Influence of plant growth regulators on morphogenic response, biomass and camptothecin production in the callus cultures of Chonemorpha fragrans (Moon) Alston

Tasiu Isah (taasmore@yahoo.co.uk)
Hamdard University New Delhi https://orcid.org/0000-0002-2112-530X

Research article

Keywords: Medicinal plants. Phytochemicals. Alkaloid. Camptothecin. Plant growth regulators. Morphogenic callus.

Posted Date: October 11th, 2019

DOI: https://doi.org/10.21203/rs.2.10713/v2

Version of Record: A version of this preprint was published at Notulae Scientia Biologicae on November 3rd, 2021. See the published version at https://doi.org/10.15835/nsb13411052.

Abstract

Background: In vitro morphogenic response of mature seed embryo-derived callus cultures of Chonemorpha fragrans was studied using solid and liquid Murashige and Skoog medium amended with cytokinins or their combinations with naphthalene acetic acid at 0.5 mg L-1.Results: The tested plant growth regulators combination and concentrations could not stimulate organogenesis after three subcultivations of the callus cultures on the same PGRs-amended solid medium, and when cultivated in the liquid but, formation of morphogenic callus was observed. Evaluation of biomass and camptothecin production showed that the PGRs influenced biomass and CPT yield of the callus cultures. The alkaloid yield of various explants of 3–4 weeks old axenic seedlings was higher in roots (0.019 % CPT) followed by mature seed embryos (0.0053 %), cotyledons (0.0039 %), hypocotyls (0.0024 %) and leaves (0.0017 %). There was no significant difference in yield of CPT from callus induced from the various explants. Camptothecin yield of morphogenic callus cultures cultivated in liquid medium was lower than that of solid due to extracellular leaching effect of the alkaloid. Amount of synthesized CPT in the callus cultures also varied with PGR type and concentration amended in the cultivation medium, and showed association with biomass production.Conclusion: Results of the present study suggest that callus cultures offer alternative tissue source for in vitro CPT yield enhancement through biotechnological approaches, with application in the large-scale production of the alkaloid to conserve the ever decimated natural population of the medicinal woody climber for CPT.

Background

Chonemorpha fragrans is an evergreen laticiferous and endangered woody perennial vine of the family Apocynaceae endemic in its distributions [1]. Medicinal value of the plant was recognized and research on phytochemicals extracted from the species showed their bioactivities. Among the phytochemicals include chonemorphine, b-sitosterol, taraxasterol and many steroidal alkaloids [2, 3]. Camptothecin (CPT), a monoterpene indole alkaloid was reported from leaf-derived callus cultures, the root and stem bark and *in vitro* regenerated shoots of *C. fragrans* [4, 5, 6, 7]. Due to slow growth of the woody climber that needs several years to reach maturity coupled with rare distribution, application of plant cell, tissue and organ culture (PCTOC) techniques offer alternative strategies for the production of CPT and other phytochemicals isolated from the species [8–10]. The technology can be deployed to conserve the ever decimated natural population of *C. fragrans* as yield of the alkaloid is very low. The extensive use of *C. fragrans* in the traditional systems of medicine coupled with many phytochemicals it produces calls for alternative strategies for its rapid clonal multiplication to meet demand of CPT raw material supply for anticancer drugs production by the pharmaceutical industry. However, application of PCTOC techniques, particularly influence of plant growth regulators (PGRs) in production of the alkaloid is yet unexplored in *C. fragrans*.

In previous studies, efforts were made in evaluating CPT yield from other natural sources of the alkaloid for the development of an *in vitro* production system to mitigate exploitation of natural sources [11–14]. However, such studies are few in the *in vitro* cultures of *C. fragrans*. In the earlier reported *in vitro* studies

of the woody climber, Kulkarni and Malpathak [5] developed simple method for clonal multiplication of the species on Murashige and Skoog [15] medium (MS) amended with N^6-benzylaminopurine (BAP), and micro shoots rooted using indole 3-butyric acid (IBA) at 100 % efficiency. Direct plant regeneration system was achieved via axillary bud proliferation along with rooting of micro shoot on solid MS medium [15]. In the study, shoot and root formation were achieved using medium amended with BAP and IBA concentrations, and addition of silver nitrate improved the *in vitro* morphogenesis parameters [16]. Kedari and Malpathak [7] developed transformed hairy roots from leaf and callus cultures of *C. fragrans* using *Agrobacterium rhizogene* strains with up to 45 % transformation frequency. In recent micropropagation experiment using nodal segment explants of *C. fragrans*, explants pretreatment, culture media strength and PGRs amended, carbon source type and concentrations, and photoperiodic incubation influenced its clonal propagation efficiency [17]. However, the effect of PGRs on CPT production and *in vitro* morphogenic response of callus derived from seed embryo and various explants of *C. fragrans* is yet unreported, given the application indirect plant regeneration offers in developing high CPT-producing transgenics. Further, because juvenile explants are more responsive to the *in vitro* morphogenesis in plants [18] and difficulty in obtaining the most juvenile explant of the species (zygotic embryo) in a season, the present experiment evaluated *in vitro* morphogenic response of *C. fragrans* callus cultures obtained from mature seed embryos. This was carried out through cultivation using solid and liquid MS medium amended with concentrations of PGRs. Camptothecin content in the various explants of 3-4-week old axenic seedlings and seed embryo axes, the resultant callus induced from the explants, and influence of PGRs on production of biomass and alkaloid from seed embryo-derived callus cultures were also analyzed.

Methods

Seeds collection and cultures establishment

Authenticated seed samples of *C. fragrans* were obtained from the Indian Institute of Horticultural Research (IIHR) Bangalore, India during March 2013. The seeds were kept in cetrimide solution for 20 minutes before it was subjected to washing under jet of running tap water for 30 minutes. Later, they were surface sterilized under laminar airflow hood chamber using 70 % ethanol for five minutes. It was followed by rinses with sterile distilled water 2–3 times. The seeds were then treated further with freshly prepared 0.1 % mercuric chloride solution for 2–3 minutes, rinsed with sterile distilled water 2–3 times, and allowed to partially dry up for few minutes to avoid possible cultures contamination. Some of the seeds were subjected to early seedling recovery treatments that involved overnight soaking of the seeds in sterile distilled water [19].

The experiments were carried out thrice using MS medium that contained 3 % sucrose and solidified with 0.8 % Agar (Agar Agar Microbiology Mumbai, India), or in liquid form. Media pH was adjusted to 5.6–5.8 using 1N NaOH or HCl before autoclaved at 121^{o}C for 21 minutes. All of the cultures were maintained under 12 hrs day/light photoperiod provided by cool fluorescent tubes (Phillips India) having photon flux

density of 40W, 50 µmol m^{-2} s^{-1} and culture room conditions of 25±2°C temperatures with relative humidity of 50–70 %.

