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Establishment and in vitro clonal propagation of the Spanish autochthonous table grapevine cultivar Napoleon: an improved system where proliferating cultures alternate with rooting ones

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Abstract

MS-1 and C_2D media both contained 2 mg/l of 6-benzyladenine (BA) led to the best results of six culture media assayed for in vitro establishment of shoot tips from the table grapevine cultivar Napoleon. The parameters considered were survival, viability, number of axillary buds and shoots developing per explant, main shoot length and percentage of explants with secondary shoots. The viability and production of axillary buds and shoots were seriously affected by the time of the year when the buds were collected. The developmental stage of the axillary buds and the position of the nodal segments on the growing shoots taken from the mother plant significantly influenced the in vitro response. The production of axillary buds and shoots, and the length of the main developed stem, significantly increased with distance from the shoot tips. A combined and sequential micropropagation protocol where proliferating cultures of axillary-bud microcuttings from in vitro-grown plants alternate with rooting cultures of the resulting shoots is described, and its commercial exploitation by the nursery industry is discussed.

Key words: *Vitis*, Shoot tips, Micropropagation, Nodal segments, Axillary-bud Microcuttings, In vitro-grown plants.

Resumen

Establecimiento y propagación clonal in vitro del cultivar autóctono español de uva de mesa Napoleón: un sistema mejorado donde los cultivos en proliferación alternan con los de enraizamiento.

Ambos medios de cultivo, MS-1 y C_2D, con 2 mg/l de 6-benciladenina (BA) dieron los mejores resultados de los seis medios ensayados para el establecimiento *in vitro* de ápices del cultivar de uva de mesa Napoleón. Se consideraron los parámetros de supervivencia, viabilidad, número de yemas axilares, desarrollo de brotes por explante, longitud del tallo principal y porcentaje de explantes con tallos secundarios. La viabilidad y producción de las yemas y brotes axilares se afectaron seriamente por la época del año en la que se recolectaron las yemas. El estado de desarrollo de las yemas axilares y la posición de los segmentos nodales

sobre los brotes en crecimiento tomados de la planta madre influenciaron significativamente la respuesta in vitro. La producción de yemas axilares y brotes, y la longitud del tallo principal desarrollado, aumentaron significativamente con la distancia desde el ápice del brote. Se describe un protocolo combinado y secuencial de micropropagación donde alternan cultivos en proliferación de explantes axilares de plántulas crecidas in vitro con cultivos de enraizamiento de los brotes resultantes, y se discute su explotación comercial en la industria viverística.

Palabras clave: *Vitis*, Ápices, Micropropagación, Segmentos nodales, Explantes axilares, Plántulas crecidas in vitro.

Introduction

The first method used to propagate *Vitis* species and cultivars in vitro was described by Galzy (1961), in which nodes containing functional buds were cultured on a medium without growth regulators. Later, Silvestroni (1981) proposed the possibility of increasing plant production by means of the propagation of nodal segments containing a leaf axillary bud in media supplemented with cytokinins to inhibit shoot tip dominance and increase the proliferation of shoots. The inclusion of cytokinins in the medium is generaly accepted as necessary for shoot proliferation from shoot tips and axillary buds (Jona & Webb 1978, Goussard 1981, Harris & Stevenson 1982, Novák & Juvová 1983, Gray & Fisher 1985, Lee & Wetzstein 1990, Torregrosa & Bouquet 1995, Mhatre et al. 2000). In a previous study, procedures of thermotherapy and in vitro culture, either separately or in combination, for the elimination of Grapevine leafroll associated virus-3 (GLRaV-3) and Grapevine fanleaf virus (GFLV) from grapevine cv. Napoleon plants were established (Valero et al. 2003). This work describes the establishment of in vitro cultures from two virus-free clones and an improved protocol for their micropropagation. A system where proliferating cultures alternate with rooting ones is described, using shoot tips and axillary-bud microcuttings from in vitro-grown plants as explants.

