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ORIGINAL PAPER

Effect of polyamines on in vitro anther culture of *Citrus clementina* Hort. ex Tan

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Abstract The improvement of the induction rate in *Citrus* anther culture is important for taking practical advantage of the haploid potential in breeding. The influence of polyamines on anther culture of *Citrus clementina*, cv Nules, with particular attention to the free, soluble and insoluble-conjugated polyamine levels, has been investigated. Putrescine, spermidine and putrescine plus spermidine, were added to the standard induction medium. Before culture, spermidine was the most abundant among the free polyamines detected in anthers. The exogenous supply of either putrescine or spermidine, either independently or combined, effected greater uptake and accumulation of polyamines. The addition of 2 mM spermidine to the medium stimulated gametic embryogenesis in clementine Nules, whereas putrescine did not influence embryo production. Regenerants were mostly tri-haploids; a few doubled-haploids and no haploid plants were obtained.

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Abbreviations

DAP	Diamino propane
PAs	Polyamines
PCA	Perchloric acid
PGI-1	Phosphoglucoisomerase
PGM	Phosphoglucomutase
PUT	Putrescine
SNK's test	Student–Newman–Keuls's test
SPD	Spermidine
SPM	Spermine

Introduction

To capitalize on the haploid potential in *Citrus* breeding we need to increase the number of responsive genotypes and to improve the induction rate (the frequency of pollen grains that form embryos) (Germanà et al. 2005). Genotype, pretreatments to the anthers, pollen developmental stage, medium composition and cultural conditions influence haploid recovery through anther culture. Since the first embryogenic calli and haploid plantlets were obtained by anther culture in *Citrus clementina* Hort. ex Tan., cv. Nules

(Germanà et al. 1994), many studies have been conducted to improve the androgenic response in *Citrus* species by using different combinations of plant growth regulators (Hidaka et al. 1979; Hidaka 1984; Chaturvedi and Sharma 1985; Chen 1985; Geraci and Starrantino 1990; Germanà et al. 1991, 1994, 2000; Germanà and Chiancone 2003).

Aliphatic polyamines (PAs), such as putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM), are low molecular mass polycations present in all living organisms (Martin-Tanguy 2001; Bagni and Tassoni 2001). These growth regulators have been implicated in several plant growth and development processes including cell division, morphogenesis, flower initiation, pollen tube growth and senescence (Bagni and Tassoni 2001). Several reports have indicated polyamine involvement in lateral and adventitious root formation (Couée et al. 2004). Endogenous PAs have been implicated in the initial stages of adventitious rooting in many woody plants, like *Prunus avium* (Biondi et al. 1990), *Populus tremula* L. × *P. tremuloides* L. (Hausman et al. 1995), *Pyrus communis* (Baraldi et al. 1995) and *Juglans regia* (Heloir et al. 1996). In *Olea europaea* L., exogenous PAs seem to have a positive effect on in vitro rooting induction of microcuttings (Rugini 1992; Grigoriadou et al. 2002). Because of their presence in meristematic and growing tissues (Torrigiani et al. 1989), PAs can also be considered, together with cytokinins, juvenility markers.

Exogenous PAs in culture medium seem to induce in vitro organogenesis and somatic and gametic embryogenesis in many species (Mengoli et al. 1989; Faure et al. 1991; Kevers et al. 2002; Rajesh et al. 2003; Bertoldi et al. 2004). Moreover, PAs in the culture medium increased the number of gynogenic embryos in *Allium cepa* L. (Martinez et al. 2000) and androgenic embryos in potato (Tiainen 1992), in some Indian wheat cultivars (Rajyalakshmi et al. 1995) and in cucumber (Ashok Kumar et al. 2004). The aim of this research was to investigate the influence of polyamines on androgenesis induction in anther culture of *Citrus clementina*, cv Nules, with particular attention to the free, soluble and insoluble-conjugated polyamine levels.

Material and methods

Plant material

Flower buds were harvested in March from 25-year-old trees of *Citrus clementina* Hort. ex Tan., cv Nules, grown at the Istituto di Genetica Vegetale, National Research Council (C.N.R.) in Lascari (Palermo, 38°N). Nules, discovered as a bud mutation on a Fina tree (Saunt 1990), is one of the most extensively cultivated clementine cultivars.

