REVIEW ARTICLE

Production of Camptothecin from *Nothapodytes nimmoniana*: An Overview

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ABSTRACT- *Nothapodytes nimmoniana* is an endangered tree endemic to the Western Ghats, India and it is the convenient source for large scale isolation of camptothecin (CPT). Since the first report of CPT detection in *N. nimmoniana*, significant work has been done on its applications. Due to heavy collection of its wood chips for CPT, population is under threat in India. Several plant tissue culture techniques offer alternative strategies for clonal propagation and CPT production to conserve the species. Various strategies are employed to enhance *in vitro* condition response through culture media optimization, elicitation, and artificial seed method. In this article, we have reviewed progress made so far on different methods of plant tissue culture techniques for production of camptothecin from *N. nimmoniana* and biosynthesis of CPT in details.

Key-words- Biosynthesis, Camptothecin, Elicitation, *Nothapodytes nimmoniana*, Plant tissue culture,

INTRODUCTION

The Western Ghats are known for their rich as well as unique flora and fauna. It is one of the richest sources for medicinal plants which are used for curing various diseases. *N. nimmoniana* (*J. Graham*) Mabberly (Syn. *Nothapodytes foetida*) belongs to the family Icacinaceae, it is a small tree which can grow up to 8 meters tall, which is allocated in Nilgiris, Annamalis, Pullneys, North Kanara and Konkan Ghats, broadly in Western Ghats of India, a global biodiversity hot spot. The tree is endemic to Western Ghats, but is also distributed in Sri lanka, China, South East Asia, North Sumatra, Taiwan and Myanmar [1-3]. The significant interest in this plant is due to the presence of anti-cancer drug, camptothecin (CPT) and 9-Methoxy CPT (Fig. 1). CPT is a monoterpene cytotoxic quinoline alkaloid, first isolated from the plant *Camptotheca acuminata* [4] which belongs to the family Nyssaceae and later in *N. nimmoniana* [5]. It is also reported in *Pyrenacantha klaineana* [6], *Ophiorrhiza* species [7], *Chenomorpha fragrance* [8], *Dysoxylum binectariferum* [9] and other related species [10]. Reason for distribution of CPT in several unrelated taxa suggests that the genes encoding enzymes involved in CPT biosynthesis evolved early during evolution, but followed switched 'ON' and 'OFF' process for a certain period [11].

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CPT is treated as third most promising anti-cancer drug of the twenty first century after taxol and vinca alkaloids [12]. The alkaloid is hoarded from different parts of *N. nimmoniana* in variable quantities with the highest concentration found in roots, followed by stem, leaves and fruits [3,13,14]. The concentration of CPT increases with increase in age of the plant after, which it stays proportionate to bark thickness [15]. CPT stabilizes the Topoisomerase I-DNA covalent complex thereby inhibiting the Topoisomerase activity. These CPT stabilized cleavage complexes act as physical barriers to DNA synthesis, chromatin structure and genes for transcription control and there by killing cells as a result of replication fork collision. Hence CPT is named as Topoisomerase 'poisons' [16,17]. Due to cytotoxicity and reduced solubility, the natural compound is difficult to synthesize, but several derivatives with better puissance than the parent compound are developed for the treatment of cancer [11]. Topotecon and Irinotecan (Fig. 1) are the two major water soluble anti-cancer drugs used for the treatment of cervical, ovarian, lung and colorectal cancers [18]. Both these semi-synthetic derivatives of CPT have been accredited by the Food and Drug Administration (FDA) of USA for various genres of cancers as well as several types of brain tumors [19]. Apart from anti-cancer activity, it has also exhibited activities such as anti-HIV, pesticidal, anti-parasitic, anti-psoriasis, antimicrobial, anti-angiogenic and neurotoxic activities [20]. The cytotoxic effect of *Plasmodium falciparum* has proven to be the target of new anti-malarial drug development system [21].

