https://doi.org/10.17221/69/2020-HORTSCI

Evaluation of ElecTIS bioreactor for the micropropagation of *Malus sylvestris* (L.) Mill., an important autochthonous species of Albania

Valbona Sota[1], Carla Benelli[2], Brunilda Çuko[1], Elektra Papakosta[3], Claudio Depaoli[4], Maurizio Lambardi[2], Efigjeni Kongjika[5]*

[1]*Department of Biotechnology, Faculty of Natural Sciences, Tirana, Albania*
[2]*Institute of BioEconomy, National Research Council (CNR/IBE), Sesto Fiorentino (Florence), Italy*
[3]*Center of Agricultural Technologies Transfer, Vlora, Albania*
[4]*Laboratory of Micropropagation Predaia (Trento), Italy*
[5]*Section of Natural and Technical Sciences, Albanian Academy of Sciences, Tirana, Albania*

**Corresponding author: carla.benelli@ibe.cnr.it*

Citation: Sota V., Benelli C., Çuko B., Papakosta E., Depaoli C., Lambardi M., Kongjika E. (2021): Evaluation of ElecTIS bioreactor for the micropropagation of *Malus sylvestris* (L.) Mill., an important autochthonous species of Albania. Hort. Sci. (Prague), 48: 12–21

Abstract: *Malus sylvestris* (L.) Mill., an economically-important fruit tree, is native to Albania and in many parts of Europe. It is cultivated as an ornamental tree, while its fruits are collected for food and a source of antioxidant substances. It is included in The IUCN Red List of Threatened Species. For these reasons, it is very important to optimise a micropropagation protocol, in order to obtain great numbers of clonal plantlets for *ex situ* conservation and production purposes. A liquid culture in a temporary immersion system (TIS) is a recently-proposed system for large-scale *in vitro* plant propagation. In this study, lateral buds of *M. sylvestris* were inoculated in MS medium with BAP (1 mg/L) and NAA (0.1 mg/L). In order to avoid oxidative stress, different antioxidants were previously tested with the culture in a gelled medium, and the combination of ascorbic acid and citric acid (both at 100 mg/L) was selected for the following culture in TIS. Stabilised explants were then cultivated in ElecTIS, an innovative TIS bioreactor, and in a semisolid medium, after which the two culture systems were evaluated. Overall, the ElecTIS showed to be more effective for all the tested parameters.

Keywords: apple; *in vitro* propagation; liquid culture; oxidative stress; temporary immersion system; TIS

Malus sylvestris (L.) Mill., an economically important fruit tree, is native to Albania, and it is also present in various parts of Europe (GRIN 2010). It is a primary wild relative and potential gene donor for the domesticated apple, *Malus domestica* Borkh. It is also widely cultivated as an ornamental tree, collected from the wild for food for humans and farmed animals and it is a source of antioxidant substances for use in pharmaceutics and cosmetics (Stojiljković et al. 2016). *M. sylvestris* is included in The IUCN Red List of Threatened Species (IUCN 2019), is considered a *DD species* (data deficient), and there are no reports about its *ex situ* conservation. The hybridisation with *M. domestica*, the com-

Supported by NASRI, Republic of Albania, and CNR,- Italy as part of a bilateral project between Academy of Sciences of Albania and Institute of BioEconomy, CNR, Italy. Contribution by Carla Benelli and Maurizio Lambardi as part of the "International Treaty on Plant Genetic Resources for Food and Agriculture – RGV-FAO, 2020–2022".

https://doi.org/10.17221/69/2020-HORTSCI

petition with invasive species, and the ecosystem stress and degradation are among the main threats worthy of mention.

