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 metabolites⁵ 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.
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.\[^9\] 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.\[^14\]

**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\textsubscript{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

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

*Figure 2:* Advantages of cell suspension culture for production of alkaloids
providing a continuous and reliable source of bioactive natural products.\textsuperscript{[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.\textsuperscript{[22]}

The fast growing suspension cultures of \textit{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.\textsuperscript{[23]}

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

\textbf{In vitro regeneration micropropagation}

\textit{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\textsuperscript{[27]} Plant regeneration from shoot, stem, and meristems has yielded encouraging results in \textit{Catharanthus roseus}, \textit{Cinchona ledgeriana}, \textit{Rehmannia glutinosa}, and \textit{Rauwolfia serpentina}.\textsuperscript{[28,29]} Propagation through axillary shoot cultures of \textit{Ranunculus asiaticus} \( \text{L.} \) was practiced using \textit{in vitro} conditions for the accelerated propagation of selected indexed genotypes to supply more performing plant materials of a superior genotype.\textsuperscript{[29]}

\textbf{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 \textit{in vitro} propagation system leading to the formation of shoot from callus, roots, and plantlets was developed from \textit{Schizanthus hookeri}. Ten alkaloids ranging from simple pyrollidine derivatives to tropane esters derived from angelic acid, tiglic acid, senecioic acid, and methyl mesaconic acid were obtained from \textit{in vitro} regenerated plantlets.\textsuperscript{[31]}

\textbf{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.\textsuperscript{[32]} Techniques for the development of somatic embryogenesis of \textit{Veratrum californicum} were developed. The \textit{in vitro} developed plantlets were found to contain steroidal alkaloids, cyclopamine and veratramine, at the various steps of somatic embryogenesis.\textsuperscript{[33]}

\textbf{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.\textsuperscript{[34]} Tropane alkaloids such as scopalamine and their transformation products have been biosynthesized successfully.\textsuperscript{[35]} \textit{Escherichia coli} has also been used successfully for the production of \( l \)-valine, an important drug precursor.\textsuperscript{[36]}

\textbf{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\textsuperscript{[37]} and opened the new avenues for the production of several biologically active natural compounds such as artemisinin, paclitaxel, scopalamine, etc.\textsuperscript{[38]} Derivatives of plant pathogens \textit{Agrobacterium tumefaciens} and \textit{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.\textsuperscript{[39]}

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

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

\textbf{Biotransformation using immobilized cell cultures}

Imbobilization 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.\textsuperscript{[43]} Increased biotransformation yields of capsacin and dihydrocapsacin, major pungent principles of chilli pepper were obtained when immobilized placental tissues of \textit{Capsicum frutescens} were fed with intermediate metabolites of the capsainoid pathway.\textsuperscript{[44]}

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

\textbf{Challenges faced by tissue culture technique}

Challenges related to technological factors, socioeconomic
Table 1: Alkaloids produced by *in vitro* culture techniques

