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Influence of cytokinins and subculturing on proliferation capacity of single-axillary-bud microcuttings of *Vitis vinifera* L. cv. Napoleón

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Abstract

The addition of cytokinins to the culture medium was essential for the sprouting and formation of multiple shoots from axillary-bud microcuttings excised from *in vitro*-grown plants of *Vitis vinifera* L. cv. Napoleón. Of three cytokinins assayed, 6-benzyladenine (BA), kinetin (K), 2-isopentenyladenine (2iP), and thidiazuron (TDZ), BA encouraged the best morphogenic response. This is especially true at concentrations of 6.67 and 8.9 μM which led to 100% sprouting, an average of 8.7 axillary buds and 2.5 shoots developed per explant, together with maximum multiplication coefficients of buds and shoots. Subculture of microcuttings to fresh media maintained sprouting rates and substantially increased the production of shoots and axillary buds per explant until the third subculture. According to the results obtained it could seem advisable to limit the multiplication cycles for cv. Napoleón to three since further transfers induced vitrification phenomema and degenerations.

Key words: In vitro subculture, Grapevine, Cultivar “Napoleón”.

Resumen

Influencia de las citoquinnias y los subcultivos en la capacidad de proliferación de microesquejes axilares de Vitis vinifera *L. cv. Napoleón.*

La adición de citoquininas al medio de cultivo fue esencial para la brotación y formación de numerosos brotes a partir de microesquejes con yemas axilares obtenidos de plántulas crecidas *in vitro* de *Vitis vinifera* L. cv. Napoleón. De las tres citoquininas ensayadas, 6-benciladenina (BA), kinetina (K), 2-isopenteniladenina (2iP), y tidiazuron (TDZ), BA fue la que mejor respuesta mofogénica dió. Las concentraciones de 6,67 y 8,9 μM de BA provocaron un 100% de brotación, una media de 8,7 yemas axilares y 2,5 brotes desarrollados por explante inicial, junto con coeficientes de multiplicación de yemas y brotes máximos. El subcultivo de los microesquejes a medio freco mantuvo las tasas de brotación e incrementó sustancialmente la producción de yemas y brotes axilares por explante hasta el tercer subcultivo. Según los resultados obtenidos, debería limitarse los ciclos de multiplicación para el cultivar Napoleón hasta tres ya que más subcultivos indujeron fenómenos de vitrificación y degeneración de los brotes.

Palabras clave: Subcultivo in vitro, Uva, Cultivar «Napoleón».

Introduction

The variable morphogenic response of *in vitro* cultured explants is a frequent problem in micropropagation. These variations occur even when the same culture media and incubation conditions are used, which suggest that there is relationship with the plant material used in each case. Shoot tips and nodal segments from vineyard plants, and greenhouse- or growth chamber-grown plants are commonly used in *in vitro* multiplication protocols involving grapevine species or varieties. They represent a heterogeneous material depending on the position of the node or shoot, shoot vigour and the time of the year when they were excised. On the other hand, the use of single-axillary-bud microcutings from *in vitro*-grown plants represents an improvement in the propagation technique due to the greater homogeneity of the plant material (Zlenko et al. 1995, Heloir et al. 1997).

In a previous study (unpublished data) we showed how the sprouting, growth and elongation of the latent buds contained in axillary-bud microcuttings obtained from *in vitro*-grown plants (vitroplants) of *Vitis vinifera* L. cv. Napoleón required the exogenous addition of BA. The main objectives of this work are to describe the comparative morphogenic effect of three cytokinins, 6-benzyladenine (BA), kinetin (K), 2-isopentenyladenine (2iP), and thidiazuron (TDZ), a phenyl-urea herbicide exhibiting a strong citokinin-like activity (Mok and others 1987, Huetteman & Preece 1993) and, also to evaluate the capacity to maintain the propagation potential of the cultures through several subcultures to fresh medium containing different concentrations of the cytokinin, which encouraged the best proliferation rate and morphological homogeneity in the developed shoots.

