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Establishment of *Mucuna pruriens* (L.) DC. callus and optimization of cell suspension culture for the production of anti-Parkinson's drug: L-DOPA

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ABSTRACT

It has become a huge challenge to satisfy the emerging demand for levo-3,4-dihydroxyphenylalanine (L-DOPA), an anti-Parkinson's drug in the international drug market. This is attributed to the conventional methods of extraction from the natural sources of *Mucuna* spp., which has a low germination rate, less viable seeds, and an irritating, itching trichomes on the pods. The need for an alternative method with continuous supply of L-DOPA without affecting the natural biodiversity has been achieved through *in vitro* procedures. However, there has not been a systematic approach to optimize the cultural conditions for the maximum productivity. Hence, in this study, we aim at optimizing the cultural conditions for high biomass and L-DOPA production. Various plant growth regulators such as auxins (indole acetic acid, indole butyric acid, picloram [Pic], naphthalene acetic acid, and 2,4-Dichlorophenoxyacetic acid), cytokinins (kinetin, benzylaminopurine, 2-isopentenyl adenine, and thidiazuron), and their combinations have been experimented to figure out the best combination to induce callus. At the same time, various factors such as growth kinetics, different media (MS, Gamborg's-B5, Chu's-N6, and Nitsch and Nitsch), media strength (0.5, 1.0, and 2.0X), effect of different macro elements and their strength (0, 0.5,1, 1.5, 2, and 3X), inoculum density, different hydrogen ion concentration (pH), ammonium/nitrate concentration, different sucrose concentrations (0–10%), and other carbon sources have been investigated in detail for optimizing the cell suspension culture. It was found out that 0.5 mg/L Pic gave the best results for callus induction. With respect to biomass, 6-week growth period (135.7 g/L fresh weight [FW]), 1.0X MS media (126.87 g/L FW), 1.5X magnesium sulfate (266.3 g/L FW), ammonium/nitrate ratio of 21.57/18.8 mM (131.4 g/L FW), pH of 6.0 (129.47 g/L FW), 100 g/L of inoculum (222.2 g/L FW), 3% sucrose concentration (125.6 g/L FW), and 3% glucose (183.4 g/L FW) as other carbon sources were found to give the highest biomass. In terms of L-DOPA production, 3-week growth period (5.90 mg/g dry weight [DW]), 0.5X B5 medium (4.27 mg/g DW), 2.0X calcium chloride (5.06 mg/g DW), ammonium/nitrate ratio of 21.57/18.8 mM (3.44 mg/g DW), pH 6.5 (4.02 mg/g DW), inoculum density of 30 g/L (4.79 mg/g DW), and 2% sucrose (5.17 mg/g DW) resulted in a higher L-DOPA yield.

1. INTRODUCTION

Mucuna pruriens (L.) DC. is commonly known as velvet bean due to the presence of fine, silky, velvety trichomes on the pods, which causes severe itching and dermatitis if comes in contact due to the secretion of *Mucunain* [1]. It is an annual climber of Fabaceae family found endemic to Asian, African, and other American regions [2]. It has been used for more than 200 indigenous tribal formulations to treat a wide array of diseases. It is mainly used to treat Parkinson's disease [3] and dopamine-induced dystonia [4] due to the production of a principal metabolite "levo-3,4-dihydroxyphenylalanine (L-DOPA)" [5]. Apart from this, it is also used as an aphrodisiac [6] as it increases the testosterone levels, it is used as a tonic for male vitality and in treatment for male impotency [7]. *M. pruriens* has shown promising antidiabetic properties and has a potential to manage type 2 diabetes [8]. It also possesses anti-venom, anti-neoplastic, anti-epileptic, and anti-helminthic properties [1]. It is also widely used as a cover crop [9]. It is also been reported to be used as a famine food [10]. L-DOPA is a non-protein amino acid which is a precursor for catecholamine neurotransmitters such as dopamine, epinephrine, and norepinephrine. Apart from these pharmacological properties, L-DOPA released in plants acts as an allelochemical and plays a defensive role against insects [11]. Although the presence of L-DOPA has been reported in all parts of the plant, extraction of L-DOPA from wild populations to satisfy the increasing demand has become difficult due to the limited availability of plants from natural conditions, allergic properties of the pods, poor germination rate, and low seed viability [12]. The

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world market for L-DOPA is 250 tons/year costing $101 billion/year [4]. Thus, an alternative approach to produce L-DOPA is very much required to satisfy its heavy demand in the international dug market and also to conserve the plants from being exploited at the same time [13].

Hence, many *in vitro* studies have been reported on plant regeneration and micropropagation [2,14-17], somatic embryogenesis [18], callus induction [20, 21, 30] and *in vitro* elicitation [19-24]. Huizing *et al.*, 1985 reported the accumulation of L-DOPA in cell suspension cultures [24]. Thereafter, a few works related to the cell suspension cultures have been reported, however, there has been no much work reported with respect to optimization of cultural conditions. Hence, in this study, we have examined callus induction using wide range of plant growth regulators (PGRs) and their combinations and established a cell suspension culture of *M. pruriens*. In addition, extensive and detailed studies have been made on optimizing the cultural conditions for the cell suspension culture such as duration of culture period, inoculum density, media composition, strength of various media and macroelements, ammonium/nitrate ratio, sucrose concentration, carbon sources, hydrogen ion concentration and their effect on the biomass production, and L-DOPA accumulation of the cell suspension cultures.

2. MATERIALS AND METHODS

2.1. Plant Material Collection and *In vitro* Seed Germination

Seeds of five different germplasm were collected from ICAR-Indian Institute of Horticulture, Bengaluru, and initially screened for the superior germplasm with respect to high L-DOPA content. Arka Shubra was selected for the *in vitro* studies because of high L-DOPA content [25]. The seeds were thoroughly washed with running water and kept in a shaker with 1% chilled ascorbic acid for 3 h. Later, they were rinsed and washed with soap water for 15 min followed by distilled water to remove the soap traces. After which, they were surface sterilized with 2% aqueous sodium hypochlorite for 8–10 min followed by rinsing with sterile distilled water thrice for 2 min interval. Then, they were treated with 0.1% (w/v) mercuric chloride for 2–3 min followed by sterile distilled water washing. The seeds were blotted and then inoculated in baby jar bottles with 50 mL modified Murashige and Skoog (MS) medium (HiMedia, India) [26] containing 3% (w/v) sucrose, 0.1 g/L ascorbic acid, 0.5 g/L activated charcoal, 300 mg/L gibberellic acid (GA_3), and solidified by 0.8% (w/v) agar. The final pH of the media was adjusted to 5.7 ± 0.2 prior autoclaving (20 min at 121°C and 15 Pa). Cultures were incubated in the dark chamber which was maintained at 25 ± 1°C. After a week, when plumule and radicle were observed, the germinated cultures were transferred to growth room and maintained at 25 ± 1°C, with a 16 h photoperiod provided with 40 W fluorescent lights. They were continuously subcultured into fresh MS medium supplemented with 0.5 g/L activated charcoal every week to overcome the phenolic exudation and browning of media. After the emergence and development of shoots, they were subcultured every 15 days.

