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Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation

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Abstract

The data presented report on trials conducted during 24 months using the Portuguese olive cultivar 'Galega vulgar'. The effectiveness of coconut water, BAP, or kinetin, as possible zeatin substitutes in olive micropropagation protocols, was investigated. In all stages of the micropropagation process, the mineral and vitamin formulation of olive medium (OM) was used. Regarding culture establishment the best results were achieved when 50 ml l^{-1} coconut water and 2.22 μM BAP were used as medium supplements. For the in vitro multiplication stage, the highest proliferation rates with an average of 3.4 new explants on each 30 days were achieved maintaining the coconut water concentration at 50 ml l^{-1} and increasing BAP up to 8.87 μM. The effects of IBA and activated charcoal on the in vitro root induction were also studied. Rooting rates of over 85% were obtained by basal immersion of the explants in IBA solution at 3 g l^{-1} for 10 s, followed by inoculation in the OM culture medium, added with 2 g l^{-1} of activated charcoal and without growth regulators. All in vitro rooted plants were transferred into Jiffy-Pots filled with vermiculite–perlite 3:1 (v/v) substrate. Those were subsequently wetted with the OM mineral solution, placed into polystyrene plates each one with 100 Jiffy-Pots capacity, which were transferred to traditional rooting mist benches, on a water-cooling equipped greenhouse. Such a simple acclimatization procedure allowed for 95% of plants survival.

Keywords: Coconut water; In vitro culture; Micropropagation; *Olea europaea*; Olive; Zeatin

1. Introduction

Olive (*Olea europaea* L.) is one of the most important fruit species of the Mediterranean region being usually propagated by leafy cuttings under mist. The olive cultivar 'Galega vulgar', known to be very difficult to multiply by this procedure, is of particular importance for Portugal, as it stands for almost 70% of all the Portuguese olive orchards.

Although the propagation of several fruit species difficult to multiply by traditional methods has been made possible by micropropagation, regarding the olive micropropagation, there is not yet enough knowledge for its use in a mass scale nursery production (Zuccherelli and Zuccherelli, 2002).

The first known scientific reports on in vitro olive propagation are from the mid 1970s, where researchers tried to optimize the mineral media formulations for all of the culture stages, in order to establish a micropropagation protocol that would suit all cultivars. From this point-of-view the OM medium (Rugini, 1984), the MSI, original MS medium (Murashige and Skoog, 1962), modified by Fiorino and Leva (1986) and the MSM, also original MS medium modified by Leva et al. (1992), are considered up-to-now as the most suitable ones for olive micropropagation, but they also proved not to be effective for all cultivars (Grigoriadou et al., 2002).

Besides the mineral formulation, growth regulators are also one of the most important components of the in vitro culture media. Since the pioneering work of Rugini (1984) that zeatin has been widely accepted as the only cytokinin capable of inducing satisfactory growth in olive cultured explants. It has been generally used at rates rising from 4.56 to 45.62 μM. However, due to its high cost, there is also a generalized opinion that an alternative replacement should be achieved for use in commercial micropropagation protocols (Mencuccini et al., 1997; Briccoli et al., 2002). One alternative to zeatin was

Abbreviations: IBA, indol-3-butyric acid; Benomyl, methyl 1-[(butylamino)carbonyl]-*H*-benzimidazol-2-yl carbamate; LSDleast significant difference; MIRugini initial medium; MSMurashige and Skoog medium; NAAα-naphthalene acetic acid; OMRugini olive medium

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recently reported by García-Férriz et al. (2002) that replaced it by thidiazuron and BAP. Nevertheless, thidiazuron is still a very expensive chemical compound and economical benefits of zeatin replacement were not yet relevant.

Another important component of all culture media is the energy source, with sucrose being widely used for such purpose. In the multiplication media for olive micropropagation Leva et al. (1992, 1994) brought a significant contribution on this subject by replacing sucrose with mannitol. They observed that the increased culture media prices due to mannitol utilization were highly exceeded by the higher multiplication rates and explant growth capacity. Similar observations of increased shoot length and breaking of apical dominance by the use of mannitol on their micropropagation trials with the 'Manzanillo' cultivar were reported by García et al. (2002).