Material and methods

Plant material and types of explant used

In vitro cultures of *Vitis vinifera* L. cv. Napoleón plants rooted in MS/2 basal medium (Murashige & Skoog 1962), with the major salts diluted to half strength, were used. Bud cultures were initiated from single-axillary-bud microcuttings without leaves and cultivated on MS basal medium supplemented with different concentrations of cytokinins or similarly acting substances. Explants obtained from newly developed shoots were used as propagules for the micropropagation protocol by routine subculturing. Twenty to thirty explants were used for each culture medium or treatment.

Media

The composition of the culture media used in this study differed as regards the type and concentration of cytokinin incorporated, but always using the MS medium as base. In the initial bud cultures, four concentrations of three cytokinins BA (2.22, 4.4, 6.67 and 8.9 μM), K (9.3, 19.6, 29.4 and 39.2 μM), 2iP (4.9, 9.8, 24.6 and 49 μM), and TDZ (0.45, 0.9, 2.25 and 4.5 μM) were used. For the propagation phase involving successive subcultures every 45 days of axillary-bud microcuttings obtained from the proliferating shoots, only BA at the above mentioned concentrations was added.

All the culture media were supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar (Technical No. 3 OXOID). The pH of the media was adjusted to 5.8. As containers we used 150x24 mm glass test tubes with plastic stoppers 25 mm in diameter (Kap-uts, BELLCO), containing 20 ml of medium. Media were autoclaved for 15 min at a pressure of 1.1 kg/cm^2 and 121ºC.

The multiplication potential of the axillary-bud microcuttings from Napoleón vitroplants was measured by estimating the sprouting percentage of buds, the number of axillary-buds and shoots produced per explant, and the length of the primary developed shoots on each of the media after 45 days' incubation. Bud and shoot multiplication coefficients (BMC and SMC respectively) were defined as:

$$B.M.C.= \frac{(\%\ \text{viable explants}) \times (\text{Average of buds/explant})}{100}$$

$$S.M.C.= \frac{(\%\ \text{viable explants}) \times (\text{Average of shoots/explant})}{100}$$

Incubation conditions

The cultures were incubated in a controlled climate chamber for 45 days at 23 ± 2^0C and with a photoperiod of 16 hours. The light was provided by fluorescent tubes of white light (Grow-lux, SYLVANIA), which provided a light intensity of 30-35 μE$m^{-2}s^{-1}$. Relative humidity varied from 55 to 60%.

Statistical Analysis

The results which needed statistical analysis were subjected to variance analysis and Tukey's comparison of means test.

Results

Effect of different cytokinins

The percentage of initial explants responding to culture varied according to the type and concentration of cytokinin (Table 1). A sprouting rate of 100% was obtained for axillary-buds cultured in presence of 6.67 and 8.9 μM BA. Acceptable results (80%) were also obtained with 2.22-4.4 μM BA and 49 μM 2iP, while the other concentrations of 2iP and all those of TDZ only resulted in about 50% viability (Table 1). The presence of kinetin, at least in the concentrations assayed, had hardly any effect on the induction of growth and development of axillary-buds.

In the culture media containing BA the number of shoots per explant increased with increasing concentrations up to 6.67 μM and then fell. The

