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Impact of plant growth regulators (PGRs) on callogenesis and artemisinin content in *Artemisia annua* L. plants

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Callus cultures were initiated from leaf explants of *Artemisia annua* L. plants for artemisinin production using Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators (PGRs), *viz*., BAP, NAA, 2,4-D and TDZ. The combination of all PGRs at different concentrations showed a better response in induction of undifferentiated friable calli in the light phase as compared to the dark phase. The calli of transgenic plants over-expressing *hmgr* gene yielded a biomass of 0.13 g dw/explant in the light in comparison to 0.06 g dw/explant in the dark, when grown on MS medium supplemented with BAP (1.0 mg L^{-1}) and NAA (2.0 mg L^{-1}). The non-transgenic (untransformed plant) calli, however, yielded a biomass of 0.10 g dw/explant in the light when grown on MS medium supplemented with the same concentrations of BAP and NAA, while yield was 0.06 g dw/explant in the dark in MS medium supplemented with BAP (1.0 mg L^{-1}) and 2, 4-D (1.0 mg L^{-1}). Further, the maximum artemisinin content (0.006% on dw basis) was recorded in both transgenic and non-transgenic calli that were grown on MS medium supplemented with BAP (1.0 mg L^{-1}) and NAA (2.0 mg L^{-1}) under light phase.

Keywords: *Artemisia annua* L., callus, explants, plant growth regulators, regeneration

Introduction

Artemisinin, a sesquiterpene lactone, is extracted from the aerial parts of *Artemisia annua* L. (Asteraceae) plant. It is rapidly becoming a drug of choice and a safe alternative therapy against multiple types of malarial infections[1,2]. WHO recommended the use of artemisinin as a first-line drug[3] and in 2010 updated its guidelines to replace oral artemisinin-based monotherapy by ACTs (artemisinin-based combination therapies)[4], which further scaled-up the demand of artemisinin. The large scale production of artemisinin through chemical synthesis/semi-synthesis is complicated and economically unviable due to low yields[5]. Different approaches were, hence, employed to improve the production of artemisinin. These include conventional breeding, cell suspension and hairy root culture techniques, and biotechnological techniques[6-9]. These approaches show potential for future development, but improvements delivered by them so far have not met the global demand. The artemisinin yield from the field grown plants are, however, very low (0.01-1.0% dw) and influenced by variations in environmental conditions, such as, temperature, humidity and sunlight during the relatively long agricultural timeframe. These factors together limit the large scale extraction of the drug from the natural source for use by the pharmacological industries[10]. Also, the conventional breeding of high artemisinin yielding plants and the manipulation of culture conditions, such as, the growth media and hormone levels, to increase the yield of artemisinin in tissue and cell cultures have not led to significant increase in the production of artemisinin[11]. Hence, there is an urgent need to develop an alternate source for the production of artemisinin throughout the year to meet out its demand. It can only be achieved either through cell suspension or hairy root culture systems with controlled culture conditions.

Callus and cell suspension cultures of the medicinal plants can produce the same compounds that exist in their parent plants[12,13]. They also have the capability to produce specific medicinal compounds at a rate similar or superior to the intact plants. Being relatively simple to control by experimental processes, they could easily be accustomed into large-scale cultures where the secondary metabolites

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could be extracted without sacrificing the whole plant, thereby reducing the cost of production[12,14-16]. The maximum artemisinin accumulation (~1.0%) have been reported in most of the field grown strains of *A. annua* at or near the onset of flowering, thereby, restricting the plant to be harvested only once annually. Further, in different studies, a low artemisinin (0.001%) has been detected in calli, which either differentiated into shoots or preferably shoots with roots[17-19]. The undifferentiated calli and cell suspension cultures, hence, could not be used for large scale production of artemisinin[17,20-22]. In order to improve the artemisinin production in cell suspension culture, we undertook the present study to develop calli from the leaf explants of normal and transgenic *A. annua* plants using different combinations and concentrations of PGRs and to evaluate their impacts on biomass of calli and artemisinin contents in the light and dark phases of cultures.

