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In Vitro Micropropagation and Somaclonal Variation for Conservation of Endangered Medicinal Plants

Dr. Rajender Gandu[1], Dr. Padala Thirupathi[2]

[1]Junior Lecturer in Botany, Government Junior College, Fort Warangal, Telangana.
[2]Asst. Professor of Botany, SRR Government Degree and P.G. College, Karimnagar, Telangana.

Abstract:
The traditional and modern pharmaceuticals obtained from many medicinal plants are diminishing due to overharvesting, loss of habitat, and slow natural regeneration. Although in vitro micropropagation is a speedy and season-independent means of propagation of threatened species, it does not necessarily prove to be a conservation approach, as the quality of the material produced may vary from what is desirable. This fidelity can be compromised by heritable change that can occur during tissue culture, known as somaclonal variation, but it can also produce useful change. This paper aims to bring together the available information regarding the existing protocols for micropropagation, somaclonal variation in plants and its detection, and the interaction of these two aspects in the conservation of endangered medicinal plants. It is based on published literature and a representative multi-species protocol framework, and contrasts explant types, culture media, and plant growth regulator regimes; beyond this, it correlates the duration of culture, concentration of the cytokinin, and the regeneration pathway with variation frequency. Representative data suggest that axillary shoot proliferation under moderate cytokinin concentration produces 6-12 shoots per explant with low variability, while callus-mediated regeneration causes a significant increase in variability. Molecular markers (RAPD - ISSR), flow cytometry, and phytochemical profiling are a useful pipeline for assessing fidelity. It suggests an integrated workflow that integrates micropropagation, cryopreservation, genetic monitoring and reintroduction, and defines research priorities for genetically safe, policy-relevant conservation of the paper.

Keywords: micropropagation; somaclonal variation; medicinal plants; ex situ conservation; genetic fidelity; plant growth regulators; cryopreservation; molecular markers.

1. Introduction

Medicinal plants remain the foundation of primary health care for a large share of the world's population and the source of many leading pharmaceutical compounds. Demand for plant-derived raw material has grown steadily with the expansion of herbal medicine, nutraceutical and cosmetic industries, and most of that material is still collected from the wild rather than cultivated (Chen et al., 2016; Schippmann et al., 2002). In India alone, hundreds of species enter trade in significant volumes each year, and a considerable proportion of these are harvested destructively, through the removal of roots, bark,

rhizomes or whole plants (Ved & Goraya, 2008). Such pressure, combined with deforestation, grazing, climate change and fragmentation of habitats, has pushed a growing number of species towards extinction. Species such as *Rauvolfia serpentina*, *Saussurea costus*, *Nardostachys jatamansi*, *Podophyllum hexandrum*, *Commiphora wightii* and *Coptis teeta* are examples of high-value medicinal plants that are now assessed as threatened in global or regional conservation assessments (International Union for Conservation of Nature [IUCN], 2023).

Conventional propagation of these species is often inadequate for conservation. Many have low seed viability, deep dormancy, long juvenile phases, poor seed set or specialised pollinator and habitat requirements. Vegetative propagation by cuttings, division or layering is slow, seasonal and yields few plants from each mother plant. Collecting large amounts of propagation material also places additional stress on the very populations that need protection. Conservation programmes therefore need technologies that multiply plants quickly from very small amounts of source tissue, work throughout the year and keep the stock free of disease.

Plant tissue culture meets several of these needs. Micropropagation, the clonal multiplication of plants from small explants under aseptic, controlled conditions, can produce thousands of plantlets from a single shoot tip or nodal segment within months (Bhojwani & Dantu, 2013). Since the pioneering work on defined media and hormonal control of organogenesis (Murashige & Skoog, 1962; Skoog & Miller, 1957), in vitro techniques have been applied to a wide range of threatened species, and they are now accepted as a component of integrated conservation strategies, alongside seed banking, field gene banks and in situ protection (Engelmann, 2011; Sarasan et al., 2006). Medicinal plants in particular have benefited from the approach because uniform, disease-free planting material also supports sustainable cultivation and reduces the incentive to harvest from natural stands (Debnath et al., 2006; Rout et al., 2000).

