High frequency somatic embryogenesis in cotton

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Abstract

A highly reproducible system for efficient somatic embryogenesis was developed to regenerate plantlets from cotton (*Gossypium hirsutum* L.) cultivars (Nazilli M-503 and Nazilli 143). Shoot apices, hypocotyls and nodes of 10-d-old seedlings were used as explants. High frequency (100 %) embryogenic calli was initiated from all tested explants on Murashige and Skoog (1962) (MS) media supplemented with 1 g dm^{-3} polyvinylpyrrolidone (PVP), 1 mg dm^{-3} 6-benzylaminopurine (BAP), 0.5 mg dm^{-3} kinetin for Nazilli M-503 and 1 g dm^{-3} PVP, 2 mg dm^{-3} BAP, 0.5 mg dm^{-3} kinetin for Nazilli-143. Globular stage somatic embryos were produced 4 months after transfer to hormone-free MS medium supplemented with 1 g dm^{-3} PVP. Subsequent subculture of globular embryos every 3 weeks on hormone-free MS medium led to the development of torpedo and cotyledonary stage embryos with the frequency of 75 and 83.2 % from hypocotyl explants of Nazilli M-503 and Nazilli-143, respectively. Afterwards, mature somatic embryos were isolated and cultured on hormone-free MS medium for germination and development into plantlets. The highest germination frequency (42.9 %) for Nazilli M-503 somatic embryos were obtained on hormone-free MS medium after 5 months with hypocotyl explants, whereas germination frequencies of Nazilli-143 embryos from hypocotyl, node and apex explants varied between 22 - 30 %.

Additional key words: *Gossypium hirsutum* L., indirect somatic embryogenesis, hypocotyls, cotyledonary node, apex.

Introduction

Direct regeneration of shoots from apical meristem (Gould *et al*. 1991), cotyledonary nodes and apex (Agrawal *et al*. 1997, Gupta *et al*. 1997, Hemphill *et al*. 1998, Bajrovic *et al*. 2001) has been reported in widely different cotton cultivars. First report on cotton somatic embryogenesis was observed in *Gossypium koltzchianum*, but no plantlet regeneration was reported (Price and Smith 1979). Afterwards, plant regeneration was obtained from two years old callus of *Gossypium hirsutum* L. cv. Coker 310 *via* somatic embryogenesis (Davidonis and Hamilton 1983). Since then, significant progress has been reported in cotton tissue culture (Zhang and Feng 1992, Zhang 1994). Although *in vitro* cultured cotton cells have been induced to undergo somatic embryogenesis the response was restricted to only a few cultivars using different strategies (Trolinder and Xhixian 1989, Firoozabady and De Boer 1993) and the efficiency of plant regeneration *via* somatic embryogenesis has been relatively low (Voo *et al*. 1991, Zhang *et al*. 1993, Zhang and Zhao 1997). In addition, the long culture time and complicated culture procedure restrict the practical application (Zhang and Zhao 1997). Recently, a highly efficient somatic embryo production and maturation procedure has been developed to regenerate plantlets from *G. hirsutum* cv. Coker 310 (Kumria *et al*. 2003). A step towards genotype-independent regeneration via

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Abbreviations: BAP - 6-benzylaminopurine; MS - Murashige and Skoog (1962) medium; PVP - polyvinylpyrrolidone.
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somatic embryogenesis (*G. hirsutum* L. cvs. Maxxa, Riata, and Ultima) was also achieved by selection for regeneration potential in commercial seed of elite cultivars by Mishra *et al*. (2003). Here, we report a new protocol for high frequency somatic embryogenesis and plant regeneration from commercial cotton cultivars.

Materials and methods

Plants: Seeds of cotton (*Gossypium hirsutum* L. cv. Nazilli M-503 and Nazilli-143 obtained from Nazilli Cotton Research Institute, Nazilli, Turkey) were got rid of lints with sulphuric acid and washed with water for 3 h. For surface sterilization, seeds were initially dipped in 70 % ethanol for 2 min, rinsed with sterile distilled water for 3 min and kept in 20 % commercial bleach for 20 min. The seeds were then rinsed 3 times with sterile distilled water. The sterilized seeds without seed coats were sown in Magenta vessels containing Murashige and Skoog (1962) (MS) medium, 30 g dm^{-3} sucrose and 2.2 g dm^{-3} *Phytagel* for germination. Hypocotyls, cotyledonary nodes and shoot apices of the 10-d-old seedlings were used as explants.

