

Research note

Putrescine enhances somatic embryogenesis and plant regeneration in upland cotton

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Abstract

Improvement in somatic embryogenesis has been achieved in several cotton lines (*Gossypium hirsutum* L.) from the Georgia and Pee Dee germplasm with culture media containing various Putrescine concentrations. The best results were obtained with the α -naphthalene acetic acid (NAA)-based treatments, S15 g.05 NAA and EMMS₂, as compared to the 2,4-dichlorophenoxyacetic acid (2,4-D)-based culture medium, EMMS₄. Inclusion of 0.5 mg l⁻¹ Putrescine improved somatic embryo (SE) induction for most treatments and lines tested. An 8- and 2-fold improvement was achieved in SE production on the EMMS₂-0.5 Putrescine treatment as compared to EMMS₂ alone for cotton lines PD 97019 and GA 98033, respectively. A significant increase in SE number (53-fold) was obtained with the addition of 0.5 mg l⁻¹ Putrescine to EMMS₂ for PD 97021, which was essentially recalcitrant without Putrescine treatment. Conversion of SEs into plants was both genotype- and culture medium-dependent.

Abbreviations: IAA – indole-3-acetic acid; BA – benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; MS – Murashige and Skoog; NAA – α -naphthalene acetic acid; SE – somatic embryo

Somatic embryogenesis is the preferred plant regeneration technique for cotton gene transformation (Kumria et al., 2003), yet the majority of cotton cultivars are recalcitrant to somatic embryogenesis (Trolinder and Xhixian, 1989). Efforts to increase the range of cotton cultivars that can produce somatic embryos (SE) have met with limited success (see review by Wilkins et al., 2000). Recently, a number of germplasm lines from the Georgia and Pee Dee breeding programs have been successfully regenerated to plants via somatic embryogenesis (Sakhanokho et al., accepted for publication). However, the regeneration potential, defined as the frequency of seedlings that are capable of inducing somatic embryogenesis (Mishra et al., 2003) and the efficiency of SE

production were significantly lower for these lines as compared to the standard cultivar for cotton transformation, 'Coker 312'.

Polyamines, which include Putrescine, Cadaverine, Spermidine, and Spermine, are naturally occurring low molecular weight, polycationic, aliphatic nitrogenous compounds that have been implicated in many important cellular processes such as cell division, protein synthesis, DNA replication, and response to abiotic stress (Kakkar and Sawhney, 2002). Recent reports have indicated that polyamines could enhance somatic embryogenesis in several plant species (Kakkar and Sawhney, 2002; Kevers et al., 2002). The objective of the present study was to determine the effect of Putrescine in three improved embryo

development/maturation media on somatic embryogenesis and regeneration potential in cotton.

The plant materials consisted of three germplasm lines (PD 97021, PD 97100, PD 97019) from the Pee Dee program, Florence, SC and one germplasm line (GA 98033) from the Georgia Agricultural Experiment Station, Tifton, GA, were provided obtained from Dr. Lloyd May. All germplasm lines were previously shown to produce SE at varying levels (Sakhanokho et al. accepted). Seeds of Coker 312, kindly provided by Dr. K. Rajasekaran, were included in this study as positive control. A completely randomized block design was used, consisting of 4 germplasm lines and one check cultivar, and 3 culture media with a factorial arrangement of 0, 0.25 and 0.50 mg l⁻¹ of Putrescine. Each treatment consisted of 10 seeds and the experiment was replicated 6 times (for a total of at least 60 seeds per medium/Putrescine combination). The following data were recorded for each treatment:

- regeneration potential, the percentage of seedling that were capable of producing SEs,
- the number of SEs produced per gram of callus, and
- the number of SEs that converted into plantlets (expressed as percentage).

The analysis of variance and mean calculations were performed using SAS v8 (SAS Inst., 2001, Cary, NC).

