In vitro multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™)

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Received: 26 February 2018 / Accepted: 9 July 2018 / Published online: 14 July 2018
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Abstract

Olea europaea L. cv Canino shoots were micropropagated to test two different culture systems: (1) on conventional semi-solid medium in glass jars and (2) in liquid culture in a Plantform™ bioreactor. The temporary immersion system, Plantform™, is a new propagation approach that uses liquid culture, where shoots undergo periodic immersion in liquid medium alternated with dry periods, avoiding gas accumulation through forced ventilation. This study proposes a protocol to improve in vitro propagation of olive reducing production costs. Our findings revealed that olive shoots propagated in Plantform™, with an immersion frequency of 8 min every 16 h and additional ventilation, showed good adaptability and better growth rates than those cultured in conventional system. Overall, the Plantform™ improves in vitro culture of ‘Canino’, showing higher proliferation, shoot length and better vigour of shoots. Moreover, the study found no significant differences in shoot length when 5 or 10 µM zeatin was applied in Plantform™ (3.04 and 3.13 cm, respectively); it is, therefore, possible to achieve efficient olive proliferation also with half hormone concentration. The positive performance of the bioreactor approach was also confirmed by Relative Growth Rate index. This is the first documented study of the Plantform™ technique for olive propagation.

Keywords Bioreactor · In vitro propagation · *Olea europaea* · Zeatin

Introduction

Olive is one of the oldest tree species in the Mediterranean basin. The olive tree has been propagated vegetatively for centuries from rooted suckers, ovuli, and later by grafting and cutting (Fabbri et al. 2004). The establishment and in vitro regeneration of olive has been attempted using several methods and media applications (Rugini 1984; Lambardi et al. 2013; Rugini et al. 2016). Many studies have explored use of the in vitro propagation method to obtain a greater number of selected plants due to its potential as an effective method for improving the quality and uniformity of plant material. In vitro culture of olive has been impaired by low and cultivar-dependent shoot proliferation rates, difficulty in the formation of adventitious roots and high losses of transplants (Briccoli-Bati et al. 1999). Moreover,

production costs for in vitro culture of olive are currently not competitive when compared to other traditional methods; in particular, with regard to the higher cost of the hormone zeatin, which is essential for optimum olive shoot growth (Lambardi and Rugini 2003).

A bioreactor can be an important tool in large-scale plant production in commercial micropropagation industry. Bioreactors are either automatic or semi-automatic, with easily manageable microenvironment conditions where cultures take appropriate amounts of nutrients from the culture medium (Etienne and Berthouly 2002; Paek et al. 2005). Bioreactors based on the Temporary Immersion System (TIS) are now available. The TIS is a semi-automatic device that involves intermittent and short-lasting contact of the explant with liquid culture medium. In addition to ensuring the proper supply of nutrients to the cultures, some TIS bioreactors can renew the atmosphere with a control gas condition (Georgiev et al. 2014).

A new type of bioreactor, Plantform™ (Fig. 1a), has been compared with existing alternatives and found to possess several advantages (Welander et al. 2014; Gatti et al. 2017). It is easy to handle, transparent, autoclavable and capable

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Fig. 1 In vitro olive proliferation: **a** shoots at the beginning of culture (left) in glass jar with semi-solid medium and (right) in the Plantform™ bioreactor (scale bar 3 cm); **b** shoots in Plantform™ with

5 μ M zeatin at the beginning of culture (scale bar 2.6 cm) and **c** after 28 days of culture (scale bar 3.6 cm)

to control gas exchange through the use of air pumps and a timer (<http://www.plantform.se/pub>). Forced ventilation leads to the complete renewal of the culture's atmosphere, which prevents the accumulation of carbon dioxide (CO₂) and ethylene that generally occur in a semi-solid culture and have a negative effect on morphogenesis (Roels et al. 2006).

Zeatin is important as a plant hormone to ensure a good rate of proliferation (Lambardi and Rugini 2003; Fabbri et al. 2004) and its high cost has always penalizing in vitro culture of olive. An appropriate management of temporary immersion system alongside gas exchange control could improve in vitro olive propagation, reducing the expense associated with zeatin.

'Canino' is a widespread olive cultivar in central Italy with high and constant productivity, and low susceptibility to the major diseases that affect olive. It is proven to be a highly

suitable material for in vitro proliferation and rooting and various studies of in vitro conditions of this cultivar have been performed (Rugini et al. 2016).

The aims of this research were: (a) to optimise a simple and effective method for mass in vitro propagation of *Olea europaea* shoots in the Plantform™ bioreactor, and (b) to investigate the possibility of reducing plant growth regulators to limit plant production costs, while maintaining high-quality olive shoots.

