In vitro production of alkaloids: Factors, approaches, challenges and prospects

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ABSTRACT

The wide diversity of plant secondary metabolites is largely used for the production of various pharmaceutical compounds. *In vitro* cell tissue or organ culture has been employed as a possible alternative to produce such industrial compounds. Tissue culture techniques provide continuous, reliable, and renewable source of valuable plant pharmaceuticals and might be used for the large-scale culture of the plant cells from which these secondary metabolites can be extracted. Alkaloids are one of the most important secondary metabolites known to play a vital role in various pharmaceutical applications leading to an increased commercial importance in recent years. The tissue culture techniques may be utilized to improve their production of alkaloids via somaclonal variations and genetic transformations. The focus of this review is toward the application of different tissue culture methods/techniques employed for the *in vitro* production of alkaloids with a systematic approach to improve their production.

Key words: Alkaloids, *in vitro* cultures, plant tissue culture, secondary metabolites

INTRODUCTION

Plants have been an important source of medicines/life-saving drugs since thousands of years for the majority of the world's population.[1] They have been used as medicinal products in two ways: (1) As traditional medicines or in formulations and (2) they are prepared and dispensed by traditional medical practitioners.[2] Approximately, one quarter of prescribed drugs contains plant extracts or active ingredients obtained from or modeled on plant substances; for example, most popular analgesic aspirin was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources.[3]

As the demand for medicinal plants is growing at a very fast pace, consequently some of them are increasingly being threatened even in their natural habitats.[4] Therefore, in search for alternatives to production of desirable medicinal compounds from plants, cell culture technologies were introduced as a possible tool for studying and producing plant secondary metabolites[5] as *in vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicines.

An alkaloid-producing plant cultured *in vitro* retained the capacity to synthesize alkaloids identical to that of an intact plant.[6] Callus culture facilitated the optimization of alkaloid production, whereas media composition was effective for the callus induction so as to enhance the alkaloid production and conservation of threatened genotype.[7]

Factors and approaches affecting the production of alkaloids using plant tissue culture

Number of factors [Figure 1] and approaches can affect the production of plant-specific bioactive metabolites resulting in accumulation of significant amounts of alkaloids in cultured cells resulting in maximization of the production with high yields suitable for commercial exploitation. The efforts have been focused on isolating the biosynthetic activities of cultured cells achieved by various techniques as cited below.

Optimization of cultural conditions

Manipulation of physical aspects and nutritional elements in a culture is the most fundamental approach for optimization of culture productivity.

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Biosynthesis of ergot alkaloids (EA) such as clavine ergot alkaloids and quinoline alkaloids (QA) such as quinocitrinines got enhanced in the medium enriched with various carbon and nitrogen sources in the presence of iron, copper, and zinc, whereas supplementation of mannitol and sucrose increased the biosynthesis of EA and QA, respectively.[8] Optimization of nitrate, ammonium, phosphate ions, and sucrose concentration increased the production of galanthamine in *Leucojum aestivum* shoot culture.[9]

Selection of high metabolite producing strains

Cell cloning methods provide a promising way for selecting cell lines and yielding the increased levels of products; for example, in cell aggregates of *Coptis japonica*, selection of cell lines increased the growth to about 6-fold in 3 weeks and the highest amount of alkaloid produced was 1.2 g/L of the medium as well as the strain was found to be very stable, producing a high level of berberine even after 27 generations.[10]

Precursor feeding

Attempts to induce or increase the yield of the final product by supplying precursors or intermediate compounds are found to be effective in many cases. The amino acids added to cell suspension culture enhanced the production of tropane and indole alkaloids[11] as well as supplementation of precursors, organic compounds and multiple feedings of loganin increased the biosynthesis followed by production of indole alkaloids in transformed roots of *Catharanthus roseus.*[12]

Influence of growth regulators

The effects of auxins and cytokinins on shoot multiplication in various medicinal plants are reported; for example, benzylaminopurine (BA) at higher concentration (1-5 ppm) stimulated the development of the axillary meristems and shoot tips in *Atropa belladonna.*[13] The content of vasicine was found to increase in the leaf and petiolar callus cultures of *Adhatoda zeylanicum* employing Murashige and Skoog (MS) medium supplemented with 10.7 μM of Naphthalene acetic acid (NAA), 4.4 μM [BA] and 2.3 μM kinetin [Kin], NAA, 4.5 μM 2,4 Di-chlorophenoxy acetic acid [2, 4-D], respectively.[14]

