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Evaluation of different carbon sources for high frequency callus culture with reduced phenolic secretion in cotton (*Gossypium hirsutum* L.) cv. SVPR-2

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ABSTRACT

An efficient protocol was developed to control excessive phenolic compound secretion during callus culture of cotton. As cotton is naturally rich in phenolic compounds factors influencing the phenolic compound secretion, callus induction and proliferation were optimized for getting high frequency callus culture. Different carbon sources such as fructose, glucose, sucrose and maltose were tested at various concentrations to control phenolic secretion in callus culture. Among them, 3% maltose was found to be the best carbon source for effectively controlling phenolic secretion in callus induction medium. High frequency of callus induction was obtained on MSB_5 medium supplemented with 3% Maltose, 2,4-D (0.90 μM) and Kinetin (4.60 μM) from both cotyledon and hypocotyl explants. The best result of callus induction was obtained with hypocotyl explant (94.90%) followed by cotyledon explant (85.20%). MSB_5 medium supplemented with 2,4-D (0.45 μM) along with 2iP (2.95 μM) gave tremendous proliferation of callus with high percentage of response. Varying degrees of colors and textures of callus were observed under different hormone treatments. The present study offers a solution for controlling phenolic secretion in cotton callus culture by adjusting carbon sources without adding any additives and evaluates the manipulation of plant growth regulators for efficient callus culture of SVPR-2 cotton cultivar.

1. Introduction

Cotton is one of the most important commercial crop plants which is cultivated worldwide with leading production by China followed by India and US. *Gossypium hirsutum* is cultivated over 90% of the agricultural fields for commercial production [24]. The yield and quality of cotton fibers are greatly influenced by various biotic and abiotic factors. These factors not only affect the growth and but also yield of the plant which results in huge economic loss. Genetic engineering has become the widely used method to generate transgenic cotton plants which can grow under adverse conditions. Various methods have been developed worldwide in order to overcome these problems. Creation of transgenic cotton plants is mainly based on efficient *in vitro* regeneration system. Genetic improvement of cotton through conventional breeding is limited by incompatibility barriers and consumes more time to develop varieties with desired characters. *In vitro* culture of cotton is an alternate way to avoid this issue for getting cotton plants with desired agronomic traits within a stipulated time. Generally cotton is recalcitrant for *in vitro* manipulation in which regeneration is somewhat difficult to achieve [28]. Several attempts have been made for getting efficient callus culture and regeneration of cotton plant for different cotton cultivars all over the world [22,33,18,3,31,17]. Various factors such as carbon sources, plant growth regulators, explants, culture conditions, culture vessels and phenolic compound secretion have been shown to positively or adversely affect the cotton callus culture. Among them phenolic compound secretion is the major problem which adversely affect the *in vitro* culture of cotton [8,14]. Addition of a suitable carbon source is required to avoid excessive phenolic secretion in culture medium. Likewise, development of an efficient regeneration

Abbreviations: 2,4-D, 2,4-dicholorophenoxy acetic acid; KIN, kinetin; BAP, 6 benzyl aminopurine; TDZ, thidiazurone; NAA, naphthalene acetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2iP, 2-isopentyl adenine; μM, micromole.

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protocol is a prerequisite to get transgenic lines of local cotton varieties as cotton regeneration is highly genotype dependent. have been made to regenerate cotton plants of local cultivars *in vitro*. Hence developing an efficient regeneration protocol is important to create superior cotton genotypes.

There are only limited reports about the role of carbon sources with reference to phenolic secretion in callus culture of cotton [8,14]. Secretion of phenolic compounds in the medium adversely affects the callus culture and regeneration capability in cotton. Hence to overcome this problem, the present study was carried out to evaluate the effect of carbon sources on phenolic secretion in callus culture of SVPR-2 cotton cultivar.

2. Materials and methods

2.1. Seed material

Seeds of *G. hirsutum* cv. SVPR-2, were obtained from Cotton Research Station, Tamil Nadu Agricultural University, Srivilliputtur, Tamil Nadu, India.

Seeds were delinted with concentrated sulfuric acid and washed under running tap water for 15 min. Subsequently the seeds were washed with Teepol solution and kept under running tap water for 1 h. The seeds were then treated with bavistin (fungicide) in a conical flask and agitated in orbital shaker for 20 min at 60 rpm. The seeds were then washed with sterile distilled water three times. The seeds were then disinfected with 70% ethanol for 50 s followed by 0.1% mercuric chloride for 5 min with intermittent washes in sterile distilled water. The disinfected seeds were germinated aseptically on half-strength MS basal medium [19]. The inoculated seeds were incubated in dark condition at $25 \pm 2\,^{\circ}C$ for 2 days and then transferred to 16:8 h light/dark photoperiod with light supplied by cool white fluorescent lamps (Philips, India) at an intensity of 80 $\mu mol\,m^{-2}\,s^{-1}$.

2.2. Influence of carbon sources on phenolic secretion

Carbon sources such as fructose, glucose, sucrose and maltose were tested at different concentrations (10–50 g/l) to evaluate their influence on controlling phenolic compound secretion and callus forming efficiency. Cotyledon and hypocotyl explants were cultured on MSB_5 medium supplemented with different concentrations of carbons sources. The results were observed after two weeks of culture and the best carbon source was used for further studies.

2.3. Callus induction

Cotyledon and hypocotyl explants were used for callus induction. Explants were excised from seven days old *in vitro* raised seedlings. Cotyledon explants were cut into small squares and hypocotyls were cut into 5 mm length and kept on MS salts with B_5 vitamins medium fortified with various concentrations of auxins and cytokinins. The explants were placed in a 15×90 mm petriplate containing approximately 30 ml medium for callus induction. MS medium supplemented with different concentrations of auxins such as 2,4-D (2,4-Dicholorophenoxy acetic acid), NAA (Naphthalene acetic acid), IBA (Indole-3-butyric acid) and IAA (Indole-3 acetic acid) was initially tested for callus induction. The results were observed after 2 weeks of culture. The best concentration of auxin was kept as standard and tried along with different concentrations of cytokinins like BAP (6 Benzyl aminopurine), 2iP (2-Isopentyl adenine), TDZ (Thidiazurone) and KIN (Kinetin). Cultures were incubated at $25 \pm 2\,^{\circ}C$ under cool fluorescent light with 16/8 h light/ dark cycle for callus induction.

