

Chapter 2

Chemical and Physical Mutagenesis in *Jatropha curcas*

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Abstract Mutagenized populations are important resources to generate and identify desirable genetic variation of useful traits for crop improvement. When the lack of genetic variability hampers the breeding progress, mutagenesis can introduce genetic variation, reveal gene function, and aid in the characterization of candidate genes involved in biological functions. Mutagenized populations are useful for screening for altered phenotypes and physiological responses, and as a genomics tool. *Jatropha curcas* is a semi-wild, economically important shrub useful as a source of biofuel or in soil reclamation, but it requires genetic improvement in order to select the best genotypes for these purposes. Therefore, this chapter describes the general methods for mutation induction (chemical and physical mutagenesis) using ethyl methanesulfonate (EMS) treatment, gamma irradiation, X-rays, and the procedures that can be used to generate large numbers of induced mutants in different tissues of *J. curcas* under in vitro and in vivo conditions.

Keywords Chemical mutagenesis • Physical mutagenesis • Gamma • X-ray • Mutation induction

2.1 Introduction

Jatropha curcas is one of the most valuable crops for its ability to produce seeds, which contain 60–63 % of protein and 30–45 % of toxic oil that renders the seedcake and oil unsuitable for animal or human consumption (Maghuly and Laimer 2013). The narrow genetic base in *J. curcas* hinders efficient genetic

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improvement (Maghuly and Laimer 2013). In fact, the ultimate breeding objectives of the *J. curcas* accessions are to reduce toxicity and improve productivity under adverse climatic conditions. To increase genetic diversity, mutagenesis can be applied for plant improvement (Maghuly and Laimer 2013; Carels 2013).

One of the most effective and commonly used chemical mutagens is ethyl methanesulfonate (EMS; $\text{CH}_3\text{SO}_2\text{OC}_2\text{H}_5$), a monofunctional alkylating agent with a formula weight of 124 able to induce chemical modification of nucleotides, resulting in base changes, breakage of the DNA backbone, and mispairing (Kodym and Afza 2003; Kim et al. 2006). The most frequent alkylating event of nitrogen occurs with guanine (G) at the 6'-O position, forming O-6-ethylguanine, which can pair with thymine (T) instead with cytosine (C), resulting in base pair errors. In this way, during DNA repair, the original G/C site is replaced by A/T in the majority of nucleotide changes (99 %) obtained with EMS (Greene et al. 2003). In addition, depurination of alkylated G will form gaps in the DNA and, therefore, after replication will result in deletions and in frameshift mutations. The gap can fill with any of four bases, obtaining a normal copy, a G/C to A/T transition, or a G/C to C/G or G/C to T/A transversion. Further, alkylation of nitrogen can occur at a lower frequency with G at N-7, forming 7-ethylguanine, representing G/C to C/G or T/A transversions, or A at N-3, forming 3-ethyladenine, representing A/T to G/C transitions, or C at N-1, forming 1-ethylcytosine (Kodym and Afza 2003; Krieg 1963; McCallum et al. 2000).

While chemical mutagenesis primarily causes base substitution, ionizing radiation causes base modification and single-/double-stranded breaks by the production of reactive oxidative species, which interact with DNA, causing oxidative damages (Morita et al. 2009). In physical mutagenesis, physical events such as ionization and extension lead to modifications of the DNA, cell membranes, lipids, enzymes, and other cellular constituents (Kodym and Afza 2003).

Physical mutagens based on linear energy transfer (LET), physical properties, and mutagenesis activity are divided into two main classes. Alpha particles, fast neutrons and heavy ion beams have high LET using subatomic particles such as electrons, protons, neutrons, deuterons, and alpha and beta particles, while gamma rays, X-rays, cosmic rays, and electron beams have a low LET and produce energy in the form of electromagnetic waves, which are the most commonly used for mutation breeding (Mba et al. 2012). Ionizing radiation causes biological injuries in higher plants through two main interactions with genetic material, and light can cause photochemical damages and is effective in producing purine or pyrimidine dimers, resulting in point mutations in the DNA (Esnault et al. 2010; Pathirana 2011; Lagoda 2012). This may result in small to large deletions, point mutations, single- and double-stranded breaks, and even chromosome deletions.

Physical and chemical mutagens have been applied to all types of plant material. Thus, soft materials such as in vivo and in vitro cuttings as well as embryogenic callus require lower doses in comparison to seeds. In fact, the water content, storage time, applied mutagen dose, and temperature represent important factors influencing mutagens in all types of plant material (Mba et al. 2010). The most important and difficult step is to screen the entire mutant population, which can be overcome

by forward and reverse genetic technologies (Maghuly and Laimer 2016). These approaches provide excellent tools for developing efficient strategies for the identification of useful alleles in a breeding program and for functional genomic analyses.

To induce variability in *Jatropha curcas*, chemical and physical techniques for crop improvement have been adapted for the treatment of seeds, as well as for in vivo and in vitro cuttings and somatic embryos (Kodym and Afza 2003; Bado et al. 2013, 2015). This chapter describes commonly used techniques for physical and chemical mutation induction on different *J. curcas* tissues. The mutated populations (M_1) are generated, and to reduce chimerism M_2 or higher populations are produced. Entire mutant populations are screened by either phenotypic evaluation for selection of phenotype of interest (forward genetics) or by genotypic evaluation for detection of novel allele in gene of interest as well as study of gene function (reverse genetics) (Fig. 2.1.).

