Al-Farabi Kazakh National University (KazNU)

Faculty of Biology and Biotechnology

Department of Molecular Biology and Genetics

Discipline: «Modern Problems of Plant Genetics»

Lecture 3

In Vitro Culture and Plant Breeding. Pollen and Microspore Culture

Totipotency, micropropagation, androgenesis, gynogenesis, chromosome elimination, and Doubled Haploid (DH) technology in crop improvement

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Aim and Structure of the Lecture

Aim: master in vitro plant tissue culture principles and haploid/doubled-haploid (DH) pathways for rapid homozygous line development

KEY FOCUS

Lecture Plan & Modules

Key sections:
1. Totipotency & Tissue Culture
2. Haploids & Doubled Haploids
3. Androgenesis Mechanisms
4. Anther vs Microspore Culture
5. Wide Crosses & In Vivo Inducers
6. Doubling & Breeding Impact

- **Totipotency & Tissue Culture:** Plasticity of plant cells, Haberlandt's concept, auxin/cytokinin balance, organogenesis vs embryogenesis
- **Haploids & Doubled Haploids:** Concept of sporophytic n plants and 100% homozygous DH (2n) lines in a single generation
- **Androgenesis Mechanisms:** Stress-induced switch from gametophytic pollen program to sporophytic microspore embryogenesis
- **Anther vs Microspore Culture:** Protocols, pretreatments, media factors, and troubleshooting albino regeneration in cereals
- **Wide Crosses & In Vivo Inducers:** Hordeum bulbosum, wheat × maize chromosome elimination, and CENH3 / MTL / DMP systems
- **Doubling & Breeding Impact:** Colchicine / oryzalin mitosis arrest, flow cytometry ploidy verification, and DH variety release

Cellular Foundations of In Vitro Plant Biotechnology

Cellular totipotency and hormonal regulation allow whole fertile plants to regenerate from single somatic or gametic cells

Part I: Core Mechanisms

Concept of G. Haberlandt (1902)

- Every nucleated plant cell retains the full genetic blueprint to dedifferentiate and regenerate a complete organism
- Validated by Steward (1958) through carrot somatic embryogenesis from single phloem cells

Auxin : Cytokinin Morphogenic Ratio

- High Auxin / Cytokinin ratio induces root differentiation (rhizogenesis); High Cytokinin / Auxin induces shoot formation
- Balanced intermediate hormone concentrations maintain undifferentiated proliferating callus tissue

Part II: Applications & Balance

Unipolar Shoot or Root Regeneration

- Sequential induction of meristematic shoot buds followed by transfer to rooting medium with IAA/IBA
- Widely used in clonal micropropagation and Agrobacterium-mediated transformation

Bipolar Embryo-Like Structures

- Single cells form globular → heart → torpedo → cotyledonary embryos with both shoot and root poles simultaneously
- Shares key developmental networks with microspore embryogenesis (BBM, WUS, LEC1/LEC2)

Why Haploid & Doubled Haploid Technology Transforms Plant Breeding

Doubled haploids fix completely homozygous pure lines in 1 generation instead of 7–8 generations of self-pollination

01

Conventional Pedigree Selfing (F1 → F8): Requires 6 to 8 consecutive generations of self-pollination or backcrossing to reach >99% homozygosity (only ~98.4% at F7); Dominant heterozygous alleles mask desirable recessive traits during early-generation field selection; Takes 8–12 years for spring/winter cereals and even longer for biennial/perennial crops like sugar beet or trees; High field labor costs and progressive environmental variance confound selection

02

Doubled Haploid (DH) Pipeline (F1 → DH1): Gametes (n) from an F1 hybrid represent unique recombinant meiotic products; chromosome doubling creates an instant 2n 100% homozygous DH line; Every recessive allele (quality traits, semidwarfism, disease susceptibility/resistance) is immediately expressed with zero dominance masking; Genetic variance among DH lines equals 2× additive variance (twice that of F2 populations), boosting selection heritability; Ideal immortalized mapping populations for QTL discovery, GWAS validation, and commercial hybrid parental lines

