BORNEO SCIENCE 7: 25-31, JUNE 2000

THE SCOPE FOR *IN VITRO* AND MOLECULAR METHODS IN GERMPLASM MANAGEMENT

K. Paranjothy & Mariam, A. L.

School of Science & Technology, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Sabah.

ABSTRACT. *The importance of germplasm conservation and management is well recognised but a lack of resources, both in financial support and technical skills, has led to fragmentary and often uncoordinated activity in germplasm science and technology. This paper examines the potential applications of in vitro and DNA based molecular methods in germplasm conservation. The rapid advances in these techniques, especially molecular methods, require a co-ordinated approach in training and dissemination of skills at national and regional levels.*

KEYWORDS. Germplasm conservation, *in vitro* culture techniques

INTRODUCTION

In situ conservation is probably the most efficient and cheapest means of ensuring the continued survival of germplasm. Human interference, disease and natural catastrophies, however, tend to minimise the advantages of *in situ* conservation. Indeed it is doubtful if any one method can be used to ensure survival of selected germplasm. Simple methods of seed storage, for example, can vastly enhance the scope of *in situ* conservation. *In vitro* methods and molecular tools also can supplement these traditional methods.

IN VITRO TOOLS IN GERMPLASM MANAGEMENT

During the last 20 years, *in vitro* culture techniques have been extensively developed and applied to more than 1000 different species (Bigot, 1987). With respect to germplasm management, tissue culture techniques are of interest in collection, exchange, multiplication and storage. The relevant techniques include embryo culture, embryo rescue, *in vitro* multiplication through enhanced axillary branching, somatic embryogenesis, slow growth *in vitro* and cryopreservation.

With some plant species, tissue culture systems result in propagation of plant material with high multiplication rates in an aseptic environment. Virus free plants can also be obtained through meristem culture. Aseptic procedures including production of disease free stocks can simplify quarantine procedures for international exchange of germplasm. The miniaturization of explants allows reduction in space requirements. Cultures can be safely kept in constant culture room conditions, minimising loss due to adverse environmental conditions and disease.

IN VITRO TECHNIQUES FOR COLLECTION

A variety of problems may be encountered when germplasm is collected:

- little or no seed may be available, requiring culture of shoot apices as an alternative
- seeds may not be fully mature, requiring culture of excised immature embryos
- on long prospecting expeditions, it may be necessary to germinate seeds *in vitro* before they deteriorate; likewise it may be necessary to culture vegetatively propagatged plant materials *in vitro* first before transport
- *in vitro* culture may be required to reduce the bulk and weight of accessions that need to be transported
- *In vitro* collections have been used successfully for germplasm of coconut, cotton and cassava and are being currently evaluated for citrus, cacao, banana, avocado and coffee (Altman *et al.* 1990, Assy Bah *et al.* 1987).

ASEPTIC GERMPLASM EXCHANGE

In vitro methods have been used extensively for the international exchange of germplasm. The advantages of *in vitro* methods include reduced weight and volume of accessions, virus free meristem cultures and quarantine friendly aseptic cultures. Procedures for international exchange of *in vitro* cultures have been developed for potato, cassava, yam and banana (Espinoza *et al.* 1992).

IN VITRO STORAGE

Two *in vitro* approaches for storage of germplasm have been researched extensively: slow-growing cultures for medium term conservation and cryopreservation for long term storage. Of the two approaches, slow growing cultures are believed to be relevant for maintaining active collections in clonal crops (Chin and Hor, 1989; Williams, 1988). Notable successes in conservation through slow growing cultures have been recorded for several root and tuber crops such as potato, cassava, yam and banana (Withers, 1999).

In vitro conservation studies are being carried out by several public organisations in Malaysia. These include the universities and crop oriented research institutes such as FRIM, PORIM, RRIM and MARDI. Techniques are being developed particularly for several forest and fruit trees that have recalcitrant seeds. Examples include citrus, jackfruit, mangosteen, *Shorea* and *Depterocarp* spp, *Hopea* and mahogany (Normah and Marzalina, 1966). Cryopreservation has been used with varying degrees of success with isolated oil palm embryos and oil palm seeds and excised embryos of rubber. The potential of cryopreservation has probably not been fully exploited for tropical plants and given more research attention it is likely that cryopreservation will be a major useful tool in germplasm management in future.

