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ORIGINAL ARTICLE

Effects of the exogenous polyamines on micropropagation of cherry rootstocks

Virginia Sarropoulou[1] · Kortessa Dimassi-Theriou[1] · Ioannis Therios[1]

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Abstract In the present study, the effects of the 3 polyamines (PAs); putrescine (put), spermidine (spd) and spermine (spm) on micropropagation in CAB-6P, Gisela 6 and MxM 14 cherry rootstocks were investigated. In CAB-6P, shoot number (1.5) and shoot multiplication percentage (50%) were highest with 1 mg/l spd whereas shoot length was maximum with put irrespective of concentration. In Gisela 6, 1 mg/l spd exhibited the maximum shoot number (2) and shoot multiplication percentage (55.56%). In MxM 14, none of the 3 PAs resulted in multiple shoot production, In CAB-6P, root number (4) and rooting percentage (45.45%) were highest with 1 and 2 mg/l put, respectively, whereas 1 mg/l spd resulted in the greatest root length (160 mm). In Gisela 6, 2 mg/l put enhanced root number and root length, whereas rooting percentage was diminished by all 3 PAs. In MxM 14, root number (6.33) and rooting percentage (100%) were greatest with 1 mg/l put and 1 mg/l spm, respectively. In all 3 cherry rootstocks, PAs did not augment chlorophyll content. In CAB-6P, PAs resulted in reduction of leaf carbohydrate and proline levels and activation of mechanism of osmoregulation and osmotic adjustment in leaves. In Gisela 6, leaf carbohydrate levels were raised with 0.5 mg/l spm or 1 mg/l spd. Among the 3 PAs, only spd raised leaf proline content, while the content in roots was increased by 1 mg/l put, showing that PAs cause some kind of stress to the explants. In MxM 14, spd and spm augmented leaf carbohydrate levels, while in roots, only spm at 0.5 mg/l increased carbohydrate content. PAs hardly affected root proline content. It seems that in Gisela 6 and MxM 14, spd and spm increase the leaf carbohydrate content, whereas in CAB-6P all 3 PAs led to depleted leaf carbohydrate levels. The different responses among the 3 cherry rootstocks to PAs concerning shoot and root attributes are genotype-dependent.

Keywords Carbohydrates · Cherry rootstocks · Chlorophyll content · Micropropagation · Polyamines · Proline

Introduction

PAs regulate different plant developmental processes. They control several cellular processes including DNA replication, cell division, protein synthesis, flower development, fruit development, senescence, abiotic and biotic stress responses, and secondary metabolism (Tun et al. 2006). PAs also affect the formation of plant architecture, such as internode elongation (Alcázar et al. 2005), root branching (Ben-Hayyim et al. 1994) and shoot apical dominance (Geuns et al. 2001). Martin-Tanguy (2001) suggested that the exogenous PAs treatment would trigger proliferation and growth of plant cells leading to adventitious shoot formation. Because of their presence in meristematic and growing tissues (Torrigiani et al. 1989), PAs can also be considered, together with cytokinins as juvenility markers.

Kuznetsov et al. (2002) have reported that PAs can be classified into two categories based on their biological effect. The first comprises put and cadaverine, which stimulate cell elongation and root formation, like auxins and gibberellins. The second includes spd and spm, which

✉ Virginia Sarropoulou
vsarrop@gmail.com

[1] Laboratory of Pomology, Department of Horticulture, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloníki, Greece

similar to cytokinins, control cell division, organogenesis and plant senescence.

The present study was undertaken in order to study the potential effects of PAs; viz. put, spd and spm on in vitro shoot proliferation and rooting for establishing an efficient micropropagation protocol.

Materials and methods

Plant material and culture conditions

The effect of PAs; put, spd and spm was studied in in vitro experiments employing the cherry rootstocks CAB-6P (*P. cerasus* L.), Gisela 6 (*P. cerasus* × *P. canescens*) and MxM 14 (*Prunus avium* × *Prunus mahaleb*). The experiment included 4 concentrations (0, 0.5, 1.0 and 2.0 mg/l) of the three PAs. As regards plant material, shoot tip explants from previous in vitro cultures of 1.5–2.5 cm in length were used. The explants were grown in glass tubes with a flat base of 25 × 100 mm containing 10 ml of MS medium (Murashige and Skoog 1962), 30 g/l sucrose and 6 g/l agar (Bacto-agar). The pH of the culture medium was adjusted to 5.8 and sterilized by autoclaving at 121 °C for 20 min. In each tube, one explant was transferred aseptically, and the tubes were closed with aluminium foil. All the cultures were incubated in a growth room under controlled environmental conditions with a light intensity of 150 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (36 W, Philips), with a photoperiod of 16 h at 22 ± 1 °C. Then, the explants were oven dried and dry weight was calculated. Data were recorded on shoot number/explant, shoot length, shoot fresh and dry weight, percentage of produced shoots (%), root number per rooted explant, root length, root fresh and dry weight, rooting percentage (%), total leaf chlorophyll, carbohydrates and proline concentration in leaves and roots. The measurements were taken 10 weeks after transferring the CAB-6P and Gisela 6 explants, and 6 weeks for MxM 14 explants to the rooting medium, to obtain full response.

