Breeding Quality Protein Maize (QPM)

Protocols for Developing QPM Cultivars

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CIMMYT®

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CIMMYT® (www.cimmyt.org) is an internationally funded, not-for-profit organization that conducts research and training related to maize and wheat throughout the developing world. Drawing on strong science and effective partnerships, CIMMYT works to create, share, and use knowledge and technology to increase food security, improve the productivity and profitability of farming systems, and sustain natural resources. Financial support for CIMMYT's work comes from many sources, including the members of the Consultative Group on International Agricultural Research (CGIAR) (www.cgiar.org), national governments, foundations, development banks, and other public and private agencies.

Correct citation: Vivek B.S., A.F. Krivanek, N. Palacios-Rojas, S. Twumasi-Afriyie, and A.O. Diallo. 2008. *Breeding Quality Protein Maize (QPM): Protocols for Developing QPM Cultivars*. Mexico, D.F.: CIMMYT.

Abstract: This manual is intended for maize breeders who would like to start developing quality protein maize (QPM) cultivars. It is a compilation and consolidation of several breeding protocols successfully used at CIMMYT over two decades of QPM development and breeding. A brief background and the basic theory of QPM genetics are explained, leading up to detailed methods and procedures of QPM development.

ISBN: 978-970-648-160-3

AGROVOC descriptors: Zea mays; Plant breeding; Breeding methods; Genetic resources; Protein quality; Protein content; Application methods; Lysine; Tryptophan; Food composition

AGRIS category codes: F30 Plant Genetics and Breeding
Q04 Food Composition

Dewey decimal classification: 633.15

Printed in Mexico.

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Foreword

Agricultural scientists have long had an interest in improving the protein quality of plants. Though not nearly as low in protein content as staple foods such as cassava, the protein content of maize is still relatively low (generally about 10%); roughly half of that protein contains almost no lysine or tryptophan, two amino acids essential for building proteins in humans and monogastric animals.

In 1963, Lynn Bates, a Ph.D. student working with Professor Edwin Mertz at Purdue University, discovered much higher levels of lysine and tryptophan than normal in two maize landraces from the Andean highlands of South America. They were able to determine that the higher levels were due to the presence of a gene called *opaque-2.*

The discovery of *opaque-2* maize stimulated considerable research interest and activity, with high hopes of substantially improving the nutritional status of maize consumers, especially in developing countries. But, as is all too often the case in plants, a highly desirable trait turned out to be closely associated with several undesirable ones, and the initial enthusiasm soon gave way to disappointment. The *opaque-2* maize kernels were dull and chalky, had 15-20% less grain weight, and were more susceptible to several diseases and insects. These formidable obstacles prompted most research programs to curtail their work on *opaque-2* maize.

Only a handful of crop research institutes continued their work, most notably the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Using conventional planting breeding methodologies, the CIMMYT interdisciplinary research team—initially led by Dr. Surinder K. Vasal, a breeder, and Dr. Evangelina Villegas, a cereal chemist—slowly overcame the original *opaque 2* defects while maintaining superior nutritional quality. They were able to convert the floury soft endosperm kernels into harder types, increase grain yield potential to the level of the best normal maize types, and endow the *opaque-2* maize with disease and insect resistance, and with utilization and storage qualities similar to those of superior normal maize materials. The new, normal-looking, normal-tasting *opaque-2* types were renamed "quality protein maize" or QPM. Given the relatively few resources that have been devoted to developing QPM, the progress that has been achieved is remarkable.

A number of national maize research programs in the developing world have also done considerable work to develop QPM. Outstanding among these are South Africa, Brazil, China, Ghana and, more recently, India. In total, more than 1.2 million ha are planted to QPM varieties and hybrids in the developing world. Of this total, more than 700,000 ha are found in 15 countries of sub-Saharan Africa. Moreover, most QPM in Africa is produced for direct human consumption.

Quality protein maize is a valuable option as an animal feed, especially for monogastric animals, since it can help reduce the requirement for additional protein sources in balanced feeds. However, my great interest in QPM has always been as a nutrionally enriched food source for impoverished people in regions of the world where maize is a primary source of energy. Infant feeding trials with QPM have repeatedly shown that QPM used as a weaning food reduces stunting and increases weight gain, thus improving child health.

Since QPM maize is a nutritionally improved food crop for the poor, the issue of type of genotype is important. Due to cost and distributional problems, an improved open-pollinated QPM variety is generally better suited to poor farmers' needs than are hybrids. However, because the *opaque-2* gene is recessive, the dilution and gradual loss of QPM's nutritional quality through pollen contamination from non-QPM maize (with normal protein) is an ongoing concern. From the standpoint of ensuring that the *opaque-2* gene is retained in the grain, hybrids would be preferable. Lack of appropriate seed multiplication and distribution systems remains one of the great challenges facing QPM breeders and advocates.

I commend the authors for producing this practical manual on QPM improvement and seed production. It will contribute to the advancement of QPM and to dealing with the quality issues that I have raised.

Norman E. Borlaug

Acknowledgments

The authors are grateful to J. MacRobert and K. Pixley for their critical review of the manual. Thanks also go to J. Lopez-Cessati and E. Nurit for their help in revising the laboratory protocols. The authors would also like to thank K. Pixley for coordinating the review of this manual, Alma McNab for editing, and Antonio Luna and Miguel Mellado for design and layout.

Many CIMMYT and partner scientists, technicians, and workers have contributed their efforts to QPM research during its more than 30-year history. Each of them has added to our current understanding, summarized in this manual, of how to effectively and efficiently breed QPM. While it is not possible to individually acknowledge each of them, the scientific leadership of CIMMYT QPM programs by Surinder Vasal, Magni Bjarnason, and Hugo Cordova was instrumental in modifying and incorporating the *opaque-2* gene into a broad and useful QPM germplasm base, developing the foundations and initiating hybrid-oriented QPM breeding efforts, and identifying and promoting competitive QPM hybrid and open-pollinated varieties. We would also like to thank CIDA-Canada and the Nippon Foundation for their generous support of research on QPM.

In addition to the scientific leadership provided by CIMMYT, hundreds of colleagues and workers around the world contributed many thousands of hours of field and laboratory work to QPM breeding. Their efforts are gratefully acknowledged.

1. Introduction and History

Maize (*Zea mays* L.) is grown on more than 96.5 million hectares in the developing world (FAOSTAT-Agric. 2004), and many millions of people worldwide are dependent on maize as a staple food. Maize accounts for 15 to 56% of the total daily calories of people in about 25 developing countries (Prasanna et al., 2001). In Africa, maize supplies at least one fifth of total daily calories consumed and accounts for 17 to 60% of people's total daily protein supply in 12 countries, as estimated by FAO food balance sheets (Krivanek et al., 2007). These values are average per capita estimates; specific groups within these countries (children being weaned, sick children, sick adults, and everyone, when crop production is low) are even more dependent on maize as the major source of dietary protein. Protein-containing foods are necessary for the rapid growth of children (Millward and Rivers, 1989), and in some countries maize is a primary weaning food for babies.

Such dependence on maize as a protein source puts people at risk for dietary protein deficiency because maize protein (as most cereal protein) is deficient in two essential amino acids,[1] lysine and tryptophan; therefore maize is a poor source of protein for both humans and monogastric animals. Thus, any maize-based diet lacking in complementary proteinaceous foods that contain greater levels of lysine and tryptophan, such as meat, pulses, and dairy products, is considered protein-deficient. Protein deficiency, especially in children, causes kwashiorkor, a potentially fatal syndrome characterized by initial growth failure, irritability, skin lesions, edema, and fatty liver. Thus, for communities that rely heavily on maize as the main staple, maize cultivars with an improved amino acid profile are a must.

Several natural maize mutants conferring higher lysine and tryptophan were identified in the 1960s and 1970s, viz., *opaque-2* (*o2*), *floury-2* (*fl2*), *opaque-7* (*o7*), *opaque-6* (*o6*), and *floury-3* (*fl3*) (Table 1).

Of these, the *o2* mutation, originally identified in a maize field located in the State of Connecticut, USA (Vietmeyer, 2000), was found to be the most suitable for genetic manipulation in breeding programs aimed at developing maize high in lysine and tryptophan. Maize homozygous for the *o2* (recessive) mutation was shown to have substantially higher lysine and tryptophan content (Table 2) than maize that was either heterozygous (*O2o2*) or homozygous dominant (*O2O2*) for the *opaque-2* locus (Crow and Kermicle, 2002). Bressani (1992) showed that increased concentration of these two amino acids in the grain endosperm can double the biological value of maize protein.[2] However, the amount of protein in such maize remains at about 10%, the same as that of common (or normal endosperm) maize. In other words, the amount of common maize that needs to be consumed to achieve amino acid equilibrium is more than twice as much as the amount of *opaque-2* maize (FAO, 1992). The nutritive value of milk protein is considered to be higher than that of maize protein (Table 3); however, milk is a protein source that very few people can afford. Maize homozygous for the *o2* mutant has a quality value equivalent to 90% that of milk.

Table 1. High lysine mutants of maize.

Gene	Allele	Researchers	Year of discovery
Opaque-2	*o2*	Mertz, Bates and Nelson	1964
Floury-2	*fl2*	Nelson, Mertz and Bates	1965
Opaque-6	*o6*	McWhirter	1971
Opaque-7	*o7*	Ma and Nelson	1975
Floury-3	*fl3*	Ma and Nelson	1975

[1] Proteins, the building blocks of life, are made up of amino acids. Of the 20 amino acids, lysine and tryptophan cannot be synthesized by the metabolism of monogastric animals such as pigs, chickens, and humans. These two amino acids thus have to be taken in through the diet to enable the completion of the amino acid profile required for protein synthesis. Hence, lysine and tryptophan are considered essential amino acids; the remaining amino acids, considered non-essential, can be synthesized by metabolic processes in monogastric animals.

[2] The biological value of protein is the proportion of absorbed protein that is successfully retained by the body for maintenance and growth.

Table 2. Comparative average percentages of lysine and tryptophan in *opaque-2* and normal (non *opaque-2*) maize.

	Normal	*Opaque-2*
	g/100 g protein	
Lysine	2.6	4.2
Tryptophan	0.4	0.9

Table 3. Comparison of the protein value of normal and *opaque-o2* maize with milk.

	Quality as % of milk
Normal maize	39
o2 maize	90
Milk	100

Bressani et al. 1969b; Viteri et al. 1972.

Note: *o2* is a natural mutant. It is not a genetically modified organism (GMO). The recessive *o2* mutation in the homozygous state confers higher quality (lysine and tryptophan) to the protein in maize, but leaves the quantity of protein unchanged.

Note: The terms "common maize" or "normal maize," or the abbreviation "NM" are used in this manual to refer to commonly available maize without enhanced levels of lysine and tryptophan.

2. Utility of High Lysine and Tryptophan Maize

Maize with high lysine and tryptophan, developed in the last 15 years, has been used in feeding studies involving (monogastric) animals and humans. Below is a brief summary of some of those studies.

2.1 Animal nutrition

Pigs raised on high lysine/tryptophan maize gain weight at roughly twice the rate of animals fed solely on normal maize with no additional protein supplements. An equal quantity of high lysine maize substituted for normal maize in pig feeds can maintain the amino acid balance and decrease the use of synthetic lysine (Burgoon et al., 1992). Smallholder farmers (who typically cannot afford balanced feeds) and commercial farmers find this extremely remunerative. In El Salvador, a farmer reported that, after 60 days, 14 pigs fed on grain of hybrid HQ-61 (a high lysine/tryptophan maize) weighed 18 kg more than pigs fed normal maize. In Guizou, one of China's poorest provinces, farmers given credit to buy pigs and raise them on lysine/tryptophan maize earned enough to build houses and conduct community development activities.

Figure 1. Pig fed high lysine/tryptophan maize (larger animal labeled QPM or Q4) compared with its sibling fed normal maize (labeled normal or N4).

Courtesy: Crops Research Institute, Kumasi, Ghana; Animal Science Department, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, and Sasakawa Global 2000.

2.2 Human nutrition

Several human nutrition studies conducted by Akuamoa-Boateng (2002) in Ghana found:

- that children fed high lysine/tryptophan maize had fewer sick days and a better chance of escaping death due to diarrhea and other infections than those fed normal maize porridge.
- reduced stunting in children weaned on high lysine/tryptophan maize as compared to those weaned on normal maize porridge.
- better growth-enhancing capabilities in children consuming high lysine/tryptophan maize compared to those fed normal maize porridge.

Based on these results, Akumoa-Boateng concluded that high lysine/tryptophan maize holds the promise of improving the nutritional status of vulnerable groups whose main staple is maize and who cannot afford protein-rich foods to supplement their diet.

In Colombia, children suffering from kwashiorkor, a severe protein deficiency disease, were restored to normal health with a diet containing only high lysine/tryptophan maize as the protein source. Recent studies have shown that, as an added benefit, increased levels of lysine aid in the assimilation of zinc and iron from maize grain.

Given the large area and the great number of farmers involved in maize production, the development, introduction, and adoption of improved, high lysine/tryptophan maize cultivars have significant potential to reduce protein malnutrition, alleviate hunger, increase incomes, and improve livelihoods.

The reader is referred to the Appendix, Section 11.1, "Potential contribution of quality protein maize to human nutrition," for a more comprehensive summary of this topic.

3. Science: Genetics of High Lysine and Tryptophan Maize

The development of high lysine/tryptophan maize involves manipulating three distinct genetic systems:

- The simple recessive allele of the *opaque-2* gene,
- Modifiers/enhancers of the *o2o2*-containing endosperm to confer higher lysine and tryptophan,
- Genes that modify the *opaque-2*-induced soft endosperm to hard endosperm.

3.1 The simple recessive allele of the *opaque-2* gene

This is a central component of the genetic system that confers higher levels of lysine and tryptophan in maize endosperm protein. The *o2* allele is inherited in a simple recessive manner (Figure 2). The presence of *o2* in the homozygous recessive (*o2o2*) state is a pre-requisite for the entire process of obtaining high lysine/tryptophan maize, discussed in the following sections.

The most abundant proteins in the grain endosperm are the zeins and, particularly, alpha-zein (Fraction II in Table 4), which are poor in lysine and tryptophan (Gibbon and Larkins, 2005). The homozygous *o2* mutant causes a decrease in the production of alpha-zein fraction of endosperm protein and a corresponding increase in the proportion of non-zein proteins (Fractions I, IV, and V) that naturally contain higher levels of lysine and tryptophan (Gibbon and Larkins, 2005) (Table 4). Therefore, in a given quantity of protein from *o2o2* maize, the proportion of non-zeins is higher, which predisposes *o2* maize to have higher lysine and tryptophan.

Table 4. Protein fraction distribution of endosperm samples of normal and soft endosperm (*o2*).

		Percentage of total protein (g/100 g protein)	
	Protein fraction		
Number	Name	Tuxpeño-1 normal endosperm	Tuxpeño-*o2* soft endosperm
I	Ablumins, globulins and soluble nitrogen	6.6	17.0
II	Zeins (alpha, beta, delta, gamma)	48.7	9.7
III	Zeinlike	14.0	13.4
IV	Glutelinlike	9.2	17.2
V	Glutelin	17.0	34.5
	Residue	4.5	8.1

Source: Cited by Bjarnason and Vasal (1992).

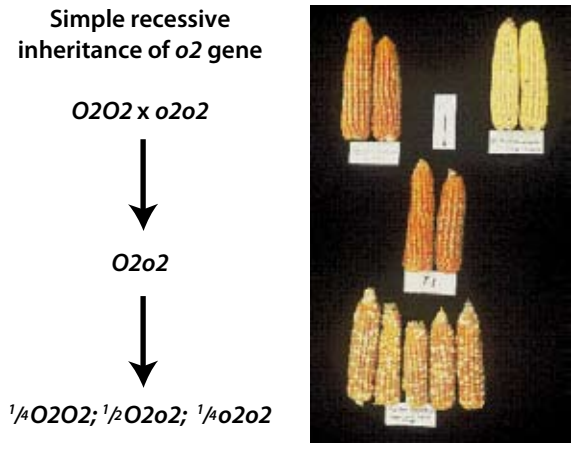


Figure 2. Simple recessive inheritance of the *o2* gene.

Note that:

- By inhibiting zein transcription, *o2o2* reduces the amount of zein protein fraction. Zein is very low in lysine content.
- As zein decreases, other protein fractions increase; for example, Fractions I and V, which have about 60 times more lysine content than zein, are doubled.

However, the presence of the *o2* allele in the recessive condition (*o2o2*) alone does not ensure high lysine and tryptophan levels, but only predisposes maize to have them. The presence of another set of genes (Section 3.2) that enhance the levels of lysine and tryptophan is required to confer higher levels of these amino acids.

3.2 Modifiers/enhancers of the *o2o2*-containing endosperm to confer higher lysine and tryptophan

This is the second essential genetic system that confers higher lysine/tryptophan in maize. It consists of minor modifying loci that affect lysine and tryptophan levels in the endosperm. Lysine levels in normal and *o2* maize average 2.0% and 4.0%, respectively, of total protein in whole grain flour. However, across diverse genetic backgrounds, these levels range from 1.5-2.8% in normal maize to 2.6-5.0% in their *o2* converted counterparts (Moro et al., 1996). Therefore, if lysine or tryptophan levels are not monitored while developing new cultivars, one could end up with a maize cultivar having the *o2o2* genotype and lysine and tryptophan levels equivalent to those in normal maize, since the lower limits of lysine and tryptophan in *o2o2* maize overlap with the upper limits in normal maize (Table 5).

3.3 Genes that modify the *opaque-2*-induced soft endosperm to hard endosperm

The *o2* mutation and the modifiers/enhancers of lysine and tryptophan are, by themselves, not sufficient to develop agronomically acceptable maize high in lysine and tryptophan. Pleiotropic effects of the *o2* allele make the maize endosperm soft and susceptible to cracking, ear rots, and weevils (Figure 3). Such negative secondary effects are obviously undesirable. This softness expresses itself as an opaque phenotype that can be viewed on a light table (Section 4.1.1.4). Therefore, breeding maize for high lysine and tryptophan requires selection based on a third, distinct genetic system, also comprised of minor modifying loci, that convert the mutant endosperm of the soft/opaque/floury phenotype to a hard/vitreous phenotype similar to normal maize.