2.4. Callus proliferation

Well developed calli were cut and transferred to MSB_5 medium fortified with different concentrations and combinations of auxins (2,4-D, NAA and IAA) and cytokinins (2iP, TDZ and KIN) for callus proliferation. The results were observed after 2 weeks of culture.

2.5. Statistical analysis

The study was carried out based on completely randomized design with ten replications and each experiment was repeated thrice. Each petridish included 10 explants and the total number of explants per treatment were 100. Control was maintained at every step of analysis. The statistical significance of the data obtained from this study was determined by one-way analysis of variance (SPSS version 17). The mean values were compared by Duncan's multiple range test ($p < 0.05$) and the percentage of response was scored on the basis of DMRT analysis. The percentage of phenolic secretion was calculated as

$$\%\ \text{of phenolic secretion} = \frac{\text{number of explants showing phenolic secretion}}{\text{total number of explants}} \times 100$$

3. Results

3.1. Development of callus induction medium

Cotyledon and hypocotyl explants excised from seven days old *in vitro* raised seedlings were used for callus induction study. Both explants formed callus under various hormone treatments. Hypocotyl explants showed quick response within 5–7 days whereas cotyledon showed slow response in callus induction under all hormonal combinations tested. The quality of the callus was considered to be good on the basis of size, color and texture.

3.2. Influence of carbon sources on phenolic secretion in callus culture

Among the different concentrations of carbon sources tested, 3% maltose was found to be the best carbon source for efficient callus culture followed by glucose. Maltose showed a minor impact on tissue browning both at lower and higher concentrations. On the other hand, MSB_5 medium supplemented with glucose at both lower and higher concentrations exhibited increased secretion of phenolic compounds. In present study, phenolic secretion was comparatively higher in cotyledons than hypocotyl explants. Moreover secretion of phenolic compounds was quite increasing both hypocotyl and cotyledon incorporated with fructose, glucose and sucrose. But maltose was found to play a significant role in controlling phenolic secretion from both explants. However, maltose also showed low amount of phenolic secretion below and above 3%, specifically in cotyledon inoculated medium. Meanwhile, fructose showed poor callusing response and increased phenolic secretion at all the concentrations tested. These results are in accordance with the earlier reports [8,11]. Hence fructose was found to play no significant role in callus culture of SVPR-2 cotton cultivar. Sucrose upto 1% showed optimum growth of callus tissues with considerable amount of phenolic secretion whereas browning of medium was observed with 3% sucrose due to high secretion of phenolic compounds (Figs. 1–3). As a result the callus growth was arrested beyond a particular stage. In contrary, 3% maltose was found to be efficient in controlling phenolic secretion and so it was used for further studies. Extracellular hydrolysis of maltose takes place at a slower rate when compared to sucrose and it is taken up and hydrolysed more slowly.

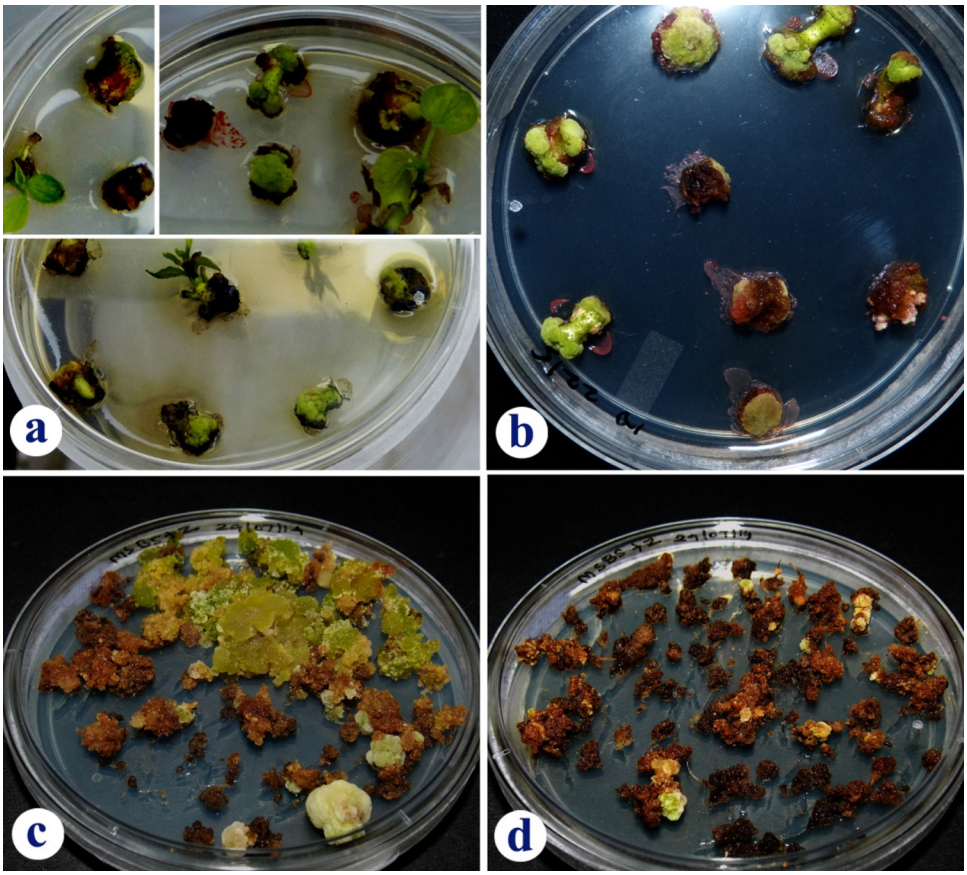

Fig. 1. Effect of carbon sources on Phenolic secretion in callus culture. (a) Phenolic secretion in callus induction medium supplemented with Sucrose, (b) Phenolic secretion in callus induction medium supplemented with Glucose, (c) Browning of callus, (d) Death of callus.