Biomass evaluation

Callus induced from seed embryos and other explants were maintained on solid MS medium amended with 2,4-D (3.0 mg L^{-1}) by regular subculture after every three weeks. For morphogenic/organogenesis response and influence of PGRs on biomass production evaluation experiments using the seed embryo-derived callus cultures, effects of BAP, TDZ or Kin at 0.5, 1.0, 2.0, 3.0, 4.0 mg L^{-1} treatments were tested. Callus cultures were cultivated on medium supplemented with the various concentrations, and when in combination with NAA (0.5 mg L^{-1}). For each subculture cycle of the callus cultures using solid or in liquid medium cultivation systems, callus samples were harvested, the weight taken in fresh form and after dried to constant mass in an oven at 70^{o}C. In the case of liquid medium cultivation, callus masses were inoculated into volume of autoclaved liquid medium contained in 50 mL capacity conical flasks, and cultures kept on orbital shaker operated at 20 rpm for 12 h/day. Fresh and absolute biomass were calculated from the relationship of callus fresh to dried weight [20]

CPT evaluation and statistical analysis

Extraction of CPT from dried callus cultures of *C. fragrans* and culture medium, and high-performance thin layer chromatographic (HPTLC) analysis of the extracts were carried out as described in the recent [21–23]. CPT yield of the tissues and culture medium were evaluated by the use of standard curve that had a linear regression line equation Y = 0.0018 X +7.2184, and expressed in percentages for tissues used without organogenesis study and in mg/g calli DW or mg/mL medium for others obtained from organogenesis study (experiments carried out at different times). Experiments were performed in triplicate sets of eight replicates (in the case of *in vitro* morphogenesis) while HPTL thrice, and obtained data analyzed using SPSS var. 21 (USA.). Significant differences between the treatments were assessed by analysis of variance (ANOVA) followed by Tukey's range test at <0.05 %, and the results expressed as mean±standard error of the replicated experiments.

Results

In the present experiment, indirect morphogenic response of *C. fragrans* mature seed embryo-derived callus cultures were evaluated using solid or liquid medium cultivation supplemented with cytokinins and their combinations with naphthalene acetic acid (NAA) at lower levels. Based on previous experience in good callus initiation achieved with another CPT-producing woody species using 2,4-dichlorophenoxy acetic acid (2,4-D) +BAP (3.0 +0.5 mg L^{-1}) [23], the concentration and combination were used for induction of callus from seed embryos and various explants of the 3–4 weeks old axenic seedlings (Fig. 1d–j), and with most of the explants the induction occured within second week of cultivation albeit at differential frequency and intensity. Callus formation was more and earlier with seed embryos followed by cotyledons, leaves, hypocotyls and root explants, and within 4–6 weeks cultivation, most of the

explants transformed to white friable callus of faster biomass accumulation (Fig. 1k). In the present study, while maintaining the induced callus cultures through regular subculture, formation and leaching of latex into the cultivation medium that resulted in media browning effect and callus necrosis occasionally occurred (Fig. 1x). However, addition of ascorbic acid (0.5 mg L^{-1}) overcame the condition in most of the cultures.

For *in vitro* morphogenic response evaluation of seed embryo-derived callus, cultivation on medium supplemented with thidiazuron (TDZ), BAP or kinetin (Kin) concentrations resulted in many callus morphologies from friable, compact to nodular-green; most of the cytokinins stimulated pigmentation at variable intensity without organogenesis, with BAP as the most efficient over TDZ and Kin (Fig. 1l–o: p–s). However, the green pigmentation showed increased intensity at higher concentration of the cytokinins, and after three subculture of callus on solid MS medium amended with the same concentration of cytokinins, *in vitro* organogenesis was not observed. Further cultivation of morphogenic callus cultures in liquid medium was insufficient in stimulating shoot/bud primordium formation or somatic embryogenesis (SE). Because cultivation of callus in the liquid medium could not stimulate organogenesis, biomass production and CPT content of the callus cultures and in the liquid medium cultivation system were analyzed. Production of CPT from the callus cultures as well as in the cultivation medium were evaluated to assess their influence on biosynthetic capacity of alkaloid in comparison with solid medium cultivation. Variation in biomass production and CPT yield observed with the solid medium cultivation were similarly obtained in the liquid. However, yield of CPT in callus cultures was reduced to about half the productivity obtained on solid medium cultivation system but, overall productivity relatively higher (Fig. 2B–F), despite the possibility that leached CPT in liquid medium cultures had gotten degraded with resultant effect in alkaloid yield [20, 23].

In all the cytokinins concentrations tested, BAP had more enhancing effect on biomass and CPT biosynthesis over Kin and TDZ. However, the production was dependent on culture medium type, i.e., solid or liquid. Corresponding higher production of biomass was achieved in liquid medium cultivation of the callus over the solid added with BAP (4.0 mg L^{-1}) and its combination with NAA (0.5 mg L^{-1}). For the solid medium cultivation of the callus cultures, maximum of 1.72g and 2.11g absolute callus biomass was produced on BAP-amended medium after 21 days culture over the lowest with TDZ where 1.31g and 1.51g were produced. Liquid medium cultivation enhanced production of the biomass with 2.39g and 2.49g absolute biomass generated by BAP-amended cultures. Correspondingly, TDZ produced 1.81g and 1.95g absolute biomass at similar amended concentration to that of BAP and culture duration (Fig. 2B–C). In this study, yield of CPT from callus biomass showed similar productivity trend between solid medium cultivation to that of liquid medium and biomass accumulation. However, productivity of callus grown in liquid medium (intracellular CPT) was relatively lower than that of solid medium cultivation, which can be explained by the leaching effect of the alkaloid (extracellular) in the case of liquid medium cultivation; overall CPT produced by liquid medium cultures was higher (Fig. 2D–F).