STORAGE OF DNA

It has often been argued that traditional germplasm accessions can be reinforced with isolated DNA or even cDNA libraries. The DNA could serve future studies requiring specific genes or studies aimed at evaluating polymorphisms. As source material for molecular and genetic studies their value is enhanced by satisfactory storage qualities even at room temperature for DNA and at 4 C or -70 C for cDNA libraries. DNA banks will not of course replace traditional germplasm banks such as seedbanks or *in situ* conservations. Any effort in this direction locally is minimal. Perhaps consideration should be given to the creation of a communal DNA bank in Sabah, at least for endangered species.

MOLECULAR TOOLS FOR GERMPLASM MANAGEMENT

In essence the conservation of germplasm aims at capturing maximum genetic diversity with a minimum number of individuals. Whilst this precept is well recognised in theory, it is exceedingly difficult to implement in practice, the difficulty stemming mainly from a lack of information regarding genetic variability. Molecular methods can provide information on genetic variability , paving a better defined path for germplasm management. When specific genes for a breeding programme are required, molecular methods again can determine individuals suited for the purpose. The same methods can again help determine which of the progeny in a breeding programme carry desired traits. Used judiciously germplasm banks therefore become useful sources of variation for both the molecular geneticist and the breeder. The integration of molecular and classical methods in breeding is advanced in some crops and it is very likely that a similar siuation will prevail with respect to germplasm collection and utilisation once molecular methods are appreciated and used more widely. It is important to emphasise that traditional or classical methods of evaluating variation through simple field measurements and visual comparisons are no less important despite the availability of molecular tools. Molecular methods, however, are precise and exploiting these methods carefully increases quantitative aspects, often with respect to special and desired genic aspects.

MOLECULAR METHODS FOR STUDYING DIVERSITY

There are a myriad DNA marker technologies in current use and new techniques are constantly being developed. Broadly these techniques fall into three categories (Karp and Edwards, 1997; for reviews see Caetgano-Anolles and Gresshof, 1997). RFLP (restriction fragment length polymorphism) is representative of the first category. In these techniques a probe is hybridised to a Southern blot of restriction enzyme digested DNA separated on an agarose gel. If the DNA being investigated is a small molecule present in high numbers, e.g. chloroplast or mitochondrial DNA, a probe may not even be necessary, as individual fragments of a restriction digest can be visualised with ethidium bromide or other dyes. In the second category we can include most other techniques that do not rely on probe hybridisation. These techniques are instead based on the polymerase chain reaction (PCR), using arbitrary primers for creating polymorphisms. There are many variations of this theme: RAPD (random amplified polymorphic DNA, DAF (DNA amplification fingerprinting), and AFLP (amplified fragment length polymorphism using primer based on restriction site sequences). When microsatellites are used as primers we have yet another subgroup of techniques: microsatellite primed PCR or MP-PCR. Arbitrary primers can also be used for amplification of DNA derived from RNA. The RNA aribitrarily primed PCR (RAP-PCR) includes differential display (DD) in which selected 10-mers (10 base long oligonucleotides) are used to selectively reveal differentially expressed RNA. Finally, in the third group we have a set of techniques in which PCR is used to amplify a single locus using allele specific oligonucleotides or a microsatellite site using primers flanking the microsatellite.

The techniques outlined above and other related techniques have two broad applications: (1) creating DNA markers for genetic mapping and tagging traits and (2) creating indicators of genetic diversity. Molecular markers enable the development of high density maps and are therefore more efficient for description of differences at the individual or species level than morphological or even biochemical traits. If a marker is linked to a trait of interest that is difficult to quantify, then selection for the marker instead of the trait itself may be more efficient. Molecular markers can also be used for QTL or quantitative trait loci. As tools for indicators of genetic diversity, molecular tools can be used to address a variety of questions. At the species level questions such as taxonomic distinctiveness, phylogenetic relationship with other species, ploidy levels and variation at organelle level can often be answered. DNA markers can also be used to bring into focus diversity related to accessions, germplasm collections and breeding populations, including *in situ* populations. Questions concerning variation are important to germplasm conservationists as much as they concern plant breeders. For example, information on genetic diversity is crucial to determining geographical collection strategies.