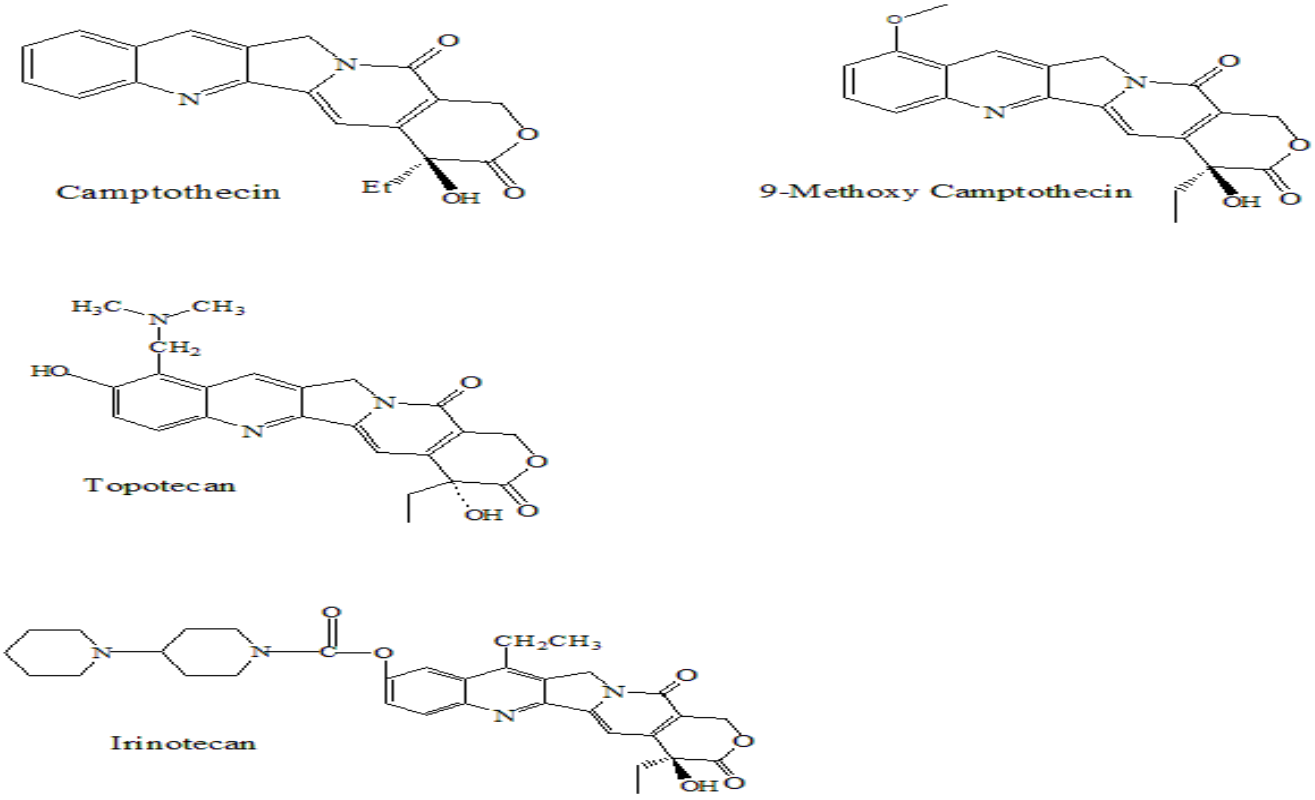

Fig. 1: Structure of CPT and its derivatives

As the trees are grown mainly in India, it is the leading exporter of CPT in the form of dried wood chips which are mainly exported to Japan, USA, and Spain for commercialization. The annual worldwide sale of CPT is reported to exceed 1000 tons but the unreported trade may be at least twice the reported one [15,22]. Whole *N. nimmoniana* trees are cut into pieces to generate biomass for extraction and export. The collectors train tribal people on the cutting of trees and drying with a pay of $ 0.16–$0.24/Kg and exporters sell the wood chips at $1500/Kg in the world market [23].