However, culturing plant cells outside the plant body is not genetically neutral. Larkin and Scowcroft (1981) coined the term *somaclonal variation* for the phenotypic and genotypic differences that appear among plants regenerated from cultured cells, tissues, and organs. Variation may arise from point mutations, chromosomal rearrangements, changes in ploidy, activation of transposable elements, and epigenetic modification such as altered DNA methylation (Kaeppler et al., 2000; Miguel & Marum, 2011; Phillips et al., 1994). This variation is sometimes taken advantage of for crop improvement, in search of new traits (Jain, 2001; Evans, 1989). The situation for conservation is different. Ex situ propagation is used to maintain the genetic diversity and adaptability of the source population, so that plants for restoration and reinforcement have the genetic diversity and adaptability of the source population. A lack of control over the variation, especially the change of secondary metabolite patterns, is detrimental to the conservation integrity and therapeutic use of the material.

This paper thus explores the two facets of micropropagation and somaclonal variation as two aspects of the same conservation problem. It summarizes the main factors affecting the efficiency of regeneration, reviews the sources and detection methods of somaclonal variation, and highlights representative comparative data that provide some indication of the effect of protocol choices on multiplication and genetic fidelity. It then suggests a process where micropropagation, genetic fidelity testing, reintroduction, and cryopreservation are connected. The objectives are:

1. To narrow down the factors controlling efficient micropropagation of endangered medicinal plants;
2. To evaluate causes, incidence, and impact of somaclonal variation in conservation culture systems; and

3. To assess the genetic and chemical fidelity of marker-based and phytochemical methods; and
4. To put forward a viable and holistic approach to the conservation of genetic safety and set research priorities.

2. Review of Literature

2.1 Status of threatened medicinal plants and the need for ex situ measures

Overexploitation is always one of the major threats identified during the assessments of medicinal plant diversity, followed closely by habitat loss (Chen et al., 2016; Schippmann et al., 2002). Species with underground storage organs, resinous exudates, or bark of therapeutic value are especially vulnerable because harvesting kills the plant. In situ conservation through protected areas, sacred groves, and community management remains the preferred strategy because it maintains evolutionary processes. However, in situ measures alone cannot rescue populations that have fallen below viable size, and they depend on enforcement and livelihood alternatives that are slow to develop. Ex situ methods, including seed banks, botanic gardens, field gene banks and in vitro collections, therefore act as an insurance policy and as a source of material for population reinforcement (Engelmann, 2011; Sarasan et al., 2006). In vitro collections are particularly useful for species whose seeds are recalcitrant, scarce or sterile, because plant material can be held as shoot cultures or, for longer periods, in cryostorage (Cruz-Cruz et al., 2013).

2.2 Principles of micropropagation

Micropropagation relies on totipotency, the capacity of differentiated plant cells to regenerate whole organisms when supplied with suitable nutrients and hormonal signals. Three routes are used. Axillary shoot proliferation uses pre-existing meristems from shoot tips and/or nodes, and is the most genetically conservative method. Adventitious organogenesis results in shoots or roots being formed directly from the tissue of the explants, usually from leaf, internode, or root fragments. The highest number of propagules can be produced through somatic embryogenesis, typically through a callus step, but with the highest variability risks (Bhojwani & Dantu, 2013). The key regulating step is the ratio of auxin to cytokinin - a relationship that was established by classic research of Skoog and Miller (1957): high ratios of cytokinin to auxin promote shoot formation, and the converse promotes rooting.

The Murashige and Skoog (1962) medium, Gamborg et al. (1968) B5, and woody plant medium (Lloyd & McCown, 1980) are the most popular formulations. Choice depends on the species' mineral tolerance, with woody and some Himalayan species responding better to reduced-salt media. Carbon source and concentration, gelling agent, pH, light quality, photoperiod, and temperature all modify responses. Common problems in medicinal species include explant browning caused by phenolic oxidation, bacterial and endophytic contamination, hyperhydricity (vitrification), and recalcitrance to rooting. These are managed by antioxidants such as ascorbic acid and activated charcoal, by surface sterilisation protocols adapted to tissue type, by adjusted agar concentrations and by liquid-phase or temporary immersion culture.

2.3 Micropropagation of endangered medicinal plants

A substantial body of work now documents protocols for medicinal species of conservation concern. Reviews of in vitro manipulation of medicinal plants record successful regeneration for hundreds of taxa, including alkaloid-yielding, glycoside-yielding and essential-oil-bearing species (Debnath et al.,

2006; Rout et al., 2000). Typically, nodal or shoot tip explants from selected mother plants are established on MS medium supplemented with a cytokinin, most commonly 6-benzylaminopurine (BAP) or kinetin, sometimes with a low concentration of an auxin such as naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA). Rooting is done on half-strength media in the presence of an auxin, and acclimatisation takes place in sterile media under high humidity conditions. Hardened plants often have survival rates above 70 percent with successful protocols, but the rate varies greatly between species (Debnath et al., 2006).