Total chlorophyll measurement

For chlorophyll extraction, 0.1 g of leaves was placed in 25 ml glass test tubes containing 15 ml of 96% (v/v) ethanol. The tubes were incubated in a water bath at a temperature of 79.8 °C for 4 h. The absorbance of chlorophyll was measured at 665 and 649 nm. Total chlorophyll was determined according to Wintermans and De Mots (1965) from the equation: chl $(a+b) = (6.10\times A_{665} + 20.04 \times A_{649}) \times 15/1000/\text{D.W.}(\text{mg g}^{-1}\text{D.W.})$.

Proline and total carbohydrates determination

0.1 g of frozen material (leaves or roots) was placed in 25 ml glass test tubes containing 15 ml of 80% (v/v) ethanol. The tubes with the plant material were incubated in a 60 °C water bath for 30 min (Khan et al. 2000). The extract was filtered with Whatman No. 1 filter paper and free proline was measured (Troll and Lindsley 1955) with acid ninhydrin solution. Total carbohydrates were measured with the anthrone reagent (Plummer 1987).

Statistical analysis

The experimental layout was completely randomized and ANOVA (Analysis of Variance) was carried out using the statistical package SPSS 17.0 (SPSS Inc, Chicago, Illinois, USA). The experiment was repeated twice and consisted of 4 treatments where each value was the mean of 20 replicates. The reported data are the means of the two experiments. To compare the means, the Duncan's multiple range test was used at $P \leq 0.05$ to establish significant differences among the treatments. For each cherry rootstock (CAB-6P, Gisela 6, MxM 14) the experiment was 3 × 4 factorial with 3 different PAs (put, spd, spm) and 4 different PAs concentrations (0, 0.5, 1, 2 mg/l). The main effect of factors (PAs type and PAs concentration) and their interaction were determined by the General Linear Model (2-way ANOVA).

Results

Effect of PAs on in vitro shoot proliferation

In CAB-6P rootstock, 1 mg/l spd significantly increased shoot number/explant (Fig. 1f) as compared to the control (Fig. 1a), whereas put and spm (0.5–2 mg/l) did not alter the above characteristic substantially (Table 1). Put gave the best results in shoot elongation (Fig. 1b–d). Spd at 0.5 or 2 mg/l and spm at 2 mg/l increased shoot length but to a lesser extent than put. The incorporation of 0.5 mg/l put into the culture medium resulted in the highest shoot fresh and dry weight followed by 2 mg/l put, and 1 mg/l spd or spm. Put at 1 or 2 mg/l and spm at 2 mg/l had no effect on shoot proliferation.

In the Gisela 6 rootstock, spm irrespective of concentration (Fig. 2h–j), spd at the two higher concentrations (1 and 2 mg/l) (Fig. 2f, g) and put at the lowest and highest concentrations of 0.5 and 2 mg/l (Fig. 2b, d), respectively meaningfully increased shoot number/explant, as compared to the control (Table 1; Fig. 2a). None of the 3 PAs had an effect on shoot length and shoot dry weight. The same

Fig. 1 Effect of the 3 polyamines (PAs); putrescine (put), spermidine (spd) and spermine (spm) on in vitro shoot proliferation and rooting of CAB-6P shoot tip explants: **a** Control (PAs-free), **b** 0.5 mg/l put, **c** 1 mg/l put, **d** 2 mg/l put, **e** 0.5 mg/l spd, **f** 1 mg/l spd, **g** 2 mg/l spd, **h** 0.5 mg/l spm, **i** 1 mg/l spm, **j** 2 mg/l spm

Table 1 Effect of polyamine type and polyamine concentration and their interaction (2-way ANOVA) on shoot number/explant, shoot length, shoot fresh weight, shoot dry weight and percentage of produced shoots (%) in CAB-6P, Gisela 6 and MxM 14 cherry rootstocks, respectively