Material and methods

Plant material

Two-year-old plants pruned to 2-3 buds in repose and protected from biological disease-transmitting vectors were used in this study. They were grown outdoors in 40 L PVC pots containing a 2:1 (v/v) mixture of peat and sand at the installations of the experimental field station CIDA (Centro de Investigación y Desarrollo Agroalimentario, La Alberca, Murcia, Spain). Plants belonged to two GLRaV-3- and GFLV-free clones of the table grapevine cv. Napoleon, were selected for their vegetative development, yield, maturing time and other fruit characteristics.

Forcing the cuttings

30-40 cm long stem cuttings with the cut ends sealed with wax were immersed in an antifungal bath containing Captan®. After drying, new cuts were made in the basal sections of the stem cuttings, which were then placed in perlite containing 6 g/l of Benlate® solution and incubated in a growth chamber, where the heat and humidity were controlled and with a 16 h photoperiod (58 $\mu Em^{-2}s^{-1}$). After 20 days the new developing shoots were large enough to be used for explant excision.

Surface disinfection of plant material and types of explants used

Tips from actively growing shoots were rinsed in 70% ethanol for 30 s and soaked for 15 min in an aqueous solution of sodium hypochlorite (1% active chlorine) to which a few drops of Tween-80 surfactant were added. After surface disinfection treatment, the plant material was washed three times in sterile distilled water to remove the sterilizing agent. Tips were then dissected and in vitro cultures of shoot tips (3 mm) or nodal segments (3-8 mm) were established. Nodal segments were divided into three groups according to the position on the growing shoots: end or distal zone (containing 1st and 2nd axillary buds), middle zone (containing 3rd-6th axillary buds) and basal zone (containing 7th-10th buds of the basal section). Twenty to thirty replicate explants were used for each treatment.

Media

Six different culture media were used to establish in vitro cultures of shoot tips: MS-0, Murashige and Skoog (1962) basal medium containing no growth regulators; MS-1, the same basal medium supplemented with 2 mg/l 6-benzyladenine (BA); C_2D as described by Chée et al. (1984), also enriched with 2 mg/l BA; MS*/2, as used by Spiegel-Roy et al. (1985) containing 1.76 mg/l of 3-indolacetic acid (IAA) and 0.346 mg/l of gibberelic acid (GA_3), AND*, as proposed by Troncoso et al. (1988) with 0.1 mg/l naphtalenacetic acid (NAA) and 1 mg/l BA added; and, finally, C1*, also used by Troncoso et al. (1990), supplemented with 0.5 mg/l BA. To establish in vitro cultures of nodal segments MS-1 medium was used only.

All the culture media were supplemented with 30 g/l sucrose as carbon and energy source, and 7 g/l 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. Sterilization was carried out by autoclaving at 121ºC, for 15 min.

Shoot multiplication

Axillary-bud microcuttings from in vitro-grown plants rooted in MS/2 basal medium with the microsalts diluted to half strength were used as propagules. The new proliferating shoots obtained in MS basal medium supplemented with different concentrations of BA were further subcultured and rooting in MS/2. In this way, it was possible to regenerate complete plants which could be used as source of new propagules.

Culture conditions

Cultures were incubated in a temperature controlled chamber at 23 ± 2ºC with 16 h photoperiod. The light was provided by fluorescent tubes of white light (Grow-lux, SYLVANIA, irradiance of 30-35 $\mu Em^{-2}s^{-1}$). Relative humidity varied from 55 to 60%. The in vitro response was evaluated after 45 days of incubation, by determining the following parameters: survival (%), viability (%), number of axillary buds and shoots developed per explant, length of the main shoot, and percentage of explants with secondary shoots.

Acclimatisation and hardening of the regenerated plants

When sufficient micropropagated plant material was obtained, the rooted plantlets were transplanted to pots containing a 1:1 (v/v) mixture of fertilized cultivation peat (NEUHAUS torf substrat, nts 1) and perlite and placed in a growth chamber with controlled temperature and humidity. A photoperiod of 16 h with a light irradiance of 58 $\mu Em^{-2}s^{-1}$ was used. After four days, the relative humidity was gradually decreased so that complete acclimatisation and active growth of plants in the new *ex vitro* conditions was achieved at the end of six weeks. Survival and plant growth were evaluated regularly every 7 days with respect to stem length and number of expanded leaves. Later, plants were placed outdoors under a mesh to provide shade in order to protect them from direct sunlight.

