Doubled Haploid Production in Maize Using *In Vivo* Maternal Haploid Induction System

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ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan (ICAR-VPKAS), Almora, is a premier institution conducting agricultural research mainly for the North-Western (NW) Himalayan region of India. However, being a multi-crop and multi-disciplinary research institute, it extends technological support to other hilly regions of the country also. The institute has made outstanding contribution to enhancing crop productivity in this region by developing 172 improved varieties of 26 crops. In its 97 years of service to the nation, ICAR-VPKAS has several pioneering achievements to its credit that include the development of landmark varieties *VL Makka 54* (the first hybrid maize variety in the country released in 1962), *Him 128* and *VL Gehun 421*, the first onion hybrid *VL Piaz 67* and the first MAS-derived single-cross Quality Protein Maize (QPM) hybrid *Vivek QPM 9* in India.

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The information presented in this manual is primarily based on our experiences while following CIMMYT's DH protocol (Prasanna et al. 2012) using haploid inducer TAILP1, and modifications introduced to adapt the protocol to the local conditions and available resources. Therefore, first of all, the editors acknowledge all the contributors to the above-mentioned source.

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PREFACE

The manual **‘Doubled Haploid Production in Maize Using *In Vivo* Maternal Haploid Induction System'** aims at providing the users with basic information on various steps involved in the process of production of doubled haploids (DH) in maize using maternal haploid inducer lines. In the first few chapters, the concept of DH production is briefly explained to familiarize the users with the underlying genetic mechanisms involved in generating homozygous lines from heterozygous source germplasm. The DH production steps described in the manual have been adapted from the CIMMYT DH production protocol to enable users to implement the protocol with available facilities and under limited resource conditions. The manual also contains information on haploid induction rate (HIR) and DH production efficiency achieved with CIMMYT haploid inducer line TAILP1, performance of DH lines generated at ICAR-VPKAS, and the comparative economics of DH and conventional breeding. Status of DH breeding at the national level, strategies for developing indigenous genetic resources for further enhancing efficiency of DH technology and enabling wider access to the generated DH germplasm, and approaches to promote wider adoption of DH technology is also discussed. Relevant publications are listed in the end for further reading.

The editors believe that this manual will help in implementation of DH programmes at other institutes and contribute to the fulfilment of ICAR's objective of promoting wider adoption of DH technology by the public maize breeding programmes in particular and the National Agricultural Research System (NARS) in general.

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Abbreviations and Terminology

DH	:	Doubled Haploid
HIL	:	Haploid Inducer Line
HIR	:	Haploid Induction Rate
TAIL	:	Tropically Adapted Inducer Line
CIM2GTAILs	:	CIMMYT Second Generation TAILs
DT-NIR	:	Diffused Transmission (DT) Technology of Near Infrared (NIR) Spectroscopy
APM	:	Amiprophos-methyl
DMSO	:	Dimethyl Sulfoxide

D_0	:	Seedlings/plants after chromosome doubling treatment of haploid seedlings
D_0 nursery	:	Nursery of D_0 populations
D_1 ear	:	Selfed ears of fertile D_0 plants
D_1 seed	:	Selfed seed from fertile D_0 plants/ears
D_1 plants	:	DH plants raised from D_1 seed
D_1 nursery	:	Nursery of DH plants/lines raised from D_1 seed

Chapter 1

Status of doubled haploid technology in maize

RK Khulbe, A Pattanayak, Devender Sharma and Lakshmi Kant

Doubled Haploid (DH) programmes in crops aim to obtain completely homozygyous lines in a shorter period than conventional breeding. Different DH development methods are employed in different crops depending on their efficiency and ease of use. For example, while anther/pollen culture is the prevalent method in rice, crossing with a related species is the preferred method in wheat. In maize, due to the lower efficiency and high genotype specificity of *in vitro* methods, the more efficient *in vivo* method is commonly used (Chaikam et al. 2012; Chaikam et al. 2019a). *In vivo* DH production in maize involves haploid induction, identification of putative haploids, chromosome doubling and generation of DH seed (Prasanna et al. 2012; Chidzanga et al. 2019). The various advantages offered by DH technology include: (i) reduced inbred generation time and consequent reduced time in hybrid commercialization (Seitz 2005; Bordes et al. 2006) (ii) more effective access to genetic diversity present in landraces and OPVs, safeguarding genetic resources and expanding genetic diversity of elite germplasm (Wilde et al. 2010; Strigens et al. 2013; Böhm et al. 2017; Melchinger et al. 2017b; Brauner et al. 2019; Hölker et al. 2019; Chaikam et al. 2019a) (iii) ideal populations for association mapping due to low population structure and quick decay of linkage disequilibrium (Strigens et al. 2013; Melchinger et al. 2017b) (iv) a powerful tool in combination with MAS for quicker fixation of favourable alleles and stacking genes (Melchinger et al. 2011; Chaikam et al. 2019a) (v) very amenable for variety registration/protection because they comply with Distinctness, Uniformity and Stability (DUS) criteria due to absence of residual heterozygosity (Röber et al. 2005).

In Europe, North America and China, *in vivo* DH technology has been adopted as a routine method by many commercial maize breeding programmes (Chaikam et al. 2019a; Nzamu 2018; Molenaar and Melchinger 2019). CIMMYT and the University of Hohenheim's collaborative efforts have helped make the technology accessible to tropical breeding programmes in both public- and private- sector organizations (Chaikam et al. 2019a). The technology is gaining popularity in sub-Saharan Africa following the establishment of the Maize Doubled Haploid Facility in Kiboko Kenya by CIMMYT in partnership with Kenya Agricultural and Livestock Research Organization (KALRO) (Chidzanga et al. 2019). Development and release of improved maize hybrids with DH lines as parents has been reported in Africa (Beyene et al. 2017; Chaikam et al. 2018).

In India, major maize breeding companies have been known to be using DH lines in their programmes, either sourced from their parent companies/associates overseas or developed in their Indian programmes. However, despite having made significant contributions in the initial developmental phases of the DH technology (Coe and Sarkar 1964; Sarkar and Coe 1966; Sarkar and Coe 1971; Aman and Sarkar 1978), Indian maize research had been slow to pursue and capitalize on the initial work, and as a result, has lagged in adopting DH technology and harvesting its benefits. Barring a few reports on some elementary work on DH (Roop

Kamal 2017; Khulbe et al. 2019, 2020; Showkath Babu et al. 2020), there is hardly anything substantial as far as large-scale production of DH lines and release of DH-based hybrids is concerned. The probable reasons are the lack of (i) knowledge and expertise in DH technology, and (ii) resources and the basic infrastructure required for DH programmes. DH production is a resource-intensive process and requires some basic facilities, especially under sub-tropical and tropical conditions. These prerequisites often inhibit maize breeders from incorporating DH technology in their breeding programmes.

Among public sector institutions, work on DH is being carried out at ICAR-VPKAS (Almora), CSKHPKV HAREC (Bajaura), PAU (Ludhiana) and ICAR-IARI (New Delhi) using CIMMYT's *in vivo* maternal haploid inducers. DH lines have been produced at these centres and are being used in breeding programmes. Efforts are in progress at ICAR level to make DH breeding an integral part of the national maize improvement programme.

Genetics of *in vivo* maternal haploid induction in maize

A Pattanayak and RK Khulbe

Spontaneous haploids are reported to occur in commercial maize inbred lines at a frequency of about 0.1% (Chase 1947), which is insufficient for DH lines production on a commercial scale. Large scale production of DH lines has been made possible by maternal haploid inducer lines (HILs) capable of inducing a higher frequency of maternal haploids, and thereby making application of DH technology in maize commercially viable. Major QTLs (*qhir1* and *qhir8*) are known to be involved in haploid induction (Prigge et al. 2012a), and recently the gene *MATRILINEAL* (*MTL*)/*ZmPHOSPHOLIPASE A1* (*ZmPLA1*)/*NOT LIKE DAD* (*NLD*) has been cloned, and its role in haploid induction validated. *MTL*/*ZmPLA1*/*NLD* gene encodes a patatin-like phospholipase A, expressed in the mature pollen. A mutation in this gene (4-bp insertion) causes a frameshift mutation leading to a truncated protein and consequent formation of haploid seed (Gilles et al. 2007; Liu et al. 2017; Kelliher et al. 2017). Despite these advancements, the mechanism underlying haploid induction is not yet fully elucidated.

Normal seeds in maize are formed as a result of double fertilization when one of the two sperm cells of the pollen grain fuses with the egg cell to create a diploid embryo, and the other fuses with the central cell to form the triploid endosperm. Disruption of or deviation from this process results in seed abortion or the formation of abnormal seed. Two possible mechanisms are hypothesized to be involved in the production of *in vivo* maternal haploids (i) single fertilization (ii) chromosome elimination after normal fertilization (Tian et al 2018; Chaikam et al. 2019a).

1. **Single fertilization**: After pollination with a haploid inducer, one sperm cell of the inducer pollen fuses with the central cell to produce triploid endosperm. The second sperm cell fails to fuse with the egg cell, but the egg cell is stimulated to develop into a haploid embryo. Evidence in favour of this mechanism includes:
    a. High frequency of hetero-fertilization (when sperm cells from two different pollen grains fuse with the egg and central cell) and endosperm/embryo abortion when using inducer pollen (Fig. 1).
    b. Presence of single fertilized ovules after pollination with haploid inducer pollen.

Fig. 1. Endosperm-aborted (left) and embryo-aborted kernels (right) in induction cross seed

2. **Chromosome elimination after normal fertilization**: Pollination with the haploid inducer results in normal double fertilization but is followed by elimination of paternal (haploid inducer) chromosomes from the zygote during subsequent cell divisions. The following evidence supports this hypothesis:
    a. Presence of inducer chromosome fragments in maternal haploids or doubled haploid derived from them
    b. Presence of B chromosomes in the haploids (albeit at a low frequency) in induction crosses using inducers with B chromosomes as cytogenetic markers
    c. Occurrence of aneuploidy, mixoploidy, lagged chromosomes and micronuclei in mitotic cells of inducer pollinated ovules and the developing embryo/endosperm
    d. Presence of haploids with embryos that are weakly pigmented with anthocyanins (Fig. 2a) and embryos with high oil when an inducer with the *R1-nj* and high oil markers is used for pollination.
    e. Presence of mosaic endosperm (consisting of normal and shrunken endosperm) in F_1 kernels in a cross between a haploid inducer with normal endosperm and a sweet corn germplasm with shrunken endosperm (Fig. 2b).

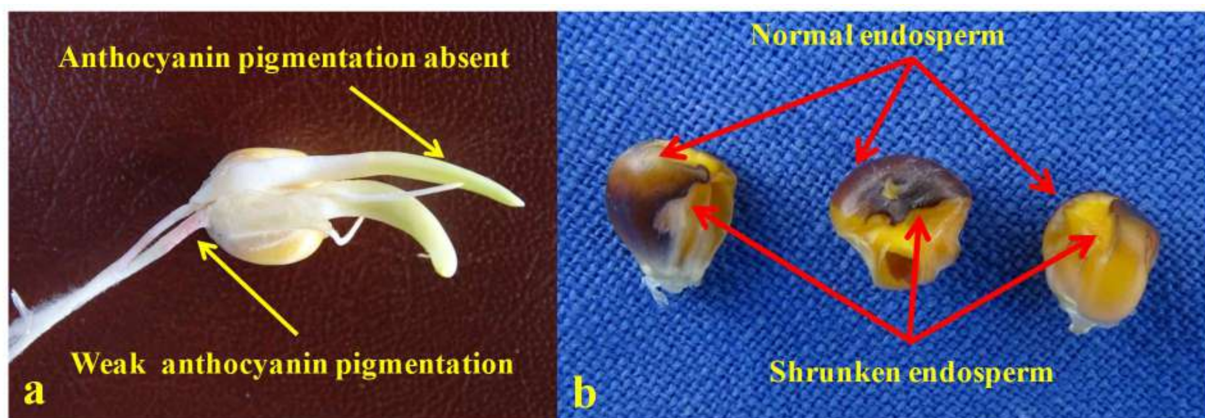

Fig. 2a. A haploid seedling with presence of weak anthocyanin pigmentation on roots b. Mosaic endosperm in F_1 kernels of a cross between sweet corn hybrid FSCH 41 and haploid inducer line TAILP1

Overall, the evidence available suggests existence of both the mechanisms, single-fertilization as well as chromosome elimination after double fertilization, in maternal haploid induction in maize.

Chapter 3

Basic DH production methodology in maize

RK Khulbe and A Pattanayak

Doubled Haploid (DH) production in crops aims to obtain completely homozygyous lines in a shorter time than the conventional method. The DH production methodology in maize differs from other crops because it is based on *in vivo* maternal haploid induction. In this method, maternal haploid inducer lines are used as the pollen parent for obtaining haploid seed of the female parent (source germplasm). Maize DH production methodology comprises a series of steps that begin with induction crosses and culminates in the generation of doubled haploid lines (Fig. 1). The steps involved in the process are as follows:

i. **Generation of induction cross**: Induction cross is a cross between the source germplasm (the population from which DH lines are intended to be derived) as the female parent and the haploid inducer line as the pollen parent.
ii. **Identification of haploid seed**: The induction cross seed is a mixture of F_1 seed, haploid seed and self-/outcross-seed/pigmentation-inhibited seed. The haploid seed is separated from the mixture on the basis of seed and/or seedling traits.

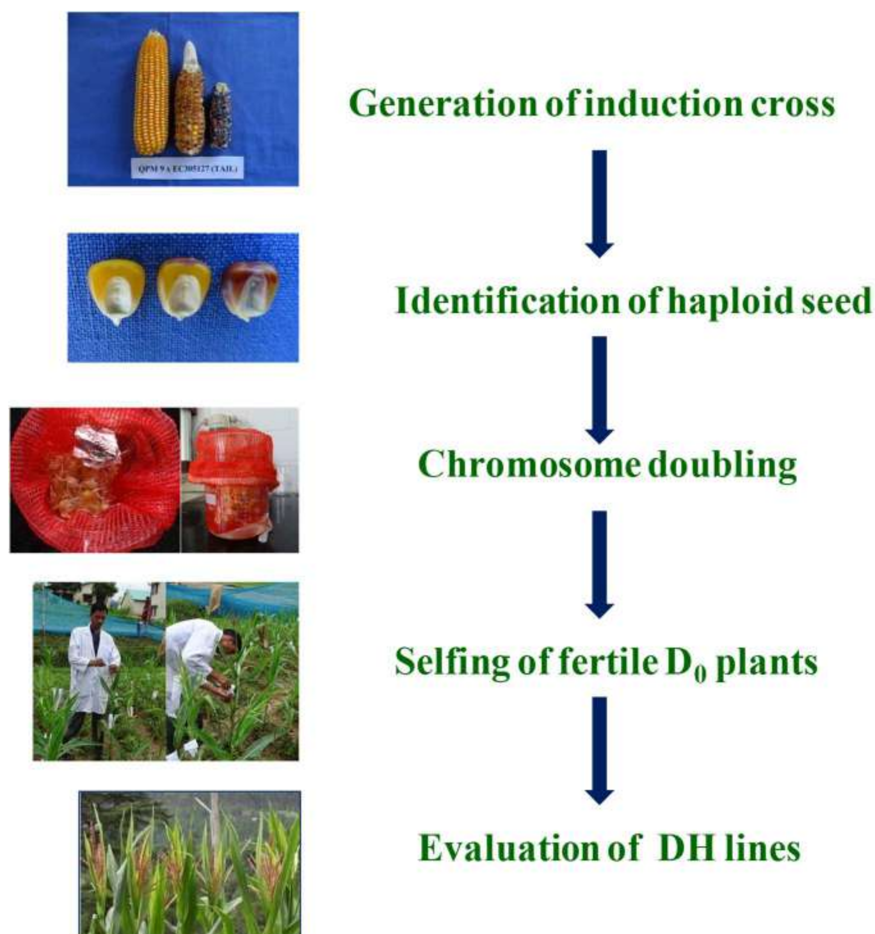

Fig. 1. Basic doubled haploid production methodology in maize

Based on seed traits

a. ***R1-nj* (*Navajo*) phenotype**: Identification of haploid seeds is based on the expression of anthocyanin pigmentation on kernel endosperm and scutellum conditioned by *R1-nj* marker (Nanda and Chase 1966; Greenblatt and Bock 1967). The haploid inducers used carry *R1-nj* marker which is expressed differentially in haploid and diploid seeds, and facilitates classification of seed. Though *R1-nj* marker is most commonly used for haploid classification, it is not effective where the source germplasm carries anthocyanin inhibitor gene(s) (Chaikam and Mahuku 2012a).
b. **Oil content**: Haploid and diploid seeds are classified on the basis of difference in oil content. This method can also be used where expression of the *R1-nj* is difficult to score or suppressed by inhibitor genes (Melchinger et al 2014). Being genotype-independent, this method is applicable in nearly all germplasm including landraces and wild relatives of maize like teosinte, with the exception of high oil maize germplasm. The use of this method may, however, be limited by the availability of inducers with high oil content (Chaikam et al. 2019a)

Based on seedling traits

a. **Red/purple root marker**: In this method, a haploid inducer with red/purple root marker is used for facilitating separation of haploid and diploid seedlings (Rotarenco et al. 2010; Chaikam et al. 2016; Chaikam et al. 2019a). This method is useful where seed-based haploid classification is not possible due to anthocyanin inhibition in the source germplasm or when *R1-nj* expression is masked by natural anthocyanin expression in the seed. A limitation of this method is that the seeds from an induction cross require to be germinated, which is time- and labour-intensive (Chaikam et al. 2019a).
b. **Seedling vigour**: Seedling traits such as radical length, coleoptile length, seminal lateral roots and root hairs have also been used for haploid classification in germplasm where *R1-nj* method cannot be employed (Chaikam et al. 2017). This method also requires germinating large number of seeds from an induction cross, which is resource-intensive.

