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ORIGINAL PAPER

Effects of exogenous application of polyamines on wheat anther cultures

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Abstract Androgenesis of wheat genotypes was evaluated by pretreating anthers or embryo-like structures (ELS) with polyamines. Anthers of the genotype DH were pretreated with different concentrations of putrescine, spermidine, and spermine for 1, 3, and 6 h, and those of drought-tolerant International Center for Agricultural Research in the Dry Areas (ICARDA) wheat accessions were treated for 1 and 3 h. ELS of two genotypes were also treated for 30 and 60 min with the same polyamines and evaluated for green plant regeneration. The pretreatment of anthers with polyamines enhanced the development of ELS in all genotypes. The formation of ELS varied significantly with genotype. Pretreated anthers showed that four treatments improved significantly green plant regeneration with the genotype ICR 17. However, two treatments (1 mM putrescine or spermine for 1 h) significantly improved green plant regeneration per 100 ELS of only two ICARDA genotypes. ELS treated with polyamines for 30 min were greener and formed more adventitious roots. The chloroplasts of these greener ELS examined with a transmission electron microscope had agranal to grana thylakoids, while those of the control had plastids with mostly starch globules. Although exogenous application of polyamines to anthers improved the production of ELS and green plants, the effects of putrescine, spermidine, and spermine was dependent on genotype and the duration of pretreatment of anthers with the polyamines.

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Keywords Androgenesis · Chloroplast · Embryogenesis · Haploid · Plant regeneration · *Triticum aestivum*

Abbreviations

ELS	Embryo-like structures
GRP	Green regenerated plants
PAs	Polyamines
put	Putrescine
spd	Spermidine
spm	Spermine

Introduction

The production of doubled haploid (DH) plants is valuable in plant biotechnology because it enables breeders to obtain homozygous lines or plants directly from hybrid individuals (Bajaj 1990; Teparkum and Veilleux 1998). Androgenesis can potentially produce a large number of doubled haploid plants due to the high number of microspores per anther. However, it is still necessary to improve the application of this technique, because many wheat cultivars of agronomic importance show very low or no response to androgenetic induction. The main factors that hinder the application of anther and microspore cultures are the low rates of embryogenesis and the high frequency of albinism among regenerated plants. Conventionally, varying sugars, auxin, and/or cytokinin in the culture medium yielded variable results in a number of crops, including wheat (Navarro-Alvarez et al. 1994; Wojnarowiez et al. 2004). However, manipulating conventional methods still remains the major approach to maximize the production of green plants.

Polyamines (PAs) are naturally occurring, low molecular weight, polycationic, aliphatic nitrogenous compounds present in all cells. Polyamines are involved in a number of plant physiological processes such as morphogenesis, flower initiation, root growth, pollen viability, and biotic and abiotic stress responses (Bais and Ravishankar 2002; Bouchereau et al. 1999; Martin-Tanguy 2001). They are involved in plant nutrition in cultured tissues and are viewed as plant growth regulators or secondary hormonal messengers (Bais and Ravishankar 2002; Kakkar et al. 2000). In most plants, the levels of PAs are significantly greater than those of plant hormones. Several reports have shown the involvement of PAs in lateral and adventitious root formation (Couée et al. 2004). Anti-senescence or greening effect of PAs has also been reported in freshly isolated oat protoplasts (Altman et al. 1977) and studies with spermine and spermidine showed the retention of chlorophyll and the stabilization of thylakoid membranes (Besford et al. 1993; Tiburcio et al. 1994).

Exogenous PAs in culture medium induced in vitro organogenesis, and somatic and gametic embryogenesis in many plant species (Bertoldi et al. 2004; Kevers et al. 2002; Rajesh et al. 2003). In culture medium, PAs have been shown to increase the number of gynogenic embryos in *Allium cepa* L. (Martínez et al. 2000), androgenic embryos in potato (Tiainen 1992), in some Indian wheat cultivars (Rajyalakshmi et al. 1995), and in cucumber (Ashok Kumar et al. 2004). However, rice culture lost regeneration capacity with increasing age (Bajaj and Rajam 1995). The application of putrescine to barley and oat anthers delayed senescence of cultured anthers, and increased embryogenesis and plant regeneration in some recalcitrant genotypes (Cho and Kasha 1992; Kelley et al. 2002). Laukkanen and Sarjala (1997) showed that the exogenous application of PAs affected the metabolism of pine callus but did not prevent senescence. In embryogenesis of *Solanum melongena* hypocotyl segments, putrescine increased at maturity (Sharma and Rajam 1995), but in *Camellia*, both soluble and conjugated spermidine increase during the development of globular embryo (Pedroso et al. 1997).

