

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

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Abstract

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. The plant growth regulators (PGRs) combination and concentrations tested 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%), and no significant difference was observed 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 was association with biomass production. 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.

Keywords: alkaloid; camptothecin; medicinal plants; morphogenic callus; phytochemicals; plant growth regulators

Abbreviations: ANOVA-analysis of variance; MS-Murashige and Skoog medium; BAP-N⁶ 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

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Introduction

Chonemorpha fragrans is an evergreen laticiferous and endangered woody perennial vine of the family Apocynaceae endemic in its distributions (Khan *et al.*, 2005). Its medicinal value was recognized and research on phytochemicals extracted from the species showed bioactivities (Clarance *et al.*, 2019). Among the phytochemicals include chonemorphine, b-sitosterol, taraxasterol and many steroidal alkaloids (Banerji and Chatterji, 1973; Rastogi and Mehrotra, 1993; Kulkarni *et al.*, 2010). 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* (Kulkarni and Malpathak, 2006; Kulkarni *et al.*, 2010). Due to slow growth of this 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 (Banerji and Chatterji, 1973; Rastogi and Mehrotra, 1993; Kedari and Malpathak, 2014). The technology can be deployed to conserve the ever-decimated natural population of *C. fragrans* as yield of the alkaloid is very low. Extensive use of *C. fragrans* in 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 natural sources of the alkaloid for the development of *in vitro* production systems, so as to mitigate exploitation of its natural sources (Liu and Adams, 1996; Kai *et al.*, 2015). 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 (2006) developed simple method for clonal multiplication of the species on Murashige and Skoog (1962) medium (MS) amended with N⁶-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 (Nishitha *et al.*, 2006). 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. Kedari and Malpathak (2014) 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 (Isah and Umar, 2018). However, the effect of PGRs on CPT production and *in vitro* morphogenic response of callus derived from seed embryos 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 *in vitro* morphogenesis in plants (von Aderkas and Bonga, 2000) and difficulty in obtaining the most juvenile explant of the species (zygotically embryo) in a season, the present experiment evaluated *in vitro* morphogenic response of *C. fragrans* callus cultures obtained from mature seed embryos. It was carried out through cultivation of the callus cultures 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, 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.

Materials and Methods

Seeds collection and cultures establishment

Authenticated seed samples of *C. fragrans* were obtained from 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, 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 (Isah and Mujib, 2015a).

The experiments were carried out in triplicate sets 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 °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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 evaluation of the morphogenic/organogenesis response of the seed embryo-derived callus cultures and influence of PGRs on biomass production, 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 °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 the 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 (Isah, 2017).

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 (Isah, 2019; Isah and Umar, 2019), modified in Zafar *et al.* (2020) and Mujib *et al.* (2020). CPT yield of the tissues and culture medium were evaluated using standard curve that had a linear regression line equation 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 \pm standard error of the replicated experiments.

Results and Discussion

In vitro morphogenesis

Cultures establishment for morphogenesis experiments

In the present study and 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) (Isah and Umar, 2019), callus initiation was achieved with mature seed embryos and explants of 3-4 weeks old axenic seedlings (Figure 1d–j), and with most of the explants, the induction observed within second week of culture at differential frequency and intensity. Formation of the callus was more and earlier in the cultured seed embryos followed by cotyledons, leaves, hypocotyls and root explants and within 4-6 weeks of cultivation, most of the explants transformed into white friable callus of faster biomass accumulation (Figure 1k). In the course of maintaining the induced callus cultures (through regular subculture), formation and leaching of latex into cultivation medium that resulted in callus browning and necrosis occasionally occurred (Figure 1x). Such brownish callus cultures (Figure 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 the cultures in subsequent cultivation duration.