Due to high demand for the CPT worldwide, there has been rampant and illegal cutting of trees in India, specifically from Western Ghats leading to deterioration in population of the trees. Owing to these factors, *N. nimmoniana* has been considered as 'Endangered' species [1,24]. To meet the demand for CPT in pharmaceutical industry and also to conserve *N. nimmoniana* population, several harvesting strategies are being employed. One of the approaches is to develop a strategy for large scale propagation of the plant without affecting natural resources [3,25]. Many researchers have investigated different geographical locations across India to find out the better species for high CPT yield through clonal propagation [26-28]. Many *in vitro* techniques are engaged for the enhanced yield of CPT such as media optimization, selection of high yielding cell lines, elicitation using different biotic and abiotic elicitors and hairy root cultures. In this review, the various strategies employed for the CPT production through differences *in vitro* techniques are discussed.

Plant Description- *N. nimmoniana* is a small tree with 3-8 m tall; young branches pale and bark is smooth, grey, wrinkled, about 5 mm in thick. Leaves are simple with alternate, slightly leathery in texture with egg shaped to elliptical-oblong, no hairs above, thin hairs beneath the leaves, crowded at the end of branches, leaf stacks are 3-6 cm in length. Petioles 0.5-1.25 inches long, glabrous or faintly pubescent. Flowers in terminal panicles 2-4 inches long, pedicels stout, densely pubescent, Calyx small, pubescent externally, 5-toothed; teeth triangular, very acute. Petals yellow, 0.25 inches long, linear-oblong, acute, densely sericeo-villos on both surfaces, tip inflexed. Stamens 5, filaments flattened and dilated below, attached to the edges of the petals at the base; anthers elliptic-oblong, not apiculate. Disk shallow, cup-shaped, villous within ovary ovoid, densely clothed with silky hairs, free, attenuated into a stout slightly conical style. Drupes ellipsoid, 0.375–0.625 inches long, pubescent when young, glabrous and purple, when ripe endocarp thin, woody, and endosperm foetid. The flowering season is during the month of July to August and ripened fruits are available during the month of November to December [29,30].

Biosynthesis of CPT- Camptothecin was isolated many years ago, but still its biosynthetic pathway is poorly inferred. Generally, CPT undergoes Shikimate pathway of (i) Common primary metabolism of tryptophan from chorismate and then moving into common secondary TIA pathway, starting from decarboxylation of tryptophan to tryptamine, in parallel, (ii) The non mevalonate 2C-methyl-D-erythritol-4-phosphate (MEP) pathway giving secologanin, from pyruvate and glyceraldehydes-3-phosphate, and the merging of tryptamine and secologanin in (iii) The specific pathway leading to CPT

but in second (TIA anabolism) and third (CPT-specific many of the biosynthetic enzymes and intermediates leading to the final product are missing. Because of the complexity of the pathway, it is divided into two main anabolism) pathways, parts (i) Pre-strictosidine part (Tryptamine and secologanin pathway), (ii) Post-strictosidine part (CPT pathway).

Pre-strictosidine part (Tryptamine and secologanin pathway)- Tryptamine pathway: Involvement of tryptamine and secologanin in CPT biosynthesis was first described in *Camptotheca acuminata* [31]. Precursor chorismate is converted to anthranilate with the help of an enzyme anthranilate synthase (β- subunit). It is the first step in tryptamine synthesis. In the next step, anthranilate with the addition of 5-phosphoribulose pyrophosphate is converted to 5-phosphoribosyl anthranilate in the subsequent steps to indole glycerol phosphate; these are intermediates in the pathway. The indole glycerol phosphate by the action of α- subunit of tryptophan synthase (TSA) gets converted to indole, which is then condensed with β- subunit of tryptophan synthase (TSB). In the final step, tryptophan is decarboxylated to form tryptamine which is catalyzed by tryptophan decarboxylase.

Secologanin pathway- The precursor for the formation of secologanin pathway is Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Secologanin is formed through several steps starting from the condensation of IPP and its isomer DMAPP to yield geranyl diphosphate (GPP). GPP is then converted to geraniol which is catalyzed by geraniol synthase. Geraniol to secologanin synthesis is mainly catalyzed by cytochrome P450 monooxygenases including geraniol 10-hydroxylase which is involved in the conversion of geraniol to 10-hydroxygeraniol. Secologanin synthase is involved in the formation of secologanin from loganin (Fig. 2).