In addition to these, haploid selection using Flow Cytometry, DT-NIR and various imagery-based methods have been reported. These methods, however, require expensive automated platforms (Chaikam et al. 2019a). Besides, the influence of genetic background and environmental factors on *R1-nj* expression may make imagery-based automated haploid seed sorting challenging (Veermani et al. 2018).

iii. **Chromosome doubling**: The seedlings of the haploid seed obtained above are subjected to chromosome doubling treatment to induce diploidization. Various treatment combinations are reported for chromosome doubling.

Chromosome doubling agents

a. **Colchicine**: Colchicine is the most commonly used chromosome doubling agent (Chaikam and Mahuku 2012b). The dose and treatment duration varies in different protocols. Colchicine, however, is hazardous and extreme care is required in its handling and disposal.

b. **Other anti-mitotic agents**: Amiprophos-methyl (APM), Trifluralin, Pronamide, Oryzalin and some other chemicals have also been recommended for chromosome duplication in maize (Wan et al. 1991; Melchinger et al. 2016). These chemicals are less toxic and have efficiency close to colchicine.
c. **Nitrous oxide (N_2O)**: Nitrous oxide has also been used as an alternative to colchicine (Molenaar et al. 2018). It is relatively safe, has no negative health effects, and chromosome doubling efficiency is similar to that of colchicine, but it requires initial investment (Chaikam et al. 2019a).

Treatment methods

a. **Germinating seed treatment**: In this method, the haploid seeds are allowed to germinate in paper towels for 72 hours at 25-28ºC. The root and shoot tissues of the seedlings are cut 2 cm and 1 cm from the tip, respectively, and the cut seedlings are immersed in a solution of 0.04% colchicine + 0.5% DMSO for 12 hours. (Chaikam and Mahuku 2012b).
a. **V2 stage seedling treatment**: In this method, the seedlings are grown for 10-12 days until they reach V2 stage. The seedlings are then removed from the growing medium carefully and their roots washed to remove the medium. The crown region of the haploid seedlings along with all the seedling roots is immersed in 0.04% colchicine + 0.1% DMSO at ambient temperature for 5 hours (Chaikam et al. 2020).

Seed and adult plant treatments have also been reported (Chaikam et al. 2019a), but the seedling treatment methods are more commonly used.

iv. **Selfing of fertile D_0 plants**: The population obtained after treating haploid seed/seedlings with chromosome doubling chemicals is designated as the D_0 population. The chromosome doubling treatments render a small proportion of the D_0 plants fertile. These fertile plants are self-pollinated for obtaining selfed seed.

v. **Evaluation of DH lines**: Selfed-seeds obtained from the fertile D_0 plants are subsequently raised for maintenance and evaluation.

Chapter 4

Choice of source germplasm

A Pattanayak and RK Khulbe

In DH breeding, the population from which DH lines are derived using a maternal haploid inducer is referred to as the 'source germplasm' or 'source population'. The choice of source germplasm is of paramount importance in a breeding programme – be it a conventional or a DH programme – as it is this choice that determines the quality and constitution of the derived product, inbred lines in case of maize. The selection of source germplasm in a breeding programme is driven by several considerations, the most important being (1) objective of the breeding programme (2) existing germplasm base.

Types of populations for use as source germplasm:

Any of the following, but not restricted to, may be used as source germplasm:

i. Open-pollinated varieties, composite and synthetic populations
ii. Local open-pollinated cultivars and land races
iii. Single-, Three way-, Double- and multiple-crosses
iv. Top-crosses and Double-top crosses
v. F_1s of pedigree crosses
vi. F_2 generations
vii. Backcross populations

The following tips may be useful for obtaining the desired output from a source germplasm:

a. The choice of source germplasm may be based on the heterotic affinity of existing elite inbred lines in a breeding programme.
b. For use in hybrid development programme, the DH lines derived from source germplasm of unknown pedigree will first require to be assigned to different heterotic groups, which is a time and resource consuming process. Use of source germplasm of known pedigree is likely to reduce the scale of this work. New source germplasm can also be synthesized to obtain DH lines with known heterotic affinity.
c. The use of distant germplasm (such as wild relatives, primitive landraces or germplasm belonging to a very different maturity group) for deriving DH lines commonly involves hybridizing them initially with suitable adapted germplasm. If wider diversity is desired in the derived DH lines, F_1 or F_2 populations may be used as the source germplasm. However, if the aim is to have only limited introgression in the DH lines, backcross populations may be used.
d. For hybridization with the distant germplasm, the adapted germplasm can be so chosen as to introduce specific traits (disease resistance, nutritional quality, or any other) into the source germplasm that the derived DH lines are intended to possess.

e. Backcrossing can also be employed to introduce known heterotic patterns into the source germplasm of unknown pedigree/background if appropriate lines are chosen for crossing with the source germplasm.

R1-nj **(*Navajo*) phenotype expression in source germplasm**

The most critical prerequisite for a potential source germplasm is its ability to express *R1-nj* (*Navajo*) phenotype in induction crosses with *R1-nj*-based haploid inducer lines. The degree of this expression determines the efficiency in haploid seed selection and, consequently, that of the entire DH production process. *R1-nj* expression in different populations may vary from 'full expression' to 'no expression' with various intermediate classes (Fig. 1). It is, therefore, essential to have prior knowledge of *R1-nj* expression of a

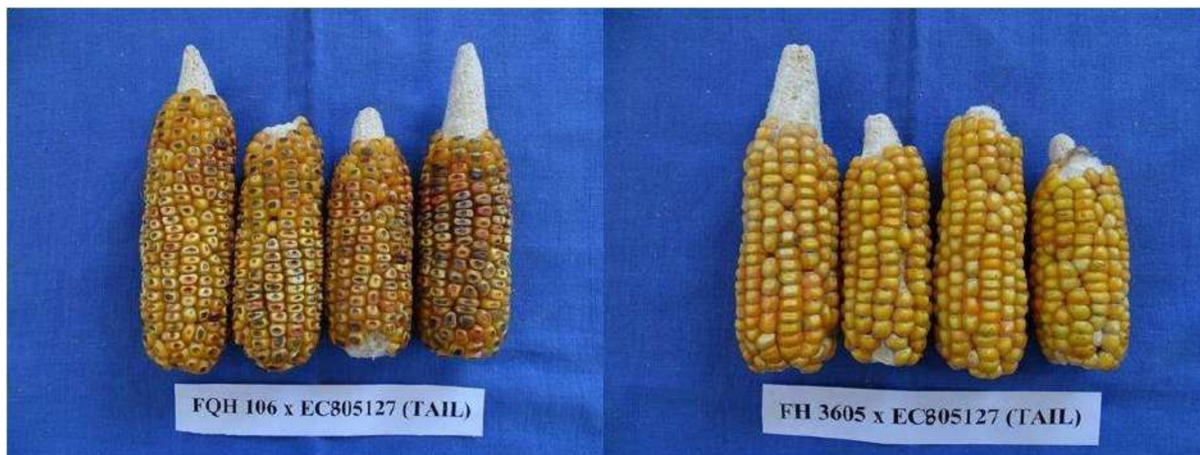

Fig. 1. Source germplasm with complete *R1-nj* expression (left) and absence of *R1-nj* expression (right)

population intended to be used as source germplasm for DH line derivation. The following methods may be used to determine *R1-nj* expression of a potential source germplasm.

1. **Use of molecular markers**: *C1-I* allele-specific diagnostic markers (a combination of two gene-specific markers; the 8 bp *C1-I* InDel and the *C1-I* SNP) for assaying *R1-nj* expression are available (Chaikam et al. 2015). These markers facilitate the identification of germplasm with dominant colour inhibitor genes and help predict amenability of source germplasm to haploid induction using *R1-nj*-based haploid inducers without having to undertake test induction crosses. The application of this method is limited by the fact that both kernel anthocyanin biosynthesis and inhibition involve more than one gene. Besides, additional facilities and resources are required to perform molecular screening.
2. ***R1-nj*** **expression in constituent germplasm**: *R1-nj* expression in the constituents of a source population may be used as a guide for predicting *R1-nj* expression in the population. Constituents with full expression are highly likely to produce fully amenable populations (Fig. 2). Since this method requires prior information on *R1-nj* expression of the constituents, it is of little help in determining the amenability of germplasm with unknown pedigree/background. Moreover, an additional crop season is required to phenotype *R1-nj* expression in the constituents if the information is not already available. The knowledge of *R1-nj* expression of the germplasm, however, is

useful for generating new source germplasm with known amenability for DH production using *R1-nj* based haploid inducers.

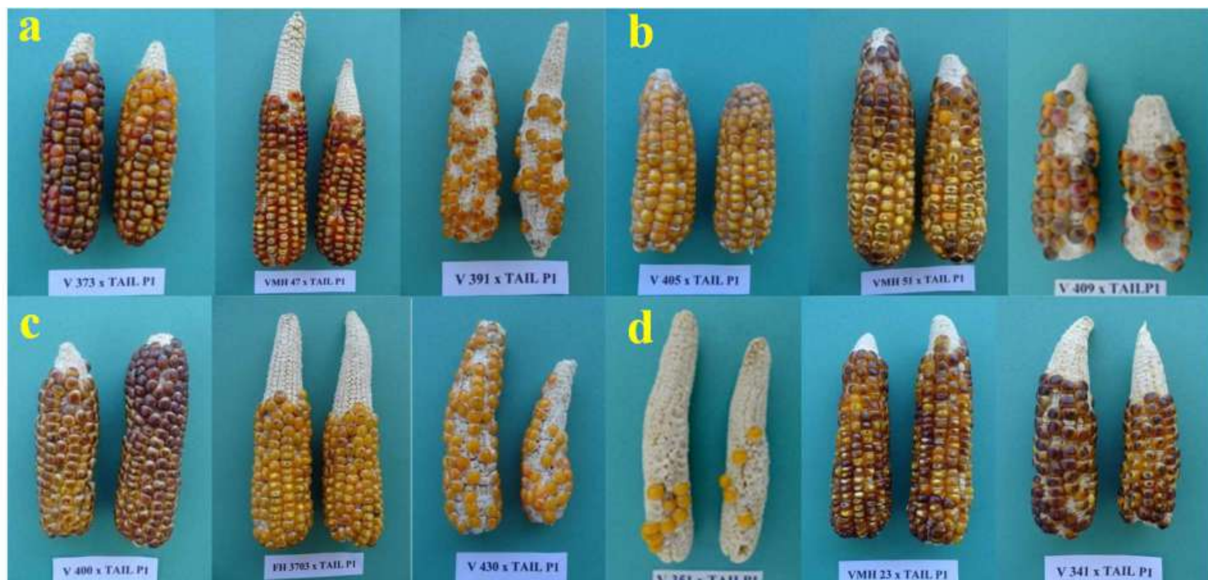

Fig. 2. *R1-nj* expression in hybrids (centre) and their parental inbreds (flanks) of a. Vivek Maize Hybrid 47 b. Vivek Maize Hybrid 51 c. FH 3703, and d. Vivek Maize Hybrid 23

3. **Test induction cross**: Before taking up a full-scale DH production programme with a set of potential source germplasm, test induction crosses of the germplasm with the haploid inducer line can be made, and the progeny phenotyped for *R1-nj* expression. This is a fool-proof method to determine potential source germplasm's amenability, including those of unknown pedigree/background. However, one additional season is required in this method as well.

The above methods have their advantages and disadvantages. Therefore, till less resource and time requiring methods with high accuracy of *R1-nj* expression prediction are available, a combination of these methods may be used as per the requirement in different cases. Genotyping and phenotypying germplasm/populations commonly used in India, as attempted by Chaikam et al. (2015) for CIMMYT germplasm, will prove significant in this direction. Information regarding amenability of released VL hybrids and their parental inbreds has been generated at Almora (Khulbe et al. 2019), which will be helpful for workers intending to use VL hybrids for deriving early maturity DH lines.

Chapter 5

Generation of induction crosses

A Pattanayak and RK Khulbe

An induction cross is a cross between the source germplasm and a haploid inducer line. Induction crosses are similar to the routine crosses made in maize breeding programmes but differ in the way that the male parent is a Haploid Inducer Line (HIL), and the crosses are generated for obtaining haploid seed for deriving doubled haploid lines. Following are the general considerations for generation of induction crosses.

Population size per induction cross

1. **Number of plants of source germplasm:**
    a. The number of plants required to be raised for a particular induction cross is primarily determined by the number of DH lines targeted to be derived and the number of haploid seeds required to obtain that number. A smaller population is needed if the inducer line's haploid induction rate (HIR) is higher and vice-versa.
    b. In general, the seed set per ear is expected to be higher in hybrids than composites and landraces. So, if the same number of DH lines is targeted, more plants would be required for the latter.
    c. Based on the DH production efficiency obtained at ICAR-VPKAS using CIMMYT haploid inducer line TAILP1 (Fig. 1) (which gave an average HIR of 5.48%), as many source germplasm plants may require to be raised as the number of DH lines targeted to be produced. For example, for a production target of 200 DH lines, 200 plants of the source germplasm may be raised. A smaller population may suffice when inducers with high HIR, such as CIM2GTAILs, are used.
2. **Number of plants of haploid inducer line:**
    a. The ratio of source germplasm plants and HIL plants is primarily determined by the inducer lines' pollen production ability. Some haploid inducer lines may be shy pollen producers at some locations, like TAILP1 is at Hawalbagh (1250 m amsl, latitude 29º36′ N, longitude 79º40′ E). Therefore, the population of inducer plants should be large enough to supply adequate pollen to the source germplasm plants.
    b. For TAILP1, a source germplam: HIL ratio of 6:1 is good enough, though it is always better to keep HIL population a little higher. In case sufficient HIL pollen is not available in the Induction Cross Block, pollen from HIL Maintenance Block can always be used. The inducer lines may require additional care, so it is important that best agronomic practices are followed to raise the HIL population, and all necessary measures are taken to ensure adequate plant stand.

