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In vitro propagation for improvement of medicinal plants: A review

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Abstract
Many plant cells exhibit totipotency; the ability to develop into whole plants via embryo formation. Plant tissue culture is the in-vitro culture of plant cells that relies on totipotency. Plant cells are grown on synthetic media and plant growth regulators such as plant hormones and their synthetic analogues are used to support and manipulate growth to user's end. Numerous studies have shown the potential of in-vitro culture in medicinal plants including micropropagation where efficient protocols of micropropagation for many various medicinal plants, enhanced production of high-value secondary metabolites and conservation of a wide range of medicinal plants, which includes endangered, rare and threatened plant species. Therefore in-vitro propagation has various advantages and many applications in plant research and production with great potential for medicinal plants as well. However, well-established efficient protocols for many medicinal plants are yet to be established before they can be commercialized.

Keywords: propagation, conservation, medicinal plants

Introduction
Micropropagation is a plant tissue culture technique used for producing plantlets through culturing cells, tissues and, organs in a well-defined culture medium under aseptic controlled conditions such as light, temperature and relative humidity. Many plant cells are totipotent; they are capable of developing into whole new plants via embryo formation (Fowler *et al.*,1993) [10]. It involves several factors, such as the composition of the culture medium, culture environment, suitable explants.It allows rapid mass propagation, including the production of virus-free plantlets among many other applications. Thecommonly used medium MS medium consists of all the essential macronutrients such as Nitrogen, Phosphorous, Potassium, Calcium, Magnesium, Sulphur and micronutrients such as Iron, Manganese, Zinc, Copper, Boron, Chlorine, Molybdenum, and Nickel, vitamins (Thiamine, nicotinic acid, pyridoxine, and myoinositol), Plant growth regulators, and a carbohydrate 2-3% Sucrose, as carbon source.Agar is most commonly used for preparing semisolid or solid culture media, but other gelling agents occasionally used to include gelatin, agarose, alginate, and gelrite. The pH of the medium (5.0-6.0) is usually more stable and better results are obtained when the medium contains both nitrate and ammonium ions sources of nitrogen. Most commonly used plant growth regulators (PGRs) are plant hormones and their synthetic analogssuch as Auxins, Cytokinin's, Gibberellins, Abscisic acid (ABA) and Ethylene.
Auxins (IAA, NAA, 2,4-D, and IBA) promote both cell division and cell growth. The most commonly used auxin is 2,4-D. Cytokinins promote cell division. 2iP (6-dimethyl-aminopurine), Zeatin, kinetin (kn), 6-benzylaminopurine (BAP) and TDZ (thidiazuron) wherein kinetin (kn) and 6-benzyl amino purine (BAP) are most frequently used cytokinins. Gibberellins (GA3) is involved in regulating cell elongation, enhances the callus growth and helps in elongation of dwarf plantlets. ABA inhibits cell division; promote distinct developmental pathways such as somatic embryogenesis. Ethylene is gaseous, naturally occurring, used in controlling fruit ripening in climacteric fruits, but is not used widely in plant tissue culture. PGRs are used to support and manipulate the growth of the plants (shoot/root ratio). If the ratio of auxin and cytokinin (Auxin: cytokinin) is high, embryogenesis, callus initiation, and root initiation occur. If low, then there is occurring of axillary and shoot proliferation.
In vitro propagation has various potential for medicinal and aromatic plants. India has a rich diversity of medicinal herbs, however, deforestation, over-exploitation of wild stock of the plant are rapidly depleted genetic resources which may result in many valuable medicinal plants are under the threat of extinction. Rare endangered and those medicinal plant species are facing regeneration failure in nature and which are difficult to propagate through other costs

effective conventional propagation and many plants do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Therefore, in-vitro propagation techniques or tissue culture offers alternatives to conventional crop improvement, large scale multiplication of disease-free plants, faster cloning, and conservation of plant genotypes, in a relatively short span of time around the year, under aseptic conditions (Yaadwinder, 2010) [43].

