Experimental Design

Roppon Picha, TINT RD

7 Feb 2025

IAEA Plant Mutation Breeding Fellowship Program

Plant mutation breeding (PMB)

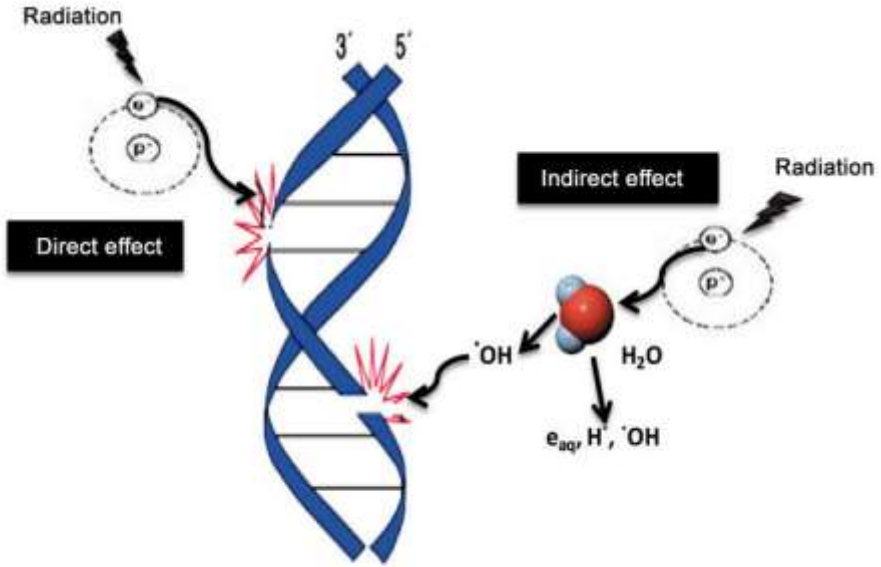
- Definition: A technique that involves inducing mutations in plants through physical or chemical mutagens to create genetic variability and develop new plant varieties with desirable traits.
- Objectives:
  - Enhancing yield
  - Improving stress resistance (biotic and abiotic)
  - Enhancing nutritional content
  - Accelerating growth
  - Modifying physical characteristics (height, branching, color, pattern, shape)

Mutagens in PMB

- Physical:
  - Radiation: gamma, X-ray, neutron, UV
  - No foreign genes are introduced – Non-Genetically Modified Organism (GMO)
- Chemical:
  - Ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), Sodium azide
  - Non-GMO
- Biological:
  - Transposons, CRISPR/Cas9 (gene editing)
  - Considered GMO

Irradiation

- Types of radiation
  - Electromagnetic wave: gamma, X-ray
  - Particle: electron, neutron, alpha, proton
- Mechanisms of mutation induction
  - Direct: radiation energy is absorbed directly by DNA molecule. This causes breaks in DNA strands (single- or double-strand)
  - Indirect: radiation ionizes water molecules, creating reactive oxygen species (ROS) such as hydroxyl radicals (•OH), superoxide ($O_2•^-$), and hydrogen peroxide (H_2O_2). These ROS cause oxidative damage to DNA, proteins, and lipids.



Rice Mutation Breeding examples

- Rice (*Oryza sativa*) varieties, developed via gamma irradiation:
  - Thailand: RD6 – glutinous rice with disease resistance, developed using gamma irradiation
  - Philippines: IR8 – short-statured and high-yield, developed using gamma irradiation
  - Myanmar: Shwe War Tun – Salinity tolerance and early maturity

Variables in mutation breeding

Independent:

- genotypes
- radiation dose
- environmental conditions

Dependent: traits

- Disease resistance
- Yield
- Etc.

Key Research Questions

What traits are usually targeted?

- Yield, disease resistance, physical characteristics

How does experimental design help?

- ensure robust evaluation of induced mutants and to handle variability effectively
- help researchers achieve reliable results and make valid conclusions.

Sources of variation in PMB

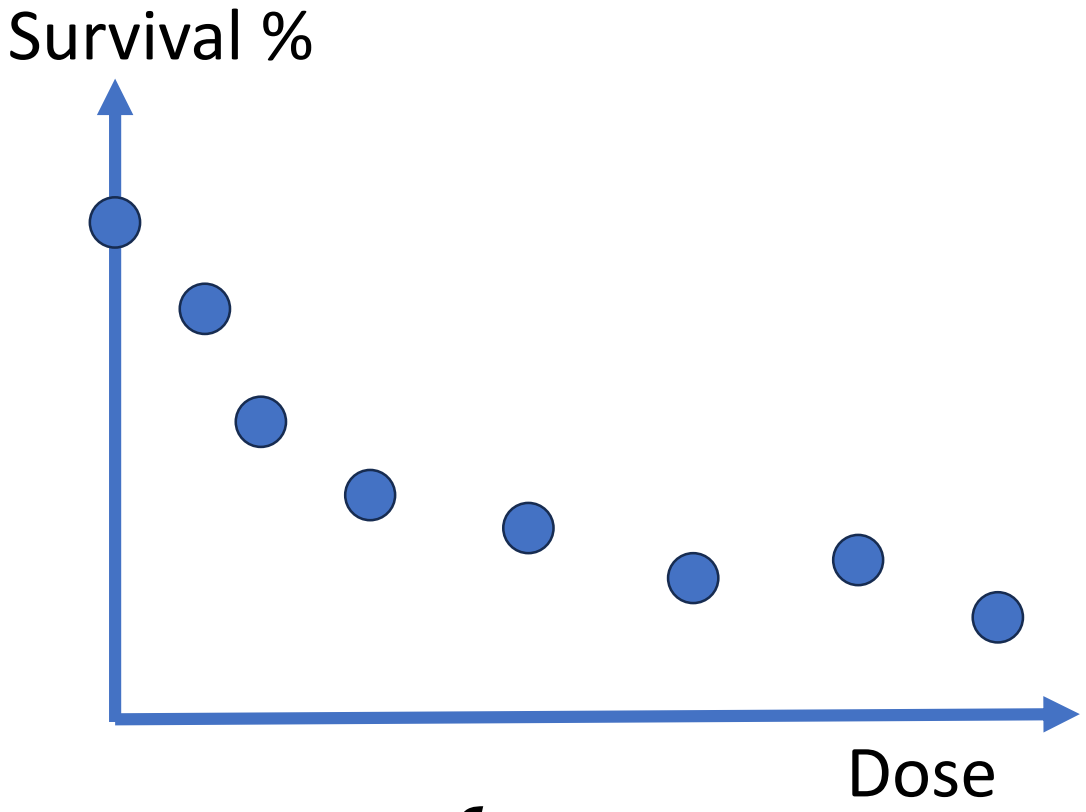
- Environmental
  - Weather: Temperature, rainfall, humidity
  - Soil: nutrient content, pH, texture
  - Water: Water supply amount and timing
  - Pests and diseases: Types and number of pests and diseases
- Genotypes (differences in genetic makeup of the plants)
  - Variation among genotypes due to inherent genetic diversity (e.g., traits like yield, drought tolerance, or disease resistance).
  - Mutation effects
  - Genotype × Environment Interaction (G×E)
- Experimental
  - Plot-to-plot variability
  - Measurement error (human error or faulty tools)
  - Sampling error (selecting unrepresentative plants, too few samples)
  - Poor experimental design