Materials and Methods

Plant Materials and Chemicals

The seeds of high artemisinin yielding strain of *A. annua* L. (~0.7% artemisinin) gifted by M/s Ipca Laboratories Pvt. Ltd., Ratlam (MP), India were stored at room temperature until use. These were germinated on solidified half-strength MS medium[23] under controlled environmental conditions to raise mother plants (~0.042% artemisinin) as a source of explants for normal (non-transgenic) callus cultures. For transgenic callus, the *in vitro*-raised *A. annua* plants (0.059% artemisinin) over-expressing *hmgr* gene from *Catharanthus roseus* (L.) G. Don was used as the source of explants.

All biology grade molecular chemicals were purchased from Sigma-Aldrich, Roche and Promega Life Science, USA. Tissue culture grade chemicals were purchased from HiMedia and Merck, India. All buffers and solutions were prepared by using autoclaved MilliQ water.

Culture Conditions

Inoculation of explants onto the medium and all other manipulations were carried out aseptically under the laminar airflow. The culture vessels were kept in culture room at 25±2ºC and under 16 h photoperiod. The light condition was provided by white florescent tubes with a light intensity of 31.08 µmol m^{-2} s^{-1}. The relative humidity of culture room was maintained at 55±5% throughout the experiment.

In Vitro Seed Germination and Preparation of Explants

The seeds of *A. annua* plant were thoroughly washed, surface sterilized using 0.1% (w/v) solution of mercuric chloride ($HgCl_2$) for 5 min and again washed thoroughly with sterile distilled water before inoculation for germination in half-strength MS basal medium (pH-5.8) supplemented with 2% sucrose (w/v). After 2 wk of germination, seedlings were transferred onto full strength MS basal medium.

Callus Induction and Growth

Leaf explants (0.5-1.0 cm) were taken from 30 d-old *in vitro*-raised seedlings of both normal and transgenic *A. annua* plants and cultured in light and dark conditions for 6 wk on callus induction medium [MS medium+3% sucrose (w/v)+0.8% agar (w/v)] supplemented with different combinations and concentrations of growth regulators as shown in Table 1.

Data were scored in terms of biomass (g dw per explant) on 2nd, 4th and 6th wk of the culture and callus morphology as well as artemisinin content (% dw) on 6th wk of the culture.

Estimation of Artemisinin

1 g dry callus was used for the estimation of artemisinin using the method as described by Zhao and Zeng[24]. The derivatized artemisinin was analyzed and quantified using reverse phase column (C18, 5 µm, 4.6*250 mm) with premix methanol and 100 mM K-phosphate buffer (pH-6.5) in the ratio of 60:40 as mobile phase at constant flow rate of 1 mL min^{-1} with the detector set at 260 nm. The artemisinin was quantified with the help of standard curve prepared by HPLC and was expressed in % dw of callus.

Results

Effect of PGRs on Callus Induction and Growth in Cultured Explants of Transgenic and Normal Plants

To study the effect of plant growth regulators (PGRs) on callus induction and growth in cultured leaf explants of both transgenic and normal *A. annua* plants, different concentrations and combinations of PGRs (BAP, NAA, 2,4-D & TDZ) were tried (Table 1).

The combination of BAP and NAA supplemented media induced a compact calli in transgenic leaf explants, but in varying colour and growth patterns. The dark brown, creamish green and green coloured calli with good growth were obtained under light regime on MS medium supplemented with BAP (0.1 mg L^{-1}) and increasing concentrations of NAA (0.5-2.0 mg L^{-1}), while on similar combinations and concentrations of PGRs, brownish, light green and

creamish green calli with moderate growth were produced under the dark regime. In the MS medium, when the concentration of BAP was increased from 0.1 to 0.5 mg L^{-1} with varying concentrations of NAA (0.5-2.0 mg L^{-1}), the callus growth was further improved in the light regime, producing dark brown, creamish green and green calli. However, the poor callus growth was observed on the MS medium supplemented with similar concentrations and combinations of PGRs under dark regime except in MS medium supplemented with BAP (0.5 mg L^{-1})+NAA (2.0 mg L^{-1}), wherein significantly faster growth was achieved in creamish green calli. Increase in concentration of BAP beyond 1.0 mg L^{-1} with increasing concentration of NAA (0.5-2.0 mg L^{-1}) showed faster growth of the calli under both light and dark regimes (Fig. 1a).