Statistical analysis of the results

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

Results

Establishment of cultures in aseptic conditions

Culture of shoot tips in different media

The main aim in the establishment phase was to obtain a high viability of explants and to promote the formation of multiple shoots. The presence of BA in the medium was the main factor affecting viability. In the absence of BA, viability was found to be 38% (Fig. 1). AND* produced a substantially higher viability rate (>90%) of explants than MS-1, C2D and C1* (about 70%). However, because AND* medium produced a large callus at the base of the explant it was not considered in micropropagation protocols. MS-1 and C_2D provided the greatest number of axillary buds per explant. C_2D produced a greater number of shoots while MS-1 promoted greater length of developing shoots (Table 1).

Influence of the time of year on the in vitro response of shoot tips

The season clearly has an influence on the physiological condition of the mother grapevine plant, which is reflected in the in vitro response of explants. A high percentage of tips (95%) responded to in vitro establisment in winter. The percentages of viable explants taken in spring or summer were clearly lower. In autumn, 80% of explants remained green and did not grow after 45 days of incubation, while the rest were quantified as non viable (Fig. 2a). Although the greatest viability response of in vitro culture was

Culture Medium	Buds per Explant x ± SD	Shoots per Explant x ± SD	Length (cm) of Primary Shoot x ± SD	Explants with Secondary Shoots % ± SE
C1*	8.7 ± 2.6 b[#]	2.4 ± 1.1 b	2.9 ± 0.9 b	70 ± 8.3
MS-I	17.2 ± 6.3 a	2.4 ± 1.0 b	4.5 ± 1.9 a	70 ± 8.7
C_2D	16.2 ± 5.1 a	3.8 ± 1.1 a	2.3 ± 0.4 bc	64 ± 8.3
AND*	8.8 ± 3.2 b	2.3 ± 0.0 b	2.1 ± 0.7 c	75 ± 7.9
P-Value	0.001	0.003	0.001	
Significance[+]	***	**	***	

Table 1. Influence of culture media on the formation of buds and multiple shoots during the establishment of shoot tip cultures from pot-grown grapevines of cv. Napoleon.
[#] Data followed by the same letter in a column are not significantly different according to Tukey's test.
[+] Significant at * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$; NS = Not significant.
Tabla 1. Influencia de los medios de cultivo en la formación de yemas y brotes múltiples durante el establecimiento de los ápices de vides del cultivar Napoleón crecidas en maceta.
[#] Los datos seguidos por la misma letra en una columna no son significativamente diferentes según el test de Tukey's.
[+] Significante a * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$; NS = No significante.

