

Research Article

Impact of phyto-hormone concentrations in optimizing cell suspension culture of flue-cured tobacco (*Nicotiana tabaccum* L.) cultivars

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Abstract

Plant cell suspension cultures are mostly used for the biochemical investigation of cell physiology, growth, metabolism and production of secondary metabolites. The study was undertaken to optimize protocol for callus induction and cell suspension in K-326 and Honghuadajinyuan (HD) cultivars of tobacco (*Nicotiana tabaccum*). The experiment was conducted at the Key Laboratory of Tobacco Genetic Improvement and Biotechnology, Chinese Academy of Agricultural Sciences (CAAS), Qingdao, P.R. China. In this study explants leaf were inoculated on the (Murashig and Skoog) media with supplementation of exogenous growth regulators of plant. For the morphogenic callus proliferation and indication form explants, (MS) medium was used with (NAA, 6-BA and 2, 4-D phytohormones. Callus derived leaves were stabilized for 4 week of period. The tobacco cell suspension culture was established initially through the culture of friable leaf derived callus in the medium of liquid callus induction. The results of growth regulator combination significantly ($P < 0.05$) affected on the calli growth. In this study between different treatments, the highest frequency of callus induction was recorded in the level (1) (i.e. NAA) (.33mg/l) and 2, 4-D (0.5mg/L, followed by the level of 03 (i.e. 0.75mg/LNAA, 2.4mg/L 6-BA and 0.5mg/L2, 4-D), respectively. The subsequently sub-culturing of friable callus on callus induction media enhanced callus biomass subculture cycle. The callus induction obtained from HD and K-326 leaf explants was 82.5% and 80%, respectively. The cell growth curve showed that, cells of HD and K-326 produced highest fresh cell mass of 38.07 and 37.67 g, respectively. The production of callus biomass became stable after three subculture cycles. The

cells subsequently grew healthy and maintained well in MS liquid medium supplement with the optimal hormone. The browning occurred when the cultures reached the highest fresh cell mass. Therefore, in order to maintain healthy cultures, sub-culturing should be done before browning occurs.

Keywords: Callus induction; Cell culture; Tobacco; Growth regulators

Introduction

In plant cell culture system callus tissue is a major material. During the introduction of it in liquid medium, plant cell disperse by liquid to form suspension of cell culture. According to researchers such type of cell are known as totipotent and must have ability to synthesize different compound particularly linked with intact plant [1]. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are desired [2]. The techniques of plant cell culture facilitate the rapid production of variant cell lines via selection procedures that are useful for research in genetics, biochemistry and biotechnology. Significant features of *In-vitro* propagation are the multiplication capacity in a relatively short span of time, the production of healthy and disease-free plants and the ability to generate propagates throughout the year independent of seasonal changes. Additionally, it is also a useful method to propagate endangered plant species with the purpose of optimizing the time and cost required to aid in preservation [3]. The suspension of culture system can be utilized on large level for plant cell culturing that secondary metabolites can be extracted from it [4]. At higher level it has been observed that tissue culture is an attractive alternate approach for traditional practices of plantation as it facilitate limited provision of biochemical, independent of plant variability [5].

In plant cell production of some secondary valuable metabolites for organ or tissue are considered as alternate attractive procedure for whole plant material extraction [6]. The suspension of cell proved in vitro platform that could be utilized an instrument for different investigation in HD and K326.

These can be utilized in protoplast isolation, transfer of gene biology, cell wall traits, mass propagation and mutant selection [7] whereas on the higher level of plant cell culturing for secondary metabolite extraction [8]. The suspension of cell culture provides a vast number of opportunities for provision of large amount of similar cell which grown in liquid form culture [8]. The two different stage of culture regime are mainly processed for production of secondary level metabolites through plant cell. In the initial stage, growth and maintained of cell at large level of density on the maintain standard media, whereas in 2nd phase these plant cell are transferred in the production media to achieve secondary metabolites [9].

It has been reported that a limited amount of in vitro cell of the *Jatropha curcas* is limited [11, 12], but provision for formation of propagation procedure is initiated from Asia, while the genetic diversity of *Jatropha curcas*. Currently studies has been described of allele known as cytokinin-hypersensitive 2 pkl, that showed a upper level of response to cytokinin exogenous in in vitro callus indication assay [13]. Different compound classes of plant for secondary metabolites including material that showed pharmaceutical function such as (vincristine and morphine, pigments, enzymes, carbohydrates latex pigments and fragrance [14]. It is reported that plant cell culture showed a super system at higher level for different secondary metabolites for production of biomass on rapid bases [15]. It is reported that an effective protocol for micro propagation established for *Kaempferia paryiflora* [16]. The lower part bud cultured on supplemented MS medium with (7 mg/ L6 benzylaminopurine produce shoots primary explants after the culture of eight weeks [17].