2.2. Induction of Callus

Leaves from *in vitro* raised plantlets were used as explant source, where the margins were excised and the leaf segments were prepared of approximately 0.5 cm^2 size and inoculated on to MS media [26] (3% sucrose, 0.8% agar) supplemented with individual auxins, cytokinins, and their combinations. Auxins such as indole acetic acid, indole butyric acid, naphthalene acetic acid (NAA), picloram (Pic), 2,4-Dichlorophenoxyacetic acid (2,4-D), and cytokinins such as kinetin (KN), thidiazuron (TDZ), 6-Benzylaminopurine (6-BAP), and 2-isopentenyl adenine (2-iP) at different concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 mg/L were used as PGRs. The final pH of the media was adjusted to 5.8 ± 0.2 prior autoclaving (20 min at 121°C and 15 Pa). Cultures were incubated in the growth chamber maintained at 25 ± 1°C, with a 16 h photoperiod provided with 40 W fluorescent lights.

2.3. Establishment of Cell Suspension Culture

Callus with high biomass, high L-DOPA production, and friable nature was selected for cell suspension culture. A 1.0 g of callus was homogenized using a sterilized homogenizer and suspended in 50 mL liquid MS media [26] supplemented with 0.5 mg/L Pic in 250 mL Erlenmeyer flasks. The cultures were continuously agitated at 100 rpm on orbital shaker at 25 ± 1°C, with a 16 h photoperiod provided with 40 W fluorescent lights.

2.4. Optimization of Culture Conditions

A growth kinetic study was performed to analyze the maximum growth and highest L-DOPA production. Initially, the cell suspension cultures were maintained for 6 weeks. The growth and metabolite contents were estimated every week. To determine the optimum inoculum required for higher biomass and metabolite production, different inoculum densities (2, 5, 10, 15, 20, 30, 40, and 60 g/L) were taken and the experiment was carried out. To study the effect of different growth medium and their strength (0.5, 1.0, and 2.0X), different media such as Gamborg's B5 medium [27], Chu's N6 medium [28], Nitsch and Nitsch medium [29], and MS medium [26] were selected. The effect of different macroelements of MS media and their strengths (0, 0.5, 1.0, 1.5, 2.0, and 3.0X) was also studied. To study the effect of nitrogen source, different ammonium/nitrate ratios in different concentrations (0.0/18.8, 7.19/18.8, 14.38/18.8, 21.57/18.8, 28.57/18.80 14.38/0.0, 14.38/9.40, 14.38/28.20, and 14.38/37.60) were taken. To determine the optimum pH, cultures were inoculated in medium with different pH levels (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5). To test the effect of different carbon sources (fructose, glucose, maltose, fructose + glucose [1:1], fructose + maltose [1:1] glucose + maltose, sucrose + glucose [1:1], sucrose + fructose [1:1], and sucrose + maltose [1:1]) and sucrose concentration of 0–10% were taken.

2.5. Determination of Cell Biomass

The callus/cell suspension culture was taken from the test tubes/flasks and the media were removed using a sterile filter paper, and fresh weight (FW) from each tube/flask was recorded. The callus/cell culture was dried in a hot air oven at 35°C for 2 days and their dry weight (DW) was recorded. The dried callus was finely ground and stored at 4°C.

2.6. Extraction and Quantification of L-DOPA using High-performance Liquid Chromatography (HPLC)

A 1.0 g DW of callus was taken and the sample was prepared for HPLC analysis as reported by Rakesh *et al.* (2021) [25] using 0.1 M orthophosphoric acid. The L-DOPA was quantified using reverse phase HPLC system (SHIMADZU) equipped with C-18 column (Sharpsil-U, 250(L) × 4.6 mm ID). The mobile phase was phosphate buffer (pH 2.5) and acetonitrile (80:20, v/v) eluted in an isocratic manner with a flow rate of 0.5 mL/min, column temperature was set at 40°C, and the wavelength of 280 nm was set. The HPLC grade standard L-DOPA (99.3% purity) was obtained from Natural Remedies, Bengaluru, India.

2.7. Statistical Analysis

All the experiments were set up in a randomized design and the results for callus induction were taken from 18 replicates and the results for optimization were taken from triplicates. The HPLC quantification of the callus was done in triplicates. The data of all the experiments were statistically analyzed using one-way ANOVA and the significance by Duncan's multiple range test (DMRT) using IBM SPSS software version 21.0. All the results are expressed as Mean ±SE.

3. RESULTS AND DISCUSSION

3.1. Effect of PGRs on Callus Induction and L-DOPA Production

To study the effect of various plant hormones on callus induction, seeds were germinated on modified MS medium, and the *in vitro* grown leaves were selected as an explant. The various stages of *in vitro* seed germination and plant growth are shown in Figure 1. Leaf was inoculated on semi-solid MS medium supplemented with different concentrations of individual auxins, cytokinins, and their combinations. The results of various hormones along with its biomass and metabolite production are represented in Tables 1 and 2. Out of all the auxins used, Pic was found to be the best while other auxins failed to induce callus. However higher concentration of NAA-induced callus. Out of the cytokinins, 2-iP and BAP favored callus induction. KN and TDZ failed to induce callus. However, when these hormones were combined with Pic, all combinations induced callus and L-DOPA was accumulated. The callus obtained from different hormonal combinations is shown in Figure 2a. The highest biomass was recorded in 0.5 mg/L Pic (3.39 g average FW) and the highest L-DOPA content was observed in 5.0 mg/L 2-iP (7.92 mg/g DW). However, the highest productivity (dry wt. × L-DOPA content) was seen in 0.5 mg/L pic. Hence, cell suspension culture was established using this hormonal concentration, where 1.0 g friable callus was inoculated in liquid MS medium and cell culture was established as shown in Figure 2b, the cells harvested from the cultures (fresh weight) are represented in Figure 2c. Janarthanam and Sumathi (2015) also reported in their study that lower concentrations of NAA and higher concentrations of BAP failed to induce callus from cotyledonary leaf and hypocotyl of *M. pruriens*. They were successful in inducing callus from 2,4-D and 2-iP concentrations [30]. In a study by Lahiri and Mukhopadhyay (2012), they reported that the rate of callus proliferation was less and the callus was hard, compact when the medium was supplemented with NAA and 2,4-D. However, on the other hand, the callus induced from BAP and 2-iP was greenish and friable [31]. These results are in concordance with our results reported here. In a study, it was reported that leaf of *M. pruriens* serves as the best explant for callus induction and thus the callus obtained from various concentrations of BAP and 2,4-D accumulated L-DOPA in the range of 3.4–6.3 mg/g DW which is in absolute agreement with our results reported here [32].