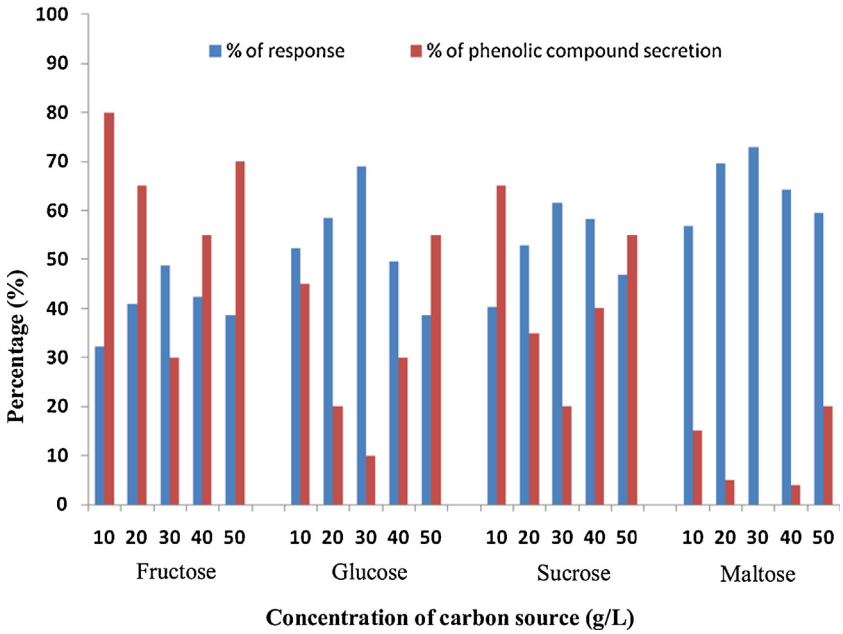

Fig. 2. Effect of different carbon sources on phenolic secretion in cotyledon explants. Cotyledon explants cultured on MSB_5 medium supplemented with different carbon sources (10–50 g/L). Data were collected after 2 weeks of culture.

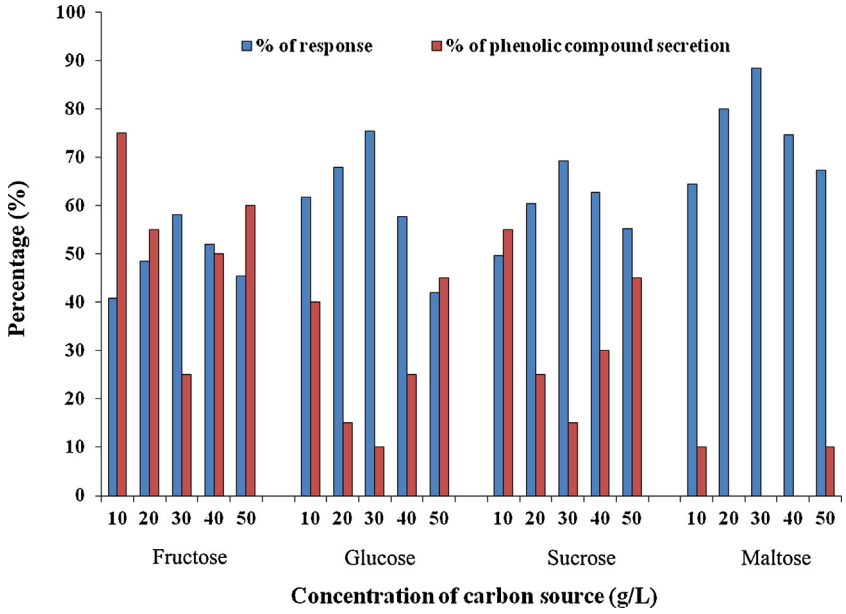

Fig. 3. Effect of different carbon sources on phenolic secretion in hypocotyl explants. Hypocotyl explants cultured on MSB_5 medium supplemented with different carbon sources (10–50 g/L). Data were collected after 2 weeks of culture.

This activity can be the major reason for the ability of maltose in controlling phenolic secretion into culture medium.

3.3. Influence of plant growth regulators on callus induction

Callus formation was noticed after two weeks of culture on MSB_5 medium supplemented with different concentrations and combinations of auxins and cytokinins. Plant growth regulators such as 2,4-D, IAA, NAA, IBA, BA, kinetin, 2iP and TDZ were used for evaluating their influence on callus induction from both hypocotyl and cotyledon explants. Among different auxins tested, 2,4-D (0.90 μM) followed by IBA (8.05 μM) resulted high frequency of callus induction (Table 1). Increasing the concentrations of NAA and IAA beyond 6.97 μM induced root formation in cotyledon explants and poor callus formation in hypocotyl explants. Among different cytokinins tested, KIN (4.60 μM) followed by 2iP (1.96 μM) showed optimum callus induction (Table 2). Among different combinations of auxins and cytokinins tested, 2,4-D

Table 1
Influence of auxins on efficient callus induction.

Plant growth regulators (μM/L)	Cotyledon		Hypocotyl	
	Percentage of response	Nature of callus	Percentage of response	Nature of callus
Control	–	–	–	–
IAA				
2.85	24.8 ± 0.13^{c}	GF	29.2 ± 0.20^{c}	GF
5.71	**34.1 ± 0.20^{a}**	**YGF**	**41.6 ± 0.24^{a}**	**YGF**
8.56	29.6 ± 0.16^{b}	GC	34.5 ± 0.22^{b}	GC
11.42	19.7 ± 0.15^{d}	GC	24.4 ± 0.18^{d}	GC
14.27	14.7 ± 0.15^{e}	BYC	21.8 ± 0.20^{e}	GYC
IBA				
2.46	24.5 ± 0.20^{c}	GC	30.2 ± 0.24^{c}	GC
4.90	29.5 ± 0.22^{b}	GF	38.7 ± 0.20^{b}	GF
7.36	**44.6 ± 0.24^{a}**	**GF**	**49.4 ± 0.26^{a}**	**YGF**
9.80	19.5 ± 0.20^{d}	GC	24.9 ± 0.22^{d}	GC
12.26	9.4 ± 0.26^{e}	GC	15.6 ± 0.20^{e}	GC
NAA				
2.68	21.9 ± 0.27^{d}	GC	29.2 ± 0.20^{d}	GC
5.37	29.7 ± 0.26^{b}	GF	38.6 ± 0.24^{b}	YGC
8.05	**39.5 ± 0.22^{a}**	**YGF**	**46.4 ± 0.20^{a}**	**GF**
10.74	24.5 ± 0.22^{c}	YGC	36.8 ± 0.22^{c}	YGC
13.42	14.7 ± 0.26^{e}	YGC	23.5 ± 0.20^{e}	YGC
2,4-D				
0.45	39.8 ± 0.20^{b}	GF	48.2 ± 0.22^{b}	GF
0.90	**55.2 ± 0.24^{a}**	**YF**	**61.9 ± 0.20^{a}**	**GYF**
1.36	35.4 ± 0.26^{c}	GF	41.6 ± 0.22^{c}	GF
1.81	21.8 ± 0.20^{d}	GC	36.4 ± 0.18^{d}	GF
2.26	15.3 ± 0.15^{e}	GC	23.8 ± 0.20^{e}	GF

GC—Green Compact, BYC—Brownish Yellow Compact, GYF—Greenish Yellow Friable, YF—Yellow Friable, GF—Green Friable, YGC—Yellowish Green Compact.
Data were collected after 2 weeks of culture on MSB_5 medium supplemented with different concentrations of auxins for callus induction. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$). Best results are indicated in bold.

