Tissue Culture and Genetic Engineering in Moringa

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Abstract

In vitro and aseptic culturing of cells, tissues, organs, and their components in artificial growth media and environments is referred as plant tissue culture, which has applications in basic and applied studies as well as in commercial production of quality planting materials. Role of plant tissue culture tools in Moringa has several folds of applications: understanding cellular activities (including cell growth and development, cytogenetics, metabolic process and nutrition, morphogenesis, embryogenesis, and pathology), manipulating plant genetic architecture and improvement, production of disease-/pathogen-free plants, in vitro germplasm storage, high-throughput clonal propagation, and product development (such as secondary metabolite production, natural color isolation, etc.). Besides, establishment of efficient Moringa in vitro culture is the critical basic requirement of genetic transformation (which provide potential of transferring gene (originated from any organism) that governs the expression of desirable traits). This chapter provides comprehensive information on Moringa tissue culture and its applications besides providing the future prospects.

8.1 Scope for Plant Tissue Culture in Moringa

Moringa is truly a tropical plant, which is largely grown in dry arid and semi-arid tracts. These areas are found to be the most suitable for its cultivation to obtain a profitable yield with minimum efforts. By realizing the huge demand for Moringa plants for commercial cultivation, all the possible methods to propagate Moringa have been tried. Conventionally, macropropagation methods such as asexual (or clonal) propagation (i.e., limb cuttings and air layering) and sexual propagation (i.e., seed multiplication) are frequently used to propagate Moringa.

Asexual or clonal propagation is usually described as production of several numbers of genetically identical copies of a cultivar by asexual means. Apomixis (which is defined as formation of seed without fertilization and meiosis) and/or vegetative propagation (reproduction of progenies from vegetative parts of the plants) are noticed as natural processes that help to induce clonal propagation.

Moringa propagation through limb cuttings is usually chosen by the farmers as it can be simply performed by them with the available limited resources. In addition, limb cutting from healthy crop develops roots very easily. Usually, cuttings

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of fairly large size stems (with a size of 1–1.35 m length and 14–15 cm girth) are planted in moist soil (preferably during the rainy season, June–August, in India) to easily establish roots, and it can attain a 2–3 m height within 3–4 months. Alternatively, in selected regions of Tamil Nadu, India, unproductive trees are trimmed to leave main stem with a length of ~1 m and maintained to grow 2–3 shoots. Once the shoots attain sufficient growth, shoots that are 2 m in length and 4–5 cm in diameter are used as additional planting materials (Ramachandran et al. 1980).

Similarly, shield budding is another successful vegetative propagation method as the budded trees start to yield after 5 months. In certain traditional varieties, this strategy was found to constantly provide significant yield at least for 13 years. At this point, it is also worth to mention that air layering, another imperative commercial and remunerative Moringa propagation technology is followed in Tamil Nadu, India. Air layering has been done on the live stem of Moringa by slicing or wounding the small part and wrap around a wounded section of the stem using moist sphagnum moss or well-composed farm yard manure.

However, all the above macropropagation technologies are useful for small-scale farming, and as you can observe from the aforementioned macropropagation protocols, large numbers of Moringa plants needed for high-throughput cultivation, cannot be multiplied using these methods within a given period of time and resources. Further, other limitations of these macropropagation methods are (i) trees from stem cuttings have shallow root system (and hence, frequently experiences water stress-related production constraints), (ii) reduces the total life span of the trees, and (iii) more importantly, results in poor yield (Islam et al. 2005). Though macropropagation methods are simple and successful for tree propagation, the current trend in Moringa cultivation for the production of leaf biomass forced to find other alternative methods of high-throughput propagation, such as seeds.

Therefore, besides vegetative propagation, Moringa is also propagated sexually using seeds. Usually, 50–90% of the fresh Moringa seeds germinate and it germinates 7–30 days after sowing. It should also be noted that Moringa seed lose its viability, if they are stored for more than 2 months. Sharma and Raina (1982) reported loss of Moringa seed germination at the rate of 50%, 48%, and 7.5% when they are sowed after 1, 2, and 3 months, respectively, of seed collection due to its high oil content (and also in certain cases, due to storage pest infestation). Thus, long-term storage of Moringa seeds critically affects the germination process, and hence, keeping the seeds even for the next season may have adverse effect on the germination.

In order to overcome these limitations, various priming strategies (such as hardening, hydropriming, osmopriming, matripriming, osmohardening, and hormonal priming) have been developed to rejuvenate Moringa seeds. Such priming treatments reduce the time required to germinate and thus increase the germination percentage or emergence index. Subsequently, increase in seed emergence leads to uniform crop stand, which ultimately results into increased yield. Moringa leaf extract is rich in cytokinin, and its application as a seed priming tool was also exploited in several crops, including Moringa. Nouman et al. (2012) found that hydropriming (soaking in water for 8 h) has promoted seed emergence, shoot vigor, and chlorophyll b contents, while priming with Moringa leaf extract for 8 h resulted in vigorous root development and enhanced levels of chlorophyll a and minerals in Moringa leaves.

Besides the problem of lower germination percentage, plants raised from seeds leads to non-uniform plants and produce fruits of inferior quality, and more imperatively, plants have invariably showed genotypic and phenotypic variations (Salem 2015). This is mainly due to the fact that Moringa is a cross-pollinated crop; the variation largely occurs owing to the segregation of undesirable traits in the subsequent generations.

Therefore, an affordable method (that can support high-throughput or large-scale production of quality Moringa planting materials) is required, and micropropagation or plant tissue culture is proposed as a method of choice at this

junction. Vegetative propagation of plants are now generally achieved by employing tissue culture or in vitro culture strategies, which involves aseptic culturing of tissues or cells under artificial chemical and physical conditions to produce huge numbers of true-to-type plantlets with relatively short time and space. This method is simply referred as micropropagation. Indeed, in several horticultural crops (such as Orchids), micropropagation is recognized as the merely viable method of propagation that has commercial value.

