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Short communication

In vitro propagation of *Vitis vinifera* L. cv. 'Monastrell'

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ABSTRACT

Background: A protocol for the micropropagation of the grape (*Vitis vinifera* L.) cultivar 'Monastrell' was developed. Initial plant material was obtained from the sanitary selection of grapevine plants performed by real-time RT-PCR to confirm the absence of *Grapevine fanleaf virus*, *Arabis mosaic virus*, *Grapevine leafroll-associated virus 1*, *Grapevine leafroll-associated virus 3*, and *Grapevine fleck virus*.
Results: The effects of the salt composition (comparing Lloyd and McCown woody plant medium and Murashige and Skoog medium 1/2 macronutrients) and the growth regulator benzylaminopurine (BAP), at 0 and 8.9 μM, on plant propagation were evaluated using nodes as explants. The most efficient procedure consisted of bud induction in the medium with Lloyd and McCown woody plant salts and 8.9 μM BAP for 30 d along with elongation in cytokinin-free medium for 60 d, which gave 22 nodes/explant (174 plants/initial plant). A second cycle of propagation in a medium without BAP for another 60 d could give approximately 10,000 nodes, which can be obtained after an additional 2 months of culture. All plants acclimatized after the second cycle of multiplication were successfully transferred to soil.
Conclusion: We developed an optimal protocol for *V. vinifera* cv. 'Monastrell' micropropagation, the first described for this cultivar.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important edible fruit crops cultivated worldwide and is mainly used in wineries [1,2]. The vast majority of quality wines around the world are made from cultivars resulting from natural or deliberate crosses between different varieties belonging to *V. vinifera* subsp. *vinifera* [3]. To maintain the resulting combination of the distinct genotypes involved in the crosses, which leads to their distinctive characteristics, vegetative propagation is the common method of grape multiplication. The use of *in vitro* culture for vegetative multiplication, termed micropropagation, offers an important alternative to conventional methods of plant propagation [4,5,6] and is an important tool to initiate breeding programs [7]. The use of efficient micropropagation protocols will result in the production of numerous plants that can be maintained under controlled conditions in a reduced space until their transfer to the field for growing or grafting.

In grapevine, virus infection is common and affects the yield and fruit quality and therefore may affect wine quality [8,9]. In addition, incompatibility problems can be acute in infected vines when grafting [10]. Considering the high cost of establishing a vineyard, it is crucial to use the best available planting material. In this context, propagation from virus-free materials by micropropagation is of great interest because currently propagation of grapevine is performed by wood cuttings. Moreover, multiplication or culture by *in vitro* procedures is of value in the application of techniques such as induced mutation and selection, *in vitro* screening, and germplasm exchange [11]. Despite the usefulness of this technique, micropropagation attempts using grapevine have had limited success [12,13]. Recently, micropropagation of several *V. vinifera* cultivars has been described: 'Malagouzia' and 'Xinomavro' by Skiada et al. [14]; 'Brasil,' 'Sun Red,' 'Pinotage,' and 'Zinfandel' by De Carvalho-Silva et al. [15]; and 'Pusa Navrang,' 'Pearl of Csaba,' and 'Julesky Muscat' by Dev et al. [16].

The work conducted using *V. vinifera*, interspecific hybrids, or grape-related species has illustrated the influence of the genotypes and the salt composition of the culture medium on the micropropagation procedure [14,16,17,18]. Therefore, this work aimed to develop a micropropagation protocol for a selected clone of 'Monastrell,' confirmed as virus free, and compare the most common salt compositions used for grapevine: MS 1/2 [17,19] *versus* Woody (W) plant salts [13,20]. 'Monastrell' is a grapevine cultivar that originated in the Valencian region of Spain, and it is very important in the Alicante designation of origin (DO), Spain. This cultivar is also commonly used in seven DOs in Eastern Spain (Valencia, Bullas, Almansa, Jumilla, Yecla, Benisalem-Mallorca, and Pla i Llevant) and in Southern France

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(Provence), where it is known as Mourvedre. This cultivar is also used to a lesser degree in five other Spanish DOs [21]. To the best of our knowledge, there are no micropropagation protocols for this cultivar.