Microspore developmental stage

Microspore developmental stage was determined in one anther per flower bud by DAPI (4,6 diamidino 2-phenylindol dichloride) staining. Anthers from buds of different sizes were squashed in a few drops of DAPI solution (1 mg ml^{-1}) and observed under a fluorescent microscope. For further experiments, only flower buds of 3.5–4.0 mm, bearing anthers with microspores at the uninucleate stage (Fig. 1A), were selected for culture.

Anther culture

After cold pre-treatment (floral buds collected and stored in the dark at 4°C for 14 days), flower buds were surface sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, 20 min in 25% (v/v) commercial bleach (about 0.5% active chlorine in water) and rinsed three times with sterile distilled water. Petals were aseptically removed with small forceps and anthers were placed in 60 mm diameter Petri dishes containing 10 ml solid medium. The induction was performed on N6 medium (Chu 1978), supplemented with Nitsch and Nitsch vitamins (Nitsch and Nitsch 1969), galactose (0.1 M), lactose (0.1 M), coconut water (5% v/v) (Sigma, Milan, Italy), caseine hydrolisate (0.05% w/v), L-glutamine (1.3 mM), biotin (2 μM), ascorbic acid (2.8 mM). Moreover, a combination of growth regulators was added: 0.1 μM 2,4-D (2,4-dichlorophenossiacetic acid), 0.1 μM NAA (α-naphthalene acetic acid), 5 μM KI (kinetin), 4 μM 6-BA (6-benzyladenine), 2 μM