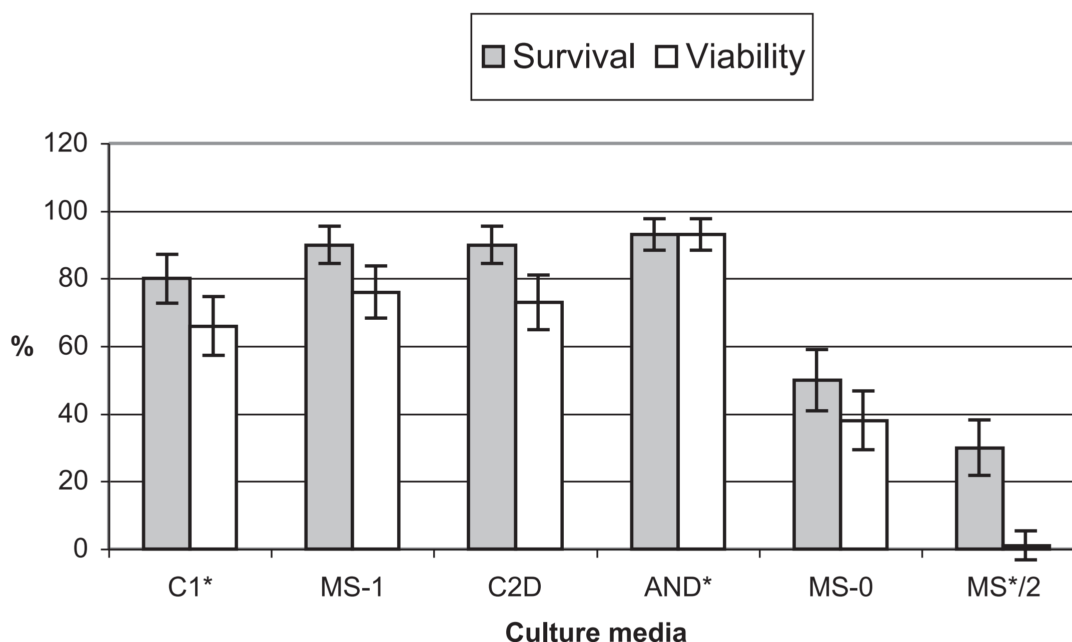

Figure 1. Survival and viability rates (%) of *in vitro* shoot tip cultures from pot-grown grapevines of cv. Napoleon.
The results are expressed as frequencies (%) ± standard error (SE), calculated as: $[p(1-p)/n]^{1/2} \cdot 100$, where p = no. of surviving or viable explants/n (n being the total number of tips belonging to each clone).
Figura 1. Tasa de supervivencia y viabilidad (%) de cultivos *in vitro* de ápices de plantas del cv. Napoleón crecidas en maceta

obtained in winter, this season provided the worst results for other micropropagation parameters such as the number of axillary buds and shoots per explant (Fig. 2b) or the length of primary shoots.

Influence of the type and position of explant on the mother plant

One of the major factors influencing the survival and in vitro proliferative response of grapevine explants is their position on the mother plant. Survival and viability obtained with both shoot tips and nodal segments taken from the apical and the intermediate zones of actively growing shoots were very similar (Table 2). The cultures derived from the nodal segments taken from basal zones of the same shoots had serious contamination problems which considerably reduced their viability. Nodal segments belonging to the zones furthest from the apex showed the highest percentage of explants with secondary shoots, the highest mean