Surface sterilization of seeds, callus initiation and liquid suspension culture were performed using a previously published procedure (Sakhanokho et al., 2001). Briefly, seeds were surface sterilized in a 100% ethanol (30–60 s) followed by 23% liquid bleach [5.25% (v/v) NaOCl] with one drop of Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma, St. Louis, MO), then rinsed 3–4 times with sterile distilled water and stored overnight at 28 °C in the final rinse. The next day, seeds were placed on MS0 culture medium containing MS salts (Murashige and Skoog, 1962), 30 g l⁻¹ glucose, 2 g l⁻¹ Gelrite (Merck & Co., Inc., Rahway, NJ) and 0.75 mg l⁻¹ MgCl₂. Four to 5 seeds were germinated in 100 × 15 mm Petri dishes (sealed with Parafilm) containing 40 ml of media and cultured in an incubator maintained at 28 ± 2 °C and under a 16/8 h (day/night) regime provided by cool-white fluorescent light (70 μmol m⁻² s⁻¹).

Hypocotyls or cotyledons from 4- to 10-day-old seedlings were cut into 4–8 5-mm segments and split longitudinally under sterile conditions before transferring onto a Petri dish containing the callus initiation medium [MS salts supplemented with 2.0 mg l⁻¹ NAA, 1.0 mg l⁻¹ kinetin, 0.4 mg l⁻¹ thiamine, 100 mg l⁻¹ myo-inositol, 30 g l⁻¹ glucose, and 0.75 g l⁻¹ MgCl₂ (pH 5.8)]. Most explants formed callus after about 4 weeks. Friable embryogenic callus (1–3 g) was transferred into 125-ml transparent polymethylpentene jars (Nalgene, Suwanee, GA) containing 70 ml of liquid embryo induction medium (MS salts with no NH₄NO₃, twice amount of KNO₃, and 10 mg l⁻¹ thiamine, 100 mg l⁻¹ myo-inositol, 1 mg l⁻¹ nicotinic acid, 1 mg l⁻¹ pyridoxine, 1 g l⁻¹ glutamine, 0.5 g l⁻¹ asparagine, 30 g l⁻¹ glucose). The jars were shaken at 130 rpm under a 16 h/8 h light/dark cycle at 28 °C.

Four- to six-week-old cell suspension cultures were strained through a 40-mesh screen and placed on development/maturation media, S15g.05NAA, EMMS₂, or EMMS₄. The culture medium S15g.05NAA consisted of MS salts supplemented with 0.05 mg l⁻¹ NAA and 15 g l⁻¹ sucrose, 2 g l⁻¹ Gelrite, and 0.75 g l⁻¹ MgCl₂. EMMS₂ culture medium was similar to liquid embryo induction medium, except it also contained 0.05 mg l⁻¹ NAA, 0.05 mg l⁻¹ kinetin, 2 g l⁻¹ Gelrite, and 0.75 g l⁻¹ MgCl₂. EMMS₄ is similar to EMMS₂ except that 0.1 mg l⁻¹ 2, 4-D and 0.5 mg l⁻¹ kinetin were used as growth regulators. The pH was adjusted to 5.8 for all the culture media before adding the gelling agent and autoclaving at 121 °C for 20 min. Each development/maturation medium was modified with 0, 0.25, or 0.5 mg l⁻¹ concentration of Putrescine. Cultures were transferred onto fresh media every 4 weeks. Germinated SEs were transferred to Magenta or Nalgene® transparent polymethylpentene jars containing the rooting culture medium (MS salts, 0.05 mg l⁻¹ IAA, 15 g l⁻¹ sucrose, 2 g l⁻¹ Gelrite, and 0.75 g l⁻¹ MgCl₂) and sealed with Parafilm. Plants were transferred into pots containing Vermiculite or Promix potting soil and covered with plastic bags with several small holes to allow for gradual acclimatization to ambient humidity. These plastic bags were removed after 1–3 weeks, depending on plantlet vigor.