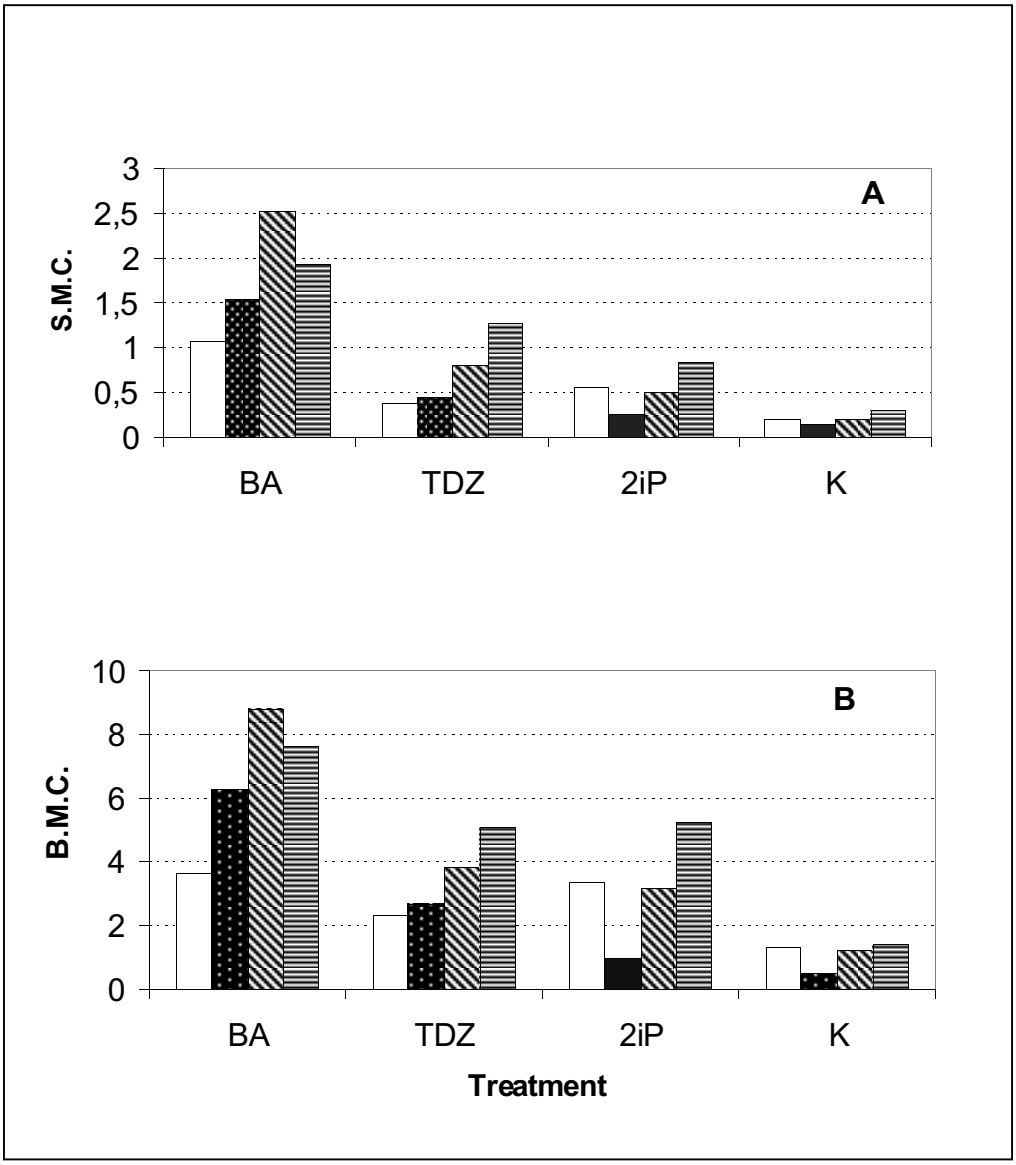

Figure 1. Effect of cytokinin type and concentration on multiplication coefficients of (A) shoots and (B) axillary-buds. BA (2.22, 4.4, 6.67 and 8.9 μM), K (9.3, 19.6, 29.4 and 39.2 μM), 2iP (4.9, 9.8, 24.6 and 49 μM), and TDZ (0.45, 0.9, 2.25 and 4.5 μM). Columns from the left to the right correspond with the lowest to the highest cytokinin concentrations for each type of cytokinin. Coefficients defined as:

$$\text{B.M.C.} = \frac{(\%\ \text{viable explants}) \times (\text{Average of buds/explant})}{100} \qquad \text{S.M.C.} = \frac{(\%\ \text{viable explants}) \times (\text{Average of shoots/explant})}{100}$$

Figura 1. Efecto del tipo de citoquinina y la concentración sobre los coeficientes de multiplicación de (A) brotes y (B) yemas axilares. BA (2.22, 4.4, 6.67 y 8.9 μM), K (9.3, 19.6, 29.4 y 39.2 μM), 2iP (4.9, 9.8, 24.6 y 49 μM), y TDZ (0.45, 0.9, 2.25 y 4.5 μM). Las columnas de izquierda a derecha corresponden con las concentraciones de citoquininas desde las más bajas hasta las más altas.