Fig. 1. Plants and mature ears of CIMMYT haploid inducer line TAILP1

3. **Planting layout:**
    a. The different source germplasm should be adequately separated from each other to avoid cross-contamination.
    b. The inducer should be planted in a separate block as it allows staggered planting, which is difficult to manage if the inducer is planted adjacent to each source germplasm.
    c. The source germplasm block and the HIL block should not be very far from each other (Fig. 2b)
    d. For large scale generation of induction crosses in isolation, CIMMYT's planting layout may be followed (Prasanna et al. 2012).

Fig. 2. Induction cross block at ICAR-VPKAS Experimental Farm, Hawalbagh, comprising a. Source germplasm block, and b. Haploid inducer block planted adjacent to the Source germplasm block

4. **Staggered planting:**
    a. Staggered planting may be required if the source germplasm and the HIL differ significantly for days to flowering. For example, TAILP1 takes 48-50 days to anthesis at Hawalbagh and, therefore, adequate staggering is required, especially if the source germplasm belongs to the mid- or full-season maturity group. Three staggered plantings of TAILP1 at weekly intervals generally serve the purpose.

5. **Pollination**
    a. Pollination is carried out in the same manner as is performed to produce bulk hybrid seed through hand-pollination.
    b. For pollination, pollen is collected from individual TAILP1 plants and is bulked for pollinating the source germplasm plants. Each ear in the source germplasm should be adequately pollinated so that seed set is good.
    c. Care should be taken to pollinate the ears at the right stage as early or late pollination leads to poor seed set.
    d. Pollination should be done carefully. Avoidance of self-pollination and cross-contamination leads to increase in the overall proportion of haploid seeds.
    e. If the objective is not to obtain a complete representation of the source germplasm in the DH lines, weaker plants or plants with undesirable traits may be avoided. Such plants may be removed timely so that the OP ears borne by them are not harvested accidentally.
6. **Harvesting of induction crosses**
    a. Each induction cross should be harvested separately and labelled correctly to avoid accidental intermixing. Intermixing of induction cross ears will lead to wrong identities of the obtained DH lines.
    b. At the time of harvesting, it is important to watch out for open-pollinated ears. These may be on the discarded plants or on plants that were not pollinated for some reason.
    c. The pollinated ears may be dehusked in the field itself or after bringing them to the threshing floor. The HIL-pollinated ears (referred to as 'induction cross ears') are easily identifiable by the pigmentation on the seeds. The degree of pigmentation may, however, vary (Fig. 3).
    d. Occasional ears with non-pigmented seeds, if found, may be discarded. However, if most of the ears are non-pigmented or weakly pigmented, the presence of anthocyanin inhibitor gene(s) in the population can be a reason.
    e. The ears should be adequately dried and safely stored in separate labelled bags till the time of haploid seed sorting.

Fig. 3. Harvested induction cross ears. Note the difference in the degree of kernel pigmentation

Chapter 6

Identification of haploid seed

RK Khulbe and A Pattanayak

Identifying and separating haploid seed is a critical step in the DH production process and is a major determinant of the overall efficiency. As mentioned in Chapter 1, various methods of haploid classification are available. Among these, *R1-nj* marker (kernel anthocyanin pigmentation) based method is presently the most popular method as it obviates the need to grow all the induction cross seed (for using seedling and plant markers), thereby saving resources considerably. The expression pattern exhibited by *R1-nj* is commonly referred

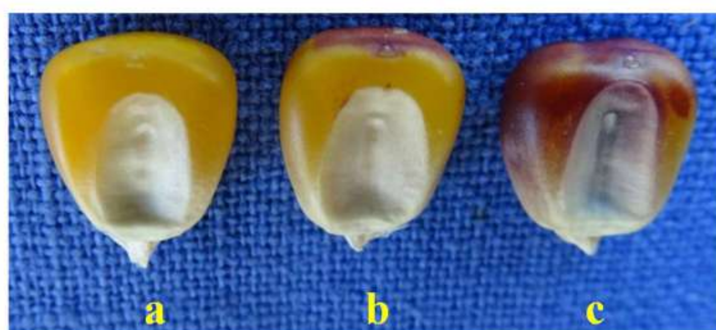

Fig 1. (Above) Seeds in induction cross ear : a. Self / out-crossed / pigmentation inhibited seed b. Putative haploid seed c. F_1 seed

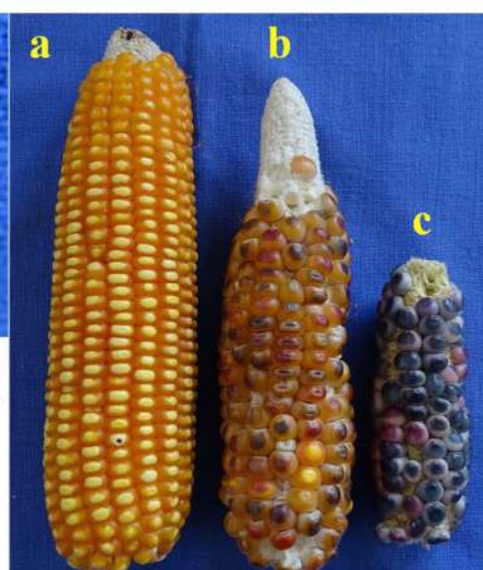

Fig 2. (right) Ear of a. F_1 hybrid Vivek QPM 9 (source population) b. Vivek QPM 9 pollinated with TAILP1 (induction cross) c. TAILP1 (haploid inducer line)

to as the '*Navajo*' phenotype (Fig. 1b) and is characterized by purple colouration in the aleurone layer on the crown region of the endosperm and the scutellum of the embryo (Nanda and Chase 1966; Greenblatt and Bock 1967). A haploid inducer line possessing *R1-nj* phenotype imparts the same to its hybrids (Fig. 2b) and enables classification of the induction cross seed into three types (Fig.1) on the basis of differences in kernel anthocyanin pigmentation expression.

The degree of kernel anthocyanin pigmentation in the induction crosses varies with the source germplasm and may range from very deep to very weak with various intermediate classes (Fig.

Fig. 3. Variation in degree of kernel anthocyanin pigmentation in induction crosses

3). Some source germplasm may altogether fail to express anthocyanin pigmentation (Fig. 3e) due to the presence of anthocyanin inhibitor gene(s). Depending on the homozygosity or heterozygosity of the inhibitor alleles in the source population, *R1-nj* expression may also exhibit variation among and within ears of the same induction cross for area marked and intensity of pigmentation (Fig. 4).

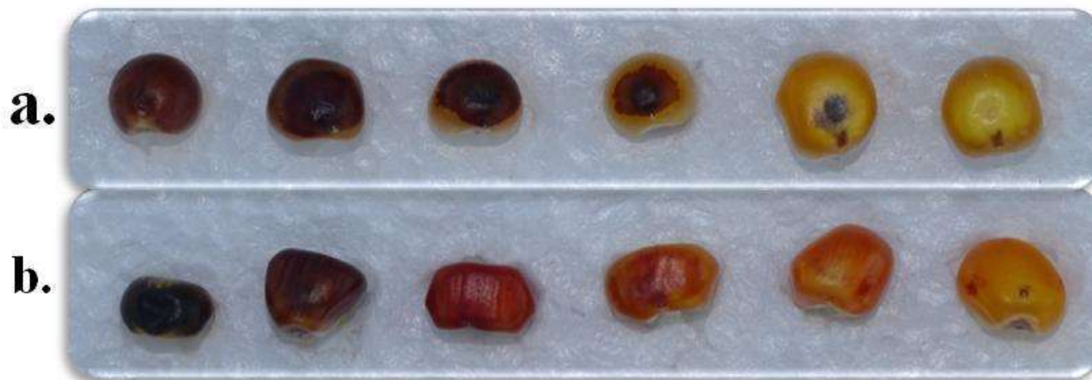

Fig. 4. Variation in induction cross for (a) area marked, and (b) intensity of colour

Apart from this, variation for scutellum pigmentation may also be found among different source germplasm (Fig 5.)

Fig. 5. Variation for scutellum pigmentation among different source germplasm a. VMH 43 b. CMVL 55 c. VMH 23, and d. V 351

Sorting of induction cross seed is done in the following way:

a. The induction cross ears can be shelled together, and the seed bulked. A handful from the bulk may be drawn each time to look for and separate the haploid seed from it. This method, however, is tedious as it often requires the seeds to be turned around and/or over to look for pigmentation on the kernel crown and the scutellum.

b. In the second method, which is more efficient, the ear is held in one hand with the proximal end (top end) facing towards the worker (Fig. 6a). The seeds are removed using the other hand one by one while rotating the cob clockwise or anti-clockwise. Removal of the first few seeds is a little difficult, but as one progresses towards the distal end (stalk end), a gentle forward pull with the thumb or the forefinger is sufficient to remove the seed from the ear (Fig. 5b). Alternatively, one complete row may be removed first, and the seeds from the remaining rows may be removed with sideways pull (Fig. 6c). The advantage of this method is that the crown region as well as the embryo region of the seed are clearly visible while it is still embedded in the ear (Fig. 6a), and a decision

on its haploid/diploid nature can be made before removing it from the ear. This method not only saves time but is also less laborious.

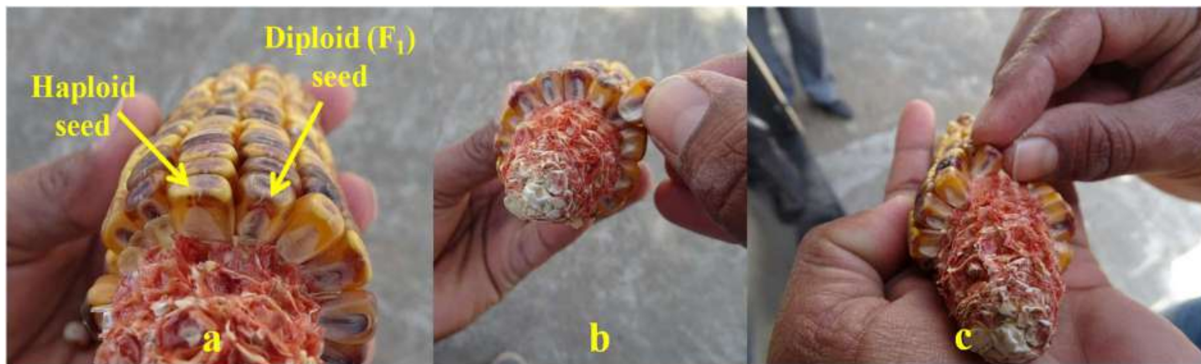

Fig. 6a. Haploid and diploid (F_1) seeds in induction cross ear; b&c. Removing seed from induction cross ear

c. For DH line generation, only haploid seeds are important. However, if any studies are intended to be performed (such as assessment of HIR of the inducer), the other three classes of seeds may also need to be retained. Therefore, before beginning seed sorting, containers labelled 'Haploid seed', 'F_1/Cross-seed', 'Selfed-seed/Pigmentation-inhibited' and 'Aborted seed' may be kept ready for different classes of seeds (Fig. 7). Care should be taken that only the correct type of seed goes into the specified container. Figure 8 shows an assortment of seeds from an induction cross.
d. After completion of sorting, the haploid seeds may be counted. If the quantity of haploid seeds is large, their approximate number can be assessed using test-weight of 2-3 randomly drawn samples. This information helps in making calculations for other resources required in subsequent steps of the process.

Fig. 7. Sorting of induction cross seed at ICAR-VPAS Experimental Farm, Hawalbagh

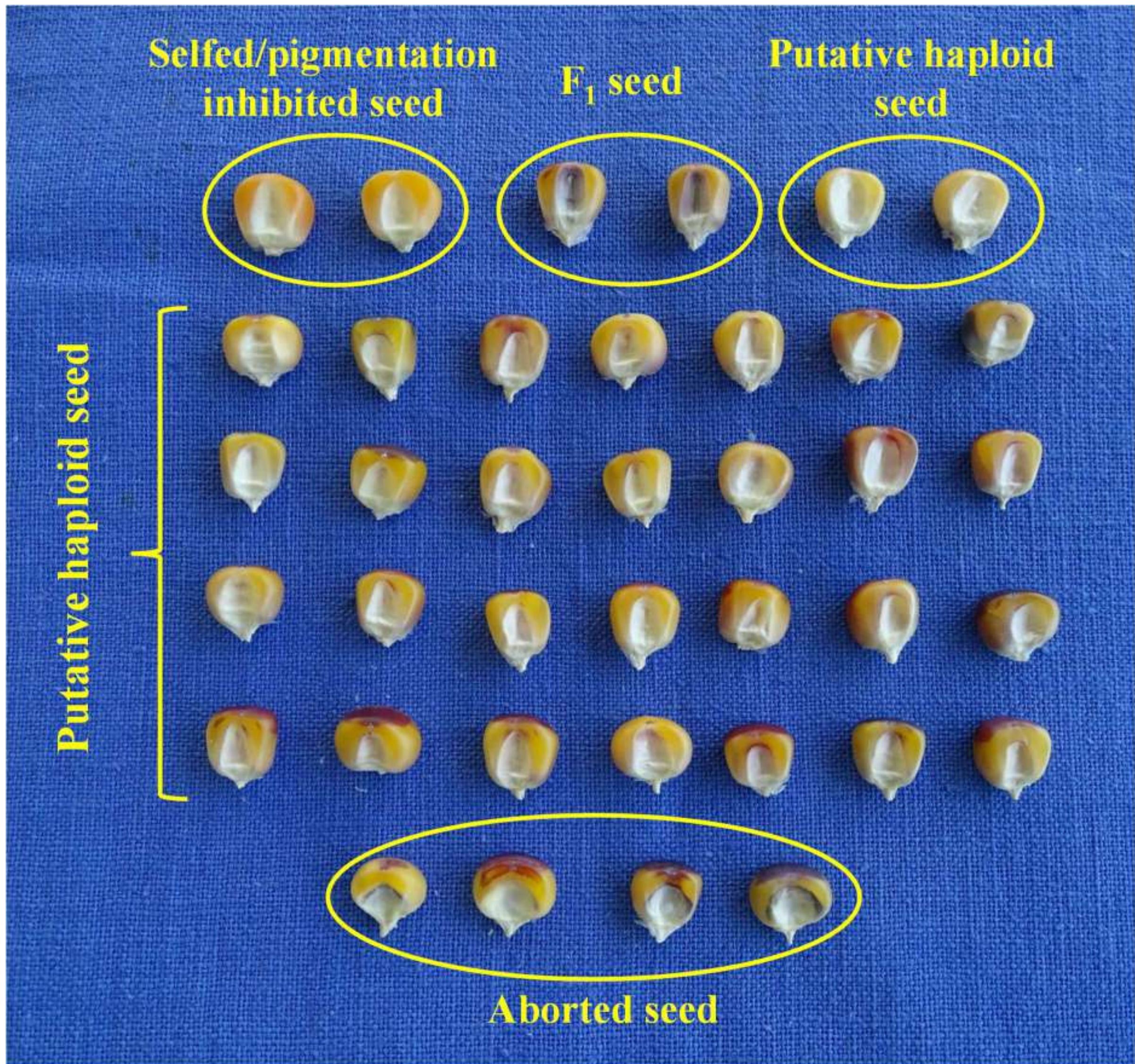

Fig 8. An assortment of different classes of seeds from an induction cross

Chapter 7

Chromosome doubling treatment

RK Khulbe, RS Pal, Vivek Pandey, Rohit Kapil and Kamal Pandey

This is a crucial step in the DH production process as any dose and/or treatment duration error can drastically affect the DH output. While sub-optimal doses can significantly reduce the frequency of diploidization, higher doses may prove lethal. Therefore, extreme caution needs to be exercised at this step of the DH production process. Chromosome doubling treatment involves:

1. Preparation of seedlings for colchicine treatment
2. Preparation of colchicine solution
3. Treatment of seedlings with colchicine

1. Preparation of seedlings for colchicine treatment

a. To prevent loss of seedlings during germination, particularly due to fungal infections (i) the haploid seeds are treated with Thiram@1.5g/kg seed before incubation, and (ii) the germination papers (size 45 x 28 cm) are soaked in 0.1% NaOCl solution for about a minute. For preparing NaOCl solution, ordinary tap water, boiled for about 10 minutes and cooled, may be used if sterilized water is not available.

b. The moist germination paper is spread out on a workbench and haploid seeds are arranged in rows on the paper. For maintaining adequate spacing among the seeds, 50 seeds (10 rows x 5 columns) per germination paper are sufficient (Fig. 1a).

c. The seeds are placed on the paper with the embryo-side facing down and the crown region oriented towards the germination paper's top side (Fig. 1b).

