

COMPARATIVE STUDY OF AGAR AND VERMICULITE ON MICROPROPAGATION AND HYPERHYDRICITY OF OLIVE (*OLEA EUROPAEA* L.) EXPLANTS

Chrysovalantou Antonopoulou^{1*}, Kortessa Dimassi¹, Ioannis Therios¹, Christos Chatzissavvidis², Olga Dichala¹

¹Laboratory of Pomology, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece

²Department of Agricultural Development, School of Agricultural and Forestry Sciences, Democritus University of Thrace, Orestiada, Greece

Abstract

Olive explants (cv. Chondrolia Chalkidikis) were cultured on Rugini olive medium (OM) which was solidified with agar added at three different concentrations: 0.6, 0.8 and 1.0%. In some treatments, agar was replaced in the test tubes with vermiculite (15 cm³) which was impregnated with the same OM nutrient solution. Moreover, in the effort of reversing hyperhydricity (or vitrification) symptoms, after four weeks of *in vitro* culture some olive explants were transferred from the agar-solidified (0.6%) medium onto the one containing vermiculite and vice versa. Replacing agar with vermiculite enhanced the production of shoots of good quality. Hyperhydricity was more intense in the solidified OM containing 0.6% agar. Increasing the concentration of agar reduced not only hyperhydricity but also *in vitro* shoot formation. Hyperhydrated shoot cultures did not recover after transferring them from the agar-solidified medium to the one containing vermiculite.

Keywords: Chondrolia Chalkidikis, *in vitro*, proliferation, reversion, vitrification

1. INTRODUCTION

Olive (*Olea europaea* L.) is one of the oldest and most extensively cultivated trees in the Mediterranean basin, while nowadays its area of cultivation has expanded worldwide. It is mostly propagated by leafy cuttings and grafting onto seedlings, although such techniques result in a limited number of new plants, since there are great differences in the rooting potential of olive cultivars [1]. These difficulties can be overcome by *in vitro* culture of olive [2]. However, olive micropropagation has not yet been fully exploited. The procedure is highly cultivar dependent and therefore there have been developed different *in vitro* protocols for each olive cultivar [3-5].

Chondrolia Chalkidikis is the main table olive cultivar of Northern Greece. Previous studies have showed that it can be *in vitro* propagated, but the explants often present low shoot proliferation and hyperhydration symptoms [6-8]. Hyperhydration (or vitrification) is a common anatomical, morphological, biochemical and physiological abnormality, due to excessive water uptake by the apoplasts and the accumulation of water in plant tissues [9,10]. Generally, it is often observed during the micropropagation of olive and other plant species [11-13]. Hyperhydrated microshoots root *in vitro* poorly and they usually do not survive at the acclimatisation phase or they result in malformed plantlets.

High relative humidity in the culture atmosphere and the water potential of the medium are the main factors responsible for *in vitro* explant hyperhydration [11,14]. Water potential is primarily regulated by both the solutes and the gelling agents found in the medium [14,15]. The higher the gelling agent concentration, the lower is the water potential, and consequently, the lower the availability of both water and dissolved substances to explants [16].

Agar is the most common gelling agent of *in vitro* culture. The severity of hyperhydration depends on the type and concentration of agar supplemented in the medium [13,17]. Some researchers have managed to reduce this problem by increasing the agar concentration in the culture medium from 0.6-0.8% up to 1.1-1.8% [18,19]. However, increasing the concentration of agar can reduce shoot

proliferation and growth, and make difficult the removal of agar from the explants during their exit from the *in vitro* environment [16,18].

The most serious symptoms of hyperhydration are usually irreversal. However, in some cases, the hyperhydrated explants recovered and grew normally, as soon as they were transferred to a more suitable *in vitro* environment [19]. In *in vitro* propagation of *Cephalotus follicularis*, hyperhydration was overcome when the shoots were transferred from an agar solidified culture medium to another, consisting of rockwool soaked with the same nutrient solution [20]. Alternative to agar, different inert substrates, such as vermiculite and perlite, could be used in micropropagation. Among their advantages are the physical support and the ventilation of the base of the explants, as well as the availability of all the components of the nutrient solution.