The combination of BAP and 2,4-D did not evoke any good growth in transgenic calli, both under light and dark regimes, except BAP (0.5 mg L^{-1}) +2,4-D (0.5 mg L^{1}) wherein light brown compact calli were produced under dark regime, and BAP (1.0 mg L^{-1})+2,4-D (0.5 mg L^{-1}) wherein brownish green and brown calli with good growth were produced under light and dark regimes, respectively (Fig. 1b). Among the combinations of TDZ and 2,4-D, higher concentration of TDZ proved better in terms of callus induction and growth. TDZ (0.5 mg L^{-1})+2,4-D (1.0 mg L^{-1}) resulted in the induction of dark green and creamish colored calli with good growth under both light and dark regimes, respectively (Fig. 1c). Similar results have also been found in the calli from normal leaf explants using the similar concentrations and combinations of PGRs (Figs 2a-c).

Table 1—Combinations and concentrations of PGRs used for callogenesis in leaf explants of *A. annua* L. plants

Treatments	Plant growth regulators (mg L^{-1})			
	BAP	NAA	2, 4-D	TDZ
BN_1	0.1	0.5	-	-
BN_2	0.1	1.0	-	-
BN_3	0.1	2.0	-	-
BN_4	0.5	0.5	-	-
BN_5	0.5	1.0	-	-
BN_6	0.5	2.0	-	-
BN_7	1.0	0.5	-	-
BN_8	1.0	1.0	-	-
BN_9	1.0	2.0	-	-
BD_1	0.1	-	0.5	-
BD_2	0.1	-	1.0	-
BD_3	0.1	-	2.0	-
BD_4	0.5	-	0.5	-
BD_5	0.5	-	1.0	-
BD_6	0.5	-	2.0	-
BD_7	1.0	-	0.5	-
BD_8	1.0	-	1.0	-
BD_9	1.0	-	2.0	-
TD_1	-	-	0.5	0.1
TD_2	-	-	1.0	0.1
TD_3	-	-	2.0	0.1
TD_4	-	-	0.5	0.5
TD_5	-	-	1.0	0.5
TD_6	-	-	2.0	0.5
TD_7	-	-	0.5	1.0
TD_8	-	-	1.0	1.0
TD_9	-	-	2.0	1.0

Effect of PGRs on Biomass Accumulation of Calli from Transgenic and Normal Plants

PGRs play an important role in the induction of calli and biomass production. In our study, we

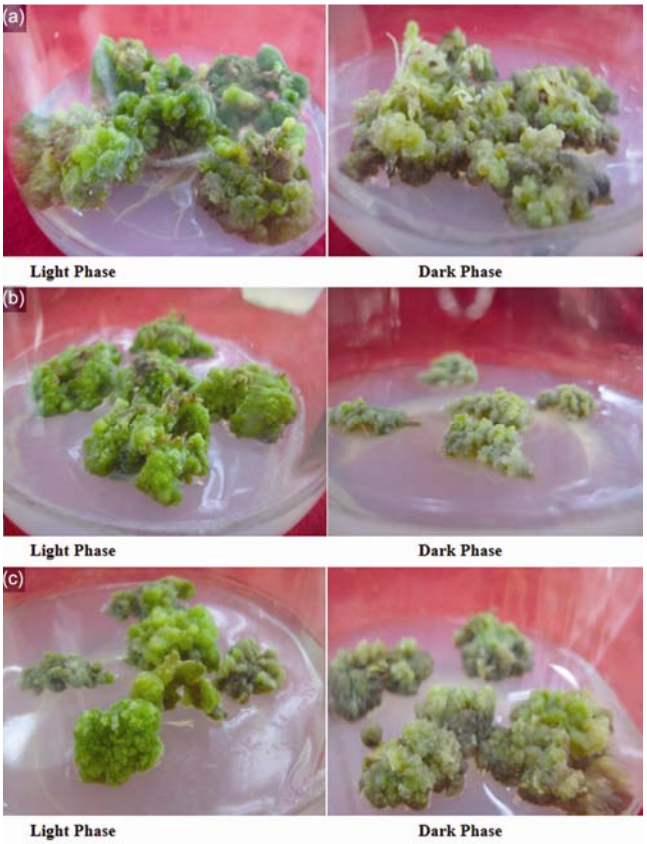

Fig. 1 (a-c)—Callogenic responses of transgenic *A. annua* L. leaf explants to MS media supplemented with different concentrations and combinations of PGRs: Effect of BAP and NAA [BAP (1.0 mg/L)+NAA (2.0 mg/L)] (a); BAP and 2,4-D [BAP (1.0 mg/L)+2 4-D (0.5 mg/L)] (b); & TDZ and 2,4-D [TDZ (0.5 mg/L)+2,4-D (2.0 mg/L)] (c) in light and dark phase on callus induction.