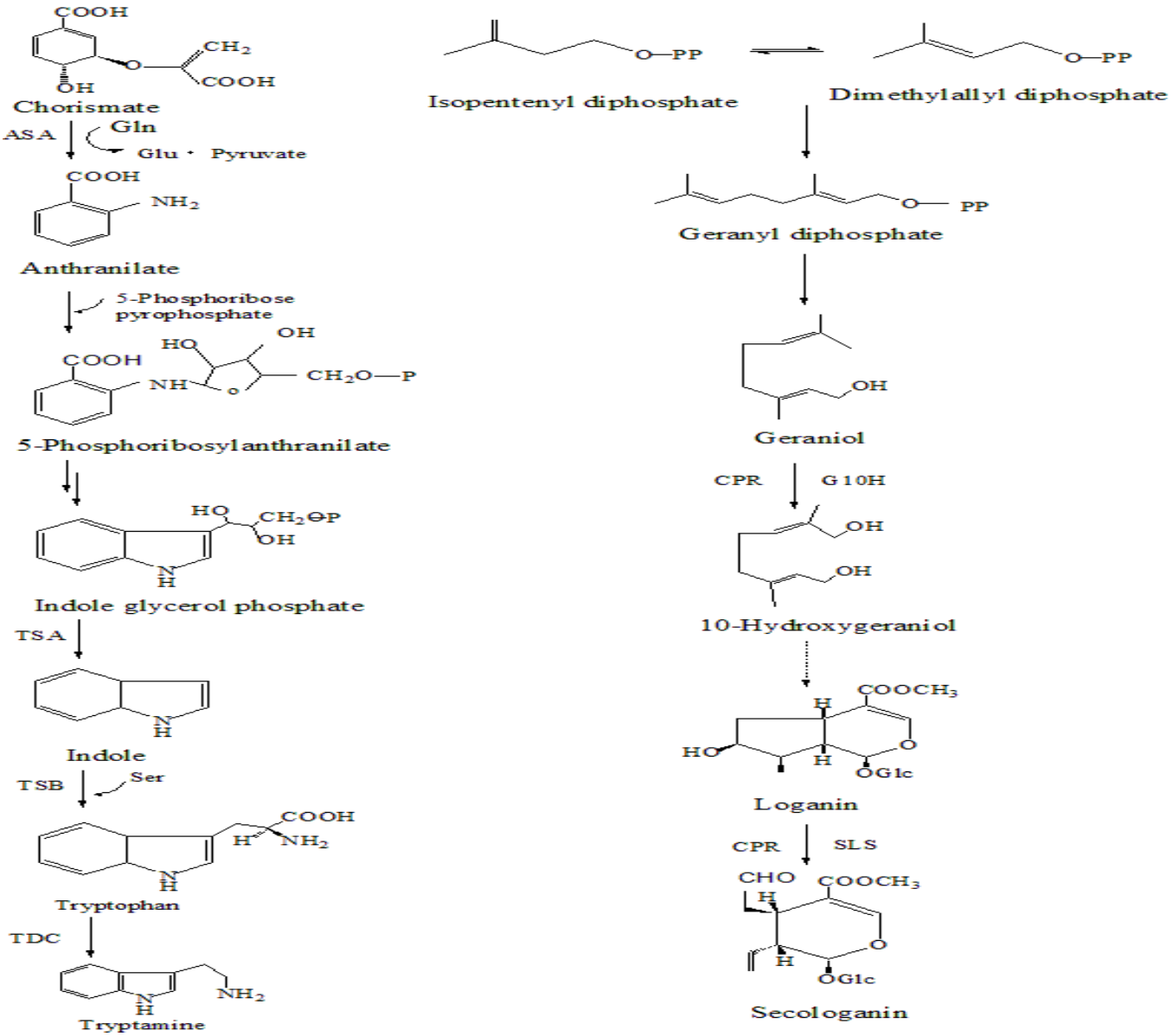

Fig. 2: Biosynthetic pathway of Tryptamine and Secologanin

[ASA-Anthranilate synthase; TSA-the alpha-subunit of tryptophan synthase; TSB-the beta-subunit of tryptophan synthase; TDC-tryptophan decarboxylase; Gln-glutamine; Glu-glutamic acid; Ser-serine; P-phosphate group. DXS-1-deoxy-D-xylulose-5-phosphate synthase; DXR-1-deoxy-D-xylulose-5-phosphate reductoisomerase; HDR-1-hydroxy- 2-methyl-2(E)-butenyl-4-diphosphate reductase; G10H- geraniol 10-hydroxylase; SLS-secologanin synthase; CPR- NADPH:cytochrome P450 reductase; PP-diphosphate group. The arrow with a dotted shaft indicates the involvement of multiple enzymatic steps].

Post strictosidine part (CPT biosynthesis)- Strictosidine synthesis: the initial step of TIA biosynthesis involves tryptamine and secologanin reaction by the action of strictosidine synthase (STR) to yield strictosidine.

Strictosidine to CPT- Intramolecular cyclization of strictosidine yields strictosamide, but more details about the exact intermediates between strictosamide and CPT are not completely known (Fig. 3). It is postulated that CPT could be formed from strictosamide by the sequential (i) oxidation-recyclization of the B and C- rings (ii) Oxidation of the D- ring and removal of the C-21 glucose moiety (iii) Oxidation of the E-ring [32].