It has been shown (Wallace et al., 1990) that an increased level of gamma zein likely contributes to the recovery of a hard endosperm phenotype, given that the *o2*-modified (hard endosperm) grains have approximately double the amount of gamma zein in the endosperm as the *o2*-only mutants. (While the proportion of zeins generally decreases in *o2* germplasm, as shown in Table 4, gamma zein increases during the recovery of hard endosperm [data not shown]). The beneficial alleles of the modifying loci that control gamma zein production can be selected using a rapid, low-cost, light-table method discussed in detail in Section 4.1.1.

Table 5. Lysine and tryptophan levels as percentages of total protein in whole grain flour of normal and *o2o2* maize.

	Normal maize	*o2o2* maize
Lysine[a]	1.6-2.6 (mean 2.0)	2.7-4.5 (mean 4.0)
Tryptophan[b]	0.2-0.6 (mean 0.4)	0.5-1.1 (mean 0.8)

[a] Moro et al. (1996).
[b] CIMMYT tropical lowland sub-program.

Figure 3. Soft endosperm *o2* ears showing splitting of pericarp.

The term quality protein maize (QPM) now refers to maize having:

- the *o2* gene in the homozygous recessive state (*o2o2*),
- high lysine and tryptophan levels, and
- an endosperm hard enough to ensure acceptable ear characteristics (Figure 4).

Thus, QPM looks like common maize and can be differentiated only through laboratory biochemical tests (Sections 4.1.2 and 11.2).

Figure 4. Ears of Pool 25, cycle 0, soft endosperm *o2* maize (left) and ears of its improved version (cycle 18) (right).

Note: Both c0 and c18 have high lysine and tryptophan levels. However, c18 is considered QPM because it also has desirable kernel characteristics.

4. Science: Breeding Quality Protein Maize

The history of QPM germplasm development is relatively short. Given the growing interest in QPM, most breeding programs should consider starting by converting[3] their elite non-QPM inbred lines and open-pollinated varieties (OPVs) to QPM either through backcrossing or through pedigree crosses between elite non-QPM germplasm and elite QPM donors. Once a breeding program has some elite QPM germplasm, one could start recycling elite-QPM germplasm with elite-QPM germplasm available in the program or from other breeding programs (i.e., use elite QPM x elite QPM crosses).

Whichever of the above breeding methods is chosen, there are two possible approaches to QPM breeding: the conventional approach and an approach that uses molecular markers to assist in *o2* selection (hereafter referred to as the molecular approach). The main method described in this manual is the conventional breeding approach. Application of the molecular approach is discussed in brief towards the end of this manual (Section 8.1).

Regardless of the breeding method and approach used, there are two unique (i.e., different from steps involved in breeding for other maize traits) and essential steps in the development of QPM germplasm:

- Identification of segregants in a family or population having the *o2* allele in the homozygous recessive (*o2o2*) condition with a hard endosperm (identified simultaneously). The conventional approach involves using a light table (see Section 4.1.1.1). In the molecular approach, leaf samples of candidate plants are analyzed using markers to identify the *o2o2* genotype, but, as in the conventional approach, a light table is needed to differentiate hard endosperm types from the *o2o2* genotypes.
- Identification and confirmation of QPM quality (percentage of tryptophan and protein in sample) through laboratory analyses (Sections 4.1.2 and 11.2).

As with any breeding program, components such as elite source germplasm, donors, and testers are required for QPM breeding. The above discussion is summarized in Table 6.

Table 6. QPM breeding approaches, methods, components, steps, and tools.

	Approaches	
	Conventional	**Molecular**
Breeding methods	Non-QPM conversion to QPM by backcrossing Non-QPM x QPM pedigree method QPM x QPM pedigree method	
Components	Elite non-QPM germplasm Good QPM donors Good testers for combining ability	Molecular markers
Step 1	Identification of *o2o2* (qualitative determination) and hard endosperm	
Tools	Light box	Molecular markers and light box
Step 2	Determination of lysine, tryptophan, and protein quantities (quantitative determination)	
Tools	Biochemical Laboratory	

The following sections are arranged in a bottom-to-top manner where:

- First, the tools used in each of the two unique steps of QPM breeding are described. An understanding of these tools is necessary to follow the unique steps of QPM breeding.
- Subsequently, the components of QPM breeding are described. Knowing the tools and the steps leads to an understanding of the components.
- Finally, we give a detailed description of the protocols of the different breeding methods (mainly backcrossing and pedigree breeding), including all the steps of QPM breeding with appropriate use of the components. Note that population improvement methods, which deal with the improvement of established populations, are not discussed in this manual.

[3] The process of transferring (either through backcrossing or by pedigree methods) the *o2* gene, the amino acid modifiers, and the kernel modifiers to any non-QPM germplasm is often referred to as conversion.

4.1 Tools for QPM breeding

4.1.1 Light table

4.1.1.1 What is a light table?

- A light table is a custom-made box used to differentiate hard endosperm maize types from soft *o2o2* genotypes.
- It is usually made of wood on all sides, except for the top surface, which is made of semi-transparent glass or plastic.
- Inside the box, one or more fluorescent (or other type) bulbs are placed in a lamp connected to an outside power source. To view kernel characteristics, place maize grain on the table and turn on the light.
- The size of a light table may vary:
  - **a)** The minimum desirable size is 27.5 cm long x 15 cm wide x 7.5 cm high (Figure 5 and 6);
  - **b)** More typically it should be 72 cm long x 63 cm wide x 11 cm high. The plastic or glass top should be 3 mm thick (Figures 7-12).
- Holes on two opposing sides of the box provide adequate ventilation and prevent overheating.
- Type (a) (Figures 5 and 6) light table typically uses one 9-watt (or higher) flourescent bulb. Type (b) (Figures 9 and 10) uses three 18-watt flourescent bulbs.

4.1.1.2 How do you use a light table?

- Connect the table to a power outlet and turn it on.
- Place kernels on top of the glass/plastic with the germ (embryo) side down (Figures 13 and 14).

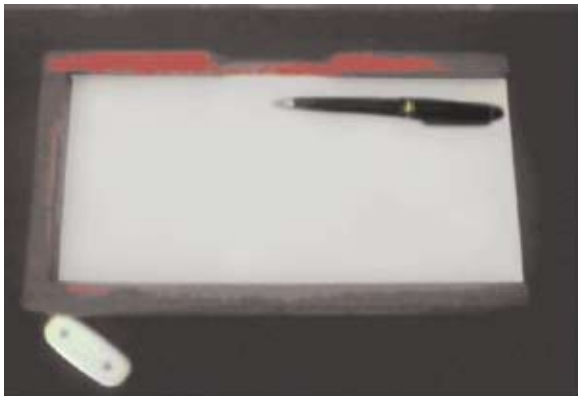


Figure 5. Top view of a type (a) light table.
Designed by the Crops Research Institute, Kumasi, Ghana.

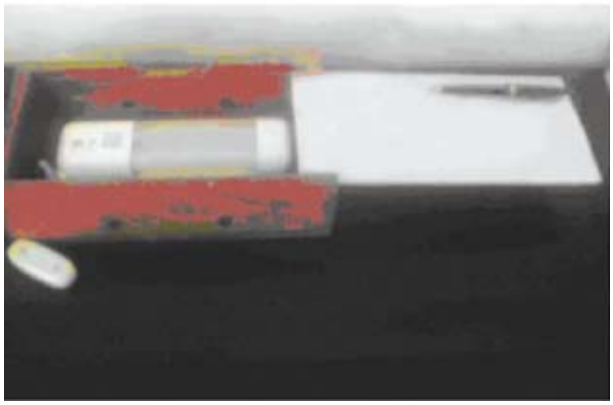


Figure 6. Type (a) light table showing the bulb inside.
Designed by the Crops Research Institute, Kumasi, Ghana.

Figure 7. Front view of a type (b) light table.

Figure 8. Back view of a type (b) light table.

Figure 9. Bulb arrangement in a type (b) light table.

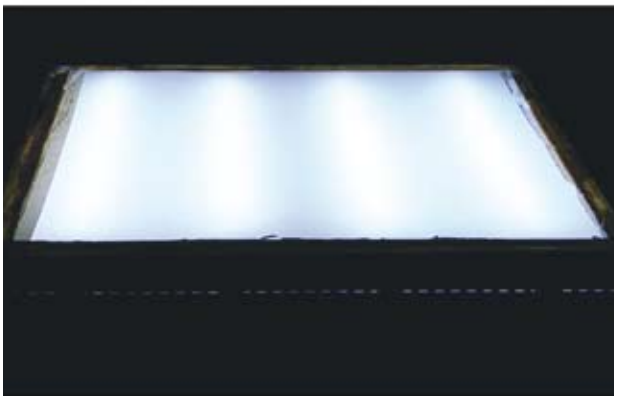


Figure 12. Illuminated light table.

Figure 10. A type (b) light table with the top open.

Figure 13. Screening maize kernels on a light table.

Figure 11. Side view of a type (b) light table with the top open.

Figure 14. Maize kernels on an illuminated light table.

4.1.1.3 What do you select on a light table?

The desired level of opaqueness (or modification) in the kernel is selected (Figure 16, Section 4.1.1.5).

4.1.1.4 What is the principle behind light table selection ("light tabling"), i.e. why do "light tabling"?

Light table selection is based on the principle that *o2o2* genotypes carry an undesirable characteristic, kernel softness, which, on a light table, is seen as complete opaqueness. Due to segregation of genes for endosperm hardness (or softness) (see Section 3.3 on modifiers), varying degrees of softness/hardness are expressed in the endosperm of segregating generations (i.e., varying levels of opaqueness are observed on a light table; Figures 15 and 16, Section 4.1.1.5). A kernel with the *O2o2* or *O2O2* genotype is normal maize, i.e., it does not have the softness and undesirable kernel characteristics associated with the *o2o2* genotype and is therefore translucent. In the absence of these undesirable traits, the counteraction of modifiers, even if present, is presumably not visible and thus not an issue.

Light tabling is done to pick out kernels with the *o2o2* genotype by using the degree of opaqueness as an indirect measure or secondary trait. Note that *O2o2* or *O2O2* genotypes may show a small but insignificant degree of softness.

4.1.1.5 How do you determine the degree of modification, and what do the modification scores mean?

Gradation in the opaqueness is scored on a 1-to-5 scale for easy descriptions of the various classes and to enable statistical analysis. The percentage of opaqueness is visually assessed and best illustrated by Figure 16.

Type (Modification score) 1: Not opaque
Type (Modification score) 2: 25% opaque
Type (Modification score) 3: 50% opaque
Type (Modification score) 4: 75% opaque
Type (Modification score) 5: 100% opaque

Less opaqueness implies higher/more action of modifiers.
Types 1 to 3 would be considered QPM, ***provided*** their protein quality is verified.

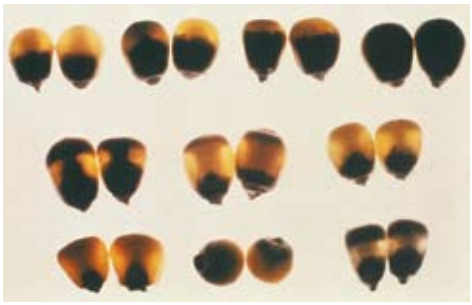


Figure 15. Maize kernels carrying the *o2* gene placed with the embryo side down on a light table. Varying degrees of opaqueness indicate varying levels of endosperm modification.
(Note that opaqueness is not always neatly graded from top to base of the kernel, as there are different modifier genes turning soft endosperm into hard endosperm**)**

- It is critical to identify the *o2o2* genotype in early generations of inbred line development.
- Light table selection is necessary to identify kernels that may carry the *o2* gene in the homozygous recessive state.
- It is preferable to use a light table, rather than molecular markers or lab analyses, as it is quick and inexpensive, especially considering the hundreds or thousands of genotypes that may have to be screened from segregating populations.
- The degree of modification is largely (but not entirely) independent of protein quality. Therefore, the light table cannot replace laboratory analysis at the final stages to confirm acceptable protein and tryptophan contents.

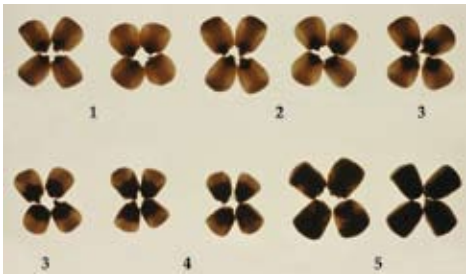


Figure 16. Kernels on a light table sorted into modification classes.

4.1.1.6 When do you use a light table?

Light table selection is done on all segregating generations when using the conventional breeding approach. It is especially important in early (F2 to F6) generations of inbred line development.

4.1.1.7 Which scale do you select?

- Select modification score 3 in the F2 generation.
- Select modification score 2 or 3 in the next two generations (F3 and F4).
- Select modification score 2 in the remaining advanced generations.

4.1.1.8 Does selecting for a particular modification score in early generations mean that the genotype is fixed?

Modifiers are a set of minor genes. Hence kernels selected for Type 3 in an early generation (e.g., F2) will very likely produce a whole range of kernel types in the next generation due to segregation of minor genes. Homozygosity increases with successive generations of inbreeding. As modification Type 2 is progressively selected, one moves towards fixation of this modification level in the kernel. Elite QPM inbred lines ideally have the modifiers fixed at a score of 2, but it is not uncommon to find elite inbred lines with a modification score of 3.

Light table opaque endosperm scores

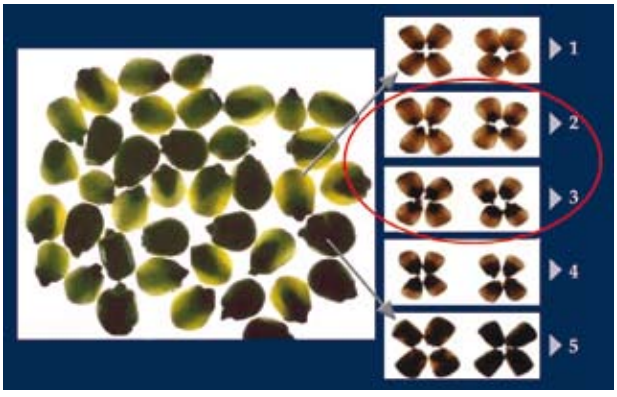


Figure 17. Kernels on a light table (left) and modification classes in F2 seed from normal x QPM donors.
Source: Adapted from Krivanek and Vivek (2006).

4.1.1.9 Why should Types 1, 4, and 5 not be selected (or why should only Types 2 and 3 be selected)?

Type 1 kernels are completely translucent, with no opaqueness. They may have the *o2* gene in the homozygous recessive condition (*o2o2*), and the modifiers may have turned the kernel endosperm completely hard (which is desirable). However, such kernels may also be heterozygous (*O2o2*) or homozygous dominant (*O2O2*), in which case the kernel is low in lysine and tryptophan, and endosperm hardness (modification) is not an issue, as *o2o2*-induced softness is simply not present (see the principle behind light table selection in Section 4.1.1.4). The only way to determine the allelic state of Type 1 kernels (other than by using molecular markers) is by doing tryptophan analysis in the lab. However, this is expensive and impractical for screening large numbers of segregants. Hence, Type 1 kernels should not be selected.

In **Type 2** kernels the presence of *o2o2* is almost guaranteed, but there is a chance that a Type 1 kernel may have been misclassified as Type 2. Also, an *O2o2* or *O2O2* genotype may have a small degree of opaqueness. Thus, selecting Type 2 kernels in early generations is not recommended, as guaranteeing the presence of *o2o2* in these generations is the priority.

In **Type 3** kernels the presence of *o2o2* is guaranteed. This kernel type is recommended for selection in early generations, as it is a compromise between the guaranteed presence of *o2o2* (a higher priority) and good modification (which can be improved in subsequent generations; see Section 4.1.1.8).

In **Type 4** kernels the presence of *o2o2* is guaranteed, but selecting this type means that the probability of obtaining well modified kernels in subsequent generations is much lower than if Types 2 and 3 are selected.

Type 5 kernels are completely opaque, just as the original *o2* mutant was. Type 5 kernels have very soft endosperm and no modifiers, which results in undesirable characteristics such as susceptibility to ear rots and weevils, and kernel cracking.

4.1.1.10 Should I select on a light table in all generations?

Yes.

4.1.1.11 At which stage do I send the samples for laboratory tryptophan analysis?

Samples are sent for laboratory analysis when the modification score of most kernels is 2. As a rule of thumb, the first tryptophan analysis is done at F3 or F4 (before the first test cross).

4.1.1.12 What are the steps to effectively select endosperm modifiers of the *o2* locus in the F2 generation?

(Note: The three steps outlined below are meant for those with less experience in QPM selection. The step by step procedure is recommended solely as a check to ensure that the desired modification classes are picked. As more experience working with QPM is gained, one may directly proceed to select the desired modification classes from the harvested and shelled lot of QPM.)

Step 1: In each F2 population only select ears that are segregating for endosperm modifiers (as shown by the bottom left circle of ears in Figure 18). Discard those that do not show segregation. In other words, selected ears must show some opaque kernels (dense soft opaque kernels seen on the cob). On ears that have a good frequency of modifiers, about 5% of the kernels will show complete soft-opaqueness (i.e., kernels that would be rated 5).

Step 2:

- Shell each segregating ear individually and separate out all kernels showing some degree of modification (i.e., modification score of 2 to 5).
- Count modified kernels vs. normal kernels. Fully modified (normal) kernels should constitute approximately 75% of all kernels, while kernels of modification Types 2 to 5 should constitute 25%.
- If modified kernels total more than the expected 25%, sort through the selected lot again and separate out the normal kernels. If modified kernels make up less than the expected 25%, sort through the normal-looking kernels again and pick the modified kernels until an approximate ratio of 3:1 is reached. This step helps refine selection and avoid mistakes.