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Phytoconstituents/alkaloids</th>
<th>Explant used/culture medium/conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucea javanica</em> (L.)</td>
<td>Canthinone alkaloids</td>
<td>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</td>
<td>[45]</td>
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<td>Merr (Simaroubaceae)</td>
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<tr>
<td><em>Catharanthus roseus</em></td>
<td>Catharanthine, ajmalicine</td>
<td>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</td>
<td>[46]</td>
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<td>(Apocynaceae)</td>
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<tr>
<td><em>Catharanthus roseus</em></td>
<td>Dimeric indole alkaloids: Vindoline, vincristine, vinblastine</td>
<td>Petiole segments of seedlings (4-day old)</td>
<td>[47]</td>
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<tr>
<td>(Apocynaceae)</td>
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<tr>
<td><em>Cereus peruvianus</em></td>
<td>Alkaloids</td>
<td>Shoots/MS+tyrosine/incubated at temp -25±1°C under dark conditions</td>
<td>[48]</td>
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<td>(Cactaceae)</td>
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<tr>
<td><em>Chonemorpha grandiflora</em></td>
<td>Camptothecin</td>
<td>Inter-nodal segments of stem/MS+2,4-D (4.52 μM)/16/8 h (light/dark) photoperiod, temp -25°C</td>
<td>[49]</td>
</tr>
<tr>
<td>(Apocynaceae)</td>
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<tr>
<td><em>Leucojum aestivum</em></td>
<td>Galanthamine</td>
<td>Bulb, shoot/MS+BAP, NAA/cultured under illumination provided by fluorescent tubes or under dark at 25°C</td>
<td>[50]</td>
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<td>(Amaryllidaceae)</td>
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<tr>
<td><em>Nandina domestica</em></td>
<td>Protoberberine alkaloids, dehydrodiscretamine, berberine, jatrophizine</td>
<td>Fruits/MS+2,4-D (1.0 mg/l), kinetin (0.1 mg/l)/constant illumination under fluorescent light, temp -25±2°C</td>
<td>[51]</td>
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<tr>
<td>(Berberidaceae)</td>
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<tr>
<td><em>Nicotiana rustica</em></td>
<td>Alkaloids, nicotine</td>
<td>Seeds and aseptically germinated seedlings there from L53 agar+2,4-D (1 μM), kinetin (1 μM)/placed at 30°C in dark</td>
<td>[52]</td>
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<td>(Solanaceae)</td>
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<tr>
<td><em>Papaver bracteatum</em></td>
<td>Thebaine</td>
<td>Seeds/MS+kinein (0.47 μM)+2,4-D (4.52 μM or 0.45 μM), sucrose (3%)/incubated at 25°C</td>
<td>[53]</td>
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<tr>
<td>(Solanaceae)</td>
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<tr>
<td><em>Pinellia ternata</em></td>
<td>Guanosine, inosine, trigonelline</td>
<td>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/m2/s light intensity at 25°C</td>
<td>[54]</td>
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<td><em>Przewalska tangutica</em></td>
<td>Tropane alkaloids</td>
<td>Seeds, germinated seedlings, leaves liquid MS; genetic transformation-MS+AS (100 μM)+30% sucrose+3.0 g/l phyto gel/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</td>
<td>[55]</td>
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<td>(Solanaceae)</td>
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<td><em>Rauwolfia serpentine</em></td>
<td>Reserpine</td>
<td>Leaves</td>
<td>[56]</td>
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<tr>
<td>(Apocynaceae)</td>
<td></td>
<td>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.</td>
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<tr>
<td><em>Schizanthus hookeri</em></td>
<td>Pyrrolidine derivatives to tropane esters derived from angelic acid, tiglic acid, senecioic acid and methyl mesaconic acid, tropane alkaloids-3α-methylmesc aonyloxytropane</td>
<td>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</td>
<td>[31]</td>
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<tr>
<td>(Solanaceae)</td>
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<tr>
<td><em>Solanum nigrum</em></td>
<td>Solasodine</td>
<td>Seeds, germinated plantlets</td>
<td>[57]</td>
</tr>
<tr>
<td>(Solanaceae)</td>
<td></td>
<td>MS+2,4-D (0.5 ppm), Kin (0.5 ppm); temp -25°C light/20°C, photoperiod of 16 h by photon flux density of 100 μmol/m2/s</td>
<td></td>
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<tr>
<td><em>Solanum tuberosum</em></td>
<td>Glycoalkaloids</td>
<td>Tubers</td>
<td>[58]</td>
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<tr>
<td>(Solanaceae)</td>
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<tr>
<td><em>Taxus</em> (Taxaceae)</td>
<td>Taxane, paclitaxel, baccatin III, 10-deacetyl-baccatin</td>
<td>Roots</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MS+I-phenylalanine (1 μM); temp -25°C in dark conditions on a gyratory shaker at 122 rpm</td>
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</tr>
<tr>
<td><em>Taxus globosa</em></td>
<td>Baccatin III, paclitaxel</td>
<td>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, ligand is provided by fluorescent lighting of 1500 W</td>
<td>[60]</td>
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</tbody>
</table>

Contd...
Ahmad, et al.: In vitro production of alkaloids

### Table 1: Contd...

<table>
<thead>
<tr>
<th>Plant name (Family)</th>
<th>Phytoconstituents/alkaloids</th>
<th>Explant used/culture medium/conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalictrum minus* (Ranunculaceae)</td>
<td>Berberine</td>
<td>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</td>
<td>[61]</td>
</tr>
<tr>
<td>Vernonia cinerea* (Asteraceae)</td>
<td>Alkaloids</td>
<td>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</td>
<td>[23]</td>
</tr>
<tr>
<td>Withania coagulans* (Solanaceae)</td>
<td>Withaferin A</td>
<td>Seeds MS+IBA (0.2 ppm), sucrose (3%); provided with continuous white light illumination at 25°C on rotary shaker at 80 rpm</td>
<td>[62]</td>
</tr>
<tr>
<td>Withania somnifera* (Solanaceae)</td>
<td>Withaferin A, withanolide D</td>
<td>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</td>
<td>[63]</td>
</tr>
</tbody>
</table>

Alkaloids produced by *suspension culture, *callus culture, *root culture, *shoot culture

### Table 2: Challenges faced by plant tissue culture techniques

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

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.[64] 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.[22] 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.[79]

**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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