	Treatments (mg/l)	Shoot number/explant	Shoot length (mm)	Shoot fresh weight (g)	Shoot dry weight (g)	Shoot multiplication percentage (%)
CAB-6P rootstock						
Control	0	1.00 a	16.00 ab	0.092 a	0.009 a	0 a
Put	0.5	1.17 a	36.96 e	0.134 d	0.014 d	16.67 e
	1.0	1.00 a	39.09 e	0.108 abc	0.011 abc	0 a
	2.0	1.00 a	35.71 e	0.121 bcd	0.012 bcd	0 a
Spd	0.5	1.13 a	20.73 cd	0.098 ab	0.010 ab	12.50 c
	1.0	1.50 b	19.63 bcd	0.127 cd	0.013 cd	50.00 f
	2.0	1.06 a	22.33 d	0.105 abc	0.011 abc	5.00 b
Spm	0.5	1.15 a	14.01 a	0.109 abc	0.011 abc	5.00 b
	1.0	1.09 a	16.71 abc	0.123 cd	0.012 bcd	14.29 d
	2.0	1.00 a	21.43 d	0.097 ab	0.010 ab	0 a
P values (2-way ANOVA)						
Polyamine type (A)		0.013* (<0.05)	***(<0.001)	0.193 ns (>0.05)	0.130 ns (>0.05)	***(<0.001)
Polyamine concentration (B)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
(A) * (B)		0.001***	***(<0.001)	0.009**(<0.01)	0.009**(<0.01)	***(<0.001)
Gisela 6 rootstock						
Control	0	1.00 a	20.50 ab	0.160 bcd	0.016 bcd	0 a
Put	0.5	1.68 cd	20.57 ab	0.156 bcd	0.016 bcd	50.00 g
	1.0	1.36 abc	19.89 ab	0.119 ab	0.012 abc	36.36 e
	2.0	1.82 d	22.15 b	0.165 cd	0.017 d	36.36 e
Spd	0.5	1.17 ab	17.92 a	0.109 a	0.011 ab	16.67 b
	1.0	1.78 cd	18.30 ab	0.178 d	0.018 d	55.56 i
	2.0	1.55 bcd	18.32 ab	0.133 abc	0.013 abc	54.55 h
Spm	0.5	1.47 bcd	20.65 ab	0.163 bcd	0.016 bcd	42.11 f
	1.0	1.44 bcd	21.15 ab	0.139 abcd	0.014 abcd	31.25 c
	2.0	1.50 bcd	19.67 ab	0.124 abc	0.012 ab	35.00 d
P values (2-way ANOVA)						
Polyamine type (A)		0.452 ns (>0.05)	0.023* (<0.05)	0.873 ns (>0.05)	0.852 ns (>0.05)	***(<0.001)
Polyamine concentration (B)		***(<0.001)	0.825 ns (>0.05)	0.312 ns (>0.05)	0.279 ns (>0.05)	***(<0.001)
(A) * (B)		0.028* (<0.05)	0.555 ns (>0.05)	0.001***	0.001***	***(<0.001)
MxM 14 rootstock						
Control	0	1.00 a	15.00 a	0.109 a	0.011 a	0 a
Put	0.5	1.00 a	28.75 b	0.272 b	0.028 b	0 a
	1.0	1.00 a	23.57 b	0.197 a	0.020 ab	0 a
	2.0	1.00 a	27.22 b	0.277 b	0.028 b	0 a
Spd	0.5	1.00 a	12.86 a	0.225 b	0.023 b	0 a
	1.0	1.00 a	12.22 a	0.283 b	0.029 b	0 a
	2.0	1.00 a	13.75 a	0.197 a	0.020 ab	0 a
Spm	0.5	1.00 a	14.44 a	0.225 b	0.023 b	0 a
	1.0	1.00 a	12.22 a	0.283 b	0.029 b	0 a
	2.0	1.00 a	15.63 a	0.197 a	0.020 ab	0 a

Table 1 continued

	Treatments (mg/l)	Shoot number/explant	Shoot length (mm)	Shoot fresh weight (g)	Shoot dry weight (g)	Shoot multiplication percentage (%)
P values (2-way ANOVA)						
Polyamine type (A)		–	***(<0.001)	0.627 ns (>0.05)	0.570 ns (>0.05)	–
Polyamine concentration (B)		–	0.039* (<0.05)	***(<0.001)	***(<0.001)	–
(A) * (B)		–	0.002** (<0.01)	0.169 ns (>0.05)	0.152 ns (>0.05)	–

Statistical analysis was conducted among the three polyamines for each rootstock separately. Treatments denoted by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at $P \leq 0.05$. ns $P \geq 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$

trend was observed in terms of shoot fresh weight with the exception of the lowest spd concentration (0.5 mg/l) (Fig. 2e), which reduced substantially the above characteristic as compared to the control. Spd at 1 mg/l induced highest shoot multiplication percentage (55.56%), which was minimum (16.67%) with 0.5 mg/l spd.