In these conditions, it is of strategic importance to develop an effective micropropagation protocol in order to obtain large numbers of clonal plantlets, which can be used for *ex situ* conservation strategies or other purposes. Indeed, micropropagation has recently become an important technique in the production of high-quality plants, particularly for vegetatively-propagated species. Satisfactory regeneration rates have been achieved for a wide number of species, and the technique is, today, considered particularly valuable for rapid clonal multiplication (Kongjika et al. 2002; Damiano et al. 2008; Lambardi et al. 2013). Notwithstanding, the micropropagation of some species still shows problems associated with its commercial application, like the exudation of phenolic compounds, and the consequent loss of the explants due to the absorption and oxidation of these substances (Thorpe, Harry 1997; Grazhdani et al. 2014). Other problems connected to the traditional culture in a semisolid medium include the hyperhydricity of the plantlets and the high labour costs in commercial micropropagation, making it indispensable to find alternative culturing techniques reducing the incidence of such drawbacks.

The large-scale cultivation of differentiated plant cultures using a liquid culture in 'Temporary Immersion Systems' (TIS), under controlled environmental conditions, recently received a rapid increase in interest worldwide (Mehrotra et al. 2007; Carvalho et al. 2019). Over time, different TIS bioreactors have been proposed (Georgiev et al. 2014; Ramírez-Mosqueda, Iglesias-Andreu 2016), among which ones based on a single container (e.g., RITA®, Plantform™), or on double containers (e.g., twin flasks, SETIS™) can be found. These bioreactors allow the plant cultures to have temporary access to the liquid medium, with other advantages regarding the nutrient absorption from the entire surface of the explant and the dilution of the phenols that can determine the oxidation and browning of the tissues (Lambardi 2012; Capuana et al. 2018). Although the experimentation that TIS bioreactors have undergone is still rather limited and has often been developed by the manufacturing companies themselves, TIS bioreactors are, today, considered a promising alternative to the traditional culture system in a gelled media. Moreover, in conventional micropropagation, the common glass or plastic vessels contain a restricted number of plants per unit, require the intense manipulation of the cultures, and involve a large amount of work for subculturing. On the contrary, the tests conducted in some research laboratories have demonstrated the effectiveness of the TIS system in promoting the proliferation phase, in terms of both quantity of the material produced, and quality of the shoots to be destined for the subsequent phases of the micropropagation cycle. TIS bioreactors use large containers with, in some devices, a very wide space for plants, allowing an increase in the subculture periods and significantly reducing the work demand under laminar flow hoods as a consequence (Takayama, Akita 1994; Lambardi 2012).

In common TIS bioreactors, the liquid substrate is the "moving" element, which occurs when the forced air is blown from outside to inside the containers through gas filters. This turns out to be a weak point of these systems which often meet contamination frequencies higher than traditional cultures in a gelled substrate.

A prototype single-container TIS bioreactor, named ElecTIS, has recently been developed and patented (European Patent No. 2617282; www.explanta.com/bioreactor-electis/). In the ElecTIS, the forced insufflation of air inside is eliminated, making the cultivation of explants mobile and stationary in the liquid substrate. Indeed, a pneumatic system allows the ascending and descending cycling movement of a plastic basket, containing the shoot culture, and makes the periodic contact with the liquid substrate, positioned at the base of the container, possible. This new TIS bioreactor has many advantages, like the simplification of the handling operations, the consistent reduction of contamination risks due to the forced insufflation of external air in the culture container, higher multiplication rates in comparison to traditional cultures in a semi-solid medium.

The aim of the present study was to develop an efficient micropropagation protocol for *Malus sylvestris*, in order to enhance the plant production and prevent oxidative stress by using an ElecTIS bioreactor. A comparison with the culture in a gelled semi-solid medium is also reported. During the processing of the two culture systems (in the gelled medium and in TIS), the amount of time and labour were recorded. Using these

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data, the general estimation of the time and labour were calculated and used to compare the relative efficiency of the two processes, with the final aim to determine which method would be best to use for the large-scale micropropagation of *Malus sylvestris*.

MATERIAL AND METHODS

Plant material collection and explant decontamination. Axillary buds of wild apple (*M. sylvestris* (L.) Mill.) were excised from scions of the population in Maminas, Durres county in western Albania at the beginning of April 2018 and used as the initial explants. The explants were disinfected with 70% ethanol for 3 min, followed by the treatments with 0.2% of 50% carbendazim (Bavistine) for 7 min and 0.01% of $HgCl_2$ for 10 minutes. Finally, the explants were rinsed three times with sterile distilled water.