8.2 In Vitro Culture

In vitro culture is a cellular life process that occurs in a test tube, culture dish, or elsewhere outside a living organism. In plant system, such process is also referred as plant tissue culture (PTC), and it offers numerous applications for Moringa propagation as well as its genetic improvement. Applications of PTC in Moringa plants includes

(i) swift production of doubled haploids that lead to develop homozygous plants from the immature wide hybridized embryos with lesser time and smaller space,
(ii) propagation of endangered *Moringa* spp., which will be used as a valuable resources for genetic and physiological studies,
(iii) evolving novel genetic variation,
(iv) inducing mutagenesis and selection of cell lines for specific traits (such as production of useful secondary metabolites),
(v) exploring protoplast fusion that promotes wide hybridization and genetic transformation (explained in detail in later part of this chapter), and
(vi) high-throughput multiplications of desired Moringa varieties or ecotypes using micropropagation strategy.

Micropropagation is an outstanding strategy for both conservation of endangered plant species and production of disease-free plants. As an effective alternative strategy to the traditional propagation methods, micropropagation supports supply of quality planting materials throughout the year with minimum cost as it employs limited space and time.

8.3 Micropropagation in Moringa

Micropropagation refers to large-scale aseptic and in vitro production of true-to-type complete plants using small part of the mother plants (referred as explant), and there are three types of micropropagation:

(1) *Somatic embryogenesis*: which refers to formation of structures comprising shoot and root that are connected by a closed vascular system (this process resembles histology, physiology, and biochemistry of zygotic embryos),
(2) *Adventitious shoot production:* This is with reference to the de novo development of shoot meristem which are arising from callus or other organized tissues such as epidermal or subepidermal cells, and
(3) *Axillary shoot production*: This process involves production of additional shoots from axillary buds and meristems under in vitro conditions.

In this chapter, micropropagation is used to refer the mass multiplication of large true-to-type plants for commercial purposes, mainly for cultivation. Though the word micropropagation is also used to describe development of shoots from transgenic callus, preservation of male sterile and other pre-breeding plant materials, development of doubled haploid plants, etc., they were not referred in this chapter. Similar to its effective and extensive applications in several horticultural crops, micropropagation has also been shown to be flourishing in Moringa. Since Moringa cultivation requires clonal propagation of thousands of propagules to cover large acreage, micropropagation in Moringa is fundamentally considered as extensive scaling-up technology.

In order to increase the yield and biotic and abiotic stress resistance, exploring the concept of heterosis and hybrids has long been explored in crop plants. Novel genes can be introduced to the

elite cultivars by employing single- or double-cross hybrids using either controlled pollination or transformation technology (which is explained below in Sect. 8.8). Thus, by sequentially crossing four lines, as shown in Fig. 8.1, a double-cross system can be established, and the consequential hybrid can be micropropagated.

8.4 Basics and Practice of Moringa Micropropagation

Micropropagation is proposed as a viable commercial method to produce large numbers of Moringa propagules, since there are insufficient seeds or cuttings available for planting. The earliest report on Moringa micropropagation was presented by Mohan et al. (1995), and they successfully multiplied *M. oleifera* from immature embryo-, seedling-, and mature tree-derived explants. Though successful reports on micropropagation have long been reported elsewhere (Islam et al. 2005; Stephenson and Fahey 2004; Xiang et al. 2007; Marfori, 2010; Saini et al. 2012), till now it has not been established as a complete commercial venture. This section provides basic procedure of micropropagation in Moringa (Fig. 8.2) besides explaining its other potential applied aspects.

The fundamental requirement for Moringa micropropagation is selection of appropriate explants. Explants (defined as any part of the Moringa plant) have the ability to grow into complete plantlets under aseptic conditions in an artificial nutrient medium. This property of the explant is referred as *totipotency* which forms the basis of micropropagation.

Published reports have frequently used nodal explants obtained from non-aseptic sources such as young seedlings or mature plants. In general, a nodal segment of 2–4 cm is taken from the mother Moringa plant and sterilized for a short period with sterilizing agents such as 0.1% mercuric chloride (w/v) for 2 min and 20% sodium hypochlorite (v/v) for 10 min. Residual sterilizing agents are removed by rinsing the explants with sterile distilled water for three times. It is preferable to perform this surface sterilization of explants inside the laminar flow hood.

Alternatively, aseptic explants can also be excised from the in vitro grown Moringa seedlings as briefly described hereunder: using the above sterilizing agents, uniformly matured Moringa seeds can be surface-sterilized. This is followed by aseptic removal of seed coats and further sterilization with 20% sodium hypochlorite (v/v) (for 5 min), and finally, the residual sodium hypochlorite are removed by rinsing in sterile distilled water at least for three times. Such sterilized seeds are inoculated aseptically in Murashige and Skoog basal medium (MS salts; Murashige and Skoog 1952) containing 30 g L^{-1} sucrose (to provide instant energy) and 5 g L^{-1} agar (which provides semi-solid environment for the growth of plants). The pH of the medium is adjusted to 5.8, and the medium is distributed to culture bottles (@ 40 ml each) which are previously sterilized by autoclaving at 121 °C for 20 min. The inoculated nodal explants are kept in the dark at 27 ± 1 °C for 15 to 30 days

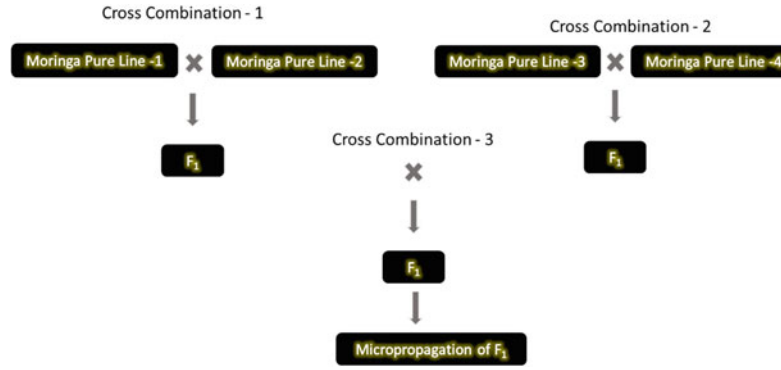

Fig. 8.1 A micropropagation scheme for large-scale production of double-cross Moringa line

