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Use of Liquid Culture with the ElecTIS Bioreactor for Faster Recovery of Blackberry (*Rubus fruticosus* L.) Shoots from Conservation at 4 °C

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Abstract: ElecTIS is a new single container bioreactor which does not require forced air blowing, instead making the culture material mobile and the liquid medium stationary. The timed up-and-down movement of the basket containing the shoot culture ensures periodic contact with the liquid medium positioned at the base of the container. In this study we tested for the first time its use in the recovery of blackberry shoot cultures (*Rubus fruticosus* L., cvs Thornfree and Chester), coming from 5 months of slow growth storage (SGS), at 4 °C and in the dark. The shoot recovery at standard culture conditions was performed on two different types of ElecTIS, i.e., one with a smaller basket ($ElecTIS_S$, 234 cm^2 of culture area), and one with a large basket ($ElecTIS_L$, 336 cm^2), comparing the culture in TIS (cycle of 8 min every 6 h, equal to 32 min/day) with the traditional one in a gelled medium in glass jars (500 cc). After each one of the three 4-week subcultures, the shoot growth parameters and the relative growth rate highlighted a clear superiority of ElecTIS in promoting the recovery of shoot cultures coming from SGS. The analyses of chlorophyll content and stoma functionality confirmed the superior quality of shoots cultured in the ElecTIS bioreactor, and these shoots were afterwards easily rooted and acclimatized ex vitro.

Keywords: bioreactor; blackberry; ElecTIS; liquid culture; *Rubus fruticosus*; slow growth storage; temporary immersion system

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1. Introduction

Commercial micropropagation in fruit species has always developed and concentrated on the use of gelled culture substrates, i.e., substrates containing a gelling substance as an aggregator of the nutrients and growth regulators necessary to support the growth and morphogenetic processes of the cultured shoots. This approach to in vitro propagation has its main weak points in the low proliferation rates and in the use of containers of limited dimensions which consequently make necessary frequent subculturing. It follows that an intensive use of hand labour in the cycle affects production costs of micropropagated plants by over 50% [1]. For this reason, for over 40 years attempts have been made to develop liquid culture systems in bioreactors with the aim of developing in vitro mass propagation in large containers that would allow a high degree of automation [2,3]. A more recent approach to liquid culture, based on the use of bioreactors that allow 'Temporary Immersion Systems' (TIS) under controlled environmental conditions, is receiving growing interest worldwide [4]. Indeed, in comparison with bioreactors for static liquid culture, the TIS culture allows temporary contact between the plants and the nutritive solutions and provides adequate oxygen transfer to the culture. Furthermore, TIS avoids the continuous

immersion and rubbing of the shoot clusters, which has been proven to be detrimental when working with fruit species, affecting their in vitro growth and morphogenesis [5]. Several TIS bioreactors have been developed over time [6,7], based on single containers (RITA®, Plantform™) or double containers (twin flasks, SETIS™). TIS bioreactors enable plant cultures to temporarily access the liquid medium, with additional benefits for nutritional absorption from the full surface of the explant and the dilution of phenols that may cause tissue oxidation and browning [8,9].

Although TIS bioreactors have so far undergone a limited number of trials, frequently carried out by the manufacturing companies themselves, they are now seen in commercial micropropagation laboratories as a promising alternative to the conventional culture in gelled media. In the traditional micropropagation, the common glass jars and plastic boxes only hold a small number of shoots per unit, require intensive culture manipulation and demand a significant amount of work for subculturing. On the contrary, TIS bioreactors contain a greater amount of material, while handling and work for subculturing is greatly reduced. Moreover, experiments carried out in research labs have shown how well the TIS system promotes the proliferation phase, in terms of both the quantity of material produced and the quality of the shoots destined for the following stages of the micropropagation cycle [8,10]. In common TIS bioreactors, the liquid substrate is the "moving" element which occurs when the forced air is blown from the outside into the containers through gas filters. This turns out to be one of the system weaknesses, since these bioreactors often encounter higher contamination frequencies than conventional cultures in a gelled substrate. A single-container TIS bioreactor prototype named ElecTIS was recently developed and patented (http://www.explanta.com/bioreactor-electis/, accessed on 7 June 2023), soon proving, soon proving very promising in the micropropagation of *Malus* germplasm [11], paulownia and chrysanthemum [9]. The innovative characteristic of ElecTIS is that the forced insufflation of air inside is eliminated, as a pneumatic system allows the ascending and descending cycling movement of a plastic basket containing the shoot culture, periodically immersing in the liquid substrate that is positioned at the bottom of the container. The main advantages of this new TIS bioreactor therefore are the simplification of the handling operations, the consistent reduction of the risks of contamination due to the forced insufflation of external air in the traditional bioreactors and the higher multiplication rates in comparison to traditional cultures in a gelled medium.