Callus morphogenic response

In vitro morphogenesis offers potentials for study of plants trans differentiation events, based on biosynthetic capacity to produce tissues and organs with application in the production of high-quality and disease-free plants and phytochemicals (Isah, 2015a; Espinosa-Leal *et al.*, 2018). 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 (Isah, 2015a). 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 (von Adekas and Bonga, 2000). In the present study, the 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. Generally, it is regarded that formation of callus opens up cellular reprogramming developmental events potential, which in turn, is determined by cultural condition for a successful organogenesis or somatic embryogenesis (Feher, 2019). Callus cultures derived from the zygotic embryo explants were cultivated on MS medium supplemented with cytokinins or their combinations with NAA at lower levels; for the morphogenic effects of BAP, TDZ or Kin on the callus cultures, callus was cultivated on MS medium supplemented with the various concentrations or when in combination with NAA (0.5 mg L⁻¹). The seed embryo-derived callus cultivation 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 (Figure 1l–o; p–s). However, the green pigmentation showed increased intensity at higher concentration of the cytokinins, and after three subcultures of the callus on solid MS medium amended with the same concentration of the cytokinins, *in vitro* organogenesis was not observed. Further cultivation of the morphogenic callus cultures in liquid medium was insufficient in stimulating shoot/bud primordium formation or somatic embryogenesis (SE). The 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 (Figure 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 (Huetteman and Preece, 1993). 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 (Zambre *et al.*, 2001; Kehie *et al.*,

2012). 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 an increase in concentration of TDZ amended in the cultivation medium (Figure 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 (Guo *et al.*, 2011). Cultivation of rhizogenic callus cultures on medium supplemented with lower levels of the TDZ stimulated shoot formation and SE at low frequency (Chhabra *et al.*, 2008) while it promoted formation of embryogenic callus from root tip, stem and leaf segments explants (Hsia and Korban, 1996). Thidiazuron at low or high concentration was found efficient in stimulating high frequency shoot morphogenesis from shoot tip-derived callus cultures (Wamaitha *et al.*, 2010). It was also found efficient for the induction of shoot from seed embryo-derived callus cultures (Ning and Bao, 2007; Gurel *et al.*, 2009). However, in an instance, cultivation on TDZ-supplemented medium induced semi-compact callus without organogenesis (Canli, 2003). 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 (Tomaszewska-Sowa, 2012; Sanputawong *et al.*, 2015). In the present study, when green non-organogenic callus cultures were cultivated in liquid medium (Figure 1 y-z, 1) with a weekly replenishment of medium nutrients, *in vitro* morphogenesis was not stimulated after four weeks and subsequent sub-cultivations of the callus cultures, 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 the 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 cytokinins concentrations tested. 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 (Li and Liu, 2003; Nishitha *et al.*, 2006; Saini *et al.*, 2012; Isah and Mujib, 2013; Sanputawong *et al.*, 2015) 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 (Mulder-Krieger *et al.*, 1982; Li and Liu, 2003; Bienaime *et al.*, 2015). In this study, transfer of the morphogenic callus cultures into liquid medium were ineffective in stimulating formation of organized structures from the callus cultures. Browning effect due to the latex and phenolics release from 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 (Figure 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 sub-cultivation of callus due to hardening and habituation (Malik *et al.*, 2007; Saini *et al.*, 2012). In *Garcinia mangostana*, BAP stimulated formation of nodular callus before shoot organogenesis from leaf-derived callus cultures (Qosim *et al.*, 2013) 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 (Verma *et al.*, 2011). Cultivars-based differential organogenesis was observed when mature seed embryo-derived callus cultures were cultivated on medium amended with different concentrations of BAP (Roy *et al.*, 2012; Sankepally and Singh, 2016) while genotypes-dependent differential regeneration efficiency occurred with callus cultivated on medium amended with combinations of BAP and Kin (Vila, 2004). In the callus cultures of *Actinidia chinensis*

var. *deliciosa* A. Chev. (A. Chev.), also known as Kiwifruit, formation of "ball-shaped" region of the calli covered with closely attached cells and membranous layer was the first indicator of shoot bud initiation while adjacent non-morphogenic calli were composed of loosely arranged cells with irregular appearance on the surface (Czernicka *et al.*, 2021). 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 (Qosim *et al.*, 2013; Miao *et al.*, 2016). 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 (mature) 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 the balanced interaction between endogenous and exogenous factors of culture conditions (Skoog and Miller, 1957; Vila, 2004; Isah, 2015b). 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 the cultivation medium is irrelevant when cultivated tissue is unresponsive to the *in vitro* morphogenesis (Miao *et al.*, 2016).