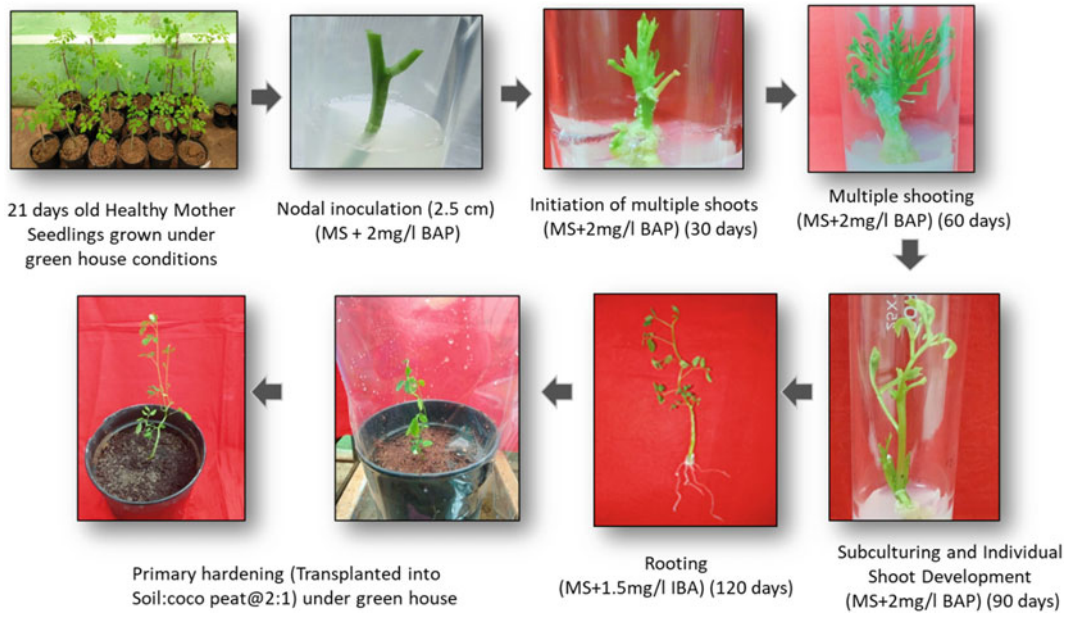

Fig. 8.2 Schematic workflow for micropropagation of Moringa cultivar PKM1

(depends on the Moringa variety/ecotype, cultural conditions, etc.), until they germinate. Once the seedlings got germinated, they are transferred under continuous light provided by cool white-fluorescent tubes (at 2,000-lx intensity). Germinated seedlings consisting of three to four nodes (which can be obtained within 3–4 weeks after inoculation) will be used in the downstream process as detailed below (Fig. 8.2).

8.4.1 Multiple-Shoot Induction

Nodal explants that were prepared either from field grown plants or grown under aseptic conditions, as above, will be subjected to induce multiple shoots by employing the following protocol: Nodal explants are planted on a multiple shoot induction medium (comprising MS salts supplemented with benzyl adenine (BA) @ 4.44 μM) for multiple axillary shoot formation.

Plant growth regulator present in the MS medium alone is not sufficient for effective development of shoots and roots, and hence, MS is supplemented with cytokinin and auxins. Usually, addition of cytokinin in the MS medium, such as BA, induces shoot formation, and auxins, such as IAA and IBA, enhance root formation in plant tissue culture protocol.

Micro shoots obtained are repeatedly subcultured (minimum of four and maximum of eight times) in MS basal medium accompanied with 4.44 μM BA at 20–30 days interval when it attained sufficient growth (usually 8–12 cm height).

8.4.2 Rooting of Micropropagated Moringa Shoots

Once sufficient number and growth of axillary shoots were developed from nodal sections, they need to be placed in a root induction medium (which is a MS medium supplemented with 2.85 μM indole-3-acetic acid (IAA) and 4.92 μM indole-3-butyric acid (IBA)). This change in media composition enhances in vitro rooting of micropropagated Moringa shoots within 7–10 days. For some of the Moringa ecotype, such high salt content of the medium may restrict growth and development of root. In that case, use of half-strength MS medium

supplemented with 0.5 mg L^{-1} IAA will be useful to induce roots. Once roots are developed (in general, 30–45 days are sufficient to develop prominent roots), such rooted plantlets are transferred to the soil for hardening.

Gupta et al. (2020) reported that complete removal of the surface contaminants was possible with the treatment of decoated seeds with 1% (w/v) Bavistin for 50 min, 0.33% (w/v) streptocycline for 30 min, and 0.1% (w/v) $HgCl_2$ for 3.5 min and maximum seed germination (89.13%) was obtained on quarter-strength MS medium. Complementary effect of plant growth regulators on axillary bud proliferation in Moringa has also been assessed by them, and it was noticed that culturing of nodal segments on MS medium combined with BA at 3 mg L^{-1} proliferated multiple shoots (maximum of 18 shoots per explants was reported in this study). Strikingly, it has been shown that lesser amounts of BA (0.5 mg L^{-1}) along with IAA (0.5 to 2 mg L^{-1}) or Kn (0.5 to 5 mg L^{-1}), showed combined effort in the shoot morphogenesis. In addition, the maximum of 100% rooting competence was accomplished with half-strength MS medium comprising either IAA or IBA. For example, average root length of 7.3 ± 0.8 cm can be induced using half-strength MS supplemented with IBA (0.1 mg L^{-1}).

8.4.3 Primary and Secondary Hardening

Acclimatizing the aseptically grown Moringa plants to the farmer's field condition is called as hardening. Initially, the rooted tissue culture plants are planted in plastic bags containing autoclaved mixture of soil, sand, and vermicompost (@ 3:1:1 v/v), and this process is called as primary hardening. During the primary hardening process, the plants require adequate care: i) plants should be kept under limited sunlight under greenhouse at surrounding temperature of ~25–28 °C and covered with transparent and perforated polythene bags, and ii) watering has to be done regularly, and if possible, it may be supplied as mist spray.

The plants can be kept under the above setup for 15 days and after that the polythene covers can be removed. However, the plants have to be maintained under the greenhouse for additional 15–20 days, and this process is referred as secondary hardening. Finally, secondary hardened plantlets are transferred to the farmer's field, and their performance is evaluated in comparison with the regularly propagated Moringa plants.