The conservation of shoot culture at low temperatures (also referred to as "slow growth storage", SGS) allows the in vitro maintenance of shoot cultures in aseptic conditions by markedly reducing the frequency of periodic subcultures (from a few months to one year or more, depending on the species) without affecting the vitality and regrowth potential of the shoots [12]. The technique is a tool of strategic importance for commercial micropropagation laboratories, which need today to counteract competition by broadening the offer of species and cultivars and achieving in general a better organization of their high-quality production. Indeed, the conservation at low temperatures (generally, 4–5 °C for temperate species [13]) allows a significant lengthening of the interval between subcultures, thus reducing the maintenance costs of stock cultures, as well as the risk of contamination during shoot handling. A problem connected with the SGS of fruit trees and shrubs also concerns the fast recovery of the full proliferative efficiency of the shoots upon return to standard culture conditions after long-term storage at a low temperature.

For the above, this study investigated the efficiency and speed of recovery from long-term storage at 4 °C of blackberry lines, comparing the return to standard culture conditions in a gelled substrate and in TIS culture using the ElecTIS bioreactor. Blackberry (*Rubus fruticosus* L.) is a berry fruit of great commercial interest and is widely reproduced by micropropagation. The numerous commercialized cultivars often make it necessary to store the material in SGS for a medium-long period of time (Battistini Vivai, Cesena, personal communication). To our knowledge, this is the first study that explored the use of the liquid culture in TIS for the return of shoot cultures at standard culture conditions after long-term storage at a low temperature.

2. Materials and Methods

2.1. Plant Material and Culture Conditions before the Transfer to ElecTIS Bioreactor

Blackberry (*Rubus fruticosus* L.) shoot culture lines, cvs Chester and Thornfree, were obtained by the Battistini Vivai of Cesena, Italy. The shoot lines came directly from the preservation in SGS (at 4 °C, in the dark) for a continuous period of 5 months. The clusters were composed of elongated shoots, etiolated in the mid-apical part, but overall they tolerated the long storage period at low temperature and in the dark sufficiently well. Before SGS, the shoot lines had been previously cultured for one year in a DKW medium [14], with the addition of 0.5 mg/L benzyl-adenine (BA), 0.01 mg/L indole-3-butyric acid (IBA) and 30 g/L sucrose and gelled with 7 g/L agar (pH 5.8), in a climatic chamber at 23 °C, for a 16 h photoperiod and with a light intensity of 40 µM m^{-2} s^{-1}. Subsequently, they were moved to SGS in the same culture medium. All the chemicals have been used in this study were from Duchefa Biochemie, Haarlem, The Netherlands.