The financial impact is a reality. The per-plant cost of in vitro propagation and preservation ranges by orders of magnitude depending on the species, labour, and scale (Pence, 2011), and this supports placing a priority on species with no alternative mode of propagation. This economic fact should mean that micropropagation should be used in conjunction with other potentially available methods, such as cryopreservation for long-term storage, to ensure that actively growing cultures are kept to the minimum necessary.

2.4 Somaclonal variation: origins and mechanisms

Larkin and Scowcroft (1981) showed that regenerants from cultured cells frequently differ from their donor plants in morphology, physiology and yield characteristics. Later work established that the sources of variation are diverse. Pre-existing variation in explant tissue, including chimeral arrangements and differences in ploidy between cell layers, can be revealed when cells are induced to regenerate independently. Culture-induced variation includes point mutations, chromosome breakage and rearrangement, aneuploidy and polyploidy, transposon activation and loss or gain of DNA methylation (Kaeppler et al., 2000; Phillips et al., 1994). Epigenetic changes are now recognised as a major component, since stress during culture, from exogenous hormones, osmotic and oxidative conditions and wounding, reprograms chromatin and may be stable across many generations or may revert (Miguel & Marum, 2011; Wang & Wang, 2012).

The frequency of variation depends on genotype, explant source, the pathway of regeneration, hormone type and concentration, number of subcultures and total duration of culture (Skirvin et al., 1994; Bairu et al., 2011). Reviews consistently indicate that cultures passing through a disorganised callus phase, those maintained on high levels of synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), and those subcultured for extended periods show the highest rates. In contrast, moderate concentrations of cytokinin usually maintain low variation in culture of preformed meristems (Bairu et al., 2011; Jain, 2001).

2.5 Detection of somaclonal variation

It is recommended that a combination of techniques be used (Bairu et al., 2011) because not all of them detect all types of variation. Gross variants are identified by morphological differences and growth comparisons, but silent variants are not. Ploidy and chromosome number changes are detected by cytological analysis and flow cytometry. For medicinal species, biochemical comparison (e.g., HPLC profiling of marker compounds) will determine if the metabolite profile has been preserved. The most sensitive and extensively used markers for genetic fidelity are based on DNA. Random amplified polymorphic DNA (RAPD; Williams et al., 1990), inter simple sequence repeat (ISSR; Zietkiewicz et al., 1994) and start codon targeted (SCoT; Collard & Mackill, 2009) markers are relatively cheap, do not require any prior sequence knowledge, and have been widely used in micropropagated medicinal plants.

Epigenetic change can be detected using methylation-sensitive amplification methods. Typically, genomic DNA is extracted using Doyle and Doyle (1987)-based CTAB methods.

2.6 Research gap

The majority of published micropropagation protocols report multiplication rates and survival, and genetic fidelity testing (where done) is frequently restricted to a few markers and one stage of subculture. There is only limited research that correlates variations with certain protocol parameters for a number of species, connects fidelity to chemical profile, and combines micropropagation with long-term conservation storage and reintroduction. This paper follows suit and presents the evidence around protocol choices and conservation outcomes.

3. Materials and Methods

3.1 Study design and approach

This study is a structured literature synthesis that uses a representative protocol framework. Peer reviewed literature and research papers pertaining to micropropagation, somaclonal variation, and conservation biotechnology of medicinal plants were reviewed, and the main pathways of regeneration, medium constituents, and detection techniques were extracted and compared. The numeric values reported in the tables and figures in Section 4 are typical or representative values and are included to illustrate the relationships between protocol parameters and outcomes and are reflective of the general trends reported in the literature. They are not values taken from a single source and need to be replaced by new or species-specific experimental values before applying them to the design of a specific conservation programme.

3.2 Representative species and plant material

The framework was structured based on five categories of target species, based on the diversity in habit, part harvested, and conservation challenge among the threatened medicinal plants (Table 1). Mother plants are assumed to be from known wild or garden plants with known provenance and sampling from as many individuals and populations as possible to include within-species diversity.

