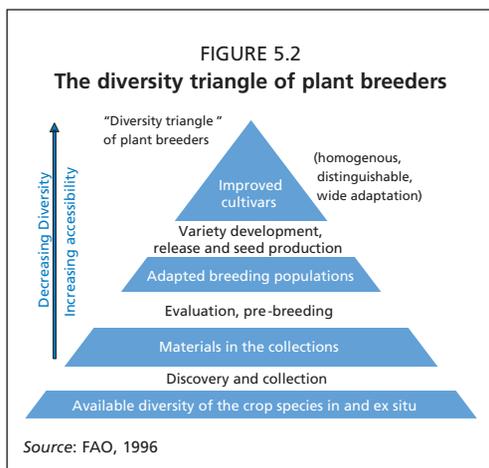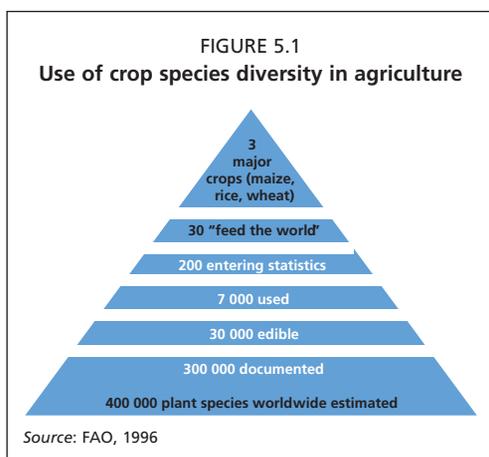# Methodologies for generating variability. Part 1: Use of genetic resources in plant breeding

Bettina I.G. Haussmann and Heiko K. Parzies

5.1 INTRODUCTION

Both inter- and intraspecific diversity is declining in our present agricultural systems. Out of an estimated total of 30 000 (FAO, 1996a) to 50 000 (Sánchez-Monge, 2002) edible plant species, only 30 "feed the world", with the three major crops being maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (FAO, 1996a; Figure 5.1). At the intraspecific level, plant breeding contributes to a diminution of diversity through development of narrow, elite breeding populations, selection of the 'best' genotypes, development of homogeneous cultivars, and promotion of a few, widely adapted varieties (Figure 5.2).

FIGURE 5.1
Use of crop species diversity in agriculture

3 major crops (maize, rice, wheat)
30 "feed the world"
200 entering statistics
7 000 used
30 000 edible
300 000 documented
400 000 plant species worldwide estimated

Source: FAO, 1996

FIGURE 5.2
The diversity triangle of plant breeders

"Diversity triangle" of plant breeders

Decreasing Diversity
Increasing accessibility

Improved cultivars (homogenous, distinguishable, wide adaptation)
Variety development, release and seed production
Adapted breeding populations
Evaluation, pre-breeding
Materials in the collections
Discovery and collection
Available diversity of the crop species in and ex situ

Source: FAO, 1996

However, the decline of inter- and intraspecific genetic variability among and within cultivated crop species bears with it several risks, including epidemics of pests and diseases due to greater genetic vulnerability; lack of adaptation to climate-change-related stresses; lack of genetic variation for specific quality traits; and reaching performance plateaus. A more efficient use of plant genetic diversity is therefore a prerequisite for meeting the challenges of development, food security and poverty alleviation (FAO, 1996b). Concrete aims of using plant genetic resources (PGR) in crop improvement are:

- to develop cultivars that are specifically adapted to abiotic or biotic stresses;
- to assure sustainable production in high-yielding environments through reduced application of agrochemicals and increased nutrient and water efficiency; and
- to open production alternatives for farmers through development of industrial, energy or pharmaceutical crops.

Methods of using PGR in crop improvement have recently been reviewed (Haussmann *et al.*, 2004). Major points will be summarized in this chapter, but for details and more examples, the reader is referred to the full review article. After the generalities concerning use of plant genetic resources (PGR) in plant breeding, this chapter will also consider more specific aspects of using plant genetic resources in participatory plant breeding, such as management of diversified populations and their potential contribution to *in situ* PGR conservation; the use of landraces as genetic resources for adaptation to stress environments, climate variability and climate change; and to better serve farmer's and end-user's diverse needs.
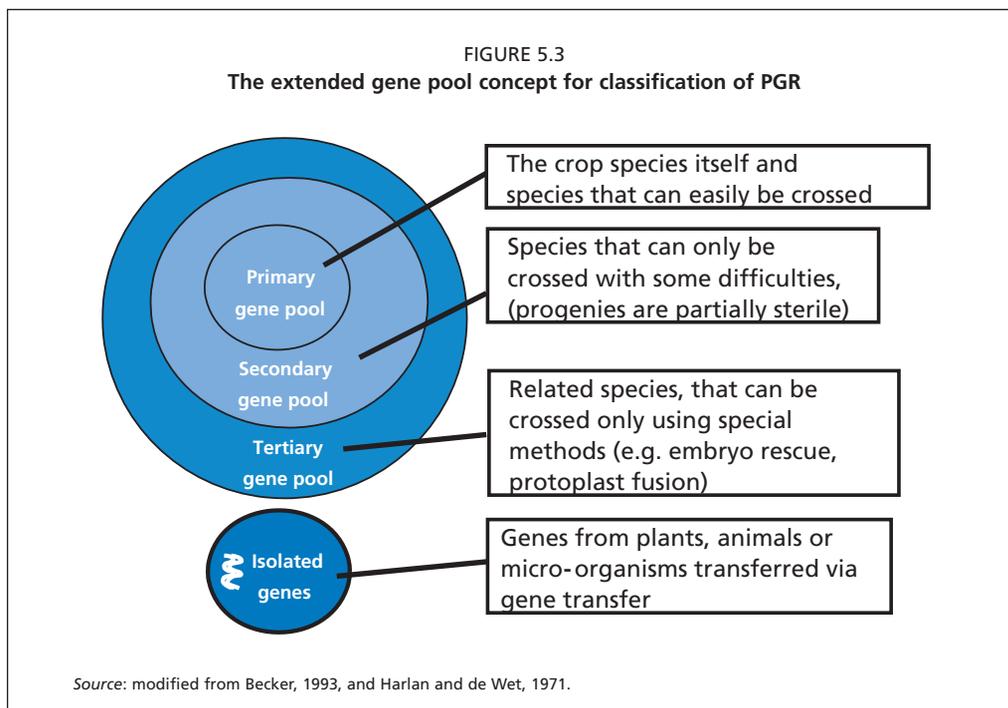
5.2 DEFINITION OF GENETIC RESOURCES FOR PLANT BREEDING

PGR can be defined as all materials that are available for modification of a cultivated plant species (Becker, 1993). PGR have also been considered as those materials that, without selection for adaptation to the target environment, do not have any immediate use (Hallauer and Miranda, 1981). According to the extended gene pool concept, genetic resources can be divided into primary gene pool; secondary gene pool; tertiary gene pool; and isolated genes (Harlan and de Wet, 1971; Becker, 1993; Figure 5.3). The primary gene pool consists of the crop species itself and other species that can be easily crossed with it. The secondary gene pool is composed of related species that are more difficult to cross with the target crop, i.e. where crossing is less successful (low percentage of viable kernels) and where crossing progenies are partially sterile. The tertiary gene pool consists of species that can only be used by employing special techniques, like embryo rescue or protoplast fusion. The fourth class of genetic resources, isolated genes, may derive from related or unrelated plant species, from animals or micro-organisms.

5.3 FACTS AND INFORMATION SOURCES

Worldwide, 1 308 gene banks are registered and conserve over 6.1 million accessions, including major crops, minor or neglected crop species, together with trees and wild plants. Of the 30 main crops, more than 3.6 million accessions are conserved *ex situ* (FAO, 1996a). Little information exists about documentation and availability of materials that are maintained *in situ*. Links to some of the most important organizations or networks dealing with PGR are listed in Box 5.1.

FIGURE 5.3
The extended gene pool concept for classification of PGR

The crop species itself and species that can easily be crossed

Species that can only be crossed with some difficulties, (progenies are partially sterile)

Related species, that can be crossed only using special methods (e.g. embryo rescue, protoplast fusion)

Genes from plants, animals or micro-organisms transferred via gene transfer

Primary gene pool

Secondary gene pool

Tertiary gene pool

Isolated genes

Source: modified from Becker, 1993, and Harlan and de Wet, 1971.
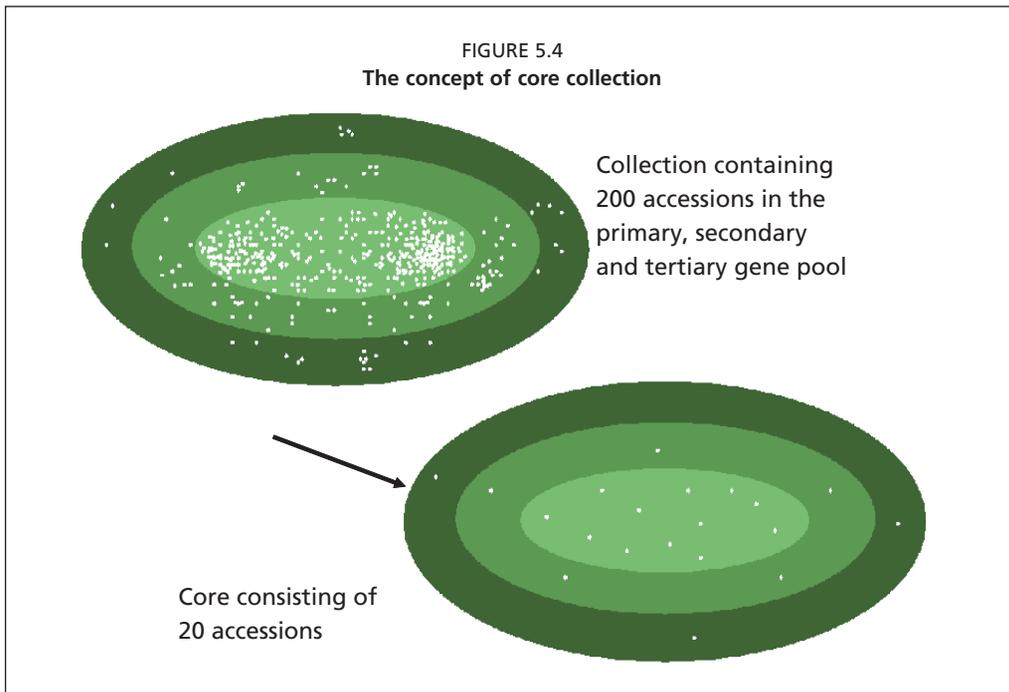
5.4 DOCUMENTATION AND EVALUATION OF PGR

Gene bank accessions are described by passport and characterization data, and to a variable extent also by evaluation data. Passport data include serial number, taxonomic name, collection site, date of collection and donor institute. Additional notes can refer to seed viability, number and mode of regenerations or reproduction, and information about the distribution of the sample. Germplasm passport information exchange is facilitated by the internationally standardized list of multi-crop passport descriptors (FAO/IPGRI, 2001).

Characterization data usually comprise scores for simple morphological traits like plant height, maturity date and thousand-seed weight. Evaluation data refer to agronomic traits like grain yield, grain quality, lodging and resistance to important pests and diseases as far as evaluated. Evaluation is a continuous process. Different people or institutions can be involved, including gene banks, breeders, pathologists or physiologists searching for or studying specific traits. Ideally, all data sets referring to an accession are stored in a central database and are made available to the public.

Systematic evaluation of germplasm conserved *ex situ* is facilitated through development of core collections. Initially, core collections were defined as a limited set of accessions representing, with a minimum of repetition, the genetic diversity of a crop species and its wild relatives (Frankel, 1984). In the context of an individual gene bank, a core collection consists of a limited number of the accessions of an existing collection, chosen to represent the genetic spectrum of the whole collection (Brown, 1995; Figure 5.4). Core collections render the evaluation process more efficient because repetition of similar entries is avoided (Hodgkin *et al.*, 1995; van Hintum *et al.*, 2000).

5.5 ACCESS TO PLANT GENETIC RESOURCES, EQUITABLE SHARING OF PROFITS AND BENEFITS, AND MATERIAL TRANSFER AGREEMENTS

The Convention on Biological Diversity (CBD) aims at the conservation and sustainable use of biological diversity, and an equitable sharing of profits and benefits generated by the use of genetic resources (www.cbd.int). One aim of the convention is to ensure recognition of the past, present and future contributions of farmers to the

FIGURE 5.4
The concept of core collection

Collection containing 200 accessions in the primary, secondary and tertiary gene pool

Core consisting of 20 accessions

conservation and development of genetic diversity (Swaminathan, 2002). To fulfil the convention, so called Material Transfer Agreements (MTAs) have been developed. The Standard MTA (SMTA, www.cgiar. org.cn/pdf/SMTA_English.pdf) protects the genetic resources of plant species listed in the Annex 1 of the International Treaty on Plant Genetic Resources in Food and Agriculture (www.fao.org/ag/cgrfa/itpgr.htm#text) against intellectual property rights and assures continuous and free availability. A special paragraph deals with the equal sharing of benefits (Figure 5.5).

MTAs from other institutions may refer to restricted plant materials, and in this case the user has to agree to use the material for research only; not to distribute or commercialize the plant material or derived materials; and to take all reasonable precautions to prevent unauthorized propagation of any of this material or derived plant materials.

5.6 METHODS OF USING GENETIC RESOURCES IN PLANT BREEDING

After identification and acquisition of potentially useful PGR, there are generally four ways of using those genetic resources in plant breeding (Simmonds, 1993; Cooper, Spillane and Hodgkin, 2001; Figure 5.6):

- introgression, which involves the transfer of one or few genes or gene complexes (chromosome segments) from a genetic resource into breeding materials;
- incorporation (also named genetic enhancement or base broadening) describes the development of new, genetically broad, adapted populations with a new range of quantitative variation and acceptable performance level;
- pre-breeding, which refers to more basic research activities with the goal of facilitating use of 'difficult' materials; and
- gene transfer.

Sometimes, the categories cannot be clearly separated one from another.

FIGURE 5.5
Some key clauses of the Standard Material Transfer Agreement (SMTA)

The Recipient may utilize and conserve the material for research, breeding or training purposes .

The Recipient shall not claim any intellectual property or other rights that limit the facilitated access to the Material provided under this Agreement, or its genetic parts or components.

In the case that the Recipient commercializes a product that is a Plant Genetic Resource for Food and Agriculture and that incorporates Material as referred to in Article 3 of this Agreement, and where such Product is not available without restriction to others for further research and breeding, the Recipient shall pay a fixed percentage of the sales of the commercialized product into the mechanism established by the Governing Body for this purpose, in accordance with Annex 2 to this Agreement. …
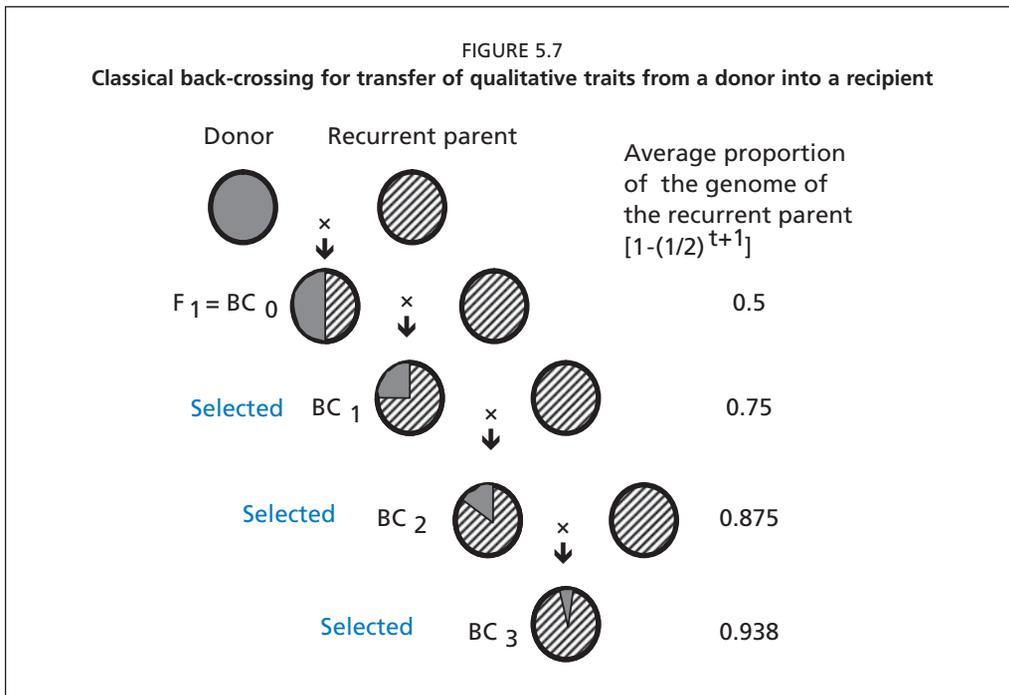
FIGURE 5.6
Overview over methods for using PGRs in plant breeding

Identification   →   Phenotypic and molecular-
(out of 6.1 million   genetic characterization,
 accessions)   data management

Transfer of superior characteristics

Introgression   →   Backcrossing of qualitative traits
                                         (possibly marker-assisted)

Incorporation   →   Population improvement for
('Basebroadening')   quantitative traits
                                         (possibly marker-assisted)

'Pre -breeding'   →   Wide crosses

Gene transfer   →   Transformation

5.6.1 Introgression

Introgression aims at improving highly heritable qualitative traits that are governed by one or a few major genes or gene complexes. Traditionally, the classical backcrossing method is used to introgress traits like resistances or restorer genes from wild relatives (= the donor) into breeding materials (= the recurrent parent) (Figure 5.7). The method is particularly effective if the trait to be transferred is dominant. In the case of recessive inheritance, all backcross
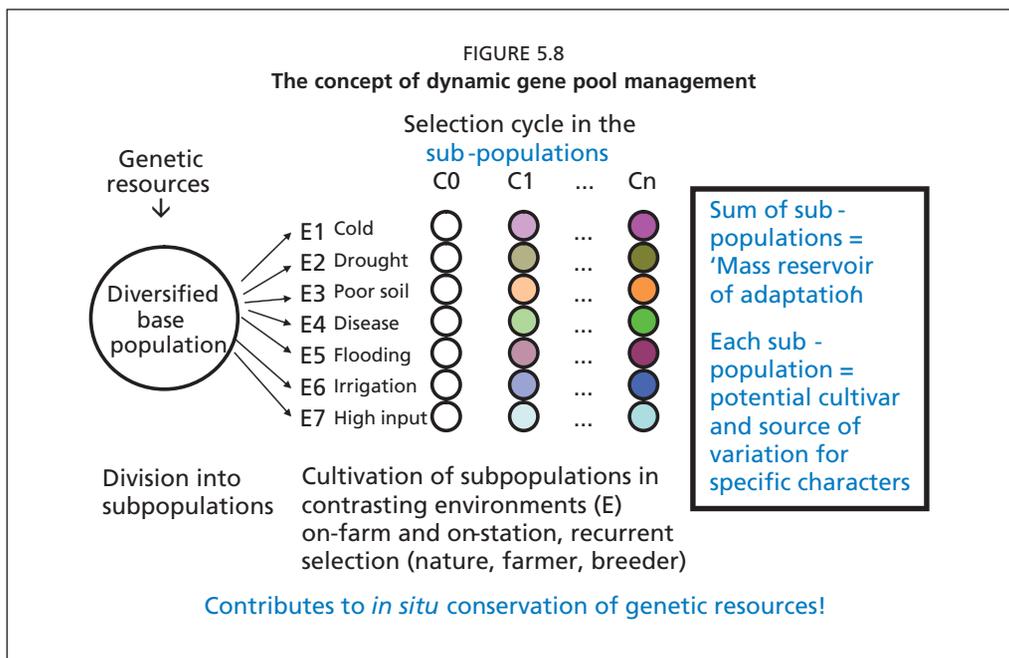
FIGURE 5.7

Classical back-crossing for transfer of qualitative traits from a donor into a recipient

Donor   Recurrent parent

Average proportion
of the genome of
the recurrent parent
$[1-(1/2)^{t+1}]$

$F_1 = BC_0$

0.5

Selected BC_1

0.75

Selected BC_2

0.875

Selected BC_3

0.938

progenies need to be selfed in order to iden-
tify the carriers of the target allele, before
the next backcross of the selected plants can
take place.

5.6.2 Incorporation

Incorporation, genetic enhancement or
base broadening aim to increase the genetic
variation for quantitative traits (i.e. traits that
are due to many gene loci with small effects)
in adapted genetic backgrounds. Various
methods of population improvement can be
used. The methods will vary depending on
the crop species (self- or cross-pollinating)
and the available time frame. Initially,
selection may concentrate on adaptation
traits that are highly heritable; performance
traits are selected at a later stage. Diversity
and recombination are maximized in
the initial phase, with minimal selection
intensities. According to the available
time frame, two main categories can be
distinguished:

- long-term development of synthetic
  or composite-cross populations and
  dynamic gene pool management; and
- short-term genetic enhancement to
  increase the actual variation in breeding
  populations.

To develop synthetic or composite-cross
populations, a large number of accessions
of different geographical origin and with
maximal genetic diversity are crossed.
The resulting population is divided into
subpopulations (effective population size
N>1000) and the subpopulations are grown
for up to 30 generations in a number
of different environments. This process
is called dynamic gene pool management.
At each site, recombination is promoted,
and both natural selection and mild mass
selection may contribute to adaptation of
the individual subpopulations to the site-
specific stresses or growing conditions. The
sum of all subpopulations has been termed
"mass reservoirs of genetic adaptability"

FIGURE 5.8
The concept of dynamic gene pool management

(Simmonds, 1993; Cooper, Spillane and Hodgkin, 2001) and is also understood as a means of *in situ* maintenance of PGR (Figure 5.8). Examples are the barley (*Hordeum vulgare*) composite cross developed at Davis, California, United States of America (Cooper, Spillane and Hodgkin, 2001), dynamic gene pool management in wheat (Goldringer *et al.,* 2001); pearl millet (*Pennisetum glaucum*) composite populations developed in Africa (Niangado, 2001); and the development of locally adapted 'farm cultivars' for ecological agriculture in Europe (Müller, 1989).

In the short term, genetic enhancement of breeding materials, genetic resources are selected for desirable agronomic traits and yield performance, but not for the highest degree of genetic diversity. They are intercrossed, recombined and then selected for adaptation to the target environment. To speed up the process, selected PGR may also be crossed with the breeding materials, and selection for yield traits carried out in the F_2 (50% exotic genome) or BC_1 (25% exotic) generation. The optimal percentage of the exotic genome of the genetic resource (100%, 50% or 25%) in a breeder's population depends on the overall objective; time available and finances; the level of adaptation of the genetic resource; and the yield difference between the genetic resource and the actual breeding population. Direct adaptation of the PGR takes usually longer than selection in F_2 or BC_1 (due to lack of adaptation of the PGR) but will result in materials that are genetically quite different from the actual breeding materials, which can be an advantage. Selection in BC_1 may be preferred over selection in an F_2 population if the PGR is highly unadapted to the target environment. At the same time, selection in the F_2 population is expected to reveal a higher genetic variance, a component of the expected gain from selection (Bridges and Gardner, 1987).

5.6.3 Pre-breeding and wide crosses

Pre-breeding includes basic research to achieve wide crosses, and activities that facilitate the use of exotic materials or wild relatives. It can refer to both qualitative and quantitative traits and the distinction between pre-breeding, introgression and incorporation is not always clear. The main objective is to provide breeders with more 'attractive' genetic resources that are easier to use, such as resistance sources in an acceptable genetic background; or inbreeding-tolerant forms of out-crossing species for hybrid breeding. An example of a very innovative use of wide crosses is the New Rice for Africa (NERICA) developed by the Africa Rice Center (WARDA, www.warda.org). Through crossing the African upland rice, *Oryza glaberrima,* with wetland Asian rice, *O. sativa*, and using embryo rescue and farmer-participatory variety selection, new rice cultivars were developed that combine positive characters (high grain yield and resistances to pests and diseases) of both rice species (www.warda.org/warda1/main/Achievements/nerica.htm).

5.6.4 Gene transfer

Gene transfer is independent of crossing barriers and may therefore increase the usable genetic variation of and beyond the tertiary gene pool. The principal steps for gene transfer from any species into cultivated crops are: gene isolation; gene cloning; gene transfer; and final expression studies in greenhouse and field trials across several generations of progeny. The details of gene transfer go beyond the scope of this chapter. Within the next 10 to 15 years, transformation research hopes to reach the following goals: controlled integration and stable expression of transferred genes; targeted manipulation of multigenic characters; efficient production of transgenes; transgenes, without or with harmless selection markers; and efficient transformation of cell organelles to ensure maternal inheritance, and thereby avoid unwanted horizontal gene transfer (Daniell, Khan and Allison, 2002). Classical examples of the use of gene transfer are the improvement of insect resistance through transfer of *bt* genes from *Bacillus thuringiensis* into crops like tobacco, tomato, maize, rice, cotton and soybean; the improvement of virus resistance through transfer of viral coat proteins in tomato and potato; and the creation of herbicide-resistant crops through transfer of bacterial or fungal genes into sugar beet, tomato and rape. There are also increasing efforts to improve stress tolerance of crops through transfer of genes for improved osmoregulation, heat shock proteins, phytohormone synthesis, and other traits from different organisms into cultivated plants. More information and numerous references on genetic engineering of stress tolerance can be found on the Web site www.plantstress.com.

5.7 UTILITY OF MOLECULAR MARKERS AND GENOME RESEARCH FOR USING GENETIC RESOURCES IN PLANT BREEDING

The utility of molecular markers and genome research in the context of using PGR for crop improvement include:

- diversity studies to distinguish genetically similar or distinct accessions, and to determine individual degrees of heterozygosity and heterogeneity within PGR populations;
- genetic mapping to identify markers in close proximity to genetic factors affecting quantitative trait loci (QTLs), followed by marker-assisted selection (MAS) of desired genotypes in segregating populations;

- exploitation of valuable QTLs from PGR by advanced backcross QTL analysis to combine QTL analysis with the development of superior genotypes or by marker-assisted, controlled introgression of PGR into breeding materials through the development of introgression libraries; and

- association studies to mine directly the allelic diversity of PGR collections and to identify those alleles that are beneficial for important agronomic traits.

5.7.1 Diversity assessment

For an efficient diversity assessment, molecular markers ideally need to be selectively neutral, highly polymorphic, co-dominant, well dispersed throughout the genome, and cost- and labour-efficient (Bretting and Widerlechner, 1995). Genetic markers complying with these requirements are protein markers (i.e. iso-enzymes) and DNA markers such as Restriction Fragment Length Polymorphisms (RFLPs) and Microsatellites or Simple Sequence Repeats (SSRs). Because the development of the latter two marker types requires prior knowledge of DNA sequences, a number of universal, dominant molecular marker types such as Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphisms (AFLPs) have also been employed in PGR diversity studies. However, the latter are not suitable for assessing factors such as mating behaviour or heterozygosity of the germplasm.

Generally, genetic diversity can be measured on three levels: in individual plants, within populations (intrapopulation) and between populations (interpopulation), while populations are considered as groups of randomly interbreeding individuals of one species. The diversity of individual plants is most commonly characterized in terms of the heterozygosity, i.e. the average number of heterozygous gene loci.

At the population level, protein markers and DNA markers are commonly used to calculate, among others, (i) allelic diversity or allelic richness (A; the mean number of alleles per locus); (ii) percentage of polymorphic loci (P; the mean proportion of polymorphic loci); (iii) Nei's average gene diversity (H_e; which denotes the probability that two randomly chosen alleles at a certain locus from a population are different. It is the generalized form of expected heterozygosity assuming Hardy-Weinberg Equilibrium and thus often abbreviated as H_e); and (iv) Shannon's index of diversity (H), which is widely used in ecology but also applied to population genetics (Lowe, Harris and Ashton, 2004).

With the employment of DNA point mutations, such as single nucleotide polymorphisms (SNPs) and small DNA Insertion/Deletions (InDels) as markers for diversity studies, a number of indices have been put forward for variants of a certain DNA sequence in a population. These are (i) the number of polymorphic (segregating) sites (S); (ii) total number of mutations (Eta); (iii) number of haplotypes (h); (iv) haplotype (gene) diversity (H_d); (v) nucleotide diversity (Pi; the average number of nucleotide differences per site between two sequences; Nei, 1987); (vi) nucleotide diversity (Pi (JC); the average number of nucleotide substitutions per site between two sequences (Lynch and Crease, 1990, cited by Rozas *et al.*, 2003); (vii) Watterson estimator Theta (Watterson, 1975); on a base-pair basis it can be interpreted as 4Nμ for an autosomal gene of a diploid organism, where N and μ are the effective population size and the mutation rate per nucleotide site per generation, respectively); and (viii) average

number of nucleotide differences (k). It seems noteworthy that indices of nucleotide diversity allow implications that go beyond quantifying the diversity of a population. For instance, the Watterson estimator Theta allows one to infer the effect of selection on a certain locus. However, detailed description of these indices is beyond the scope of this chapter. For further reading refer to Rozas *et al.* (2003).

Diversity between populations is commonly illustrated through graphical presentation of results of multivariate methods (cluster analyses) in the form of dendrograms (e.g. based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) or Neighbour-Joining algorithms) and two- or three-dimensional plots (e.g. Principal Coordinate Analyses). The bases for all cluster analyses are pairwise dissimilarity coefficients (distance/similarity measures) between all respective populations of a study. Some important dissimilarity coefficients for co-dominant marker data are (i) Euclidean Distance; (ii) Modified Rogers' Distance; (iii) Nei's genetic distance; and (iv) Reynolds' dissimilarity (which is based on the co-ancestry coefficient).

Some important similarity coefficients for dominant marker data are (i) Simple matching; (ii) Jaccard (1908, cited by Reif, Melchinger and Frisch, 2005); and (iii) Dice (1945, cited by Reif, Melchinger and Frisch, 2005). A comprehensive account of the dissimilarity indices mentioned here is given by Reif, Melchinger and Frisch (2005) and also by Mohammadi and Prasanna (2003). Considering the partitioning of diversity within and between populations, Wright's Fixation index (F_{ST}), which is calculated from allele frequencies, plays an important role in diversity studies (Lowe, Harris and Ashton, 2004). Besides measuring the par-

titioning of diversity between and within populations, it can be interpreted as a measure of differentiation between subpopulations, and also as the reduction of heterozygosity of subpopulations due to random genetic drift. In this respect, F_{ST} offers the possibility to calculate gene flow (N_m) between populations according to the formula $N_m = (1 - F_{ST}) / 4F_{ST}$, which can be interpreted as the number of migrants between populations per generation. As the latter indices only apply to co-dominant marker types, Excoffier, Smouse and Quattro (1992) developed a variance-based technique—analysis of molecular variance (AMOVA)—to calculate analogous indices to F_{ST}, which they called Phi$_{ST}$. AMOVA can also be used to characterize the diversity of populations in terms of variances regardless of the marker type.

