (Plair and Pence 1999; 2000b).

Needle collecting of stem tissue

In vitro collecting using the needle collecting technique had a much lower rate of contamination than the leaf punch method, even though antimicrobial agents were not used in the medium (Pence 1996). The rate of growth and regeneration from that tissue, however, was also generally lower than with the leaf punch method. Species regenerated into whole plants from needle collections include S. *assurgens* and *M. glabra*.

Bud collecting

Once methods to control contamination were developed using the leaf punch procedure, these methods were adapted to controlling contamination in buds collected from rare species. Table 12 lists the rare US species for which *in vitro* collecting has been attempted in

this laboratory. The technique has been effective in obtaining viable tissue from several species. These cultures have subsequently been used to propagate the species and to provide material for shoot tip cryopreservation for long term germplasm storage (Plair and Pence 2000b). With other species, tissue browning has resulted in the loss of cultures completely, or in a substantial decrease in the growth potential of the tissues, such that only callus was recovered.

Discussion and conclusion

As has been emphasized (Chapter 1), *in vitro* collecting is a flexible technique, which can and should be adapted to maximize success with the species being collected. The three different procedures described here may each find use in particular circumstances. Bud collection is generally the method of choice, since growing plants from a preformed meristem minimizes the chance of genetic variation. It has been successfully used for collecting a number of the species described in this volume (Chapters 4–11), as well as several species of conservation concern in the United States. As with all *in vitro* collecting, the selection of young, growing tissue should improve the chances for success. Such tissues are generally less contaminated than older tissues and will be most likely to continue growing *in vitro*. Some species produce large amounts of phenolic compounds upon wounding and these can inhibit growth of the shoot tip (e.g. *Dicerandra frutescens*), but recent work in this laboratory with antioxidants has shown promise and should help reduce browning in future collections.

Methods which rely on the formation of adventitious buds can be a second choice for *in vitro* collecting. In some species, young leaf tissue can successfully regenerate shoots and leaf tissue is generally the most abundant material for collection. It can be valuable as a supplement to bud collection, particularly if only one or a few buds are available. Contaminants in leaf collections are often similar to those in bud collections from the same plant and thus, if time permits, preliminary work with leaves may help direct the design of media to control contamination in buds (Yidana *et al.* 1987; Gochenauer and Pence, in prep.).

Results with the needle collecting method indicate that its use may be more limited than that of the leaf punch. As with other methods, young, growing tissue is the most desirable, but there is a minimum stem size needed for collecting with this method. On the other hand, when it is a possibility, it can provide relatively clean tissues and it may be a useful method for some species with fleshy stems. Further research on media which will stimulate growth more efficiently from these small pieces of tissue could make the technique

Table 12. Examples of plant species which have been the object of *in vitro* germplasm collecting