3.2. Growth Kinetic Study

Time course accumulation of biomass and L-DOPA is shown in Table 3. The highest biomass was observed at the end of the 6th week (135.70 g/L FW), while the highest metabolite content was

Figure 1: Different stages of *in vitro* seed germination and growth of *Mucuna pruriens* (L.)DC. on modified Murashige and Skoog medium.

Table 1: Effect of individual PGRs on callus induction and L-DOPA quantification from *in vitro* leaf of *Mucuna pruriens* (L.) DC. as an explant.

S. No.	PGRs		Concentration (mg/L)	Percentage of callus induction (%)	Nature of callus	FW (g)	DW (g)	Conc. of L-DOPA (mg/g DW)
1.	Auxins	IAA	0.1	-	-	-	-	-
2.			0.5	-	-	-	-	-
3.			1.0	-	-	-	-	-
4.			2.0	-	-	-	-	-
5.			5.0	-	-	-	-	-
6.			10.0	-	-	-	-	-
7.		IBA	0.1	-	-	-	-	-
8.			0.5	-	-	-	-	-
9.			1.0	-	-	-	-	-
10.			2.0	-	-	-	-	-
11.			5.0	-	-	-	-	-
12.			10.0	-	-	-	-	-
13.		Pic	0.1	90	Brown, friable	0.476±0.02e	0.063±0.003g	3.00±0.14d
14.			0.5	100	Golden brown, friable	3.39±0.12a	0.244±0.0023a	3.70±0.14d
15.			1.0	100	Yellowish-white friable	2.97±0.07b	0.145±0.043ab	3.06±0.18d
16.			2.0	95	Yellowish-brown, friable	3.11±0.16a	0.154±0.034ab	2.11±0.19de
17.			5.0	95	Dark brown, friable	1.61±0.05c	0.109±0.06cd	3.60±0.21d
18.			10.0	90	Dark brown, compact	1.38±0.03cd	0.108±0.0034cd	4.56±0.2c
19.		2,4-D	0.1	-	-	-	-	-
20.			0.5	-	-	-	-	-
21.			1.0	46.1	Greenish-white, friable	0.63±0.00d	0.053±0.0043gh	4.68±0.16c
22.			2.0	-	-	-	-	-
23.			5.0	-	-	-	-	-
24.			10.0	-	-	-	-	-
25.		NAA	0.1	-	-	-	-	-
26.			0.5	-	-	-	-	-
27.			1.0	-	-	-	-	-
28.			2.0	-	-	-	-	-
29.			5.0	-	-	-	-	-
30.			10.0	-	-	-	-	-
31.	Cytokinins	KN	0.1	-	-	-	-	-
32.			0.5	-	-	-	-	-
33.			1.0	-	-	-	-	-
34.			2.0	-	-	-	-	-
35.			5.0	-	-	-	-	-
36.			10.0	-	-	-	-	-
37.		BAP	0.1	-	-	-	-	-
38.			0.5	61.5	Green, compact	0.78±0.01d	0.096±0.0034c	5.97±0.05bc
39.			1.0	68.8	Green, compact	0.85±0.07d	0.076±0.006ef	4.07±0.01c
40.			2.0	33.3	Greenish-white, compact	1.15±0.03cd	0.126±0.034bc	6.93±0.03a
41.			5.0	38.4	Golden-green compact	1.90±0.07c	0.188±0.027ab	4.81±0.04c
42.			10.0	22.2	Whitish compact	0.68±0.01d	0.062±0.008g	1.91±0.01e
43.		2-iP	0.1	-	-	-	-	-
44.			0.5	46.1	Greenish-golden friable	1.26±0.07cd	0.095±0.0012d	6.73±0.28a
45.			1.0	76.9	Greenish friable	1.48±0.03cd	0.098±0.0018d	7.21±0.08a

(Contd...)

Table 1: *(Continued)*.

S. No.	PGRs	Concentration (mg/L)	Percentage of callus induction (%)	Nature of callus	FW (g)	DW (g)	Conc. of L-DOPA (mg/g DW)
46.		2.0	100	Greenish friable	0.89±0.01d	0.044±0.0084h	5.42±0.33bc
47.		5.0	46.1	Greenish-white compact	1.57±0.04c	0.065±0.004g	7.92±0.10a
48.		10.0	77.7	Greenish compact	1.22±0.05cd	0.088±0.006e	6.27±0.18ab
49.	TDZ	0.1	-	-	-	-	-
50.		0.5	-	-	-	-	-
51.		1.0	-	-	-	-	-
52.		2.0	-	Greenish, compact	1.87±0.03c	0.102±0.06d	2.94±0.25de
53.		5.0	-	-	-	-	-
54.		10.0	-	-	-	-	-

Data in the table represent the mean values±SE of nine replicates. Each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test. PGRs: Plant growth regulators, FW: Fresh weight, DW: Dry weight, IAA: Indole acetic acid, IBA: Indole butyric acid, NAA: Naphthalene acetic acid, Pic: Picloram, 2,4-D: 2,4-Dichlorophenoxyacetic acid, KN: Kinetin, TDZ: Thidiazuron, BAP: Benzylaminopurine, 2-iP: 2-isopentenyl adenine, L-DOPA: Levo-3,4-dihydroxyphenylalanine.

Table 2: Effect of combination of PGRs on callus induction and L-DOPA quantification from *in vitro* leaf of *Mucuna pruriens* (L.) DC. as an explant.