If there is any deviation in the performance of the micropropagated Moringa, it is advisable to reduce the number of subculturing or change the explant source. It was noticed that micropropagated plants were found to be nutritionally richer when compared with conventionally grown plants and Moringa also has witnessed such trends (Saini et al. 2012). Greater amount of phytochemicals and nutrients in the tissue culture-derived plants might be due to progressive shoot development of the tissue cultured plant (i.e., vigorous vegetative growth) that prevails during the planting which helps to reach better growth and development.

8.4.4 Clonal Fidelity Analysis and Confirmation of Authenticity of Micropropagated Moringa

Plantlets

The authenticity of micropropagated Moringa propagules can be typically verified by using molecular markers. Somaclonal variation is the rare phenomenon found during the micropropagation and other in vitro culture procedures. It is generally referred to changes in genetic elements (i.e., DNA) of plant tissue due to the micropropagation and such changes are attributed to:

(1) Employing several sources of explants,
(2) Physical and Chemical status of cultural conditions,
(3) Regeneration methods employed during in vitro culturing, and
(4) Long course of subcultural practices.

Additionally, induction of several chromosomal abnormalities owing to excess concentration of plant growth hormones is also found to curtail production of true-to-type plants. Thus, when Moringa micropropagation is employed to produce true-to-type quality plants, somaclonal variation is recognized as a major concern. Therefore, evaluating the genetic stability of micropropagated Moringa becomes mandatory.

In order to test the genetic authenticity of the micropropagated plants, simple and robust polymerase chain reaction (PCR)-based molecular markers such as Inter Simple Sequence Repeats (ISSR), Start Codon Targeted (SCOT) marker, Randomly Amplified Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP) can be employed (Boopathi 2020). If the banding patterns (that are produced by a set of random primers used in the above marker classes) are found to be identical, then it is used to confirm the clonality of all the investigated micropropagated plants.

It is generally recommended that use of more than one type of molecular marker could increase the efficiency of the genetic fidelity assessment (Behera et al. 2019) of micropropagated Moringa plants.

The procedure for testing the genetic fidelity using RAPD marker (Boopathi 2020) is briefly provided here: Extract the DNA from the leaf tissues of micropropagated plantlets that are randomly selected from each batch of in vitro culture using cetyl trimethylammonium bromide (CTAB) method (Doyle 1991) or using commercial kits. Select a set of 20–25 random primers (the sequence of each primer can be obtained from the website of Operon Technologies, Alameda, CA) and artificially synthesize it. PCR can be performed by preparing a total cocktail volume of 25 μl (by mixing 1 μl of the DNA sample (which is prepared as 50 ng/μl concentration), 2.5 μl of 10X *Taq* Buffer, 200 μM of each dNTPs, 2.5 mM MgCl2, 0.75 U of *Taq* polymerase, 0.8 μM primer, and make up to 25 μl with deionized water) using the commercially available Thermal Cyclers with the following amplification conditions: initial denaturation at 95 °C for 5 minutes to denature the entire Moringa DNA. This is followed by programming 35 cycles of 95 °C for 50 s, 37 °C for 45 s, and 72 °C for 45 s. Finally, a final extension of PCR products at 72 °C for 5 minutes is also programmed to complete the amplification of all the PCR products.

The PCR products are analyzed using 1.5% (w/v) agarose gel electrophoresis. The banding pattern is documented using a Gel Documentation System after staining the gel with ethidium bromide. Verification of all the micropropagated propagules that have produced the same banding pattern as that of mother plant confirms the fidelity of the micropropagated products. Variations in the banding pattern denote there is a somaclonal variation and the investigated micropropagation lot needs to be discarded to ensure that every micropropagated plants are true-to-type to the mother plant.

Fidelity of the uniform genetic content of micropropagated plants of drumstick tree was demonstrated with different molecular markers in several occasions. For example, Avila-Treviño et al. (2017) employed Randomly Amplified Microsatellite Polymorphism (RAMP) markers. They have also demonstrated that somaclonal variation was minimal in shoot tip-based clonal propagation of *M. oleifera* by validating true-to-type nature of in vitro raised plantlets using RAMP.

Similarly, clonality of the Moringa was assessed by Gupta et al. (2020) using RAPD and ISSR markers and found that there was an amplification of distinct, identical, and reproducible bands. Upon scrutinizing the micropropagated plants with RAPD and ISSR markers, they have found that there was a high monomorphism (95.39%) among the investigated plants. This confirms the high genetic stability and clonal fidelity among the tissue cultured plants.

8.5 Chief Applications of Micropropagation in Moringa

1. Mass propagation of genetically uniform planting materials: Instead of obtaining

~10,000 practically utilizable plants per year through vegetative propagation (such as limb cuttings that measures ~1 m with 12–15 cm girth) from a hectare of Moringa field, micropropagation can provide more than 1 million genetically uniform plants per year from 1000 s of explants (preferably nodal segments that measures 2–3 cm).

2. In vitro culture can be initialized from small parts of plants with relatively smaller space: 100 m^2 space of micropropagation unit (including culture room and greenhouse) is sufficient for annual production of 1 million plants.
3. Mass multiplication of disease- and virus-free plantlets make things easier during the international exchange of plants, besides offering huge reduction in cultivation cost involved in management of seed- and/or soil-borne pest and diseases during the establishment of Moringa garden.
4. Micropropagation also facilitates introduction of disease-free elite cultivars to the new production zone, elsewhere in the world.
5. Sterile hybrids or multiple cross hybrids (Fig. 8.1) can be easily multiplied using vegetative tissues using micropropagation.
6. Conservation of endangered *Moringa* spp., and their ecotypes is need of the hour. Cryopreservation of Moringa in vitro cultures will be very much useful to store such germplasm for long periods.
7. Genetic improvement of Moringa can be completed with lesser time than it requires usually through conventional breeding methods as micropropagation shortens the breeding cycle.