have observed the effect of different combinations and concentrations of PGRs (BAP, NAA, 2, 4-D & TDZ) on the callus biomass at 2nd, 4th and 6th wk of the culture of both transgenic and normal leaf explants of *A. annua*. The biomass yield of transgenic calli was significantly higher (0.13 g dw/explant) in the light as compared to the yield (0.06 g dw/explant) in the dark regime on the 6th wk of culture on MS medium supplemented with BAP (1.0 mg L^{-1}) +NAA (2.0 mg L^{-1}). It was followed by MS medium supplemented with BAP (1.0 mg L^{-1}) +2, 4-D (0.5 mg L^{-1}), wherein the 0.08 g dw of biomass/explant and 0.06 g dw of biomass/explant were recorded under light and dark regimes, respectively. However, the least biomass accumulation was recorded in the calli from the explants that were cultured on MS medium supplemented with TDZ (0.5 mg L^{-1}) and 2,4-D (2.0 mg L^{-1}) (Figs 3a-c).

Similarly, the maximum dry biomass 0.1g dw/explant of normal calli was obtained on MS medium supplemented with BAP (1.0 mg L^{-1})+NAA (2.0 mg L^{-1}) in the light; whereas on the similar combination and concentration of PGRs in the dark, 0.05 g dw/explant was observed. It was followed by 0.07 g dw/explant in the light on MS medium supplemented with BAP (1.0 mg L^{-1})+2,4-D (1.0 mg L^{-1}); while on the similar concentration and combination of PGRs, 0.06 g dw/explant was obtained in the dark (Figs 4a-c). In case of MS medium supplemented with TDZ (0.5 mg L^{-1}) and 2, 4-D (1.0 mg L^{-1}), a biomass of 0.07 g dw of callus/explant was recorded in the light and 0.04 g dw/explant in the dark conditions.

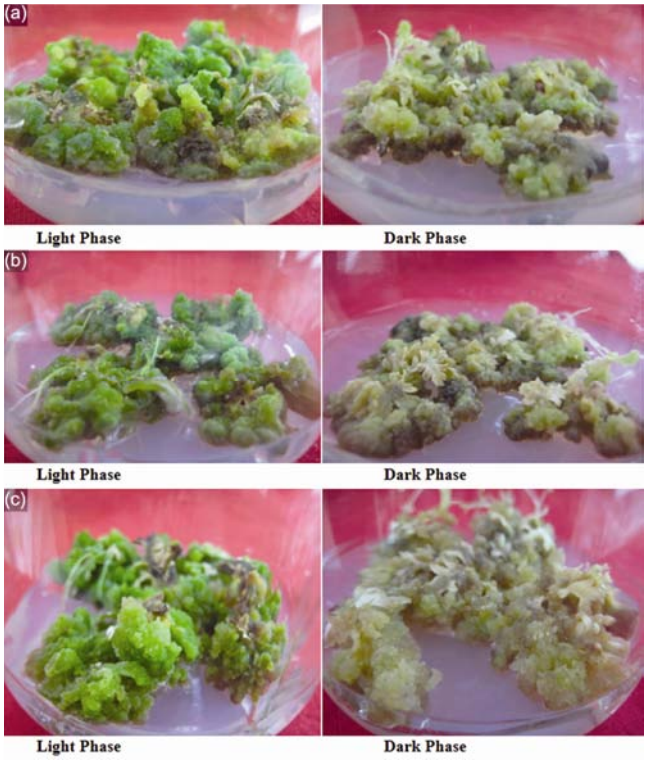

Fig. 2 (a-c)—Callogenic responses of normal (non-transgenic) *Artemisia annua* L. leaf explants to MS media supplemented with different concentrations and combinations of PGRs: Effect of BAP and NAA [BAP (1.0 mg/L)+NAA (2.0 mg/L] (a); BAP and 2, 4-D [BAP (1.0 mg/L)+2,4-D (1.0 mg/L] (b); & TDZ and 2,4-D [TDZ (0.5 mg/L+2,4-D (1.0 mg/L)] (c) in light and dark phase on callus induction.