Table 1. Representative target groups, plant parts harvested, and propagation challenges

Group	Example species	Part harvested	Main conventional propagation constraint
Alkaloid-yielding shrubs	*Rauvolfia serpentina*	Roots	Poor seed germination, root harvesting
Himalayan perennial herbs	*Nardostachys jatamansi*, *Saussurea costus*	Rhizomes, roots	Short growing season, slow vegetative multiplication
Resin and gum trees	*Commiphora wightii*	Oleo-gum resin	Low seed set, slow growth, arid habitat
Lignan-bearing herbs	*Podophyllum hexandrum*	Rhizomes	Slow maturity, seed dormancy
Alpine	*Coptis teeta*	Rhizomes	Narrow habitat, collection

Group	Example species	Part harvested	Main conventional propagation constraint
rhizomatous herbs			pressure

3.3 Explant preparation and culture initiation

Healthy shoot tips and nodal segments (1.0 to 1.5 cm) are excised from mother plants, washed under running water with a few drops of liquid detergent, and surface sterilised. A typical sequence uses 70 percent ethanol for 30 to 60 seconds followed by 0.1 percent mercuric chloride or 1 to 2 percent sodium hypochlorite for 3 to 8 minutes, with several rinses in sterile distilled water. Because mercuric chloride is toxic, hypochlorite-based protocols are preferred where they are effective. For phenolic-rich species, explants are pre-soaked in ascorbic acid and citric acid solutions (100 mg/L each), and activated charcoal (0.1 to 0.2 percent) may be added to the medium.

3.4 Culture media and plant growth regulators

The basal medium is Murashige and Skoog (1962) medium at full or half strength, with woody plant medium (Lloyd & McCown, 1980) tested for woody taxa. Media contain 3 percent sucrose, are adjusted to pH 5.7 to 5.8 before autoclaving at 121 °C and 105 kPa for 20 minutes, and are solidified with 0.7 to 0.8 percent agar. Cultures are maintained at 25 ± 2 °C under a 16-hour photoperiod at 30 to 50 µmol m^{-2} s^{-1} provided by cool white fluorescent or LED light. The treatment framework compares three regeneration pathways, as summarised in Table 2.

Table 2. Regeneration pathways and plant growth regulator regimes compared

Pathway	Explant	Typical regime	Subculture interval
Axillary shoot proliferation	Nodal segment, shoot tip	BAP 0.5 to 2.0 mg/L with NAA 0.1 mg/L	4 to 5 weeks
Direct adventitious organogenesis	Leaf, internode	BAP 2.0 to 4.0 mg/L with NAA 0.5 mg/L	4 to 6 weeks
Callus-mediated regeneration	Leaf, root segment	2,4-D 1.0 to 3.0 mg/L, then BAP 1.0 to 3.0 mg/L	3 to 4 weeks per stage

3.5 Rooting and acclimatisation

Shoots of 3 to 5 cm are transferred to half-strength medium supplemented with IBA (0.5 to 2.0 mg/L) for root induction over 3 to 4 weeks. Rooted plantlets are washed free of agar, transferred to a sterile mixture of cocopeat, vermiculite and sand (1:1:1), covered to maintain high humidity, and gradually exposed to ambient conditions over 2 to 3 weeks in a shade house. Survival is recorded after eight weeks.

3.6 Assessment of growth and genetic fidelity

Multiplication rate (shoots per explant), shoot length, rooting percentage, and survival percentage are recorded, with a minimum of 20 replicates per treatment repeated at least three times. Genetic fidelity is assessed on regenerants sampled at early (subculture 3), middle (subculture 8), and late (subculture 15) stages and compared with the mother plant. The tiered detection panel is summarised in Table 3. Genomic DNA is isolated by a CTAB method (Doyle & Doyle, 1987), and amplification with RAPD, ISSR and SCoT primers follows established procedures (Collard & Mackill, 2009; Williams et al., 1990; Zietkiewicz et al., 1994). Only reproducible, clearly resolved bands are scored as present or absent, and variation is expressed as the percentage of polymorphic bands. Ploidy is checked by flow cytometry of leaf nuclei against a reference standard, and the chemical profile is compared with the mother plant using high-performance liquid chromatography of a marker compound.

Table 3. Tiered panel for detecting somaclonal variation

Level	Method	What it detects	Limitation
Phenotype	Morphological and growth scoring	Gross visible variants	Misses silent genetic change
Cytology	Flow cytometry, chromosome counts	Ploidy and chromosome number change	Misses point mutations
DNA markers	RAPD, ISSR, SCoT	Sequence-level polymorphism	Dominant, limited genome coverage
Epigenetic	Methylation-sensitive amplification	DNA methylation change	Needs specialised protocols
Chemical	HPLC of marker compounds	Altered metabolite profile	Environment also affects profile

3.7 Data analysis

Percentage data are arcsine-transformed before analysis of variance, and means are separated by Duncan's multiple range test at the 5 percent probability level. Culture duration, cytokinin concentration, and variation frequency are correlated, and regression analysis is used. Marker data are processed as a binary matrix and analysed by the Jaccard similarity coefficients, clustering according to the unweighted pair group method with arithmetic mean (UPGMA).