Cytokinin and concentration (μM)		Sprouting % ± SE	Buds per explant ± SD	Shoots per explant ± SD	Length (cm) of main shoot ± SD
BA	2.22	86 ± 7.3	4.2 ± 1.4 b[#]	1.2 ± 0.4 c	0.9 ± 0.3
	4.4	84 ± 8.4	7.2 ± 2.3 a	1.8 ± 0.7 bc	1.1 ± 0.2
	6.67	100	8.7 ± 2.8 a	2.5 ± 1.1 a	0.9 ± 0.2
	8.9	100	7.6 ± 3.7 a	1.9 ± 1.0 ab	0.9 ± 0.2
TDZ	0.45	35 ± 10.7	6.5 ± 1.8	1.1 ±0.3 b	1.7 ± 0.3
	0.9	45 ± 11.1	5.8 ± 2.1	1.0 ± 0.0 b	1.4 ± 0.4
	2.25	40 ± 11.2	7.62± 3.5	1.6 ± 0.7 ab	1.4 ± 0.3
	4.5	50 ± 10.9	8.5 ± 4.4	2.1 ± 0.9 a	1.2 ± 0.3
2iP	4.9	55 ± 11.1	6.1 ± 2.5	1.0 ± 0.0	1.8 ± 0.9
	9.8	25 ± 9.7	3.8 ± 1.7	1.0 ± 0.0	1.1 ± 0.4
	24.6	50 ± 11.2	6.3 ± 2.0	1.1 ± 0.3	1.4 ± 0.2
	49	80 ± 8.9	6.5 ± 1.9	1.0 ± 0.2	1.6 ± 0.4
K	9.3	20 ± 8.2	6.5 ± 2.8	1.0 ± 0.0	2.3 ± 1.4
	19.6	15 ± 6.7	3.3 ± 0.5	1.0 ± 0.0	0.8 ± 0.1
	29.4	20 ± 8.2	6.0 ± 2.3	1.0 ± 0.0	2.0 ± 1.1
	39.2	30 ± 10.2	4.6 ± 2.1	1.0 ± 0.0	1.0 ± 0.3
Cytokinin (P and significance)			0.034 *	0.000 ***	0.002 **
Concentration (P and significance)			0.033 *	0.002 **	0.000 ***
Cytok x Conc (P and significance)			0.014 *	0.004 **	0.024 *

Comparison of means by Tukey´s test, P ≤ 0.05.

Table 1. Effect of different cytokinins and concentrations on proliferation capacity of axillary bud cultures of the table grapevine cultivar Napoleón.
Tabla 1. Efecto de las concentraciones y tipos de citoquinina en la capacidad de proliferación de yemas axilares del cultivar de uva de mesa Napoleón.

number of shoots also increased with increasing concentrations of TDZ. The maximum response was observed at the highest concentration assayed (4.5 μM). In general, the use of 2iP or kinetin only resulted in the production and growth of one shoot per sprouted bud. These shoots were longer than those obtained with BA and similar in length to those obtained with TDZ. The maximum number of axillary-buds developed per explant was about eight when 4.4-8.9 μM BA or 2.25-4.5 μM TDZ was added to the culture medium.

All the above differences were much more apparent when the multiplication coefficients were estimated for the buds (B.M.C.) and shoots (S.M.C.) developing in each of the media assayed. BA was by far the most successful cytokinin for eliciting a response, especially at 6.67 μM, when maximum coefficients of 8.7 axillary-buds and 2.5 shoots were obtained. The use of 8.9 μM BA also produced acceptable results (Fig. 1 A and B).