Somatic embryogenesis is an *in vitro* morphogenic program by which somatic plant cells acquire competence for the formation of embryogenic tissue which further differentiates into somatic embryos through its various developmental stages (Zimmerman, 1993; Feher, 2019). Strategies that involve manipulating culture medium composition and conditions can be employed to switch somatic program to the embryogenic in a plant *in vitro* culture system (Carman, 1990; Ikeuchi *et al.*, 2013; Isah, 2016). In the present 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 ($0.25, 0.5, 1.0 \text{ mg L}^{-1}$) and their combinations with BAP, TDZ or Kin ($0.25, 0.5, 1.0 \text{ mg L}^{-1}$) were found insufficient for stimulating embryogenic tissue formation and SE expression, 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.



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; **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).

Production of camptothecin

Because cultivation of the callus in liquid medium could not stimulate organogenesis, biomass production and CPT content of the callus cultures and in the liquid medium cultivation system (of triplicate experiments) 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 (Figure 2B-F), despite the possibility that leached CPT in liquid medium cultures had gotten degraded with resultant effect in alkaloid yield (Isah, 2017; Isah and Umar, 2019).

In all the cytokinins concentrations tested, BAP had more enhancing effect on biomass and CPT biosynthesis over Kin and TDZ up to certain levels. 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).

¹). For the solid medium cultivation of the callus cultures, maximum of 1.72 g and 2.11 g absolute callus biomass was produced on BAP-amended medium after 21 days culture over the lowest with TDZ where 1.31 g and 1.51 g were produced. Liquid medium cultivation enhanced production of the biomass with 2.39 g and 2.49g absolute biomass generated by BAP-amended cultures. Correspondingly, TDZ produced 1.81 g and 1.95 g absolute biomass at similar amended concentration to that of BAP and culture duration (Figure 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 (Figure 2D-F).

Production of camptothecin in explants and resultant callus cultures

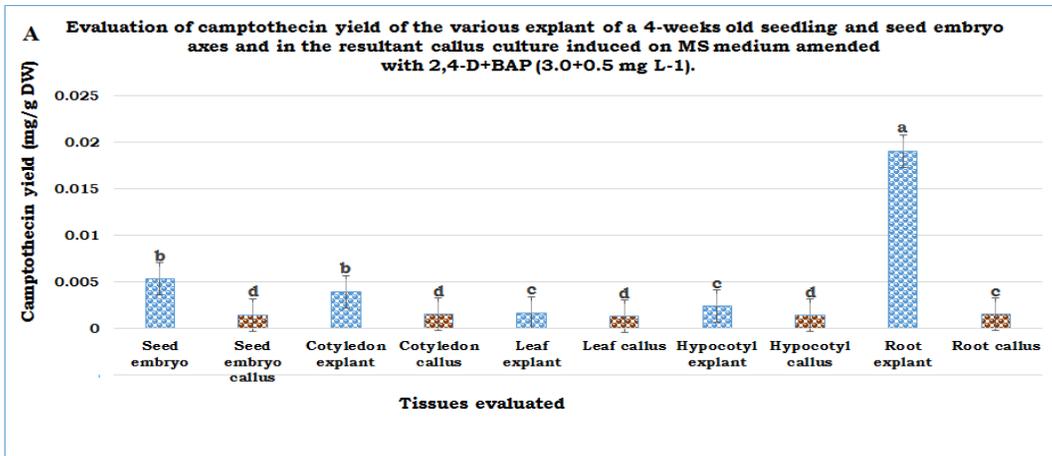
Capacity to produce a bioactive molecule produced by *ex vitro* grown plant when cultivated in the *in vitro* condition at differential yield was recognized decades ago but, exploiting endangered plant sources of CPT is relied upon for its supply to pharmaceutical industry for anticancer drugs production (Isah *et al.*, 2018). 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 (Isah and Mujib, 2015b, c; Isah *et al.*, 2018). 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 (Kai *et al.*, 2015). In the present 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 (Figure 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 (Ikuta *et al.*, 1974; Murthy *et al.*, 2014; Isah and Mujib, 2015b, c;) and includes anticancer alkaloids such as CPT (Facchini and Bird, 1998; Yamazaki *et al.*, 2010; Sankar-Thomas *et al.*, 2011; Kai *et al.*, 2015). For instance, biosynthesis of cardenolides and benzylisoquinoline alkaloids in the callus cultures was reported to be dependent on organ redifferentiation (Ikuta *et al.*, 1974; Hagimori *et al.*, 1980; Facchini and Bird, 1998), as in morphine alkaloid production as well (Yoshikawa *et al.*, 1985). 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 the level of morphine production increased as tissue differentiates (Yoshikawa *et al.*, 1985), similar to observation made in the present study.