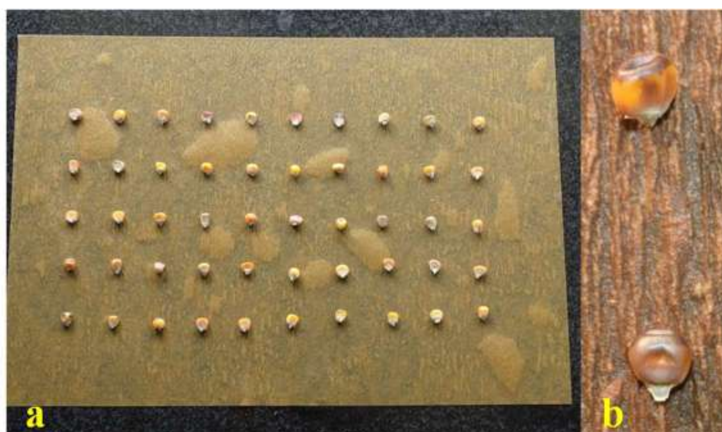

Fig. 1a. Haploid seed arranged in rows b. Seed with embryo-side facing down

d. A second germination paper is laid on top of the first germination paper, and the two are rolled up. The ends of the roll are secured with rubber bands to prevent the seeds from falling out. Four to five such rolls may be fastened together into a bundle using rubber bands. A rectangular piece of the germination paper with the identity of the source germplasm and date written on it using water-proof ink may be rolled together and fastened with them to

serve as a label. The bundles are then placed in a plastic tray containing about 1 cm level of water. The purpose of this is to keep the germination paper bundles adequately moist (Fig. 2a). In place of water, 0.1% NaOCl solution may also be used. Seed germinator, if available, can also be used for germinating seeds.

Fig. 2a. Germination paper rolls placed in water tray b. Germinated seed

e. The germination papers are opened after 4-5 days depending upon the prevailing ambient temperature (Fig. 2c). The seedlings with coleoptiles having attained a length of about 2 cm are removed for cutting. The remaining seeds are rolled up back in the germination paper. At Almora, we start incubating seeds from mid-May when the average minimum and maximum temperature is 23ºC and 38ºC, respectively.
f. The selected seedlings are placed in a water tray to keep them moist. The seedlings are then prepared for colchicine treatment by cutting their shoot and root tissues. The root and shoot tissues are recommended to be cut at about 2 cm and 1 cm from the tip, respectively (Chaikam and Mahuku 2012b). However, at Almora we cut the coleoptile about 2-3 cm above the coleoptile node and roots are so cut as to retain a length of about 2 cm (Fig. 3a). For cutting,

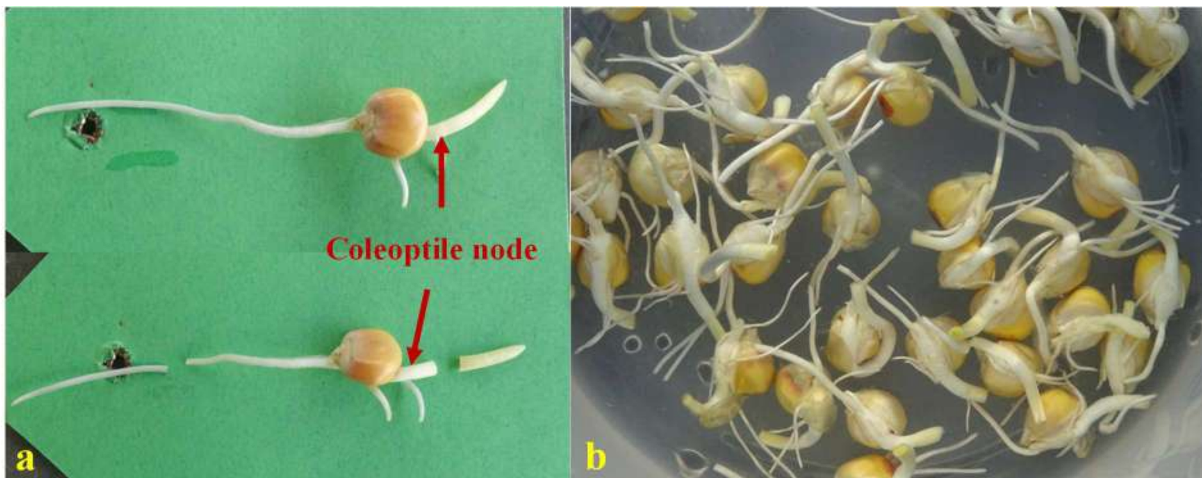

Fig. 3a. Cutting of seedlings a little above the coleoptile node b. Cut seedlings ready for colchicine treatment

a surgical blade (No. 22 size) or a stationery knife may be used. The cut seedlings are kept immersed in water in a separate tray until they are removed for colchicine treatment (Fig. 3b).

g. Variation for coleoptile node position may be found between seedlings of different source germplasm (Fig. 4a). Some degree of variation may also be observed among seedlings of the same source germplasm. In some seedlings, the coleoptile node is diffused, making it difficult to locate it. Therefore, while cutting the seedlings, care should be taken not to make the cut at or below the coleoptile node. A cut at the node or below the node it will cause cessation of seedling growth, eventually leading to mortality of the seedling.

h. As mentioned in Chapter 4, some diploid seeds may also be classified as haploid due to error in classification due to weak kernel anthocyanin pigmentation in some induction cross seeds. The misclassified F_1 seeds can be identified at the seedling stage based on anthocyanin pigmentation on the root(s) and the shoot tip (Fig. 4b). Such seedlings should be discarded at this stage. Differentiating haploid and misclassified selfed/pigmentation-inhibited seed is difficult at this stage as both lack root and shoot anthocyanin pigmentation.

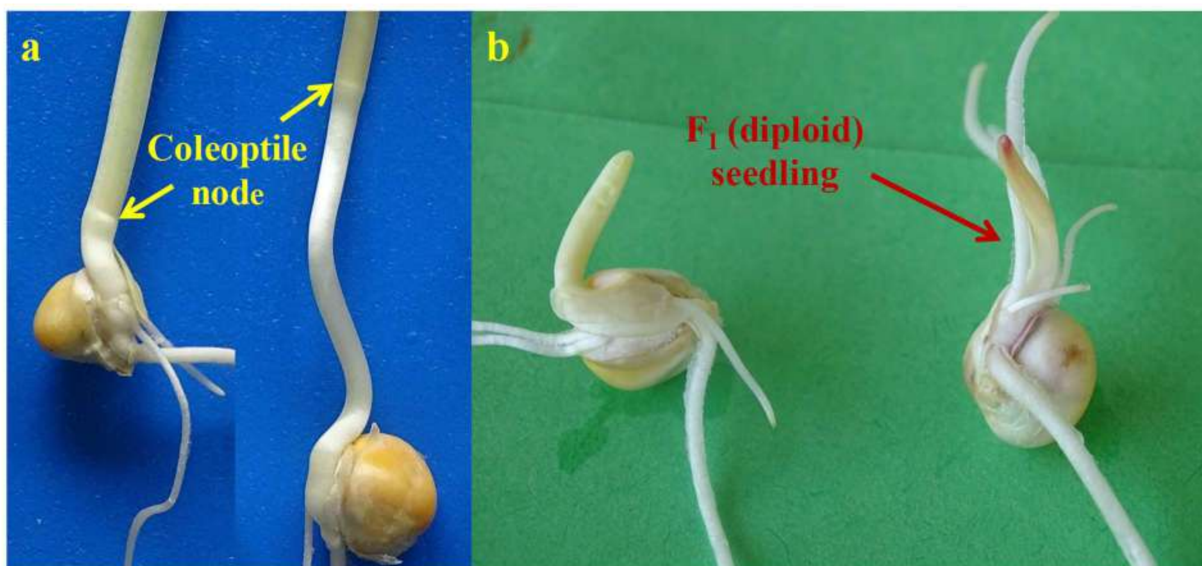

Fig. 4a. Variation for coleoptiles node position b. Mis-classified diploid seedling

2. **Preparation of colchicine solution**
    a. Colchicine should be procured from a reputed source, and changing the source frequently should be avoided
    b. The required quantity of colchicine should be calculated based on the number of seedlings to be treated and should be procured in advance
    c. Colchicine is a hazardous chemical, so all necessary precautions should be taken during its handling
    d. It is recommended to prepare the required quantity of colchicine solution fresh every time
    e. For seed treatment, colchicine solutions of 0.04% and 0.06% are recommended for 12h and 8h treatments. Colchicine solution is prepared in 0.5% DMSO.

f. The used colchicine solution should be collected in a labelled container and disposed of as per biosafety norms

3. **Treatment of cut seedlings with colchicine**

   a. Depending upon the protocol being followed, colchicine treatment involves keeping the cut seedlings immersed in colchicine solution for 8h or 12h. At Almora, treatment of the seedlings with 0.04% colchicine solution for 12h is followed.

   b. For colchicine treatment, the cut seedlings from the water tray are gently transferred to nylon mesh bags. The mesh bags are then placed in the colchicine tank, as explained by Chaikam and Mahuku (2012b). If a colchicine tank is not available, the mesh bags may be placed in glass beakers, the size of which depends upon the number of seedlings to be treated (Fig. 5). A steel tag with the name of the cross embossed on it is placed inside each bag for identification.

   c. Thereafter, the colchicine solution is poured into the beaker in an amount that is sufficient to immerse all the seedlings.

   d. The beakers containing the cut seedlings in colchicine solution are then kept for 12h in the dark to complete the treatment.

Fig. 5. Treatment of cut seedlings with colchicine

Chapter 8

Transfer of colchicine-treated seedlings into cups/pro-trays

GS Bisht, MC Pant, Vivek Pandey, Rohit Kapil and NC Mishra

This process requires very delicate execution as colchicine treatment renders the cut seedlings brittle, making them vulnerable to handling damage. Besides, the part of the coleoptiles left exposed after cutting becomes prone to fungal infections. The seedling transfer process involves the following steps:

1. **Preparation of potting medium**
    a. A mixture of cocopeat and vermicompost in 1:3 ratio by weight may be used as the potting medium. This combination allows unobstructed growth of the roots and, at the same time, supplies nutrients to the seedlings in the initial days.
    b. The potting medium is prepared 2-3 days in advance. Copper oxychloride@2gm/kg is added to the cocopeat-vermicompost mixture to keep it free of fungal and bacterial propagules.
    c. A day before seedling transfer, disposable paper cups in required numbers are filled with the potting medium. Before filling the medium, a small hole is made at the bottom of the cups to allow excess water to drain out. The medium is filled only up to 3/4th of the volume of the cups so that there is space for accommodating a small quantity of the medium after seedling transfer.
2. **Transfer of seedlings**
    a. In the morning, colchicine solution from the seedling treatment container is drained in the colchicine disposal can. The seedlings are washed twice with plain water to remove colchicine from their surface. The water from these washes also is drained into the colchicine disposal can.
    b. Before transferring the seedlings, the potting medium is teased with the help of a pencil or a pencil-thick stick so as to make space to accommodate the seedling. The seedling is then slowly lowered into the space created for it. Since the seedlings are delicate (Fig. 1a) and even a little extra pressure can cause the shoot or roots to break, the seedlings are held by the seed during the process. After placement of the seedling, the surrounding space is filled with the potting medium (Fig. 1b). The

Fig. 1a. Treated seedling b. Potting of seedling in cup c. Cups potted with seedlings

seedling should be so placed that the roots are fully inside the potting medium and the shoot is completely outside (Fig. 1c). This prevents the roots from drying and the coleoptiles from rotting.

c. After potting the seedling, a small amount of water is added to the cups to moisten the potting medium and fill any gaps around the seedling root. Addition of water makes the medium compact and prevents it from spilling out during watering by fountain-can.
d. Once potting is completed, the potted cups are arranged in trays, which are labelled and numbered, and transferred inside a polyhouse or a shade-house so that the tender seedlings remain protected from the harsh conditions outside. If not protected, high seedling mortality may occur.
e. It is important to clear out rodents from the shade-house/polyhouse. Rodents come for the seed which is still attached to the seedling and can cause significant loss of seedling population. Baiting with zinc phosphide-treated soybean seeds and trapping can effectively control rodents in the shade-house/polyhouse.
f. Ants and grass-hoppers also cause damage to the seedlings inside the shade-house/polyhouse. Early-stage damage can result in complete loss of the seedlings. Foliar spray of Deltamethrin (0.1%) may be used to prevent damage to seedlings from ants and grasshoppers.

3. Post-potting maintenance of seedlings

a. The potted seedlings need to be handled with great care to prevent mortality of the seedlings.
b. Sufficient moisture should be maintained in the potted cups. Watering lightly 2-3 times a day is considered better than watering heavily once in a day. Excess watering should be avoided.
c. A regular watch must be kept for cups that are losing moisture too fast or holding water for too long. While the former condition may be due to a tear at the bottom of the cup, the latter may be caused by blockage of the hole. Seedling from such cups should be transferred to new cups.

Fig. 2. Established treated seedlings

d. From the 4th day onwards, in place of plain water, the seedlings may be watered with a solution of readymade NPK fertilizer mix (20:20:20)@2g/litre of water. This promotes growth of the seedlings.
e. The occurrence of fungal infections in the seedlings is common (Fig. 3a). The infected seedlings should be drenched with 0.2% solution of contact fungicide Copper oxychloride 50% WP or a combination of systemic and contact fungicide (Metalaxyl 8% + Mancozeb 64% WP@2g/litre of water) to check the infection.
f. Deltamethrin (0.1%), a systemic insecticide, is applied at seven days interval to prevent insect damage to seedlings.
g. Besides infections, some seedlings may exhibit malformation or stagnated growth (Fig. 3b), and some may be chlorotic (Fig. 3c). Such seedlings eventually die.

Fig. 3a. Infected seedling b. Malformed seeding c. Chlorotic seedling

Chapter 9

Transplanting of D_0 seedlings in the field

GS Bisht, MC Pant, Vivek Pandey and Kamal Pandey

The next step in the process is to transfer D_0 seedlings from the cups/pro-trays to the field. In order to have a sufficient population of D_0 plants in the field, damage to the plants during this step should be kept to a minimum. The following are some general recommendations.

1. **Selection of field**
    a. The field selected for raising D_0 seedlings should be in the central area of the research farm so that it is easy to keep a regular watch on the crop. Peripheral fields should be avoided to protect the crop from wild animals' damage and pilferage by passers-by. Ideally, the entire DH breeding block may be fenced to restrict access and prevent damage from external agencies.
    b. The field should be well levelled and have good fertility and organic matter content. Fields with a history of higher weed population and rodent infestation should be avoided.
    c. Further, if possible, the D_0 nursery should be at a distance from the induction cross block or other diploid populations, which produce copious pollen and may serve as a source of contamination for the D_0 plants.
2. **Field preparation**
    a. Compost or FYM (@20 t/ha) may be incorporated at least two weeks before transplanting, and the field should be cultivated to a fine tilth.
    b. Since maize plants are particularly susceptible to waterlogging at the seedling stage, the field should have good drainage.
    c. The recommended dose of fertilizers (NPK@25:60:40 at Almora) should be applied at last ploughing. The remaining 75N is applied in three equal splits later.
    d. A wide range of insects attack maize crop at the seedling stage. Chlorantraniliprole 0.4% GR (25.0 kg/ha) may be mixed with basal fertilizers to control soil–inhabiting insects such as white grubs and cutworm, which often damage the crop in the initial stages.
3. **Spacing**
    a. Row-to-row spacing of 60 cm and plant to plant distance of 20 cm may be followed. Though haploid plants are shorter and weaker than diploid plants, very close planting should be avoided as sufficient space is needed to move around, especially during pollination.
    b. At Almora, a row length of 3 m is maintained, which accommodates 14-15 haploid plants. Row length may vary according to the location and dimensions of the field.

c. Separate blocks may be demarcated in the D_0 nursery for seedlings of different induction crosses depending upon the population size in the induction crosses.