It seems noteworthy that comparing data achieved with different molecular marker types, or even measured at different marker loci of the same type, is ambiguous, as diversity measures are relative rather than absolute (Ennos, 1996). For this reason, some authors give diversity indices for a certain marker locus as polymorphism information content (PIC), which provides an estimate of the discriminatory power of a locus (Botstein *et al.,* 1980). The use of PIC values allows the direct comparison of population diversity from different studies, provided that the same marker loci have been used.

A different objective of molecular diversity studies is heterotic grouping of genotypes suitable for hybrid breeding approaches. The principle behind this approach is the search for a correlation between genetic distance and heterosis, i.e. the more distant two genotypes of a crop species are genetically, the more heterozygosity, and therefore heterosis, can

be expected in the hybrid resulting from a cross between them (Melchinger, Coors and Pandey, 1999; Reif *et al*., 2003a, b). Yet, the effect on heterosis and hybrid performance needs to be distinguished, since high heterosis does not necessarily mean high hybrid yield. Recent studies have shown that the correlation between diversity measures and hybrid performance gets stronger when the markers used for diversity assessment are linked to performance QTLs, rather than from using neutral markers (Vuylsteke, 1999; Vuylsteke, Kuiper and Stam, 2000; Jordan *et al.*, 2003).

5.7.2 Genetic mapping and marker-assisted selection

Marker-assisted selection (MAS) can help (i) to select individuals carrying molecular markers that are linked to the trait of interest, instead of performing extensive phenotypic tests (foreground selection); and (ii) to reduce undesired parts of the donor genome, including the linkage drag (background selection). Foreground selection requires a tight linkage between the trait of interest and its flanking markers for which one is selecting. Background selection necessitates genotyping with a larger number of markers, which cover the whole genome.

MAS has proven efficient for the transfer of simply inherited qualitative traits from genetic resources into elite materials using backcrossing procedures. It is particularly useful for traits that are recessive, that can be assessed only after flowering or that are very difficult and expensive to assess. By using a combination of foreground and background selection, the transfer of a monogenic trait from a genetic resource into a breeding line may be completed within three to four generations, instead of the usual six generations of classical

backcrossing with the same proportion of the recurrent parent genome (Ragot *et al.*, 1995; Frisch, Bohn and Melchinger, 1999).

MAS for multigenic, quantitative traits at first requires the identification of the genomic regions (QTLs) that affect the trait of interest. In classical QTL mapping, a segregating population (e.g. F_2, F_3 or recombinant inbred population) is developed from two inbred lines. This mapping population is evaluated for the trait(s) of interest. Simultaneously, the population is genotyped with a number of markers and a genetic map is constructed from the marker data. In the final QTL analysis, data is analysed for co-segregation of particular markers with the trait of interest. QTL analysis is then followed by transfer of favourable QTL alleles into elite materials via pure MAS or MAS combined with phenotypic selection.

However, for complex, quantitative traits, the efficiency of QTL mapping and MAS is contested. There are a number of risks that can render MAS inefficient. For example, there may be no selection gain because of: unreliable QTL estimates (too few QTLs, with highly over-estimated effects); QTLs not being expressed in new genetic backgrounds; recombination between marker and QTL; unfavourable alleles of other genes linked to good QTL alleles; or too-high costs for marker analyses. It is therefore essential to use large mapping populations; genotype the mapping population with good genome coverage; assess phenotypic values in multi-environment field trials; cross-validate the gained data; verify QTL effects, using independent population samples, near-isogenic lines or different genetic backgrounds; ensure close linkage between marker and QTL, and verify the linkage by a phenotypic test in all 3 or 4 generations;

increase the marker density around the QTL to allow reduction of the linkage drag; and to optimize individual procedures while taking into account economic parameters. For quantitative traits, where many loci of minor effects are responsible, it is very difficult to obtain reliable, unbiased QTL estimates (e.g. Beavis, 1998; Melchinger, Utz and Schoen, 1998; Utz, Melchinger and Schön, 2000). Prospects for MAS are therefore more promising for traits that are determined by few QTLs with large effects (Melchinger, 1990).

5.7.3 Advanced backcross QTL analysis and introgression libraries

QTL analysis can also be performed in backcross generations derived from crosses of exotic PGR with elite materials. The Advanced Backcross QTL Analysis (AB-QTL; Tanksley and Nelson, 1996) combines QTL analysis with the development of superior genotypes and has been shown to be particularly useful for a trait transfer from poorly adapted germplasm. AB-QTL is therefore of special importance in the use of PGR for crop improvement. The starting point is a segregating generation of a cross between an exotic parent and an elite line that is analysed with as many molecular markers as possible. QTL mapping procedure is delayed until one of the advanced backcross generations ($\geq BC_2$) when lines or testcrosses are evaluated across environments.
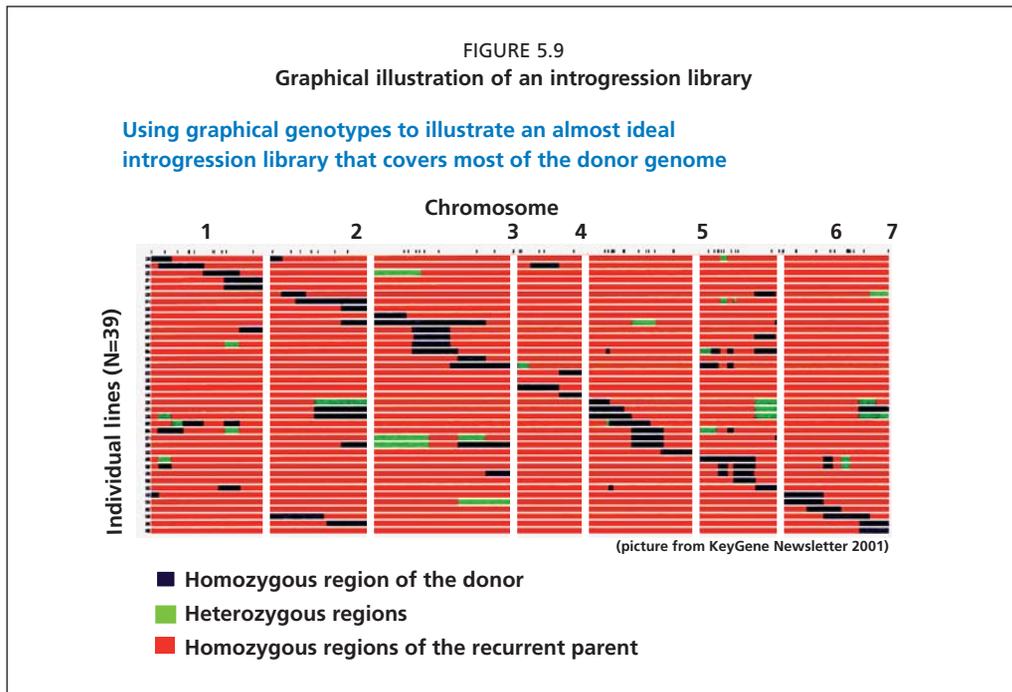
To date, the AB-QTL strategy has been applied in several crops, including tomato, rice and barley (Tanksley *et al.,* 1996; Fulton *et al.*, 1997, 2000; Bernacchi *et al.,* 1998; Xiao *et al.,* 1996, 1998; Moncada *et al.*, 2001; Pillen, Zacharias and Léon, 2003; von Korff *et al.*, 2008). Once favourable QTL alleles from an exotic donor are identified, one or two additional backcrossing

and selfing generations are needed to derive QTL-bearing near-isogenic lines (QTL-NILs). These carry recurrent parent alleles throughout their genome except for the specific target QTL (Tanksley and Nelson, 1996). QTL-NILs can be used to verify observed QTL effects as well as commercial lines improved for one or more quantitative traits compared with the original recurrent elite line.

In contrast to the AB-QTL method, Eshed and Zamir (1994, 1995) suggested the approach of establishing a population of NILs such that the donor chromosome segments are evenly distributed over the whole recipient genome. Ideally, the total genome of the exotic donor is comprised in the established set of NILs (Figure 5.9). This NIL population, termed an introgression library, consists of a set of lines, each carrying a single marker-defined donor chromosome segment introgressed from an agriculturally unadapted source into the background of an elite variety (Zamir, 2001).

The procedure of establishing an introgression library implies systematic transfer of donor chromosome segments from a PGR (donor) into an elite line (recurrent parent) by marker-aided backcrossing. Additional self-pollination and marker-based selection lead to NILs homozygous at donor chromosome segments. Such NILs differ from the elite line by only a small, defined chromosomal segment, and phenotypic differences between a line in the library and the nearly isogenic elite line are associated with the single donor chromosome segment (Šimić *et al.*, 2003).

Both introgression library and AB-QTL approaches provide a valuable opportunity to extract quantitative trait alleles for modern crop varieties from exotic PGR. Their main advantage is that the exotic genome is

FIGURE 5.9
Graphical illustration of an introgression library

**Using graphical genotypes to illustrate an almost ideal
introgression library that covers most of the donor genome**

Chromosome

1   2   3   4   5   6   7

Individual lines (N=39)

(picture from KeyGene Newsletter 2001)

■ **Homozygous region of the donor**
■ **Heterozygous regions**
■ **Homozygous regions of the recurrent parent**

introgressed into the elite line only as small, well defined donor chromosome segments. This reduces unfavourable effects that often impede the use of PGR in practical breeding programmes.
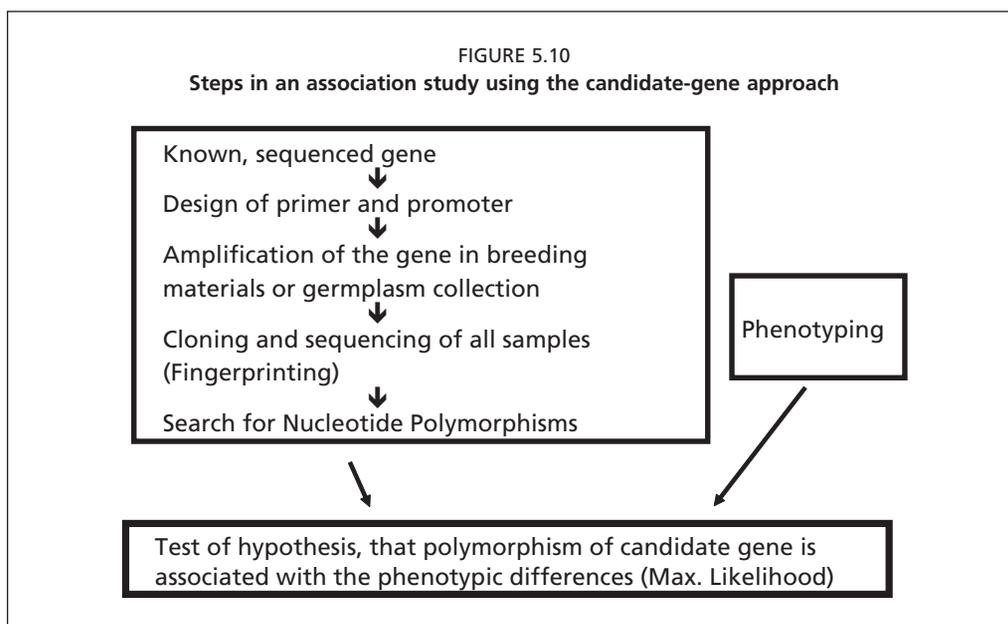
5.7.4 Association studies and direct allele selection

Increased insight into the molecular organization and sequence of plant genomes has led to new methods to mine directly the allelic diversity of PGR. The aim of such studies is to associate sequence polymorphisms within genes or across genomes with phenotypic variants to detect superior alleles affecting agronomically important traits. Such valuable alleles detected within germplasm collections can subsequently be transferred to elite breeding materials via marker-assisted backcrossing using allele-specific markers (direct allele selection; Sorrells and Wilson, 1997) or marker-assisted recurrent selection (D. Hoisington,

pers. comm.). The major advantages of association studies over classical QTL mapping experiments is that no segregating population has to be established from two inbred lines, and that the results are not limited to the specific mapping population but can cover the full allelic variation available in natural or breeding populations or gene bank accessions (Jannink, Bink and Jansen, 2001; Jannink and Walsh, 2002).

Associations between DNA sequence polymorphisms and phenotypic trait variation can occur either when the polymorphisms are directly responsible for the functional differences between the alleles of the respective genes, or when the analysed polymorphisms are in linkage disequilibrium (LD) with the functional alleles. LD is defined as a non-random association of alleles at different loci within a population (Falconer and Mackay, 1996).

The basic idea of association mapping can be investigated using two strategies.

FIGURE 5.10
Steps in an association study using the candidate-gene approach

Known, sequenced gene
⬇
Design of primer and promoter
⬇
Amplification of the gene in breeding materials or germplasm collection
⬇
Cloning and sequencing of all samples (Fingerprinting)
⬇
Search for Nucleotide Polymorphisms

Phenotyping

Test of hypothesis, that polymorphism of candidate gene is associated with the phenotypic differences (Max. Likelihood)

One approach is first to identify candidate genes (i.e. from available databases or gene expression studies) and to re-sequence those candidate genes in plants derived from diverse germplasm accessions (Figure 5.10). The maize gene *dwarf8*, a candidate gene for flowering time and plant height, was used by Thornsberry *et al.* (2001) in a first association study with a crop species. They sequenced *dwarf8* in a representative set of 92 inbred lines and found polymorphisms within the gene to be strongly associated with flowering time. This group of researchers also developed a software suite, TASSEL, (http://www.maizegenetics.net/bioinformatics/index.htm) for analysing LD and for performing association mapping in populations of inbred lines.

A second approach is to analyse a set of randomly chosen molecular markers, evenly distributed across the genome. If such markers are in LD with the genes controlling the trait variation, one will also detect a significant association. The practicability of this approach strongly depends on the level and structure of LD. Low levels of LD would be favourable for high resolution fine mapping within candidate genes, but limit the feasibility of genome-wide association studies. A first attempt to use the genome-wide approach in plants was reported for *Beta vulgaris* subsp. *maritima* using 440 AFLP markers in 106 individual plants from four natural populations (Hansen *et al.*, 2001). Two markers were detected showing significant association with the bolting gene, which is responsible for the vernalization requirement.

Population structure in germplasm collections, which may be unknown to the researcher, can cause spurious associations. Statistical methods were developed by Pritchard, Stephens and Donnelly (2000) and Falush, Stephens and Pritchard (2003) to detect such population structures using a few molecular markers evenly spread across the genome. Removing the effects of population structure increases the power of the association study to detect useful markers.

5.8 THE USE OF GENETIC RESOURCES IN PARTICIPATORY PLANT BREEDING

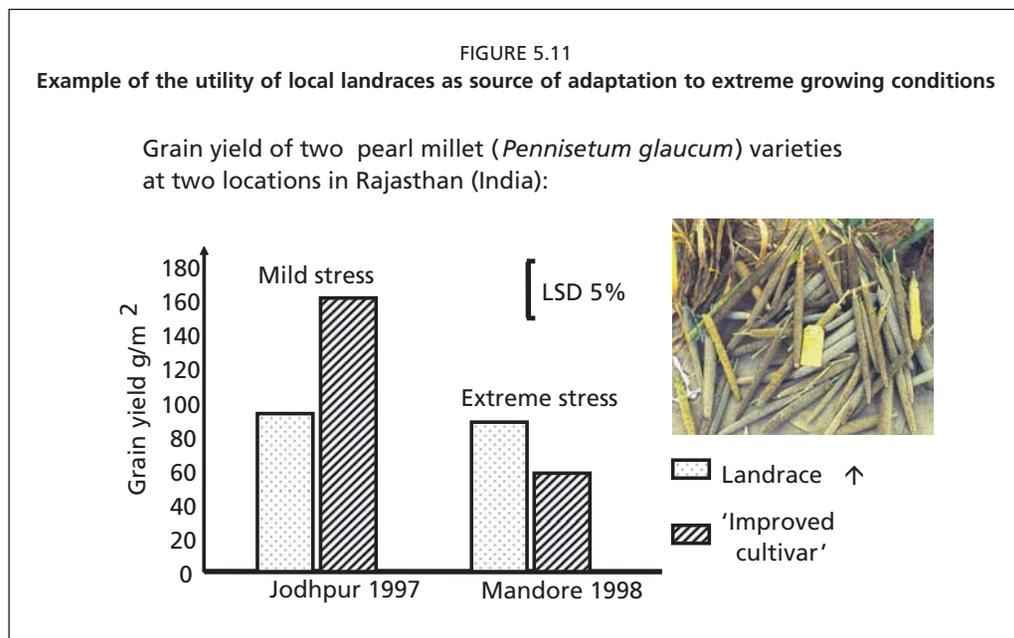Genetic resources can be used in a number of ways in participatory plant breeding programmes.

Participatory improvement of diversified populations and potential contribution to in situ *conservation of PGR*

Farmer-participatory improvement of diversified populations combines *in situ* conservation with genetic improvement of PGR to meet farmer's diverse needs as well as the challenges of adaptation to site-specific conditions, climatic variability and climate change. In a first step, farmers may evaluate a range of diverse varieties or germplasm accessions of the target crop and chose accessions that carry traits of interest to them. The diversified base population will then be built through crossing and recombining the farmer-selected materials. Representative seed lots of targeted base populations will be distributed to farmers in contrasting sites with specific selection

pressures of a target region (see Figure 5.8 above). Natural and recurrent selection by farmers and breeders will act on the distributed material and lead to the development of new subpopulations that can be excellent sources of variation for specific adaptation and farmer-preferred traits, as well as new trait combinations (via recombination) not previously available. Such a dynamic gene pool approach provides the best opportunity to "offer a wide diversity of material to the wide diversity of farmers" for effective participatory plant breeding (Weltzien *et al.*, 2000).

Use of landraces as genetic resources for specific adaptation to stress environments, climate variability and climate change, and to better serve farmer's and end-user's diverse needs

Breeding for wide adaptation has been found to be inappropriate for extreme stress environments, because of cross-over genotype × environment interactions appearing at low yield levels (e.g. Simmonds, 1991;

FIGURE 5.11

Example of the utility of local landraces as source of adaptation to extreme growing conditions

Grain yield of two pearl millet (*Pennisetum glaucum*) varieties at two locations in Rajasthan (India):

Ceccarelli *et al.,* 2001; vom Brocke *et al.,* 2002a, b). Cross-over genotype × environment interactions represent the situation where newly bred 'widely adapted' cultivars are inferior to local, indigenous varieties under extreme environmental conditions. An example is given in Figure 5.11. Such interactions may be considered as a hindrance to crop improvement in a target region, but they also offer new opportunities, e.g. selecting and using genotypes that show positive interaction with the location and its prevailing environmental conditions (exploitation of specific adaptation), or genotypes characterized by low frequency of crop failure (Annicchiarico, 2002).

Landraces grown in extreme areas, such as semi-arid to arid regions in Asia and Africa, can represent important PGR in breeding for specific adaptation (Hawtin, Iwanaga and Hodgkin, 1997). They can be donors for individual monogenic traits; sources of new quantitative variation for specific adaptation to stress conditions; and breeding population or crossing partner in the development of improved, locally adapted cultivars for the same or other marginal areas. Strategies for the development of locally adapted germplasm include (Ceccarelli *et al.*, 2001; Witcombe, 2001; Ceccarelli and Grando, 2007):

- decentralization of the breeding process from the international to the national level, and from stations to farmers' fields;
- crossing of elite materials with locally adapted, farmer-preferred cultivars;
- development of different breeding populations for different regions;
- distribution of segregating materials to national programmes; and
- farmer-participatory selection, to increase final acceptance of the improved cultivars.

5.9 OUTLOOK

Numerous methods are available for the use of PGR in crop improvement. The choice mainly depends on the crop, the trait(s) of interest, availability of molecular markers, the chosen time frame, and the finances available. A combination of advanced, molecular techniques with classical and farmer-participatory breeding methods will most probably achieve the desired impact. In order to enhance the utilization of PGR in crop improvement, the Global Plan of Action (FAO, 1996b) proposed a number of measures, among them expanded creation, characterization and evaluation of core collections; increased genetic enhancement and base-broadening efforts; development and commercialization of underutilized species; development of new markets for local varieties and 'diversity-rich' products and concomitant efficient seed production and distribution; comprehensive information systems for PGR; and promoting public awareness of the value of PGR for food and agriculture.

REFERENCES

Annicchiarico, P. 2002. Defining adaptation strategies and yield stability targets in breeding programmes. *In* M.S. Kang, ed. *Quantitative Genetics, Genomics and Plant Breeding.* pp. 365–383. Wallingford, UK, CAB International.

Beavis, W.D. 1998. QTL analyses: power, precision, and accuracy. *In* A.H. Paterson, ed. *Molecular Dissection of Complex Trait*s, pp. 145–162. New York, USA, CRC Press.

Becker, H. 1993. *Pflanzenzüchtung*. Stuttgart, Germany, Verlag Eugen Ulmer.

Bernacchi, D., Beck-Bunn, T., Eshed, Y., Lopez, J., Petiard, V., Uhlig, J., Zamir, D. & Tanksley, S. 1998. Advanced backcross QTL analysis in tomato. I. Identification of QTLs for traits of agronomic importance

from *Lycopersicon hirsutum*. *Theoretical and Applied Genetics,* 97: 381–397.

Botstein, D., White, R.L., Skolnick, M. & Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics,* 32: 314–331.

Bretting, P.K. & Widerlechner, M.P. 1995. Genetic markers and horticultural germplasm management. *Hortscience,* 30: 1349–1356.

Bridges, W.C. & Gardner, C.O. 1987. Foundation populations for adapted by exotic crosses. *Crop Science,* 27: 501–506.

Brown, A.H.D. 1995. The core collection at the crossroads. *In* T. Hodgkin, A.H.D. Brown, T.J.L. van Hintum & E.A.V. Morales, eds. *Core Collections of Plant Genetic Resources*, pp. 3–19. Rome, IPGRI; and UK, John Wiley/Sayce Publishing.

Ceccarelli, S., Grando, S., Amri, A., Asaad, F.A., Benbelkacem, A., Harrabi, M., Maatougui, M., Mekni, M.S., Mimoun, H., El-Einen, R.A., El-Felah, M., El-Sayed, A.F., Shreidi, A.S. & Yahyaoui, A. 2001. Decentralized and participatory plant breeding for marginal environments. *In* H.D. Cooper, C. Spillane & T. Hodgkin, eds. *Broadening the Genetic Base of Crop Production*, pp. 115–135. Wallingford, UK, CABI Publishing, in cooperation with FAO and IPGRI.

Ceccarelli, S. & Grando, S. 2007. Decentralized-participatory plant breeding: an example of demand driven research. *Euphytica,* 155: 349–360.

Cooper, H.D., Spillane, C. & Hodgkin, T. 2001. Broadening the genetic base of crops: an overview. *In* H.D. Cooper, C. Spillane & T. Hodgkin, eds. *Broadening the Genetic Base of Crop Production*. pp. 1–23. Wallingford, UK, CABI Publishing, in cooperation with FAO and IPGRI.

Daniell, H., Khan, M.S. & Allison, L. 2002. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology [Review]. *Trends in Plant Science,* 7(2): 84–91.

Ennos, R.A. 1996. Utilising genetic information in plant conservation programmes. *In* M.E. Hochberg, J. Clobert & R. Barbault, eds. *Aspects of the Genesis and Maintenance of Biological Diversity*, pp. 278–291. Oxford, UK, Oxford University Press.

Eshed, Y. & Zamir, D. 1994. A genomic library of *Lycopersicon pennellii* in *L. esculentum*: A tool for fine mapping of genes. *Euphytica,* 79: 175–179.

Eshed, Y. & Zamir, D. 1995. An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. *Genetics,* 141: 1147–1162.

Excoffier, L., Smouse, P.E. & Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics,* 131: 479–491.

Falconer, D.S. & Mackay, T.F.C. 1996. *Introduction to Quantitative Genetics*. 4th Edition. New York, USA, Longman Scientific and Technical.

Falush, D., Stephens, M. & Pritchard, J.K. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics,* 164: 1567–1587.

FAO. 1996a. *Report on the State of the World's Plant Genetic Resources for Food and Agriculture.* Prepared for the International Technical Conference on Plant Genetic Resources, Leipzig, Germany, 17–23 June 1996. FAO, Rome, Italy (Available at http://www.fao.org/ag/AGP/AGPS/PGRFA/pdf/swrfull.pdf; accessed 26 Dec. 2008).

FAO. 1996b. *Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources* and the Leipzig

Declaration, adopted by the International Technical Conference on Plant Genetic Resources, Leipzig, Germany, 17–23 June 1996. FAO, Rome (Available at: ftp://ftp.fao.org/ag/cgrfa/GS/gpaE.pdf; accessed 26 Dec. 2008).

FAO/IPGRI [International Plant Genetic Resources Institute]. 2001. FAO/IPGRI Multi-Crop Passport Descriptors (Available at http://apps3.fao.org/wiews/mcpd/MCPD_Dec2001_EN.pdf; accessed 26 Dec. 2008).

Frankel, O.H. 1984. Genetic perspectives of germplasm conservation. Paper No. 15, *in* W. Arber, K. Llimensee, W.J. Peacock & P. Starlinger, eds. *Genetic Manipulation: Impact on Man and Society*. Part III. Cambridge, UK, Cambridge University Press.

Frisch, M., Bohn, M. & Melchinger, A.E. 1999. Minimum sample size and optimal positioning of flanking markers in marker-assisted backcrossing for transfer of a target gene. *Crop Science,* 39: 967–975.

Fulton, T.M., Beck-Bunn, T., Emmatty, D., Eshed, Y., Lopez, J., Petiard, V., Uhlig, J., Zamir, D. & Tanksley, S.D. 1997. QTL analysis of an advanced backcross of *Lycopersicon peruvianum* to the cultivated tomato and comparisons with QTLs found in other wild species. *Theoretical and Applied Genetics,* 95: 881–894.

Fulton, T.M., Grandillo, S., Beck-Bunn, T., Fridman, E., Frampton, A., Lopez, J., Petiard, V., Uhlig, J., Zamir, D. & Tanksley, S.D. 2000. Advanced backcross QTL analysis of a *Lycopersicon esculentum × Lycopersicon parviflorum* cross. *Theoretical and Applied Genetics,* 100: 1025–1042.

Goldringer, I., Enjalbert, J., David, J., Paillard, S., Pham, J.L. & Brabant, P. 2001. Dynamic management of genetic resources: a 13-year experiment on wheat. *In* H.D. Cooper, C. Spillane & T. Hodgkin, eds. *Broadening the Genetic Base of Crop Production*. pp. 245– 260. Wallingford, UK, CABI Publishing, in cooperation with FAO and IPGRI.

Hallauer, A.R. & Miranda, J.B. 1981. *Quantitative Genetics in Maize Breeding*. Iowa, USA, Iowa State University Press.

Hansen, M., Kraft, T., Ganestam, S., Säll, T. & Nilsson, N.O. 2001. Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. *Genetical Research,* 77: 61–66.

Harlan, J.R. & de Wet, J.M.J. 1971. Toward a rational classification of cultivated plants. *Taxon,* 20: 509–517.

Haussmann, B.I.G., Parzies, H.K., Presterl, T., Susic, Z. & Miedaner, T. 2004. Plant genetic resources in crop improvement (Review Article). *Plant Genetic Resources – Characterization and Utilization,* 2(1): 3–21.

Hawtin, G., Iwanaga, M. & Hodgkin, T. 1997. Genetic resources in breeding for adaptation. *In* P.M.A. Tigerstedt, ed. *Adaptation in Plant Breeding*, pp. 277–288. Amsterdam, The Netherlands, Kluwer Academic Publishers.

Hodgkin, T., Brown, A.H.D., van Hintum, T.J.L. & Morales, E.A.V., eds. 1995. *Core Collections of Plant Genetic Resources*. Rome, IPGRI; and UK, John Wiley/Sayce Publishing.

Jannink, J., Bink, M.C. & Jansen, R.C. 2001. Using complex plant pedigrees to map valuable genes. *Trends in Plant Science,* 6: 337–342.

Jannink, J.L. & Walsh, B. 2002. Association mapping in plant populations. *In* M.S. Kang, ed. *Quantitative Genetics, Genomics and Plant Breeding*. pp. 59–68. Wallingford, UK, CAB International.

Jordan, D., Tao Ygodwin, I., Henzell, R., Cooper, M. & McIntyre, C. 2003. Prediction of hybrid performance in grain sorghum using RFLP markers. *Theoretical and Applied Genetics,* 106: 559–567.

Lowe, A., Harris, S. & Ashton, P. 2004. *Ecological Genetics. Design, Analysis and Application*. Oxford, UK, Blackwell Publishing.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. *Plant Breeding,* 104: 1–19.

Melchinger, A.E., Utz, H.F. & Schoen, C.C. 1998. Quantitative trait locus (QTL) mapping using different testers and independent population samples in maize reveals low power of QTL detection and large bias in estimates of QTL effects. *Genetics,* 149(1): 383–403.

Melchinger, A.E., Coors, J.G. & Pandey, S. 1999. Genetic diversity and heterosis. In *The genetics and exploitation of heterosis in crops*. pp. 99–118. Proceedings of an international symposium, CIMMYT, Mexico City, Mexico, 17–22 August 1997. CIMMYT, Mexico.

Mohammadi, S.A. & Prasanna, B.M. 2003. Analysis of genetic diversity in crop plants—Salient statistical tools and considerations. *Crop Science,* 43: 1235–1248.

Moncada, P.P., Martínez, C.P., Borrero, J., Chatel, M., Gauch, H. Jr, Guimaraes, E., Tohme, J. & McCouch, S.R. 2001. Quantitative trait loci for yield and yield components in an *Oryza sativa* × *Oryza rufipogon* BC$_2$F$_2$ population evaluated in an upland environment. *Theoretical and Applied Genetics,* 102: 41–52.

Müller, K.J. 1989. Die Entwicklung von Basispopulationen bei Getreide für biologisch-dynamische Betriebe. *Zeitschrift Lebendige Erde,* 5(89): 364–367.

Nei, M. 1987. *Molecular Evolutionary Genetics.* New York, USA, Columbia Univ. Press.

Niangado, O. 2001. The state of millet diversity and its use in West Africa. *In* H.D. Cooper, C. Spillane & T. Hodgkin, eds. *Broadening the Genetic Base of Crop Production*. pp. 147–157. Wallingford, UK, CABI Publishing, in cooperation with FAO and IPGRI.

Pillen, K., Zacharias, A. & Léon, J. 2003. Advanced backcross QTL analysis in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics,* 107: 340–352.

Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics,* 155: 945–959.

Ragot, M., Sisco, P.H., Hoisington, D.A. & Stuber, C.W. 1995. Molecular-marker-mediated characterization of favorable exotic alleles at quantitative trait loci in maize. *Crop Science,* 35: 1306–1315.

Reif, J.C., Melchinger, A.E. & Frisch, M. 2005. Genetical and mathematical properties of similarity and dissimilarity coefficients applied in plant breeding and seed bank management. *Crop Science,* 45: 1–7.