Discussions

In vitro morphogenesis

In vitro morphogenesis offer potentials for study of plant transdifferentiation developmental events, based on biosynthetic capacity to produce tissues and organs with application in the production of high-quality and disease-free plants and phytochemicals [9–11]. The system involves formation of meristemoids from single or globular mass of cells to produce organized structures such as somatic embryos, shoot or root primordium, and has been achieved with many plant species [9]. In the case of *in vitro* morphogenesis that involves a woody species, explant juvenility plays key role in the morphogenic response, and only possible with many species when a juvenile is used [9, 8, 23], the reason callus cultures derived from zygotic embryo explants were cultivated on MS medium supplemented with cytokinins or their combinations with NAA at lower levels in the present experiment. Callus initiation was achieved with 2,4-D +BAP (3.0 +0.5 mg L^{-1}) using explants of 3–4 weeks old axenic seedlings (Fig. 1d–j). With most of the explants, the induction was observed within second week of culture at differential frequency and intensity. Formation of the callus was more and earlier in seed embryos followed by cotyledons, leaves, hypocotyls and root explants, and within 4–6 weeks of cultivation, most of the explants transformed to white friable callus of faster biomass accumulation (Fig. 1k). In the course of maintaining induced callus cultures, formation and leaching of latex into cultivation medium that resulted in callus browning and necrosis occasionally occurred (Fig. 1x). Such brownish callus cultures (Fig. 1x) remained brown and non-morphogenic over several subcultures. However, addition of ascorbic acid (0.5 mg L^{-1}) overcame the condition in most of these cultures, without organogenesis achieved from the callus in subsequent cultivation duration.

For morphogenic effects of BAP, TDZ or Kin on callus cultures of *C. fragrans,* the callus was cultivated on MS medium supplemented with their various concentrations or when in combination with NAA (0.5 mg L^{-1}). Callus cultures were cultivated on the same PGRs-amended medium for at least three passages, and *in vitro* morphogenesis in the form of shoot bud/primordial or somatic embryogenesis (SE) not stimulated. However, variable growth and degree of callus morphologies were observed in most of the cultures (Fig. 1l–w). Due to its high cytokinin activity, TDZ is regarded most effective PGR for promoting *in vitro* morphogenesis in woody plants, and even at low concentration can facilitate efficient *in vitro* response of recalcitrant plants by initiating shoot bud primordial formation, SE or callus induction [24, 25]. The intricate morphogenesis signal routes induced by PGRs are yet unclear but, treatment with their concentrations had stimulated formation of nodular-green morphogenic callus in many plants [e.g, 26, 27]. In the present experiment, cultivation of *C. fragrans* callus cultures on solid medium added with 0.5 mg L^{-1} of the cytokinins could not induce differential callus morphology about PGRs-free media cultivation. However, with the TDZ slight change in morphology of the callus cultures into dark brown was observed. The dark browning showed increased intensity accompanied by increased compactness of callus cultures with the increase in concentration of TDZ amended in the cultivation medium (Fig. 1t–w). In many reported studies in the literature lower levels of the TDZ was found sufficient for stimulating shoot organogenesis from cotyledonary explant while higher concentrations promoted SE [28]. Cultivation of rhizogenic callus cultures on medium supplemented with lower levels of the TDZ

stimulated shoot formation and SE at low frequency [29] while it promoted formation of embryogenic callus from root tip, stem and leaf segments explants [30]. Thidiazuron at low or high concentration was found efficient in stimulating high frequency shoot morphogenesis from shoot tip-derived callus cultures [31]. It was also found efficient for the induction of shoot from seed embryo-derived callus cultures [32, 33]. However, in an instance, cultivation on TDZ-supplemented medium induced semi-compact callus without organogenesis [34].

For other cytokinins, cultivation of *C. fragrans* callus cultures on medium supplemented with BAP or Kin concentrations resulted in differential callus morphologies from friable, compact to nodular-green; most of the concentrations stimulated pigmentation at variable intensity without organogenesis, and BAP was the most efficient over Kin (Fig. 1l–o: p–s). The green pigmentation showed increased intensity at higher concentration of the cytokinins, and after three subcultures of the callus on solid medium supplemented with the same concentration of the cytokinins, *in vitro* morphogenesis was not observed. Due to unhindered diffusion of media components permitted in liquid medium cultivation, morphogenic response of *in vitro* cultures can be initiated by an enhanced uptake of nutrients and supplements, as found efficient in stimulating *in vitro* morphogenesis with many species [e.g, 35, 36]. In the present study, when green non-organogenic callus cultures were cultivated in liquid medium (Fig. 1y–z, 1) with a weekly replenishment of medium nutrients, *in vitro* morphogenesis was not stimulated after four weeks and subsequent subcultivations, suggesting that *C. fragrans* is recalcitrant to indirect organogenesis, at least from seed embryo-derived callus cultures under the experimental conditions of the present study. The fact was supported by additional experiment to assess synergistic action of cytokinins with auxin and its capacity to stimulate formation of organized structures. Callus cultures were cultivated on solid medium amended with low levels of NAA in addition to the cytokinins concentrations. Such cultivation could not result in substantial difference in morphogenic response observed with the other cytokinins concentrations. Even at low levels, the addition of BAP or Kinetin (Kin) has been found efficient for shoot initiation with many medicinal and woody plants [16, 36–39] and with some of the plants, it promoted cell growth along with morphogenic callus formation without organogenesis. For instance, it occurred at higher rate in liquid over solid medium cultivation with synergistic response obtained when auxin was supplemented along with the cytokinins [37, 40–42]. In this study, transfer of the morphogenic callus cultures into liquid medium were ineffective in stimulating formation of organized structures from callus cultures. Browning effect due to latex and phenolics release from the callus cultures into cultivation medium that resulted in callus necrosis was observed but, amending ascorbic acid (1.0 mg L^{-1}) into the cultivation medium overcame the effect without organogenesis capacity shown by the cultures (Fig. 1x, 1–2). In other reported studies in the literature, indirect shoot morphogenesis from callus induced from various explants involved formation of hard, green and compact calli on cytokinins-amended medium, and occurred at very low frequency to absent even after second subcultivation of callus due to hardening and habituation [38, 43]. In *Garcinia mangostana*, BAP stimulated formation of nodular callus before shoot organogenesis from leaf-derived callus cultures [44] while shoot morphogenesis and SE were stimulated by its different concentrations when cotyledonary leaf segment-derived callus cultures of *Digitalis lamarckii* were cultivated on medium added with the PGR [45]. Cultivars-based differential

organogenesis was observed when mature seed embryo-derived callus cultures were cultivated on medium amended with different concentrations of BAP [46, 47] while genotypes-dependent differential regeneration efficiency occurred with callus cultivated on medium amended with combinations of BAP and Kin [48]. Although Kin has been used to stimulate indirect organogenesis with many species, it is less efficient than BAP in most of the reported literature due to higher efficiency of plant tissues to metabolize BAP over Kin and enhanced stimulating effect it has on biosynthesis of endogenous hormones in plant tissues [44, 49]. Superior degree of callus pigmentation stimulated by BAP over Kin concentrations observed in the present experiment supports the explanation. However, low and higher concentrations of the two cytokinins was found insufficient in stimulating formation of organized structures from the callus cultures, possibly due to genetic background of *C. fragrans* that lack capacity for indirect regeneration from seed embryo-derived callus cultures as competence for indirect organogenesis is species and explant-specific in some plants. Ability of *in vitro* cultivated plant cells, tissues or organ to respond to exogenous applied PGRs in a cultivation medium with resultant morphogenic response is dependent on interaction between endogenous and exogenous factors of culture conditions [9, 48]. Therefore, inherent capacity to synthesize and respond to exogenous applied cytokinins is essential for successful induction of *in vitro* morphogenesis in plants; higher capacity to synthesize cytokinins imply more responsiveness to shoot organogenesis. Increasing concentration of the PGRs in cultivation medium is irrelevant when cultivated tissue is unresponsive to the *in vitro* morphogenesis [50].