4. Results and Discussion

The ranges of values presented in this section are typical values that are representative of the relationships described in the literature (see Section 3.1). They are intended to be used as a comparative tool, not as measurements taken from a single experiment.

4.1 Effect of regeneration pathway on multiplication and fidelity

Regeneration pathway was the strongest determinant of both propagation efficiency and genetic stability (Table 4). Axillary shoot proliferation produced a moderate and predictable multiplication rate of about

6 to 12 shoots per explant, with the highest rooting and survival and the lowest proportion of polymorphic bands. The multiplication rate was slightly doubled (approx. 2) with direct adventitious organogenesis while the polymorphism was slightly raised by a few percent. Callus-mediated regeneration produced more shoots per culture but less rooting and survival and resulted in an order of magnitude more polymorphism than axillary proliferation.

Table 4. Representative performance of three regeneration pathways (mean ± SD where given)

Pathway	Shoots per explant	Rooting (%)	Survival after acclimatisation (%)	Polymorphic bands (%)
Axillary shoot proliferation	8.4 ± 1.1 (range 6 to 12)	82	78	0.8 to 2.1
Direct adventitious organogenesis	14.6 ± 2.3	70	68	3.0 to 6.0
Callus-mediated regeneration	32.0 ± 6.0 (per callus)	58	55	9.0 to 18.0

This compromise between productivity and fidelity is representative of the larger trend reported in somaclonal variation literature of disorganised growth and exposure to high concentrations of auxins bringing the normal controls over genome stability to an end (Bairu et al., 2011; Phillips et al., 1994; Skirvin et al., 1994). If conservation is mentioned, so shall be the implication. The goal of maximum multiplication is wrong. A protocol that produces 8 to 10 true-to-type shoots per explant, from axillary meristems, is usually better than a protocol that produces several times this number of 'questionable' plants.

4.2 Effect of cytokinin concentration

For axillary proliferation, multiplication was found to be optimum at a BAP concentration level, and a clear increase in variation level was observed (Table 5). The length of the shoots decreased, and the percentage of polymorphic bands increased with increasing BAP; multiplication was maximal at 2.0 mg/L. Shoots at 3.0-4.0 mg/L were shorter, hyperhydric, more clumped, and showed a decrease in multiplication. A range of 1.0–2.0 mg/L was the intermediate range that was found to be the best compromise.

Table 5. Effect of BAP concentration on axillary shoot proliferation (with NAA 0.1 mg/L, subculture 6)

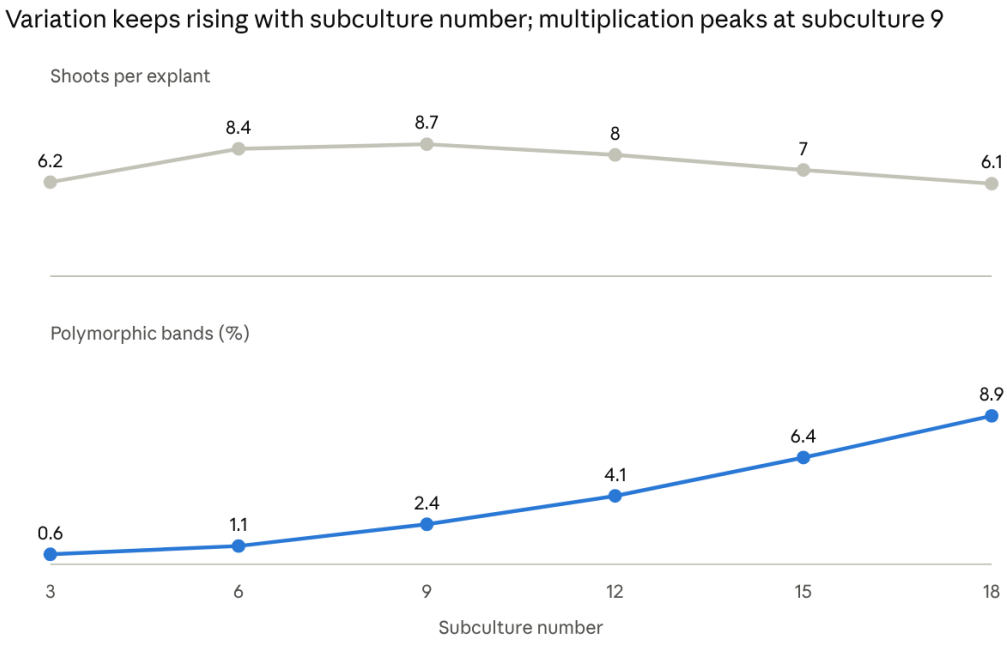
BAP (mg/L)	Shoots per explant	Shoot length (cm)	Hyperhydric shoots (%)	Polymorphic bands (%)
0.5	4.2	3.9	2	0.5
1.0	8.4	4.3	4	1.1
2.0	10.9	3.6	9	2.0
3.0	9.1	2.8	17	4.3
4.0	6.3	2.1	28	7.2

