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In vitro response of *Actinidia deliciosa* explants to different BA incubation periods

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Abstract

Actinidia deliciosa apical shoots were cultured in MS liquid medium with cellulose plugs as support for the explants. Different BA (4.4 μM) incubation periods were tested in order to improve the effectiveness of the micropropagation system by reducing the cytokinin incubation period. At the end of 3 successive subcultures, the explants were analysed and a number of parameters (number, weight and length of shoots, presence and weight of callus, multiplication index, etc.) were measured. Different BA incubation periods have a long-term effect since the best results at the end of multiplication stage were not followed by better growth at the end of the acclimatised period studied. The highest quality plants were those obtained from culturing in the presence of BA for 1 day. Our results show that BA not only has an important effect on the different phases of micropropagation, but will also regulate the future development of the regenerants.

Abbreviations: BA – benzyladenine; MS medium – Murashige and Skoog medium

Introduction

In vitro culture of some species occasionally presents serious problems due to the production of shoots with abnormal morphologies (hyperhydration or apical necrosis). There are many studies which describe how the number of abnormal microshoots increases with successive subcultures with some subcultures having as high as 50 and 80% of anomalous shoots (Ziv et al., 1983; Leshem et al., 1988; Safrazbekyan et al., 1990). Moreover, conventional methods of micropropagation cannot always be used due to the limited growth of the explants, the difficulties of microshoot rooting and the low percentage of explant survival after transfer to soil.

Exogenous cytokinin application is essential for micropropagation (Biondi et al., 1984; Chen et al., 1985). Benzyladenine is the most frequently used cytokinin in commercial micropropagation (Thomas and Blakesley, 1987) as it induces more proliferation than zeatin or kinetin (Marino and Bertazza, 1990). However, high concentrations and long incubation periods with BA are reported to be damaging to shoot growth and leaf expansion (Dunstan et al., 1985), as well as causing a higher percentage of hyperhydrated shoots (Ziv, 1991).

In cotyledons of Gymnosperms, the ability to induce adventitious buds depends on the age of the mother plant as well as the application method of plant growth regulators (Jansson and Bornman, 1981; Bornman, 1983). Consequently, cytokinin concentration and explant exposure time are two important factors which must be carefully selected since they affect the subsequent development of the plant.

The rate and amount of BA absorbed from the medium and the pattern of BA metabolism varies considerably among species, depending on the physical nature of the culture medium (Nordström and Eliasson, 1986; Blakesley, 1991; Blakesley and Constantine, 1992; Feito et al., 1994; Moncaleán et al.,

1999). In previous studies (Moncaleán et al., 1999), we have observed that most BA uptake by kiwifruit explants cultured in a liquid medium, with cellulose plugs as support, occurs during the first 30 min of culture. Furthemore, others have demonstrated that 70% of the BA taken up by the explants is metabolized during the first 24 h (Blakesley, 1991; Feito et al., 1994).

Hormonal stimulation of cell division begins during the first 30 min, and the whole process is concluded within the first 24 h (Trewavas, 1979). Hence, the incubation period with BA in liquid medium should presumably be kept as short as possible to decrease the problems associated with cytokinins.

We studied the effects of different application patterns of BA in a liquid culture system, using cellulose plugs as explants support, with the aim of improving the micropropagation of *Actinidia deliciosa* by minimizing the exposure period to cytokinins without losing the advantages these offer. Moreover, we established optimal conditions for BA application during the multiplication phase in order to obtain good rooting and survival percentages when planlets were transferred to *ex vitro* conditions.