Fixation of Genetic Homozygosity: Conventional Selfing vs Doubled Haploids

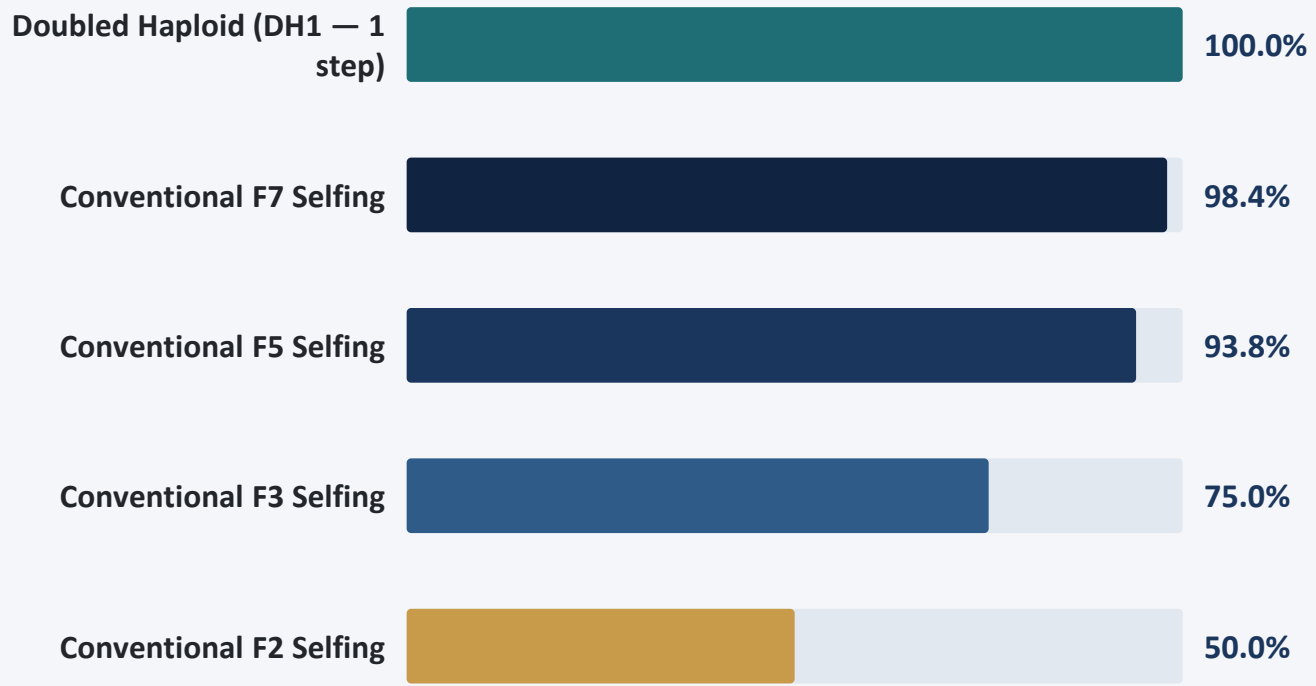
Percentage of homozygous loci (%) achieved across generations starting from a heterozygous F1 cross

100%
Homozygosity in DH (1 gen.)

7–8 gen.
Saved via DH pipeline

2x
Additive genetic variance

Percentage of Homozygous Loci Achieved by Method & Generation (%)



Breeding Economics

- Each DH line derived from an F1 microspore has 100% genetic uniformity across every locus
- In major maize, wheat, barley, and canola programs, DH pipelines shorten variety release by 4–6 years
- Recessive alleles appear at frequency p (instead of p^2 in F2), slashing population sizes needed to pyramid multiple genes

Four Biological Pathways for Generating Haploid (n) Plants

Haploids may originate from the male gametophyte (androgenesis) or the female gametophyte (gynogenesis / parthenogenesis)

KEY FOCUS

Core Analytical Focus

Key sections:
1. Anther & Isolated Microspore Culture
2. Unpollinated Ovary / Ovule Culture
3. Uniparental Chromosome Elimination
4. CENH3, MTL/ZmPLA1 & DMP Stocks