2.2 Materials

2.2.1 *In Vivo Material*

1. High quality, disease-free seeds, clean and uniform in size (*see Note 1*).
2. In vivo stem cuttings (*see Note 1*).
3. Sterilized soil mixture.
4. Glasshouse facility.

2.2.2 *In Vitro Material*

1. High-quality embryogenic callus cultures and cuttings of in vitro grown cultures in defined genotypes (*see Note 1*).
2. Laminar flow cabinet for in vitro work (in vitro tissue culture facility).
3. Tissue culture media.
4. 70 % ethanol.
5. Parafilm.
6. Whatman filter paper circles (90 mm diameter).
7. Petri dishes (90 mm diameter).
8. Regeneration media.

2.2.3 *Mutagenesis by Chemical Agents (See Note 2)*

1. Ethyl methanesulfonate (EMS) AR grade (*see Note 3*).
2. Dimethyl sulfoxide (DMSO).

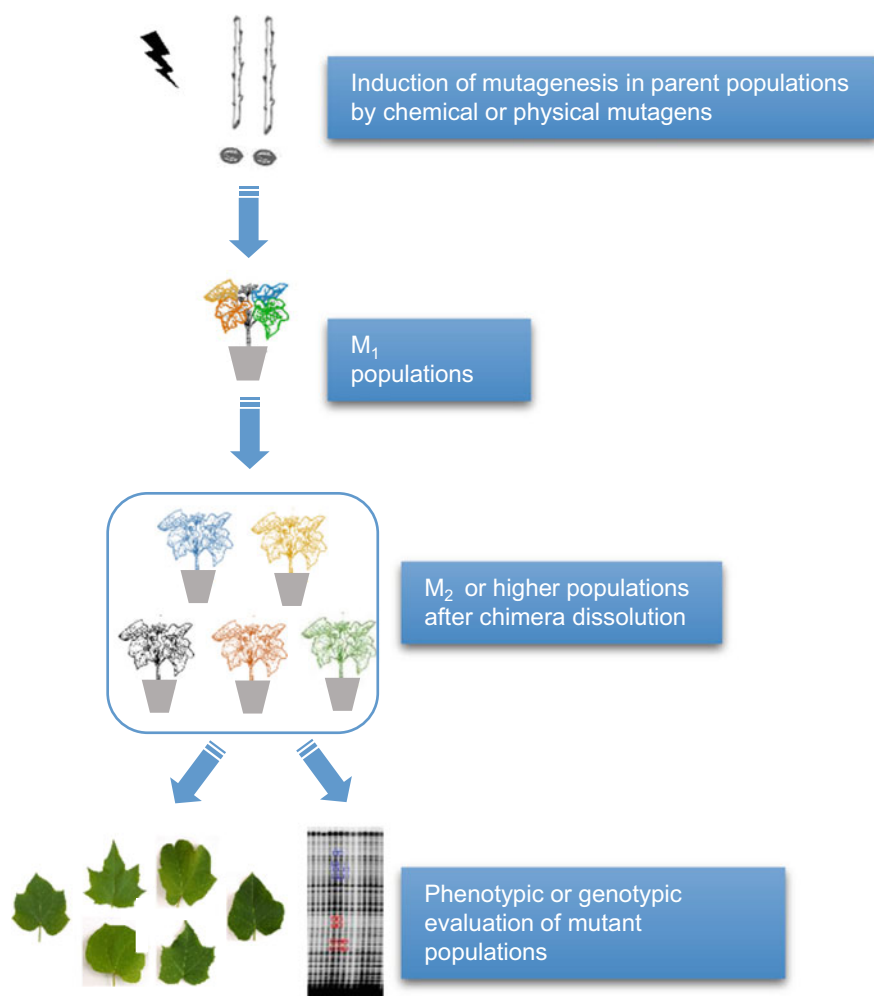


Fig. 2.1. Schematic diagram of the basic steps in physical and chemical mutagenesis of *Jatropha curcas* and characterization of mutant plants

3. 10 % (w/v) sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) for decontamination of EMS solution and tools (*see Note 4*).
4. Sterile deionized water (*see Note 5*).
5. Beakers (500 ml and 1000 ml).
6. Wash bottles.
7. Sterile sieves (metal, 70 mm diameter, 70–100 μm pore size).
8. Forceps.
9. Flat containers for mutagenesis of in vivo cuttings.

10. Sterile membrane filters for filtering the EMS solution (25 mm diameter, 0.2 μm pore size).
11. Syringe.
12. Laboratory fume hood for solution preparation.
13. Polyethylene mesh bags (ca. 11 \times 7 cm in dimension).
14. Orbital shaker.
15. Personal protective equipment (dedicated laboratory coat, protective eyewear, shoe protection, nitrile gloves).
16. Hazardous liquid waste receptacle (collection vessels for EMS waste solution).
17. Box for dry hazardous material disposal.

2.2.4 Mutagenesis by Physical Agents

1. Gamma radiation source.
2. X-ray irradiator RS-2400.
3. Paper envelopes (air and water permeable).
4. Vacuum desiccator.
5. Petri dishes (90 mm diameter).
6. Whatman filter papers for 90 mm Petri dishes.
7. 60 % glycerol (v/v).
8. Sterile and non-sterile deionized water.
9. Parafilm.