Materials and methods

Multiplication phase

Branches were cut in the winter from a commercial kiwi (*Actinidia deliciosa* Chev. Cv. Hayward) plantation in Pravia (Asturias). They were washed with a Captan solution (0.4%, w/v) and ferrous sulphate (8%, w/v) in water, and the cut ends were protected with paraffin. Subsequently the branches were kept in storage for 1 month at 4 °C. After this period, shoot portions with one bud and 0.5–1 cm of stem were surface sterilized by immersion in 80% (v/v) ethanol for 5 min, followed by one rinse in sterile distilled water. They were subsequently transferred to a 2.5 g l^{-1} calcium hypochlorite solution and then washed three times in sterile distilled water. Primary explants were cultured in KH medium (Cheng, 1977) without plant growth regulators (Figure 1). After 7 days, uncontaminated explants were transferred to a shoot initiation medium for 20 days (Figure 1), as described by Standardi (1983). Subsequently, the explants were subcultured on MS medium (Murashige and Skoog, 1962) with 4.4 μM BA, 0.6 μM gibberellic acid (GA_3), 2% (w/v) sucrose and 0.7% (w/v) agar (multiplication medium) for 35 days (Figure 1). The pH was adjusted to 5.6 before autoclaving. Each Magenta box culture vessel (GA7, pat. pen., Magenta Corp., USA) contained 5 shoots growing on 70 ml of culture medium. All cultures were maintained in a growth chamber at 25±2 °C under a 16-h photoperiod provided by cool-white fluorescent lamps (TDL 58 W/33, Phillips, France) at a photon flux density of 33 μmol m^{-2} s^{-1}.

Once the micropropagation system was established, apical shoots consisting of the apical meristem and three axillary buds were cultured in MS liquid medium, supplemented with sucrose (2%, w/v), 0.6 μM GA_3 and 4.4 μM BA, for different periods: 30 min, 1, 4 and 8 h and 1, 2, 4, 8 and 15 days. Cellulose plugs (Sorbarod, Baumgartner Papiers S.A., Lausanne, Switzerland) were used to support the explants. Explants cultured for short incubation periods (30 min, 1, 4 and 8 h and 1 and 2 days) were transferred to initiation medium; whereas a corrected medium was used for the explants treated with BA for longer periods (4, 8 and 15 days). This correction was based on the macronutrient and carbohydrate absorption undergone by the kiwi explants over these culture periods (Moncaleán, 2000). These BA treatments were applied at the beginning of three successive subcultures (35 days each) using explants from the previous subculture and maintaining the explant characteristics described for the first subculture (Figure 1). In addition, explants cultured in the absence and presence of BA were used as controls.

The following variables were measured in all the explants after each subculture: shoot number, shoot length and number of those longer than 1 cm, presence or absence of hyperhydrated shoots, callus weight, presence of roots, aerial part weight and multiplication index (number of explants obtained per initial explant). Furthermore, the multiple multiplication index, a theoretical value obtained by multiplying the three multiplication indices in the three subcultures, was calculated.

Rooting phase

After 3 successive subcultures, apical shoots consisting of the apical bud and three axillary buds were immersed in a filter-sterilized solution of indole-3-butyric acid (IBA) 4.9 mM and then cultured for 20 days in KH liquid medium (Cheng, 1977) (Figure 1) under the same conditions as those previously mentioned for the multiplication phase. After that,