8.6 Key Issues in Micropropagation in Moringa

Though commercial viability of micropropagation in Moringa has been established, below points encapsulates key issues of micropropagation in Moringa:

1. Success in Moringa micropropagation is largely depending on type and source of explants and type of cytokinin and auxin. There is no simple universal combination of these factors for Moringa micropropagation, and they need to be standardized across the laboratories and ecotypes (Table 8.1). This requires significant initial investment in infrastructure development due to expensive laboratory equipment and service.
2. It has often been found that Moringa explants are continuously exposed to stress under in vitro culture conditions and that led to induce certain antioxidant enzymes (such as superoxide dismutase, catalase, and peroxidase) in the explants to combat such stresses. This is an extra burden to the Moringa growth and development under in vitro conditions and all the ecotypes are not responding to this stress uniformly.
3. Explant variations in the efficiency of somatic embryogenesis: Owing to the variations in endogenous plant hormones among the explants that are derived from different sources, all such explants are not uniformly responding to the tissue culture conditions. Therefore, it is recommended to use explants derived from in vitro grown Moringa (see Sect. 8.4) as they have superior ability to get into organogenesis when compared with explants excised from farmer's field (i.e., in vivo grown plants).
4. Degree of somaclonal variation: As explants derived from different sources have different rates of in vitro regeneration potentials, genetic variation among regenerated plants is widespread in the micropropagation process. This necessitates vigorous testing of genetic fidelity of the in vitro plants, and it is, thus, highly essential to obtain true-to-type plants. Such endeavor further enhances the efforts, time, and money that involved in micropropagation of Moringa.
5. Cost of production: In order to find the finest in vitro culture conditions for Moringa micropropagation, it requires several costlier studies and efforts (such as ascertaining the

Table 8.1 Standardization of culture conditions with respect to different explants of Moringa (only selected references are shown here to represent the need for standardization of micropropagation in Moringa)

Explants employed	Sterilizing agent employed	Media and supplements	Remarks or additional works employed	References
Stem cuttings from the in vitro germinated seeds	70% ethanol for 2 min + 15 min in 25% Clorox™ bleach	MS + 1 mg L^{-1} BAP, 1 mg L^{-1} GA3, 0.25% activated charcoal for shoot multiplication Half-strength MS + 0.5 mg/L NAA (93.1 mM) for primary and secondary root development	The success rate was 73% and the multiplication rate averaged 4.7 shoots per culture	Stephenson and Fahey (2004)
Cotyledonary node of decapitated seedlings	1% sodium hypochlorite solution mixed with a drop of Tween-20 for 20 min	Full strength MS for multiple shoot induction Half-strength MS for rooting	Attempted micropropagation in *Moringa Oleifera* Lamk., *Moringa stenopetala* (Baker f.) Cufod, and *Moringa peregrine* Forssk. ex Fiori and established method to secure biodiversity; establishment of vegetative clones and avoided laborious and time-consuming usage of plant growth regulators	Steinitz et al. (2009)
Nodal segments from in vitro germinated seeds	Seeds surface-sterilized by wiping them with 95% ethanol and flaming for 30 s	MS + 2.5 μM BAP produced 4.5 axillary shoots per explant MS + 0.25 μM NAA for rooting	Only 80% plants survived	Marfori (2010)
Nodal segments from in vitro germinated seeds	Sterilizing seeds: 0.1% mercuric chloride (w/v) for 2 min and 20% sodium hypochlorite (v/v) for 10 min	MS + 4.44 μM BA produced 9.0 ± 1.0 axillary shoots per explant MS + 2.85 μM IAA + 4.92 μM IBA for 100% rooting	Eighty percent of the tissue cultured plants survived after transplanting and they were found nutritionally superior over conventionally grown plants	Saini et al. (2012)
Nodal segments obtained from pot grown seedlings	70% ethyl alcohol (v/v) for 30 s and 20% sodium hypochlorite (v/v) for 3 min	Shoot multiplication: MS + 2.5 μM BAP under mild ventilation Root Induction: MS + 4.92 μM IBA for adventitious	Under salinity, in vitro shoots showed symptoms of vitrification	Salem (2015)
Nodal segments and shoot cuttings (shoots and shoot tips)	Consequent treatment with 70% ethyl alcohol and 0.1% HgCl2 for 3 min in each step	MS + 0.55 mg L^{-1} of BAP produced 9.57 ± 2.08 shoots. Half-strength MS + 0.5 mg L^{-1} IAA for successful root formation	Impact of reactive oxygen species on vitrification was studied	Hassanein et al. (2019)

(continued)

Table 8.1 (continued)

Explants employed	Sterilizing agent employed	Media and supplements	Remarks or additional works employed	References
Nodal segments from in vitro germinated seeds	1% (w/v) Bavistin for 50 min followed by 0.33% (w/v) streptocycline for 30 min and 0.1% (w/v) $HgCl_2$ for 3.5 min	Multiple shoot proliferation: MS + 5-benzyladenine (BA) (3 mg L^{-1}) Rooting Media: half-strength MS + IAA (0.1 mg L^{-1}) + IBA (0.1 mg L^{-1})	Multiplication ratio of 18 shoots per explant and 100% rooting efficiency was reported. Genetic uniformity of the micropropagated plants was confirmed using molecular markers	Gupta et al. (2020)

type and source of explants, best concentration and type of growth regulators, and affordable and optimal media type that stimulate better organogenesis).

6. Another common problem in Moringa micropropagation is vitrification of the regenerated shoots. Vitrification (also well recognized as Hyperhydricity) is a physiological malformation found in the in vitro regenerated Moringa plants due to disproportionate hydration, little lignifications, weakened stomatal activity, and decreased mechanical strength of stems. Therefore, the regenerated Moringa plants that experiences hyperhydricity often possess poor acclimatization to the farmer's field, which is already characterized as a resource limited environment. Therefore, in order to realize the potential of the micropropagation in Moringa, problems of both vitrification and somaclonal variation have to be circumvented. To this end, Hassanein et al. (2018) proposed the addition of silver nitrate and salicylic acid in the culture medium to avoid vitrification and for durable maintenance of vigorous shoots.

8.7 Untapped Applications of Plant Tissue Culture Strategies in Moringa

Besides micropropagation, other strategies of plant tissue culture (including protoplast fusion and cybridization, exploiting desirable somaclonal variation, in vitro germplasm conservation, production of double haploids, cell suspension culture and secondary metabolite production, and fabrication of synthetic seeds) may offer numerous applications in Moringa. Though such applications have not been fully realized in Moringa, their potentials are briefly described hereunder anticipating their utility in Moringa.