2. Materials and methods

2.1. Plant material, virus analysis, and in vitro culture

The sanitary status of a single asymptomatic plant of cv. 'Monastrell' was evaluated as described by López-Fabuel et al. [22] to test for *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), Grapevine leafroll-associated virus 1 (GLRaV-1), *Grapevine leafroll-associated virus 3* (GLRaV-3), and *Arabis mosaic virus* (ArMV). Viral isolates of each of these virus species, maintained in a screened greenhouse at the Instituto Valenciano de Investigaciones Agrarias, were used as positive controls. Data acquisition and analysis were performed using StepOne Plus 2.0 software. The cv. 'Monastrell' was cultured *in vitro* in basal medium B [Murashige and Skoog salts (1/2 macronutrients) plus vitamins (DUCHEFA, The Netherlands)] that contains 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 36.7 mg/L FeNaEDTA, 6.20 mg/L H_3BO_3, 0.83 mg/L KI, 16.90 mg/L $MnSO_4 \cdot H_2O$, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, and 8.60 mg/L $ZnSO_4 \cdot 7H_2O$ as micronutrients; 166 mg/L $CaCl_2$, 85 mg/L KH_2PO_4, 950 mg/L KNO_3, 87.86 mg/L $MgSO_4$, and 825 mg/L NH_4NO_3 as macronutrients; 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 0.1 mg/L thiamine HCl as vitamins; 20 g/L sucrose; 7.5 g/L plant agar; polyvinylpyrrolidone (0.1 g/L); and 0.1 mg/L indolebutyric acid (IBA). Afterward, clones of virus-free plants were obtained and cultured in tubes. The pH of the medium was adjusted to 5.8 before sterilization at 121°C for 20 min. The cultures were incubated in a growth chamber at 26 ± 2°C under a 16-h photoperiod with cool white light.

2.2. Shoot multiplication: effects of mineral salts and benzylaminopurine on growth and proliferation

Four 'Monastrell' plants (7–9 cm tall), grown in *in vitro* culture for 45 d and obtained from the initial virus-free plant, were used as the source of nodes. Eight nodes per plant (each bearing a single axillary dormant bud) were obtained and cultured (one node per tube; Fig. 1a) in tubes containing 16 mL of medium B or W [similar to B but with Lloyd and McCown woody plant salts (DUCHEFA, The Netherlands): 0.25 mg/L $CuSO_4 \cdot 5H_2O$, 36.7 mg/L FeNaEDTA, 6.2 mg/L H_3BO_3, 22.30 mg/L $MnSO_4 \cdot H_2O$, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, and 8.6 mg/L $ZnSO_4 \cdot 7H_2O$ as micronutrients; 72.5 mg/L $CaCl_2$, 471.26 mg/L $Ca(NO_3)2 \cdot 4H_2O$, 170 mg/L KH_2PO_4, 990 mg/L K_2SO_4, 180.54 mg/L $MgSO_4$, and 400 mg/L NH_4NO_3 as macronutrients; 2 mg/L glycine; 100 mg/L myo-inositol; 0.5 mg/L nicotinic acid; 0.5 mg/L pyridoxine HCl; and 1 mg/L thiamine HCl as vitamins] supplemented with 0 or 8.9 μM benzylaminopurine (BAP) (Fig. 1b). On day 30 of culture, explants cultured on media containing BAP were transferred to baby food jars containing medium B or W (depending on their initial medium) (Fig. 1c). The number of sproutings and yield (number of nodes obtained/initial plant, from the