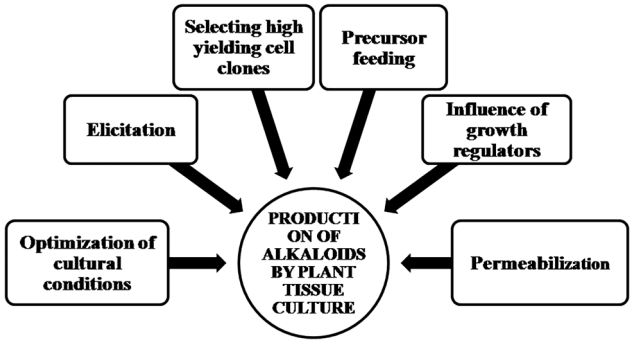

Figure 1: Factors affecting *in vitro* production of alkaloids

Elicitation

Elicitors are signals triggering the formation of secondary metabolites and classified as biotic and abiotic, both of them being used to stimulate the formation of secondary metabolites, thereby reducing the time to attain their high concentrations;[15] for example, incorporation of yeast extract and salicylic acid in the nutrient medium resulted in the elicitation of hyoscyamine and scopolamine content in the *in vitro* root cultures of *Datura metel* to the levels comparable to the intact parent plant.[16] The production of indole alkaloids enhanced in catharanthus cultures by employing amino acid, casein hydrolysate supplementations along with irradiation treatments.[17] The rise in KNO_3 concentration up to 35 mM increased the tropane alkaloidal content up to 3-20 times with improved ratio of hyoscyamine/scopolamine.[18] *In vitro* grown tissues (non-regenerative callus, regenerative callus, and microshoot-derived leaves) of *Solanum nigrum* cultured under salinity stress (0-150 mM) were found to yield maximum of solasodine with 150 mM of sodium chloride.[19]

Permeabilization

Since, the alkaloids may be highly toxic for plant cells and permeabilization might be a factor enhancing the alkaloid production. The permeability effect of tween-80 was studied on the production of tropane alkaloids from transformed roots of *Datura innoxia*. The permeabilization with tween modulated the tropane alkaloid accumulation by the release of alkaloid into the medium and the restoration of hyoscyamine content.[20] The immobilized callus cultures of *Tinospora cordifolia* were subjected to cell permeabilization with chitosan that showed a 10-fold increase in production of arabinogalactans as compared to respective controls devoid of resin and chitosan.[21]

Use of cell suspension cultures

Cell suspension culture systems can be utilized for large-scale culturing of plant cells with additional advantages [Figure 2], from which alkaloids could be extracted and thus ultimately

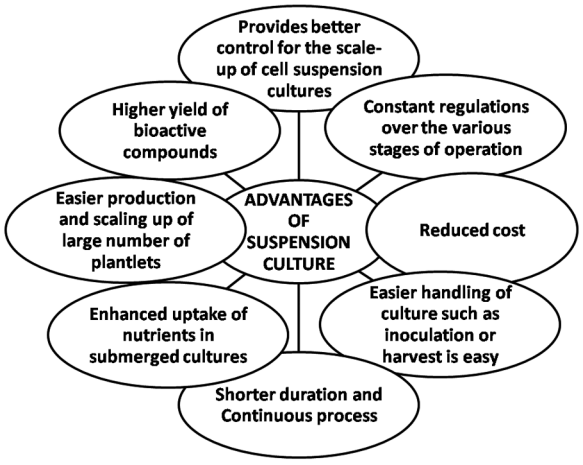

Figure 2: Advantages of cell suspension culture for production of alkaloids

providing a continuous and reliable source of bioactive natural products.[5] Screening, selection, and medium optimization of suspended cells resulted into a 20-30-fold increase in alkaloid concentrations in the case of high strain-producing cultures.[22]

The fast growing suspension cultures of *Vernonia cinerea* developed at the different combinations of BA and NAA for the production of alkaloids with maximum of biomass in 20 days and alkaloid content up to 1.15 mg/L.[23]

The significant amounts of sanguinarine were produced in cell suspension cultures of *Papaver somniferum* using bioreactors.[24] A bench top bioreactor allowing continuous extraction of secondary metabolites was designed for *Catharanthus roseus* and *Santalum album* plant cell suspensions, yielding various secondary metabolites.[25] Similarly, production of tropane alkaloids, scopolamine and hyoscyamine, has been achieved from the adventitious roots of *Scopolia parviflora* by employing bubble column bioreactor followed by elicitation using *Staphylococcus aureus* results in their enhanced production.[26]