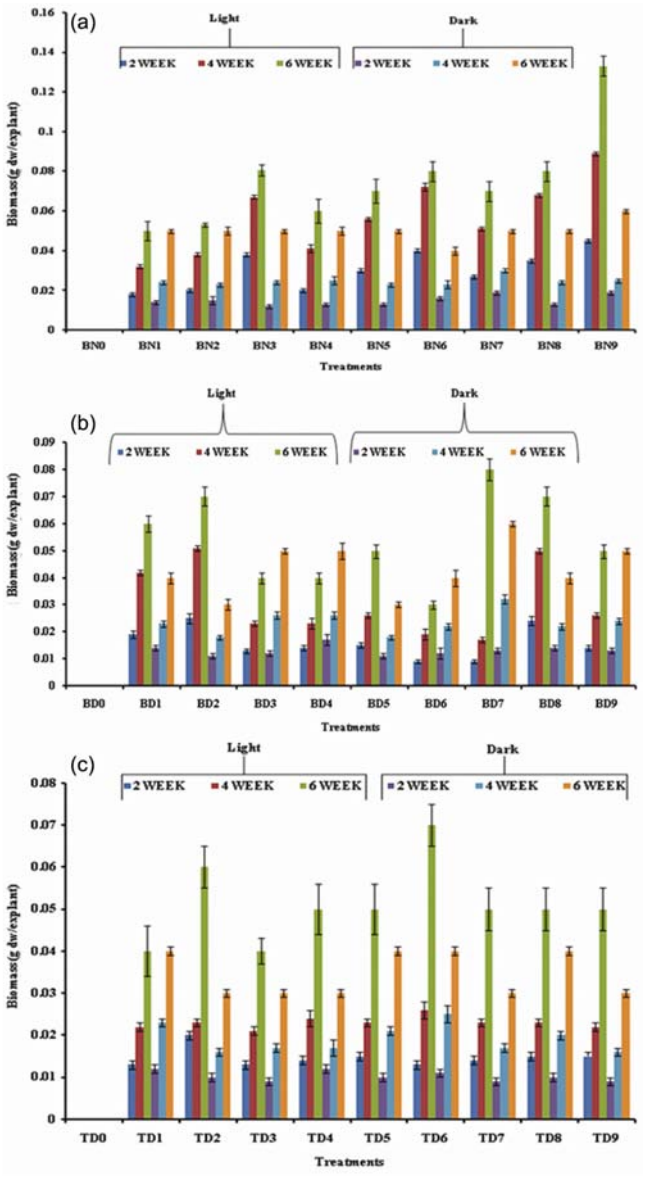

Fig. 3 (a-c)—Biomass accumulation (g dw/explant) in transgenic calli cultured on MS media supplemented with various concentrations and combinations of BAP and NAA (a); BAP and 2,4-D (b) ; & TDZ and 2,4-D (c). Bar represents ±SE (n= 10). [Treatments correspond to Table 1].

Effect of PGRs on Artemisinin Content of Calli from Transgenic and Normal Plants

Since the highest biomass of calli from both transgenic and normal plants of *A. annua* was observed at the 6th wk of the culture, the artemisinin content in the calli was determined at the same period of culture on MS medium supplemented with various concentrations and combinations of PGRs. The higher artemisinin content (0.006% dw) was recorded in the transgenic calli cultured in the light condition as compared to those cultured in the dark (0.004% dw) on the MS media supplemented with BAP (1.0 mg L^{-1}) and NAA (2.0 mg L^{-1}) (Figs 5a-c). The other hormonal treatments could not evoke any further increase in the artemisinin content of transgenic calli cultured in light/dark regimes. Further, no significant differences in the artemisinin content were observed between transgenic and normal calli grown on similar PGRs under the light/dark regimes (Figs 6-c).