In the MxM 14 rootstock, none of the 3 PAs had an effect on the induction of multiple shoots. Among the 3 PAs, only put increased the length of the initial shoot (Fig. 3b–d) as compared to the control (Table 1; Fig. 3a). The shoot fresh and dry weight were substantially increased when 0.5 or 1 mg/l spd (Fig. 3e, f), 0.5 or 1 mg/l spm (Fig. 3h, i) as well as put (0.5 and 2 mg/l) were applied.

Effect of PAs on in vitro rooting

In CAB-6P rootstock, the maximum root number/rooted explant (4), root length (160 mm) and root fresh weight (0.038 g) were recorded with 2 mg/l put (Fig. 1a, d; Table 2). In this treatment, root number and root fresh weight were doubled and root length was higher by 0.98 cm as compared to the control. Among the 3 PAs, put at 1 mg/l induced the highest rooting percentage (45.45%) (Fig. 1c). Put promoted more rooting ability than spd or spm. Spm at 1 mg/l caused complete inhibition of rooting (Fig. 1i).

In Gisela 6 rootstock, spd inhibited rooting completely (Fig. 2e–g). The same trend was observed with the lowest put (0.5 mg/l) (Fig. 2b) and the highest spm concentration (2 mg/l) (Table 2; Fig. 2j). Root number was increased from 2 to 3 by intergrading 2 mg/l put into the culture medium (Fig. 2d) whereas 1 mg/l put or spm caused a reduction in root number as compared to the control. Root length and fresh weight were increased when 0.5 mg/l spm (Fig. 2h) were incorporated into the culture medium whereas put, spd or spm at higher concentrations had an inhibitory effect. In this treatment, root length was greater by 2.09 cm as compared to the control. The root dry weight and rooting percentage were maximum in the control treatment (Fig. 2a). All 3 PAs adversely affected the explants rooting ability.

In MxM 14 rootstock, PAs, irrespective of type and concentration significantly increased root number (4–6 times), root length (2–3½ times), root fresh and dry weight (4–11 times) as compared to the control (Table 2) with the exception of spm at 1 mg/l (Fig. 3i) which did not alter root number considerably and put at 1 mg/l (Fig. 3c) which did not increase root length and root fresh weight. The rooting percentage was maximum (100%) with the addition of 1 mg/l spm to the culture medium, as compared to the control.

Effect of PAs on total chlorophyll, carbohydrate and proline content

In CAB-6P rootstock, a reduction in chlorophyll content was observed with 0.5 mg/l spm or 0.5–1 mg/l spd (Table 3). All PAs treatments, led to a decrease in total leaf carbohydrates as compared to the control. The same trend was observed for endogenous leaf proline levels, except 0.5 mg/l put which did not alter this biochemical parameter significantly. Spm at 0.5 mg/l and to a lesser extent put at 2 mg/l significantly increased root carbohydrate concentration, whereas 1 mg/l put had the opposite result. Root proline levels were raised to a greater extent by adding put (1 or 2 mg/l), and spm 0.5 mg/l.

In Gisela 6 rootstock, 0.5 mg/l put or spd and 1 or 2 mg/l spm led to a substantial decrease in leaf chlorophyll content (Table 3). Spd at 1 mg/l and spm at 0.5 mg/l resulted in higher leaf carbohydrate levels as compared to the control. Among the 3 PAs, only spd at 1 mg/l enhanced leaf proline levels. Supplementing the culture medium with 1 mg/l put or 0.5 mg/l spm resulted in reduction in root carbohydrate levels. Put at 1 mg/l raised root proline content by 3.2–3.3 times.

Fig. 2 Effect of the 3 polyamines (PAs); putrescine (put), spermidine (spd) and spermine (spm) on in vitro shoot proliferation and rooting of Gisela 6 shoot tip explants: **a** Control (PAs-free), **b** 0.5 mg/l put, **c** 1 mg/l put, **d** 2 mg/l put, **e** 0.5 mg/l spd, **f** 1 mg/l spd, **g** 2 mg/l spd, **h** 0.5 mg/l spm, **i** 1 mg/l spm, **j** 2 mg/l spm

In MxM 14 rootstock, the application of 2 mg/l put or spd (0.5 or 1 mg/l) led to decrease in leaf chlorophyll levels, as compared to the control (Table 3). Spd and spm, regardless of concentration, and put at 1 mg/l raised leaf carbohydrate content. Among the 3 PAs, only spm at 0.5 mg/l increased leaf proline levels by 6.1 times. In roots,

