IN VITRO TECHNOLOGY FOR MUTATION BREEDING


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FOREWORD

Biotechnology is currently arousing a great deal of attention in both developed and developing countries. There appeared a great trend to use these new techniques to develop better varieties of crop plants.

The FAO and the IAEA, through their Plant Breeding and Genetics Section in the Joint FAO/IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and Agricultural Development have for more than 20 years devoted a great deal of attention to the need for further improving crop cultivars through induced mutations. In recent years, it has been learned that mutations occur during in-vitro culture of plant material without application of mutagenic treatment. It has called for the examination of this 'somaclonal variation' for its potential value in relation to the mutagenesis with radiation and chemical mutagens.

Another matter of interest for plant breeders is the use of mutagens in combination with in-vitro cultures. Further, in-vitro culture techniques seem to offer certain advantages in the screening of plant cells or cultured tissues for useful variants. These points are, however, to be carefully assessed before these techniques become a dependable means of plant breeding.

It is also desirable to look into future developments in genetic engineering, even though this area seems somewhat 'immature' right now, so as to formulate effective, practical applications aimed at solving the food problems in the developing world.

All of these matters were dealt with in detail during the symposium organized jointly by the FAO and the IAEA in Vienna from 19–23 August 1985. A Co-ordinated Research Programme on the In-Vitro Technology for Mutation Breeding was also organized and the First and Second Research Co-ordination Meetings were held from 31 October to 4 November, 1983 and from 22 to 28 August, 1985, respectively. Here is the report of these two Research Co-ordination Meetings.
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REPORT OF THE
FIRST RESEARCH CO-ORDINATION MEETING

(VIENNA, 31 OCTOBER—4 NOVEMBER 1983)
1. INTRODUCTION

The Joint FAO/IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and Agricultural Development has recently established a Co-ordinated Research Programme on In vitro Technology for Mutation Breeding. The ultimate aim of this programme is to provide new effective tools for plant breeders to construct new cultivars, thus increasing agricultural production of food, feed and industrial raw material, particularly in developing countries. The first Research Co-ordination Meeting was held by the Joint FAO/IAEA Division in Vienna from 31 October to 4 November 1983. The participants considered the potential of new advances of agricultural biotechnology, especially the use of in vitro techniques for mutation breeding.

In vitro technology has developed rapidly and extensively in the last few years. It has a recognized potential to broaden the scope of mutation breeding. The purpose of the meeting was to discuss the present state of art with a view to fundamental and applied research. The participants felt that important progress can be made in the near future in fundamental research in plant biotechnology. They expect that this technology will make a significant contribution to crop improvement by introducing new variation, and improving breeding technology. The latter will ultimately lower the costs in breeding programmes by shortening the period for the introduction of new cultivars and reducing space requirements.

Selection methods for alternatives in many plant characters are often easier to apply to cells in culture than to whole plants. Several traits that were hitherto unknown in whole plants have been isolated rapidly in vitro, suggesting that induced mutagenesis in plant cell culture could provide new sources of variation for plant breeders. However, the immediate possibilities for modifying crop plants in vitro are limited by poor knowledge of the biochemical basis of agronomic characters, inadequate data on mutagen treatment of cells in culture and unsatisfactory cell culture procedures for most of the major crop plants.

2. GENETIC VARIABILITY IN THE IN VITRO SYSTEM

It is now well established that genetic variability may occur in tissue culture without the application of known physical or chemical mutagens (somaclonal variation). There is a range of observable changes: alterations in the number of chromosomes (polyploidy and aneuploidy), chromosome breakages and nuclear fragmentations, point mutations, and epigenetic changes. The incidence of variants is as yet unpredictable and appears to depend on species, genotype and culture conditions. There is also some evidence for pre-existing variation in plant tissues in situ, e.g. endopolyploidy, differential gene activity (e.g. gene amplification) and perhaps accumulated radiation damage that may contribute to the spontaneous variation recorded. Variants useful for agronomic purposes do appear in in vitro populations.

Chemical and/or physical agents can significantly raise the level of genetic variability over and above that seen in control populations. Evaluation of induced variation must be weighed against the background of spontaneous variation. While occasional observations
suggest that mutagenic agents or conditions may alter the spectrum and frequency of variants, there is no clear cut confirmatory evidence on this point.

2.1 Recognition of Genetic Variability from In vitro Culture

Systematic studies are needed to compare somaclonal variability and induced mutations at both the tissue culture and whole plant levels and where agronomically important traits must be expressed.

For basic studies of induced variation, it is desirable to control spontaneous variation. It may be difficult to prevent variability, especially if it is an inherent feature of the explant. The goal here is to control the extent of variability within the design of the experiment.

There are some indications of the parameters that affect the levels of spontaneous variations. However, these are only indications and detailed studies are needed to either confirm or reject the roles of these parameters and to elucidate others. Concomitantly, an understanding of the underlying mechanisms is essential and research in this realm is also recommended.

2.1.1 Genotype or species

There is some evidence that certain genera, species, genotypes, ploidy conditions, etc. are more prone to the occurrence of spontaneous variation than others. Careful choice of the starting plant material is therefore advised.

2.1.2 Explant source

Use of meristematic or embryonic cells derived from shoot apices, inflorescences and embryos may provide a more genetically uniform source of cells than differentiated tissue. A similar advantage may be gained by using the young tissue of seedlings or leaves.

2.1.3 Stability of the differentiated state in vitro

The induction and growth of undifferentiated cells in unorganized callus or cell suspension cultures appears to foster variation. Confirmatory studies are needed. Therefore, it is recommended that the initial material be maintained as meristem cultures, that organized growth such as adventitious buds or somatic embryos be promoted, and that cells be returned to the differentiated state as swiftly as possible after manipulation.

2.1.4 Culture conditions and maintenance

(a) Attention should be paid to avoiding stress conditions that may promote changes such as temperature and light regimes which need to be properly controlled.

(b) Special attention should be paid to the precise timing of the subculture regime to avoid additional stress.

(c) If cultures need to be maintained over long periods without experimental manipulation then cryopreservation, cold storage, or other means of establishing minimal growth may be useful. How-
ever, there are indications that mutant or wild type lines may have variable responses to the protocols presently employed. (d) To maintain recognizable and reproducible cell lines experiments should use known genotypes or varieties and in particular those internationally available in large numbers in seed banks or germplasm collections. This would permit periodic re-initiation of fresh cultures. Alternatively, meristem or shoot tip cultures can be maintained to provide a stable reference source as well. The possibility of meristem banks should be considered.

2.1.5 Composition of the medium

The hormonal content itself may generate variability and especially changes in the ploidy level. There are some indications that 2,4D may be especially active. However, this is just a general impression; good evidence is lacking. Among research efforts controlled experiments can compare the effects of different hormones at different concentrations.

For the moment, we suggest:
(a) Use of the lowest concentration of hormone compatible with the aims of the experiment.
(b) In this vein, the lowest level of other media components, e.g. major salts, micronutrients, may also prove beneficial in the stabilization of the genotype.

Recommendation: The factors affecting somaclonal variation and the underlying mechanisms through which they operate need to be investigated.

2.2 Confirmation of the Genetic Nature of Variation

If in vitro selection has been made for a desirable characteristic, the most convincing evidence for confirmation of the genetic nature of the variation comes from progeny testing following plant regeneration. Only by performing reciprocal crosses and/or test crosses can cytoplasmic, chromosomal and genomic variation be distinguished. It is also important to follow variants through some sequence of sexual cycles, since an effect may be due to gene amplification or some unstable change rather than stable point mutation. For regenerated plants that cannot reproduce sexually, or where sexual reproduction is inappropriate, indication of a permanent stability of the altered trait over a number of vegetative propagation generations would have to be checked. Genetic confirmation of the altered character would have to be established at the cellular level, especially in the cell lines without plant regeneration capacity. Two basic techniques for this confirmation are available:

(a) Chemical and biochemical analyses
Analyses of specific gene products such as amino acids, isozyme profiles and enzyme activities.
(b) Cell fusion to check for complementation
The test may use nucleate or enucleate protoplasts (cybrid formation). One additional benefit of this procedure may be to promote eventual regeneration in the variant line, i.e. if fusion involves a second line capable of regeneration.
Other methods for confirmation of the genetic nature of variability induced in vitro need to be developed.

2.3 Quantification of Genetic Variability

In quantifying genetic variability at the cellular level the ideal procedure would be to start with single cells, e.g. protoplasts, macerated single cell or genuine single cell suspensions. If this is unobtainable, then there must be an effort to minimize the size of cell clusters that distort estimates of cell number and therefore mutation frequencies and rates. The criteria to be considered are survival rate, plating efficiency and eventually lethality. At some point confirmed genetic variation must be determined vis-a-vis total variation per se.

The cells used in the initial manipulations should ideally be of a maximum regenerative capacity as determined by prior experimentation to quantify the effect at the whole plant level. Standardized procedures for mutagen testing should be designed.

Quantification is difficult to obtain in experiments with multicellular units such as explants, callus tissue and meristems. Therefore, in reporting such results, specific procedural details must be given.

2.4 Use of Marker Genes

Experiments illustrating the potential use of specific marker genes have only recently been performed. Ideally, one would expect the development of marker genes (expressed at both the cellular and whole plant level) which can also be correlated with agronomic traits. Indications in this direction are the work being carried out in the United States of America and Israel on tentoxin resistance and organelle inheritance in tobacco, in Hungary on streptomycin resistance and cytoplasmic male sterility and in the USSR on amino acid analogous resistance and alkaloid content.

3. MUTAGEN APPLICATION IN THE IN VITRO SYSTEM

Material to be treated in vegetatively and seed propagated species:

(a) Protoplasts: before or at first division
(b) Cell suspension: individual cells or clumps (in log phase or growth)
(c) Callus culture: in log phase of growth
(d) Adventitious buds from original explant or from callus material
(e) Anther at microspore uninucleate stage
(f) Shoot tip or meristem tip culture
(g) Axillary bud: at initiation of meristem development (few cell stage)
(h) Explants of various plant organs or tissues before or during primary in vitro culture.

The preculture of the material has to be standardized. The in vitro culture period should be as short as possible to prevent loss of the regeneration capacity.
3.1 **Choice of Mutagens**

The aim is to develop methods which give the highest rate of gene mutations with the lowest chromosome and physiological damage.

### 3.1.1 Physical mutagens (ionizing radiations and UV)

**Advantages:**
- high reproducibility
- high penetration in multicellular systems (not valid for UV)

**Disadvantages:**
- possibly high degree of sterility in plants regenerated from treated culture
- difficulties in reproducibility which may be overcome by standardizing application methods

To achieve these advantages the effects of the following procedures should be investigated.

(a) Application of increasing radiation doses  
(b) Application of fractionated radiation doses  
(c) Comparison of low dose rate irradiation with high dose irradiation

Standardized methods of irradiation have to be used to enable comparison of data obtained by different research groups. For detailed information on mutagen application refer to the *Manual on Mutation Breeding* (2nd ed., Technical Reports Series No. 119, IAEA, Vienna (1977)). Specific interaction of mutagenic radiation with culture media should be assessed. Post-treatment conditions of cultured material should also be considered.