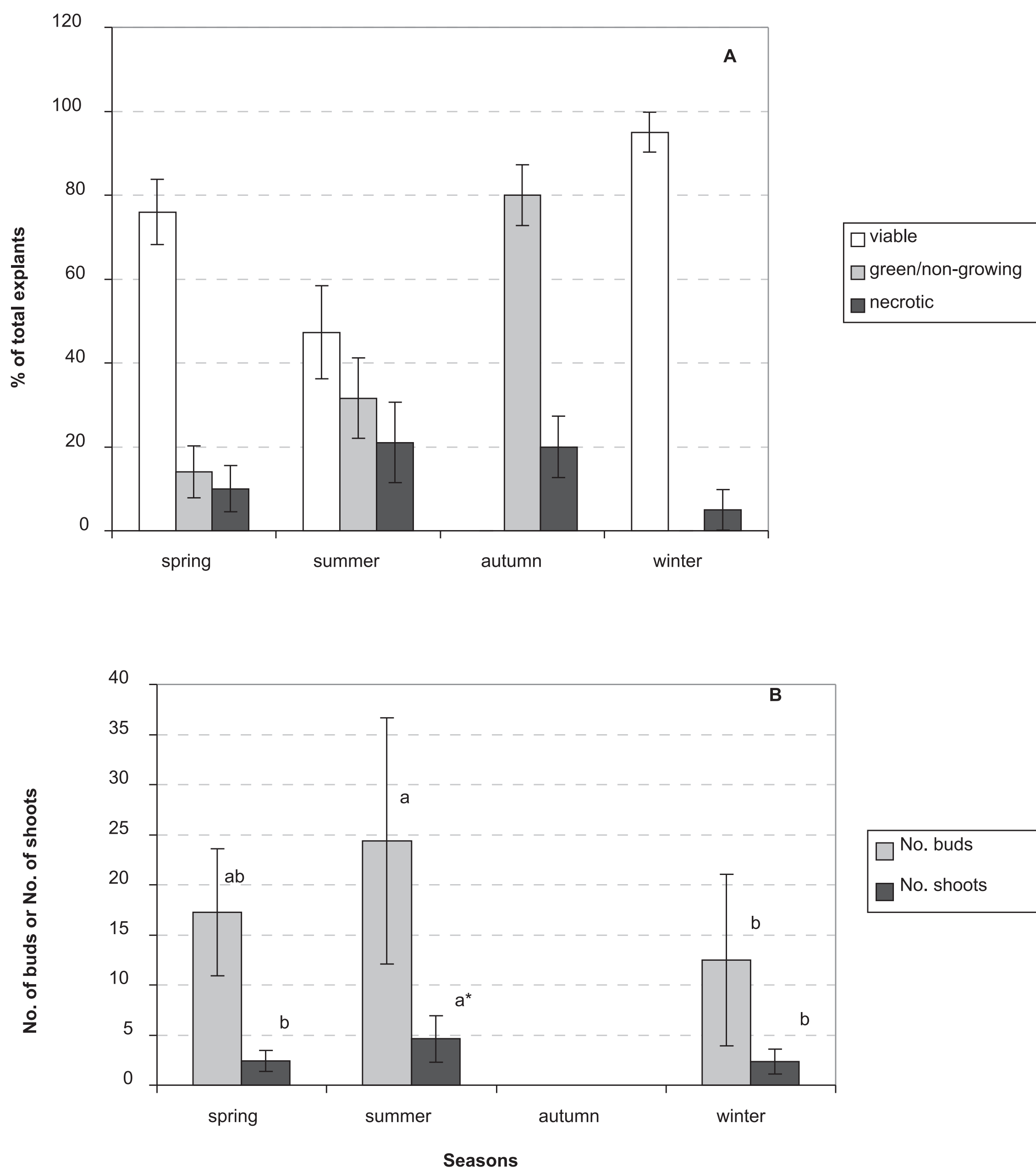

Figure 2. Season influence on the establishment of *in vitro* MS-I shoot tip cultures from pot-grown grapevines of cv. Napoleon. The results in A are expressed as frequencies (%) ± standard error (SE) and in B as means ± standard deviation (SD). * Comparison of means by Tukey's test, $P \leq 0.05$.

Figura 2. Influencia de la estación del año sobre el establecimiento *in vitro* de ápices de vides del cv. Napoleón crecidas en maceta.

number of axillary buds and shoots per explant and the greatest mean length of the shoots developed (Table 2). In the tip cultures, the mean values for the different parameters were in general very close to those determined for the nodal segment cultures established from the middle zone.

Shoot multiplication

Sprouting of buds and the subsequent formation of shoots by culturing axillary-bud microcuttings from in vitro-grown plants was achieved. The presence of BA in the culture medium was a decisive factor

	EXPLANT TYPE			
PARAMETER	**Shoot tips**	**1st and 2nd buds (Apical zone)**	**3rd-6th buds (Middle zone)**	**7th-10th buds (Basal zone)**
Survival % ± SE	90 ± 5.4	88 ± 6.9	96 ± 4.4	42 ± 10.2
Viability % ± SE	76 ± 7.7	75 ± 8.8	81 ± 8.3	38 ± 9.8
Explants not growing % ± SE	14 ± 6.2	13 ± 6.6	15 ± 7.1	4 ± 5.5
Necrotic explants % ± SE	10 ± 5.5	12 ± 6.6	2 ± 4	2 ± 4
Contaminated explants % ± SE	-	-	2 ± 4	56 ± 10.2
Buds per explant X ± SD	17.2 ± 6.3 b	8.7 ± 6.9 c	16.3 ± 6.7 b	28.5 ± 6.7 a*
Shoots per explant X ± SD	2.4 ± 1.0 b	1.7 ± 0.9 c	2.9 ± 1.4 b	4.1 ± 2.1 a
Length (cm) of primary shoot X ± SD	4.5 ± 1.9 a	1.9 ± 0.8 d	2.9 ± 0.7 c	3.6 ± 0.9 b
Explants with secondary shoots % ± SE	70 ± 8.7	43 ± 10.1	82 ± 7.8	95 ± 4.4

Table 2. Quantification of different growth parameters for several grapevine explants established in MS-1 medium.
* Data followed by the same letter in a file are not significantly different according to Tukey's test, $P \leq 0.05$.
Tabla 2. Cuantificación de diferentes parámetros de crecimiento de varios tipos de explantes de vid establecidos en medio MS-1.
* Los datos seguidos por la misma letra en una columna no son significativamente diferentes según el test de Tukey's.

in axillary bud growth and elongation (Table 3). In media containing 1 or 2 mg/l BA, the mean number of axillary buds and shoots developed per explant were significantly greater than in its absence. Very few axillary-bud microcuttings (20%) were able to sprout and grow without BA supply.