2.3 Methods

2.3.1 In Vivo Material

1. Select clean, homogeneous, and disease-free seeds (*see Note 1*).
2. Check the viability and homogeneity by performing a germination test (*see Note 6*).
3. Select seeds with good viability (i.e. >90 %) that allow for the assessment of the mutagen effects.
4. Separate scions of *Jatropha* according to the stage of maturity (from bottom to top: hardwood, semihard, and softwood). This classification is recommended when cuttings are used to perform the radiosensitivity test (*see Note 7*).
5. Cut scions to ten nodes (meristems) per cutting (*see Note 8*).
6. Tie 10–15 cuttings with a rope into a pack (or put in a transparent plastic bag). Package volume should not exceed the irradiator chamber.
7. Place plant material in Petri dishes or appropriate containers for irradiation (*see Note 9*).

2.3.2 *In Vitro Material*

1. Prepare Petri dishes with two wet Whatman filter papers.
2. Prepare one-node cuttings from freshly micropropagated plantlets (subculture intervals of 3 weeks).
3. Place cuttings into Petri dishes with wet filter paper (20–30 cuttings per 94×16 mm Petri dish).
4. Seal Petri dishes with Parafilm to avoid contamination outside the tissue culture laboratory.
5. Prepare the regeneration media (MS medium supplemented with growth regulators).
6. Collect the plant material for mutagenesis (rescued mature embryos, shoot tips from micropropagated cultures, leaves, meristems, and cotyledons).

2.3.3 *Mutagenesis by Chemical Agents*

2.3.3.1 EMS Mutagenesis of In Vivo Material (*See Note 10*)

1. Mix the required volumes of distilled water and 2 % (v/v) DMSO and autoclave at 120 °C for 15 min. Let the mixture cool to room temperature (RT) (*see Note 11*).
2. Prepare decontamination solution consisting of 10 % (w/v) sodium thiosulfate (*see Note 4*).
3. Prepare the concentration series of EMS in water-DMSO mixture (Table 2.1), including a pure water control treatment.
4. Use a sterile syringe and a 0.2 μ m filter to add the required volume of EMS solution to the sterile water-DMSO mixture (*see Note 12*). Commence with the lowest concentration.
5. Shake the EMS-DMSO solution vigorously to form a homogeneous emulsion, and decant approximately equal volumes into each of bottles (for seeds, *see Note 16*) and flat containers (for stem cuttings) labeled with the tissue type, EMS concentration, and incubation time (*see Note 17*).

Table 2.1 Example of EMS concentrations chosen for mutagenic treatment (T) of seeds and stem cuttings of *J. curcas* (*see Note 13*)

Plant material	T 1	T 2	T 3	T 4	T 5	T 6
Seeds	–	–	0.8 %, 1.5 h	1.6 %, 1.5 h	0.8 %, 3 h	1.6 %, 3 h
Stem cuttings	0.4 %, 1.5 h	0.4 %, 3 h	0.8 %, 1.5 h	1.6 %, 1.5 h	0.8 %, 3 h	1.6 %, 3 h

Incubation times were 1.5 h and 3 h (*see Note 14*). Each treatment should include a control (water-DMSO) (*see Note 15*)

6. Note the time and immerse a separate treatment batch of material into each solution (0 %, 0.4 %, 0.8 %, and 1.6 %) for the selected incubation time (*see Note 18*).
7. After 1.5 h of incubation, quickly but carefully decant each of the 1.5 h treatment batches, and rinse thoroughly with tap water (at least 3–5 times). Capture the residual EMS solution and any wash water for disposal in a hazardous waste container labeled as “Hazardous waste.”
8. Continue incubations of the treatment material selected for 3 h incubation time.
9. After 3 h of incubation, quickly but carefully decant each of the 3 h treatment batches, and rinse thoroughly with tap water (at least 3–5 times). Capture the residual EMS solution and any wash water for disposal in a hazardous waste container labeled as “Hazardous waste.”
10. Detoxify the waste and all unused EMS solution by adding 10 % (w/v) sodium thiosulfate in a 3:1 ratio by volume. Pour into a designated container (marked with “Hazardous waste”) and let stand for at least six half-lives (*see Note 19*).
11. Decontaminate the working area with sodium thiosulfate, and dispose EMS-contaminated items according to laboratory safety rules.
12. Wash plant seeds or stem cuttings in the glasshouse.
13. Mutated seeds and stem cuttings can be immediately planted.
14. Collect data on the germination rate (seed) or sprouting of cuttings and the survival rate after transplants on a weekly basis.
15. Reduce chimerism by producing M_2 or higher mutation populations.
16. Screen mutant populations by either phenotypic or genotypic evaluation (Fig. 2.1.).

2.3.3.2 EMS Mutagenesis of In Vitro Material (*See* Notes 10 and 20 and Fig. 2.2)

1. Prepare containers able to accommodate stem cuttings and bottles for seed mutagenesis. Label them with the incubation time and EMS concentration.
2. Prepare decontamination solution consisting of 10 % (w/v) sodium thiosulfate (*see Note 4*).
3. Calculate the final volume of EMS solution needed (Table 2.2, *see Note 13*). Mix the required volumes of water and 2 % (v/v) DMSO (*see Note 11* and step 1 of Sect. 2.3.3.1.).
4. Prepare the concentration series of EMS in water-DMSO mixture, including a control water treatment (*see Note 12*, and step 3 of Sect. 2.3.3.1.).
5. Shake the solution vigorously to form a homogeneous emulsion, and decant approximately equal volumes into each beaker/bottle labeled with the tissue type, EMS concentration, and incubation time.
6. Note the time. Immerse separate treatment batches of material into each of the four solutions (0 %, 0.4 %, 0.8 %, and 1.6 %) for three incubation times.