S. No.	Concentration of PGRs in combinations (mg/L)		Percentage of callus induction (%)	Nature of callus	FW (g)	DW (g)	Conc. of L-DOPA (mg/g DW)
1.	Pic (0.5) +	KN (0.5)	77.7	Golden-white friable	1.43±0.05cd	0.125±0.003bc	3.49±0.17c
2.		KN (1.0)	100	White friable	1.75±0.06cd	0.127±0.004bc	4.56±0.05b
3.		KN (2.0)	100	Whitish callus	1.33±0.05cd	0.126±0.005bc	4.76±0.02b
4.		BAP (0.5)	100	Greenish compact	0.62±0.01d	0.073±0.007f	1.83±0.04e
5.		BAP (1.0)	88.8	Greenish compact	1.07±0.06cd	0.110±0.03cd	3.87±0.02c
6.		BAP (2.0)	33.3	Greenish compact	1.47±0.02cd	0.142±0.007b	4.96±0.19b
7.		2-iP (0.5)	100	Whitish-brown compact	1.47±0.06cd	0.146±0.008ab	4.61±0.05b
8.		2-iP (1.0)	100	Whitish-brown compact	1.52±0.09cd	0.157±0.006ab	3.44±0.08c
9.		2-iP (2.0)	100	Whitish compact	2.38±0.02b	0.181±0.012ab	3.12±0.01c
10.		TDZ (0.5)	100	Greenish compact	1.11±0.07d	0.110±0.018cd	4.10±0.03bc
11.		TDZ (1.0)	77.7	Greenish compact	0.91±0.01d	0.094±0.0084e	3.29±0.01c
12.		TDZ (2.0)	100	Greenish-white compact	0.88±0.06d	0.106±0.005cd	2.06±0.07de
13.	Pic (1.0) +	KN (0.5)	100	Golden-white compact	1.66±0.05cd	0.133±0.07bc	2.81±0.05d
14.		KN (1.0)	100	Golden-white compact	1.71±0.01cd	0.150±0.003ab	2.59±0.03d
15.		KN (2.0)	100	Golden-white compact	1.63±0.05cd	0.139±0.07b	2.37±0.04d
16.		BAP (0.5)	100	Greenish friable	1.20±0.09d	0.123±0.005c	4.98±0.008b
17.		BAP (1.0)	100	Greenish compact	0.86±0.01d	0.098±0.034d	5.31±0.01a
18.		BAP (2.0)	100	Greenish-brown compact	0.84±0.01d	0.089±0.03de	4.72±0.86b
19.		2-iP (0.5)	100	Golden-white compact	1.91±0.045cd	0.173±0.045ab	6.95±0.04a
20.		2-iP (1.0)	55.5	Golden-brown compact	1.46±0.067cd	0.132±0.049bc	4.00±0.13bc
21.		2-iP (2.0)	100	Golden-brown compact	1.09±0.035cd	0.111±0.0012cd	5.85±0.09a
22.		TDZ (0.5)	100	Greenish-white compact	0.53±0.006e	0.069±0.043f	2.31±0.02d
23.		TDZ (1.0)	100	Greenish compact	0.88±0.003d	0.104±0.034cd	4.66±0.05b
24.		TDZ (2.0)	100	Greenish compact	0.89±0.05d	0.106±0.06cd	5.04±0.02ab
25.	Pic (2.0) +	KN (0.5)	100	Golden-yellow compact	1.64±0.034c	0.098±0.0034cd	1.67±0.04e
26.		KN (1.0)	100	Yellowish-brown compact	2.36±0.05bc	0.118±0.027c	3.68±0.01c
27.		KN (2.0)	100	Golden-yellow compact	2.67±0.01b	0.127±0.0034bc	4.14±0.01bc
28.		BAP (0.5)	66.6	Whitish friable	1.46±0.03cd	0.110±0.027cd	3.05±0.02cd
29.		BAP (1.0)	44.4	Golden-white compact	1.05±0.045d	0.098±0.08cd	2.83±0.01d
30.		BAP (2.0)	100	Whitish compact	1.05±0.049d	0.094±0.06e	5.08±0.05ab

(Contd...)

Table 2: *(Continued)*.

S. No.	Concentration of PGRs in combinations (mg/L)	Percentage of callus induction (%)	Nature of callus	FW (g)	DW (g)	Conc. of L-DOPA (mg/g DW)
31.	2-iP (0.5)	100	Brownish compact	2.99±0.12b	0.132±0.012bc	6.28±0.01a
32.	2-iP (1.0)	55.5	Brownish-white compact	1.94±0.045c	0.110±0.086cd	3.67±0.02c
33.	2-iP (2.0)	100	Golden-brown compact	2.42±0.09bc	0.112±0.005cd	2.05±0.01de
34.	TDZ (0.5)	100	Whitish compact	0.89±0.003d	0.094±0.005de	2.58±0.01d
35.	TDZ (1.0)	100	Whitish compact	0.89±0.023d	0.097±0.0056cd	4.80±0.02b
36.	TDZ (2.0)	100	Whitish compact	0.65±0.043d	0.072±0.0086f	3.16±0.01c
37.	2,4-D (1.0) + KN (0.5)	100	Golden-yellow compact	1.20±0.034cd	0.102±0.02cd	1.35±0.006e
38.	KN (1.0)	100	Golden-brown compact	1.33±0.06cd	0.107±0.065cd	1.06±0.05ef
39.	KN (2.0)	100	Golden-brown compact	2.24±0.34bc	0.152±0.005ab	4.54±0.01b
40.	BAP (0.5)	80	Whitish friable	1.35±0.027cd	0.130±0.07bc	0.88±0.01f
41.	BAP (1.0)	100	Whitish-brown compact	1.21±0.08cd	0.127±0.03bc	1.04±0.01ef
42.	BAP (2.0)	100	Whitish compact	1.03±0.06cd	0.102±0.07cd	1.63±0.03e
43.	2-iP (0.5)	100	Golden-yellow friable	2.87±0.12b	0.152±0.005ab	3.36±0.06c
44.	2-iP (1.0)	55.5	Golden-white friable	2.01±0.18bc	0.138±0.034b	2.87±0.06d
45.	2-iP (2.0)	100	Golden-brown compact	2.14±0.084bc	0.150±0.07ab	3.62±0.008c
46.	TDZ (0.5)	100	Whitish compact	0.73±0.04d	0.082±0.003ef	4.69±0.005b
47.	TDZ (1.0)	44.4	Golden-whitish compact	1.09±0.06cd	0.106±0.006cd	4.84±0.001b
48.	TDZ (2.0)	33.3	Whitish compact	0.99±0.05d	0.102±0.004cd	4.56±0.17b

Data in the table represent the mean values±SE of nine replicates. Each experiment was repeated twice. Mean values with common letters are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test. PGRs: Plant growth regulators, FW: Fresh weight, DW: Dry weight, IAA: Indole acetic acid, IBA: Indole butyric acid, NAA: Naphthalene acetic acid, Pic: Picloram, 2,4-D: 2,4-Dichlorophenoxyacetic acid, KN: Kinetin, TDZ: Thidiazuron, BAP: Benzylaminopurine, 2-iP: 2-isopentenyl adenine, L-DOPA: Levo-3,4-dihydroxyphenylalanine.