Step 3: From the 25% modified kernels, select well-modified kernels with a score of 3. In advanced generations, select kernels with a score of 2.

4.1.2 Protein quality laboratory

Samples are usually first sent to the laboratory for protein content and tryptophan analysis at the F3 or F4 stage. This section gives an introduction to laboratory analyses, while Section 11.2 contains

Figure 18. Illustration of F2 ears segregating for endosperm modification (bleached white kernels are completely opaque).

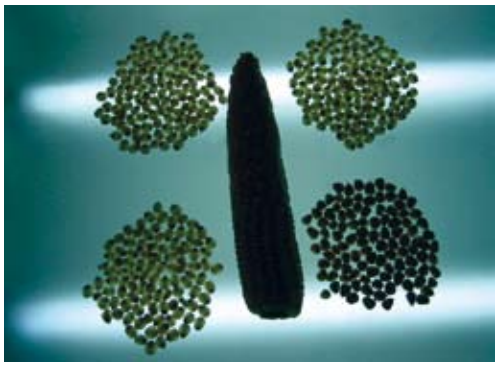


Figure 19. Kernels on a light table sorted by modification scores.

detailed laboratory protocols for protein and tryptophan analysis.

Both lysine and tryptophan concentrations are increased in QPM materials. These increases need to be monitored during the breeding process, but only tryptophan is analyzed on a routine basis. This is because lysine (Lys) and tryptophan (Trp) values are highly correlated. Normally, the value of lysine is four times that of tryptophan. Due to the well-established relationship between these amino acids in the protein of *opaque-2* maize endosperm (Hernandez and Bates, 1969; Villegas et al., 1992), tryptophan can be used as a single parameter for evaluating the nutritional protein quality of the protein. As illustrated in Figure 20, a high correlation between lysine and tryptophan has more recently been found (H. Cordova and A. Krivanek, 2006, personal communication), confirming that it is unnecessary to measure both amino acids.

Another disadvantage of trying to measure lysine is that the colorimetric reaction for lysine determination in maize kernels (Tsai et al., 1972) is time-consuming, and its reproducibility is affected by many factors. This makes it difficult to use it to analyze large numbers of samples; lysine analysis is therefore not recommended for use in a practical breeding program.

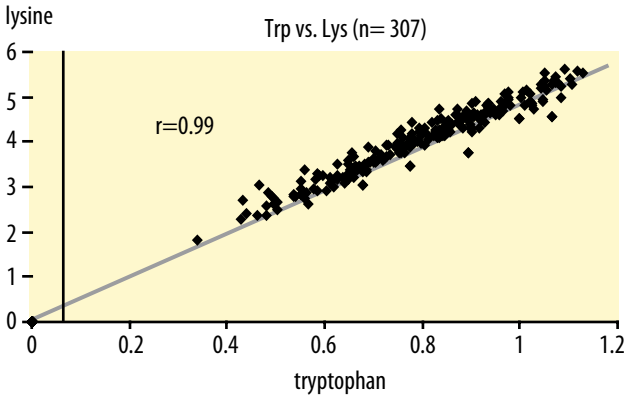


Figure 20. Correlation between tryptophan and lysine content in 307 samples of tropical germplasm.
Source: H. Cordova and A. Krivanek, 2006, personal communication.

Laboratory analysis requires using samples consisting of 20-50 maize kernels each (see Section 7). The analysis can be done on the whole grain or only the endosperm. Maize endosperm protein is generally deficient in the amino acids lysine and tryptophan, while the germ has a relatively constant and well-balanced amino acid composition regardless of genetic background. A comparison between the two types of analyses is given in Table 7.

Section 11.2 contains detailed laboratory protocols for protein and tryptophan analysis. The HPLC method of lysine determination (Huang et al., 2006) is the most reliable but also the most expensive method. If samples need to be tested for lysine, the possibility of having HPLC analysis done at a reputable lab should be explored.

Table 7. Laboratory analyses of endosperm vs. whole grain.

Endosperm analysis	Whole grain analysis
Pericarp and embryo are removed before grinding the grain; hence, more time- and resource-consuming (Table 8).	Whole grain is ground; less time- and resource-consuming (Table 8).
Estimated tryptophan, protein, and lysine values are lower.	Estimated tryptophan, protein, and lysine values are slightly higher.
Ideally used on elite germplasm and final products.	Ideally used on segregating generations.

Table 8. Typical costs of laboratory analyses.

	Cost (US $ per sample)	
Analysis	Whole grain	Endosperm
Sample preparation	2.2	3.8
Nitrogen	3.3	
Tryptophan	2.5	
Lysine	2.9	

Note:
- Costs may vary between laboratories.
- Typically two people can analyze 160 duplicate samples for tryptophan in a single day using the colorimetric method.

4.2 Interpreting laboratory results

Protein or tryptophan values obtained from the laboratory are for a given unit of samples and expressed as a percentage. If the tryptophan value is 0.08%, this indicates the amount of tryptophan in the sample and NOT the tryptophan percentage in the protein.

4.2.1 Quality index (QI)

The quality index is the tryptophan-to-protein ratio in the sample, expressed as a percentage. For example:

- Tryptophan in sample = 0.08%
- Protein in sample = 10%
- QI = 100 * 0.08/10, which is = 0.8

4.2.2 Relationship between QI, protein quantity, and protein quality

Due to the relationship described in 4.2.1, there is a negative correlation between:

- protein quality and quantity
- % protein and QI

4.2.3 Laboratory values to be considered during selection

It is by now obvious that QI does not indicate the quantity of protein. Hence, when interpreting lab results for making selections, make sure that the protein, tryptophan, and QI are above the acceptable limits described in Table 9.

Table 9. Ready reckoner for interpreting laboratory results.

(All values in %)		QPM		Non-QPM
	Protein	>=8		>=8
In protein	Lysine	4		2
	Tryptophan	>0.65		<0.60
		Whole grain	***Endosperm***	
In sample	Tryptophan	>0.075	>0.07	
	Quality index	>0.8	>0.7	

4.2.4 In conclusion

- Do whole grain analysis on early segregating generations and endosperm analysis on elite germplasm and final products.
- Lysine and tryptophan values are correlated. Lysine may be tested (but is not required) in the final product only if tryptophan values are acceptable.
- There are no absolute values that "define" QPM.
- Always compare QPM values with non-QPM checks.
- Look at combining ability and agronomic characters (Section 4.4.3).
- Select the best of what you have and keep your standards high.

4.3 Issues with laboratory analysis of tryptophan in QPM (as described in Section 11.2.3)

4.3.1 Tryptophan analysis in brief

The process for determining the percentage of tryptophan in a QPM sample is as follows:

1. Grind either the whole grain or just the endosperm.
2. Defat the ground samples using a Kjeldahl apparatus.
3. Add papain (an enzyme) to hydrolyze (i.e., cut) the proteins.
4. Add a mixture of glacial acetic acid and H_2SO_4 (sulphuric acid) to induce color development (a purplish color) in the sample.
5. Measure color intensity with a spectrophotometer at 560 nm. Increased color intensity indicates more tryptophan. Use a formula to convert the reading from the spectrophotometer to % tryptophan in the sample.

The critical and procedurally demanding step in the above process is color development. Good color development is guided by the chemical reactions described below.

acetic acid + ferric chloride → glyoxylic acid

glyoxylic acid + sulfuric acid + tryptophan (in sample) → color (detectable at 560 nm)

4.3.2 The problem

Due to chemical reactions, acetic acid normally contains glyoxylic acid, which may be considered an impurity. However, in this case, the impurity (glycoxylic acid) reacts with tryptophan in the sample and ensures color development. Thus, in this instance, the "impurity" is desirable.

However, the "impurity" in a batch of acetic acid is not controlled. Therefore, different batches of acetic acid do not give the same color intensity (absorbance with a spectrophotometer) with a given amount of tryptophan (or with the same sample). Hence different batches of acetic acid would need to be tested for color development before deciding which batch should be used for analysis.

In summary, improper color development due to different degrees of "impurity" found in acetic acid is a problem in tryptophan laboratory analyses. Larger laboratories—for example, those at CIMMYT and IITA—have overcome this problem by

- purchasing small quantities of different batches of acetic acid and testing them for proper color development;
- and once the right batch of acetic acid is identified, purchasing larger quantities (sufficient for doing lab analyses for at least a year) of the same batch for use in the lab.

The above solution is not viable for all laboratories and especially not for labs in Africa because of:

- the lack of trained staff, and
- the logistics of ordering and testing multiple batches of acetic acid, and then re-ordering the identified batch. This involves many non-technical considerations such as local availability, importation across international borders, customs regulations, time, and money.

The problem of having to test different batches of acetic acid is the main reason why an alternative tryptophan determination method is being validated at CIMMYT (Nurit et al., 2007, in preparation). For further details, please contact Natalia Palacios (n.palacios@cgiar.org).

4.4 Components of QPM breeding

4.4.1 Elite source germplasm[4]

Pedigree breeding or recycling is the process of deriving, extracting, and developing inbred lines using crosses among elite lines (and/or elite OPVs) as source germplasm. Backcrossing is a more specific term that refers to the process by which an elite line is improved for a specific trait (e.g., a non-QPM line improved for the QPM trait) while maintaining its original background to a large extent.

The choice of which cross to make while recycling or backcrossing should be made in a careful and informed manner, as this will determine the product profile, the cost:benefit ratio, and, hence, the success of the breeding effort years down the road.

(i) In any pedigree breeding program, genetic progress is determined by the eliteness of the source germplasm. Using the best germplasm in a pedigree breeding project ensures maximum genetic gain. Of all the advanced lines that may be available in a breeding program, the few that have proven to be the best, both agronomically and in terms of combining ability, should be used to start pedigree breeding projects, in this case with the aim of deriving QPM having either partial or full background of the non-QPM recipient.

(ii) Ensure that the germplasm used in a cross make up for each other's deficiencies in the hybrid generated (from now on referred to as the source hybrid), as it will be subjected to inbreeding in the process of deriving inbred lines. In other words, the parents used in a cross must complement each other. For example, although each inbred line may lack a specific trait (disease resistance or, in this case, the QPM components), the source hybrid must have the full complement of target traits. As this hybrid is subjected to inbreeding during several generations of selfing, desirable segregants are picked up.

(iii) Ideally, the germplasm used in a cross should belong to the same heterotic group (if known). Extracting new inbred lines from within-heterotic-group crosses increases the chances that the new lines will show maximum heterosis when they, in turn, are crossed with new lines developed similarly from the opposite heterotic group. If the heterotic groups are unknown or unclear, crosses

[4] Elite germplasm is among the best germplasm in a breeding program; it has proven agronomic performance, combining ability, and resistance/tolerance to relevant biotic and abiotic stresses.

may be made among germplasm sources without violating points (i) and (ii) above, to generate the source hybrid (it may be determined later on that such a cross is heterotic, i.e., the lines belong to opposite heterotic groups).

The issues in QPM development will become apparent in the following sections; therefore, to increase the likelihood of success, projects should start with at least five elite entries (inbred lines or OPVs), if many are available.

4.4.2 QPM donors

The choice of a QPM donor is just as critical as that of the recipients, and sometimes more, as donors tend to be used repeatedly to initiate pedigree projects within a breeding program. The choice of a poor donor could prove to be very expensive and wasteful.

In a non-QPM program, a donor is an elite line with a trait that makes up for the deficiency of the recipient. Even in a QPM program, a QPM line or OPV chosen as a donor for a conversion[5] project is usually the most elite, but with an additional requirement: it must confer good endosperm modification to the recipients. This point may seem counter-intuitive, given that a line (or OPV), by virtue of being elite, should possess good modifiers and the ability to pass them on when crossed. However, the process works as follows:

- *o2* is a simple recessive gene and, as such, very easy to transfer to any germplasm. A cross to a QPM donor gives the F1; its subsequent selfing generates segregants for the *o2* locus in a 1:2:1 ratio (Figure 2). Transferring kernel texture modifiers, however, is more difficult because it involves many minor genes. Breeders have frequently found that some inbred lines are easy to convert to QPM (i.e., transfer the *o2* gene and its associated amino acid and kernel modifiers), and others are not. As expressed by QPM breeders, the difficulty lies mainly in the transfer of kernel hardness modifiers (see Section 3.3) and, to a lesser extent, amino acid modifiers (Section 3.2) (even though amino acid modifiers are a set of many minor genes).
- Although researchers are just beginning to study this difficulty in conversion, it may be assumed that the genetic composition of the recipient or the donor, or both, could be at work here. One must be very prudent when choosing a QPM donor because it will be used repeatedly. Since a donor is likely to be better characterized than other elite germplasm, exercising this prudence with a donor is easier.

Although a QPM line may be elite and have good protein quality, it may not be the best choice as a donor.

The ability of a line or OPV, once converted to QPM, to perform as a donor in subsequent projects is assessed based on how difficult it was to convert it to QPM in the first place. When used as QPM donors, inbred lines (or OPVs) that were easily converted to QPM transfer their modifiers more readily than lines (or OPVs) that were difficult to convert. Thus new QPM lines (or OPVs) may show varying degrees of "donor" ability (from excellent to good to poor).

An alternate and more systematic study of donors would involve crossing a series of elite QPM lines (or OPVs) (those showing good combining ability and protein quality) to non-QPM recipients using the North Carolina Design II. Hybrids are selfed to form F2 populations. The proportions of Type 1, 2, and 3 kernels (as seen on a light table, Sections 4.1.1.5 and 4.1.1) in F2 populations derived from crosses between a particular donor and all non-QPM recipients are compared with those in F2s generated by crossing other QPM donors with the same non-QPM recipients. A good donor would be one that has a high combined percentage (about 90%) of 1, 2, or 3 modification scores over a range of recipient lines.

Performing the described procedure to differentiate good donors from bad every time new elite lines are generated should not become an academic exercise for breeding programs, unless that is the specific objective. Rather, from a practical perspective, the above exercise should be a step (arrived at systematically) in pedigree projects aimed at generating inbred lines. The whole procedure involves the following steps:

1. Cross a set of elite recipients to a set of donors with unknown donor abilities.
2. Study the F2 populations for modification, as above.

[5] The process of transferring (through backcrossing or pedigree methods) the *o2* gene, amino acid modifiers, and kernel hardness modifiers to non-QPM germplasm is often referred to as conversion.

3. Identify good and bad donors.
4. At the same time, identify F2s having a high frequency of modifiers, and classify lines into donor types (excellent, good, fair, and poor).
5. Continue inbreeding selected F2s with high frequency of modifiers. At this point, there may be some F2s that have a high frequency of modifiers, but derived from crosses with lines wich may subsequenty be classified as bad donors. In other words, bad donors may generate a high frequency of modification with specific recipients, perhaps due to the contribution to modifying genes from the recipient. Continue inbreeding such specific F2 crosses.

However, do not use lines now classified as bad donors to start other conversion projects. Only good donors should be used to start new projects; eventually newer inbred lines identified as good donors using the above procedure will replace the current set of donors. Due to the unpredictable specific incompatibilities that may exist between donors and recipients, it is always preferable to cross a number of good donors with several recipients when starting pedigree projects.

It is important to reiterate that a donor must not only be good in its ability to modify kernel phenotype, but also "elite" in all other respects.

Based on the above processes, several QPM lines were classified in the following four groups:

- Group 1 = Excellent donors: CML144, CML181f, CML176, CML150, CL-RCWQ83, CML491. These donors work well with most lines.
- Group 2 = Good donors: CML173, CML154, CML175.
- Group 3 = Fair donors: CML181d, CML182.
- Group 4 = Poor donor: CML159.

Since this classification, several newer lines have been developed at CIMMYT, some derived from good donors (e.g., from CML176). Such lines have a background of good donors, but have been improved in other respects, and are recommended for immediate use in breeding programs. (Section 11.3)

Bear in mind that the presence of diversity in nature is what makes it possible to select the good and discard the bad (i.e., without diversity, gene tic improvement would not be possible). Thanks to genetic diversity, plants have been endowed, among other things, with resistance/ tolerance to biotic and abiotic stresses. Hence, long-term conservation of genetic diversity is essential and use of only one or a few QPM lines as donors could narrow the genetic base. On the other hand, using poor donors just to preserve diversity leads to slow genetic progress. Hence, it is important to strike a balance between the two.

4.4.3 QPM testers

Every hybrid breeding program has to invest considerable effort in choosing an appropriate tester for assessing the combining ability of segregating lines. A tester may be an inbred line, an OPV, or a single-cross hybrid. A desirable tester must facilitate discrimination among genotypes for combining ability and desirable traits, simultaneously identify useful hybrid products for direct use, and be compatible with a practical maize breeding program (Vasal et al., 1997). The choice of testers involves a mix of theoretical and practical considerations (Bänziger et al., 2000), such as:

- how genetically broad-based the tester should be;
- whether it should be high or low yielding;
- whether it should have a high or low frequency of target traits;
- whether it has good or poor general combining ability;
- how many testers will be used;
- how many heterotic groups are being handled; and
- whether testers should be related or unrelated.

In practice, a tester in a breeding program is typically an elite line, an elite OPV, or a within-heterotic-group single cross of elite lines. A breeding program usually has at least two heterotic groups.

Explaining the actual process of tester identification is beyond the scope of this manual. Interested readers should therefore refer to maize breeding text books and journal articles dealing with combining ability.

Listed below are practical considerations to be kept in mind when identifying/choosing testers.

General checklist on use of heterotic groups:

- ✓ Use a minimum of two heterotic groups, plus one tester from each group.
- ✓ Heterotic groups must be carefully chosen to ensure maximum heterosis between them.

Checklist for choosing an inbred or OPV tester:

- ✓ Use an inbred line or OPV tester if the final product being targeted is a single- or top-cross hybrid. This makes it possible to simultaneously identify the final product during early generation testing.
- ✓ Testers should have good general combining ability (GCA) effects.
- ✓ The tester's per se performance (yield, standability, disease resistance) must be reasonably good.