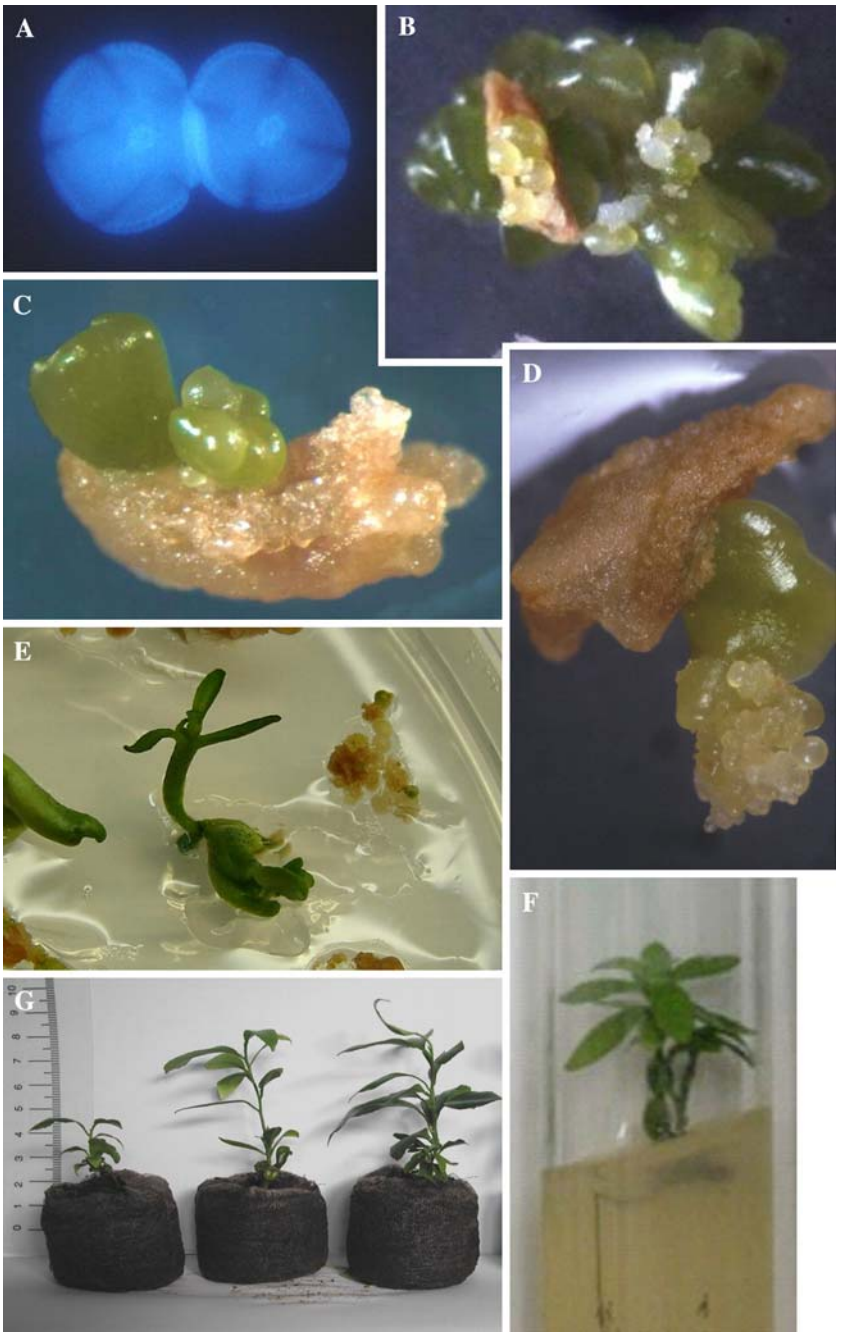

Fig. 1 Microspores of *C. clementina* Hort ex Tan. at the uninucleate stage (**A**); clumps of gametic embryos from a clementine''Nules'' anther (**B**); direct embryogenesis from a single anther (**C**); secondary embryogenesis (**D**); embryo germination (**E**); haploid plantlet of ''Nules'' obtained from embryo germination (**F**); homozygous plantlets transferred in vivo (**G**)

ZEA (zeatin), 2 μM TDZ (thidiazuron). The pH was adjusted to 5.8 with 1 N KOH and 0.8% (w/v) agar (Type Washed, Sigma) was added. For polyamine treatments, 0 mM (control), 2 mM putrescine (PUT), 2 mM spermidine (SPD) or 1 mM each of putrescine and spermidine (PUT + SPD), were added to the standard medium before autoclaving (20 min, 120°C). Petri dishes with anthers (60 anthers per Petri dish) were incubated at 27 ± 1°C for 15 days in the dark, and then placed under cool white fluorescent lamps (Philips TLM 30W/84, France) with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a photoperiod of 16 h. A randomized

complete block design was used for the experiment. Ten Petri dishes, containing anthers from 3 to 4 flower buds, were used for each experimental treatment (about 600 anthers per treatment, approximately 2,400 anthers in total). After 10 months of culture without subculture, anther response was recorded in terms of callus production, swollen anthers, and non-responding anthers. The significance of the treatments was determined using analysis of variance (ANOVA). Percentage data were subjected to arcsin-square root transformation before analysis. The variation among treatment means was analyzed using Student–Newman–Keuls's test (SNK's test).

Embryo germination

Embryogenic calli and germinated embryos were transferred in MS medium (Murashige and Skoog 1962) containing MS vitamins, 0.1 M galactose, 0.1 M lactose, 3 μM gibbereltic acid (GA_3), 0.1 μM NAA and 0.8% (w/v) agar. As soon as embryos reached 2–3 mm in size, they were transferred to Magenta boxes or test tubes with the same medium. Embryos were incubated in the light at 27 ± 1°C (with a 16-h photoperiod).

Plant recovery

Plantlets 4–5 cm high were transferred, for the hardening phase, to the greenhouse in Jiffy pots or in pots containing sterilized peat moss, sand and soil in the ratio of 1:1:1, with 100% humidity for 40–50 days.

Ploidy analysis

Tissues (200 mg) from calli, embryos, plantlets or plants were chopped with a razor blade in the presence of 1 ml nuclei extraction buffer (CyStain® UV Solution A, Partec, Münster, Germany). Tissue from the mother plant, known to be diploid, was added as a control. The suspension was filtered through 30 μm partec CellTrics® (Partec, Münster, Germany) and mixed with 4 ml of DAPI stain (CyStain® UV Solution B, Partec, Münster, Germany). Relative DNA content of the sample was determined using a Partec Cell analyser PA II (Partec GmbH, Münster, Germany). A total of 40 regenerants was analyzed: ten each from the control, PUT medium, SPD medium and PUT + SPD medium.

Isozyme analysis

Isozyme techniques enable us to distinguish androgenic vs. somatic tissue when an enzyme is heterozygotic in the diploid donor plant (Germanà 2003). For example, *Citrus clementina* is heterozygous for PGI-1 and PGM. To identify the origin of calli, embryos and plantlets obtained, we analyzed their crude extracts using two enzyme systems: phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM), as reported by Grosser et al. (1988). Numbering for isozymes (PGI-1) and lettering for different allozymes are according to Torres et al. (1978).