Somatic embryogenesis, an *in vitro* morphogenic program by which somatic plant cells acquire competence for the formation of embryogenic tissue that further differentiates into somatic embryos through its various developmental stages [51–53]. Strategies that involve manipulating culture medium composition and conditions can be employed to switch somatic program to the embryogenic in a plant *in vitro* cultures [54–57]. In this study, callus cultures were cultivated on solid medium amended with 2,4-D (3.0 mg L^{-1}) for at least three passages before used in the SE study. Cultivation of the callus cultures on PGRs-free medium (control), lower concentrations of 2,4-D or NAA (0.25, 0.5, 1.0 mg L^{-1}) and their combinations with BAP, TDZ or Kin (0.25, 0.5, 1.0 mg L^{-1}) were found insufficient for stimulating embryogenic tissue formation or SE, suggesting that at least for mature seed embryo-derived callus cultures and under the experimental condition of the study, SE is not attainable in *C. fragrans*. Although indirect *in vitro* plant regeneration is the most appropriate developmental pathway for genetic improvement of high-value medicinal plants, the explant and genotype, PGRs type and concentration amended in the cultivation medium, and conditions of physical environment determine the morphogenic response. With some of the plants, the response is not attainable, as observed in an experiment with mature seed embryo-derived callus cultures of *C. fragrans* in the present study.

Production of camptothecin

Production of camptothecin in explants and resultant callus cultures

Capacity to produce a bioactive molecule produced by *ex vitro* grown plant when cultivated in the *in vitro* condition at differential yield was recognized decades ago [11, 58]. However, exploiting endangered plant

sources of CPT is relied upon for its supply to pharmaceutical industry for anticancer drugs production [6, 11]. Production of the alkaloid from plant sources is yet to reach commercial application using PCTOC techniques, which has resulted in an endangered status to most plant sources and increasing interest to apply biotechnological strategies of PCTOC in producing CPT [6, 11, 59, 60]. Biosynthesis of the plant secondary metabolites that include CPT shows close correlated expression with morphological and cytological differentiation. However, the degree of the production and its dependence on development is unknown, whether it is genetic or physiological phenomenon in plant cells and tissues [61, 62]. In this study, evaluation of CPT content in the various explants of 4–weeks–old aseptic seedling, mature seed embryo axes and resultant callus cultures revealed that maximum production of the alkaloid was in roots explant (0.019 %). This was followed by mature seed embryos (0.0053 %), cotyledons (0.0039 %), hypocotyls (0.0024 %) and leaves (0.0017 %). Yield of the alkaloid showed significant difference between most of the explants, except in seed embryos and cotyledons (Fig. 2A). However, production of the alkaloid was reduced in callus induced from the explants, and no variation in CPT content of callus cultures derived from various explants was observed. In the reported literature, many studies have shown variations in biosynthetic capacity for alkaloids in differentiated and undifferentiated *in vitro*-raised cells and tissues [59, 60, 62, 63] and includes anticancer alkaloids such as CPT [64–67]. For instance, biosynthesis of cardenolides and benzylisoquinoline alkaloids in the callus cultures was reported to be dependent on organ redifferentiation [63, 64, 68], as in morphine alkaloid production as well [69]. Green callus cultures that differentiated epidermis and vascular bundles produced alkaloids while cells with limited degree of differentiation contained codeine as the principal alkaloid but, level of morphine production increased as the tissue differentiate [69], similar to observation made in the present study.

Influence of PGRs on production of camptothecin in seed embryo-derived callus biomass

Plant growth regulators are instrumental composition of culture medium that have profound effect on cell growth, cyto-differentiation and alkaloid production in the *in vitro* plant cell cultures [70] and their type, concentration and modification is an effective tool for optimizing cultures condition and improved *in vitro* biosynthesis of alkaloid [41, 62]. In this study, production of CPT from callus cultures (after dried to constant mass) and in the culture medium were evaluated to assess its influence on biosynthetic capacity about solid medium cultivation. Variation in biomass production and CPT content yield observed with the solid medium cultivation was similarly obtained in the liquid. However, yield of CPT in the callus cultures were reduced to about half the productivity obtained on solid medium cultivation system but, overall productivity relatively higher (Fig. 2B–F), despite the possibility of degradation of the leached CPT in liquid medium cultivation system [20, 71]. For evaluation of the influence of PGRs on biomass production and biosynthesis of CPT in *C. fragrans* callus cultures, absolute biomass production in callus obtained from *in vitro* morphogenesis study and CPT production were evaluated by assessment of fresh and dried weight, alkaloid extracted and HPTLC analysis performed [21]. The PGRs amended in the cultivation medium had influential effect on biomass and CPT biosynthetic capacity of callus cultures over the control, and the substantial effect was based on type and concentration of PGRs amended in the cultivation medium (Fig. 2D–E). Control callus cultures cultivated on PGRs-free medium showed lowest biomass and CPT production when compared with the cytokinins-amended medium