Checklist for choosing a single-cross tester:

- ✓ Use a single-cross tester if the final product being targeted is a three-way cross, as this makes it possible to identify the final product during early generation testing.
- ✓ Inbred lines constituting a single-cross tester should have good GCA effects.
- ✓ Inbred lines constituting a single-cross tester should belong to the same heterotic group.
- ✓ Per se performance of inbred lines constituting a single-cross tester should be reasonable.
- ✓ Inbreeding depression of the hybrid tester should be bearable => a single-cross tester must yield reasonably well to qualify for use as a female parent in successful three-way and double-cross hybrids.

Once testers are identified, germplasm has to be classified into heterotic groups. In established breeding programs, germplasm is generally well categorized into heterotic groups; newer breeding programs that may not have done this must strive to do so.

The process of testing combining ability also makes it possible to classify newer inbred lines into heterotic groups, if unknown, or to confirm the heterotic patterns of lines developed from within-heterotic-group crosses. Usually combining ability is first tested at the S1 (F3) or S2 (F4) stage. Breeding programs may have hundreds of early generation lines being test-crossed and evaluated across at least five target locations. The best 5-20% of the lines are selected and advanced. By the time combining ability is tested for the second time (usually at the S3 [F5] or S4 [F6] stage), the chosen inbred lines are fairly fixed and may be test-crossed to more testers in order to identify final products. More locations are generally used for testing and identifying hybrids at this stage.

Once a promising QPM hybrid has been identified based on combining ability in the advanced test-crosses, grain from this hybrid (a product that farmers will utilize) needs to be tested for protein quality. The grain to be tested may come from the F1s in a trial. Two to three plants within the selected plots are shoot-bagged, and plant-to-plant pollination (sib mating) is performed among these plants. This simulates the cross-pollination that occurs among a population of hybrid plants growing in a farmer's field; the harvest represents the grain a farmer would harvest. Grain harvested from these pollinations is sampled and sent for tryptophan and protein analysis in the laboratory (Section 11.2).

Remember:

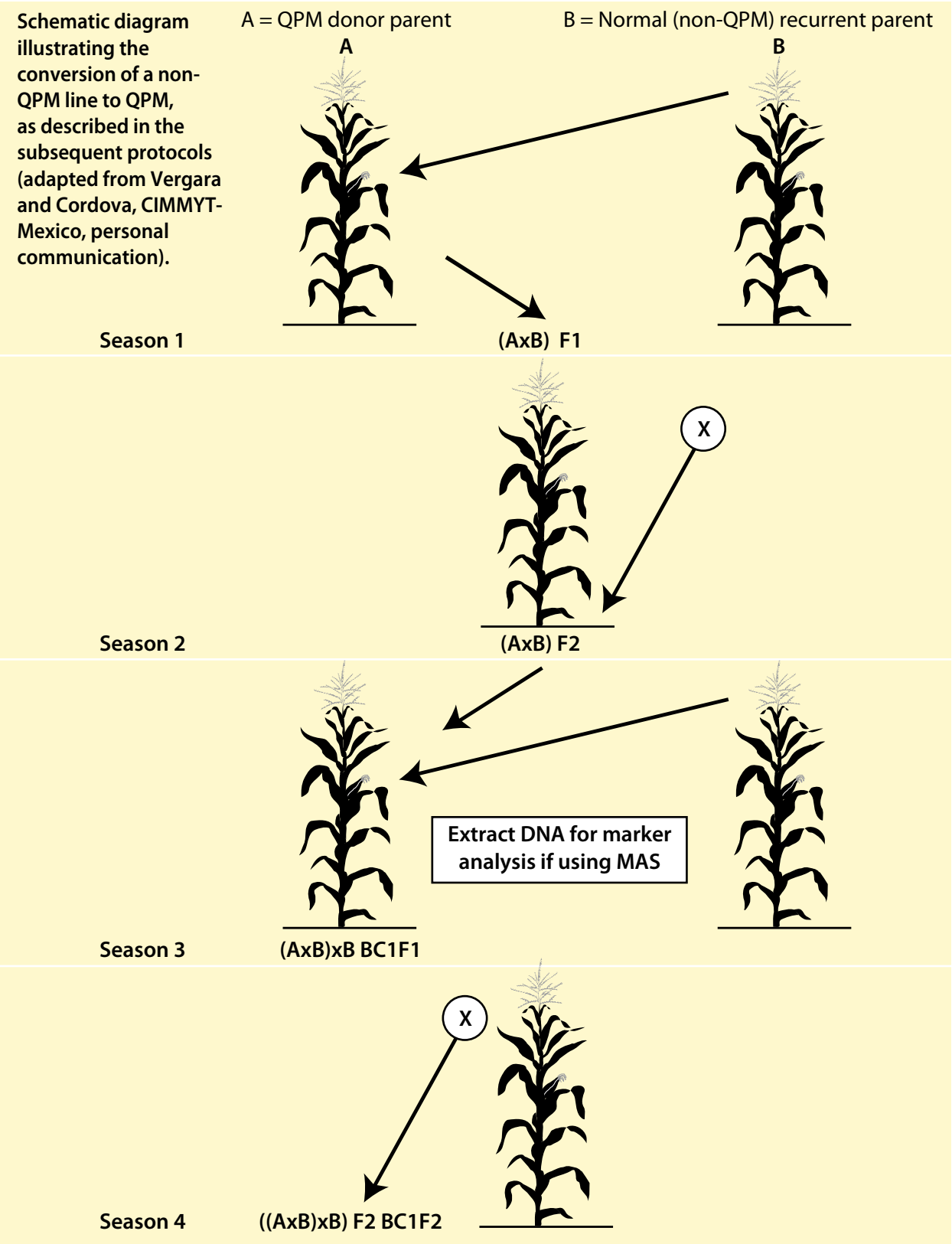
- **Heterotic groups are man-made (and thus subjective), and classification of groups and inbred lines into groups is a constantly evolving process.**
- **Classification into heterotic groups is important, as proper use of heterotic groups increases the probability of success. Breeding is a "numbers game," and heterotic groups increase the odds of identifying a good genotype. This determines the rate of genetic progress and the return on the investment (costs of identifying the final product).**
- **All breeding programs must, in the medium-to-long term, move towards classifying germplasm into heterotic groups.**
- **In the short term, lack of knowledge of the heterotic pattern of a germplasm should not prevent its use. An unclassified elite germplasm may be used in pedigree crosses with elite germplasm from various heterotic groups. Although the probability of success is lower in such cases, it would be seemingly worse if the germplasm were not used at all.**
- **Therefore, use heterotic groupings as a tool, rather than as a restrictive rule.**

4.5 QPM breeding methods

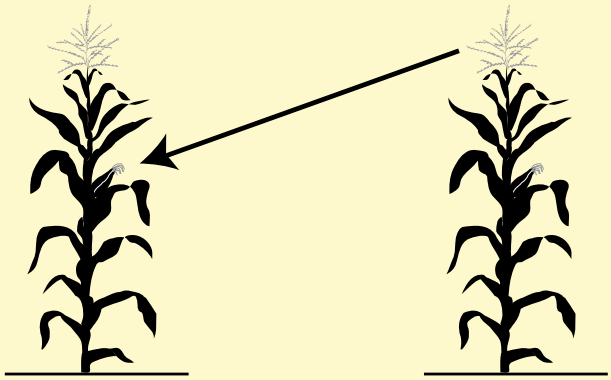
Many of the procedures described in this section are, in principle, the same as those used to breed non-QPM cultivars, except that QPM breeding protocols have to be geared towards increasing/ maximizing the frequency of modifiers at each step. The following protocols are modifications and adaptations of protocols developed at CIMMYT, as reported (through personal communication and in several CIMMYT internal reports) at different times by several scientists (Cordova, Pixley, Vasal, Vergara).

What follows is a schematic illustration of the conversion of a non-QPM line to QPM, which should help the reader visualize the whole process (described in Tables 10 and 11).

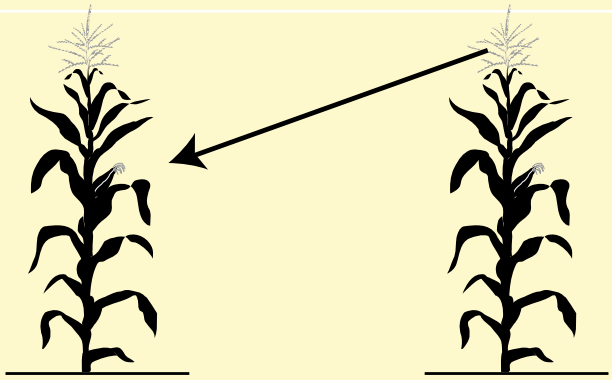
Schematic diagram illustrating the conversion of a non-QPM line to QPM, as described in the subsequent protocols (adapted from Vergara and Cordova, CIMMYT-Mexico, personal communication).



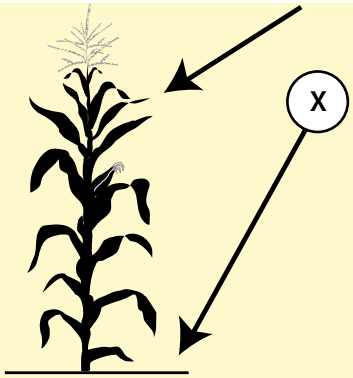
((AxB)xB) BC1F2 B



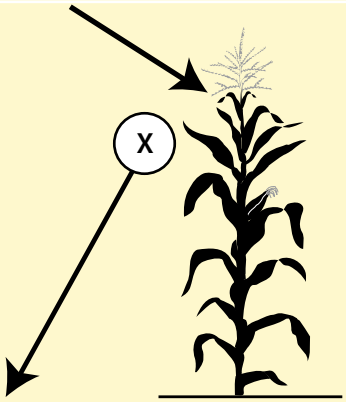
Season 5 (((AxB)xB)xB BC2F1



Season 6 (((AxB)xB)xB)xB BC3F1



Season 7 (((AxB)xB)xB)xB)) BC3F2 = Line converted to QPM



Season 8 (((AxB)xB)xB)xB))=BC3F3 = Seed increase of converted line

4.5.1 Conversion of a normal OPV to QPM.

Table 10. Conversion of a normal OPV to QPM.

Ideal season	Objectives	Materials to plant	Instructions
1) Off	Form F_1 (BC_0F_1)	If using inbred line donor: Plant 1 row (17-26 plants) of the QPM donor (Q) line, and 20 rows (at least 250 plants) of the recurrent parent (N) OPV.	Bulk pollen from 75 plants of the recurrent parent (N) and pollinate 5 plants in the donor parent (Q), to form F_1. Break the tassels of the recurrent parent plants once pollen has been bulked. Repeat the process of bulking the pollen and pollinating the donor parent two more times. Use at least 200 plants of the recurrent parent OPV in the process. Harvest and select at least 10 clean F1 ears (no ear rot). From the selected ears, bulk F1 seed from each family.
		(If uncertain of a good donor line, plant 3-5 potential donor parents.)	(If you have multiple donor parents, form 3-5 respective F1s. Harvest and select at least 10 clean F1 ears from each family. Separately, bulk F1 seed from each family.)
1) Off	Form F1 (BC_0F_1)	If using OPV donor: Plant 20 rows (at least 250 plants) of the recurrent parent (N) OPV. Plant 20 rows (at least 250 plants) of the QPM donor (Q) OPV.	Bulk pollen from 75 plants of the donor parent OPV and pollinate 75 plants in the recurrent parent (N), to form F_1. Break the tassels of the donor plants once pollen has been bulked. Repeat the process of bulking the pollen and pollinating the recurrent parent two more times. Use at least 200 plants of the recurrent parent OPV and 200 plants of the donor OPV in the process. Harvest and select at least 20 clean F1 ears (no ear rot). From the selected ears, bulk F1 seed from each family.
		(If uncertain of a good donor line, plant 3-5 potential donor parents.)	(If you have multiple donor parents, form 3-5 respective F1s. Harvest and select at least 20 clean F1 ears from each family. Separately, bulk F1 seed from each family.)
2) Main	Advance to F_2 (BC_0F_2)	Plant 15 rows (255-390 plants) of bulked F_1 (or if 3-5 F1 families, plant 15 rows of each).	Select healthy plants that are resistant to diseases and have other desirable agronomic traits. Self all selected F_1 plants to advance to F_2. Pollinate at least 100 plants. At harvest, select at least 30 healthy ears, bulk F_2 seed, and save remnant seed to represent the F_2 population. On a light table, select endosperm modification scale 3 (you may select scale 2 if you do not have enough scale 3 seed). Thus, ~80% of the seed will be rejected on scale 1 (~75% normal *O2O2* or *O2o2* and ~ 5% *o2o2* completely modified and thus indistinguishable) and another ~10% poorly modified/opaque *o2o2* (scale 4-5). On average only ~10% of the seed will make it through this selection (depending on the germplasm). If more than one QPM donor was used, send a 20-seed sample (selected for modification 3) from each F_2 family to the laboratory for whole grain tryptophan analysis for among-family selection. Lab analysis takes at least 1 month; eliminate all F2 families with tryptophan levels below 0.075%.
3) Off	Form BC_1F_1	Plant 5 rows (85-130 plants) of the selected *o2o2* F_2 family with the highest tryptophan content. Plant only 1 seed per station (hill).	
		Plant 10 rows (170-260 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Select within the family the plants whose phenotypic traits are similar to those of the recurrent parent (genetic background selection based on phenotype). Bulk pollen from 75 plants of the recurrent parent N and pollinate the selected F2 plants to form BC_1F_1. Break the tassels of the recurrent parent plants once pollen has been bulked. Repeat the process of bulking the pollen and pollinating the BC_0F_2 two more times. Use at least 200 plants of the recurrent parent OPV in the process. At harvest, select 20 healthy ears. Make a balanced composite and save remnant seed to represent BC_1F_1.
4) Main	Advance to $BC1F_2$	Plant 5 rows (85-130 plants) of the BC_1F_1 balanced composite.	Select vigorous plants that are disease resistant and have other desirable agronomic traits. Self all selected plants to advance to BC_1F_2. Pollinate at least 75 plants to increase the frequency of modifiers.

Table 10. continued...

Ideal season	Objectives	Materials to plant	Instructions
			At harvest, select at least 30 ear rot resistant ears; shell each ear in a separate envelope. Each individual BC_1F_2 ear must be screened on the light table by selecting modified kernels grades 2 and 3. Selected ears must have a higher frequency of grains with good modification. Take a 20-kernel sample from each individual BC_1F_2 ear (selected for modification 2 and 3) and send to the lab for whole grain tryptophan analysis.
5) Off	Form BC_2F_1	Plant each selected BC_1F_2 family ear-to-row (~30 rows). Plant only 1 seed per station (hill).	Eliminate all rows with tryptophan levels below 0.075%, leaving 8-10 families. Next select within families disease resistant plants with desirable agronomic traits and phenotypic traits similar to the recurrent parent (for genetic background selection based on phenotypic markers).
		Plant 10 rows (170-260 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Bulk pollen from 75 plants of the recurrent parent (N) and pollinate selected plants in selected BC_1F_2 family rows to form BC_2F_1. Once pollen is collected from a plant, detassel the plant to avoid using it twice. Repeat the process of bulking the pollen and pollinating the recurrent parent two more times. Use at least 200 plants of the recurrent parent OPV in the process. At harvest, select at a minimum the 10 best ear rot resistant ears in one BC_1F_2 family. Make a balanced composite with the same number of kernels from the ears selected from each family to represent BC_2F_1.
6) Main	Advance to BC_2F_2 [a]	Plant 5 rows (85-130 plants) of the BC_2F_1 balanced composite.	Select vigorous plants that are resistant to diseases and have other desirable agronomic traits. Self all selected plants to advance to BC_2F_2. Pollinate at least 75 plants to increase the frequency of modifiers. At harvest, select at least 30 ear rot resistant ears; shell each ear in a separate envelope. Each individual BC_2F_2 ear must be screened on the light table to select modified kernels grade 2. Selected ears must have a higher frequency of grains with good modification. Take a 20-kernel sample from each individual BC_2F_2 ear (selected for modification 2) and send to the lab for whole grain tryptophan analysis.
7) Off	Form BC_3F_1	Plant each selected BC_2F_2 family ear-to-row (~30 rows). Plant only 1 seed per station (hill).	Select families first based on tryptophan analysis and eliminate all families with tryptophan below 0.075 %, leaving 8-10 families. Next select among and within families the plants resistant to lodging and diseases whose phenotypic traits are similar to those of the recurrent parent (for genetic background selection based on phenotypic markers).
		Plant 5 rows (85-130 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Compare each BC_2F_2 agronomically to the recurrent parent and only pollinate the best families that look like the recurrent parent. Bulk pollen from 75 plants of the recurrent parent (N) and pollinate all plants selected in BC_2F_2 family rows to form BC_3F_1. Detassel all plants used to avoid using them twice. Repeat the process of bulking the pollen and pollinating two more times. At harvest, select at least 20 ears from each family. Make a balanced composite using all ears selected in each BC_2F_2 family to represent BC_3F_1.
8) Main	Advance to BC_3F_2	Plant 5 rows (85-130 plants) from each BC_3F_1 balanced composite.	Select plants with disease resistance and other desirable agronomic traits. Self all selected plants in each BC_3F_1 family to advance to BC_3F_2. Compare each family agronomically to the recurrent parent, and self the best families that are similar.

[a] One may form the BC_3F_1 at this stage by crossing the BC_2F_1 back to the recurrent parent. BC_3F_2 is then formed and used as the final conversion product. The breeder may decide to make a third backcross depending on whether sufficient recovery of the recurrent parent has been achieved by this stage.

Table 10. continued...