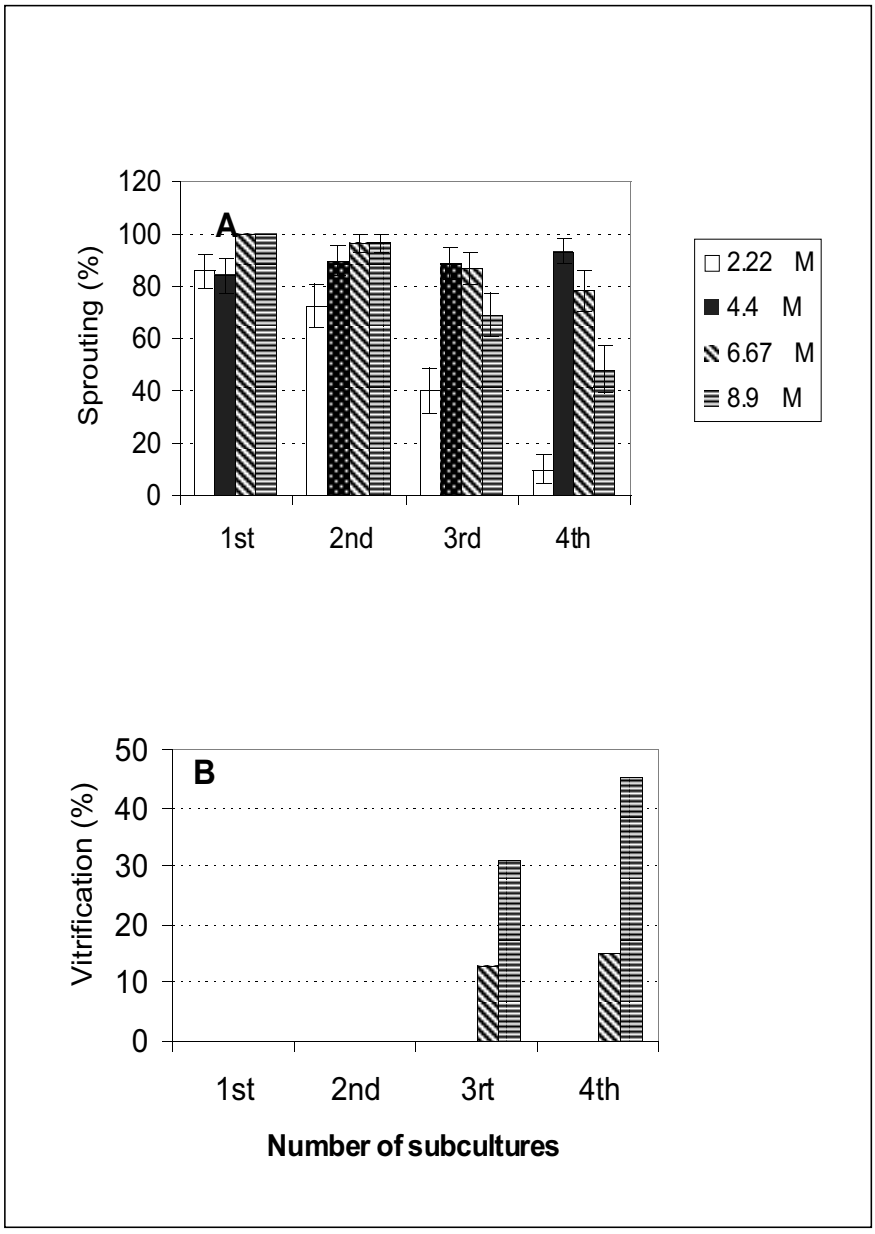

Figure 2. Percentage of (A) sprouting and (B) vitrification observed during four subcultures according to the concentration of BA in the propagation medium.
Figura 2. Porcentaje de (A) brotación y (B) vitrificación observados durante cuatro subcultivos, según la concentración de BA en el medio de propagación.

Shoot proliferation by subculturing

Following the above step, propagation was continued by realizing successive subcultures of axillary-buds on fresh culture medium of identical composition in order to determine the maximum number of times this could be done to obtain a large number of shoots without their appearance and quality affected. BA which was determined above as the better cytokinin was used.

The sprouting rate of axillary-buds remained stable (around 90%) during the four subcultures made on media containing 4.4 µM BA, with a slight tendency to increase as the number of transfers grew (Fig. 2A). Similar behaviour was observed for the second subcultures growing in media containing 6.67 or 8.9 µM BA while vitrification set in during the last two subcultures. On media with 8.9 µM BA, vitrification in the third subculture affected 31% of explants and in the fourth subculture 45%. At 6.67