Plant	Reference
Aconitum noveboracense Gray & Coville	Pence *et al.* Ch. 12, this vol.
Agalinis novasotensis Dubrule & Canne-Hilliker	Pence *et al.* Ch. 12, this vol.
Astragalus cremnophylax Barneby var. cremnophylax Barneby	Pence *et al.* Ch. 12, this vol.
Bryophyllum pinnatum (Lam.) S. Kurz	Pence *et al.* Ch. 12, this vol.
Butia capitata (Mart.) Becc.	Sossou *et al.* 1987
Caryota urens L.	Sossou *et al.* 1987
Cistus salvifolius L.	Predieri, pers. comm.
Citrus L. sp.	Brenes *et al.* Ch. 7, this vol.
Clematis socialis Kral	Pence *et al.* Ch. 12, this vol.
Cocos nucifera L.	Sossou *et al.* 1987; Assy-Bah et al. 1987
Coffea arabica L.	Lozoya *et al.* Ch. 4, this vol.
Colocasia esculenta (L.) Schott.	Taylor, Ch. 9, this vol.
Cynodon Rich sp.	Ruredzo 1989
Digitaria Haller sp.	Ruredzo 1989
Dicerandra frutescens Shinners; *D. thinicola* H.A. Mill.	Pence *et al.* Ch. 12, this vol.
Erythrina L. sp.	Sandoval and Villalobos, Ch. 8, this vol.
Gonzalagunia hirsuta (Jacq.) Schum.,	Pence *et al.* Ch. 12, this vol.
Gossypium hirsutum L.	Altman *et al.* 1990
Hedeoma todsenii Irving	Pence *et al.* Ch. 12, this vol.
Houstonia purpurea (Small) *Terrell var. montana* (Small) Terrell	Pence *et al.* Ch. 12, this vol.
Hippobroma longiflora (L.) G. Don f	Pence *et al.* Ch. 12, this vol.
Ipomoea batatas (L.) Lam.	Huaman *et al.* 1995
Jubaea chilensis (Molina) Baillon	Sossou *et al.* 1987
Juniperus phoenicea L.	Predieri, pers. Comm.
Lobelia boykinii Torr. & Gray ex A.DC.	Clark and Pence 1999
Manihot esculenta Crantz	Chavez *et al.* 1987
Merremia glabra Hallier	Pence *et al.* Ch. 12, this vol.
Mespilis canescens Phipps.	Pence *et al.* Ch. 12, this vol.
Miconia virescens (Vahl.) Triana, *M. lacera* (Bonpl.) Naud., *M. nervosa* (Sm.) Triana, *M. acinodendron* (L.) Sweet	Pence *et al.* Ch. 12, this vol.
Myrtus communis L.	Predieri, pers. Comm.
Musa L. sp.	Montoya *et al.* Ch. 6, this vol.
Persea americana Miller	Sandoval and Villalobos Ch. 8, this vol.
Phyllirea angustifolia	Predieri, pers. comm.
Piper marginatum Jacq., *P. aequale* Vahl., *P. hispidum* Sw. and *P. aduncum* L.	Pence *et al.* Ch. 12, this vol.
Pistacia lentiscus L.	Predieri, pers. Comm.
Pouteria aublet sp.	Sandoval and Villalobos Ch. 8, this vol.
Prunus L. sp.	Elías 1988
Rhexia aristosa Britt.	Clark and Pence, 1999;
Schoenocrambe suffratescens (Rollins) Welsh & Chatterley	Pence *et al.* Ch. 12, this vol.
Shorea leprosula Miq.	Krishnapillay *et al.* Ch. 11, this vol.
Sida acuta Burm. f.,	Pence *et al.* Ch. 12, this vol.
Smilax cumanensis Humb. & Bonpl.ex Willd.,	Pence *et al.* Ch. 12, this vol.
Spermacoce assurgens Ruiz & Pavon	Pence *et al.* Ch. 12, this vol.
Theobroma cacao L.	Yidana *et al.* 1987; Alvarenga *et al.* Ch. 5, this vol.
Vanilla planifolia Jackson	Sandoval and Villalobos, Ch. 8, this vol.
Vitis L. sp.	Elías 1988
Zygopetalum maxillare Lodd.	Warren 1983

more widely applicable. Alternatively, with very thin, fragile stems, slices may be cut with a small scissors, although these slices tend to have contamination rates similar to those of leaf discs.

Controlling contamination is the first priority in *in vitro* collecting, since a contaminated culture will generally preclude any further growth or development. A high proportion of contamination has been observed to originate with the explant and appears to arise from endophytes which escape the action of surface sterilization (Chapter 3, this volume). The fungicide benlate, incorporated into the medium, in combination with an antibiotic solution of cefotaxime and vancomycin, added after collecting, consistently reduced the contamination in the cultures with very little apparent toxicity.

In vitro collecting has been successfully applied to several endangered species, when seeds were not available or when seeds had proven to be non-viable or difficult to germinate. In several cases, it has been performed by collaborators from other botanical gardens, using an '*in vitro* collecting kit'. By following simple instructions, they have successfully collected tissues and returned them by overnight courier to this laboratory, where the tissues were monitored and many were grown into propagating cultures. Because it is minimally invasive, the original plants, which are few in number, were left unharmed.

Thus, with its simplicity and flexibility, *in vitro* collecting should prove to be an important tool in the conservation of endangered plant germplasm. When propagation by seeds is not possible, *in vitro* collecting can facilitate collecting and transport of tissues of endangered species. It can provide material for propagation and for germplasm storage and thereby improve the chances of survival for species that are threatened with extinction in the wild.