- **Anther & Isolated Microspore Culture:** Stress pretreatment redirects uninucleate microspores from pollen maturation into embryogenesis; Highest throughput in barley, wheat, rapeseed (Brassica), tobacco, pepper, and rice
- **Unpollinated Ovary / Ovule Culture:** Activated egg cells, synergids, or antipodal cells divide into haploid embryos without fertilization; Essential alternative when species suffer extreme albinism in anther culture (e.g., onion, sugar beet, cucumber)
- **Uniparental Chromosome Elimination:** Cross barley × Hordeum bulbosum or wheat × Zea mays; paternal chromosomes are preferentially extruded during early mitoses; Immature haploid embryo rescued on MS/B5 nutrient medium ~14 days post-pollination
- **CENH3, MTL/ZmPLA1 & DMP Stocks:** Specialized pollen inducer lines trigger ~5–15% maternal haploid kernels directly on the ear/spike; CRISPR-engineered CENH3 tailswap and ZmDMP mutants extend in vivo induction to dicots and wheat

Anther Culture: Direct Haploid Callus and Embryoid Induction

Pioneered by Guha & Maheshwari (1964, Datura); widely applied in wheat, rice, barley, and triticale



Fig. 1. Cultured wheat anthers producing embryogenic androgenic callus structures in vitro

- **Explants & Developmental Window:** Harvest tillers when microspores are at the mid-to-late uninucleate stage (before first pollen mitosis), verified by acetocarmine staining
- Intact anthers provide somatic anther-wall nursing factors (hormones, amino acids, L-glutamine) that support dividing microspores
- **Technical Limitations & Mixed Ploidy Risk:** Risk of regenerating unwanted diploid heterozygous plants from somatic connective/filament tissue — requires SSR/ploidy screening
- Lower microspore uniformity and crowding inside the anther locule compared to purified isolated microspores

Isolated Microspore Culture (IMC): High-Efficiency Pure Gametic Embryogenesis

Mechanically releasing millions of synchronous uninucleate microspores eliminates somatic anther-wall contamination

- **1. Stress Reprogramming Switch:** Cold (4 °C, 7–21 days), heat shock (32 °C in Brassica), or mannitol/carbon starvation triggers dedifferentiation
- Symmetrical mitotic division replaces normal asymmetrical pollen division; cell fate shifts via BBM/LEC expression
- **2. Liquid Culture & Nurse Co-Culture:** Density-gradient purified microspores are cultured in NLN, N6, or FHG liquid medium (with arabinogalactan proteins/ovary co-culture in cereals)
- Generates 100–1,000× more embryoids per spike than anther culture with zero somatic tissue contamination

