Chapter 9
Field Evaluation of Mutagenized Rice Material

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Abstract Since the discoveries of the beneficial effects of some mutations on plants, scientists have made use of both physical and chemical mutagens to develop new varieties of crops and ornamentals. More than 3200 varieties have been produced through mutation induction, and these have contributed towards improving food security in many countries.

Proper field techniques can greatly enhance the chances of identifying desirable phenotypes. The protocol described here deals mainly with field techniques successfully employed and developed in the evaluation of rice mutants for over 8 years in the uplands and the hydromorphic lowlands at the Rokupr Agricultural Research Centre (RARC) of the Sierra Leone Agricultural Research Institute (SLARI) between 2005 and 2012 to evaluate gamma and X-ray-treated rice seeds.

Keywords Mutation breeding • Field observation • Bulk selection • Single panicle selection • Observational yield trials • Replicated yield trials • Generation of mutant population

9.1 Introduction

Since the discoveries by Muller and Stadler, plant scientists have made use of the effects of induced mutations to develop new varieties of crops and ornamentals using both physical and chemical mutagens (Muller 1927; Stadler 1928). More than

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3200 new varieties have been produced through mutation induction, and these have contributed towards improving food security in many countries (MVD 2016; Jankowicz-Cieslak and Till 2015). Breeding was, and is still, a game of numbers where phenotypes play a central role, whether routine hybridization is involved or reverse genetics such as Targeting Induced Local Lesions IN Genomes (TILLING) is contemplated (Kurowska et al. 2011). The end result of a high-throughput approach or simple hybridization is to produce desirable phenotypes that meet the demands of end users.

When employed properly, field techniques can greatly enhance the chances of identifying desirable phenotypes and therefore should be well planned before starting. The use of radiation techniques to produce mutants is increasingly becoming important in the development of high yielding, disease tolerant crop varieties as well as ornamentals (Ahloowalia et al. 2004).

Whilst the literature on mutation breeding dealing directly with a methodology for field evaluation of mutants is scarce (El-Degwy 2013), field evaluation of mutants is routinely conducted in many research institutes around the world to identify mutants with desirable traits for incorporation into existing varieties to improve yields and confer resistance or tolerance to new threats of diseases or pests in crop varieties as well as flowers and ornamentals. Evaluation of mutant phenotypes is also increasingly employed in reverse-genetic approaches such as TILLING (Kurowska et al. 2011).

The protocol presented in this chapter deals mainly with field techniques successfully used for the evaluation of rice mutants over a 5-year period in the uplands and the hydromorphic lowlands at RARC, one of seven SLARI centres in Sierra Leone. Field evaluations are especially important when screening mutant populations. This is because the frequency of induced mutations, whilst orders of magnitude higher than natural mutations, is still relatively low. The most densely mutagenized populations have been produced with chemical mutagens. For example, a mutation density of ~ 1/280 kb in rice has been reported (Till et al. 2007; Tsai et al. 2011). Physical mutagens such as gamma irradiation have been shown to produce large genomic indels with only a few such lesions reported per line (Yuan et al. 2014; Henry et al. 2015). This means that the chance of mutating a gene that would alter the desired trait is quite low, and thousands of plants need to be routinely screened for a chance of success. Field evaluations should accommodate large numbers and ensure that rare phenotypic variations can be easily spotted. Once phenotypes are detected, efforts can be made to develop molecular markers, thus reducing the workload for producing new varieties.

Prior to field evaluation, care should be taken for the development of a suitable mutant population (Fig. 9.1). Homogeneous seeds are selected and tested for viability and treated with the appropriate mutagen. Mutagenesis procedures and important considerations are described in Chaps. 2, 3, 4 and 6 of this book. Importantly, the first generation after irradiation of seed (called the M_1) is chimeric and not suitable for phenotyping. In rice and other sexually propagated species, meiotic propagation removes chimeras (*see* Chap. 1). Phenotypic selection can therefore begin in the second (M_2) generation. When M_1 plants are grown, a choice