In anther and microspore culture, the type of pretreatment and the carbohydrate used in culture medium are factors that affect barley and wheat androgenesis (Cistué and Kasha 2006; Devaux and Kasha 2009; Redha and Talaat 2008). In green plant regeneration, however, osmotic potential and the carbohydrate source also played significant roles in the switch from gametophytic to sporophytic phase of barley and wheat anthers. Most of the research conducted on the effects of polyamines were on somatic culture, and only a few investigated the influence of PAs on gametic embryogenesis (Martínez et al. 2000). Based on the various roles or functions of PAs and the limited studies on the effect of PAs in androgenesis, particularly in wheat, we hypothesized that the application of PAs to anthers in the initial phase of wheat androgenesis and/or to embryo-like structures (ELS) could influence green plant regeneration or chloroplast development, thus, enhancing the greening of regenerated plants.

The objective of this study was, therefore, to evaluate the effects of putrescine (put), spermidine (spd), and spermine (spm) as pretreatments of anthers or in the initial phase of wheat anther culture on ELS and regeneration of green plants.

Materials and methods

Planting material

Seeds of donor plants (DH, ICR 1, ICR 4, ICR 17, ICR 33, and ICR 39) were germinated at room temperature on moistened germination paper (Anchor paper #38, St. Paul, Minnesota, USA). After 7 days, seedlings were transferred to pots (10 cm in diameter) containing peat moss and placed in growth chambers at 18 ± 1°C, under 16-h photoperiod, and 290–310 μmol m^{-2} s^{-1} light intensity. After 3 weeks, the plants were transferred to 15-cm-diameter pots. The actively growing plants were fertilized every fortnight with a complete and balanced fertilizer until flowering stage. Spikes were collected when most of the microspores were at mid- to late-uninucleate stage (Schmid 1990). The developmental stage of microspores was determined using acetocarmine stain. Developed tillers were selected and kept at 4°C for 7–10 days.

Anther culture

The media used in anther culture were liquid anther induction medium (AM), embryo medium (EM), and plant regeneration medium (PM) (Schmid 1990). Phytagel (3 gl^{-1}) was added to the EM and PM media. Donor spikes were sterilized in 70% ethanol for 1 min, and the anthers were dissected from flowers as described previously (Redha et al. 1998), and then plated in 60 × 15-mm Petri dishes containing 10 ml of liquid anther induction medium. The number of replicates was ten per treatment, with 36 anthers in each Petri dish. Anthers were incubated in the dark at 28°C for 7 weeks. ELS produced were counted and transferred to the PM at 27°C at a photoperiod of 16 h and light intensity of 55 μmol m^{-2} s^{-1}. The criteria used to determine ELS was described by Büter et al. (1991). An ELS structure comprised of callus and/or embryo. Embryos readily stained in acetocarmine squashed preparation, while callus cell consisted of thick-wall parenchymatous cells with translucent or faintly stained cytoplasm. Young

embryos consisted of isodiametric, meristematic cells with intensely stained nucleus and cytoplasm (Shumann 1990). After 4 weeks, regenerated green plants with well-developed roots and shoots were recorded and transferred to peat moss for further development.

Screening of genotypes for green plant regeneration

A total of 50 plant accession genotypes provided by the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, were screened for androgenetic performance with respect to the formation of ELS and green plant regeneration capacity. These genotypes were selected for their tolerance to drought conditions. The genotype, DH83Z118.32 (DH), provided by the Swiss Federal Institute of Technology, ETH, Zurich, was included for its high androgenetic response. Donor plants were grown and anthers cultured as described above.

Pretreatments of anthers with PAs

Anthers of the genotype DH were pretreated with put, spd, and spm alone at 1 mM of each PA or 0.5 + 0.5 mM combinations of each of the three PAs. The durations of the pretreatments were 1, 3, and 6 h. The two best pretreatment periods were used in the anther culture of ICARDA genotypes. Anthers of the genotypes ICR 1, ICR 4, ICR 17, ICR 33, and ICR 39 were pretreated with put, spd, and spm alone at 1 mM or in combinations of two of the three PAs at 0.5 mM. The durations of pretreatments were 1 and 3 h, determined in a previous test using DH genotype, after which anthers were placed in AM containing sucrose (0.29 M).