Advantages of in-vitro propagation

Some advantages of *In vitro* micro propagation of the medicinal plant are; rapid and mass propagation, availability of plants all year round (off-season)**,** production of disease and virus-free plants, enable embryo rescue to overcome seed dormancy and assess seed viability, production of secondary metabolites, safe and easier exchange of germplasm, conservation of genetic resources and endangered plants through cryopreservation**,** exploiting somaclonal variation and genetic engineering techniques for crop improvement.

Limitation of in-vitro propagation

The appearance of somaclonal variation; genetic variation among progenies of plants regenerated from somatic cells cultured *in vitro*. A monoculture is produced after micropropagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same pathogen. Not all plants can be successfully tissue-cultured, often because the proper medium for particular growth of the plant is not known or the plants produce secondary metabolic chemicals that stunt or kill the explant.

The different stages of micropropagation or invitro propagation for most of the plant:

Stage 0: Selection of explants: This is the initial step in micropropagation, and involves the selection and growth of stock plants for about 3 months under controlled conditions.

Stage 1: Establishment of explants: Introduction of the surface disinfected explants is cultured in a suitable culture medium, preferably agar-based media for tissue activation, followed by the initiation of shoot growth and multiplication. Usually, it takes 4 to 6 weeks to complete and generate explantsthat are ready to be moved to Stage II (Hartmann *et al.*, 2002) [14].

Stage II: Proliferation and Multiplication: Shoot proliferation and multiple shoot production are done in this stage. Multiple shoots are separated and transplanted to a new culture medium (Hartmann *et al.*, 2002) [14]. Shoots are sub-cultured every 2 to 8 weeks and also sub-cultured several times to a new medium to maximize shoots proliferation which largely depends upon the combination of growth regulators. The duration of this stage is unlimited and largely depends on the choice of the propagator.

Stage III: Shoot elongation and Root formation: In this stage, the selected plants are forced for root formation, which can be achieved by media modification and modifying the concentration of growth regulators. The concentration of Cytokinins and sugars is reduced and the concentration of auxins and light intensity in the laboratory is increased to start with photosynthesis and other physiological activities. This stage may involve not only the rooting of shoots but also the conditioning of the plants to increase their potential for acclimatization and survival during transplanting.

Stage IV: Acclimatization or hardening: Transfer of regenerated plants to the soil under natural environmental conditions. Transplantation of in-vitro derived plants to the soil is often characterized by lower survival rates. Before the transfer of soil rooted plants to their final environment, they must be acclimatized in a controlled environment room or in mist chambers where the relative humidity is maintained at higher-order (Rohr *et al.*, 2003) [31]. Plants also form a protective epicuticular wax layer over the surface oftheir leaves and regenerated plants gradually become adapted to survival in their new environment. Once new growth is seen, the plants may be slowly transferred to outside to increased light intensity in stages.

Types of in-vitro culture

1. Shoot tip or meristem culture
2. Callus culture
3. Somatic embryogenesis
4. Protoplast culture
5. Suspension culture
6. Anther culture

1. **Shoot tip or meristem culture:** Shoot tip culture may be described as the culture of terminal (0.1-1.0 mm) portion of a shoot comprising the meristem (0.05-0.1 mm) together with primordial and developing leaves and adjacent stem tissue. Young meristems are used for producing virus-free plantlets as the phloem does not extend to young meristem. Another advantage is the genetic stability inherent in this technique. Shoot development, directly from the meristems, avoids callus formation and adventitious organogenesis thus ensuring that genetic instability and or somaclonal variation are minimized.
2. **Callus culture:** A callus is an unorganized and undifferentiated mass of cells. It is induced from plant tissue cultured on the appropriate solid medium, with both an auxin and a cytokinin medium supplemented with PGRs such as Auxins, Cytokinins, and Gibberellins.There is four points highlight of significances of callus culture they are (a) *Source of Tissue for Plant Regeneration.* (b) *Source of Chromosomal Variation (Somaclonal Variation)* (c) *Source of Secondary Metabolites* (d) *Source of Tissue for Cell Suspension Culture.*
3. **Somatic embryogenesis:** A plant or embryo is derived from a single somatic cell. Somatic embryos differ from zygotic embryos in that they develop from vegetative or somatic cells instead of zygotes. There are two types of embryo development through the culture of somatic cells they are: (a) *Direct embryogenesis:* Embryos started directly from explant tissue creating an identical clone, (b) *Indirect embryogenesis:* Explants produced undifferentiated or partially differentiated cells maintained or differentiated into plant tissues such as leaf, stem or roots.Successful plant regeneration has been achieved in Aloe and *Centella asiatica* by somatic embryogenesis (Garro-Monge *et al.*, 2008; Joshee *et al.*, 2007) [11, 18].
4. **Protoplast culture:** Protoplasts, cell without cell wall used to regenerate plantlets. This culture is produced experimentally by the removal of the cell wall by either enzymatically or mechanical means from the artificially plasmolyze plant cells are known as isolated protoplasts (Bhojwaniand Razdan,1996) [6]. The basic principle of

