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Dikegulac-Sodium Effect on Micropropagation and Biochemical Parameters in the Cherry Rootstocks CAB-6P and Gisela 6

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Abstract

The effects of dikegulac-sodium (DS) alone and in combination with benzyladenine (BA) on the morphogenetic and biochemical responses in the cherry rootstocks CAB-6P (*Prunus cerasus* L.) and Gisela 6 (*Prunus cerasus* x *Prunus canescens*) were investigated. In the CAB-6P rootstock, DS did not promote shoot proliferation whereas its application at 40, 120 and 150 µM suppressed shoot length. In the Gisela 6 rootstock, the number of shoots per explant and shoot proliferation rate were greater with 80 µM DS. Furthermore, DS significantly stimulated rhizogenesis in both rootstocks. Leaf chlorophyll concentration of CAB-6P microshoots was maximum in the control treatment, whereas in the Gisela 6 rootstock, 40 µM DS had a positive effect on it. In the CAB-6P explants, DS decreased leaf (20-150 µM) and root (80-150 µM) carbohydrate concentration as well as proline concentration in roots (40-150 µM). All DS concentrations, especially 80 µM increased leaf carbohydrate concentration of the Gisela 6 explants. Carbohydrate and proline concentrations in roots were 2.5 times greater with 80 µM DS, compared to the control. In the CAB-6P rootstock, a synergistic effect was found between BA and 250 µM DS regarding shoot fresh weight (FW). Best rooting results in terms of root number per rooted explant and rooting percentage were obtained with 500 µM DS. In the Gisela 6 rootstock, 250 µM DS promoted the positive effect of 4.4 µM BA concerning shoot number per explant. DS seems to be a promising growth regulator in micropropagation of the cherry rootstocks CAB-6P and Gisela 6.

Keywords: Carbohydrates, cherry rootstocks, chlorophyll content, dikegulac-sodium, growth retardants, proline

Introduction

CAB-6P (*Prunus cerasus* L.) is a widely used rootstock for cherry plants. All cherry varieties grafted on this rootstock present less vigor (-30%), earlier cropping, better fruit quality and color and higher yield efficiency in comparison to those grafted on seedlings. Gisela 6 (*Prunus cerasus* x *Prunus canescens*) is less demanding than Gisela 5 and tolerates soils of poorer quality and less water supply. The vigor of this clone is between Gisela 5 and *Prunus avium* (Dimassi-Theriou and Therios, 2006).

Micropropagation protocols have been described in *Prunus* species such as in *P. cerasus* L. (Borkowska, 1983), *P. avium* L. (Hammatt and Grant, 1996) and in several cherry rootstocks, including *P. cerasifera* x *P. munsoniana* (Dalzotto and Docampo, 1997). Rapid propagation of sour cherry (*P. cerasus* L. cultivar 'Chios' and its selection 'Ben-Zion') was obtained by shoot-tip culture (Snir, 1983). In the explants of sour cherry rootstock (*P. cerasus* L.), maximum shoot multiplication was recorded on Woody Plant Medium supplemented with 2 mg L^{-1} BA plus 0.1 mg L^{-1} IBA. Medium fortified with 2 mg L^{-1} IBA recorded the highest rooting percentage and root number per explant (Dar et al., 2010).

CAB-6P (*P. cerasus* L.) and Gisela 6 (*P. cerasus* x *P. canescens*) are two sweet cherry rootstocks of great importance. However, their in vitro shoot proliferation rate should be increased in order to reduce the cost of their commercial production. For shoot proliferation of these rootstocks, BA is the most frequently used cytokinin. BA concentrations from 8.87 to 12.82 µM gave optimal shoot proliferation in

chokecherry (*Prunus virginiana* L.), 'Garrington', and 4.44 µM BA in both cultivars of pincherryl(*P. pensylvanica* L.f), 'Mary Liss' and 'Jumping Pound' (Pruski et al., 2000). In an attempt to increase the efficiency of shoot proliferation, various synthetic growth regulators were tested, such as thidiazuron (TDZ), which induced short shoot formation and big size of callus at the base of the explants. However, BA was more effective than TDZ in regenerating shoots from leaves in sweet (*P. avium* L.) and sour cherry (*P. cerasus* L.) cultivars (Tang et al., 2002). In this process, DS [2,3:4,6-bis-O-(1-methylethylidine)-alpha-L-xylo-2-hexulofuranosonic acid sodium salt] alone or in combination with BA was used to break apical dominance and to promote lateral branching, as was shown in some other plants (Das et al., 2006; Sansberro et al., 2006).

DS under the commercial name Atrimmec or Atrinal is a growth inhibitor and its action was counteracted by gibberellic acid (GA_3) in peas (*Pisum sativum* L.) (Bocion and de Silva, 1977). Furthermore, DS was shown to stimulate in vitro shoot multiplication of the olive cultivars Canino, Frantoio and Moraiolo but not of the cultivars Rosciola and Plantone di Moraiolo. An optimum number of olive shoots was obtained with 66.7 µM DS (Mendoza de Gyves et al., 2008). DS is transported from the leaves to the apex in very small quantities and affects only certain cell types (Arzee et al., 1977).

Very few reports exist in the literature concerning the use of DS in in vitro propagation (Mendoza de Gyves et al., 2008). In *Zantedeschia aethiopica* (calla lilly), Ebrahim (2004) noted an increase in shoot multiplication rate by increasing the DS concentration up to a certain value, above of which shoot number was similar to the control. Under certain conditions, DS acts as anti-auxins do, inhibiting polar auxin transport and reducing apical dominance (Cline, 1997).