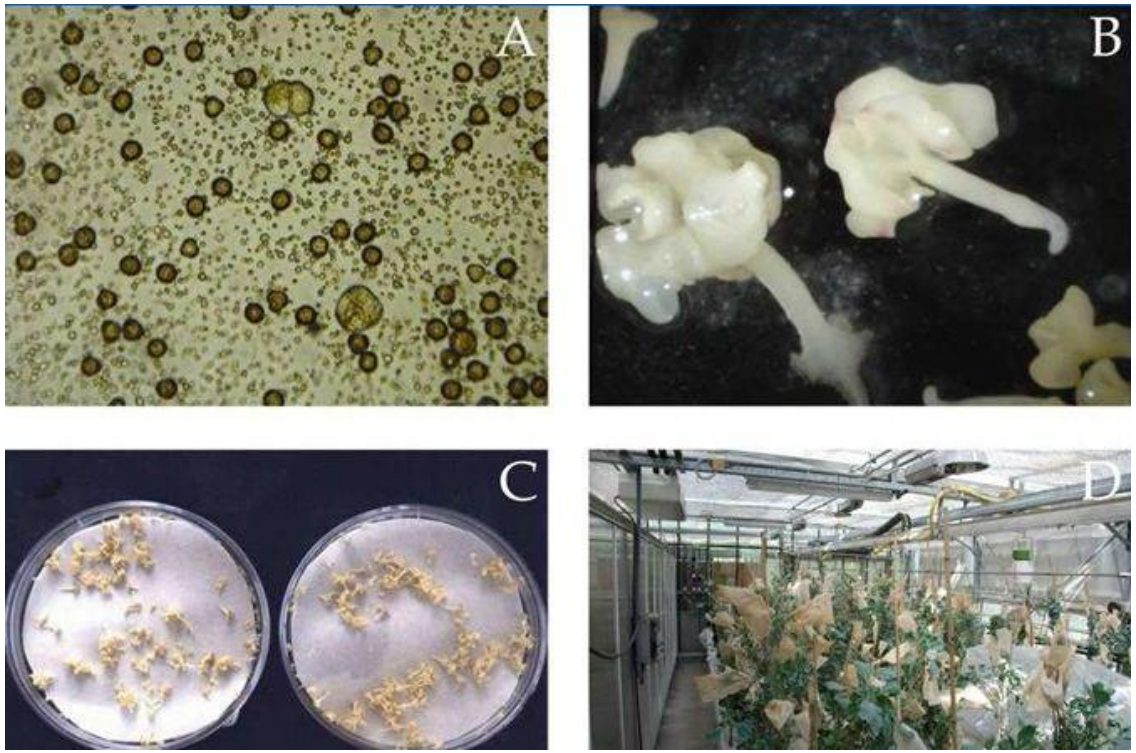


Fig. 2. Microspore division (A), regenerated DH embryos (B), green plantlet calli (C), and DH plants (D)

Step-by-Step Microspore Culture Protocol to Doubled Haploid Seed

Operational workflow from donor F1 plants grown in controlled environments to fertile homozygous DH progeny

1 1. Donor Plant & Bud Harvest

Grow F1 plants under optimal light/temperature; select spikes at late uninucleate stage

2 2. Cold/Starvation Pretreatment

Apply mannitol/cold pretreatment (4 °C) or heat shock to arrest gametophytic fate

3 3. Isolation & Purification

Blend/macerate buds, sieve (40–100 µm), and purify viable microspores on maltose/Percoll gradient

4 4. Embryogenesis & Germination

Incubate 3–4 weeks in dark → transfer multicellular embryos to light regeneration medium

5 5. Doubling & Vernalization

Verify ploidy, double n plantlets with colchicine, acclimate in greenhouse to harvest DH seed

Carbohydrate source matters: replacing sucrose with maltose in cereal anther/microspore media dramatically suppresses premature browning and improves green plant yield.

Major Physiological Bottlenecks: Cereal Albinism and Chromosome Doubling

Overcoming chlorophyll-deficient regenerants and converting sterile haploids into fertile DH plants

Albinism in Monocot Androgenesis (Barley, Wheat, Rice)

- Chlorophyll-less albino plantlets cannot photosynthesize and die after depleting medium sucrose (ranging 10–90% in recalcitrant genotypes)
- **Molecular cause:** massive deletions (~70%) and rearrangements in plastid DNA (cpDNA) during the proplastid-to-amyloplast transition before pretreatment
- **Mitigation strategies:** optimize donor growth conditions, shorten cold pretreatment, supplement $CuSO_4$, replace sucrose with maltose, or use wheat × maize crosses

Spontaneous vs Chemical Chromosome Doubling

- Haploids (n) are sterile because unpaired chromosomes segregate randomly at meiosis — chromosome doubling to 2n restores full fertility
- Spontaneous doubling via nuclear fusion occurs naturally in 40–80% of barley/rice microspore regenerants, vs only 5–20% in maize/wheat
- **Chemical doubling:** treat seedlings/tillers at 3-leaf stage with colchicine (0.05–0.1% + 2% DMSO for 4–5 h), or safer alternatives oryzalin / amiprophos-methyl
- Colchicine binds α/β-tubulin, blocking mitotic spindle microtubule assembly (C-mitosis) and doubling chromosome number in dividing meristem cells

In Vivo Gynogenesis & Haploid Inducer Systems in Crop Breeding

Pollination with specialized inducer lines triggers haploid embryo formation directly inside developing seeds