### 3.1.2 Chemical mutagens

The following chemical mutagens should preferably be investigated: Ethyl-nitroso-urea (ENUA), Methyl-nitroso-urea (MNUA), Ethyl-methane-sulphonate (EMS), Di-ethylsulphate (d ES), Ethyleneimine (EI), Sodium azide (SA), \( (\text{NaN}_3) \).

**Advantages:**
- point mutations predominate
- less chromosome damage
- possibly different mutation spectra compared with physical mutagens
- high mutation rates are known in certain systems.

**Disadvantages:**
- penetration difficulties in multicellular systems
- difficulties in reproducibility which may be overcome by standardizing application methods
- care has to be exercised as most chemical mutagens have cancerogenic properties.

The following parameters have to be investigated to establish procedures for experiment standardization:

(a) Dose (concentration x time)  
(b) pH  
(c) Physical and chemical properties of the agent, e.g. 'half-life'
(d) Interactions with the culture medium
(e) Post-treatment conditions (for details refer to Manual on Mutation Breeding).

3.2 Recommendations

3.2.1 Reference System

Protoplasts from experimental systems, e.g. *Nicotiana* sp. and *Petunia hybrida* are recommended as a reference system for investigating in vitro mutagen methodology. The following parameters have to be established to study the mutagenic effectiveness and efficiency:

(a) Preculture of the donor plants has to be standardized.
(b) Culture conditions of the protoplasts have to be standardized.
(c) The effect of the cell cycle stage has to be investigated.
(d) Mutagen application conditions:
   - physical mutagens – (see Section 3.1.1)
   - chemical mutagens – (see Section 3.1.2).

3.2.2 Criteria for assessment of mutation effectiveness and efficiency of the reference system

(a) Number of treated protoplast
(b) Number of surviving protoplasts (colony forming)
(c) Number of mutants
(d) Estimation of chromosome damage
(e) Effects on plant regeneration capacity.

3.2.3 Additional recommendations

It is recommended that research involving in vitro mutation induction in plant species and systems other than the reference systems should take into account the criteria laid down above for the reference system to establish mutagenic effectiveness and efficiency in their systems.

Application of the results of the reference system will require studies on the effects of the mutagenic agents on regeneration and expression of the mutations in the regenerated plants.

4. CHIMERISM

In conventional mutation breeding, chimera formation has been one of the major obstacles in recovering mutations both in seed and vegetatively propagated crop plants after mutagenic treatment of a multicellular structure (seed, buds).

Mutation breeding using the in vitro culture technique may be regarded as a new tool to better overcome the chimera problem and to enhance the recovery of induced mutations.

Initially, it is necessary to make a clear distinction between mosaicism and chimerism:

Mosaicism: instability in the progeny of a cell, due to chromosomal, genic or cytoplasmic cause
Chimerism: character of a plant, organ or tissue formed of cell lines differing in their caryological or genetical contents.

In many vegetatively propagated species, spontaneous or induced chimeras are very useful for agriculture and horticulture. The interaction between two genetically different cell types may be positive, for example, in carnation (White Sim).

Grafting and mutagenesis are used to create new chimeras. After mutagenic treatment, mericlinal chimeras must be transformed into periclinal ones.

4.1 Chimerism of In Vitro System

Some spontaneous or induced chimeras, periclinal or sectorial (mericlinal), are very stable, others are more or less unstable.

Using in vitro culture, it is possible to regenerate adventitious shoots and to recover genetically homogeneous plants derived from the different histogen layers of a chimeric individual.

When a plant regenerates after mutagen treatment of a group of cells, chimerism may be expected. In general, the size of the mutated sector will depend on the number of treated initial cells that participate in organ formation.

The treatment applied on an apical meristem will give rise to a mericlinal chimera. Periclinal structures can be obtained by repeated culture of the axillary meristems derived from the treated apex. In vitro propagation could probably accelerate the isolation of periclinal chimeras and the usefulness of the method probably depends on the species.

Shoot meristem from a plant grown in in vitro culture may have a reduced number of meristematic cells facilitating induced somatic mutations.

4.2 Types of Explants and Consequences for Chimerism

To decrease the recurrence of chimerism, it is preferable to irradiate single cells (protoplasts), clusters or calli. The incidence of chimerism will generally be higher from organized meristems.

The possibility of regenerating plants from different layers of a chimera leads to two important consequences:

(a) Sorting out solid mutated plants
(b) Mutations present in a periclinal chimera cannot be transmitted through seed if they are not located in the sporogenic layer; the transfer of a character from another tissue is possible after regeneration of a solid plant.

4.3 Intrasomatic Diplontic Selection

Intrasomatic cell competition may in some way be controlled by modifying in vitro conditions and an improved survival rate of mutated
cells. We may expect that by controlling the physical and chemical factors, the frequency or spectrum of recovered mutations would be influenced. Research on this problem should be encouraged.

4.4 Recommendations

(a) Research on anatomy, histology and cytology of initial explants and on their behaviour after culture should be encouraged in order to elucidate the origin and nature of regenerated plants and to understand the development of mutated sectors in chimeras.

(b) It would be preferable to apply mutagenic treatment to apical meristems, axillary buds or, if possible, on single cells. The assumption that adventitious buds arising from single cells in vitro cannot be chimeric could be an over-simplification. It should be investigated whether, as would be expected from semi-conservative DNA replication, mutated single cells give rise to chimeric structures beginning with the first cell division after the mutagenic event. The progeny resulting from various mutagen treatments and cells of different cell cycle phases should be examined for chimerism.

(c) Studies should be encouraged to develop techniques for somatic embryogenesis, which could prevent chimeral development.

(d) Studies should be initiated on the chimeric structure of mutagen-treated shoot meristems with a view to finding out how to dissolve or stabilize a chimeric sector by successive in vitro clonal propagation.

5. MUTANT SELECTION IN THE IN VITRO SYSTEM

It is no longer necessary to discuss only the potential of in vitro cultures for revealing mutations in agronomic traits. Many ideas about the types of selection pressures that could be applied to tissue cultures of crop plants have been put to the test over the last ten years. The experience gained now allows us to classify current ideas for selection into 'do-able' (i.e. established methods) and 'not do-able' (i.e. selection schemes needing a re-examination of the approach and more basic research). The established methods have (so far as we are aware) not yet contributed to increased agricultural efficiency, although some plant lines isolated in vitro by application of specific selection pressures are part of current breeding programmes. Attitudes to selection pressures in vitro have been influenced by the more recent demonstration of random variation among regenerated plants that can be screened in field conditions.

5.1 Selection Pressures

5.1.1 Pesticide resistance

Mutants resistant to various herbicides have been isolated both from model systems and crop plant tissue cultures by incorporating the selective agent in the medium. The traits are monogenic and mainly dominant. As monogenic traits are acceptable to plant breeders and as resistance mechanisms isolated in vitro are operating in regenerated plants, selection for pesticide resistance is an established method and can be applied to all types of tissue culture from protoplast - derived
clones, suspension cultures, callus pieces and whole cultured organs. It needs to be established whether mutagen treatment can increase the frequency of such mutations.

5.1.2 Disease resistance

Plant lines resistant to pathogen toxins or culture filtrates have been isolated. The genetics of the mutations is largely unknown as most examples are in vegetatively propagated species and the best investigated mutation occurred in the cytoplasmic genome. Although the mechanisms of resistance selected for in vitro did translate to the plant level, too few examples are known to establish rules. More knowledge of pathogenic agents and their modes of action must be obtained. Various types of tissue culture can be used for selection and therefore the method can be considered established even for crop plants with poor tissue culture. The usefulness of the expected monogenic resistance is doubtful and repeated rounds of selection using high numbers of cells should be tried. Screening tissue culture variants in the field with repeated tissue culture cycles is also to be recommended. Information on the effectiveness of mutagens in the induction of disease resistance in vitro should be collected.

5.1.3 Nutritional quality.

Selection for (soluble) amino acid overproduction in vitro can also be considered an established technique. By including toxic levels of amino acids or amino acid analogues into the culture medium single dominant mutations have been recovered in both model species and crop plants using tissue cultures of various types. Amino acid overproduction has been detected in various organs, e.g. in the grains of cereals.

The best examples involve overproduction of threonine, methionine and lysine. Such selection methods have resulted in the isolation of plants with increased storage protein levels. Again, repeated induction and selection should be tried in the hope of collecting several mutations affecting the soluble amino acid pool size. This technique can be applied to most types of tissue culture. Information about the effect of mutagen on the induction of these traits should be collected.

5.1.4 Heavy metal tolerance

Because only a few examples are known of plants regenerated from heavy metal stressed tissue cultures, this is not an established technique. It is encouraging that tolerance in culture is expressed in two cases at the plant level. One case of exclusion has been described. No genetics of tolerant plants is reported. The importance of the tissue culture type should be investigated. There is no known way to select for the specific mechanisms of resistance known amongst plant lines. The influence of mutagens is not known. Heavy metal tolerance should also be sought in the field using tissue culture régénérants.

5.1.5 Salt tolerance

The fact that there are no completely reliable reports of salt tolerant mutants by in vitro selection even in model species is a cause for concern. Application of a selection pressure by adding NaCl or
even sea water may be naive. More information is needed on the ways in which whole plants tolerate salt stress. Salt resistant lines should be tested for expression of the trait in tissue cultures of various types from suspension culture to organ culture. The capacity of cells for adaptation produces background "noise". The adaptation level must be established and used as the starting line.

An approach should be designed which assumes that salt tolerance is a polygenic trait. Such an approach would be based upon repeated rounds of mutation and selection with high numbers of diploid cells. Sustained attempts must be made to induce salt tolerance by mutagen treatment. This trait may be better sought amongst tissue culture regenerants.

5.1.6 Cold tolerance

The situation is similar to that of salt tolerance, i.e., there is no established method of finding cold tolerant mutants in plants in vitro despite numerous attempts using model species and crop plants. Some results, however, suggest a capacity for altered responses to cold that could be genetically controlled. In certain cases differential frost resistance of plants was demonstrated as cold tolerance in tissue culture.

5.1.7 Other traits

Some attempt has been made to improve photosynthetic efficiency by eliminating photorespiration from crop plants. Single gene dominant mutations giving resistance to various analogues have been reported that should have altered photorespiration. Neither the tissue cultures nor the whole plants have been analysed biochemically. This is an example of attempts to isolate mutants that jump ahead of biochemical knowledge as the exact influence of photorespiration on plant efficiency is imperfectly understood.

At this moment we are unable to detect in in vitro other characters related to plant growth and development. For example, day length requirements, vernalization, apical dominance, germination. However, some traits isolated at the whole plant level involving germination, plant height and wilting are being exposed as single gene alterations in the biosynthesis or accumulation of plant hormones. Selection pressures should be designed that could be applied in vitro to mutagen treated cells in order to isolate a wide variety of alterations to plant hormonal biochemistry and the influence of these mutations on plant growth and development should be studied.