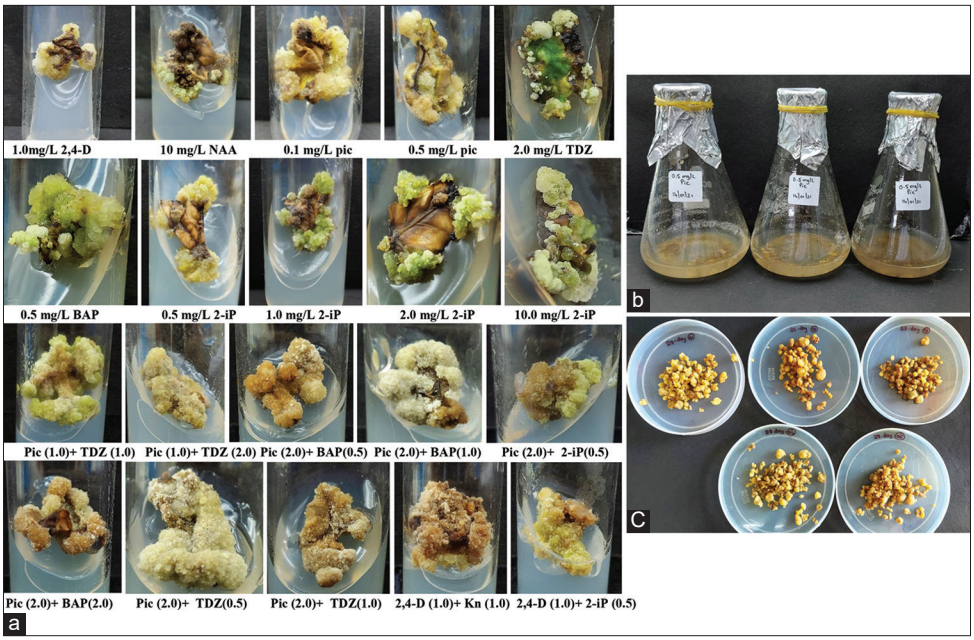

Figure 2: (a) Callus induced from leaf as an explant on semi-solid MS medium supplemented with different hormones and their combinations; (b) Establishment of cell suspension culture in liquid MS medium with 0.5 mg/L picloram; (c) Fresh weight of cell cultures induced from liquid MS medium + 0.5 mg/L picloram.

accumulated at the end of the 3rd week (5.90 mg/g DW), and later, the metabolite content decreased as the growth period increased. The highest productivity was achieved at the end of the 4th week (28 days); hence, all the experiments were carried up to 28 days of growth period. In a study by Janarthanam and Sumathi (2015), they reported the highest biomass at the end of 24th day after which the biomass started to decline [30]. Stevioside production from *Stevia rebaudiana* (Bertoni) cultures was reported to be high in between 7 and 14 days growth period and slightly decreased on the 21st day after which it drastically reduced. However, the biomass production was high from the 21st to 28th day [33]. A similar trend has been observed in our study reported here. The growth kinetic studies on *Withania somnifera* cell

cultures revealed that at the end of the 4th week, they observed highest biomass and withanolide A production [34].

3.3. Effect of Different Media and their Strength

The effect of various strengths of different media is represented in Figure 3. The highest biomass was obtained in 1.0X MS medium (126.87 g/L FW) followed by 1X Gamborg's B5 medium (119.2 g/L FW) when 20 g/L FW friable callus was inoculated into liquid medium. The highest metabolite was accumulated in 0.5X B5 medium (4.27 mg/g DW) followed by 1X MS medium (3.77 mg/g DW). The least biomass production (30.87 g/L FW) and metabolite accumulation (0.29 mg/g DW) were found to be in 2.0X Chu's N6 medium and 0.5X Nitsch and Nitsch medium, respectively. In terms of productivity, MS medium was found to be superior to the other medium followed by Gamborg's B5 medium. In a study by Karwasara and Dixit (2013), they found out that MS medium was favorable both in terms of biomass and camptothecin production followed by B5 medium from cell cultures of *Nothapodytes nimmoniana* (J. Grah.) [35]. In another study, 1X MS medium was found to be the best for higher biomass and withanolide A production from *W. somnifera* cultures [34].

3.4. Effect of Macroelements and their Strength

The effect of different macroelements in MS medium and their concentration on biomass and metabolite production is represented in Figure 4. The highest biomass (266.3 g/L FW) was seen when 1.5X magnesium sulfate (MS) was added in the medium. 1.0X ammonium nitrate (AN), 1.5X potassium nitrate (PN), 3.0X calcium chloride (CC), and 1.5X potassium phosphate (PP) also resulted in higher biomass production. The least biomass (33.5 g/L FW) was seen in 3.0X AN. Higher concentrations of AN reduced the callus growth. The higher metabolite content (5.06 mg/g DW) was observed when 2.0X CC was added in the medium. Increase in calcium concentration favored metabolite production. 1.5X AN, 1.0X PN, 1.0X MS, and 1.0X PP also resulted in higher metabolite content. Least L-DOPA production (0.63 mg/g DW) was seen in 0X AN. 0X PN, 0X CC, 0X MS, and 0X PP also resulted in low metabolite production. In the cell cultures of *S. rebaudiana*, it was observed that 1X AN, 3X PN, 3X MS, and 3X PP resulted in a higher biomass for a 21-day growth period [36]. Similar results are also observed in our study reported here. In a study on *W. somnifera* cell cultures, 0.5X AN, 2.0X PN, 1.0X CC, 1.5X MS, 2.0X PP, and 0X AN, 2.0X PN, 2.0X CC, 1.0X MS, and 2.0X PP resulted in a higher biomass and metabolite production, respectively [37]. Calcium plays an important role in both, cell growth and metabolite formation. Higher concentrations showed an increase in biomass and rosmarinic acid production in *Anchusa officinalis* cultures. It was also found that the higher levels of phosphate suppress the secondary metabolite formation in cell cultures of *Catharanthus roseus*, *Nicotiana tabacum*, *Peganum harmala* [38], and camptothecin production in *N. nimmoniana* [35]. Similar trend has been observed in our studies where increase in concentration of calcium increases both the biomass and L-DOPA production while increase in phosphate concentration decreases the metabolite production.