The objectives of this research was to appraise DS as a potential growth regulator which would facilitate micropropagation by evaluating its effects on shoot proliferation and rooting and also in chlorophyll, carbohydrate and proline concentration in the commercial cherry rootstocks CAB-6P (*P. cerasus* L.) and Gisela 6 (*P. cerasus* x *P. canescens*). The experimental material was shoot tip explants from previous in vitro cultures of the cherry rootstocks CAB-6P (*P. cerasus* L.) and Gisela 6 (*P. cerasus* x *P. canescens*) established in vitro the previous year and maintained by sub-culturing every 30 days. The nutrient medium used was the Murashige and Skoog (MS) (Murashige and Skoog, 1962) supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids. The culture medium was supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar (Bacto-agar). The pH of the medium was adjusted to 5.8 before adding agar and afterwards the medium was sterilized at 121 ^{o}C for 20 min. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16h light duration (150 µmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 1 ^{o}C. Apical explants with a node and two leaves (1.5 to 2.5 cm in length) were excised from the 30 day old plantlets originated by sub-culturing and transferred into flat base test tubes (25 x 100 mm) containing 10 mL of MS culture medium. DS (Sigma-Aldrich chemical company USA) was filter-sterilized and added to the culture medium after autoclaving.

Table 1. Effect of dikegulac-sodium (DS) concentration on shoot number/explant, shoot length, shoot fresh weight, percentage of sprouting, root number/rooted explant, root length, root fresh weight and rooting percentage in the rootstocks CAB-6P and Gisela 6, respectively. Analysis of Variance (2-way ANOVA, 2x6) Effect of rootstock and DS concentration as well as their interaction on shoot proliferation and rooting characteristics.

DS (µM)	Shoot number/ explant	Shoot length (mm)	Shoot fresh weight (g)	Percentage of sprouting (%)	Root number /rooted explant	Root length (mm)	Root fresh weight (g)	Rooting percentage (%)
CAB-6P								
0	1.00±0.00 a	21.25±0.38 de	0.150±0.004 abc	0 a	2.25±0.16 bc	87.47±5.14 g	0.051±0.002 c	32.00 f
20	1.00±0.00 a	22.50±1.13 e	0.118±0.007 a	0 a	2.33±0.18 bc	55.72±3.67 e	0.048±0.004 bc	50.00 g
40	1.00±0.00 a	14.58±0.67 a	0.148±0.009 abc	0 a	3.00±0.06 d	30.56±0.53 bc	0.045±0.001 bc	25.00 e
80	1.00±0.00 a	21.25±1.40 de	0.135±0.017 abc	0 a	1.75±0.11 a	57.19±2.45 e	0.053±0.002 c	25.00 e
120	1.00±0.00 a	16.25±1.21 ab	0.135±0.010 abc	0 a	5.50±0.20 e	38.20±0.83 d	0.053±0.001 c	12.50 c
150	1.00±0.00 a	17.89±1.27 bc	0.144±0.013 abc	0 a	6.33±0.19 f	25.92±0.52 ab	0.042±0.002 b	15.79 d
Gisela 6								
0	1.00±0.00 a	19.67±0.89 cde	0.165±0.014 c	0 a	1.75±0.09 a	36.88±1.91 cd	0.073±0.006 d	26.67 e
20	1.13±0.07ab	19.06±1.28 bcd	0.121±0.008 a	12.50 b	3.00±0.00 d	35.00±0.00 cd	0.021±0.000 a	6.25 a
40	1.25±0.09 b	17.66±1.09 bc	0.127±0.012 ab	25.00 d	2.50±0.18 c	32.50±1.46 cd	0.027±0.001 a	25.00 e
80	1.44±0.15 c	20.05±1.60 cde	0.160±0.015 bc	31.25 e	2.25±0.18 bc	22.00±1.05 a	0.051±0.007 c	25.00 e
120	1.16±0.09ab	19.08±1.31 bcd	0.121±0.012 a	16.67 c	2.00±0.08 ab	35.00±1.15 cd	0.023±0.001 a	11.11 bc
150	1.16±0.09ab	19.47±1.20 cde	0.161±0.025 bc	15.79 c	2.00±0.08 ab	64.17±0.32 f	0.050±0.002 bc	10.53 b
P-values								
Rootstock (A	***	0.718 ns	0.537 ns	***	***	***	***	***
DS (B)	***	***	0.004**	***	***	***	***	***
(A)*(B)	***	0.005**	0.234 ns	***	***	***	***	***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$.
ns $P \geq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Table 2. Effect of dikegulac-sodium (DS) concentration on total leaf chlorophyll (a+b), total carbohydrate and proline concentration in leaves and roots of the rootstocks CAB-6P and Gisela 6, respectively. Analysis of Variance (2-way ANOVA, 2x6) Effect of rootstock and DS concentration as well as their interaction on biochemical measurements.