***In vitro* stabilisation stage of shoot cultures.** All the explants were cultivated in agarized MS nutrient medium (Murashige, Skoog 1962), added with 3% sucrose, 1 mg/L 6-benzylaminopurine (BAP), 0.1 mg/α-naphthaleneacetic acid (NAA) and 0.7% agar (Sigma-Aldrich, USA). The pH was adjusted to 5.7 before autoclaving the medium at 120°C for 20 minutes. At this stage, small glass tubes (ST), 30 mL in total volume, were used as culture vessels.

In order to control excess browning, the explants were kept for 48 h in darkness and transferred twice into fresh medium. Afterwards, 3 different antioxidant combinations were compared in the MS medium [ascorbic acid (200 mg/L); citric acid (200 mg/L); ascorbic acid (100 mg/L) plus citric acid (100 mg/L)], in combination with 3 treatment times (5, 24, and 48 hours).

ElecTIS description. This bioreactor consists of one large plastic container (2 000 cc), with a cover and a basket entering in the inner space (Figure 1A). The basket has two pierced parts where two bellow pistons are connected (Figure 1B). The basket is prepared with the shoot culture inside and is connected to the cover, before being inserted in the container (Figure 1C). The cover is then hermetically connected to the container. The retraction and expansion of the pistons is allowed by a connection with an aspirant pump, and results in the up and down movement of the basket. The air flow only occurs inside the pistons, while no air is pressed inside