In vitro rooting of proliferated shoots

Shoots obtained by in vitro culture did not in general show any problems as regards rooting. Good root development was obtained by transferring shoots to the basal MS/2 medium. In this way, percentages 85% rooted plants were obtained, with an average of three roots each (data not shown).

Acclimatisation of micropropagated plants

The *ex vitro* transfer of rooted plants was extremely successful with more than 90% surviving. After one week of acclimatisation, the direct observation of a random sample of plants showed that most of the roots formed in vitro presented longitudinal growth and formation of secondary roots. The plants continued to grow vigorously during the acclimatisation period (Fig. 3).

Discussion

Many authors have indicated that the ideal composition of grapevine culture medium depends on the species and cultivar in question so that the results obtained with one genotype in a given medium may differ from those obtained with other genotypes (Reisch 1986, Botti et al. 1993). This explains the differences in the mineral composition and growth regulators used for the establishment and initiation of in vitro shoot tip cultures of *Vitis vinifera* cv. Napoleon.

The results obtained show that BA was crucial for stimulating explant growth and development. Moreover, the concentration of this cytokinin strongly influenced the number of axillary buds developed per explant. This might be of great importance if the next stage were the propagation by successive subcultures of nodal segments excised from the developed shoots (Silvestroni 1981). The inclusion of auxins in the cul-

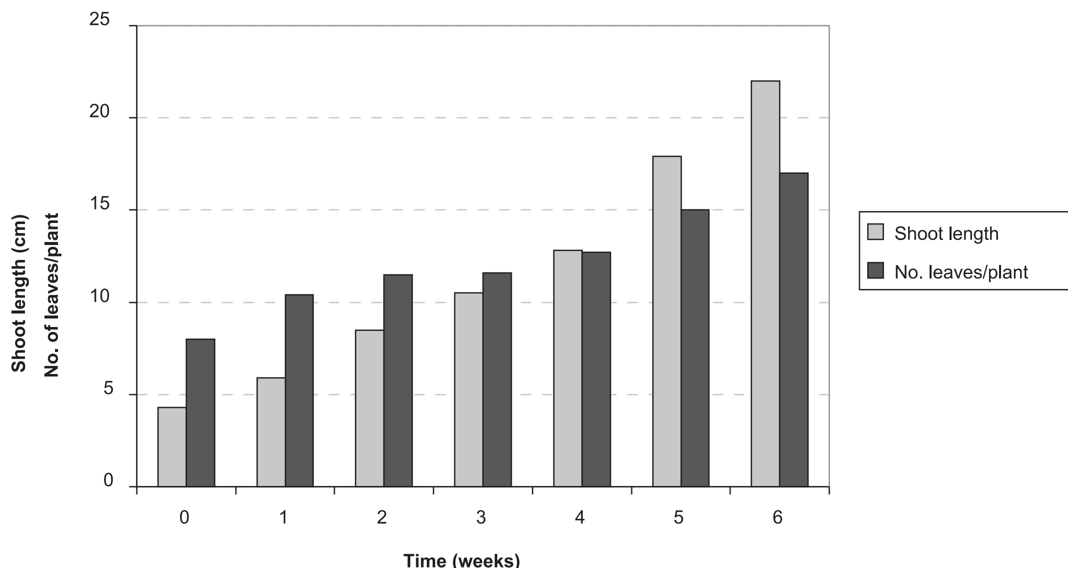

Figure 3. Growth of micropropagated plants during six weeks of acclimatisation and hardening.
Figure 3. Crecimiento de plantas micropropagadas durante seis semanas de aclimatación y endurecimiento.

ture media was not necessary for the establishment of shoot tip cultures since it formed unwanted calli. A possible explanation for this might be that the explants would act as active centers of auxin biosynthesis.