5.2 Recommendations

(a) There are some established techniques for the isolation of agronomic traits in vitro. They make use of very easily applied selection pressures that can be applied directly to the cells in culture.

(b) These routine techniques could be applied to any crop plant for which basic tissue culture methods exist, and could be used in developing countries with specific problems, especially disease and environmental stress resistance.
(c) Many other traits, unfortunately related to the environmental factors causing most crop losses, cannot be routinely isolated in vitro and need more basic research such as:

- more information on the biochemistry and physiology of such traits
- a more organized effort with various mutagens to induce such traits and
- consideration of whether more rapid progress would be achieved by screening tissue culture regenerants in the field

(d) The poor state of crop plant tissue culture knowledge has in some cases reduced the possibilities for applying in vitro selection pressures. Those techniques that are established are not dependent upon the type of tissue culture. However, quantitative studies to optimize conditions require the use of suspension cultures or protoplast derived clones. In general, populations of the smallest morphogenic unit are recommended, with a minimum surviving population after mutagenesis of \(10^5\) units.

(e) The influence of tissue culture type on the expression of traits is unknown and should be investigated.

(f) There is evidence of enhanced somatic crossing even in tissue cultures and this should be investigated further, including the effect of irradiation on crossing-over frequencies.

6. GENERAL CONCLUSIONS AND RECOMMENDATIONS

For mutation breeding using in vitro techniques the most important facet is the eventual regeneration of whole plants possessing the desired agronomic alterations. For a number of the most important crops, usable techniques do exist but they are often not optimal. For a large number of crops and especially some of the most important ones, e.g. within the cereals and legumes, good reproducible methods for going from cells to plants still need to be developed. This must be considered the first priority.

To maximize the effectiveness of current research in both advancing technology and in producing agronomically useful variants, the ideal choice of crop species would be those that are capable of growth as single cells, where the mutagen can effectively be applied, where spontaneous variation can be to some degree regulated and where whole plants can be regenerated at high frequency. Haploid cells offer a special advantage in facilitating selection of recessive mutations at the cellular level. The majority of these criteria, if not all, cannot be met for any particular crop and more fundamental research is needed. Agronomically important traits that appear amenable to current selection are disease resistance, herbicide resistance and perhaps salt tolerance.

If properly applied, in vitro techniques could be equally important for solving serious agricultural problems in developing and industrialized countries. However, this technology has a place only if the goal cannot be accomplished by conventional breeding, or if a breeding aim can be reached in a shorter period of time.
In certain crop species high priority should be given to the development of efficient in vitro systems before they can be integrated into breeding schemes, including mutation induction. However, the achievements of plant regeneration in many cereals and legumes during the past period are very promising for breeding programmes of these crops. Material derived from in vitro culture should be subjected to conventional genetic analysis and to normal breeding procedures.

Emphasis should be placed on the application of in vitro technologies to economically important crops. Furthermore, the existence of an appropriate infrastructure for conventional breeding should be guaranteed and in vitro techniques should be used to strengthen such programmes.

7. SPECIFIC TASK OF THE FAO/IAEA AGRICULTURAL BIOTECHNOLOGY LABORATORY AT SEIBERSDORF AND TRAINING ACTIVITY

In the field of mutation induction the Group recognizes the work of the FAO/IAEA Agricultural Biotechnology Laboratory at Seibersdorf. It is also realized that in vitro systems might offer prospects for studies related to induction of mutations and recovery of mutants. Studies should be directed towards elucidating the efficiency of various mutagens and applying efficient selection procedures.

The laboratory should not concentrate on improving plant tissue culture methodology or design selection pressures in vitro. The laboratory should focus on adapting or developing the methodology of in vitro plant breeding as a basis for more effective and efficient methods of mutation induction, mutant selection, mutant propagation and genetic engineering.

The FAO/IAEA Joint Division should initiate training programmes for geneticists, plant pathologists and breeders in in vitro technology for mutation breeding of crop plants, where practical application seems feasible.

The FAO/IAEA Joint Division training course on the induction and use of induced mutations in plant breeding should also include specific training in tissue and cell culture methodology, shoot tip and embryo culture in vitro plant regeneration, anther culture, etc. In addition, a specific course, dealing with in vitro technology for mutation breeding, should be organized by the FAO/IAEA Joint Division to provide intensive training to scientists from Member States actively engaged in plant breeding. The aim of course would be to provide information and practical experience in the use of in vitro techniques for mutation breeding of crop plants. Preference would be given to candidates from developing countries to enable them to carry out in vitro mutation technology as a part of regular national plant breeding programmes for crop improvement.

The Joint FAO/IAEA Division could provide advice to breeders, consult with experts on specific items, and give advice on mutation breeding of crops and related in vitro techniques.

The FAO/IAEA Division should also promote a frequent exchange of experience and international dissemination of knowledge through Research Co-ordination Meetings and scientific meetings.
LIST OF PARTICIPANTS

AUSTRIA
Dr. E. Haunold
Ost. Forschungszentrum
Abtl. für Landwirtschaft
Seibersdorf
Dr. J. Schmid
Ost. Forschungszentrum
Abtl. für Landwirtschaft
Seibersdorf

BELGIUM
Dr. J. Bouharmont
Catholic University of Louvain
Laboratory of Cytogenetics
Place de l'Universite 1
Louvain
Dr. I. Negrutiu
Vrije Universiteit Brussel
Instituut voor Moleculaire Biologie
Paardenstraat 64
B-1640 St. Genesius-Rode
Brussels

BULGARIA
Dr. A. Atanassov
Tissue Culture Laboratory
Institute of Genetics
Academy of Sciences
Ul. Nezabravka bl. 50
Sofia 1113

CANADA
Dr. K.K. Kartha
National Research Council of Canada
Prairie Regional Laboratory
Saskatoon, Saskatchewan S7N 0W9

GERMANY, FEDERAL REP.
Dr. H. Lörz
Max-Planck-Institut für Züchtungs-
forschung
Englspfad
D-5000, Köln
and
Dr. W. Nitzsche

Dr. W. Preil
Federal Research Centre for
Horticultural Plant Breeding
Borns-kamps weg
D-2070 Ahrensburg

and
Dr. F. Walther

Dr. F. Kohler
Biologische Bundesanstalt
Institut für Land und Forstwirtschaft
Messeweg 11-12
D-3300 Braunschweig

ITALY
Dr. V. Nuti-Ronchi
Instituto di Mutagenesi e Differenziamento
Consiglio Nazionale delle Richerche
Via Svezia 10
I-56100 Pisa

Dr. G. Picciurro
U. di Bioagricoltura, ENEA
Centro Richerche Nucleari Casaccia
00060 S. Maria de Galeria, Roma

JAPAN
Dr. Yasuo Ukai
Institute of Radiation Breeding
Ohmiya, Naku-gun
Ibaraki-Ken
NETHERLANDS
Dr. A.J. Kool
Department of Genetics
Vrije Universiteit
De Boelelaan 1087
1007MC Amsterdam

SWITZERLAND
Dr. A. Blonstein and
Dr. P.J. King
Friedrich Miescher Institut
Postfach 273
CH-4002 Basel

UNITED KINGDOM
Dr. B.W.W. Grout
North East London Polytechnic
Department of Biology
Romford Road, London E15 4LZ

USA
Dr. P.V. Ammirato
Plant Technology Corporation
B-2611 Branch Pike
Cinnaminson, New Jersey 08077

Dr. J.P. Helgeson
USDA-ARS North Central Region
Department of Plant Pathology
1630 Linden Drive
University of Wisconsin
Madison, Wisconsin 53706

ORGANIZATION

INTERNATIONAL ATOMIC ENERGY AGENCY

Mr. M. Zifferero
DDG, Head of the Department of Research and Isotopes

Mr. B. Sigurbjörnsson
Director, RIFA

Mr. A. Micke
Head, Plant Breeding and Genetics Section

Mr. M. Maluszynski
Plant Breeding and Genetics Section

Mr. L. Edquist
Animal Production and Health Section

Mr. T. Hermelin
Seibersdorf Laboratory

Mr. H. Brunner
Seibersdorf Laboratory

Mr. S. Daskalov
Seibersdorf Laboratory

Mr. G. Hardarson
Seibersdorf Laboratory

Mr. B. Donini
Plant Breeding and Genetics Section

Mr. F. Novak
(Scientific Secretaries) Seibersdorf Laboratory
REPORT OF THE
SECOND RESEARCH CO-ORDINATION MEETING

(VIENNA, 22—28 AUGUST 1985)
I. INTRODUCTION

The Joint FAO/IAEA Division has organized the 2nd Research Co-ordination Meeting on "In Vitro Technology for Mutation Breeding" in Vienna, August 26-28, 1985, in conjunction with the symposium on "Nuclear Techniques and In Vitro Culture for Plant Improvement" held there on August 19-23, 1985 (published in the Proceedings Series, STI/PUB/698, IAEA, Vienna, 1986).

The Research Co-ordination Meeting (RCM) had several objectives: review progress achieved by members of the Co-ordinated Research Programme (CRP) since 1983, discuss it and co-ordinate plans for the next phase of the CRP, assess in conjunction with the above symposium the potential impact on plant breeding of novel technologies, such as those making use of somaclonal variation, cell hybridization, and molecular genetics. The areas covered were the extent and origin of genetic variability under in vitro conditions and assessment of the nature of heritable variation and its utilization in breeding programmes of sexually and asexually propagated plants.

It was concluded that appropriate exploitation of in vitro technologies, including induction of mutations, could provide the desired variability, shorten breeding cycles and reduce breeding costs. This is particularly essential for crops where classical plant breeding approaches are inadequate such as perennial vegetatively propagated species.

After comprehensive discussions of the research and in view of the reports and reviews presented at the symposium, the participants arrived at the conclusions and recommendations presented in this document. These conclusions confirm and extend those adopted in previous meetings, viz. "Mutation Breeding of Crops and Related In Vitro Techniques and Problems", FAO/IAEA RCM, Vienna, October 31 - November 4, 1983, which is included in this document, and "Mutation Breeding for Disease Resistance Using In Vitro Culture Techniques", FAO/IAEA Advisory Group Meeting, Vienna, October 8-12, 1984, IAEA-TECDOC-342, 1985.

II. GENETIC VARIABILITY

1. ORIGIN UNDER IN VITRO CONDITIONS

1.1. Somaclonal variation

Plants regenerated from organized meristematic tissues such as shoot tips or axillary buds are usually true to type and uniform; occasional deviants can be epigenetic variants or somatic mutants. However a callus phase preceding differentiation often leads to genetic variation (somaclonal variation) among the regenerated plants. This somaclonal variation is now recognized as a rather general phenomenon occurring in dedifferentiated, unorganized, callus tissues. Some of the genetic changes could have pre-existed in the original organized explant (e.g. leaf, stem). However, it is clear now that genetic changes can occur much more readily during the callus phase. These genetic changes persist through in vitro differentiation and plant regeneration. The frequency and spectrum of the genetic changes which persist probably vary with the species and the nature of changes, but these aspects have not been studied adequately so far.
The heritable nature of various phenotypic changes observed among in vitro regenerants has been demonstrated in a range of plant species. In vitro generated mutations were recovered in selfed progenies of regenerants of cereals and other sexually propagated crops (e.g. maize, wheat, oat, alfalfa, tomato). In vegetatively propagated crops, the genetic nature of the somaclonal variants is deduced from the stability of the new traits in long-term clonal propagation (e.g. in potato, garlic, sugarcane). The number of species in which somaclonal variation has been demonstrated is increasing rapidly but so far it has had little impact on plant breeding. Also, the causes of somaclonal variation and its molecular basis have not been established so far and further investigations are required.