2.2. Description of the ElecTIS Bioreactor

Two ElecTIS bioreactors were tested, differing in the size of the container, i.e., one with a smaller basket (18 × 13 cm, equal to 234 cm^2, named here 'ElecTIS$_S$'), and one with a large basket (21 × 16 cm, equal to 336 cm^2, named here 'ElecTIS$_L$'). ElecTIS$_S$ was previously described [11]. The ElecTIS$_L$ bioreactor consists of a larger plastic container (3000 cc; Figure 1a), with a cover and a basket entering in the inner space (Figure 1b). The cover has a central piston in the inner part (Figure 1c), connected to a pump, which pushes the basket down to the container to activate the immersion phase of the culture. When the pump stops working, in response to the TIS cycle regulated by a clock, a spring underneath the basket pushes the basket up again (Figure 1d), activating the dry phase of the cycle. The basket is prepared with the shoot culture inside before being inserted in the container. The cover is then hermetically connected to the container. The retraction and expansion of the piston is allowed by a connection with a pressing pump and results in the up and down movement of the basket. Air flow occurs inside the piston, while no air is pressed inside the container.

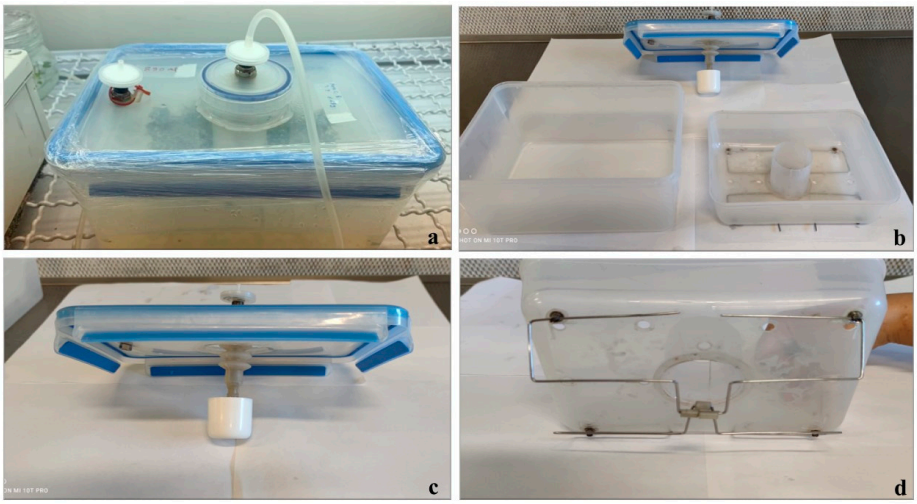

Figure 1. (**a**) The assembled bioreactor ElecTIS$_L$ (basket size, 21 × 16 cm); (**b**) the three components of the bioreactor: lid, container and mobile basket; (**c**) the lid with the central piston which determines the descent of the basket; (**d**) the system of springs under the basket determines its elevation.

2.3. Shoot Multiplication in Post-Conservation with ElecTIS Bioreactors and Gelled Medium

Shoot lines from each cultivar were cultured in ElecTIS bioreactors in the same liquid medium as reported above, with ElecTIS$_S$ containing 300 mL and ElecTIS$_L$ 500 mL of medium. The culture in glass jars (500 cc) was performed as a control in a gelled medium, with the same composition of liquid medium but solidified with 7 g L^{-1} agar. The pH

was adjusted to 5.8. Glass jars with medium, ElecTIS bioreactors and liquid medium in Erlenmeyer flasks were autoclaved (120 °C/1 bar/20 min) before the culture preparation under a laminar flow hood. Before subculturing, the shoots were excised, weighed, counted and then transferred to the different culture systems ($ElecTIS_S$, $ElecTIS_L$ and glass jars). Thirty shoot clusters were transferred to both ElecTIS bioreactors and glass jars. Previous trials were conducted to select an effective cycle of immersion. Afterwards, the cycle applied to the liquid culture in ElecTIS biorectors was of 8 min every 6 h (equal to 32 min/day). All cultures were incubated at 23 ± 2 °C with a 16 h photoperiod and a light intensity of 40 μM m^{-2} s^{-1}.