Table 3: Biomass and L-DOPA production at different time period interval.

Time period	Fresh wt. (g/L)	Dry wt. (g/L)	L-DOPA conc. (mg/g DW)	Productivity (Conc. × Dry wt.)
Week-1	35.36±0.51d	2.32±0.10e	3.80±0.11b	0.44
Week-2	47.56±0.69c	3.72±0.15d	5.32±0.18a	0.99
Week-3	71.64±0.71b	5.28±0.16c	5.90±0.14a	1.56
Week-4	124.16±0.28a	9.20±0.12b	3.51±0.20b	1.61
Week-5	133.28±0.50a	10.48±0.25a	2.79±0.08c	1.46
Week-6	135.70±0.27a	10.65±0.30a	2.59±0.06c	1.38

Data in the table represent the mean values±SE of 5 replicates. Mean values with common letters are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test. L-DOPA: Levo-3,4-dihydroxyphenylalanine.

3.5. Effect of Ammonium/Nitrate Ratio

The effect of different concentrations of ammonium and nitrate ions in the MS medium is tabulated and represented in Table 4. The highest biomass (131.4 g/L FW) and highest L-DOPA production (3.44 mg/g DW) were observed when the ammonium/nitrate ratio was 21.57/18.8 mM. Higher concentrations of ammonium ions in the media gradually increased the callus growth and metabolite content while the nitrate ions in the media neither increased the biomass nor the metabolite as compared to control. It was found that 15 mM nitrate ion concentration was favorable for both cell growth and rosmarinic acid production in *A. officinalis* cultures [38]. In a study, it was observed

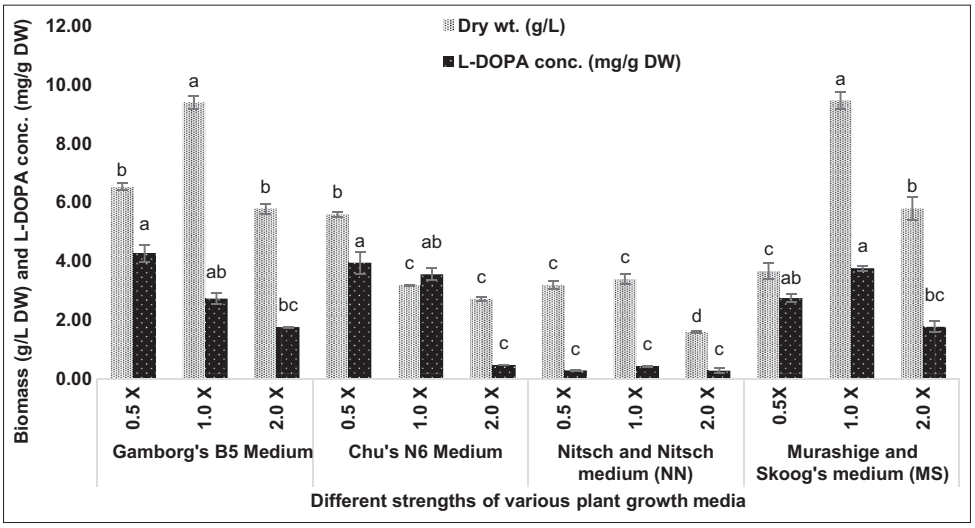

Figure 3: Effect of different media and their strengths in the biomass production and L-DOPA accumulation. Data in the figure represent the mean values of triplicates. Bar represents the standard error. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

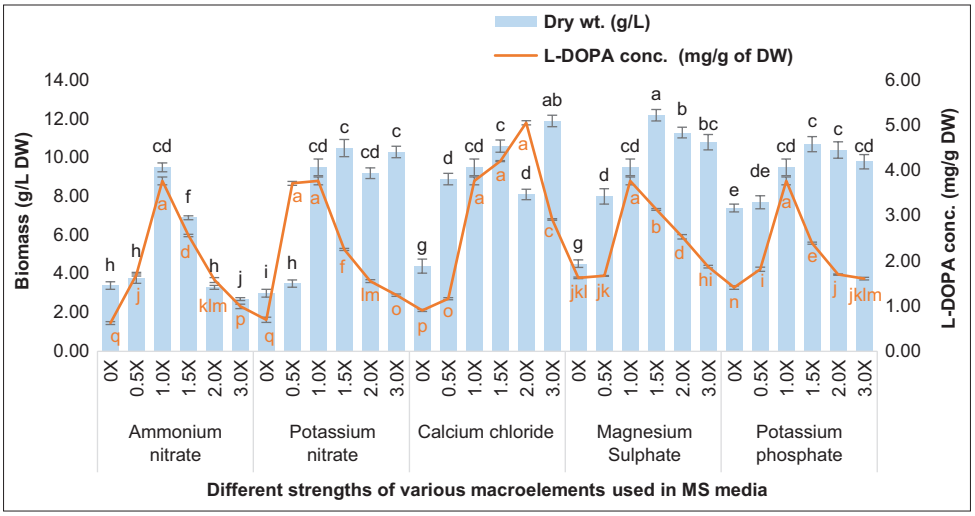

Figure 4: Effect of different concentrations of macroelements in the MS medium on biomass and L-DOPA accumulation. Data in the figure represent the mean values of triplicates. Bar represents the standard error. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Table 4: Biomass and L-DOPA production at different ammonium/nitrate ratios in the Murashige and Skoog medium.

Ammonium/nitrate ratio in mM	Fresh wt. (g/L)	Dry wt. (g/L)	L-DOPA conc. (mg/g DW)
0.0/18.8	50.6±1.2c	2.80±0.20c	0.61±0.02c
7.19/18.8	76.8±1.6b	7.70±0.53b	0.96±0.03c
14.38/18.8	98.6±2.4b	5.90±0.35b	2.16±0.01b
21.57/18.8	131.4±2.7a	10.20±0.64a	3.44±0.02a
28.57/18.80	92.1±1.5b	7.20±0.40b	1.18±0.02c
14.38/0.0	39.3±0.9c	3.20±0.40c	0.56±0.02c
14.38/9.40	48.7±1.1c	3.10±0.30c	0.72±0.02c
14.38/28.20	91.5±1.3b	6.80±0.38b	2.40±0.05b
14.38/37.60	46±0.9c	3.00±0.20c	1.21±0.04c
Control (20.0/18.8)	121.8±2.3a	9.50±0.30a	3.20±0.06a

NH_4^+/NO_3^- = NH4Cl/KNO3 (mM/mM). Data in the table represent the mean values±SE of triplicates. Mean values with common letters are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test. L-DOPA: Levo-3,4-dihydroxyphenylalanine.