Reif, J.C., Melchinger, A.E., Xia, X.C., Warburton, M.L., Hoisington, D.A., Vasal, S.K., Beck, D., Bohn, M. & Frisch, M. 2003a. Use of SSRs for establishing heterotic groups in subtropical maize. *Theoretical and Applied Genetics,* 107: 947–957.

Reif, J.C., Melchinger, A.E., Xia, X.C., Warburton, M.L., Hoisington, D.A., Vasal, S.K., Srinivasan, G., Bohn, M. & Frisch, M. 2003b. Genetic distance based on simple sequence repeats and heterosis in tropical maize populations. *Crop Science,* 43: 1275–1282.

Rozas, J., Sanchez-Delbarrio, J.C., Messeguer, X. & Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics,* 19: 2496–2497.

Sánchez-Monge, E. 2002. *Diccionario de plantas de interés agrícola.* Ministerio de Agricultura, Pesca y Alimentación, Madrid.

Šimić, D., Presterl, T., Seitz, G. & Geiger, H.H. 2003. Comparing methods for inte-

grating exotic germplasm into European forage maize breeding programs. *Crop Science,* 43: 1952–1959.

Simmonds, N.W. 1991. Selection for local adaptation in a plant breeding programme. *Theoretical and Applied Genetics,* 82: 363–367.

Simmonds, N.W. 1993. Introgression and incorporation. Strategies for the use of crop genetic resources. *Biological Reviews,* 68: 539–562.

Sorrells, M.E. & Wilson, W.A. 1997. Direct classification and selection of superior alleles for crop improvement. *Crop Science,* 37: 691–697.

Swaminathan, M.S. 2002. The past, present and future contributions of farmers to the conservation and development of genetic diversity. *In* J.M.M. Engels, V.R. Rao, A.H.D. Brown & M.T. Jackson, eds. *Managing Plant Genetic Diversity,* pp. 23–31. Rome, IPGRI, and Oxford, UK, CABI Publishing.

Tanksley, S.D. & Nelson, J.C. 1996. Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. *Theoretical and Applied Genetics,* 92: 191–203.

Tanksley, S.D., Grandillo, S., Fulton, T.M., Zamir, D., Eshed, Y., Petiard, V., Lopez, J. & Beck-Bunn, T. 1996. Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium. Theoretical and Applied Genetics,* 92: 213–224.

Thornsberry, J.M., Goodman, M.M., Doebley, J., Kresovich, S., Nielsen, D. & Buckler IV, E.S. 2001. *Dwarf8* polymorphisms associate with variation in flowering time. *Nature Genetics,* 28: 286–289.

Utz, H.F., Melchinger, A.E. & Schön, C.C. 2000. Bias and sampling error of the estimated proportion of genotypic variance explained by quantitative trait loci determined from experimental data in maize using cross validation and validation with independent samples. *Genetics,* 154: 1839–1849.

Van Hintum, T.J.L., Brown, A.H.D., Spillane, C. & Hodgkin, T. 2000. Core collections of plant genetic resources. *IPGRI Technical Bulletin,* No. 3: 5–48.

vom Brocke, K., Christinck, A., Presterl, T., Weltzien-Rattunde, E. & Geiger, H.H. 2002a. Effects of farmers' seed management on performance and adaptation of pearl millet in Rajasthan, India. *Euphytica,* 130: 267–280.

vom Brocke, K., Presterl, T., Christinck, A., Weltzien-Rattunde, E. & Geiger, H.H. 2002b. Farmers' seed management practices open up new base populations for pearl millet breeding in a semi-arid zone of India. *Plant Breeding,* 121: 36–42.

von Korff, M., Wang, H., Léon, J. & Pillen, K. 2008. AB-QTL analysis in spring barley: III. Identification of exotic alleles for the improvement of malting quality in spring barley (*H. vulgare* ssp. *spontaneum*). *Molecular Breeding,* 21(1): 81–93.

Vuylsteke, M.J.R. 1999. Genetic analysis of maize by using the AFLP® method. PhD Thesis, University of Wageningen, Netherlands.

Vuylsteke, M., Kuiper, M. & Stam, P. 2000. Chromosomal regions involved in hybrid performance and heterosis: their AFLP-based identification and practical use in prediction models. *Heredity,* 85(3): 208–218.

Watterson, G.A. 1975. On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology,* 7(2): 256–276.

Weltzien, E., Smith, M.E., Meitzner, L. & Sperling, L. 2000. Technical and institutional issues in participatory plant breed-

ing – from the perspective of formal plant breeding. A global analysis of issues, results and current experience. Working Document No. 3. CGIAR Systemwide Program on Participatory Research and Gender Analysis for Technology Development and Institutional innovation. 106 p.

Witcombe, J.R. 2001. The impact of decentralized and participatory plant breeding on the genetic base of crops. *In* H.D. Cooper, C. Spillane & T. Hodgkin, eds. *Broadening the Genetic Base of Crop Production*, pp. 407–417. Wallingford, UK, CABI Publishing, in cooperation with FAO and IPGRI.

Xiao, J., Grandillo, S., Ahn, S.N., McCouch, S.R., Tanksley, S.D., Li, J. & Yuan, L. 1996. Genes from wild rice improve yield. *Nature,* 384: 223–224.

Xiao, J., Li, J., Grandillo, S., Ahn, S., Yuan, L., Tanksley, S.D. & McCouch, S.R. 1998. Identification of trait-improving quantitative trait loci alleles from a wild rice relative, *Oryza rufipogon. Genetics,* 150: 899–909.

Zamir, D. 2001. Improving plant breeding with exotic genetic libraries. *Nature Reviews Genetics,* 2: 983–989.

CHAPTER 6

Methodologies for generating variability Part 2: Selection of parents and crossing strategies

John R. Witcombe and Daljit S. Virk
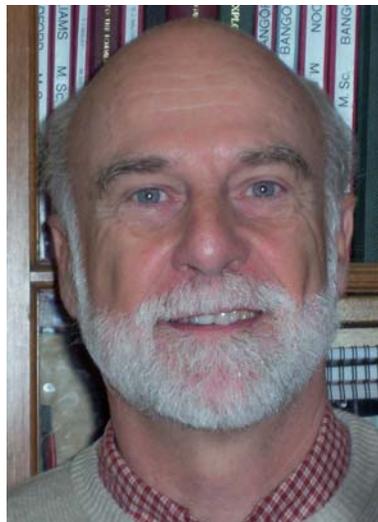
6.1 INTRODUCTION

More client-oriented approaches to plant breeding actively involve farmers in either consultative and collaborative roles, or both, in early generations in the breeding process (see Witcombe *et al.,* 2005; IPGRI, 1996). Little attention has been paid as to how the methods might differ from classical breeding in the number of crosses that are made and hence the strategy for the selection of parents.

In conventional breeding of inbred crops on research stations, breeders have the capacity to deal with the progeny of hundreds of crosses each season. Even with fairly limited resources, they can test many hundreds, or even thousands, of F_4 or F_5 lines in a nursery that has no, or few, replicates. In cross-pollinating crops, dozens of composites can be handled and trials can be conducted on hundreds of progenies (for population improvement) or inbred lines (for hybrid breeding).

Farmers can only grow variable material in their own fields if there are many fewer crosses, entries and plots than in classical breeding, because, without help from scientists, individual farmers cannot be expected to grow trials of hundreds of entries. However, in a participatory breeding programme it is inexpensive for a farmer to grow a very large population of any entry when it replaces the usual cultivar. The cost to the farmer of replacing his or her usual variety is only any decrease in the value of the crop, not the total cost of growing the crop. Indeed, when the segregating population is superior to the customary variety it provides a benefit, whereas, in classical breeding, the full cost of the area under an increased population size is borne by the breeding programme. Hence, in a participatory programme it is cost effective to have a farmer grow large

bulk populations, and this can easily be replicated by collaboration with several farmers.

6.2 NUMBER OF CROSSES

How many crosses are used in a breeding programme has crucial impacts on success and efficiency. However, the outcomes of theoretical calculations to determine the optimum number of crosses vary greatly with the assumptions that are made on how well the breeder can predict the value of crosses. If the breeder can predict the best cross with certainty, then only one cross is needed, but, assuming the breeder has no power of prediction at all, very many are required. These are extreme assumptions but neither experimental data nor theories exist that determine where the balance lies between the two. Hence, most breeders have inclined, undoubtedly with much success, towards what seems to be a more risk adverse strategy of the latter extreme, with many programmes having hundreds of crosses per year (Witcombe and Virk, 2001). Can efficiency be improved by moving towards the former, rarely-tested, extreme of using only a few crosses?

This question cannot be answered from experimental approaches on the optimization of cross number and population size. We have found no such reports in the literature, presumably because the required experiments are too large and expensive. An ideal comparison is clearly very difficult: it would compare the results of two parallel breeding programmes conducted over many years that use the same total number of plants (*K*), identical selection methods and environments, but contrasting values for *m* (number of crosses) and *n* (population size).

Although data and practical theory are lacking on how many crosses to use, the

theory is clear that the optimum population size of any cross has to be large if desirable transgressive segregants are to be obtained for traits that involve several loci (Allard, 1999). However, most breeding programmes use much smaller population sizes than theory dictates to accommodate the many crosses that are made.

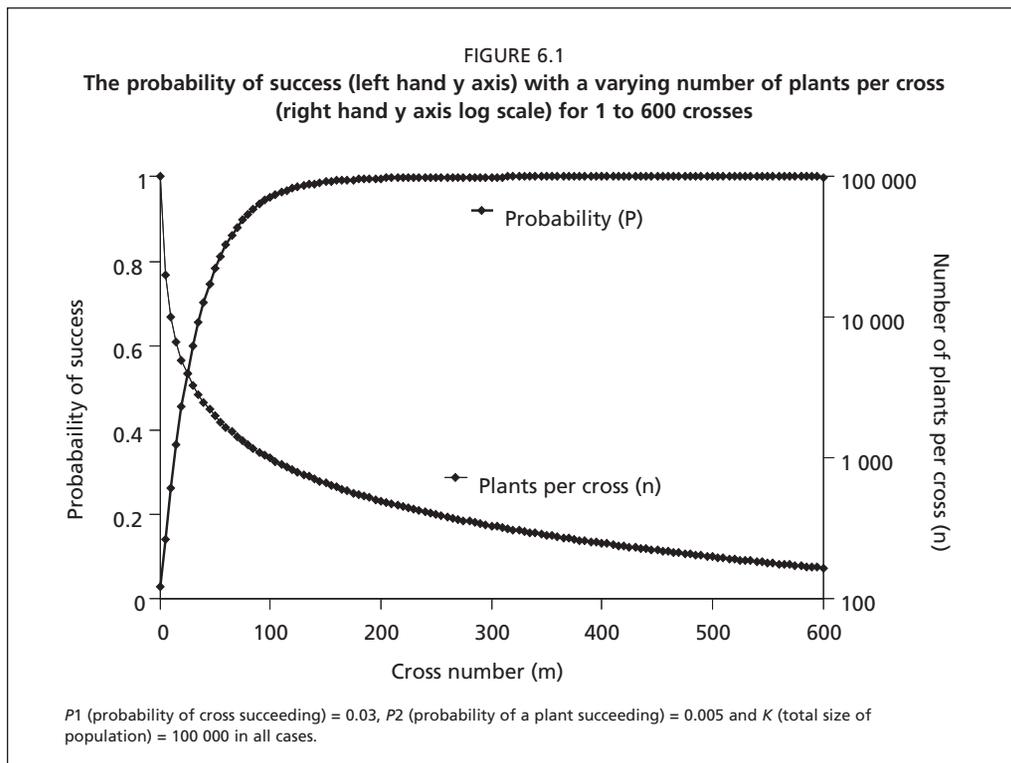### 6.2.1 A re-examination of the theory on the optimum cross number

In theoretical determinations of the optimal number of crosses (m) and population size (n) per cross, given a limit of K plants, two approaches have been used: either (i) minimizing the risk of excluding superior genotypes, or (ii) maximizing the response to selection.
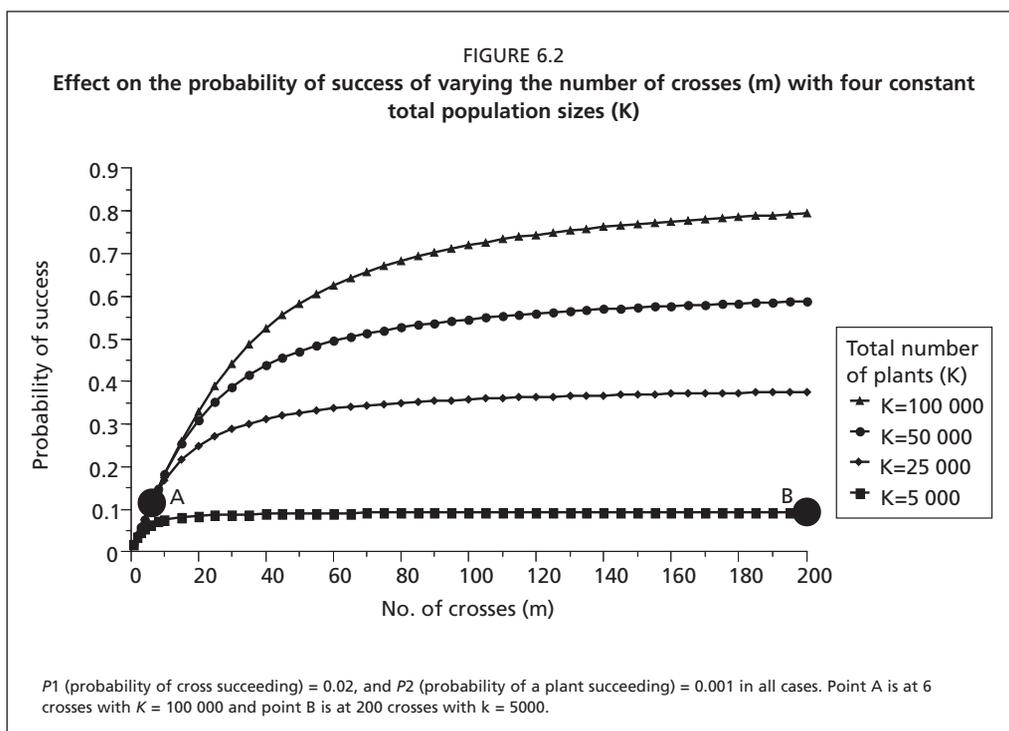
Using the first approach, Yonezawa and Yamagata (1978), Wricke and Weber (1986) and Hüehn (1996) suggested increasing the

number of crosses rather than increasing the population size of each cross in order to minimize the risk of missing the favourable plant. They found that each of the m crosses should be represented by only one F_2 plant ($n = 1$) to give the lowest risk of failure. However, this did not consider all of the possible scenarios. The probability of success can increase to 1 long before n falls to 1. This happens when:

• the probability of a cross succeeding ($P1$) is high, but still much lower than what we have achieved in our few-cross breeding programmes;
• the probability of a plant succeeding ($P2$) is also reasonably high; and
• K is large (Figure 6.1).

In the second approach, the magnitude of the response to selection among and within crosses is considered (Baker, 1984; Wricke and Weber, 1986; Hüehn, 1996;

FIGURE 6.1
The probability of success (left hand y axis) with a varying number of plants per cross (right hand y axis log scale) for 1 to 600 crosses

$P1$ (probability of cross succeeding) = 0.03, $P2$ (probability of a plant succeeding) = 0.005 and K (total size of population) = 100 000 in all cases.

FIGURE 6.2

Effect on the probability of success of varying the number of crosses (m) with four constant total population sizes (K)

P1 (probability of cross succeeding) = 0.02, and P2 (probability of a plant succeeding) = 0.001 in all cases. Point A is at 6 crosses with K = 100 000 and point B is at 200 crosses with k = 5000.

Bernardo 2003) and fewer crosses where n is always >1 maximizes the chance of success. For example, Baker (1984) concluded that for a total of 2 000 families the maximum response is found with 50 to 100 crosses having 40 to 20 families. Therefore, Bernardo (2003) concluded that the two approaches give conflicting results. However, we have found that by examining the response to increasing the cross number (m) the conflict is not as great as it might first appear. If the values of K are high, then adding more crosses is not very effective in increasing the probability of success, and a fewer-cross strategy with a larger population size would be more cost effective (see below for considerations of cost). Using the model of Yonezawa and Yamagata (1978), the rate of increase in the probability of success by adding more crosses declines dramatically after about 50 to 100 when values of K are ≤50 000 and values of $P1$ and

$P2$ are not extreme (Figure 6.2). Moreover, when values of K are greater than this, e.g. 100 000, then adding crosses improves the probability of success very little after about 15 crosses are made (Figure 6.2).

Even though the model of Yonezawa and Yamagata (1978) has been used to argue the case for more crosses, the argument for increasing K is just as powerful. A few crosses where K is large have a much lower risk over many crosses when K is small. For example, 6 crosses (point A in Figure 6.2) where K = 100 000 are less risky than 200 crosses (point B in Figure 6.2) where K = 5 000.

The relative costs of making crosses and growing plants are ignored in the model of Yonezawa and Yamagata (1978). Hence, with one cross or many crosses K remains the same, even though it is easier and cheaper to make one cross and grow 10 000 plants from it than make 10 000 crosses and only grow one plant from each. This applies

to all other values, such as 200 crosses with 50 plants each compared with 100 crosses with 100 plants each. Not only is there the additional cost of making more crosses, but there is an additional cost in record keeping, planting and labelling the more crosses there are within any given K.

In all of these models we have not considered the ability of breeders to choose superior crosses rather than making random ones. If the declining probability of success of each cross is considered—the first choice of a breeder should be better and more carefully considered than the hundredth—then the optimum number of crosses falls. This is simply a quantitative and realistic extension of the argument that if the best cross is known then only one needs to be made. The optimum number of crosses also becomes smaller as more resources are spent on evaluating parents and more time is spent on choosing crosses.

6.3 CHOICE OF PARENTS
6.3.1 Selection of parents
Little consensus exists among plant breeders on how best to choose parents for crosses that will produce high yielding progenies and it remains a debated issue (Qualset, 1979; Baker, 1984). The strategies for selection of parents for desired progeny performance fall into two categories: methods based on parental performance *per se,* or methods that assess the value of parents estimated from progeny performance. The first category includes selection based on: mid-parental values; divergence coefficients among parents (Murphy, Cox and Rodgers, 1986); character complementation or the geometric approach (Grafius, 1964, 1965; Lupton, 1965); and multivariate analysis and parental distances (Bhatt, 1970; Pederson, 1981). These methods have the advantage that they use data from a

single generation. However, the efficacy of these methods can only be evaluated by progeny tests.

In the second category of methods, parents are evaluated on the basis of the performance of their progeny. Such tests require time as at least two generations of plants need to be grown and evaluated to determine means and variances. The evaluation can be of combinations of F_1, F_2 and later generations (Allard, 1956; Busch, Hanke and Frohberg, 1974; Cox and Frey, 1984) or the evaluation of progeny produced from mating designs such as diallel and line × tester (Lupton, 1965). The relationship between predicted and actual progeny performance provides empirical evidence of the value of selecting parents by these methods, but the results of such experiments provide no consensus.

Other authors have concluded that gathering experimental data to estimate the value of possible crosses using progeny tests demands so many resources that it is unwarranted (Wricke and Weber, 1986; Lupton, 1965). We would agree with this as participatory methods provide simpler and effective methods of choosing parents by using genotypic performance *per se* as a prediction of parental value (Baenziger and Peterson, 1992). This is done without using the formal quantitative analysis described above for the first category of methods. Much information is already available for genotypes that have already been adopted by farmers. If a breeder's knowledge of such genotypes can allow the prediction of the more useful cross combinations, then only a few would need to be made, each with a higher probability of success. We have demonstrated in practical, participatory breeding programmes that this is the case in rice (Joshi *et al.*, 2007; Virk *et al.*, 2003) and that the equivalent in maize to a few

crosses—a single composite population—is also effective (Witcombe, Joshi and Goyal, 2003; Virk *et al*., 2005).

The breeder can predict that a cross is more likely to give rise to desirable segregants when the parents have complementary attributes suitable for the target environment. It is advantageous if they are also unrelated, to increase the extent of possible transgressive segregation. A few-cross, participatory strategy emphasizes the role of the plant breeder in the evaluation of introduced and collected germplasm as potential parents and the collective skills of farmers and breeders in selection, rather than emphasizing the skill of the breeder in selecting superior genotypes from within many crosses.

In participatory, i.e. decentralized, breeding at least one of the parents should be adapted to the target environment and have traits that farmers like. Participatory varietal selection (PVS) efficiently identifies locally adapted parents: a range of germplasm can be evaluated by farmers in their own fields that can include local landraces, recommended cultivars and introduced varieties. A variety selected by PVS is an ideal parent since it has local adaptation and traits that farmers prefer. Witcombe *et al.* (1996) suggested several types of crosses following PVS: a variety selected by farmers in the PVS trials is crossed with either a local landrace, another variety selected by participatory methods, or an exotic variety. This allows crosses to be made both between adapted × adapted parents or between adapted × unadapted parents. Of these options, an adapted variety identified by PVS crossed with an unadapted exotic variety will often have the greatest genetic dissimilarity. When breeding for marginal environments, the exotic variety can have adaptation to more favourable environ-

ments and hence be selected for its high yield potential and multiple disease and pest resistance. Client-oriented, participatory methods then benefit from classical breeding for adaptation to favourable environments.

Another participatory method conceptually closely related to PVS is to exploit current varietal adoption. D.N. Duvick (pers. comm.) has pointed out how farmers do a tremendous amount of selection for maize breeders in the United States of America, because the inbred-line parents of the most successful cultivars are used as parents in breeding programmes. This is participatory research—those cultivars have been grown over thousands of locations for several years providing a multilocational testing system far beyond the capacity of a formal breeding programme. In our rice breeding programmes in Nepal, widely adopted varieties such as CH 45 and Sabitri have been used as parents. Although extent of current adoption is clearly a useful criteria for parental selection, in many marginal areas PVS will sometimes quickly and cheaply identify varieties superior to those that farmers are currently growing, e.g. Joshi and Witcombe (1996).

Landraces may sometimes offer specific characteristics that are preferred by farmers. However, in our breeding programmes in rice, using landraces as parents has been less productive than using high-yielding modern varieties. Perhaps this is unsurprising, since improved varieties often have higher yields, and are more disease resistant than landraces. It makes no sense to use landraces just because they are landraces (Wood and Lenné, 1997), but rather to use them only when identified as having superior attributes. The argument that a landrace is 'locally adapted' and has post-harvest qualities that farmers appreciate is

insufficient when the best PVS variety also has these traits.

The strategy for choosing parents may differ little across target environments and the scale of the breeding programme. More favourable environments are highly diverse and participatory approaches are also needed for them (Witcombe, 1999). In breeding for favoured mega-environments, the same crosses are used to cover several or many countries, but this process is better decentralized by matching crosses to target countries (Ceccarelli *et al.*, 1994).

6.4 EVIDENCE AND CONCLUSIONS

We have used a few-cross approach in our client-oriented breeding (COB) programmes (Witcombe *et al.*, 2005). Using few crosses was effective; we made only three crosses in our breeding programme for rice targeted at Nepal and India by 1998, two of which were clearly successful, compared with a success rate of <1 percent in classical breeding programmes (Witcombe and Virk, 2001). The first cross we made, between the tall upland rice variety Kalinga III and the dwarf-statured lowland IR64, produced successful varieties, including three that had been released by 2008: two in India (Virk *et al.*, 2003) and one in Nepal (Gyawali *et al.*, 2006). The third cross we made, Radha 32/Kalinga III, produced Judi 582 and Judi 572 that have been adopted in Bangladesh (Joshi *et al.*, 2007). The second cross we made, Kalinga III/IR36, also produced high yielding lines, but these were not promoted as they were inferior in grain quality to those from the IR64 cross.

A fewer-cross strategy greatly simplifies the breeding scheme and saves resources. Some resources were re-allocated to increase the probability of obtaining desirable segregants (and reduce the risk from using only a few crosses) by using a much larger F_2 population from which several generations of large, early-generation, bulk populations were derived before lines were produced (Witcombe and Virk, 2001). Thus we avoided using resources on selection in the early generations, when it is less efficient for low-heritability traits compared with selection in later ones (Fahim *et al.*, 1988). Instead, we concentrated resources on selecting in later generations when higher between-line genetic variance increased efficiency (Kearsey and Pooni, 1996). Mass selection in the advanced bulk populations produced rice varieties as uniform as those from line selection (Virk, Steele and Witcombe, 2007).

There is further evidence that the few-cross approach is effective. Another rice breeding programme in Nepal funded by the International Plant Genetic Resources Institute (IPGRI, now Bioversity International) has also relied on only a few crosses. One parent was always a local landrace because landrace utilization was an objective of the programme. Even with this constraint on the choice of parents, of only 8 crosses, 4 have resulted in varieties that are in the release or pre-release stage (Gyawali, unpublished). At the Africa Rice Center (WARDA; formerly the West Africa Rice Development Association), a 'wide-cross' breeding programme between *Oryza sativa* and *O. glaberrima* placed considerable effort on choosing the parents of the crosses (Jones *et al.*, 1997). Only eight parents of *glaberrima* and five of *sativa* were chosen on the basis of their best combination of traits, and only seven of the crosses set seed. All of the seven 'New Rice for Africa' (NERICA®) varieties that were released in 2000 (WARDA, 2006) were from just one of these crosses, a success rate of 14 percent. As was the case for our crosses, this is a considerable improvement over normal success

rates and our experience suggests that this was due to the great attention paid to choosing parents, necessitated by the high cost of making these wide crosses. In maize, the parallel of a few-cross approach is to make only a single composite population, and we tested this in western and eastern India. Two populations were made, one for each region, and both have produced a released variety (Witcombe, Joshi and Goyal, 2003; Virk *et al.*, 2005).

What if all breeders used only a few crosses? This would restrict the amount of germplasm used in crosses but not restrict the amount used in successful crosses. In conventional programmes, although many crosses are made, most produce neither released varieties nor progeny that could be used in crosses to eventually produce a released variety. However, the exceptions are valuable: for example, IR64 has an extremely complex parentage with 20 original farmer varieties from eight countries as parents (IRRI, 1985). Clearly, not all of them would have previously been released varieties or parents of released varieties. To deliberately broaden the genetic base of crops, more crosses have to be made. This is particularly so when it involves parents about which little is known or, as is the case for little-grown landraces or wild relatives, when performance *per se* gives poor indications that the parents are valuable. Using few crosses is certainly suitable for breeding that is entirely targeted at rapidly producing varieties for client farmers in national programmes that have limited resources. The few crosses allow better market orientation and increase efficiency.

REFERENCES

Allard, R.W. 1956. Estimation of prepotency from lima bean diallel cross data. *Agronomy Journal,* 48: 537–543.

Allard, R.W. 1999. *Principles of Plant Breeding.* Second Edition. New York, USA, John Wiley and Sons.

Baenziger, P.S. & Peterson, C.J. 1992. Genetic variation: Its origin and use for breeding self-pollinated species. *In* H.T. Stalker and J.P. Murphy, eds. *Plant breeding in the 1990s.* pp. 69–92. Wallingford, UK, CABI.

Baker, R.J. 1984. Quantitative genetic principles of plant breeding. *In* J.P. Gustafson, ed. *Gene manipulation in plant improvement I*, pp. 147–176. New York, USA, Plenum Press.

Bernardo, R. 2003. Parental selection, number of breeding populations, and size of each population in inbred development. *Theoretical and Applied Genetics,* 107: 1252–1256.

Bhatt, G.M. 1970. Multivariate analysis approach to selection of parents for hybridization aiming at yield improvement in self-pollinated crops. *Australian Journal of Agricultural Research,* 21: 1–7.

Busch, R.H., Hanke, J.C. & Frohberg, R.C. 1974. Evaluation of crosses among high and low yielding parents of spring wheat (*Triticum aestivum* L.) and bulk prediction of line performance. *Crop Science,* 14: 47–50.

Ceccarelli, S., Erskine, W., Hamblin, J. & Grando, S. 1994. Genotype environment interaction and international breeding programmes. *Experimental Agriculture,* 30: 177–187.

Cox, D.J. & Frey, K.J. 1984. Combining ability and the selection of parents for interspecific oat matings. *Crop Science,* 24: 963–967.

Fahim, M., Dhanapala M.P., Senadhira, D. & Lawrence, M.J. 1988. Quantitative genetics of rice II. A comparison of the efficiency of four breeding methods. *Field Crops Research*, 55: 257–266.

Grafius, J.E. 1964. A geometry for plant breeding. *Crop Science,* 4: 241–246.

Grafius, J.E. 1965. A geometry for plant breeding. *Research Bulletin, Michigan Agricultural Experiment Station,* No. 7.

Gyawali, S., Witcombe, J.R., Joshi, K.D., Devkota, K.P., Tripathi, M., Subedi, M., Sunuwar, S., Chaudhary, B.N., Chaudhary, D., Adhikari, N.P., Akhtar, T., Yadav, R.B., Khatiwada, S.P., Subedi. D. & Mishra, H.D. 2006. A proposal for the release of rice variety Barkhe 3004 (a rice variety developed using client-oriented breeding (COB) approaches). LI-BIRD, Pokhara, Nepal; NRRP, Hardinath, Nepal; Jaskelo Youth Club, Chitwan, Nepal; CAZS-NR, University of Wales, Bangor, UK. See www.dfid-psp.org

Hüehn, M. 1996. Optimum number of crosses and progeny per cross in breeding self fertilizing crops. I. General approach and first numerical results. *Euphytica,* 91: 365–374.

IPGRI [International Plant Genetic Resources Institute]. 1996. *Proceedings of the IDRC/IPGRI Workshop on Participatory Plant Breeding.* International Agricultural Research Centre, Wageningen, Netherlands. 28–30 July 1996. Edited by P. Eyzaguirre & M. Iwanaga. IPGRI, Rome.

IRRI [International Rice Research Institute]. 1985. Parentage of IRRI crosses IR1 – IR50,000. Los Baños, Philippines, IRRI.

Jones, M.P., Dingkuhn, M., Aluko, G.K. & Semon, M. 1997. Interspecific *Oryza sativa* (L.) × *O. glaberrima* (Steud.) progenies in upland rice improvement. *Euphytica,* 94: 237–246.

Joshi, A. & Witcombe, J.R. 1996. Farmer participatory crop improvement. II. participatory varietal selection, a case study in India. *Experimental Agriculture,* 32: 461–477.

Joshi, K.D., Musa, A.M., Johansen, C., Gyawali, S., Harris, D. & Witcombe, J.R. 2007. Highly client-oriented breeding, using local preferences and selection, produces widely adapted rice varieties. *Field Crops Research,* 100: 107–116.

Kearsey, M.J. & Pooni, H.S. 1996. *The genetical analysis of quantitative traits*. Cheltenham, UK, Stanley Thornes.

Lupton, F.G.H. 1965. Studies in the breeding of self pollinating cereals. 5. Use of the incomplete diallel in wheat breeding. *Euphytica,* 14: 331–352.