2.4. Rooting and Shooting of Clusters in ElecTIS Bioreactors and Gelled Medium and Transfer to Acclimatization

To investigate the in vitro rooting of blackberry, following the multiplication phase shoot clusters from ElecTIS bioreactors and glass jars were weighed, cut to 0.5-cm segments and subcultured in the rooting medium. The rooting liquid medium of ElecTIS bioreactors contained 1/2-strength MS medium [15], supplemented with 20 g L^{-1} sucrose, 0.5 mg/L IBA and 0.2 g/L FEDDAH. The applied immersion cycle was the same of the multiplication phase (8 min/6 h). For the control in glass jars, the medium was solidified with 7 g L^{-1} agar. ElecTIS bioreactors and glass jars were kept under the same climatic conditions as described above. The rooting phase lasted 5 weeks.

Rooted shoot clusters were finally acclimatized in ex vitro condition by transferring them in preforma trays (360 holes per tray) in mini-greenhouses under plastic tunnels that were gradually opened in a period of 3 weeks, after which the plantlets were moved under a classic greenhouse for the final hardening.

2.5. Data Collection

After 4 weeks of culture in ElecTIS bioreactors or in glass jars, the following parameters were determined:

Growth parameters

(i) The number of shoots/clusters, (ii) average shoots length/cluster (cm) and (iii) fresh weight (FW) of the whole culture for each container were determined, after which the culture was dried in an oven at 70 °C for 72 h to calculate the dry weight (DW).

Water content

The water content (WC) was calculated as:

$$WC = (FW - DW)/FW \times 100$$

Relative Growth Rate

The relative growth rate (RGR) at successive subculture intervals was calculated as:

$$(\text{Ln } W_2 - \text{Ln } W_1) \times 100/(t_2 - t_1)$$

where W_1 is the initial FW (g) and W_2 is the final FW (g) after each subculture, at starting time t_1 and finishing time t_2, respectively.

Photosynthetic pigments

The photosynthetic pigments (chlorophylls a and b, total chlorophyll, carotenoids) were determined in fresh blackberry leaves. The extraction was performed in test tubes with 200 mg of fresh leaves in 10 mL ethanol 80% (v/v) and heated in a water bath to 60 °C until the sample colour completely turned white; the pigment concentrations were determined by a spectrophotometer with the absorbance at 663 nm for chlorophyll a, 644 nm for chlorophyll b and 452 nm for carotenoids, and they were calculated according

to Lichtenthaler [16]. The pigment contents were calculated in leaves as mg/g fresh weight using the following equations:

$$\text{Chlorophyll a} = (13.36 \times A_{663}) - (5.19 \times A_{644})$$

$$\text{Chlorophyll b} = (27.49 \times A_{644}) - (8.12 \times A_{663})$$

$$\text{Carotenoids} = [(1000 \times A_{452}) - (2.13 \times \text{Chl.a}) - (97.6 \times \text{Chl.b})]/209$$

Stomatal study

In order to investigate the effect of the two different systems of culture on stoma density and morphology, after thoroughly cleaning the leaf abaxial epidermis with a cotton pad the mid-section between the central vein and the leaf edge was carefully swiped with nail polish and left to dry for about 20 min. The fine film was peeled off from the leaf surface, put on a glass slide, quickly covered with a cover slip, and then gently pressed with fine point pincers. The total number of stomata was counted in each film strip of known dimension under a photomicroscope system (Nicon Eclipse 90i). The methodology for leaf stomatal density determination followed what was described by Xu and Zhou [17] and was expressed as the number of stomata per mm^{-2} [18]. The stomatal size in length (i.e., the distance measured in micrometers between the guard cell junctions at each end of the stoma) and in width was also determined. These measures indicate the stomatal pore maximum potential opening, but not its functionality [19,20]. Therefore, the percentage of closed and open stomata was also calculated.

Rooting and shoot development in clusters

After 5 weeks of culture in the rooting medium, the characteristics of clusters to be directed to the acclimatization phase were evaluated by calculating the following parameters: (i) average number of roots per cluster, (ii) average number of roots $\geq$0.5 cm per cluster, (iii) average number of shoots forming the cluster and (iv) average shoot length.