This study was designed with the objectives of synthesis of tissue culture environment for callus formation and production of cell suspension culturing through the use of various contributed concentrations of (cytokinin NAA, 6 BA and auxin 2, 4 D). The cell culture conditions optimized to obtained through this will further be utilized in present target for secondary metabolite production.

Materials and methods

Plant materials and culture condition

In this study two cultivars (HD and K-326) of tobacco (*Nicotiana tabacum* L.) provided by Germplasm Bank of Tobacco Research Institute of Chinese Academy of Agricultural Sciences (CAAS), Qingdao, P.R. China were evaluated for establishment of cell culture. The seeds of tobacco were subjected to *In-vitro* germination in Murashige and Skooge; MS (1962) medium.

Before MS medium inoculation the plant seed were grown 3 times for thirty seconds with (75%) ethanol then 3 times were sterilized by water. The seeds were again surface sterilized for fifteen minutes in (15%) H₂O and then sterilized 5 time in water. This sterilized seed were de coated partly as well as aseptically grown in the bottle of glass that contain (500 ml) in MS media autoclaved. The Ph was adjusted 5.7. The dry seed were inoculated in plates that contain MS media solid. All cell culture was inoculated in the culture in cell culture room with white fluorescent light with intensity of (3000) lux with sixteen hours at photoperiod. The temperature of rom was maintained at 26⁰ C + 2. Germinal seedling then transferred to glass bottles that contain MS medium and showed similar growth. The seedlings of eight week leaves old *in vitro* utilized as the explants for callus induction.

Callus induction

The seedlings of leaf mentioned in (Fig. 1) extracted by *In vitro* plantlets, then used as explants. Leaf material was harvested from the plants under sterile conditions. Leaf parts

were cut into segments (0.5 cm) and cultured on the plates (9 cm in diameter, 1.5 cm deep) and (four pieces per plate) The Petri dishes contains (20 ml) MS media with the supplementation of various concentration of growth regulators including (NAA, 6 BA and 2, 4 D) with various combinations and concentrations for callus induction and sealed with Para film. Medium of pH was maintained 5.8 prior to autoclave at 121^o C till twenty minutes. The each culture was incubated in dark with 26±2°C for 28 days (Table 1). MS basal medium and cultures were monitored daily. After 28 days of incubation period, calli appeared on the leaf explants. The data was recorded on days to callus initiation, percentage of callus induction, and size and color of callus. The different concentration combinations of growth regulators were using U₆^{*} (6⁴) uniform design experimentation included 6 levels NAA, 6 levels 6-BA, 6 levels 2,4-D, totally being 6 treatments with 3 replicates (Table 1). Data collected in the experiments were analyzed using SAS (version 9.1) data analysis software for researchers. The best combination of growth regulators was screened using the partial least-squares regressive way.

Sub-culture

The calli were aseptically shifted to freshly prepared MS medium containing appropriate hormonal supplements to obtain an optimal growth. The sub-culturing media was MS containing corresponding different concentration combinations of NAA, 6-BA and 2, 4-D. The subculture was again incubated in the dark at 26 ± 2°C for 42 days. The culture vessels showing the sign of contamination were discarded. Repeated sub-cultures were incubated three times at 2 week intervals which formed the creamy color calli under the same temperature as mentioned previously for maintenance of calli and organogenesis.



Figure 1. Eight weeks old *In-vitro* seedling of tobacco cultivars (K-326 and HD)

Table 1. The influence of phytohormones on callus induction and growth of tobacco cultivars

cv. HD Levels	Phytohormones (mg/l)			Callus size (cm)	Callus induction (%)	Morphology of callus
	NAA	6-BA	2,4-D			
1	(1)0.5	(2)0.8	(3)0.4	2.26667bc	82.5a	+++
2	(2) 1.0	(4) 1.6	(6) 1.0	2.1515c	36.25bc	---
3	(3)1.5	(6) 2.4	(2) 0.2	2.66667a	72.5a	++
4	(4)2.0	(1) 0.4	(5) 0.8	2.14375c	43.75b	+
5	(5)2.5	(3) 1.2	(1) 0	2.44667b	70.0a	++
6	(6)3.0	(5) 2.0	(4) 0.6	2.15375c	25.0c	---
cv. K326						
1	(1)0.5	(2)0.8	(3)0.4	1.69a	80.0a	+++
2	(2) 1.0	(4) 1.6	(6) 1.0	1.63a	66.7ab	---
3	(3)1.5	(6) 2.4	(2) 0.2	1.67a	68.8ab	++
4	(4)2.0	(1) 0.4	(5) 0.8	1.29a	65.0ab	--
5	(5)2.5	(3) 1.2	(1) 0	1.46 ab	75.0a	-
6	(6)3.0	(5) 2.0	(4) 0.6	1.22b	43.3b	---