Medium composition and culture conditions: The basal MS medium consisted of MS mineral salts and B_5 vitamins (Gamborg *et al*. 1968) with 30 g dm^{-3} sucrose, 2.2 g dm^{-3} *Phytagel* and 1 g dm^{-3} PVP. The pH was adjusted to 5.8 before sterilization by autoclaving at 121 °C for 20 min. The growth regulators (BAP, kinetin) were filter sterilized prior to addition to the culture media. All cultures were maintained in a growth chamber at 25 °C, 70 % relative humidity and irradiance of 50 - 60 µmol m^{-2} s^{-1} (cool-white fluorescent tubes) with a 16-h photoperiod, except that explants for inducing callus were incubated at very low irradiance (5 µmol m^{-2} s^{-1}).

Induction of embryogenic callus: For callus induction hypocotyl, cotyledonary node and shoot apices were placed on basal MS medium supplemented with various concentrations of BAP and kinetin (Table 1). Frequency was scored after 4 weeks of culture and induced calli were detached from the explants and proliferated onto the same fresh media for long-term maintanence of embryogenic potential.

Differentiation of somatic embryos and plant regeneration: After one month, callus tissues on MS1 - MS8 media initiated from different explants were transferred onto hormone-free basal MS media. The frequency of calli producing somatic embryos was recorded as percentage of embryogenic calli with embryos after 4 months. The growth index of callus was calculated as [(final callus mass) - (initial callus mass)]/(initial callus mass).

Further development of globular embryos to heart-shaped and torpedo stages were performed by subculturing every 3 weeks onto the same medium. Afterwards, mature somatic embryos were isolated and cultured on hormone-free basal MS medium for germination and development into plantlets. After 5 months on hormone free basal MS medium, the frequency of conversion of somatic embryos into plantlets was calculated.

Statistical analysis: Each treatment consisted of 30 hypocotyls, 20 cotyledonary nodes, 12 shoot apices and each experiment was repeated three times. The data shown represents the mean ± standard error of the three independent experiments. *ANOVA* was performed on the results of each experiment and the data means were compared using Duncan's multiple range test ($P < 0.05$).

Results

Induction of callus: Calli were observed after one month of culture in both cultivars of Nazilli M-503 and Nazilli 143 on basal MS media supplemented with different concentrations of BAP and kinetin from hypocotyl, cotyledonary node and apex explants. The proliferation of callus was better when the explants were incubated under low irradiance. The best concentrations were 1 mg dm^{-3} BAP and 0.5 mg dm^{-3} kinetin (MS4) for Nazilli M-503, 1 mg dm^{-3} BAP and 2 mg dm^{-3} kinetin (MS6) or 2 mg dm^{-3} BAP and 0.5 mg dm^{-3} kinetin (MS7) for Nazilli 143. Percentage of calli induction was 100 % for Nazilli M-503 on MS4 or MS6 from hypocotyls and node explants whereas it was 91.6 % for apex explants on MS6 medium (Table 1). However, the frequency of calli induction for Nazilli-143 was highest on MS7 medium (100% in the hypocotyl and cotyledonary node explants and 91.6 % in apex explants). Several types of callus were distinguishable based on their appearance. Although the calli were compact and green on MS1, MS2, MS3, MS5 and MS8, they were grayish yellow or gray and friable on MS4, MS6 and MS7. After 4 months of subculture, the friable calli differentiated into white clumps of embryogenic calli on MS1 - MS8 media. Initially most of the embryogenic cultures showed a high degree of friability with many yellowish green globular structures.

Table 1. Frequency of callus induction, callus mass and frequency of somatic embryo producing calli in cotton cv. Nazilli M-503 and Nazilli 143. Frequency of callus induction was calculated after one month in basal MS media supplemented with various concentrations of BAP and kinetin, frequency of somatic embryogenesis was calculated 4 months after transfer of calli to hormon free basal media. Means ± SD. For each treatment, 30, 20 and 12 hypocotyl, node and apex explants were recorded, respectively, in 3 replicates.