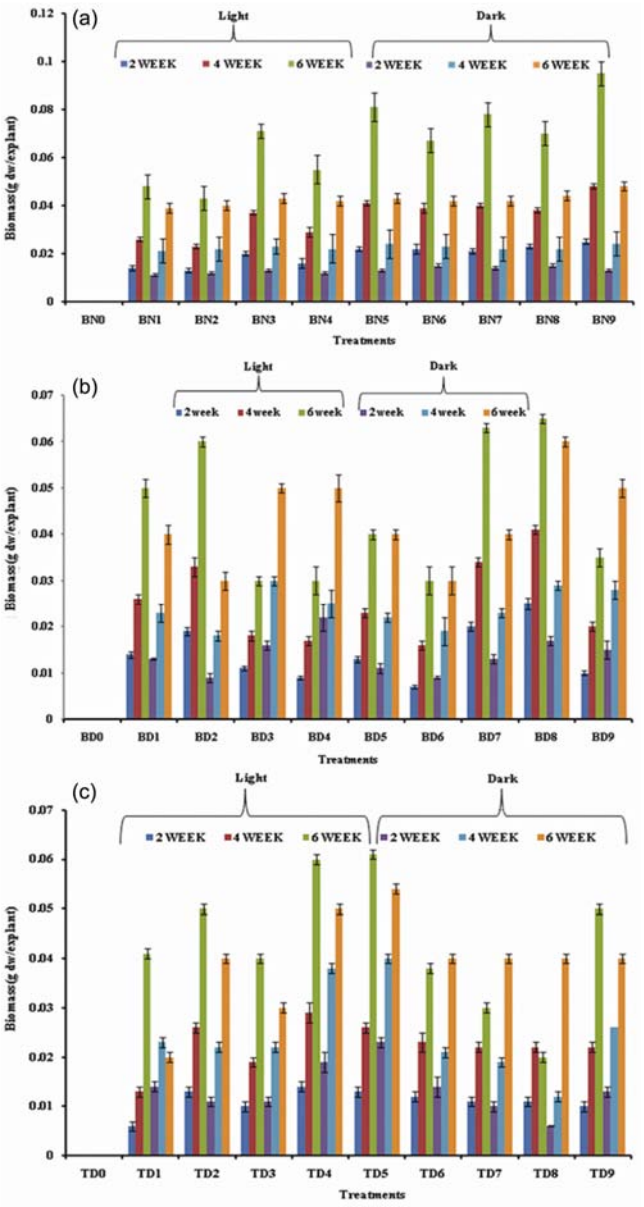

Fig. 4 (a-c)—Biomass accumulation (g dw/explant) in normal (non-transgenic) calli cultured on MS media supplemented with various concentrations and combinations of BAP and NAA (a); BAP and 2, 4-D (b); TDZ and 2,4-D (c). Bar represents ±SE (n=10). [Treatments correspond to Table 1].