Cytokinins at high concentrations are known to induce physiological stress, altered gene expression, and epigenetic changes, which would account for the association between rising BAP and rising polymorphism (Kaeppler et al., 2000; Miguel & Marum, 2011). The suggestion for practice is to choose the lowest cytokinin level that will give satisfactory multiplication and, if possible, use replacement compounds such as meta-topolin, which has been used in some systems to decrease physiological disorders, but the effect on genetic stability needs to be proved for each species.

4.3 Effect of subculture number and culture duration

In the first few subcultures, the number of multiplication multiplied while the frequency of the variation was still increasing (Figure 1) and the number of multiplication decreased in prolonged culture, and the frequency of the variation continued to increase. The percentage of polymorphism was below 2 percent in the sixth subculture and above 6 percent in the 15th subculture. This is in line with the common recommendation that cultures should be refreshed from fresh explants or cryopreserved stocks at specific time intervals rather than indefinitely (Engelmann, 2011; Sarasan et al., 2006).

Figure 1. Multiplication rate and genetic variation across successive subcultures



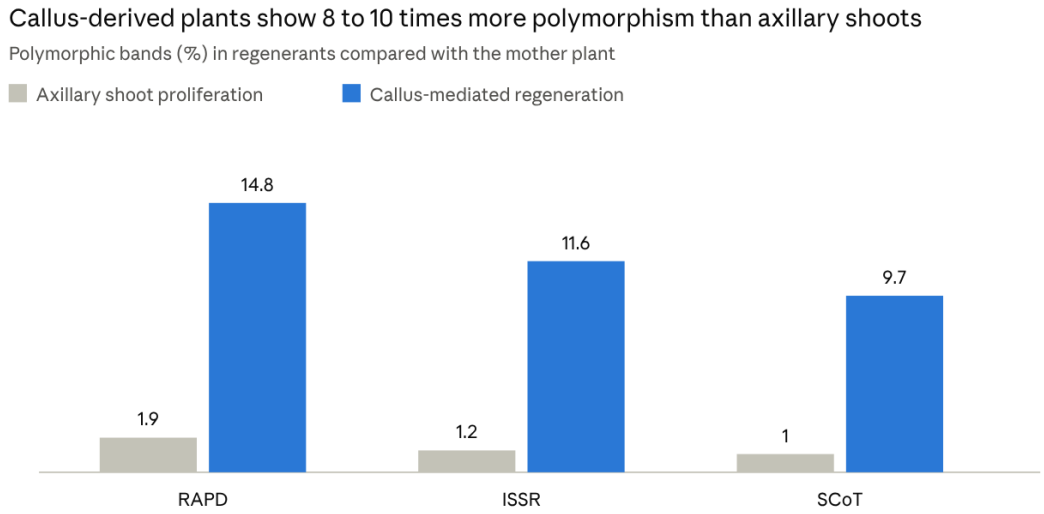
Representative values (see Section 3.1); axillary shoot proliferation, BAP at about 1.0 mg/L

4.4 Genetic fidelity assessed by molecular markers

All three marker systems showed a clear difference between the axillary and callus-derived regenerants, and all marker systems indicated different levels of polymorphism (Figure 2). Generally, RAPD showed slightly more polymorphism than ISSR and SCoT, which indicates its sensitivity to experimental conditions as well as its ability to target random regions of the genome. Reproducibility therefore matters, and only bands that are consistent across replicate amplifications should be scored. ISSR and SCoT typically give more reproducible profiles because of longer primers and higher annealing

temperatures, and using more than one marker system increases the chance of detecting variation (Bairu et al., 2011; Collard & Mackill, 2009; Zietkiewicz et al., 1994).