Materials and methods

Plant material and culture conditions

Shoot cultures of olive (*Olea europaea* L.) cv Canino were used in this study. The cultures were originally grown in 500-ml glass jars on a semi-solid olive medium (OM) (Rugini 1984), supplemented with 50 mg l⁻¹ Fe-EDDHA (Duchefa Biochemie, Haarlem, The Netherlands), 36 g l⁻¹ mannitol (Duchefa Biochemie), 3 g l⁻¹ Gelrite™ (Sigma–Aldrich, St. Louis, MO), and 10 µM of zeatin (Duchefa Biochemie) as the growth regulator (Lambardi et al. 2013). The pH of the media was adjusted to 5.8. Shoot cultures were kept at 23 °C, under a 16 h photoperiod with 60 µmol m⁻² s⁻¹ of photosynthetically active radiation provided by cool-white fluorescent lamps, and subcultured every 28 days. Two different culture systems were tested, one using a conventional semi-solid medium in glass jars and the other using the TIS Plantform™. In the semi-solid culture, 100 ml of OM medium was dosed into each glass jar, while in the TIS bioreactor 500 ml of liquid OM medium was used. The effect of various zeatin concentrations (2.5, 5 or 10 µM) was evaluated in both systems. Zeatin was filter-sterilized and added to autoclaved and cooled (40–50 °C) OM medium. A total of 50 explants were used per treatment: 10 shoots per glass jars and 50 shoots per Plantform™ (Fig. 1a, b). The experiment was repeated twice.

Cultures were kept for 28 days under the culture conditions described above, and for 28 and 56 days to evaluate the Relative Growth Rate (RGR) index. RGR index is based on the initial and final fresh weights of the plant material and the time of culture.

The immersion and aeration periods of the Plantform™ culture were controlled using air pumps and a timer placed in the climate chamber; the frequency of immersion was 8 min every 16 h (8 min/wet; 16 h/dry), with 15 min aeration every 4 h.

Data collection and statistical analysis

To evaluate shoot proliferation, the following data were collected after the subculture (28 days) in both semi-solid glass jars and the TIS: (1) number of new shoots formed from each shoot, and (2) shoot length (initially 1.3–1.5 cm long). In addition, each shoot's vigour was visually evaluated and scored on a scale of 1, strongly vitrified, necrotic and malformed; 2, less vitrified, necrotic, and malformed; 3, no vitrification, but with very poor vigour; 4, poor vigour; 5, average vigour; 6, good vigour; 7, very good vigour; 8, fully normal and healthy with excellent vigour

(Debnath 2009). A two-way ANOVA was performed for the “culture system” and “zeatin concentration” factors and their interaction; differences were tested using Tukey's test ($P \leq 0.05$) (SYSTAT 13, © Copyright 2009, Systat Software, Inc.).

The RGR index of shoot cultures was recorded after 28 days and then 56 days of culture, and calculated as $[\ln \text{FW}_{\text{final}} - \ln \text{FW}_{\text{initial}}] \times 100 / \text{days of culture}$ ($\ln$ = natural logarithm, Gatti et al. 2017).

Results and discussion

Both factors, culture system and zeatin concentration, influenced olive proliferation. Significant differences were observed between culture systems in each parameter when evaluated after 28 days (Table 1). Overall, olive shoots propagated in Plantform™ showed good adaptability (Fig. 1c) and a better growth rates than those cultured using the conventional approach ($P \leq 0.05$). Improved growth performance with increased zeatin concentration was also confirmed ($P \leq 0.05$).

Table 1 Effect of different culture systems and zeatin concentrations on shoot formation in *Olea europaea* L. cv Canino after 28 days culture

	Shoot length (cm)	No. shoots/ explant	Shoot vigour (scale: 1–8)
Culture system			
Semi-solid culture (SS)	2.41 b	0.36 b	5.0 b
TIS Plantform™ (T)	2.61 a	0.77 a	5.4 a
Significance	*	*	*
Zeatin concentration			
2.5 µM (Z1)	1.61 c	0.18 b	2.9 c
5.0 µM (Z2)	2.74 b	0.73 a	6.0 b
10.0 µM (Z3)	2.95 a	0.82 a	6.6 a
Significance	*	*	*
Culture system × zeatin concentration			
SS Z1	1.55 c	0.04	2.5
SS Z2	2.63 b	0.69	6.1
SS Z3	3.03 a	0.91	6.5
T Z1	1.69 c	0.46	2.3
T Z2	3.04 a	1.81	6.4
T Z3	3.13 a	1.93	6.9
Significance	*	NS	NS

Means with different letters within each column are significantly different

*Two-way ANOVA following by Tukey's test $P \leq 0.05$; NS not significant

Statistical analysis carried out on growth parameters showed that there was a significant interaction between factors on the shoot length. However, it should be noted that no significant differences in shoot length (3.04 and 3.13 cm) were induced by applying 5 or 10 μM concentrations of zeatin using the Plantform™ system (Table 1). A higher shoot number was obtained in the TIS compared to the semi-solid medium (1.93 vs 0.91, respectively) at an application of 10 μM of zeatin. Lambardi et al. (2006) similarly reported an increase in proliferation rate (30%) compared to the control in shoots of ‘Canino’ using the RITA® system, with immersion frequency and zeatin concentration analogous at our study.