	Chlorophyll (a+b)		Leaves		Roots	
DS (µM)	Chl(a+b) mg g^{-1} FW	Chl(a+b) mg g^{-1} DW	Carbohydrates µmol g^{-1} FW	Proline µmol g^{-1} FW	Carbohydrates µmol g^{-1} FW	Proline µmol g^{-1} FW
CAB-6P						
0	3.268±0.308 cd	28.949±2.651 ef	58.801±4.418 e	3.645±0.332 a	46.858±1.620 c	1.850±0.345 cde
20	2.902±0.038 abcd	21.384±1.165 bc	28.983±2.001 a	3.908±0.869 a	44.496±1.452 c	2.296±0.598 def
40	2.478±0.224 a	21.411±3.983 bc	33.317±2.225 ab	5.091±0.998 a	42.891±1.369 c	0.671±0.124 ab
80	2.650±0.194 abc	21.141±1.020 bc	43.167±2.032 c	11.101±2.216 b	35.300±2.378 b	0.148±0.055 a
120	2.897±0.155 abcd	22.850±0.761 bcd	33.285±2.257 ab	5.617±0.521 a	34.873±2.258 b	1.235 ±0.281bc
150	2.954±0.238 abcd	27.628±1.547 def	42.276±3.296 c	12.091±3.517 b	35.155±2.286 b	0.783±0.167 ab
Gisela 6						
0	3.412±0.412 d	20.985±1.781 bc	30.186±1.301 a	2.395±0.108 a	113.996±0.577 d	2.872±0.058 ef
20	3.136±0.366 bcd	18.448±1.548 ab	49.857±2.354 d	3.545±0.523 a	-[w]	3.021±0.097 f
40	4.630±0.599 e	30.865±2.632 f	49.857±2.941 d	5.046±1.024 a	5.240±0.058 a	1.392±0.127 bcd
80	3.283±0.397 cd	27.358±2.143 def	60.172±3.953 e	3.741±0.369 a	277.946±0.996 e	7.613±0.857 g
120	3.113±0.354 abcd	23.948±1.965 cde	55.015±3.214 de	3.611±0.421 a	31.109±0.526 b	2.088±0.293 cdef
150	2.533±0.221 ab	14.902±1.257 a	37.550±1.854 bc	2.784±0.485 a	-[w]	-[w]
P-values						
Rootstock (A)	***	0.233 ns	***	***	***	***
DS (B)	0.005**	0.007**	***	0.005**	***	***
(A)*(B)	***	***	***	0.003**	***	***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$.
ns $P \geq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$
-[w]: determination of carbohydrate and proline content was not conducted in these treatments due to low availability of plant material (roots).

Table 3. Effect of dikegulac-sodium (DS) and BA separately or combined on shoot number/explant, shoot length, shoot fresh weight, percentage of sprouting, root number/rooted explant, root length, root fresh weight and rooting percentage in CAB-6P and Gisela 6 rootstocks, respectively. Analysis of variance (3-way ANOVA, 2x3x2). Effect of BA and DS concentrations, rootstock and their interactions on shoot proliferation and rooting characteristics.

Treatments BA / DS (µM)	Shoot number/ explant	Shoot length (mm)	Shoot fresh weight (g)	Percentage of sprouting (%)	Root number/ rooted explant	Root length (mm)	Root fresh weight (g)	Rooting percentage (%)
CAB-6P								
Control	1.00±0.00 a	16.00±1.00 de	0.092±0.026 a	0 a	2.00±0.10 b	62.50±6.54 c	0.017±0.002 b	10 b
4.4 BA	3.30±0.21 b	9.96±0.86 a	0.255±0.041 cd	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
250 DS	1.00±0.00 a	17.00±1.11 de	0.075±0.008 a	0 a	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
500 DS	1.10±0.10 a	18.75±1.00 ef	0.084±0.007 a	10 b	3.00±0.15 c	56.88±6.06 c	0.017±0.001 b	20 c
4.4 BA+250 DS	3.50±0.40 b	10.97±0.84 ab	0.395±0.079 f	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
4.4 BA+500 DS	3.70±0.37 b	11.22±0.99 ab	0.226±0.024bcd	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
Gisela 6								
Control	1.00±0.00 a	20.50±1.38 f	0.160±0.020 abc	0 a	2.00±0.15 b	37.67±4.57 b	0.033±0.004 c	20 c
4.4 BA	3.70±0.47 b	12.66±0.53 abc	0.281±0.036 de	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
250 DS	1.60±0.31 a	14.75±1.64 cd	0.136±0.021 ab	40 c	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
500 DS	1.80±0.29 a	16.88±1.43 de	0.148±0.028 abc	60 d	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
4.4 BA+250 DS	5.90±0.74 c	13.93±0.76 bcd	0.374±0.060 ef	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
4.4 BA+500 DS	4.30±0.45 b	14.53±0.63 cd	0.2270.020 bcd	100 e	0.00±0.00 a	0.00±0.00 a	0.000±0.000 a	0 a
P-values								
BA (A)	***	***	***	***	***	***	***	***
DS (B)	0.012*	0.295 ns	0.017*	***	***	***	***	***
Rootstock (C)	***	0.013*	0.116 ns	***	***	***	0.795 ns	***
(A)*(B)	0.134 ns	0.069 ns	0.003**	***	***	***	***	***
(A)*(C)	0.089 ns	0.022*	0.140 ns	***	***	***	0.795 ns	***
(B)*(C)	0.034*	0.065 ns	0.868 ns	***	***	***	***	***
(A)*(B)*(C)	0.148 ns	0.031*	0.925 ns	***	***	***	***	***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. ns $P \geq 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Figure 1. Rootstock CAB-6P: **(a)** Minus DS treatment with the longest roots, **(b)** 40 µM DS with the smallest root number, **(c)** Maximum root number with 150 µM DS, **(d)** 4 times less root length with 150 µM DS; Rootstock Gisela 6: **(e)** Minus DS treatment (control) with no shoot proliferation, **(f, g)** Shoot multiplication with 80 µM DS **(h)** 20 µM DS produced the greatest root number, **(i)**
Production of multiple shoots and roots in the
same explant with 80 µM DS