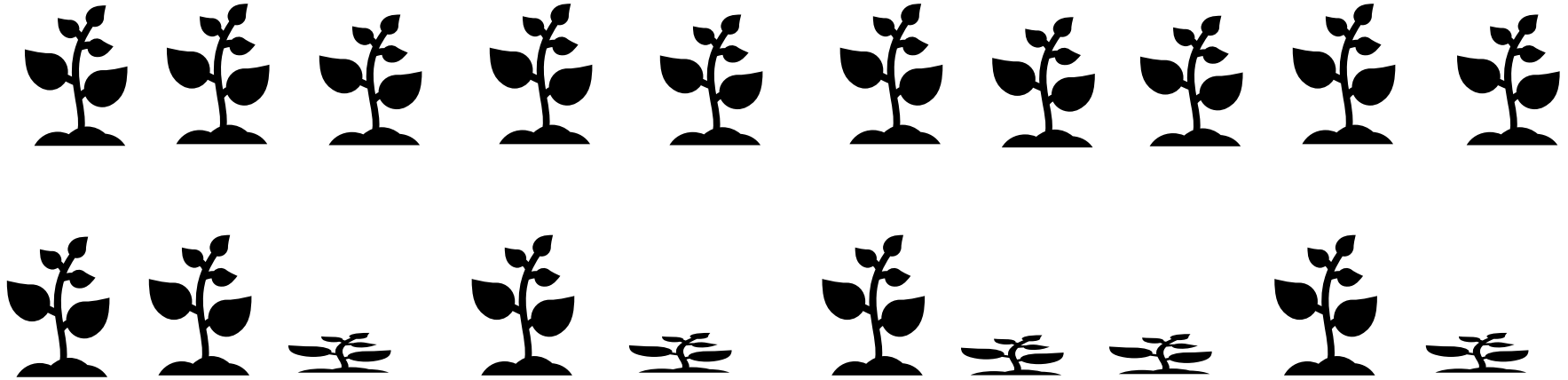
Plant selection criteria

- Biological Suitability - Reproductive mode, diploid vs polyploidy (chromosome sets), growth cycle.
- Genetic characteristics - Diversity, homozygosity, sensitivity to gamma rays.
- Economic importance - Traits of interest, market demand, and economic value.
- Radiation tolerance - Ability to survive optimal radiation doses.
- Screening feasibility - Traits easily observable or measurable, field compatibility.

Dose determination

- LD50 concept
- Dose-response tests





Gamma irradiation equipment

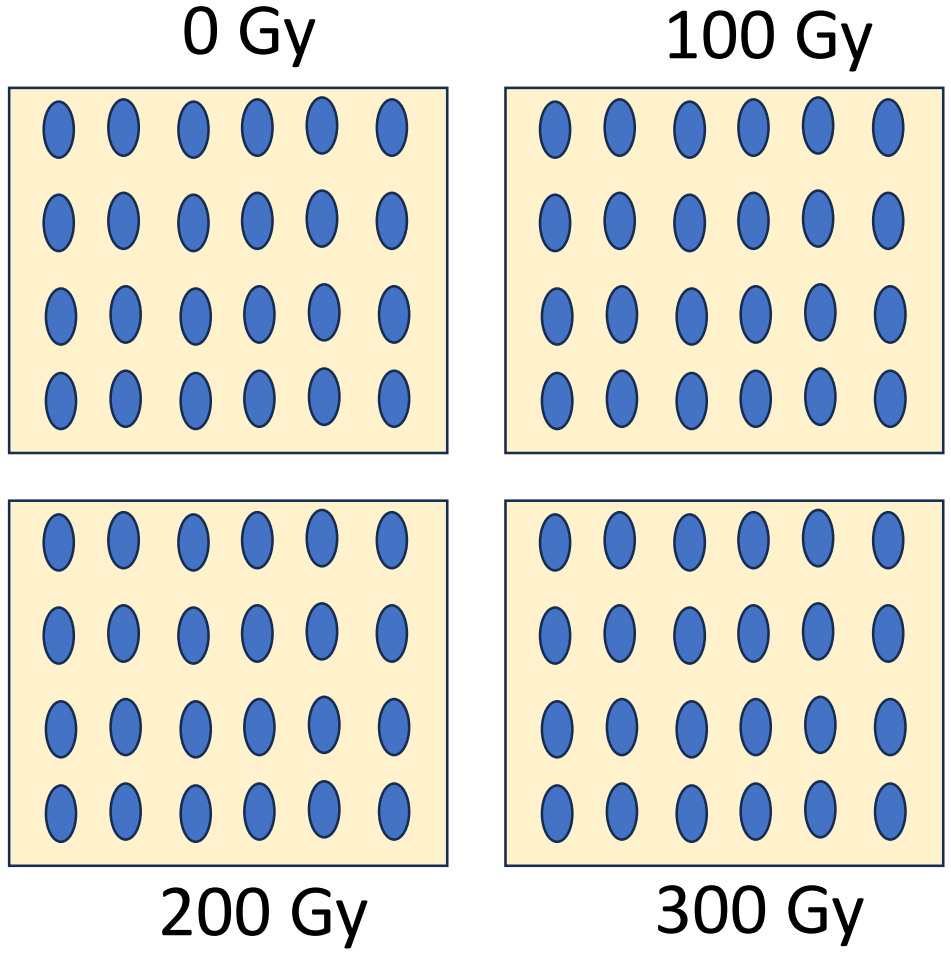
- Sources (Co-60, Cs-137)
  - Energy
  - Dose rate
- Safety consideration: ALARA

Why experimental design matters

- Ensuring reliability, reproducibility, meaningful results
- A well-designed experiment minimizes bias, reduces experimental error, and allows valid comparisons.
- Poor experimental design, on the other hand, can lead to misleading conclusions, wasted resources, and invalid research findings.
- Example: Testing X-ray (0 Gy, 100 Gy, 200 Gy, 300 Gy) effects on rice seed germination.