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

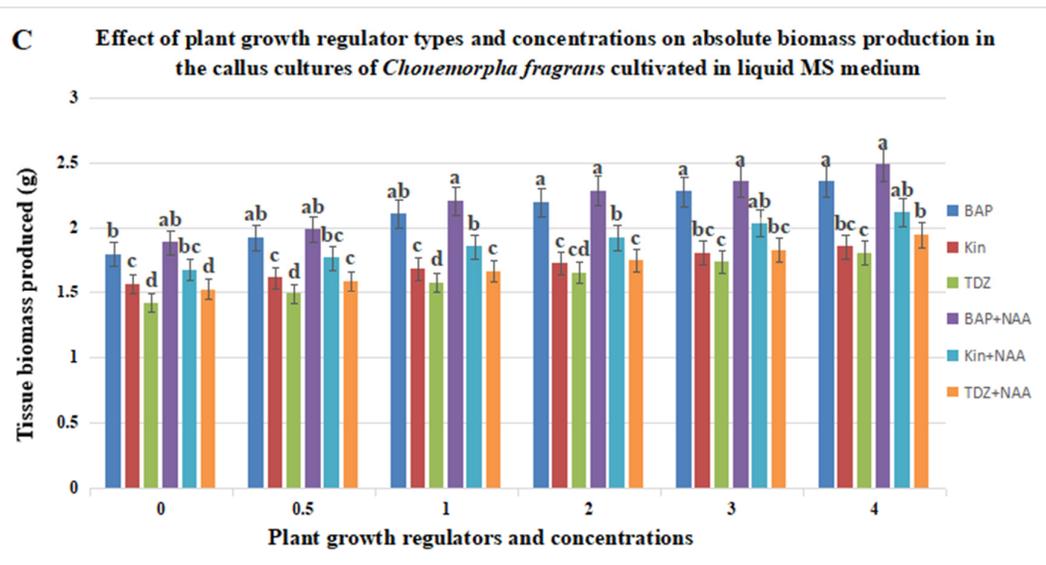
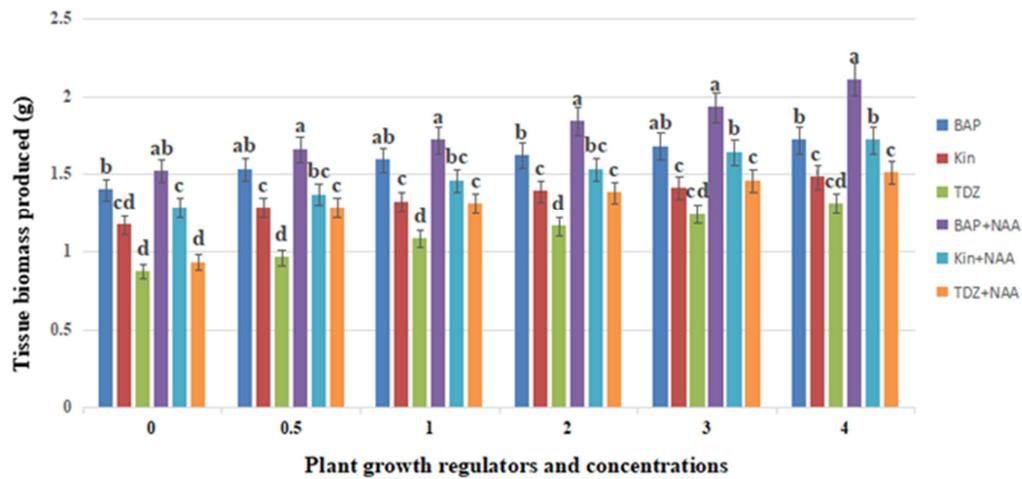
Plant growth regulators are instrumental composition of culture medium that have profound effect on cell growth, cyto-differentiation and alkaloid production in the *in vitro* plant cell cultures (Jaleel *et al.*, 2009) and their type, concentration and modification is an effective tool for optimizing cultures condition and improved *in vitro* biosynthesis of alkaloid (Murthy *et al.*, 2014; Bienaime *et al.*, 2015). In the present 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 system 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 (Figure 2B-F), despite the