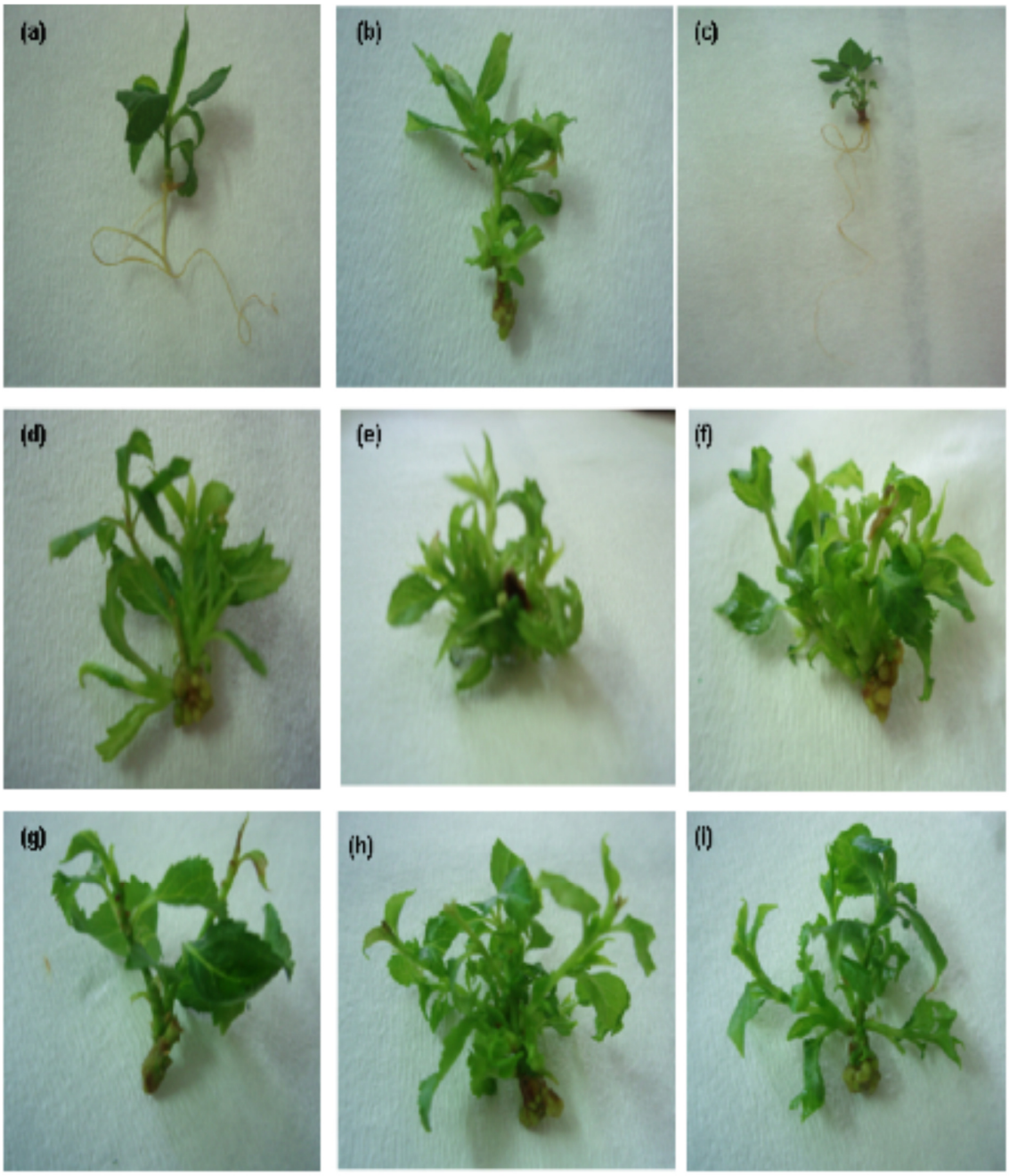

Figure 2. Rootstock CAB-6P: **(a)** Control (-DS), formation of roots but not of multiple shoots, **(b)** 4.4 µM BA, **(c)** 500 µM DS (-BA) Rooting and shoot proliferation, **(d)** BA+DS, increase of shoot proliferation rate, **(e)** Inhibition of shoot elongation of the initial explant and of the produced new shoots in the combined effect of BA+DS; Rootstock Gisela 6: **(f)** Increased shoot number but reduced shoot length with 4.4 µM BA DS), **(g)** DS (-BA), promotion of shoot induction but insignificant increase of shoot number/explant, **(h)** BA+250 µM DS resulted in greater shoot number compared to BA (-DS), **(i)** Production of new shoots with BA+500 µM DS

Materials and Methods

Plant material and culture conditions

Two experiments were conducted and repeated twice and the reported data are the means. In the first experiment, the effects of 6 DS concentrations (0, 20, 40, 80, 120 and 150 µM) were studied in order to break apical dominance and to increase production of multiple shoots or roots under in vitro conditions. The MS culture medium was free of growth regulators. The duration of the first experiment was 12 weeks, consisted of 6 treatments and each treatment included 13 replications (tubes). In the second experiment, the effects of 2 benzyladenine (BA) concentrations (0 and 4.4 µM) alone or in combination with 3 DS concentrations (0, 250, 500 µM) on shoot proliferation, were tested. The duration of the second experiment was 7 weeks, consisted of 6 treatments and each treatment included 12 replications. Shoot number/explant, shoot length, shoot FW, shoot multiplication rate (%), root number, root length, root FW and rooting percentage (%) were recorded at the end of each experiment. Furthermore, total leaf chlorophyll (a+b), total carbohydrate and proline concentrations in both leaves and roots, were measured.

Chlorophyll determination

For chlorophyll measurement, 0.1 g of frozen leaf material was taken and placed in glass test tubes of 25 mL volume. 15 mL of 96% ethanol was added in each tube, which was covered with aluminum foil to reduce ethanol evaporation. The tubes were incubated in a water bath of 79.8 ^{o}C, until complete sample discoloration and chlorophyll extraction. After chlorophyll extraction, the samples (tubes) were allowed to cool at room temperature and the level of 96% ethanol was completed to be 15 mL volume. The absorbance of chlorophylls a and b was measured at 665 and 649 nm, respectively, in a visible spectrophotometer. The decolorized leaf sample was dried for 24h at 68 ^{o}C and its dry weight (DW) was measured. Chlorophyll concentration was determined according to Wintermans and De Mots (1965) from the following equations:

chl(a + b) = (6.10 × A_{665} + 20.04 × A_{649}) × 15/1000/FW (mg g^{-1} FW),

chl(a + b) = (6.10 × A_{665} + 20.04 × A_{649}) × 15/1000/DW (mg g^{-1} DW).