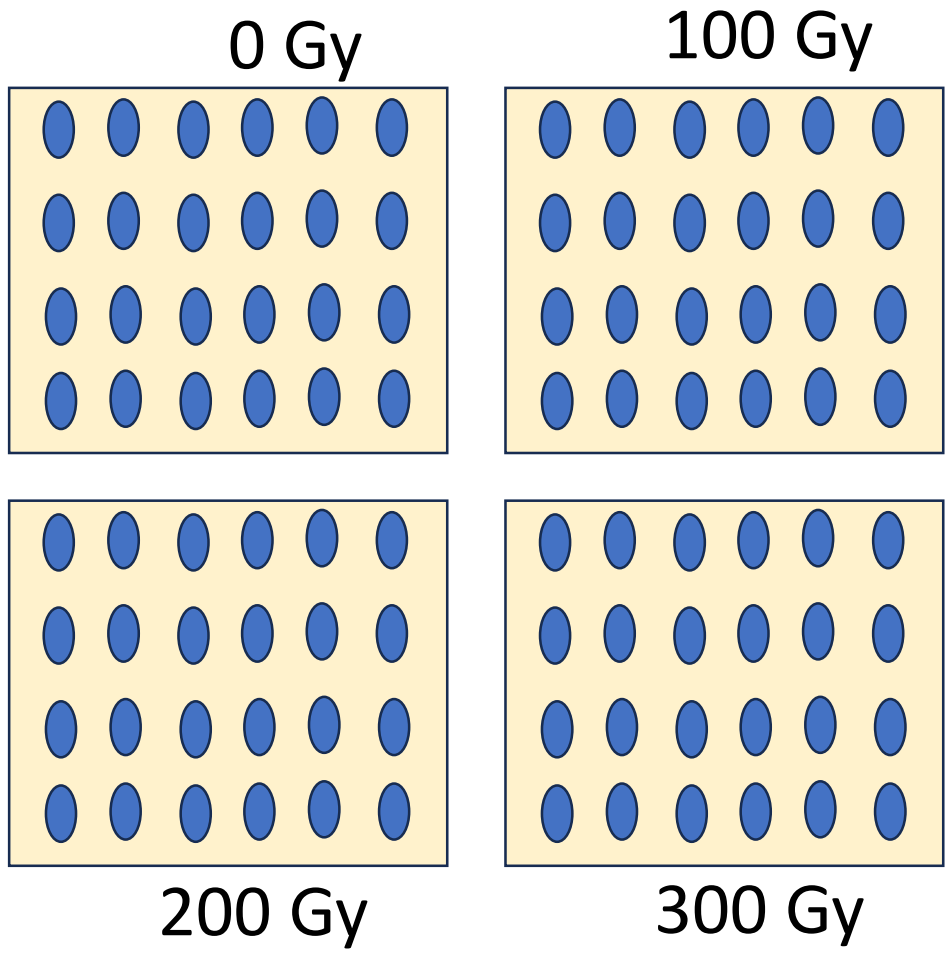
Example design 1

- Setup:
  - Place seeds treated with different doses on separate trays, but trays are exposed to varying light and temperature conditions.
  - No randomization or replication is used.
  - Germination is observed on just one tray per treatment.



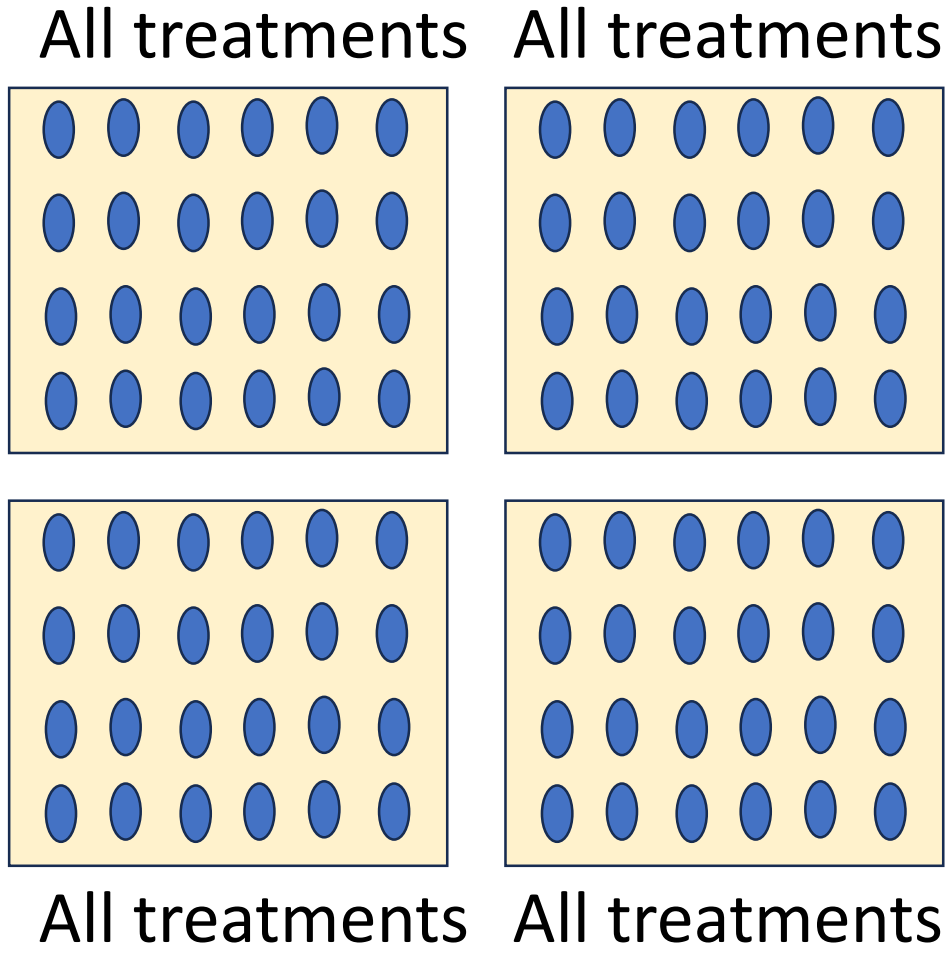
Example design 1

- Problems:
  - Confounding Factors: The observed differences in germination rates may be due to light or temperature differences, not irradiation dose.
  - Lack of Replication: Without multiple trays, there is no way to estimate variability or ensure that results are consistent.
  - Bias: Treatments are not randomized, potentially introducing systematic bias.
  - Invalid Comparisons: Differences cannot be reliably attributed to irradiation dose.