cultures, suggesting substantial effect of PGRs on the production of biomass and CPT biosynthesis in *C. fragrans* (Fig. 2B–F). In all the tested concentrations, BAP had more enhancing effect on biomass and CPT biosynthesis over Kin and TDZ. However, the production was dependent on the culture medium type, i.e., solid or liquid medium cultivation system. Corresponding higher production of biomass was achieved in liquid medium cultivation of the callus over the solid added with BAP (4.0 mg L^{-1}) and its combination with NAA (0.5 mg L^{-1}). In the case of solid medium cultivation of the callus cultures, maximum of 1.72g and 2.11g absolute callus biomass was produced on BAP-amended medium after 21 days culture over the lowest with the TDZ where 1.31g and 1.51g were produced (respectively) on medium added with their concentrations. Liquid medium cultivation enhanced production of the biomass with maximum of 2.39g and 2.49g absolute biomass generated by BAP-amended cultures. Correspondingly, TDZ produced 1.81g and 1.95g absolute biomass at similar amended concentration to the BAP and combinations, and culture duration (Fig. 2B–C). Yield of the alkaloid showed similar productivity trend to that of the callus cultures cultivated on solid and in the liquid medium systems. However, the productivity of callus cultures grown in liquid medium (intracellular CPT) was relatively lower than that of solid medium cultivation, which can be explained by the leaching effect of the alkaloid (extracellular) in the case of liquid medium cultivation systems. However, the overall CPT produced by liquid medium cultures were relatively higher (Fig. 2D–F); maximum production of CPT was achieved with BAP and its combination with the NAA where combined intracellular (0.00063+0.00072 mg/g calli DW) and extracellular (0.00071+0.00079 mg/2 mL medium) were biosynthesized, which is relatively higher to that of the solid medium cultivation. Solid medium cultures produced maximum of 0.00093 mg/g calli DW and 0.00098 mg/g calli DW respectively. Correspondingly, TDZ and its combination with NAA produced lowest with combined intracellular (0.00035 mg/g calli DW, 0.00047 mg/g calli DW) and extracellular CPT (0.00044 mg/2 mL medium, 0.00053 mg/2 mL medium) yield, been higher than that produced by solid medium cultivation of the callus cultures (0.00064 mg/g calli DW and 0.00077 mg/g calli DW), respectively (Fig. 2D–F). Cytokinins have enhancing effect on secondary metabolites production [41, 42] and enhance the biosynthesis in the plant *in vitro* cell cultures by their type and concentration amended in the cultivation medium. Purine-type of the cytokinins have beneficial effect on alkaloids production in the *in vitro* cultures without adverse effect on callus growth while phenylurea derivatives either inhibit growth of cultures or highly improve biomass production at the expense of alkaloids biosynthesis [41]. Therefore, modification of their combination and ratio is an effective strategy for studying plant secondary metabolites biosynthetic capacity of cultures during *in vitro* morphogenesis [41, 71]. For instance, in the tender-stem induced callus cultures of *Securinega suffruticosa*, production of biomass and virosecurinine alkaloids was influenced by sucrose concentration and PGRs amended in the cultivation medium, and combination of Kin with NAA was the best for the productivity [72]. Biosynthesis of indolizidine alkaloids in the cultivated callus cultures of the same species was also enhanced when cytokinins were amended in the cultivation medium, and when in combination with auxins [42]. Kinetin enhanced production of the alkaloids but, no correlation between concentration amended in the culture medium and alkaloids production was established. In the study, about two-fold enhanced biosynthesis of securinine was stimulated in the callus cultures by TDZ treatment. However, allosecurinine level was negatively affected while PGR-free medium cultivation resulted in low production of the indolizidine alkaloids, suggesting the substantial effect of

PGRs on biosynthesis of alkaloids [42, 73]. In previous study with *N. nimmoniana* callus cultures, biomass and CPT production were influenced by the culture medium type and cultivation duration [20]. In the study, significant proportion of the synthesized CPT was leached into the culture medium, resulting into lower yield in its callus cultures. However, cultivating callus cultures on cytokinins supplemented-medium such as Kin or BA influenced biosynthetic capacity of alkaloids, and higher concentrations inhibited the yield [40]. Biosynthesis of indolizidine alkaloids in the callus cultures of *S. suffruticosa* was enhanced by the type and concentration of cytokinins amended in the cultivation medium, as well as its synergistic action with auxins [42]. In *Phyllanthus glaucus* shoot cultures, cytokinins types, their combination with auxins or auxins-alone and concentrations supplemented in the cultivation medium influenced the levels of securinega-type alkaloids and biomass production in differential manner [74]. Only BAP enhanced production of the alkaloids while other cytokinins showed inhibitory effect to the securinega-type alkaloids biosynthesis. In the present study, the observed low production of CPT in *C. fragrans* callus cultures cultivated in liquid medium could be explained by the leaching effect of the alkaloid into cultivation medium and its possible degradation. In callus cultures of *Cinchona pubescence*, PGRs and culture conditions affected biosynthesis of alkaloids [40] while culture medium of cultivation and PGRs influenced CPT yield in *Camptotheca acuminata* [75]. Application of BA enhanced yield of the alkaloid and morphogenic parameters in *C. acuminata* callus cultures [38]. Rhizome explant-derived morphogenic callus cultures of *Alpinia galanga* obtained on BAP-added medium produced significant phytoconstituents at higher level over the non-morphogenic [76].

Conclusion

In conclusion, results of the present study suggests that cytokinins and their combination with auxin exerts substantial influence on biomass and CPT production along with morphogenic callus formation without organogenesis/SE response from mature seed embryo-derived callus cultures of C. fragrans when cultivated on solid or in liquid medium supplemented with the PGRs. Supply of the CPT raw material to pharmaceutical industry for production of its analog drugs approved for chemotherapy of cancer is relied upon exploitation of plants, leading to endangered status to the natural plant sources. Production of the alkaloid from the plant sources is affected by many barriers such as collection season and extraction difficulties among others. However, the barriers can be overcome by developing biotechnological production system due to the ease at which alkaloids can be obtained from in vitro cultures when compared to field grown plants. Complex tissue structure of plants makes indirect organogenesis an alternative strategy for developing transgenic plants, with application in the production of CPT. However, the morphogenic pathway could not be achieved with C. fragrans using mature seed embryo-derived callus cultures, at least under the experimental conditions tested in the present study. Thus, callus cultures offer alternative tissue-source for the large-scale production and isolation of CPT to overcome the seasonal and other barriers in sourcing from plants, so as to conserve natural population and meet demand of pharmaceutical industry for CPT raw material supply used in production of its analog drugs.

List Of Abbreviations

MS–Murashige and Skoog medium

BAP–N^6 benzylaminopurine

PGRs–plant growth regulators

TDZ–thidiazuron

Kin–kinetin

NAA–naphthalene acetic acid

IAA–indole acetic acid

IBA–indole butyric acid

2,4-D–2,4-dichlorophenoxy acetic acid

CPT–camptothecin

SE–somatic embryogenesis

ANOVA–analysis of variance

Declarations

Ethics approval and consent to participate

The work was carried out in compliance with scientific ethical conduct that did not involve the use of humans.

Consent for publication

Authors declare having right to publish the research work reported in the manuscript

Availability of data and material

All the data used in the present study have been included in the manuscript.

Competing interest

Author declares that competing interest does not exist in the manuscript contents.

Funding

Not applicable

Authors contribution

TI conceived the research idea, performed experiments and wrote the manuscript.

Acknowledgement

Thanks to Hamdard University New Delhi, India for providing research facilities and Indian Institute of Horticultural Research for the permission and gift of *Chonemorpha fragrans* germplasm used in the experiment.

Authors' Information

Department of Botany, School of Chemical and Life Sciences,

Hamdard University New Delhi -110 062, India.