Subculture	BA concentration (μM)	Buds per explant	Shoots per explant	Length (cm) of main shoot
1st	2.22	4.2 ± 1.4 b[#]	1.2 ± 0.4 c	0.9 ± 0.3
	4.4	7.1 ± 2.3 a	1.8 ± 0.7 bc	1.1 ± 0.3
	6.67	8.7 ± 2.8 a	2.5 ± 1.1 a	0.9 ± 0.2
	8.9	7.6 ± 3.7 a	1.9 ± 1.0 b	0.9 ± 0.2
2nd	2.22	3.7 ± 2.4 b	1.2 ± 0.5 b	0.7 ± 0.3 b
	4.4	8.8 ± 5.0 a	2.3 ± 1.7 b	1.2 ± 0.5 a
	6.67	9.9 ± 5.3 a	2.5 ± 1.1 ab	1.3 ± 0.5 a
	8.9	11.9 ± 9.8 a	3.7 ± 3.2 a	1.5 ± 0.4 a
3rd	2.22	2.2 ± 1.6 c	1.0 ± 0.0 b	0.7 ± 0.5 b
	4.4	12.7 ± 6.9 b	2.0 ± 1.2 b	2.1 ± 0.5 a
	6.67	18.6 ± 8.0 a	3.8 ± 1.9 a	2.0 ± 0.3 a
	8.9	22.2 ± 7.9 a	4.3 ± 1.9 a	2.0 ± 0.3 a
4th	2.22	5.5 ± 2.1 b	1.0 ± 0.0 b	1.7 ± 0.3 a
	4.4	5.5 ± 1.6 b	1.2 ± 0.3 b	1.3 ± 0.2 a
	6.67	7.1 ± 2.2 a	1.4 ± 0.6 ab	1.3 ± 0.2 a
	8.9	6.0 ± 1.8 ab	1.6 ± 0.5 a	1.0 ± 0.1 b
Subculture (P and significance)		0.000 ***	0.000 ***	0.000 ***
Concentration (P and significance)		0.000 ***	0.000 ***	0.000 ***
Subcul x Conc (P and significance)		0.000 ***	0.000 ***	0.000 ***

Comparison of means ± SD by Tukey's test, $P \leq 0.05$.

Table 3. Effect of subculture number of axillary-bud microcuttings and cytokinin concentrations on proliferation capacity of axillary bud cultures of the table grapevine cultivar Napoleón.
Tabla 3. Efecto del número de subcultivos y la concentración de BA sobre la capacidad de proliferación de yemas axilares del cultivar de uva de mesa Napoleón.

μM BA, vitrification was about 15% in the last two subcultures (Fig. 2B). With 2.22 μM BA the sprouting percentage of axillary-buds rapidly decreased to reach 10% after four subcultures (Fig. 2A).

As regards the other parameters quantified, the variance analysis pointed to a high degree of significance both for the principal factors considered (number of subcultures and BA concentration) and their interaction (subculture x concentration) (Tables 2 and 3). In general, with relatively high BA concentrations (4.4-8.9 μM) the production of axillary-buds and shoots per explant increased gradually from the first to second subculture and then sharply in the third before falling to levels below the starting levels. At 2.22 μM BA the proliferation of shoots and the number of axillary-buds developed per explant remained practically stable and always low during the subcultures.

The multiplication coefficient estimated for the axillary-buds in each of the four subcultures carried

Factors	Buds per explant	Shoots per explant	Length (cm) of main shoot
Subculture			
1st	6.9 ± 3.2 c[#]	1.8 ± 1.0 c	1.0 ± 0.2 c
2nd	8.6 ± 6.9 b	2.3 ± 1.5 b	1.2 ± 0.5 b
3rd	13.9 ± 9.5 a	2.8 ± 2.0 a	1.7 ± 0.6 a
4th	6.0 ± 2.0 c	1.3 ± 0.5 c	1.3 ± 0.2 b
BA Concentration (μM)			
2.22	3.9 ± 2.0 c	1.1 ± 0.4 c	1.0 ± 0.4 b
4.4	8.5 ± 5.1 b	1.8 ± 1.2 b	1.4 ± 0.5 a
6.67	11.1 ± 6.6 a	2.5 ± 1.5 a	1.4 ± 0.5 a
8.9	11.9 ± 8.6 a	2.7 ± 1.7 a	1.4 ± 0.5 a

Comparison of means ± SD by Tukey´s test, $P \leq 0.05$.