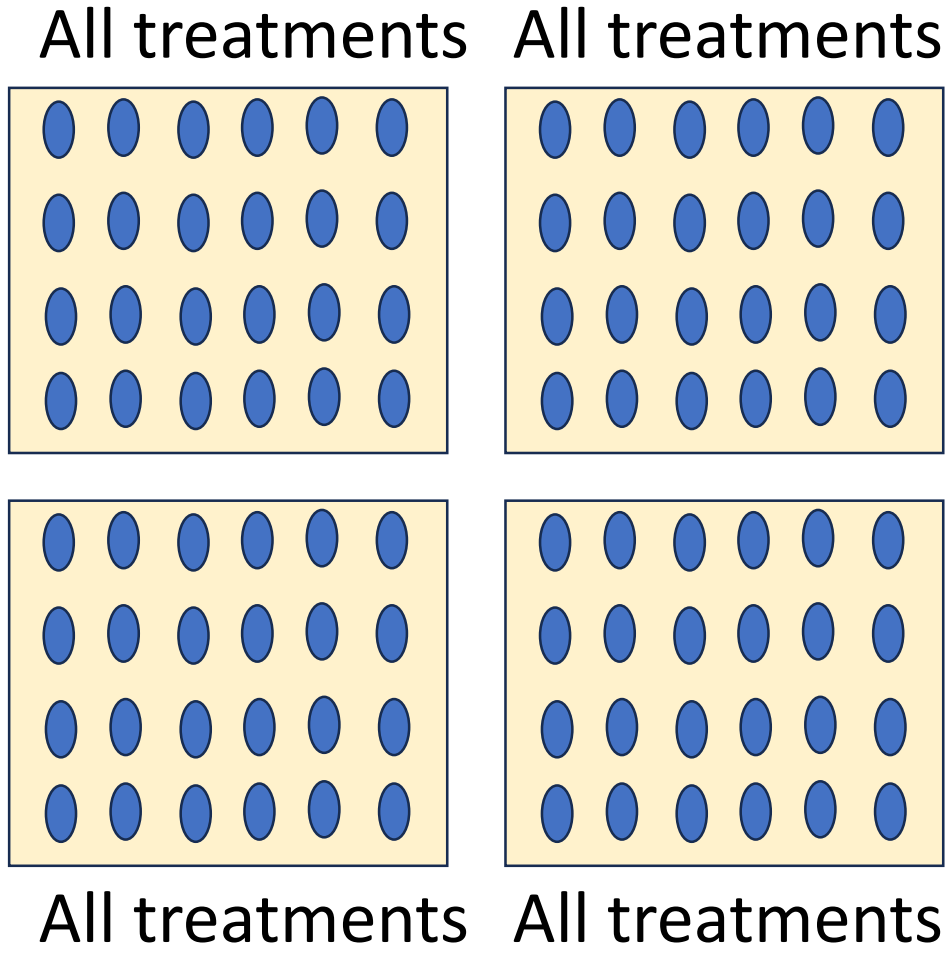
Example design 2

- Setup:
  - Randomize seeds from each dose treatment across trays to avoid confounding environmental effects (e.g., light, temperature).
  - Use replication by having multiple trays (e.g., 3 replicates per dose).
  - Treat trays as blocks to account for variability in light and temperature within the growth chamber.



Example design 2

- Features:
  - Randomization: Ensures treatments are spread across trays and reduces bias.
  - Replication: Provides multiple observations for each treatment, allowing estimation of variability.
  - Blocking: Accounts for environmental gradients across trays.
  - Statistical Rigor: Enables valid statistical analysis to determine if observed differences are due to treatment.



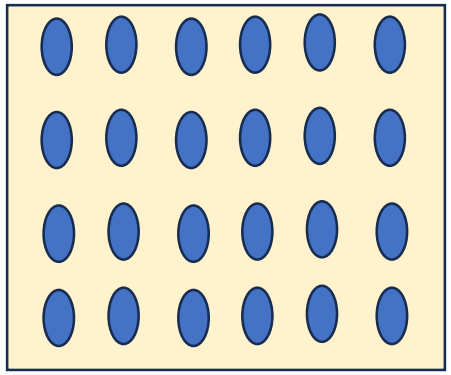
Example results

- Design 1 germination rates:
  - 0 Gy: 90%
  - 100 Gy: 70%
  - 200 Gy: 50%
  - 300 Gy: 40%

- Can we conclude anything about X-ray dose effect from this?

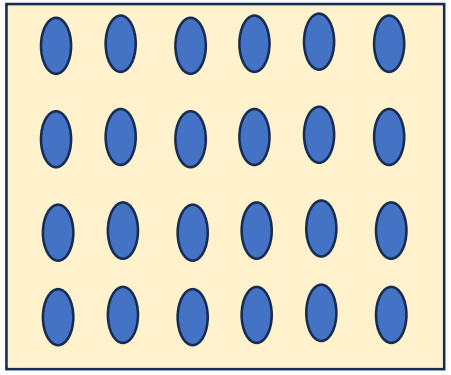
Optimal condition

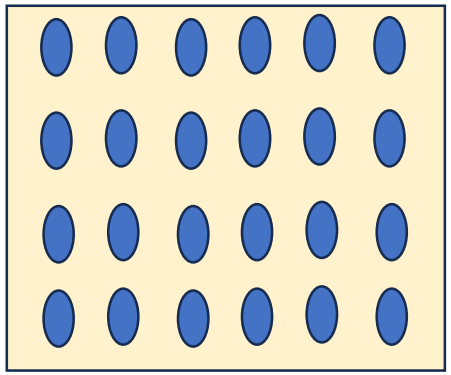
0 Gy



Good sunlight

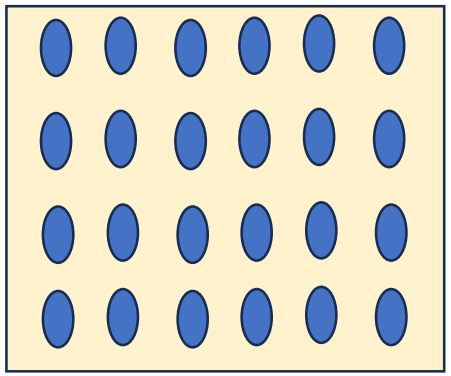
100 Gy





200 Gy

Dark, cold spot



300 Gy

Near windy window

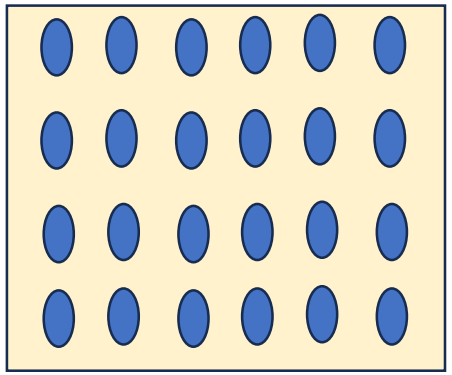
Example results

- Design 2 germination rates:
  - 0 Gy: 90%, 85%, 86%, 92%
  - 100 Gy: 70%, 72%, 69%, 73%
  - 200 Gy: 62%, 60%, 65%, 58%
  - 300 Gy: 50%, 55%, 48%, 51%

- Results are consistent across replicates and can be attributed to X-ray doses.