Polyamine determination

The anther polyamine content was analyzed at time zero (just after collection from the tree and before culture and cold pretreatment) and after 10 months of culture in both control medium and those supplemented with the different polyamines. Polyamine analyses were performed according to Tassoni et al. (2000). Anther samples (about 0.2 g FW) were homogenized in 10 volumes of 4% (v/v) cold perchloric acid (PCA) and centrifuged at 20,000*g* for 30 min at 4°C. The pellet was washed three times and resuspended to the original volume with PCA 4%. Triplicates of this suspension and of the supernatant were hydrolysed with 6 N HCl in flame-sealed glass ampoules at 110°C for 20 h in order to release PAs from their conjugates. Aliquots (0.2 ml) of supernatant (free PAs), hydrolysed supernatant (PCA-soluble conjugated PAs) and hydrolyzed pellet (PCA-insoluble-conjugated PAs) were derivatized with dansyl-chloride (3 mg ml^{-1} acetone), extracted with toluene and analyzed by HPLC (JASCO, Tokyo, Japan) with a reverse phase C_{18} column (Spherisorb ODS2, 5 μM particle diameter, 4.6 × 250 mm, Phenomenex, Torrance, CA, USA) as described by Tassoni et al. (2000). Experiments were repeated twice with similar results.

Results and discussion

Embryogenesis and plantlet recovery

After 1 week of culture, most of the anthers were swollen, and after 1 month two types of calli, type A and type B, were produced. Type A calli appeared compact and green, while type B were friable and white, highly embryogenic and differentiated into a clump of embryos (Fig. 1B). Sometimes direct embryo formation without callus production was observed (Fig. 1C). Well-structured embryos showed normal dicot developmental patterns: globular, heart, torpedo and cotyledonary stages. Secondary embryos were also obtained (Fig. 1D). Embryo could be converted into vigorous plantlets (Fig. 1E–G).

Polyamine treatments

The addition of SPD gave the best results. In fact, in this medium the statistically highest percentage of anthers producing embryogenic callus and embryos (4.7%) was observed (Table 1). Moreover, after four subcultures of 40 days in the germination medium, the calli on this medium produced the greatest number of embryos (763). This finding builds upon previous results when 4% of anthers gave embryogenic callus (Germanà and Chiancone 2003). The medium-containing PUT produced the greatest percentage of non-responding anthers (22%) and of anthers with callus (51.4%) and the lowest percentage of swollen anthers (25.5%). The results obtained on medium containing both PAs as well as the control, were similar with respect to non-responding anthers (12%) and swollen anthers (37%); the percentage of anthers with callus (49%) was similar to the PUT-supplemented ones. Medium containing both PAs was less effective in producing anthers with embryogenic callus and embryos, with only 0.9% of anthers induced to embryogenesis and only 90 embryos (Table 1).

Ploidy analysis

Flow cytometry showed that all the analyzed regenerants coming from control, PUT and PUT + SPD media were triploid (100%) (Fig. 2A). Most (80%) of the regenerants from SPD medium were triploid, the rest were diploid (20%) (Fig. 2B). Further studies are in progress to identify the mechanisms responsible (nuclear fusion, endoreduplication, etc), and the possible genetic controls because the way in which microspores regenerate into tri-haploids is still not well known.

Isozyme analysis

Because of the spontaneous polyploidization of the haploid calli during culture, ploidy analysis cannot always be used to determine androgenic plants. In this experiment both enzyme systems, PGI-1 and PGM of all the examined samples confirmed the gametic origin of regenerants. In fact, the regenerant zymogram was characterized by single bands, compared to three bands for PGI-1 (Fig. 3A) and two bands for PGM for the anther donor (Fig. 3B), both in calli and leaf tissues. In previous papers, isozyme analyses have been employed to identify the androgenic origin of the calli and plantlets (Germanà et al. 1991, 1994; Germanà and Reforgiato Recupero 1997).

Table 1 Influence of two polyamines added to the medium on the anther response recorded after 10 months of in vitro culture. The PAs were supplied at 2 mM concentration or 1 mM each in the PUT + SPD combined treatment

Treatment	No. of anthers	Anthers with embryogenic callus		Embryos obtained after 4 subcultures
		No.	%	
Control	600	9[b]	1.5	179[b]
SPD	628	30[a]	4.7	763[a]
PUT	701	8[b]	1.1	286[b]
PUT + SPD	741	7[b]	0.9	90[b]

Values within each column followed by the same letter (a or b) are not significantly different at $P \leq 0.05$ (SNK's test)