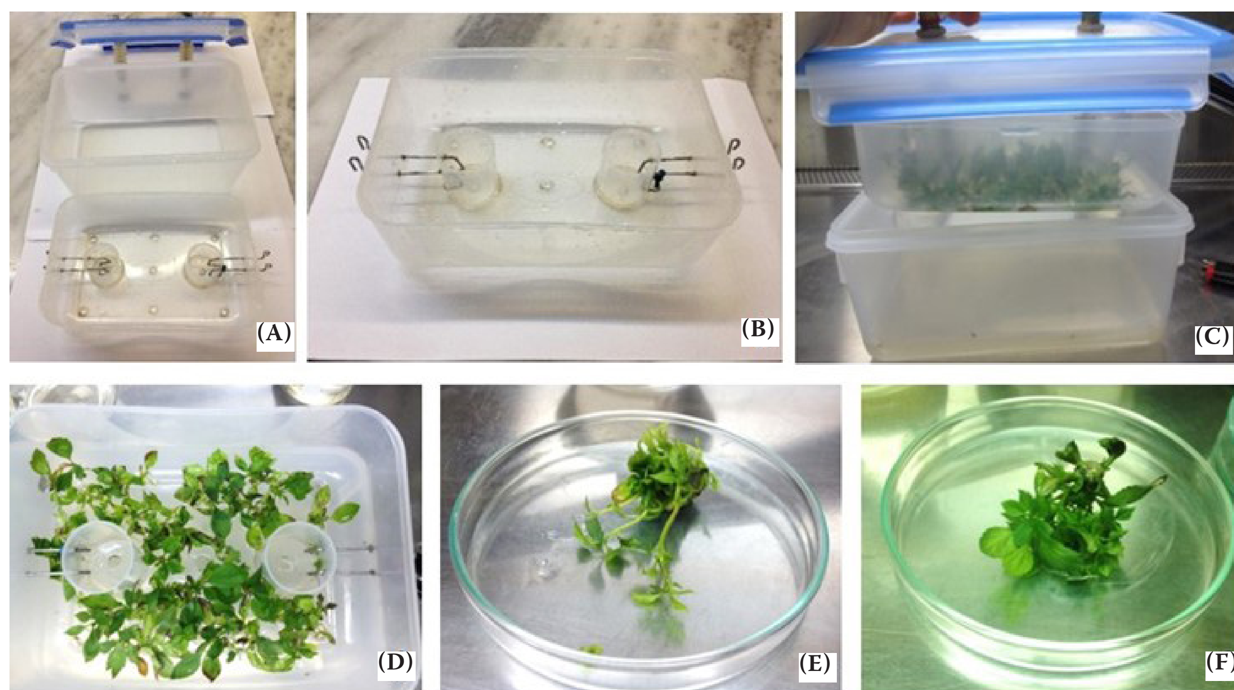

Figure 1. ElecTIS components and assembly. (A) the components of the ElecTIS: cover (top), box (middle) and basket (bottom); (B) the basket, with the cylinders where the pistons are allocated; (C) the basket, with the shoot culture inside, before the closure of the ElecTIS; (D) no sign of oxidation was observed in the shoots cultured in the TIS in the ElecTIS system; (E) a shoot cluster grown in a semisolid medium, showing long shoots and leaves with small surfaces; (F) a shoot cluster grown in the ElecTIS, showing short shoots and leaves with larger surfaces

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the container. 250 mL of a liquid nutrient medium is poured into the base of the container and the shoots are placed into the basket. The basket with shoots is immersed in the nutrient medium when the two pistons are expanded, while it is suspended in the container when the pistons retract. The immersion cycle of the shoots is controlled by an electronic timer. All the ElecTIS parts are made by a transparent, heat resistant polypropylene material and can be easily autoclaved.

Liquid and gelled medium for shoot micropropagation. The above-described MS medium was tested for micropropagation (1) as a liquid medium, using the ElecTIS bioreactor, and (2) as a gelled semi-solid medium, with the addition of 0.7% agar. In both cases, the pH of the medium was adjusted to 5.7 before autoclaving.

In the ElecTIS, the shoots (2–3 cm of length) were measured, the fresh weight of all the shoots (30 per container) was recorded before inserting them horizontally into the basket. Three immersion frequencies (immersion period/dry period) were tested in the ElecTIS: (i) 4 min/6 h (with a total of 16 min every 24 h); (ii) 4 min/8 h (12 min/24 h); (iii) 8 min/12 h (16 min/24 hours).

For the culture in the gelled medium, 3 types of culture vessels were used (each of them closed with non-hydrophilic cotton): 1) small glass tube (ST), 30 mL total volume and one shoot per tube; 2) big glass tube (BT), 100 mL total volume, with one shoot per BT; 3) Erlenmeyer flask (EF), 100 mL total volume and 5 shoots per EF.

The amount of MS medium and shoots in each container, the consequent amount of nutrient medium per shoot, and the total number of replications for each container type are reported in Table 1. The procedure for the preparation of all the culture systems' liquid was performed under an aseptic condition of a laminar air-flow cabinet. All the containers were maintained in a climatic chamber, at the temperature of 23 ± 2 °C, a photoperiod of 16 h and a light intensity of 43.4 μmol/m^2·s.

Data collection and elaboration. As for the oxidative stress, the percentages of explants showing evident signs of oxidation were recorded. The statistical analysis of the percentages was carried out with the non-parametric χ_2 test for pairwise comparisons for each treatment time ($P < 0.05$). As for the culture trials, the total fresh weight of the explants was recorded at the moment of inoculation and after the period of 28 days of culture.

The relative growth rate (RGR) index of the shoot cultures was recorded after 28 days and calculated as: [ln FW final – ln FW initial] × 100 /days of culture, where ln is the natural logarithm, and FW is the fresh weight (Gatti et al. 2017).

Finally, in order to compare the efficacy of the two propagation systems (the micropropagation in the gelled semi-solid medium and in the ElecTIS bioreactor) the following parameters were recorded: (1) the time required for the preparation of the nutrient medium, (2) the time required for the medium distribution in the culture vessels, (3) the time required for the explant inoculation, and (4) the time required to clean the different culture containers.