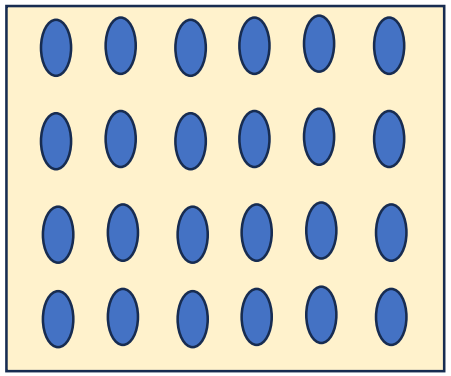
Good condition

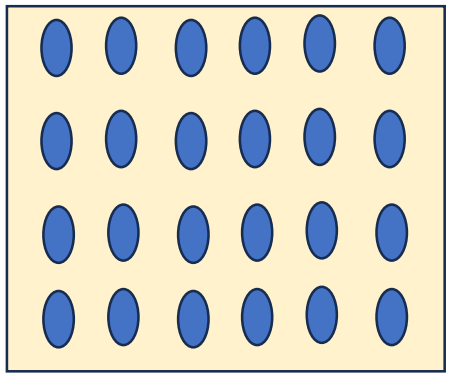
All treatments



Similar condition

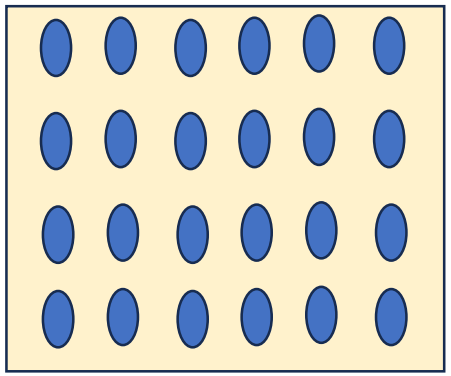
All treatments





All treatments

Similar condition



All treatments

Similar condition

Key principles of experimental design

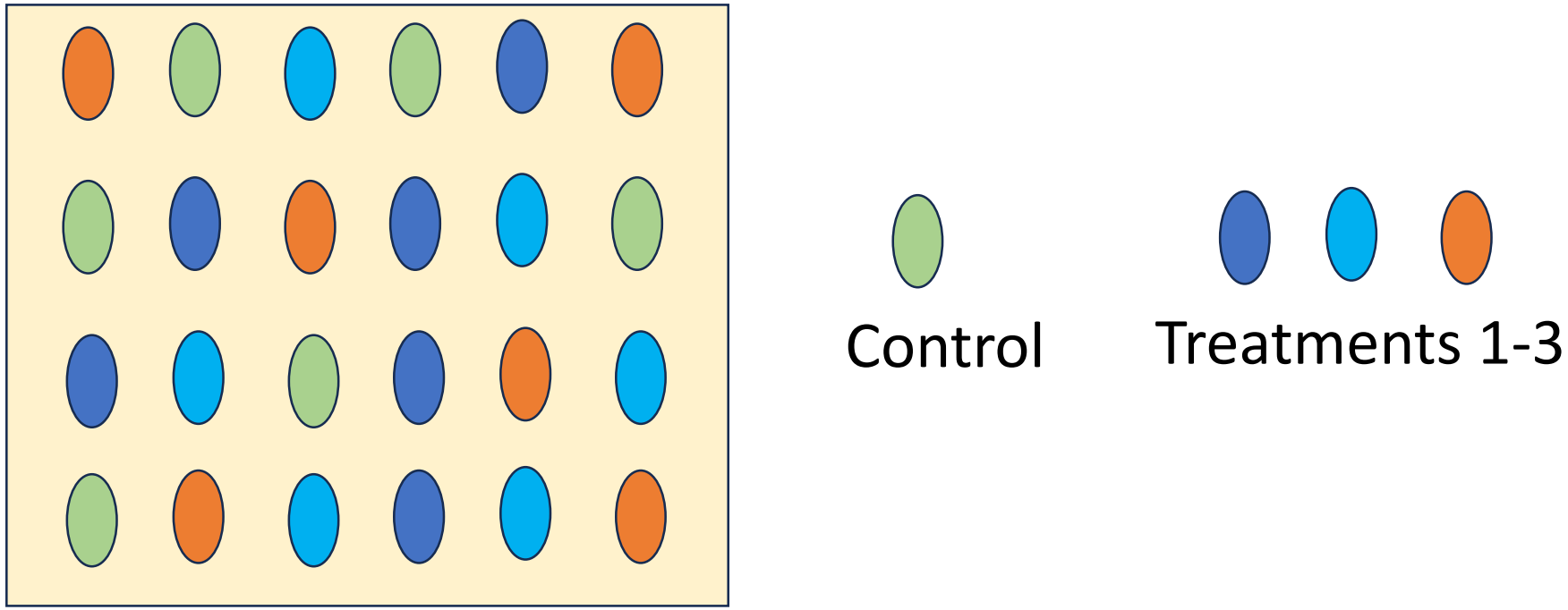
- **Replication:** Repeating treatments across multiple experimental units to estimate variability.
  - Example: Testing three doses of gamma irradiation on plant growth with 5 replicates for each dose.
- **Randomization:** Randomly assigning treatments to experimental units to eliminate bias.
  - Example: Randomly placing plants with different irradiation doses in a greenhouse to avoid effects of location-based variability (e.g., light or temperature).
- **Blocking:** Grouping experimental units into blocks that are as homogeneous as possible and randomizing treatments within each block.
  - Example: Grouping plots in a field trial into blocks based on soil type and randomizing treatments within each block.
- **Control:** Including a baseline treatment (control) for comparison with other treatments.
  - Example: Including non-irradiated plants (0 Gy) as a control when testing gamma irradiation doses.

Key principles of experimental design

- **Independence:** Ensuring that observations from one experimental unit do not influence those of another.
  - Example: Preventing cross-contamination between treated and control plants.
- **Reproducibility:** Designing experiments so they can be repeated by others under similar conditions.
  - Example: Documenting precise experimental protocols, such as radiation dose rates and plant growth conditions.
- **Simplicity:** Avoiding unnecessary complexity in experimental design.
  - Example: Focusing on two or three key factors instead of testing many variables simultaneously.

Completely Randomized Design (CRD)

- CRD randomly assigns treatments to experimental units without considering any grouping.