The objective of the present research was to enhance shoot proliferation of olive explants of the cultivar Chondrolia Chalkidikis and to diminish or control hyperhydration symptoms that occurred during their *in vitro* culture.

2. MATERIALS AND METHODS

Binodal olive explants of the cv. Chondrolia Chalkidikis were transferred onto the Olive Medium (OM) [21] containing BA 2 mg l⁻¹, IBA 0.05 mg l⁻¹ and sucrose 20 g l⁻¹. The culture medium was solidified with agar (Oxoid No3) at three different concentrations: 0.6 (control), 0.8 and 1.0%. Additionally, in some treatments, the agar solidified medium in the vessels was replaced by vermiculite (15 cm³) which was impregnated with the same OM nutrient solution (10 ml) and then sterilized. The duration of the experiment was 8 weeks, with an intermediate subculture at the end of the fourth week. Each treatment included 10 replications (tubes). In an attempt to reverse the hyperhydration symptoms, during the subculture some explants were transferred from the agar solidified medium (0.6%) to the vessels containing vermiculite-nutrient solution and vice versa.

The pH of the nutrient media was set to 5.8 before sterilization at 121°C for 20 min. The cultures were grown at 23 ± 2°C, under cool white fluorescent lamps (90 μmol m⁻² s⁻¹), with a 16-h photoperiod. At the end of the experiment, shoot proliferation and hyperhydrated explants were recorded. The experimental layout was completely randomized and the experiment was repeated twice. The reported data are the means of the two experiments. Means were compared using the Duncan's multiple range test ($P \leq 0.05$ in the SPSS 8.0 statistical program).

3. RESULTS AND DISCUSSION

Olive micropropagation can be an important tool for the production of a large number of new plants and for genetic improvement [2]. In our attempt to develop an efficient micropropagation protocol for the Greek olive cultivar Chondrolia Chalkidikis, the hyperhydration problem was encountered.

Kevers et al. [11] assumed that hyperhydration can be the morphological reaction to stress when explants are cultured in an unsuitable medium. The agar-solidified culture medium is the most common micropropagation substrate of woody plant species. Water potential of the culture medium is one of the main hyperhydration factors [14]. This abnormality has been reported when explants are cultured in media containing a small amount of agar or are liquid [22]. Alternatively, the physical support that is provided to explants by the agar-solidified medium can also be achieved by using inert substrates, such as perlite, vermiculite or sand.

At the end of the experiment, hyperhydration symptoms were observed at all treatments. Hyperhydrated explants had a 'wet' appearance, smaller internodes and their leaves were thicker than normal, dark green, twisted and fragile. The problem was particularly intensive (~60%) when 0.6% agar was added in the nutrient medium and did not improve when explants subsequently were transferred onto the vermiculite medium (Fig 1). According to Debergh [23], the less agar that a nutrient medium contains the greater is the absorption of cytokinin by plant tissues. However, the increase of the endogenous levels of cytokinins can be the trigger for the appearance of this problem. On the other hand, increasing

the concentration of agar in the culture medium it decreased the percentage of hyperhydrated explants (~40%), which remained almost stable at the two highest levels of agar (Fig 1). Some possible explanations of this positive effect are the slight decrease of the relative humidity in the culture vessel and a reduction in the absorption of nutrients and growth regulators [23]. Similarly, Rugini et al. [18] mention that when they increased the concentration of agar to 0.9%, hyperhydration symptoms of olive explants were limited. Specifically, supplementing the nutrient medium with agar 0.6%, resulted in 30% of hyperhydrated shoots, which furthermore hyperhydrated up to 90% at the next subculture. When the amount of agar increased to 0.9%, the hyperhydration percentage was stable at 30-40%.