From the breeders point of view both genetic instability and stability are important. Instability is desired in order to obtain genetic variation, but too high a degree of variation resulting from multiple events may be difficult to handle in a breeding programme and may require extensive cross breeding. On the other hand, stability of cultures and regenerants is essential for most stages in breeding programmes, for commercial propagation of established cultivars (clones) and for maintenance of germplasm. Thus, it is very important to understand the causes of somaclonal variation and to learn how to control them.

The degree of genetic variability obtained in in vitro systems appears to depend on the species and also on the genotype (variety) used. The genotype-dependent differences in the frequencies and spectra of somaclonal variants suggest that it is not advisable to focus on one variety only; it is unpredictable which variety will yield the desired range of variation. For the same reasons, it may be easier to maintain stability in some genotypes than in others. This has particular implications for the preservation of germplasm.

The choice of tissue, i.e. meristematic vs. differentiated, merits careful consideration. In cereals such as wheat, maize and rice, undifferentiated embryonic or meristematic explants provided the sources of regenerating or embryogenic tissue cultures where somaclonal variation occurred. On the other hand, in many of the dicotyledonous species, regenerating tissues and cell cultures can be obtained also from various differentiated tissues, e.g. leaves.

Further studies must be conducted in order to find if the genetic variation pre-existed in the source tissues before culturing began or was induced subsequently by the culture conditions, ingredients and/or metabolites. For such studies, true breeding seeds, stable meristems or stable shoot cultures should be maintained in order to provide reference sources and permit reinitiation if necessary.

The incidence of variants with altered chromosome numbers, chromosomal rearrangements and/or gene mutations is reported to be much higher in unorganized callus cultures, than in differentiating ones (embryonic cultures, embryogenic suspensions). Cells maintaining genetic stability in cultures can be recognized by their dense cytoplasm (no vacuole), small size and high nucleus/cytoplasm ratio. Such relatively stable cultures should be used for mutant selection experiments as well as source (reference) materials to obtain cells for callus tissues giving somaclonal variation for further breeding work.

Hormonal regimes and/or culture media composition may affect the genetic stability of the cell cultures. However, it has been demonstrated
that plant hormones such as different cytokinins and auxines are not directly mutagenic; the higher or lower observed rates of genetic variation could be due to indirect effects such as changes in cell metabolism. In this connection, it should be noted that older cultures (after a higher number of subcultures) have a higher degree of variability.

1.2. Genetic changes induced by mutagenic treatments

In view of the limited experience gained so far, only general guidelines can be given to help researchers choose the mutagen or dose to use. This clearly depends on the type of cell used and the desired types of mutations. This point has already been discussed in much detail in the first Research Co-ordination Meeting in 1983 and the conclusions remain largely valid (see appended, Section 3, pages 4-6: Mutagen application in the in vitro system).

The nitroso-urea compounds have been used successfully both in seed and protoplast treatments to obtain cytoplasmic mutations in several plant species (Antirrhinum, maize, tobacco and tomato) e.g. albinism, resistance to streptomycin or lincomycin. Other chemicals or physical mutagenic treatments were not very successful in this respect. These results may indicate that a very potent mutagen (as the nitroso-urea compounds) is required to get a selectable quantity of cytoplasmic mutations.

Findings from mutation induction experiments with cell and protoplast cultures of Nicotiana plumbaginifolia may be instructive. Stable revertants to normal in protoplast cultures of mutants deficient in nitrate reductase occurred in similar frequencies following UV or gamma-irradiation. However, significantly higher reversion rates were obtained when DNA of high molecular weight (approx. 20-50 kbp) was applied. Thus, DNA application gave the same results as a potent mutagen in terms of mutant reversions. With respect to treatment severity, it is suggested that too high doses should be avoided. Thus high doses of nitroso-ethylurea (0.1 mM or higher) caused sterility in N. plumbaginifolia regenerants. On the other hand, in Petunia hybrida cell cultures, most HgCl2-resistant or 6-fluorotryptophane resistant mutants were obtained from rather low nitrosoguanidine concentrations that did not affect plating efficiencies significantly.

It has been shown in synchronized cell cultures of N. plumbaginifolia that the frequency of recessive mutations induced by UV-irradiation is significantly higher when the cells are in the S-phase. Therefore, it is advisable to use, whenever possible, synchronized cultures and treat at the S-phase in order to obtain high frequencies of monogenic recessive mutations.

Similarly, when employing chemical mutagens that interact with DNA replications, the stage in the cell cycle or the phase of the tissue culture where active DNA synthesis is expected should be used.

There is no clear information whether the best time for mutagenic treatments is before or during culturing. However, to avoid chimerism, mutagen treatments during culturing would not be advisable if the culture consists of multi-cellular units. Treatment of single cells, e.g. protoplasts, would be optimal; if not possible, treatment of the explant before culturing would be preferred.

The radiosensitivity of differentiated leaf cells in two species of Nicotiana, has been shown to be significantly higher than that of
proliferating cells of young calli. Whether this also leads to differences in mutation frequencies has not been investigated so far.

For efficient sorting out of induced cytoplasmic mutations, the mutagenic treatments should be applied at a phase of low organelles' number, i.e. cells containing only a few chloroplasts or mitochondria. Few chloroplasts per cell are found in actively dividing cell systems such as meristems or in chlorophyll-deficient (white) cell cultures. However, cells in such tissues usually contain high numbers of mitochondria. Therefore, to obtain mutants in the mitochondrial DNA the opposite type of cells should be mutagenically treated, e.g. differentiated green tissue such as leaf mesophyll. Nevertheless, it should be noted that chloroplast mutations have been obtained from mutagen-treated leaf mesophyll protoplasts. Thus, even in such less favourable material (with many chloroplast copies), sorting-out can occur in tissue cultures and result in progenies with mutant chloroplast DNA.

The summary guidelines on tissue types and time of mutagen application established by the 1983 Research Co-ordination Meeting (see appended, pages 4-5) are still valid.

1.3. Somatic Cell Hybridization

Fusion of protoplasts from different plant species is a feasible approach to produce genetically modified cells. However, it is applicable only if plants can be regenerated from the cultures. Furthermore, it appears that true hybrids can be obtained only between plant species that are fairly closely related. If the species are taxonomically distant, the limited gene transfer approach might be more appropriate, where the chromosomes in the protoplasts of the donor are fragmented by irradiation before protoplast fusion. The identification and selection of hybrids is a major bottleneck in this protoplast fusion approach. Markers such as resistance to antibiotics and to amino acids' analogs or hormone autotrophy have been successfully used to select the hybrid cells. In breeding programmes, where such markers are not available in the taxa to be combined by protoplast fusion, it may take too long to first introduce such markers by crossing or mutation induction. Also, such procedures and more prolonged culturing will most probably generate some undesirable genetic background variation. In these cases, micromanipulation to pick up the fusion products could be better, but at present it is still of limited practical value. It should be noted that the selection of hybrids with just one or a few donor genes of economic value, which is the usual objective of plant breeders, requires suitable selection methods for the particular gene(s) under in vitro conditions.

This situation is somewhat more promising when the objective is to transfer only chloroplasts or mitochondria carrying a known trait, which can be achieved by using nucleus-free donor protoplasts or donor protoplasts that had their nuclei inactivated by irradiation. In this way, cytoplasmic male sterility, a mitochondrial trait, has been transferred in Brassica and Nicotiana. Chloroplast encoded properties, e.g. cold-resistance (in Brassica) and atrazine-resistance (in Solanum tuberosum), have also been successfully transferred by "cytoplast fusion". This approach is expected to provide a rapid method to transfer cytoplasmic organelles into various breeding lines. It can make such "crosses" more viable since the nearly complete or total absence of donor chromosomes will circumvent the problem of nuclear-cytoplasmic incompatibility, often encountered in fusions between nuclei-containing protoplast partners of unrelated plant species. Since transfer of cell organelles
and recombination between the mitochondrial genomes can be obtained by cytoplasmic fusion and not by sexual crosses, cytoplasmic fusion can provide a valuable addition to plant breeding techniques.

2. ASSESSMENT OF HERITABLE VARIATION

Available data suggest that somaclonal variation results from events occurring at relatively high rates, basically similar to those found after mutagenic treatments. The spectra of phenotypic variation appear similar too, but large scale analyses of the mutant genotypes may show differences. Assessment of the nature, type and rate of genetic variation induced by mutagens and obtained from in vitro cultures is very important for plant breeders who have to choose the best method.

Confirmation of the genetic nature of the variation comes mainly from progeny testing following plant regeneration. Molecular methods (restriction fragment length polymorphism, pattern of highly repetitive species specific DNA sequences, etc.) can be employed at earlier stages, but they require expertise and well equipped high-cost laboratories.

In model systems such as N. plumbaginifolia where the single cell origin of regenerated plants can be ascertained, screening for pigmentation mutations or resistance to 5-methyl-tryptophane, as well as one-or-two dimensional protein pattern analysis of such clones can be used to measure the rate of genetic variation induced by the various approaches.

Where the regenerants have a multicellular origin and may therefore be chimeric, such methods are inappropriate, with the possible exception of protein pattern analysis. In such cases, direct screening for the desired breeding objectives (e.g. resistance to herbicides or pathogens) can be performed on single cells or regenerants, but it should be followed by progeny testing.

Exposure to various mutagenic treatments generally inhibits cell division and growth. Available information suggests that the impact of such treatments on the ability to regenerate plants from callus and cell cultures, or to produce shoots from cultured meristems may indicate the efficacy of the treatments, but correlations with gene mutation rates must still be established. Male sterility in regenerated plants can also indicate the degree of genetic, especially chromosomal, damage.

Gross chromosomal modifications due to the in vitro passage can be identified by chromosome counting, cytophotometric determinations, Giemsa banding, etc. Culture conditions generating such abnormalities at high rates should be avoided in breeding programmes.

Efforts should be made to establish procedures which produce mainly discrete changes. Where higher incidence of variability or a maximum spectrum of variation is desired, it is recommended to combine mutagen treatments with in vitro culture-induced somaclonal variation.