Means followed by different small letters in a same column are significantly different at $P=0.05$ according to Duncan's multiple range tests. +++ very creamy, ++ creamy, + little creamy, ---very green, -- little green –green

Cell suspension culture establishment

The suspension of cell culture started by friable, soft and healthy cali sub cultured till 42 days were transferred aseptically to (MS medium) with supplementation of 3% sucrose contain maximum level of hormone with 3 lots in the conical flask that contain 150 ml Erlenmeyer 30 ml in MS media. These flask were transferred in rotary shaker and rotated at 120 rpm without air till seven days. After passing the seven days liquid media that contain micro cali and cali were filter in a (200 micron mesh sieve. This filter material contain laboratory maintained cells. To find out efficiency of growth, the liquid medium

containing flask were placed in orbital shaker. The growth of cell were observed through the weighing of cell for 3 ml in liquid medium were taken every day (Fig 2). Other side, to receive callus few cell were distributed in flask that contain semi solid media at 26°C for fourteen days of incubation in dark place. The Micro cali were prepared in flask from aggregated cells for initiating induction of callus. In this way during days' time period fresh cell mass was observed. The biomass cell data were statistically analyzed to find out the growth regulator pattern with favorable harvest time. The cell morphology aggregate was also finding out.

Results and discussion

Influence of growth hormones on callus formation and proliferation

Large size of aseptic seed were received through the protocol of surface sterilize and (95%) seed were germinated after the 3 weeks culturing period on basal MS media. Seedling were placed to grow in similar medium in absence of (phytohormones till 28 days (Fig. 1) which were utilized at plant material for the formation of cell suspension and callus cultures. Seed of two cultivars were grown on MS medium at same days but different responses were observed where variety HD grew quickly than variety K-326. The supplemented growth of plant hormones particularly (cytokinin in culture media after encouraging the organogenesis callus formation. The callus induction formation response for explant %, nature and size were significantly affected due to different concentrations of phytohormones (Table 1). Analysis of variance showed significant differences ($p \leq 0.05$) among different treatments (Table 2). Highest callus

induction 82.3% with a mean callus size of 2.27 cm was obtained in HD variety when the explants were cultured on MS media at level 1.

The calli were friable in texture and creamy in color as shown in (Fig. 3A). Whereas at level 3, the second highest callus induction 72.5% with a mean callus size of 2.67 cm was induced in HD variety. The calli produced were more compact in texture and green in color (Fig. 3B). In case of K-326 highest callus induction 80% with a mean callus size of 1.69 cm was obtained at level 1 and calli were friable in texture and creamy in color (Fig. 3C). Whereas at level 5, the second highest callus induction 75% with a mean callus size of 1.46 cm was induced and calli were more compact in texture and green in color (Fig. 3D). Moreover, the best combination of phytohormones for callus induction, size, texture and color in variety HD was 0.33mg/LNAA and 0.5mg/L2,4-D and that of for variety K-326 was 0.75mg/LNAA, 2.4mg/L6-BA and 0.5mg/L2,4-D.



Figure 2A. HD



Figure 2B. K-326

Figure 2. Cell suspension culture of tobacco cultivars

Table 2. ANOVA for callus induction (%) of tobacco cultivars

Source of variation	Mean square			
	Callus induction percentage			
	F value	p value	R-Square	contribution
Model	19.26	0.0001	76.75%	76.75%
Error				23.25%
Treatments	19.15	0.0001		63.61%
Varieties	19.79	0.0001		13.14%



Figure 3A. HD



Figure 3B. HD



Figure 3C. K326



Figure 3D. K326

Figure 3. Green and friable creamy color of callus induction

Formation of cell suspension culture

Early proliferation of white friable calli 1g weight were grown the solid media that were consider good agent for starting of cell suspension culture in both cultivators (Table 3).