Ideal season	Objectives	Materials to plant	Instructions
		Plant 5 rows (85-130 plants) of the recurrent parent (N) on either side for phenotypic comparison.	At harvest, select the best 30 ear rot resistant ears in each BC_3F_1 family; shell each ear in a separate envelope. Every BC_3F_2 ear should be screened on the light table; select only those with high frequency of modification 2. Ears with no modified kernels should be discarded. Take a 20-kernel sample from each individual BC_3F_2 ear (selected for modification 2) and send to the lab for endosperm tryptophan and protein analysis.
9) Off	Advance to BC_3F_3	Plant each selected BC_3F_2 family ear-to-row (~30 rows). Interplant 3 rows of the recurrent parent (N) every 10 families for phenotypic comparison.	Select only families with high protein and tryptophan content. Self the best 8 plants in BC_3F_2 to advance to BC_3F_3. At harvest, select ears that are resistant to ear rot. Compare each BC_3F_2 agronomically to the recurrent parent (N), and select the best family that also has high protein and tryptophan content.
10) Main	Test cross formation	Plant the selected BC_3F_3 families and the normal line.	Cross a normal line and a converted QPM line to an appropriate tester from the opposite heterotic group.
11 & 12)	Yield trial	Test crosses	Compare the performance of the normal line vs the QPM line in 3-5 locations to confirm equivalent performance.

Table 10a. Approximate costs of converting an OPV to QPM.

	Inbred donor		OPV donor	
Approximate costs	**Rows**	**Rows**	**Lab samples**	**Lab samples**
Total row or sample number	216	235	120	120
Cost per row or sample (US$)	6	6	4.7	4.7
Total cost (US$)	1296	1410	654	654
Total cost of converting a line (US$)	1950	2064		

Cost of tryptophan analysis is $2.20 for sample preparation plus $2.50 for determination. Protein content determination is $3.30. A more reliable value of protein quality would be to use % tryptophan of total protein (rather than % of flour wt), but this adds cost to the conversion process.

Time to conversion is 4.5 years (in locations where 2 cycles per year are possible). Can be reduced to 4 years if cycle 6 is removed and a direct backcross is made from $BC2F_1$ to $BC3F_1$; however, modification of endosperm hardness and protein quality may be lost.

4.5.2 Conversion of a normal line to QPM.

Table 11. Conversion of a normal line to QPM.

Ideal season	Objectives	Materials to plant	Instructions
1) Off	Form F_1 (BC_0F_1)	If using inbred line donor: Plant 1 row (17-26 plants) of each of the progenitors: QPM donor (Q) line, recurrent parent (N) line.	Bulk pollen from plants of the recurrent parent (N) and pollinate all plants in the donor parent (Q), to form F_1. Harvest and select at least 2-3 clean F1 ears (no ear rot). From the selected ears, bulk F1 seed from each family.
		(If uncertain of a good donor line, plant 3-5 potential donor parents.)	(If you have multiple donor parents, form 3-5 respective F1s. Harvest and select at least 2-3 clean F1 ears from each family. Separately, bulk F1 seed from each family.)
	Form F_1 (BC_0F_1)	If using OPV donor: Plant 1 row (17-26 plants) of recurrent parent (N) line. Plant 20 rows (at least 250 plants) of QPM donor (Q) OPV.	Bulk pollen from 75 plants of the donor parent OPV and pollinate 5 plants in the recurrent parent (N), to form F_1. Break the tassels of the donor plants once pollen has been bulked. Repeat the process of bulking the pollen and pollinating the recurrent parent two more times. Use at least 200 plants of the donor parent OPV in the process. Harvest and select at least 20 clean F1 ears (no ear rot). From the selected ears, bulk F1 seed from each family.
		(If uncertain of a good donor, plant 3-5 potential donor parents.)	(If you have multiple donor parents, form 3-5 respective F1's. Harvest and select at least 20 clean F1 ears from each family. Separately, bulk F1 seed from each family.)
2) Main	Advance to F_2 (BC_0F_2)	If using inbred donor: Plant 1 row (17-26 plants) of bulked F1 (or if 3-5 F1 families, plant 1 row of each).	Select healthy plants that are disease resistant and have other desirable agronomic traits. Self all selected F_1 plants to advance to F_2. Pollinate at least 12 plants. At harvest, select at least 10 healthy ears, bulk F_2 seed (~5000), and save remnant seed to represent the F_2 population.
		If using OPV donor: Plant 15 rows (255-390 plants) of bulked F1 (or if 3-5 F1 families, plant 10-15 rows of each).	Select healthy plants that are disease resistant and have other desirable agronomic traits. Self all selected F_1 plants to advance to F_2. Pollinate at least 100 plants. At harvest, select at least 30 healthy ears, bulk F_2 seed (~5000), and save remnant seed to represent the F_2 population. On a light table, select endosperm modification scale 3 (you may select scale 2 if you do not have enough scale 3 seed). Thus, ~80% of the seed will be rejected on scale 1 (~75% normal O2O2 or O2o2 and ~ 5% o2o2 completely modified and thus indistinguishable) and another ~10% poorly modified/opaque o2o2 (scale 4-5). On average, only ~10% of the seed will make it through this selection (depending on the germplasm). If more than one QPM donor was used, send a 20-seed sample (selected for modification 3) from each F_2 family to the laboratory for whole grain tryptophan analysis for among-family selection). Lab analysis takes at least 1 month; eliminate all F2 families with tryptophan levels below 0.075%.
3)	Form BC_1F_1	Plant 5 rows (85-130 plants) of the selected o2o2 F2 family with the highest tryptophan content. Plant only 1 seed per station (hill).	
		Plant 2 rows (34-52 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Select within the family the plants whose phenotypic traits are similar to those of the recurrent parent (for genetic background selection based on phenotype). Bulk pollen from 34-52 plants of the recurrent parent N and pollinate the selected F_2 plants to form BC_1F_1. At harvest, select 20 healthy ears. Make a balanced composite and save remnant seed to represent BC_1F_1.
4) Main	Advance to BC_1F_2	Plant 5 rows (85-130 plants) of the BC_1F_1 balanced composite.	Select vigorous plants that are disease resistant and have other desirable agronomic traits. Self all selected plants to advance to BC_1F_2. Pollinate at least 75 plants to increase the frequency of modifiers.

Table 11. continued...

Ideal season	Objectives	Materials to plant	Instructions
			At harvest, select at least 30 ear rot resistant ears; shell each ear in a separate envelope. Each individual BC_1F_2 ear must be screened on the light table to select those with modified kernels grades 2 and 3. Select at least 20 ears that have a higher frequency of grains with good modification.
			Take a 20-kernel sample from each individual BC_1F_2 ear (selected for modification 2 and 3) and send to the lab for whole grain tryptophan analysis.
5) Off	Form BC_2F_1	Plant each selected BC_1F_2 family ear-to-row (~20 rows). Plant only 1 seed per station (hill).	Eliminate all rows with tryptophan levels below 0.075%, leaving 2-4 families. Next select within families the plants that are disease resistant and have desirable agronomic traits with phenotypic traits similar to those of the recurrent parent (for genetic background selection based on phenotypic markers).
		Plant 2 rows (34-52 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Bulk pollen from 34-52 plants of the recurrent parent (N) and pollinate all plants selected from BC_1F_2 family rows to form BC_2F_1. Once pollen is collected from a plant, detassel the plant to avoid using it twice.
			At harvest, select at a minimum the 10 best ear rot resistant ears in one BC_1F_2 family. Make a balanced composite with the same number of kernels from the ears selected in each family to represent BC_2F_1.
6) Main	Advance to BC_2F_2 [a]	Plant 5 rows (85-130 plants) of the BC_2F_1 balanced composite.	Select vigorous plants that are disease resistant and have other desirable agronomic traits. Self all selected plants to advance to BC_2F_2. Pollinate at least 75 plants to increase the frequency of modifiers.
			At harvest, select at least 30 ear rot resistant ears; shell each ear in a separate envelope. Each individual BC_2F_2 ear must be screened on a light table to select those with modified grade 2 kernels. Select at least 20 ears that have a higher frequency of grains with good modification.
			Take a 20-kernel sample from each individual BC_2F_2 ear (selected for modification 2) and send to the lab for whole grain tryptophan analysis.
7) Off	Form BC_3F_1	Plant each selected BC_2F_2 family ear-to-row (~20 rows). Plant only 1 seed per station (hill).	Select families first based on tryptophan analysis, and eliminate all families with tryptophan below 0.075 %, leaving 2-4 families. Next select among and within families the plants resistant to lodging and diseases with phenotypic traits similar to those of the recurrent parent (for genetic background selection based on phenotypic markers).
		Plant 2 rows (34-52 plants) of the recurrent parent (N) on either side for phenotypic comparison.	Bulk pollen from 34-52 plants of the recurrent parent (N) and pollinate all plants selected from BC_2F_2 family rows to form BC_3F_1. Detassel all plants used to avoid using them twice. Compare each BC_2F_2 agronomically to the recurrent parent, and pollinate only the best families that look like the recurrent parent.
			At harvest, select at least 10 ears from each family. Make a balanced composite using all ears selected from each BC_2F_2 family to represent BC_3F_1.
8) Main	Advance to BC_3F_2	Plant 5 rows (85-130 plants) from each BC_3F_1 balanced composite.	Select plants that are disease resistant and have other desirable agronomic traits. Self all selected plants in each BC_3F_1 family to advance to BC_3F_2. Compare each family agronomically to the recurrent parent, and self the best families that are similar to the recurrent parent.
		Plant 2 rows (34-52 plants) of the recurrent parent (N) on either side for phenotypic comparison.	At harvest, select the best 20 ear rot resistant ears in each BC_3F_1 family; shell each ear in a separate envelope. Every BC_3F_2 ear should be screened on a light table to select only those with high frequency of modification 2 kernels. Ears with no modified kernels should be discarded.

[a] One may form the BC_3F_1 at this stage by crossing the BC_2F_1 back to the recurrent parent. BC_3F_2 is then formed and used as the final conversion product. The breeder may decide to make a third backcross depending on whether sufficient recovery of the recurrent parent has occurred by this stage.

Table 11. continued...

Ideal season	Objectives	Materials to plant	Instructions
			Take a 20-kernel sample from each individual BC_3F_2 ear (selected for modification 2) and send to the lab for endosperm tryptophan and protein analysis.
9) Off	Advance to BC_3F_3	Plant each selected BC_3F_2 family ear-to-row (~20 rows).	Select only families with high protein and tryptophan content. Self the best 8 BC_3F_2 plants to advance to BC_3F_3. At harvest, select ears that are resistant to ear rot.
		Interplant 1 row of the recurrent parent (N) every 10 families for phenotypic comparison.	Compare each BC_3F_2 agronomically to the recurrent parent (N) and select the best family that also has high protein and tryptophan content.
10) Main	Test cross formation	Plant the selected BC_3F_3 families and the normal line.	Cross a normal line and a converted QPM line to an appropriate tester from the opposite heterotic group.
11 & 12)	Yield trial	Test crosses	Compare the performance of the normal line vs the QPM line in 3-5 locations to confirm equivalent performance.

Table 11a. Approximate costs of converting a normal line to QPM.

	Inbred donor		OPV donor	
Approximate costs	**Rows**	**Lab samples**	**Rows**	**Lab samples**
Total row or sample number	117	64	234	74
Cost per row or sample (US$)	6	4.7	6	4.7
Total cost (US$)	702	360	1404	408
Total cost to convert a line (US$)	1062		1812	
Cost of tryptophan analysis is $2.20 for sample preparation plus $2.50 for the determination. Protein content determination is $3.30. A more reliable value of protein quality would be to use % tryptophan of total protein (rather than % of flour wt) but this adds $211 to the conversion costs.				
Time to conversion is 4.5 years (in locations where 2 cycles per year are possible). Can be reduced to 4 years if cycle 6 is removed and a direct backcross is made from BC_2F_1 to BC_3F_1; however, modification of endosperm hardness and protein quality may be lost.				

4.5.3 Recycling non-QPM OPVs and lines with QPM donors (Scheme 1).

Table 12. Recycling non-QPM OPVs and lines with QPM donors (Scheme 1).

Season	Objectives	Materials to plant	Instructions
1	Form F_1	Plant 17-26 plants of the non-QPM line or 300 plants of the non-QPM OPV. Plant 400-500 plants of each QPM donor OPV or 50 plants of the donor line. Using 3-4 donors (minimum of 2) is recommended.	**OPV donor:** Bulk pollen from at least 70-80 good plants (perform mild selection) of the QPM OPV and pollinate 5 plants in each of the non-QPM lines or 75 plants of a non-QPM OPV. Break the tassels of plants used to make the pollen bulk to avoid using them again. Repeat above process on at least 2 additional dates. A total of at least 200 plants of the QPM OPV should be included in the pollen bulks; 12 plants of the non-QPM line or 200 plants of the non-QPM OPV should be pollinated. Select ear rot resistant ears. Make a balanced composite to form the F_1. **Inbred line donor:** Bulk pollen from at least 5 good plants (perform mild selection) from each QPM line and pollinate 5 plants in each of the non-QPM lines or pollinate 75 plants of the non-QPM OPVs. Break the tassels of plants used to make the pollen bulk to avoid using them again. Repeat above process on at least 2 additional dates. A total of at least 12 plants of the QPM line should be included in the pollen bulks and at least 12 plants of the non-QPM line or 200 plants of the non-QPM OPV should be pollinated. Select ear rot resistant ears. Make a balanced composite to form the F_1.
2	Form F_2 (S_1) [a]	Plant 17-26 plants (if only inbreds were crossed) or 170-260 plants (if an OPV was involved in the cross) of the F_1 balanced bulk (formed using equal numbers of seed from each F_1 ear harvested in season 1).	Select plants for good disease reaction and other agronomic traits. Self-pollinate at least 12 selected plants. At harvest, select the best ears.
3	Form S_1 (S_2)	Shell each F_2 ear individually and screen the kernels on a light table; keep and plant only kernels with modification 3 (1 to 5 scale). Plant 1 row for each harvested ear.	Select the best lines (best 20%) for disease reaction and phenotype. Pollinate the best 5 plants in each selected line. Keep 2 to 3 good ears of each selected line.
4	Form S_2 (S_3)	Shell each S_1 (S_2) ear individually and screen kernels on a light table; keep and plant only kernels with modification 2 or 3 (1 to 5 scale). Plant 1 row for each harvested ear. Plant a second set of lines that were screened on the light table.	Select the best lines (best 20%) for disease reaction and phenotype. Pollinate the best 5 plants in each selected line. Keep 2 to 3 good ears of each selected line. Take a 20-kernel sample of each individual ear, and send for whole grain protein and tryptophan analysis. Eliminate ears with low quality. Cross to tester; if the heterotic group is known, cross to tester from the opposite group; otherwise, cross to testers of two groups that are heterotic to each other (e.g., A & B).

[a] Generation name in parentheses is a frequently used alternate name for the same generation.

Table 12. continued...

Season	Objectives	Materials to plant	Instructions
5	Form S_3 (S_4)	Shell each S_2 (S_3) ear individually and screen kernels on a light table; keep and plant only kernels with modification 2 or 3 (1 to 5 scale). Plant 1 row for each harvested ear.	Select the best lines (best 20%) for disease reaction and phenotype. Pollinate the best 5 plants in each selected line. Keep 2 to 3 good ears from each selected line.
	Evaluate TCs	Plant the test crosses.	Evaluate the test crosses in 4 to 6 locations, making sure the chosen sites represent the target environments.
6	Form S_4 (S_5)	Shell ears in bulk; plant only those lines with ability; good protein quality and good combining screen kernels on a light table; plant only kernels with modification 2 (1 to 5 scale).	Increase seed of selected lines by selfing.
		Plant another set of the selected lines.	Cross to other elite germplasm (either lines or single crosses, depending on the desired final product).
			Send 20 kernels of the selected lines for a second whole grain analysis of tryptophan and protein. Eliminate families with poor protein quality.
7	Form S_5 (S_6)	Plant a bulk of the lines from season 6.	Self-pollinate and advance only those lines with good protein quality.
		Plant test crosses.	Evaluate hybrids at multiple locations, making sure the target environments are covered.
8	Form S_6 (S_7)	Plant a bulk of lines with good combining ability, based on test in season 7. Plant at least 10 rows of the selected lines. This is the first big increase of elite lines.	Self-pollinate and advance the lines.
			Send the best hybrids from season 7 for tryptophan and protein analysis, preferably on an endosperm basis.
		Plant a second set of the chosen lines.	Cross to other elite germplasm to generate more experimental hybrids.
		Plant crosses.	Continue testing process as in season 7.

4.5.4 Recycling non-QPM OPVs and lines with QPM donors (Scheme 2).

Table 13. Recycling non-QPM OPVs and lines with QPM donors (Scheme 2).