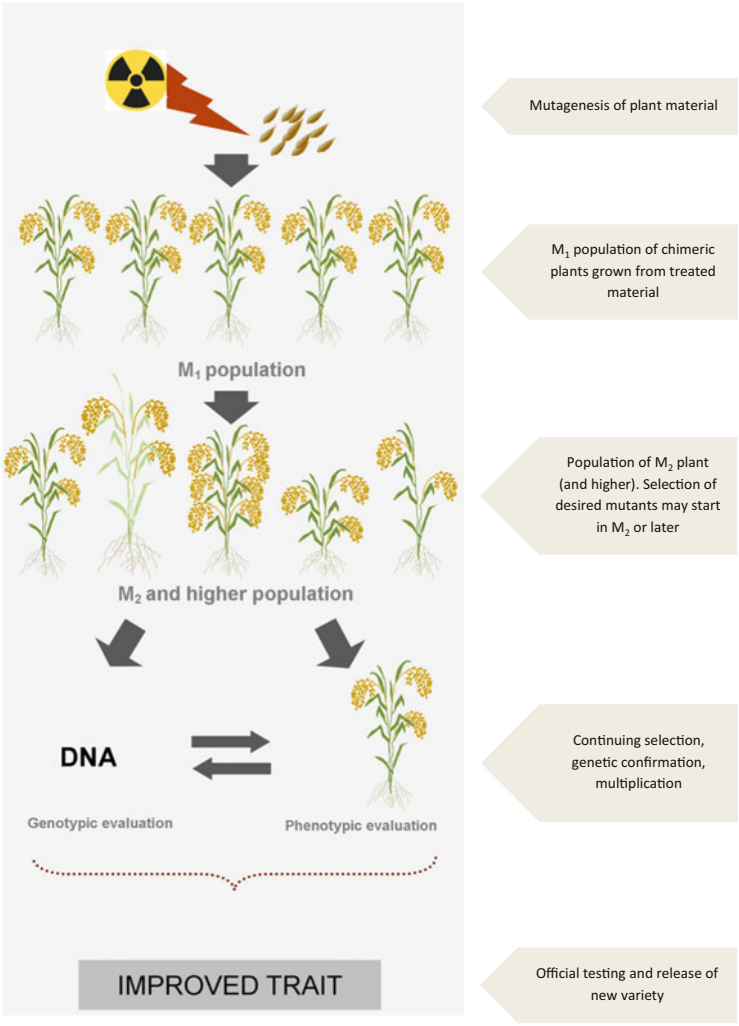

Fig. 9.1 An overview of the production and screening of mutant crop populations. Mutations are induced in seed or other plant materials. The first generation after mutagenesis is termed the M_1. When multicellular tissues like seed are mutagenized, the resulting plants are chimeric with different cells harbouring different mutations. Chimeric material is not suitable for screening. Plants are propagated and screening methods can be applied to the first non-chimeric generation (the M_2) in seed mutagenized material. Phenotypic and/or genotypic selections are applied to identify plants with desired improved characteristics. Further propagation and validation of traits are usually performed to make pure-breeding material. This material is tested prior to release as a new variety

must be made regarding organization of the mutant population. The two main options are between a bulk or pedigree population structure. Bulked approaches, as the name implies, involve collecting all seeds from the M_1 together and sowing M_2 progeny together. Here it is impossible to trace back an observed trait to a specific founding mutant plant. The advantage is that it requires little work in organizing and tracking seed. The disadvantage is that it is difficult to estimate mutation densities, and efforts must be made to determine if two plants showing similar phenotype are allelic. Pedigree approaches track the lineage as early as the M_1. Information is collected and each generation and the genetic relationship of each seed can be tracked. This approach is especially important in TILLING as described in Chap. 18. The disadvantage is that it is very labour intensive. In addition, a computerized system and database for seed organization and tracking is advised when handling large populations. The advantage is that genetic relationship of plants with similar phenotypes is known, and precise estimations on mutation density can be calculated. The choice of strategy depends on the research objective, and the two need not be mutually exclusive as lines can be developed in later generations through single plant selections from bulk populations. The protocol below describes this combined approach.

9.2 Materials

9.2.1 Plot Design

1. Mutagenized rice seeds (*see* **Note 1**).

9.2.2 Field Preparation and Planting

1. Heavy equipment for land preparation.

9.2.3 Rice Culture

1. Equipment for weeding.
2. Herbicides.
3. Fertilizers.

9.2.4 Field Observations and Trait Evaluation

1. Equipment for evaluating traits of interest.
2. Data collection equipment (e.g. laptop or tablet computer, tape recorder).
3. Field equipment (land preparation and planting).
4. Field maintenance supplies (e.g. fertilizers, herbicides).
5. Equipment for evaluations (e.g. data recorders, specific instruments for phenotyping particular traits if needed).
6. Equipment and supplies for harvesting and processing seeds (e.g. threshers, drying equipment, storage supplies like envelopes).