protoplast culture is the aseptic isolation of a large number of intact living protoplasts removing their cell wall and cultures them on a suitable nutrient medium for their requisite growth and development.

5. **Suspension culture:** It is a type of culture in which single cells or small aggregates of cells multiply while suspended in the agitated liquid medium. It is used to Understand of biosynthesis pathway, mutant selection, secondary metabolite production and use of suspension cultures in plant propagation. It is normally initiated by transferring pieces of undifferentiated and friable calli to a liquid medium and also it can be started from sterile seedlings or embryos. It could be used for the large-scale culture of plant cells from which secondary metabolites can be extracted from medicinal plants.
6. **Anther culture:** Anther culture is a technique by which the anthesis obtained from an unopened flower bud and is cultured on a nutrient medium under aseptic condition. callus tissue or embryoids from anther, that give rise to haploid plants of several species (Rout *et al.*2000) [32]. They are used in plant breeding and crop improvement as well as to obtained secondary metabolites of medicinal plants through this culture.

Table 1: Some important examples of In-vitro propagation of medicinal plants

Plant name	Explant source	Response	References
Atropa belladona	Axillary meristem	Multiple shoots, plantlet formation	Benjamin *et al.*, 1987 [4].
Azadirachta indica	Shoot tip, root tip	Multiple shoots, plantlet formation	Shahin-uz-zaman *et al.*, 2008 [34].
Catharanthus roseus	shoot-tip, nodal segment	Multiple shoots, planlet formation	Begum and Mathur 2014 [3].
Withania somnifera	Nodal explant,	Multiple shoots and roots, Plantlet formation	Mir *et al.*, 2014; Saema *et al.*, 2013) [25, 33].
Plantago ovata	shoot-tip	Multiple shoots, plantlet formation	Barna and Wakhlu, 1988 [2].
Rauwolfia serpentina	Nodal, leaf explant	Multiple shoots, plantlet formation	khan *et al.*, 2018 [21].
Valeriana wallichii	apical meristem	Multiple shoots, plantlet formation	Mathur *et al.*, 1988 [22].
Centella asiatica	shoot-tip, leaf	Callus, shoot bud, plantlet	Joshee *et al.*, 2007 [18].
Aloe vera	Shoot tips, Aux. bud	Shoot, root and plantlet forming	Molsaghi *et al.*, 2014 [26].

Application of plant tissue culture in medicinal plants

Micropropagation: Efficient protocols form in crop propagation of various important medicinal plants such as *Aloe vera* (Molsaghi *et al.*, 2014) [26]. *Artemisia annua* L. (Gopinath *et al.*,2014) [12], *Catharanthus roseus* and *Bacopa monnieri* (Vandana *et al.*, 2020; Begum and Mathur. 2014)[40, 3], *Withania somnifera* (Patel and Krishnamurthy, 2013; Saema *et al*., 2013) [29, 33], *Chlorophytum borivilianum* (Kemat *et al.*,2010) [20], *Rauwolfia serpentina* (Bhatt *et al.*,2008) [5], *Gloriosa superba* L. (Yadav *et al.*,2015) [44], *Picrorhiza kurrooa* (Jan *et al.*, 2010; Chandra *et al.*,2006) [16, 7] are established.