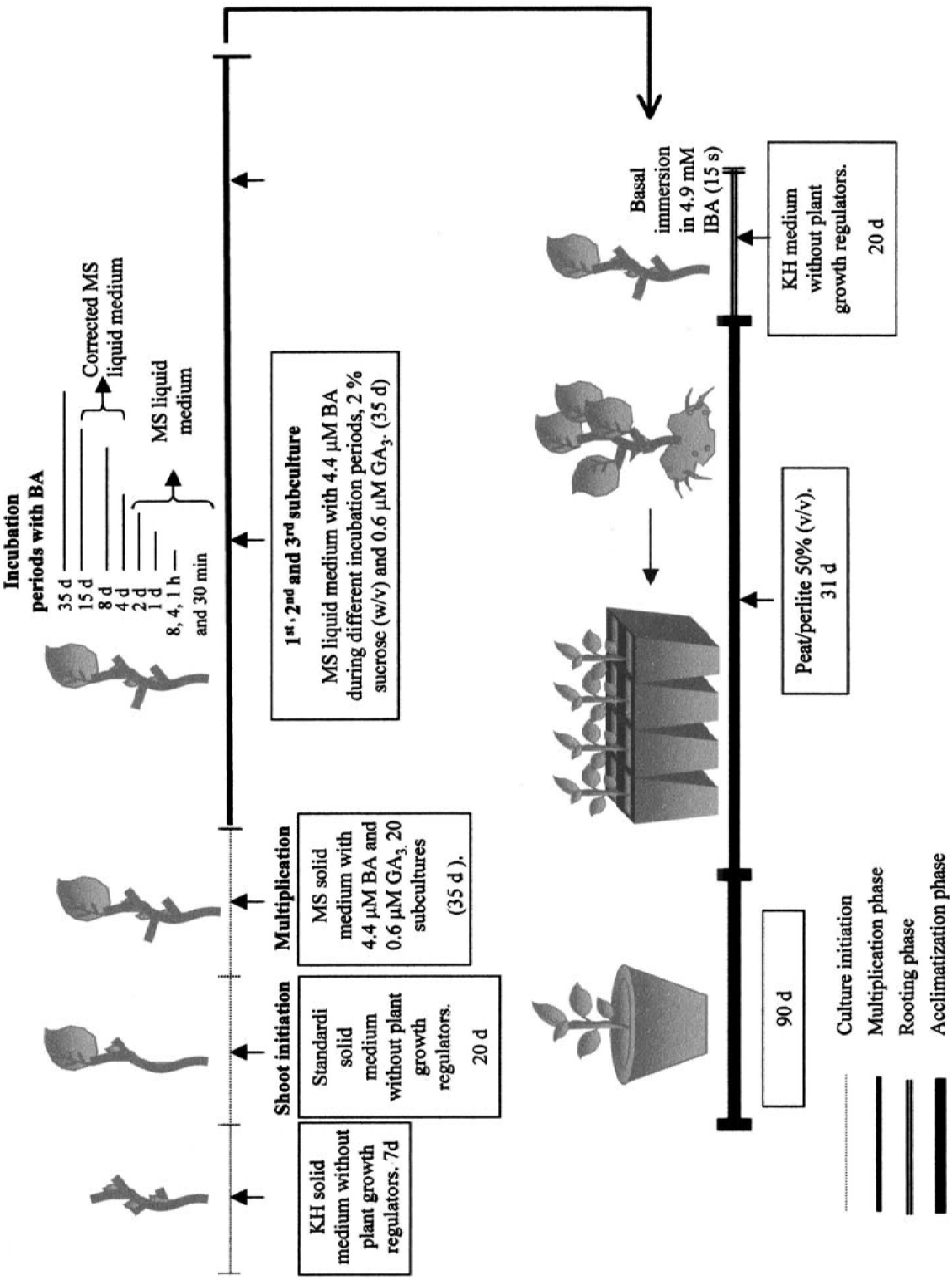

Figure 1. Scheme of the successive phases of *Actinidia deliciosa* micropropagation.

Table 1. Callus weight (g) in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods in their *in vitro* multiplication phase during 3 successive subcultures

Incubation period with BA (4.4 μM)	Callus weight (g)		
	1st Subculture	2nd Subculture	3rd Subculture
0	[1,2]0.99±0.14 [ab]	[2]0.76±0.10 [abc]	[1]1.26±0.07 [a]
30 min	[1]0.92±0.10 [abc]	[1]0.73±0.08 [abc]	[1]0.93±0.12 [b]
1 h	[1]0.55±0.07 [ef]	[1]0.39±0.12 [c]	[1]0.37±0.10 [fg]
4 h	[1]0.68±0.08 [cde]	[1]0.60±0.12 [abc]	[1]0.81±0.08 [b]
8 h	[1]0.94±0.14[abc]	[1]0.75±0.10 [abc]	[1]0.88±0.13 [b]
1 d	[1]0.85±0.07 [abcd]	[1]0.81±0.08 [a]	[2]0.47±0.06 [defg]
2 d	[1]0.77±0.09 [bcde]	[1]0.61±0.07 [abc]	[1]0.75±0.07 [bc]
4 d	[1]0.59±0.04 [def]	[1]0.50±0.05 [c]	[1]0.55±0.06 [cdefg]
8 d	[1]0.55±0.04 [ef]	[1]0.54±0.08 [bc]	[1]0.45±0.04 [efg]
15 d	[2]0.40±0.04 [f]	[1]0.79±0.12 [ab]	[2]0.35±0.03 [g]
35 d	[1]1.08±0.13 [a]	[2]0.59±0.06 [abc]	[1,2]0.73±0.07 [bc]

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column. Significant differences (α=0.05) among subcultures are indicated by different numbers in each row.