The physiological condition of the mother plant has a marked influence on the in vitro culture response, as manifested by the different results obtained with cultures established at different seasons of the year. This influence has been commented by other authors (Botti et al. 1993, Dalal et al. 1993), although there is little agreement as to the best way of setting about culture establishment. For some authors (Yu & Meredith 1986, Dalal et al. 1993) forcing cutting in repose is the best way, while for others spring (just after sprouting) is the best time to carry out such operation (Padeliev et al. 1990). Our results (Figs. 2a, b) do not help in this respect, since the greater degree of viability in explants obtained from forced material is offset by the greater number of axillary buds obtained per explant during spring. Although most shoot tips set to in vitro culture during autumn (80%) showed no growth and remained green after 45 days of incubation, their transfer to fresh medium stimulated growth and development in 71% of subcultivated shoot tips, while 21% showed symptoms of vitrification and 8% remained green and did not grow. This shows that it is possible to achieve a moderate-high level of in vitro established cultures throughout the year.

Another determining factor in the morphogenic response of the in vitro cultured explants is the position that they occupy on the mother plant. In general, when nodal segments are cultured, shoot production

BA (mg/l)	**Sprouting % ± SE**	**Buds per Explant x ± SD**	**Shoots per Explant x ± SD**	**Length (cm) of Primary Shoot x ± SD**
0.0	20 ± 10.2	3.1 ± 1.7 b[#]	1.0 ± 0.2 b	0.8 ± 0.7
1.0	84 ± 8.4	7.2 ± 2.3 a	1.8 ± 0.7 a	1.1 ± 0.2
2.0	100	7.6 ± 3.7 a	1.9 ± 1.0 a	0.9 ± 0.2

Table 3. Effect of BA on the behavior of axillary-bud microcutting cultures from *in vitro*-grown plants of grapevine cv. Napoleon, grown on MS basal medium.
[#] Data followed by the same letter in a column are not significantly different according to Tukey's test, $P \leq 0.05$.
Tabla 3. Efecto de BA en el comportamiento de explantes axilares de plantas del cv. Napoleón crecidas *in vitro*, en medio base MS.