Proline determination

Leaf or root frozen tissue (0.1g), was chopped into small pieces and placed in glass test tubes of 25 mL. In each tube, 10 mL of 80% (v/v) ethanol was added and placed in a water bath of 60 ^{o}C for 30 min (Khan et al., 2000). The tubes were covered with aluminum foil to reduce evaporation. The extracts were filtered and 80% (v/v) ethanol was added until the total volume (ethanol extract) to be 15 mL. After extraction, the aluminum foil was removed and the tubes were allowed to cool at room temperature. In each tube, 4 mL of toluene was added and mixed well with a vortex. Two layers were visible in each tube. The supernatant (toluene layer) was removed with a pasteur pipette and was placed in a glass cuvette. The optical density of the extract was measured at 518nm. The extract was filtered with Whatman No. 1 filter paper and free proline was measured (Troll and Lindsley, 1955) with acid ninhydrin solution. Proline concentrations were calculated from a standard curve by using L-proline (Sigma Chemical Company) at 0-0.2 mM concentrations.

Carbohydrate determination

Carbohydrate determination of plant tissue was conducted by using the anthrone method (Plummer, 1987). For reagent preparation, 1g of anthrone was diluted to 500 mL concentrated sulfuric acid (96%). The extract (plant ethanolic extract) for carbohydrate determination was the same as that used for proline, with the only difference that it was diluted 10 times with 80% (v/v) of ethanol. In each test tube, 2 mL of anthrone reagent were placed and maintained in an ice bath. Subsequently, the diluted extract (10% of the initial) was added dropwise in contact with the test tube walls in order to avoid blackening of the samples. After shaking the tubes with a vortex, the samples were incubated in a water bath of 95 ^{o}C for 15 min. Afterwards, the tubes were placed in a cold water bath for cooling and optical density was measured at 625nm. Carbohydrate concentrations were calculated from a standard curve by using 0-0.2 mM sucrose concentrations.

Statistical analysis

The experiments were completely randomized and analyzed by ANOVA (Analysis of Variance) using the statistical program SPSS 17.0

(SPSS Inc., Illinois, New York, USA) at P≤0.05, according to the Duncan's multiple range test ± S.E. The first experiment was a 2x6 factorial with 2 rootstocks (CAB-6P and Gisela 6) and 6 DS concentrations. The main effect of factors (rootstock, DS concentration) and their interaction were determined by the General Linear Model (2-way ANOVA). The second experiment was a 2x3x2 factorial with 2 BA concentrations, 3 DS concentrations and 2 rootstocks (CAB-6P and Gisela 6). The main effect of factors (BA, DS, rootstock) and their interactions were evaluated by the General Linear Model (3-way ANOVA). Furthermore, in each rootstock separately with the same model, the main effect of factors (BA, DS) and their interaction (BA x DS) were also evaluated.

Results

Experiment 1. Effect of DS on shoot proliferation and rooting in vitro, total leaf chlorophyll, carbohydrate and proline concentration in leaves and roots

DS effect on in vitro shoot proliferation

In the CAB-6P rootstock, DS did not enhance shoot proliferation and shoot FW of the initial explant. However, the shoot length of the explants at the termination of the experiment was significantly greater with 0, 20 and 80 μM DS in comparison with 40, 120 and 150 μM DS. In the Gisela 6 rootstock, the number of shoots per explant was significantly greater with 80 μM DS (Figure 1f, 1g) in comparison to the control (Figure 1e). The effects of DS concentration on shoot length and shoot FW were not significant. The shoot proliferation rate was greatest when 80 μM DS were incorporated into the culture medium. On the contrary, DS at 120 and 150 μM significantly reduced shoot proliferation. A positive correlation was recorded between 0-80 μM DS and percentage of sprouting (Table 1).

It is worth mentioning that Gisela 6 reacted positively regarding the number of produced shoots/explant in the presence of 40 and 80 μM DS in the culture medium, whereas DS had absolutely no effect on CAB-6P explants. On the contrary, CAB-6P responded better than Gisela 6 concerning shoot length with 20 μM DS. The opposite happened with 40 μM DW. The shoot FW was not significantly affected by the rootstock. Furthermore, the interaction of rootstock x DS concentration was not significant (Table 1).

DS effect on in vitro rooting

The number of roots per rooted explant in the CAB-6P rootstock was increased substantially with 40, 120 and 150 μM DS (Figure 1b, 1c) and it was 3 times greater than the control. DS was inhibitory to root length as its maximum value was achieved in the control treatment (- DS). Therefore, at 150 μM DS, root length was 3 to 3.5 times lower (Figure 1d) than the control (Figure 1a). Additionally, 150 μM DS significantly reduced root FW in comparison to the control. The rooting percentage was greatest (50%) with 20 μM DS compared to the control (32%). DS concentrations higher than 20 μM negatively affected the explants' rooting ability (Table 1).

In the Gisela 6 rootstock, the root number was significantly increased with 20, 40 and 80 μM DS. With 20 μM DS, the above characteristic was 2 times greater compared to the control (Figure 1h). Although the highest DS concentration (150 μM) doubled the length of the roots, the greatest root FW was recorded in the control treatment. Therefore, DS negatively affected root FW, which was 2 to 3 times lower than it was in the control. Additionally, the rooting percentage was greatest in the control plantlets (- DS). A significant decrease in the rooting percentage was recorded with low (20 μM) and high DS concentrations (120 and 150 μM) (Table 1). It is innovative that the explants produced concurrently multiple shoots and roots with DS, especially with 80 μM (Figure 1i).