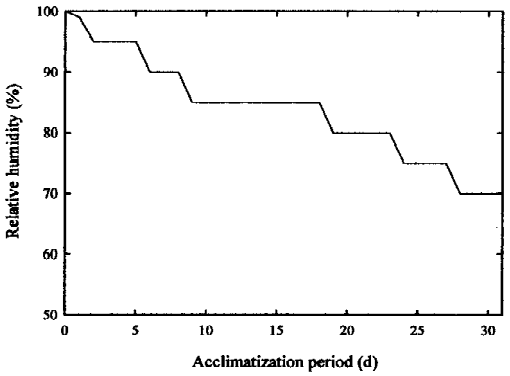

Figure 2. Relative humidity (%) in the *Actinidia deliciosa* acclimatization chamber during 31 days under *ex vitro* conditions.

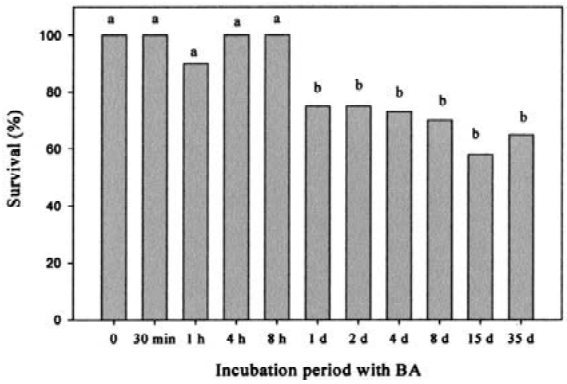

Figure 3. Survival (%) in *Actinidia deliciosa* explants treated with different BA pulses (4.4 μM) during their *in vitro* multiplication stage after 90 days under *ex vitro* conditions. Significant differences (α=0.05) among BA treatments are indicated by different letters.

the following parameters were determined in all the explants: shoot length, percentage of explants with basal callus, number of leaves, percentage of rooted explants.

Acclimatization phase

After the rooting phase, the basal parts from rooted and non-rooted explants were washed in distilled water and microshoots were transplanted to soil (peat:perlite, 1:1, v/v) in multipot bowls which were maintained in a chamber with a programmable relative humidity (±4%). Figure 2 shows the decline of the relative humidity during the acclimatization period.

After 31 days in the acclimatization phase, the following variables were determined in all the microplants: shoot length, percentage of rooted microshoots and number of leaves.

Subsequently, the microplants were transplanted into 15 cm diameter containers and cultured in a greenhouse (Figure 1).

After 90 days under *ex vitro* conditions, leaf number, shoot length and survival percentage of each plant were analysed.

Statistical analyses

The data shown is the mean of 3 experiments using ten explants per treatment. Statistical analysis of quantitative data was carried out using the ANOVA test and

Table 2. Aerial part weight (g) in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods in their *in vitro* multiplication phase during 3 successive subcultures

Incubation period with BA (4.4 μM)	Aerial part weight (g)		
	1st Subculture	2nd Subculture	3rd Subculture
0	$^{2}0.33\pm0.04^{\ cd}$	$^{2}0.37\pm0.04^{\ bcdef}$	$^{1}0.60\pm0.06^{\ ab}$
30 min	$^{1}0.49\pm0.05^{\ a}$	$^{1}0.47\pm0.04^{\ a}$	$^{1}0.61\pm0.05^{\ a}$
1 h	$^{1}0.38\pm0.04^{\ abcd}$	$^{1}0.33\pm0.04^{\ def}$	$^{1}0.46\pm0.05^{\ bcdef}$
4 h	$^{2}0.37\pm0.01^{\ bcd}$	$^{2}0.42\pm0.04^{\ abcde}$	$^{1}0.55\pm0.05^{\ ab}$
8 h	$^{1}0.37\pm0.04^{\ bcd}$	$^{1}0.42\pm0.04^{\ abde}$	$^{1}0.41\pm0.04^{\ def}$
1 d	$^{1}0.41\pm0.03^{\ abc}$	$^{1}0.43\pm0.02^{\ abd}$	$^{1}0.37\pm0.03^{\ ef}$
2 d	$^{1}0.45\pm0.04^{\ ab}$	$^{1}0.41\pm0.03^{\ abcde}$	$^{1}0.41\pm0.03^{\ cdef}$
4 d	$^{1}0.37\pm0.04^{\ bcd}$	$^{1}0.32\pm0.02^{\ ef}$	$^{1}0.35\pm0.02^{\ f}$
8 d	$^{1}0.40\pm0.02^{\ abcd}$	$^{2}0.29\pm0.02^{\ f}$	$^{1,2}0.33\pm0.02^{\ f}$
15 d	$^{2}0.31\pm0.03^{\ d}$	$^{1}0.40\pm0.03^{\ abcde}$	$^{1,2}0.33\pm0.02^{\ f}$
35 d	$^{1}0.45\pm0.04^{\ abc}$	$^{2}0.32\pm0.02^{\ cdef}$	$^{1,2}0.35\pm0.04^{\ f}$

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column. Significant differences (α=0.05) among subcultures are indicated by different numbers in each row.

comparison of means was performed by the LSD test. Percentages analyses were carried out using the chi-square test. All statistical analyses were performed at the 5% level using the SPSS© statistical package software.