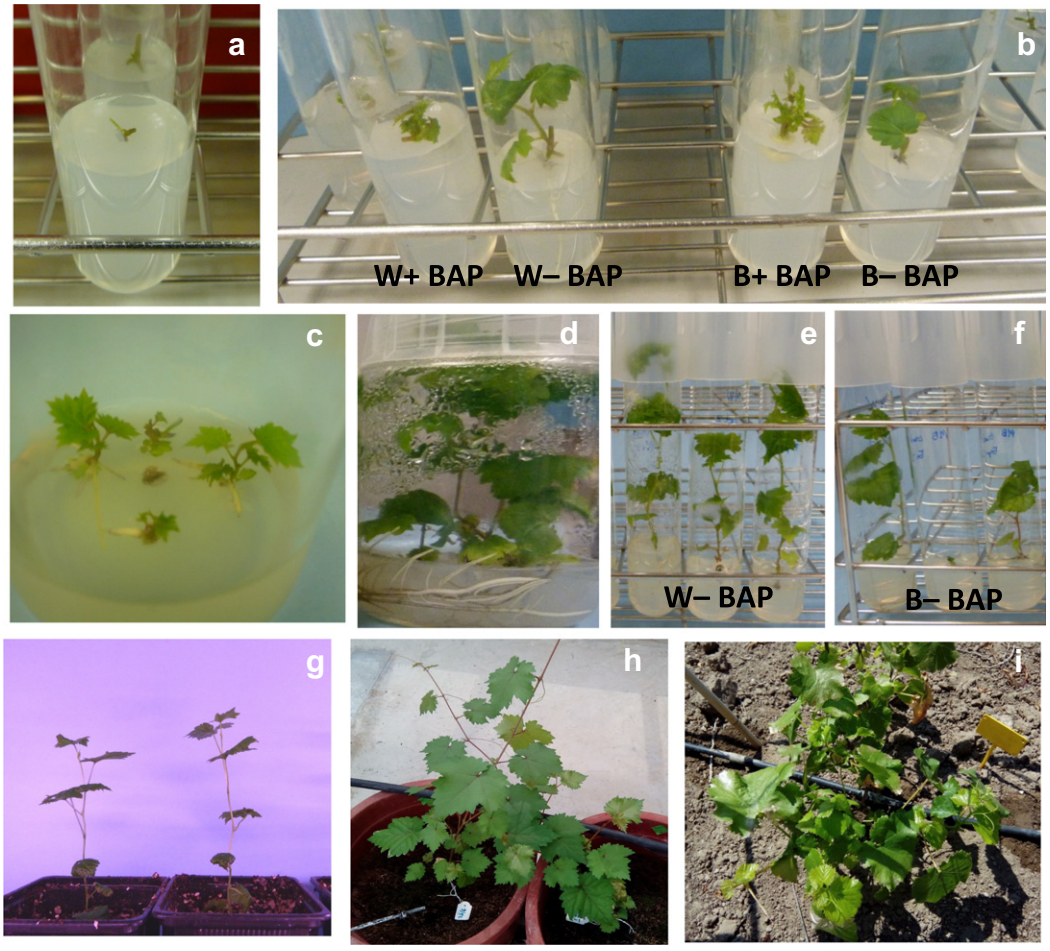

Fig. 1. (a) Nodes on culture media at day 0. (b) Explants grown on medium W or B supplemented or not with BAP (8.9 μM) after 30 d of culture. (c) Shoots induced in BAP-containing media and developing in media without growth regulators. (d, e, f) Shoots developed after 90 d of culture. (g) Plants, 15 d after acclimatization. (h, i) Micropropagated plants grown under greenhouse (h) and field conditions (i).

eight initial nodes) were measured 30 and 90 d after the start of the initial culture (Fig. 1d). This assay was performed twice.

Chi-square test was used to analyze the percentage of sprouting at 30 and 90 d of culture. The effect of the media on yield (number of nodes per initial plant after 60 and 90 d of culture) was analyzed using ANOVA. As significant differences were found, the means were separated by a post-hoc Tukey HSD test ($P < 0.05$). The Statgraphics program was used for all the analyses.

For a second multiplication cycle, four plants (10 nodes per plant) obtained from the best procedures (media with W + BAP) were cultured on medium W without cytokinin for another 60 d (Fig. 1e). The percentage of sprouted buds and the number of nodes of the developed plants were noted at the end of this period.

2.3. Acclimatization and growth in greenhouse conditions

Twenty plants obtained after the second cycle of multiplication were acclimatized in pots containing soil and vermiculite (1:1). The plants were covered with a plastic vessel for 1 week and were grown in a chamber with 70–80% humidity, 26 ± 2°C, and 1160 lx luminance for 20 d. Then they were transplanted to pots and grown under hydroponic conditions in a greenhouse. A sample of these plants was transferred to the field.

3. Results and discussion

The analysis of the mother plant, a clone of cv. 'Monastrell,' was performed to confirm the absence of GFLV, ArMV, GFkV, GLRaV-1, and GLRaV-3 in the starting plant material. Only positive controls gave a successful amplification by real-time RT-PCR, while the mother plant tested negative for all five viruses. Subsequently, four plants were obtained from this initial virus-free mother plant to use as sources of nodes and to determine the effects of the mineral salt composition and/or BAP addition on 'Monastrell' *in vitro* plant growth and bud induction. The effect of culture medium mineral composition on the *in vitro* culture of grapevine has been reported by different authors [14,16,17,18]. With regard to the addition of cytokinin to the culture medium, which is essential to increase multiplication in micropropagation procedures, the BAP concentration chosen in this study was similar to that used by Alizadeh et al. [23] for the micropropagation of four grape rootstocks (8.9 μM) and by Abido et al. [24] for the grapevine cv. 'Muscat de Alexandria.' In addition, this dose of BAP was reported as adequate with regard to inducing new buds with good development in other species. For instance, Bhatt et al. [25] considered this concentration optimal for five *Alocasia* species; higher concentrations (22.2 or 44.4 μM) induced pale and stunted shoots.

After 30 d of culture (Fig. 1b) in media without cytokinin, bud break was observed in approximately 45% of the explants (precisely, 37.5% of those cultured on B and 50% of those cultured on W), whereas in the BAP-containing media, new bud induction was observed in 87.5% of the nodes cultured on B + BAP and in all the nodes (100%) cultured on W + BAP. The Chi-square test comparing the two media without cytokinin showed no significant difference (P-value = 0.78). Similarly, no difference was obtained when comparing the two media with cytokinin (P-value = 0.85). Adventitious buds were transferred to media without growth regulators for elongation (Fig. 1c); the remaining nodes were maintained in the corresponding tubes for sprouting or elongation. After 90 d of culture, 62.5% sprouting was achieved on medium B and 87.5% on medium W; no significant difference between media B and W was found (P-value = 0.84). For both media with BAP, 100% of the nodes had new shoots.