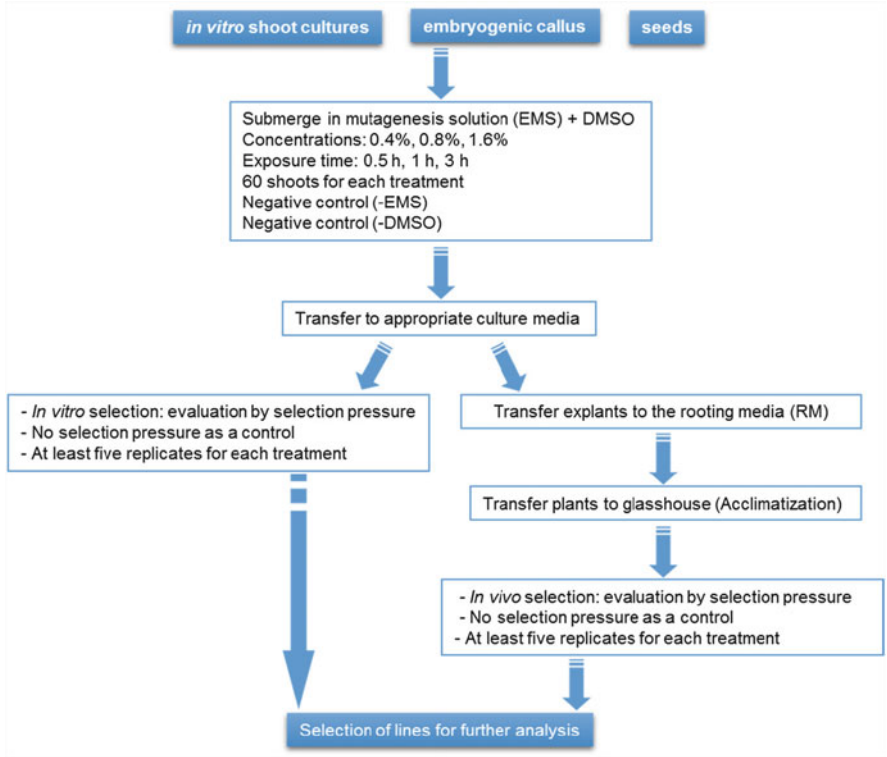


Fig. 2.2 Schematic representation of EMS mutagenesis of *in vitro* material

Table 2.2 Example of EMS concentrations chosen for mutagenic treatment of seed, embryogenic callus, and *in vitro* cuttings of *J. curcas* (see **Note 13**)

Plant material	T 1	T 2	T 3	T 4	T 5	T 6	T 7	T 8
Seeds	–	–	–	–	0.8 %, 1.5 h	0.8 %, 3 h	1.6 %, 1.5 h	1.6 %; 3 h
Embryogenic callus	0.4 %; 0.5 h	0.4 %; 1.5 h	0.8 %, 0.5 h	0.4 %, 3 h	0.8 %, 1.5 h	0.8 %, 3 h	–	–
In vitro shoot cultures	0.4 %, 0.5 h	0.4 %, 1.5 h	0.8 %, 0.5 h	0.4 %, 3 h	0.8 %, 1.5 h	0.8 %; 3 h	–	–

The chosen incubation times of 1.5 h and 3 h resulted in four treatment (T) combinations for each type of mutagenized tissue (see **Note 14**). Each treatment includes control (water-DMSO) (see **Note 15**)

7. After 0.5 h of incubation, quickly but carefully decant each of the treatment batches labeled with the 0.5 h incubation time, and rinse thoroughly with sterile water (at least 3–5 times). Capture the residue EMS solution and any waste-water for disposal in a hazardous waste container labeled as “Hazardous waste.”

8. Continue the same way with longer incubation times of 1.5 h and 3 h.
9. Detoxify the waste and unused EMS solution by adding 10 % (w/v) sodium thiosulfate in a 3:1 ratio by volume. Pour into a designated container (marked with “Hazardous waste”) and let stand for at least six half-lives (*see Note 19* and step 9 of Sect. 2.3.3.1.).
10. Decontaminate working area with sodium thiosulfate, and dispose of EMS according to laboratory safety rules.
11. After all treatment batches are dispensed, arrange the treated material in each Petri dish/magenta box containing MS media.
12. Take the cultures to the incubation room with 28 °C and 12 h light.
13. Transfer mutagenized tissue to culture media. After 24 h transfer it again to a fresh media.
14. Transfer cultures weekly into fresh media to reduce possible accumulation of phenolic compounds due to the stress of mutagenesis.
15. Thirty days after the treatment, record survival rates of the mutagenized population.
16. Subculture the growing embryogenic callus and in vitro cutting cultures for chimera dissolution.
17. Transfer plants to rooting media for acclimatization phase and subsequently to the glasshouse for further mutant evaluation (Fig. 2.2).