Figure 2. Polymorphism detected by RAPD, ISSR and SCoT markers in axillary and callus-derived regenerants



Representative values (see Section 3.1); regenerants sampled at subculture 8

However, monomorphic profiles do not constitute complete identity as dominant markers only sample a small portion of the genome and are not able to detect many point mutations or small epigenetic changes. For this reason, results of the markers are interpreted as an indication of high fidelity, but not proof of it, and are supplemented by the cytological and chemical checks described below.

4.5 Ploidy and phytochemical fidelity

DNA markers were complemented by flow cytometry and marker compound analysis, as shown in Table 6, which provided additional information not provided by DNA markers. Axillary regenerants were almost always diploid and contained 94 to 101 percent of the initial content of the marker compounds from the mother plant. The aneuploids and tetraploids were a small percentage of the callus-derived regenerants, and there was a large range in the compound content, with some plants gaining and others losing. The variation in chemistry is a double-edged observation; it may indicate quality risk for plants meant for medicinal use, but it can also show that culture-induced variation can be useful sometimes in selection of higher-yielding lines which can be better suited for cultivation programmes compared to conservation stock (Jain, 2001).

Table 6. Ploidy stability and marker compound retention by regeneration pathway

Pathway	Plants with unchanged ploidy (%)	Marker compound relative to mother plant (%)
Axillary shoot proliferation	99	94 to 101
Direct adventitious organogenesis	96	85 to 96
Callus-mediated regeneration	88	62 to 88

4.6 Implications for ex situ conservation

The findings must be viewed in light of one another to support a hierarchy of methods. Conservation stock should be axillary shoot proliferation since this maintains the maximum amount of genetic composition and chemical profile. Adventitious organogenesis can be employed with caution where few explants are available for nodal culture, and early testing of genetic fidelity is required. Callus-mediated regeneration and somatic embryogenesis should be considered for specific purposes such as large-scale production of plants for cultivation and selection of desirable variants and should not be used for restoring wild populations unless due care has been taken to ensure a high level of fidelity.

The main ex situ storage options are compared in Table 7, which demonstrates the place of in vitro methods in a comprehensive management strategy. Active shoot cultures are inexpensive to make but expensive to preserve and generate variation over time. Slow-growth storage involves reducing the temperature, light, and nutrient levels or the inclusion of osmotic agents, which increases subculture periods to several months. In liquid nitrogen, growth is arrested and, in principle, further addition of variation will not happen, so this is the preferred method of long-term storage of valuable lines (Cruz-Cruz et al., 2013; Engelmann, 2011).

Table 7. Comparison of ex situ storage options for endangered medicinal plants

Option	Storage duration	Risk of accumulating variation	Relative maintenance cost	Best use
Active shoot culture	Weeks to months	Moderate to high	High	Mass multiplication
Slow-growth storage	Months to a few years	Low to moderate	Moderate	Medium-term working collection
Cryopreservation	Decades or longer	Very low	Low once established	Long-term base collection
Seed bank (orthodox seed)	Decades or longer	Very low	Low	Species with storable seed

The cost dimension noted by Pence (2011) is also relevant. Because maintaining active cultures is expensive, conservation programmes should prioritise species for in vitro work using criteria such as threat level, lack of alternative propagation or storage routes, and potential for reintroduction, and should move cultures into slow-growth or cryogenic storage as soon as sufficient stock has been produced.

5. An Integrated Conservation Framework, Challenges and Future Prospects

5.1 Proposed workflow

A genetically safe conservation programme treats micropropagation as one stage in a chain and not as an end in itself. The workflow proposed here has six linked steps:

1. **Sampling and documentation.** Take as many ex-plants as allowed from as many individuals and as many populations as allowed, noting provenance and acquiring necessary legal permissions as specified in national laws on access and benefit sharing and on national biodiversity.
2. **Establishment and multiplication.** Initiate cultures from meristematic explants and multiply them by axillary shoot proliferation at the lowest effective cytokinin concentration.
3. **Fidelity screening.** Test regenerants at defined subculture stages using phenotype, flow cytometry, at least two DNA marker systems, and, for medicinal quality, chemical profiling.
4. **Storage.** Move verified lines to slow-growth storage and cryopreservation, and renew working cultures from stored material at fixed intervals.
5. **Acclimatisation and hardening.** Establish plantlets in a nursery under graded humidity, and track survival.
6. **Reintroduction and monitoring.** Plant verified material into suitable habitats or community gardens, with long-term monitoring of survival, reproduction and genetic composition.