Treatment of ELS with PAs

The ELS of two genotypes, ICR 4 (recalcitrant in terms of GRP/100 ELS produced) and ICR 39 (moderate response), were treated with PAs for 30 and 60 min. PA treatments were with 1 mM of put, spd, spm, and 0.5 mM mixtures of PAs.

Microscopy

ELS tissues obtained were randomly sampled for transmission electron microscopy (TEM). Tissues were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed three times with cacodylate buffer, and post-fixed in 1.0% osmium tetroxide in the same buffer. The samples were dehydrated in a graded concentration of ethanol and embedded in epoxy resin. Semi-thin sections were cut and stained with toluidine blue and examined under a light microscope. Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT) and stained with uranyl acetate, followed by lead citrate. The stained sections were examined in a transmission electron microscope (JEOL JEM-1200EX II). Micrographs were taken at 80 kV on Kodak 4489 ESTAR thick-base photographic EM film.

Experimental design and data analysis

The experiment was designed to determine the influence of PAs on wheat genotypes. Firstly, 50 wheat accession genotypes were evaluated as described earlier to determine androgenesis response. Then, five genotypes were selected for the pretreatment of anthers with PAs. The DH genotype was used as a standard and the anthers were pretreated for 1, 3, and 6 h to determine how long anthers should be pretreated with PAs to improve plant regeneration. Then, anthers of five ICARDA genotypes were pretreated with 1.0 mM of putrescine, spermidine, and spermine, and 0.5 mM mixtures of each PA. Two genotypes (i.e., one recalcitrant and the other moderate in response with respect to GRP/100 anthers) were selected from the five and their ELS were pretreated with PAs. The effects of PAs on the genotype response was evaluated on the basis of ELS produced and green plant regeneration. ELS were recorded per 100 anthers (ELS/100 anthers), as well as green regenerated plants (GRP/100 anthers or ELS) (Broughton 2008; Büter et al. 1991; Redha et al. 2000). Each experiment was repeated three times. The data were pooled and subjected to analysis of variance (ANOVA), and the means were separated according to Fisher's protected least significant difference (LSD) using MSTAT-C. Data in percentages were arcsine transformed prior to analysis.

Results

Screening of genotypes

The androgenetic response of the 50 ICARDA genotypes showed significant differences with respect to ELS and GRP production among genotypes. Twenty-five genotypes formed less than 10% ELS (genotypes not listed, Table 1), including ICR 35, which did not produce any ELS. Approximately 40% of anthers of the genotypes ICR 4, ICR 5, ICR 17, ICR 18, ICR 36, and ICR 44 developed ELS, but most of these ELS differentiated into albino plants. Six genotypes (ICR 6, ICR 32, ICR 34, ICR 41, ICR 22, and ICR 2) regenerated 9–20% green plants from their ELS (Table 1). Generally, albinism was a problem with all genotypes; at least 60% of all ELS of the genotypes developed albino plants.

Table 1 The androgenic response of ICARDA genotypes with respect to ELS and GRP formation

Embryogenesis			Green plant regeneration		
No. of genotypes[a]	ICARDA wheat accession number[b]	% ELS	No. of genotypes[a]	ICARDA wheat accession number[b]	% GRP
25	N.L.	0–10	33	N.L.	0–4
12	N.L.	11–20	11	N.L.	5–8
7	ICR 1, 13, 33, 38, 39, 46, 49	21–30	4	ICR 6, 32, 34, 41	9–12
0	0	31–40	1	ICR 22	13–16
6	ICR 4, 5, 17, 18, 36, 44	41–60	1	ICR 2	17–20

N.L. Genotypes not listed

[a] Represents the number of genotypes that produced ELS and GRP

[b] Shows the list of genotypes that produced ELS and GRP

Effect of PAs on anthers of DH genotype

The effects of PAs on DH anthers are presented in Figs. 1, 2, and 3. Androgenic response was within 3–4 weeks for all treatments. Treatment differences were significant for GRP/100 anthers and GRP/100 ELS at $P = 0.05$. The 1-h treatment showed that 1 mM spm enhanced the formation of GRP/100 ELS (Fig. 1). There was no significant difference between the control and 1 mM spd and 0.5 mM (put +spd) with respect to GRP/100 ELS. Anther culture showed that spd and spm at 1 mM enhanced the production of green plants in comparison to the control. All other PA treatments for 1 h yielded significantly fewer GRP/100 anthers. The 3-h treatments did not improve the formation of GRP/100 anthers, while 0.5 mM (put + spd) only improved the development of GRP/100 ELS (Fig. 2). For 6-h treatments, 1 mM spm and 0.5 mM (put + spm) produced a significantly higher number of GRP/100 ELS (Fig. 3). The green plants produced per 100 anthers by all treatments were significantly less than the control.