9.3 Methods

9.3.1 Plot Design

1. Conduct bulk irradiation of seeds at desired dosages or obtain mutagenized seeds from a service lab (*see* **Notes 1** and **2**).
2. Establish plot layout. Choose the number of varieties and treatment dosages to be evaluated based on available field space and labour (*see* **Note 3** and Fig. 9.2).

9.3.2 Field Preparation and Planting

1. Prepare field for planting using standard practices for your region (*see* **Note 4**).
2. Sow seeds in rows with and intra-row spacing of 20 cm between seeds (single seed per hill) and an interrow spacing of 20 cm (*see* Fig. 9.1 and **Note 6**).

9.3.3 Rice Culture

1. Field management. As with other field crop species, the two major management issues during rice culture are weed management and fertilizer application (*see* **Note 5**). Additionally, disease and pest management can be of significant concern.

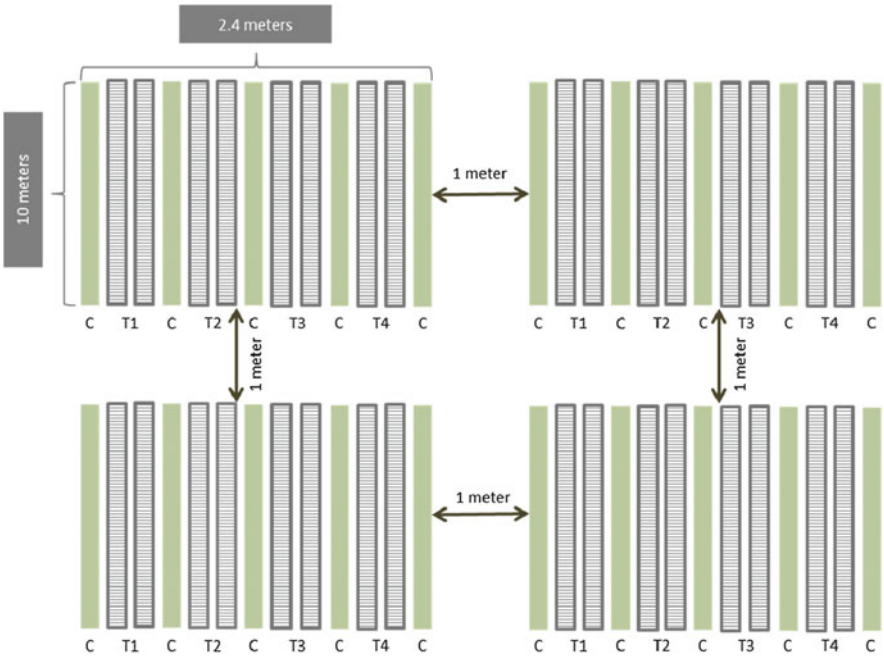

Fig. 9.2 Planting scheme of mutant populations. An individual genotype is evaluated in a single plot. Four plots are diagrammed in this figure. Plants treated with different dosages of mutagen are evaluated in the same plot (labelled T1, T2, T3 and T4 for the different treatments). Treated materials are flanked by control plants of the same genotype that have not been irradiated. Interspersing nonirradiated material helps control for GxE interactions when phenotypically selecting mutants (e.g. variations in field drainage). Plots are 10 m long with seed spacing of 20 cm within and between rows. Each plot is separated by 1 m. Care should be taken when organizing field experiments so that there is sufficient time and labour to evaluate all the planted materials. Phenotypic evaluation should not begin before the second (M_2) generation after mutagenesis

9.3.4 Field Observations and Trait Evaluation

1. Conduct regular field observations. All events observed in the field environment that are likely to affect the outcome of the activities should be recorded (*see* **Note 6**).
2. Evaluate traits of interest. Field evaluation of mutants is conducted to identify those with desirable/superior traits for varietal release or incorporation in other breeding procedures (*see* **Note 7**). Procedures for collection of trait data are well established for rice (e.g. Standard Evaluation System for Rice 1996). Standard practices (including the type of traits and the methods employed for evaluation) for the growing region should be employed. It is preferable for at least two people to be involved in data collection (*see* **Note 8**). Initial analysis of data collected can be restricted to the use of simple averages and standard errors (*see* **Note 9**).