RESULTS AND DISCUSSIONS

Explant disinfection and avoidance of polyphenolic oxidation

Regarding the contamination rates, the data collected from the experiment with the wild apple *Malus sylvestris* buds, isolated at the beginning of April, showed that the sterilisation protocol was effective with a high percentage of non-contaminated explants (97%). According to some authors, apple *in vitro* cultures are better established from explants collected from stock plants in the active growth phase (Teixeira da Silva, Dobránszki 2013). Hence, the success of establishment of the cultures is highly dependent on the time of the collection, spring often being the ideal

Table 1. Culture parameters for the different tested containers [ST, small glass tube (30 ml); BT, big glass tube (100 ml); EF, Erlenmeyer flask (100 ml)]

Container	Medium	Medium (ml per container)	No. of shoots (per container)	No. of containers (per 30 shoots)	Amount of medium (ml per 30 shoots)
ElecTIS	liquid	250	30	1	250
ST	semisolid	15	1	30	450
BT	semisolid	30	1	30	900
EF	semisolid	45	5	6	270

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Table 2. Time required for the main steps of the culture, using the different containers (the calculations do not include the sterilisation time in an autoclave, as it was invariable in all the culture systems)

	Time required for medium preparation (500 ml)	Time required for medium distribution in the containers	Time required for explant inoculation	Time required for cleaning the containers
ElecTIS	5–7 min	1 min (1 box)	3–4 min	5 min
ST	15 min (agar melting)	10 min (30 vessels)	20 min	20 min (agar removal)
BT	15 min (agar melting)	10 min (30 vessels)	20 min	20 min (agar removal)
EF	15 min (agar melting)	5 min (6 vessels)	20 min	20 min (agar removal)

season to establish *in vitro* cultures with minimum contamination (Hutchinson 1984; Modgil et al. 1999).

The accumulation of polyphenols represents a problem for both the culture stabilisation and the multiplication phase. The explants of the wild apple, cultured in the MS medium without the addition of antioxidants, exhibited evident browning at the cut ends just 2–3 h after their *in vitro* inoculation. In this study, the explants' stabilisation on the MS medium added with the ascorbic acid and citric acid (at 200 mg/L), alone or in combination (at 100 mg/L each), and maintenance for 48 h in darkness, was effective in reducing the oxidative stress of the explants (Figure 2), although a limited browning of the basal part of the shoots and of the surrounding medium continued for a long time, sometimes being evident even after culturing for 28 days. The explants cultured on the MS medium supplemented with a combination of 100 mg/L of ascorbic acid and 100 mg/L of citric acid were significantly less affected by the oxidation stress after each detection time. After 48 h of culture, only 36% of the explants released exudates into the medium, in comparison to the explants cultured in the MS media containing either 200 mg/L citric acid, or 200 mg/L ascorbic acid (59% and 61%, respectively).

Many experiments on the micropropagation of different apple accessions show the browning of the culture, due to the exudation of polyphenols, to be one of the major bottlenecks for the establishment