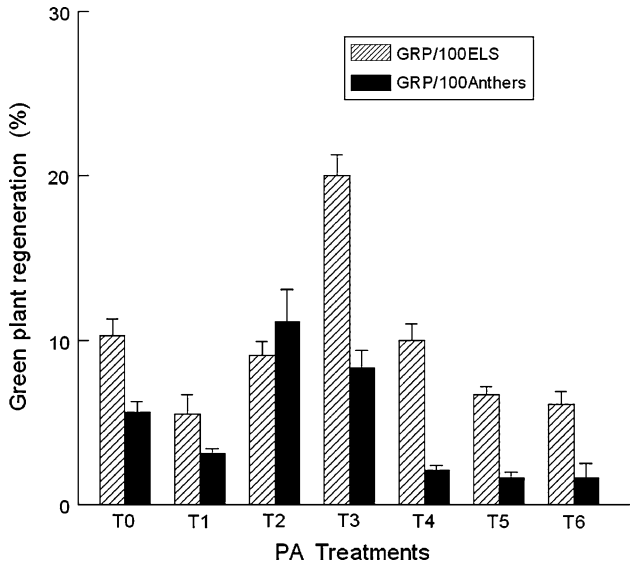

Fig. 1 The effect of PAs applied to DH anthers for 1 h. Treatment abbreviations: T0 = control; T1 = 1 mM putrescine; T2 = 1 mM spermidine; T3 = 1 mM spermine; T4 = 0.5 mM (putrescine + spermidine); T5 = 0.5 mM (putrescine + spermine); and T6 = 0.5 mM (spermidine + spermine). The *bars* represent the standard error of the mean

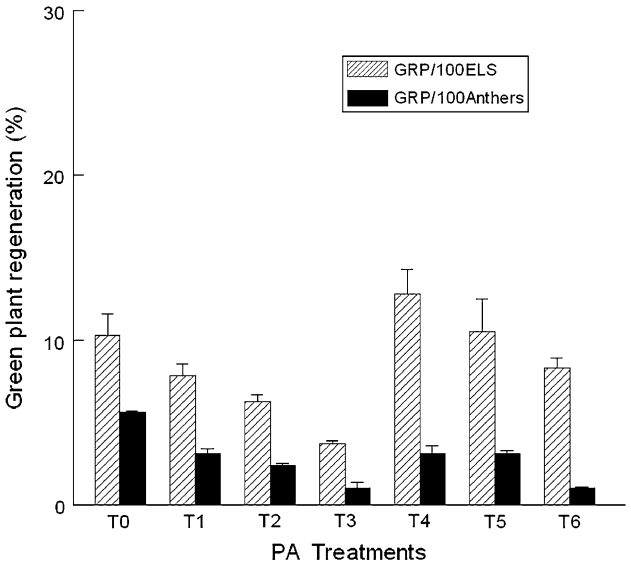

Fig. 2 The effect of PAs applied to DH anthers for 3 h. Treatment abbreviations: T0 = control; T1 = 1 mM putrescine; T2 = 1 mM spermidine; T3 = 1 mM spermine; T4 = 0.5 mM (putrescine + spermidine); T5 = 0.5 mM (putrescine + spermine); and T6 = 0.5 mM (spermidine + spermine). The *bars* represent the standard error of the mean

Effect of PAs on anthers of ICARDA accession genotypes

Tables 2, 3, and 4 show the effects of PA treatments on the anthers of five selected ICARDA genotypes. The selection was based on the results of the screening test where the genotypes showed at least 20% ELS production or about 20% GRP. The effects of the treatments were variable for the genotypes. Compared to the controls, most of the PA