In vitro regeneration micropropagation

In vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines and the same can be achieved through micropropagation[27] Plant regeneration from shoot, stem, and meristems has yielded encouraging results in *Catharanthus roseus, Cinchona ledgeriana*, *Rehmannia glutinosa,* and *Rauwolfia serpentina.*[28,29] Propagation through axillary shoot cultures of *Ranunculus asiaticus* L. was practiced using *in vitro* conditions for the accelerated propagation of selected indexed genotypes to supply more performing plant materials of a superior genotype.[30]

Callus-mediated organogenesis

The induction of callus growth, followed by differentiation and organogenesis is accomplished by the differential application of growth regulators along with the controlled conditions in the culture medium. A rapid *in vitro* propagation system leading to the formation of shoot from callus, roots, and plantlets was developed from *Schizanthus hookeri.* Ten alkaloids ranging from simple pyrollidine derivatives to tropane esters derived from angelic acid, tiglic acid, seneciocic acid, and methyl mesaconic acid were obtained from *in vitro* regenerated plantlets.[31]

Somatic embryogenesis

Efficient development and germination of somatic embryos are pre-requisites for the plantlet production and are achievable on MS medium even without the addition of growth regulators.[32] Techniques for the development of somatic embryogenesis of *Veratrum californicum* were developed. The *in vitro* developed plantlets were found to contain steroidal alkaloids, cyclopamine and veratramine, at the various steps of somatic embryogenesis.[33]

Metabolic engineering

Metabolic engineering plays an important role in production of drugs which are difficult to synthesize and are operated at whole cell level; for example, strain development for the production of artemisinin and benzylisoquinoline alkaloids.[34] Tropane alkaloids such as scopolamine and their transformation products have been biosynthesized successfully.[35] *Escherichia coli* has also been used successfully for the production of l-valine, an important drug precursor.[34]

Genetic transformations

The stable introduction of foreign genetic information into the plants represents one of the significant developments in recent advances of plant biotechnology including high volume production of pharmaceuticals[36] and opened the new avenues for the production of several biologically active natural compounds such as artemisinin, paclitaxel, scopolamine, etc.[37] Derivatives of plant pathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have been proved to be efficient and highly versatile vehicles for the production of genes into the plant genome resulting in the transfer and integration of genes of the plasmids from the bacteria into the plant DNA, transformed neoplastic tissues, crown galls, and hairy roots.[38]

An efficient protocol has been developed for the root cultures of transgenic opium poppy (*Papaver somniferum* L.) and California poppy (*Eschscholzia californica* Cham.) using *A. rhizogenes.*[39] Similarly, solasodine production was found to be regulated by *A. rhizogenes* induced transformed roots of *Solanum aviculare.*[40] In another instance, transformed roots of *Artemesia annua* yielded a sesquiterpene named artemisinin.[41]

A binary vector system in conjunction with the use of the R_i plasmid had been widely used for the integration of foreign genes into medicinal plants. The shooty teratomas of *Catharanthus roseus* epicotyls and stem nodal explants exhibited a 10-fold increased production of vincristine as compared to untransformed control cultures.[42]

Biotransformation using immobilized cell cultures

Immobilization or entrapment of the cells may produce a microenvironment that resembles the organized tissue in the intact cells causing differentiation and production of secondary metabolites.[43] Increased biotransformation yields of capsaicin and dihydrocapsaicin, major pungent principles of chilli pepper were obtained when immobilized placental tissues of *Capsicum frutescens* were fed with intermediate metabolites of the capsaicinoid pathway.[44]

Some of the examples for the *in vitro* production of different nucleus of alkaloids by adopting the various tissue culture techniques have been cited in Table 1.