Effect of DS on total leaf chlorophyll, carbohydrate and proline concentration

Chlorophyll concentration of CAB-6P leaves (mg g^{-1} FW or DW) was maximum in the control treatment, it was reduced with 40 μM DS and it was constant with 120 and 150 μM DS. Total carbohydrate concentration in both leaves and roots was maximum in the absence of DS from the culture medium. DS, irrespective of concentration significantly reduced 1 to 1.5 times leaf carbohydrate concentration, compared to the control. Low DS concentrations (20 and 40 μM) did not alter significantly carbohydrate concentration in roots whereas higher DS concentrations (80-150 μM) were inhibitory. The endogenous leaf proline was

increased 2 to 3 times, compared to the control with 80 and 150 µM DS, while the other DS concentrations did not affect substantially this biochemical parameter. On the contrary, in roots, 80 µM DS decreased 12 to 13 times proline concentration, in comparison to the control, while in the other DS treatments proline concentration in roots was not affected meaningfully (Table 2).

In the Gisela 6 rootstock, the lowest (20 µM) and the highest (150 µM) DS concentrations decreased total chlorophyll content whereas the intermediate concentrations (40-120 µM) increased the level of chlorophyll, in comparison to the control. Leaf chlorophyll concentration was maximum with 40 µM DS and minimum with 150 µM DS. Considering carbohydrates and proline at all tested DS concentrations, their level were increased 1.5 to 2 times with 80 µM DS as compared to the control. A positive relationship was observed between carbohydrate and proline concentration in leaves and DS concentrations up to 80 µM and a negative one at concentrations greater than 80 µM DS. Carbohydrate and proline concentrations in roots were minimum and maximum with 40 and 80 µM DS, respectively. Carbohydrate and proline concentrations in roots were 2.5 times greater with 80 µM DS, in comparison to the control, while at 40 µM DS the endogenous proline level was 50% of that of the control's and the carbohydrate content 21 to 22 times lower than the control. (Table 2).

Experiment 2. Effect of DS and BA concentrations on in vitro shoot proliferation and rooting

In the CAB-6P rootstock, shoot number/explant and shoot proliferation rate (100%) were maximum by applying BA alone or combined with DS. The control and the DS treatments (-BA) produced the longest shoots. The maximum shoot FW was achieved with 4.4 µM BA plus 250 µM DS. BA (4.4 µM) alone significantly promoted shoot proliferation (Figure 2b) compared to the control (Figure 2a), whereas shoot length was substantially decreased. DS alone in the culture medium (-BA) did not significantly affect the number, the length and the FW of the shoots. In the control plants and in the 250 µM DS treatment, no shoot proliferation was recorded whereas in the presence of 500 µM DS, a very low shoot proliferation rate (10%) and rooting percentage (20%) were achieved (Figure 2c). Furthermore, DS did not promote the positive effect of BA on shoot proliferation concerning shoot number/explant, shoot length and percentage of sprouting. On the contrary, a synergistic effect between BA and 250 µM DS concentration was found regarding shoot FW (Table 3; Figure 2d). The combined effect of BA with DS resulted in the inhibition of elongation of the initial explant (Figure 2e).

Considering the Gisela 6 rootstock, the maximum shoot number and FW were recorded in the BA (4.4 µM) plus DS (250 µM) treatment. The shoot length was found to be greatest in the control (- BA, - DS), BA alone or BA plus DS treatments. DS without BA did not significantly affect shoot number and shoot FW, in comparison to the control. On the other hand, shoot length was substantially reduced. The increment of DS concentration from 250 µM to 500 µM resulted in the increase of the sprouting percentage from 40% to 60% (Figure 2i). A synergistic effect between BA and DS (250 µM) was observed concerning the number of shoots/explant (Figure 2h). BA (-DS) significantly increased shoot number/explant, shoot FW and percentage of sprouting but reduced shoot length (Figure 2f). DS and BA did not interact regarding the number and FW of shoots/explant and DS alone (Figure 2g) does not play any role concerning shoot length or FW. The percentage of sprouting is a function of BA, DS and their interaction (Table 3).

Discussion

DS is used mainly on woody plants as a growth regulator in order to suppress apical dominance and to enhance development of lateral buds. We tested the effectiveness of DS in the cherry rootstocks CAB-6P (*P. cerasus* L.) and Gisela 6 (*P. cerasus* x *P. canescens*) as a shoot and root promoting agent under in vitro conditions. In both rootstocks, all DS concentrations tested significantly affected not only shoot proliferation and rooting characteristics but total leaf chlorophyll (a+b), carbohydrate and proline content in both leaves and roots as well.

In the Gisela 6 rootstock, the increased shoot number per explant produced with 80 µM DS is ascribed to the high cytokinin concentrations followed by low IAA and GA_3 concentrations resulting in breaking of apical dominance, as proposed by Puglisi (2002) in the *Clematis* species, and by Ebrahim (2004) in