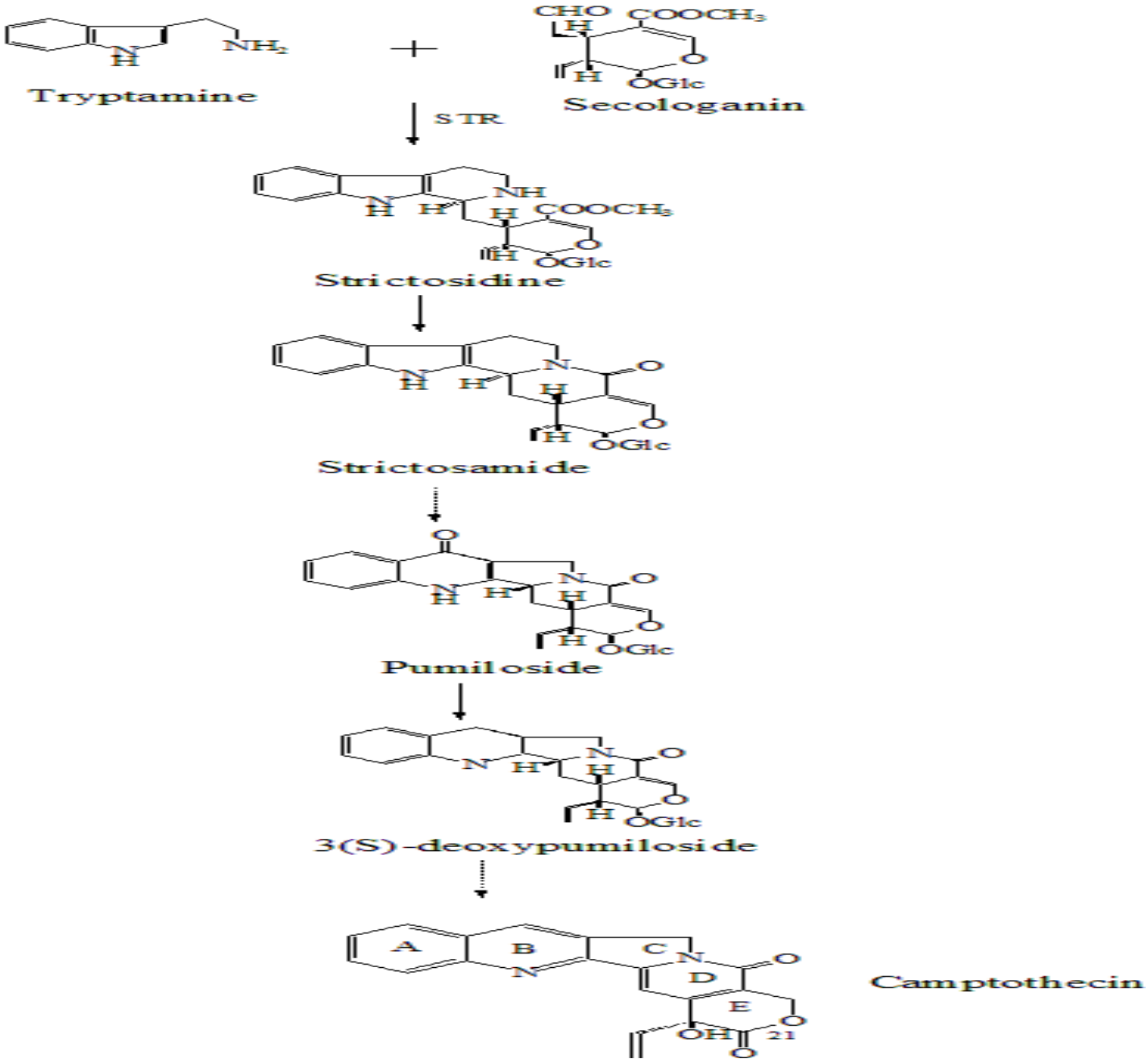

Fig. 3: Biosynthetic pathway for production of camptothecin

[STR-strictosidine synthase; Glc-glucose moiety. Arrows with a dotted shaft represent the involvement of multiple enzymatic steps].

Production of CPT from various tissue culture techniques- The complex structures of most secondary metabolites make their chemical synthesis an Augean and

economically inconvenient process, and moreover, most of the plants producing valuable secondary metabolites are in endangered condition or their habitat is laborious to access. Therefore, biotechnological production methods constitute an alternate and efficient way for obtaining specific secondary metabolites. The abatement in the diversity of plant by uncommon cutting and exploitation leads to various tissue culture methods to preserve the plant diversity and production of concerned products from it [33].

Callus cultures- Camptothecin (CPT) yield from different explants of *in vitro* regenerated and field grown plants varied (Table 1). Roja and Heble [34] were the first to produce CPT through *in vitro* culture techniques from *N.nimmoniana*. The protocol involved taking various explants from tree *i.e.* Immature embryos, stems and leaves. These explants were inoculated onto different growth hormones and incubated at appropriate conditions. Immature embryos showed better response among others with callus development on MS medium supplemented with BAP and 2, 4-D, multiple shoot development was observed when cultured on BAP and NAA. Low levels of 2, 4-D (0.2 and 3.0 ppm) showed brownish hard callus with embryo like structure. Whereas, high levels of 2, 4-D (5.0 ppm and 10.0 ppm) gave friable callus. Ciddi *et al.