Correspondence: taasmore@yahoo.co.uk

References

1. Khan S, Karnat NM, Shankar D. India's foundation for the revitalization of local health traditions, Pioneering *In Situ* conservation strategies for medicinal plants and local cultures. Herbalgram. 2005;68:34–
2. Banerji J, Chatterji A. A new steroidal alkaloid from *Chonemorpha fragrans* (Moon) Alston. Iranian J Chem. 1973;56:1056–57.
3. Rastogi RP, Mehrotra BN. Compendium of Indian medicinal plants, Vol. II. New Delhi: CDRI, Publication Information Directorate. 1993;178.
4. Kulkarni AV, Patwardhan AA, Lele U, Malpathak NP. Production of camptothecin in cultures of *Chonemorpha grandiflora.* Pharmacogn Res. 2010;2(5):296–99. doi:10.4103/0974-8490.72327
5. Kulkarni AV, Malpathak NP. Micropropagation of Chonemorpha fragrans (moon) Alston: an endangered medicinal plant. Indian Drugs Bombay. 2006;43(12):1001.
6. Isah T. Anticancer alkaloids from trees: development into drugs. Pharmacogn Rev.2016a;10(20):90. doi:4103/0973-7847.194047
7. Kedari P, Malpathak NP. Hairy cultures of *Chonemorpha fragrans* (Moon) Alston.: a potential plant for camptothecin production. Ind J Biotechnol. 2014;13(2):231–
8. Kedari P, Malpathak NP. Screening of *Chonemorpha fragrans* bioactive extracts for cytotoxicity potential and inhibition studies of key enzymes involved in replication. Pharmacogn Mag. 2016;12(46):297. doi:4103/0973-1296.185708

9. Isah T. Adjustments to *in vitro* culture conditions and associated anomalies in Plants. Acta Biol Cracov ser. Bot. 2015a;57(2):9– doi:10.1515/abcsb-2015-0026
10. Isah T. Rethinking *Ginkgo biloba*: medicinal uses and conservation. Pharmacogn Rev.2015b;9(18):140. doi:10.4103/0973-7847.162137
11. Isah T, Umar S, Mujib A, et al.. Secondary metabolism of pharmaceuticals in the plant *in vitro* cultures: strategies, approaches, and limitations to achieving higher yield. Plant Cell Tiss Organ Cult. 2018;132(2):239–65. doi:10.1007/s11240-017-1332-2
12. Liu Z, Adams J. Camptothecin yield and distribution within *Camptotheca acuminata* trees cultivated in Louisiana. Can J Bot. 1996;74(3):360– doi:10.1139/b96-045
13. Fulzele DP, Satdive RK. Somatic embryogenesis, plant regeneration, and evaluation of camptothecin content in *Nothapodytes foetida*. In Vitro Cell Dev Biol – Plant. 2003;39(2):212–16. doi:10.1079/IVP2002368
14. Mingzhang A, Jing W, Yue S, Wentao G, Longjiang Y. Camptothecin distribution and content in *Nothapodytes nimmoniana*. Nat Prod Comm. 2011;6(2):197–200. doi:10.1177/1934578X1100600210
15. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473–97. doi:1111/j.1399-3054.1962.tb08052.x
16. Nishitha K, Martin KP, Ligimol, Beegum AS, Madhusoodanan PV. Micropropagation and encapsulation of medicinally important *Chonemorpha grandiflora.* In Vitro Cell Dev Biol – Plant. 2006;42(5):385– doi:10.1079/IVP2006762
17. Isah T, Umar S. Influencing *in vitro* clonal propagation of *Chonemorpha fragrans* (moon) Alston by culture media strength, plant growth regulators, carbon source and photoperiodic incubation. J For Res doi:10.1007/s11676-018-0794-3
18. von Aderkas P, Bonga JM. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Physiol. 2000;20(14):921– doi:10.1093/treephys/20.14.921
19. Isah T, Mujib A. Enhanced *in vitro* seedling recovery in *Nothapodytes nimmoniana*. British Biotechnol J. 2015a;6(1):2231– doi:10.9734/BBJ/2015/15368
20. Isah T. Production of camptothecin in the elicited callus cultures of *Nothapodytes nimmoniana* (J. Graham) Mabberly. Chem Papers. 2017;71(6):1091–1106. doi:10.1007/s11696-016-0056-9
21. Isah T. Proteome study of somatic embryogenesis in *Nothapodytes nimmoniana* (J. Graham) Mabberly. 3 Biotech 2019:9(4);119.doi: 10.1007/s13205-019-1637-4.
22. Scott RPW. Chromatographic detectors: design, function, and operation. Chromatographic Science Series 70, Marcel Dekker Inc. New York, Basel, Hong Kong. 1996
23. Isah T, Umar S. Proteome study of embryogenic competence acquisition in the callus cultures of *Nothapodytes nimmoniana* (J. Graham) Mabberly. Acta Physiol Plant 2019;41:96. doi:10.1007/s11738-019-2896-8

24. Hemanthakumar AS, Preetha TS, Krishnan PN, Seeni S. Utilization of zygotic embryos of an economic rattan palm *Calamus thwaitesii*(Arecaceae) for soma plant regeneration and cryo-banking. 3 Biotech. 2013;3(3):195–203. doi: 10.1007/s13205-012-0083-3
25. Huetteman CA, Preece JE. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss Organ Cult. 1993;33(2):105–19. doi:10.1007/BF01983223
26. Kehie M, Kumaria S, Tandon P. *In vitro* plantlet regeneration from nodal segments and shoot-tips of *Capsicum chinense* cv. Naga King Chili. 3 Biotech. 2012;2(1):31–5. doi:10.1007/s13205-011-0025-5
27. Zambre M, Geerts P, Maquet A, Montagu MV, Dillen W, Angenon G. Regeneration of fertile plants from callus in *Phaseolus polyanthus*. Ann Bot. 2001;88(3):371– doi:10.1006/anbo.2001.1468
28. Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH. Thidiazuron: a multidimensional plant growth regulator. Afri J Biotechnol. 2011;10(45):8984–9000. doi:10.5897/AJB11.636
29. Chhabra G, Chaudhary D, Varma M, Sainger M, Jaiswal PK. TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris*). Physiol Mol Biol Plants. 2008;14(4):347–53. doi:10.1007/s12298-008-0033-z
30. Hsia CN, Korban SS. Organogenesis and somatic embryogenesis in callus cultures of *Rosa hybrida* and *Rosa chinensis* Plant Cell Tiss Organ Cult. 1996;44(1):1–6. doi:10.1007/BF00045906
31. Wamaitha MJ, Suwa K, Fukuda KI, Mii M, Daimon H, Mishiba KI. Thidiazuron-induced rapid shoot regeneration via embryo-like structure formation from shoot tip-derived callus culture of sugarcane. Plant Biotechnol. 2010;27(4):365– doi:10.5511/plantbiotechnology.27.365
32. Gurel F, Karakas O, Albayrak G, Ari S. Regeneration capacity of mature embryo-derived callus in barley (*Hordeum vulgare*). Acta Biol Hungaric. 2009;60(3):309–19. doi:10.1556/ABiol.60.2009.3.8
33. Ning GG, Bao MZ. Plant regeneration from callus derived from immature embryo cotyledons of *Prunus mume*. Hortic Sci. 2007;42(3):744– doi:10.21273/HORTSCI.42.3.744
34. Canli FA. Effects of dark and TDZ on callus formation of rose leaf explants. Pak J Biol Sci.2003;6(19):1672–74. **doi:**3923/pjbs.2003.1672.1674
35. Tomaszewska-Sowa M. Effect of growth regulators and other components of culture medium on morphogenesis of sugar beet (*Beta vulgaris*) in unfertilized ovule *in vitro* cultures. Acta Agrobot. 2012;65(4). doi:10.5586/aa.2012.025
36. Sanputawong S, Raknim T, Benchasri S. Influence of different type of culture media and activated charcoal on callus induction and shoot multiplication of *Cadamine lyrata*. J Agric Technol. 2015;11(8):1697–
37. Li Z, Liu Z. Effects of benzyladenine and naphthalene acetic acid on growth and camptothecin accumulation in *Camptotheca acuminata* J Plant Growth Regul. 2003;22(3): 205–16. doi:10.1007/s00344-003-0015-x
38. Saini RK, Shetty NP, Giridhar P, Ravishankar GA. Rapid *in vitro* regeneration method for *Moringa oleifera* and performance evaluation of field grown nutritionally enriched tissue cultured plants. 3 Biotech. 2012;2(3):187– doi:10.1007/s13205-012-0045-9