Fig. 3 Effect of the 3 polyamines (PAs); putrescine (put), spermidine (spd) and spermine (spm) on in vitro shoot proliferation and rooting of MxM 14 shoot tip explants: **a** Control (PAs-free), **b** 0.5 mg/l put, **c** 1 mg/l put, **d** 2 mg/l put, **e** 0.5 mg/l spd, **f** 1 mg/l spd, **g** 2 mg/l spd, **h** 0.5 mg/l spm, **i** 1 mg/l spm, **j** 2 mg/l spm

Table 2 Effect of polyamine type and polyamine concentration and their interaction (2-way ANOVA) on root number/rooted explant, root length, root fresh weight, root dry weight and rooting percentage (%) in CAB-6P, Gisela 6 and MxM 14 cherry rootstocks, respectively

	Treatments (mg/l)	Root number/rooted explant	Root length (mm)	Root fresh weight (g)	Root dry weight (g)	Rooting percentage (%)
CAB-6P rootstock						
Control	0	2.00 d	62.50 h	0.017 d	0.002 b	10.00 c
Put	0.5	1.00 b	12.50 c	0.003 b	0.000 a	33.33 h
	1.0	2.40 e	20.76 d	0.014 c	0.002 b	45.45 j
	2.0	4.00 g	24.70 e	0.016 cd	0.002 b	42.86 i
Spd	0.5	3.00 e	38.60 f	0.030 e	0.005 e	12.50 e
	1.0	1.00 b	160.00 i	0.038 f	0.004 d	6.25 b
	2.0	1.50 c	44.88 g	0.016 cd	0.003 c	11.11 d
Spm	0.5	2.00 d	23.30 e	0.044 g	0.005 e	15.00 g
	1.0	0.00 a	0.00 a	0.000 a	0.000 a	0 a
	2.0	1.33 c	5.02 b	0.005 b	0.000 a	14.29 f
P-values (2-way ANOVA)						
Polyamine type (A)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
Polyamine concentration (B)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
(A) * (B)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
Gisela 6 rootstock						
Control	0	2.00 c	51.61 d	0.033 d	0.006 e	20.00 e
Put	0.5	0.00 a	0.00 a	0.000 a	0.000 a	0 a
	1.0	1.00 b	40.00 c	0.012 b	0.002 c	4.55 b
	2.0	3.00 d	40.00 c	0.019 c	0.002 c	4.55 b
Spd	0.5	0.00 a	0.00 a	0.000 a	0.000 a	0 a
	1.0	0.00 a	0.00 a	0.000 a	0.000 a	0 a
	2.0	0.00 a	0.00 a	0.000 a	0.000 a	0 a
Spm	0.5	2.00 c	72.50 e	0.053 e	0.005 d	5.26 c
	1.0	1.00 b	20.00 b	0.010 b	0.001 b	6.25 d
	2.0	0.00 a	0.00 a	0.000 a	0.000 a	0 a
P-values (2-way ANOVA)						
Polyamine type (A)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
Polyamine concentration (B)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
(A) * (B)		***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)	***(<0.001)
MxM 14 rootstock						
Control	0	1.33 a	10.51 a	0.010 a	0.001 a	30.00 a
Put	0.5	5.17 bc	37.01 c	0.057 b	0.006 b	75.00 c
	1.0	6.33 c	20.80 ab	0.043 ab	0.005 b	85.71 e
	2.0	5.00 bc	37.12 c	0.066 b	0.008 bc	77.78 d
Spd	0.5	5.80 c	22.32 b	0.060 b	0.008 bc	71.43 b
	1.0	5.13 bc	22.12 b	0.054 b	0.006 b	88.89 f
	2.0	5.33 bc	21.92 b	0.061 b	0.006 b	75.00 c
Spm	0.5	5.29 bc	29.99 bc	0.074 bc	0.007 b	77.78 d
	1.0	3.22 ab	29.44 bc	0.073 bc	0.007 b	100 g
	2.0	4.00 bc	34.32 c	0.105 c	0.011 c	75.00 c
P-values (2-way ANOVA)						
Polyamine type (A)		0.092 ns	0.007**	0.017**	0.068 ns	***
Polyamine concentration (B)		***	***	***	***	***
(A) * (B)		0.328 ns	0.074 ns	0.547 ns	0.270 ns	***

Statistical analysis was conducted among the three polyamines for each rootstock separately. Treatments denoted by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at $P \leq 0.05$. ns $P \geq 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Table 3 Effect of polyamine type and polyamine concentration and their interaction (2-way ANOVA) on total leaf chlorophyll content, total carbohydrate and endogenous proline content in leaves and roots in CAB-6P, Gisela 6 and MxM 14 cherry rootstocks, respectively