* [35] established callus cultures from MS media supplemented with Picloram at 2 mg/l, the response was observed in 3 weeks, after 4 passages, the cultures were extracted for quantitative analysis for the presence of CPT and 9-methoxy CPT. Fulzele *et al.* [36] quantified CPT from suspension cultures of *N. nimmoniana.* Stem parts were used as explants and inoculated onto MS media with different concentrations of 2, 4-D and NAA. After 3 weeks of incubation under appropriate conditions, callus cultures were inoculated into liquid media containing NAA (10.74 µM) and BAP (2.22 µM) and incubated for 20 days. A considerable amount of CPT (0.035 mg/ml) and 9-methoxy CPT (0.026 mg/ml) were found in the medium. Thengane *et al.* [37] analysed the production CPT from callus cultures of *N. nimmoniana* cotyledon explants, authors experimented using different concentrations of growth hormones. Callus induction was observed within 10 days of inoculation of explants, then the CPT content was analyzed using HPLC and found to be 1.306% of DW in MS medium supplemented with 2, 4-D and BAP. Karwasara *et al.* [38] studied the effect of culture media nutrients on the growth and production of camptothecin by varying sugar, nitrate and ammonium concentrations. The modified MS medium was prepared which contained 0.5 mM phosphate, a nitrogen source feeding ratio of 50/10 mM NH_4^+/NO_3^- and 3 % sucrose with additional 2 % sucrose feeding (added on day 12 of the cell culture cycle) with 10.74 µM NAA and 0.93 µM KN. The results showed 1.7 fold increase in intracellular CPT content and 2.3 fold increase in extracellular CPT content.