PROSPECTS FOR THE FUTURE

In the field of germplasm storage there is no doubt that seed banks and *in situ* accessions will continue to dominate. However, many important tropical seeds are recalcitrant to simple storage techniques of low temperature and low humidity. It is clearly essential that techniques for cryopreservation of germplasm, including recalcitrant seeds and embryos be developed with priority. Much of the research in cryopreservation has been limited to empirically exploring variations in cryoprotectants and cooling rates and thawing rates for optimal storage and recovery. Hardly any research, on the other hand, has been carried on stress induced proteins, for example, that might be related to improved cryostorage. Techniques concerning germplasm storage sometimes require concomitant use of tissue explants and tissue culture techniques. In these cases, it would be prudent therefore to first of all determine if explants are amenable to culture before attempting to develop cryopreservation protocols.

There is a wide array of molecular techniques that seem relevant to gemplasm management and it must be a difficult task determining which technique suits a particular situation best. It is perhaps more important formulating clear research questions first before trying to identify techniques. Many of these techniques are costly (Table 1), especially in developing countries. In fact within developing countries many of these techniques are confined to well funded research institutes concerned with nationally important plantation or agricultural crops. At the present time there is little expenditure on molecular marker assisted germplasm conservation in developing countries.

Table 1. Comparison of the main features of different DNA marker techniques. (Modified from Karp and Edwards, 1977)

Feature	RFLP	MAAP	AFLP	STMS	Sequencing
Development Cost ($/Sample)	100	0	0	500	500
Running Cost ($/Sample)	2	1	1.5	1	2
Samples/day (Research lab)	20	50	50	50	20
Level of skill required	low	low	medium	low/ medium	high
Automation	difficult	yes	yes	yes	yes
Reliability	high	Low/ medium	high	high	high
Dominant or codominant	codominant	dominant	dominant (codominant)	codominant	codominant
Polymorphism	medium	medium	medium	high	medium-low (sequence dependent)

At the technology level it is likely that even newer methods will emerge. In the near future, it is possible that the microchip based hybridization systems now available commercially may become widely available for routine work after further improvement and cost reductions. Other new technologies may hopefully be automation and cost friendly. Germplasm prospection should be a precise and cost effective science and the availability of suitable molecular methods can help to make this possible.

REFERENCES

Altman, D. W. , Fryxell, P. A., Koch, S. D. and Howell. C. R. 1990. *Gossypium* germplasm conservation augmented by tissue culture techniques for field collecting. Econ. Bot. **44**:106 113.

Assy-Bah, B., Durrand-Gasselin, T., and Pannetier, C. 1989. Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L). FAO/IBPGR GEN. RES. NEWSL. **71**: 4 10.

Bigot, C. 1987. *In vitro* manipulation of higher plants: Some achievements, problems and perspectives. In: Boccon-Gibod, J., Benbadis, A and Short, K. C. (Eds). Proceedings of IAPTC French-British Meeting, 8 9 October, 1987. Cell culture techniques applied to plant production and plant breeding. Angers. France. : 5 17.

Caetano-Anolles, G and P. M. Gresshoff (Eds.). 1997. DNA markers. Protocols, applications and overviews. Wiley-Liss, New York. 364 Pp.

Chin, H. F. and Y. L. Hor. 1989. Seed and *in vitro* storage of genetic resources. : 78 84 In: Proceedings of the 6th International Congress of SABRAO.

Espinoza, N., Lizarraga, R., Siguenas, C., Buitron, F., Bryan, J. and Doods, J. H. 1992. Tissue Culture, micropropagation, conservation and export of potato germplasm. CIP Research Guide 1. International Potato Center, Lima, Peru. : 19.

Karp, A. and K. Edwards. 1997. DNA markers: a global overview. In: Caetano-Anolles, G and P. M. Gresshoff (Eds). DNA markers. Wiley-Liss, Inc., New York. : 1- 14.

Krishnapillay, B. and Marzalina, M. 1993. *In vitro* conservation of bamboo germplasm. Proceedings of conference on forestry and forest products research.

Normah, M. N. and Marzalina, M. 1996. Achievements and prospects of in vitro conservation for tree germplasm. In: Normah, M. N., Narimah, K. K. and Clyde, M. M (Eds). *In vitro* conservation of plant genetic resources. Proceedings of the International Workshop on *in vitro* conservation of plant genetic rescources. July 4 6, 1995. Kuala Lumpur. : 253 261.

Williams, J. T. 1988. Recent changes in emphasis in international genetic resources work. : 7 12 In: S. Suzuki. (Ed). Crop Genetic Resources of East Asia, IBPGR, Rome.

Withers, L. A. 1991. Biotechnology and plant genetic resources conservation. In: Paroda, R. S. and Arora, R. K. (Eds). Plant genetic resources: Conservation and management. NBPGR, New Delhi, India. : 273 297.