The cells were observed for their continuous growth in 6 flask (3 flasks for HD and three flasks for K-326) with three media combinations of 6-BA, NAA and 2,4-D. The maximum fresh cell mass of 38.07 g for variety HD was obtained at the concentrations of 0.33mg/LNAA, and 0.5mg/L2,4-D. Whereas for variety K-326 maximum fresh cell mass of 37.76 g was noted at concentration of 0.75mg/LNAA, 2.4mg/L 6-BA and 0.5mg/L2,4-D (Table 4). All three hormone concentration and

combination described same type growth of cell at peak level growth of cell in HD variety was record on eight day (Fig. 4 & 5). After two weeks, cell growth was found in stationary. The callus induction at time of explant cultured in MS media contains cytokinin and auxin. The maximum explant 82.5% with 80% of callus induction in varieties including K 36 and HD, when growth regulators were used (NAA, 6 BA and 2, 4 D in MS media supplementations and treatments were 01 (NAA + 0.5 mg/ l), (2, 4 D + 0.4 mg/ l). The same findings were described by (Kumari et al., 2008) in *Ricinus communis* L. and they received induction of callus by cotyledon explant used with combination of (6 BA 2.0 mg/1) and (0.8mg/1 NAA). In the governing

callogenesis the composition of callus culture medium is highly important factor. Another study was conducted by [18], who had reported maximum callus induction percentage on MS medium. The MS media is important for induction of callus Tetrandra were grown in various basal medium to get highest callogenesis with used of fold MS [19,

20], reported that Phalaenopsis callus induction the dark movement play a major part in plantlet regeneration as well as callus induction. It has been reported that influence of 4PU 30 and kinetin on the content of polyphenols and growth in tobacco callus, at 26 C darkness showed excellent results [20].

Table 3. Effect of different phytohormones on fresh cell biomass of tobacco cultivars

DAYS	HD variety				K-326 variety			
	Phytohormones (mg/l)			Fresh cell biomass (mg)	Phytohormones (mg/l)			Fresh cell biomass (mg)
	Flask-1	Flask-2	Flask-3		Flask-1	Flask-2	Flask-3	
1	30.4	30.33	29.82	30.18333	31.12	31.3	30.11	30.84333
2	31.34	30.9	31.69	31.31	30.4	32.22	31	31.20667
3	31.56	32.23	33.44	32.41	31.12	32.22	32.88	32.07333
4	32.78	33.45	34.99	33.74	32.34	32.1	32.65	32.36333
5	34.11	36.45	38.22	36.26	33.78	35.24	34.29	34.43667
6	36.68	38.34	36.99	37.33667	35.24	34.99	36.89	35.70667
7	38.78	38.58	35.88	37.74667	36.87	37.78	37.72	37.45667
8	38.11	40.11	35.99	38.07	36.78	39.49	36.74	37.67
9	34.41	40.1	37.45	37.32	34.34	39.12	34.88	36.11333
10	35.88	39.44	36.12	37.14667	33.8	37.22	35.11	35.37667
11	35.11	38	36.99	36.7	34.23	36.1	35.25	35.19333
12	35.32	34.44	38	35.92	34.68	36.1	35.34	35.37333
13	34.56	33.61	37.11	35.09333	34.81	35.6	35.34	35.25
14	34	33.2	36.44	34.54667	34.22	33.11	35	34.11

Table 4. ANOVA for fresh cell biomass of tobacco cultivars

Source of variation	Mean square							
	1st week, Cell fresh mass (g/ml)				2nd week, Cell fresh mass (g/ml)			
	F value	p value	R-Square	contribution	F value	p value	R-Square	contribution
Model	0.09	0.7821	2.14%	2.14%	0.15	0.716	3.67%	3.67%
Error			97.86%					96.33%
Varieties			2.14%					3.67%

The formula of callus taken fragment reported initial of vigorous, while and soft color tissue [21]. The formation of callus *Tinospora cordifolia* showed from leaf explant nodal when they were grown in MS media that contain growth regulators (NAA and BAP, then callus was observed only from explant leaf [17]. The findings of our study are in agreement with (Kuo et al., 2011) that

showed an effective in vitro induction of callus system in *Stephania tetrandra* was formed in MS media with supplementation of three % sucrose in plant growth regulators such as cytokinin (BA, Zeatin, TDZ and kinetin) and auxin (NAA, 2, 4, D and IAA) in dark movement. The MS media supplemented with (1.0 mg/ L BA and 0.5 mg/ L TDZ favors the growth of cell with its

proliferation. A large amount of callus size was formed in 28 days after the culture. The same explant leaf showed brown friable callus in various basal medium with supplementation of (2, 4 D, NAA and 6 BA in both cultivators (Table 6). The percentage of callus induction was observed superior in solidified MS media with supplementation of (NAA, 0.33 mg/l and 0.5 mg/L) then followed by (0.75 mg/ LNAA, 2.4 mg/L 6 BA and 0.5 mg/L 2, 4 D). While in all conditions the continuous cell growth till 14 days. The highest growth of cell was observed on 8 days. Similar statement reported [22] in brinjal that showed maximum peak growth in 7 to days of incubation. Further studies have also reported

maximum peak growth of cell in *Abrus precatorius* where the cell got maximum peak level growth within 7 days. In this study the rate of cell growth in both cultures was slow during initial stage but later it was increased due to culture continuously contained significantly increased with accumulation of biomass till 14 days. Similar results reported by [13] in sugar beet the required time to formation of cell suspension culture varies and depend on the tissues of different plant species and media composition. The use of better suspension culture showed great opportunity for checking of cell origin of somatic embryo and asymmetry of 1st cell division that initiate process.