Concerning in vitro rooting, success rates ranging from 25 to 85% were reported by Rugini (1984), Fiorino and Leva (1986) and Rama and Pontikis (1991) depending on the cultivar tested and on the time of the year when the experiments were carried out. For this culture stage, most researchers directly supplement the culture medium with IBA (indol-3-butyric acid) or NAA (α-naphthalene acetic acid), at rates rising from 1 to 4 mg l^{-1}.

Besides growth regulators, medium illumination is also of relevant importance for the in vitro rooting of olive explants. Significant increases on the rooting rates were achieved by Rugini et al. (1993) by darkening the basal explants after painting in black the outside of the vessels and by placing black sterile polycarbonate granules on the surface of the solidified medium. Looking into replacing Rugini's interesting but cumbersome technique with a simpler and more economic alternative method, Mencuccini (2003) reported rooting rates between 86 and 100% on its trials with three Italian olive cultivars using the Brilliant Black commercial dye from Sigma® for medium darkening. Still on the subject, Canas et al. (1992) reported that medium darkening it is not of such importance for the adventitious root induction stage but it seams crucial for its further development. This opinion it is not corroborated by Mencuccini (2003), which reported that in vitro olive explants carry over the in vivo seasonal rooting ability of the olive cultivars and the medium darkening process is the only known way to cancel this effect.

In spite of the extensive studies carried out in the last years aiming to improve the culture conditions for some olive cultivars, olive micropropagation success rates are still in general limited. The shoot proliferation rate is generally low and cultivar dependent (Dimassi-Theriou, 1994; Bartolini et al., 1990), the formation of adventitious roots in many micropropagated olive cultivars is still difficult, and the percentage of post-transplanting losses remains high (Briccoli-Bati et al., 1999; Rugini et al., 1999).

The aim of the present study was to report on the development of an efficient micropropagation protocol for an economically important and difficult to root cultivar like it is the 'Galega vulgar'.

2. Materials and methods

2.1. Plant material

Single node segments each with two opposite buds (Fig. 1) were prepared at the region between the second and fifth nodes from vigorous growing shoots. The shoots were collected in June 2003 from 8-year-old trees, growing in the 'Galega vulgar' mother field of the olive nursery company Plansel S.A. The trees were grown on an alluvial soil with homogeneous chemical and morphological characteristics. Trees were very close to each other (1 m × 1.25 m) and were systematically submitted to severe pruning. From the 17 'Galega vulgar' clones planted, only one was used for these trials and shoots were randomly collected from each one of the ten trees of the clone.

2.2. Explant sterilization

The single node explants were surface sterilized in a three step procedure: washing in tap water for 3 h; immersion in 1% (w/v) Benomyl solution for 50 min, followed by a three-times wash in bi-distillate water; immersion in 0.2% (w/v) $HgCl_2$ with three drops of Tween 20 on 500 ml water for 10 min, followed by a three-times wash in sterile water. During the immersion periods, both in Benomyl and $HgCl_2$, the explants were continuously shaken on an electric rotary shaker.

2.3. Culture establishment

After surface sterilization, the explants were inoculated in Ø 9 cm Petri dishes containing 15 ml of the MI basal culture medium and vitamin complex, as proposed by Rugini (1984). The growth regulators BAP, kinetin or zeatin were tested alone, or mixed with 50 ml l^{-1} coconut water, at 2.22, 2.32 and 2.28 μM, respectively. All media were supplemented with 7 g l^{-1} commercial agar-agar and 30 g l^{-1} sucrose.

Cultures were kept for 30 days in a growth chamber with 24 °C/21 °C (±1 °C) day/night temperature and 15 h light period, under cool white fluorescent light (36 μmol m^{-2} s^{-1}).

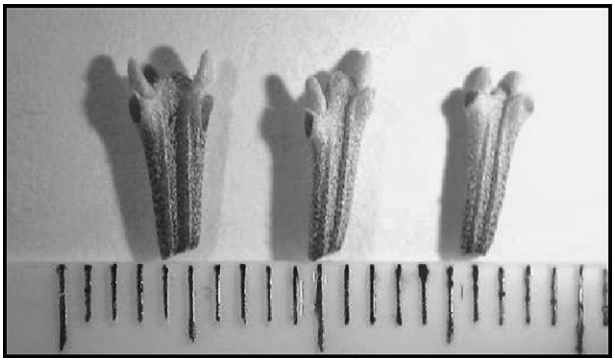

Fig. 1. Uninodal cuttings with 5–7 mm and two axillary buds, were used to initiate the in vitro propagation process.