Table 3. Effect of subculture number of axillary-bud microcuttings and cytokinin concentrations on proliferation capacity of axillary bud cultures of the table grapevine cultivar Napoleón.
Tabla 3. Efecto del número de subcultivos y la concentración de BA sobre la capacidad de proliferación de yemas axilares del cultivar de uva de mesa Napoleón.

out (Fig. 3) corroborate the analysis of the data shown in Tables 2 and 3. In short, the production of axillary-buds at high cytokinin concentrations increased substantially up to the third subculture and then fell sharply. At low BA doses proliferation fell continually. The best propagation rates were obtained during the third subculture in the media containing 6.67 or 8.9 μM BA, with no significant differences between them.

Discussion

The stimulatory effect of cytokinins on the *in vitro* proliferation and development of shoots from shoot tips and axillary-buds of grapevine species is well known (Pool & Powell 1975, Novák & Juvová 1983, Goussard 1981, Goussard 1987). The experiment described in this study set out to ascertain the type of cytokinin and its concentration which led to the greatest proliferation of shoots and formation of axillary-buds in cultures of axillary-bud microcuttings from *in vitro*-grown plants of the table grapevine cultivar Napoleón. Other factors, such as callus formation, colour and general appearance of the shoots obtained or the occurrence of vitrification phenomena, were also taken into account. All these criteria are commonly used for establishing *in vitro* propagation protocols in different species and hybrids of grapevine (Chée & Pool 1985, Chée & Pool 1987, Chée et al. 1984, Safadi & Abu Irmalleh 1987, Torregrosa & Bouquet 1995).

Of the three cytokinins assayed, BA produced the best results especially at 6.67 and 8.9 μM BA. These results are in accordance with those obtained by other authors (Barlass & Skene 1980, Silvestroni 1981, Chée & Pool 1983, Safadi & Abu Irmalleh 1987), which suggest that 2.22 to 11.1 μM is the optimum concentration for the micropropagation of different plant materials of *Vitis* spp. The shoots developed were of good appearance with an intense green colour and of uniform size. However, a preliminary study had shown that BA concentrations above 8.9 μM (data not shown) caused vitrification, abnormal leaves and shorter shoots (see also Lee & Wetzstein 1990). It is to be expected that the optimum concentration of BA shows very slightly between cultivars and that it should be determined in each individual case.

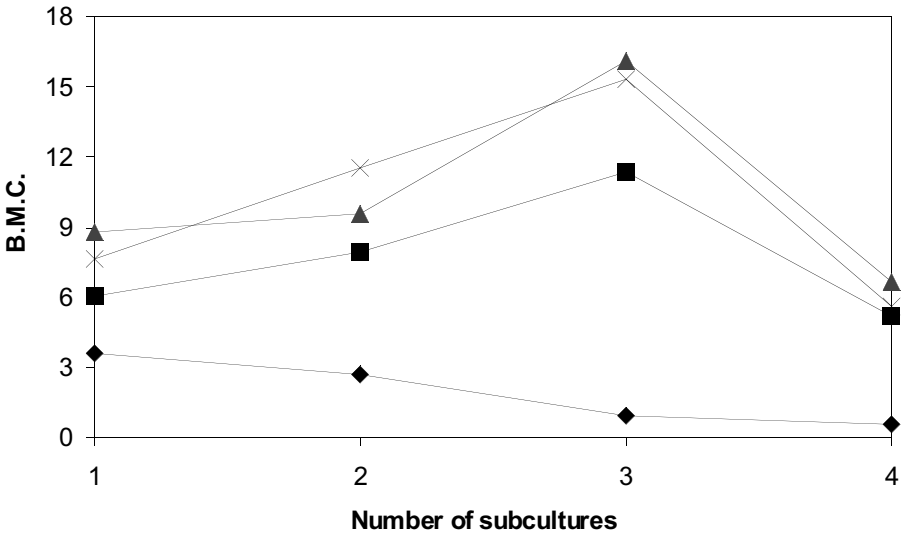

Figure 3. Effect of successive subcultures on the estimated bud multiplication coefficient (B.M.C.) with different concentrations of BA: ◆ 2.22 μM , ■ 4.4 μM, ▲ 6.67 μM, ✕ 8.9 μM.
Figura 3. Efecto de los sucesivos subcultivos sobre el coeficiente de multiplicación de yemas axilares con diferentes concentraciones de BA.