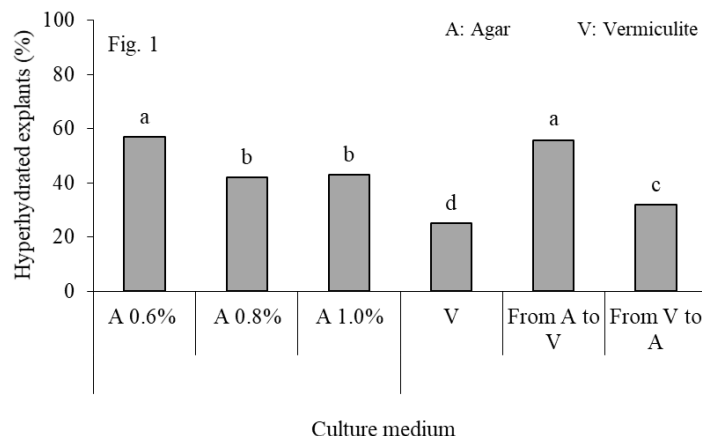


Fig. 1. The effect of *in vitro* culture medium types on the percentage of hyperhydrated explants of olive cv. Chondrolia Chalkidikis. (A: agar solidified medium, V: vermiculite and nutrient solution, From an agar solidified medium to another containing vermiculite, and vice versa) (Values with different letters are significantly different at $P \leq 0.05$)

Less hyperhydration symptoms were observed in the presence of vermiculite, probably due to the better aeration of the culture medium. Additionally, this positive effect of vermiculite was maintained even when the explants were subsequently subcultured in the agar-solidified medium (0.6%). Specifically, when the initial medium was solid hyperhydration was more intense compared to vermiculite as an initial medium (Fig. 1). During *in vitro* culture of some plant species, normal shoots become hyperhydrated after a few weeks when they are transferred from a solid to a liquid nutrient medium, but return to normal when repositioned in agar [24]. This did not occur in the present study, since when explants of Chondrolia Chalkidikis were transferred from the agar-solidified culture medium to the one containing vermiculite they showed almost the same hyperhydration percentages as those that were continuously developed in the culture medium solidified with 0.6% agar. Therefore, if we consider that low concentrations of agar contribute to the appearance of hyperhydration, the transfer of olive explants to a more suitable *in vitro* environment (vermiculite) did not help them recover.

The explants of Chondrolia Chalkidikis cultured *in vitro* in the vessels containing vermiculite, developed the most new shoots (3.7), while those grown in the medium solidified with agar 0.6% produced several new shoots (3.0) (Fig. 2) This amount of agar has been effective in the micropropagation of several olive cultivars [3,4,25]. In contrast, increasing the concentration of agar it reduced the number of new shoots formed (1.2) (Fig 2). Similarly, when Rugini et al. [18] increased the concentration of agar to 0.9%, although it eliminated hyperhydration symptoms of olive explants, it also reduced *in vitro* shoot proliferation and growth. This negative effect was attributed probably to the limited contact of the explants with the nutrient medium and the subsequent limited absorption of nutrients. Casanova et al. [16] found that the increase in agar concentration resulted in the reduction of hyperhydration, water content of the tissues, shoot fresh weight, number and size of the parenchymal cells, and the number of

new shoots. The alternation of culture media, from agar-solidified to vermiculite and vice versa, neither positively nor negatively affected *in vitro* proliferation of Chondrolia Chalkidikis (Fig 2).