2.6. Statistical Analysis

For each cultivar, each experiment was repeated 3 times. Discrete data were analysed by ANOVA, followed by the Duncan test at $p \leq 0.05$. A one-way ANOVA was also applied to assess the significance of variations in photosynthesis pigments using SPSS v.16. [21]. A statistical analysis of the stomata was carried out using the χ^2 test at $p \leq 0.05$. Bars in Figure 3 represent standard errors (SE) of the means.

3. Results

3.1. Effect of Shoot Culture in ElecTIS and in Gelled Medium Systems on Vegetative Growth Parameters

For both the tested blackberry cultivars, data reported in Table 1 show a notable difference from the culture in the ElecTIS bioreactors and in the traditional gelled medium in terms of vegetative growth parameters. For both the cultivars cultured in the ElecTIS bioreactors, the average shoot length was always in the range of 3.1 ('Thornfree' in ElecTIS$_L$) and 3.7 cm ('Chester' in ElecTIS$_L$), with no significant difference between the two sizes of bioreactors. Conversely, the average shoot length was consistently and significantly lower when the culture was inside the glass jars on the gelled medium (1.0 and 0.9 cm for 'Thornfree' and 'Chester', respectively). Moreover, the culture in the TIS bioreactors produced a much higher shoot proliferation in comparison to the traditional culture in the gelled medium. The shoots of the two cultivars proliferated in a very similar way when in the bioreactors, with 31.4–31.5 average shoots per cluster for the 'Thornfree' and 30.2–30.4 for 'Chester', with no significant difference between the two ElecTIS bioreactors. In the gelled medium, the average number of shoots per cluster was almost five times lower, resulting in 6.6 for 'Thornfree' and 6.4 for 'Chester'. The superior growth of the shoot cultures was also confirmed from the average FW and DW of clusters. Indeed, both these physiological parameters were about 1/3 in the clusters grown in the gelled medium in

comparison with the liquid culture in the ElecTIS, regardless of the size of the containers. The water content (%) was similar in the material cultured in liquid or in gelled media. Indeed, it was at the highest value in the cv Thornfree cultured in ElecTIS$_S$ (92.06%), while the lowest value was showed by 'Chester' cultured in the gelled medium (86.61%).

Table 1. Vegetative growth parameters of blackberry shoots after subculture in ElecTIS compared to gelled medium (control). In each cultivar, as for shoot length and number of shoots per cluster, different superscripts indicate significant differences by the ANOVA, followed by the Duncan test at $p \leq 0.05$. As for FW, DW and water content, data are average values ± SE.

Cultivar/ Culture System	Growth Parameter				
	Shoot Length (Average, cm)	Shoots/Cluster (Average, n°)	FW (Avg, g)	DW (Avg, g)	Water Content (%)
Thornfree					
ElecTIS$_L$	3.1 ^{a}	31.4 ^{a}	1.99 ± 0.2	0.17 ± 0.1	91.14 ± 4.1
ElecTIS$_S$	3.4 ^{a}	31.5 ^{a}	2.10 ± 0.2	0.16 ± 0.1	92.06 ± 5.0
Gelled medium	1.0 ^{b}	6.6 ^{b}	0.65 ± 0.1	0.06 ± 0.0	89.52 ± 4.6
Chester					
ElecTIS$_L$	3.7 ^{a}	30.4 ^{a}	1.99 ± 0.2	0.18 ± 0.0	90.96 ± 3.9
ElecTIS$_S$	3.4 ^{a}	30.2 ^{a}	2.27 ± 0.4	0.21 ± 0.1	90.39 ± 4.1
Gelled medium	0.9 ^{b}	6.4 ^{b}	0.64 ± 0.4	0.08 ± 0.0	86.61 ± 6.2