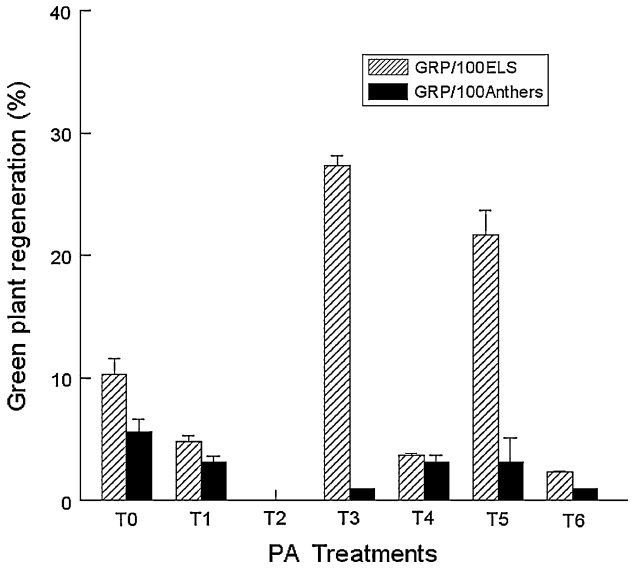

Fig. 3 The effect of PAs applied to DH anthers for 6 h. Treatment abbreviations: T0 = control; T1 = 1 mM putrescine; T2 = 1 mM spermidine; T3 = 1 mM spermine; T4 = 0.5 mM (putrescine + spermidine); T5 = 0.5 mM (putrescine + spermine); and T6 = 0.5 mM (spermidine + spermine). The *bars* represent the standard error of the mean

treatments improved the production of ELS/100 anthers for all of the genotypes (Table 2). spm at 1 mM for 3 h enhanced ELS/100 anthers in ICR 4 and ICR 33. Other treatments that showed significant improvement in ELS/100 anthers were 1 mM put for 3 h in ICR 1, 0.5 mM (put + spm) for 3 h in ICR 17, and 0.5 mM (put + spd) for 1 h in ICR 39.

Generally, 4 out of the 5 ICARDA genotypes showed improvement in the GRP formation/100 ELS, except for ICR 33 (Table 3). There were no significant differences between the treatments for ICR 1 and ICR 4 with respect GRP/100 ELS. A significant difference in PA treatments was observed in the genotypes ICR 17 and ICR 39. The best treatments for these two genotypes were 1 mM spm for 1 h and 1 mM put for 1 h, respectively.

Effect of PAs on ELS of ICR 4 and ICR 39

ELS of ICR 4 treated with 1.0 mM put and spd, and those of ICR 39 treated with 0.5 mM (put + spm) for 30 min produced slightly larger ELS and numerous adventitious roots compared to the control (Fig. 4a). In general, adventitious root production was enhanced with the combined PA treatments (data not shown). Treatment with put + spm at 0.5 mM for 30 min produced the most greenish ELS in genotype ICR 39 (Fig. 4a). All ELS treated with PAs for 60 min showed reduction in the growth and root proliferation (Fig. 4b). The only treatment that produced green plants (both roots and green shoot) in ICR 4 was 0.5 mM (spd + spm) after treatment for 30 min (Fig. 4c), but the same treatment produced only albino plants with ICR 39.

Microscopy

Microscopy showed that the initial stage in the formation of ELS of both treated and untreated anthers was characterized by the formation of irregular cells, some of which appeared highly vacuolated. As the cells of the ELS from untreated anthers continued to proliferate, they accumulated mostly plastids with starch globules (Fig. 5a). The starch globules were depleted as the cells aged. put and

Table 2 The effect of PAs on the production of ELS/100 anthers in ICARDA wheat genotypes

Treatments	ICR 1	ICR 4	ICR 17	ICR 33	ICR 39
Control	60.2 f	46.1 d	38.7 e	19.7 c	10.6 c
1 mM put for 1 h	67.5 f	46.8 d	36.1 e	25.2 bc	17.6 b
1 mM spd for 1 h	69.4 ef	58.2 c	21.3 f	40.6 a	14.2 c
1 mM spm for 1 h	47.5 g	42.8 d	26.6 f	27.9 b	21.6 ab
0.5 mM put + 0.5 mM spd for 1 h	63.9 f	36.5 e	51.7 cd	28.6 b	24.8 a
0.5 mM put + 0.5 mM spm for 1 h	114.1 b	60.4 b	48.9 d	29.8 b	23.6 a
0.5 mM spd + 0.5 mM spm for 1 h	105.8 c	47.2 d	63.4 b	28.0 b	20.4 ab
1 mM put for 3 h	125.6 a	53.2 c	56.6 c	22.8 c	17.3 b
1 mM spd for 3 h	72.0 e	50.3 c	38.2 e	24.9 bc	23.8 a
1 mM spm for 3 h	95.5 d	70.8 a	62.2 b	47.6 a	15.6 b
0.5 mM put + 0.5 mM spd for 3 h	63.9 f	49.7 cd	65.9 b	43.1 a	11.1 c
0.5 mM put + 0.5 mM spm for 3 h	93.4 d	53.7 c	76.1 a	29.5 b	17.7 b
0.5 mM spd + 0.5 mM spm for 3 h	66.2 f	38.9 e	38.7 e	21.3 c	12.1 c