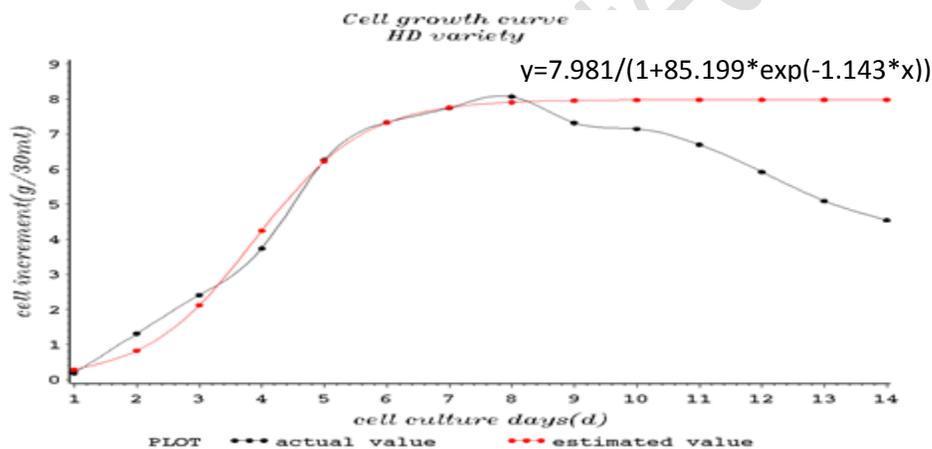


Figure 4. Cell growth curve (HD Variety)

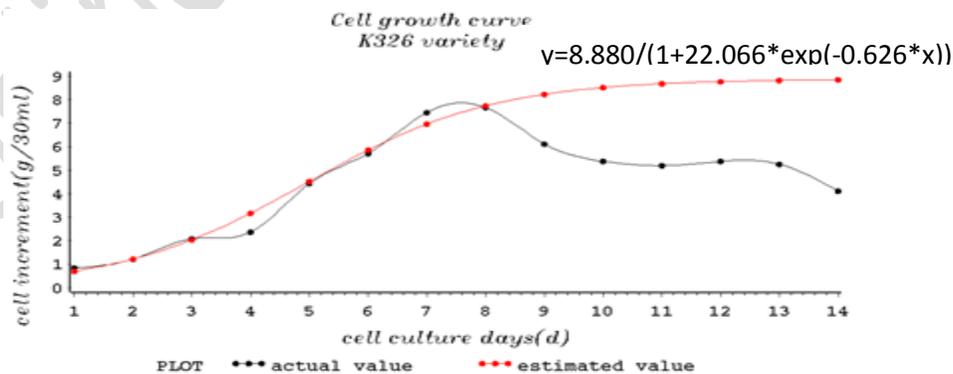


Figure 5. Cell growth curve (K-326 variety)



Figure 6A. HD



Figure 6B. K-326

Figure 6. Friable subcallus culture of tobacco cultivars

Conclusion

It is concluded from this study that (auxin NAA, 6 BA and 2, 4 D) showed significant role in callus size, color and induction. Concentrations of 0.33mg/LNAA and 0.5mg/L2,4-D proved the highest favorable combination for the induction of embryonic calli by HD variety whereas 0.75mg/LNAA, 2.4mg/L 6-BA and 0.5mg/L2,4-D for K-326 variety. The phytohormones NAA, 6-BA in combination with 2, 4-D were observed favorable medium composition for increasing the tobacco cell growth. The study also demonstrated that in tobacco cells initiate to grow between 7 to 8 days of culture that showed potential for cell culture development and production of major secondary metabolites as important enzymes.

Authors' contributions

Conceived and designed the experiments: QD Jogi & A Chen, Performed the experiments: QD Jogi, M Sun, S Wang & MN Kandhro, Analyzed the data: AH Soomro, MA Ansari & NA Pahnwar, Contributed materials/ analysis/ tools: MH Hulio, ZA Abbasi & R Kumar, Wrote the paper: QD Jogi & N Babar.

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