possibility of degradation of the leached CPT in liquid medium cultivation system (Karwasara and Dixit, 2013; Isah, 2017). In order to evaluate the influence of PGRs on biomass production and CPT biosynthesis capacity in the *C. fragrans* callus cultures, absolute biomass production in callus obtained from *in vitro* morphogenesis study and CPT production were evaluated by the assessment of fresh and dried weight, alkaloid extracted and HPTLC analysis performed (Isah, 2019; Isah and Umar, 2019). The PGRs amended in the cultivation medium had influential effect on biomass and CPT biosynthetic capacity of the callus cultures over control, and the substantial effect was based on type and concentration of PGRs amended in the cultivation medium (Figure 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* (Figure 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 1.72 g and 2.11 g absolute callus biomass was produced on BAP-amended medium after 21 days culture over the lowest with the TDZ where 1.31 g and 1.51 g were produced (respectively) on medium added with their concentrations. Liquid medium cultivation enhanced production of the biomass with maximum of 2.39 g and 2.49 g absolute biomass generated by BAP-amended cultures. Correspondingly, TDZ produced 1.81 g and 1.95 g absolute biomass at similar amended concentration to the BAP and combinations, and culture duration (Figure 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 (Figure 2D-F); maximum production of CPT was achieved with BAP and its combination with the NAA where combined intracellular ($0.00063+0.00072 \text{ mg/g calli DW}$) and extracellular ($0.00071+0.00079 \text{ mg/2 mL medium}$) were biosynthesized, which is relatively higher to that of the solid medium cultivation. Solid medium cultures produced maximum of $0.00093 \text{ mg/g calli DW}$ and $0.00098 \text{ mg/g calli DW}$ respectively. Correspondingly, TDZ and its combination with NAA produced lowest with combined intracellular ($0.00035 \text{ mg/g calli DW}$, $0.00047 \text{ mg/g calli DW}$) and extracellular CPT ($0.00044 \text{ mg/2 mL medium}$, $0.00053 \text{ mg/2 mL medium}$) yield, been higher than that produced by solid medium cultivation of the callus cultures ($0.00064 \text{ mg/g calli DW}$ and $0.00077 \text{ mg/g calli DW}$), respectively (Fig. 2D–F). Cytokinins have enhancing effect on secondary metabolites production and enhance the biosynthesis in the plant *in vitro* cell cultures by their type and concentration amended in the cultivation medium (Bienaime *et al.*, 2015; Raj *et al.*, 2015). 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 (Bienaime *et al.*, 2015). Therefore, modification of their combination and ratio is an effective strategy for studying plant secondary metabolites biosynthetic capacity of cultures during *in vitro* morphogenesis (Karwasara and Dixit, 2013; Bienaime *et al.*, 2015). 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 (Yuan *et al.*, 2007). 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 (Raj *et al.*, 2015). 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 2-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 (Verpoorte *et al.*, 1991; Raj *et al.*, 2015). In previous study with *N. nimmoniana* callus cultures, biomass and CPT production were influenced by the culture medium type and cultivation duration (Isah, 2017). 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 (Mulder-Krieger *et al.*, 1982). 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 (Raj *et al.*, 2015). 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 (Sparzak-Stefanowska *et al.*, 2019). 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 (Mulder-Krieger *et al.*, 1982) while culture medium of cultivation and PGRs influenced CPT yield in *Camptotheca acuminata* (van Hengel *et al.*, 1992). Application of BA enhanced yield of the alkaloid and morphogenic parameters in *C. acuminata* callus cultures (Saini *et al.*, 2012). Rhizome explant-derived morphogenic callus cultures of *Alpinia galanga* obtained on BAP-added medium produced significant phytoconstituents at higher levels over the non-morphogenic (Rao *et al.*, 2011).