Murphy, J.P., Cox, T.S. & Rodgers, D.M. 1986. Cluster analysis of red winter wheat cultivars based upon coefficients of parentage. *Crop Science,* 26: 672–676.

Pederson, D.G. 1981. A least-squares method for choosing the best relative proportions when intercrossing cultivars. *Euphytica,* 30: 153–160.

Qualset, C.O. 1979. Mendelian genetics of quantitative characters with reference to adaptation and breeding of wheat. *In* S. Ramanujam, ed. *Proceedings 5th International Wheat Genetics Symposium.* Vol. 2, pp. 577–590. Indian Society of Genetics and Plant Breeding, New Delhi.

Virk, D.S., Steele, K.A. & Witcombe, J.R. 2007. Mass and line selection can produce equally uniform rice varieties. *Field Crops Research,* 100: 341–347.

Virk, D.S., Singh, D.N., Prasad, S.C., Gangwar, J.S. & Witcombe, J.R. 2003. Collaborative and consultative participatory plant breeding of rice for the rainfed uplands of eastern India. *Euphytica,* 132: 95–108.

Virk, D.S., Chakraborty, M., Ghosh, J., Prasad, S.C. & Witcombe, J.R. 2005. Increasing the client-orientation of maize breeding using farmer participation in eastern India. *Experimental Agriculture,* 41: 413–426.

WARDA [Africa Rice Center]. 2006. Upland NERICA®. Nerica for Africa. (available at www.warda.org/warda/uplandnerica.asp; accessed 27 Dec. 2008).

Witcombe, J.R. 1999. Do farmer-participatory methods apply more to high potential areas than to marginal ones? *Outlook on Agriculture,* 28: 43–50.

Witcombe, J.R. & Virk, D.S. 2001. Number of crosses and population size for participatory and classical plant breeding. *Euphytica,* 122: 451–462.

Witcombe, J.R., Joshi, A. & Goyal, S.N. 2003. Participatory plant breeding in maize: A case study from Gujarat. *Euphytica*, 130: 413–422.

Witcombe, J.R., Joshi, A., Joshi, K.D. & Sthapit, B.R. 1996. Farmer participatory crop improvement. I: Varietal selection and breeding methods and their impact on biodiversity. *Experimental Agriculture,* 32: 445–460.

Witcombe, J.R., Joshi, K.D., Gyawali, S., Musa, A.M., Johansen, C., Virk, D.S. & Sthapit, B.R. 2005. Participatory plant breeding is better described as highly client-oriented plant breeding. I. Four indicators of client-orientation in plant breeding. *Experimental Agriculture,* 41: 299–319.

Wood, D. & Lenné, J.M. 1997. The conservation of agrobiodiversity on-farm: questioning the emerging paradigm. *Biodiversity and Conservation*, 6: 109–129.

Wricke, G. & Weber, W.E. 1986. *Quantitative genetics and selection in plant breeding*. Berlin, Germany, and New York, USA, W. de Gruyter.

Yonezawa, K. & Yamagata, H. 1978. On the number and size of cross combinations in a breeding programme of self-fertilizing crops. *Euphytica,* 27: 113–116.

Methodologies for generating variability Part 3: The development of base populations and their improvement by recurrent selection

John R. Witcombe
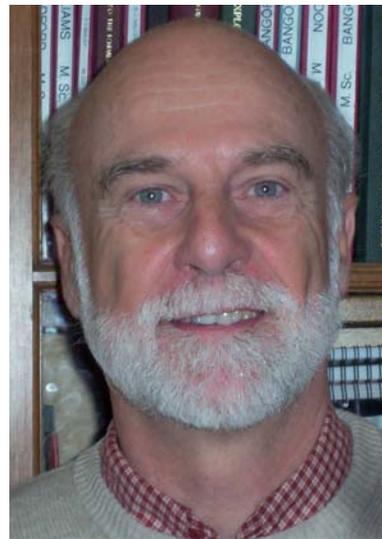
7.1 INTRODUCTION

This chapter presents a detailed account of the methodology of population improvement in open-pollinated crops using maize (*Zea mays* L.) and pearl millet (*Pennisetum glaucum* (L.) R. Br.) as examples. These are examples of highly cross-pollinated crops, for which population improvement breeding methods are very suitable. Appreciable gains for grain yield have been realized by recurrent selection in maize (Hallauer and Miranda, 1988) and in pearl millet (Govil, Pokhriyal and Murty, 1982, 1986; Kapoor *et al.*, 1983; Khadr, 1977).

Population improvement methods can be used both in the breeding of hybrids and in the breeding of open-pollinated varieties (OPVs) or the two can be combined. Hybrids tend to be higher yielding, but OPVs are easier to breed, their seed production is cheaper and quicker, their disease resistance is more stable over seasons and locations, and low-resource farmers can cultivate the crop from farm-saved seed without paying a large yield penalty. In this chapter, neither hybrid breeding nor combinations of hybrid breeding and open-pollinated variety breeding are considered.

Useful farmer participatory techniques that improve the client orientation of the programme and its effectiveness are considered. These techniques increase the possibilities of successful use of the new varieties by the client farmers.

7.2 FORMATION OF BASE POPULATIONS
7.2.1 How many parents

Few or many parents can be intercrossed to form base populations for recurrent selection. The number will depend on the genetic diversity among them and the balance desired between high initial yield (to increase short-term gains) and high genetic variance (to increase the potential for

long-term genetic advancement). Because of the need to balance short- and long-term gains, there is no generally applicable rule for determining the optimum number of parents. An additional factor is the degree of inbreeding in the parents; to avoid inbreeding depression, more inbred lines are needed than if open-pollinated cultivars are parents.

7.2.2 Choosing parents

Methods that actively involve the target clientele can quickly identify the traits needed in parents. This is done poorly in many breeding programmes, as shown by farmers continuing to grow landraces or obsolete cultivars while officially-released cultivars lack the traits demanded by them. Surveys of farmers are needed to elicit information on what is needed. In the public sector, such surveys are often referred to as participatory rural appraisals (PRA) (Chambers, 1997) and are equivalent to the market research approach of the private sector (Sumberg and Reece, 2004). For a breeding programme, well-applied PRA techniques or customer profiling result in better client orientation and make possible efficient goal setting (e.g. Weltzien, Whitaker and Anders, 1996). In pearl millet, for example, Kelley *et al.* (1996) identified the importance of traits such as straw production and quality. At the end of this process a product specification can be made where the traits of the desired variety are known for all of the major adaptive, yield and quality traits (Witcombe *et al.*, 2005).

The above is a consultative process, but the active collaboration of farmers in participatory varietal selection (PVS) helps greatly when parents are selected for their performance *per se*. Techniques that delay the creation of the base population by, for example, running yield trials or conducting genetic analyses on candidate parents (e.g.

Gardner and Eberhart, 1966) are usually not warranted. This is particularly true for client-oriented approaches where often the breeding is targeted at farmers who have had limited benefits from modern plant breeding. The base population then needs to be developed from a viewpoint of speed and simplicity, because the task is not the difficult one of improving upon a recent modern variety but to improve upon a landrace or an obsolete variety.

The biggest gains will be made when the population has a high initial yield and a high genetic variance. For decentralized breeding, we have found that the recombination of locally adapted varieties with high-yielding exotic varieties can produce this desirable combination. High genetic variance can be achieved by crossing unrelated germplasm, such as white- and yellow-endosperm material (Witcombe, Joshi and Goyal, 2003; Virk *et al*., 2005). Tiwari (2001) tried several types of crosses, and the one that involved both local and improved germplasm and white- and yellow-endosperm types was the most successful.

In client-oriented breeding programmes that are decentralized to a given target region, a high proportion of the base population parents should be adapted to it and have traits that local farmers like (Witcombe *et al*., 1996). To increase diversity, less-well-adapted parents should also be included for their complementary attributes in order to produce desirable segregants. When breeding for marginal environments, these complementary parents can be adapted to more favourable environments and are chosen for high yield potential and superior disease and pest resistance. They are likely to be unrelated to the locally adapted parents, thereby increasing the genetic variance of the population. In pearl millet, the value of such an approach of

crossing locally adapted landraces with complementary, high yielding parents (modern varieties) was demonstrated (vom Brocke *et al*., 2002, 2003). The introgression of modern varieties into landraces increased genetic diversity, led to broader adaptation than either landraces or modern varieties alone, and under high rainfall conditions still yielded as much as modern varieties.

In maize, in western India (Witcombe, Joshi and Goyal, 2003) and in eastern India (Virk *et al*., 2005) the traits that farmers wanted in new maize varieties (white grain (endosperm), with early maturity and with tolerance to the most common abiotic constraints of drought encountered in these regions) were determined from interviews and from the results of participatory trials. More detailed requirements emerged during the course of these programmes, such as a need for high cob-placement to avoid damage by jackals. In Africa, Bänziger and Cooper (2001) have targeted the two major constraints identified by farmers, namely drought and the ability to yield well even under low nitrogen conditions. In pearl millet, a survey of farmers (ICRISAT, 1987) in Maharashtra showed the importance that farmers placed on large individual grain size and early maturity. A breeding programme was based on the creation of a composite with bold grains and earliness (the Bold Seeded Early Composite). This produced highly acceptable cultivars for farmers in both India and several African countries (ICRISAT, 1997).

Participatory varietal selection is a very efficient way of identifying locally adapted parents with traits that meet specific client needs. A closely related method, because it also relies on farmer acceptance of varieties, is to exploit knowledge of current varietal adoption. Duvick (2002) points out that farmers do a tremendous amount of selection

for maize breeders in the United States of America, because the inbred-line parents of the most successful cultivars are used as parents in breeding programmes. However, although current adoption is clearly a helpful criterion for parental selection, in more marginal agricultural areas it may be less useful. There may be considerably better varieties than those that farmers are currently growing and these can be quickly and cheaply identified by PVS (e.g. Joshi and Witcombe, 1996).

7.2.3 Population size

Population size has to be large to provide a reasonable probability of finding rare or infrequent desirable transgressive segregants. In cross-pollinated crops, small population sizes cause significant inbreeding depression, so population sizes need to be sufficiently large to avoid this; an effective population size of more than 500 plants in each generation is sufficiently large to avoid significant inbreeding depression (this is calculated from a standard formula, e.g. Falconer, 1981; see also Chapter 2 in this volume). The resources available also dictate the size of each population. The more base populations that are created and improved, the fewer the resources that can be devoted to each one. In the breeding of open-pollinated crops, creating and improving even a single population still requires many resources. The strategy used in our client-oriented breeding programmes has therefore been to minimize the use of resources by improving a single population for each group of target clients (Witcombe, Joshi and Goyal, 2003; Virk *et al.*, 2005). Not only does this reduce the resources required, but it more carefully focuses the base population to an identified target group of clients. It has proven to be a successful strategy.

7.2.4 Making the initial cycle (C_0) bulk
General considerations
Adequate genetic recombination between the parents of a composite will produce a diverse range of recombinants for the first cycle of recurrent selection. How early this occurs will depend on how inbred the parents are. When the parents are inbred, the third generation of random mating is equivalent to the F_2 in an inbreeding crop in that it is the first in which transgressive segregation can occur. In this case a third generation of random mating is needed before selection should commence. Transgressive segregants occur earlier if the parents are heterozygous, open-pollinated varieties. In this case, mass selection after a single generation of random mating can be expected to result in a genetic advance, but possibly at the expense of an early reduction in genetic diversity that reduces the potential for long-term gains. A third generation of random mating remains desirable before any progeny testing is started.

Using maternal ancestry to aid recombination
In crops where the occurrence of natural selfing is low, the simplest method of recombination is to allow random mating in a bulk grown from equal amounts of parental seed. However, the maintenance of some form of population structure is desirable as it allows a visual estimate to be made of the extent of recombination. When parental numbers are not too high, this can be done by maintaining sub-bulks derived from the individual parents of the base population and growing them in an isolated plot (Figure 7.1). To aid randomness of recombination a pollinator bulk is used that is made from aliquots of seed of the original parents. The pollinator bulk is preferably also planted on the borders of the isolated

FIGURE 7.1
Planting layout design for hills in maize. Detasselled plants in grey

6	3	2	5	8	2	1	7	1	5	1	2	4	2	8	5	7	8	6	3
4	9	4	6	4	8	9	8	9	3	7	1	8	1	6	1	6	1	4	9
7	8	2	9	7	5	4	5	6	8	4	7	1	3	5	8	5	2	7	8
2	7	1	5	1	3	7	3	4	5	6	2	3	8	7	3	1	7	2	7
5	4	6	2	9	7	3	1	8	6	3	9	7	9	2	9	4	3	5	4
1	9	2	8	2	9	8	9	5	7	6	4	8	5	3	6	2	3	1	9
9	2	3	9	5	6	3	2	1	2	9	3	2	3	8	4	1	7	9	2
3	8	2	7	3	4	9	5	6	9	2	1	5	2	1	7	8	1	3	8
8	5	4	3	4	8	4	6	2	1	7	3	4	7	9	2	9	5	8	5

plot. When, for example, 50 percent of the area is planted with the pollinator bulk, the entries and the bulk are planted in alternate ridges and beds so that every entry is surrounded by the pollinator bulk. In the second and subsequent random mating generations, the pollinator is either recreated by bulking aliquots of seed taken from the open-pollinated entries, or is advanced through the generations by harvesting its open-pollinated seed (Figure 7.1). Random mating is repeated until the entries lose all or most of their identity relative to each other and the pollinator bulk. In maize, unlike in pearl millet, the entry rows and the pollinator bulk can be made to cross by detasselling the entry rows. In this case, the pollinator bulk is best made up afresh each time from aliquots of the female entries.

If necessary, only the entry rows can be planted, either to reduce the land requirement or because there is insufficient seed to sow both the entry rows and the pollinator bulk. The extent of recombination is then assessed by the between-entry phenotypic differences.

When recombination appears complete, equal amounts of seed are taken from the entry rows to make the C_0 bulk of the composite. A portion of the C_0 bulk should be retained for use as the base population in trials for evaluating the progress made by selection.

Using forced crossing in the first generation of random mating

When there are few parents, it is much more efficient to employ diallel crossing in the first generation of random mating. This greatly increases randomness of mating by avoiding the high proportion of sibbing within the parental entries that occurs under natural random mating. It also avoids the need for an isolated plot. When there are too many parents to make a complete diallel, a half diallel can be used, or they can be crossed in, for example, a partial diallel using systematically selected crosses, or a randomly made partial diallel (designs for such random crossings are discussed below). If there are not too many crosses in the diallel, then progress of extent of recombination can be assessed, in the second and subsequent random matings, by planting the entry rows according to the maternal parents of the cross that were made in the first generation.
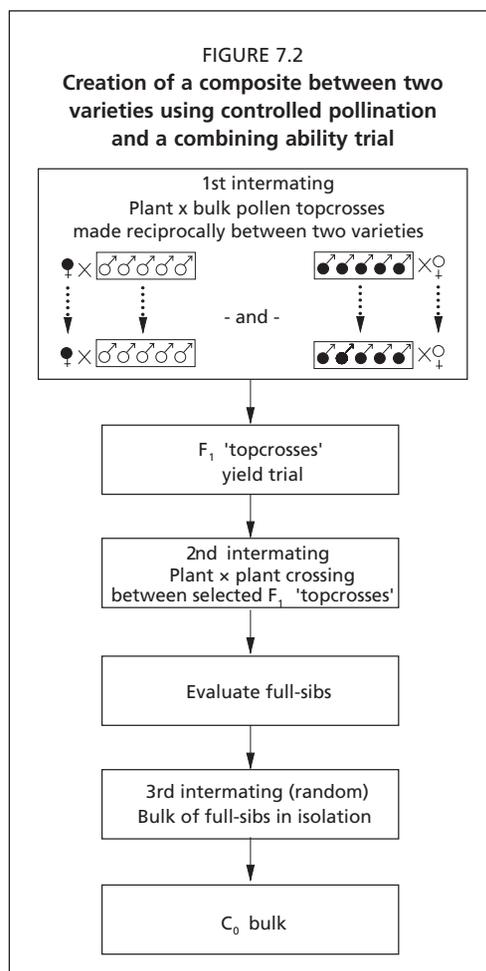
Hill designs in maize

In maize, the prevention of selfing is simply done by detasselling plants. This can lead to very effective methods of increasing

recombination. In the breeding of maize using client-oriented methods only a single composite was made per target environment so resources allowed quite sophisticated methods to be used in the creation of the base population. Witcombe, Joshi and Goyal (2003) described the making of a base population based on crossing three varieties with white endosperms and three with yellow endosperms. All nine possible white by yellow crosses between the varieties were made by hand pollination in a reciprocal fashion. In this generation, selfing was avoided by selecting only grains of the colour of the pollen parent; endosperm colour is a highly heritable trait and xenia makes the pollen genotype apparent in the seed in the maize ear. In subsequent generations, hills were planted of the nine crosses in a pseudo-random design: the hills were randomized but plants derived from the same cross were not allowed to occur in adjacent hills in either the horizontal or the vertical rows. Where adjacent hills in a diagonal row were from the same cross the plants were detasselled and further plants were randomly selected for detasselling to bring the proportion of detasselled plants to 50 percent. An example of part of the planting design is shown in Figure 7.1, with detasselled plants in bold, before the addition of the randomly selected plants. Hills were individually harvested and labelled with the cross number to repeat the design in the following generations.

There are simpler ways of attempting to maximize recombination, such as using the row-planting model shown in Figure 7.1. However, given that with client-oriented methods only one base population is used, more resource-consuming methods are feasible and reduce the perceived risk of relying on a single base population.

Recombination with progeny testing

In the most complex methods, progeny are tested during the random mating generations. For example, when only two varieties or composites are merged to form a new composite, then the combining ability of individual plants of one entry can be assessed by using the other entry as the tester. The topcross hybrids produced by individual plant × bulk pollen crosses are assessed in a yield trial, and the selected topcross hybrids are themselves used as parents for the second generation of random mating (Figure 7.2). The topcross test is preferable to making full-sibs between

FIGURE 7.2
Creation of a composite between two varieties using controlled pollination and a combining ability trial

1st intermating
Plant x bulk pollen topcrosses made reciprocally between two varieties

- and -

F_1 'topcrosses' yield trial

2nd intermating
Plant × plant crossing between selected F_1 'topcrosses'

Evaluate full-sibs

3rd intermating (random)
Bulk of full-sibs in isolation

C_0 bulk

plants of the two entries. A full-sib test determines the specific combining ability of the pairs of plants that are crossed, whereas a topcross test determines the general combining ability of individual plants.

7.2.5 Recombination in selection cycles
Overview
Recombination in selection cycles differs from recombination to make the base population. The parents of the base population are diverse and unrelated, so crossing has to take this into account by using formal crossing designs. The parents for recombination during selection are all from the same population so all families are equivalent. Creating a base population requires a single random-mated population to be produced, whereas it is helpful during the recurrent selection cycles to also produce a family structure, such as full-sibs. Hence, in describing how recombination can be made during the selection phase, attention is paid to the resultant family structure.

Perfectly random recombination is where each individual plant produces half-sib seed by randomly crossing to the remainder of the population. However, in methods of recurrent selection where the test units are families (the only exception being mass selection), perfect randomness is undesirable as sibbing within families will cause inbreeding and create non-heritable between-plant variation. Natural outcrossing in pearl millet and outcrossing in maize, even when forced by detasselling, will produce half-sib families but cannot avoid within-family crossing as some of the pollen will unavoidably come from the same family as the female parent. Although certain planting designs will reduce this effect, it cannot be eliminated, particularly because within-family crossing inevitably occurs as a result of assortative mating caused by relatively higher intermating within early-

flowering or later-flowering groups of families than among families with differing flowering times.

As a result of such limitations and the inefficiency of half-sib family selection, it is more cost effective to use forced crossing. The desirability of forced crossing increases the fewer the selected families and the more inbred they are. The forced intermating can produce topcrosses (individual plants crossed with bulk pollen) but sibbing among families is not completely avoided. Making full-sib families (individual plant × plant crossing) avoids sibbing entirely. Rope ladder crossing designs can be used to make this process more efficient (see below).

7.2.6 Production of full-sibs
The production of full-sibs provides the greatest control over pollination and avoids both within-family sibbing and selfing, but demands most labour. However, the increase in labour requirement to make full sibs is less than might be expected, because the work involved in making a pollen bulk by collecting and mixing aliquots of pollen is no longer required. A real disadvantage of full-sibs is that the effective population size is reduced over half-sibs because each entry in a full-sib nursery has only two parents, whilst a half-sib has many. The effective population size in the full-sib nursery can be maximized by using each plant in the full-sib mating only once as either a male or a female. Progress made by full-sib selection is expected to be much higher in the subsequent generation. Full-sib families have twice the additive genetic variance between them as half-sib families, making them more efficient as a selection unit, and the correlation between their performance *per se* (which is assessed in the trial) and their general combining ability (which determines their genetic value in the
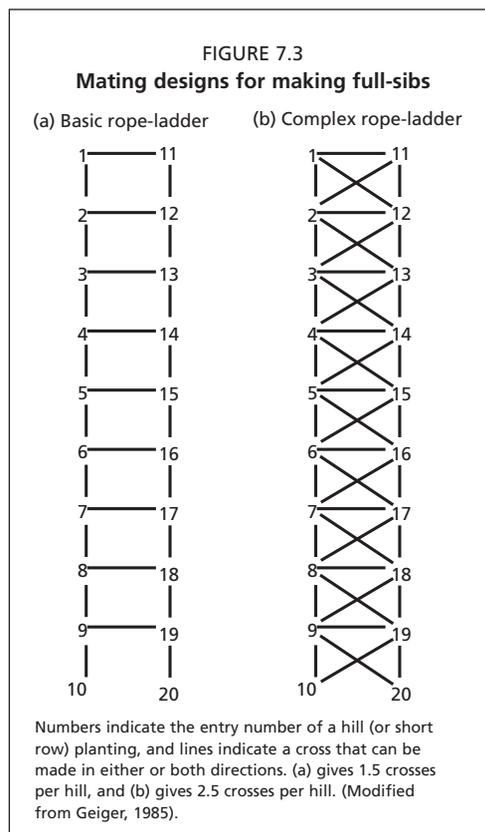
next cycle of the population) is expected to be higher in full-sibs than in half-sibs unless very large dominance effects are present.

The production of full-sibs can be facilitated by using an appropriate planting plan (Figure 7.3) that avoids the complications of making the unnecessary full diallel between the selected families. 'Rope ladder designs' (Figure 7.3) can be used, and to increase the number of crosses that are possible using this design, the rope ladders are replicated. The number of replications required, r, is easily obtained from the specified number of crosses, n, and the number of selected families, s. After rounding up to the nearest whole integer $r = n/s/1.5$ (for the basic stepladder) and $r = n/s/2.5$ (for the complex stepladder). A random arrangement of the families within each replication is employed, and when the number of families is reasonably high there is little risk of repeating any particular cross to an excessive extent.

7.2.7 Improvement of base populations

Matching the selection environment to the target environment

In typical breeding programmes, selection in the segregating generations is conducted on-station in well-managed conditions. In many countries there are recommended packages of practices that require high standards of management and high levels of purchased inputs. They maximize yield, but farmers in marginal areas invariably apply lower inputs than are recommended for several good reasons: it matches better their limited capacity to procure resources, reduces their risks and maximizes their longer-term benefit:cost ratios by reducing or avoiding negative returns from purchased inputs in poor years. Hence, a common criticism of public sector-breeding programmes

FIGURE 7.3
Mating designs for making full-sibs

(a) Basic rope-ladder   (b) Complex rope-ladder

Numbers indicate the entry number of a hill (or short row) planting, and lines indicate a cross that can be made in either or both directions. (a) gives 1.5 crosses per hill, and (b) gives 2.5 crosses per hill. (Modified from Geiger, 1985).

targeted at less favourable agricultural environments is that unrealistically favourable selection environments (SE) are employed (Almekinders and Elings, 2001; Ceccarelli, Grando and Booth, 1996; Virk *et al*., 2003; Witcombe *et al*., 1996). M. Bänziger (pers. comm.) has shown a typical mismatch between the SE and the target population of environments (TPE), where only the poorer research station environments match those of the farmers' fields (Figure 7.4). Hence, if the SE is to match the true TPE (Fischer *et al*., 2003) then the SE must not be optimal but encounter similar stresses to the TPE, such as low fertility and limited water.

Reducing the levels of on-station crop management can reduce the gap between the SE and the TPE. This approach has been used in client-oriented breeding programmes

in maize in India, where the applied levels of fertilizer were significantly reduced from the recommended levels (e.g. Witcombe, Joshi and Goyal, 2003). A second strategy is to manage stress environments on-station to breed for tolerance to abiotic stresses commonly encountered in farmers' fields, such as drought and low nitrogen (Bänziger and Cooper, 2001). By testing progeny in both managed-stress environments and more favourable environments, families can be selected that not only tolerate these common stresses but also respond to more favourable environments. Since the stresses are managed, they can be carefully controlled so that the heritability of the trait under selection is increased and genetic gains are enhanced. A third strategy is to carry out selection in farmers' fields by breeders, or farmers, or both. If the selection programme is unreplicated (as is also typically the case on the research station), then a typical farmer needs to be chosen whose field is representative of the target area.

In more favourable agricultural environments the risk of a significant mismatch between the SE and the TPE is lower – both scientists and farmers manage the crop well, with applied, purchased inputs. Nonetheless, there are possible pitfalls. A mismatch still occurs when higher levels of purchased inputs are applied on the research station because, as in the case of marginal environments, the full economic cost and risk to farmers of applying them have not been considered. A mismatch can also result from disparities in the on-station cropping system used; researchers may employ more fallow or green manuring, because they are recommended rotations, even when farmers rarely adopt them (see also Chapter 6 in this volume).

Whether the selection is done by breeders, farmers, or breeders and farmers work-

FIGURE 7.4
An idealized diagram of the gap between the selection environments and the farmers' environments (top) and the elimination of this gap (bottom)
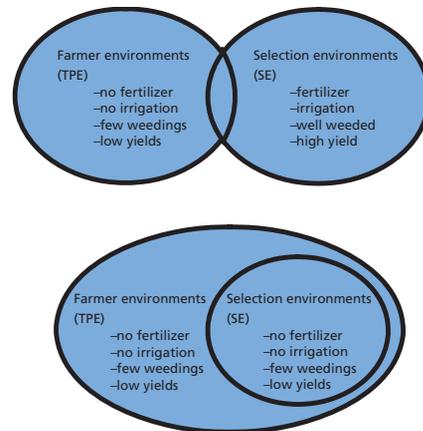
Modified from M. Bänziger (pers. comm.).

ing together, will depend on circumstances (Witcombe *et al.*, 2005). In many cases, it will be easier and more efficient for the breeders to do the selection, provided they have correctly identified the traits required by the target clientele. In some breeding methods that combine hybrid with OPV breeding, it makes no sense to involve farmers if most of the effort is on the development of inbred lines and on trials that assess their combining ability. In other circumstances, it may be easier for farmers to do the selection. In pearl millet, vom Brocke *et al.* (2002) argue that farmers' seed management practices can be incorporated into breeding programmes. Also in pearl millet, Monyo *et al.* (2000) described a breeding programme based on a farmers' deliberate selection within a cross the farmer had allowed to occur between the improved variety Okashana-1 and a local landrace.

If selection in the segregating generation is optional, it is essential to involve farm-

ers in selection among the varieties that are produced by the programme. This process is commonly called participatory varietal selection (PVS) and involves farmers testing material in their own fields (Witcombe *et al.*, 1996, 2005). The methods used involve some form of 'mother-and-baby' design (Snapp, 1999), where all of the entries are tested in relatively simple designs in the mother trials and subsets or individual entries are tested in baby trials. In this system, researchers and farmers evaluate the varieties. Particular attention is paid to the perceptions of the farmers for a range of traits and their overall preferences among the varieties.
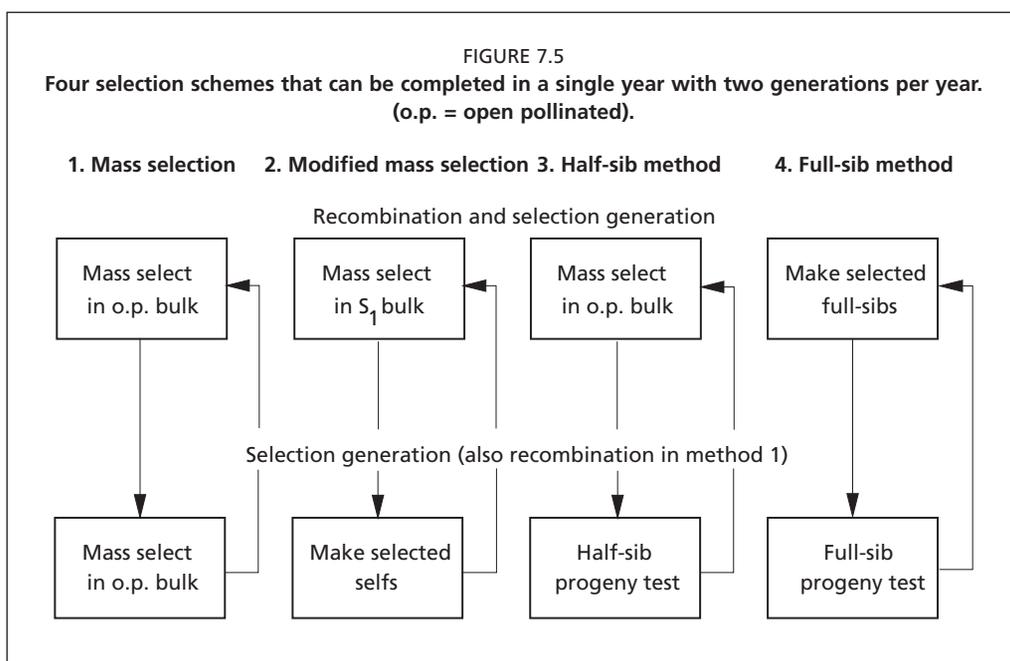
7.3 POPULATION IMPROVEMENT METHODS REQUIRING ONE YEAR PER CYCLE

Mass selection, modified mass selection, half-sib family selection and full-sib family selection can be completed in a single year in locations where two generations can be grown in the field in a year (Figure 7.5).

7.3.1 Mass selection methods
General considerations
It seems to be an unavoidable fact that most plant breeders dislike simple methods. In self-pollinated crops most breeders employ elaborate forms of line selection whereas, when experimental comparisons are made, it is the simplest methods, such as bulk-population breeding or single-seed-descent methods, that show the highest efficiencies (e.g. Fahim *et al.*, 1998, in rice). The same may apply to outbreeding crops where simple mass selection is rarely used, perhaps because it is considered to be an under-exploitation of the breeder's knowledge and skills. However, in outbreeding crops there is some justification for more elaborate methods because evidence shows that greater genetic gains per year can be made with progeny testing. Nonetheless, as can be seen below, significant genetic progress can be made from mass selection, and that progress requires the smallest investment in resources per unit of gain.