2.3.4 Mutagenesis by Physical Agents

2.3.4.1 Gamma Irradiation of In Vivo Material (See Notes 21–22)

1. Pack seeds in paper envelopes (*see Note 23*), or tie cuttings with a rope into a pack (optionally put in a transparent plastic bag) (*see Note 7*).
2. Calculate the exposure time based on the dose rate of gamma cell irradiator that will be used (Gy/s or Gy/min) (*see Note 24*).
3. Label envelopes according to the dose required (Table 2.3) and genotype when handling more than one genotype. An untreated bag (control) with same number of seeds will be held in the same conditions as the treated ones.
4. Place the packed seeds in a vacuum desiccator (with 60 % glycerol) for moisture equilibration.
5. Keep the seeds in vacuum desiccator for 3–7 days. This equilibrates the seed moisture content to approximately 12–14 %, which is the ideal moisture for efficient induction of mutation (*see Note 25*).
6. Apply the required dose (Table 2.3) by placing bags into irradiator chamber for the exposure time to produce the dosage (*see Note 7*).
7. Start gamma cell irradiator (*see Note 26*).
8. Open the lead shielding collar (when gamma cell is at the loading stage).

Table 2.3 Example of 139 Gy/min gamma irradiator doses chosen for mutagenic treatment (T) of seeds and stem cuttings of *J. curcas*

Plant material	T 1	T 2	T 3	T 4	T 5
Seeds	100 Gy/40s	200 Gy/83 s	300 Gy/126 s	400 Gy/169 s	500 Gy/212 s
Stem cuttings	15 Gy/3 s	20 Gy/5 s	25 Gy/7 s	30 Gy/9 s	35 Gy/12 s

Each treatment also included untreated samples

9. Open the sample chamber door.
10. Place the plant material into the irradiator chamber (Fig. 2.3).
11. Close the sample chamber door and the lead shielding collar.
12. Set the exposure time to produce the required dose (*see* **Notes 24 and 27** and Table 2.3). When the applied dose for the genotype is unknown, a radiation test may be performed (*see* **Note 7**).
13. Put the chamber at the irradiation stage by lowering the elevator.
14. Start the countdown of exposure time.
15. Raise the chamber to the loading stage when exposure time is completed.
16. Open the lead shielding collar and then the sample chamber door.
17. Remove the irradiated plant material (*see* **Note 28**).
18. If necessary, repeat the treatment at defined time intervals to reach the required mutation induction dose.
19. Irradiated seeds and stem cuttings can be planted immediately.
20. Collect data on a weekly basis on the germination rate of seeds, sprouting of cuttings, and the number of survival.
21. Reduce chimerism by producing M₂ or higher mutant populations.
22. Screen mutant populations by performing either phenotypic or genotypic evaluation (Fig. 2.1.).

2.3.4.2 Gamma Irradiation of In Vitro Material

1. Label each Parafilm-sealed Petri dish containing plant material with the selected dose (*see* **Note 29**).
2. Transfer Petri dishes to physical mutagen source laboratory.
3. Apply the required dose for mutation induction (*see* **Notes 7 and 30**).
4. Transfer irradiated samples to tissue culture laboratory.
5. Surface-sterilize each Petri dish with 70 % ethanol before removing the Parafilm (*see* **Note 31**).
6. Transfer the irradiated material into an appropriate tissue culture media.
7. Take the cultures to the incubation room (28 °C and 12 h light).
8. Record survival rates of the mutagenized population 30 days after treatment.
9. Subculture growing embryogenic callus or in vitro shoot cultures for chimera dissolution.