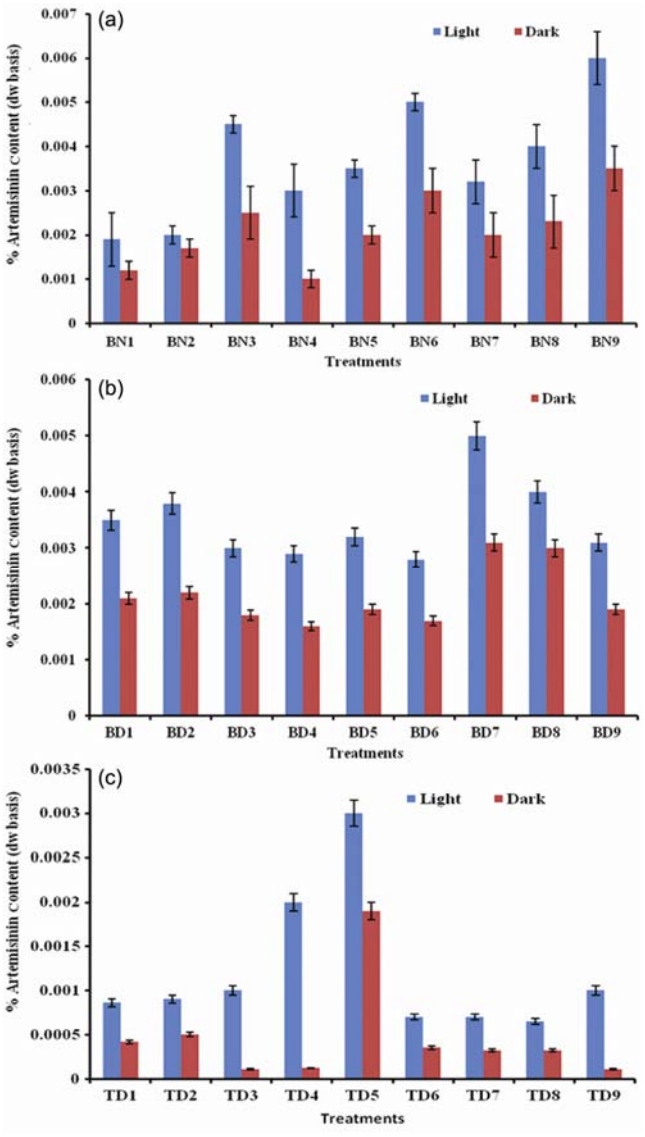

Fig. 5 (a-c)—Artemisinin content in transgenic calli cultured on MS media supplemented with various concentrations and combinations of BAP and NAA (a); BAP and 2,4-D (b): & TDZ and 2,4-D (c). Bar represents ±SE (n=10). [Treatments correspond to Table 1].

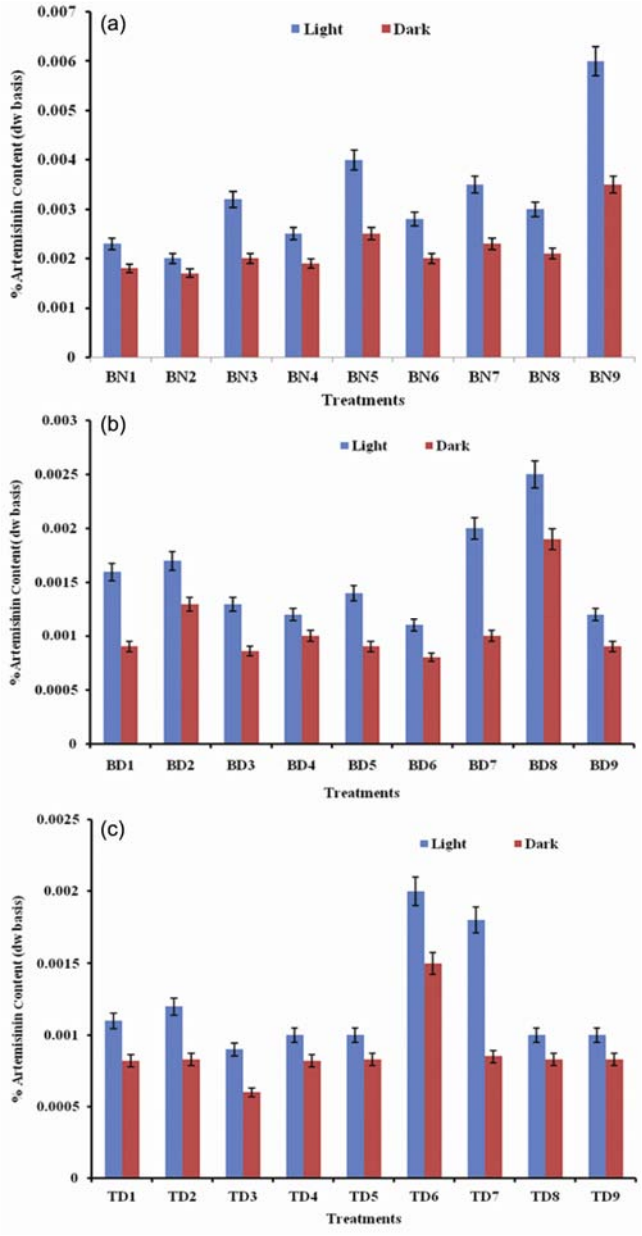

Fig. 6 a-c)—Artemisinin content in normal (non-transgenic) calli cultured on MS media supplemented with various concentrations and combinations of BAP and NAA (a); BAP and 2,4-D (b); & TDZ and 2,4-D (c). Bar represents ±SE (n=10). [Treatments correspond to Table 1].