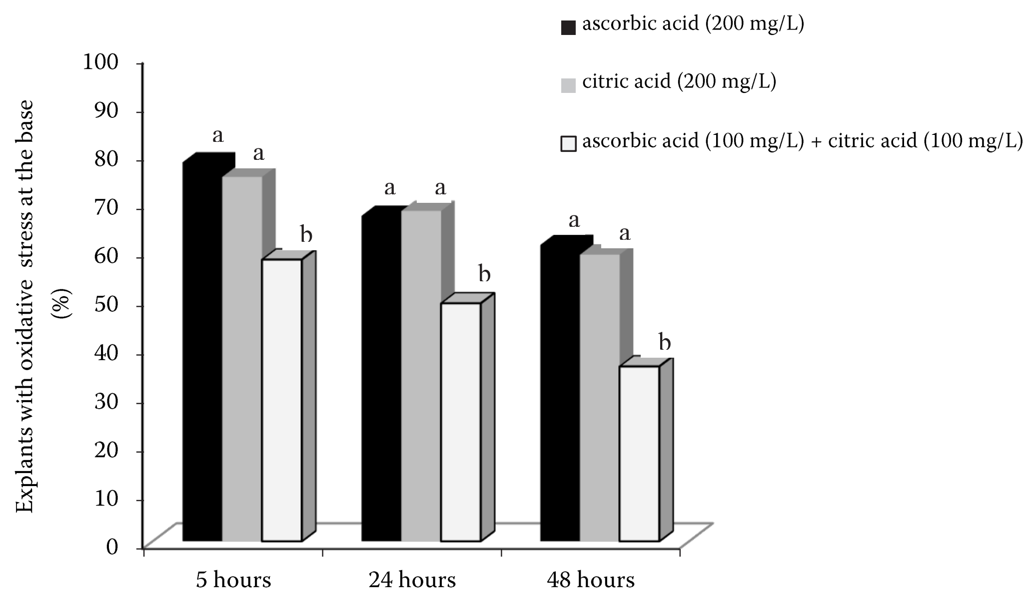

Figure 2. Percentage of *M. sylvestris* explants showing oxidative stress of the shoots cultured in a semisolid MS medium added at different concentrations and antioxidant combinations. For each detection time, the bars with the different superscripts are significantly different at the $\chi 2$ test for the pairwise comparison ($P < 0.05$)

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of aseptic cultures (Jafarkhani Kermani et al. 2009; Boudabous et al. 2010; Volz, McGhie 2011), together with the microbial contamination. The growth of the explants is strongly inhibited by the accumulation of toxic substances in the medium near the cut ends of the explants, and this problem is substantial on a semisolid medium culture. According to other authors, the most critical phase is between the first 24–48 h in the *in vitro* culture conditions. (Laimer da Câmara Machado et al. 1991). To counteract the problem, several antioxidants have been used for the initiation of apple cultures. Similar to this study, other authors (e.g., Wang et al. 1994; Modgil et al. 1999; Kereša et al. 2012) applied different antioxidant concentrations of ascorbic acid, citric acid, and cysteine, either alone or in combination or with other adsorbing agents, for the initiation of apple cultivars' cultures.

Comparison of shoots multiplication in ElecTIS bioreactor and in semisolid medium

Efficacy in implementation and usage. For this first study, different aspects of the ElecTIS implementation were analysed. The ElecTIS was easy to use both for its sterilisation (the bioreactor was autoclaved in the normal steam-sterilisation mode for 20 min at 121°), and in the preparation and pouring of the liquid nutrient medium. The ElecTIS bioreactor required 250 mL of the liquid nutrient medium for the growth of 30 shoots. This amount was similar to the quantity used in the EF for the same shoot number, while for the other test tubes, a higher amount was necessary, i.e., 1.8 times higher for the ST and 3.6 times higher for the BT (Table 1).

The use of larger amounts of both the nutrient medium and culture vessels increases the cost of production per plant, due to the higher consumption of chemical reagents and electricity for the sterilisation step. Furthermore, the addition of 0.7% agar increases the cost of the micropropagation in the semisolid medium. It is important to underline that the use of agar for the jellification is one of the important cost items in the traditional micropropagation process. Hence, a modern strategy in improving the *in vitro* commercial propagation includes the reduction or even the total elimination of the use of agar, in order to break down the final production costs.

Comparison of time and labour required by the different culture systems. The amount of time and labour necessary to carry out the two different systems were recorded during the culture process. Table 2 shows the calculation of the time required for the main steps of the procedure using the liquid medium in the ElecTIS or the semisolid medium in the 3 culture vessels. In the ElecTIS, the medium preparation and its distribution in the container, and the inoculation of the shoots in the basket (after the measure of the shoot weight) required 8–12 min, including the assembly of all the pieces of ElecTIS in the laminar air-flow cabinet. The time is a very important parameter during the inoculation stage because it reduces the level of contamination and the cost of the manual labour. In the semisolid medium system, comparing the number of vessels necessary to culture the same quantity of shoots of one ElecTIS bioreactor, the time required for the same main steps ranged from a minimum of about 40 min (with the EF) and a maximum of 45 min (with both the ST and BT); the longer time required was due to the time needed to melt the agar and to distribute the shoots in the culture vessels. On the other hand, it should be underlined that when working with the ElecTIS bioreactor, the quality control of the starting material is fundamental, as even a single contaminated shoot can cause the contamination and consequent loss of a high quantity of material cultured in a single container.