Stabilization of the material, and/or sorting out of chimeric events, can be done by separation under in vitro conditions and clonal multiplication using suitable selection pressures. Homozygosity of the induced variation can be obtained by several cycles (at least two) of selfing. Self-incompatible species can be selfed with established special techniques.
3. UTILIZATION OF HERITABLE VARIATION

At the first Research Co-ordination Meeting held in 1983 the rapidly developing approaches in biotechnology were discussed and several ways to assess the value of these techniques in mutation breeding were outlined (see Mutation Breeding of Crops and Related In Vitro Techniques and Problems: Conclusions and Recommendations, p. 9-12, attached). It is satisfying to note that in the short elapsed period, much additional information was gained on induced genetic variability in vitro. Furthermore, a few crop varieties obtained via in vitro cultures were already released, some are about to be released and additional lines in different crops are in various stages of testing.

Some examples from cereals and ornamentals are described below:

**Rice**: Lines with improved yield, with tolerance to bacterial blight, cold and high salt concentrations and with wider adaptability were obtained.

**Wheat**: Lines with more vigorous tillering, earliness, increased cold and salt tolerance and improved grain qualities were selected.

**Weigela**: A variety with deeper red flowers ("Rubirif") was released following gamma irradiation of in vitro cultures. Two other promising lines are in advanced testing stages.

**Pelargonium**: A variety with a special aroma ("Velvet Rose") was developed through somaclonal variation.

**Chrysanthemum**: Cold tolerant mutants were obtained from irradiated suspension cultures while no such mutants were obtained in the controls. One cold resistant line has been released for commercial propagation to replace the original variety ("Puck"). Additional mutant lines are in advanced testing stages in several locations.

Further examples of improved tolerance to environmental stresses, herbicides and diseases in several species follow:

**Salt tolerance**: Lines with tolerance to high salt (Na Cl) concentrations were selected from in vitro cultures in tobacco (3 lines) and oat (2 lines).

**Tolerance to aluminum**: Tolerant lines were obtained in tomato and in *N. plumbaginifolia* using protoplast cultures.

**Herbicide resistance**: Clones resistant to electron transport inhibition by herbicides (bleaching) were obtained in *N. plumbaginifolia* by culturing freshly isolated protoplasts in nitroso ethylene. Resistance to paraquat was obtained in tomato. Herbicide resistance was obtained also in soybean and birdsfoot trefoil.

**Virus resistance**: Resistance to the garlic mosaic virus was discovered following nitroso methylurea treatment of garlic shoot cultures. No mutation was found in a large control population. A TMV resistant mutant of *N. sylvestris* was isolated using in vitro techniques.

**Fungal diseases resistance**: Resistant progenies were obtained from in vitro cultures, in maize to *Helminthosporium maydis* toxin, in *Brassica napus* (spring rape) to *Phoma lingam* toxin and germinating conidia, in *N. tabacum* to *Pseudomonas tabaci* toxin, and in *Nicotiana* spp. to *Alternaria*. Plants resistant to culture filtrate of *Fusarium oxysporum* were derived from in vitro cultures of tomato and potato.

The potentials and problems of selecting for disease resistance under in vitro conditions were discussed in detail at the Advisory Group Meeting on "Mutation Breeding for Disease Resistance Using In Vitro Culture Techniques" in Vienna, 8-12 October, 1984. The conclusions and recommendations were published in IAEA-TECDOC-342, 1985.
III. UTILIZATION OF IN VITRO TECHNIQUES IN MUTATION BREEDING: GENERAL

In view of the reports and discussions during the FAO/IAEA International Symposium on "Nuclear Techniques and In Vitro Culture for Plant Improvement", held in Vienna 19-23 August, 1985 (published in the Proceedings Series, STI/PUB/698, IAEA, Vienna, 1986) and in view of the deliberations of the second Research Co-ordination Meeting the participants adopted suggestions for terminology and several schemes for in vitro mutation breeding which are summarized in Appendices I-IV and reached the following conclusions and recommendations on the utilization of in vitro techniques in mutation breeding:

a) Selection in in vitro systems proved effective for simply inherited traits (e.g. dwarfism) as well as for complex characters (e.g. cold tolerance). In view of the success in the past two years, the utilization of in vitro cultures should be encouraged and the development of effective screening procedures should be emphasized.

b) Mutagenic treatment enhances genetic variability and should be employed judiciously: physical mutagens may be preferred for in vitro cultures because of the ease of application. Among the chemical mutagens, nitroso compounds proved successful in increasing variability in in vitro cultures particularly for cytoplasmic traits; further studies with various chemical mutagens are required.

c) Further studies are needed to identify the optimal source of explants for cultures and optimal culture conditions as well as regeneration approaches to maximize mutation yields.

d) To enhance the probability of success, in vitro mutation breeding programmes should be initiated with two genotypes or more, preferably not closely related.

e) Studies identifying markers, physiological indicators or criteria and reliable correlations useful for in vitro screening should be intensified since many of the economically desired characters are not usually expressed in culture conditions.

f) Studies on the effects of mutations derived from somaclonal variation or in vitro mutagenesis on hybrid performance should be expanded in view of preliminary reports on the improvement of combining ability of sublines within maize inbreds.

g) It is important to develop systems in agricultural crops, especially in asexually propagated ones, where the mutants could be followed through sexual generations to verify the precise genetic nature of the changes.

h) In vegetatively propagated crops, in vitro cultures proved useful in separating the components of chimeras rapidly and should be used more widely for that purpose.

i) Combining in vitro culturing with mutagenic treatments is expected to enhance the frequency and broaden the spectrum of mutations. Breeders may however prefer to apply mutagens to stable cultures and utilize somaclonal variation separately in order to avoid excessive genetic variation.

j) In vitro culturing combined with mutagenic treatments may be utilized to produce markers within promising breeding lines, lacking other adequate means for cultivar identification.

k) Suitable approaches for differential mutagenic treatments of nuclear vs. cytoplasmic targets should be further developed.

l) In sexually (seed) propagated crops, haploids can be very advantageous in the identification of mutants and their rapid advancement to homozygosity. In asexually (vegetatively) propagated crops, the use of chemicals which can induce partial
chromosome elimination (e.g. para-fluorophenylalanine = PFP, griseofulvine) should be investigated.

m) The nature of the observed stimulation of regeneration by low doses of radiation or EMS and by stress caused by high temperatures or higher salt concentrations, should be investigated.

IV. UTILIZATION OF IN VITRO MUTATION BREEDING: SEXUALLY PROPAGATED CROPS

1. CEREALS

1.1. Explant and type of culture

Various explants have been used successfully in cereals for the initiation of in vitro cultures (anthers, microspores, immature and mature sexual embryos, young inflorescences, the basal parts of young leaves). Plant regeneration could be achieved from these in vitro cultures in all species, but not with every genotype. Examples of the successful applications of in vitro culture techniques for breeding purposes are found in wheat, rice, maize and oat where varieties derived from anther culture have been released, and promising lines were developed in in vitro culture (see Section II. 3, above, p. 9-11).

It is encouraging that in all the major cereal species, plants have been regenerated from callus cultures. However, reproducible and efficient plant regeneration from single cells and protoplasts still remains a goal to strive for.

1.2. Generation of variation

Somaclonal variation is found following passage of the cells through in vitro culture. Variability for qualitative and quantitative traits among the regenerated plants has been observed in all cereal species investigated (e.g. maize, oat, rice, wheat).

Chromosome variation has been described in régénérants of barley, maize, oat, triticale and wheat. Nevertheless, the origins and causes of somaclonal variation are still unknown and the mechanisms which promote or inhibit such genetic variability are not understood.

Stability in in vitro régénérants is required in order to improve one trait in a cultivar by mutagenic treatments during tissue culturing, without changing other characters. To date the experience in cereals with combined mutagens' application and in vitro cultures followed by plant regeneration is limited. Investigations in this area are necessary to assess and compare the nature, frequency and spectra of the mutations obtained.

In model systems, other procedures for genetic engineering of plants were developed, e.g. protoplast fusion (including gene transfer), direct gene transfer into protoplasts, microinjection of DNA into plant cells and utilization of Agrobacterium or viruses as vector systems for the introduction of foreign genes. One of the major limitations of genetic manipulation in cereals is the present inability to achieve reproducible regeneration of protoplasts to whole plants. Thus the procedures mentioned above based on the use of protoplasts are not yet applicable for cereal breeding.
Viruses that infect Gramineae are intensively studied at present as potential vector systems for the transfer of foreign genes into Gramineae species.

The use of Agrobacterium Ti plasmid for cereal transformation is still restricted by the limited host range. So far, Agrobacterium infection of Gramineae has not been demonstrated.

Procedures for DNA microinjection which will facilitate penetration of plant cell walls and delivery of the DNA segments into the nucleus have to be developed. Potential recipient cells for the transfer of foreign genes by microinjection could be regenerating structures such as somatic embryos, meristematic cell clusters in embryogenic cell suspensions or unfertilized ovules.

The advantage of increased variability among the regenerated plants is in obtaining plants with useful variation in agronomically important traits without mutagen applications. However, since the causes of somaclonal variation are still unknown it is too early to forecast whether manipulation of this type of variation towards the desired traits is possible. On the other hand, mutagenic treatments cause a wide array of variation, which can be narrowed to some extent by the particular treatment procedures (e.g. radiation vs. chemical mutagenesis) and from which the desired mutants must be selected. Induced mutations from treatments with radiation or chemical mutagens have been used successfully in breeding many improved commercial cereal cultivars (See Mutation Breeding Review No. 3, IAEA, Vienna, 1985). Such varieties have not been produced by somaclonal variation so far.

1.3. Selection approaches

The feasibility of selection under in vitro cultures (e.g. callus cultures) was demonstrated by selecting for resistance to disease, salt and cold; and among regenerated plants for higher tillering, improved yield and quality (see II. 3, above, p. 9-11 and "Mutation Breeding for Disease Resistance Using In Vitro Techniques", IAEA-TECDOC-342, Vienna, 1985). However, it was noted during the discussion that generally applicable protocols for selection are lacking and procedures have to be worked out to meet specific objectives. In addition it must be verified that the character selected for in the cultures will be expressed in the regenerated plants.

1.4. Research recommendations

a) The development of efficient and reproducible in vitro totipotent systems for cereals: all factors (choice of explant, culture media, subculture, etc.) should be assessed with regard to the final aim, i.e. plant regeneration.

b) Careful evaluation is needed in order to determine whether in vitro culture procedures or mutagen treatments or their combinations lead to more desirable genetic variation for specific characters.

c) Studies should be carried out to clarify the mode of action of the genes controlling regeneration capacity, observed in some tissue culture systems.
2. LEGUMES AND VEGETABLES

This section deals with several widely divergent groups of crops; Grain Legume: soybean, pea, chickpea, peanut, Vigna, beans, faba bean, lentil; Forage legume: alfalfa, vetch, clovers, birdsfoot trefoil, sainfoin; Vegetables: tomato, pepper, eggplant, Brassica spp., cucumber, lettuce, onion, celery, carrot, garlic, etc. Examples of the successful application of in vitro techniques for breeding purposes are available in several of these species (see also Section II.3, above).