	Leaves		Leaves		Roots	
	Treatments (mg/l)	Chl (a + b) mg/g D.W.	Carbohydrates µmol/g F.W.	Proline µmol/g F.W.	Carbohydrates µmol/g F.W.	Proline µmol/g F.W.
CAB-6P rootstock						
Control	0	28.949 de	58.801 b	3.645 b	46.858 b	1.850 a
Put	0.5	19.585 bcd	17.131 a	3.572 b	–	–
	1.0	29.766 de	25.386 a	2.666 a	33.001 a	2.724 b
	2.0	30.201 e	26.727 a	2.001 a	64.783 c	3.300 b
Spd	0.5	6.208 a	30.064 a	2.432 a	–	–
	1.0	17.199 bc	27.460 a	2.159 a	–	–
	2.0	26.439 cde	26.934 a	2.386 a	–	–
Spm	0.5	15.017 ab	22.495 a	1.788 a	93.471 d	7.085 d
	1.0	19.905 bcd	17.102 a	1.837 a	–	–
	2.0	23.434 bcde	28.648 a	2.298 a	–	–
P values (2-way ANOVA)						
Polyamine type (A)		0.006**	0.363 ns	0.019*	–	–
Polyamine concentration (B)		***	***	***	–	–
(A) * (B)		0.206 ns	0.510 ns	0.018*	–	–
Gisela 6 rootstock						
Control	0	28.949 c	30.186 abc	2.395 a	59.530 c	3.918 a
Put	0.5	16.590 ab	23.761 a	2.772 a	–	–
	1.0	25.049 bc	36.986 bcd	2.753 a	16.922 a	12.716 b
	2.0	20.300 abc	35.758 abcd	2.420 a	–	–
Spd	0.5	14.643 a	26.215 ab	4.465 b	–	–
	1.0	20.354 abc	43.994 d	6.681 d	–	–
	2.0	21.976 abc	39.838 cd	5.735 c	–	–
Spm	0.5	26.648 c	45.072 d	2.540 a	37.354 b	3.779 a
	1.0	14.917 a	37.639 bcd	3.108 a	–	–
	2.0	16.311 ab	33.264 abcd	2.821 a	–	–
P values (2-way ANOVA)						
Polyamine type (A)		0.760 ns	0.092 ns	***	–	–
Polyamine concentration (B)		0.016*	0.073 ns	0.007**	–	–
(A) * (B)		0.008**	0.004**	0.012*	–	–
MxM 14 rootstock						
Control	0	25.694 c	21.227 a	2.182 a	–	–
Put	0.5	26.398 c	19.756 a	3.252 b	41.609 a	2.935 a
	1.0	25.435 c	32.868 bc	5.141 c	54.399 ab	1.852 a
	2.0	15.127 ab	24.687 ab	4.456 bc	51.112 ab	2.843 a
Spd	0.5	15.013 ab	43.752 d	2.635 ab	61.065 abc	1.724 a
	1.0	9.150 a	35.714 cd	3.263 b	54.604 ab	1.739 a
	2.0	21.047 bc	43.840 d	3.147 b	66.491 bc	2.853 a
Spm	0.5	23.435 c	41.307 cd	13.387 e	71.410 bc	1.783 a
	1.0	18.821 bc	36.470 cd	7.015 d	77.598 c	2.100 a
	2.0	19.762 bc	38.247 cd	6.743 cd	67.399 bc	1.443 a

Table 3 continued

	Leaves		Leaves		Roots	
	Treatments (mg/l)	Chl (a + b) mg/g D.W.	Carbohydrates µmol/g F.W.	Proline µmol/g F.W.	Carbohydrates µmol/g F.W.	Proline µmol/g F.W.
P values (2-way ANOVA)						
Polyamine type (A)		0.003**	***	***	0.001***	0.245 ns
Polyamine concentration (B)		0.092 ns	0.371 ns	0.018*	0.696 ns	0.561 ns
(A) * (B)		0.002**	0.017*	0.040*	0.395 ns	0.338 ns

Statistical analysis was conducted among the three polyamines for each rootstock separately. Treatments denoted by the same letter in each column are not significantly different according to Duncan's Multiple Range Test at $P \leq 0.05$. ns $P \geq 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

the carbohydrate content was maximum in the presence of 1 mg/l spm and minimum with 0.5 mg/l put. None of the 3 PAs had an effect on root proline content.