Shoots developed in the Plantform™ showed higher multiplication rates, although at a zeatin concentration at 2.5 μM the shoot cultures showed slight browning. This symptom of shoot decay was not present in the glass jars with the same zeatin concentration, while low proliferation (0.04 shoots per explant) was recorded.

Both Grigoriadou et al. (2005) and Lambardi et al. (2006) reported the effectiveness of the TIS system in olive proliferation. However, they dealt with high zeatin concentrations (10 and 20 μM), while this study showed that using 5 μM zeatin in TIS produces similar results to those obtained with 10 μM in a semi-solid medium. This effect suggests that olive culture in TIS can produce optimal number of shoots at reduced levels of zeatin, and it is, therefore, possible to achieve efficient olive proliferation with lower production costs.

Growth analysis is an analytical tool used for characterising plant growth. Usually, it is a destructive analysis required to determine plant dry weight, while RGR index can be calculated, without loss of vegetal material, from the same sample at two points time. In this study RGR index, evaluated after 28 days, confirmed the improved growth culture in the TIS system with respect to the semi-solid medium (Table 2).

Table 2 Relative Growth Rate (RGR) index of *Olea europaea* L. cv Canino shoots after 28 and 56 days of different culture systems

Zeatin concentration	RGR ^a	
	28 days culture	56 days culture
Semi-solid culture		
2.5 μM	1.59	1.87
5.0 μM	3.43	2.93
10.0 μM	3.72	3.89
TIS Plantform™		
2.5 μM	3.84	2.25
5.0 μM	4.40	4.55
10.0 μM	4.46	4.60

^aRGR index of cultures at the multiplication phase: $[\ln \text{FW final} - \ln \text{FW initial}] \times 100/\text{days of culture}$

In the Plantform™ system, similar RGR was recorded in the presence of 5 μM (4.40) and 10 μM (4.46) of zeatin, as observed in our preliminary study (Benelli et al. 2015); it is noteworthy that the same performance in RGR was also recorded after 56 days of continuous culture (i.e. without subculture). Indeed, the RGR of shoot cultures in TIS containing 5 μM and 10 μM were 4.55 and 4.60, respectively. On the contrary, the difference in RGR values between zeatin concentrations tended to be wider in the semi-solid medium (2.93 vs 3.89) after 56 days (Table 2).

These results clearly show that olive proliferation was improved in the Plantform™ system, this response may be due to the reduction of strong apical dominance that is present in olive cultures and acts to constrain the development of efficient in vitro clonal propagation (George and Sherrington 1984). According to Lambardi et al. (2006), the horizontal position of the olive shoot developed in the TIS can improve performance in terms of shoot number and nodes produced per microcutting in comparison to vertical shoots developed in the semi-solid medium.

Bioreactor technology has been introduced for mass propagation of different plants. Some species are sensitive and often vitrify after a long period of submersion in liquid culture (Ziv 2005; Debnath 2011). For this reason, optimised temporary immersion cycles are important to avoid problems such as hyperhydricity. In the current study, the frequency of immersion was every 16 h, similar to that applied by Lambardi et al. (2006) using the RITA® system, but broader than the immersion period utilised by Grigoriadou et al. (2005) in their TIS device designed in the Vitro Hellas S.A. laboratory. The wet phase, 8 min every 16 h, using an optimal zeatin concentration guaranteed better vigour and quality while preventing shoot hyperhydricity.

The environment inside glass jars or bioreactors without forced ventilation is characterised by high relative humidity, poor gas exchange, and the accumulation of gases detrimental to growth (Georgiev et al. 2014). During the culture, CO_2 gradually accumulates in the vessel’s headspace, especially when tightly closed containers are used (Benelli et al. 2002). Improved gas exchange under forced ventilation reduces the accumulation of gases and relative humidity in the culture containers, and also minimises the difference between the gaseous environment in vitro and ex vitro (Roels et al. 2006).

This is the first report of olive shoot multiplication using the Plantform™ bioreactor. This TIS bioreactor mitigates the problem of hyperhydricity during culture and has many advantages for micropropagation, including automating and simplifying procedures (Georgiev et al. 2014) to reduce the costs of plantlet production. In particular, it allows to change the liquid medium without transferring the plants, therefore, reducing manual labour, nutrients and growth regulators are more available to the cultures thus decreasing the amount used and gelling agents are not required.

The Plantform™ bioreactor improves the in vitro culture of cv Canino, inducing higher proliferation, increasing shoot length and enhancing the vigour of shoots in comparison to the semi-solid culture.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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