

Does dikegulac affect *in vitro* shoot proliferation and hyperhydricity incidence in olive explants?

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Abstract

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Dikegulac was tested as a lateral shoot-inducing agent on micropropagated olive (*Olea europaea* ‘Chondrolia Chalkidikis’) shoots. Rugini olive medium was supplemented with dikegulac at 0, 16.9, 33.8, 66.7, 100.5 or 133.4 µM. Dikegulac was not phytotoxic and the explants treated with 100.5 µM had higher number, length, and weight of new shoots. Hyperhydricity (or vitrification) symptoms were diminished by increasing dikegulac concentration in the medium dose (66.7–133.4 µM). Also, dikegulac stimulated the production of large amounts of callus at the base of olive explants.

Keywords: apical dominance; growth retardant; micropropagation; *Olea europaea*, vitrification

Olive (*Olea europaea* L.) is one of the oldest and most extensively cultivated trees in the Mediterranean basin. It is commonly propagated by grafting and leafy cuttings, although such methods produce a limited number of plants, with great differences in rooting potential of cultivars. *In vitro* culture of olive can overcome some difficulties experienced with conventional propagation techniques, and offers a valuable tool for genetic improvement (RUGINI et al. 1999). However, micropropagation of olive has not yet been very successful. Shoot proliferation rate is generally low and cultivar-dependent; the formation of adventitious roots in many micropropagated olive cultivars is difficult and the percentage of post-transplanting losses is high (GRIGORIADOU et al. 2007).

‘Chondrolia Chalkidikis’ is the major table olive cultivar in Northern Greece. It can be propagated *in vitro*, although previous studies have shown that the explants frequently present hyperhydric symp-

toms (vitrification), and produce new shoots of low quality (ANTONOPOULOU et al. 2006; ANTONOPOULOU 2009). Leaves of vitrified (hyperhydrated) shoots are thicker than normal, dark green, twisted, and fragile. Vitrified shoots have a ‘wet’ appearance, smaller internodes and poor rooting ability. Among the factors that may induce vitrification is the presence of plant growth regulators in the medium, especially cytokinins (KATAEVA et al. 1991; MARTIN et al. 2006). The severity of vitrification depends on the type and concentration of cytokinin supplemented in the medium (MARTIN et al. 2006). Zeatin was found to be more effective for *in vitro* shoot proliferation of *Olea europaea* ‘Chondrolia Chalkidikis’, but benzyladenine (BA) also gives good results. Explants that were exposed either to zeatin or to BA have presented vitrification, mostly at higher cytokinin concentrations (ANTONOPOULOU 2009). BA has been used in olive micropropagation with good results (DIMASSI-

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THERIOU 1994; DIMASSI 1999; SGHIR et al. 2005), but it often produces short and thin shoots, with an abundance of callus (RUGINI 1990). Since BA is cheaper and can be autoclaved at 121°C without significant deterioration, in order to increase *in vitro* proliferation there have been conducted successful trials where relatively low concentrations of BA were combined with other components, such as thidiazuron (TDZ), gibberellic acid (GA_3), indole butyric acid (IBA) and coconut milk (DIMASSI-THERIOU 1994; GRIGORIADOU et al. 2002; ZUCCHERELLI, ZUCCHERELLI 2002; PEIXE et al. 2007).