Table 2
Influence of cytokinins on efficient callus induction.

Plant growth regulators (μM/L)	Cotyledon		Hypocotyl	
	Percentage of response	Nature of callus	Percentage of response	Nature of callus
Control	–	–	–	–
BAP				
2.22	19.7 ± 0.15^{d}	GC	28.3 ± 0.20^{d}	GC
4.40	37.6 ± 0.33^{b}	GC	42.9 ± 0.22^{b}	GC
6.62	**41.9 ± 0.23^{a}**	**GC**	**54.2 ± 0.20^{a}**	**YGC**
8.90	26.0 ± 0.21^{c}	GC	36.6 ± 0.24^{c}	GC
11.12	16.1 ± 0.23^{e}	BYC	21.8 ± 0.19^{e}	BYC
Kinetin				
2.32	31.6 ± 0.22^{c}	GC	38.9 ± 0.24^{c}	GC
4.60	**49.4 ± 0.22^{a}**	**GYF**	56.6 ± 0.22^{a}	**GF**
6.97	42.3 ± 0.21^{b}	YF	49.8 ± 0.26^{b}	GF
9.30	25.5 ± 0.16^{d}	GC	34.2 ± 0.18^{d}	GC
11.61	18.8 ± 0.29^{e}	GC	24.4 ± 0.20^{e}	GC
2iP				
0.98	31.9 ± 0.23^{c}	GC	38.8 ± 0.20^{c}	GF
1.96	**45.2 ± 0.20^{a}**	**GF**	**51.2 ± 0.22^{a}**	**GF**
2.95	34.9 ± 0.17^{b}	GF	40.4 ± 0.19^{b}	GF
3.93	23.6 ± 0.22^{d}	GC	28.2 ± 0.20^{d}	GF
4.92	17.2 ± 0.29^{e}	GF	20.8 ± 0.24^{e}	GF
Zeatin				
0.46	19.9 ± 0.17^{c}	YGF	24.4 ± 0.19^{c}	YGF
0.91	**35.5 ± 0.22^{a}**	**YGF**	**42.8 ± 0.20^{a}**	**YGF**
1.36	24.7 ± 0.15^{b}	GF	30.2 ± 0.17^{b}	YGF
1.82	17.8 ± 0.24^{d}	GF	21.6 ± 0.22^{d}	YGF
2.28	12.5 ± 0.22^{e}	GC	16.7 ± 0.24^{e}	YGF

GC—Green Compact, BYC—Brownish Yellow Compact, GYF—Greenish Yellow Friable, YF—Yellow Friable, GF—Green Friable, YGC—Yellowish Green Compact.
Data were collected after 2 weeks of culture on MSB_5 medium supplemented with different concentrations of cytokinins for callus induction. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$). Best results are indicated in bold.

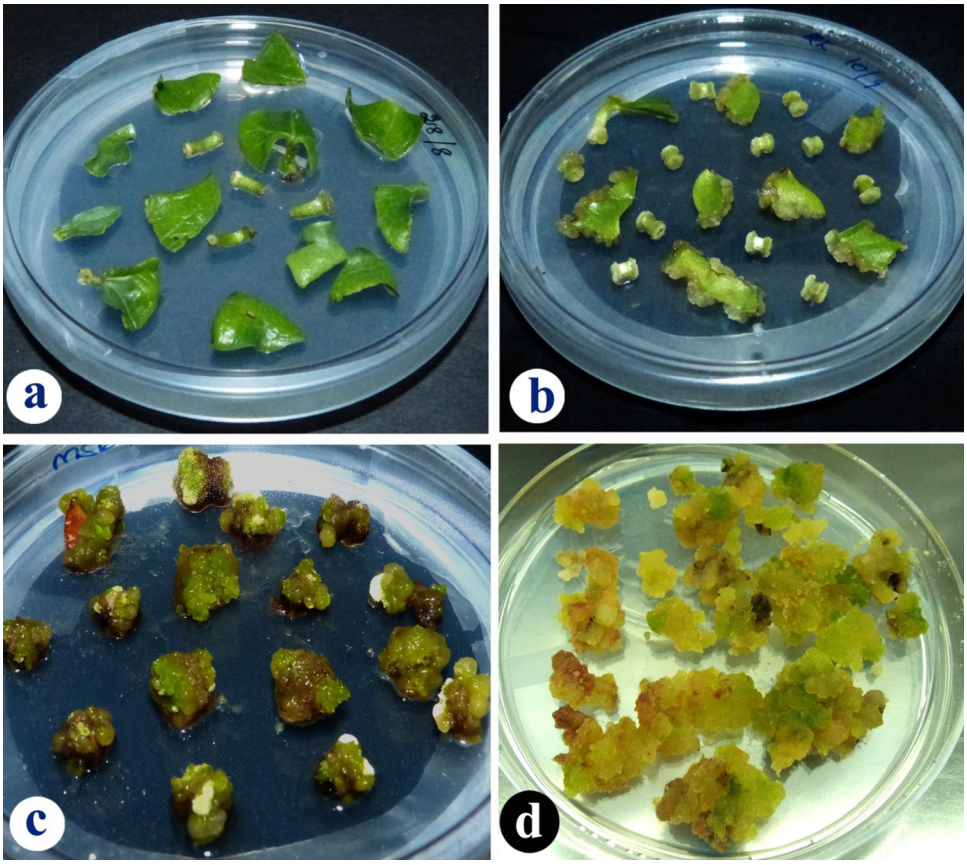

Fig. 4. Influence of maltose on reduced phenolic secretion and efficient callus culture. (a) Cotyledon and hypocotyl explants, (b) Callus induction on MSB_5 medium supplemented with maltose (30 g/L), (c) Callus development, (d) Callus proliferation.

Table 3
Combined effect of auxins and cytokinins on callus induction.