39. Isah T, Mujib A. *In vitro* plant regeneration of coffee senna (*Senna occidentalis*) from hypocotyl-derived callus. Acta Biologica Cracov ser. Bot. 2013;55(2):120– doi:10.2478/abcsb-2013
40. Mulder-Krieger T, Verpoorte R, De Graaf YP, van Der Kreek M, Baerheim-Svendsen A. The effects of plant growth regulators and culture conditions on the growth and the alkaloid content of callus cultures of *Cinchona pubescens*. Planta Med. 1982;46(09):15– doi:10.1055/s-2007-970009
41. Bienaime C, Melin A, Bensaddek L, Attoumbre J, Nava-Saucedo E, Baltora-Rosset S. Effects of plant growth regulators on cell growth and alkaloids production by cell cultures of *Lycopodiella inundata*. Plant Cell Tiss Organ Cult. 2015;123(3):523–33. doi:10.1007/s11240-015-0856-6
42. Raj D, Kokotkiewicz A, Drys A, Luczkiewicz M. Effect of plant growth regulators on the accumulation of indolizidine alkaloids in *Securinega suffruticosa* callus cultures. Plant Cell Tiss Organ Cult. 2015;123(1):39– doi:10.1007/s11240-015-0811-6
43. Malik S, Zia M, Chaudhary MF. *In vitro* plant regeneration from direct and indirect organogenesis of *Momordica charantia*. Pak J Biol Sci. 2007;10(22):4118– doi:10.3923/pjbs.2007.4118.4122
44. Qosim WA, Purwanto R, Wattimena GA. Indirect organogenesis and histological analysis of *Garcinia mangostana* Asian J Plant Sci. 2013;12(6):279. doi:10.3923/ajps.2013.279.284
45. Verma SK, Yucesan BB, Gurel S, Gurel E. Indirect somatic embryogenesis and shoot organogenesis from cotyledonary leaf segments of *Digitalis lamarckii*, an endemic medicinal species. Turkish J Biol. 2011;35(6):743–50. doi:10.3906/biy-1007-45
46. Roy A, Aich SS, Mukherjee S. Differential responses to indirect organogenesis in rice cultivars. Intl J Sci Res Public. 2012;2(9):1–
47. Sankepally SSR, Singh B. Optimization of regeneration using differential growth regulators in indica rice cultivars. 3 Biotech. 2016;6(1):1– doi:10.1007/s13205-015-0343-0
48. Vila SK, Rey HY, Mroginski LA. Influence of genotype and explant source on indirect organogenesis by *in vitro* culture of leaves of *Melia azedarach* Biocell. 2004;28(1):35–42.
49. Malik SK, Chaudhury R, Rajwant KK. Rapid *in vitro* multiplication and conservation of *Garcinia indica*: a tropical medicinal tree species. Sci Hortic 2005;106(4):539–53. doi:10.1016/j.scienta.2005.05.002
50. **Miao Y, Zhu Z, Guo Q, et al.** Dynamic changes in carbohydrate metabolism and endogenous hormones during *Tulipa edulis* stolon development into a new bulb. J Plant Biol 2016;59(2):121– doi:10.1007/s12374-016-0456-y
51. Catterou M, Dubois F, Smets R, Vaniet S, Kichey T, van Onckelen H, et al.. hoc: an *Arabidopsis* mutant overproducing cytokinins and expressing high *in vitro* organogenic capacity. The Plant J. 2002;30(3):273–87. doi:10.1046/j.1365-313X.2002.01286.x
52. Mujib A, Ali M, Tonk D, Isah T, Zafar N. Embryogenesis in ornamental monocots: plant growth regulators as signaling element. In: Mujib A (Ed.), Somatic embryogenesis in ornamentals and Its applications, Springer India. 2016;pp.187-201. doi:10.1007/978-81-322-2683-3_12
53. Fehér A. Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology?. Front Plant Sci 2019;10:536. doi: 10.3389/fpls.2019.00536