Another important aspect is the cleaning process because, using the glass vessels, the agar should be removed from the containers and thrown away before the subsequent washing process. Considering the number of culture vessels equivalent to one ElecTIS, in terms of shoots, this time resulted in about 20 min, independently by the type of container (ST, BT or EF). Working with the ElecTIS, the cleaning step consists of removing the liquid medium and washing the pieces under tap water, and only requires about 5 min. Hence, from this study, it was evident that the use of the ElecTIS is a time-effective procedure in comparison to the traditional micropropagation in a semisolid medium.

The liquid culture is considered an important step towards the better automation of the commercial micropropagation process, as well as a system which allows one to reduce the costs of production (Firoozabady, Gutterson 2003; Be, Debergh 2006; Pence 2011). In literature, few studies dealing with the micropropagation of *Malus* spp. in a liquid culture are available. Mehta et al. (2014) observed an 8-fold reduction in the costs for the production

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Table 3. Initial and final weight and relative RGR index of the shoots grown in the different types of containers, calculated after 28 days of culture [ST, small glass tube (30 ml); BT, big glass tube (100 ml); EF, Erlenmeyer flask (100 ml)]

Container type	Initial weight (g)	Final weight (g)	RGR index	Immersion frequency
ElecTIS	1.38	36.02	11.61	4 min/6 h = 16 min/24 h
ElecTIS	1.40	9.04	6.58	4 min/8 h = 12 min/24 h
ElecTIS	1.10	7.12	6.67	8 min/12 h = 16 min/24 h
ST	1.15	7.95	6.90	–
BT	1.50	18.10	8.89	–
EF	1.01	13.50	9.25	–

of apple rootstocks (MM106 and B9) using nodal segments cultured in 250-cc Erlenmeyer flasks with a liquid medium. Moreover, the shoot growth and multiplication were higher after 30 days, with respect to the culture in a semisolid medium, and the plantlet survival rate was also higher after the transfer in the field. The liquid culture also significantly reduced the cultivation costs of the M9 apple rootstock, together with a high shoot proliferation and the production of well rooted plantlets, which could be efficiently transferred to the field (Bhatt et al. 2012).

Oxidative stress and shoot morphology after the culture in different containers. Different methodologies have been proposed over time to overcome the phenolic oxidation affecting the *in vitro* propagation of some fruit species and, among them, Pierik (1987) suggested the use of a liquid medium to promote the easy and quick dilution of toxic compounds. Ziv (2005) stated that the one main advantage of liquid culture is that it allows the dispersion of harmful phenolic exudates into the medium. Indeed, in this study, differently from what was observed with the culture in semisolid medium, the culture in TIS using the ElecTIS system produced no phenolic oxidation, and the shoots proliferated without any oxidative symptoms (Figure 1D).

In all four containers, the leaves of the shoots maintained their typical shape and colour. However, some morphological differences in the size of the shoots and leaves could be observed. The shoots cultured in ST, BT and EF on the semisolid medium had longer shoots and leaves with smaller surfaces (Figure 1E), while the liquid cultured in the TIS in the ElecTIS produced shorter shoots and leaves with larger surfaces (Figure 1F). Cytokinins are fundamental for the proliferation of apple shoots (Dobranszki, Teixeira da Silva 2010). In the present study, the same kind and concentration of BAP was used in all the culture systems. However, in the ElecTIS bioreactor, the shoots are periodically fully immersed in the liquid medium, so the contact surface of the material with the nutritive medium and the included BAP is greater, and the liquid can diffuse inside the tissues not only from the shoot base, as it does in the culture in the semisolid medium, but it can occur from the whole shoot surface.