Table 2 shows that after 4 weeks of culture, the RGR calculated with the shoots grown in the gelled medium ranged between a minimum of 5.4 ('Thornfree' in the second subculture) and a maximum of 7.9 ('Thornfree' in the first subculture), with an increment of proliferating material in the range of 5.0–10.2% times the initial weight. The index confirmed the superiority of the ElecTIS bioreactors in promoting the proliferation of shoot cultures coming from long-term conservation in slow growth storage. In fact, as regarded by the cv Thornfree, the RGR index after 4 weeks of culture ranged from a minimum of 7.8 (ElecTIS$_S$ in the second subculture) to a maximum of 10.2 (ElecTIS$_S$ in the first subculture; Figure 2a,b), corresponding to an increment of proliferating material of 10.4 and 21.2 times, respectively. Considering the pool of three consecutive subcultures, the two ElecTIS bioreactors performed similarly with an RGR of 8.7 (ElecTIS$_L$) and 9.0 (ElecTIS$_S$), related to an increment of proliferating material of 13.2 and 14.7 times the initial weight, respectively, significantly higher that what was obtained with the culture in the gelled medium (8.4).

Table 2. Effect of the culture system on the expression of the proliferation potential of *Rubus fruticosus* L. shoot cultures. RGR, relative growth rate; PM, proliferating material. As for the increment of proliferating material, different superscripts indicate significant differences by the ANOVA, followed by the Duncan test at $p \leq 0.05$.

Cultivar *Subculture* Culture System	Growth Parameter		
	Initial/Final Weight (g)	Increment of PM	RGR
Thornfree			
1st subculture			
ElecTIS$_L$	4.6/72.9	15.8 ^{a}	9.2
ElecTIS$_S$	2.7/57.3	21.2 ^{a}	10.2
Gelled medium	1.2/12.2	10.2 ^{b}	7.9
2nd subculture			
ElecTIS$_L$	5.1/75.4	14.8 ^{a}	9.0
ElecTIS$_S$	5.3/55.2	10.4 ^{a}	7.8
Gelled medium	2.3/11.4	5.0 ^{b}	5.4
3rd subculture			
ElecTIS$_L$	5.1/71.6	14.0 ^{a}	9.4
ElecTIS$_S$	4.3/54.2	12.6 ^{ab}	9.1
Gelled medium	1.8/11.1	10.1 ^{b}	6.5

Table 2. *Cont.*

Cultivar ***Subculture*** **Culture System**	**Growth Parameter**		
	Initial/Final Weight (g)	**Increment of PM**	**RGR**
Average			
ElecTIS$_L$	---	*13.2* [a]	*8.7*
ElecTIS$_S$	---	*14.7* [a]	*9.0*
Gelled medium	---	*8.4* [b]	*6.6*
Chester			
1st subculture			
ElecTIS$_L$	6.7/88.6	13.2 [a]	8.6
ElecTIS$_S$	4.5/51.0	11.3 [ab]	8.1
Gelled medium	0.6/5.5	9.1 [b]	7.4
2nd subculture			
ElecTIS$_L$	4.4/72.9	16.6 [a]	9.3
ElecTIS$_S$	5.0/52.4	10.5 [a]	7.8
Gelled medium	1.1/5.6	5.1 [b]	5.5
3rd subculture			
ElecTIS$_L$	8.4/84.2	10.0 [a]	8.2
ElecTIS$_S$	6.6/50.5	7.7 [ab]	7.3
Gelled medium	1.0/5.5	5.5 [b]	6.2
Average			
ElecTIS$_L$	---	*13.3* [a]	*8.7*
ElecTIS$_S$	---	*9.8* [b]	*7.7*
Gelled medium	---	*6.6* [c]	*6.4*