PAs polyamines; *ELS* embryo-like structures; *put* putrescine; *spd* spermidine; *spm* spermine

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ as determined by LSD

Table 3 The effect of PAs on the production of GRP/100 ELS in ICARDA wheat genotypes

Treatments	ICR 1	ICR 4	ICR 17	ICR 33	ICR 39
Control	0.3*	0.3*	15.5 a	9.2 a	3.5 b
1 mM put for 1 h	0.3	0.3	9.5 b	2.5 b	7.4 a
1 mM spd for 1 h	1.8	0.7	3.7 d	2.7 b	0.0 c
1 mM spm for 1 h	2.0	0.7	18.1 a	3.3 b	0.9 c
0.5 mM put + 0.5 mM spd for 1 h	0.8	0.0	10.0 b	1.1 b	1.4 c
0.5 mM put + 0.5 mM spm for 1 h	1.2	0.0	5.7 d	3.1 b	2.9 bc
0.5 mM spd + 0.5 mM spm for 1 h	0.4	0.3	12.7 ab	2.5 b	1.0 c
1 mM put for 3 h	2.7	0.5	12.5 ab	1.7 b	2.3 bc
1 mM spd for 3 h	0	0.0	11.1 b	1.2 b	0.0 c
1 mM spm for 3 h	0	0.0	11.4 b	2.0 b	3.6 b
0.5 mM put + 0.5 mM spd for 3 h	1.2	0.0	5.8 d	3.9 b	5.4 ab
0.5 mM put + 0.5 mM spm for 3 h	0.7	0.0	8.9 c	2.9 b	0.9 c
0.5 mM spd + 0.5 mM spm for 3 h	1.8	0.0	9.0 c	1.5 b	2.7 bc

PAs polyamines; *GRP* green regenerated plants; *ELS* embryo-like structures; *put* putrescine; *spd* spermidine; *spm* spermine

* Not significantly different

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ as determined by LSD

Table 4 The effect of PAs on the production of GRP/100 anthers in ICARDA wheat genotypes

Treatments	ICR 1	ICR 4	ICR 17	ICR 33	ICR 39
Control	0.2*	0.2*	6.0 b	1.8*	0.4*
1 mM put for 1 h	0.2	0.1	3.4 d	0.6	1.3
1 mM spd for 1 h	1.2	0.4	0.8 e	1.1	0.0
1 mM spm for 1 h	0.9	0.3	4.8 c	0.9	0.2
0.5 mM put + 0.5 mM spd for 1 h	0.5	0.0	5.1 b	0.3	0.4
0.5 mM put + 0.5 mM spm for 1 h	1.4	0.0	2.8 e	0.9	0.7
0.5 mM spd + 0.5 mM spm for 1 h	0.4	0.1	8.0 a	0.7	0.2
1 mM put for 3 h	3.3	0.3	7.1 a	0.4	0.4
1 mM spd for 3 h	0	0.0	4.2 c	0.3	0.0
1 mM spm for 3 h	0	0.0	7.1 a	1.0	0.6
0.5 mM put + 0.5 mM spd for 3 h	0.7	0.0	3.9 c	1.7	0.6
0.5 mM put + 0.5 mM spm for 3 h	0.6	0.0	6.8 ab	0.9	0.2
0.5 mM spd + 0.5 mM spm for 3 h	1.2	0.0	3.4 d	0.3	0.3

PAs polyamines; *GRP* green regenerated plants; *put* putrescine; *spd* spermidine; *spm* spermine

* Not significantly different

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ as determined by LSD

spd-treated anthers or ELS developed chloroplasts with thylakoid systems that were well organized into grana. The chloroplasts from put appeared to be well developed with plastoglobules with apparently no starch granules (Fig. 5b). spm treatment had more agranal-like chloroplasts with thylakoids that were not well organized as grana, or had few grana with a couple of starch grains (Fig. 5c). These chloroplasts (spm-treated) were not only slow in their development, but they appeared to be smaller in size compared to those from put and spd treatments. ELS treated with PAs also developed more meristemoids or centers of meristematic cells with dense cytoplasm. These meristemoids appeared to give rise mostly to adventitious roots.