Dikegulac (2,3:4,6-di-*O*-isopropylidene- α -L-xylo-2-hexulofuranosonic acid) is a synthetic compound which was found to inhibit gibberellin biosynthesis, reduce apical dominance, promote lateral branching, and flower-bud formation in some plants (SACHS et al. 1975; NORCHINI et al. 1994; COCHRAN, FULCHER 2013; SARROPOULOU et al. 2014; SUN et al. 2015). It has also been tested as a chemical pinching agent in ornamental species (JACYNA et al. 1994; SANSBERRO et al. 2006), as a growth retardant for trees by trunk injection (WRIGHT, MORAN 1988) and as a fruit abscission agent in citrus (POZO et al. 2004). Dikegulac was also found to increase fruit set in field-grown adult olives as a result of the temporary plant growth reduction and increase in the proportion of short shoots (NIR et al. 1983; RUGINI, PANELLI 1993). As regards micropropagation, it was suggested that dikegulac can increase shoot proliferation rates by stimulating axillary shoot production (CHOUDHURY, GUPTA 1999; BURNS et al. 2002; EBRAHIM 2004; MENDOZA-DE GYVES et al. 2008). According to LITWINCZUK and PROKOP (2010), if such an effect, like producing more shoots, is achieved could lower costs of micropropagation thanks to the replacement of expensive cytokinins with the much cheaper dikegulac.

The objective of this study was to determinate whether dikegulac could reduce the apical dominance of *Olea europaea* 'Chondrolia Chalkidikis' explants, enhance the effect of BA on lateral shoot production, and diminish vitrification symptoms of new shoots induced by BA.

MATERIAL AND METHODS

Leafless single node segments of *Olea europaea* 'Chondrolia Chalkidikis' were taken from stock

plants grown in pots in a greenhouse, disinfected according to ANTONOPOULOU et al. (2012) and established *in vitro* on a hormone-free Olive Medium (OM) (RUGINI 1984). New, healthy shoots produced were used as the explant source for the present experiment. Binodal microshoots (approximately 1.5 cm) were transferred to OM supplemented with 8.9 μ M BA, 20 g/l sucrose and 6 g/l agar (Oxoid No. 3), in testing tubes (25 × 100 mm) containing 10 ml of the nutrient medium. The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 20 minutes. Dikegulac (Sigma-Aldrich, USA) was filter-sterilized and added to the nutrient medium at six concentrations (0, 16.9, 33.8, 66.7, 100.5 or 133.4 μ M), after autoclaving. Each treatment included 15 replications (tubes). Cultures were placed at 23 ± 2°C under cool white fluorescent lamps (45 μ mol/m²/s), with a 16-h photoperiod. Shoot proliferation and vitrified shoots were recorded after 4 weeks. The experimental layout was completely randomized and the experiment was repeated twice. Reported data are the means of two experiments. Means were compared using the Duncan multiple range test for $P \leq 0.05$.

RESULTS AND DISCUSSION

It was reported that dikegulac, besides its positive effect on lateral shoot development, both *in vitro* or *in vivo*, it can also induce phytotoxicity, depending on its concentration and the plant species (JACYNA et al. 1994; SANSBERRO et al. 2006; GROSSMAN et al. 2013). After exposing *Olea europaea* 'Chondrolia Chalkidikis' microshoots to 0–134.4 μ M dikegulac for four weeks, no explant losses or toxicity symptoms on leaves were recorded. Similar results were reported with dikegulac on the *Olea europaea* 'Canino', 'Moraiolo' and 'Frantoio' (MENDOZA-DE GYVES et al. 2008) or azalea grown *ex vitro* (BI, GU 2015). On the contrary, dikegulac reduced explant survival of the *Olea europaea* 'Rosciola' and 'Piantone di Moiano' (MENDOZA-DE GYVES et al. 2008).

At the base of *Olea europaea* 'Chondrolia Chalkidikis' explants large callus was formed, especially when dikegulac was present at relatively higher concentrations (66.7–133.4 μ M). On the other hand, vitrification symptoms were more severe when dikegulac was absent or supplemented at low concentrations (0–33.8 μ M) (Fig. 1A). By increasing dikegulac concentration in the nutrient me-