8.7.1 Protoplast Fusion and Cybridization

Sexual breeding is the key process by which genetic materials are transferred from one species to another eukaryotic species. Though transfer of genetic materials has been realized among distantly related plant species, barriers such as sexual incompatibility restrict the development of complete and viable hybrids between desired and distantly related plant species. It is also well documented in Moringa that even within species and intra-varietal cross combinations, success has been found to be difficult due to several factors (including small flowers that occur in fragile tree branches). To this end, somatic hybridization resulting from fusion of unrelated cells (for example, *M. oleifera* and *M. drouhardii* or *M. ovalifolia* and *M. stenopetala*) can be employed as a novel approach to execute distant hybridization in Moringa, and fusion can be accelerated through the plasma membrane.

However, plasma membrane in plants is surrounded by strong cellulosic walls, which are

further strengthened by pectin-rich matrix that tightly joins neighboring cells. Therefore, advances in animal somatic cell genetics is stronger (as there is no such boundaries in animal cells that surrounds plasma membrane) than plants. To break this barrier, in 1950, E. C. Cocking has first reported the successful removal of the plant cell walls using enzymes and demonstrated a procedure that acquired hefty numbers of protoplasts (which is actually viable naked cells).

Besides being able to fuse with each other, higher plant protoplasts can also take up foreign DNA, through their naked plasma membrane under specific chemical and physical treatments. Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.

However, utilization of these isolated protoplasts has been realized only when they were fused with each other, though they are isolated from different species. The process of fusing two differently sourced protoplasts was named as *somatic hybridization* as this technique totally bypassed the sexual means of hybridization. Another uniqueness of somatic hybridization is it also brings together cytoplasmic organelles from both the parents as opposed to the sexual reproduction (where only female parent contributes organelle genomes).

However, it should also be noted that though recombination in chloroplast genome of somatic hybrids was found to be rare as compared to mitochondrial genome (in which recombination happens frequently), but chloroplast segregation from either one of the parent would lead to unique and desirable nuclear-cytoplasmic genetic amalgamation and such process is called as *cybridization*. Thus, the cybridization will lead to the development of cybrid, which is referred as a cell line that has the nucleus of one parent and extra-nuclear genome(s) of the other parent.

Therefore, harnessing both the potentials of the cytoplasmic genes of one species and nuclear and cytoplasmic genes of another species is the key objective of the cybrid development. However, proofs on distribution of chloroplasts and mitotic segregation of plasma genes indicated that only a small proportion of the regenerated plantlets can be denoted as cybrid, and majority of the plantlets possess plasma genes of either one of the species.

Even though poor efficiency of cybrid development in plants has been reported, cybrid offers the following applications in plants including Moringa:

1. Simple and efficient transmission of plasma genes from one species into another (irrespective of the species, whether they are sexually compatible or not) within one generation.
2. Efficient revival of recombination occurred between the DNAs of parental mitochondria or chloroplasts.
3. Evolving different arrays/permutation of parental and recombinant chloroplasts with the various parental or recombinant mitochondria.
4. It is also possible to evolve a cybrid with mitochondria from one parent and chloroplast from another.
5. Cytoplasmic male sterility (CMS) has been successfully transferred from *Nicotiana tabaccum* to *N. sylvestris* and *Petuniahybrida* to *P. axillaries* using cybridization, since this process offered several advantages: (i) simple procedure as it is a single step procedure, (ii) the property of the elite cultivar is conserved as the nuclear genotype is unaltered and (iii) more importantly, all the progenies of cybrid definitely will be a CMS line.

8.7.2 Somaclonal Variation

Morphological and genetic variation found in the in vitro regenerated plants are described as somaclonal variation, and it has been found that such variations are mainly due to chromosomal rearrangement, modifications in ploidy level, simple and/or complex mutations in DNA or due to epigenetic factors such as exposure to plant growth regulators (PGRs), and prolonged culture time. Thus, there could be two kinds of somaclonal variations:

(1) Heritable or Genetic variability which is due to changes in DNA including mutations, chromosomal aberrations, and
(2) Non-heritable or Epigenetic variability that are found as transitory morphological changes due to in vitro culture conditions but cannot be found in the next generation.

There are several other features that are also responsible for induction of somaclonal variation during micropropagation and they are comprehensively narrated below.

8.7.2.1 Physiological Causes of Variation

Physiological status of the explants, such as response to PGR in the growth medium and environmental conditions during the micropropagation protocol, may induce somaclonal variation. However, such factors are epigenetic, and hence, those variations would not be identified in subsequently subcultured plants and/or may not be found as Mendelian inheritance. For example, variation in regenerated plants were often found when the explants were grown in strong auxins such as phenoxyacetic acid (e.g., 2, 4-D, or 2, 4, 5-T).

8.7.2.2 Genetic Causes of Variation

Changes in nucleotide sequence or even at chromosomal level some time result in somaclonal variation. Though the regenerated plantlets are morphologically similar, the tissues of such plants originate from different sources of cell types. Hence, those regenerated plants are cytologically different as they regenerated from the dissimilar explants.

8.7.2.3 Biochemical Causes of Variation

Simple changes in biochemical pathway will lead to large implications in cellular process. For example, changes in carbon metabolism will result in poor photosynthesis (which may cause albinos in regenerated plants), and this will also have great impact in starch biosynthesis and carotenoid pathway. Variations due to changes in biochemical pathways are the most frequently found somaclonal variations during micropropagation.

8.7.2.4 Benefits of Somaclonal Variation

Though it is a major menace to micropropagation (see Sect. 8.5), such variations are sometimes beneficial. The importance of somaclonal variation has been recognized in the following areas:

1. Somaclonal variation can be used as a novel strategy to evolve desirable genetic variation in crop plants. For example, herbicide-resistant crop cultivars can be evolved by culturing its explants in the media mixed with the target herbicide. Other potential areas are developing plants resistant to disease or patho-toxins and tolerant to environmental or chemical stresses. On the other hand, identification of desirable somaclonal variation warrants careful experimental design, drawing systematic information, and utilizing the pre-breeding materials in the regular breeding program.
2. Understanding the mechanisms behind the somaclonal variation may open up new avenues in plant breeding strategies that aim to develop novel genetic variation in crop plants. It also provides fundamental molecular mechanism behind the somaclonal variation and why the clonal uniformity fails, even though it is believed that micropropagation produce true-to-type plants.
3. Somaclonal variation can also be explored to produce novel culture that has ability to produce large quantity of secondary metabolites (see Sect. 8.7.5).