A comparison of the results obtained with BA and TDZ contrast strongly with those of Sudarsono & Golgy (1991) using the same cytokinins with *Vitis rotundifolia* Michx., who found a higher sprouting percentage and greater number of shoots developed with TDZ. Our results are nearer those of Gray & Klein (1989), and Gray & Benton (1991), who found that although the number of shoots was similar using both cytokinins, those formed in the presence of TDZ were smaller and less vigorous. In the range of TDZ concentrations assayed in our experiment (0.45-4.5 μM) the shoots obtained were of normal appearance and uniform size. Nevertheless, in a preliminary experiment (data not shown) using very much higher concentrations of TDZ (9 and 18 μM), despite the substantial increase in explant sprouting (85-90%), a no less important increase in negative symptoms was observed. Among these were the callus formation at the base of the explants and the vitrified, stunted and swollen appearance of shoots with clustered axillary-buds and reduced leaf expansion. Such negative effects were even more drastic in axillary bud cultures of cultivar Barbera (Gribaudo & Fronda 1991), where a concentration of only 0.9 μM TDZ was sufficient to produce signs of vitrification, swelling and leaf malformation in the growing shoots.

Except at the highest concentration assayed, the use of 2iP induced sprouting in approximately half of the explants cultivated and the subsequent growth of the resulting shoots. However, it was poor at promoting the formation of multiple shoots from the axillary-buds produced. Perhaps a 2-4 times increase in concentration would have improved both parameters although, even so, Safadi & Abu Irmallech (1987) found the number of shoots per explant remained low.

The use of kinetin, too, had a similar effect although, in this case, very few axillary-buds sprouted and those that did sprout were frequently irregularly formed. This does not mean that kinetin is not effective since, in combination with other cytokinins (Sasahara et al. 1981, Yamakawa et al. 1986, Sudarsono & Golgy 1991), it can be useful for establishing and propagating certain genotypes.

The second objective was to determine the maximum number of subcultures to which the plant material could be submitted without the quality of the regenerated material being affected. Successive subcultures in cytokinin-rich media increase the propagation rates of several plant species although the cultures may degenerate (Koruza & Jelaska 1993, Torregrosa & Bouquet 1995) and these may be a greater risk of somaclonal variation (Silvestroni 1981). Moreover, when the number of subcultures increases the exogenous cytokinin demand is reduced as a result of the rejuvenation and gradual habituation of the cultures during *in vitro* culture. The use of four BA concentrations was intended to throw light on this matter. The results obtained confirm the positive effect of relatively high doses of BA (6.67 and 8.9 μM) on the production of shoots and axillary-buds up to the third subculture and the sharp drop in production thereafter. At low doses (2.22 μM) the propagation of shoots and buds varied considerably during the successive subcultures but always remained low. An increase in the number of shoots and axillary

buds was also observed in routine subculturing of *Vitis vinifera* L. cv. Chenin blanc shoot tips cultures (Goussard 1982) and axillary buds cultures of the grapevine hybrid rootstock 1103P (*V. berlandieri x V. rupestris*) (Safadi & Abu Irmalleh 1987). The behaviour of the cultivated grapevine cultivars Cabernet Sauvignon, Chardonnay and Xarel.lo varied during subcultures (Gras et al. 1997).

The growing problem of vitrification during the third and fourth subcultures in the presence of high BA concentrations (6.67 and 8.9 μM) suggest that it was the high concentration of cytokinin that was responsable for this phenomenon. Others causes might also have been involved, such as the presence of ethylene and/or the high level of humidity inside the culture container, the partial content of agar and certain ions, the time taken to make the transfers, etc. Whatever the reason, Torregrosa & Bouquet (1995) observed that more than three transfers of *Vitis x Muscadinia* hybrids in the propagation medium led to the appearance of vitrified shoots and a substantial reduction in the propagation capacity of the axillary-bud cultures. The continuous subcultures of *Vitis vinifera* L. cv. Refosk explants in a cytokinin-supplemented medium also inhibited shoot elongation and accelerated aging (Koruza & Jelaska 1993).

According to the results obtained it would seem advisable to limit the number of propagation cycles to three. It might be possible to increase the number of subcultures by using different concentrations of BA (Harris and Stevenson 1982; Safadi & Abu Irmalleh 1987) and even modifying the cytokinin content of propagation medium during subcultures (Grass et al. 1997). A subculture period possibly shorter than that used in this study might play an important role in this respect.

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