Results and discussion

Multiplication phase

Developed microshoots never showed hyperhydratation symptoms, regardless of the BA incubation period.

Independently of the BA incubation period and the considered subculture, explants developed more than one shoot only occasionally, in contrast with the results obtained in previous work (Moncaleán et al., 1999).

The effectiveness of BA as a caulogenic inductor for *in vitro* cultured explants of conifers, when media formulations with low ammonium concentration are used, is well documented (Abdullah et al., 1985; Flinn et al., 1986; Pérez-Bermudez and Sommer, 1987; Paranjothy et al., 1990). Since kiwifruit culture medium is rich in ammonium, we attribute the absence of bud proliferation to a high amount of this ion. However, with this composition more than one shoot was obtained in the past (Moncaleán et al., 1999); this suggests that results might be dependent on the age of the micropropagation system.

In contrast, the spontaneous rooting percentage decreased in parallel with the increase in the BA incubation period. Explants grown in BA-free medium showed 20% spontaneous rooting, which was also found in all the explants treated for less than 8 h with BA.

The BA incubation period affected callus weight (Table 1). At the end of the third subculture, explants cultured in the absence of BA throughout the entire culture period had the greatest callus weight, showing significant differences with respect to the values presented by the rest of the explants. Aerial part weight (Table 2) increased from the 1st to the 3rd subculture in those explants treated with BA incubation periods shorter than 8 h. Explants cultured in the presence of BA for more than 8 h showed constant or slightly lower values between the 1st and 3rd subculture.

The BA incubation period also had a significant effect on shoot length (Table 3). A progressive increase in this parameter from the 1st to the 3rd subculture in all the treatments except in 2 days and 35 days-treated explants was found. This effect was more evident for incubation periods shorter than 2 days. At the end of the 3rd subculture, explants cultured in the absence or in the presence of BA during 30 min showed the highest length, followed by 1 day-BA treated explants, although significant differences were not observed between these treatments. In contrast, explants grown 1 day in the presence of BA had

Table 3. Shoot length (cm) in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods in three successive subcultures during their *in vitro* multiplication phase

Incubation period	Shoot length (cm)		
with BA (4.4 μM)	1st Subculture	2nd Subculture	3rd Subculture
0	^{2}2.35±0.19 ^{defg}	^{1,2}2.95±0.24 ^{abc}	^{1}3.61±0.3 ^{abc}
30 min	^{1}2.92±0.31 ^{abcde}	^{1}3.40±0.24 ^{a}	^{1,2}3.68±0.25 ^{ac}
1 h	^{2}1.92±0.16 ^{g}	^{2}2.15±0.16 ^{e}	^{1}3.05±0.15 ^{abcdef}
4 h	^{2}2.65±0.18 ^{bcdef}	^{1,2}2.89±0.24 ^{abce}	^{1}3.30±0.23 ^{abcd}
8 h	^{1}2.37±0.25 ^{cdefg}	^{1}3.05±0.32 ^{abc}	^{1}3.05±0.24 ^{cde}
1 d	^{1}3.02±0.24 ^{abcd}	^{1}3.25±0.23 ^{abc}	^{1}3.30±0.19 ^{abcd}
2 d	^{1}3.35±0.46 ^{a}	^{1}2.67±0.14 ^{cde}	^{1}3.00±0.17 ^{bcde}
4 d	^{2}2.02±0.11 ^{fg}	^{1}2.60±0.16 ^{cde}	^{1}2.70±0.20 ^{defg}
8 d	^{1}2.32±0.14 ^{efg}	^{1}2.21±0.16 ^{de}	^{1}2.37±0.16 ^{fg}
15 d	^{2}2.02±0.18 ^{fg}	^{1}2.75±0.27 ^{bcde}	^{1,2}2.16±0.20 ^{g}
35 d	^{1}2.69±0.25 ^{abcdef}	^{1}2.62±0.18 ^{cde}	^{1}2.55±0.25 ^{efg}

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column. Significant differences (α=0.05) among subcultures are indicated by different numbers in each row.