The regeneration potential of Coker 312 was very high, ranging from 92 to 100%, while that of

Table 1. The effect of putrescine concentrations on somatic embryogenesis and embryo conversion for 5 Upland cottons cultured on different embryo initiation/maturation media

Medium (Put conc.)	Coker 312		PD 97019		PD 97021		PD 97100		GA 98033	
	RP	Conversion ^a	RP	Conversion ^a	RP	Conversion ^a	RP	Conversion ^a	RP	Conversion ^a
S15g.05NAA										
(Put-0)	95 (85)	42.3 ± 2.1	7 (60)	23.6 ± 8.2	5 (64)	13.6 ± 3.1	3 (63)	11.6 ± 5.6	12 (62)	41.3 ± 4.2
(Put-0.25)	92 (63)	48.6 ± 5.9	8 (60)	28.9 ± 4.0	8 (60)	8.9 ± 6.0	5 (61)	12.9 ± 3.0	10 (60)	37.9 ± 6.0
(Put-0.50)	97 (70)	51.9 ± 4.6	12 (69)	32.8 ± 2.8	5 (60)	18.9 ± 2.8	7 (60)	12.9 ± 2.8	13 (60)	53.9 ± 9.2
EMMS ₂										
(Put-0)	100 (60)	33.5 ± 9.3	15 (60)	26.4 ± 8.2	5 (60)	13.6 ± 2.8	0 (60)	0 ± 0	8 (68)	43.8 ± 3.3
(Put-0.25)	93 (60)	28.9 ± 8.3	17 (68)	20.9 ± 4.0	7 (62)	16.2 ± 5.3	0 (62)	0 ± 0	12 (60)	60.2 ± 6.3
(Put-0.50)	93 (60)	38.9 ± 2.8	17 (61)	28.9 ± 3.4	3 (60)	18.9 ± 1.4	0 (60)	0 ± 0	15 (60)	58.5 ± 1.7
EMMS ₄										
(Put-0)	92 (65)	66.5 ± 5.2	10 (60)	38.4 ± 6.1	0 (60)	0 ± 0	0 (60)	0 ± 0	10 (61)	50.1 ± 2.3
(Put-0.25)	97 (60)	55.9 ± 1.3	12 (60)	68.5 ± 2.2	0 (60)	0 ± 0	0 (65)	0 ± 0	15 (60)	49.9 ± 8.8
(Put-0.50)	92 (78)	58.8 ± 9.8	8 (60)	62.9 ± 9.8	0 (60)	0 ± 0	0 (60)	0 ± 0	17 (62)	58.9 ± 1.8

RP = regeneration potential: the percentage of seedlings producing somatic embryos, with the total number of seeds tested for each treatment given in parenthesis.

^aMean and standard error for the percentage of somatic embryos converted to plantlets.

Georgia and Pee Dee lines was low, ranging from 0 to only 17%, for all treatments (Table 1). This low percentage was not a surprise as seed-to-seed variability in embryogenesis ability was observed in almost all cotton cultivars tested when commercial or breeder's seed sources were used (Sakhanokno et al., 2001; Mishra et al., 2003). The high regeneration potential observed in Coker 312 was expected since the seed source was specifically selected for this character (K. Rajasekaran, personal communication). Our data indicated that regeneration potential was medium-dependent in some breeding lines. For example, the explants from PD 97100 produced SEs only on the S15g.05NAA-based media (Table 1), which contains sucrose as the carbon source with no kinetin. Similarly, PD 97021 produced no SEs on EMMS₄-based media where 2,4-D was used as the growth regulator. Both PD 97019 and GA 98033 responded similarly to all media with 0 Putrescine, with regeneration potential levels ranging from 7% on S15g.05NAA to 15% on EMMS₂. The inclusion of Putrescine in the media may have promoted somatic embryogenesis induction for some breeding lines. For example, a 0.5 mg l⁻¹ Putrescine concentration almost doubled the percentage of seedlings that produced SEs for GA 98033 on both the EMMS₂ and EMMS₄-based media, and for PD 97100 on S15g.05NAA-based media. However, this positive effect was not consistently observed in other lines with the same media combinations. Further study is needed to address this inconsistency, as a low regeneration potential can constitute a major hindrance to the use of these lines in genetic transformation.