4. **Appropriate stage for transplanting**

a. The appropriate stage for transplanting D_0 seedlings in the field is 4-leaf stage. At Almora, the 4-leaf stage is usually attained in 12-14 days after transferring the colchicine treated seedlings into the cups (Fig. 1a).

Fig. 1a. Hardening of D_0 seedlings b. Transfer of D_0 seedlings to the field in labelled trays

b. Seedlings of the same lot often show some growth variation, therefore, transfer of seedlings of the same lot to the field may require to be spread over 3-6 days, depending upon the size of the lot. Keeping the weak seedlings in cups beyond 20 days rarely helps as the growth of the seedlings remains stagnated and the seedlings begin to turn yellow. Therefore, by the 20th day, all seedlings should be transplanted in the field.

5. **Transplanting**

a. The seedlings should be carried from the shade-house/polyhouse to the field in properly labelled plastic trays (Fig. 1b).

b. About 30 minutes before transplanting, the seedlings are watered for compacting the potting medium. Thereafter, the seedlings are treated with Deltamethrin (0.1%) to prevent damage from ants, grasshoppers and other insects in the field.

c. Before transplanting, spots are marked in the rows at specified distance (20 cm). The soil at these spots is loosened and a small pit (6-8 cm depth) is prepared.

d. The cups containing the seedlings are arranged in the rows with one cup at each spot (Fig. 2a). For transplanting, the seedling is carefully removed from the cup along with the potting medium to keep the roots intact.

e. The seedling along with the potting medium is placed in the pit and firmly embedded in the soil.

f. After transplanting, the seedlings are watered with a solution of carbendazim@2g+copper oxychloride@2g/l water (Fig. 2b). From the second day onwards, the plants are watered at regular interval using fountain-can or garden pipe until the plants have attained enough growth to allow basin irrigation.

Fig. 2a. Transplanting of D_0 seedlings b. Watering newly transplanted seedlings

g. Drip irrigation or sprinkler system may be installed for effective management of irrigation in large D_0 nurseries.
h. Timely intercultural operations and plant protection measures are important for raising healthy D_0 plants (Fig. 3a&b and Fig. 4a&b)

Fig. 3. Young D_0 plants of source populations a. CMVL 55, and b. FSCH 41

Fig. 4a. D_0 plants at pre-flowering stage, and b. Flowering stage

Chapter 10

Management of D_0 nursery

GS Bisht, MC Pant and RK Khulbe

D_0 plants are haploid plants and, therefore, need more care than diploid maize plants. So, once D_0 plants have established in the field, it is important to maintain them in a healthy state through proper nutrient management and plant protection measures. Haploid plants being weaker, a close and regular watch is required so that necessary remedial measures may be taken timely in the event of any stress.

In the post-transplanting period, the D_0 plants are exposed to damage from different external agencies:

1. **Diseases**: Haploid plants have low vigour, which makes them more vulnerable to different maize diseases. Under Almora conditions, phythium rot, turciucm leaf blight (TLB) and banded leaf & sheath blight (BLSB) pose serious threat. Adequate and timely plant protection measures, therefore, need to be taken to prevent loss of plants due to diseases.
2. **Insects**:
    a. **Shoot-fly**: Shoot-fly can cause considerable damage during the early stages, especially under drier conditions. In locations with regular occurrence of the insect, shoot-fly traps may be installed 1-2 days before transplanting. Remommended measures should be taken as soon as insect damage is observed.
    b. **Aphids:** Adequate measures should be taken to control aphids in the crop. If not detected and prevented timely, aphids can suck the leaves and tassels dry.
    c. **Shoot/stem borers:** Early attack by shoot borers can result in plant mortality. Damage at later stages results in plants with multiple shoots, which generally remain vegetative. Stem borer attack can lead to damaged tassel. Sometimes complete tassel may be lost. In the last two years, Fall army worm (FAW) has emerged as a major pest of maize. The recommended management practices, therefore, should be followed to prevent damage to the D_0 plants.
    d. Blister beetles and some insect larvae feed on maize silks. While early damage to silks can preclude pollination, later stage damage can reduce seed set.
3. **Physical injury**: Physical injury may occur during field operations such as weeding, earthing-up, sprays, irrigation, etc. Therefore, post-transplanting field operations should be performed carefully so that no physical injury is caused to the plants.
4. **Wild animals**: Crop planted in fields close to forests is particularly prone to damage from wild animals. Adequate safeguards (regular watch-and-ward, fencing, etc.), therefore, need to be put in place to prevent the damage.

Nutrient, weed and water management

a. In addition to the recommended doses of fertilizer (At Almora - NPK@100:60:40 with N in four equal splits), additional nutrients should be supplied to the D_0 plants through foliar sprays of micronutrient mixtures. The

sprays may be administered at weekly intervals. Apart from these, plant growth-promoting formulations may also be used at regular intervals.

b. For about a week after transplanting, the plants should be watered manually. Basin irrigation should be given only after the plants have established in the field. Irrigation should be light and the frequency may be adjusted depending upon the requirement. Drip irrigation or sprinkler-system may be installed for better water management in the crop.
c. The D_0 nursery should be kept free of weeds to allow proper growth of the haploid plants. Though effective post-emergence weedicides are available in maize, it is advised to exercise care in using them on haploid populations.

Removal of diploid plants

As explained in the previous chapters, due to the variable expression of *R1-nj* kernel anthocyanin pigmentation marker, some degree of seed misclassification is unavoidable. This results in some plants, which otherwise are diploid, being transferred to the D_0 nursery along with haploid plants. Detection of such diploid plants at pre-transplanting stage or in the first few days after transplanting is difficult. However, about two weeks after transplanting, the diploid plants can be easily distinguished from haploid plants on the basis of morphological characteristics and growth differences (Fig. 2 to Fig. 4). The characters that may be used to differentiate haploid and diploid plants (referred to as 'Gold Standard') are as follows:

Character / parameter	**Haploid plant**	**Diploid plant**
Vigour	Generally lack vigour	Vigorous
Plant stature	Shorter	Taller
Stem	Narrow	Thicker
Leaf colour	Lighter green	Dark green/green
Leaf blade and length	Narrow and shorter	Broad and longer
Angle between leaf and stem	Narrow	Wide
Tassel	Usually small	Large
Fertility	May or may not be fertile	Always fertile
Ear size	Small	Large

It is important to remove the diploid plants from the D_0 nursery before they reach tasseling stage for the following two reasons:

1. If not removed, the diploid plants may be self-pollinated along with the fertile haploid plants, and at the time of harvesting, distinguishing diploid ears (with kernel pigmentation absent) from selfed ears of fertile haploid plants may be difficult.
2. The diploid plants produce copious pollen which may become a source of contamination for the fertile D_0 plants, which generally produce small amount of pollen.

Diploid plants which can be readily identified should be removed at the vegetative stage itself. However, roguing of plants that exhibit intermediate features may be delayed until tasseling stage.

Fig. 1a&b. Diploid plants at early vegetative stage

Fig. 2a&b. Diploid plants at vegetative stage

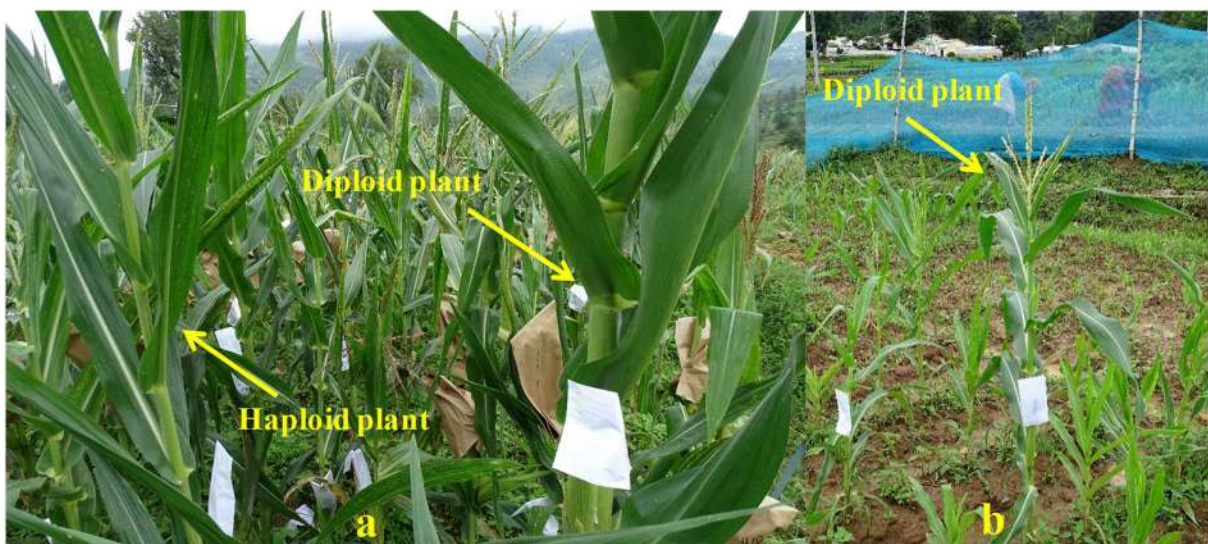

Fig. 3a. Differences in stem and leaf characteristics between haploid and diploid plants b. Early flowering in diploid plants

Though D_0 plants are weaker than the diploid plants, sometimes vigorous and fertile D_0 plants may also be found (Fig. 4b).

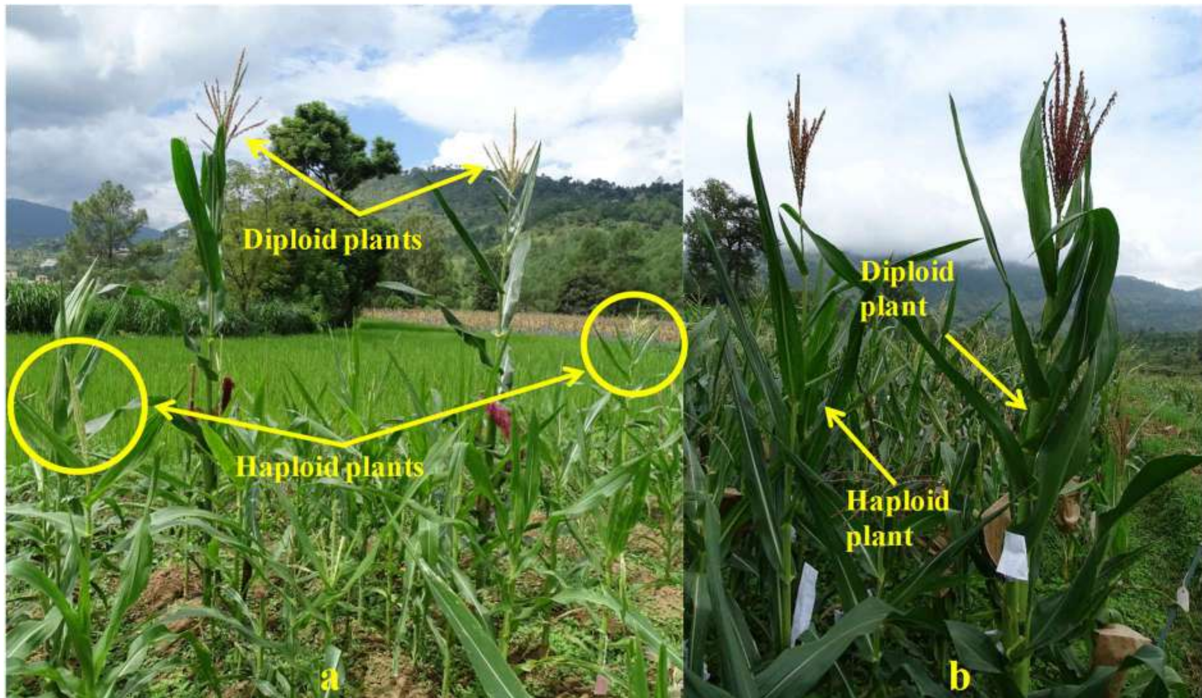

Fig. 4a. Difference in tassel size of haploid and diploid plants b. A vigorous and fertile haploid plant in D_0 population derived from TA 5024

Apart from the diploid plants, it is common to find abnormal plants such as those with tassel silk, rudimentary tassel, malformed tassel, or other abnormalities in the D_0 nursery (Fig. 5a-c).

Fig. 5. Abnormal plants with a. Tassel silk b. Malformed tassel c. Rudimentary tassel in D_0 nursery

Chapter 11

Self-pollination of fertile D_0 plants

GS Bisht, MC Pant, RK Khulbe, Vivek Pandey and Rohit Kapil

Identification of fertile D_0 plants is a critical step in DH production process as it is the selfed seed obtained from the fertile D_0 plants that eventually gives rise to DH lines. Since in the D_0 nursery, a large percentage of plants is sterile, it is important to not lose any fertile D_0 plant.

Identification of fertile plants

1. Though the confirmation of a D_0 plant being fertile or sterile is possible only at anthesis, a preliminary idea can be obtained by the appearance of the spikelets in the emerging tassel. Plump spikelets are indicative of a plant being fertile (Fig. 1a), whereas plants with flat spikelets are generally sterile (Fig. 1b).
2. A range of variation may be observed in tassel development among the sterile plants (from barren tassels to partially developed tassels to fully developed tassels).

Fig. 1a. Fertile spikelets b. Sterile spikelets c. Sterile plant with no anther extrusion d. Plant with anther extrusion but without pollen

3. In sterile plants, anther extrusion often does not take place (Fig. 1c). Even if anthers emerge, they are thin and flat and do not contain pollen (Fig. 1d). A D_0 plant should, however, not be dismissed as sterile until the tassel has fully emerged, as sometimes smaller branches near the tassel base may produce 1-2 fertile anthers, which are enough to obtain a few selfed seeds.
4. Vigour of the D_0 plants is no indication of their being sterile or fertile
5. Like sterile plants, a wide variation can be observed among fertile D_0 plants for tassel size and fertility (Fig. 2a-f):
    a. Fully fertile tassel as in normal maize inbreds
    b. Fully fertile tassel but with only a few small branches
    c. Normal-sized tassel with both sterile and fertile branches
    d. Tassel with all branches bearing anthers but of smaller size and lesser plumpness. Such anthers contain very little pollen but it is sufficient for obtaining some selfed seeds

e. Tassel with only one fertile branch
f. Tassel with only a few fertile anthers

Besides these, other variant combinations of the above types may be observed.