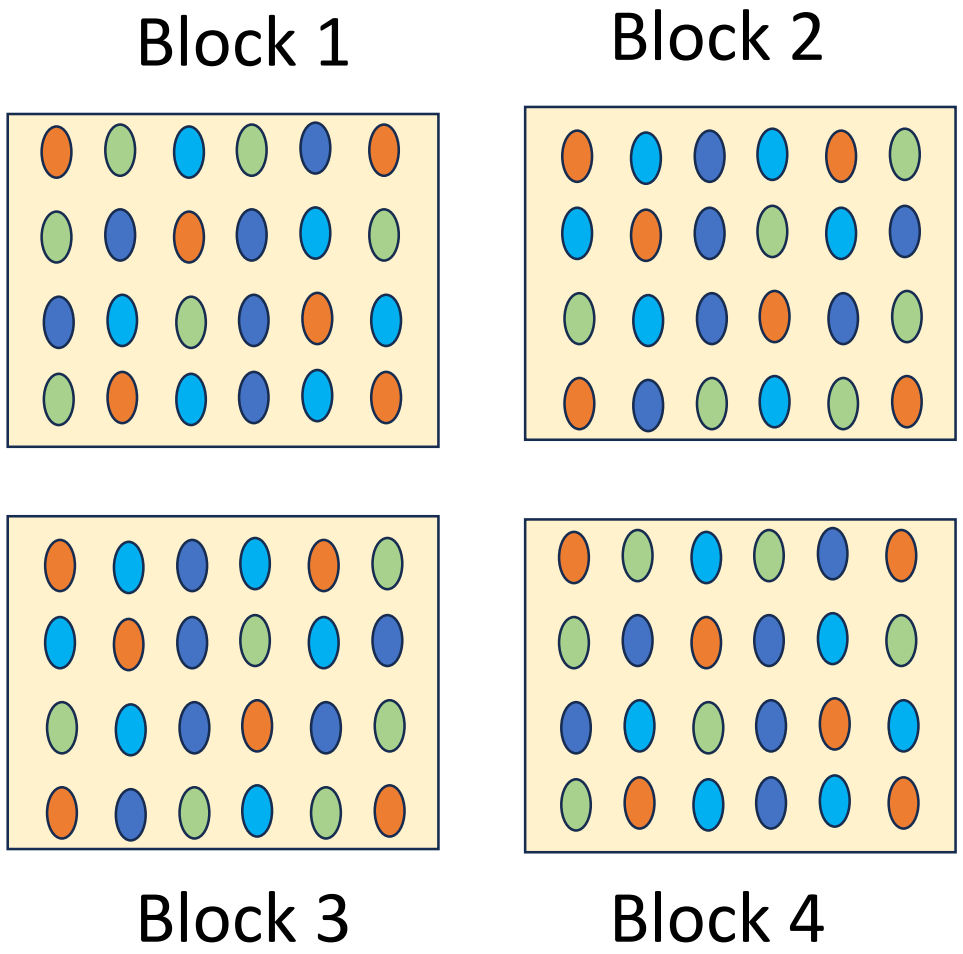
Example of CRD

- To evaluate the effects of gamma irradiation on rice (or another crop) at the **M1 (first mutant) and M2 (second mutant) generations** to identify promising mutant lines for further breeding.
- Resource: Has a growth chamber with minimal variation in environment
- Factors:
  - Factor 1: generation: M1 (first-gen mutants) and M2 (second-gen mutants)
  - Factor 2: Gamma dose (0 Gy, 100 Gy, 200 Gy)
  - Total treatments = 2 gens x 3 doses = 6 treatments
- Replications = 3
- Total experimental units = 6 x 3 = 18

M1, 0 Gy, rep 1	M1, 100 Gy, rep 1	M1, 200 Gy, rep 1
M2, 0 Gy, rep 1	M2, 100 Gy, rep 1	M2, 200 Gy, rep 1
M1, 0 Gy, rep 2	M1, 100 Gy, rep 2	M1, 200 Gy, rep 2
M2, 0 Gy, rep 2	M2, 100 Gy, rep 2	M2, 200 Gy, rep 2
M1, 0 Gy, rep 3	M1, 100 Gy, rep 3	M1, 200 Gy, rep 3
M2, 0 Gy, rep 3	M2, 100 Gy, rep 3	M2, 200 Gy, rep 3

Randomized Complete Block Design (RCBD)

- The overall layout is first divided into blocks.
- Inside each block the treatments are randomized.



Example of RCBD

- To evaluate the effects of gamma irradiation on rice (or another crop) at the **M1 (first mutant) and M2 (second mutant) generations** to identify promising mutant lines for further breeding.
- Resource: Field trials with environmental difference (e.g., soil condition, moisture, slope)
- Factors:
  - Factor 1: generation: M1 (first-gen mutants) and M2 (second-gen mutants)
  - Factor 2: Gamma dose (0 Gy, 100 Gy, 200 Gy)
  - Total treatments = 2 gens x 3 doses = 6 treatments
- Replications = 3
- Total experimental units = 6 x 3 = 18

Block 1	M1, 0 Gy, rep 1	M1, 100 Gy, rep 1	M1, 200 Gy, rep 1
	M2, 0 Gy, rep 1	M2, 100 Gy, rep 1	M2, 200 Gy, rep 1
Block 2	M1, 0 Gy, rep 2	M1, 100 Gy, rep 2	M1, 200 Gy, rep 2
	M2, 0 Gy, rep 2	M2, 100 Gy, rep 2	M2, 200 Gy, rep 2
Block 3	M1, 0 Gy, rep 3	M1, 100 Gy, rep 3	M1, 200 Gy, rep 3
	M2, 0 Gy, rep 3	M2, 100 Gy, rep 3	M2, 200 Gy, rep 3