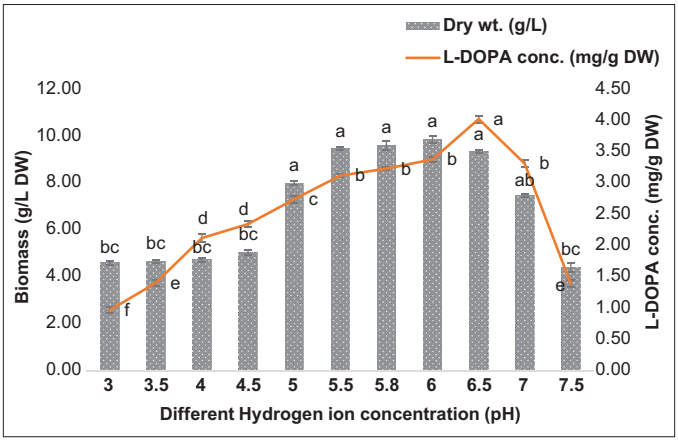

Figure 5: Effect of hydrogen ion concentration (pH) in the MS medium on biomass and L-DOPA accumulation. Data in the figure represent the mean values of triplicates. Bar represents the standard error. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

that 7.19/18.80 and 14.38/37.60 mM ammonium/nitrate ratio resulted in a higher biomass and withanolide A production, respectively, in the cell cultures of *W. somnifera* [37]. In the cultures of *Gymnema sylvestre*, it was noted that 7.19/18.80 resulted in both higher biomass and gymnemic acid production [39].

3.6. Effect of pH

The effect of hydrogen ions in the MS medium on biomass production and L-DOPA accumulation is represented in Figure 5. The highest biomass (129.47 g/L FW) and L-DOPA content (4.02 mg/g DW) were seen at pH 6.0 and 6.5, respectively. The least biomass (61.13 g/L FW) and L-DOPA production (0.97 mg/g DW) were seen in pH 7.5 and 3.0, respectively. Increase in the pH of the medium above 6.0 drastically reduced the cell growth, however, there was a slight increase in the metabolite production. In terms of productivity, pH 6.5 shows best result (37.55). However, pH in the range of 5.5–6.5 favors both the cell growth and metabolite production and remains the most desired hydrogen ion concentration for the medium. Similar results were observed in a study by Sundaram and Anupama, 2013, where they obtained a FW of 181 g/L when the pH of MS medium was maintained at 6.15 [20]. Huizing *et al.*, (1985), reported that the growth of *M. pruriens* callus was better at a pH range of 5.5–6.0 [24]. Inamdar *et al.*, (2013), reported that in *M. monosperma* cultures, the maximum L-DOPA yield was reported from pH 4.2 to 5.8; this is due to the pH optima of the enzyme tyrosinase involved in conversion of tyrosine to L-DOPA whose pH optima is at 5.4 [21]. It was reported that pH between 5.5 and 6.0 resulted in a higher biomass and withanolide A production in *W. somnifera* cell cultures [34] while pH 4.5 was reported to be favorable for higher biomass and bacoside production in *B. monnieri* cell cultures [40].

3.7. Effect of Inoculum Density

The effect of inoculum density on biomass and L-DOPA production is shown in Figure 6. The highest biomass (222.2 g/L

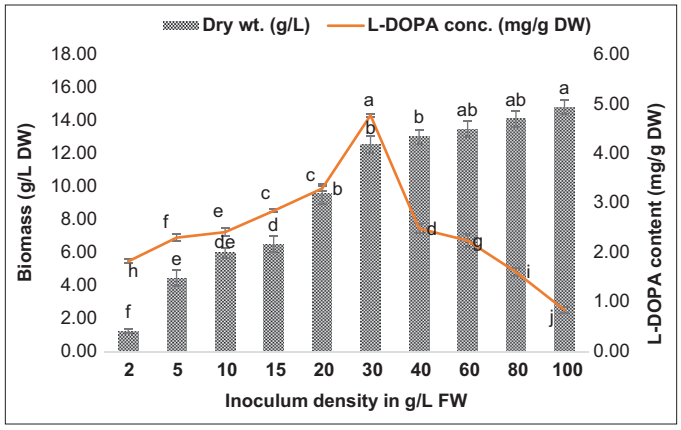

Figure 6: Effect of inoculum density on biomass and L-DOPA production. Data in the figure represent the mean values of triplicates. Bar represents the standard error. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

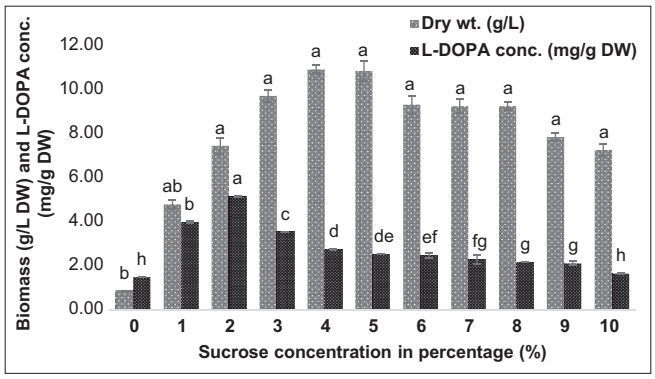

Figure 7: Effect of sucrose concentration in MS medium on biomass and L-DOPA production. Data in the figure represent the mean values of triplicates. Bar represents the standard error. Mean values with common letters are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

FW) was observed when 100 g/L of callus was inoculated into cell suspension culture. Higher L-DOPA production (4.79 mg/g DW) was seen when 30 g/L was inoculated into the medium. However, the highest fold increase (14.16-fold higher than the inoculum) in biomass was observed when 5.0 g/L was added followed by 10.0 g/L (11.5-fold higher). As the inoculum density increased, the increase in biomass fold and L-DOPA concentration was highly reduced. This can be attributed to the depletion of the nutrients and competition in the medium. The least biomass (19.07 g/L FW) and L-DOPA production (0.85 mg/g DW) were seen in 2.0 and 100g/L inoculum density, respectively. In terms of productivity, 30 g/L of the inoculum gave the best results (60.34) followed by 20g/L (32.60). In a study, it was noted that increasing the inoculum density up to 100 g/L FW increased the ajmalicine production while it did not affect much the catharanthine production in the cell cultures of *Catharanthus roseus* [41]. While, reported that 20 g/L FW of inoculum density showed a faster growth response in the *S. rebaudiana* cell suspension cultures [36]. In another study, 10 g/L FW resulted in a higher biomass and withanolide A production from *W. somnifera* cell cultures [34].