Table 4. Multiplication index in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods during 3 successive subcultures and at the end of these

Incubation period	Multiplication index			
with BA (4.4 μM)	1st Subculture	2nd Subculture	3rd Subculture	Multiple
0	^{2}1.55±0.13 ^{bcd}	^{2}1.50±0.15 ^{bcd}	^{1}2.00±0.18 ^{abe}	4.65
30 min	^{1}1.85±0.13 ^{abc}	^{1}1.80±0.20 ^{abcd}	^{1}2.16±0.16 ^{a}	7.15
1 h	^{1}1.50±0.13 ^{cd}	^{2}2.30±0.16 ^{ad}	^{2}2.10±0.11 ^{abe}	7.25
4 h	^{1,2}1.80±0.13 ^{abc}	^{2}1.47±0.17 ^{c}	^{1}2.10±0.11 ^{abe}	5.42
8 h	^{1}1.50±0.15 ^{cd}	^{1}1.65±0.18 ^{abcd}	^{1}1.66±0.16^{bdegh}	4.10
1 d	^{1}2.10±0.14 ^{a}	^{1,2}1.83±0.14 ^{abcd}	^{2}1.55±0.13 ^{degh}	5.95
2 d	^{1}2.10±0.17 ^{a}	^{2}1.50±0.11 ^{bcd}	^{1,2}1.75±0.14 ^{eg}	5.51
4 d	^{1} 1.25±0.09 ^{d}	^{1}1.57±0.13 ^{abcd}	^{1}1.50±0.13 ^{gh}	2.94
8 d	^{1}1.75±0.09 ^{abc}	^{2}1.23±0.10 ^{d}	^{2}1.30±0.10 ^{h}	2.79
15 d	^{1}1.55±0.11 ^{bd}	^{1}1.60±0.19 ^{abcd}	^{2}1.10±0.07 ^{fgh}	2.72
35 d	^{1}2.05±0.18 ^{a}	^{1,2}1.65±0.10 ^{abcd}	^{2}1.35±0.16 ^{cdfgh}	4.56

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column. Significant differences (α=0.05) among subcultures are indicated by different numbers in each row.

longer shoots than those exposed to BA throughout the whole culture period and significant differences were observed for this parameter. Reduction of the exposition to BA increased shoot length. This was especially clear in explants grown in the absence of BA during the three subcultures, which could increase the action of the GA_3 added to the culture medium. The stimulatory effect of GA_3 in plant development is well known, and George and Sherrington (1984) postulated that this plant growth regulator promotes cellular division and elongation in the shoot's subapical zone. As we previously observed (Moncaleán, 2000), when the explants grew in a BA-free medium, GA_3 absorption was higher than when BA was present in the culture medium. Therefore, this high uptake of gibberellin could be responsible for the increased shoot length observed.

Our results are in agreement with those reported by Dunstan et al. (1985), who postulated that increasing the incubation period with BA led to a reduction in shoot elongation. On the other hand, Sujatha and Reddy (1998), in a study of *Ricinus communis*, ob-

Table 5. Callus (%), number of leaves, rooting (%) and shoot length (cm) in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods during their *in vitro* multiplication stage, after 20 day in rooting medium

Incubation period with BA (4.4 μM)	Rooting stage			
	Callus (%)	Number of leaves	Rooting (%)	Shoot length (cm)
0	57 ^{bc}	2.84±0.21 ^{bc}	100 ^{a}	2.44±0.12 ^{a}
30 min	35 ^{c}	1.85±0.19 ^{f}	85 ^{abc}	2.1±0.18 ^{abc}
1 h	30 ^{c}	2.3±0.21 ^{cdef}	90 ^{abc}	2.1±0.17 ^{abc}
4 h	75 ^{b}	1.95±0.18 ^{ef}	90 ^{ab}	1.9±0.14 ^{bcd}
8 h	60 ^{b}	2.7±0.36 ^{bcd}	100 ^{ab}	1.75±0.21 ^{cde}
1 d	80 ^{b}	3.4±0.16 ^{a}	95 ^{ab}	2.22±0 .13 ^{ab}
2 d	100 ^{a}	1.9±0.06 ^{f}	60 ^{c}	1.27±0.09 ^{fg}
4 d	100 ^{a}	2.8±0.21 ^{ab}	100 ^{a}	1.6±0.11 ^{defg}
8 d	100 ^{a}	1.9±0.14 ^{f}	75 ^{bc}	1.6±0.08 ^{defg}
15 d	100 ^{a}	2.11±0.14 ^{def}	88 ^{abc}	1.23±0.14 ^{g}
35 d	100 ^{a}	2.65±0.30 ^{bcde}	80 ^{bcd}	1.35±0.12 ^{efg}