2.1. Explant and type of culture

The choice of explant has important implications. Less differentiated explants from juvenile organs are preferred for the initiation of organogenic or embryogenic cultures. For example, embryos, ovules, parts of young seedlings, young leaves and stem meristems can be used for most legume and vegetable crops.

For maintenance of germplasm (lines, land races, cultivars) genetic stability, meristem cultures are considered best, because they can be stored at low and ultra-low temperatures. Direct shoot or embryo formation, or indirect formation via callus culture, are appropriate ways to produce somaclonal variation in legumes but more efforts are needed in faba bean, Vigna, lentil, etc.

Highly embryogenic cultures, as those developed for alfalfa, clover, soybean, eggplant and carrot are suitable for the selection of desired mutants. However, more attention should be given in various legumes and vegetables to the development of reliable techniques for selection, preferably in unicellular suspensions and protoplast cultures; and to synchronization, dormancy, maturation and genetic stability of the embryogenic suspensions. Highly embryogenic callus and cell-suspension cultures are convenient source materials for the isolation of protoplasts.

Routine techniques for obtaining whole plant regeneration from protoplasts are available only in alfalfa, clover, tomato, Brassica spp., carrot and eggplant.

Haploid production via anther culture (Appendix IV) has been developed for breeding purposes so far only in Brassica species, pepper, eggplant and, to some extent, in tomato. Therefore, other available techniques such as ovule and pollen culture, should be examined for haploid production in the other species.

A successful application is the rescue via embryo culture of inter-specific hybrids achieved already in many vegetable crops and some legumes (e.g. clover, alfalfa, lentil, cabbage, tomato). This technique is now ready for breeding applications, but not yet fully exploited.

2.2. Generation of variation

Somaclonal variation may result from alterations in chromosome number, chromosome breakage, nuclear fragmentation and point mutations as well as epigenetic changes. Gross alterations are believed to have little value in breeding terms. The incidence of variants with potential use in agriculture (e.g. high solids content, jointless pedicle and male sterility in tomato; carotene content in carrot; improved morphology in celery and cabbage) is fairly high, but unpredictable and varies with species, genotype and culture conditions. Combination of somaclonal variation and induction of mutations by mutagen treatments may be useful.

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2.3. **Selection approaches**

The breeding objectives for any crop are based on the preferences of the farmers, processors, consumers, etc. Success in breeding depends largely on clear and feasible objectives, broad genetic variability and selection or screening efficiency.

*In vitro* screening or selection of mutants can be performed on cells (single cells or callus), differentiated structures (meristems, somatic embryos) and/or regenerated plants, as shown in Appendices II and III. Examples where mutants or variants were produced from leaf explants (e.g. via callus and plantlet regeneration in tomato) or dissected embryos (as in cereals) are also known. Since the advantage of using *in vitro* systems lies in the fact that a large population of cells can be screened, cell selection is the central issue of *in vitro* breeding systems. However, selection at the cellular level is applicable only when the genetic changes are expressed in the cultured cells and are correlated with the desired whole plant characters.

Mutant cells are usually selected by applying specific selection pressures. The approaches to selection *in vitro* which have been summarized in the Conclusions and Recommendations of the first Research Co-ordination Meeting of this programme, Vienna, 1983, remain valid and are appended to this document.

2.4. **Research Recommendations**

**a)** Information should be collected on the effectiveness of mutagens for *in vitro* induction of resistance to herbicides and diseases, and other agents.

**b)** Mutants for many economically important traits cannot be routinely identified because the appropriate screening methods have not been developed yet, or because the genes responsible for such traits are not expressed in cell cultures. Studies should be undertaken on modulation of gene expression, and correlations between plant-expressed traits and molecular or other markers expressed at the cell level which can aid selection. Thus, the spectrum of agronomically important traits for selection under *in vitro* culture systems might be expanded.

**c)** The utilization of somaclonal variation for breeding purposes should be explored, but it should be remembered that the various agents responsible for it, their mode of action, and the molecular bases of the process are not yet understood. The ability to modulate its action and control undesired side-effects is thus reduced. The interest in somaclonal variation, both basic and applied, has increased markedly in recent years. This will have obvious consequences in both a better understanding of the intimate nature of somaclonal variation events, and in the production of commercial varieties in an increasing number of crops. However, the participants draw the attention of all those concerned with breeding policies to the danger of believing that somaclonal variation alone can solve all the problems of genetic variability in world agriculture. Integration within active breeding programmes is essential for useful exploitation of somaclonal variation.

**d)** Classical mutation breeding has played an important part in the development of improved cultivars and will continue to play a significant role in the future. The use of *in vitro* techniques can enhance the efficiency of mutation breeding programmes. As noted above, both mutation induction and somaclonal variation yield random
and non-specific variation. Experimental control is limited to the choice of explants, culture conditions, mutagens and selection methods. It should be remembered that breeders are often interested only in adding or altering a single specific trait in an otherwise well adapted, high yielding cultivar; and not in very high degrees of variability which disrupt genotypic combinations of proven merit.

e) Specific gene transfer methods, currently under development, are likely to be extremely useful in the future. The two main approaches for adding foreign or in vitro modified genes are:
   (i) Direct introduction of the specific, cloned (or in vitro modified and cloned) sequences by micro-injection or direct DNA uptake by protoplasts,
   (ii) Utilization of "disarmed" Ti or Ri plasmids of Agrobacterium.
   Most of the legume and vegetable species can be transformed using the Agrobacterium system.

The major problem however is the identification of the desired DNA sequences (genes). Rapid developments in this area can be expected in the near future as reported during the FAO/IAEA Symposium on "Nuclear Techniques and In Vitro Culture for Plant Improvement", Vienna, August 19-23, 1985, which preceded the Research Co-ordination Meeting (Proceedings Series, STI/PUB/698, IAEA, Vienna, 1986).

f) Somatic hybridization by protoplast fusion is already possible for a number of vegetables and legumes such as tomato and alfalfa. This approach should be encouraged; it is expected to facilitate the transfer of genes from one plant species into another, even though they cannot be crossed sexually. At present though, this approach is still of limited practical value because techniques for obtaining and fusing protoplasts may be lacking, nuclear-cytoplasmic incompatibilities may occur, and suitable methods for the early selection of the genes to be transferred and for plantlet regeneration may be unavailable.

g) The transfer of cytoplasmic traits encoded by chloroplasts and/or mitochondria which can be achieved by fusion of nucleus-free "donor" protoplasts or "donor" protoplasts that have their nuclei inactivated by irradiation with nucleated "recipient" protoplasts appears promising. This method is expected to become very useful in the transfer of cytoplasmic organelles between breeding lines within species and also to make quite distant "crosses" possible. The nearly complete or total absence of donor chromosomes will circumvent the often encountered problem of nuclear incompatibility. Therefore it is recommended that cell fusion and organelle transfer in legumes and vegetables will be further developed and investigated.

h) For fuller exploitation of tissue culture in future breeding programmes of legumes and vegetables, the established techniques of embryo culture or clonal propagation using meristem culture should be augmented by tissue culture systems with good regeneration ability, especially somatic embryogenesis. Then it would be possible to explore more fully the potentials of somaclonal variation, in vitro mutagenic treatments and genetic engineering. For legumes, the symbiotic interactions between the plants and the Rhizobium bacteria are very important. Tissue culture methodology could clarify many physiological and genetic problems. Hopefully, tissue culture approaches will be useful in the selection of better symbiotic combinations in legumes.

i) For better utilization of in vitro methodology in certain groups of crop species the following problems should be solved in the near future:
Grain legumes:
- Regeneration in callus, cell and protoplast cultures (via shoot morphogenesis and somatic embryogenesis).
- Somatic hybridization and hybrid selection.
- Selection for improved symbiosis and N₂ fixation.

Forage legumes:
- Selection for quality (protein, anti-metabolites).
- Selection for seed production.
- Interspecific somatic hybridization.
- Selection for improved symbiosis and N₂ fixation.

Vegetables:
- Seed propagated: haploid production, somatic hybridization, cell organelle transfer (male sterility).
- Vegetatively propagated: somaclonal variation and somatic hybridization (see below).

V. UTILIZATION OF IN VITRO MUTATION BREEDING:
VEGETATIVELY PROPAGATED PLANTS

In comparison with mutation breeding of sexually reproduced annual species there are special problems in dealing with asexually (vegetatively) propagated species, many of which are also perennial.

a) Often a long time elapses between the mutational event and the expression of the character of interest e.g. flower colour or fruit characteristic, particularly for some woody species with juvenile phases which may last several years. Even after mutations are detected, 2-3 years of vegetative propagation must follow and further field testing is required for confirmation.

b) It is not possible to distinguish between genetic and epigenetic changes in the early stages. Long-term field evaluation is essential.

c) At the time of the mutagenic treatments or culturing, chimeras (including cryptochimeras) due to prior mutations in somatic tissues (genetic mosaicism) may already exist, thus cultivars (clones) chosen for mutagenesis may be chimeric. Knowledge on clone performance in this regard should be obtained from breeders and commercial propagators. Also, in vitro control cultures can reveal many of the chimeras and some derived clones may indeed be useful.

1. WOODY PLANTS, ORNAMENTALS AND ROOT AND TUBER CROPS

1.1. Explant and type of culture

The explants and types of culture suitable for mutation induction are species specific. They should be chosen considering also regenerative competence. Callus or single cell suspension cultures are useful only where regeneration is feasible. More research is needed to establish efficient in vitro culture systems for species in which in vitro shoot regeneration and propagation has not been achieved so far. Where such in vitro systems are available, the following points should be considered with respect to in vitro mutagenesis:

a) In certain explants, such as shoot tips and axillary buds, the probability of chimeral rearrangement or separation may be lower. This would be especially important where the economic value of a cultivar depends on a characteristic chimeral organization.
b) Explants involving tissues formed earlier in ontogenesis, e.g. lower internodes, may lead more readily to the "release" of pre-existing variability (mosaicism) as a source of variation.

c) In forest trees and other woody species, variability can arise when adventitious juvenile shoots arising from the base of the trees are used for in vitro micropropagation and mutagenesis. Studies assessing this potential source of variability should be encouraged. Although these shoots have a juvenile phenotype and perhaps greater regenerability in culture, they may also be a source of accumulated mutations.

1.2. Generation of variation

Generation of variability under in vitro conditions has been documented for various traits including flower colour (e.g. Weigela and carnation), plant morphology (e.g. Forsythia and Gerbera), cold tolerance (e.g. Chrysanthemum) (see also Section II.3, above). It is important to develop suitable methodologies for the selection of physiological characters. Selection for stress tolerance had some success, but it must be emphasized that the mechanisms controlling such characters are not understood as yet. Thus, for instance, salt tolerance can involve several mechanisms e.g. ion exclusion at the root level or osmotic adjustment. In vivo, these characters can be expressed differently in juvenile and mature tissues, and therefore long-term field testing must follow in vitro selection. Examples of elimination with time of variant types obtained in vitro are known (e.g. potato). The causes may be genetic instability, chimeral rearrangement or epigenetic changes. This reaffirms the necessity for long term field evaluation, and for several cycles of vegetative propagation in order to confirm that the variants are stable mutations. Analytical methods such as isozyme analyses and chromosome observations may help in ascertaining the genetic nature of the variation.