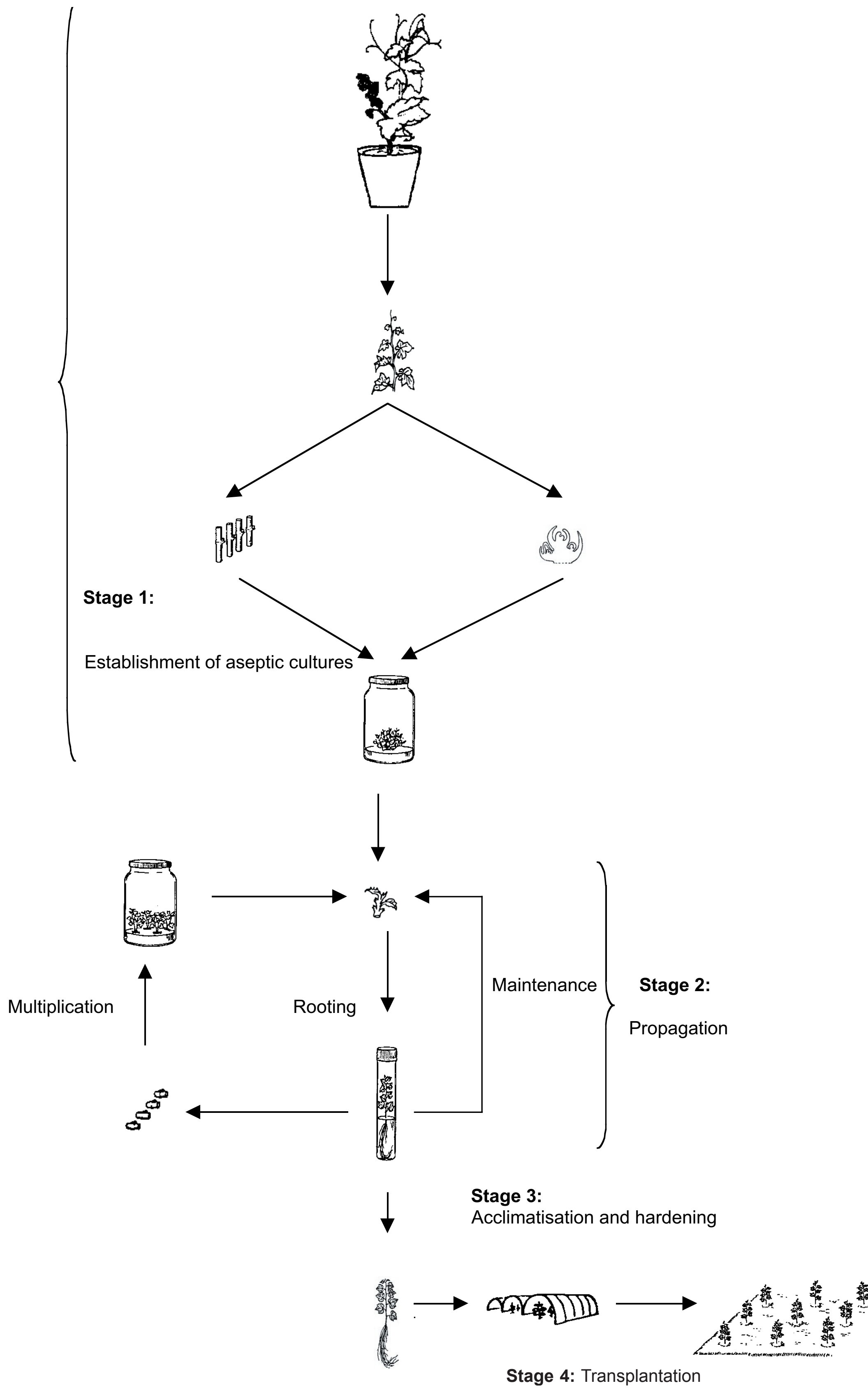

Figure 4. Micropropagation protocol for *Vitis vinifera* L. cv. Napoleon.
Figura 4. Protocolo de micropropagación de *Vitis vinifera* L. cv. Napoleón.

increases with the distance from the tip (Pandeliev et al. 1990, Nicolaou 1991, Sudarsono & Goldy 1991), although other authors have obtained different results (Cholvadová 1989). In our case, we found that not only the number of shoots per explant increased with the distance from the tip but also the number of explants with secondary shoots, the number of axillary buds developed and the length of the formed shoots. However, the best explants were those corresponding to the nodal segments from the middle zones of the actively growing shoots containing the 3rd to 6th axillary buds. The nodal segments of the basal area had too high level of contamination under the disinfection conditions used for us. Shoot tip cultures provided a very similar response to the nodal segments of the middle zone.

The use of nodal segments containing axillary buds as propagules is common in in vitro multiplication protocols for different species and varieties of grapevine, since they provide genetic stability and only require the growth induction of the preformed buds, which contain all the elements of a shoot in miniature. The use of nodal segments from actively growing shoots of both field and greenhouse or growth chamber-grown grapevines as initial explants is a common micropropagation practice despite that this plant material is morphologically and physiologically heterogeneous, depending on the position of the node, the vigour of the shoot and the season when it is taken. On the other hand, the use of nodal segements excised from in vitro established plants might improve multiplication because of the greater homogeneity of the material used (Heloir et al. 1997).

The addition of 1 or 2 mg/l of BA to the propagation medium was essential to stimulate sprouting, growth and lengthening of axillary-bud microcuttings from in vitro-grown plants. After rooting of new formed shoots, the good development of the regenerated plants with more than 22 axillary buds gave us the idea that these buds might be used as starting material to carry out successive propagation cycles. An improved micropropagation procedure where proliferating cultures alternate with rooting ones is proposed here (Fig. 4). Multiplication coefficients of 2 and 22 could be achieved for both axillary bud propagation and rooting phases, respectively. The commercial success of a propagation technique depends on obtaining the greatest number of plants in a short period of time. Taking into consideration the results obtained in this study as a whole, this combined and sequential micropropagation method will permit about 5×10^6-10^7 in vitro plants to be obtained annually.

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