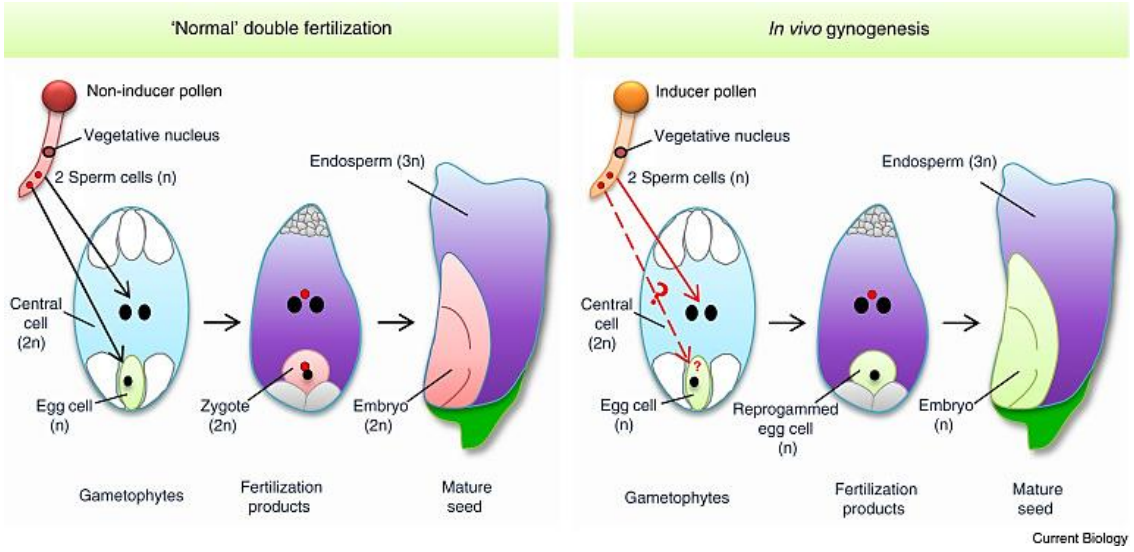


Fig. 3. Normal double fertilization (2n embryo, 3n endosperm) vs In vivo gynogenesis (n embryo, 3n endosperm)

- **Maize MATRILINEAL (MTL / ZmPLA1) & ZmDMP:** Frameshift mutation in pollen-specific phospholipase MTL (Stock6) causes fragmented paternal chromosomes after fertilization or single fertilization
- Produces normal 3n endosperm + viable haploid maternal (n) seed at 10–15% frequency; purple R1-nj kernel marker sorts haploids automatically
- **Engineering CENH3 Inducers Across Crops:** Modifying centromeric histone H3 (GFP-tailswap CENH3) delays kinetochore assembly of inducer chromosomes in the zygote
- Selective post-zygotic elimination of inducer chromosomes works in Arabidopsis, wheat, maize, and dicots

Wide Crossing Systems: Uniparental Chromosome Elimination

Bypassing genotype-recalcitrance and albinism of anther culture through interspecific chromosome extrusion

01 **BARLEY × H. BULBOSUM — The Bulbosum Method (Kasha & Kao, 1970):** Cultivated barley (Hordeum vulgare, 2n=14) pollinated by wild bulbous barley (Hordeum bulbosum, 2n=14); During early zygotic divisions, H. bulbosum chromosomes fail to attach properly to spindle centromeres (inactive CENH3) and are extruded into micronuclei

02 **WHEAT × MAIZE / IMPERATA —** Zea mays Pollination of Wheat (Laurie & Bennett): Pollinating bread wheat spikes with maize (Zea mays) or cogon grass pollen avoids crossability inhibitor genes Kr1/Kr2; Maize chromosomes are rapidly eliminated over the first 3 cell divisions, leaving a 21-chromosome polyhaploid wheat embryo (ABD)

03 **EMBRYO RESCUE (2,4-D + GA3) —** In Vitro Rescue of Developing Haploids: Because hybrid endosperm degenerates early, spikes are treated with auxin (2,4-D / dicamba) to sustain caryopsis growth; 14–16 days post-pollination, tiny haploid embryos are excised aseptically and cultured on B5/MS medium

04 **GENOTYPE INDEPENDENCE — Advantage Over Anther Culture:** Unlike anther culture, wheat × maize wide crossing shows minimal genotype bias and yields ~100% green plantlets; Widely used in Canadian, European, and Kazakhstani spring wheat breeding programs

Comparative Performance of Major Doubled Haploid Induction Methods

Choosing the optimal platform depending on crop species, donor genotype recalcitrance, and laboratory infrastructure