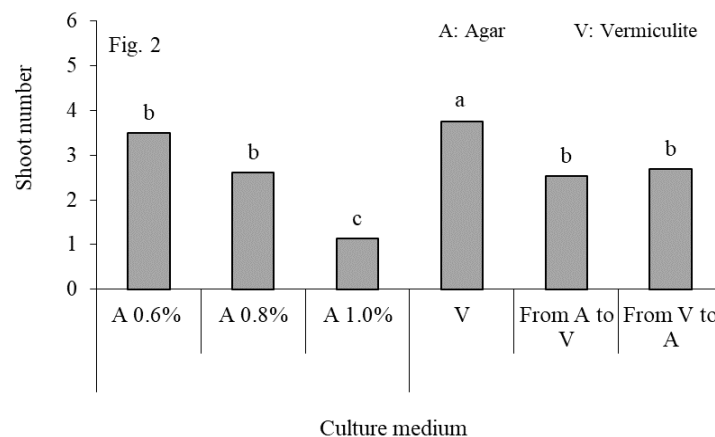


Fig. 2. The effect of *in vitro* culture medium types on shoot number of olive cv. Chondrolia Chalkidikis explants. (A: agar solidified medium, V: vermiculite and nutrient solution, From an agar solidified medium to another containing vermiculite, and vice versa) (Values with different letters are significantly different at $P \leq 0.05$)

The highest shoot length was achieved when the explants were cultured in the medium solidified with the lowest agar concentration and also in the medium containing vermiculite (Fig. 3). Increasing the concentration of agar decreased dramatically shoot length. Both fresh and dry weight of the new olive shoots produced presented their lowest value in the densest agar-solidified culture medium (1.0% agar) and the highest value in the presence of vermiculite and in the medium containing the smallest amount of agar (Fig. 4).

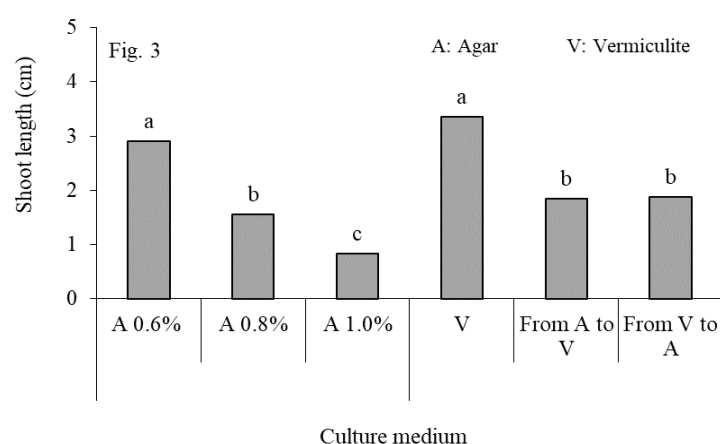


Fig. 3. The effect of *in vitro* culture medium types on shoot length of olive cv. Chondrolia Chalkidikis explants. (A: agar solidified medium, V: vermiculite and nutrient solution, From an agar solidified medium to another containing vermiculite, and vice versa) (Values with different letters are significantly different at $P \leq 0.05$)

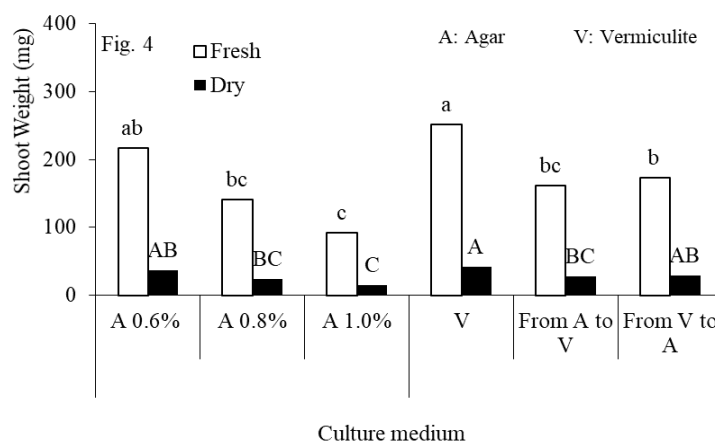


Fig. 4. The effect of *in vitro* culture medium types on shoot fresh and dry weight of olive cv. Chondrolia Chalkidikis explants. (A: agar solidified medium, V: vermiculite and nutrient solution, From an agar solidified medium to another containing vermiculite, and vice versa) (Values with different letters are significantly different at $P \leq 0.05$)