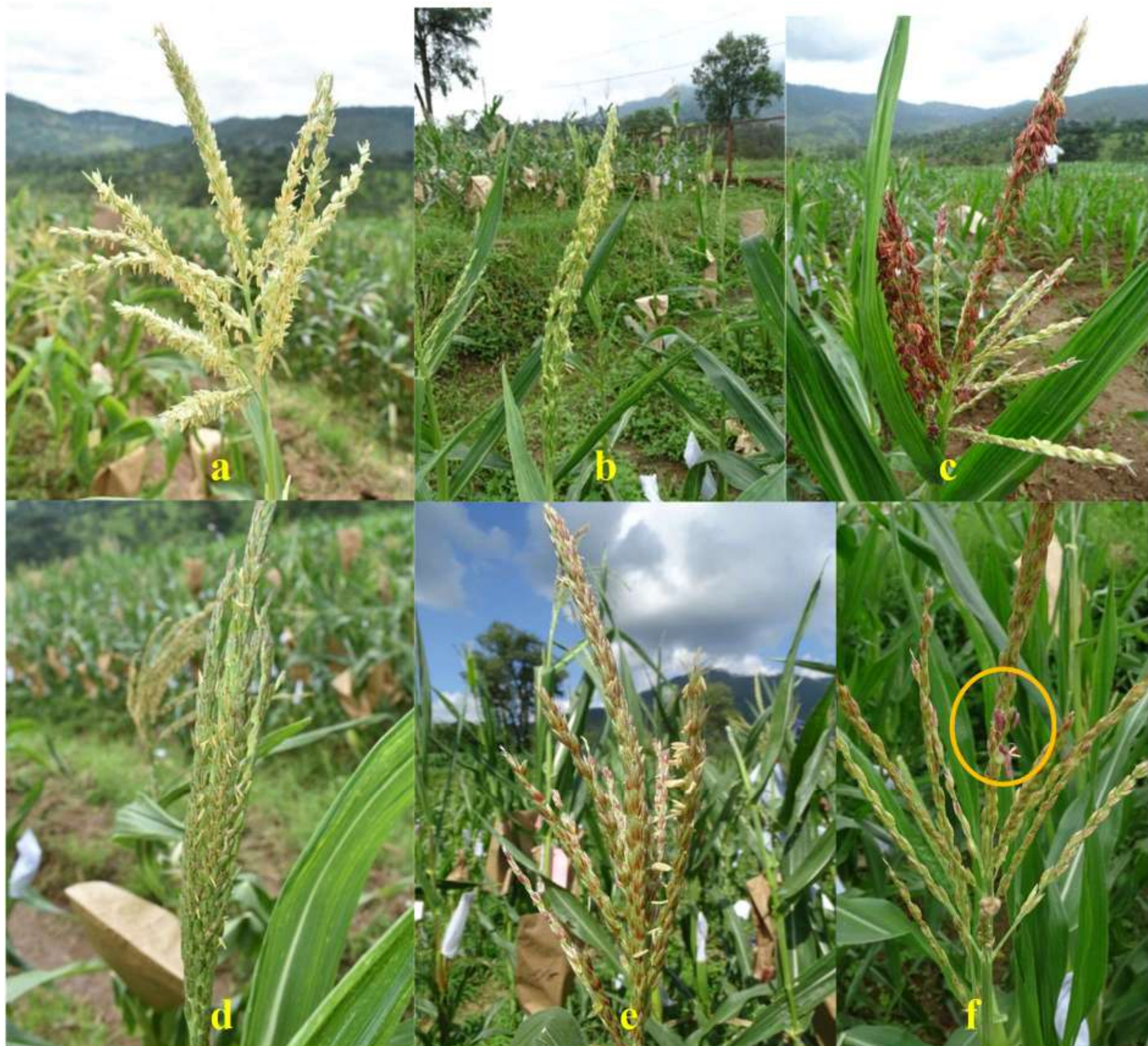

Fig. 2. Variation for tassel size and fertility among fertile D_0 plants

6. As mentioned at (f) above, in some plants the tassels may produce only 1-2 anthers before being over. These anthers can appear at any stage and in any branch of the tassel. Therefore, constant watch may be kept even on apparently fully sterile plants so that even a single fertile anther may be utilized for self-pollination.

Self-pollination in fertile plants

1. D_0 plants are self-pollinated in the same manner as normal maize lines. For self-pollination, ears in fertile D_0 plants are covered with the regular size butter paper bags at pre-silk emergence stage.
2. For pollen collection, tassel bags of smaller size may be used to cover the tassels since tassels in D_0 plants are mostly smaller in comparison to normal maize lines. Using regular-size tassel bags may cause breakage of tassel when rains or strong winds occur.

If tassel bags of smaller size are not available, the regular-size tassel bag may be shortened from the open end and used (Fig. 3a).

3. In the D_0 nursery, many plants often remain unpollinated despite being fertile due to larger ASI (Anthesis-Silking Interval). It is, therefore, important to constantly monitor silk emergence and anthesis in fertile plants and ear shoots may be cut, if required.

Fig. 3a. Covering fertile tassel b. Self-pollination c. Tagging self-pollinated D_0 plant

4. In D_0 plants with normal fertile tassel, single pollination is sufficient (Fig. 3b). However, partially fertile plants may need to be pollinated more than once, depending upon pollen availability. Therefore, extra care needs to be taken while collecting pollen (to avoid damaging the tassel) and carrying out pollination (to prevent contamination) in such plants.
5. In D_0 plants bearing only 1-2 anthers, the anthers can be collected in petri-plates a little before dehiscence and ruptured to release pollen. The pollen then can be used for self-pollination using a small brush.
6. After pollination, the date(s) of pollination may be mentioned on the tassel bag (which is used to cover the pollinated ear). This is particularly important in the case of partially fertile plants which require additional rounds of pollination.
7. After pollination, each self-pollinated D_0 plant may be marked with a tag for identification (Fig. 3c).

Fig. 4. D_0 nursery at Experiental Farm, Hawalbagh

It may be noted that the percentage of fertile plants in a well-maintained and healthy D_0 nursery (Fig. 4) is likely to be higher than in a poorly managed nursery (Kleiber et al. 2012). Good maintenance also increases the chances of seed set in self-pollinated fertile plants.

Chapter 12

Post-pollination management of D_0 nursery

GS Bisht and MC Pant

The self-pollinated D_0 plants need careful handling to prevent damage from diseases, insects, rodents and wild animals.

1. **Diseases:** After pollination, the D_0 nursery requires additional care due to the increased vulnerability of the pollinated plants to diseases. Adequate plant protection measures, therefore, need to be taken to prevent loss of plants due to infections.
2. **Insects**:
    a. **Aphids**: Freshly pollinated ears covered with tassel bags offer a congenial environment for aphids to thrive. If not detected and controlled timely, aphids can sap the developing ears.
    b. **Borers**: The larvae of *Spodoptera* sp. and *Helicoverpa* sp. feed on developing seeds. Therefore, proper control measures need to be taken in advance, because once the ears have been pollinated and covered with tassel bags, detecting and controlling the insects becomes difficult.
2. **Rodents**: Rats can climb up maize plants and eat developing seed. The damage is difficult to detect as the pollinated ears remain covered by tassel bags. The damage is noticed only at the time of harvesting, by which time it is too late. It is therefore recommended to maintain regular watch for rodents in the field and, if observed, required control measures must be taken.
3. **Wild animals**: Damage from monkeys and wild animals may occur in fields that are close to forests. Adequate safeguards need to be put in place to prevent the damage. For monkeys, watch-and-ward during the day is sufficient as monkeys do not venture out after sunset. However, for nocturnal animals like wild boars, strong fencing around the field and watch-and-ward during the night is required.

Fig. 1a. Wild boar damage in D_0 nursery b. Protection by chain-link fence

Chapter 13

Harvesting and handling of D_1 ears

RK Khulbe, GS Bisht and MC Pant

The self-pollinated plants in the D_0 nursery need careful handling so that they remain safe and healthy up to maturity and produce diploid seeds. The importance of post-pollination handling can be understood from the fact that even a single mature seed from a self-pollinated D_0 plant is a potential DH line. The following points may be kept in mind during the harvesting of D_1 ears.

1. The number of days from pollination is a good indicator of the harvesting time of the pollinated ears from haploid plants. At Almora, the D_1 ears are ready for harvest in 40-45 days after pollination (Fig. 1). However, it is always safer to check a couple of ears to decide the appropriate harvesting time.

Fig. 1. Harvesting of D_1 ears at ICAR-VPKAS Experimental Farm, Hawalbagh

2. Th presence of set seeds in maize ears can typically be felt from outside without dehusking the ear, and a decision to harvest (or not to harvest) a particular ear may be taken accordingly. However, in the D_0 nursery, it is essential to harvest and dehusk each and every pollinated ear because sometimes ears with just one or two seeds set in the basal portion of the ear may escape detection from outside.
3. Dehusking should be done gently, layer by layer. Application of force in removing the husk may sometimes result in the seed coming away along with the husk and getting lost.

4. During harvesting, it is important to watch out for (i) ears with seeds showing pigmentation, and (ii) vigorous ears with full seed set (Fig. 2). Such ears are produced by misclassified diploid plants which have escaped roguing in the D_0 nursery and should be discarded outright.
5. In addition to the above, D_1 ears more vigorous than normal haploid ears and with full seed set (but without seed pigmentation) are also sometimes present (Fig. 2d). These may be produced by (i) haploid plants with higher vigour and fertility or (ii) cross-pollination or (iii) misclassified selfed plants. Such ears may be marked and kept under special observation in the D_1 nursery. High vigour and/or lack of uniformity will be indicative of their not being DH. The genetic constitution of such lines can also be determined using molecular markers.

Fig. 2. Ears from F_1 seed (a, b & c) and selfed seed (d) mis-classified as haploid during sorting of induction cross seed

6. Sometimes germinating seed can be found in some harvested D_1 ears (Fig. 3). If all the seeds in an ear have germinated, the seeds should be gently removed and immediately potted in cups. The seedlings can later be transplanted in the field/polyhouse, and the DH lines can thus be saved from permanent loss.
7. Seed with fungal infection can also be handled in the above manner. Before potting, infected seed should be treated with suitable fungicides.

Fig. 3. Fungal infection and *in situ* germination of seed in D_1 ears

8. Seed set in D_1 ears may range from a single seed to almost a completely filled ear (Fig. 4) and depends upon the amount of pollen produced by the plant, among other factors.
9. The dehusked ears should preferably be kept in cloth bags to allow quicker drying. Nylon bags may also be used. The mesh size, however, should be small enough to not allow the seed to pass through. Each bag should have a tag bearing the identity of the plant and the induction cross.
10. The harvested ears should be properly dried before shelling, and the shelled seed should be kept in paper seed packets and properly stored.
11. It is important to count the number of healthy seeds in each harvested ear and record it. The number of seeds can be written on the seed packet also. This information helps in deciding whether the seeds may be planted in cups (for transplanting in the field later to avoid loss of seed) or sown directly in the field in the D_1 nursery.

Fig. 4. Range of seed set in D_1 ears of sweet corn hybrid Sugar 75 (left) and normal corn hybrid VMH 45 (right)

Chapter 14

Raising and handling of D_1 nursery

RK Khulbe, Rakesh Bhowmick, GS Bisht and MC Pant

D_1 nursery comprises DH lines/plants raised from seeds obtained from the D_1 ears (Fig.1). Depending upon the availability of seed, the D_1 nursery can be raised for maintenance alone or for maintenance as well as a preliminary evaluation of the DH lines.

Fig. 1. D_1 nursery at ICAR-VPKAS Experimental Farm, Hawalbagh

As mentioned in the previous chapter, the seed harvested from D_1 ears may range from one to over 100. For convenience, four groups may be formed depending upon the number of D_1 seeds obtained: Group I (< 5 seeds), Group II (6-10 seeds), Group III (11-20 seeds) and Group IV (>20 seeds). The D_1 seed in each group may be handled differently as suggested below.

Group I: It is strongly recommended to raise the plants (D_1 plants) of this group in the cups first and thereafter transfer them to the field to minimize the risk of losing a DH line completely due to germination related problems in the field. If space is a constraint, plants of more than one DH line can be accommodated in a single row in the field. Sufficient distance, however, should be maintained between the plants. In addition, the plants should be marked with labelled sticks. The labels can be shifted to the plants after they have grown up. Raising the seedlings in cups requires additional space and manpower. Besides, proper record needs to be kept to avoid mixing up during seeding transfer and transplanting in the field.

Group II: The plants of this group may be raised as in Group I or the seed may be sown directly in the field. However, since some seeds would require to be retained to serve as a back-up in the event of unexpected loss of seed/plants in the field, raising the seeds in cups is advisable.

Group III: The seed of this group can be sown directly in the field for maintenance. However, if preliminary evaluation (in ABD or other suitable design) is also intended to be conducted

simultaneously, the seedlings may be raised in cups. This not only saves seeds but also allows maintenance of required plant to plant distance in a row without the need to thin out plants (as generally is required in direct sowing).

Group IV: The seed of putative DH lines in this group can be sown directly in the field for maintenance as well as preliminary evaluation.

In Group III and Group IV also, some seed may be retained as sometimes it may not be possible to maintain some lines due to poor vigour or some abnormalities. The remnant seed from such lines may be planted again under congenial conditions for maintenance.

Maintenance of D_1 nursery

Since all the plants in the D_1 nursery are essentially diploids, the same package of practices as recommended for raising conventional inbred lines may be followed. Though the selfed seed harvested from the D_1 ears is expected to produce uniform progeny (Fig. 2), there

Fig. 2. Uniformity within DH lines in D_1 nursery

is always a possibility of some fertile haploid plants in the D_0 nursery having set seed (partially or fully) with external pollen. This happens mainly because many fertile plants in the D_0 nursery produce very few anthers and, consequently, very little pollen, which increases the chances of contamination if adequate care is not taken at the time of selfing. Apart from this, some weak diploid plants in the D_0 nursery (from selfed/contaminated induction cross seed misclassified as haploid) may also be mistaken as haploids and selfed. Such lines need to be identified and handled as described below.

Identification and handling of contaminated lines/off-type plants in D_1 nursery

The non-DH/contaminated lines in the D_1 nursery may belong to the following two categories:

1. **Lines from D_1 ears with all seed from external pollen**: Such lines exhibit uniformity and hybrid-like vigour and can, therefore, be distinguished from the DH lines without much difficulty (Fig. 3a). Once their hybrid status (tall stature, bigger

tassel and ears) is confirmed, such lines should be removed from the field so that they do not become a source of contamination for the true DH lines.

2. **Lines from D_1 ears with seed from self + external pollen**: Such lines show variation for plant growth and vigour (Fig. 3b&c). In such lines, the inbred-like plants may be selfed, and the resulting seed raised again in the next season for confirmation.

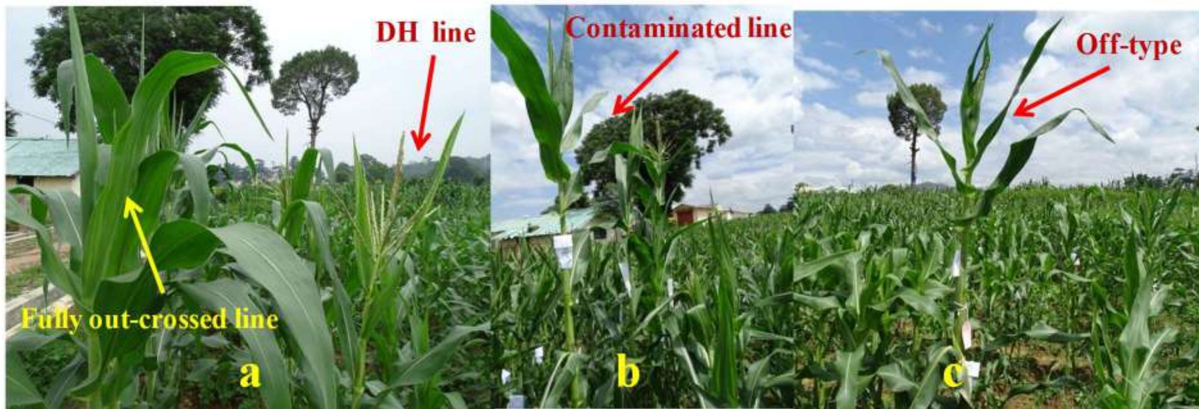

Fig. 3a. Fully contaminated line (left) and a true DH line (right) b. Contaminated line with DH and non-DH plants c. DH line with an off-type (non-DH) plant

In addition to these, some within line variation, as is observed in conventional inbreds, is seen in DH lines also. Some lines may show variation of a kind (minor variation in height, glume/pollen colour, kernel traits) that is not characteristic to DH lines but does not appear to be a result of contamination either. In such lines, the selfed seed of individual plants may be raised again for confirmation.