Plant growth regulators (μM/L)		Cotyledon		Hypocotyl	
		Percentage of response	Nature of callus	Percentage of response	Nature of callus
Control		–	–	–	–
IAA	Kin				
5.71	2.32	43.4 ± 0.20^{c}	YGF	55.2 ± 0.24^{c}	GF
	4.60	$\mathbf{54.9 \pm 0.16^{a}}$	**GF**	$\mathbf{67.5 \pm 0.22^{a}}$	**GF**
	6.97	47.1 ± 0.22^{b}	GC	56.6 ± 0.22^{b}	GF
	9.30	36.8 ± 0.18^{d}	GC	48.6 ± 0.26^{d}	GC
	11.61	29.9 ± 0.20^{e}	GC	40.3 ± 0.18^{e}	GC
IBA	Kin				
7.36	2.32	36.7 ± 0.24^{c}	YGC	46.1 ± 0.18^{c}	GC
	4.60	43.9 ± 0.20^{b}	YGC	52.1 ± 0.18^{b}	GC
	6.97	$\mathbf{49.2 \pm 0.22^{a}}$	**YGC**	$\mathbf{57.7 \pm 0.15^{a}}$	**GF**
	9.30	30.4 ± 0.20^{d}	GC	39.7 ± 0.26^{d}	GC
	11.61	28.8 ± 0.19^{e}	GC	34.7 ± 0.15^{e}	GC
NAA	Kin				
8.05	2.32	39.8 ± 0.26^{e}	YF	45.2 ± 0.20^{e}	GF
	4.60	46.9 ± 0.20^{c}	YGF	58.5 ± 0.22^{c}	YGF
	6.97	$\mathbf{58.2 \pm 0.24^{a}}$	**YGF**	$\mathbf{69.8 \pm 0.20^{a}}$	**YGF**
	9.30	50.8 ± 0.22^{b}	GF	61.1 ± 0.27^{b}	YGF
	11.61	42.9 ± 0.20^{d}	GC	54.3 ± 0.26^{d}	GF
2,4-D	NAA				
0.90	2.68	32.8 ± 0.22^{e}	GF	45.1 ± 0.18^{e}	GF
	5.37	39.3 ± 0.24^{d}	GF	49.8 ± 0.20^{d}	GF
	8.05	58.6 ± 0.18^{b}	GYF	64.4 ± 0.22^{b}	GF
	10.74	$\mathbf{65.9 \pm 0.22^{a}}$	**GF**	$\mathbf{74.7 \pm 0.15^{a}}$	**GF**
	13.42	48.2 ± 0.20^{c}	GC	59.9 ± 0.18^{c}	GF
2,4-D	Kin				
0.90	2.32	64.1 ± 0.20^{c}	YGF	75.6 ± 0.22^{c}	YGF
	4.60	$\mathbf{85.2 \pm 0.22^{a}}$	**GF**	$\mathbf{94.9 \pm 0.18^{a}}$	**YGF**
	6.97	70.4 ± 0.18^{b}	YGF	79.7 ± 0.15^{b}	YGF
	9.30	52.3 ± 0.22^{d}	YGF	64.7 ± 0.21^{d}	YGF
	11.61	39.8 ± 0.24^{e}	YGC	45.1 ± 0.18^{e}	YGF
2,4-D	Zeatin				
0.90	0.46	42.4 ± 0.20^{e}	GF	55.9 ± 0.23^{e}	GF
	0.91	54.2 ± 0.22^{c}	WF	65.9 ± 0.27^{c}	GF
	1.36	$\mathbf{74.8 \pm 0.23^{a}}$	**WPF**	$\mathbf{85.2 \pm 0.20^{a}}$	**WPF**
	1.82	69.2 ± 0.26^{b}	WPC	70.6 ± 0.22^{b}	WPF
	2.28	48.9 ± 0.20^{d}	WC	60.0 ± 0.29^{d}	WPF

YGF—Yellowish Green Friable, GF—Green Friable, GC—Green Compact, YGC—Yellowish Green Compact, YF—Yellow Friable, GYF—Greenish Yellow Friable, WPC—Whitish Pink Compact, WPF—Whitish Pink Friable, WC—White Compact.
Data were collected after 2 weeks of culture on MSB_5 medium supplemented with different concentrations and combinations of auxins and cytokinins for callus induction. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$). Best results are indicated in bold.

(0.90 μM) along with KIN (4.60 μM) was found to be the best combination for high frequency of callus induction with 85.2% and 94.9% of response in cotyledon and hypocotyls (Fig. 4) (Table 3). Organogenic calli were produced with increased concentrations of cytokinins and regeneration was not achieved from these types of calli. In all the experiments tested, the frequency of callus formation was comparatively lower in cotyledon explants than hypocotyls which formed well developed callus within a month. This observation clearly indicates the callus forming potential of the hypocotyl explants in cotton tissue culture.

3.4. Influence of plant growth regulators on callus proliferation

Different colors and textures of calli were observed on medium supplemented with various concentrations and combinations of plant growth regulators. Callus obtained from both cotyledon and hypocotyls showed good proliferation on medium supplemented with 2,4-D (0.45 μM) + 2iP (2.95 μM) with 89.8% of response followed by IBA (7.36 μM) + TDZ (6.80 μM) combination with 78.6% of response (Table 4). This observation clearly indicates that the role of auxin along with a cytokinin is necessary for efficient callus proliferation. Highest percentage of callus proliferation was observed after 3 weeks of subculture. The production of anthocyanin pigment in callus tissues under 2,4-D+2iP and IBA +TDZ combinations indicating the cease of cell division and entry of the cells into somatic embryogenesis pathway as previously reported by Khan et al. [14]. Different colors and textures of callus were observed under various hormone treatments (Fig. 5) which clearly indicate the influence of different plant growth regulators on determining callus morphology.

4. Discussion

Generally cotton plants are rich in phenolic compounds which hinder the growth of the explant tissues in tissue culture medium and eventually result in their death. It is undesirable for the growth of any plant tissues under *in vitro* condition. Phenolic compounds are usually secreted in medium from cut part of the plants while preparing explants for *in vitro* culture. Phenolic secretion affects the tissue growth, results in browning of medium and finally leads to death of the explants. This is the serious problem in cotton tissue culture which makes its regeneration a difficult one. In order to

Table 4
Combined effect of auxins and cytokinins on callus proliferation.