This effect, together with the larger available space, helped to create a larger leaf surface in the wild apple shoots, an important factor for speeding up and increasing the photosynthetic rate of plantlets during the subsequent acclimatisation stage. This observation is in accordance to other reports (Dey 2005; Vyas et al. 2008; Mbiyu et al. 2012) which evidenced that, in various species, the culture with floating explants facilitates the nutrient uptake by the explants of several species. It is also noteworthy that the *M. sylvestris* shoots cultured in the ElecTIS bioreactor do not show any sign of hyperhydricity.

Effect on shoot proliferation of the different culture systems

Table 3 shows that, after 28 days of culture, the RGR calculated with the shoots grown in the semisolid medium ranged between a minimum of 6.90 (in the ST) and a maximum of 9.25 (EF). The higher RGR detected in the shoots grown in the Erlenmeyer flasks was probably due to the higher microenvironment volume which allows a lower gas accumulation, in comparison to the ST and BT. When using the ElecTIS bioreactor, the great importance of selecting the best immersion cycle was proven. Indeed, the cycle based on 4 min of immersion every 6 h (with a total 16 min every 24 h) determined the best vigour and quality of the cultures, preventing any shoot hyperhydricity. The RGR index after 28 days of culture was 11.61. The other immersion frequencies tested

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resulted in lower RGR indexes. In comparison, although the immersion cycle of 8 min every 12 h produced the same daily immersion (16 min), was not likewise effective, producing an RGR of 6.67. With the ElecTIS, the minimum RGR (6.58) was detected with the immersion cycle of 4 min every 12 min (corresponding to 12 min every 24 h), which resulted in inferior RGR, also in comparison to all the cultures in a semisolid medium.

In the M6 apple rootstock cultured in RITA bioreactor, for the shoot proliferation phase, a better immersion frequency of 16 times per day with a 2 min immersion time, in combination with a lower BAP concentration (4.4 μM) was assessed (Zhu et al. 2005). These authors also evidenced that a decrease in the immersion frequency, combined with an increase in the BAP concentration in the liquid medium, was accompanied by an increase in the shoot hyperhydricity. With the best immersion cycle, the multiplication rate in the RITA system was higher than the culture one on a gelled media. Nodal segments of the M9 EMLA apple rootstock were tested by Chakrabarty et al. (2003) in a liquid culture, comparing the permanent and the temporary immersion. Also, in this study, the frequency of the immersion was a decisive parameter to achieve the best results: too long and too frequent immersions induced the shoots' hyperhydricity. Moreover, the shoot hyperhydricity was lower in the TIS in comparison to the liquid culture in a permanent immersion. Working with the ElecTIS bioreactor, the present study confirmed the importance of the optimisation of the immersion cycle (length and frequency) when the liquid culture in the TIS is used, in order to achieve a high RGR index, with a large biomass production of wild apple plantlets with good morphological and physiological characteristics. Several other reports have demonstrated that the immersion time is a fundamental factor affecting the efficacy of the liquid culture in the TIS, since it influences the nutrient uptake, the control of the hyperhydricity, the RGR index and, in general, the quality of the shoot subcultures (Etienne, Berthouly 2002; Alabarrán et al. 2005; Gatica-Arias et al. 2008; Lyam et al. 2012; Venutolo, Aguilar 2015; Benelli, De Carlo 2018).

CONCLUSION

The present study demonstrated that, working with *M. sylvestris*, the ElecTIS is an optimal bioreactor to achieve all the advantages of a liquid culture in a TIS, among which the availability and uptake of the mineral and organic nutrients, and the dilution of the inhibiting compounds, mainly phenolic exudates, should be considered strategic. When comparing the shoot proliferation in the ElecTIS and in a semisolid medium, all the observed parameters evidenced that the micropropagation in the ElecTIS were more effective in terms of the implementation of the system, the time and costs required, the oxidative stress prevention, the morphological aspects of the shoot culture, and the achieved RGR index. In summary, the ElecTIS bioreactor is a promising device to reduce the production costs in a large scale commercial micropropagation plan.

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Received: May 2, 2020
Accepted: October 6, 2020