Season	Objectives	Materials to plant	Instructions
1	Form F_1	Plant 17-26 plants of the non-QPM line or 300 plants of the non-QPM OPV. Plant 400-500 plants of each QPM donor OPV or 50 plants of the donor line. Using 3-4 donors (minimum of 2) is recommended.	**OPV donor:** Bulk pollen from at least 70-80 good plants (perform mild selection) of the QPM OPV and pollinate 5 plants of each of the non-QPM lines or 75 plants of a non-QPM OPV. Break the tassels of plants used to make the pollen bulk to avoid using them again. Repeat above process on at least 2 additional dates. A total of at least 200 plants of the QPM OPV should be included in the pollen bulks; 12 plants of the non-QPM line or 200 plants of the non-QPM OPV should be pollinated. Select ear rot resistant ears. Make a balanced composite to form the F_1. **Inbred line donor:** Bulk pollen from at least 5 good plants (perform mild selection) from each QPM line and pollinate 5 plants of each of the non-QPM lines or pollinate 75 plants of the non-QPM OPVs. Break the tassels of plants used to make the pollen bulk to avoid using them again. Repeat above process on at least 2 additional dates. A total of at least 12 plants of the QPM line should be included in the pollen bulks and at least 12 plants of the non-QPM line or 200 plants of the non-QPM OPV should be pollinated. Select ear rot resistant ears. Make a balanced composite to form the F_1.
2	Form F_2 (S_1)[a]	Plant 17-26 plants (if only inbreds were crossed) or 170-260 plants (if an OPV was involved in the cross) of the F_1 balanced bulk (formed using equal numbers of seed from each F_1 ear harvested in season 1).	Select plants with good disease reaction and other agronomic traits. Self-pollinate at least 12 selected plants. At harvest, select the best ears.
3	Form S_1 (S_2)	Shell each F_2 cob individually and screen the kernels on a light table; keep and plant only kernels with modification 3 (1 to 5 scale). Plant 1 row of harvested each cob.	Select the best lines (the best 20%) for disease reaction and phenotype. Pollinate the best 5 plants in each selected line. Keep 2 to 3 good ears of each selected line.
4	Form S_2 (S_3)	Shell each S_1 (S_2) ear individually and screen kernels on a light table; keep and plant only kernels with modification 2 or 3 (1 to 5 scale). Plant 1 row of each harvested ear.	Select the best lines (the best 20%) for disease reaction and phenotype. Pollinate the best 5 plants of each selected line. Keep 2 to 3 good ears of each selected line.
5	Form S_3 (S_4)	Shell each S_2 (S_3) ear individually and screen kernels on a light table; keep and plant only kernels with modification 2 or 3 (1 to 5 scale). Plant 1 row of each harvested ear. Plant a second set of lines that were selected on the light table.	Select the best lines (th ebest 20%) for disease reaction and phenotype. Pollinate the best 5 plants of each selected line. Keep 2 to 3 good ears of each selected line. Take a 20-kernel sample of each individual ear, and send for whole grain protein and tryptophan analysis. Eliminate ears with low quality. Cross to tester; if heterotic group is known, cross to tester from the opposite group; otherwise cross to testers of two groups heterotic to each other(e.g., A & B).
6	Form S_4 (S_5)	Shell each ear individually, and screen kernels on a light table; keep and plant only kernels with modification 2 (1 to 5 scale). Plant 1 row of each harvested ear. Plant only those progenies whose progenitor has good protein quality.	Select the best lines (the best 20%) for disease reaction and phenotype. Pollinate the best 5 plants of each selected line. Keep 2 to 3 good ears of each selected line.

[a] Generation name in parentheses is a frequently used alternate name for the same generation.

Table 13. continued...

Season	Objectives	Materials to plant	Instructions
		Plant test crosses.	Evaluate test crosses in 4 to 6 locations making sure the chosen sites represent the target environments.
7	Form S_4 (S_5)	Shell the ears in bulk; plant only those lines with good protein quality and good combining ability; screen kernels on a light table; plant only kernels with modification 2 on a 1 to 5 scale.	Increase seed of selected lines by selfing.
		Plant another set of the selected lines.	Cross to other elite germplasm (either lines or single crosses, depending on the desired final product). Send 20 kernels of the selected lines for a second whole grain analysis of tryptophan and protein.
8	Form S_5 (S_6)	Plant a bulk of the lines from season 6.	Self-pollinate and advance only those lines with good protein quality. Evaluate hybrids at multiple locations making sure the target environments are covered.
		Plant crosses.	
9	Form S_6 (S_7)	Plant a bulk of lines with good combining ability, based on test in season 7. Plant at least 10 rows of the selected lines. This is the first big increase of elite lines.	Self-pollinate and advance the lines. Send the best hybrids from season 7 for tryptophan and protein analysis, preferably on an endosperm basis.
		Plant a second set of chosen lines.	Cross to other elite germplasm to generate more experimental hybrids.
		Plant the crosses.	Continue with the testing process as in season 7.

4.5.5 Recycling elite QPM with elite QPM

The process of recycling elite QPM with elite QPM is very similar to recycling non-QPM OPVs and lines with QPM donors. This method has the advantage that the *o2* allele is fixed even in segregating generations. All the principles of good selection discussed in this manual have to be borne in mind, and careful selection for good modification has to be performed. However, this method can be used only when breeding programs have sufficient elite QPM germplasm. Its protocol is very similar to protocols discussed in Tables 12 and 13. The only change recommended is to start selecting Modification Type 2 or 3 (as opposed to selecting only Type 3) in the F_2, and to select Type 2 in subsequent generations.

5. Seed Production

Production of QPM seed is no different from non-QPM seed production. The same strict standards must be followed to ensure reproduction of true-to-type, high-quality seed. Several manuals (e.g., Beck, 1999) give a detailed description of maize seed production, and the reader should consult such manuals for an in-depth review. Some specific recommendations for QPM seed increase are made in Sections 5.1, 5.2, and 7.1. The only additional requirement to meet the standards for QPM seed is that seed increases must be sent to the laboratory for tryptophan and protein analysis to ensure that values are above the required minimum.

5.1 OPV breeder seed production

To ensure protein quality and endosperm modification of seed, it is recommended that QPM OPV breeder's seed be produced in half-sib isolation blocks.

Cycle 1:

- Plant 300-500 representative plants in isolation in a breeder's seed production field.
- At harvest, shell each ear separately.

Cycle 2:

- Plant each ear as a single female row.
- Bulk equal quantities of each ear to plant as male rows (sole pollen source).
- Plant 3 female rows to 1 male row in a half-sib isolation block. Detassel the female rows.
- Conduct mild selection by rejecting all rows that deviate from the variety description. However, ensure that not more than 20% of female rows are rejected.
- At harvest, select the best representative rows and plants within rows of the variety under production to provide seed for the next seed production cycle.
- Select 1-2 ears from representative rows to constitute the 300-500 female rows for the next production cycle.
- Conduct lab analyses of seed samples of the selected ears. Use only ears that have acceptable protein and tryptophan levels for the bulk seed to be used as the pollen source. Reject female rows that have unacceptably low protein quality. Note that lab analyses could be limited to every third cycle of breeder's seed production to save time and money.

Cycle 3:

- Proceed to plant the third cycle of seed production as in cycle 2.
- ***After counting seed to plant as females, keep remnant seed of each female in storage, and bulk the rest of the seed together with the remaining seed of the male rows to constitute the breeder's seed for seed producers.***

In cases where a cultivar is suspected of having lost protein quality:

- Grow out at least 10 rows of breeder's seed of the inbred line and self-pollinate 3-5 ears in each row.
- At harvest, select only ears showing segregation for endosperm modification (modification types 2 to 5); reject normal looking ears (modification 1).
- Shell each ear separately.
- Use the light table to select seeds showing modification 2-3 to represent each ear.
- Send samples of seeds for lab analysis.
- Reconstitute the breeder's seed using ears selected for good protein quality.

5.2 OPV foundation seed production

Foundation seed producers should produce their seed from a fresh stock of breeder's seed after two cycles of foundation seed production. They should maintain a quality check on their foundation seed blocks by sending representative bulk samples for regular protein quality analyses. This will not only ensure that the benefits of protein quality are passed on to client seed producers and, eventually, to farmers and consumers, but will also help breeder seed producers maintain the required quality standards of the variety.

6. QPM and Its Benefits to Farmers and Communities

The *o2* is a recessive gene, and the homozygous recessive condition, *o2o2,* is a prerequisite for high lysine and tryptophan in maize. Pollination of a QPM cultivar with non-QPM pollen immediately renders the grain harvested non-QPM—i.e., grains on a QPM ear that are fertilized by extraneous pollen from non-QPM maize will be non-QPM grain. This is similar to what happens when yellow endosperm maize is planted in the vicinity of white endosperm maize. The extent of contamination in the white endosperm maize is immediately visible at harvest due to the xenia effect of endosperm color and because yellow is dominant over white. It is very likely that a farmer's field sown to a QPM cultivar for grain production will be surrounded by plots of non-QPM cultivars. This could be a setback for a farmer hoping to:

a) derive nutritional benefit from the consumption of QPM, or
b) save seed for planting the following crop.

Another difficulty is that QPM cultivars look like non-QPM cultivars. Markets in most of the developing world are non-specialized. Since a heap of QPM grain cannot be visually differentiated from non-QPM grain, a premium price cannot be set for QPM grain unless a quality analysis is performed. This is another perceived drawback:

c) for the farmer, especially one looking to obtain added returns by selling QPM grain solely for its enhanced nutritional quality, and
d) for the sensitized consumer looking to purchase QPM.

A third difficulty is that many farmers grow maize under conditions of low soil fertility (especially low nitrogen), as they either do not have the resources to purchase nitrogenous fertilizers or do not have access to them. In addition, according to some reports, endosperm modification apparently breaks down when crops are affected by drought. This has led many skeptics to wonder:

e) whether QPM grown under drought conditions or in soil with less than optimum nutrients still has good protein quality, or
f) whether QPM may lose its quality even when grown under optimal conditions and not contaminated by non-QPM pollen.

The above arguments have been put forth in debates that question whether QPM really benefits the target group (i.e., resource-poor farmers and people who cannot afford a protein-rich diet), by increasing their financial returns or improving their nutrition. These criticisms are addressed in the following sub-sections.

6.1 QPM contamination in farmers' fields

a) What nutritional benefits will the farmer obtain if his/her field is surrounded by non-QPM cultivars? Assuming the worst case scenario, the following arguments can be made in support of QPM production:

- If one row of QPM is sown next to a non-QPM cultivar, 50% of the grain harvested from the QPM cultivar should still be high in lysine and tryptophan. This is because non-QPM pollen competes about evenly with QPM pollen to pollinate the QPM cultivar.
- When a block of QPM is surrounded by non-QPM, the proportion of non-QPM pollen to QPM pollen pollinating the QPM should be about 4:1, and the farmer can expect at least 25% of harvested grain to be QPM.
- In an even worse situation, when the proportion of non-QPM:QPM pollen is higher, a small portion of the harvested grain will be QPM and will nutritionally benefit (however small) the farmer and his household. The proportion of QPM:non-QPM grain increases towards the middle of the field. As awareness of QPM spreads, and as more farmers and entire communities start growing QPM cultivars, the nutritional benefits can only increase.

In fact, several studies conducted to directly address the above concerns present an even more favorable picture. In a QPM contamination study conducted in Ghana (Ahenkora et al., 1999; Twumasi-Afriyie et al., 1996b), a one-acre field of white grained QPM variety Obatanpa was completely surrounded by a 5-meter band of a

yellow-grained, non-QPM cultivar of the same maturity. Results of the experiment showed that the maximum contamination was 11% of the entire harvest. The greatest contamination occurred within the first 6 meters of the QPM field; thereafter contamination rapidly decreased towards the middle of the field, 30 meters from the non-QPM, where there was virtually no contamination. Wind direction was also important, with the highest contamination in the windward direction to the QPM.

Findings from the study clearly showed that the extent of contamination depends on:

- proximity of the non-QPM maize;
- synchrony of flowering between QPM and non-QPM cultivars;
- wind direction towards or away from the QPM during pollination; and
- competitiveness of the non-QPM pollen that lands on the QPM silks.

In practical terms, therefore, planting a QPM field next to a non-QPM field does not completely render the entire harvest non-QPM, but rather induces levels of contamination ranging from 0 to 11%.

When physical mixtures of QPM and non-QPM grains in varying proportions were assessed both in lab analyses and rat-feeding studies, it was found that contamination caused the loss of QPM benefits only after the introduction of more than 20% of non-QPM grain into the QPM (Ahenkora et al., 1999), a contamination level higher than what was observed in the field (maximum contamination: 11%).

The above studies suggest that farmers do not lose the entire benefit of QPM under normal farming conditions, when there are non-QPM plots in the vicinity.

b) What should a farmer do if he wishes to save seed from his/her grain production for the following cycle? This is an issue even for farmers growing an improved non-QPM cultivar among unimproved local varieties. The above studies clearly suggest that:

- farmers should select OPV seed from the middle of their fields, away from possible contamination sources;
- farmers should purchase fresh seed from seed producers after three planting cycles to renew varietal purity; and
- these recommendations should become extension messages not only for QPM growers but for all OPV growers to ensure that they benefit from the use of improved OPVs.

6.2 QPM adoption and marketing

c) Can farmers expect to receive premium prices for their QPM grain?

- Farmers should not expect to receive premium prices for QPM grain in a non-specialized market. Any QPM cultivar available to the farmer must be able to compete agronomically with a non-QPM check (i.e., it must yield as much or more than non-QPM cultivars). Thus the sale of QPM grain generates higher income only if the QPM cultivar yields more, because the nutritional benefit remains hidden. Figure 21 shows that it is possible to develop QPM cultivars that perform

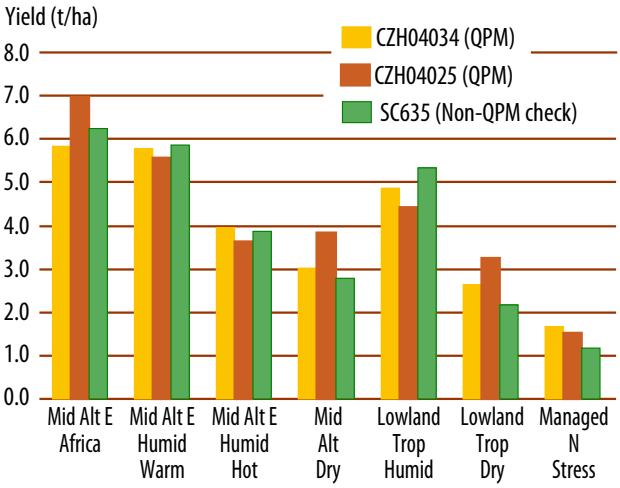


Figure 21. Comparison of yield performance of two QPM hybrids with a non-QPM commercial check across 25 locations in eastern and southern Africa in 2005.

better than non-QPM check cultivars. This should be the primary strategy for every QPM breeding program targeting areas with non-specialized markets. Nevertheless, where relief organizations prefer QPM to normal maize, farmers at least have a more assured market for their products, though perhaps not at a premium.

d) The following strategies were used effectively in Ghana in the early 1990s (Twumasi-Afriyie et al., 1996a) to ensure that QPM purchasers and other commercial users received the full benefit of its quality. They foster QPM adoption, especially in a country that is just beginning to do so:

- Selected communities should be targeted for saturation with their preferred QPM cultivar.
- Commercial food and feed producers wishing to use QPM should engage only purchasing agents who agree to deliver QPM of the required quality.
- QPM purchasing agents should be linked to saturated QPM-growing communities.

In general it has been observed that once farmers identify QPM as the preferred maize cultivar based on their own experience or on extension messages, they always save enough QPM grain to satisfy their household needs and sell other non-preferred grain types.

6.3 Stability of QPM quality under poor soil fertility conditions

e) Several studies have shown that QPM grown under low soil nitrogen conditions has lower protein quantity. However, the quality of the protein (lysine and tryptophan levels) is unaffected. In other words, QPM is still QPM when grown under low soil nitrogen. Studies have also shown that protein quality is also maintained when QPM is grown under drought conditions.

6.4 Stability of QPM quality with no contamination by non-QPM maize

f) In conclusion, it is fair to say that QPM does not lose protein quality when it is not contaminated by non-QPM.

7. Protein Quality Standards

7.1 Breeding standards

7.2 Selection criteria and standards

Table 14. Selection criteria and standards.

				Scheme 1 Breeding Scheme		Scheme 2	
Generation to plant Naming convention A	B	Season number	Select under light table every season; use modification scale of	Testcrossing	Lab analysis (always light table for the appropriate modification score and then send for analysis)	Testcrossing	Lab analysis (always light table for the appropriate modification score and then send for analysis)
S1	F2	3	3				
↓	↓						
S2	S1	4	2 to 3	Cross to tester; if heterotic group known cross to tester from the opposite group; else cross to both A and B testers	First tryptophan analysis of inbred lines		
↓	↓						
S3	S2	5	2 to 3	Advance and test only those lines that show good quality		Cross to tester; if heterotic group known cross to teser from the opposite group; else cross to both A and B testers	First tryptophan analysis
↓	↓						
S4	S3	6	2	Cross to other elite germplasm (either lines or single crosses depending on what product is being targeted)	Second tryptophan analysis of inbred lines	Advance and test only those lines that show good quality	
↓	↓				Do analysis of tryptophan and protein of best hybrids that have been remade for wide testing (also include parents of hybrdis)		
S5	S4	7	2	Advance and test only those lines that show good quality and perform well in the first test		Cross to other elite germplasm (either lines or single crosses depending on what product is being targeted) or remake best hybrids from above test for wide testing	Second tryptophan analysis of all lines
↓	↓						
S6	S5	8	2	Cross to other elite germplasm (at least 3 lines or single crosses depending on what product is being targeted)		Advance and test only those lines that show good quality; wide-test only those hybrids that are good for lab quality	Do analysis of tryptophan and protein of best hybrids that have been remade for wide testing (also include parents of hybrdis)
↓	↓						
Sn	S6	9	2	Advanced generation test	Check tryptophan level of hybrid seed	Cross to other elite germplasm (at least 3 lines or single crosses depending on what product is being targeted) or remake best hybrids from above test	

7.1 continued...

Generation to plant				Scheme 1 Breeding Scheme		Scheme 2	
Naming convention		Season number	Select under light table every season; use modification scale of	Testcrossing	Lab analysis (always light table for the appropriate modification score and then send for analysis)	Testcrossing	Lab analysis (always light table for the appropriate modification score and then send for analysis)
A	B						
↓	↓					Advanced generation test	Check tryptophan level of hybrid seed
Sn	Sn	10	2	Do not increase an inbred line too many times, i.e. increase in big lots. For each increase have at least 10 rows (target 10 kgs of seed per increase). Select (per se) best row of the 10 rows as your long-term breeder's stock.* Bulk the remaining seed and send bulked seed and breeder's seed to lab. Use bulk seed for any further increase. Check tryptophan level for each increase.		Do not increase an inbred line too many times, i.e. increase in big lots. For each increase have at least 10 rows (target 10 kgs of seed per increase). Select (per se) best row of the 10 rows as your long-term breeder's stock. Bulk the remaining seed and send bulked seed and breeder's seed to lab. Use bulk seed for any further increase. Check tryptophan level for each increase.	
OPVs and Sn hybrids	OPVs and Sn hybrids				Analyze tryptophan and protein of hybrid and inbred parents. For OPVs take 5-10 samples for analyzing.		Analyze tryptophan and protein of hybrid and inbred parents. For OPVs take 5-10 samples for analyzing.