Discussion

PAs are compounds involved in a number of developmental processes in plants and, although much research has been conducted on the effects of PAs on tissue culture, only a few researchers have studied their influence on gametic

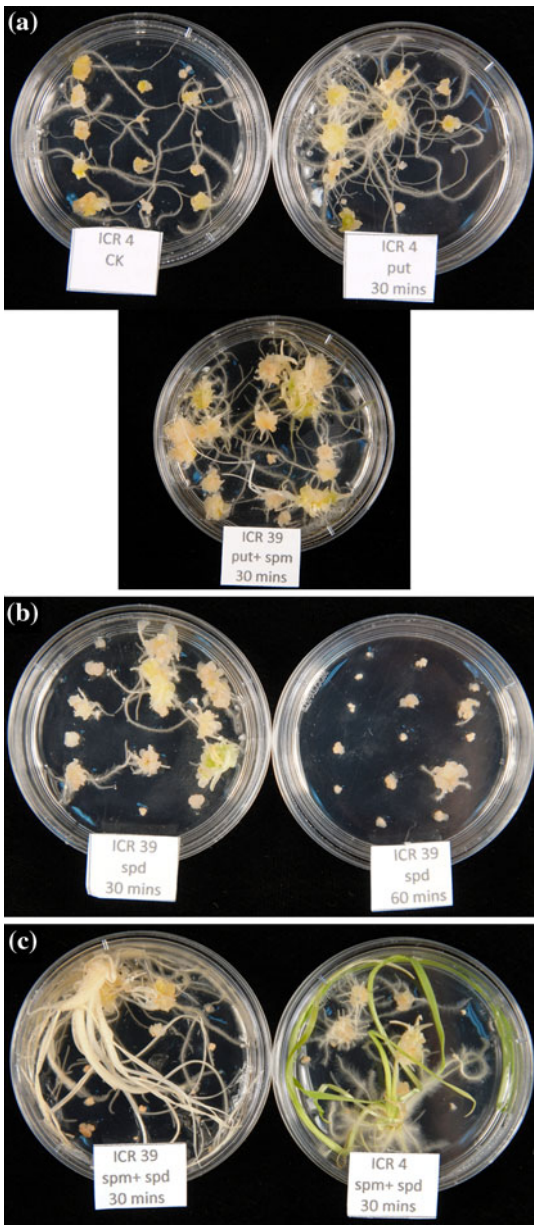

Fig. 4a–c The effect of PAs on ICARDA wheat genotypes. **a** The effect of 1 mM putrescine (put) and 0.5 mM (put + spm) on ELS of ICR 4 and ICR 39 for 30 min. *CK* = control. **b** The effect of 1 mM spermidine (spd) on ELS of ICR 39 wheat genotype for 30 and 60 min. **c** The effect 0.5 mM (spm + spd) on ELS of ICR 39 and ICR 4 wheat genotypes for 30 min. *spm* = spermine and *spd* = spermidine

embryogenesis (Martínez et al. 2000). In this study, the effects of PAs on anthers and the duration of pretreatment showed that the ELS and GRP formation varied among genotypes. Exogenous application of PAs to anthers for 1 h appeared to exert a weak plant growth regulator-like effect on anthers with the formation of calli and regenerants having more adventitious roots. Shariatpanahi et al. (2006) reported similar observations in cultured wheat microspore, in which the medium was supplemented with 1 mgl^{-1} 2,4-D plus 0.2 mgl^{-1} kinetin. In this study, ELS or GRP/100 anthers was enhanced particularly with put, spd alone, or a combination of put, spd, or spm (Kelley et al. 2002; Meijer and Simmonds 1988; Phillips et al. 1987; Rajyalakshmi et al. 1995). Similar results were observed with the application of put to some recalcitrant barley (Cho and Kasha 1992), oat anther cultures (Kelley et al. 2002), and rice anther culture (Dewi and Purwoko 2008).

The production of ELS for some of the genotypes increased over 100-fold and exogenous application of the PAs to these ELS resulted in more root proliferation, greener ELS, and/or green plants. This study corroborates the observation of Couée et al. (2004), who reported the involvement of PAs in lateral and adventitious root formation. The greening or anti-senescence effects of PAs was also reported in freshly isolated oat protoplasts and rice anther culture (Altman et al. 1977; Dewi and Purwoko 2008), and studies with spm and spd showed the retention of chlorophyll and stabilization of thylakoid membranes (Besford et al. 1993; Tiburcio et al. 1994).