Challenges faced by tissue culture technique

Challenges related to technological factors, socioeconomic

Table 1: Alkaloids produced by *in vitro* culture techniques

Plant name	Phytoconstituents/alkaloids	Explant used/culture medium/conditions	References
Brucea javanica (L.) Merr[a] (Simaroubaceae)	Canthinone alkaloids	Fruits/MS+2,4-D (1 mg/l), kinetin (0.1 mg/l), sucrose (5%)/cultures maintained under illumination on an orbital shaker with 2 cm stroke, temp -25±2°C	[45]
Catharanthus roseus[a] (Apocynaceae)	Catharanthine, ajmalicine	Leaf, stem/MS+NAA (2 mg/l), IAA (2 mg/l), kinetin (0.1 mg/l), sucrose (3%)/incubated on rotary shaker at 23±2°C in darkness	[46]
Catharanthus roseus[b] (Apocynaceae)	Dimeric indole alkaloids: Vindoline, vincristine, vinblastine	Petiole segments of seedlings (4-day old) MS+NAA (0.1, 5, 10, 20 ppm), Kin (0.1,5,10,20 ppm); maintained in dark for first 2 weeks followed by 24 h light period (fluorescent, cool white light, 7 W/m^2) for next 2 weeks at temp -35°C	[47]
Cereus peruvianus[b] (Cactaceae)	Alkaloids	Shoots/MS+tyrosine/incubated at temp -25±1°C under dark conditions	[48]
Chonemorpha grandiflora[b] (Apocynaceae)	Camptothecin	Inter-nodal segments of stem/MS+2,4-D (4.52 μM)/16/8 h (light/dark) photoperiod, temp -25°C	[49]
Leucojum aestivum[b] (Amaryllidaceae)	Galanthamine	Bulb, shoot/MS+BAP, NAA/cultured under illumination provided by fluorescent tubes or under dark at 25°C	[50]
Nandina domestica[b] (Berberidaceae)	Protoberberine alkaloids, dehydrodiscretamine, berberine, jatorrhizine	Fruits/MS+2,4-D (1.0 mg/l), kinetin (0.1 mg/l)/ constant illumination under fluorescent light, temp -25±2°C	[51]
Nicotiana rustica[b] (Solanaceae)	Alkaloids, nicotine	Seeds and aseptically germinated seedlings there from/LS agar+2,4-D (1 μM), kinetin (1 μM)/placed at 30°C in dark	[52]
Papaver bracteatum[b] (Solanaceae)	Thebaine	Seeds/MS+kinetin (0.47 μM)+2,4-D (4.52 μM or 0.45 μM), sucrose (3%)/incubated at 25°C	[53]
Pinellia ternata[a]	Guanosine. inosine, trigonelline	Tubercles, shoots/MS+NAA (0.5 ppm), 6-BA (1.0 ppm); NAA (02 ppm), 6-BA (1.0 ppm)/cultured under 16 h photoperiod providing 135 μmol/m^2/s light intensity at 25°C	[54]
Przewalskia tangutica[c] (Solanaceae)	Tropane alkaloids	Seeds, germinated seedlings, leaves liquid MS; genetic transformation-MS+AS (100 μmol/l)+30% sucrose+3.0 g/l phytogel/250 mg/l; germination of seeds at 25±0.5°C in dark conditions. Bacterial preparation carried out in orbital shaker at 180 rpm at 28°C for 30 min, culturing of hairy roots at 110 rpm at 25±1°C	[55]
Rauwolfia serpentine[b] (Apocynaceae)	Reserpine	Leaves MS+PABA (1 ppm)+NAA (4 ppm); temp -25±2°C, photoperiod of 16/8 light and dark cycle; some leaf explants incubated under total dark conditions to observe root growth.	[56]
Schizanthus hookeri[b] (Solanaceae)	Pyrrolidine derivatives to tropane esters derived from angelic acid, tiglic acid, seneciocic acid and methyl mesaconic acid, tropane alkaloids-3α-methylmesc aonyloxytropane	Roots, callus for generating shoots MS+ + NAA (2.69 μM), BA (2.22 μM) BA (4.44 μM)+NAA (0.54 μM); temp -22±1°C, light regimen of 14 h at 48 μmol/m2/s	[31]
Solanum nigrum[b] (Solanaceae)	Solasodine	Seeds, germinated plantlets MS+2,4-D (0.5 ppm), Kin (0.5 ppm); temp - 25°C light/20°C dark, photoperiod of 16 h by photon flux density of 100 μmmol/m^2/S	[57]
Solanum tuberosum[b] (Solanaceae)	Glycoalkaloids	Tubers MS+BA (0.5 ppm), IAA (2 ppm) Kinetin; temp - 26°C+16/8 light/dark cycle	[58]
Taxus (Taxaceae)	Taxane, paclitaxel, baccatin III, 10-deacetyl-baccatin	Roots MS+l-phenylalanine (1 μM); temp -25°C in dark conditions on a gyrorotary shaker at 122 rpm	[59]
Taxus globosa[a] (Taxaceae)	Baccatin III, paclitaxel	Stem, internodes, leaves, meristmatic tissues modified gamborg B5+methyl jasmonate (0.1, 1.0, 10, 100 μM)/incubated at 25±1°C under dark conditions or 16 h photoperiod, light is provided by fluorescent lighting of 1500 W	[60]

Contd...