Acknowledgements

Part III.
Prospects

Chapter 13. The future of *in vitro* collecting

Valerie C. Pence, Victor M. Villalobos A. and Jorge A. Sandoval

The efficient use of plant genetic resources requires the careful collecting of germplasm, its conservation, evaluation, documentation and exchange. Despite the interest of governments and other public organizations in these activities, the collecting, conservation and access to plant genetic resources is a complex task that involves cultural, ecological, technical and political considerations. Guidelines provided by international conventions, such as the Convention on Biological Diversity, form a framework around which to equitably build programs and relationships to facilitate these activities (Wyse Jackson and Sutherland 2000).

The tools of biotechnology can supplement traditional approaches, in order to expand the opportunities for propagating, preserving and improving plants. Over the last 30 years, plant tissue culture has been used to propagate hundreds of plant species. The use of this technique has been particularly important for the conservation and multiplication of plants that produce recalcitrant seeds, are parthenocarpic or propagate vegetatively, or have unpredictable seed production or germination. More recently, *in vitro* techniques have also been adapted to the propagation and preservation of endangered plant germplasm of uncultivated, wild species. Several programes have focused on using these methods for conservation (e.g. Royal Botanic Gardens, Kew, UK; Kings Park Botanic Garden, Perth, Australia; Cincinnati Zoo and Botanical Garden, Cincinnati, OH, USA).

In vitro collecting is a natural outgrowth of this work. Tissue culture, a basic component of plant biotechnology, can expand the possibilities for obtaining plant germplasm. It can supplement seed collecting, providing an alternative source of material for propagation and preservation when seeds are not available. The chapters in this volume have demonstrated the flexibility and broad applicability of *in vitro* collecting techniques. The variety of methods illustrated at each step in these examples can provide a basis for developing the technique further for future applications.

The choice of explant can range from buds and embryos (the most common choices) to leaves, stems, flower buds and other parts which can regenerate buds or embryos. Meristems, apices and embryos are more genetically stable than other types of explants, which need to undergo a developmental change before regenerating plants. Even so, 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 explant can provide more opportunities for recovering plants. When dealing with rare or endangered species, judgements must be made as to the most appropriate explant, not only from the standpoint of collecting, but from that of the species, as well. The effects of removing a bud from a monopodial plant, or embryos from a very small population must be considered and alternative tissues may be less harmful in terms of maintaining the species *in situ*.

Asepsis is a requisite for *in vitro* culture and is perhaps the greatest constraint in developing *in vitro* collecting protocols. Sodium hypochlorite has been widely used for surface sterilizing, but other compounds such as ethanol at 70% and solutions of antibiotics and fungicides have also been employed. 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 will 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 used in the studies in this volume. The use of non-traditional compounds, such as PPM, is increasing, as well. The problem of contamination is fundamental to the technique of *in vitro* collecting 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. *In vitro* collecting 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 should also be emphasized that, while *in vitro* collecting reduces the level of pathogens in explants, it does not guarantee their elimination. Therefore, *in vitro* collecting must also comply with phytosanitary regulations, including those for asepsis.

Although *in vitro* germplasm collecting is technically relatively easy, its 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* collecting 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 (Chapter 1). Advances in this area are dependent upon basic work in plant tissue culture directed at understanding the factors which limit growth *in vitro* for some species. One such factor, prominent in a number of the reports in this volume, is the oxidative browning of explants. The use of antioxidants or other methods to reduce browning should improve the success of *in vitro* collecting with a number of species. Similarly, a better understanding of the basic biology of regeneration would likely improve the ability to regenerate plants from *in vitro* collected leaves, stems, etc., thereby increasing the possibilities for collecting. Currently, the number of species that have been the object of *in vitro* collecting is limited (Table 12), but as this number grows, improvements in the technique will naturally follow.

Conclusions

The chapters in this volume have demonstrated that *in vitro* collecting can be an important tool for solving a number of the problems associated with germplasm collecting. The *in vitro* method is especially useful for collecting tissues from remote sites when seeds are not available. It can also be used to distribute that germplasm both within and outside the country of collection.

Currently only 37 000 accessions around the world are conserved *in vitro* (FAO 1994). *In vitro* collecting is one way of increasing this number. A major priority is, therefore, to prepare proposals for analyzing this possibility in other tropical species that produce recalcitrant seeds or propagate asexually. Another theoretical-practical meeting, similar to the one on which this volume is based, should be taken up to refine principles and methods, to update existing knowledge and to integrate *in vitro* collecting 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. The studies presented in this volume constitute examples of creativity oriented towards adapting tissue culture to the field of germplasm collecting and conservation. 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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