FIGURE 7.5
Four selection schemes that can be completed in a single year with two generations per year. (o.p. = open pollinated).

Because of this cost effectiveness and because of its simplicity, mass selection methods are attractive. They are particularly useful for decentralized breeding in difficult environments where complex breeding methods are beyond locally available capacity.

Mass selection is most effective when the initial base population yield is high and there is high genetic variance. Given these circumstances, high rates of genetic gain can be achieved in response to selection. As was discussed earlier, in the development of base populations, a combination of locally adapted material with high yielding exotic genotypes can produce this desirable combination.

Mass selection can also be made more attractive by simple modifications. The most commonly suggested modification is stratified mass selection, but personal experience has not shown this to be either simple to do or effective. Instead, alternative improvements to mass selection are considered after a brief consideration of the evidence of its effectiveness in pearl millet and maize.

7.3.2 Evidence of the effectiveness of mass selection
Pearl millet
Mass selection can be effective in improving pearl millet (Govil, Pokhriyal and Murty, 1986; Rattunde, Singh and Witcombe, 1989; Singh *et al.,* 1988). Rattunde, Singh and Witcombe (1989) showed in four composites that heritabilities from single plants were appreciable. Averaged across composites, the estimated heritabilities for 19 traits varied from 0.29 for yield, 0.45 for flowering time, to 0.64 for panicle length, indicating that mass selection will be effective for many important traits. Singh *et al*. (1994) demonstrated a significant gain from

mass selection in the New Elite Composite (NELC) to produce a high-yielding open-pollinated variety, ICMV 155, that was released for cultivation in India.

Maize
Hallauer and Miranda (1988) report many studies on mass selection in maize in the 1960s and 1970s. The responses to mass selection were high given the simplicity of the method, but few of the experiments were conducted over more than three cycles. Examples of longer-term experiments are gains in grain yield of 19.1 percent per cycle over ten cycles (Genter, 1976) and gains of 2.1 percent per cycle over six cycles (Lonnquist, Cota and Gardner, 1966). More recent studies on mass selection are few. Weyhrich, Lamkey and Hallauer (1998) report on a comparison of selection methods in a population. Over ten cycles of mass selection an average gain of 0.6 percent per cycle was achieved, the lowest of the gains per cycle in the various selection methods (from mass selection to S_2). However, it was superior to half-sib family selection in gains per year. It was also the most superior of the methods in terms of cost-effectiveness; the costs per unit of gain were the lowest and the returns on investment the highest.

7.4 IMPROVEMENTS OVER SIMPLE MASS SELECTION
7.4.1 Gridded mass selection – do grids really help?
Various authors have suggested improvements over mass selection. Gardner (1961) developed an improved method of mass selection—gridded (or stratified) mass selection—and demonstrated its effectiveness in improving grain yield in maize. Burton (1974) reported on recurrent restricted phenotypic selection in Pensacola Bahiagrass (*Paspalum notatum*) that differs from mass

selection by having five restrictions, the two most important being stratification (grids) as well as the control of pollination so that both male and female parents are selected (see below).

Rattunde, Singh and Witcombe (1989) examined heritability values in four pearl millet composites, using non-stratified and stratified data for many traits. Stratification was not worthwhile since the improvements in heritability with stratification were low and erratic. For stratification to be effective, uniform conditions are required within the strata, with a gradient in one or more environmental variables across them. In practice, such conditions seem to occur rarely in typical pearl millet experimental fields, which tend to be either uniform or have random variation. Experience of mass selection in maize has shown that this was a common problem in this crop as well.

7.4.2 Improving mass selection by simple modifications to selection procedure

Discarding poor areas of the plot
When the principles of client-oriented breeding are followed, only one base population is improved per target domain. Given the small number of base populations, this allows sufficient resources to grow them in large plots. Hence, it is possible to remove all of the plants before flowering from patches and field margins where the crop has grown poorly and still leave a large population.

Equal plant spacing
Equal spacing of plants eliminates an important source of environmental variability and improves the between-plant heritability. In maize, the population is hill planted (two plants per hill) and thinned to one plant per hill. In pearl millet, the crop can be sown at a more than adequate seed rate and then thinned to a uniform spacing.

Realistic selection differentials
In many mass selection schemes, very high selection pressures are applied by selecting, for example, the 100 best plants from 10 000 (e.g. Weyhrich, Lamkey and Hallauer, 1998). Instead, we apply strong roguing (removal of undesirable plants). Although the selection differential applied will be lower, it will be more reliable; when only a few phenotypically best plants are selected the risk increases that a high proportion of the selected plants are 'mistakes' because they happen to be in environmentally better situations.

Moving grids
The removal of the undesirable or poor plants is done by the breeder walking between alternate rows and removing plants in the rows on either side that are inferior to their neighbours. This can be likened to a form of 'moving grid' where selection is done on the relative performance of plants in a small area and better accounts for random (patchy) variation than grids.

Avoiding selfing
In mass selection in maize, the selected plants are allowed to random mate with the rest of the population. However, any maize breeder looking at the ear of a single white-endospermed maize plant grown amongst many yellow-endospermed plants (or vice versa) cannot fail to be surprised by how numerous are the grains with the maternal grain colour that result from selfing. Even though reports in the literature report selfing rates of about 5 percent, this is not based on any extensive experimental data. The proportion of selfing may be considerably higher, particularly when wind speeds
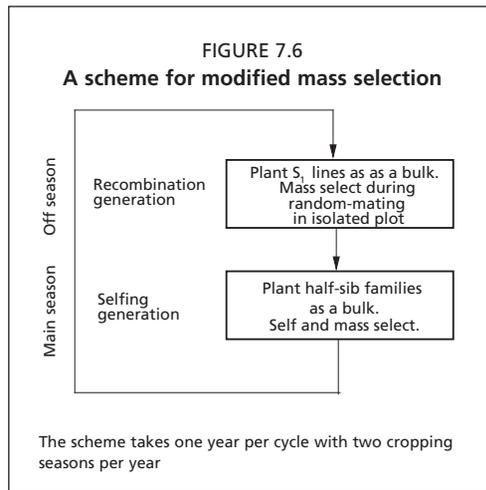
are low during anthesis. Because selfing may be high it makes good sense to see that the population to be mass selected is entirely outcrossed. The avoiding of selfing means that the mass selection is more efficient. Even with only 5 percent outcrossing, 5 percent of the plants have to be removed for inferior phenotypic performance because of inbreeding, without any genetic gain being made, and with higher selfing rates the problem increases.

Selfing is avoided by detasselling 50 percent of the population and after mass selection (which is done on all of the population of plants whether detasselled or not) only the ears from the detasselled plant contribute to the next generation. The detasselling does not add greatly to the labour involved as selection, which has to be done frequently to eliminate poor pollinator plants as early as possible, can be combined with detasselling. Alternatively, unskilled workers can be employed to do the detasselling.

Selection before pollen shed

Frequent, early selection, rather than selection just at maturity, means that many plants can be eliminated before pollen shed. Hence, selection is exerted not only on the female parent but to a lesser extent on the male parents as well (so $c > 0.5$ but < 1 in the equation below).

One form of modified mass selection is to employ alternate selfing and recombination generations (Figure 7.6). This is feasible in pearl millet as selfing only involves bagging panicles, whereas controlled crossing between the tassel and silk is required in maize. The major advantages of employing alternate selfing generations is control of pollination in the selfing generation, which doubles the efficiency of mass selection as it is applied to both the male and the female parents. In the subsequent recombination

FIGURE 7.6
A scheme for modified mass selection

The scheme takes one year per cycle with two cropping seasons per year

generation, higher selection efficiency is achieved because the between-plant heritability between S_1 plants is higher than between S_0 plants.

Of practical interest is the question of in which generation is selection the most efficient? Predicted gain with varying degrees of pollination control can be estimated from the following equation (Hallauer and Miranda, 1988):

$$\Delta G = ic\sigma_A^2//\sigma_A^2 + \sigma_D^2 + \sigma_E^2$$

where: i is the selection intensity or standardized selection differential; and c is a coefficient where $c = 0.5$ (no control over pollination and selection after pollination) or $c = 1$ (control over both male and female gametes as in the case of selfing). The remaining terms refer to the square roots of additive (σ^2A), dominance (σ^2D) and environmental (σ^2E) variances. Selfing controls the source of both male and female gametes ($c = 1$). In simple mass selection, the selection takes place after pollination, so selection is only on the female parent ($c = 0.5$). Hence, controlling pollination by selfing always doubles the selection efficiency over simple mass selection.

The efficiency of selection in the S_1 bulk is increased over simple mass selection by

1.2 to 2.4 (Dhillon, 1991). This is because the inbreeding caused by selfing increases the additive genetic variance between plants and recessive alleles are more frequently expressed phenotypically. However, values that are at least 2.0 (note 2.0 is always achieved in the selfing generation) are rare and occur only when dominance is high (the additive genetic variance increases more) and when gene frequency is high (for the same reason). Hence, under most circumstances the selection efficiency will be highest in the selfing generation and it makes sense to grow this generation, rather than the S_1 bulk, in the season where the crop is most frequently grown. Further justification comes from studies in maize that showed that the correlation between performance *per se* of the S_1 plants (on which selection is based), and their general combining ability (that determines their contribution to the performance of the population), is not as high as theoretically expected (reviewed by Seitz, 1989). This reduces the expected efficiency of selection amongst S_1 plants.

7.5 HALF-SIB METHODS
7.5.1 Overview
We strongly recommend to any maize or pearl millet breeder not to use half-sib family methods of selection. The theoretical advantages of half-sib family selection are limited and experimental evidence supports the theory. The possibilities are too great of being misled by the effects of non-random mating in the production of half-sibs to make the method attractive. If the degree of selfing was higher in the crossing block in some half-sib families than in others (a likely occurrence because selfing will be higher in early and late flowering plants) then the non-heritable variation in between family means will be high.

7.5.2 The method
The genetic variance among-sib families accounts for only 1/4 of the additive genetic variance, whilst the remainder is within them. Hence, most of the selection has to be exerted within families where it is equal to the efficiency of simple mass selection. Unfortunately, many of the modifications to simple mass selection suggested above will not be practical in the context of a half-sib family trial.

The selection efficiency, however, is not entirely related to the proportions of additive variance among and within families. Entries can be replicated in the progeny trial to increase the heritability of the half-sib family means, whereas no replication is possible for the selection of individual plants within the families. However, since within-family selection is essential, no matter how many replications are used, it is desirable to have spaced plants that demand additional land. Once spaced plants are combined with replication of families the method becomes resource demanding.

7.6 FULL-SIB METHOD
Schipprack (1992) has discussed the high efficiency of full-sib selection compared with other methods. One cycle can be completed in two generations in a single year (Figure 7.4). This progeny testing method allows one cycle per year where the families are evaluated in the field and, unlike the case of half-sib families, the additive genetic variance between families is at least as high as the additive genetic variance within them. The full-sib method does not require an isolated plot for recombination. However, hand control of pollination is required to make crosses and, in pearl millet, this needs more labour than making selfs, whereas in maize the labour requirements are almost the same. There has to

be a sufficient number of these crosses to avoid inbreeding in later cycles.

7.7 METHODS REQUIRING INBREEDING

In general, if the breeding of open-pol-linated varieties is not combined with the breeding of hybrids, then any method that goes beyond the S_1 stage will not be efficient. The correlation between performance *per se* and general combining ability worsens with the degree of inbreeding. In methods that use inbred lines to make OPVs (Bänziger and Cooper, 2001) then combining ability trials of the inbred lines are required. This is efficient if hybrids are being bred, but much less so if the purpose is only to breed OPVs.
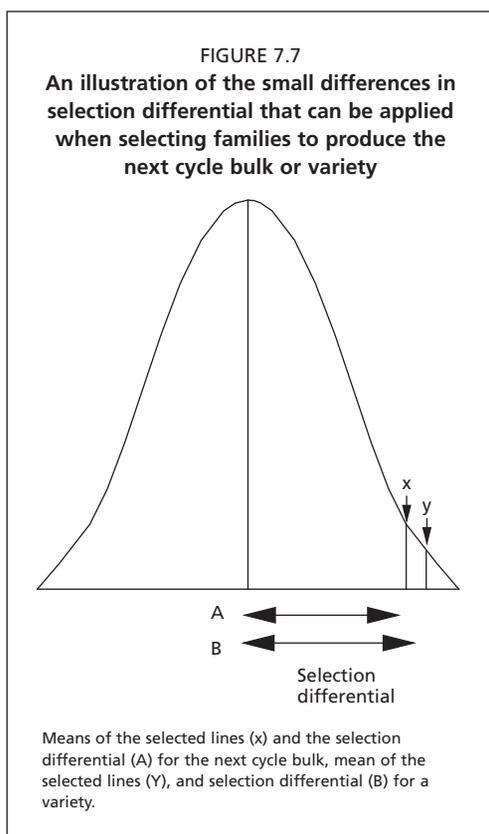
However, in all selection methods where there is no inbreeding (mass, half-sib and full-sib methods), selection for dominant alleles is less efficient compared with when some degree of inbreeding is used (e.g. S_1 or S_2). In pearl millet, when selecting for resistance to downy mildew, which is a dominant trait and determined by loci of large effect (Jones *et al.,* 1995), the efficiency of full-sibs as a test unit is lower than that of S_1 lines. With loci of large effect and dominant gene action, full-sib and S_1 testing can be directly compared from the predicted gains from these two methods (Hallauer and Miranda, 1988) without the need to determine the expected correlation between the performance of S_1 lines *per se* and their general combining abilities. The smallest disadvantage for full-sib selection will be when the recessive susceptible allele is at intermediate frequencies, but its greatest disadvantage will be when the allele is at a low frequency, since inbreeding will be required to uncover it. A good strategy, therefore, in breeding for disease resistance controlled by major resistance genes would be full-sib selection in the early stages

when the frequency of recessive alleles is high, followed by S_1 selection when some progress has been made in reducing the frequency of the susceptible alleles.

7.8 PRODUCING VARIETIES

The underlying theory behind the production of varieties involves two main parameters, the selection differential that can be applied to the entries in the progeny test, and the resultant inbreeding depression of the variety. A higher selection differential is applied to make a variety by selecting fewer lines than when the next cycle of the composite is recombined. The higher yield and reduced genetic variance expected from using fewer lines is desirable in a variety. However, the difference between the selection differential applied to produce a variety and to produce the next cycle bulk is inevitably small (Figure 7.7). Moreover, if a high selection differential is to be applied to making varieties, then even different subsets of entries selected according to varying criteria, such as location-specific adaptation, will inevitably have in common entries that are high-yielding in all locations. Greater genetic gains can be made by making and improving new composites than by increasing the number of varieties made from the same cycle of a composite. Consequently, only one or very few varieties should be made from every cycle of the composite.

As a small number of varieties (1 to 4) are made each cycle, larger numbers of selected families (10 to 25) are used to make varieties. Larger numbers give a more predictable selection response since sampling error is reduced; the smaller the number of lines the greater the proportional contribution of any line misidentified as high-yielding because of experimental error. The reduced selection differential that is applied

FIGURE 7.7
An illustration of the small differences in selection differential that can be applied when selecting families to produce the next cycle bulk or variety

Means of the selected lines (x) and the selection differential (A) for the next cycle bulk, mean of the selected lines (Y), and selection differential (B) for a variety.

when a larger number of lines are selected is compensated, at least in part, by the reduced level of inbreeding expected.

Inbreeding increases when fewer lines are used for making a variety. It also increases with the degree of inbreeding of the selected lines, since the more inbred they are, the greater the expected inbreeding of a variety made from them.

For S_1 lines, about 15 is optimal (Busbice, 1969), and in practice this gives good results. Since most composites are created and selected to have distinct morphological and phenological characteristics, the larger numbers of lines do not cause undesirable increases in variability.

Mass selection avoids any inbreeding caused by selecting a subset of lines, and avoids the error involved in selecting only

a few of them. In pearl millet, several high-yielding varieties have been bred from ICRISAT composites by mass selection within a population, of which the released variety ICMV 155 is one example (Singh *et al.,* 1994).

7.9 SUMMARY AND CONCLUSIONS

In the formation of composites, choice of initial parental material is dependent on the intended use of the population. Parents can be carefully chosen by understanding the needs of the clients. These can be determined by using farmer-participatory approaches. Information on farmers' selective adoption and the results of PVS trials can be used to identify useful parents. A compromise has to be achieved between high initial yield and genetic variance, and the number of parents used will vary greatly according to this compromise

Methods of making the initial cycle bulk vary from the simple to the elaborate. Ideally, the method should allow a visual assessment of the degree of recombination. When there are not too many parents, it is most efficient to use some form of diallel crossing in the first generation of random mating. Combining ability can be tested when forming a new base population by combining two varieties or populations.

Recombination of selected entries from the progeny trials during recurrent selection to produce half-sib families is fraught with difficulties, and makes the production of full-sibs an attractive alternative. This has the added advantage of providing a more efficient test unit in the subsequent generation.

Particular attention is paid to population improvement methods that take one year per cycle. Selection is carried out in an appropriate environment for traits that are important to the target group of farmers. Appropriate environments can be achieved

by making the research station environment match those of farmers' fields. Alternatively, selection can be decentralized to the farmers' fields. Managed stress nurseries can be used to select for abiotic stresses commonly encountered in the farming systems of the client farmers.

Plant breeders often disregard simple methods in favour of the more elaborate, even when simpler ones are more cost effective. Mass selection is very cost effective and there are many simple ways of increasing its effectiveness. In maize, these include the complete avoidance of selfing by detasselling to eliminate the errors involved in selecting among plants with differing degrees of inbreeding. In pearl millet, they include alternate selfing and recombination generations to allow control of pollination and to increase between-plant heritability.

Of the methods that use family testing, half-sib methods are theoretically the least efficient and experimental evidence supports this. The full-sib method is the only one that permits a field test with families that have a high between-family additive genetic variance, and completes a cycle of selection in only one year. Methods that involve a degree of inbreeding, such as S_1 and S_2, are best combined with hybrid breeding. However, even in the breeding of OPVs, S_1 testing can assist in the fixation of dominant alleles that determine disease resistance.

The theory relevant to the production of varieties from composites indicates that differences in yield between varieties will not be high, since all of them will be created employing similar selection differentials. A minimum number of entries are required to make a variety to avoid inbreeding depression and reduce the sampling error involved in the selection. Mass selection from the source population is a simple and effective way of making varieties.

REFERENCES

Almekinders, C.J.M. & Elings, A. 2001. Collaboration of farmers and breeders: Participatory crop improvement in perspective. *Euphytica,* 122: 425–438.

Bänziger, M. & Cooper, M. 2001. Breeding for low-input conditions and consequences for participatory plant breeding: Examples from tropical maize and wheat. *Euphytica,* 122: 503–519.

Burton, G.W. 1974. Recurrent restricted phenotypic selection increases forage yields of Pensacola bahiagrass. *Crop Science,* 14: 831–885.

Busbice, T.H. 1969. Inbreeding in synthetic varieties. *Crop Science*, 9: 601–604.

Ceccarelli, S., Grando, S. & Booth, R.H. 1996. International breeding programmes and resource-poor farmers: crop improvement in difficult environments. *In* P. Eyzaguirre & M. Iwanaga, eds. *Participatory plant breeding,* pp. 99–116. Proceedings of a workshop on participatory plant breeding. Wageningen, Netherlands, 26–29 July 1995. Rome, IPGRI.

Chambers, R. 1997. Learning to learn. In *Whose reality counts? Putting the first last*, pp. 102–129. London, Intermediate Technology Publications.

Dhillon, B.S. 1991. Recurrent mass selection based on selfed-plant evaluation in allogamous species. *Crop Science,* 31: 1075–1077.

Duvick, D.N. 2002. Theory, empiricism and intuition in professional plant breeding. *In* D.A. Cleveland & D. Soleri, eds. *Farmers, Scientists and Plant Breeding: Integrating Knowledge and Practice,* pp. 189–212. Wallingford, UK, CABI.

Fahim, M., Dhanapala, M.P., Senadhira, D. & Lawrence, M.J. 1998. Quantitative genetics of rice. II. A comparison of the efficiency of four breeding methods. *Field Crops Research,* 55: 275–266.

Falconer, D.S. 1981. *Introduction to Quantitative Genetics.* 2nd edition. London and New York, USA. Longman Group Limited. See pp. 214–218.

Fischer, K.S., Fukai, S., Lafitte, R. & McLaren, G. 2003. Know your target environment. *In* K.S. Fischer, R. Lafitte, S. Fukai, G. Atlin & B. Hardy, eds. *Breeding rice for drought-prone environments*, pp. 5–11. Los Baños, Philippines, IRRI.

Gardner, C.O. 1961. An evaluation of the effects of mass selection ands seed irradiation with thermal neutrons on the yield of corn. *Crop Science*, 1: 241–245.

Gardner, C.O. & Eberhart, S.A. 1966. Analysis and interpretation of the variety cross diallel and related populations. *Biometrics,* 22: 439–452.

Genter, C.F. 1976. Mass selection in a composite of intercrosses of Mexican landraces of maize. *Crop Science*, 16: 556–558.

Geiger, H.H. 1985. *Hybrid breeding in rye.* Proceedings of the cereal section on rye. Svalöf, Sweden. Part 1: 237–265. EUCARPIA.

Govil, J.N., Pokhriyal, S.C. & Murty, B.R. 1982. Full-sib and reciprocal recurrent selection in relation to pearl millet improvement. *Theoretical Applied Genetics*, 62: 25–30.

Govil, J.N., Pokhriyal, S.C. & Murty, B.R. 1986. Effect of recurrent mass selection on grain yield in pearl millet. *Crop Improvement,* 13: 67–70.

Hallauer, A.R. & Miranda, F.O. 1988. *Quantitative genetics in maize breeding*. 2nd edition*.* Iowa, USA, Iowa State University Press.

ICRISAT [International Crops Research Institute for the Semi-Arid Tropics]. 1987. *ICRISAT Annual Report 1986*. ICRISAT, India. See pp. 116–118.

ICRISAT. 1997. *Southern and Eastern Africa Region Highlights 1996*. ICRISAT, India.

Jones, E.S., Liu, C.J., Gale, M.D., Hash, C.T. & Witcombe, J.R. 1995. Mapping quantitative trait loci for downy mildew resistance in pearl millet. *Theoretical and Applied Genetics*, 91: 448–456.

Joshi, A. & Witcombe, J.R. 1996. Farmer participatory crop improvement. II. Participatory varietal selection, a case study in India. *Experimental Agriculture,* 32: 461–477.

Kapoor, R.L., Singh, F., Yadav, H.P. & Dass, S. 1983. Recurrent selections in pearl millet. *Haryana Agricultural University Journal of Research,* 13: 424–428.

Kelley, T.G., Rao, P.P., Weltzien, R.E. & Purohit, M.L. 1996. Adoption of improved cultivars of pearl milet in an arid environment: Straw yield and quality considerations in Western Rajasthan. *Experimental Agriculture*, 32: 161–171.

Khadr, F.H. 1977. Recurrent selection in pearl millet *Pennisetum typhoides* in Northern Nigeria. *Zeitschrift fur Pflanzenzüchtung,* 79: 145–153.

Lonnquist, J.H., Cota, O. & Gardner, C.O. 1966. Effect of mass selection and thermal neutron radiation on genetic variances in a variety of corn (*Zea mays* L.). *Crop Science*, 6: 330–332.

Monyo, E.S., Ipinge, S.A., Heinrich, G.M. & Chinhema, E. 2000. Participatory breeding: Does it make a difference? Lessons from Namibian Pearl Millet farmers. *In* N. Lilja, J.A. Ashby & L. Sperling, eds. *Assessing the impact of participatory research and gender analysis*. Cali, Colombia, International Center for Tropical Agriculture (CIAT).

Rattunde, H.F, Singh, P. & Witcombe, J.R. 1989. Feasibility of mass selection in pearl millet. *Crop Science,* 29: 1423–1427.

Schipprack, W. 1992. Estimation of population parameters and optimization of recurrent selection in pearl millet. PhD Thesis. University of Hohenheim, Germany.

Seitz, G. 1989. Experimentelle und theoreitische untersuchungen zur neziehung

zwiscen linieneigenleistung und allge-
meiner kombinationsfahigkeit bei silomais.
Dissertation. University of Hohenheim,
Germany.

Singh, P., Rai, K.N., Witcombe, J.R. &
Andrews, D.J. 1988. Population breed-
ing methods in pearl millet improvement
Pennisetum americanum. *L'Agronomie
Tropicale*, 43:185–193.

Singh, P., Witcombe, J.R., Raj, A.G.B.R. &
Rao, M.N.V.R. 1994. Registration of 'ICMV
155' pearl millet. *Crop Science*, 34: 1409.

Snapp, S. 1999. Mother and baby trials:
a novel trial design being tried out in
Malawi. *TARGET. The Newsletter of the
Soil Fertility Research Network for Maize-
Based Cropping Systems in Malawi and
Zimbabwe*. Jan. 1999 issue. CIMMYT,
Zimbabwe.

Sumberg, J. & Reece, D. 2004. Agricultural
research as new product development.
Experimental Agriculture, 40: 295–314.

Tiwari, T.P. 2001. Participatory crop improve-
ment for maize/millet intercropping with
trees in the middle hills of Nepal. PhD
Thesis, University of Wales, Bangor, UK.

Virk, D.S., Singh, D.N., Kumar, R., Prasad,
S.C., Gangwar, J.S. & Witcombe, J.R. 2003.
Collaborative and consultative participatory
plant breeding of rice for the rainfed uplands
of eastern India. *Euphytica*, 132: 95–108.

Virk, D.S., Chakraborty, M., Ghosh, J., Prasad,
S.C. & Witcombe, J.R. 2005. Increasing
the client-orientation of maize breeding
using farmer participation in eastern India.
Experimental Agriculture, 41: 413–426.

vom Brocke, K., Presterl, T., Christinck, A.,
Weltzien E., & Geiger, H.H. 2002. Farmers'
seed management practices open up new
base populations for pearl millet breeding
in a semi-arid zone of India. *Plant Breeding,*
121: 36–42.

vom Brocke, K., Weltzien, E., Christinck, A.,
Presterl, T. & Geiger, H.H. 2003. Effects of
farmers' seed management on performance
and adaptation of pearl millet in Rajasthan,
India. *Euphytica,* 130: 267–280.

Weltzien, R.E., Whitaker, M.L. & Anders, M.M.
1996. Farmer participation in pearl millet
breeding for marginal environments. *In* P.
Eyzaguirre & M. Iwanaga, eds. *Participatory
Plant Breeding,* pp. 99–116. Proceedings of
a workshop on participatory plant breed-
ing. Wageningen, The Netherlands, 26–29
July 1995. Rome, IPGRI.

Weyhrich, R.A., Lamkey, K.R. & Hallauer,
A.R. 1998. Responses to seven methods of
recurrent selection in the BS11 maize popu-
lation. *Crop Science,* 38: 308–321.

Witcombe, J.R., Joshi, A. & Goyal, S.N. 2003.
Participatory plant breeding in maize: A
case study from Gujarat, India. *Euphytica,*
130: 413–422.

Witcombe, J.R., Joshi, A., Joshi, K.D. &
Sthapit, B.R. 1996. Farmer participatory
crop improvement. I. Varietal selection
and breeding methods and their impact
on biodiversity. *Experimental Agriculture,*
332: 445–460.

Witcombe, J.R., Joshi, K.D., Gyawali, S., Musa,
A.M., Johansen, C., Virk, D.S. & Sthapit,
B.R. 2005. Participatory plant breeding is
better described as highly client-oriented
breeding. I. Four indicators of client ori-
entation in plant breeding. *Experimental
Agriculture,* 41: 299–319.

CHAPTER 8

Methodologies for generating variability Part 4: Mutation techniques

Miroslaw Maluszynski, Iwona Szarejko, Chittaranjan R. Bhatia, Karin Nichterlein and
Pierre J.L. Lagoda

8.1 INTRODUCTION – ECONOMIC IMPACT OF INDUCED MUTATIONS

The use of various mutagens to generate genetic variation in crop plants has a history almost as long as that of conventional breeding. Induction of variability by irradiation of barley seeds with X-rays was already demonstrated in 1928 by Stadler. The application of this phenomenon has come a long way to become a real tool, not only in crop breeding but also in basic research on the plant genome, its structure and function. Breeders were the first to recognize the potential of induced mutations through analogy with spontaneous mutants, often selected as new plant types in many crops, from cereals to apples, not to mention ornamental and decorative plants. Many mutants with desired traits were selected in the second or third generation after mutagenic treatment and subsequently released as new cultivars after agronomic evaluation in regional and national trials. These or other mutants developed with mutations in desired traits, even though not released as new cultivars, have been used in cross-breeding programmes as a source of particular alleles, often allelic to the spontaneous ones, but in a desired genotype. Among them were sources for characters such as short stature and lodging resistance; disease resistance; oil quality; and increased nitrogen fixation. These mutated genes are especially valuable as the best currently grown cultivar was usually selected for mutagenic treatment. A desired mutation in a good genetic background is a very attractive component in breeding programmes. This approach is much simpler and faster than crossing with an exotic source, and it is one of the main reasons for the wide use of mutated alleles in the breeding of numerous species.

8.2 CULTIVARS OBTAINED BY MUTATION BREEDING

By 2000, the FAO/IAEA Mutant Varieties Database (MVD) had collected information on 2 252 cultivars obtained by mutation and officially released in 59 countries world-wide, mainly in Asia (1 142), Europe (847) and North America (160). Almost half of these cultivars (1 019) were released after 1985. The list of crop species with induced mutant cultivars reached 175 in 2000, compared with 154 crop species in 1995, which indicates increased dynamics in the contemporary application of this technique in plant breeding. This list included many important crops, including rice, wheat, cotton, oilseed rape, sunflower, sesame and grapefruit. Of the 2 252 cultivars, 75 percent (1 700) are in crops and the rest (552) in ornamental and decorative plants. In sexually propagated crops, with 1 603 mutant cultivars released, cereals (1 072) dominate, followed by legumes (311), industrial crops (81), vegetables (66), oil crops (59) and others (111). About 70 percent of cultivars from this group were released as direct mutants; the remaining 30 percent were developed as recombinants from crosses with mutants. Of 1 585 directly developed mutant varieties, the great majority (1 411) were selected from mutated generations following the use of radiation, mainly gamma rays, as the mutagen (Maluszynski *et al*., 2000).

Most of the desired genetic variation explored in breeding programmes has occurred naturally and is preserved in germplasm collections. However, when these collections fail to provide a source for a particular trait, it is necessary to resort to other sources of variation. In such cases, mutation techniques provide tools for the rapid creation of desired traits. Even though the great majority of induced mutations are recessive and deleterious from a breeder's

point of view, it is possible, with proper selection tools, to find desired genotypes from adequately large mutated populations. As a result of these unique possibilities, mutation techniques have significantly contributed to plant improvement worldwide, and have had an outstanding impact on the productivity of some crops.