4. CONCLUSION

The results of this study demonstrated that during the micropropagation of olive cv. Chondrolia Chalkidikis, solidification of the culture medium with increased amount of agar did eliminate hyperhydration symptoms of explants but it also reduced shoot proliferation. In contrast, replacing the agar solidified medium with a combination of vermiculite and nutrient solution (OM) it enhanced the *in vitro* production of new shoots of good quality, with less symptoms. Alternation of culture media, subculturing explants from an agar-solidified to a vermiculite containing medium and vice versa, did not reverse hyperhydration. Since there are several factors that interact with each other and contribute to the appearance of hyperhydration, this abnormality is not completely predictable and it is not easy to find a solution for all cases.

REFERENCES

1. Therios, IN 2009, Olives (Vol. 18). CABI.
2. Rugini, E, Gutiérrez-Pesce, P, Spampinato, PL, Ciarmiello, A & D'ambrosio, C 1999, 'New perspective for Biotechnologies in olive breeding: morphogenesis, *in vitro* selection & gene transformation', *Acta Horticulturae*, vol. 474, pp. 107-10, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.1999.474.18>.
3. Dimassi, K 1999, 'Micropropagation studies of the cv. Kalamon olives (*Olea europaea* sativa L.)', *Acta Horticulturae*, vol. 474, pp. 83-86, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.1999.474.13>.
4. Roussos, PA & Pontikis, CA 2002, 'In vitro propagation of olive (*Olea europaea* L.) cv. Koroneiki. *Plant growth regulation*, vol. 37, no. 3, pp. 295-304, viewed 25 April 2020, <https://doi.org/10.1023/A:1020824330589>.
5. Mendoza-de Gyves, E, Mira, FR, Ruiu, F & Rugini, E 2008, 'Stimulation of node and lateral shoot formation in micropropagation of olive (*Olea europaea* L.) by using dikegulac', *Plant cell, tissue and organ culture*, vol. 92, no. 2, pp. 233-38, viewed 25 April 2020, <https://doi.org/10.1007/s11240-007-9314-4>.