Plant Growth Regulators (μM/L)		Cotyledon		Hypocotyl	
		Percentage of response	Nature of callus	Percentage of response	Nature of callus
Control		–	–	–	–
IBA	TDZ				
7.36	2.26	58.4 ± 0.22^{b}	CWF	69.8 ± 0.24^{b}	CWF
	4.53	64.8 ± 0.20^{a}	CWF	78.6 ± 0.16^{a}	CWF
	6.80	$\mathbf{52.2 \pm 0.24^{c}}$	**WPF**	$\mathbf{64.5 \pm 0.16^{c}}$	**WPF**
	9.07	39.8 ± 0.18^{d}	WPF	49.0 ± 0.24^{d}	WPF
	11.34	29.6 ± 0.24^{e}	WC	41.9 ± 0.21^{e}	WC
NAA	2iP				
8.05	0.98	31.9 ± 0.20^{d}	GC	42.4 ± 0.22^{d}	GC
	1.96	$\mathbf{48.8 \pm 0.18^{b}}$	**GF**	$\mathbf{54.3 \pm 0.20^{b}}$	**GF**
	2.95	54.1 ± 0.20^{a}	YGF	63.8 ± 0.15^{a}	YGF
	3.93	34.6 ± 0.24^{c}	GC	49.7 ± 0.16^{c}	GC
	4.92	27.2 ± 0.20^{e}	GF	39.5 ± 0.18^{e}	GF
2,4-D	Kin				
0.45	2.32	20.1 ± 0.20^{e}	GC	28.7 ± 0.26^{e}	GC
	4.60	28.6 ± 0.18^{c}	GF	35.3 ± 0.21^{d}	GF
	6.97	34.5 ± 0.24^{b}	GF	45.0 ± 0.21^{b}	GF
	9.30	$\mathbf{45.9 \pm 0.20^{a}}$	**GYF**	$\mathbf{58.3 \pm 0.15^{a}}$	**GYF**
	11.61	26.4 ± 0.18^{d}	GC	38.6 ± 0.26^{c}	GC
2,4-D	2iP				
0.45	0.98	46.9 ± 0.18^{e}	GPF	55.4 ± 0.26^{e}	GPF
	1.96	60.4 ± 0.20^{c}	GPF	74.7 ± 0.15^{c}	GPF
	2.95	$\mathbf{74.2 \pm 0.24^{a}}$	**GPF**	$\mathbf{89.8 \pm 0.20^{a}}$	**GPF**
	3.93	69.1 ± 0.20^{b}	GPF	78.5 ± 0.22^{b}	GPF
	4.92	56.3 ± 0.22^{d}	GPF	64.7 ± 0.15^{d}	GPF

CWF—Creamy White Friable, WPF—Whitish Pink Friable, WC—White Compact, GC—Green Compact, GF—Green Friable, YGF—Yellowish Green Friable, GYF—Greenish Yellow Friable, GPF—Greenish Pink Friable.
Data were collected after 2 weeks of culture on MSB_5 medium supplemented with different concentrations and combinations of auxins and cytokinins for callus proliferation. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$). Best results are indicated in bold.

avoid this major issue, it is necessary to find an optimum carbon source for efficient *in vitro* culture of cotton. Secretion of phenolic compounds as a wounding response inhibited *in vitro* explant growth in sugarbeet and garden beet [10]. Sucrose as a carbon source supports growth of plant cells under in vitro condition [7]. Medium supplemented with 3% sucrose showed highest phenolic concentration while the lowest was observed from 0% sucrose in callus culture of Sugar beet [32]. The same observation was reported by [13] about the influence of sucrose on phenolic concentration in avocado. Enzymes such as polyphenoloxidase (PPO) and peroxidase (POD) were reported to be responsible for blackening *in vitro* cultures and catalyzing oxidation of phenolic compounds [20,5,30]. Kahn [13] suggested that the degree of blackening is related to phenolic concentration and activity of the PPO enzyme. Andersone and Ievinsh [2] suggested that reduced peroxidase and polyphenoloxidase activity increases the ability of tissues to initiate growth *in vitro*. Chemical, enzymatic and physical treatments can be used to prevent PPO-catalyzed tissue blackening [16]. However these treatments often cannot be used in *in vitro* culture. PVP has been added to the medium to alleviate the effects of browning in *Haworthia* cultures [29] and to control phenolic secretion during multiple shoot induction of *Ricinus communis* [9]. In contrary to the above said reports, addition of the anti-oxidants PVP and ascorbic acid to the medium did not visibly reduce the browning in the cultures of *Aloe polyphylla* and browning increased within hours of subculturing on medium supplemented with PVP [1].

Increasing the sucrose concentration of the culture media resulted increase of phenolics in willow [12]. Curtis and Shetty [4] reported that elevated phenolic levels were associated with increased sucrose concentration in oregano. Thus the observations and results obtained from our present study also coincide with the above said reports stating that increasing the overall sucrose concentration leading to cell death due to secretion of phenolic compounds. [17] used 4% glucose as the sole carbon source for efficient callus induction in cotton Shoemaker [22] used 3% glucose for embryogenic callus initiation and 3% sucrose for callus proliferation in order to avoid the loss of embryogenic potential of the callus. 3% Glucose was used as sole carbon source for callus culture of an elite Chinese cotton variety [31]. Glucose has been proven to control tissue browning of explants due to phenolic oxidation by [23]. However maltose was used as the sole carbon source for callus induction, proliferation and somatic embryogenesis in cotton [15] in which browning of cells was not observed at any stage of the callus culture. Maltose was used as an effective carbon source in callus culture of Narasimha cotton cultivar to control secretion of excessive phenolics [14]. The results of the present study also comply with the above statement for callus culture of SVPR-2 cotton cultivar in which 3% maltose was effectively used as the sole carbon source until callus proliferation to avoid phenolic secretion.

Earlier experiments were performed with different hormonal combinations for callus induction in cotton using different explants. Price and Smith [21] successfully initiated callus from hypocotyl explant on MSB_5 medium supplemented with IAA and KIN and 3% glucose and subculture on MS medium supplemented with 2iP and NAA. Wu et al. [31] described obtained best callus induction in Chinese cotton varieties with a combination of IBA and Kinetin. Induction of embryogenic callus with 2,4-D and KIN combination was well documented for Coker varieties and other local cotton cultivars.