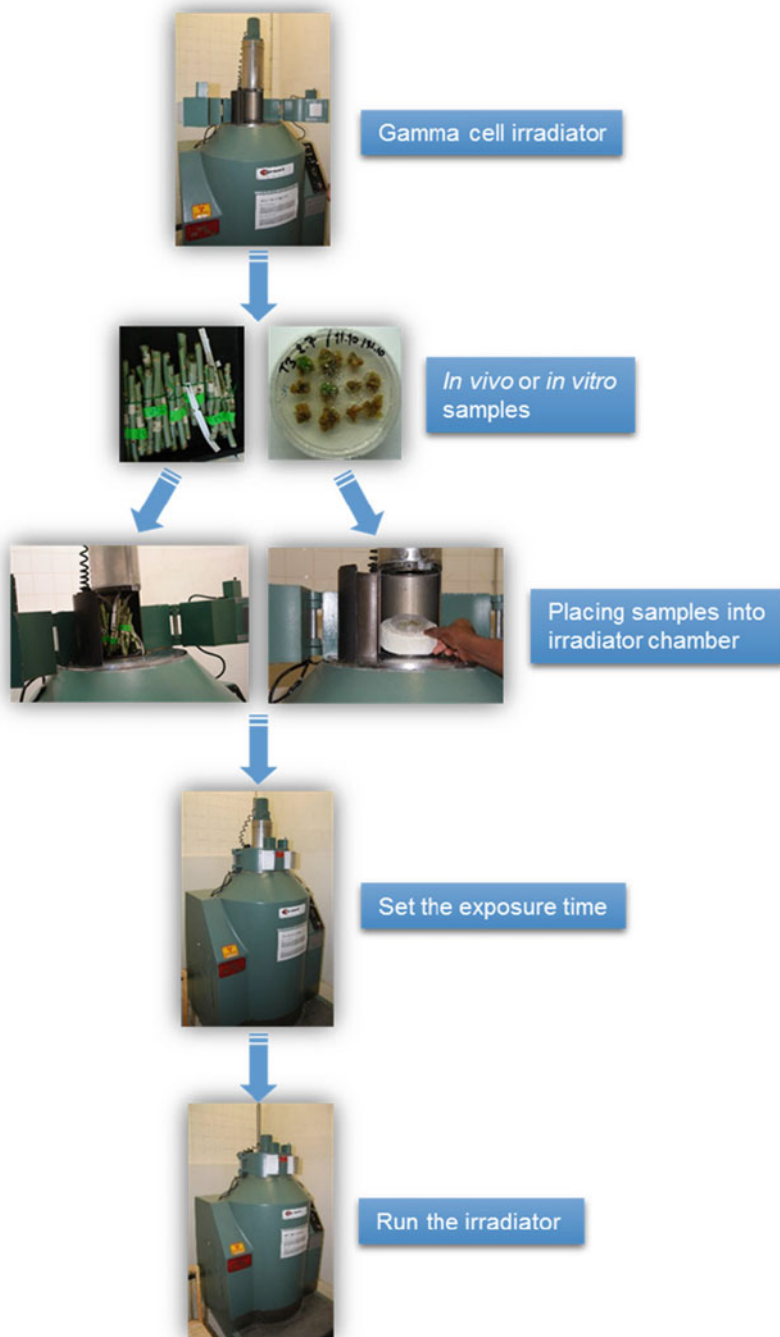


Fig. 2.3 Process of irradiation using gamma cell irradiator cobalt-60 source

10. Transfer plants to rooting media for acclimatization and subsequent transplant to the glasshouse for further mutant evaluation (Fig. 2.2).

2.3.4.3 X-Rays (See Note 32, Fig. 2.4)

1. Label each Petri dish containing plant material with the selected dose.
2. Place samples into containers and fix them with brackets and move into the canister.
3. Place canister with adaptors into the center of the irradiator.
4. Create a vacuum by filling the empty space with calibration samples (i.e., rice).
5. Close the canister with the lid.
6. Open the shielding window of the irradiator chamber.
7. Take out the sample platform.
8. Place the canister in one of the five canister holders (make sure that all canisters are present including the sample canister).
9. Place the platform at the optimal position.
10. Close the shielding window of irradiator chamber.
11. Set the exposure time by putting amount (kW) needed to produce the required dose (see Note 33).
12. Run the irradiator until the monitor displays zero kW.
13. Open the shielding window of the irradiator chamber.
14. Take out the canister containing the irradiated samples.
15. Take off the canister lid.
16. Remove the vacuum samples by dumping the instant rice and do not disturb the adaptor holding plant material.
17. Remove the samples from the canister and adaptors (see Note 34).
18. Take the plant material to appropriate culture media, soil, or storage conditions.

2.4 Further Analyses

The second generation (and higher) after chimera dissolution of *in vivo* and *in vitro* plants can be screened for the selection of candidate genes based on phenotypes or genotypes (Figs. 2.5 and 2.6). Mutations can be detected with various direct and indirect methods such as denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis (DGGE), temperature gradient capillary electrophoresis (TGCE), heteroduplex analysis (HD), the analysis of single-stranded DNA conformation polymorphism (SSCP), chemical or enzymatic cleavage of mismatches (CECMs), and Targeting Induced Local Lesions in Genome (TILLING) (Till et al. 2007). Methods such as whole genome sequencing,