Medium	BAP + kinetin [mg dm^{-3}]	Explant	Nazilli M-503			Nazilli-143		
			Callus induction [%]	Callus mass [g $explant^{-1}$]	Somatic embryo [%]	Callus induction [%]	Callus mass [g $explant^{-1}$]	Somatic embryo [%]
MS1	0.5 + 0.5	hypocotyl	33.3 ± 1.6	2.4	40.0 ± 1.2	56.6 ± 2.6	2.1	100.0 ± 0.0
		node	65.0 ± 3.1	2.5	46.0 ± 1.4	60.0 ± 2.9	3.4	100.0 ± 0.0
		apex	58.3 ± 2.9	1.9	71.4 ± 2.0	41.6 ± 4.8	2.1	35.7 ± 1.1
MS2	0.5 + 1.0	hypocotyl	40.0 ± 4.7	4.0	41.6 ± 1.3	53.3 ± 2.4	3.7	100.0 ± 0.0
		node	70.0 ± 0.9	2.4	42.8 ± 1.3	70.0 ± 0.9	1.5	100.0 ± 0.0
		apex	50.0 ± 2.4	3.4	83.3 ± 0.3	66.6 ± 3.2	2.8	66.6 ± 1.8
MS3	0.5 + 2.0	hypocotyl	53.3 ± 2.8	1.9	31.2 ± 1.2	40.0 ± 4.7	2.2	83.3 ± 1.8
		node	20.0 ± 2.3	2.6	60.0 ± 1.6	70.0 ± 0.9	2.9	42.0 ± 1.3
		apex	41.6 ± 4.8	2.3	66.6 ± 1.8	50.0 ± 2.4	3.5	100.0 ± 0.0
MS4	1.0 + 0.5	hypocotyl	100.0 ± 0.0	2.9	66.6 ± 1.8	86.6 ± 3.2	2.6	76.9 ± 2.1
		node	100.0 ± 0.0	3.5	100.0 ± 0.0	60.0 ± 2.9	1.9	88.8 ± 1.9
		apex	100.0 ± 0.0	3.1	100.0 ± 0.0	91.6 ± 3.2	0.9	73.3 ± 2.0
MS5	1.0 + 1.0	hypocotyl	46.6 ± 5.5	4.2	85.7 ± 1.8	60.0 ± 2.9	2.2	55.5 ± 1.4
		node	70.0 ± 0.9	2.8	71.4 ± 2.0	55.0 ± 2.5	3.9	54.5 ± 1.4
		apex	41.6 ± 4.8	2.5	20.0 ± 0.7	66.6 ± 3.2	1.0	75.0 ± 2.0
MS6	1.0 + 2.0	hypocotyl	100.0 ± 0.0	1.9	43.3 ± 0.3	83.3 ± 3.0	2.5	100.0 ± 0.0
		node	100.0 ± 0.0	2.5	100.0 ± 0.0	95.0 ± 3.3	2.8	100.0 ± 0.0
		apex	91.6 ± 3.2	3.9	100.0 ± 0.0	91.6 ± 3.2	3.0	48.8 ± 1.4
MS7	2.0 + 0.5	hypocotyl	86.6 ± 3.2	2.5	46.1 ± 1.4	100.0 ± 0.0	2.3	100.0 ± 0.0
		node	80.0 ± 2.9	2.2	50.0 ± 1.3	100.0 ± 0.0	2.3	100.0 ± 0.0
		apex	75.0 ± 0.4	3.2	88.8 ± 1.9	91.6 ± 3.2	1.5	55.5 ± 1.4
MS8	2.0 + 1.0	hypocotyl	33.3 ± 1.6	1.8	20.0 ± 0.7	20.0 ± 2.3	1.1	50.0 ± 1.3
		node	30.0 ± 1.4	2.6	16.0 ± 0.7	20.0 ± 2.3	3.1	25.0 ± 0.6
		apex	33.3 ± 1.6	2.4	25.0 ± 0.6	16.6 ± 1.9	4.8	50.0 ± 1.3

Table 2. Effects of cultivars and explant types on growth index of embryogenic calli, frequency of somatic embryogenesis (after one month, callus tissues on MS1 - MS8 media initiated from different explants were transferred onto hormone-free basal MS media for 4 months) and germination frequency of somatic embryos (after one month, callus tissues on MS1 - MS8 media initiated from different explants were transferred onto hormone-free basal MS media for 5 months). Means followed by the same letters are not significantly different at $P < 0.05$ according to Duncan multiple range test.

Cultivar	Explant	Growth index	Somatic embryo [%]	Germination [%]
Nazilli M-503	hypocotyl	1.57 ± 0.1	75.0 ± 3.0b	42.9 ± 1.8c
	node	1.47 ± 0.1	69.4 ± 2.8ab	27.0 ± 0.9b
	apex	1.40 ± 0.1	60.8 ± 2.5a	25.1 ± 0.7ab
Nazilli-143	hypocotyl	1.68 ± 0.2	83.2 ± 3.2c	29.8 ± 1.1b
	node	1.53 ± 0.1	76.2 ± 3.0b	24.5 ± 0.8a
	apex	1.77 ± 0.2	62.7 ± 2.6a	22.2 ± 0.6a