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column.

tained the best elongation results using a mix of BA (0.88 μM) and GA_3 (0.3–3 μM). Furthermore, Gómez and Segura (1994) observed a decrease in shoot length in *Juniperus oxicedrus* in explants grown in a BA-free medium compared to those grown in the presence of cytokinin throughout the entire culture period.

With respect to the multiple multiplication index (Table 4), the best results were observed for explants treated for 1 h and 30 min with BA, although good results were also obtained for treatments of 1 and 2 days with this hormone. At the end of the 3rd subculture, explants cultured in the presence of BA for less than 8 h presented the highest values of this parameter, showing significant differences with respect to those obtained with the rest of the treatments. A similar improvement caused by a reduction in the BA incubation time has been described in strawberries by López-Aranda et al. (1994) after 8 subcultures.

It is important to point out that the micropropagation efficiency of explants grown in the absence or in the presence of BA throughout the entire culture period was similar. Likewise, we observed a decrease in the multiplication index (Table 4) throughout the three subcultures for explants cultured in the presence of the cytokinin for 35 days, significant differences being observed between the 1st and 3rd subculture. The contrary effect was observed in explants cultured in the absence of BA. These results suggest, in agreement with Williams (1992), that BA can have more detrimental side effects in liquid than in a solid media because, in the former, the availability of the plant growth regulator is higher and the accumulation of cytokinins inside the tissues might provoke a progressive decay in the cultures.

Shoot length and multiplication indices increased in BA-free cultured explants throughout the 3 subcultures analysed. This may indicate that explants had a better hormonal balance.

In summary, the best results in this stage were achieved with explants grown for less than 2 days in the presence of BA.

Rooting phase

After 20 days in the rooting medium, all the explants which had been grown with BA in the culture medium for 2 or more days had a significantly higher basal callus formation rate than obtained in the rest of the treatments. In explants cultured with BA during periods of less than 2 days, this percentage varied between 30 and 80% (Table 5).

We observed the highest number of leaves in 1 day BA-treated explants, although no clear trend was noted.

In general, the rooting percentage was high for all the BA incubation periods studied.

Explants grown in the absence of BA throughout all the culture period presented the highest shoot length (Table 5). With longer exposure, the shoot length obtained decreased significantly. Similar results were achieved by Polanco and Ruiz (1997) when they

Table 6. Rooting (%), number of leaves and shoot length in *Actinidia deliciosa* explants treated with different BA (4.4 μM) incubation periods during their multiplication stage, after 31 days under *ex vitro* conditions

Incubation period with BA (4.4 μM)	Acclimatization stage		
	Rooting (%)	Number of leaves	Shoot length (cm)
0	100 ^{a}	4.84±0.11 ^{a}	2.78±0.10 ^{defg}
30 min	100 ^{a}	4.00±0.17 ^{defg}	2.70±0.13 ^{efg}
1 h	100 ^{a}	4.00±0.29 ^{efg}	2.10±0.12 ^{i}
4 h	100 ^{a}	4.45±0.16 ^{abcdef}	2.15±0.13 ^{i}
8 h	100 ^{a}	4.20±0.32 ^{f}	2.40±0.10 ^{ghi}
1 d	100 ^{a}	4.56±0.15 ^{abcef}	3.71±0.17 ^{a}
2 d	77 ^{c}	3.22±0.19 ^{h}	2.25±0.21 ^{hi}
4 d	100 ^{a}	4.14±0.20 ^{cdef}	3.17±0.22 ^{bcd}
8 d	95 ^{abc}	4.25±0.16 ^{bcdef}	3.07±0.14 ^{cde}
15 d	80 ^{bc}	3.53±0.19 ^{gh}	2.23±0.17 ^{hi}
35 d	90 ^{abc}	4.35±0.15 ^{bcdef}	2.55±0.10 ^{fgh}

M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters in each column.

cultured lentil seeds *in vivo* with and without BA, finding longer shoots and roots in the former treatment. It seems clear that shoot length in kiwifruit decreases in parallel with an increase in the incubation period with BA.