B Effect of plant growth regulator types and concentrations on absolute biomass production in the callus cultures of *Chonemorpha fragrans* cultivated on solid MS medium



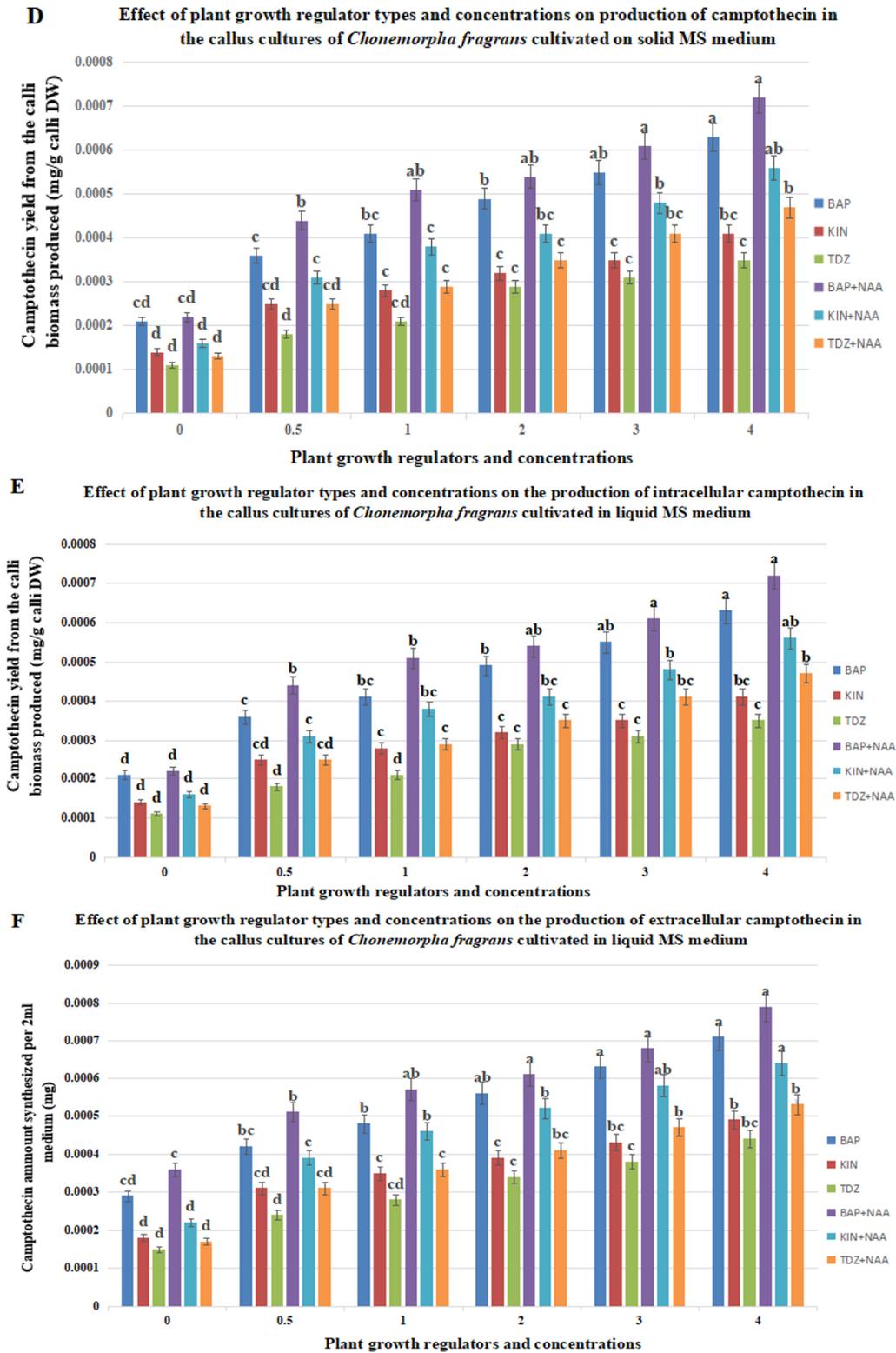


Figure 2. A-F evaluation of biomass and camptothecin production in the explants and resultant callus cultures of *C. fragrans*

Conclusions

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 systems, 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.

Authors' Contributions

Both authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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