When the objective of the tissue culture experiment is producing true-to-type plants or genetic transformation, it is important to reduce or completely minimize the somaclonal variation. To this end, the following strategy can be followed:

1. Frequent molecular assay (such as RAPD, SSR, ISSR; see Sect. 8.4.4) with the batch-wise subcultured plants and confirming the clonal fidelity when the objective is micropropagation of elite genotypes.

2. Somaclonal variation increases as the numbers of subculture, and hence, minimum numbers of subcultures are essential, and it is desirable to start fresh cultures using new explants. Besides, use of strong PGR such as 2,4-D in the culture medium may be avoided as it may induce variation frequently.

At this point, it should be noted that though somaclonal variation has great potential in Moringa genetic improvement program, it has not yet been reported.

8.7.3 Synthetic Seeds

In majority of crops, it has been found that the seeds are simple and affordable way of national and international exchange as storage and transportation of micropropagated plants require sophisticated transportation facility. On the other hand, seed propagation has several other inherent limitations (see Sect. 8.1). These limitations can be avoided when synthetic seeds are used.

Synthetic seeds are prepared by encapsulating somatic embryos with a suitable matrix (such as sodium alginate) enriched with mycorrhizae, insecticides, fungicides, and herbicides.

In India, Bhaba Atomic Research Center (BARC), Bombay, standardized and demonstrated the preparation of synthetic seeds in sandalwood and mulberry as early as in 1990. However, it has not yet been practiced in Moringa, and exploring such possibilities may have many applications as described below:

(i) Devoid of losing the viability, synthetic seeds can be kept for one year,
(ii) Synthetic seeds can be easily produced and simply exchanged among national and global organizations, and
(iii) As that of normal seeds, synthetic seeds can be grown in soil as direct sowing, and it does not require any hardening procedure as that of micropropagated planting materials.

However, production of synthetic seeds involves costlier efforts. As novel strategies and cost-reduction procedures are being developed, the cost of synthetic seed production will be reduced in the near future, and hence, it is expected that synthetic seeds will catch up Moringa commercial sector shortly.

8.7.4 In vitro *Germplasm Conservation*

With respect to seed storage for long period of time, seeds are grouped as orthodox seeds and recalcitrant seeds. The seeds that can tolerate the dehydration up to 5% (or sometimes even less than 5%) and retain the viability are referred as orthodox seeds and they can be stored in very low temperature with that moisture content.

On the other hand, seeds that possess highest moisture and cannot tolerate desiccation are grouped as recalcitrant seeds; the plant species living in tropical or subtropical regions produce such kind of seeds and such seeds can be maintained only in wet medium. Such maintenance will evade dehydration injury, and it also imperative to maintain them under reasonably warm environment as these species experience damage due to cold environment. Therefore, recalcitrant seeds (e.g., Moringa, oil palm, coconut, cacao coffee) can be stored for a short period (maximum of few months) even under appropriate moisture conditions and environment.

Another issue with recalcitrant seeds (e.g., gymnosperms and angiosperms) is their life span is lengthy and needs several years to set the seeds. Similarly, preserving the vegetatively propagated plants such as cassava, potato, and yams also have certain issues in long-term storage. Additionally, as there is continuous reduction in wild areas due to urbanization, in situ conservation is also impracticable, and efforts through ex situ conservation are also found to be complex as collection and conservation of all the ecotypes that represent the complete genetic diversity existing in the given species is a herculean task.

To circumvent the above issues, conservation through in vitro strategies has been proposed and

several procedures for storing thousands of tropical plant species have been standardized. In vitro tissue culture techniques have been applied for not only to conserve recalcitrant and vegetatively propagated species for long time but also possess following other applications:

(1) High-throughput production of quality planting materials.
(2) As it involves aseptic means of production system, the plants produced from in vitro strategies are devoid of fungi, bacteria, viruses, and insect pests.
(3) It requires little space when compared with other conservation strategies.
(4) There is nil genetic erosion as the plants are maintained under controlled conditions.
(5) It drastically reduces the production cost as it involves less workforce.
(6) Conservation through in vitro tissue culture system simplifies the international exchange of germplasm as the sample size can be reduced to minimum (which facilitates easy shipment under sterile conditions).

Though the use of in vitro germplasm conservation in Moringa species has been reported earlier, its potential has not yet been fully explored in conservation of all the Moringa species and their ecotypes due to the following limitations of this strategy:

(i) Initial investments in establishing the in vitro tissue culture protocol for long-term storage and imparting skills to manpower to maintain the stock,
(ii) Cautious management of subcultured materials of Moringa as it involves skillful exercise, and
(iii) As the duration of in vitro storage increases, the threat to genetic variation among the stored Moringa subculture also increases, and hence, it greatly affects the uniformity of the genetic materials.

However, as new tools and technologies develop in plant tissue culture, such limitations will be avoided, and Moringa germplasm can be conserved for longer period for the people of coming century.

8.7.5 Doubled Haploids

It is always preferable to have homozygous lines of the cross-pollinating species and hybrids as it simplifies the procedure of selection and further multiplication of uniform plants. Moringa is a cross-pollinated crop. Development of homozygous lines in Moringa is a time-consuming and laborious process and requires at least seven to eight generations of self-pollinated progenies. Further, such effort is impossible in self-incompatible, male sterile, and tree species.

Alternatively, by employing diploidization of the haploid tissues, homozygous lines can easily be produced within a single generation. This process of producing homozygous dihaploids (or doubled haploids; DH) through plant tissue culture procedure is considered to be stable and equivalent to Fα generation of the conventional breeding strategy, and hence, it is considered as fastest method of producing homozygous lines.

In order to diploidize the pollen obtained from the target crop, colchicine is usually employed, and it is applied for this purpose in several ways: (i) immersing the pollen derived plants (also called as anther cultured plants) in colchicine, (ii) smeared as lanolin paste, (iii) introducing colchicine into the secondary buds, and (iv) feeding colchicine through roots.