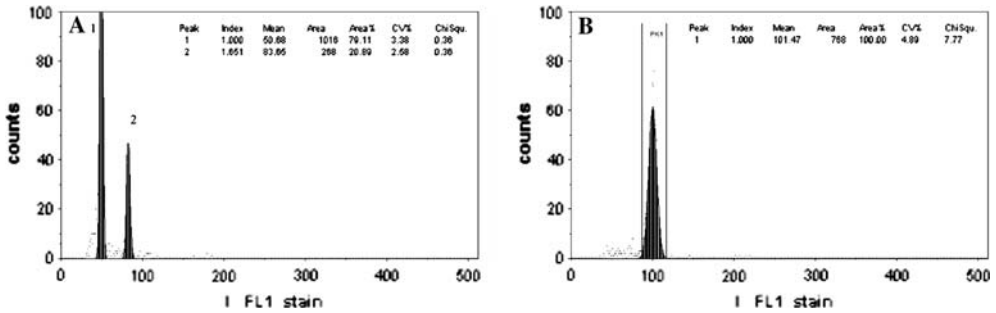

Fig. 2 (**A**) Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid leaf tissue of *Citrus clementina* mother plant (peak 1) and triploid embryo of *Citrus clementina* ''Nules'' anther culture regenerant (peak 2). (**B**) Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid leaf tissue of *Citrus clementina* mother plant and diploid embryo of *Citrus clementina* ''Nules'' anther culture regenerant (peak 1)

Citrus clementina is heterozygous for phosphoglucoisomerase (PGI-1) and phosphoglucomutase (PGM). According to Torres et al. (1978), the heterozygous clementine mother plant is FI (F = allele that specifies fast migration toward the anode enzyme; I = intermediate) in PGM, and WS (W = allele that specifies an enzyme migrating faster than F; S = allele that specifies a slowly migrating enzyme) in PGI-1.

Polyamine content in anthers

Few data are available for polyamine content in anthers in contrast to those for ovaries (Antognoni et al. 2002) and isolated pollen from both in vivo and in vitro culture (Biasi et al. 1999). At time zero, SPD was the most abundant among the free PAs detected in anthers (Fig. 4A), whereas PUT was mainly present both in the PCA-soluble and in particular insoluble conjugated forms (Fig. 4B, C). SPM was present only in trace amounts (data not shown).

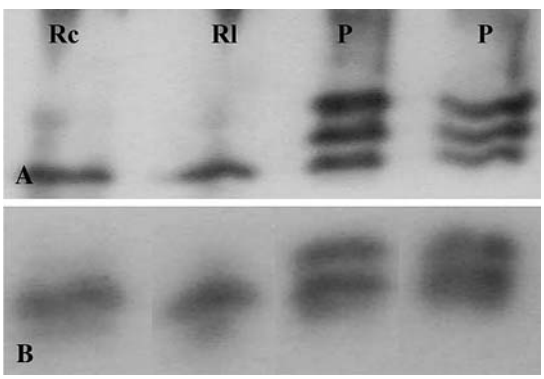

Fig. 3 Isozyme pattern of PGI-1 (**A**) and PGM (**B**) of calli and leaves. The last two lanes are zymograms of the heterozygous Nules parent (P), the first two are those of the homozygous regenerants: callus (Rc) and leaves (Rl)

After 10 months of culture, anthers on the control medium displayed little PUT and SPD in all three forms, with a 10-fold decrease in the soluble-conjugated PUT amount and 18-fold decrease of the insoluble-conjugated PUT amount in comparison with time zero samples (Fig. 4B, C).

The exogenous supplementation of either 2 mM PUT or SPD or PUT + SPD (1 mM each) correlated with uptake and accumulation of polyamines, which reached a total final concentration of 4.9 mM in anthers treated with PUT and 0.9 mM in those supplemented with SPD. When PUT and SPD were given in combination, the level of PUT was about 2/3 with respect to the accumulation of SPD. At the same time, the occurrence of a small DAP in the free fraction, both in the SPD and PUT + SPD treated anthers was an indication of the probable presence of polyamine oxidase activity (PAO) (Federico and Angelini 1991). DAP in the conjugated form was also detected both in SPD and PUT + SPD treated anthers (Fig. 4B, C). Some exogenous PUT and SPD was conjugated in the soluble and insoluble-conjugated PCA fractions (Fig. 4B, C). Conjugated PAs are mostly covalently bound with hydroxycinnamic acid monomers (PCA-soluble fraction), dimers or trimers branched or unbranched (PCA-insoluble fraction) (Bagni and Tassoni 2001). The latter are often conjugated