* Elite lines could be checked for purity by "growing out" 100-200 plants until they are knee height. The level of contamination can be determined by this method.

7.2 Selection Criteria and Standards

Preparing Samples for lab analysis

Code the samples (e.g., Entry 1, Entry 2, etc.) sent to the lab so that the pedigree or name of the sample does not bias the person doing the analysis.

Send 20 kernels for inbred lines and hybrids and 50 seeds of OPVs.

Send the samples in 2 or 3 replicates (recommended: OPVs should be sent in 5 replicates).

Always include a non-QPM check and a known QPM check.

Selection Criteria and Standards

	Minimum levels	
	Whole grain	Endosperm
Tryptophan in sample	0.075%	0.070%
Protein	8%*	8%*
Quality index (QI)**	0.8	0.7

*Protein level higher than 8% is desirable; but ensure QI is above minimum
**(Tryptophan in sample / protein in sample)

8. New Methods

During backcross conversion of normal lines, a selfing generation between each backcross is required to recover the recessive *o2* allele before selecting for endosperm hardness and amino-acid-modifying loci. Thus, it takes seven seasons to complete QPM conversion and obtain BC3 seed, compared to the typical four seasons (Krivanek and Vivek, 2006) required for breeding a non-QPM line, for example, for disease resistance. One of the potential solutions to this problem is to use molecular markers to identify the *o2* gene, as will be discussed in this chapter.

8.1 Marker-assisted selection (MAS)

8.1.1 Molecular markers for the *opaque-2* gene[6]

It has recently become possible to use MAS to accelerate selection for the *opaque-2* allele in QPM breeding work. There are three publicly available (SSR) markers for this purpose, phi57, phi112, and umc 1066, (J.M. Ribaut, personal communication) the sequences of which are in the maize database (http://www.agron.missouri.edu/ssr.html) and can be ordered from any company that synthesizes oligos (e.g., Research Genetics: info@resgen.com). The three markers are located within the *opaque-2* gene, which means there is very high correlation between marker data and phenotypic expression.

The *opaque-2* gene has about 4000 DNA bases considering introns and exons, and the difference between an *opaque-2* mutant allele and a "normal" allele varies from 50 to perhaps only 2 or 3 bases. Such differences in DNA composition are located at different places in the gene sequence. The three PCR markers amplify up to 300 bases at three different spots and, depending on the position of the DNA difference between the two alleles of a given cross, a polymorphism may be detected.

To use molecular markers to screen for the presence of *o2o2* in a segregating family or population, you must have a polymorphism for one of the three available SSRs, that is, a difference between DNA fragment size (length) of the donor (QPM) and recipient (recurrent parent). Currently CIMMYT does marker-assisted selection for *opaque-2* on crosses between two inbreds; therefore, we need a polymorphism between two alleles, one from each line. In any particular cross, it is critical that the markers be run first on the two parents, to confirm the polymorphisms (size differences) for the marker alleles. Occasionally the two parents may share the same marker alleles and, in such cases, one of the two remaining markers must be tried.

In the case of an OPV, which is a mixture of genotypes, there may be between 3 to 10 alleles at the marker locus in the population, because it is not fixed. Therefore, the probability that a single allele from a QPM line is different from the numerous alleles present in an OPV is lower than the probability of finding a polymorphism between lines. In the former case, one allele is compared with an average of six alleles; in the latter case, one allele is compared with another one. This means that more than one SSR marker may have to be assessed to define a "haplotype" (or the specifically different alleles at three different markers) characteristic of the QPM parent.

One of the markers, phi112, is dominant and identifies genotypes that do not contain a recessive *o2* allele. This means that the marker will identify normal (*O2O2*) and heterozygous (*O2o2*) genotypes; the breeder can then assume that all other genotypes are of the desired homozygous recessive type (*o2o2*). However, in diverse backgrounds of CIMMYT maize germplasm, some *o2o2* lines also present a band with this marker. Therefore, whenever markers are used to distinguish *o2o2* genotypes from *O2o2* and *O2O2*,

[6] Much of the following discussion is excerpted from personal (e-mail) communication between J.M. Ribaut (CIMMYT Applied Biotechnology Center, Mexico) and Kevin Pixley (CIMMYT-Mexico), and from personal communication with Kevin Pixley and D.J. Skinner (CIMMYT Applied Biotechnology Center, Mexico).

the donor parent and the recipient parent (be it a line or an OPV) must be clearly defined by marker genotyping prior to selection, and multiple SSR markers may be needed to select *o2o2* progeny in the subsequent segregating family.

8.1.2 Constraints in using molecular markers for breeding

The advantage of MAS is that leaf tissue from seedlings is used to extract DNA and conduct the assay; therefore, selection of the desired genotypes can be completed prior to flowering, and only the desired plants need to be pollinated. While MAS for the *o2o2* genotype has been applied at CIMMYT and evaluated relative to other procedures (Dreher et al., 2003), MAS for the QPM genotype currently presents two problems: effectiveness and cost (Krivanek et al., 2007).

As mentioned above, one of the three available markers is dominant and does not amplify the *opaque-2* allele. Therefore, this marker is suitable for identifying the homozygous *opaque-2* mutant alleles that are needed for QPM. The marker will not distinguish between heterozygous and homozygous normal allele genotypes, because both cases will amplify DNA and produce a band. Based on CIMMYT's experience, this marker works with most tropical QPM lines, but we also found a few QPM lines that present a band with this marker. This means that there may be more than one *opaque-2* alleles in CIMMYT germplasm, as the lines used in those few crosses were QPM.

Marker-assisted selection for the *o2* allele has been reported in a study aimed at achieving faster backcross conversion of normal endosperm genotypes to the *o2o2* genotype (Babu et al., 2005), and would appear to be an appropriate use of the technique. However, without concurrent selection for amino acid modifiers, protein quality can drop considerably even in *o2o2* backgrounds. In the study by Babu et al. (2005), tryptophan content as a percentage of total protein decreased from 1.05% in the QPM donor line to 0.78-0.85% in the BC_2F_2 families. Reduction in protein quality when not selecting for amino acid modifiers has also been observed in CIMMYT's QPM breeding program; this is not surprising, considering the wide variation of lysine levels in *o2o2* genotypes of different genetic backgrounds (Moro et al., 1996). Loss of hard endosperm modification has also been found to occur in lines resulting from direct backcrossing procedures evaluated at CIMMYT. In order for QPM MAS to be fully effective (by reducing breeding time, enabling selection in non-target environments, etc.), a suite of effective markers linked to modifying loci of both amino acid levels and endosperm hardness needs to be identified (Krivanek and Vivek, 2006). Also, additional traits will likely need to be coupled with selection for QPM to become cost competitive with traditional methods.

Currently, any maize seed may be planted in the field, self pollinated, and genotyped for seed traits (such as QPM) at a cost of US$ 0.24 per plant (assuming $6 and 25 plants per row). Current costs of marker genotyping are considerably higher at US$ 0.50-2.50 per plant. If the goal of multiple trait selection can be met, then total MAS costs will be much more in line with traditional screening methods.

Bear in mind that there are no markers available for endosperm modification. Modification has to be selected using the light table.

9. Concluding Thoughts

Breeding is a continuous, never-ending process with a time lag between initiation of a project and product delivery. In a good breeding program, there is always germplasm at various stages of development (from breeding starts, F2s, S1, ….up to final products being tested and seed being increased) in every season. Any break in this chain (e.g., failure to generate a breeding start in a particular season) could lead to a gap in product delivery down the road. Thus, "the worst one can do in plant breeding is to do nothing."

There is no plant breeding method that is absolutely right or absolutely wrong. But it is essential that the chosen breeding method maximize the odds of identifying a good cultivar ***with the available resources***.

Breeding is as much (or more) of an art as a science. A good understanding and correct application of the science of breeding and genetics help boost the probability of success. Nonetheless, there are several examples of great successes in plant breeding achieved by people with minimal formal training but who have applied the plant breeding skills they gained through practical experience.

"The success of a breeding program depends on how much you throw out."
Anonymous

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11. Appendix

11.1 Potential contribution of quality protein maize to human nutrition[1]

The following gives a brief insight into published references on the benefits of quality protein maize (QPM) for human nutrition. The literature on the importance of various forms of human malnutrition and the best remedial interventions is vast and complex, and seemingly simple questions are often difficult to answer due to the relationship among poverty, various forms of malnutrition, other health issues, and the great variation in diets. However, as outlined below, there is significant scientific evidence that QPM is making an important and relevant contribution to human nutrition.

11.1.1 The biological value of QPM

The biological value of protein is estimated based on the average proportion of absorbed protein that is successfully retained by the body for maintenance and growth. Biological value is closely related to protein quality, which in the case of maize is limited mostly by low concentrations of the amino acids lysine and tryptophan. Quality protein maize contains *opaque-2*, a single-gene mutation that alters the protein composition of the endosperm portion of the kernel, and nearly doubles lysine and tryptophan concentrations. At least four studies on children and four on adults have found that subjects eating QPM had significantly higher nitrogen retention than those who ate normal maize (Bressani, 1991), indicating that QPM protein is more "bioavailable" (NRC, 1988). The biological value of QPM protein is about 80%, that of milk is about 90%, and that of normal maize is about 45% (FAO, 1992).

Experts concluded that:
"*The data demonstrating the nutritional superiority of QPM over normal maize in human nutrition are overwhelming*" (Bressani, 1991).
"*The evidence presented from studies in both children and adults clearly indicates the superiority of opaque-2 maize over common maize*" (FAO, 1992).

11.1.2 The link between wealth, quality of diet, protein, and lysine malnutrition

The link between wealth and quality of diet is both intuitive and well documented. Comparisons of per capita gross national product (GNP) with food balance sheets indicate that increased wealth is associated with the consumption of more calories, more protein, more animal protein, less cereal protein, and much more lysine (Pellet and Ghosh, 2004; Young and Pellet, 1990). Animal protein has higher biological value than cereal protein, and while 60-70% of the protein people in wealthy countries consume comes from animals and less than 20% from cereals, this proportion is often reversed in poorer countries.

It has been argued that improved—and more diverse—diets offer a better solution than high-lysine cereals such as QPM. However, diets of poor consumers in regions where maize is a staple food are not in all cases improving. In Bangladesh, for example, prices of staple grains have declined by about 40%, while those of fish, animal, and non-staple plants have increased 75-100% in the past three decades. Poor people who obtain most of their protein from cereals can afford relatively little meat and legumes, and are particularly likely to suffer lysine deficiency.

Experts concluded that:
"*QPM can provide a partial safety net for poor consumers when economic, social, or environmental reasons drive their diets toward increased relative consumption of staple cereals"* (Rahmanifar and Hamaker, 1999).

11.1.3 Protein malnutrition in countries where maize is the staple

Per capita consumption of maize is particularly high in eastern and southern Africa and Central America, but maize is also an important staple to the poor in several countries of West Africa, Asia, and South America. In recent decades nutritionists have debated and modified their recommendations on protein and lysine requirements for healthy

[1] Flyer developed to spread awareness of the benefits of QPM for humans. Kevin Pixley and Marianne Banziger, 2006, personal communication.

human diets. Based on 1991 FAO/WHO recommendations, as well as on soon-expected revisions to these, it has been estimated that "…a significant proportion of those in Africa might be at risk of lysine deficiency" (Pellet and Ghosh, 2004). It is important to recognize that dietary guidelines are set for healthy individuals, whereas protein requirements may vary. For example, they are substantially higher for catch-up growth or recovery from infections common among children in poor communities (Chupad et al., 2003; Rahmanifar and Hamaker, 1999).

Per capita maize consumption in Malawi (nearly 150 kilograms per year) is among the highest in the world. Recent studies report that in Malawi 70-83% of low-income children 2 to 5 years old are stunted, and a 12-month study found that severe malnutrition was the principal diagnosis for 11% of all children admitted to hospitals in the southern Malawian city of Blantyre (Brewster et al., 1997). Milk is the principal treatment for these malnourished children, but when supplies of powdered milk were cut, hospitals had to seek alternative dietary interventions. Although this study did not use it, QPM was cited as "another approach" both as a milk-replacing ingredient in diets for recovering children and for its possible role in reducing malnutrition, if QPM were to replace normal maize as the Malawian staple.

Recent studies in China and Pakistan demonstrated that lysine fortification of cereal-based diets (wheat, in those cases) improved growth in children and various health indicators for children and adults, confirming that lysine enrichment of cereal-based diets remains beneficial to some populations (Hussain et al., 2004; Pellet and Ghosh, 2004; Zhao et al., 2004). Similarly, recent studies on children in Ghana and Mexico have documented growth and health benefits for children eating QPM instead of normal maize (Akuamoa-Boateng, 2002; Morales-Guerra, 2002).

Experts concluded that:

"*The nutritional advantages of quality protein maize vs common maize are of a magnitude that must be exploited for the advantage of children in maize-consuming poor countries*" (Graham et al., 1989).

"*…there appears to be a substantial rationale for continuing research directed towards increasing the production of legumes and of cereals with higher lysine content as well as for improving the output and efficiency of production of animal protein foods*" (Young and Pellet, 1990).

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For further information:

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11.2 Laboratory protocols

The following protocols are currently used in CIMMYT's Soil and Plant Analysis Laboratory (SPAL). However, methodologies are being upgraded, including modification of the colorimetric method for tryptophan analysis based on the Hopkins-Cole reaction (Nurit et al., 2007, in preparation) and use of NIR (near infrared reflectance) for protein and amino acid composition (Orman and Schumann, 1991). The main advantage of the modified tryptophan method is that it obviates the need to use acetic acid, a chemical that has proven very difficult for many laboratories to obtain within the required quality (purity) standards. Higher throughput, improved sensitivity, and decreased analysis costs are additional factors driving the upgrading of the procedure. Anyone interested in detailed results of the updated methodologies should contact the SPAL laboratory at CIMMYT (n.palacios@cgiar.org).

11.2.1 Nitrogen determination

Nitrogen determination is based on a colorimetric method in which an emerald-green color is formed by the reaction of salicylate and hypochloride with ammonia.

11.2.1.1 Nitrogen determination with the Technicon Autoanalyzer II method

Sample digestion:

1. Weigh between 40 mg ground sample. Include two check samples.
2. Transfer the sample to the bottom of a 75 ml digestion tube.
3. Include one or two tubes as blanks (no samples) for digestion.
4. Add 2.0 g of the catalyst mixture to each tube and 2.5 ml concentrated H_2SO_4. Let stand until the reaction ceases.
5. Digest under the fume hood in a pre-heated digester block at 380 °C for 90 minutes.

Sample analysis:

6. Remove the rack of tubes from the digester, let them cool to room temperature and add 75 ml of distilled water to avoid crystal formation. Make sure the digestion solution is totally clear.
7. Close tubes tightly with a rubber cap and mix by inverting the tubes several times.
8. Transfer 2 ml of the solution to Technicon vials and place samples in the Technicon Autoanalyzer.
9. Establish the baseline by pumping each of the four reagents: reagent mixture 1, reagent mixture 3, reagent mixture 4, and sodium hypochloride.
10. Set at 0% on the chart using the blank digestion solution.
11. Run four vials of blank digestion solution and recheck the 0% baseline.
12. Run four vials of 20 µg N/ml standard and set the peak at 70% on the chart.
13. Run check samples and unknown samples.

Preparing the N standard:

1. Prepare 100 µg/ml of ammonium sulphate solution in distilled water.
2. Every time you analyze samples, make a dilution of 20 µg/ml ammonium sulphate in blank digestion solution.

Calculating the nitrogen percentage:

20 µg N/ml is set at 70% on the chart, where:
1% on the chart = 0.2857 µg N/ml in digest
µg N/ml in digest = % chart reading x 0.2857 µg N/ml
or
µg N in 75 ml digest = % chart reading x 0.2857 µ x 75

$$\text{Calculation factor} = \frac{20 \mu gN/ml \text{ X Digestion volume x } 100\%}{\text{Set of chart divisions x } 1000}$$

$$\%\,N = \frac{2.1427 \text{ x chart reading}}{\text{Weight of sample (mg)}}$$

Special recommendations:

1. Soap may be used to clean the digestion tubes, but be sure to remove all residue with deionized water.
2. If necessary, digested samples can be stored at room temperature, protected from air, for a maximum of 7 days before doing the sample analysis. However, the sooner the digested samples are analyzed, the better.
3. Clean the Technicon vials by washing 3-4 times with deionized water only. Do not use soap.
4. Always include at least two standards with every 34-sample set analyzed.
5. Calibrate the Technicon every time measurements are started.

Appendix Table1. Reagents used for nitrogen determination with the Technicon Autoanalyzer.