It is established that colchicine induces chromosome duplication and changes in genic and non-genic elements and instabilities in chromosomes. Chromosome duplication is the widespread impact of colchicine, and hence, it has been explored in duplication of chromosomes in haploid chromosomes of regenerated plant as well as callus cells to generate homozygous and fertile diploid plants.

During this process, callus is induced by culturing the explants (pieces of vegetative parts such as stem, root, or petiole segments) in an appropriate medium. Though the very few diploid callus are found during the initial period, the frequency of obtaining desirable diploid cells will be increased as the subculturing number increases. The selected diploid calli are subjected to the regeneration process and the plants derived from this method are generally diploid. Upon

confirming the diploid status, the plants can be used in the further downstream experiments.

It takes nearly a decade to release a novel variety (require 4–5 years for backcrossing and 4–5 years of inbreeding) in a routine conventional breeding program especially under hybridization strategy. However, fixing of desirable F_1 hybrids is possible by employing anther culture of the hybrid gametes within a single generation. Further, novel recombinations may also be generated during anther culture and such recombinations can also be concurrently fixed.

Despite such potential benefits, DH lines have not yet been explored in Moringa, and it is strongly believed that upcoming Moringa breeding program will explore this potential avenue.

8.7.6 Cell Suspension Culture and Secondary Metabolite Production

Nutritional, medicinal, and therapeutic content of various plant parts of *M. oleifera* has been reported repeatedly in several scientific journals, and Mahmud et al. (2014) provided a comprehensive metabolite database specific to each tissue of *M. oliefera*. This database provides details on metabolite spectra (using one-dimensional proton nuclear magnetic resonance) of different secondary metabolites and their biochemical pathways.

However, minimum studies have attempted to isolate desirable secondary metabolites from *M. oleifera* using cell suspension culture even though large numbers of studies have focused on its medicinal and nutritional values. Further studies should be concentrated on production of bioactive secondary metabolites using cell culture as it offers an opportunity to produce round the year production in a controlled environment which is not affected by climatic and soil conditions.

As an effective system of secondary metabolite production, cell suspension culture also ensures uniform quality, high quantity and purity, and continuous supply when compared with other isolation procedures. Such products have potential applications in medical industries as they are shown to possess anti-mutagenic, anti-carcinogenic, anti-aging properties.

However, the final yield of secondary metabolites by cell suspension culture through regular procedure is found to be low, and hence, use of certain enhancing procedures such as light irradiation with UV light, addition of jasmonic acid, ozone, heavy metal, ethylene, and sucrose seem to be increasing the yield of secondary metabolites.

Though industrial level production of food additives such as anthocyanins, shikonin compounds, safflower yellow, saffron, and other colorants have been reported using cell culture in other crops (Misawa 1994), incredibly rare reports are available in Moringa secondary metabolite production using plant cell suspension culture as detailed below.

As a first attempt, Mustafa et al. (2020) reported isolation of secondary metabolites using suspension cultures of Moringa tissues (especially leaves and seeds). They have also confirmed the specific antioxidant and antitumor activity of the isolated secondary metabolites against three cell lines, and highlighted that the Moringa callus extracts have presented higher antioxidant and cytotoxic activities than extracts obtained from the Moringa leaves and seeds as the Moringa calli were found to have higher amount of the secondary metabolites. This study provides the experimental proof that production of secondary metabolites from Moringa cell culture would be more productive than directly using Moringa leaf and seeds. In line with this, more experiments with Moringa cell suspension culture will shoot up soon as there are enough reports on importance of Moringa metabolites in therapeutic industries.

8.8 Genetic Engineering and Its Applications in Moringa

Crop improvement program generally utilizes introduction and selection, hybridization, and mutation strategies as the main methodologies.

However, transfer of desirable alleles through hybridization or increasing the genetic variation through mutagenesis requires several years of continuous work and huge resources.

Further, selection pressure on the breeding for desirable crop cultures for hundreds of years has also resulted in narrow genetic diversity in the cultivated crop gene pool and greatly hinders the further improvement in agronomically important traits. Though mutation breeding by employing both chemical and physical mutagens has been explored to expand genetic variation, it was found to be a random procedure and again this procedure is limited by its complicated and uncontrollable molecular mechanisms. Further, it is a labor- and time-intensive task as it needs to develop and screen huge numbers of mutants. Therefore, conventional breeding strategies seem to be exhausting, lengthy, and having untargeted results and cannot meet out the current demand on food, fodder, and fiber production. To this end, transgenic breeding or genetic engineering of crop plants is proposed as a viable strategy as it helps to evolve novel cultivars by transferring genes/traits from any exogenous biological system, which is not possible through conventional breeding.

Promises of genetic engineering or recombinant DNA (rDNA) technology towards increasing the agriculture productivity have been realized in several occasions (https://www.isaaa.org/kc/default.asp). Though it is not a standalone technology, but it has unique ability to transfer genes between entirely different organisms, and thus, it helps to ensure higher crop yields and enhanced nutritional value of the plant products. Actually, rDNA technology is considered as a complementary tool to traditional plant breeding approaches by facilitating fast track transmission of agronomically important traits among the crop plants.

Transgenic technology has revolutionized the plant breeding approaches, and globally 191.7 million hectares are planted with transgenic crops with improved traits (such as traits associated with herbicide resistance, pest and disease resistance, and quality) in 25 countries by 17 million farmers (www.isaaa.org accessed on 17thAugust, 2020). Another promising and more précise improvement in transgenic technology is *CRISPR/Cas* genome editing, which enable efficient targeted modification at nucleotide level. Recent developments in *CRISPR/Cas* systems and novel approaches in base editing may have great impact in breeding resilient crop cultivar as such developments will reduce the cost and skill required to develop efficient genome edited plants in several crops (Chen et al. 2019).

Despite such benefits shown in several economically important crops, transgenic technology has not yet been explored in Moringa. Transferring appropriate genes that control pest and diseases in Moringa would require immediate attention, as there are new pests (e.g., tea mosquito bug) and diseases emerging in some pockets of Moringa cultivation. At the same time, it should also be kept in mind that releases of genetically engineered organisms are not accepted everywhere in the world since it is speculated that they may have objectionable ecological, social, and economic effects. Further, lengthy and expensive regulatory process of evaluating transgenic crops and general acceptability of transgene(s) will limit the immediate commercialization of genetically modified Moringa.

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