Plant regeneration- Regeneration of plants from different explants has been reported in many articles; Thengane *et al.* [37] reported the regeneration of adventitious shoots from different explants such as Cotyledons, hypocotyls and leaves which was cultured on MS media augmented with various concentrations of TDZ ranging from 0.45-4.54 µM. the rooting was observed in shoots with 3/4-strength MS medium supplemented with 2.22 µM BAP + 0.49 µM IBA. Plants obtained were planted in polythene bags containing 1:1 ratio of sand and soil mixture, 50% survival rate was observed. Regeneration from the callus cultures were first reported by Tejavathi *et al.* [28], they inoculated the embryo explants on three different Media i.e., MS, Modified MS and Philips and Collins (L2) media which was augmented with different growth hormones. Among the growth hormones used, TDZ showed better growth with multiple shoots and also shoot differentiation was reported from callus cultures. L-glutamine was added which showed enhanced results with dark green callus and other salutary effects on morphological features. The generated shoots were excised and inoculated onto MMS supplemented with IBA at 2.46 µM for rooting, initiation of roots started after 4–5 weeks of incubation. The generated plantlets were transferred to plastic cups containing soilrite and covered with perforated polythene bags for 4-5 weeks and transferred to pots containing soil, manure and sand in the ratio of 1:1:1. The survival rate observed was 40%. Dandin and Murthy [26] were reported that liquid cultures are better than solid cultures in terms of shoot induction and multiplication. They investigated using embryo explants with MS media containing TDZ at 2 µM after callus formation, it was split into 2 batches, one batch was cultured in liquid medium and the other was cultured on solid medium with the same concentration of MS media with BAP 2 µM. The CPT content was found to be 0.028% and 0.021% for leaves and stem respectively in liquid medium whereas, in semisolid medium the content was 0.082% and 0.037% for leaves and stem respectively. Ugraiah *et al.* [39] established multiple shoot plantlets by supplying a variety of growth hormones in to the MS medium, among the growth hormones used, BAP at 8.87 µM showed better response in both leaf (25.4±0.54) and nodal (28.0±0.37) explants respectively within 60 days. Further proliferations of obtained cultures were done in MS medium in combination with 4.44 µM BAP and 0.87 µM gibberellic acid (GA3). The obtained shoots were rooted on half strength MS medium containing 4.9 µM IBA and showed 90% survival rate. The obtained plants from *in vitro* technique showed better CPT content ranging from 0.08% to 0.2%. Kaveri and Srinath [40] was reported the observation of multiple shoots as well as callus, which was friable from mature embryos and hypocotyl explants when inoculated onto MS medium supplemented with TDZ at 0.1 mg/l (14.60±0.32/explant). It was reported that number of shoots decreased with increase in the concentration of growth hormone. TDZ was shown dual character by performing both auxin and cytokinin like function, when supplemented in the medium during the *in vitro* culturing of tree species.

Induction of Untransformed (Adventitious) and transformed (Hairy roots) roots for camptothecin production- For large scale production of the secondary metabolite, hairy root transformation is one of the approaches to be studied. Further, it can be upgraded to bioreactors. Untransformed hairy root cultures were developed on semi-solid medium supplemented with high concentration of NAA [41], while transformed hairy root cultures were developed from nodal and leaf segments co cultivated with *Agrobacterium rhizogene*s at different transformation efficiency, strain type, plant part and *N. nimmoniana* clone [42]. The transformed hairy root cultures showed a high frequency of growth on semi-solid medium without PGR's and grew 1.8 times faster in liquid medium with the release of CPT into the media after 30 days.