The main reasons for utilizing induced mutations in breeding vegetatively propagated plants are to generate genetic variability (which cannot be obtained otherwise) and to shorten the time required to develop new cultivars. The breeding objectives are usually rather specific but in general, resistance to pathogens and to environmental stress factors is of outstanding importance. Through induced mutations, it is often possible to obtain specific changes in already well accepted, existing varieties and to improve them without affecting other important characteristics, e.g. disease resistance in an otherwise suitable but susceptible variety. This is important where varieties have a high consumer preference and also in situations where regulations permit only certain varieties in certain areas.

Somaclonal variation is of potential value to breeders in generating variability, but it is still unproven in terms of recovering novel traits. It is another approach to obtaining mutations, complementing other breeding and mutagenic approaches.

1.3. Selection approaches

Terminology for mutation breeding of vegetatively propagated crops is proposed in Appendix I and used in the breeding schemes which apply more directly to vegetatively propagated crops (Appendices II and III and in certain cases also IV). It should be recognized that axillary and adventitious bud micropropagation can be used to convert mericlinal or sectorial chimeras into stable periclinal chimeras or homohistonts.
There are two types of shoot tip cultures (Appendix II):

(i) A continuing growth of a single shoot following irradiation of the shoot tip; two zones on the regenerated plants can then be distinguished:

Zone A: The basal portion which may carry visible abnormalities pre-existing in the buds or caused by the primary physiological effects of the mutagen.

Zone B: The upper portion with reduced or no visible abnormalities.

Selection of axillary buds for further culturing should not be restricted to Zone A. It should be stressed that Zone A buds require one more cycle of vegetative propagation prior to screening compared to Zone B buds.

(ii) A multiple shoot growth pattern of adventitious and axillary buds. All shoots from the culture should be screened, including shoots derived from subsequent proliferations.

1.4. Research recommendations

In vivo and in vitro mutation breeding technology for vegetatively propagated plants is fairly well established and has yielded economically useful cultivars. Genetic engineering technology is still in the developmental stage, using mostly model species and not agriculturally important plants. Important potential applications for it with vegetatively propagated crops can be envisaged; therefore it is recommended that research in this field will be intensified.

Two research areas should be given immediate priority in a wide range of crops:

(i) Development of reproducible cell and protoplast cultures from which plantlets can be routinely regenerated. Such cultures are the preferred material for in vitro mutagenesis.

(ii) Development of cell and tissue selection systems for resistance to environmental stresses or diseases which correlate with whole plant performance in the field.

2. BANANA AND PLANTAIN

Bananas and plantains are somewhat unique among the major crop plants in that the clones (varieties) cultivated are seedless triploids. The banana variety originally grown in the Caribbean area for export, Gros Michel, was destroyed in the 1930's by the Panama disease which is caused by race 1 of Fusarium oxysporum f. cubense. Fortunately, the varieties of the Cavendish group are resistant to race 1 of Fusarium and could replace Gros Michel. While there are several different Cavendish varieties, all are closely related and provide no genetic variability for protection against new disease epiphytotics. No varieties are known in the existing germ plasm of banana which have the necessary agronomic traits and market qualities to replace the Cavendish varieties, should the need arise.

At present, two relatively new banana diseases pose serious threats to the continued cultivation of the Cavendish varieties. The first, a new race of Fusarium, race 4, capable of attacking the Cavendish types, is currently a serious problem in Taiwan and is spreading in South Africa and Australia. The only control for this disease is genetic resistance. The second, black sigatoka leaf spot, caused by Mycosphaerella fijiensis var. difformis is expected to spread to all banana growing regions in a few years. It has greatly increased the costs of producing bananas in many...
countries. Currently its chemical control on the export bananas in Central and South America costs approximately $ US 100 million annually.

Plantains are resistant to the original yellow sigatoka pathogen, *Mycosphaerella musicola*, but are susceptible to black sigatoka, *M. fijiensis* var. *difformis*. Chemical control of black sigatoka on plantains is not feasible because they are grown in small plots and it is also too expensive. A great part of the current annual production of some 20 million tons of plantains is in danger of destruction by this disease.

While there are other pests and diseases of bananas and plantains, such as the burrowing nematode (*Radopholus similis*), moko disease (*Pseudomonas solanacearum*) and bunchy top virus, the major research efforts should be aimed at the control of race 4 of *Fusarium* and of *M. fijiensis* var. *difformis*. The latter two could completely eliminate production in some areas, if resistant varieties are not developed in the near future.

The commercial varieties of bananas and plantains are vegetatively propagated allotriploids. Considerable conventional breeding efforts have yielded little progress in them. Mutation breeding offers a promising approach to obtain disease resistance in the currently cultivated clones, without disrupting their commercially valuable genetic constitution.

In vitro mutation breeding of bananas and plantains is feasible as shown by the following discussion of the known procedures and suggested investigations:

a) Shoot tips of the commercially grown Cavendish and plantain varieties are now cultured in vitro routinely for propagation in many laboratories around the world. Somaclonal variation is considered an attractive area of study, but as yet no plants have been regenerated from protoplasts or somatic cells and no protocol has been devised to regenerate the necessary quantities of plants from callus. Moreover, different *Musa* genotypes and plant parts should be examined as sources of protoplasts, somatic cell culture and callus with regeneration capacity.

b) A low amount of natural variation has been observed in plantlets derived from shoot tips. Preliminary studies have established the dose range of gamma rays which can be applied to shoot tips for mutation induction. Various in vitro cultural procedures and mutagenic treatments leading to high levels of useful variability should be investigated.

c) The primary breeding objectives are to induce resistance to the most destructive and costly diseases, race 4 of *F. oxysporum f. cubense* and *M. fijiensis* var. *difformis*. Young plants can be screened for resistance to the former, while for the latter older plants in the field will be necessary. Further investigations should be undertaken to make earlier screening for *Mycosphaerella* possible. Evaluations for agronomic qualities would be carried out on the plants which survive the disease screenings. At least initially, screening should be done for one disease at a time, since the two diseases do not co-exist in all the banana producing countries.

d) The recommended breeding procedures and terminology to be used in the in vitro breeding scheme are described in Appendix V.

e) Somaclonal variation may receive more attention in future investigations. At present, it is not useful because ways to regenerate large quantities of plants from single cells are not known.

f) Mutation breeding is considered preferable at present to other means of genetic engineering since little is known about the application of the latter techniques in bananas and plantains.
g) Priority should be given to efficient ways to obtain high proportions of mutant plants from shoot tips cultured \textit{in vitro} and to proper procedures for laboratory screening of large numbers of regenerated plants for resistance to race 4 of \textit{Fusarium} and to \textit{M. fijiensis} var. \textit{difformis}. See Appendices VI and VII.

VI. \textbf{INTERNATIONAL CO-OPERATION AND ROLE OF THE JOINT FAO/IAEA DIVISION AND THE AGRICULTURAL BIOTECHNOLOGY LABORATORY AT SEIBERSDORF}

The group recognizes the activities of the Joint FAO/IAEA Division and the Plant Breeding Unit at the Agricultural Laboratory at Seibersdorf in enhancing mutation induction for plant improvement, utilizing \textit{in vivo} and \textit{in vitro} techniques. Methodological research, training and dissemination of information have contributed substantially to the utilization of induced mutations in plant breeding.

\textit{In vitro} systems might offer additional prospects for the induction of mutations and recovery of desirable mutants. Studies should be intensified and directed towards improving the effectiveness of mutagen applications in different types of \textit{in vitro} cultures and improved \textit{in vitro} selection procedures for mutation breeding of important crop plants where practical application seems feasible.

The Laboratory should specifically concentrate on adapting or developing for crops relevant to Member States methods of \textit{in vitro} and \textit{in vivo} mutation induction, mutant selection and mutant propagation as well as genetic engineering based on the use of radiation. More attention should be given to the application of induced mutation techniques in tissue culture and to anther culture and related techniques, especially for plants that are important in developing countries; also to the utilization of radiations in preparing nucleus-free protoplasts for fusion.

The preparation of protocols for the handling of cultures, for mutation induction and for selection of mutants at the tissue culture level in important crops will be very important for the transfer and dissemination of these technologies to developing countries. In the future the Laboratory may be involved also in recombinant DNA technology in order to share the knowledge with developing countries. Thus, the Agricultural Laboratory at Seibersdorf should play an important role in the development of methodology for mutation induction and selection, the transfer of technology to Member States via training courses and fellowships.

The FAO/IAEA Training Course on the Induction and Use of Induced Mutations in Plant Breeding, organized annually at the Seibersdorf Laboratory, should continue to include also specific training in \textit{in vitro} mutation breeding. Additional courses should be held on a regional basis as until now; they should emphasize the crops of those regions and the genetic improvements required in them.

In addition, the Joint FAO/IAEA Division should consider initiating training programmes for qualified breeders, geneticists, agronomists, pathologists, etc. from Member States who are actively engaged in plant breeding, on \textit{in vitro} technology for mutation breeding of agronomically important crops. For that purpose, specific training courses, dealing with \textit{in vitro} technology for mutation breeding, should be organized by the Joint FAO/IAEA Division in the Seibersdorf Laboratory and/or elsewhere.
Preference should be given to plant breeders from developing countries to enable them to utilize, where applicable, in vitro mutation technology as a part of the regular national crop improvement programmes.

The Joint FAO/IAEA Division should provide advice through the IAEA Technical Co-operation Programme to breeders and assist them in solving crop improvement problems and in attaining important objectives by a combination of nuclear and biotechnological techniques. The IAEA Research Contract Programme should continue to facilitate the development of the methodologies of in vitro mutation breeding through Co-ordinated Research Programmes, and to foster international co-operation.

Co-operation with international and national organizations related to crop improvement should be sought in order to foster the integration of induced mutations and in vitro technology into plant breeding programmes and to facilitate exchange and transfer of in vitro plant materials, e.g. disease free propagules. Laboratory facilities using in vitro techniques for mutation breeding can also be utilized for germplasm conservation. Therefore, more co-operation should be sought with the FAO/AGP, AGP Seed Service, IBPGR and national and other international bodies interested in germplasm exchange and preservation using tissue culture techniques. Close co-operation with other international agricultural research institutes active in crop improvement such as CIAT, CIMMYT, ICARDA, ICRISAT, IITA and IRRI and with other international organizations such as IPGNET would be desirable. Very close contacts should be established between the Joint Division and ICGEB established recently by UNIDO in Trieste and New Delhi.