Rice

Among 434 mutant cultivars of rice, cvs. RD6 and RD15, developed after gamma ray treatment of an old local variety, KDML105, have had enormous economic impact. They were released in Thailand in the late 1970s. According to the Bureau of Economic and Agricultural Statistics in Bangkok, during 1989–1998 these two varieties were planted on a total of nearly 24 million hectares and yielded 42 million tonne of paddy, or 26.9 million tonne of milled rice, worth US$ 16.9 billion. More than 20 years after their release, both varieties are still grown extensively in Thailand. The gamma ray-induced sd_1 mutation, allelic to the spontaneous 'Green Revolution gene' sd_1 in rice, led to the release of the mutant cultivar Calrose 76 in California in 1976 (Rutger, 1992). This mutated gene has been extensively transferred by crosses into other genetic backgrounds, which resulted in the release of 20 new cultivars in countries on three continents. This includes the leading Australian mutant cv. Amaroo, released in 1987. This semi-dwarf cultivar covered 60-70 percent of the rice cultivation area, and on average yielded 8.9 t/ha. Gamma ray-induced cv. Zhefu 802 was the most extensively planted conventional rice cultivar in China between 1986 and 1994. This variety has a short growing period and high yield potential even under infertile conditions and poor management, which contributes to its wide adaptability.

Barley

In barley, two short-stature mutant cultivars have made a major impact on the brewing industry in Europe: cv. Diamant, released in 1965 in the former Czechoslovakia, and cv. Golden Promise, developed in Scotland in 1966 following gamma ray treatment. Both have added billions of dollars to the value of the brewing and malting industry. The X-ray-induced gene *denso* from Diamant, allelic to some spontaneous sources for semi-dwarfness, has been transferred to about 180 cultivars in Europe and other continents. Cv. Golden Promise has stiff straw, high yield and improved malting quality. It was widely used in the UK and Ireland for the production of whisky and beer. This variety contributed US$ 417 million to grain production in Scotland between 1977 and 2001. It is still popular for its high quality. It was recently discovered that Golden Promise is also salt tolerant. The FAO/IAEA MVD listed 269 barley mutant cultivars officially released in 28 countries (MVD, no date).

Wheat

In wheat, there are 197 officially released mutant cultivars. The most impressive is the mutant cv. Creso, released in 1975 in Italy. This durum wheat variety was obtained by crosses with a semi-dwarf mutant from variety Capelli, following thermal neutron treatment. Creso was grown on 400 000 ha and shared 53.3 percent of the market of certified durum wheat seeds in Italy as early as 1984. The estimated additional grain yield over the decade 1983–1993 of its cultivation was valued at US$ 1 800 million. The gene for semi-dwarfness from Creso is still used in breeding programmes in Italy, Austria, Bulgaria and other European countries where durum wheat is cultivated. More recently, due to some problems

with fertility of bread wheat cultivars with semi-dwarfness genes *Rht-B1* and *Rht-D1* in temperate climates, the mutant line 'Krasnodarskii karlik' with gene *Rht11* has become more widely used in cross breeding programmes in Europe and Australia.

Cotton

In cotton, mutagenic treatment with gamma rays led to the development of two very important cultivars – NIAB 78 in Pakistan and Lumian No. 1 in China. NIAB 78 was released in 1983 in Pakistan and during the following ten years it doubled cotton production - contributing more than US$ 3.0 billion. The added income to cotton growers due to the cultivation of this cultivar from the year of its release onwards has been estimated at US$ 486 million. NIAB 78, due to its wide adaptability, tolerance to heat and escape from bollworm attack because of early maturity, saved the textile industry of Pakistan, which was threatened by reduced cotton production. Annual cultivation of the high yielding mutant cultivar Lumian No. 1 exceeded one million hectares by the late 1980s. It was the most widely grown cotton cultivar in China.

Vegetatively propagated crops

Important results have also been obtained in breeding vegetatively propagated crops. Two outstanding examples are mutant cultivars of grapefruit and of Japanese pear. Budwood of grapefruit mutant cv. Star Ruby irradiated with thermal neutrons led to the release of the 'Rio Red' cultivar. This mutant cultivar was released in 1984 in Texas. It is seedless and has red flesh, red and stable juice colour, and good yield. The fruits of these cultivars are known under the trademark 'Rio Star'. They are grown on 75 percent of the grapefruit-producing area in Texas. The mutant cultivar 'Gold

Nijisseiki' of Japanese pear was developed with chronic irradiation in the gamma field in Japan. The cultivar is more resistant to Black spot disease and needs only one or two applications of fungicides per season. The additional annual income for growers is almost US$ 30 million (Ahloowalia, Maluszynski and Nichterlein, 2004).

8.3 MUTAGENIC TREATMENT
8.3.1 Radiation

The FAO/IAEA Database on Officially Released Mutant Varieties (MVD, no date) indicates that radiation, especially gamma rays, has been the most often used treatment for inducing mutations of crop plants. The reason for this is the simplicity of the treatment rather than to any higher efficiency of mutation induction. Seeds or other organs of the plant have to be delivered for irradiation to a nuclear centre. Such centres have been established in most countries. Nuclear centres are usually supervised by the Ministry of Energy or directly by the Prime Minister's Office. They will inform the plant breeder which centre is providing a 'seed irradiation service'. Mutagenic treatment is free of charge in most developing countries. Dry seeds (M_0 generation), disease free, with good germination ability and about 12–13 percent moisture content, can be sent for irradiation by regular mail. As the process of acute irradiation is very short, they should be returned to the breeder in the same way – by mail, within one or two weeks. This is possible because irradiated seeds (M_1 generation) or other plant organs are not radioactive and can be used directly for sowing or kept refrigerated awaiting the proper sowing period, even for a few months, depending on the crop species. A free-of-charge seed irradiation service is also provided by the FAO/IAEA Agriculture

Laboratory, Plant Breeding Unit, A-2444 Seibersdorf, Austria (<*Official.Mail@iaea. org*>). Any plant breeder can send seeds for gamma ray irradiation to this address. The scientists working there can advise on the dose of gamma rays or apply the dose requested by the breeder. They will also adjust seed moisture content if necessary.

Physical mutagens are also very useful for inducing mutations in vegetatively propagated crops and in *in vitro* cultures. Cuttings, immature spikes or Petri dishes with explants, calli, somatic embryos or microspores are often subjects of irradiation by ultraviolet (UV), gamma or X-rays.

8.3.2 Chemical mutagenesis

The use of chemical mutagens is also very simple and can be done in any biological laboratory with basic equipment. However, it should be kept in mind that most chemical mutagens are also strong carcinogens. For this reason, all steps of mutagenic treatment should be carried out wearing gloves and under a Biohazard flow-hood. These safety conditions are not necessary for treatment with sodium azide, which is a very powerful mutagen, but only for a limited number of species, including barley, rice, maize, oat, sorghum, sesame, jute and soybean. Numerous chemical mutagens have been successfully used for crop improvement (Table 8.1).

The mutagenic action of a chemical mutagen induces somatic and genetic effects in a treated cell, tissue or organ. After treatment of seeds, only unrepaired damage to the DNA in initial cells of the sporogenic layer (germline cells) are transferred as mutations to the next generation. Other mutations in somatic cells of the embryo, including mitotic chromosomal aberrations, together with toxic action of a mutagen on all components of cytosol, affect plant growth and development, and are called the 'somatic effect' of the mutagen.

The steps generally followed in mutagenic treatment of seeds with chemical mutagens are:
- pre-soaking in distilled water;
- pre-treatment rinsing in tap water;
- treatment with the mutagen;
- post-treatment rinsing in tap water; and
- drying (if necessary) on a filter paper.

All steps of mutagenic treatment should be done using glass beakers to avoid any interaction of chemical mutagens with even trace quantities of metallic cations or other active reagents. Seeds for each dose of mutagenic treatment (M_0 generation) and for the untreated control—usually the parent variety—are put into beakers that are visibly labelled with the applied concentration of mutagen.

As dry seeds are usually used for treatment, pre-soaking in distilled water should

TABLE 8.1
Chemical mutagens used most commonly in plant mutagenesis

Name	Abbreviation	Molecular weight[1]
Ethyleneimine	EI	43.07
Dimethyl sulfate	DMS	126.13
Diethyl sulfate	dES (DES)	154.19
Ethyl methanesulphonate	EMS	124.20
N-ethyl-N-nitrosourea	ENU (ENH)	117.11
N-methyl-N-nitrosourea	MNU (MNH)	103.08
N-methyl-N[1]-nitro-N-nitrosoguanidine	MNNG	147.09
Sodium azide	NaN3	65.01

Notes: (1) data from SIGMA, 2005

be applied to activate seeds physiologically before treatment with mutagen. The amount of water used in pre-soaking should be at least two to three times the volume of dry seeds. The beakers with pre-soaked seeds should be gently shaken a few times to remove air bubbles, which can block access of mutagen to embryos. Duration of pre-soaking depends on the biology of germination of a particular crop species. For example, in barley and other major cereals, 8–10 hours of pre-soaking in room temperature (20–24°C) is usually applied. Pre-soaking significantly reduces the somatic effect of chemical mutagen. Short washing, 2–3 times in room-temperature tap water should be applied after soaking to remove water-soluble substances leaching from the seed.

Such prepared seeds are ready for mutagenic treatment. It is advisable to use three doses of mutagen for a large-scale field experiment. This is especially desired for regions with very variable and unpredictable weather conditions during the growing period of mutagenetically treated material. Drought, cold and heat can significantly modify the somatic effect of a mutagen and influence the final effect of treatment.

The concentration of mutagen, its duration and temperature of treatment are understood under the term 'dose' in chemical mutagenesis. A temperature of mutagenic solution of 22–24°C is most often applied for the seed treatment of various crop species. The use of other temperatures is also possible. However, it should be noted that the increased temperature will significantly shorten the half-life of chemical mutagen and generate products of hydrolysis that can increase undesired somatic effect of a mutagen. This is especially relevant to treatment with mutagens such as dES or EMS. To obtain equal penetration of a mutagen through the cells of a seed embryo, it is necessary to treat seeds in a water solution of the mutagen for 3 to 5 hours. Similar to the pre-soaking, the treatment should be done with a significant surplus of mutagenic solution, some 2 to 3 times the volume of the dry seeds. In cereals, about 1–1.5 ml of mutagenic solution is applied per seed. The concentration of the mutagen should be considered, together with duration of the treatment. A shorter treatment time with higher concentration of mutagen can increase somatic effects and could be insufficient to penetrate equally all cells in the plant material. A gentler treatment requires a lower concentration but longer period of application.

Extensive post-treatment rinsing several times in room-temperature tap water is necessary to stop action of the mutagen and to remove its residues from the surface of the seeds. To facilitate sowing, the treated seeds can be dried on filter paper under a fume hood. However, too intensive drying, especially with increased air temperature, can enhance somatic effects of the mutagen. Surface-dry seeds are ready for sowing and are termed the M_1 generation. In a well organized laboratory, pre-soaking is done overnight and mutagenic treatment in the early morning. This allows the M_1 seeds to be sown the same day. Should this be impossible, due to prolonged pre-soaking or mutagenic treatment, the mutagen-treated seeds, after brief drying, can be kept in a refrigerator at a temperature of around 6 to 8°C.

Some mutagens are active in a particular acidity of a treatment solution. This is the case for sodium azide, which is a very efficient mutagen in several species if applied at low pH. For this reason, sodium azide is dissolved in a phosphate buffer at pH 3 and this solution is used for treatment.

8.4 INDUCED MUTATIONS IN CROP PLANTS

8.4.1 Determination of treatment dose

Choosing the treatment dose is probably the most important decision in mutation breeding or genomic research using induced mutation. As the selection of mutants is done in large mutated populations, any mistake in choosing the right dose of mutagen will determine the success or failure of the entire breeding programme. The description of generations after mutagenic treatment is given in Figure 8.1. Before deciding on the use of mutation techniques, a number of parameters need to be carefully considered, and they are discussed below.

The objectives

These can be divided into two groups. The first is the programme of improvement of one particular character in a promising cultivar or breeding line, and is most often chosen for the direct release of desired mutant line as a new cultivar, without the use of a cross-breeding approach. The second programme deals with basic research or with the development of new gene sources, not present in available germplasm, which have to be transferred by crosses into other breeding lines.

The plant material

The objective of induced mutations determines the plant material. Contrary to basic research on plant mutagenesis, where homogenous and homozygous lines are preferable, various breeding materials have been successfully used for mutagenic treatment to obtain new, improved cultivars. Most often, already released cultivars are

FIGURE 8.1
Schema of induced mutations project with description of mutated generations

Notes: a) seed before mutagenic treatment – M_0 generation; b) the same seed after mutagenic treatment – M_1 generation; c) M_1 generation in the field; d) individual M_1 plant with visible chimeric chlorophyll sectors; e) spike of M_1 plant with M_2 seeds; f) M_2 chlorophyll mutants at seedling stage; g) mutant selected in M_2 generation and its parent variety; h) M_3 generation; i) small plots of selected mutants in M_4 generation.
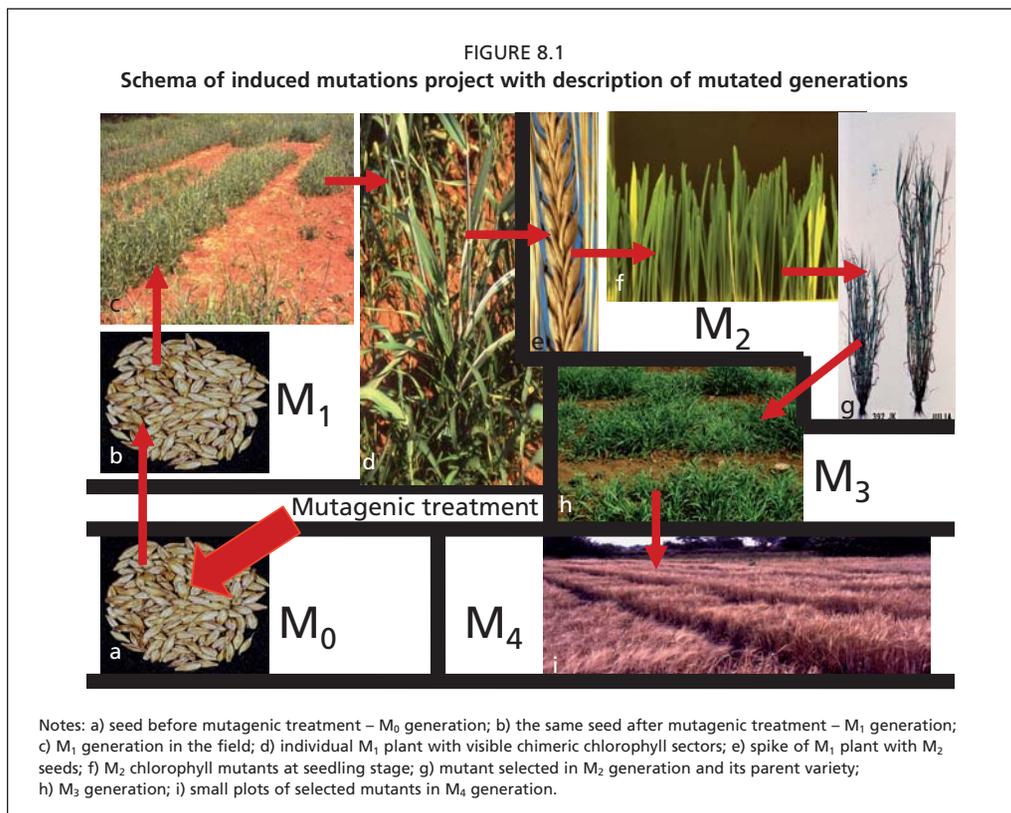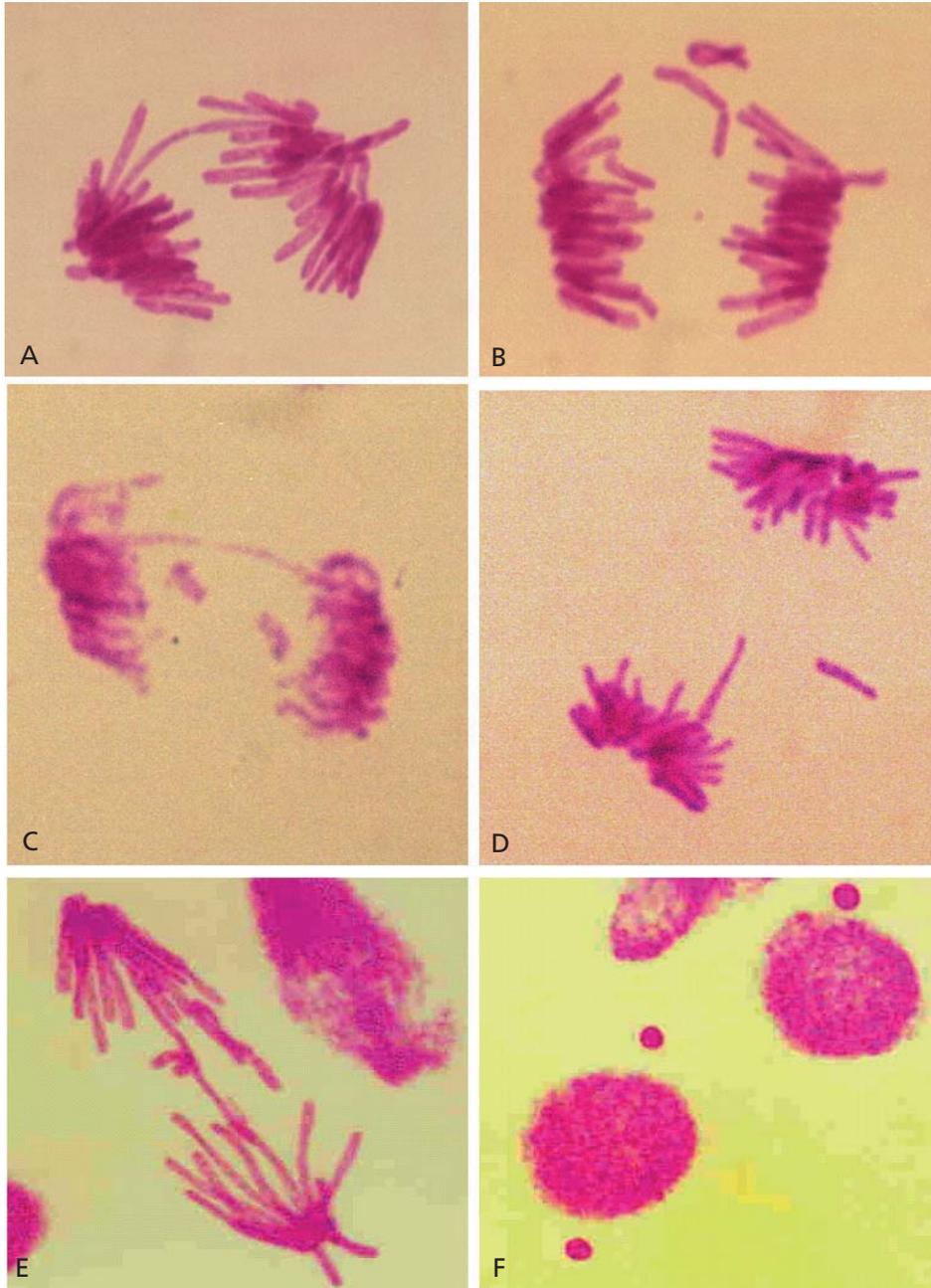
FIGURE 8.2
Structural chromosomal aberrations in anaphase of root meristems

A–B *Vicia faba* and C–D *Hordeum vulgare* after maleic hydrazide treatment; E–F *Vicia faba* after MNU treatment; a) bridge; b) two fragments; c) bridge and two fragments; d) fragment and delayed chromosome; e) bridge and numerous fragments: f) interphase with micronuclei *(courtesy of Dr. J. Maluszynska).*

mutagen-treated, to improve one or two characters that can significantly increase their agronomic value. Very often, promising breeding lines, F_1, F_2 and later generations of various crosses, doubled-haploid (DH) lines or even natural populations, have been used as material for treatment.

The mutation frequency of the gene responsible for desired character

Knowledge of the natural mutation frequency of the gene responsible for a desired character will help very much in choosing the mutagen and its dose. In the case of inducing short stature in rice and other cereals, a population of 10 000 to 30 000 M_2 plants is usually sufficient to find a desired phenotype. Unfortunately, in most cases, the frequency of mutation at the locus of interest in particular species is unknown, or a previously used mutagen unavailable. However, it should be clearly remembered that the frequency of mutations observed in one species or even in another cultivar of the same species differs markedly from another cultivar or species. The induction of mutation has two major steps: damage of DNA; and its subsequent repair. Both these processes, and especially DNA repair, depend on many cellular factors involving numerous enzymes. Diversity of the DNA repair machinery, among other factors, generates the variation in somatic and genetic effects of mutagenic treatment.

Somatic effects of mutagenic treatment in M_1

As a large mutated population at the M_2 or M_3 stage is necessary to select the phenotype desired, the level of somatic effects in the M_1 generation determines the amount of mutated seeds which can be used in the next generations. The level of somatic effects after mutagenic treatment can be evaluated on the basis of various param-

eters, including delay in seed germination; level of disturbances in the cell cycle; frequency of chromosomal aberrations in meristematic tissues (Figure 8.2); reduced seedling emergence; reduced seedling and plant growth; appearance of chlorophyll defects; and reduced fertility and plant survival. The term 'reduced' indicates change in expression of a particular character in relation to the control, usually the parent cultivar or the breeding line whose seeds were treated with a mutagen. The reduction is expressed as a percentage. In other words, the control has 100 percent of the value of any parameter and 0 percent of its reduction. It should be noted that all steps of mutagenic treatment should be the same for treated material and for parent variety, except for the use of mutagenic solution, which should be replaced by distilled water or pH buffer, if used.

Desired and possible size of the M_1, M_2 and M_3 populations

The size of the M_1 population is rather small in comparison with the following generations. In cereals, a few thousand seeds per treatment dose should be enough to obtain 10 000 to 30 000 seeds for the M_2 generation, if the applied mutagen dose was not too high. For other crops, the knowledge of seed production by an individual plant of the parent variety in the particular experimental field is very helpful for this calculation. The field size of M_2 and M_3 generations, together with the degree of difficulties in selection techniques, in great part determines the cost of the programme.

Selection technique to be applied

The change of a morphological character is most often the subject of selection. Easily visible characters such as plant height, til-

lering, flower and fruit shape and colour, but also resistance to herbicides, allows the screening of very large populations under field conditions. What is usually not possible or too costly is selection using any laboratory technique.

In summary, the chosen doses for mutagenic treatment should be relatively low for the improvement of a parent material with favoured genetic background. It should be noted that mutagenic treatment generates mutations in many other genes in the genome of each cell, not only at the desired locus. One result of using too high a dose is a high frequency of desired mutations, unfortunately also accompanied by a high frequency of deleterious mutations in other important loci. The deleterious mutations negatively influence the agronomic value of selected mutants. Such mutants selected in M_2 or M_3 generations usually have problems of low fertility, late maturity or susceptibility to stresses, i.e. the characters that directly influence yielding capacity. The use of high doses of mutagens, in the era of application of induced mutations termed as 'mutation breeding', was the reason that many programmes in the 1960s and 1970s did not yield the expected results. A dose causing 50 percent lethality (LD_{50}) was often suggested as the optimal for breeding programmes, resulting in too high a frequency of deleterious mutations. Nevertheless, it should be noted that low doses of mutagen decrease the frequency of mutations and in consequence a larger population of M_2 or M_3 is necessary to find the most desirable mutants in a promising genetic background. It is a good breeding practice to cross a selected mutant with its parent variety and select desired recombinant from the segregating F_2 generation. This approach, known as the 'cleaning method', helps in elimina-

tion of undefined deleterious mutations from a mutated genotype. Significant yield improvement of the selected recombinant with the mutated phenotype in relation to the original mutant is the best illustration that the elimination of deleterious mutations has been achieved, at least partly.

For the breeder without experience in the use of mutation techniques, the most difficult problem is to identify, in practice, a proper dose for mutagenic treatment, keeping in mind the considerations discussed above. There are several laboratory tests to help define a critical dose for both physical and chemical mutagens. The term 'critical dose' implies the dose of a mutagen beyond which the somatic effects in the M_1 generation are too high. In sexually propagated crops, doses of LD_{50} and above are definitely considered to be critical doses in current approaches to induced mutations for breeding purposes.

The simplest and cheapest laboratory test, suitable for the evaluation of somatic effects of mutagenic treatment in mono- and dicotyledonous crop species with any size of seeds, is a pot test for the measurement of emergence and seedling growth reduction. To perform this test, ceramic or plastic pots, 18–22 cm diameter, are all that are needed. The pots can be replaced by any other plastic container of a similar volume and size. Metal and wood containers should be avoided as they can release ions or active reagents influencing germination and seedling growth. For very small seeds, such as poppy, much smaller pots or containers can be used. The pots are two-thirds filled (about 4 cm below the top of the pot) with garden soil. One hundred seeds are sown on the soil surface of each pot and are covered by a few centimetres of sand. The depth of the covering layer depends on the size of the seeds. Smaller seeds should

be covered with less sand. However, the sandy layer should not be less than 1 cm. For cereals, a 4 cm sand layer is usually applied. Pots are transferred into a greenhouse, growth chamber or light room with a temperature typical for seed germination of the particular crop. Seeds of each treatment combination, including control (parent cultivar), should be sown in three pots, composing three replications. They should be watered according to the normal practice for the crop. All emerged seedlings should be counted in each pot when the number of seedlings in the control treatment is stable and no longer increasing. The average number of emerged seedlings from three pots of the control combination is taken as 100 percent of emergence. The reduction of the emergence in treated combinations is calculated relative to the control. For example, when the average emergence in the control is 96.2 seedlings and the average emergence in the lowest dose is 84.6, the emergence reduction is equal to 12.1 percent, according to the formula:

Emergence reduction (percent) = 100 - ((Average emergence in the dose × 100) / Average emergence in the control)

It is useful to present the emergence reduction data in the form of a figure (Figure 8.3).

In cereals, the seedling growth reduction can be measured when the second leaf becomes visible in the majority of seedlings of the control combination. For this purpose, the seedlings are cut on the sand surface and the height of each seedling should be measured with an accuracy ±0.5 cm (Figure 8.4). The calculation of results is similar to the previous one:

Seedling growth reduction (percent) = 100 – ((Average height of seedlings in the dose × 100) / Average height of seedlings in the control)

FIGURE 8.3
Emergence reduction of buckwheat (*Fagopyrum esculentum*) seedlings after seed treatment with 0.75-3.75 mM MNU (3 h, 24^{0}C)
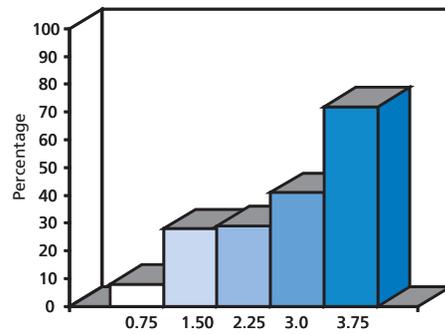
FIGURE 8.4
Seedling growth reduction of barley cultivar 'Maresi' after seed treatment with 0.5-3.0 mM MNU (3 h, 24^{0}C)
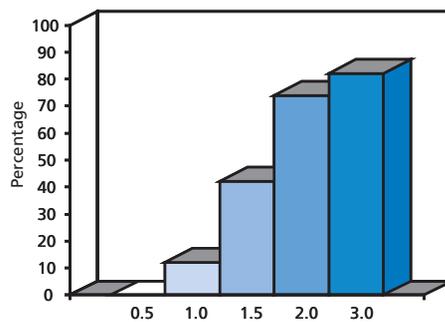
In tropical countries, this test can be done in a nursery bed or in hydroponics. The experiment should be organized in three blocks with randomly distributed small plots, each for 100 seeds. For cereal seedling growth reduction, a method called 'blotter sandwich' or 'growing-rack' can also be used. The method was developed by Professor C. Konzak in the early 1960s (FAO/IAEA, 1977). However, it needs a filter paper and special, but simple, equipment.

Pilot experiment

Regardless of the test used, any dose causing a reduction in emergence or seedling growth of greater than 30 percent is considered too high for a large-scale breeding programme with mutants. However, this is rather a general recommendation, as all somatic and genetic effects of the mutagen very much depend on the genetic background of the material treated, with strong differences not only between species but also between cultivars or breeding lines of the same species. For this reason, it is worthwhile to organize a pilot experiment to help compare the somatic and genetic effects induced by a range of doses in one or a few of the genotypes chosen for the breeding programme. The pilot experiment will delay the programme for one season, but it helps in proper selection of the dosages for further experiments with particular genotype(s) and protects the breeder from the failure of a large field experiment.

The best approach is to choose a few genotypes that are promising from the breeder's point of view, and to treat this material with at least four to five different doses of the mutagen, selected on the basis of the results from the pot test. Seeds from each dose and parent should be sown in plots (three replications, randomized blocks) according to normal agronomic practice. It depends on the crop, but usually one hundred seeds per plot should be enough for this purpose. The somatic effects listed earlier can be observed in the M_1 generation. However, it is most important to measure the growth, fertility and survival reduction at maturity. For survival reduction, all plants from the plot should be harvested and counted, except fully sterile plants. Based on the average number of surviving plants in relative to the control, the reduction in survival for each

dose is calculated, using the same formula as for emergence reduction, described above. Similarly, the measurement of the plant height of randomly selected 20–30 plants from each plot gives growth reduction. The simplest way to evaluate fertility reduction is to thresh all plants from the plot and weigh all seeds. In most species, these seeds, which are the M_2 generation, can be sown in containers to evaluate the genetic effect of the applied dose of mutagen on the basis of the frequency of point (gene) mutations. In practice, this can be done on the basis of the frequency of chlorophyll mutants among seedlings of the M_2 generation, as suggested by Ake Gustafsson for barley in the early 1940s (e.g. Gustafsson, 1941). From many cereal mutants with chlorophyll defects, three are very easily recognized, as the entire seedling has the same colour. These are the mutants: *albina* (white), *xantha* (yellow) and *viridis* (pale green). It is possible to find a similar type of mutation in other crops, including dicotyledonous species. For example, chlorophyll mutants can be easily observed in tomato M_2 seedlings at the cotyledon stage, before the first leaf develops. The observation of the mutants should be done a few days after emergence, as most of the chlorophyll mutations are lethal. The frequency of mutations (as a percentage) on a seedling basis (M_{sd}) is calculated according to the simple formula:

M_{sd} = (number of mutated seedlings
   (*albina* + *xantha* + *viridis*) ×100)/N

where N is the number of all M_2 seedlings analysed for a particular dose. An M_{sd} value greater than 3–4 percent should be considered as very high.