Aydin et al. [3] obtained 100% of callus induction from shoot apices, cotyledonary nodes and hypocotyls on MS medium supplemented with BAP and Kinetin. Shoemaker et al. [22]

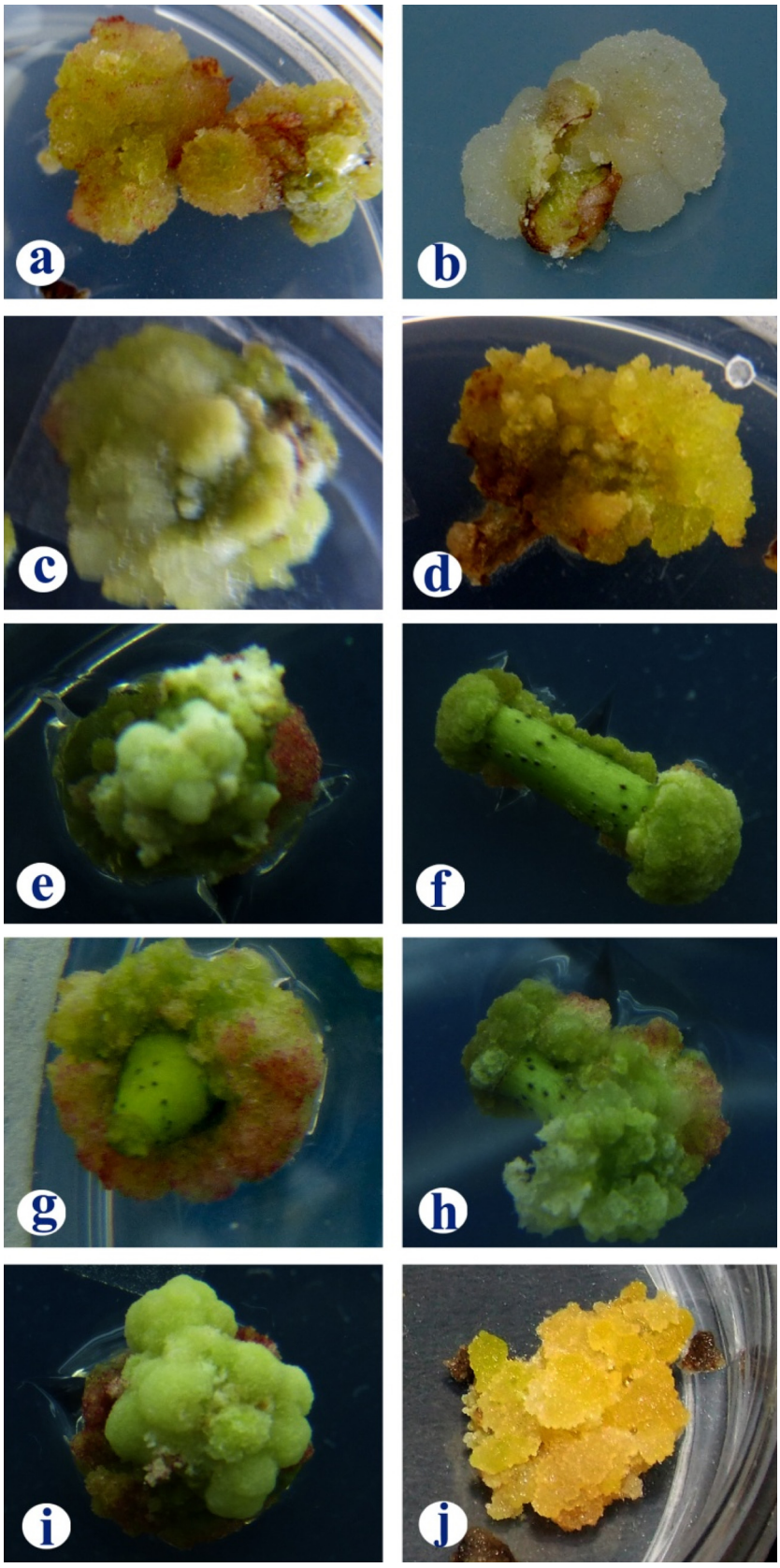

Fig. 5. Colors and Textures of callus on medium supplemented with different plant growth regulators. (a) Greenish Yellow Friable, (b) Creamy White Friable, (c) White Compact, (d) Yellowish Green Friable, (e) Whitish Green Compact, (f) Green Compact, (g) Greenish Pink Compact, (h) Green Friable, (i) Green Compact, (j) Yellow Friable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggested that *Gossypium* sp. contains much genotypic variation for callus initiation, proliferation and regeneration capacity. 2,4-D has been proved to be essential for optimum callus induction from hypocotyl explants of cotton and NAA has also been shown to be beneficial for good quality callus formation [18]. Earlier studies reported the use of 2,4-D at lower concentration (0.1 mg/l) for efficient callus induction in cotton [25,26,27,28,33,15] and supplementation of 2iP with auxin has been well reported for efficient callus proliferation [21,23,6,22]. Different hormonal combinations of auxins (NAA and 2,4-D) and cytokinin (kinetin) were reported for effective callus induction [18]. Proliferation of the induced callus on hormone free MS medium was tested to check its proliferating capacity in the absence of growth regulators. The callus showed no remarkable changes in callus size and texture even after 20 days but the callus remained in its original condition until it was transferred to proliferation medium. This observation revealed the importance of plant growth regulators in callus proliferation.

5. Conclusion

As *in vitro* culture and regeneration of cotton is highly genotype dependent it is necessary to standardize a specific protocol for each cotton cultivar. Likewise SVPR-2 cotton cultivar which is cultivated widely in southern part of Tamil Nadu is also recalcitrant for *in vitro* manipulation and hence it is desirable to generate a suitable protocol for this cultivar to get high frequency regeneration. Till date there are only a few reports about the influence of carbon sources on phenolic secretion in cotton callus culture. The present study offers a simple solution to alleviate the problem of phenolic secretion in cotton callus culture by adjusting carbon source without adding any antioxidants into the culture medium.

Author contribution

GP contributed in doing experiments and preparing manuscript, SS, GS, SV, MV and MK helped in experimental analysis and discussion, TS and NJ contributed in designing the experiments.