Adequate elongation of shoots was produced for all treatments (Fig. 1d). In grape, difficulties in shoot elongation [13] or deficiencies such as vitrification [23] in BAP-containing media have been described. Difficulties in shoot elongation or vitrification were not observed during the protocol developed here, possibly because of the use of another genotype, differences in the composition of the culture media, or the transfer of shoots induced in a BAP-containing medium to a medium without growth regulation for elongation.

Yield, measured as the number of nodes obtained from an initial plant after a period of culture, was calculated after 60 and 90 d of initial culture. Statistical differences were found between the media at both times of initial culture (P-value = 0.0022 at 60 d and 0.0001 at 90 d). The yield observed from the explants cultured on medium W was approximately double that of explants cultured on medium B, in the absence or presence of BAP, on both days of scoring (Fig. 2). Therefore, it was concluded that medium W is better than B for the *in vitro* growth of the grape cv. 'Monastrell.' The most efficient multiplication was obtained from nodes cultured on medium W supplemented with BAP and elongated in medium W; 174 shoots—5–15 cm tall—were obtained from each initial plant (8 nodes) at day 90 of the initial culture, averaging 21.75 nodes/explant. This result is better than that obtained by De Carvalho-Silva et al. [15] using a lower BAP concentration and similar time of culture for four cultivars of *V. vinifera* (ranging from 1.9 to 2.8 nodes/explant). Medium W has an auxin, indole-butyric acid (IBA), that favors rooting and also contains polyvinylpyrrolidone that may favor rooting induction [26]. Concerning the mineral composition of the media, the main differences were the higher levels of SO_4^{2-}, PO_4^{3-}, and Ca^{2+} and lower NO_3^- in medium W than in medium B. Moreover, the thiamine HCl concentration was 10 times higher in medium W.

After the initial propagation step, the number of clones can be increased by using a second cycle of multiplication. Of the 40 nodes (extracted from four plants) obtained in the first cycle of propagation and cultured on medium W without cytokinin for 60 d, 38 shoots sprouted and grew (each with 6.53 ± 0.21 nodes/shoot). Therefore, in this second cycle of multiplication, approximately 62 nodes were obtained per plant (6.53 nodes/shoot × 38 shoots/4 plants). Considering that we obtained 174 plants in the first cycle, overall approximately 10,000 nodes (174 plants × 62 nodes/plant) could have been produced to start a third multiplication step.

Finally, the acclimatized plants were 8.4 ± 0.40 cm tall, on average, 20 d after transplanting. All the plants transferred for growing under greenhouse and field conditions were adapted (Fig. 1g–i).

In conclusion, the salt composition of medium W doubled the yield with respect to medium B, with and without the addition of BAP. By following the most efficient micropropagation procedure of those tested (nodes of the mother plant cultured on medium W containing

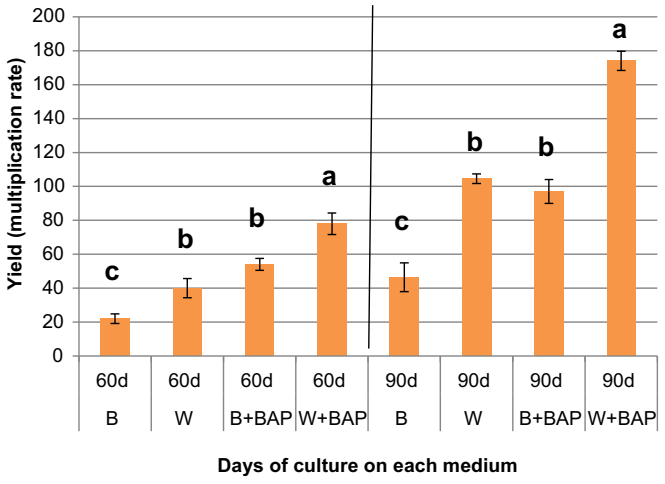

Fig. 2. Mean values of the propagation rate (yield) after 60 and 90 d of culture on media B and W, supplemented or not with BAP (8.9 μM). Yield: number of nodes obtained per initial plant after a period of culture. Mean values separated by different letters are significantly different ($P < 0.05$) according to Tukey's test.

8.9 μM BAP for 30 d; then, transfer of the induced buds to medium W for elongation for 60 d and a second cycle of multiplication in medium W for another 60 d), approximately 10,000 clones of cv. 'Monastrell' rooted plants that can be transferred to soil with high efficiency could be obtained from one initial plant after approximately 7 months of culture.

Conflict of interest

The authors declare no conflict of interest.

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