Somatic embryo development and germination: Embryogenic calli after being transferred to hormone-free MS medium developed into globular, heart-shaped and torpedo stages of somatic embryos. The concentrations of BAP and kinetin in MS medium for callus induction affected the number of embryos after transfer to hormone-free MS medium. The frequency of somatic embryo formation on hormone-free MS medium when transferred from MS4 or MS6 media was highest (100 %) from cotyledonary node and apex explants while it was

reduced to 93.3 % from hypocotyl explants for Nazilli M-503 (Table 1). However, the frequency of somatic embryo formation was highest from the hypocotyl and cotyledonary node explants of the embryogenic calli obtained on MS1, MS2, MS6 or MS7 media for Nazilli-143. In contrast, when embryogenic calli from hypocotyl explants obtained on MS3 medium were used the frequency of somatic embryo formation was 100 % for Nazilli-143 (Table 1). When transferred to hormone-free MS medium the frequency of somatic embryogenesis of Nazilli-143 (83.2 %) was slightly higher than Nazilli M-503 (75 %). Frequency of calli undergoing somatic embryogenesis from node and apex explants of Nazilli M-503 was 69.38 - 60.77 %, respectively. It was 76.2 and 62.7 % for Nazilli-143 node and apex explants on hormone free MS medium (Table 2).

Subculturing of somatic embryos on hormone-free MS medium led to the development of complete plantlets within 5 months. The conversion frequency of Nazilli M-503 somatic embryos from hypocotyl explants were 42.9 %, whereas it was 27.03 and 25.1 % from node and apex explants, respectively (Table 2). Germination frequency of Nazilli-143 somatic embryos was 29.78, 24.46 and 22.17 % from hypocotyl, node and apex explants, respectively (Table 2).

Discussion

We have developed a new and effective protocol for induction of somatic embryogenesis and plant regeneration in cotton cultivars within 5 and 6 months, respectively. However, Firoozabady and De Boer (1993) obtained regenerated plants from embryogenic calli in cvs. GSA 25, GSA 71, GSA 75, GSA 78, G 8160, SS-2 (except cvs. Coker 201 and Coker 310) after 7 to 8 months. Kumar and Pental (1998) obtained regenerated plants *via* somatic embryogenesis from an Indian cultivar MCU-5 in 6 - 7 months. Hoever, Zhang *et al*. (1999), obtained regenerated plants from Chinese elite cultivar CRI 12 in 4 - 5 months and Zhang *et al*. (2000) obtained high frequency somatic embryogenesis and regenerated plants from cv. Coker 201 within 2 to 3 months. To compare previously published methods, the method described here is more simple and efficient.

We also demonstrated (unpublished results) that low irradiance enhanced the percentage of callus induced from all tested explants. Similarly, low irradiance enhanced regeneration in strawberry from leaf explants (Predeiri *et al*. 1989, Korban *et al*. 1992), and the number of shoots induced *via* organogenesis *in vitro* from shoot apex of cotton (Gupta *et al*. 2000).

In previous reports, 2,4-D was an essential hormone for the induction somatic embryogenesis in cotton and other plants (Trolinder and Goodin 1987, Chen *et al*. 1987, Zhang and Feng 1992, Davidonis and Hamilton 1983, McKersie and Brown 1996, Kumar and Pental 1998, Zhang *et al*. 1999, 2000, Ogburia 2003/4, D'Onofrio and Morini 2003/4). Recently, Zhang *et al*. (2001) found that zeatin can also induce embryogenic callus in cotton. In present study, BAP and kinetin were found efficient for the induction and proliferation of embryogenic callus, however, the frequency of somatic embryo formation varied according to the explant type and concentrations of BAP and kinetin. Many reports indicated that embryo development was usually associated with a reduction or the omission of auxin from the medium (Carman 1990, Ammirato 1987). In this study, embryo maturation and germination was obtained when embryogenic calli was transferred onto hormone-free MS medium. Both, cultivars and explant types influenced the frequency of cotton somatic embryogenesis (*e.g.*, germination frequency of Nazilli M-503 somatic embryos derived from hypocotyl explants was higher than that of Nazilli-143).

The addition of PVP to media for callus induction and somatic embryo development media protected the browning of explants and enhanced induction, development and germination of embryos (data were not given). *In vitro* tissue browning is a serious problem often associated with woody and perennial species (Ndoumou *et al*. 1997, Choi *et al*. 1998). Browning is a consequence of many enzymatic reactions where chlorogenic acid (Cheng and Crisosto 1995) or other phenolic compounds are oxidized (Block and Lankes 1995).

The simple system of somatic embryogenesis and plant regeneration described here will facilitate the development of transgenic cotton plants. This system has recently been employed in our laboratory to successful propagation of cotton plants with foreign genes and analyses of gene expression profiles during somatic embryogenesis.

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