3. Make selections of individuals (*see* **Note 10**). Seeds should be harvested, cleaned, dried to a moisture content of approximately 14 % and stored in plastic or paper bags in a cool, dry area. Additional seed treatments (e.g. for insect pests) may be needed.
4. Repeat field evaluation process to determine stability of selected mutants and to assess other agronomic traits. These observational and replicated yield trials are performed using standard recommended designs (*see* **Note 10**).

9.4 Notes

1. Mutagenized rice seed may be generated by employing caesium-137 or cobalt-60 gamma irradiators to induce mutations. A homogeneous seed source should be used for mutagenesis to prevent contamination from other varieties or landraces which could be mistaken for induced mutants. Seeds can be irradiated and stored for months before sowing. Cold storage of seed is preferable. However, when yield trials are being performed (*see* **Note 11**), seed should be stored at ambient temperatures to mimic conditions used by farmers.
2. The Plant Breeding and Genetics Laboratory of the FAO/IAEA provides irradiation mutagenesis services to member states. For groups who are able to perform irradiation mutagenesis locally, it should be noted that the dosage rate of the irradiator may affect the spectrum and density of induced mutations. Seed irradiation and dosage optimization should be performed according to published protocols (Mba et al. 2010). Special care should be taken when conducting dosage optimization using X-ray irradiators as the physical variations of different machines (e.g. fixed versus orbital rotation) may have an impact on actual dosage delivered to the seeds. Dosage depends on the responses of species and variety types. It is recommended that a preliminary varietal sensitivity test be performed to identify the appropriate dose range. At the Sierra Leone Agricultural Research Institute, initial varietal sensitivity of the local *O. glaberrima*, the institute's released *O. sativa* and NERICA rice varieties showed that favourable responses could be obtained from 100 to 400 Gy using both gamma rays using cobalt-60 and X-ray sources. Dosages above 500 proved fatal. The LD50 was between 350 and 440 Gy.
3. It is good practice to have a reasonably large population to facilitate the identification of beneficial mutants. It is however necessary to limit the total number of treatments to handle. For example, starting with ten varieties, the number of irradiation treatments including the controls (i.e. M_0) should not exceed five, resulting in 50 different treatment combinations. Assuming 100 seeds are irradiated per treatment, this will provide a working population (M_0 and M_1) of 650 seeds per variety and 6500 seeds for ten varieties. Using the

recommended intra-row spacing of 20 cm and an interrow spacing of 20 cm, the total amount of space required for one variety with five irradiation treatments including the control is 2.4 × 10 m plus 1 m separation between different mutagenic sources as shown in Fig. 9.2. Each variety should be sown in separate rows according to the level of irradiation commencing with control (nonirradiated seeds) followed by irradiated seeds. The principle of randomization is adopted in assigning treatments. Since each treatment consists of variety by levels of irradiation, varieties are initially randomized using a randomization table, or other method of randomization, followed by randomizing irradiation levels within varieties, essentially a split plot design. Intra- and interrow spacing 20 cm is considered adequate as segregation is expected after irradiation treatment.