As expected, significant differences ($p < 0.01$) in the number of SEs produced were observed among the cotton lines in all development/maturation culture medium tested. The inclusion of Putrescine in each of the media significantly ($p < 0.05$) enhanced the induction of embryogenic calluses to differentiate into SEs. For example, the 0.5 mg l⁻¹ Putrescine treatment in the S15.05NAA-based culture medium consistently outperformed the two treatments with a lower Putrescine concentration for all lines (Figure 1). The level of improvement was almost 2-fold (99.2 ± 2.2 versus 187.9 ± 3.8) and 4-fold (2.5 ± 1.0 versus 9.6 ± 3.5) in SE production when comparing S15g.05NAA without and with

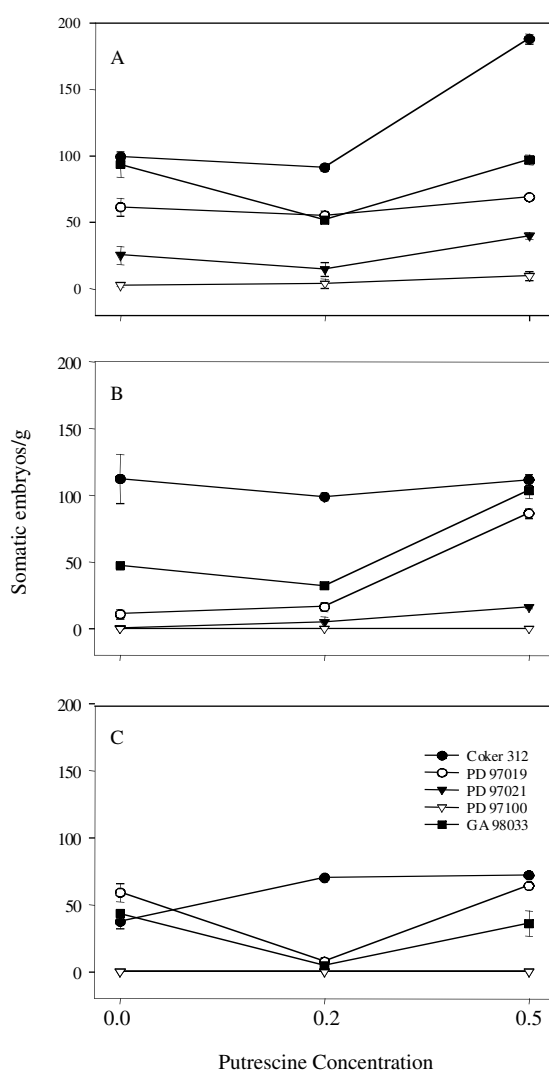


Figure 1. The effect of Putrescine treatments on somatic embryogenesis from calluses of cotton lines cultured on three media systems: (A) S15g.05NAA, (B) EMMS₂, and (C) EMMS₄.

0.5 mg l⁻¹ Putrescine for Coker 312 and PD 97100, respectively. Similarly, a noticeable improvement in SE production for PD 97019, PD 97021 and GA 98033 was observed with the addition of Putrescine to EMMS₂. In this culture medium, an 8- and 2-fold increase was obtained in SE production on 0.5 mg l⁻¹ Putrescine as compared to 0 Putrescine for PD 97019 and GA 98033, respectively. The most dramatic increase (53-fold) was achieved with the addition of 0.5 mg l⁻¹ Putrescine to EMMS₂ for PD 97021, which was essentially recalcitrant in this culture medium with no Putrescine treatment.

Addition of Putrescine to NAA-based treatments (EMMS₂ and S15g.05NAA) had a synergistic effect in promoting SE production. For both culture media, the best results were obtained with the treatments containing 0.5 mg l⁻¹ Putrescine.

A low conversion of SEs to plantlets can constitute a serious hurdle as it reduces the likelihood of recovering transformation events. Therefore, a good regeneration protocol should not only induce a high frequency of SEs but also subsequently convert those SEs into plantlets. To test the number of embryos converted to plantlets, mature embryos were transferred to new plates and the number germinated embryos with well-developed root systems and at least two well-developed cotyledons were counted at the end of a 4–6 week period. Although there were line effects in the percentage of plantlets regenerated, in general the treatments containing 0.5 mg l⁻¹ Putrescine generated the greatest percentage of plantlets (Table 1), the exceptions being Coker 312 and PD97019 on EMMS₄-based media, and GA98033 on EMMS₂-based media. This result suggests that the inclusion of Putrescine promotes SE germination and the recovery of plantlets. The percentage of plants obtained for GA 98033 was comparable to that of Coker 312, suggesting that this germplasm line could be another source of regenerable genotype useful for development of transgenic cotton.

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