The results obtained from the pilot experiment guides selection of the dose for a large field experiment. The frequency of mutations in applied doses should be

the most important criterion, followed by the breeding objective of the programme. It should also be considered that genes controlling other, often useful, characters can mutate with lower frequency than the genes controlling chlorophyll. After choosing the dose, it is very important to calculate the size of the M_1 generation. In this step, the knowledge of survival and fertility reduction for a particular dose is necessary. These parameters help to calculate how many plants will survive after mutagenic treatment and how many seeds will be harvested as a result of the reduction in fertility in surviving plants. As the pilot experiment was performed under the climatic conditions of one season, it should be also considered that the level of somatic effects under the conditions of the next season could be different. For this reason, is very advisable, for the large-scale experiment, to grow the M_1 generation not only with the selected dose, but also with slightly lower and higher doses to be sure that even under different climatic conditions it will be possible to collect enough mutated seeds for the desired size of the M_2 generation. It is also a good practice to have a small plot of the parent cultivar in the close neighborhood to have a control comparator for the reaction of plants to growing conditions and management.

8.4.2 Handling mutated generations and mutant selection

M_1 generation

Mutagen-treated seeds should be sown in fertile soil and grown under good management practices, including the use of fertilizers. It is very important to maintain the M_1 crop at the proper soil moisture level, as plants with somatic effects are much more sensitive to stresses, especially drought. The use of herbicides should be avoided and replaced by mechanical means as some active components of herbicides, often also mutagenic, can influence the growth and development of injured plants. Sowing at double spacing in rows is often applied to avoid competition between M_1 plants with different levels of somatic effects. Good tillering will also allow exploitation of all mutations from different initial cells. When a high dose of mutagen is used, a significant delay in maturity should be expected.

As a multicellular tissue was the subject of mutagenic treatment and a different spectrum of mutations was induced in each cell, the tissues developing from the embryo carry cells with differently modified genomes and are chimeric. Induced genetic polymorphism among initial cells of the sporogenic layer influences the segregation ratio in the M_2 generation. The mutations in cells of somatic tissues are not transferred to the next sexual generation. Some morphological mutations in somatic tissues, such as chlorophyll defects, are often visible and they clearly illustrate the chimeric structure of the M_1 plant (Figure 8.5). The appearance of chlorophyll defects is a good indicator of genetic action of the mutagen. Somatic tissue chimeras are a valuable source of genetic variation in breeding of vegetatively propagated crops.

The method of harvesting M_1 is a key issue in exploiting induced genetic variation in sexually propagated crops. The method applied reflects various factors, including the biology of reproduction of a particular crop species, the cost of labour and of the selection method, the objective of breeding, and the possible size of the M_2 generation. Different methods can be chosen, ranging from the collection of only one seed per plant, to bulk harvesting of entire M_1 plots for development of the M_2 population. From a theoretical point of view, the best

FIGURE 8.5
Chlorophyll defects in M_1 generation after MNU treatment

Notes: a) xantha sector in barley; b) albina and xantha sectors in maize; c) large albina sector in narrow lupine; d) xantha sector in faba bean; e) xantha sector in pea; f) albina, xantha and viridis sectors in backwheat.

way is to harvest separately and thresh the spikes, pods or fruits from each individual M_1 plant and sow seeds using a spike-, pod- or fruit-to-row method. However, this is a rather unrealistic approach in practice. More often, in breeding programmes, a few spikes, pods or fruits from each M_1 plant are harvested, threshed and sown together.

M_2, M_3 and M_4 generations

Normal agronomic practices should be applied for cultivation of the M_2 and M_3 generations. Double-spaced sowing or planting can be used if changes in some morphological characters are the subject of selection in the M_2 generation. However, it should be considered that the small size of the sector built by a single initial cell and the high level of sterility in spike, pod or fruit of the M_1 plant can lead to few recessive forms in the segregating M_2 generation. Numerous mutations are not recognized in the M_2 generation, being obscured by the heterozygous stage. However, they will give Mendelian segregation in the M_3 generation. For this reason, it is good practice to postpone selection to the M_3 generation. Additionally, selection in the M_3 is done on a row or plot basis of homozygous plants, which significantly helps in distinguishing plants with only small, but often agronomically very important, morphological and especially quality characters. This is also the best way to evaluate resistance to biotic and abiotic stresses.

Depending on the generation in which the selection was performed, the homozygosity test can be done in M_3 or M_4. The preliminary evaluation of agronomic traits can be done with selected mutant lines in the M_4 generation. Crosses with a parent variety, other mutants or promising breeding lines are also often initiated in the M_4 generation.

Advanced generations

The selected mutants along with the parent cultivar should be entered into national yield, disease and pest nurseries wherever possible. Growing the mutants in multi-location trials and as off-season crops helps in advancing the generations, while evaluating performance in agroclimatic environments different from that of the main experimental site helps in selecting mutants with wider adaptability. The breeders should also ensure availability of adequate quantity of seed to enter in the regional or national trials. It is also desirable to use these homozygous mutants in the conventional cross-breeding programme of the station. Information on official release of new cultivars derived through mutation techniques should be sent to the MVD at the Joint FAO/IAEA Division, Vienna, Austria. Such information can help other breeders to determine appropriate dosages or selection procedures for developing new, improved cultivars.

8.4.3 Induced mutations in doubled-haploid systems

Doubled-haploid (DH) techniques, such as anther and microspore cultures, wide hybridization, and ovary and ovule cultures have become well established in a range of economically important crop species, including major cereals and the brassicas (Maluszynski *et al.*, 2003). Application of DH system in a conventional breeding programme saves many generations for the production of pure breeding lines. It also enhances effectiveness of selection of desired recombinants, especially when quantitative traits are evaluated.

The same benefits are evident when a DH system is employed in the process of mutant induction and selection. The most important advantages of applying DH systems in mutagenesis include the shortening of time needed for selection of true-to-type mutants; immediate fixation of mutated genotypes in the homozygous stage; screening for recessive mutations in the first generation after mutagenic treatment; and avoiding chimeric structure in M_1 plants. Additionally, if mutant selection

can be carried out *in vitro*, the haploid cells or embryos provide an extremely large mutagenized population, increasing the probability of identifying a rare mutation event. The advantages of combining mutation techniques with DH systems are apparent only when an efficient procedure of DH production is available for a particular crop. The recent progress in developing effective protocols for DH production, especially through isolated microspore culture, has made possible the application of mass-scale *in vitro* mutagenesis and selection methods in major cereals: wheat, barley and maize, similar to the previous achievements in oilseed rape. However, DH protocols are difficult to directly transfer between laboratories, and the success in producing a sufficient number of DH plants depends very much on the breeder's ability to grow high quality donor plants.

There are two main approaches for the use of haploid systems for mutant production. Most often, mutagenic treatment is applied to haploid cells (microspores) or organs containing haploid cells (anthers, spikes, panicles or flower buds) at, or before, *in vitro* culture. Mutagenic treatment of isolated microspores with gamma, X-ray or UV radiation proved to be an efficient method for mutation induction in oilseed rape. Chemical mutagens can also be applied to haploid cells *in vitro*, but the mutagenized cultures are more difficult to handle because of the requirement for extensive washing in order to remove the mutagen residues. The application of mutagenic treatment to the haploid cells or tissues in *in vitro* culture usually drastically decreases their regeneration ability. For this reason it is advisable to perform a mutagen sensitivity test for the haploid cell or embryo survival and regeneration capacity before setting up a large-scale experiment.

It should be noted that the doses of mutagens applied to cells *in vitro* should be at least an order of magnitude lower than the doses used for seed treatment. In *Brassica napus*, the irradiation doses used for mutation induction in isolated microspore cultures ranged from 5 to 15 Gy for gamma rays, 10 to 40 Gy for X-rays, and a dose rate of 33 erg mm^{-2} s^{-1} for 10 to 60 s with UV rays. In microspore cultures of barley treated with sodium azide, the mutagenic treatment lasted only 1 hour, and the concentration of the applied mutagen has not exceeded 1^{-4} M.

Another approach to mutagenic treatment with the use of a DH system relies on using M_1 plants derived from mutagenized seeds as donors for haploid production. In this method, seeds are treated with the doses of physical or chemical mutagens used in conventional seed mutagenesis. Treatment of dormant seeds instead of haploid cells in culture allows for application of much higher doses of mutagens, which provide the higher frequency of mutations in the DH population. Avoiding the somatic effects of mutagenic treatment on *in vitro* regeneration ability is another advantage of this procedure in comparison with the treatment of microspores in culture. Use of M_1 plants as donors for anther culture has been successfully demonstrated in barley and rice. In Peruvian barley cultivars, numerous DH mutants were produced from anther culture of M_1 plants developed by treatment with MNU and sodium azide. In rice, anther culture of gamma-irradiated M_1 plants resulted in the development of a short-duration upland rice mutant line, which in Myanmar matured 19 days earlier than the parent variety.

The mutagenic treatment of haploid cells can be followed by selection applied at the *in vitro* stage. If the selected trait is

expressed equally at the haploid cell or embryo and the plant levels, it is possible to apply a selection factor at the *in vitro* stage, maximizing the population size of individuals (cells or embryos) screened for a particular mutation. The selective agent, e.g. herbicide, should be used at a concentration near to LD_{100}. The feasibility of this system has been verified by recovering herbicide- and disease-resistant mutants in oilseed rape after *in vitro* mutagenesis and selection applied to isolated microspore cultures.

Oilseed rape haploid or DH embryos provide an excellent target for another early selection technique. Microspore-derived and zygotic embryos proved to have almost identical fatty acids composition and glucosinolate content. This was used to identify haploid embryos with the desired fatty acid composition, based on the analysis of one cotyledon. This non-destructive method of analysis allowed isolation of homozygous oilseed rape mutants with increased level of oleic acid and accompanying reduction of linoleic acid.

8.5. INDUCED MUTATIONS IN MOLECULAR BREEDING – TILLING

Recent advances in plant genomics, especially large-scale genome sequencing, have opened new possibilities for application of mutation techniques in crop improvement. Using the reverse genetic strategy called TILLING (Targeting Induced Local Lesions In Genomes), it is possible to induce a series of alleles in a target locus, providing that its sequence is known (McCallum *et al.*, 2000). The TILLING strategy was initially developed for model plant and animal species as a discovery platform for functional genomics, but soon it became a valuable tool in crop breeding as an alternative to the transgenic approach. The TILLING technique relies on a high

frequency of mutations induced by chemical mutagenesis, combined with a high-throughput screening method for single-nucleotide polymorphisms (SNPs) in the targeted sequence. The feasibility of this technology for generating a series of new alleles in a gene of interest has been already demonstrated in barley, maize and wheat, not to mention model organisms such as *Arabidopsis thaliana*, fruit fly, zebrafish and rat. Identification of 246 alleles of the *waxy* gene among EMS-treated M_2 individuals of bread and durum wheat is the best example of the potential of TILLING in creating new alleles of a gene responsible for an economically important character.

The basic TILLING methodology has the following steps:
• creation of a mutated population (M_2);
• isolation of the DNA from M_2 plants;
• PCR amplification of the targeted DNA segment using pooled DNA from M_2 plants as a template;
• denaturation and re-annealing of PCR products to form heteroduplexes between mutated and wild-type DNA strands;
• detection of mismatches in the heteroduplex using different procedures, e.g. cleavage by the specific endonuclease or denaturing high performance liquid chromatography (DHPLC); and
• sequencing the targeted DNA region in M_2 individuals composing the positive pool, for detection of the mutant.

As the first step in the TILLING procedure, large-scale mutated populations are generated. Most often, the chemical mutagen EMS is used for mutation induction, although sodium azide and MNU has also been used in barley and rice. Both these mutagens are known to induce a high frequency of point mutations. Usually, M_1 populations of 10 000–20 000 individuals are grown under good conditions after treat-

ment with two or three doses of mutagen. M_1 plants are harvested individually. Taking into consideration the chimeric structure of M_1 plants, seeds from different spikes, panicles or pods are often threshed separately.

Screening for mutations is performed in the M_2 population. Depending on the programme objective, M_2 populations consisting of several hundreds to 20 000 individuals are created. To prevent redundancy, usually one M_2 plant from each selfed M_1 individual or from one M_1 spike, panicle or pod is sampled. DNA from each M_2 seedling is isolated separately and the M_2 plant is grown to maturity. Each M_2 plant is harvested individually and M_3 seeds are carefully stored.

Screening for mutations in a target gene is based on detecting heteroduplexes between the wild-type and mutant-DNA fragments in the pooled DNA samples. In the first step, polymerase chain reaction (PCR) amplification of the targeted genome region is performed using DNA pooled from 5 to 8 M_2 individuals as a template. Next, the amplified DNA fragment is denatured and re-annealed, which allows formation of heteroduplexes between the wild-type and mutant DNA strands. Many approaches for detecting the mismatched sites within the heteroduplexes have been tried, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), denaturating high-performance liquid chromatography (DHPLC) or cleavage by the specific endonuclease. DHPLC was the first high throughput technology applied for the detection of heteroduplexes with the mismatched sites. However, cleavage of the mismatches with *Cel*I endonuclease (the novel plant enzyme isolated from celery), followed by the analysis of DNA fragments on denaturing polyacrylamide gels has

now become the most popular system for mutation detection. The advantage of this procedure is that it eliminates the number of false-positive mutation identifications, as both cleaved DNA fragments are labelled with different fluorescent dyes. With two-colour imaging, a true mutation has two mutant bands below the wild-type band in the same lane. The sum of the length of the mutant bands in a lane must equal the length of the wild-type band in order for the mutation to be confirmed.

Once the mutation is detected in a DNA pool, the target gene region in all M_2 individuals comprising this pool is sequenced and the M_2 plant carrying the mutation is identified. M_3 progeny of the identified mutant are then used for phenotypic evaluation of the mutated trait.

8.5.1 Case study: Development of waxy mutants in bread and durum wheat (Slade *et al.,* 2005)

Development of cereal varieties with waxy starch, composed almost entirely of amylopectine with little or no amylose, has been one of the most important objectives of commercial plant breeding. Waxy starch wheat has broad potential commercial uses in the food, paper and adhesive industries for making better quality products. Despite many breeding efforts over the last two decades, there are no wheat varieties with fully waxy starch. Using the TILLING approach for mutation generation and discovery, the research team from Anavah Inc., USA, induced and identified 246 mutated *waxy* alleles in two elite wheat varieties through only one experiment.

Development of TILLING libraries
Seeds of bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum* subsp. *durum*) were treated with two doses of

EMS. Elite commercial cultivars were used as the genetic material for mutation induction. For the bread wheat cv. Express, 0.75 percent and 1.2 percent EMS treatment was applied, while for the durum cv. Kronos the EMS concentrations were 0.75 percent and 1 percent. Each treatment lasted 18 hours and was preceded by a short (4 minutes) vacuum infiltration (pre-soaking) with H_2O. After treatment, M_1 seeds were washed for 4 to 8 hours and sown to produce M_1 plants, which were allowed to self-pollinate and were harvested individually. TILLING populations of about 10 000 M_2 individuals of hexaploid wheat and about 8 000 M_2 plants of durum wheat were created. To prevent redundancy, only a single M_2 progeny from each M_1 selfed plant was used in the study.

Screening for mutations at the waxy loci
The target sequences in the bread wheat involved 2 114 kb at the *Wx-A1* locus and 1 345 kb of the *Wx-D1* locus. In the durum wheat, 1 232 kb sequence of the *Wx-A1* and 487 kb of the *Wx-B1* genes were screened. DNA from 1 152 bread wheat and 768 durum wheat M_2 seedlings was used for mutation screening. Most of bread wheat individuals (768 M_2) and all M_2 durum plants analysed came from treatment with 0.75 percent EMS. DNA isolated from individual M_2 plants was pooled two to sixfold. The PCR amplification was carried out with the use of specific primers labelled with fluorescent dyes IRD700 and IRD800. The PCR products were digested with the *Cel*I enzyme, denatured and re-annealed. The samples were then separated on denaturing polyacrylamide gel using the LI-COR[2] DNA sequencer. Images were analysed visually for the presence of the cleaved DNA fragments indicating a mutation in the target region.

Allelic series of mutations in the target loci
In total, 246 new alleles at three *waxy* loci were identified in a population of 1 920 M_2 individuals used in the survey: 196 alleles in hexaploid and 50 in tetraploid wheat. The majority of mutations were G to A, or C to T transitions. Among the identified changes there were 84 missense, 3 truncation and 5 splice junction mutations. These new alleles encode waxy enzymes ranging in their activity from near wild-type to almost zero. A null mutant containing mutations in all three *waxy* homologues, highly desirable for wheat starch improvement, was also isolated. As the authors (Slade *et al.*, 2005) pointed out, the series of alleles created through TILLING in one experiment represent more genetic diversity than had been described in the preceding 25 years.

8.6. MUTATION TECHNIQUES FOR THE IMPROVEMENT OF MAJOR CROPS
8.6.1 Induced mutations in cereals
Cereals, of all crop species, have most often been the subject of improvement through the use of mutation techniques. In addition to major cereals such as rice, barley, wheat and maize, other species, including some exotic ones, have also been the subject of mutagenic treatment in several countries (Table 8.2). The majority of more than 1 000 mutant cultivars were obtained after radiation, especially gamma ray treatment, and directly released. However, the ease of mutagenic treatment rather than the kind of mutagenic specificity determined the use of radiation rather than chemical mutagens. Additionally, the bio-hazardous nature of most mutagenic components prevents their use in simply equipped laboratories. Nevertheless, numerous valuable mutants have been obtained with the use of MNU, EMS and sodium azide. Another tendency, observed more recently, is to transfer the

TABLE 8.2
Species and the number of officially released cereal mutant cultivars (FAO/IAEA Mutant Varieties Database – actualized)

Crop species	Common name	No. of released mutant cultivars			No. of countries with released mutant cultivars
		Total	Direct	Cross	
Avena sativa	Oat	21	5	16	4
Coix lachrymal-jobi	Job's tears	1	1		1
Fagopyrum esculenthum; F. sagittatum	Buckwheat	8	6	2	1
Hordeum vulgare	Barley	269	53	216	28
Oryza sativa	Rice	434	291	143	31
Panicum spp.; *Setaria* spp.	Millet	25	22	3	4
Pennisetum spp.	Pearl millet	5	3	2	1
Psathyrostachys juncea	Russian wildrye	1	1		1
Secale cereale	Rye	4	4		2
Sorghum bicolor	Sorghum	13	12	1	4
Sorghum durra	Durra	1	1		1
Triticum aestivum	Wheat	197	148	49	18
Triticum turgidum subsp. *durum*	Durum wheat	30	10	20	5
Zea mays	Maize	68	12	56	6

mutated gene to other cultivars by crosses. This is especially evident in barley, where 216 out of 269 mutant cultivars were developed by introduction of a mutated genetic source, mainly genes for semidwarfness, into new genetic backgrounds, and in maize, where only 12 out of 68 mutant cultivars were directly released.

Mutagenic treatment

Seed treatment is most often applied for mutagenesis of cereals. The pollen treatment method was developed by the team of Professor M.G. Neuffer from the University of Missouri, Columbia, USA, and successfully applied in maize to establish the largest mutant collection widely utilized in breeding and basic research (Bird and Neuffer, 1987). More recently, due to the success in DH production, microspore or anther cultures have become the subject of mutagenic treatment in major cereals.

All steps described in Section 8.3, 'Mutagenic treatment', should be followed in the treatment of seeds. For major cereals, 8 to 10 hours of pre-soaking in water are usually applied before treatment with chemical mutagen. To facilitate the work of plant breeders starting a mutation breeding programme, the doses of physical and chemical mutagens employed for seed treatment in some cereal species are listed in Table 8.3. These data can be helpful when planning experiments to estimate the critical dose and for the subsequent pilot programme or large-scale experiment.

Growing and handling the early generations

Growing the M_1 generation on fertile soil free of biotic and abiotic stresses is important for the production of adequate seeds for the M_2 generation. In rice, M_1 seeds are sown in the nursery, and seedlings are transplanted to the field according to the local practice. In cross-pollinated species, the M_1 plants should be kept isolated from untreated material. To grow plants under good conditions is a relatively easy task as the area needed for the M_1 population is small,

TABLE 8.3
Doses of physical and chemical mutagens used for seed treatment in cereals

Crop species	Mutagen	Range of doses
Rice	Fast neutrons	3–8 Gy
	X-rays	95–250 Gy
	Gamma rays	100–350 Gy
	MNH (MNU)	(0.7–1.5 mM) × (3–5 h)
	ENH (ENU)	(1.7–2.5 mM) × (3–5 h)
	EMS	(0.2–0.5 percent) × (8–20 h)
	NaN_3	(0.5–2 mM) × (3–5 h)
	EI	(0.01–0.03) × (3–6 h)
	DMS	(0.01–0.05 percent) × (4–6 h)
	Streptomycin	(800–2400 ppm at 15°C) × 40 h
Wheat	Fast neutrons	2–6 Gy
	Thermal neutrons	$1×10^{11}$ N/cm^2 – $8×10^{12}$ N/cm^2
	X-rays	150–250 Gy
	Gamma rays	50–350 Gy
	MNH (MNU)	(0.75–1.5 mM) × 5 h
	EMS (ENU)	(0.01–0.04 percent) × (10–30 h)
	NaN_3	(0.5–2.0 mM) × 5 h
	DMS	(0.005–0.04 percent) × 5 h
	DES	(0.4–1.0 percent) × 5 h
	EI	(0.04–0.09 percent) × (3–5 h)
	Streptomycin	(0.1–0.2 mg/ml) × (12–48 h)
Barley	Fast neutrons	2–5 Gy
	Thermal neutrons	$4.0×10^7$ N/cm^2 – $6.5×10^7$ N/cm^2
	X-rays	60–200 Gy
	Gamma rays	150–400 Gy
	MNH (MNU)	(0.5–1.0 mM) × 5 h
	ENH (ENU)	(1.0–2.5 mM) × 5 h
	EMS	(0.02–2.5 percent) × (8–20 h)
	NaN_3	(0.5–1.5) × 5 h
	Ethylene oxide	(0.02–0.04 percent) × (15–20 h)
	DMS	(1.0–1.5 percent) × (8–12 h)
	EI	(0.03–0.06) × (8–12 h)
Millet	Gamma rays	200–400 Gy
	DMS	(0.02–0.05 percent) × (15–20 h)
	MNH (MNU)	(1.0–1.7 mM) × (3–5 h)
	Streptomycin	(800–2 400 ppm at 15°C) × 40 h
Sorghum	Gamma rays	150–300 Gy
	NaN_3	(1.0–4.0 mM) × 4 h

Notes: The chemical mutagenesis was usually applied at a temperature of 20–24°C.

even if three doses of mutagen have been applied. Treatment of 2 000–4 000 seeds per dose should be sufficient to obtain a large M_2/M_3 generation. However, the size of the M_2 depends on the method of seed collection from the M_1 generation. If M_1 has been set up in three doses, the breeder can choose a population with a significant number of seeds during the harvest. They should consider the level of somatic effects, such as mature plant growth reduction, and fertility and survival reduction. The final decision on which dose seeds should be chosen for the M_2 generation can be taken based on the genetic effect of the mutagen. As M_1 plants are usually harvested individually (pulled with roots), the spikes or panicles can be collected from a few hundred plants and planted, without threshing, in boxes for evaluation of the frequency of chlorophyll mutants. Depending on the objective of breeding, and considering somatic and genetic parameters of mutagenic treatment, the breeder can choose from which dose to take the seeds for the M_2 generation. Plants from the other two doses can be threshed and seed stored as a reserve of treated material.

In classical work, one or a few spikes or panicles from each individual M_1 plant are treated separately: collected, threshed and sown as plant progenies, head-to-row method. Various modifications of this approach have been applied, such as harvesting single heads from each M_1 plant in bulk and sowing seeds in rows after threshing. Should these approaches be too laborious, mechanical harvesting in bulk, threshing and sowing can be also used. If selection is initiated in the M_2 generation, i.e. on a single-plant basis, seeds are usually sown double spaced. However, taking into consideration the low occurrence of recessives in the M_2 generation, it is strongly

suggested to postpone selection to the M_3 generation, which will allow selection to be carried out on a progeny basis. This is especially important if selection for biotic and abiotic stress tolerance or resistance is done under field conditions in the stress-prone area. In this approach, as many as possible, randomly chosen, M_2 plants are harvested, threshed and sown on a plant-progeny basis. This method is more laborious but increases the probability of selecting numerous plants homozygous for a desired character and helps avoid selecting false-positive mutants. To avoid having to thresh individual heads, the most laborious part of this method, interesting modifications were successfully used for the selection of semi-dwarf barley and salt-tolerant rice. In both cases, heads from each M_2 plant were harvested and sown—without threshing—the next season directly in the field. In the case of rice, the panicles were sown in a saline area. Numerous surviving lines, with mutants homozygous for these characters, could be directly selected, in addition to segregating progenies. Several hundred thousand M_2 plants could be characterized in the M_3 progenies using this approach.

Generally, both M_2 and M_3 generation are grown using normal agronomic practices. Any modification in plant cultivation depends mainly on the selection method. If the selection of desired mutants was initiated in the M_2, the seeds from selected plant (M_3) are sown on a progeny basis to check mutant homozygosity.

8.6.2 Case study: Development of malting barley cultivar 'Diamant' (Source: Based on FAO/IAEA, 1977)

The variety 'Valicky' was chosen for mutagenic treatment to improve lodging resistance and yield. This cultivar with high malting quality was first released (under the

name 'Valticky pivovarsky') in Moravia in the early 1920s, as a landrace selection of a shorter form of local cultivar 'Proscovcuv hanacky'. Valticky was re-released after the Second World War as a synthetic population of two types of this cultivar called types A and B, at this time grown in Moravia.

1956   6 000 dry seeds of cv. Valticky irradiated with 100 Gy of X-rays. The M_1 plants harvested individually.

1957 M_2 grown as plant progenies. Selection initiated in M_2. In one progeny, No. 228, higher tillering, short-straw mutants were detected.

1958–61   Progeny testing and seed increase of previously selected mutant line, designated as VR_Z.

1962–64   State variety trials including line VR_Z at multiple locations, demonstrating 10 percent higher average yields than other cultivars in the 31 trials.

1965   Official registration of new cultivar 'Diamant' in Czechoslovakia, differing from the parent cv. Valticky by the following characters: culm 10–15 cm shorter due to shorter internodes; about 10 percent higher yield, but equal quality of grain and malt; number of fertile tillers increased by 2–3; and tillering delayed by 10–14 days. Genetic investigation indicated that mutation at *denso* (*sdw1*) locus, mapped to chromosome 3 (3H), was responsible for the changed characters.

8.6.3 Induced mutations in legumes

Faba bean (*Vicia faba* L.) was extensively used in the early period of plant mutagenesis for investigating the effect of ionizing radiation and chemical mutagens on chromosomes. 'N.C. 4-X' was the first groundnut cultivar released for cultivation in the United States of America in 1959. It was developed by Gregory at North Carolina after exposing seeds to X-rays. Since then, several mutant breeding derived cultivars of legume crops have been released for commercial cultivation, increasing from 265 in 1999 to 337 to date (MVD, no date). The most common plant characters altered in the new cultivars are listed in Table 8.4. Theoretically, it should be possible to obtain mutation at any of the 25–30 000 loci currently estimated for plants, provided there are means to identify the induced changes, and can be used for screening large populations. Most of the mutation experiments are limited to the identification and selection of the 'visible' mutants in the field. These include mutations affecting the characteristics listed in Table 8.4. In addition, a large number of mutants altering symbiosis with nitrogen fixing micro-organisms have been identified in legumes using appropriate screening methods. Such mutants have been isolated in pea, soybean, common bean, faba bean, chickpea, groundnut and pigeon pea, and include mutants that either do not produce, have few or have ineffective root nodules. Hypernodulating and mutants that produce nodules even at otherwise inhibitory levels of nitrate concentration have been isolated after mutagenizing seeds in pea, soybean and common bean (Bhatia, Nichterlein and Maluszynski, 2001). A new soybean cultivar 'Nitrobean 60' that gave higher yield and contributed a greater amount of fixed N to the following cereal crop was developed in Australia after crossing an induced hypernodulating mutant. A day-length insensitive mutant in *Sesbania rostarata,* a green manure plant

TABLE 8.4
Most frequent and other characters altered in legume crops

Character most frequently modified	Yield, plant type, erect habit, dwarfness, branching habit, phytomass (biological) yield, leaf size and shape, flowering time, maturity, flower colour, number of flowers, pod and fruit characters (size, length, number of seeds per pod or fruit, non-shattering pods), seed and kernel size, seed coat colour
Other characters modified	Day length insensitivity in pigeon pea, mungbean and sesbania
	Afila-type mutant in pea resulting in modification of leaflets into tendrils which facilitates mechanical harvesting of green peas
	Terminal inflorescence in pigeon pea and faba bean
	Higher shelling percentage, thick or thin pod cover, harvest index, seed dormancy in groundnut
	Cotyledon colour in dry seeds
	Cold and drought tolerance in soybean and pea
	Resistance to bacterial, fungal, and viral diseases in several crops, and insect resistance in some
	Lodging resistance
	Drought resistance
	Superior nutritive value and protein content in cowpea and pea
	Fodder quality in lupin
	Nodulation mutants with hypernodulation, nitrate-tolerant nodulation, no nodulation and ineffective nodules have been isolated in specially designed experiments

TABLE 8.5
Successful gamma or X-ray doses for dry seed exposure in legume crops[1]

Crop species	Common name	Successful dose range (Gy)
Arachis hypogaea	Groundnut	150–250 (11)
Cajanus cajan	Pigeon pea	160 (1)
Cicer arietinum	Chickpea	100–200 (5)
Dolichos lablab	Hyacinth bean	240 (1)
Glycine max	Soybean	100–200 (20)
Phaseolus vulgaris	Common bean	100–200 (5)
Pisum sativum	Pea	100–200 (5)
Vigna radiata	Mungbean	100–200 (7)

Notes: (1) 'Successful doses' defined as the doses that led to the development, registration and release of a mutant cultivar directly without using the mutant as a parent in crossbreeding. The number of released cultivars is in brackets.

that produces aerial nodules on the stem, was identified when the crop was grown in the off season. The mutant produces tall plants with large phytomass and N-fixing nodules irrespective of planting time.

Mutagenic treatment
Gamma ray or X-ray exposure of the dry seeds is the most convenient method for creating genetic variability in legume species. Successful dose ranges—defined as the dose that led to the development, registration and release of mutant cultivars directly without resorting to cross breeding—are given in Table 8.5. Exposures of seeds to 100–200 Gy, except for faba bean, resulted in 49 out of 111 legume cultivars developed as direct mutants. Chlorophyll mutated sectors appearing on the first true leaves after seed germination in leguminous plants can be used to monitor the effect of radiation and chemical mutagens.