Zantedeschia aethiopica cv. Spreng. Similar explanation was proposed in olives, where 66.7 and 100.5 μM DS in the cultivars Canino and Moraiolo and 33.8-100.5 μM in the cultivar Frantoio increased the number of shoots/explant (Mendoza de Gyves et al., 2008). Similar promotory effects of DS were recorded in the Gisela 6 explants regarding shoot number. DS in the Gisela 6 rootstock, breaks apical dominance by reducing the growth of the apical meristem of the explants or by differentiation of the vascular tissue, both of which promote lateral shoot production. The same explanation was given by Schilling (1985) for the mode of action of cycocel. An opposite response was recorded in the CAB-6P rootstock, where DS did not promote shoot proliferation. The different response of the two rootstocks is probably genotype-dependent. Furthermore, DS reduced growth in length of CAB-6P explants at high concentrations (120 and 150 μM) in comparison to the control due to a reduced rate of cell division and/or cell expansion of the explants cells. Our data are in agreement with Mendoza-de Gyves et al. (2008) where 100.5 and 133.4 μM DS had a negative effect on shoot length in the olive cultivars Canino, Frantoio and Moraiolo. According to Thetford and Berry (2000), DS exerted an inhibitory effect on height in euonymus, forsythia, Chinese privet, waxleaf privet and azalea. The decrement of shoot length is ascribed to inhibition of gibberellins biosynthesis due to DS addition, by inhibiting oxidation of ent-caurene to ent-kaurenic acid, which is a prerequisite for GA biosynthesis. Opposite results were reported by Ebrahim (2004) where DS (0.85-6.67 μM) increased shoot length. DS inhibites gibberellin biosynthesis which is responsible for shoot elongation and therefore for the greater height of the explants. Furthermore, DS reduces number and dimensions of xylem cells connected to mineral transport to tops (Fletcher et al., 2000). Considering the Gisela 6 rootstock, DS did not exert any significant effect on shoot length which agrees with the results reported for the species *Christia subcordata* Moench (Whiting, 2007).

The shoot FW was not affected by DS concentration. Opposite results were reported by Ebrahim (2004) for the species *Zantedeschia aethiopica* cv. Spreng, where 0.85 and 1.69 μM DS increased shoot FW. The different response of the two cherry rootstocks in comparison to *Zantedeschia aethiopica* is due to significantly greater DS concentrations (20-150 μM) used in our experiment and to different genotype effect. DS promoted rooting and increased the number of roots/rooted explant at high (120 and 150 μM) in the CAB-6P and at intermediate concentrations (20-80 μM) in the Gisela 6 rootstock. This rooting response is related to increased chlorophyll level and transport of photosynthates to roots. However, our data disagree with Ebrahim (2004) where in the species *Zantedeschia aethiopica* cv. Spreng (calla) 1.69 μM DS inhibited rooting.

The root FW was reduced by the application of DS, irrespective of its concentration in the Gisela 6 and at 150 μM in the CAB-6P rootstocks respectively, due to decreased endogenous level of IAA ascribed to activation of IAA-oxidase (Bekheta et al., 2003). Therefore, tryptophan is not transformed to IAA. The same explanation was proposed for wheat plants treated with paclobutrazol (El-Kady 2002), another plant growth retardant.

DS at 20 μM improved the rooting ability of the CAB-6P explants. Furthermore, promotory were its results in the Gisela 6 rootstock regarding shoot and root number as well as root length. These data are in concurrence with Ebrahim (2004) for the species *Zantedeschia aethiopica* cv. Spreng. In the Gisela 6 rootstock, DS reduced the rooting potential. Negative was also the effect of DS on the rooting percentage of *Vaccinium corymbosum* L. 'Herbert' in vivo (Litwińczuk and Prokop, 2010).

Chlorophyll concentration of Gisela 6 leaves treated with 40-120 μM DS was increased. Similar results were obtained in **Vigna sinensis** (L.) Walp. cv. Pusa Phalguni (Biswas et al., 1989), in pea (*Pisum sativum* L.) and in horse gram (*Dolichos biflorus* L.) leaves (Kanp et al., 2009) where DS increased their chlorophyll content. This happens by speeding up chlorophyll biosynthesis, chloroplasts development and activation of photosynthetic enzymes. The growth inhibitors increase chlorophyll concentration by transformation of leucoplasts to chloroplasts. The decrease of cell expansion due to DS may explain its beneficial effect on chlorophyll concentration, since more cells are present per leaf blade. Therefore, although the content of chlorophyll per cell is constant, the chlorophyll content/leaf blade increases. The decrease of chlorophyll content with 40 μM DS in the CAB-6P explants and at 150 μM in the Gisela 6 rootstock is due to a decrease in the number of leaves/explant and the total

leaf area (cm^2) per explant. Our findings are in agreement with those exhibited by Bhattacharjee and Gupta (1981) and Choudhury and Gupta 1998 for sunflower (*Helianthus annus* L. cv. EC 68414) and *Catharanthus roseus* leaves, respectively. The possible reasons for this decrease are the biosynthesis of chlorophyllase, which may participate in chlorophyll degradation, destruction of chloroplast, mitochondria and plasmalema structure and production of ROS (Dolatabadian and Jouneghani, 2009).

In the CAB-6P rootstock, the negative effect of DS concerning carbohydrate concentration in leaves and roots is attributed to a decrease in photosynthetic efficiency. A similar decrease was also reported in sunflower (*Helianthus annus* L. cv. EC 68414) (Bhattacharjee and Gupta, 1981) and in mung bean leaves (Bhattacharjee et al., 2006). However, contradictory results were obtained by Das et al. (2006) regarding carbohydrate content of *Cucumis sativus* L. fruits. The decreased content of leaves and roots in carbohydrates indicates that DS participates in carbohydrate metabolism leading to their transformation to other substances or to their consumption during aerobic respiration and inhibition of their transport from the leaves to the roots. The opposite was recorded in the Gisela 6 rootstock regarding total leaf carbohydrate concentration which agrees with the findings of Bhattacharjee and Jana (1990) for sunflower cotyledons after 120h of germination. The increment of total leaf carbohydrates in the Gisela 6 explants is due to an increase of leaf area, and also to elevated chlorophyll concentration and activation of photosynthesis (Ibrahim et al., 2007).