Discussion

Effect of PAs on in vitro shoot proliferation

In CAB-6P and Gisela 6 rootstocks, PAs led to multiple shoot induction whereas in MxM 14 rootstock they had absolutely no effect. Put and spm did not improve the rate of new shoots production from apricot leaves (Petri et al. 2005). In CAB-6P explants, PAs significantly promoted shoot induction as 0.5 mg/l put, 1 mg/l spd or spm increased the shoot number (except for spm), fresh and dry weight of shoots and spm also increased shoot length, in higher concentrations. Spd but not put or spm enhanced the differentiation of multiple shoots from shoot tips in cucumber (Vasudevan et al. 2008). In Gisela 6 explants, shoot length was not significantly affected by PAs. The same trend was observed for shoot fresh and dry weight with respect to put and spm, while 1 mg/l spd showed an upward trend. Put and spd but not spm increased shoot number. PAs promoted shoot regeneration from Passiflora leaves, cotyledons of *Brassica campestris* species and cucumber shoot tips (*Cucumis sativus*) (Shankar et al. 2011). In MxM 14 explants, spd and spm did not differ in shoot length, in contrast to put, while all 3 PAs increased shoot fresh and dry weight. It has been reported that exogenous put increased shoot length in flax (*Linum usitatissimum* L.) (El-Lethy et al. 2010), myrtle (*Catharanthus roseus* L.) (Talaat et al. 2005), onion (*Allium cepa* L. cv. 'Giza 20') (Amin et al. 2011), artichoke (*Cynara scolymus* L.) (El-Abagy et al. 2010) and bean seedlings *(Phaseolus vulgaris* L. cv. Giza) (Zeid 2004).

A possible explanation for the positive effect of PAs on vegetative growth is attributed to the strengthening of cell division and cell expansion (Cohen 1998). The reduction in shoot length due to PAs application according to Smith (1985) has been associated with the shortening of the internodes through reduction of elongation of internodes, since PAs can play an important role in causing cell division but not cell expansion. In CAB-6P rootstock, among the 3 PAs, put enhanced more shoot elongation whereas spd (1 mg/l) gave the best results in terms of shoot number and frequency of produced new shoots. In the Gisela 6 rootstock, put at low concentrations and spd at higher ones enhanced more the explants ability to induce multiple shoots than spm. MxM 14 microshoots reacted better to put regarding shoot elongation. In *Achras sapota* species, among the 3 PAs, spm proved to be the best one with respect to shoot number, put affected shoot elongation to a greater extent while spd did not result in shoot formation (Purohit et al. 2007).

Effect of PAs on in vitro rooting

PAs resulted in root formation in all the 3 cherry rootstocks studied except for spd, which resulted in complete inhibition of rooting in Gisela 6 explants. Similar findings were recorded in *Arabidopsis thaliana* L., where 0.2–0.8 mM spd reduced root length whereas 1–1.4 mM spd resulted in complete inhibition of rooting (Tassoni et al. 2000). In the dwarf apple MM 106 rootstock, put and spd promoted rooting (72–98%) in the absence of IBA (Naija et al. 2009). In *Tectona grandis* L. species, 160 mg/l put exhibited 70% rooting (Mendoza de Gyves et al. 2007). In the absence of IBA, 1 mM put or spd increased rooting of *Fraxinus angustifolia* microshoots from 76 to 100% during the induction phase while spm had an inhibitory effect (Tonon et al. 2001).

PAs promoted rhizogenesis of MxM 14 explants, improving all the individual rooting characteristics. The same trend was recorded with spd in the Gisela 6 rootstock. PAs promoted early rooting and increased rooting

percentage and root number in olive (Rugini et al. 1997), hazel (Rey et al. 1994) and vitiligo (Hausman et al. 1997). Negative was the effect of put on root length, fresh and dry weight in CAB-6P and Gisela 6 rootstocks, and a positive one on root number only when applied at 2 mg/l. Nag et al. (2001) found that 0.1 mM put increased root number and root length in bean cuttings (*Vigna radiata* L. cv. 105). In addition, put enhanced and inhibited, respectively, the ability of explants to form roots in CAB-6P and Gisela 6 rootstocks, respectively, while spm negatively affected root number. Jarvis et al. (1983) observed that spm favored rooting of bean microcuttings by increasing root number. A decreasing trend was noted in terms of root length in CAB-6P rootstock and regarding rooting percentage of Gisela 6 explants, due to spm application. Spm has been reported to inhibit rooting in many plants, including cherry (*Prunus avium* L.) (Biondi et al. 1990), walnut (*Juglans regia* L.) (Kevers et al. 1997) and vitiligo (*Populus tremula* L. × *P. tremuloides* L.) (Hausman et al. 1994). In contrast, 0.5 mg/l spm stimulated root elongation of Gisela 6 and rooting percentage of CAB-6P explants, while higher spm concentrations had inhibitory effects. Tarenghi et al. (1995) showed that pretreatment of strawberry microcuttings with 1 mM put led to increased root number and root length. Locke et al. (2000) reported that 1 μM put, spd or spm improved root growth of barley seedlings.