Production of secondary metabolites: Medicinal plants are the valuable source of industrially important natural plant-derivedcompounds called secondary metabolites which include many terpenes, polyphenols, cardenolides, steroids, alkaloids and glycosides (Matkowski A. 2000) [23].These compounds are used as pharmaceutical, agrochemicals, aromatics and food additives. There has been a tremendous success in the production of high-value secondary metabolites such as shikonin from cell cultures of *Lithospermum erythrorhizon*, berberine from *Coptis japonica* and sanguinarine from *Papaver somniferum.* These are produced at industrial levels (Ramachandra and Ravishankar, 2002) [30]. The production of secondary metabolites from medicinal plants is called Plant-Derived Medicinal Compounds (PDMC). PDMC can be produced *in vitro* by *callus* induction and growth, cell culture, shoot proliferation, and the inducing hairy roots using transgenic techniques.

Callus induction and multiplication have been extensively used in PDMC *in vitro* production and it is an efficient approach to produce PDMCs in large scale when compared with other techniques, mainly because the *in vitro* callus induction is a straightforward and rapid system of cell multiplication (Kapoor *et al.*,2018) [19], highly consistent, and used commercially already in tissue culture. The callus (friable) obtained *in vitro* also can be used also for establishing new suspensions of cell cultures. Researchers have reported secondary metabolites production through callus culture such as more stevioside present in callus cultured cells as compared with extracts from leaves of mother plants *Stevia rebaudiana* (Janarthanam *et al.*,2010) [17]. Plant cell culture holds much promise as a method for producing complex secondary metabolites *in vitro*. Researchers have reported considerable effect so manipulation of media formulations, growth hormones, temperature and photoperiod on the secondary metabolites production (Stafford *et al.*, 1986) [39]. Rout *et al.*, 2000 [32] reported that 12-fold increase of solasodine content was obtained from *in vitro* plantlets of *Solanum trilobatum*. In suspension cultures of *Catharanthus roseus*, MS medium was reported to be best for producing serpentine. 2,4-D was reported to cease metabolite production (DiCosmo and Misawa 1995) [9]. Wu *et al.* 2003 [42] was reported the production of Rosavin as a product of glycosylation in *Rhodiolarosea* (roseroot). In *Catharanthus roseus*, auxins promoted callus induction but cytokinins alone were found to be increased in secondary metabolites production (Di Cosmo and Misawa 1995) [9]. The production of secondary metabolites through invitro cultures such as Artemisinin (*Artemisia annua*) in cell suspension and hairy root cultures (Ikram *et al.*, 2017) [15].

Grzegorczyk-Karolak *et al.* (2015) [13] reported PDMCs produced by shoot culture in *Scutellaria alpine*. Few examples in the special literature that focus on secondary metabolite production from shoot cultures, e.g. *Spathiphyllum cannifolium* and *Stevia rebaudiana*. Regarding the use of elicitors, Sharma *et al.* (2010) [37] reported that maximum bacoside contents on *in vitro* shoot cultures of *Bacopa monnieri* by using 45 mg L^{-1} of $CuSO_4$ in the culture medium, combined with a shoot elicitor incubation period of 6 to 9 days.

Hairy root cultures are those cultures in which the genetic transformation mediated bacteria *Agrobacterium rhizogenes* is used to induce and obtain hairy-root lines (Chen *et al.*, 2018) [8] in tissues already established *in vitro*. Hairy-root lines are important sources of stable cells specialized in the production of useful PDMCs in amounts higher than those

obtained from cell cultures in several species McCown BH. (1986) [24].