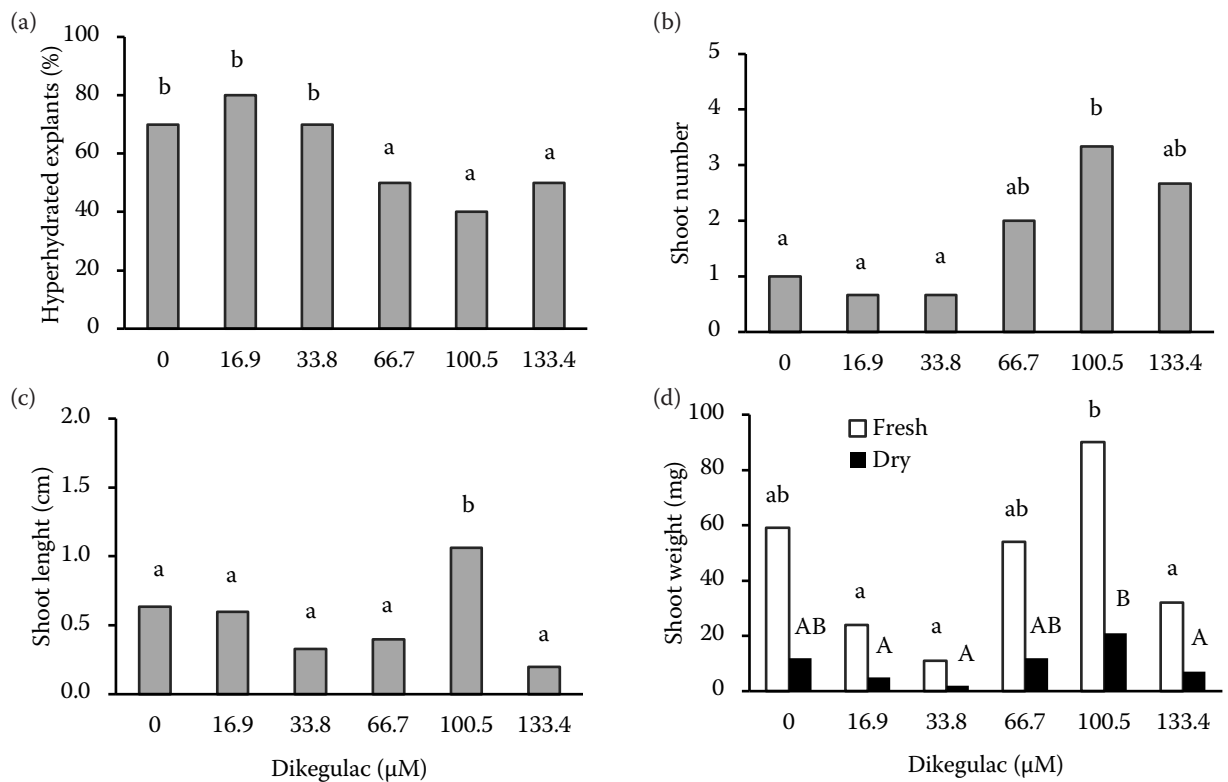


Fig. 1. The effect of dikegulac on the percentage of hyperhydrated explants (a), shoot number (b), shoot length (c) and the fresh and dry weight of olive shoots (d) of the *Olea europaea* 'Chondrolia Chalkidikis'. Significant differences are indicated with by different letters (Duncan multiple range test, $P \leq 0.05$)

dium, vitrification symptoms were diminished and 50–60% of all the explants produced well developed shoots (Fig. 1a). There have been very few reports concerning the effect of dikegulac on either inducing or eliminating *in vitro* vitrification symptoms.