The work of the Agricultural Laboratory at Seibersdorf on banana should be enhanced in view of the importance of banana to the economic and social stability of many developing countries, its clonal propagation and the grave danger of a wipeout of banana and plantain due to rapidly spreading new races of destructive diseases. Close communication should be established with regional banana improvement organizations such as ACIAR (Australia), IITA (West Africa), ACORBAT and INIBAP (Caribbean and Tropical America).
LIST OF PARTICIPANTS

AUSTRALIA
Dr. Martin Barlass
CSIRO, Division of Horticultural Research
G.P.O.Box 350
Adelaide, South Australia 5001

AUSTRIA
Dr. J. Schmid
Austrian Research Centre
Plant Biotechnology Department
A-2444, Seibersdorf

BELGIUM
Dr. J. Bouharmont
Catholic University of Louvain
Laboratory of Cytogenetics
Place de l'Universite 1
Louvain
Dr. P. Lepoivre
Faculté des Sciences Agronomiques
Laboratoire de Pathologie Végétale
13, Avenue Maréchal Juin
B-5800 Gembloux

Dr. I. Negrutiu
Vrije Universiteit Brussel
Instituut voor Moleculaire Biologie
Paardenstraat 64
B-1640 St. Genesius-Rode
Brussels

BRAZIL
Dr. A. Ando
Centro de Energia Nuclear na Agricultura
Departamento de Genetica, ESALQ
Universidade de Sao Paulo
C.P. 96, 13400 Piracicaba, SP

BULGARIA
Dr. A. Atanassov
Tissue Culture Laboratory
Institute of Genetics
Academy of Sciences
Ul. Nezabravka bl. 50
Sofia 1113

CZECHOSLOVAKIA
Dr. L. Havel
Institute of Experimental Botany
Czechoslovak Academy of Sciences
Sokolovska 6
772 00 Olomouc

FRANCE
Dr. L. Decourtye
Institut National de la Recherche Agronomique
Station Arboriculture Fruitière
Beaucouze - 49000 Angers

GERMANY, FED. REP.
Dr. E. Göbel
Max Planck Institut für Züchtungsforschung
Engelspfad
D-5000, Köln

GERMANY, FED. REP.
Dr. W. Prell
Federal Research Centre for Horticultural Plant Breeding
Bornskampsweeg
D-2070 Ahrensburg

HONDURAS
Dr. P.R. Rowe
Programa Mejornamiento Genetico del Banana en Honduras, FAO
La Lima

HUNGARY
Dr. L. Marton
Institute of Plant Physiology
Biological Research Center
Hungarian Academy of Sciences
P.O. Box 521
Szeged H-6701

IRELAND
Dr. G. Douglas
The Agricultural Institute
Kinsealy Research Centre
Malahide Road, Dublin

JAPAN
Dr. T. Kawai
877 Shimosato Takimamuro
Konosu-shi, Saitama-ken 365

NETHERLANDS
Dr. A.J. Kool
Department of Genetics
Vrije Universiteit
De Boelelaan 1087
1081 HV Amsterdam

PHILIPPINES
Dr. R.R.C. Espino
University of the Philippines at Los Banos (UPLB)
College, Laguna 3720

POLAND
Dr. I. Szarejko
Department of Genetics
Silesian University
Jagiellonska 28
40-032 Katowice

THAILAND
Dr. B. Silayoi
Department of Horticulture
Faculty of Agriculture
Kasetsart University
Bangkok 10903
<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
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<tbody>
<tr>
<td>Dr. B. Sigurbjörnsson</td>
<td>Director, IAEA</td>
</tr>
<tr>
<td>Dr. H. Brunner</td>
<td>Seibersdorf Laboratory</td>
</tr>
<tr>
<td>Dr. S. Daskalov</td>
<td>Seibersdorf Laboratory</td>
</tr>
<tr>
<td>Dr. T. Hermelin</td>
<td>Seibersdorf Laboratory</td>
</tr>
<tr>
<td>Dr. F. Novak</td>
<td>Seibersdorf Laboratory</td>
</tr>
<tr>
<td>Dr. A. Ashri</td>
<td>Plant Breeding and Genetics Section</td>
</tr>
<tr>
<td>Dr. M. Maluszynski</td>
<td>Plant Breeding and Genetics Section</td>
</tr>
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<td>Dr. A. Micke</td>
<td>Plant Breeding and Genetics Section</td>
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Scientific Secretary:
Dr. B. Donini               Plant Breeding and Genetics Section
APPENDICE

APPENDIX I. PROPOSED TERMINOLOGY AND SYMBOLS FOR MUTAGENIC TREATMENTS IN VIVO AND IN VITRO

I. $M_x$ — mutagen applied in vivo, generation $x$.
   $M_x$ — mutagen applied in vitro, generation $x$.
   $V_x$ — vegetatively propagated cycle in vivo, vegetative generation $x$.
   $V_x$ — vegetatively propagated cycle in vitro, vegetative generation $x$.

II. In all cases $x=1$ denotes the treated generation.

III. In special situations, SC for plantlet regeneration from somatic cells and GC for plantlet regeneration from gametophytic cells can be judiciously used.
APPENDIX II. IN VITRO MUTATION BREEDING SCHEME

Shoot tip culture and micropropagation via axillary and adventitious bud proliferation

A. Initial explant

Shoot tip

B. Apical and axillary bud meristems with chimeric structure (mericlinal, sectorial)

Shoot growth

M_1 V^c

Shoot growth from axillary and adventitious buds

M_1 V^c, V_n (n=3-4)

C. Establishment of somatically uniform mutated shoot (periclinal or homohistont situation)

D. Vegetative propagation:

in vitro

M_1 V^c V^n
or

in vivo

M_1 V^c V_1

D. Vegetative propagation: Self-pollination (if possible)

Hybridization

M_1 V^c x P

F_1

F_2

SELECTION C: INDUCED GENETIC VARIABILITY

1. MUTAGENIC APPLICATION
Time of treatment
- e.g. before in vitro culture
- e.g. during in vitro culture

2. MICROPROPAGATION of axillary buds to eliminate chimerism
   a) nodal position of axillary bud
   b) no. of axillary buds to be propagated
   c) no. of cycles of micropropagation to overcome chimera

3. SELECTION OF INDUCED MUTATIONS
   Selection pressure at
   a) stage of organ or plant established

P = Cultivar used in the cross
   c = in vitro culture

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**APPENDIX III. IN VITRO MUTATION BREEDING**

Various initial explants, callus and cell cultures and plant regeneration via organogenesis or somatic embryogenesis

A. Initial explant

| Cell, tissue, organ |

B. Assuming a regeneration

| Shoot organogenesis from single cell, a non-chimeric mutated shoot or somatic embryo is expected |

| Full plant established |

| $M_1$ SC$^1$

C. Vegetative propagation

| Self-pollination (if possible) |

| Hybridization |

| $M_1$ SC$^1$ V$^c_1$
| $M_1$ SC$^1$ V$^c_2$
| $M_1$ SC$^1$ V$^c_n$
| $M_1$ SC$^1$ V$^c_1$ V$^n_1$
| $M_1$ SC$^1$ V$^c_n$ V$^n_1$

| Hybrid $M_1$ SC$^1$ x P |

| $F_1$
| $M_2$ SC$^1$

| $F_2$

---

$P$ = Cultivar used in the cross  
$SC$ = Plantlets regenerated from somatic cells
APPENDIX IV. IN VITRO MUTATION BREEDING

Anther cultures, haploid - doubled haploid plant regeneration*

A. Initial explant
   i) Anther from M₁
   ii) Anther

B. Direct andro-ogenesis

1. a) MUTAGENIC TREATMENT
   Time of treatment
   i) before meiosis from seed to PMC (diplophase)
   ii) after the meiosis (all at haplophase)

1. b) GAMETOCLONAL VARIATION

2. SELECTION OF INDUCED MUTATIONS
   Selection pressure at
   a) haploid plant phase
   b) diploid plant phase

(i)
M₂ GC₁
Segregation at haplophase

(ii)
M₁ GC₁

CHROMOSOME DOUBLING

Homozygous diploid plant

M₂ GC₁ (DH) → M₃ GC₂ (DH)
M₁ GC₁ (DH) → M₂ GC₂ (DH)

Genetic variation among homozygous diploid plants

DH = Dihaploid plants
GC = Plantlets regenerated from gametophytic cells
* = A similar scheme could be applied to ovule culture
APPENDIX V: BANANA IN VITRO MUTATION BREEDING SCHEME

Mutagenesis in shoot tip culture and micropropagation via axillary and adventitious buds proliferation

A. Initial plant(s)  Shoot tip(s)

Micropropagation via axillary and adventitious bud proliferation  
(M&S medium + cytokinin, e.g. 20 uM BAP)

B. Multiple shoot culture  
Meristem tips from in vitro growing buds  
M_1 V^c

MUTAGENIC APPLICATION:  
gamma-rays (20-40 Gy)  
EMS (0.7 - 1.2%, 2 hr)

Axillary and adventitious bud proliferation  
(M&S medium + cytokinin, e.g. 20 um BAP)

\[ M_1 V^c \rightarrow M_1 V_2 \]

C. In vitro subcultures in proliferation medium  
M_1 V_4  
\[ M_1 V_4 \rightarrow M_1 V_n \]

IN VITRO SELECTION:  
Leaf spot diseases - dual culture with pathogen  
- toxin and/or filtrate resistance  
IN VITRO INOCULATION

D. In vitro rooting (M&S medium, hormone free) and transplantation into soil  
\[ M_1 V_4 \rightarrow M_1 V_n \]

IN VIVO SELECTION  
Fusarium

E. Screening in young plants' stage

F. Screening and evaluation in adult plants' stage and selection of plants with desirable character(s).

G. Vegetative propagation in vitro  
\[ M_1 V_4 V_n \]

H. New clones released for testing
<table>
<thead>
<tr>
<th>Stage where selection can be applied</th>
<th>Screening agents</th>
<th>Procedures</th>
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<tbody>
<tr>
<td>Plantlets in <em>in vitro</em> culture</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Young plants *</td>
<td>Pathogen itself **</td>
<td>Artificial inoculation with spore suspension</td>
</tr>
<tr>
<td>Plants in field</td>
<td>Pathogen itself</td>
<td>Plants planted in infected field</td>
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* Studies are necessary to determine the correlation between resistance in young and field grown plants.

** For *F. oxysporum* only
### APPENDIX VII. BANANA AND PLANTAIN: PROPOSED SCREENING SYSTEMS TO SELECT FOR RESISTANCE TO FUSARIUM OXYSPORUM AND MYCOSPHAERELLA FIJIENSIS VAR. DIFFORMIS

<table>
<thead>
<tr>
<th>Stage where selection can be applied</th>
<th>Screening agent *</th>
<th>Proposed procedures</th>
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<tbody>
<tr>
<td>Multiple shoot culture</td>
<td>Toxic metabolites</td>
<td>Crude extracts of liquid culture, double layer technique, purified toxin(s)</td>
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<tr>
<td>Dual host-pathogen in vitro culture **</td>
<td>Pathogen itself (exclusively for M. fijiensis var. difformis)</td>
<td>Inoculation of plantlets by spores' suspension</td>
</tr>
</tbody>
</table>

* It is absolutely necessary to study the relation between the level of field resistance of known cultivars and their reactions in the proposed screening systems.

** The growth of the fungus on the medium must be inhibited, e.g. by fungistatic compounds or by a paraffin coated sand layer.
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