TABLE 8.6
Method for isolation and induction of mutations for use in breeding of grain legume crops

Generation	Operations
M_1	Expose 5000 to 10 000 seeds of the best available cultivar to 100–200 Gy of gamma or X-rays.
	Plant the M_1 generation following normal cultivation practices.[1]
	Harvest the first five pods, or all the pods, from each of the M_1 plants.
M_2	Grow the M_2 population as plant-progeny rows (minimum about 50 000 M_2 plants).
	Look for all morphological and physiological changes in each M_2 plant from seedling stage to harvest, and harvest the selected plants individually.
M_3	Grow the selected mutants as single-plant progenies and check for the segregation of the desired trait. Continue selection for the desired trait on single-plant basis. Uniform, non segregating mutant progenies, if any, can be bulked at this stage to hasten the breeding cycle.
M_4	Evaluate the expression of the selected trait and yield of the bulk lines in comparison with the parent as well as with the best check cultivar. Record observations on all agronomic parameters, disease and insect resistance in comparison with the parent cultivar. Repeat the procedure for the single-plant selections as outlined above.
M_5 onwards	Follow normal plant breeding procedures with the selected progenies. Evaluate the selected lines at more than one location. Enter one or two of the best lines at a time in national and regional evaluation trials, or to local farmers in participatory breeding programmes. Initiate seed multiplication to meet the demand of the mandatory trials for official approval and release of the cultivar.

Notes: (1) Outcrossing is increased in the M_1 plants due to pollen sterility induced by mutagenic treatments. It is desirable to grow the M_1 plants in isolation for facultative crosspollinating species. Selfing of the M_1 plants is essential for genetic experiments.

Growing and handling of the early generations

The general procedures for growing and handling the early generations are outlined in Table 8.6. These are based on over forty years of mutation experiments with groundnut, black gram, mung bean, pigeon pea, soybean and *Sesbania* sp. at the Bhabha Atomic Research Centre, Bombay, India, where over twenty new cultivars have been released for cultivation that have been developed using induced mutations. The procedures shown in Table 8.6 were used for the selection of early flowering, plant type, pod and seed size, and for other yield component traits. It was observed that it is relatively easy to find mutants for one of the yield components, such as number of pods per plant, pod size and length, number of seeds per pod or seed weight. Such mutants may not be superior to the parent in yield *per se*. Hybridization between mutants individually superior in

yield components resulted in selection of genotypes significantly higher in grain yield over the parent cultivar.

8.6.4 Case study: Development of black gram (*Vigna mungo*) cultivar 'TAU-1' (Information provided by Drs R.G. Thakare and S.E. Pawar)

'No. 55' was the best prevailing cultivar in the state of Maharashtra, India, with the drawback of small seed size (low hundred-seed weight of about 4 g). Constraint analysis indicated that increase in seed weight might enhance yield.

1974	500 seeds each exposed to 100, 200, 300, 400 and 500 Gy gamma rays. M_1 was grown and harvested as single plants.
1975	Single M_1 plant progenies grown as M_2 population. Approximately 400 progenies, 4 radiation doses and 30 plants per progeny, totalling to 48 000 plants, were screened for

mutants with alteration in plant type, yield components and seed size. Three large seed size and other putative mutants identified. Large-seed mutants were obtained following 300 Gy exposure.

1976   All mutants were progeny tested, and further selection of single plants continued in the M_3 and M_4 generations. True breeding lines were isolated, three with large seed size and 65 for other characters.

1977-78 Large seed size mutants evaluated in yield trials with parent No. 55 and a newly approved cultivar 'T-9'. Large seed size mutant yields were superior to the parent No. 55, but less than T-9 with still smaller seed size (hundred seed weight <4 g).

1979   Large seed size mutant lines 'UM-196' and 'UM-201' hybridized to T-9.

1980-81 F_1 and F_2 populations were grown, and selections made for large seed size, T-9 plant type and yield components, and advanced to F_3 and F_4.

1982-84 Selection '80-7' was found to give the highest yield in on-station trials.

1985-87 Line 80-7 was entered in the evaluation trials of the Punjabrao Agricultural University, and subsequently in the trials of the Coordinated Pulses Improvement Programme of the Indian Council of Agricultural Research. 80-7 gave 24 percent and higher yield over No. 55, and 9 percent over T-9, the national check cultivar. Its mean hundred seed weight was 4.8 to 5.0 g. It was first released for the Vidarbha region of Maharashtra State, India, in 1987, and later for the entire state, and also for Karnataka State in India.

At the time of official release as TAU-1, 150 kg of Breeder's, 3 560 kg of Foundation and 1 440 kg of Certified seed were available. Foundation seed production reached 40 tonne during 1994–95. Two crops per year were grown for experimental work.

8.6.5 Induced mutations in oil and fibre crops

Mutation techniques have been used for improvement of annual oil crops and crops providing bast and seed fibres (Table 8.7). Breeding objectives for using induced mutations are similar to other crops, although for oil crops there are unique objectives for the modification of oil quality (Table 8.8). Most of the oilseed mutant-derived cultivars released have been developed directly from mutants, but 50 percent of groundnut and 67 percent of linseed mutation-derived varieties were developed from

TABLE 8.7
Oil crops and fibre plants improved through induced mutations

Latin name	Common name
Oil crops	
Arachis hypogaea	Groundnut
Brassica campestris	Turnip rape
Brassica juncea	Indian mustard
Brassica napus	Rapeseed
Euphorbia fulgens	Euphorbia
Glycine max	Soybean
Helianthus annuus	Sunflower
Linum usitatissimum	Linseed
Ricinus communis	Castor bean
Papaver somniferum	Opium poppy
Sesamum indicum	Sesame
Fibre plants	
Boehmeria nivea	Ramie
Corchorus capsulari	Jute
Corchorus olitorius	Tossa jute
Gossypium sp.	Cotton
Linum usitatissimum	Flax

TABLE 8.8
Most frequent and other characters altered with induced mutations in oil and fibre crops

Oil crops	Fibre crops
Characters most frequently modified	
Higher yield	Higher yield
Altered plant type	Plant vigour
Early flowering	Earliness
Early maturity	
Higher oil content	
Other characters modified	
Modified oil quality (such as high oleic, low linolenic acid content)	Disease resistance
Increased resistance or tolerance to diseases and pests	Improved stress tolerance
Resistance to shattering	Lateness
Improved drought tolerance	

crosses with mutants. In fibre crops, 75 percent of the varieties have been directly developed, mainly through treatment with gamma radiation or X-rays, and 25 percent through crosses with mutants. For some of these varieties, in both oilseeds and fibre crops, remarkable economic gains have been reported. In breeding of oilseed rape and sunflower, spontaneous and induced mutants have been used in combination with conventional breeding methods to modify oil composition and increase yield.

Traditional oilseed rape has high erucic acid content in the oil and high glucosinolate levels in the meal, and both components are nutritionally undesirable. They have been reduced by breeders after identifying and using spontaneous mutants in the development of canola with less than 2 percent erucic acid and less than 30 µM/g of aliphatic glucosinolates in the meal. This process was facilitated by the development of analytical methods for quality assessment suitable for screening individual plants or single seeds of large breeding populations. Canola quality has been developed for *Brassica campestris* (turnip rape), *Brassica napus* (oilseed rape) and *Brassica juncea* (Indian mustard). Further improvements

were made using crosses with EMS-induced low-linolenic-acid mutants and radiation-induced high-oleic-acid mutants. The expansion of canola cultivation in Canada and Europe is primarily due to its modified fatty acid composition, i.e. low erucic acid, low linolenic acid and high oleic acid, combined with a low content of glucosinolates, which improved the nutritional quality of the oil for human nutrition, processing and storage, and the meal for livestock feed. Microspore mutagenesis has also been used in combination with *in vitro* screening for the development of *B. napus* tolerant to the herbicides imidazoline and chlorosulfuron. In Canada, Australia and Europe, many *Brassica* varieties with a modified oil-profile are based on mutant germplasm (Bhatia, Nichterlein and Maluszynski, 1999).

A number of mutant varieties with changed oil composition have also been developed in other species. In sunflower, after mutagenic treatment with dES, the mutant cultivar 'Pervenets' with high oleic and low linolenic acid content was developed, and widely used for hybrid production with high oleic acid content in the United States of America and Europe, to produce oil with the high oxidative stability preferred for

food processing (e.g. frying). The oil with high oleic acid content has steadily gained market acceptance, leading to increasing cultivation areas of high-oleic sunflower cultivars. In linseed, a doubled mutant has been developed after crossing two individually selected mutants with reduced linolenic acid obtained after EMS treatment, and used in further crosses, resulting in the release of a number of low-linolenic-linseed cultivars in Australia and Canada.

In cotton, the development of 'NIAB-78', a gamma-ray induced, high yielding mutant cultivar, released in 1983, had great economic impact in Pakistan. Developed at the National Institute of Agricultural Botany, NIAB-78 had a marked influence on sustaining the textile industry of Pakistan, and contributed to the national economy in several ways. The variety, ideal for a cotton-wheat rotation, had a shorter stature, determinate growth habit, tolerance to heat and escaped bollworm attack due to its early maturity. Within five years of release, its cultivation doubled cotton production in Pakistan, and even after 14 years of its first release, nearly 25 percent of the area under cotton in

Pakistan is planted to this cultivar. The new cultivar 'NIAB Karishma', released in 1996, and derived from a cross of the mutant cultivar NIAB-86 with an American strain 'W 83-29', is early maturing, has improved heat tolerance, high yield potential and has been cultivated on 486 000 ha.

Mutagenic treatment
Both ionizing radiation (gamma rays or X-rays) and chemical treatments have been applied to dry, dormant seeds of oil and fibre crops. The successful dose ranges for radiation, defined as the dose that led to the development, registration and release of varieties derived directly from mutants without using mutant crosses are given in Table 8.9. The doses given should be considered as an orientation, because tolerance to seed irradiation can differ between varieties. The recently released variety 'Abasin-95' was developed after gamma ray treatment of the Canadian variety 'Tower' using a much higher dose, 1 400 Gy, than for the development of most of the other oilseed rape varieties. Chemical mutagens were much less used; however, they led to the development of important commercial

TABLE 8.9
Successful gamma or X-ray doses for dry seed exposure in oil seeds and fibre crops

Crop species	Successful dose range (Gy)
Oil crops	
Arachis hypogaea (groundnut)	150–250 (11)
Brassica juncea (Indian mustard)	700
Brassica napus (rapeseed)	600–800
Glycine max (soybean)	100–200 (20)
Linum usitatissum (linseed)	100 (1)
Papaver somniferum (opium poppy)	50 (1)
Ricinus communis (castor bean)	400 (1)
Sesamum indicum (sesame)	100–200 (5)
Fibre crops	
Corchorus capsularis (jute)	250 (1)
Gossypium spp. (cotton)	200–400 (5)

Notes: Successful doses defined as the doses which led to the development, registration and release of mutant variety directly without using the mutant as a parent in cross breeding. The number of released cultivars is in brackets.

varieties with altered oil composition, e.g. high-oleic-acid sunflower (dMS), low-linolenic oilseed rape (EMS) and low-linolenic linseed, the so-called linola (EMS).

Growing and handling of the early generations

The general procedures for growing and handling the early generations of oil and fibre crops are outlined in Table 8.10. For the detection of plants with altered oil com-

position, the identification of mutants can be done using half-seed methods, screening M_2 seeds harvested from M_1 plants. For traits such as tolerance to biotic and abiotic stresses, it is recommended to do the mutant screening not on a single-plant level in the M_2 but in M_2 progeny rows (M_3). Mutants (M_4) with low agronomic performance, but valuable mutant traits, can be improved through backcrossing to the parent or by other crosses.

TABLE 8.10
Method for isolation and induction of mutations for use in breeding of oil and fibre crops

Generation	Operations
M_1	Expose 5 000 to 10 000 seeds of the best available variety or homozygote line to gamma or X-rays
	Plant the M_1 generation under optimal conditions to produce M_2 seeds, following normal cultivation practices, growing them in isolation or bagging before flowering.
	Harvest the first two to five pods, capsules, bolls or heads; the M_1 plants can be harvested individually if the M_2 will be grown in progeny rows, or in bulk if the M_2 will be grown in bulk.
M_2	For traits expressed and visible at the single-plant level: grow the M_2 population as plant-progeny rows (30–50 plants from each M_1 plant), and remaining seed can be stored for sowing in subsequent seasons.
	Look for all morphological and physiological changes in each M_2 plant from the seedling stage to harvest, and harvest the selected plants individually.
	For traits expressed at the seed level (e.g. oil composition in oil seeds): cut part of the M_2 seed (linseed, sunflower) or germinate M_2 seed (*Brassica* spp.) and cut one cotyledon for fatty acid analysis, then continue cultivating the seed or seedlings with desired fatty acid composition.
	For traits not expressed at the single-plant level: multiply seed of each M_2 plant, and harvest single plants for row evaluation in M_3.
M_3	Grow the selected mutants as single plant progenies and check for the segregation of the desired trait.
	Continue selection for the desired trait on a single-plant basis.
	Uniform, non segregating mutant progenies, if any, can be bulked at this stage to speed the breeding cycle.
	Select on an M_2 plant-progeny basis for traits such as resistance to stresses and quality (not reliably expressed on a single-plant basis).
M_4	Evaluate the expression of the selected trait and yield of the bulk lines in comparison with the parent as well as the best check cultivar.
	Record observations on all agronomic parameters, disease and pest resistance compared with the parent variety.
	Repeat the procedure for the single-plant selections, as outlined above,
	Mutants with valuable traits but undesirable characters should be 'backcrossed' with parent or used in crosses.
M_5 generation and beyond	Follow normal plant breeding procedures with the selected progenies: preliminary yield trials, multilocation evaluation of mutants or lines derived from crosses with mutants, submission of one or two of the best lines, at a time, for national or regional evaluation trials, or to local farmers in participatory breeding programmes.
	Initiate seed multiplication to meet the demand of the mandatory trials for official approval and release of the variety.

8.6.6 Oil crop Case study 1: Development of the high yielding oilseed rape variety 'Abasin-95' (Nuclear Institute for Food and Agriculture, Peshawar, Pakistan)

The objectives were to improve productivity and resistance or tolerance to biotic and abiotic stresses.

1988   10 000–15 000 dry seeds of oilseed rape (*Brassica napus*) cv. Tower with 10 percent moisture were irradiated with 1 000, 1 200 and 1 400 Gy gamma rays (60°C), and planted directly in the field in isolation, as M_1. At maturity, four pods from every primary branch were harvested and seeds were bulked on a dose basis.

1989–90 M_2 population was grown in rows and after every 20 rows the parent variety was included for comparison. Individual M_2 plants were selected on the basis of their short stature, early maturity, heavy bearing, long pods, more grains per pod, bold seed, stress tolerance or a combination. The mutant 'RM-152-2' (1 400 Gy treatment) and other mutants were selected, and further advanced to M_5.

1995   RM-152-2 officially released as a new cultivar: Abasin-95.

Two generations a year were grown from M_2 to M_4 to speed up the breeding process (winter in Peshawar; summer in Kaghan).

8.6.7 Oil crop Case study 2: Improving nutrition value.

Another case study describes the re-orientation of breeding objectives for linseed and use of induced mutations as a response to shrinking traditional markets for the traditional oil with good drying properties, as a result of the advent of plastic paints. A programme to change the oil quality from industrial (high linolenic acid content) to edible (low linolenic acid content) was conceived and proved to successful. Various steps—see Table 8.11—applied before and during the programme illustrate well the general principles that should be followed in the use of induced mutations in improvement of industrial crops.

8.6.8 Mutation induction enhanced breeding of asexually or vegetatively propagated crops

According to the FAO/IAEA Mutant Variety Database (MVD, no date), about three-quarters of all released mutant-derived cultivars are sexually propagated species, while only a quarter are asexually or vegetatively propagated (AVP) crops such as ornamentals, trees and shrubs, fruits, root and tuber crops, and sugar cane. Mutation induction shows its most promising aspects in AVP crops compared with cross-breeding methods due to its ability to change only a very few characters of an otherwise good cultivar without significantly altering the remaining, and often unique, genotype. Coupled to biotechnologies such as somatic embryogenesis or micropropagation, mutation induction might be considered an obvious means of conventional plant breeding, and as a possible shortcut for inducing desired genetic alterations in asexually propagated cultivars. Obviously, mutation induction is the only means for producing genetic variability in vegetatively propagated sterile crops and in obligate apomicts (Broertjes and van Harten, 1988).

As good mutation practice, the cultivar to be mutagenized is generally chosen for its outstanding agronomic performance and good adaptation, and the least number of

TABLE 8.11
Breeding linseed (*Linum usitatissimum*) varieties for new uses with altered oil quality

Step	Description of breeding procedure
Screening of crop germplasm	Previous screening for low linolenic acid in linseed germplasm, but none was found.
Screening of wild relatives	Screening in wild species for low linolenic genotypes, and some were found, but crossing with cultivated linseed failed.
Mutagenic treatment of linseed	Seeds of locally adapted variety 'Glenelg' were treated with gamma rays (300 to 900 Gy) and two doses of EMS (0.3 percent and 0.4 percent, 2 h at 2°C and 2 h at 20°C
Screening method development	A rapid, efficient half-seed screening method was developed using a colour reaction.
Mutant screening	Large M_1 and M_2 populations were grown, representative samples of M_2 embryos (seeds produced on M_1 plant) of each M_1 plant were screened.
Mutant confirmation	Reserve half-seeds of those with positive reaction were planted, selected and progeny tested; most proved false positive.
Genetic study	Two independent recessive mutants were induced by 0.4 percent EMS; both showed reduced linolenic acid contents (from 46 reduced to 28 percent linolenic acid) and proved to be at different loci; recombination of the two mutated genes.
Identification of double mutant	Identification of double mutant 'Zero' from recombination of the two single mutants, with only 2 percent linolenic acid (C18:3), and from 19 to 63 percent increased linoleic acid (C18:2).
'Backcrossing' of mutants to parent	Initial mutants showed low yield, and were backcrossed to parent 'Glenelg' to eliminate undesirable mutations. After four BC's, some lines exceeded parent.
Crossing of 'Zero' with other varieties	'Zero' was crossed with a number of varieties and breeding lines, allowing selection of high yielding recombinants with 'Zero' quality.
Release of varieties with altered oil quality	Release of low linolenic acid ('Linola TM') varieties 'Wallaga' (1992), 'Eyre' (1992), 'Argyle' (1994) in Australia; 'Coniston' (1992) and 'Derwent' (1992) in UK; and 'Linola 947' (1993) and 'Linola 989' (1993) in Canada.

Sources: From data presented by A.G. Green (CSIRO, Australia) and co-authors in numerous publications during 1984–2003.

genetic modifications required. Classically, plant material used when treating AVP crops are bud wood, bulbs, corms, dormant cuttings, rooted cuttings, scion wood, stolons, suckers, tubers or even whole growing plants (Table 8.12). AVP crop breeding programmes based on mutation induction face a major technical problem in chimera formation after irradiation of a multicellular meristem. In order to dissociate chimeras easily, the primordia to be used for treatment ought to consist of as few cells as possible. A mutation is a one-cell event, but multicellular apices generally consist of a number of fairly autonomous groups

of cell layers, comprising amongst others the epidermis and subepidermis, and have a number of meristematic cells in each layer. More or less small sectors of mutated tissue develop, restricted to one of those cell layers. Thus, chimera formation in most cases results in mericlinal chimeras, which only subsequently develop periclinal branches, shoots or tubers.

Different parameters influence the chances of a mutated cell developing into a sector or layer and to manifest itself. The major one is the position of the mutated cell within the apex. It follows that, after mutagenic treatment and before selection

TABLE 8.12
Radiation doses and plant material used for the induction of somatic mutations in AVP crops

Genus or crop	Plant material treated	Dose
Ornamentals		
Achimenes	Detached leaves	30 Gy
Alstroemeria	Rhizomes	4–6 Gy
Azalea	Rooted cuttings	10–20 Gy
Begonia	Detached leaves	15–25 Gy
Buddleia	Plants	20–30 Gy
Canna	Rhizomes	10–30 Gy
Chrysanthemum	Rooted cuttings	15–20 Gy; $6–12×10^{12}$n(th)/cm^2
Clematis	Rooted cuttings	2–5 Gy
Conifers	Rooted cuttings	0.5–5 Gy
Cosmos	Rooted cuttings	20 Gy
Crocus	Dormant bulbs, directly after harvest	10–15 Gy
Dahlia	Freshly harvested tubers	15–25 Gy
Dianthus	Rooted cuttings	40–60 Gy
Dianthus	Unrooted cuttings (base shielded)	80–100 Gy
Endymion	Detached leaves	1–5 Gy
Euphorbia	Rooted cuttings	30–50 Gy
Forsythia	Rooted cuttings	40–80 Gy
Gladioulus	Dormant corms (2n)	40 Gy
Hyacinthus	Bulbs, before-wounding basis	2–5 Gy
Iris	Freshly harvested corms	10 Gy
Kalanchoë	Detached leaves	15–20 Gy
Laburnum	Plants	20–30 Gy
Lilium	Bulb scales	2.5 Gy
Malus	Just-grafted plants	20–30 Gy
Muscari	Detached leaves	10–15 Gy
Narcissus	Dormant bulbs, directly after harvest	5–10 Gy
Ornithogalum	Detached leaves	5–10 Gy
Potentilla	Rooted cuttings	60–80 Gy
Prunus	Just-grafted plants	20–30 Gy
Rhododendron	Rooted cuttings	30–50 Gy
Roses	Budding wood	20–40 Gy
	Dormant plants	40–100 Gy
Saintpaulia	Detached leaves	30–40 Gy
Scilla	Detached leaves	1–5 Gy
Streptocarpus	Detached leaves	30 Gy
Syringa	Plants	30 Gy
Tulip	Dormant bulbs, directly after harvest	3–5 Gy
Fruit crops		
Apple	Grafts	30–40 Gy
		$4–7×10^{12}$ n(th)/cm^2
Banana	Corms	25–50 Gy
Blackberry	Young dormant plants	60–80 Gy
Blackcurrant	Dormant woody cuttings	30 Gy
Cherry	Grafts	20–30 Gy
		$4–7×10^{12}$n(th)/cm^2
Grape	Dormant buds	10–30 Gy
Lemon tree	Cuttings	20–70 Gy
Orange tree	Dormant scions	50 Gy

TABLE 8.12
Continued

Genus or crop	Plant material treated	Dose
Peach	Summer buds	10–40 Gy
Pear	Grafts	40–50 Gy
Plum (European)	Dormant scions	40–60 Gy
Raspberry	Spring suckers	10 Gy
Strawberry	Young runner plant	150–250 Gy
Other crop plants		
Cacao	Buds	10–20 Gy
Cassava	Nodes	30 Gy
Hevea	Dormant green buds	5–20 Gy
Potato	Dormant tuber parts	20–30 Gy
Sugar cane	Buds	20–60 Gy
Sweet potato	Detached leaves	30–40 Gy
Tea	Rooted cuttings	40–60 Gy

can be started, the mutated cells should participate in the formation of a shoot or plant. If some type of adventitious bud technique has been used, many of the mutants will be solid and early selection is possible. Work on irradiated multicellular apices necessitates the mutated sector size to be increased in order to quickly obtain completely homohistont tissue, or at least stable periclinal chimeras. These conditions can be reached in not fewer than 3 to 4 vegetative propagation cycles. The development of advanced *in vitro* techniques, and the extension of these techniques to otherwise neglected species, has created new potentials and opportunities to induce mutations in any AVP crop. *In vitro* plant material, such as apical meristems, adventitious or axillary buds, embryogenic calli, micro-cuttings, cell suspensions or protoplasts, compared with *in vivo* starting material, allows the treatment of larger populations in less space, and plantlets are maintained in a controlled, disease-free environment, facilitating the recovery of mutants. Avoidance or dissociation of chimera and rapid clonal propagation of useful mutants, as well as the production of a large number of plants for further evaluation based on *in vitro* plant material translates into an important gain in efficiency.

Mutagenic treatment

The most common mutagen used with AVP crops is radiation. Bulky material, like bulbs, stolons or scions for grafting, is difficult to treat in a reproducible way with chemicals. The chemical mutagen must penetrate to the meristematic zones, and the excess of chemical has to be removed after treatment. The procedures for acute or chronic irradiation are rather simple, with good repeatability and high mutation frequency. All types of ionizing radiation are effective; in practice, however, it is likely that only an X-ray machine or a gamma-ray source (e.g. [60]Co) are available. The dose to be applied depends on the radiosensitivity of the species, cultivar, plant development stage and also of the plant part to be treated. Plant parts that have developed new, adventitious roots and shoots, e.g. unrooted cuttings or freshly detached leaves, are more sensitive than plant parts with existing root and shoot meristems. Thus it is difficult to predict the dose that would be efficient in any new mutation experiment, even for the same cultivar. The best practice is empiri-

TABLE 8.13
Actual *in vitro Musa* mutation induction enhanced breeding process using shoot-tip culture and selection in the field

Steps	Time (months)	Additional information
Sucker from the field Shoot tip culture	T_0	1986 ; cv. Grande Naine (AAA) ; ITC collection
Radiosensitivity test	$T_0 + 6$	1987–1988; FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria
^{60}Co γ irradiation: M_1	$T_0 + 12$	Radiosensitivity tests on a minimum of 200 shoot-tips
Micropropagation M_1V_1	$T_0 + 13$	Irradiation of a minimum of 2000 shoot tips with an LD_{50} dose of γ-rays
Micropropagation M_1V_2	$T_0 + 14$	
Micropropagation M_1V_3	$T_0 + 15$	
Rooting M_1V_4	$T_0 + 16$	
Acclimatization to soil	$T_0 + 17$	1988–1990; line 'GN-60A'; putative early flowering mutant; glasshouse of the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria
Field Selection	$T_0 + 24$	
Stability confirmation and agronomic evaluation	$T_0 + 48$	
Micropropagation of desired plants	$T_0 + 60$	1990–1993; vegetative progeny sent to Honduras, Australia, South Africa and Malaysia for field-testing under commercial plantation conditions. Not all the plants were demonstrating earliness. The chimeric constitution of the original plant could explain this behaviour as only progenies deriving from the mutated sector gave rise to the putative early mutant.
Multilocation trials	$T_0 + 84$	September 1993, in Malaysia, only the early flowering plants obtained in the field were tissue cultured again to produce about 2000 plants for commercial evaluation in the United Plantations Bhd.
Cultivar release	$T_0 + 100$	1995; Malaysia cv. Novaria flowering about 10 weeks earlier than the original parental clone

Notes: LD_{50} = Lethal dose of 50 percent survival; V_x = Vegetative generation.
Source: N. Roux on the basis of his own and F. Novak's experiments at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria.

cal. Based on empirical data gathered over more than forty years, some dose ranges may be inferred as guidance for a test series (Table 8.12). For vegetatively propagated crops, doses less than LD_{50} are usually employed. A moderate dose that permits good growth and propagation of the material is to be preferred. Too many mutations per cell may be induced at high dose levels, with the risk that a favourable mutation is accompanied by one or more that are unfavourable. In AVP crops, it is very difficult—if not impossible—to separate favourable mutations from unfavourable ones occurring in the same cells because recombination through crossing or selfing

is greatly reduced, if not impossible. Which dose level should or can be applied depends on the crop, the type of propagation available, the numbers that can be handled and the selection method.

8.6.9 Case study: Banana mutation induction using in vitro plant material

Based on information from N. Roux on his own and F. Novak's experiments at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria.

1986   Initiation of shoot-tip culture. Suckers from the field of cultivar 'Grande Naine' (AAA), ITC collection.

1987–88   Radiosensitivity tests on a mini-
          mum of 200 shoot-tips, ^{60}Co γ
          irradiation, M_1, micropropaga-
          tion M_1V_1 to M_1V_3, rooting M_1V_4
          plants.
1988–90   Acclimatization to soil, field eval-
          uation, selection of 'GN-60A,'
          putative early flowering mutant,
          stability confirmation and agro-
          nomic evaluation in glasshouse of
          the FAO/IAEA Laboratory.
1990–93   Micropropagation of selected
          plants, multilocation trials, vegeta-
          tive progeny sent to Honduras,
          Australia, South Africa and
          Malaysia for field-testing under
          commercial plantation conditions.
          Not all the plants of GN-60A
          were demonstrating earliness. The
          chimeric constitution of the origi-
          nal plant could explain this behav-
          iour, as only progenies deriving
          from the mutated sector gave rise
          to the putative early mutant.
1993   In Malaysia, only the early flow-
          ering plants obtained in the field
          were tissue cultured again to pro-
          duce about 2 000 plants for com-
          mercial evaluation in the United
          Plantations Bhd.
1995   Cultivar 'Novaria' released in
          Malaysia, flowering about 10
          weeks earlier than the original
          parental clone.

$V_{\#}$ = Vegetative generation

Because the integrated use of muta-
tion induction and *in vitro* technology
speeds up the whole procedure, it is pos-
sible to increase the propagation rate and
generations per unit time and space, and
thereby enhance the economic efficiency
of the process. However, some bottle-
necks remain. With the increased use of

shoot-tip culture for *Musa* micropropaga-
tion, somaclones are being detected among
regenerated plants. This—mostly undesir-
able—variation interferes with the induced
mutations and makes the selection of useful
mutants more difficult. The actual process
is summarized in Table 8.13.

REFERENCES

Ahloowalia, B.S., Maluszynski, M. & Nichterlein, K. 2004. Global impact of mutation-derived varieties. *Euphytica*, 135(2): 187–204.

Bhatia, C.R., Nichterlein, K. & Maluszynski, M. 1999. Oilseed cultivars developed from induced mutations and mutations altering fatty acid composition. *Mutation Breeding Review*, 11: 1–36.

Bhatia, C.R., Nichterlein, K. & Maluszynski, M. 2001. Mutations affecting nodulation in grain legumes and their potential in sustainable cropping systems. *Euphytica*, 120(3): 415–432.

Bird, R.M. & Neuffer, M.G. 1987. Induced mutations in maize. *In* J. Janick, ed. *Plant Breeding Reviews*. Vol. 5, pp. 139–180. New York, USA, Van Nostrand Reinhold Company.

Broertjes, C. & van Harten, A.M. 1988. *Applied Mutation Breeding for Vegetatively Propagated Crops*. Amsterdam, Netherlands, Elsevier. 345 p.

FAO/IAEA. 1977. Manual on Mutation Breeding. 2nd edition. *Technical Report Series*, No. 119. Vienna, IAEA. 288 p.

Gustafsson, A. 1941. Mutation experiments in barley. *Heriditas,* 27: 225–242.

Maluszynski, M., Nichterlein, K., van Zanten, L. & Ahloowalia, B.S. 2000. Officially released mutant varieties – the FAO/IAEA Database. *Mutation Breeding Review,* 12: 1–84.

Maluszynski, M., Kasha, K.J., Forster, B. & Szarejko, I., eds. 2003. *Doubled Haploid*

Production in Crop Plants. A Manual. Dordrecht, Netherlands: Kluwer Academic Publishers. 428 p.

McCallum, C.M., Comai, L., Greene, E.A. & Henikoff, S. 2000. Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology*, 123: 439–442.

MVD [Mutant Variety Database]. No date. FAO/IAEA Mutant Variety Database. See: http://www-infocris.iaea.org/MVD/

Rutger, J.N. 1992. Impact of mutation breeding in rice - a review. *Mutation Breeding Review,* 8: 1–24.

Slade, A.J., Fuerstenberg, S.I., Loeffler, D., Steine, M.N. & Facciotti, D. 2005. A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology,* 23(1): 75–81.