Method	Target Crops	Advantages	Key Limitations
Isolated Microspore Culture (IMC)	Brassica, barley, wheat, tobacco	Highest throughput (thousands of embryos/flask); zero somatic diploid contamination	Genotype-dependent response; albinism in cereals; delicate optimization
Anther Culture	Rice, wheat, triticale, pepper, potato	Simple dissection; intact anther wall supplies native growth factors	Risk of diploid regenerants from anther somatic tissues; lower yield than IMC
Wheat × Maize Embryo Rescue	Bread & durum wheat, triticale	Genotype-independent; virtually zero albino plants; overcomes Kr1/Kr2 barriers	Labor-intensive floret emasculation and microscopic embryo excision
In Vivo Maternal Inducers (MTL/CENH3)	Maize, wheat (TaMTL), Brassica, tomato	Field/greenhouse-based without tissue culture; scalable via automated seed sorting	Requires reliable haploid kernel markers (R1-nj / oil / fluorescent) and doubling step
In Vitro Gynogenesis (Ovary/Ovule)	Onion, sugar beet, cucumber, squash	Bypasses severe androgenic recalcitrance and cereal albinism	Lower embryo induction efficiency (1–5 embryos per flower)

Quality Control: Ploidy Verification and Homozygosity Validation of DH Lines

Ensuring regenerated plants are true doubled haploids rather than escaped heterozygous somatic regenerants

1 1. Stomatal Guard Cell Assay

Rapid morphological screen: haploids show smaller guard cells and fewer chloroplasts per pair

2 2. Flow Cytometry (2C DNA)

DAPI/propidium iodide nuclear staining cleanly distinguishes 1C (haploid) vs 2C (diploid/DH) peaks

3 3. Colchicine Doubling

Confirmed n plantlets undergo controlled meristem doubling; chimeric sectors form fertile spikes

4 4. Molecular Marker Audit

Codominant SSR or KASP SNP markers confirm 100% homozygosity (single allele) across loci

5 5. Agronomic Selection & Seed

DH lines advance directly to multi-location yield, quality, and stress trials or CRISPR editing

Microspore transformation (electroporation/Agrobacterium or RNP transfection of uninucleate microspores) enables direct production of homozygous edited DH plants in 1 generation.

Control Questions and Key Takeaways

Consolidating Lecture 3 concepts

? Control & Review Questions

- 1. Define cellular totipotency and explain how the auxin-to-cytokinin ratio controls organogenesis vs callus growth.
- 2. Why do Doubled Haploids (DH) offer twice the additive genetic variance and save 6–8 generations compared to conventional pedigree selfing?
- 3. Compare anther culture and isolated microspore culture (IMC), noting stress pretreatments and causes of cereal albinism.
- 4. Explain chromosome elimination in Hordeum bulbosum / wheat × maize crosses and the mechanism of in vivo MTL/CENH3 haploid inducers.
- 5. How are chromosome doubling (colchicine/oryzalin) and quality control (flow cytometry, SSR markers) carried out before field release?

✓ Key Takeaways

- Haploid induction followed by chromosome doubling fixes 100% homozygous DH lines in a single generation with zero dominance masking.
- Stress pretreatments (cold, heat, starvation) reprogram uninucleate microspores from pollen maturation into sporophytic embryogenesis.
- Albinism in cereal anther/microspore culture arises from cpDNA deletions, whereas wide crosses (wheat × maize) bypass genotype bias.
- Modern in vivo haploid inducer lines (MTL, DMP, CENH3) and microspore gene editing accelerate Breeding 4.0 pipelines.

Recommended Literature

Sources on plant tissue culture, androgenesis, gynogenesis, and doubled haploid breeding

1 Main Literature

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- 2. Touraev A., Forster B.P., Jain S.M. Advances in Haploid Production in Higher Plants. — Springer, 2009.
- **3. Dwivedi S.L., Britt A.B., Tripathi L. et al. Haploids:** constraints and opportunities in plant breeding // Biotechnology Advances. — 2015. — Vol. 33. — P. 812–829.
- 4. Jacquier N.M.A. et al. Puzzling out plant reproduction by haploid induction for innovations in plant breeding // Nature Plants. — 2020.

2 Additional Literature & Databases

- 1. Guha S., Maheshwari S.C. In vitro production of embryos from anthers of Datura // Nature. — 1964. — Vol. 204:497.
- 2. Gilles L.M. et al. Loss of pollen-specific phospholipase NOT LIKE DAD / MATRILINEAL triggers gynogenesis in maize // EMBO Journal. — 2017.
- 3. International Association for Plant Biotechnology (IAPB) Resources: https://iapb-home.org
- **4. CIMMYT Doubled Haploid Manual for Wheat & Maize Breeding:** https://www.cimmyt.org

Thank you for your attention! • Department of Molecular Biology and Genetics, KazNU