4. Standard methods for preparing fields for planting of rice *via* direct seeding should be employed. This may involve the use of pre-emergent herbicides and pre-plant fertilizers which typically consist of major nutrients nitrogen (N), phosphorus (P) and potassium (K). It may be necessary to supplement with various mineral nutrients (e.g. zinc, iron). The methods, quantities, types of fertilizers and when to apply depend on the soil native fertility, the recommended rates and the cropping history. Whether the investigation is located on the uplands or irrigated lowlands, split application of N, P and K is usually recommended. Mini bunds are normally erected around plots to avoid fertilizer drift from plot to plot.
5. Depending on the available resources and local cultural practices, weed management may be conducted using application of herbicides during early stages of growth as well as manual weeding. Potential sensitivity of mutants to herbicides is a consideration. Midseason (i.e. mid-tillering stage of development) application of nitrogen may be desirable. When applying fertilizers, it is important not to go beyond what is optimally required. The need for application of fertilizers is justified as the genotypes should not suffer from nutrient stress. Excessive fertilization is also not recommended as the mutants should be exposed to as normal a condition as what operates in other field evaluations. Undue environmental effects that could mask the expression of mutant phenotypes should be limited to the extent possible. It is therefore important that the experimenter applies what normally operates in the region. Mutants should not be given any special treatment from the normal population.
6. After irradiation, it is best to sow one seed per hill except where the number of mutants is large as in M_2 and M_3, in which case two seeds could be placed per hill. If both seeds emerge, one would have to be transplanted and labelled. It is recommended to store mutants and controls in a refrigerator or a cold room especially in warm and humid regions to avoid climatic/weather conditions on seed viability. This is necessary to distinguish the effect of mutagenesis from local storage conditions. Monitoring of field conditions (e.g. air and water temperature) which may affect development of the plants should be conducted on a regular basis. If available, data loggers that are able to record thousands of measurements of temperature and relative humidity are ideal although manual

measurements or data from nearby weather stations are alternatives for obtaining information.

7. In the evaluation process, mutants which are sufficiently different from the controls are identified. Labelling is expected, but it should not be done at the expense of objectivity. Rather than have all the details, labels should be clear but coded, and when a mutant is spotted with desirable traits, its real identity can be verified later. This is necessary to reduce bias in selection. Observers should be open-minded and not be restricted towards the objectives of the investigation as mutants with traits other than those set out to look for could be spotted and noted on the field by tagging. The Standard Evaluation System for Rice (1996) is useful in making observations on agronomic traits, crop damage (such as diseases, insects, rodents and birds), physiological stresses including acidity and drought, morphological characters and grain quality. Some important selection criteria should include tiller number, panicle number, panicle size, grain size and quality, number of filled spikelets per panicle, adaptation to local conditions and acceptable plant height and physiological maturation period.
8. It is preferable to have more than one observer to assess the plots at different stages of plant growth. Regular visits are essential with the growth phases of the crop providing a guide as to the number of visits. Three visits during each growing period are normally adequate: emergence, tiller production, reproductive and ripening stages. Where there are clear disagreements between observers, the team can visit the field together to arrive at some objective conclusion. Field activities are normally fraught with many predators ranging mostly from mammals, insects and birds. Effort should be made to erect barriers to restrain pests such as rodents and birds. Rodents usually attack the crop throughout the growing period, birds usually at the seeding and reproductive stages. A minimum of two people should be involved in data collection. Always write down as much details on the field before leaving as it is quite easy to forget details that were not recorded. Data obtained on the field should be transferred to a computer either from field notebooks or the use of data loggers. The raw data should be transferred; no transformation should be made on the field. It is always a good practice to have a spare copy of the raw data kept separately just in case of natural disasters like fire. It may be necessary to save seeds from each treatment for possible laboratory investigations, for example, Near-Infrared Reflectance Spectroscopy (NIRS) or physico-chemical analysis.
9. Analyses of data collected should be restricted to using simple averages and standard errors. This can be done without losing information on selected mutants as the analysis does not interfere with the selections made. Data on individual mutants selected are computed and compared with the controls (M_0) and other mutants not selected to obtain trends in plant characters amongst nonselected mutants, selected mutants and controls. Such information is useful and could provide benchmarks for future investigations.
10. Whilst selection could commence at the M_2 stage, mutant plants with interesting traits should be harvested separately. Single panicle selection is the preferred method of harvesting. Other plants are harvested according to treatment.

At the M_3, M_4 and M_5 stages, panicles of selected mutants are also harvested, labelled and stored separately (Fig. 9.3). The rest of the materials are bulked by treatment. A number of options exist for selected mutants between M_2 and M_5. Some of the selections could be fixed to produce double haploids (Maluszynski et al. 2003) in tissue culture, whilst others could be utilized in reverse-genetics activities such as TILLING (Till et al. 2006); the rest of the selected mutants, when fixed, could be incorporated in yield trials.