Reagent/ mixture	Specific reagents	Preparation	Special recommendations
100 ug/ml ammonium sulphate		Weigh 1.179 g and dissolve in 250 ml distilled water.	Store at 4 °C for one month maximum. Keep in a light-protected container.
Sulfuric acid (analytical grade, 98%)			Store at room temperature in a light-protected container.
Catalyst mixture	Potassium sulphate Selenium	Mix 1 kg of K_2SO_4 very well with 5 g of selenium.	Handle very carefully; selenium is extremely dangerous. Store at room temperature.
Reagent mixture 1	Sodium chloride Sulfuric acid Brij 35 purified	Dissolve 200 g of sodium chloride, 15 ml of sulfuric acid and 2 ml of Brij 35. Complete volume to 2 l with distilled water.	Store at room temperature.
Reagent mixture 2	Sodium phosphate dibasic anhydrous Sodium hydroxide	Dissolve 71 g of sodium phosphate dibasic anhydrous and 20 g sodium hydroxide. Complete volume to 1 l with distilled water.	Store at room temperature.
880 mM potassium tartrate	Potassium L- tartrate tetra-hydrated	Dissolve 200 g potassium tartrate in 1 l distilled water.	Store at room temperature.
5 M sodium hydroxide	Sodium hydroxide	Dissolve 200 g sodium hydroxide in 1 l distilled water.	Store at room temperature.
Reagent mixture 3	Reagent mix 2 880mM potassium L-tartrate tetra-hydrated 5 M sodium hydroxide Brij 35	Mix 400 ml of reagent mixture 2, 500 ml of 880 mM potassium tartrate, 500 ml of 5 M sodium hydroxide, and 1ml of Brij 35. Complete volume to 2 l with distilled water.	Store at room temperature in darkness for a maximum of 15 days.
Reagent mixture 4	Sodium salycilate (99.5%) Sodium nitro-prusiate Brij 35	Dissolve 300 g sodium salycilate (99.5%) and 600 mg sodium nitro-prusiate. Add 2 ml Brij 35 and complete volume to 2 l with distilled water.	Store at room temperature in darkness.
Sodium hypochloride	Sodium hypochloride	Use 6 ml sodium hypochloride and complete volume to 100 ml with distilled water.	Store at room temperature in darkness.
Ammonium sulphate (100 μg/ml)	Ammonium sulphate	Dissolve 10 mg of ammonium sulphate in 100 ml distilled water.	Store at 4 °C. Stable for one month.

Appendix Table 2. Troubleshooting for nitrogen determination with the Technicon Autoanalyzer.

Problem	Troubleshooting
Baseline too high or variable	Check that all reagents are being pumped in the system. If a new reagent was prepared, make sure it was done properly. Prepare new reagents.
Changes in values of check samples	Weigh samples accurately. Make sure sample digestion was complete. Be sure reagent mixture 3 is not oxydized. If it is, prepare a new one. Verify quality of reagents. Prepare new ones.
Digestion solution not clear	Make sure sample is placed at the bottom of the tube. Make sure catalyst mixture is placed at the bottom of the tube. When adding sulfuric acid, do it carefully. Try to wash the wall of the tube with the sulfuric acid being introduced.
Black/yellow spots in the digestion solution	Check temperature of digester. Check that digester wells are clean. Extend the digestion time 20 min.

11.2.1.2 Nitrogen determination with the Micro-Kjeldahl method

(AOAC, 1980)

Although this procedure is precise, it is very time consuming and, therefore, not recommended for breeding programs that need to analyze many samples in a short time.

Sample digestion:

1. Weigh 40 mg of ground sample. Include two check samples.
2. Transfer sample to the bottom of a 75-ml digestion tube.
3. Include one or two tubes as blanks (no samples) for digestion.
4. Add 2.0 g of catalyst mixture to each tube and 2.5 ml concentrated H_2SO_4. Let stand until reaction ceases.
5. Digest under the fume hood in a pre-heated digester block at 380 °C for 90 minutes.

Sample distillation and titration:

1. Add 20 ml distilled water to dissolve any crystals that may have formed.
2. Transfer this solution to the distillation system, washing the digestion tube 5 or 6 times with about 2 ml distilled water.
3. Add 6 ml of 4% boric acid and 4 drops of indicator solution to a 125 ml Erlenmeyer flask and place it under the condenser. Make sure the condenser terminal is inside the solution.
4. Add 10 ml of 50% sodium hydroxide to the distillation system and distill at about 80-90 °C.
5. Distill until 50 to 75 ml are obtained.
6. Titrate with 0.02 N hydrochloric acid, standardized until violet color is obtained.
7. Make the determination for blanks, checks, and samples.

Calculating the nitrogen percentage:

$$\% \text{ nitrogen} = \frac{\left[\begin{array}{c}\text{(Volume of HCl used for titration in sample (ml)}\end{array} - \begin{array}{c}\text{(Volume of HCl used for titration in blank)}\end{array}\right] \times \text{normality of HCl} \times \frac{14.0067}{1000} \times 100}{\text{Sample weight (mg)}}$$

Appendix Table 3. Reagents used for nitrogen determination with the Micro-Kjeldahl method.

Reagent/mixture	Specific reagents	Preparation	Special recommendations
Sulfuric acid (analytical grade, 98%)			Store at room temperature in a light-protected container.
Catalyst mixture	Potassium sulphate Selenium	Mix 1 kg K_2SO_4 very well with 5 g selenium.	Handle very carefully; selenium is extremely dangerous. Store at room temperature.
4% boric acid solution		Dissolve 4 % boric acid in 100 ml solution.	Store at room temperature.
Methyl red solution	Methyl red (pH 4.2-6.2) Absolute ethanol	Dissolve 20 mg methyl red in 10 ml ethanol.	Prepare every time indicator reagent is needed.
Bromocresol green solution	Bromocresol green (pH 3.6-5.4) Absolute ethanol	Dissolve 100 mg bromocresol green in 50 ml ethanol.	Prepare every time indicator reagent is needed.
Indicator reagent		Mix 10 ml methyl red solution and 50 ml bromocresol green solution.	Prepare in a light-protected bottle. Store at 4 °C for a maximum of 3 months.
0.02 N hydrochloric acid	Hydrochloric acid	Dissolve 1.65 ml HCl in 1 l water. Normality must be checked and verified for each preparation.	Store at room temperature.

11.2.2 Protein determination

The Kjeldahl method has been widely used for protein estimation in cereals because: (1) it allows quantifying nitrogen from either soluble or insoluble samples; (2) the nitrogen from cereal samples is mainly derived from protein; and (3) the amino acid composition of endosperm protein is constant enough to have a relatively fixed nitrogen: protein ratio within a given cereal (Nkonge and Balance, 1982). Protein can be estimated from the nitrogen value; in the case of maize, it is calculated as follows:

% protein = % nitrogen x 6.25 (conversion factor for maize)

11.2.3 Tryptophan determination

(Villegas et al., 1984)
Various analytical methods for determining tryptophan content—in the areas of ion exchange chromatography (Huang et al., 2006), spectrophotometry (Piombo and Lozano, 1980), and microbiology (Scott et al., 2004)—have been studied extensively. They have proved to be complicated, laborious and, therefore, unsuitable for screening large numbers of samples. For several years the CIMMYT laboratory has effectively utilized, for its simplicity and reproducibility, the Opienska-Blauth et al. (1963) colorimetric method modified by Hernandez and Bates (1969). This protocol is described below.

Appendix Table 4. Troubleshooting for nitrogen determination with the Micro-Kjeldahl method.

Problem	Troubleshooting
Distillation time too long	Increase temperature, being careful that it does not boil.
Changes in values of check samples	Weigh samples accurately.
	Make sure sample digestion was complete.
	Be sure reagent mixture 3 is not oxidized. If it is, prepare a new one.
	Verify quality of reagents. Prepare new ones.
Digestion solution not clear	Be sure sample is placed at the bottom of the tube.

Principle:
The protocol is based on the Hopkins-Cole reaction, in which 1 molecule glyoxylic acid and 2 molecules tryptophan form a colored compound with a maximum absorption at 560 nm.

This method is actually based on the amount of glyoxylic acid that may be present as an impurity in the acetic acid. Therefore, several batches of acetic acid must be tested until one identifies the batch that gives constant and close optical density (OD) values for the standard curve (such as the ones presented below), and the values of control samples (known normal and known QPM) are as expected. This limitation of having to test different batches of acetic acid is the main reason why an alternative tryptophan determination method is being validated at CIMMYT (Nurit et al., 2007, in preparation). For further details, please contact Natalia Palacios (n.palacios@cgiar.org).

Sampling and grinding:

1. Take a random sample of 20-30 seeds that is representative of your material.
2. Be sure all seed samples have similar moisture content.
3. If the seeds have been treated, wash thoroughly with tap water and then rinse with distilled water. Let the seeds dry.
4. Grind each sample to a very fine powder. If possible use the 0.5 mm setting of a cyclone mill.

Defatting:

5. Place each sample in a commercial filter paper envelope (size: 10 x 11 cm, for example).
6. Defat samples with approximately 300 ml of hexane per balloon in a Soxhlet-type continuous extractor for four hours.
7. Air dry samples and be sure all hexane has evaporated.

Digestion:

8. For each sample, weigh 80 mg of defatted powder in a 15-ml falcon tube.
9. Add 3 ml of papain solution.
10. Always include at least 2 blank controls, 4 checks (of known tryptophan concentration: 2 QPM, 2 normal), and the standard curve (see details below).

11. Close tubes to make sure no evaporation takes place during incubation.
12. Vortex the samples thoroughly and place them in an oven at 65ºC for 16 hours (overnight). If possible, vortex them twice more, one hour after being placed in the oven and one hour before they complete the 16-h incubation period.
13. Take tubes out of the oven and let them cool to room temperature.
14. Vortex the tubes immediately before centrifuging them at 3600 g for 10 min. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.

Colorimetric reaction:

15. Remove the hydrolysate from the oven; shake and let cool to room temperature.
16. Centrifuge at 2,500 rpm for 5 min.
17. Transfer 1 ml of the hydrolysate to a new tube.
18. Add 4 ml of reagent C by slowly pouring it down the inner wall of the tube.
19. Vortex thoroughly and incubate at 63± 2 ºC for 15 min for color development.
20. Take samples out of the oven and let them cool down at room temperature.
21. Read absorbance at 560 nm in a spectrophotometer.

Appendix Table 5. Reagents used for tryptophan determination.

Reagent/mixture	Specific reagents	Preparation	Special recommendations
Acetate solution: 0.165 M NaH_3CCOOH	Sodium acetate	Weigh 13.6 g of sodium acetate for 1 liter of distilled water. Adjust to pH 7.0 with NaOH.	Keep as stock at 4°C. Stable for several weeks.
Papain solution 4 mg/ml	Papain (crude extract: 2.5 units/mg)	Weigh 40 mg of papain for 10 ml of solution. Always prepare a fresh batch that is more than what you need (3 ml per sample). Dissolve the papain in the sodium acetate solution at room temperature.	Prepare before every use. Be sure the phosphate buffer is at room temperature. Be sure papain powder is completely dissolved.
Reagent A	Ferric chloride six-hydrated Glacial acetic acid	Dissolve 270 mg of FeCl3-6H2O in 1 liter glacial acetic acid.	Each bottle of acetic acid must be tested for color development in the presence of tryptophan, as some aldehyde-free acetic acid does not produce enough glyoxylic acid to react with tryptophan and produce the colored compound.
Reagent B: 30 N sulfuric acid	Sulfuric acid (analytical)	Place a bottle on ice. Mix at the same time 833.3 ml of sulfuric acid (98%) and 166.7 ml of distilled water to prepare a 30 N H_2SO_4 solution. Complete final volume with distilled water.	Keep as stock at room temperature; Stable for several weeks.
Reagent C	Reagents A and B	Prepare a volume to volume mixture of reagents A and B at least one hour prior to use.	
Tryptophan 100 µg/ml	DL-Tryptophan	Prepare a stock solution of 100 µg/ml tryptophan in 0.1 M sodium acetate solution pH 7.	Prepare weekly and store at 4ºC.

Special recommendations:

1. Defatting of maize flour is important to improve accuracy and repeatability of results. When samples are not defatted, an average of 0.8% less tryptophan is detected using this protocol.
2. Make sure there are no sample particles stuck to the wall of the tube or floating in the supernatant after centrifuging samples in step 14. If there are some particles, vortex the sample again and centrifuge it for 15 min.
3. The reaction, as in any analytical method, is very sensitive to pipetting precision. Be sure your pipettes and/or dispensers are properly calibrated.
4. Always include one standard curve for every set of samples analyzed in a day.
5. Always measure the papain blank from the same batch. Papain is a protein that contains large amounts of tryptophan itself (every papain molecule contains 7 tryptophan units). This has to be subtracted when making the calculations for each sample.

Standard curve:

1. Prepare a stock solution of 100 µg/ml tryptophan in 0.1 M sodium acetate solution pH 7 (prepare it weekly and store at 4 ^{0}C).
2. In 15-ml falcon tubes, prepare daily 0, 10, 20, 15, and 30 µg/ml dilutions (in 0.1 M sodium acetate solution pH 7). Vortex properly before further use.
3. Do a colorimetric reaction (steps 16 to 20) using 1 ml of those dilutions.

Appendix Table 6. Tryptophan standard curve preparation.

Tube no.	Stock: Try 100 µg/ml (ml)	Sodium acetate 0.1 N, pH 7.0 (ml)	Total volume (ml)	Concentration (µg Try/ml)
1	0.0	10.0	10.0	0.0
2	1.0	9.0	10.0	10.0
3	1.5	8.5	10.0	15.0
4	2.0	8.0	10.0	20.0
5	2.5	7.5	10.0	25.0
6	3.0	7.0	10.0	30.0

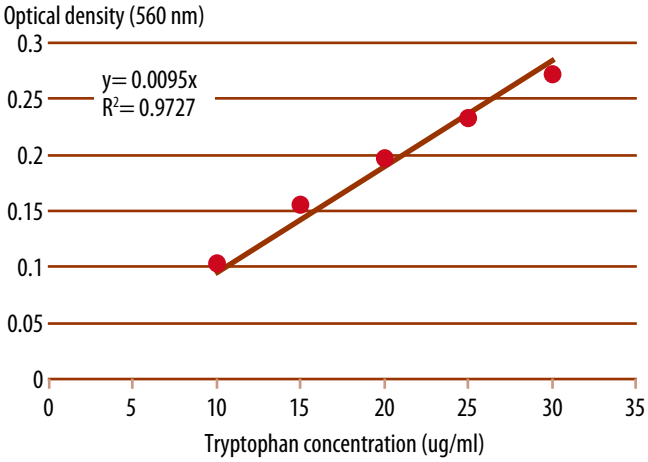
Standard curve for tryptophan (calibration curve):
Develop a calibration curve using known amounts of tryptophan, ranging from 0 to 30 µg/ml. Plot the absorbance readings at 560 nm as a function of concentration and calculate the slope (y) of that standard curve. Note that the slope has the unit of OD*ml/µg.

Calculation of tryptophan percentage:
The amount of tryptophan (trp) in each sample is estimated using the following equation:

$$\% \text{ trp} = \frac{OD_{560\text{ nm}}}{\text{slope}} \text{ X } \frac{\text{hydrolysis volume}}{\text{sample weight}} \text{ X } 100\%$$

Example:

$$\% \text{ trp } (\mu g/\mu g) = \frac{0.5}{0.0095 \frac{OD}{\mu g/ml}} \text{ X } \frac{3 \text{ ml}}{80000 \text{ µg}} \text{ X } 100\%$$



Appendix Figure 1. Example of a standard curve for tryptophan (calibration curve).

However, this amount includes the tryptophan in the sample plus the tryptophan from the papain. To calculate the trp content in the biological material (defatted grain powder), subtract the papain value.

Therefore, % trp should be calculated from the corrected absorption value:

$$\textbf{\% trp} = \textbf{OD}_{\textbf{560 nm corrected}} \textbf{ x Factor}$$

Where:

$$OD_{560\text{ nm corrected}} = OD_{560\text{ nm sample}} - OD_{560\text{ nm average of papain blanks.}}$$

$$Factor = \frac{0.00375}{\text{slope}}$$

$$Note\ that: \frac{3\text{ ml}}{80000\ \mu g} = 0.00375$$

In general, a sample with more than 0.070% of tryptophan is considered QPM. However, this also depends on the protein content and, therefore, the quality index value (% trp/protein).

Appendix Table 7. Troubleshooting for tryptophan determination.

Problem	Troubleshooting
No color development in the reaction	1. Add 2-4% acetic anhydride to acetic acid. 2. Test another batch of acetic acid.
Changes in factor curve values/ OD measurements of tryptophan standard curve	1. Verify quality of tryptophan standard curve. 2. Test another batch of acetic acid. 3. Make sure that sulfuric acid is 30N. 4. Verify quality of all reagents. Prepare new ones. 5. Make sure all quantities of reagents are properly measured.
OD for the papain blank is too high	1. Verify that the amount of papain is correct. 2. Use another batch of papain.
OD for the papain blank is too low	1. Be sure the amount of papain is correct. 2. Use another batch of papain. 3. Test another batch of acetic acid. 4. Add 2-4% acetic anhydride to acetic acid.
Low values of control samples	1. Make sure sample digestion is done properly: a) Be sure there are no particles on tube wall after sample digestion. If there are, vortex the sample and centrifuge again for 15 min. b) Verify that incubation was done at 65 °C for 16 h. 2. Verify quality and quantity of the reagents used. 3. Verify quality of the tryptophan standard curve: a) Be sure that stock solution of tryptophan is properly dissolved before doing the dilutions. b) Mix the tryptophan stock solution well before doing the dilutions. c) Prepare new tryptophan stock solution.
OD measurements between replicates vary too much	1. Verify accuracy of sample weights. 2. Make sure replicates are analyzed equally, using the same batch of reagents. 3. Be sure samples have cooled to room temperature before reading. 4. Set the spectrophotometer to "zero" again, and be sure it is stable before reading samples.
Papain does not dissolve	Be sure the acetate solution is at room temperature.

11.2.4 References

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11.3 QPM donors available from CIMMYT

QPM Donor	Heterotic Group	Source	Grain type	Adaptation
CML144	B	P62	White Flint	Lowland tropical
CML147	B	P63	White Dent	Lowland tropical
CML150	A	G24 QPM	White Dent	Lowland tropical
CML176	B	P63/P67	White Dent / Flint	Lowland tropical
CML491	A	P62	White Flint	Lowland tropical
CML492	B	P62	White Flint	Lowland tropical
CML502	AB	P63/P67	White Dent / Flint	Lowland tropical
CML503	B	P63/P67	White Dent / Flint	Lowland tropical
CLQ-6315	BA	P63	White Dent	Lowland tropical
CLQ-6316	B	P63	White Dent	Lowland tropical
CLQ-RCWQ83	AB	P63/G24 QPM	White Dent	Lowland tropical
CML511	B	CML176	White Semi-Flint	Mid altitude

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