Table 1: Contd...

Plant name	Phytoconstituents/alkaloids	Explant used/culture medium/conditions	References
Thalictrum minus[a] (Ranunculaceae)	Berberine	Leaf segments/LS+NAA (60 μM), BA (10 μM)/ cultures were agitated on reciprocal shaker at speed of 100 strokes/min at 25°C in dark	[61]
Vernonia cinerea[b] (Asteraceae)	Alkaloids	Young leaves MS+NAA (1, 1.5 ppm), BA (1, 5 ppm)/cultures incubated at 25±2°C with 16/8 h (light/dark) photoperiod under cool white fluorescent tubes	[23]
Withania coagulans[c] (Solanaceae)	Withaferin A	Seeds MS+IBA (0.2 ppm), sucrose (3%); provided with continuous white light illumination at 25°C on rotary shaker at 80 rpm	[62]
Withania somnifera[d] (Solanaceae)	Withaferin A, withanolide D	Single shoot tips/MS+BA (1 mg/l), sucrose (3%)/ incubated at 16 h/8 h (light/dark) photoperiod with rel. humidity of 55-60% maintained at temp -25±2°C	[63]

Alkaloids produced by [a]suspension culture, [b]callus culture, [c]root culture, [d]shoot culture

Table 2: Challenges faced by plant tissue culture techniques

Challenges	Issues	Remedial measures	References
Technological	Somaclonal variation Hyper-hydricity	*Pseudomonas* (bacteria) mediated approach to control hyper-hydricity	[64,65]
Socioeconomic	Inadequate infrastructure Lack of skilled manpower Inadequate funds Lack of interest	Management of micropropagation processes by software development Improvement of space and manpower scheduling	[66-69]
Dissemination of research outputs	Lower market values research Maximum emphasis on impacted publications	Concentrating on eco-friendly research Plantation of energy plants, e.g., Jatropha, a source of biodiesel	[70,71]
Policy issues	Implementation at national/global level	Commercialization of tissue culture outputs Alternative plant source or endangered medicinal plants, e.g., camptothecin by *Chonemorpha grandiflora, Ophiorrhiza pumila*	[49, 68,71]
Research priorities	Irrelevant research projects	Beneficial and relevant research projects Production of therapeutically important secondary metabolites	[68,49]
General perception about technique	Lack of scientific knowledge about biotechnological principles	Updation of knowledge about biotechnological principles Conveying scientific information to the general public	[68]

factors, public perception of biotechnology, policy issues, and aspects related to research priorities and dissemination of research outputs are among the bottle necks affecting the productivity of plant tissue culture techniques and they have been summarized in Table 2.

FUTURE PROSPECTS

Plant tissue culture has remained an art because of the unique culture conditions required for each medicinal plant. To accommodate a genotype or species that has not been manipulated in culture previously, one must adapt an established protocol or create a new one bearing in mind about the efficiency imperatives. Plant tissue culture provides valuable tool for synthesizing same range of chemicals as that of natural plant as well as novel compounds are also synthesized via biotransformations.[68] The ability of *A. rhizogenes* to induce hairy roots in a range of medicinal plants has been exploited as a source of root-derived pharmaceutical compounds.[72] Over the past two decades, the concept of plant-based production of high volume-based pharmaceutical proteins such as vaccines and antibodies has received growing research interest and offered critical advantages over traditional bacterial and mammalian cell-based systems.[73] The production of recombinant proteins using either whole plant[74] or *in vitro* plant cell cultures commonly known as molecular framing is emerging as promising prospects for the pharmaceutical industry. Similarly, metabolomics and dereplication of medicinal plants and their cultures may lead to development of new drug molecules from tissue culture techniques since several times it produces novel molecules unrelated to its parent plant.[75]

CONCLUSION

This review gives an insight to the different aspects of tissue culture for the production of alkaloids under *in vitro* conditions. The review gives an insight to the various factors that may affect their production along with the

suitable examples. An update has been provided to the different approaches utilized for increasing the production of alkaloids. Different challenges posed to tissue culture techniques at various stages were discussed along with their respective remedial measures. At the end, future perspective has been framed in lieu of their advanced applications to utilize them at industrial and commercial scales.

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