The greener ELS observed in this study was due to the development of agranal-like to normal chloroplasts in the undifferentiated ELS cells. In wheat androgenesis, this is the first report of this phenomenon of greening of ELS. This led to the speculation of the involvement of paternal inheritance of chloroplasts in the genotypes used in this study. Paternal inheritance of chloroplasts was observed in some crosses of agronomic crops such as *Hordeum* × *Secale* (Soliman et al. 1987), and chloroplast inheritance of this nature is mostly common in interspecific hybrids (Hansen et al. 2007).

The effects of PAs in this study was not generalized in the genotypes, since some of the genotypes still showed no improvement in the development of ELS or GRP/100 anthers. During androgenesis of *Triticum aestivum* (Bothma and Duberry 1991), *Nicotiana tabacum*, and *Datura innoxia* (Villanueva et al. 1985), put and spd were found to be the major PAs, and could be relevant in anther culture. Recent studies with wheat have also shown genetic variability in androgenic traits with respect to embryo induction and plant regeneration (Liu et al. 2002; Zheng 2003).

In summary, this study showed that low concentrations of put or together with spd or spm could be used in androgenesis of some recalcitrant wheat genotypes to improve ELS induction and regeneration of green plants. Green plant regeneration was enhanced when anthers or ELS at the initial stage of development were pretreated with put, spd, and spm for 1–3 h. The application of PAs appeared to improve androgenesis by increasing cell proliferation of cultured anthers and ELS production that regenerated to green plants. This study also indicated that the treatment of anthers at the induction phase with PAs is

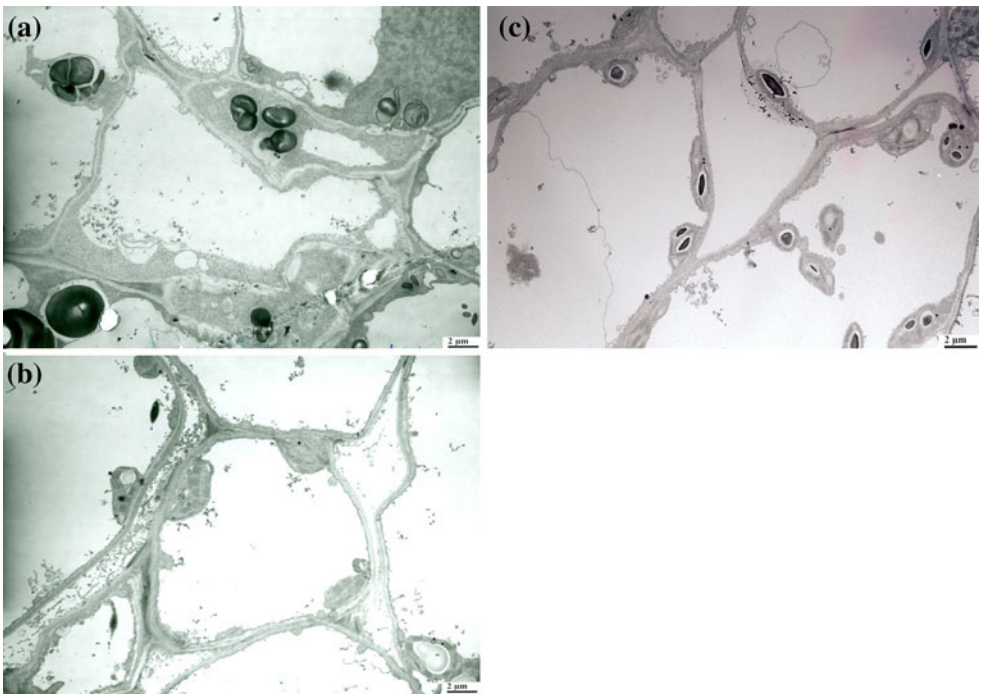

Fig. 5 Electron micrographs of untreated and PA-treated ELS. **a** Cells of control treatment with plastids of starch globules. **b** Cells of ELS from putrescine treatment with well-developed chloroplasts and plastoglobules. **c** Cells of ELS from spermine treatment with agranal-like chloroplasts

preferable to treating ELS, even in genotypes poor or recalcitrant in anther culture response.

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