6. Grigoriadou, K, Vasilakakis, M & Eleftheriou, EP 2002, 'In vitro propagation of the Greek olive cultivar 'Chondrolia Chalkidikis'', *Plant Cell, Tissue and Organ Culture*, vol. 71, pp. 47–54, viewed 25 April 2020, <https://doi.org/10.1023/A:1016578614454>.
7. Antonopoulou, C, Dimassi, K, Chatzissavvidis, C, Therios, I & Papadakis, I 2006, 'Effect of BA and GA₃ on the micropropagation and vitrification of olive (*Olea europaea* L.) explants (cv. Chondrolia Chalkidikis)', *Proceedings of the second International Seminar, Biotechnology and quality of olive tree products around the Mediterranean basin*, Olivebioteq, Marsala-Mazara, Sicily, vol. I, pp. 477-80.
8. Antonopoulou, C, Dimassi, K, Chatzissavvidis, C, Papadakis, I & Therios, I 2012, 'The effect of the explant type and nutrient medium on the *in vitro* proliferation of olive (*Olea europaea* L., cv. Chondrolia Chalkidikis)', *Acta Horticulturae*, vol. 949, pp. 185-89, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.2012.949.25>.
9. Rojas-Martínez, L, Visser, RG & De Klerk, GJ 2010, 'The hyperhydricity syndrome: waterlogging of plant tissues as a major cause', *Propagation of Ornamental Plants*, vol.10, no. 4, pp. 169-75.
10. Van den Dries, N, Giannì, S, Czerednik, A, Krens, FA & de Klerk, GJ 2013, 'Flooding of the apoplast is a key factor in the development of hyperhydricity', *Journal of Experimental Botany*, vol. 64, no. 16, pp. 5221–30, viewed 25 April 2020, <https://doi.org/10.1093/jxb/ert315>.
11. Kevers, C, Franck, T, Strasser, RJ, Dommès, J & Gaspar, T 2004, 'Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state', *Plant Cell, Tissue and Organ Culture*, vol. 77, no. 2, pp. 181-91, viewed 25 April 2020, <https://doi.org/10.1023/B:TICU.0000016825.18930.e4>.
12. Grigoriadou, K, Eleftheriou, EP & Vasilakakis, M 2007, 'Hidden hyperhydricity may be responsible for abnormal development and acclimatization problems of micropropagated olive plantlets: an anatomical leaf study', *Acta Horticulturae*, vol. 748, pp. 103–06, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.2007.748.10>.
13. Bayraktar, M, Hayta-Smedley, S, Unal, S, Varol, N & Gurel, A 2020, 'Micropropagation and prevention of hyperhydricity in olive (*Olea europaea* L.) cultivar 'Gemlik'', *South African Journal of Botany*, vol. 128, pp. 264-73, viewed 25 April 2020, <https://doi.org/10.1016/j.sajb.2019.11.022>.
14. Ziv M 1991, 'Vitrification: morphological and physiological disorders of *in vitro* plants', in PC Debergh & R Zimmerman (eds.): *Micropropagation. Technology and Application*, Kluwer Academic Publishers, Dordrecht, pp. 45-69.
15. Hazarika, BN & Bora, A 2008, 'Hyperhydricity-A bottleneck to micropropagation of plants', *Acta Horticulturae*, vol. 865, pp. 95-101, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.2010.865.11>.
16. Casanova, E, Moysset, L & Trillas, MI 2008, 'Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots', *Biologia Plantarum*, vol. 52, no. 1, pp. 1-8, viewed 25 April 2020, <https://doi.org/10.1007/s10535-008-0001-z>.
17. Ivanova, M & Van Staden, J 2011, 'Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*', *Plant Cell, Tissue and Organ Culture*, vol. 104, no. 1, pp. 13-21, viewed 25 April 2020, <https://doi.org/10.1007/s11240-010-9794-5>.
18. Rugini, E, Tarini, P, Rossodivita, ME & Costanzo, S 1985, 'Control of shoot "vitrification" of almond and olive grown *in vitro*', *Acta Horticulturae*, vol. 212, pp. 177-84, viewed 25 April 2020, <https://doi.org/10.17660/ActaHortic.1987.212.28>.
19. Pérez-Tornero, O, Egea, J, Olmos, E & Burgos, L 2001, 'Control of hyperhydricity in micropropagated apricot cultivars', *In Vitro Cellular & Developmental Biology-Plant*, vol. 37, no. 2, pp. 250-54, viewed 25 April 2020, <https://doi.org/10.1007/s11627-001-0044-8>.

20. Adams, RM, Koenigsberg, SS & Langhans, RW 1979, 'In vitro propagation of *Cephalotus follicularis* (Australian Pitcher Plants)', *HortScience*, vol. 14, pp. 512–13.
21. Rugini, E 1984, 'In vitro propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos', *Scientia Horticulturae*, vol. 24, pp. 123-34, viewed 25 April 2020, [https://doi.org/10.1016/0304-4238\(84\)90143-2](https://doi.org/10.1016/0304-4238(84)90143-2).
22. Wawrosch, C 2012, 'Temporary immersion systems for efficient mass propagation of medicinal and aromatic plants', *Proceedings of the second International Symposium on Horticulture in Europe*, International Society for Horticultural Science, Angers, France, pp. 443-46.
23. Debergh, PC 1983, 'Effects of agar brand and concentration on the tissue culture medium', *Physiologia Plantarum*, vol. 59, no. 2, pp. 270-76, viewed 25 April 2020, <https://doi.org/10.1111/j.1399-3054.1983.tb00770.x>.
24. George, EF 1996, *Plant propagation by tissue culture. In practice. Part II*. Exegetics Limited.
25. Dimassi-Theriou, K 1994, 'In vitro propagation of cv. Kalamon olives (*Olea europaea sativa* L.)', *Advances in Horticultural Science*, pp. 185-189.