Production of Camptothecin through Elicitation- Elicitation is the process of adding trace quantity of substance to ameliorate the bio synthesis of specific compounds. Gamma irradiation can be used to improve the production of secondary metabolites. Fulzele *et al.* [43] established Callus cultures using MS medium in combination with NAA and BAP (10.74 µM+2.22 µM) and giving gamma radiation doses ranging from 5 to 30 Gy at room temperature. Low doses of gamma radiation found to increase the product quantity by 20 folds than non-irradiated callus cultures at 20 Gy.
Isah [27] was reported the elicitation of CPT production in hypocotyl derived calli with yeast extract (YE) and vanadyl sulfate (VS), both the elicitors enhanced CPT production more in liquid medium than in solid medium; YE increased the production by 4.2 fold whereas VS increased the production by 3.03 fold in liquid medium.

Production of artificial Seeds- Because of low seed germination rate of *N. nimmoniana* even at favorable conditions, artificial seed production would be one of the approaches to combat this issue. Rajta *et al.* [44] established callus cultures using leaf explants with combination of IBA (2 mg/l) and KN (1 mg/l) giving the response at 50–55 days. Then the mature embryos from callus cultures were taken for artificial seed formation by using 3% sodium alginate and 100 mM Calcium chloride and allowed for germination on MS medium supplemented with IBA+KN+GA_3 (1 mg/l+3 mg/l + 2 mg/l), the response was observed in 10–13 days. The advantage of using artificial seeds is that their availability throughout the year irrespective of the seasons.

Table 1: Camptothecin yield from various *in vitro* raised *N. nimmoniana* tissues

Explant	PGR	Tissue used	CPT yield (%)
Immature embryo [34]	BAP+NAA	Regenerated plant Callus	High
Zygotic embryo [35]	Picloram (8.28 µM)	Callus	0.00095
Zygotic embryo [36]	NAA+BAP (10.74 µM+2.22 µM)	Suspension culture	0.035
Zygotic embryo [36]	NAA+BAP (10.74 µM+2.22 µM)	Callus Somatic embryos	Traces 0.011
Zygotic embryo [41]	NAA+BAP (71.36 µM+8.87 µM)	Untransformed root cultures Regenerated plant root	0.01 0.12
Mature and immature cotyledons [37]	2,4-D+BAP (0.90–13.57 µM+2.22 µM)	Callus Callus	0.126–1.30 –
Plantlet [22]	Picloram+BAP+GA3 (8.28 µM+4.44 µM +5.20 µM)	Regenerated plant	2.893±2.38
Stem and leaf Segment [45]	NAA+BAP (8.055 µM +13.35 µM) 2,4-D+Kin (2.26 µM+4.64 µM) NAA (5.37 µM) PGRs-free	Callus Cell suspension culture Untransformed root culture Somatic embryos (globular)	0.0030 0.00087 0.1196 0.0082
Zygotic embryo [46]	NAA+BAP (10.74 µM+2.22µM)	Callus	0.4903
Nodal segment [26]	TDZ (2 µM)	Regenerated leaves in liquid medium Stems of regenerated plant in liquid medium Regenerated leaves on semi-solid medium Stems of regenerated plant on semi-solid medium	0.028 0.021 0.082 0.037
Leaf and stem Explants [38]	NAA+Kin (10.74 µM+0.9 µM)	Cell suspension culture	0.005171

CONCLUSIONS

Interest in *N. nimmoniana* has increased over the years due to its medicinal properties. Extracts of this plant is used in heterogeneous forms for the treatment of several diseases. Sufficient information is known on *in vitro* clonal propagation of *N. nimmoniana* through direct regeneration but less is known on indirect regeneration. The production of camptothecin (CPT) is affected by *in vitro* culture conditions, concentration of PGR's and also by concentration of other nutrients. The influences of these factors are already known, but other external factors such as light and physical factors are yet to be studied. Molecular studies to find out intermediates and other regulatory factors which are crucial in the biosynthesis of CPT are also studied. Large scale multiplication of the plant through artificial seed method, enhanced production of CPT through irradiation and elicitation using both biotic and abiotic compounds are also promising fields to explore.

AUTHOR CONTRIBUTION STATEMENT

Mithun P R has collected and studied the literature and drafted the Research article. Praveen N was involved in editing of article. Jobi Xavier and Jayarama reddy assisted in compilation of the article. All the authors agreed on article content.

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