Conservation of plant genetic resources

Conservation and preservation of threatened medicinal plant genetic resources through ABA, low-temperature storage and cryopreservation is a novel application of *in vitro* culture in medicinal plants. The maintenance of the germplasm collections in the field is costly, requires large areas, and can be affected by adverse environmental conditions. Seed storage is ideal for crops with normal seed set, however, a large number of medicinal plants cannot be propagated and conserved in this manner as the seeds may be dormant, non-viable, or require extremely long periods for germination. For example, Loss of seed viability in plants such as Neem, *Rauvolfia serpentine*, and which are not easily stored in seed banks (Murthy and Saxena 1998) [28]. *Panax ginseng* seed germination is a long and involved process requiring several stratification steps that make seed banking difficult. Such seed conservation may not be feasible and in case of costly field gene banks. Tissue culture protocols were applied for the conservation of a wide range of medicinal plants, which includes endangered, rare and threatened plant species such as, *Saussaurealappa, Picorrhizakurroa, Ginkgo biloba, Swertiachirata and Gymnemasylvestre, Tinospora cordifolia, Salaca oblonga, Holostemma, Celastru spaniculata, Oroxylum indicum* (Sharma *et al.*, 2010)[37]. Slow growth conservation using ABA; Abscisic acid is an endogenous growth retardant and it was often used for growth reduction of *in vitro* conserved cultures. During the storage, ABA tends to modify carbohydrate metabolism of cells to increase the ability of conserved tissues to sustain higher residual water content and yet to decreases the adverse effects of osmotic stress and dehydration (Wakhluk *et al*., 1989) [41]

Sharma and Chandel. (1992) [36] reported low-temperature incubation of *in vitro* shoot and node cultures of Sarpagandha (*R. serpentine*) could be maintained even upto 9-15 months of storage at 15°C. shoot cultures of *Saussurea lappa*, an endangered, medicinal plant is stored at 5°C in the dark for 12 months without an intervening subculture survived with 100% viability. The shoots cold-stored for 6 months or more showed higher rates of multiplication under culture room conditions than the untreated shoots (Arora and Bhojwani. 1989) [1].

Cryopreservation is a safe and cost-effective technique for the preservation of germplasm and the management of *in vitro* produced materials for biotechnological applications. It offers a unique opportunity for the long-term preservation of endangered species as well as cultured cells and somatic embryos with unique attributes (Sakai and Englemann, 2007)[45]. Cryopreservation is the non-lethal storage of plant material or tissues at ultra-low temperature (-196 ºC) usually that of liquid nitrogen (Wen and Wang, 2010) [46]. It was always reported to be advantageous over most other conservation methods in terms of simplicity and the applicability to a wide range of genotypes (Paunescu, 2009)[47]. Moreover, the cryogenic storage is practiced in a small volume, which excludes contamination risks and minimizes maintenance requirements.

Cryopreservation techniques would be applicable to various plant material types such as cultured cells, shoot tips and somatic embryos (Sakai and Englemann, 2007) [45]. The different procedures for cryopreservation, like encapsulation-dehydration, vitrification, encapsulation vitrification, and droplet-vitrification (Sakai and Engelmann, 2007) [45]. Some of the Invitro cryopreserved of derived shoot tips of the medicinal plants such as *Bacopa monnieri* (Sharma *et al.*, 2011) [38], *Dioscoreabulbifera, Dioscoreaalata* (Mukherjee *et al.*, 2009) [27] and *Picrorhizakurroa* (Sharma and Sharma, 2003) [35] using verification technique.

In situ, ex-situ and *in vitro* conservation approaches are comprising the three pillars of the conservation plan triangle for medicinal plants. *In vitro* conservation is offering a package of techniques that are widely used for the conservation of plant germplasm. Slow growth conservation is a tissue culture technique based on reducing the growth rates of the stored explants by modifying the growth media composition (addition of elevated levels of osmotic agents or ABA) or storage under minimal growth conditions, such as low temperature and dark incubation. Moreover, cryopreservation is consisting of a collection of conservation techniques that are simple, applicable to a wide range of genotypes and able to maintain the genetic stability of the stored plant material.

Conclusion

Invitro propagation of medicinal plants is useful for rapid mass production of quality planting materials, enhanced production of secondary metabolites and efficient germplasm conservation of genetic resources of rare endangered medicinal plants. *In vitro* culture has various advantages and applications in plant research and production with great potential for medicinal plants as well. However, well-established protocols are not available for many medicinal plants yet.

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