Table 5: Biomass and L-DOPA production for different carbon sources supplemented in the Murashige and Skoog medium.

S. No.	Carbon source	Fresh wt. (g/L)	Dry wt. (g/L)	L-DOPA conc. (mg/g DW)
1.	Glucose	183.4±2.4a	11.0±0.52a	1.78±0.07f
2.	Fructose	99.5±1.2bc	6.40±0.41bc	2.49±0.06cd
3.	Maltose	101.1±1.6bc	6.73±0.48abc	2.35±0.05d
4.	Sucrose	125.60±1.2ab	9.80±0.41ab	3.56±0.01a
5.	Glucose+Fructose	87.6±1.5bc	5.60±0.22cd	2.93±0.06b
6.	Glucose+Maltose	115.1±1.2b	8.40±0.47abc	2.55±0.05c
7.	Fructose+Maltose	88.5±1.4bc	5.93±0.35cd	3.42±0.04a
8.	Sucrose+Glucose	85.9±0.8bc	5.67±0.39cd	1.45±0.07g
9.	Sucrose+Fructose	33.4±0.4c	2.62±0.46d	0.51±0.08h
10.	Sucrose+Maltose	144.8±1.6ab	10.93±0.18a	2.03±0.06e

Data in the table represent the mean values±SE of triplicates. Mean values with common letters are not significantly different at $P \leq 0.05$, according to Duncan's multiple range test. L-DOPA: Levo-3,4-dihydroxyphenylalanine.

3.8. Effect of Sucrose Concentrations

The effect of sucrose in the MS medium on biomass and L-DOPA production is represented in Figure 7. The highest biomass (125.6 g/L FW) and highest L-DOPA (5.17 mg/g DW) production were seen when 3% and 2.0% sucrose were added in the medium, respectively. As the sucrose concentration increased in the medium, the biomass reduced gradually and after 6%, it started to saturate showing minimal effect on the biomass. While, on the other hand, low sucrose concentration favored L-DOPA production, when the sucrose concentration increased the metabolite concentration decreased. The least biomass (25.8 g/L FW) and L-DOPA concentration (1.49 mg/g DW) were observed when no sucrose was added in the medium. In terms of productivity, 3% sucrose in the medium gave the best result (38.53) followed by 3% (34.62). Our results are in exact agreement with the study done by Sundaram and Anupama (2013), where they obtained highest biomass of *M. pruriens* cell cultures at 3% sucrose concentration in the medium [20]. In *N. nimmoniana* cultures, the highest biomass and camptothecin production were seen at 3 and 2% sucrose, respectively, for a 27-day growth period which are in concordance to our study reported here [35]. In a study on the cell cultures of *W. somnifera,* it was found out that 4 and 3% of sucrose resulted in a higher biomass and withanolide A production, respectively [34] while 2 and 0% sucrose resulted in a higher biomass and bacoside A production, respectively, in *B. monnieri* cell cultures [40].

3.9. Effect of Different Carbon Sources

Other than sucrose, other carbohydrates were added as a source of carbon in the MS medium, the effect of those compounds on biomass and metabolite production has been represented in Table 5. The highest biomass (183.4 g/L FW) was obtained when 3% glucose was added in the MS medium which resulted in a 1.47-fold higher biomass production than sucrose. When 1.5% sucrose + 1.5% maltose was added in combination, there was a 1.15-fold increase in biomass. Other carbon sources individually or in combination resulted in a lower biomass than sucrose. The highest metabolite content was found in the media with sucrose. All other carbon sources decreased the L-DOPA production significantly. However, 1.5% Fructose + 1.5% Maltose produced L-DOPA which was close to the sucrose. However, the highest productivity (34.86) was observed in sucrose alone, hence, no sugars can replace sucrose in the medium. In a study by De-Eknamkul

and Ellis (1985), they noticed not much difference in the growth and rosmarinic acid production from *A. officinalis* cell suspension cultures when glucose, fructose, and their combination were added into the medium [38]. It was found out that sucrose was the best carbohydrate source for camptothecin production from *N. nimmoniana* cultures out of glucose, fructose, and maltose [35]. Similarly, sucrose was also found to be the best carbohydrate source in the cell cultures of *W. somnifera* for higher biomass and metabolite production [34].

4. CONCLUSION

From the above studies, the optimized protocol can be followed for all the *in vitro* cell suspension cultures to obtain higher biomass and high L-DOPA yield. The optimum condition varies according to the parameters we choose (biomass or L-DOPA production) for the experiment. When both are taken into consideration, 30 g/L FW of callus inoculated in 1.0X liquid MS medium with ammonium/nitrate concentration of 21.57/18.8 mM, 1.5X CC, and 1.5X MS supplemented with 2% sucrose and the pH of medium maintained at 6.5 for a 28-day growth period will give the best results according to this study. However, these are subjective to somaclonal variations and mutations. Hence, care must be taken to observe the callus for its yield and properties at every stage. With this background information, one can enhance the metabolite production using other biotechnological approaches such as precursor feeding, elicitation, and gene manipulation. The author's laboratory is currently investigating the different biotic and abiotic elicitors to enhance the L-DOPA production from cell suspension cultures of *M. pruriens*. Since L-DOPA is present in all parts of the plant, other *in vitro* techniques such as hairy root culture and *in vitro* shoot multiplication can be employed to increase the L-DOPA production naturally in the plant system.

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6. AUTHORS' CONTRIBUTIONS

Rakesh. B: Performing experiment, data analysis, drafting manuscript, and statistical analysis. Praveen. N: Concept and design, critical revision of manuscript, supervision, and final approval.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors Rakesh. B and Praveen. N declare no conflicts of interest.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated or analysed during this study are included in this manuscript. Apart from these, there are no datasets that were generated or analyzed during the study.

11. PUBLISHER'S NOTE

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