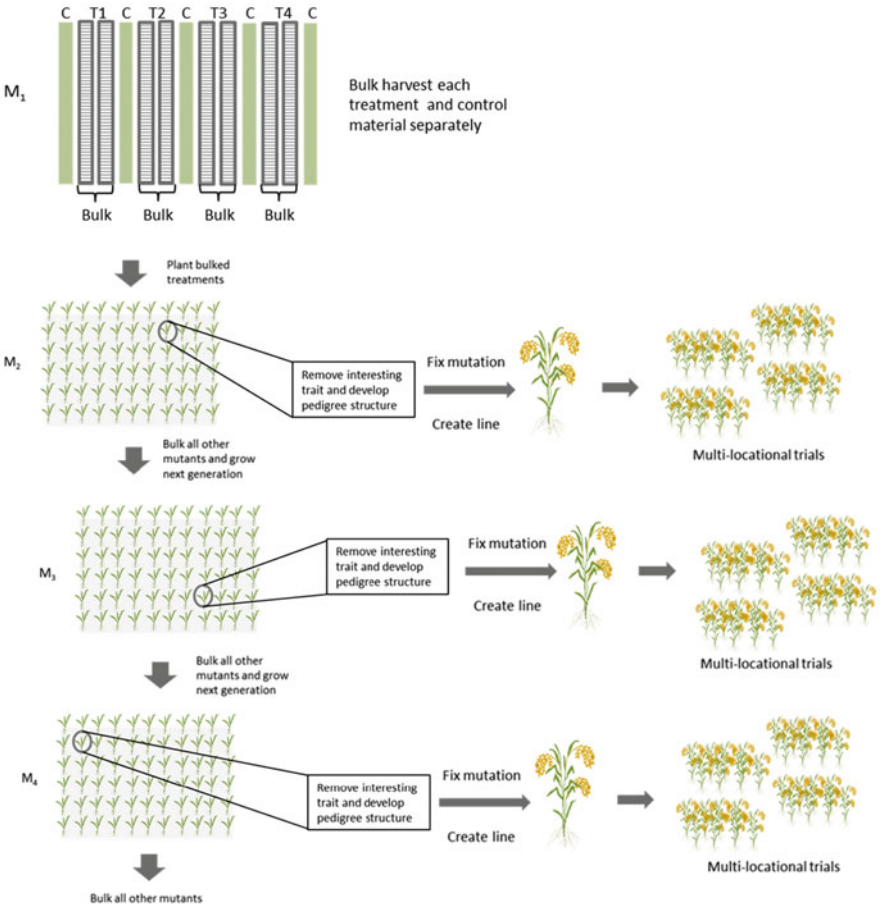

Fig. 9.3 Plot design, bulk screening and mutant selection of rice. Different treatments should be planted in rows (*grey bars*) flanked by non-mutagenized controls (*green bars*). Seed from a single treatment/genotype are bulked and phenotypic evaluation begins in the M_2 generation (*middle panel*). Plants with interesting phenotypes are selected and individually harvested. A pedigree approach is followed with these plants whereby mutations are fixed so that traits are pure-breeding and lines are created. This material is then evaluated in multi-location trials prior to varietal release. The rest of the seed from the plot (similar treatment/genotype) is bulked and the next generation planted and the selection process repeated (*bottom panel*). This is continued for several generations or until no further interesting plants with novel characteristics are recovered

11. At the M_5 generation, mutants are generally stable enough for observational yield trials (OYTs) and replicated yield trials (RYTs) using any of the recommended standard designs. Many field designs are available for replicated yield trials, ranging from completely randomized design, randomized complete block, partially incomplete blocks, split plots to fan designs to select from for trial establishment. Nonirradiated controls should be included in the evaluation of observational and replicated trials to determine superiority of mutants over parents. Selection of the appropriate or relevant design depends on the objective of the evaluation, and assistance could be sought from a biometrician or consulting recognized books on statistics and experimental designs (e.g. Cochran and Cox 1968; Cox 1958; Gomez and Gomez 1984).
12. Irradiation could be successfully used to produce beneficial changes in plant traits. This method is a lot safer, and seeds can be easily handled in the field with little or no risk to the environment or individuals involved in handling them. This method could be combined with in vitro techniques to reduce the period necessary for traits to be fixed. From experience, it is necessary for at least 4–6 growth cycles before one could be fairly certain that any traits observed on the phenotypes could be fixed.
13. It would be advisable in future for the number of varieties to be limited as the number of plant materials increased exponentially after the M_2 stage. Also with a larger number of varieties, the amount of data that could be recorded would be limited.

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