It is important to note that all vigourous DH lines are not necessarily contaminated lines as many DH lines exhibit vigorous growth, which may be attributed to transgressive segregation and the source germplasm used (Fig. 4). In case of any doubt about the DH status of any line, molecular markers may be used, or the selfed seed may be raised again in the next season for confirmation.

Fig. 4. Vigorous DH lines in D_1 nursery

In cases where only one or two plants of a DH line are available, the DH status and uniformity can be confirmed only through screening with molecular markers and/or raising the selfed seed again in the next season. However, maximum care should be taken during selfing such plants since cross-pollination can lead to contamination or their complete loss.

Use of molecular markers for identifying contaminated lines/off-type plants

1. **Foreground markers**: Screening with foreground markers is useful when the DH lines are derived from a source germplasm that is homozygous for trait(s) for which molecular markers are available. For example, in maize, robust markers are available for *opaque2* (high tryptophan/lysine), *crtRB1* (provitamin A) and *lpa* (low phytate) and many other genes. Gene-specific molecular markers may be used for screening DH lines derived from source germplasm carrying these genes (Fig. 5a&b).
2. **Background markers**: The DH status of lines can be confirmed using a set of polymorphic background markers. The true DH lines are expected to carry a single parental allele (either P1 or P2) and never both. A line showing presence of both the parental alleles is a confirmation of its not being a DH line (Fig. 5c).

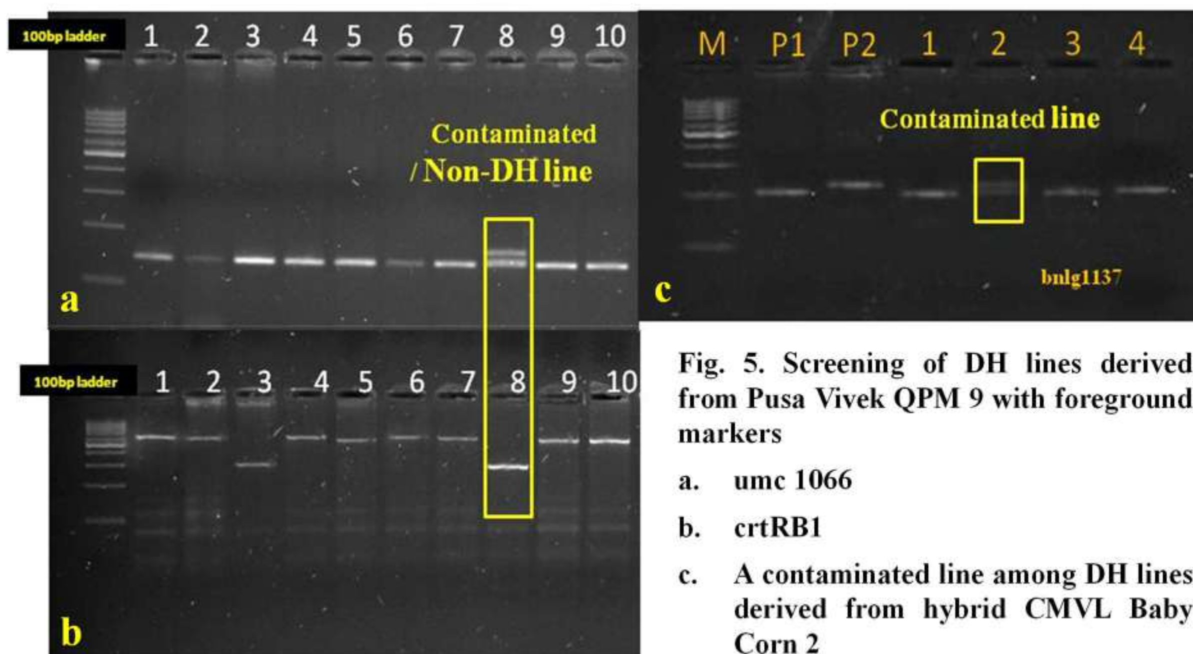

Fig. 5. Screening of DH lines derived from Pusa Vivek QPM 9 with foreground markers

a. umc 1066

b. crtRB1

c. A contaminated line among DH lines derived from hybrid CMVL Baby Corn 2

Unless generated for specific purposes, the need to screen all DH lines with molecular markers, before utilizing them in breeding programmes, generally does not arise. However, molecular markers may be used for confirmation of the presence of the desired trait(s) in the DH lines selected for further use in a breeding programme.

In the D_1 nursery, it is common to find lines with abnormal characters (Fig. 6a-c). Some of these may not survive up to the flowering stage, and some may not allow self-pollination.

Fig. 6. Abnormal DH lines in D_1 nursery a. Severe whorl twisting b. Chlorosis c. Rudimentary tassel

These lines may not be of direct use in breeding but may serve as important stocks for studying genetic behaviour of such characters. Therefore, all possible efforts should be made to maintain these lines. If selfing is not possible due to the absence of functional tassel or ear, another line may be used as seed or pollen parent so that the character is not permanently lost and is preserved in a heterozygous state for further use. However, the lines with undesirable traits (Fig. 7a-d) are best discarded.

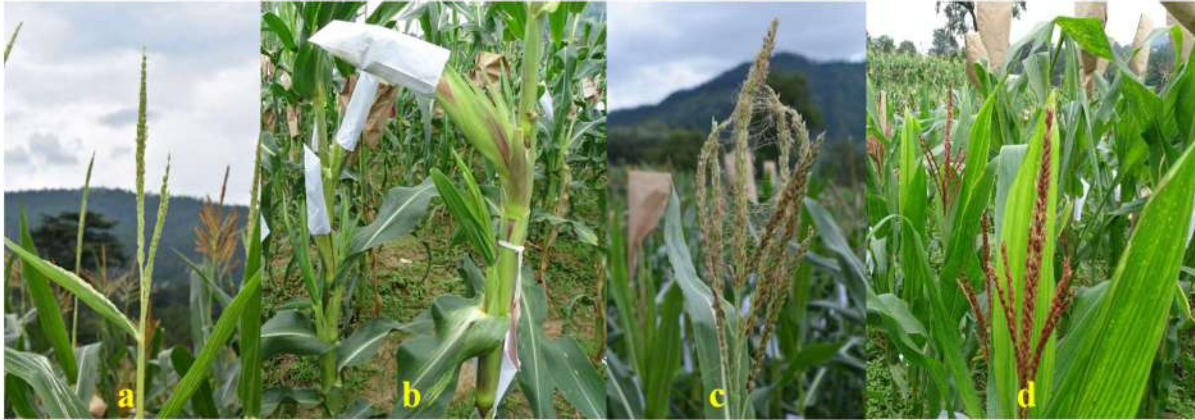

Fig. 7. Lines with undesirable traits a. Poor tassel b. Rabbit ears c. Tassel silk d. Very short stature

Few elite inbreds/DH lines and parental constituents of the source germplasm (if available) may be included in the D_1 nursery to serve as checks and aid in the selection of superior DH lines for use in the breeding programme.

At maturity, the selfed ears of each DH line should be harvested individually and screened for ear and grain characteristics before bulking.

Chapter 15

Success rates at different DH production steps

RK Khulbe, A Pattanayak, GS Bisht, MC Pant and Vivek Pandey

Among the key factors determining the success of a DH programme, the efficiency of haploid induction, referred to as Haploid Induction Rate (HIR), is of critical importance. The haploid inducers developed by CIMMYT are accessible to public and private maize programmes. The first set of Tropically Adapted Inducer Lines (TAILs) developed by CIMMYT in collaboration with the University of Hohenheim has HIR of 6-13% (Chaikam 2012). Haploid inducer line TAILP1 belongs to the first set of CIMMYT inducer lines.

In a study conducted at ICAR-VPKAS, Almora involving eight source populations and CIMMYT haploid inducer TAILP1 (Khulbe et al. 2020), the relative percentage and per se percentage at different working steps were calculated as follows:

1. ***Per se* percentage:** (No. of plants at a working step/Total seeds in the induction cross) x 100
2. ***Relative percentage:*** (No. of plants at a working step/Total plants in the previous step) x 100

Haploid induction rate (HIR) and Mis-classification percentage were worked out as follows:

1. HIR: No. of putative haploid seeds/Total seeds in the induction cross x 100.
2. MCP: 100 x Total diploid plants/Total plants in the field

Haploid Induction Rate (HIR): The average HIR of TAILP1 was 5.48%, with a range of 2.01-10.03%.

The relative percentage and *per se* percentage obtained at different working steps are as follows:

1. **Transfer to cups**: The average relative percentage of seedlings transferred to cups after germination and colchicine treatment was 89.9 with a range of 82.7 to 94.1, whereas the *per se* percentage was 4.93.
2. **Transfer to field**: The relative percentage ranged from 53.8 to 96.6, with an average of 75.0. The average *per se* percentage at this step was 3.7.
3. **Total plants in the field**: Survival in the field ranged from 49.2 to 85.1 per cent, with an average of 66.1 per cent. The average *per se* survival percentage was 2.44 per cent.
4. **Total haploid plants in field**: The relative percentage of total haploid plants in the field ranged from 94.5 to 99.06 with an average of 98.1. The *per se* average percentage was 2.40.
5. **Self-pollinated D_0 plants**: The relative percentage of self-pollinated D_0 plants ranged from 36.2 to 73.3 with an average of 60.9. The average *per se* percentage at this step was 1.46.
6. **Total D_1 ears harvested**: Relative percentage of harvested D_1 ears ranged from 41.2 to 90.8 with an average of 73.7. The *per se* percentage of harvested D_1 ears was 1.07, which was considered as the **overall efficiency** of the DH production method.

Mis-classification percentage (MCP): The percentage of misclassified plants in the field was small. The average relative percentage was 1.93 with a range of 0.94 to 5.48.

Comparison of population loss occurring at different working steps (Table 1) indicated that maximum loss was due to lower frequency of normal and fertile D_0 plants (39.1%, Step 4-5) followed by lower field survival (33.9%, Step 2-3), lower frequency of plants with seed set (26.6%, Step 5-6) and toxic effect of colchicine (25%, Step 1-2).

Table 2. Loss of population at different working steps starting with haploid seeds

Working step	Total Putative Haploid Seed (THS)	Total Transferred to Cups (TTC)	Total Transferred to Field (TTF)	Total Plants in Field (TPF)	Total Haploid Plants in Field (THPF)	Total Self-Pollinated D_0 Plants (TSPP)	Total harvested D_1 ears (THDE)
	0	**1**	**2**	**3**	**4**	**5**	**6**
Total population	5235	4708	3532	2336	2291	1396	1025
% Loss		10.1	25.0	33.9	1.9	39.1	26.6

In the study, the number of DH lines obtained per plant of the source population (assuming a loss of about 15% plants due to non-germination or post-germination mortality) worked out at 1.18, which broadly translates to one DH line per plant of the source population. This efficiency was higher than that worked out for the standard CIMMYT protocol using CIMMYT's considerations for determining the number of induction crosses per source population (Chaikam et al. 2012). The higher efficiency in our study resulted from higher field survival, lower misclassification percentage (lower number of diploid plants in the field), a higher proportion of fertile plants and a higher number of plants with seed set. Higher field survival is attributable to good agronomic management and favourable climatic conditions at Almora. The proportion of diploid plants (misclassification percentage) was very low as all the source populations used in the study exhibited complete *R1-nj* expression allowing precision in the identification of haploid seed. Good agronomic management and proper and adequate pollination are likely to have contributed to the higher proportion of seed set in D_1 ears.

However, as regards the higher proportion of fertile haploid plants in the present study, it is pertinent to mention that genotypic differences exist among different maize germplasm for spontaneous fertility restoration in haploids (Kleiber et al. 2012; Ren et al. 2017; Wu et al. 2017; Ma et al. 2018; Chaikam et al. 2019b). Spontaneous fertility restoration is caused by a spontaneous doubling of the chromosome complement and results in haploid plants producing pollen and seed without being specially treated to stimulate doubling of the chromosome complement (Chase 1949), and may range from 0 to 70% (Kleiber et al. 2012; Chaikam et al. 2019b).

Chapter 16

Performance of DH lines produced at ICAR-VPKAS, Almora

RK Khulbe, A Pattanayak, Devender Sharma, GS Bisht and MC Pant

Doubled haploid (DH) lines differ from conventionally-bred lines chiefly in the method of their development and in being completely homozygous. In terms of agronomic performance, DH lines do not differ much from lines developed through the conventional line development methods (Chidzanga et al. 2019).

Although doubled haploid (DH) technology enables the production of completely homozygous lines, the DH lines might present several drawbacks. Their heterotic response, *per se* productivity, adaptation to different environments, agronomic performance, biotic, and abiotic stress reactions are not known (Silva-Vinancio et al. 2019). Though broad predictions can be made if genetic background of the source germplasm is known, the worth of the lines for use in breeding programmes can be determined only after evaluation. Therefore, like inbred lines developed using conventional methods, evaluation of DH lines is essential for identifying lines with good agronomic performance, tolerance to diseases, and presence of specific traits for use in breeding programmes. Besides, characterization of genetic diversity in the DH germplasm set is essential for using the lines in heterosis breeding programmes.

In a set comprising 465 DH lines (derived from six early maturing F_1 hybrids at ICAR-VPKAS, Almora), five parental inbreds and seven elite inbred lines as checks, wide variation

Fig. 1. DH lines derived from Vivek QPM 9 (left) and FQH 106 (right) exhibiting variability and transgressive segregation for ear and kernel traits

was observed for the eight yield contributing traits (plant height, ear height, number of days to 50% silking, days to maturity, cob length, cob girth, number of kernel rows and 100-grain weight) (Fig. 1). Transgressive segregation for all the traits was observed in both directions (Table 1), and a number of DH lines showed significantly higher trait values than the inbred

checks. Cluster analysis grouped the 465 DH lines into five clusters, indicating a substantial amount of genetic diversity among them.

Table 1. Number of DH lines statistically significant over the best check for different traits

Sl. No.	Trait	Best check	Trait Value	No. of DH lines statistically significant over the best check
1.	Plant height (cm)	V412	187.0	19
		V407	139.0	105
2.	Ear height (cm)	V495	103.0	4
		VQL1	67.0	48
3.	Days to 50% silking	V495	59.0	67
		VQL2	51.0	5
4.	Maturity days	V400	102.0	22
		VQL1	86.0	Nil
5.	Cob length (cm)	V407	18.76	2
6.	Cob girth (cm)	V400	14.44	14
7.	Kernel rows	VQL1	17.60	17
8.	100-seed weight (g)	V412	329.52	24

Fig. 2. Ears of normal corn DH lines (left) and sweet corn DH lines (right) derived from public and private sector hybrids

Continuous infusion of new and diverse inbred lines into maize breeding programmes is essential for ensuring sustained development of high performing hybrids. DH technology has emerged as a very effective means of achieving that. The DH lines generated at Almora will contribute to augment the existing germplasm base (Fig. 2). Besides, the promising DH lines belonging to divergent clusters and differing in pedigree may be directly used as parents in hybrid development programmes.

Chapter 17

Economics of DH line production vis-à-vis conventional breeding

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Maize breeding programmes require a continuous supply of superior and potentially heterotic homozygous lines (inbreds). The speed with which new inbred lines can be produced determines the efficiency of breeding programmes. Faster advancement of segregating material leads to early generation of inbred lines, enabling quicker development and commercialization of hybrids, thereby reducing breeding cost. However, the development of inbred lines using conventional methods is a time-consuming process (6-8 generations of selfing) and involves considerable expense. Therefore, in order to reduce the time and cost involved in inbred line development, doubled haploid (DH) technology is being widely adopted as an alternative approach. Though the use of DH technology allows the production of homozygous lines in 2-3 generations compared to 6-8 generations in conventional breeding, the process requires additional facilities and resources. A cost analysis of maize lines produced using the DH method and the conventional method performed at ICAR-VPKAS, Almora is detailed in this chapter.