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References

[1] A.L. Abrie, J.V. Staden, Micropropagation of the endangered *Aloe polyphylla*, Plant Growth Regul. 33 (2001) 19–23.
[2] U. Andersone, G. Ievinsh, Changes of morphogenic competence in mature *Pinus sylvestris* L. buds *in vitro*, Ann. Bot. 90 (2002) 293–298.
[3] Y. Aydin, Z. Ipekci, T. Talas-Ora, A. Zehir, K. Bajrovic, N. Gozukirmizi, High frequency somatic embryogenesis in cotton, Biol. Plant. 4 (2004) 491–495.
[4] O.F. Curtis, K. Shetty, Growth medium effects on vitrification, total phenolics, chlorophyll, and water content of *in vitro* propagated oregano clones, Acta Hort. 426 (1996) 489–504.
[5] P.W. Down, R.A. Norton, Browning-associated mechanism of resistance to insects in corn callus tissues, J. Chem. Ecol. 21 (1995) 583–600.
[6] J. Finer, R.H. Smith, Initiation of callus and somatic embryos from explants of mature cotton (*Gossypium klotzschianum* Anderss), Plant Cell Rep. 3 (1984) 41–43.
[7] O.L. Gamborg, G.C. Phillips, Media preparation and handling, in: O.L. Gamborg, G.C. Phillips (Eds.), Plant Cell, Tissue Organ Cult: Fudamental Methods, Springer, Germany, 1995, pp. 21–34.
[8] M. Ganesan, N. Jayabalan, Carbon source dependent somatic embryogenesis and plant regeneration in cotton *Gossypium hirsutum* L. cv. SVPR2 through suspension cultures, Indian J. Exp. Biol. 43 (2005) 921–925.
[9] K. Ganesh kumari, M. Ganesan, N. Jayabalan, Somatic organogenesis and plant regeneration in *Ricinus communis*, Biol. Plant. 52 (2008) 17–25.
[10] C.T. Harms, I. Baktir, J.I. Oertli, Clonal propagation *in vitro* of red beet (*Beta vulgaris* L.) by multiple adventitious shoot formation, Plant Cell Tissue Organ. Cult. 2 (1983) 93–102.
[11] R.K. Jain, M.R. Davey, E.C. Cocking, Ray Wu, Carbohydrate and osmotic requirements for high frequency plant regeneration from protoplast derived colonies of *indica* and *japonica* rice varieties, J. Exp. Bot. 48 (1997) 751–758.
[12] R. Julkunen-Tiitto, Defensive efforts of *Salix myrsinifolia* plantlets I photomixotrophic culture conditions: the effect of sucrose, nitrogen and pH on the phytomass and secondary phenolic accumulation, Ecoscience 3 (1996) 297–303.
[13] V. Kahn, Polyphenoloxidase activity and browning of three avocado varieties, J. Sci. Food Agric. 26 (1975) 1319–1324.
[14] T. Khan, V.S. Reddy, S. Leelavathi, High-frequency regeneration via somatic embryogenesis of an elite recalcitrant cotton genotype (*Gossypium hirsutum* L.) and efficient *Agrobacterium*-mediated transformation, Plant Cell Tissue Organ. Cult. 101 (2010) 323–330.
[15] R. Kumria, V.G. Sunnichan, D.K. Das, S.K. Gupta, V.S. Reddy, R.K. Bhatnagar, S. Leelavathi, High-frequency somatic embryo production and maturation into normal plant in cotton (*Gossypium hirsutum*) through metabolic stress, Plant Cell Rep. 21 (2003) 635–639.
[16] E. Laurila, R. Kervinen, R. Ahvenainen, The inhibition of enzymatic browning in minimally processed vegetables and fruits, Postharvest News Inf. 9 (1998) 53–66.
[17] Z. Michel, K.T. Hilaire, K. Mongomaké, A.N. Georges, K.Y. Justin, Effect of genotype explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.), Aust. J. Crop Sci. 2 (2008) 1–9.
[18] R. Mishra, H. Wang, R. Yadav, A. Wilkins, Development of a highly regenerable elite Acala cotton (*Gossypium hirsutum* cv. Maxxa)—a step towards genotype-independent regeneration, Plant Cell Tissue Organ Cult. 73 (2003) 21–35.
[19] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[20] F. Pizzocaro, D. Torreggiani, G. Gilardi, Inhibition of apple polyphenoloxidase (PPO) by ascorbic acid, citric acid and sodium chloride, Food Nutr. Press. 17 (1993) 21–30.
[21] H.J. Price, R.H. Smith, Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anderss, Planta (1979) 305–307.
[22] R.C. Shoemaker, L.J. Couche, D.W. Galbraith, Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.), Plant Cell Rep. 3 (1986) 178–181.
[23] R.H. Smith, H.J. Price, J.B. Thaxton, Defined conditions for the initiation and growth of cotton callus *in vitro* I. *Gossypium arboreum*, In vitro. 13 (1977) 329-334.
[24] C. TianZi, W. ShenJie, Z. Jun, G.W. Zhen, Z. TianZhen, Pistil drip following pollination: a simple *in planta Agrobacterium*-mediated transformation in cotton, Biotechnol. Lett. 32 (2010) 547–555.
[25] L. Trolinder, J.R. Goodin, Somatic embryogenesis and plant regeneration in cotton *(Gossypium hirsutum* L.), Plant Cell Rep. 6 (1987) 231–234.
[26] L. Trolinder, J.R. Goodin, Somatic embryogenesis in cotton (*Gossypium*)I. Effects of source of explant and hormone regime, Plant Cell Tissue Organ Cult. 12 (1988) 31–42.
[27] L. Trolinder, J.R. Goodin, Somatic embryogenesis in cotton (*Gossypium*)II. Requirements for embryo development and plant regeneration, Plant Cell Tissue Organ Cult. 12 (1988) 43–53.
[28] L. Trolinder, C. Xhixian, Genotype specificity of the somatic embryogenesis response in cotton, Plant Cell Rep. 8 (1989) 133–136.
[29] D.C.J. Wessels, E.G. Groenewald, A. Koeleman, Callus formation and subsequent shoot and root development from leaf tissue of *Haworthia planifolia*. Z, Plant Physiol. 78 (1976) 141–145.
[30] J.R. Whitaker, C.Y. Lee, Recent advances in chemistry of enzymatic browning, in: C.Y. Lee, J.R. Whitaker (Eds.), Enzymatic Browning and Its Prevention, vol. 600, ACS Symp. Series, Washington, D.C., USA, 1995, pp. 2–7.
[31] J. Wu, X. Zhang, Y. Nie, S. Jin, S. Liang, Factors affecting somatic embryogenesis and plant regeneration from a range of recalcitrant genotypes of Chinese cottons (*Gossypium hirsutum* L.), In Vitro Cell Dev. Biol. Plant. 40 (2004) 371–375.
[32] M. Yildiz, S. Önde, M. Özgen, Sucrose Effects on phenolic concentration and plant regeneration from sugarbeet leaf and petiole explants, J Sug Beet Res. 44 Nos. (2007) 1 & 2.
[33] B. Zhang, F., Liu, C. Yao, Plant regeneration via somatic embryogenesis in cotton, Plant Cell Tissue Organ Cult. 60 (2000) 89-94.