54. Mujib A, Ali M, Isah T, Dipti T. Somatic embryo mediated mass production of *Catharanthus roseus* in culture vessel (bioreactor)–A comparative study. Saudi J Biol Sci.2014;21(5):442– doi:10.1016/j.sjbs.2014.05.007
55. Carman JG. Embryogenic cells in plant tissue cultures: occurrence and behavior. In Vitro Cell Dev Biol – Plant. 1990;26(8):746–53. doi:10.1007/BF02623615
56. Ikeuchi M, Sugimoto K, Iwase A. Plant callus: mechanisms of induction and repression. The Plant Cell. 2013;25(9):3159–173. doi:10.1105/tpc.113.116053
57. Isah T. Induction of somatic embryogenesis in woody plants. Acta Physiol Plant. 2016b;38:118. doi:10.1007/s11738-016-2134-6
58. Ali M, Isah T, Mujib A, Dipti T. Climber plants: medicinal importance and conservation strategies. In: Shahzad A, Sharma S, SiddiquiSA (Eds.), Biotechnological strategies for the conservation of medicinal and ornamental climbers, Springer International Publishing, Switzerland. 2016;pp101– doi:10.1007/978-3-319-19288-8
59. Isah T, Mujib A. *In vitro* propagation and camptothecin production in *Nothapodytes nimmoniana*. Plant Cell Tiss Organ Cult. 2015b;121(1):1–10. doi:10.1007/s11240-014-0683-1
60. Isah T, Mujib A. Camptothecin from *Nothapodytes nimmoniana*: review on biotechnology applications. Acta Physiol Plant. 2015c;37:106. doi:10.1007/s11738-015-1854-3
61. Wierman R. Secondary plant products and cell and tissue differentiation. In: Conn EE (Ed.), The Biochemistry of Plants, 1981; Vol.7, p.85–116, Academic Press, USA.
62. Murthy HN, Lee EJ, Paek KY. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tiss Organ Cult. 2014;118(1):1– doi:10.1007/s11240-014-0467-7
63. Ikuta A, Syono K, Furuya T. Alkaloids of callus tissues and redifferentiated plantlets in the Papaveraceae. Phytochem. 1974;13(10):2175–179. doi:10.1016/0031-9422(74)85023-5
64. Facchini PJ, Bird DA. Developmental regulation of benzylisoquinoline alkaloid biosynthesis in opium poppy plants and tissue cultures. In Vitro Cell Dev Biol – Plant. 1998;34(1):69–79. doi:10.1007/BF02823126
65. Kai G, Wu C, Gen L, Zhang L, Cui L, Ni X. Biosynthesis and biotechnological production of anti-cancer drug Camptothecin. Phytochem Rev. 2015;14(3):525–39. doi:10.1007/s11101-015-9405-5
66. Yamazaki M, Asano T, Yamazaki Y, Sirikantaramas S, Sudo H, Saito K. Biosynthetic system of camptothecin: an anticancer plant product. Pure Applied Chem. 2010;82(1):213– doi:10.1351/PAC-CON-09-02-08
67. Sankar-Thomas YD, Lieberei R. Camptothecin accumulation in various organ cultures of *Camptotheca acuminata* Decne grown in different culture systems. Plant Cell Tiss Organ Cult. 2011;106(3):445– doi:10.1007/s11240-011-9942-6
68. Hagimori M, Matsumoto T, Kisaki T. Studies on the production of *Digitalis* cardenolides by plant tissue culture I. Determination of digitoxin and digoxin contents in first and second passage calli and

organ redifferentiating calli of several *Digitalis* species by radioimmunoassay. Plant Cell Physiol. 1980;21(8):1391–404. doi:10.1093/pcp/21.8.1391

69. Yoshikawa T, Furuya T. Morphinan alkaloid production by tissues differentiated from cultured cells of *Papaver somniferum* (1). Planta Med. 1985;51(2):110– doi:10.1055/s-2007-969421
70. Jaleel CA, Gopi R, Gomathinayagam M, Panneerselvam R. Traditional and non-traditional plant growth regulators alter phytochemical constituents in *Catharanthus roseus*. Process Biochem. 2009;44(2):205– doi:10.1016/j.procbio.2008.10.012
71. Karwasara VS, Dixit VK. Culture medium optimization for camptothecin production in the cell suspension cultures of *Nothapodytes nimmoniana* (J. Graham) Mabberley. Plant Biotechnol Rep. 2013;7(3):357–69. doi:10.1007/s11816-012-0270-z
72. Yuan W, Zhu P, Cheng K, Meng C, Ma L, Wu F, Zhu H. Callus of *Securinega suffruticosa*, a cell line accumulates dextro Securinega alkaloids. Nat Prod Res 2007:21(3);234– doi:10.1080/14786410701189781
73. Verpoorte R, van der Heijden R, van Gulik WM, ten Hoopen HJG. Plant biotechnology for the production of alkaloids: present status and prospects. In: Brossi A (Ed.). The alkaloids: chemistry and pharmacology, 1991; vol. 40, pp.1–187, Academic Press San Diego USA. doi:10.1016/S0099-9598(08)60268-0
74. Sparzak-Stefanowska B, Krauze-Baranowska M, Hałasa R. Influence of plant growth regulators on the shoot culture of *Phyllanthus glaucus* and accumulation of indolizidine alkaloids with evaluation of antimicrobial activity. Acta Physiol Plant 2019:41;6. doi:10.1007/s11738-018-2796-3
75. van Hengel AJ, Harkes MP, Wichers HJ, Hesselink PG, Buitelaar RM. Characterization of callus formation and camptothecin production by cell lines of *Camptotheca acuminata*. Plant Cell Tiss Organ Cult. 1992;28(1):11– doi:10.1007/BF00039910
76. Rao K, Chodisetti B, Gandi S, Mangamoori LN, Giri A. Direct and indirect organogenesis of *Alpinia galanga* and the phytochemical analysis. Applied Biochem Biotechnol. 2011;165(5–6):1366– doi:10.1007/s12010-011-9353-5

Figures

Figure 1

a surface sterilized mature seed with a slanted cut at the radicular end, b in vitro germination of the seed on half strength basal MS medium amended with GA3 (1.0 mg L-1), c seed embryo axes excised from surface sterilized seeds, d aseptic seedling obtained from seed embryo axes after four weeks culture, e induction of callus on MS medium added with 2,4-D +BAP (3.0 +1.0 mg L-1) using seed embryo axes; f cotyledons; g leaf; h hypocotyl and i-j root explants. k seed embryo-derived callus biomass production on MS medium amended with same PGRs as the induction medium, l morphology of the callus cultures

cultivated on MS medium amended with Kin (1.0 mg L-1); m Kin (2.0 mg L-1); n Kin (3.0 mg L-1); o Kin (4.0 mg L-1); p BAP (1.0 mg L-1); q BAP (2.0 mg L-1); r BAP (3.0 mg L-1); s BAP (4.0 mg L-1); t Thidiazuron (1.0 mg L-1); u TDZ (2.0 mg L-1); v TDZ (3.0 mg L-1) and w TDZ (4.0 mg L-1). x formation and leaching of latex into the culture medium leading to the callus browning, y morphogenic callus (green) cultivation in liquid medium amended with BAP (3.0 mg L-1), z callus browning after two weeks cultivation in liquid medium; 1 three weeks cultivation; 2 five weeks cultivation. (Fig.1 a–b, d–x, 2 scale bars 1.5 cm, c scale bars 0.8 cm, y–1 scale bars 1.9 cm).

Figure 2

A–F evaluation of biomass and camptothecin production in the explants and resultant callus cultures of C. fragrans.