Discussion

The callus culture provides the advantage of continuous and reliable source of plant pharmaceuticals, and could be used for large scale extraction of these compounds. However, for the controlled growth and morphogenesis in a culture medium, plant growth regulators and their concentration are critical factors. Generally, high concentrations of auxins and low concentrations of cytokinins in medium promote abundant cell proliferations with the formation of callus[25,26]. The nature of tissue, degree of totipotency and composition of medium with respect to micronutrients and hormones also affect the callogenesis.

With the cell growth and differentiation, PGRs are also one of the most significant factors that affect the metabolite formation[27]. The suitable concentration of the medium determines growth and metabolite production in callus. Thus, it is imperative to found the optimal culture conditions for the specific plant species used to obtain a good amount of cell dry mass, in addition to the secondary metabolites. The concentration of auxins and cytokinins individually or in combination used in the media generally influences the growth and regulation of cell metabolism[28].

The different concentrations and combinations of PGRs, used in our study, were effective to induce calli in the leaf explants from both transgenic and normal *A. annua* plants and their growth. The combinations of BAP and NAA supplemented medium induced compact calli in the leaf explants of transgenic plant, but they varied in colour and growth pattern. The dark brown, creamish green and green coloured calli with good growth were observed under light regime on MS medium supplemented with BAP (0.1 mg L^{-1}) and increasing concentrations of NAA (0.5-2.0 mg L^{-1}); while on similar combinations and concentrations of other PGRs, *viz.*, 2,4-D and TDZ, brownish, light green and creamish green calli with moderate growth were produced under the light and dark regimes. This is because of the fact that NAA is less inhibitory to the chlorophyll biosynthesis than TDZ and 2, 4-D that makes the calli greener[29,30].

Further, the biomass of transgenic calli was increased 1.5 times more on MS medium supplemented with BAP (1.0 mg L^{-1}) and NAA (2.0 mg L^{-1}) as compared with the transgenic calli cultured on medium supplemented with BAP (1.0 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}). Similar observations were also made by several investigators in *Pistacia vera* and *P. atlantica* subsp. *kurdica*[31,32].

Light has a significant effect on increasing the biomass of calli in terms of fresh weight in different explant derived calli, such as, leaf, hypocotyl, cotyledon, etc., as reported by George *et al*[33] and others[29,31]. Our results are in agreement with the previous studies and 1.5-2.0 times higher biomass was recorded in those calli cultured in the light conditions irrespective of the combinations and concentrations of PGRs. In both transgenic

and normal lines, the calli under light phase were found much greener than those under the dark phase[29].

Secondary products are usually found in the well differentiated parts of the plant and make an important feature of differentiation. Secondary metabolites production from undifferentiated plant cell cultures has been intensively studied[34-37], although very few compounds have been commercially manufactured *in vitro*. The metabolite production is also dependent on plant growth regulators. Earlier, artemisinin concentration ranging from 0.78 to 1.13 mg g^{-1} dw was reported in *A. annua* calli cultured on MS media supplemented with different concentrations of BAP, NAA and 2, 4-D[38]. However, the concentration of artemisinin declined to zero after three subcultures[13,16]. Further, the yield of artemisinin was found quite low or sometimes even not detectable in the dedifferentiated cells, such as, callus or suspension cultured cells[19,39-42]. We have also reported artemisinin (0.006% dw basis) in both transgenic and normal calli of *A. annua* that were cultured in light on MS medium supplemented with BAP (1.0 mg L^{-1})+NAA (2.0 mg L^{-1}), but these calli were undifferentiated, friable and green. Hence, they can be used to develop photosynthetically active cell suspension culture for the production of artemisinin[43-45].

Many reports have shown that light is an important physical factor influencing the growth of calli and formation of primary and secondary metabolites[46-51]. Besides, illumination was also found to affect the composition of sesquiterpenes in callus culture of *Marticaria chamomile*[13,52,53]. The results of these studies support our results, where higher artemisinin content was observed in the calli grown in light (1.5 times higher) as compared to the calli grown in dark. The friable, green calli of *A. annua* developed by us could be used as a sustainable calli-based platform for the production of artemisinin. Furthermore, this callus culture may also be used to initiate cell cultures that could be extrapolated to the bioreactor level for artemisinin extraction in the near future. This system could also be used for the clonal propagation of high artemisinin yielding strain of *A. annua* for large scale cultivation.

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