Comparing CRD and RCBD

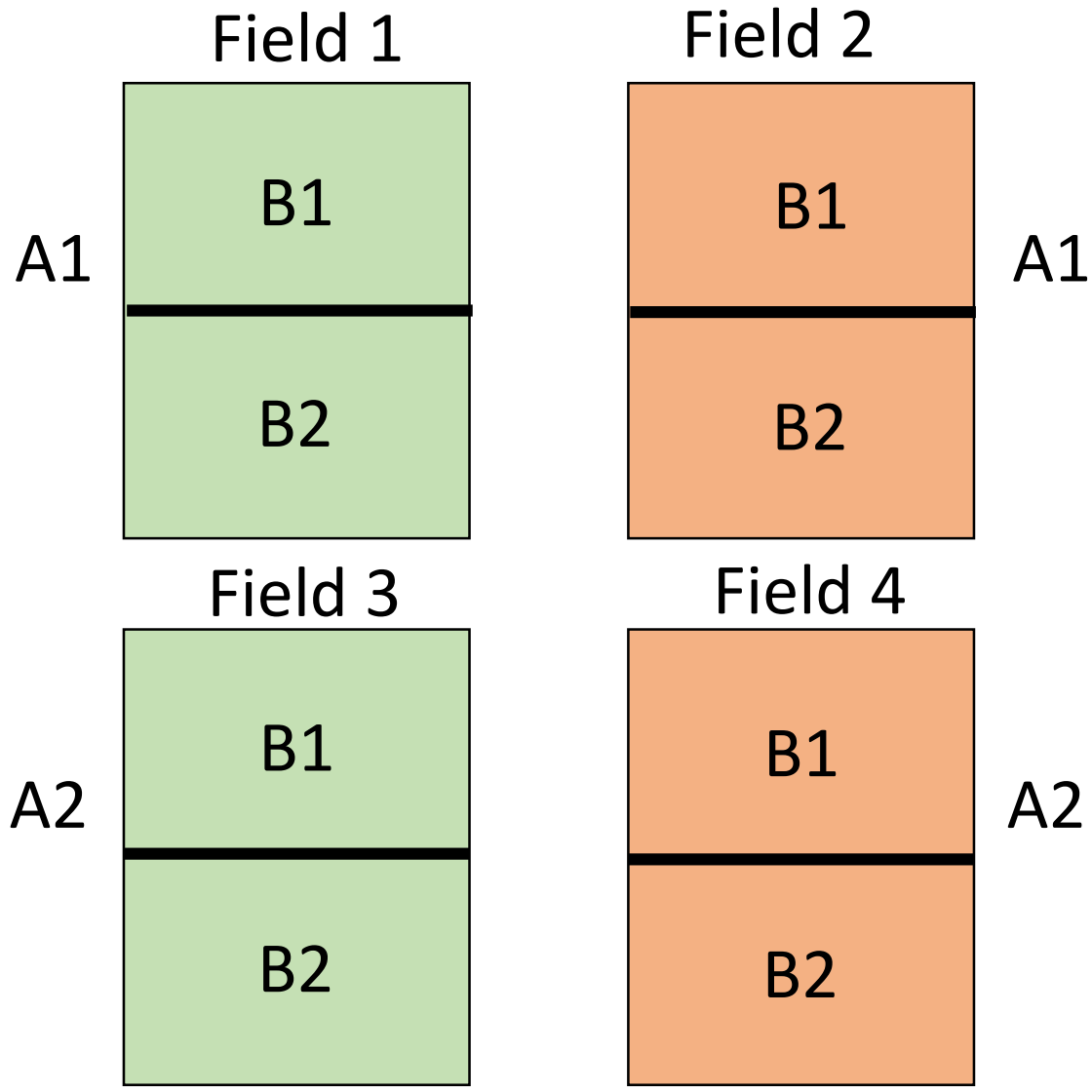
Aspect	CRD	RCBD
Definition	All treatments are randomly assigned to experimental units without grouping.	Experimental units are grouped into blocks, and treatments are randomized within each block.
Suitability	Best for homogenous experimental conditions (e.g., laboratory or greenhouse).	Ideal for field conditions with variability (e.g., soil fertility, water availability).
Control of Variation	Minimal control of variability; assumes conditions are uniform.	Controls variation by grouping similar experimental units into blocks.
Efficiency	Less efficient in controlling environmental effects.	More efficient in reducing experimental error.
Analysis Complexity	Easier statistical analysis.	Requires additional steps to account for blocking in analysis.

Other designs: Split-Plot design

- Split-plot design is an experimental layout used when there are two or more factors to investigate, and one of the factors requires a larger experimental unit due to practical or logistical constraints.
- More complex than RCBD (typically 1 main factor)
- This design is particularly useful for agricultural and plant breeding experiments where treatments like irrigation levels or radiation doses are difficult to randomize on a small scale.
- For multi-factor experiments (e.g., dose × genotype interactions)
- Two Levels of Experimental Units:
  - Main Plot: Larger units for treatments that are difficult to randomize, such as irrigation levels or gamma radiation doses.
  - Sub-Plot: Smaller units within each main plot for treatments that can be randomized, such as plant genotypes.

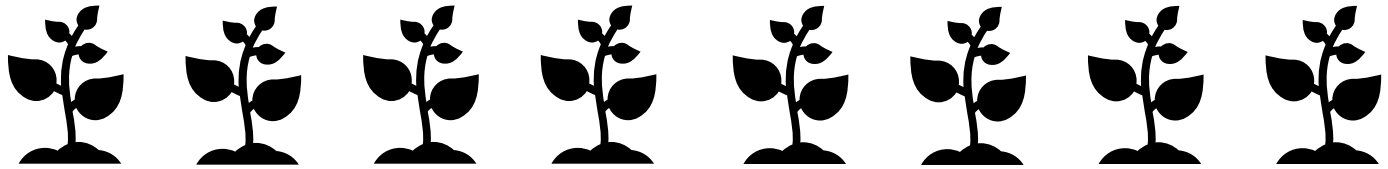
Split-Plot

- Two variables: irrigation level (A) and dose (B)
- Select irrigation method (‘hard to change’) for each field (plot)
- Then split each plot and randomly assign dose (‘easy to change’) to each subplot.