DS, irrespective of concentration increased endogenous proline level of Gisela 6 rootstock leaves and roots but only of CAB-6P leaves. It is well known that high proline accumulation is a significant mechanism of stress tolerance (Hare et al., 1998). It is probable therefore that DS creates a kind of stress leading to proline accumulation. The place of proline accumulation indicates that the osmoregulatory mechanism is located only in the leaves of the CAB-6P rootstock but in both leaves and roots in the Gisela 6 one.

A decrease in root proline content was observed when CAB-6P explants were treated with 80 µM DS and Gisela's with 40 and 120 µM DS. Similar results were reported for **Vigna sinensis** (L.) Walp plants treated with DS (Biswas et al., 1989). Both a decrease and an increase of proline concentration are indications of one critical point in growth and development of plants (Watanabe et al., 2001). Hence, proline is overproduced only when the degree of stress is greater than a critical point for plant growth. The effect of DS on proline levels in our experiment indicates that DS exerts osmoregulatory roles (Haq et al., 2011). Furthermore, our data indicate that DS participates in photosynthesis and biosynthesis and metabolism of carbohydrates and proline, has osmoregulatory properties and constitutes an adaptation mechanism to environmental stresses. Concerning shoot proliferation and rooting, DS promoted shoot multiplication in the Gisela 6 cherry rootstock, enhanced rooting of the CAB-6P explants at 20 µM and increased root number at 120 and 150 µM in the CAB-6P and at 20-80 µM in the Gisela 6 rootstock.

The second experiment was planned to clarify the effects of greater concentrations of DS alone or simultaneously with BA, a substance routinely used for shoot proliferation in tissue culture studies. Addition of BA (4.4 µM) without DS resulted in shoot proliferation in both rootstocks but reduced the explants' shoot length. The beneficial effect of BA is due to a promotion of cell division (Davies, 2004) and hindrance of ethylene biosynthesis produced with BA (Pech et al., 2004). Our data concerning the number and FW of shoots are in agreement with others (Soad et al., 2010)

In both cherry rootstocks, shoot number or FW was not significantly differentiated from the control when DS (-BA) was applied in the culture medium at 250 and 500 µM concentrations. However, DS significantly reduced shoot length in the Gisela 6 explants. This indicates that the applied DS concentrations (250 and 500 µM) were high, reaching toxic levels.

In the CAB-6P rootstock, 250 µM DS combined with 4.4 µM BA did not affect substantially shoot number/explant in comparison to BA alone, whereas in the Gisela 6 rootstock the effect was positive. A reduced number of shoots was reported for the in vitro culture of *Vaccinium corymbosum* L. cv 'Herbert' when DS combined with 5 mg L^{-1} 2-isopentenyladenine (2ip) (Litwińczuk and Prokop, 2010). In our study, 250 µM DS increased the positive effect of BA on shoot FW in the CAB-6P and on shoot number/explant in

the Gisela 6 rootstock and this effect was synergistic. However, the DS concentration of 500 µM did not exert any effect.

In both cherry rootstocks, the percentage of explants producing new shoots was 100% in the combined effect of BA with DS. In the olive cultivar Chondrolia Chalkidikis, DS concentrations ranging between 16.9 and 100.5 µM promoted the activity of cytokinin in producing new shoots, whereas concentrations higher than 133.4 µM had negative effect (Antonopoulou, 2009). Complete rooting inhibition due to DS addition (-BA) was observed in the Gisela 6 explants and with 250 µM DS in the CAB-6P rootstock. This is explained based on the tendency of DS to reduce endogenous level of IAA by increasing the activity of IAA-oxidase and by reducing the transformation of tryptophan to IAA (El-Kady, 2002). Furthermore, the inhibitory action of DS in rooting is due to high levels of cytokinins which are followed by low levels of IAA and GA_3. The same explanation was proposed for the growth inhibition in wheat by uniconazole (El-Kady 2002), another plant growth retardant. Under certain conditions, DS acts as an anti-auxin, like TIBA, inhibiting polar auxin transport from the apex to the base of the explants. This explanation was previously expressed by Cline (1997). Anti-auxins were reported to promote or modify morphogenetic processes in vitro by inactivating the excess of exogenous or endogenous auxins. Furthermore, it was proposed that anti-auxins act as inhibitors of the basipetal auxin transport, regulating the cytokinin/auxin ratio required for in vitro propagation of explants (Singh and Syamal, 2000). DS only at the highest concentration (500 µM) substantially increased root number/rooted explant in the CAB-6P rootstock. Opposite results were reported for the olive cultivars Canino, Frantoio and Moraiolo, where 66.7 µM DS combined with 4.5 µM zeatin did not modify significantly the previous characteristic (Mendoza de Gyves et al., 2008).

Conclusion

In the first experiment it was found that between the two cherry rootstocks, Gisela 6 reacted positively to DS application concerning shoot proliferation, while CAB-6P did not react at all. Furthermore, DS did not modify substantially the length of the Gisela 6 microshoots but it was inhibitory to CAB-6P plants regarding the shoot length of the initial explant. Both rootstocks reacted positively to DS application i.e. the CAB-6P concerning the number of roots/rooted explant and rooting percentage and the Gisela 6 regarding root number and root length. In the Gisela 6 rootstock, low DS concentrations increased chlorophyll concentration, while high ones exerted an inhibitory effect. On the contrary, in the CAB-6P rootstock DS reduced or did not change significantly leaf chlorophyll concentration. DS reduced the level of total carbohydrates and proline in leaves and roots of the Gisela 6 explants whereas in the CAB-6P rootstock had a stimulatory effect. In both rootstocks, proline leaf concentration was increased as a result of DS effect.

To our knowledge, this is the first study which reports the use of DS as a growth regulator on cherry rootstocks under in vitro conditions with aim to increase shoot proliferation and concurrently to promote rooting. Therefore, the use of DS appears to be a promising substance in plant tissue culture by reducing propagation stages and time to end with rooted explants.

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