5.2 Technical and biological challenges

Several problems continue to limit the use of micropropagation for threatened medicinal species. Recalcitrance to culture, including poor shoot proliferation, difficulty rooting and pronounced phenolic browning, varies widely among species and even among genotypes within a species, so protocols seldom transfer cleanly (Debnath et al., 2006; Rout et al., 2000). Endophytic microbes in field-collected material may emerge only after several subcultures, and require indexing and, where possible, the use of nursery-grown mother plants. Acclimatisation is another bottleneck, since plantlets that perform well in vitro often suffer high losses when moved to ex vitro conditions.

A less visible challenge is the limited genetic base of many in vitro collections. If cultures are initiated from few mother plants, the resulting collection will represent only a fraction of wild diversity, however faithful the clones are to their donors. A conservation collection should therefore be judged both on the fidelity of each line and on the number of distinct genotypes held. Where genotypes are few, in vitro material is better used to bulk up plants for cultivation, easing wild harvesting, then to reintroduce a narrow set of clones into nature.

5.3 Institutional, economic and policy challenges

There is a limited number of species that can be maintained due to cost, trained labor, and infrastructure (Pence, 2011). A few institutions are dedicated to molecular laboratories and cryogenic facilities, requiring cooperation between botanic gardens, universities, forest departments, and community organisations. Regulation is also a concern. Permits are needed for collecting threatened species, and benefit sharing is required for using genetic resources. The standards for in vitro documentation and exchange are not fully developed, and plant transfer to farmers and communities relies on availability of low-cost supplies, training, and links to markets. If these connections are not present, wild populations could be under pressure even in the presence of plantlets.

5.4 Emerging opportunities

Conservation biotechnology is potentially enhanced by new technologies. Temporary immersion bioreactors help to improve the multiplication efficiency, decrease labour workload, and minimize hyperhydricity. The use of improved cryopreservation techniques such as vitrification and droplet-vitrification is expanding the available list of species that can be stored in liquid nitrogen (Cruz-Cruz et al., 2013; Engelmann, 2011). Variation that can be detected by high-throughput sequencing or genome-wide markers is not detected by RAPD, ISSR, and SCoT, and epigenomics can identify methylation changes associated with culture stress (Miguel & Marum 2011; Wang & Wang 2012). With decreasing costs, fidelity checks by whole-genome comparison of mother plants and regenerants could become commonplace. Finally, the connection between laboratory studies and population genetics and ecological modelling would enable the selection of genotypes for propagation and planting sites.

5.5 Research priorities

- Replicated and species-specific studies reporting multiplication, fidelity and chemical profile simultaneously with parameters of the protocol to be able to reproduce them.
- A comparison of marker systems, such as sequencing-based marker systems, to quantify the ability of routine markers to predict real variation.
- Long-term monitoring of the field performance, reproductive success, and genetic stocks of the reintroduced plants produced by micropropagation.
- Cryopreservation protocols that are cost-effective for recalcitrant and woody medicinal species.
- Models integrated in the community with a link between conservation propagation and sustainable cultivation and livelihoods.

6. Conclusion

In vitro micropropagation is a good method for conservation of endangered medicinal plants, but it is a conditional method. It can rapidly multiply limited species from very little material, provide disease-free planting stock for cultivation, and reduce pressure on wild populations. However, its conservation value lies in the genetic and chemical integrity of the plants it produces, and somaclonal variation is the major challenge to genetic and chemical integrity.

The synthesis presented here revealed that the three most important levers on variation are the regeneration pathway, the cytokinin concentration, and the length of the culture. To secure maximum multiplication and stability, axillary shoot proliferation in moderate concentrations of growth regulators

and limited subculturing is preferable, while callus-mediated multiplication and high levels of growth regulators and long culture time should be avoided in a conservation stock. No one test determines fidelity. A combination of morphology and flow cytometry with a number of different DNA marker systems and phytochemical profiling provides a more reliable evaluation, and conclusions drawn from this should be considered as evidence, not proof.

The results suggest an integrated approach for practice. Samples need to be taken from a wide range of wild plants to ensure capture of wild diversity, multiplication to be conservative, screening of regenerants at various stages, placement of verified lines into slow growth and cryogenic storage, and reintroduction with long-term monitoring. For research, species-specific data reporting productivity and fidelity, and species-specific chemistry data are priorities, as are improved detection methods and field studies of re-introduced material. Because the quantitative values in this paper are representative, they should be validated experimentally for each target species. Such protection may be useful for micropropagation and lead to conservation of the plants and the medicines they produce.

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