Acclimatization

After 31 days in a humidity controlled chamber, all the microshoots which had been cultured 1 day or less with BA rooted (Table 6). The lowest rooting percentage (77%) was found in 2 days BA-treated explants.

In most of the treatments tested, all the explants showed a similar leaf number (Table 6). Plants grown in the absence or in the presence of BA for 1 day showed the highest values for this parameter, but significant differences were not observed. One day BA-treated explants also developed the longest shoots during the acclimatization stage, showing significant differences in shoot size with respect to the other microplants.

After 90 days under *ex vitro* conditions, a decrease in the survival percentage (Figure 3) in parallel with an increase in the BA incubation period was observed.

Explants grown in the presence of BA for less than 8 h showed near 100% survival after transfer to soil. The longest BA treatments (15 and 35 days) had the most negative effect.

Those explants which generated callus during the rooting phase (Table 5) showed the lowest root development after the acclimatization period and the lowest survival percentage 90 days after their transfer to *ex vitro* conditions.

After 90 days under *ex vitro* conditions, the influence of the BA incubation period during the *in vitro* multiplication stage was evident. The highest leaf number and shoot length (Figure 4 A, B) was found in explants cultured in the presence of BA for 1 day. These differences were not expressed until 3 months after the transplanting of the kiwi plants to *ex vitro* conditions, indicating that the pattern of BA application has a long time effect.

The data presented in this paper suggests that plants 'remember' the treatments to which they were exposed during the initial phases of their growth, thus conditioning their future development. A follow-up of the plants *ex vitro* is necessary to select the best growth regulator treatment to be applied during the multiplication stage.

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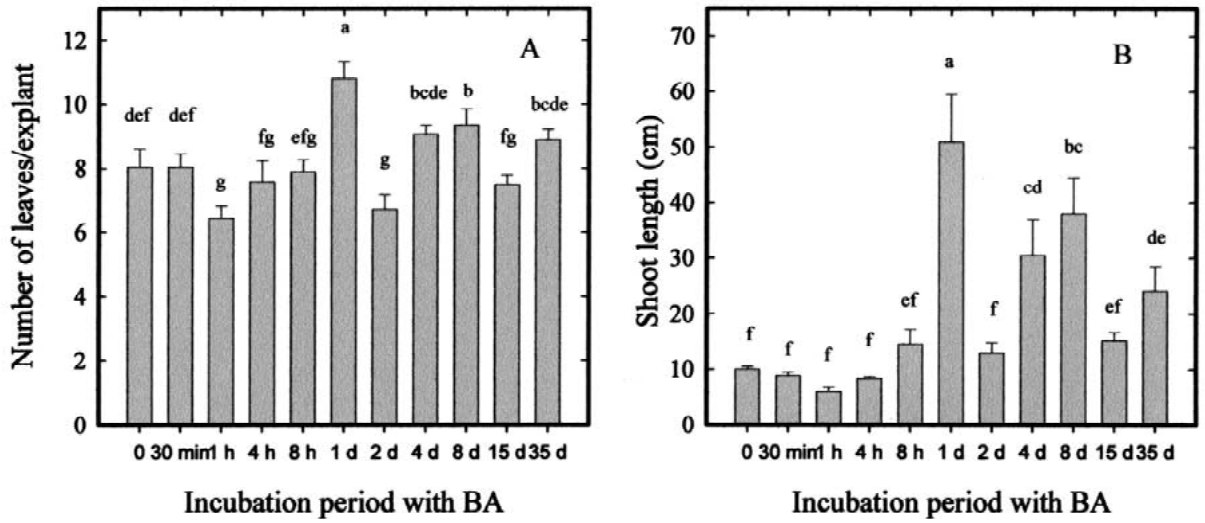

Figure 4. Number of leaves per explant (*A*) and shoot length (cm) (*B*) in *Actinidia deliciosa* explants treated with different BA (4.4 μM) pulses during the multiplication phase after 90 days under *ex vitro* conditions. M±S.E. Significant differences (α=0.05) among BA treatments are indicated by different letters.

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