The effect of dikegulac on the reduction of *in vitro* apical dominance of *Olea europaea* 'Chondrolia Chalkidikis' was positive up to a certain concentration of dikegulac. Similarly, SARROPOULOU et al. (2014) reported that dikegulac broke apical dominance in Gisela 6 (cherry rootstock; *Prunus cerasus* × *canescens*) by suppressing the growth of the apical meristem of the explants or by differentiation of the vascular tissue, both of which promote lateral shoot production. Lower dikegulac concentrations (16.9–33.8 µM) did not significantly affect shoot number while the increase of dikegulac concentration in the medium significantly enhanced the production of new olive shoots (Fig 1b). The optimal concentration of dikegulac for the formation of shoots was 100.5 µM (Fig. 1b, Fig. 2). In line, SARROPOULOU et al. (2014) who found increased shoot number per explant in Gisela 6 treated with 80 µM dikegulac, ascribed it to the high cytokinin concen-

trations followed by low IAA and GA_3 concentrations, resulting in breaking of apical dormancy, as proposed by PUGLISI (2002) and EBRAHIM (2004). Dikegulac did not exert any significant effect on shoot elongation, except for the treatment with 100.5 µM (Fig. 1c), which agrees with the results reported for the genotypes *Christia subcordata* and Gisela 6 (*Prunus cerasus* × *canescens*) (WHITING 2007; SARROPOULOU et al. 2014). New shoot fresh and dry matter weight were not affected significantly by dikegulac concentration (Fig. 1d). These data are in concurrence with SARROPOULOU et al. (2014) for two cherry rootstocks treated with 20–150 µM of dikegulac. According to PAQUES and BOXUS (1987), shoots with mild vitrification symptoms may grow faster than normal, but this advantage disappears when the problem becomes more severe.

Dikegulac at 16.9 to 100.5 µM probably enhances cytokinin action on shoot formation while at higher concentrations (133.4 µM) inhibits it (MENDOZA-DE GYVES et al. 2008). Also, early studies on peas (*Pisum sativum* L.) have shown that the action of dikegulac is counteracted by GA_3 (BOCION, de SIL-

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Fig. 2. Formation of callus and new shoots of *Olea europaea* 'Chondrolia Chalkidikis' explants placed in Olive Medium modified with 100.5 μM dikegulac.

VA 1977). Moreover, the decrement in some woody species of shoot length was ascribed to the inhibition of gibberellin biosynthesis due to dikegulac addition by inhibiting oxidation of ent-kaurene to ent-kaurenic acid, which is a prerequisite for gibberellic acid biosynthesis (THETFORD, BERRY 2000). Hence, since dikegulac inhibits gibberellin biosynthesis, its inclusion in the nutrient medium at low doses could have similar results to chemical pinching, while at higher concentrations it inhibits shoot growth. CLINE (1996; 1997) proposed that dikegulac can act in a similar fashion to anti-auxins, such as tri-iodo benzoic acid (TIBA) which blocks auxin translocation and reduces the apical dominance in some plant species. Similarly, MENDOZA-DE GYVES et al. (2008) observed that dikegulac and zeatin stimulated *in vitro* shoot multiplication of the *Olea europaea* 'Canino', 'Moraiolo', and 'Frantoio'. However, in the above study the optimal dikegulac concentration was lower (74 μM) than the one observed in the present study for *Olea europaea* 'Chondrolia Chalkidikis' (100.5 μM), but in both studies a further increase of dikegulac did not stimulate additional shoot formation. However, dikegulac did not promote shoot proliferation of the *Olea europaea* 'Rosciola' and 'Piantone di Moiano' explants suggesting that the positive effect of dikegulac was cul-

tivar-dependent (MENDOZA-DE GYVES et al. 2008). The above discrepancies are not unexpected since using plant growth regulators to enhance branching has much potential but many inconsistencies, as well (LATIMER, FREEBORN 2010).

CONCLUSION

To sum up, including both 8.9 μM BA and 100.5 μM dikegulac in OM it resulted not only in enhancing shoot proliferation of *Olea europaea* 'Chondrolia Chalkidikis' microshoots, but also in producing new shoots of good quality, diminishing vitrification symptoms. However, it should be stressed that the obtained results should be considered as preliminary since the current study was performed only on one cultivar, while different reactions of various olive genotypes to dikegulac is common. The possible application of dikegulac, in practice, demands more detailed and complex studies.

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