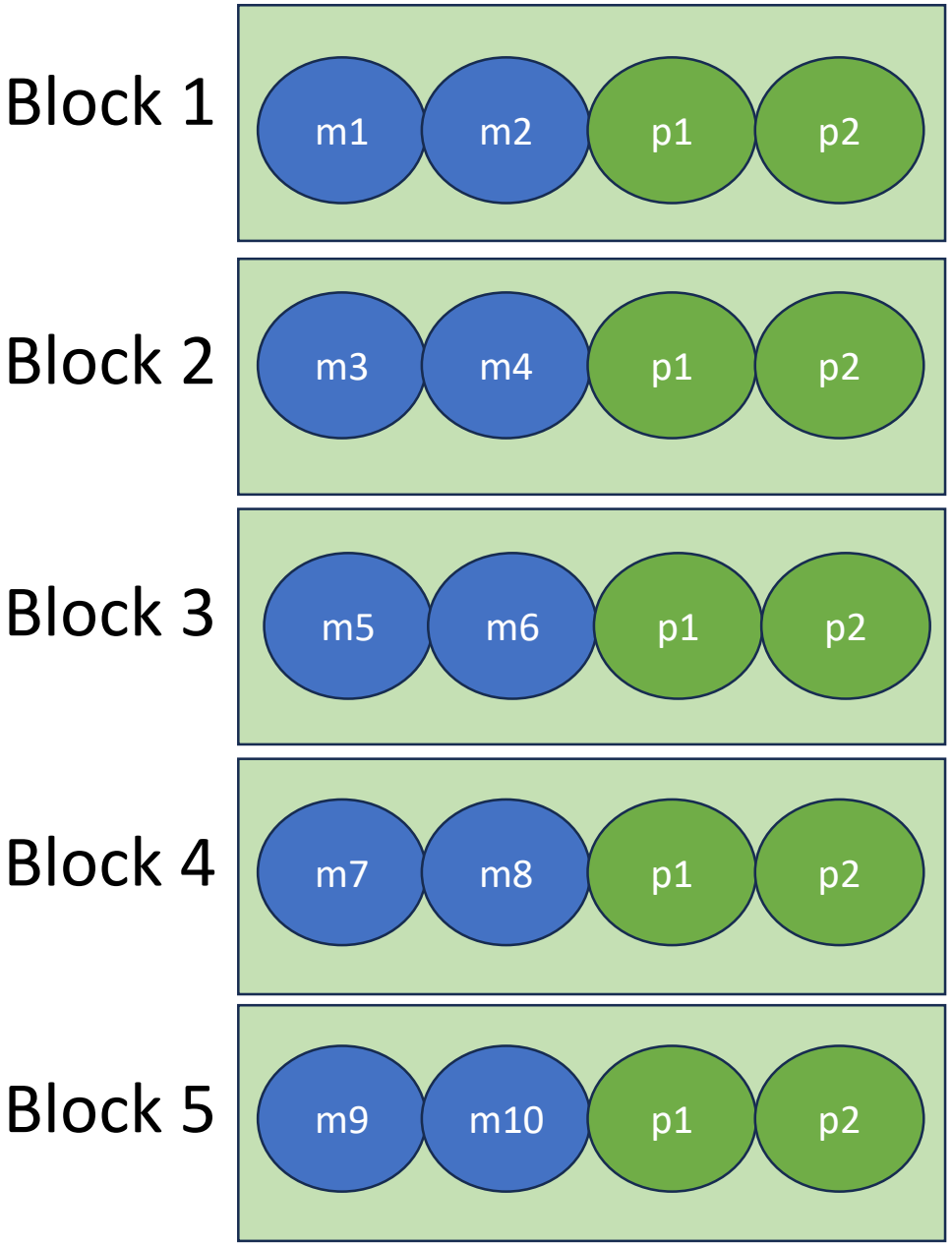
Other designs: Augmented designs

- Augmented designs are experimental layouts used when there is a need to evaluate many treatments or genotypes but with limited replication due to constraints such as time, resources, or experimental space.
- This is particularly useful in plant breeding, including mutation breeding, where many mutant lines or genotypes need preliminary evaluation before detailed testing.



Augmented design

- Test Treatments (Unreplicated):
  - 10 mutant (m) lines developed through gamma irradiation.
- Check Treatments (Replicated):
  - 2 parent (p) lines (e.g., non-irradiated controls).
- Blocks:
  - Divide the field into blocks based on environmental homogeneity (e.g., soil type or elevation).
- Randomization:
  - Randomly assign the mutant lines and parent lines within each block.
  - Ensure the parent lines are present in every block (replicated).

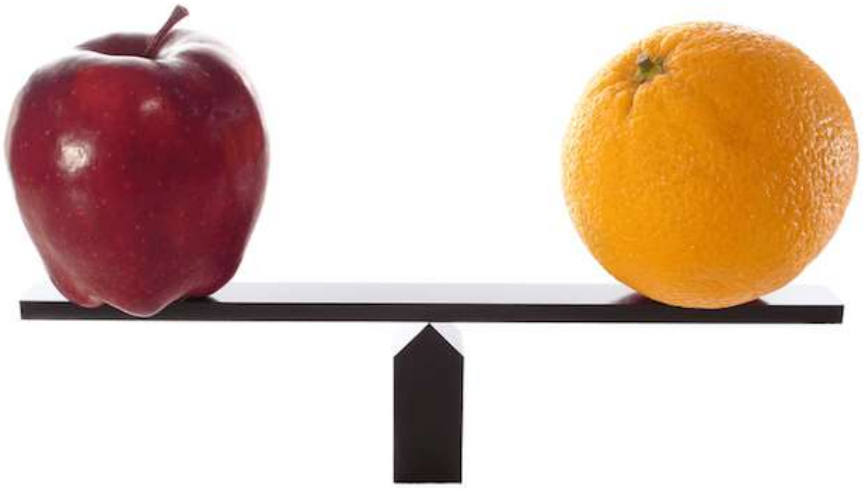


Comparing designs

Aspect	Split-Plot	Augmented
Purpose	Designed to study two or more factors, especially when one factor requires larger plots (e.g., gamma dose × genotype).	Designed to study two or more factors, especially when one factor requires larger plots (e.g., gamma dose × genotype).
Treatment Replication	Both main plot treatments and sub-plot treatments are replicated.	Control treatments are replicated, but test treatments are unreplicated.
Treatment Replication	Both main plot treatments and sub-plot treatments are replicated.	Control treatments are replicated, but test treatments are unreplicated.

Data analysis

- After results are obtained, we can compare to see if there is any significant difference among groups.
- ANOVA (Analysis of Variance) to determine if there are significant difference among group means (as a whole). It is used when there are more than 2 groups.
  - Answers: “Is there a difference?”
- If ANOVA shows significant differences, a post-hoc test can be used to compare means between treatments (pairwise).
  - Answers: “Which groups are different?”



Challenges in PMB

- Mutagen Selection and Application
  - Dose Optimization: balancing mutation frequency vs plant survival
  - Mutagen-Specific Effects: Different mutagens affect the genome in different ways
  - Uniform Application: Ensuring uniform exposure of plant material to the mutagen
- Plant Material Constraints
  - Complex genetic structures (e.g., polyploidy) may mask mutations.
- Screening and Evaluation of Mutants
  - Identifying beneficial mutations is labor-intensive and time-consuming.
  - Many mutations are non-heritable.
- Experimental Design
  - Requires robust replication and controls to reduce bias.
  - Large populations are needed, demanding extensive resources.

Exercise: Designing an experiment

- Time: 10 min
- Group size: 2 people
- Goal: Create a field experiment layout for a given scenario.
- Objective: Evaluate the yield performance of 8 rice genotypes under 3 irradiation conditions (control, 100 Gy, 200 Gy).
- Tasks:
  - Decide an appropriate experimental design based on your resource
    - Case 1: a green house with uniform environment
    - Case 2: a large field, with 3 environmental conditions
  - Visualize the field layout and label the plots with genotypes and treatments