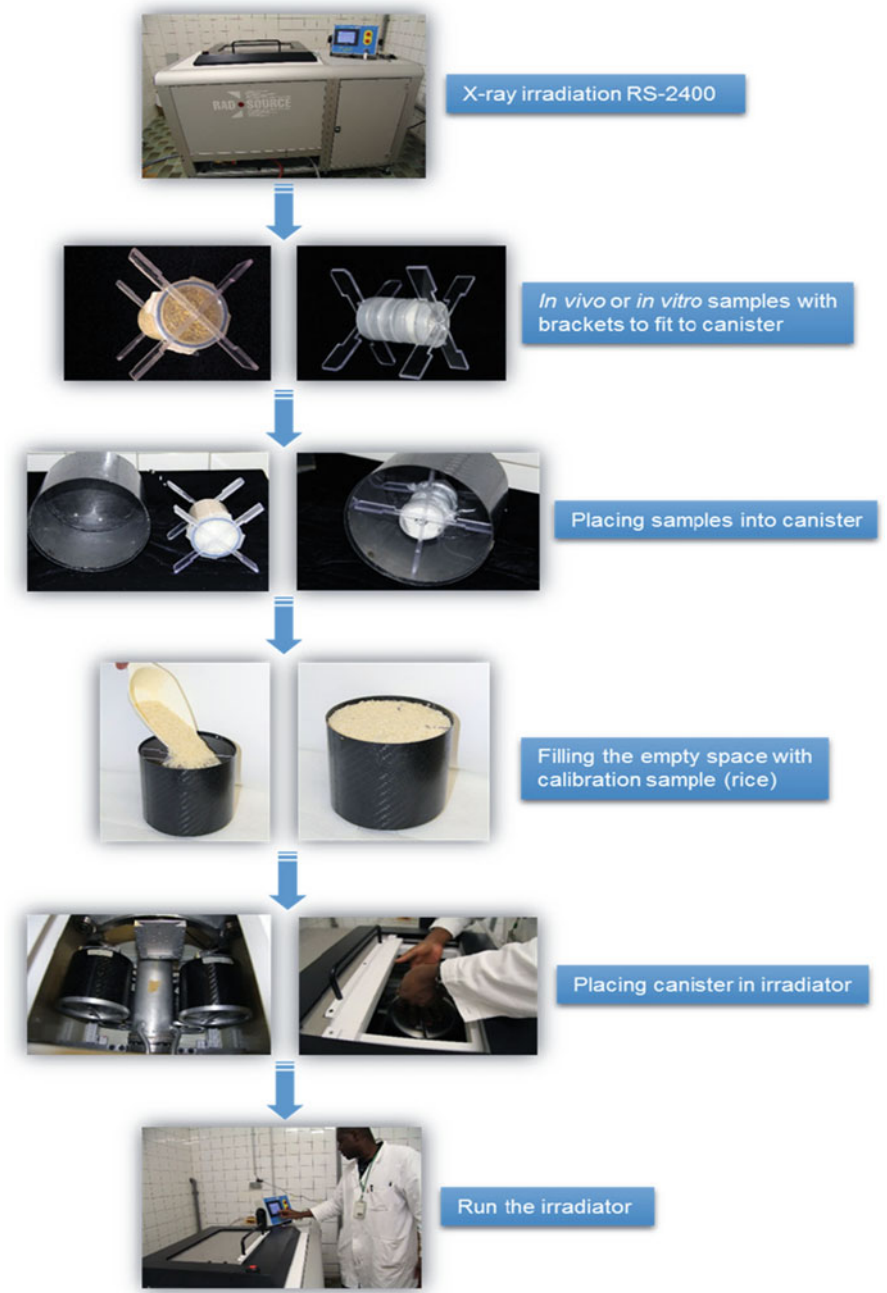


Fig. 2.4 Process of sample preparation for irradiation using X-ray RS-2400 source

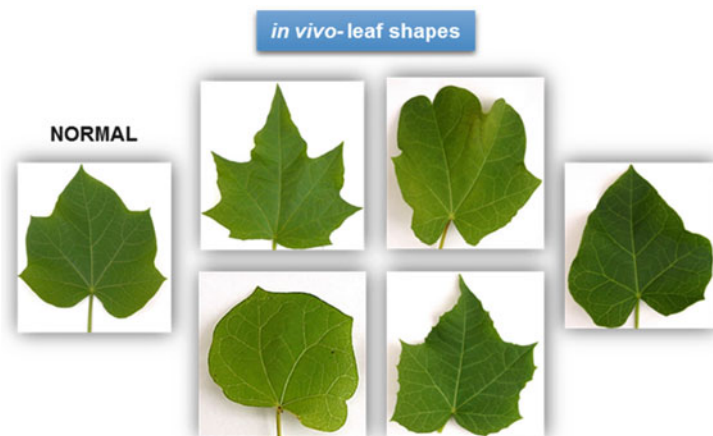


Fig. 2.5 Phenotype analysis of in vivo leaf shapes of mutagenized *Jatropha*

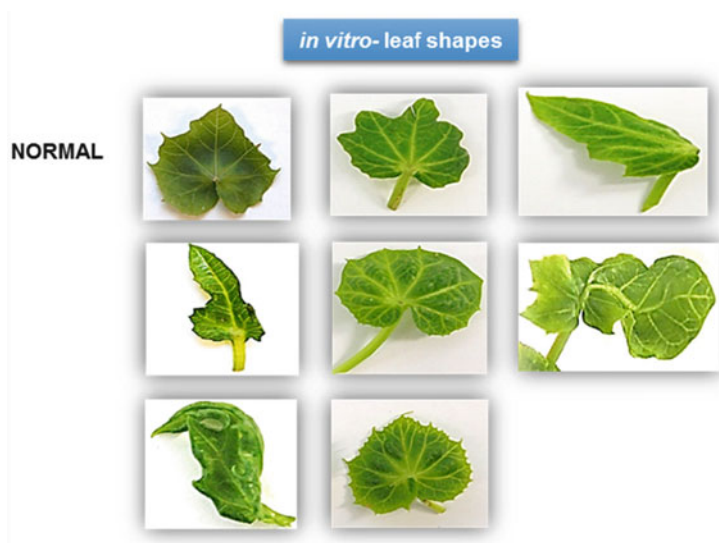


Fig. 2.6 Phenotype analysis of in vitro leaf shapes of mutagenized *Jatropha*

exome capture sequencing, restriction-site-associated DNA (RAD) sequencing, and genotyping by sequencing (GBS) provide the necessary information for mutation confirmation, which cannot be achieved through indirect methods (Maghuly and Laimer 2016).