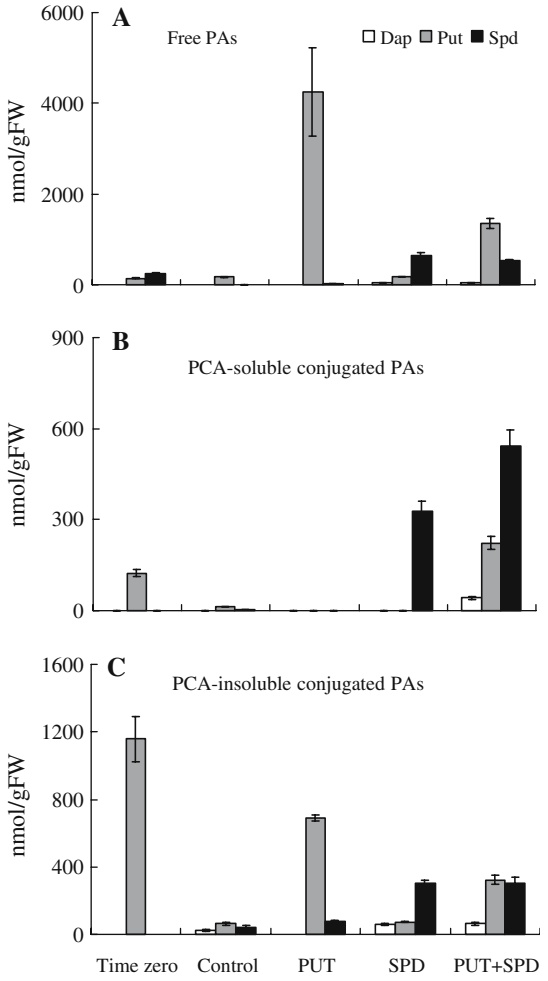

Fig. 4 Free (**A**), PCA-soluble conjugated (**B**) and PCA-insoluble conjugated (**C**) polyamine content (nmol gFW^{-1}) in anthers of *Citrus clementina* at time zero and after 10 months of culture in control and polyamine supplemented medium. The PAs were supplied at 2 mM concentration or 1 mM each in the PUT + SPD combined treatment. Values (±SD) represent the means of two experiments each of three determinations

with cell wall components such as hemicelluloses (Markwalder and Neukon 1976). Generally greater insoluble-conjugated polyamines, especially SPD, was related to flower differentiation (Scaramagli et al. 1999; Tassoni et al. 2000), as observed in kiwi ovaries (Biasi et al. 1999), since polyamine interaction with cell wall components can be responsible for a transition from relaxation to rigidity (Berta et al. 1997). Our results, in particular when exogenous PAs were supplied in the culture medium, support previous studies.

Conclusions

Although much research has been conducted on the effect of polyamines on tissue culture, only a few have studied their influence on gametic embryogenesis (Faure et al. 1991; Martinez et al. 2000). Moreover, there are very few reports that investigate the influence of PAs on tissue culture of trees. Here, we find that SPD added alone (2 mM) to the medium was more effective in inducing embryogenic callus formation than the other tested treatments. Moreover, exogenous SPD induced its endogenous accumulation in anthers. In fact, previous studies demonstrated that SPD is present in a physiologically significant amount that increases during kiwi microsporogenesis (Biasi et al. 1999). Spermidine has been implicated in somatic embryogenesis in tissue cultures of *Daucus carota*, *Vigna*, *Hevea* and *Panax ginseng* (Kevers et al. 2002) in coadjuvated gynogenesis in *Allium cepa* (Martinez et al. 2000) and androgenesis in *Solanum tuberosum* (Tiainen 1992) and somatic embryogenesis in ginseng (Monteiro et al. 2002). In addition, the major effect of SPD with respect to PUT, also shown in seven different wheat cultivars (Rajyalakshmi et al. 1995) as well as in *Cucumis sativus* (Ashok Kumar et al. 2004), suggests that in in vitro anther culture there could be a reduced capacity of synthesis of the two enzymes responsible for SPD formation, namely S-adenosylmethionine decarboxylase (SAMDC) and spermidine synthase. For this reason an exogenous supply of polyamines seems to be necessary. To our knowledge, this is the first study where PAs, particularly SPD, added to the medium positively stimulates gametic embryogenesis in woody plants.

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