The information presented primarily aims to give a broad idea to the maize breeders as to the cost expected to be incurred if the same research team that is engaged in conventional maize breeding seeks to introduce DH production in their breeding programme. The cost of DH line production worked out, therefore, excludes the salaries of the regular staff (scientists, technical and field staff) and the already existing resources and facilities (land, farm machinery, irrigation, and other infrastructure and facilities). In addition, DH production requires trained workforce for efficient execution of the DH protocol. The cost also excludes the expenditure incurred in training the scientific/technical staff in DH production and/or time and resources spent developing adequate skills in DH production through self-learning and practicing. Therefore, the DH line production cost worked out may not be regarded as the representative cost of DH line production under Indian conditions.

DH (D_1) seed production requires two seasons - one for the generation of induction crosses and the second for raising and diploidization of the haploid plants. To be precise, D_1 seed obtained at the end of the second season when raised in the third season produces DH plants. In other words, it is the third season (D_1 nursery) when the DH lines can be characterized and evaluated for the first time, and may be used in the breeding programme. The cost of DH line production has, however, been calculated only up to D_1 seed generation stage as it is the D_1 seed that is supplied to the indentors by organizations offering DH production services.

1. DH Method

First season

A. Raising of source populations and generation of induction crosses: As explained in Chapter 4, for the generation of induction crosses, the source germplasm is raised in the same manner as any other breeding material. For generating about 1000 induction

cross ears (@200 ears each of 5 source populations), around 1100 plants of source germplasm and 220 plants of haploid inducer line are needed. In terms of rows@10 plants/row, it means110 rows of source population + 22 rows of haploid inducer. A higher number of source germplasm plants are required because induction cross ears are harvested from only about 90 per cent of the source germplasm populations as about 10% of the plant population is lost for various reasons. The sorting of haploid seed can be done at any time in the period intervening the two seasons. In the present analysis, the cost of haploid seed sorting has been added to the first season's expenditure. The resources required for generating 1000 induction cross ears are detailed in Table 1.

Net area required : 220 m^2 Gross area required : 300 m^2

Table 1. Field operations/inputs and associated cost in generation of induction crosses

Particulars		Approx. cost (Rs.)
1. Haploid Inducer Line*		-
2. Inputs (FYM, fertilizers, weedicide, fungicides, insecticides)		2,300.00
3. Field operations (sowing, weeding, earthing-up, sprays, watch-and-ward, pollination)		8,700.00
4. Breeding supplies (silk bags, tassel bags, cloth bags, nylon bags)		9,480.00
5. Harvesting, shelling, haploid seed sorting, seed packeting		7,900.00
	Total (A)	**28,380.00**

***Cost of the haploid inducer line is not included as TAILP1was provided by ICAR-IIMR, Ludhiana free of cost. However, the acquisition of new high HIR haploid inducer lines (CIM2GTAILs) from CIMMYT may cost up to 15000.00 INR**.

Second season

B. Colchicine treatment and maintenance of treated haploid seedlings: In the second season, the haploid seedlings are treated with colchicine and transplanted in the field. From 1100 source population plants (~1000 induction cross ears), approximately 6960 haploid seeds are expected to be obtained (based on results obtained in our study: 127 kernels per induction cross ear and average HIR of 5.48%). The cost involved in this step is detailed in Table 2.

Table 2. Cost involved in colchicine treatment and maintenance of haploid seedlings

Particulars		Approx. cost (Rs.)
1. Inputs (germination paper, colchicine, cocopeat/vermi-compost, paper cups, plastic trays)		49,100.00
2. Colchicine		80,000.00
3. Field operations (seed germination, seedling clipping, transfer to cups and maintenance in shade-house)		26,000.00
	Total (a)	**1,55,100.00**
4. Shade-house (15 x 12 m)		60,000.00
5. Iron step-benches		44,000.00
	Total (b)	**74,000.00**
	Total (B) (a+b)	**2,59,100.00**

Fig. 1. Temporary shade-house and iron step-benches

C. **Maintenance of D_0 nursery and generation of D_1 seed:** Before transplanting in the field, the colchicine-treated haploid seedlings are maintained in a shade-house until they reach the transplanting stage (Fig. 1). This period is about two weeks at Almora. From 6960 haploid seeds, approximately 6261 seedlings will be transferred into the cups and approximately 4700 final plants will be transferred to the field. The approximate cost of transplanting 4700 seedlings in the field is as below (Table 3):

Net area required : 800 m^2 Gross area required : 1000 m^2

Table 3. Approximate cost of raising D_0 nursery and generation of D_1 seed

Particulars		Cost (Rs.)
1. Inputs (FYM, fertilizers, vermi-compost, weedicide, fungicides, insecticides)		9,595.00
2. Field operations (transplanting, manual watering, weeding, earthing-up, sprays, watch-and-ward)		60,000.00
3. Breeding supplies (silk bags, tassel bags, cloth bags, nylon bags)		28,900.00
4. Pollination		80,000.00
5. Harvesting, shelling and seed packeting		11,000.00
	Total (C)	**1,89,495.00**

Total cost (A+B+C) in DH production	**4,76,975.00**

Out of total 4700 D_0 plants, D_1 seed (which will produce DH plant/line in the next season) is expected to be obtained from about 1000 plants, and the cost of producing one DH line thus comes to INR 477.00.

2. Conventional Method

The conventional method of inbred development requires at least 7 generations of selfing (S_0-S_6) to recover homozygous lines. The number of progenies raised and advanced varies from one generation to another, depending upon the objective and the source population. The details of operations involved in generating inbred lines through the conventional method are given in Table 4.

Table 4. Field operations and associated cost for developing inbred line by conventional breeding method

Generation	Area (m^2)	Expenditure (Rs.)							Progenies / lines retained
		Farm inputs	Field operations	Breeding supplies	Pollination	Harvesting, shelling, seed packeting	Watch-and-ward**	Total	
S_0	200	500	2600	5895*	3000	600	1900	14395	75
S_1	600	1500	7800	2205	9000	1200	5700	27405	150
S_2	1100	2750	14325	5535	15000	1600	10400	49910	200
S_3	1400	3500	18200	7200	21000	1200	13000	64900	150
S_4	700	1750	9100	1050	10500	600	6600	30000	75
S_5	400	1000	5200	715	6000	400	3800	17315	50
S_6	250	625	3100	450	3750	400	2400	10775	45
Total		11625	60325	23050	68250	6000	43800	**217700**	

***Including additional onetime cost of Rs. 5000/- for nylon bags, cloth bags and seed packets**
****May be excluded if the farm area is already protected**

Based on the above calculations, the cost of developing a single homozygous line through conventional breeding was found to be INR 4838.00.

Comparison of cost of DH method and conventional breeding

In our analysis, the cost of developing a single homozygous line was found to be INR 477.00 and INR 4838.00 in the DH and conventional method, respectively. Thus, even allowing for the cost of haploid inducer line and other miscellaneous expenses likely to have missed inclusion in DH production cost, the cost of producing one DH line (D_1 seed) is expected to be within INR 500.00.

There, however, are other considerations that may be taken into account in making a cost comparison of the two methods:

1. While the conventional lines can be used for breeding in the S_6 generation itself, one more (third) season is required to raise DH plants from the D_1 seed harvested in the second season. Raising of the D_1 nursery for characterization, evaluation and maintenance is estimated to cost another about INR 80,000.00.
2. In the conventional method, S_6/S_7 generation lines are ready-to-use as they have already undergone multiple cycles of selection in the course of their development. In contrast, the number of DH lines that can actually be used in the breeding programme becomes known only after their evaluation.

However, even assuming that only 20 per cent of the DH lines produced are promising and, therefore, usable in the breeding programme, the cost per usable DH line (INR 2385.00) still comes to about half that of a conventional inbred.

Comparison with outsourcing of DH line production

Some organizations also offer DH line production services at a cost. For example, CIMMYT has DH production facilities at Agua Fría, Mexico and Kalro-Kiboko, Kenya, which provide DH production services. Generally, about 150 DH lines (D_1 seed) per source population are produced, and the cost per DH line comes to USD 10-14 (approx. INR 750-

1000) (Personal communication with Dr Vijay Chaikam, CIMMYT, Kenya). Based on the DH line production cost obtained in our analysis, an in-house DH line production programme is likely to cost less than outsourcing DH line production.

Timeline of homozygous lines' development in DH and Conventional methods

In the DH method, D_1 seed can be obtained in the second season itself, and completely homozygous DH lines can be obtained in the third season. The use of DH lines in the breeding programme can begin at this stage (Fig. 2). In contrast, 6-7 seasons are required for developing a homozygous line using the conventional method. In terms of time, it means at least three years, if two generations can be raised in a single year, which is possible only if the climatic conditions of the location allow two crops per year, or off-season nursery or polyhouse facility is available. In its absence, 6-7 years may be required to develop a conventional inbred line.

Season and Year	DH Method (One generation / year)	DH Method (Two generations / year)	Conventional Method (Two generations / year)
I Season – I Year (*Kharif*)	Induction Crosses	Induction Crosses	S_0 generation
II Season – I Year (*Rabi*)	-	D_0 Nursery	S_1 generation
III Season – II Year (*Kharif*)	D_0 Nursery	D_1 Nursery (Utilization) ✓	S_2 generation
I V Season – II Year (*Rabi*)	-	-	S_3 generation
V Season – III Year (*Kharif*)	D_1 Nursery (Utilization) ✓	-	S_4 generation
VI Season – III Year (*Rabi*)	-	-	S_5 generation
VI I Season – IV Year (*Kharif*)	-	-	S_6 generation (Utilization) ✓

Fig. 2. Timeline of production of maize lines in DH and Conventional methods

Even in the DH method, the time required to obtain a DH line depends on the ability, or otherwise, to raise more than one generation per year. If the crop can be raised in successive seasons, the DH lines can be obtained in the second year. Otherwise, the DH lines will be available only in the third year. In the absence of climatic conditions favouring two crops in a year, the time to obtain DH lines can be reduced by raising off-season crop in polyhouses. However, growing large off-season populations requires a large number of polyhouses, which

will add enormously to the cost of DH line production. Polyhouses, if available, can be used to advance few very promising populations.

In summary, the cost of DH line production is a direct function of the efficiency of a DH production protocol. This, in turn, depends on various factors, such as the haploid induction rate of the inducer line, efficiency in haploid classification, chromosome doubling frequency, and handling of the material, among others. Each factor influences the cost of DH line production by affecting the recovery of DH lines. Therefore, judicious choice of the haploid inducer line and the source germplam, and trained workforce for efficient handling of the material at various steps of the DH production protocol is important for maximizing DH lines' recovery and minimizing the cost. Regarding advantage in terms of time, the benefit can be availed if two or more generations can be raised in a year, without which DH method does not seem to offer significant advantage over the conventional method. At some locations, raising up to three generations per year may be possible by adopting special crop management practices, which could further shorten DH generation time. Considering the cost advantage of DH production, the technology has excellent potential for developing commercial production units or business incubators in public research institutes.

Chapter 18

Future Prospects for National Maize Breeding Programme

RK Khulbe and A Pattanayak

The use of doubled haploids as parental lines of commercial hybrids goes as far back as the early 1960s in the US. DeKalb 640 was the first widely planted commercial hybrid developed from a double-cross of three DH and one conventional inbred parent lines (Liu et al. 2016). Its tremendous success helped pave the way for the widespread use of DHs for inbred line development. Public maize breeding programmes in many countries and international and national private sector maize breeding programmes have been using DH technology for quite some time now and commercial DH based hybrids have been released in many countries. Despite various advantages offered by DH technology, its initial progression was moderate due to lower DH production efficiency, owing mainly to the low haploid induction rate (HIR) of the early haploid inducer lines. The technology gathered pace with the advent of high HIR inducers in superior temperate backgrounds and tropicalization of the inducer lines (TAILs), making them amenable for use in the semi-tropical and tropical countries as well. Simultaneously efforts were made to improve the HIR as well as agronomic features of the inducers lines, and as a result, inducer lines with high HIR and better agronomic performance are now available. CIMMYT's second-generation inducer lines (CIM2GTAILs), which have higher HIR and better agronomic performance compared to the first-generation inducer lines, are examples of that. The CIMMYT inducer lines are readily accessible for use in public breeding programmes.

Initially, public maize breeding programmes in India may use CIMMYT TAILs and DH production protocols available in the public domain for DH production. However, a long-term research strategy would be required for generation of indigenous resources. Presently available haploid inducers have restrictions associated with their use, which necessitates concerted efforts at the national level to develop indigenous haploid inducer lines. This may be achieved through different strategies. In sorghum, an extensive search for genotypes with haploid induction trait among 4000 germplasm entries resulted in the identification of haploid inducers with 1–2% HIR (Hussain and Franks 2019). The same strategy can be used for maize. The indigenous maize germplasm collections, particularly from diversity-rich regions such as the North-East, can be initially screened using molecular markers specific for the haploid induction trait to create a smaller set carrying variations for the gene. This set may then be phenotyped for haploid induction ability using liguleless/glossy tester genotypes (Prigge et al. 2012b). Germplasm with even very low HIR can be a significant starting resource because even the founder source (Stock 6) of the present-day high HIR inducer lines had an induction rate of only 2.3% (Coe 1959). In the absence of natural variation for the trait, the creation of novel variation for the trait using advanced methods for mutation induction and detection can be employed. Haploid formation in maize has also been achieved by manipulating the *CENH3* gene (Kelliher et al. 2016) and inducing parthenogenesis using the *PsASGR-BBML* gene (Conner et al. 2017), and mutation in the *ZmDMP* gene (a non-Stock 6-originating gene in

qhir8) has been shown to increase HIR 5–6-fold in the presence of *MTL/ZmPLA/NLD* (Zhong et al. 2019). Indigenous haploid inducer lines without usage related restrictions would go a long way in promoting the adoption and refinement of DH technology in Indian public maize breeding programmes. CIMMYT and the University of Agricultural Sciences-Bangalore (UAS-Bangalore) have signed a collaboration agreement for establishing a maize DH facility at the Agricultural Research Station in Kunigal (Tumkur, Karnataka, India) to accelerate maize breeding and hybrid development and significantly increase genetic gains through maize breeding in Asia (CIMMYT, 2019). This facility is expected to cater to the need of national maize breeding programmes for the next few years, but as adoption increases, low-cost regional DH facilities may require to be established for catering to the growing needs of regional maize breeding programmes more effectively. Human resource development in DH technology is a crucial aspect of efforts to promote use of DH technology as trained human resource is the key to the success of maize DH programmes. NARS maize scientists may be trained at national and overseas facilities to help them pursue DH programme at their respective centres.

DH breeding at ICAR-VPKAS, Almora was initiated in 2015 and up-scaled following receipt of a competitive grant from ICAR-National Agriculture Science Fund (NASF) in 2018. The number of DH lines evaluated during 2019 and 2020 was >500 and >900, respectively, and >1200 new DH lines are in the pipeline for evaluation during 2021. This number of DH lines, though is minuscule compared to that generated annually by major national and international commercial maize breeding programmes, demonstrates the feasibility of incorporating DH technology in public sector maize programmes in the country. The major factor behind successful production of DH lines at Almora centre is its mild climate which (i) allows TAILP1 to grow well and produce a decent amount of pollen for making induction crosses, and (ii) minimizes mortality of haploid plants in the field. While pollen production may not be an issue if CIM2GTAILs are used, centres located in the warmer regions may require additional facilities to minimize post-transplanting losses. *Rabi* season may be more suitable in these regions for raising haploid populations (D_0 nursery).

Fig. 1. Hi-tech polyhouse at ICAR-VPKAS Experimental Farm, Hawalbagh, for off-season generation advancement

In summary, trained human resource equipped with requisite infrastructural facilities and financial support from funding agencies, andcollaboratively functioning in a network mode can help realize the integration of DH technology in public maize breeding programmes to the benefit of the national maize programme.

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