2.5 Notes

1. Select a population size that assures success of the experiment, bearing in mind that an M_2 population of at least several thousand (5000–10,000) individuals is considered suitable (Kodym and Afza 2003; Van Harten 1998; Brock 1997). Consider that the different explant types, e.g., seeds, in vivo or in vitro cuttings, or embryogenic callus, have different requirements and capacities. This is especially important in the case of long-lived organisms like trees and has consequences at the level of population size, dissolution of chimerism, and frequency of mutation.
2. The mutagenesis should be conducted in the dedicated chemical mutagenesis laboratory.
3. EMS is an irritant and highly carcinogenic compound. Avoid any skin contact and use disposable gloves. All body parts or laboratory coats contaminated with EMS should be washed thoroughly with water and detergent and further neutralized with 10 % (w/v) sodium thiosulfate (*see* step 3 of Sect. 2.2.3).
4. Alternatives to sodium thiosulfate have also been used, such as sodium hydroxide, to inactivate the EMS.
5. Deionized water prevents undesired effect of metallic ions (Kodym and Afza 2003).
6. A germination test should be performed with at least ten seeds.
7. When the applied dose for the genotype is unknown, a radiation test should be conducted. To perform radiosensitivity test on vegetative tissue (e.g., cuttings), select 30 cuttings per dose with a wide range from 0 to 100 Gray (Gy). However, the ranges of 0, 10, 20, 30, 40, 50, and 60 Gy of gamma and X-rays may be sufficient to establish the optimal dose due to the high moisture content in comparison to seeds. The Gy is the unit used to quantify the absorbed dose of radiation (1 Gray = 1 J/kg).
8. Cuttings should not exceed the size of the irradiator's chamber. About 20 cm length per cutting is optimal for the gamma irradiator chamber (Fig. 2.1.). It is important to take meristematic dominance into consideration and thus to perform the mutagenesis with two-node cuttings which allows the production of many putative mutants.
9. Plant materials should occupy the same position along the exposure time. Petri dishes of in vitro material should be wrapped with Parafilm in order to avoid surface contamination.
10. Use only freshly prepared EMS solution.
11. This preparatory step may be carried out in advance.
12. This step and any further steps must be carried out in a laminar flow hood.
13. While reviewing the literature and reports on EMS concentrations used in mutagenesis experiments, both percentage (%) (v/v) and molarity concentrations are listed. Table 2.4 lists the percentage (%), corresponding molarity, and dilutions for an example of 10 ml volume of mutagen. The standard EMS stock from Sigma is 9.7 M.

Table 2.4 Examples of the concentration EMS in molarity or percentage in 10 ml final volume

Molarity (mM)	%	Amount to add to a final volume of 10 ml (in microliters)
9.7	0.10	10
19.4	0.20	20
22.5	0.23	23
25	0.26	25.7
27.5	0.28	28.3
29	0.30	30
48.5	0.50	50

14. Estimate the time needed to conduct the entire experiment with various tissue types. In case you are not confident in doing the mutagenesis on all tissue types, split the plant material into batches for separate days treating one tissue type per day.
15. Some protocols call for the addition of DMSO, which may make the mutagen more miscible.
16. Presoaking seeds increases infusion of the mutagen into the embryo. The time of presoaking depends on the seed coat.
17. The amount of mutagen solution should be enough to cover the whole tissue, to provide a constant concentration of mutagen throughout the treatment, and to expose all samples similarly.
18. If duration of treatment is longer than half-life of the EMS (*see Note 19*), the solution should be replaced with a freshly prepared mutagen solution.
19. The half-life of EMS in water at pH 7 at 20 °C is 93 h and at 30 °C is 26 h (Kodym and Afza 2003). For EMS in a 10 % sodium thiosulfate solution, the half-life is 1.4 h at 20 °C and 1 h at 25 °C.
20. Somatic embryogenesis is an excellent system for plant propagation and mutation induction because somatic embryos originate from single cells. Somatic embryos can be produced on a large scale in cell suspension cultures in Erlenmeyer flasks or in bioreactors. Somatic embryogenesis limits chimeras among in vitro plantlets making embryogenic callus an ideal plant material for mutation induction. Somatic embryos of *Jatropha* can be induced by direct somatic embryogenesis or indirect somatic embryogenesis (Kalimuthu et al. 2007). The difference between the two strategies is whether plants are regenerated directly from embryos or from an intervening callus phase.
21. Gamma ray mutagenesis may be performed using different facilities, such as gamma cell irradiator, gamma phytotron, gamma house, or gamma field. The gamma cell irradiator with cobalt-60 or cesium-137 as radioactive source is the most commonly available equipment worldwide.
22. Radioactivity is mutagenic and carcinogenic. It should be handled by trained personnel in a suitable laboratory. The safety precautions for exposing plant material to a gamma irradiation source must be strictly observed.
23. Perform radiosensitivity test for minimum of ten seeds per bag with at least three replications per dose, or 100 seeds per bag and per dose.

24. Exposure time is equal to the required dose divided by the dose rate of the day.
25. The amount of water has an important impact on the irradiation efficiency. A decreased amount of seed moisture content will increase the mutation frequency. Therefore, it is necessary to equilibrate the seed moisture content before the irradiation process.
26. Take care to observe all safety precautions before exposing tissues to irradiation.
27. For each exposure, dosimetry was performed following the Gafchromic dosimetry system. One 20 by 20 mm (for X-ray) film dosimeter is placed in a small paper envelope labeled with requested dose and then placed in the center of samples before each exposure. The optical density measurement is performed on a radiochromic reader 24 h after irradiation, and the dose was calculated according to the calibration Gafchromic dosimetry film with their optical density. That allows the estimation of the absorbed dose by the sample exposed to X-ray.
28. The irradiated samples are safe to be held in hands because the sample chamber isolates the plant material from the source and there is no surface contamination.
29. A Petri dish with untreated cuttings (control) has to be prepared and kept in the same conditions as the treated samples.
30. Incubate somatic embryos at the appropriate conditions according to direct or indirect techniques.
31. Observe general rules for plant tissue culture practices.

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