Among the three PAs, in the CAB-6P rootstock, put was considered the best regarding rooting percentage (put: 1 mg/l) and root number (put: 2 mg/l), spd (1 mg/l) influenced better root elongation while spm (0.5 mg/l) exhibited the best results concerning root fresh and dry weight. In microcuttings of the vine rootstock 41 B (*Vitis vinifera* cv. Chasselas x *Vitis berlandieri*), put and spd (0.06–0.25 mM) positively affected root number and root length (Martin-Tanguy and Carre 1993). Gisela 6 explants responded better to spm (0.5 mg/l) as compared to put or spd in relation to root number, root length and root fresh weight. However, 1 mM put or spd increased root number and root length in strawberry (*Fragaria x ananassa* Duch.) microshoots, while 1 mM spm resulted in complete rooting inhibition (Tarenghi et al. 1995). In MxM 14 rootstock, spm (1 mg/l) enhanced the explants capacity for rooting to its maximum (100%), while put increased root number to a greater extent (put: 1 mg/l) as well as root length (put: 0.5 or 2 mg/l). Bais et al. (1999) reported that pretreatment of chicory roots (*Cichorium intybus* L. cv. Lucknow Local) with 1.5 mM put increased the length of primary roots, the number of secondary and tertiary roots and the total fresh weight of the roots. Put is implicated in root induction, increasing the activity of total peroxidases in the base of the explants, promoting the rapid growth of roots and increasing the rate of rooting (Rugini et al. 1997).

Effect of PAs on total chlorophyll, carbohydrate and proline content

In Gisela 6 rootstock, 1–2 mg/l spm and 0.5 mg/l put or spd led to reduction in chlorophyll levels. In CAB-6P, chlorophyll loss was reported for 0.5 mg/l spm or 0.5–1 mg/l spd. Subhan and Murthy (2001) reported that, spm delayed loss of chlorophyll and protein over that of spd and spd more than put, suggesting the importance of the strength of organic cations. Several studies have focused on the effects of PAs on functional activity of the thylakoid membranes, the degradation of chlorophyll and the production rate of photosynthetic oxygen (Bograh et al. 1997). In MxM 14, a decline in chlorophyll content occurred in the presence of 0.5–1 mg/l spd or 2 mg/l put. Cohen et al. (1979) reported that PAs between 0.05 and 1 mM caused destruction of chloroplasts and reduced activities of photosystems I and II. The beneficial effect of PAs on chlorophyll content may be due to stabilization of the thylakoid membranes of chloroplasts (Besford et al. 1993).

In the MxM 14 rootstock, PAs increased total leaf carbohydrate content and in roots higher carbohydrate levels were recorded with exogenous spm, followed by spd and lower with put. Similar results were recorded in wheat (*T. aestivum* var. Giza 168) (El-Bassiouny et al. 2008). In CAB-6P, all 3 PAs reduced the content of total carbohydrates in leaves, whereas in roots spm resulted in elevated carbohydrate levels. In Gisela 6, 0.5 mg/l spm or 1 mg/l spd raised endogenous leaf carbohydrates, while in roots both put and spm caused a decline in its content. On the contrary, put increased the content of total carbohydrates in chrysanthemum flowers (*Chrysanthemum indicum* L.) probably due to an increase in the photosynthetic process (Mahros et al. 2011).

Proline is thought to play adaptive roles in mediating osmotic adjustment and protecting sub-cellular structure in stressed plants (Ashraf and Foolad 2007). In CAB-6P, although PAs raised free proline content in roots it caused a substantial reduction of its content in leaves. Hussein et al. (2006) found that foliar application of put in pea shoots caused a highly significant increase of proline content. In Gisela 6, among the 3 PAs only spd led to augmented proline levels in leaves and only put did the same in roots. Increased accumulation of proline was reported in cucumber roots (Duan et al. 2008) and in rice leaves (Sultana et al. 1999) exogenously treated with spd. In MxM 14, no significant alterations were recorded on free proline in roots among the 3 PAs. All three PAs significantly increased the leaf proline content of MxM 14 explants with the increase being higher with spm, followed by put and spd.

In conclusion, taking all the above into consideration it is obvious that the different responses among the three cherry rootstocks to shoot proliferation and rooting attributes in tissue culture systems as well as to the various biochemical parameters evaluated are dependent on the main effect of PAs type, PAs concentration and genotype as well as to their interactions.

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Author contributions VS performed all the experimental work, statistics and wrote the first draft of the manuscript. KDT and IT contributed by scientific advices, designed and managed the experiments and participated in writing. All the authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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