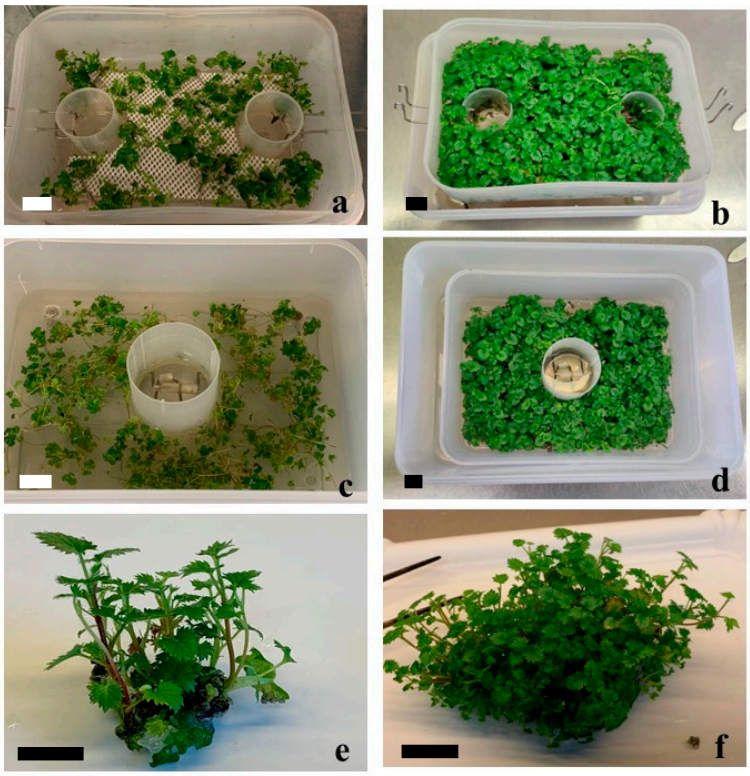

Figure 2. (**a**) The cv Thornfree in ElecTIS$_S$ at the beginning of the second subculture (bar, 2 cm); (**b**) after 4 weeks of culture (bar, 2 cm); (**c**) the cv Chester in ElecTIS$_L$ at the beginning of the second subculture (bar, 2 cm); (**d**) after 4 weeks of culture; (**e**) a single shoot cluster after 4 weeks of culture in glass jars on gelled medium (cv Thornfree) (bar, 1 cm); (**f**) the same, but after the liquid culture in ElecTIS (bar, 1 cm).

The results obtained with the cv Chester substantially confirmed the superiority of the culture in the ElecTIS compared to that in glass jars, albeit with a lower average proliferation potential than in the 'Thornfree' (Figure 2c,d). It is interesting to note that, with the 'Chester' culture coming from long-term conservation at low temperature and in darkness, the ElecTIS$_L$ bioreactor proved to be more efficient than the ElecTIS$_S$ in stimulating shoot proliferation, both in the single subcultures and in the average.

The observation of the single clusters evidenced the superiority of the TIS culture in stimulating proliferation in comparison to the one in glass jars (Figure 2e,f).

3.2. Effect of the Different Culture Systems on Chlorophyll Content

Figure 3 shows that throughout the two sizes of the ElecTIS bioreactors and the culture in the gelled medium, the differences in the chlorophyll contents were evident, especially in chlorophyll a. Both blackberry cultivars in the ElecTIS$_L$ bioreactor had the highest values of chlorophyll a concentration (Figure 3a), without a significant difference with ElecTIS$_S$, but with significant differences from the chlorophyll a detected in the gelled medium. By contrast, the chlorophyll b content was similar in the three different culture systems (Figure 3b). Figure 3c shows that the total chlorophyll was at the highest amount in ElecTIS$_L$ in both cultivars, with 0.096 and 0.095 mg g^{-1} FW in 'Chester' and 'Thornfree', respectively, with no significant difference between the two size bioreactors. In contrast, shoots cultured in the gelled medium showed a significant reduction of total chlorophyll concentrations, with values of 0.85 and 0.84 mg g^{-1} FW in 'Chester' and 'Thornfree', respectively. The highest values of carotenoids were in the ElecTIS bioreactors, resulting equally in the two different cultivars (0.25 and 0.24 mg g^{-1} FW in ElecTIS$_L$ and ElecTIS$_S$, respectively; Figure 3d). Moreover, the content of carotenoids was significantly lower in the shoots cultured in the gelled medium, with 0.23 and 0.24 mg g^{-1} FW in 'Thornfree' and 'Chester', respectively.

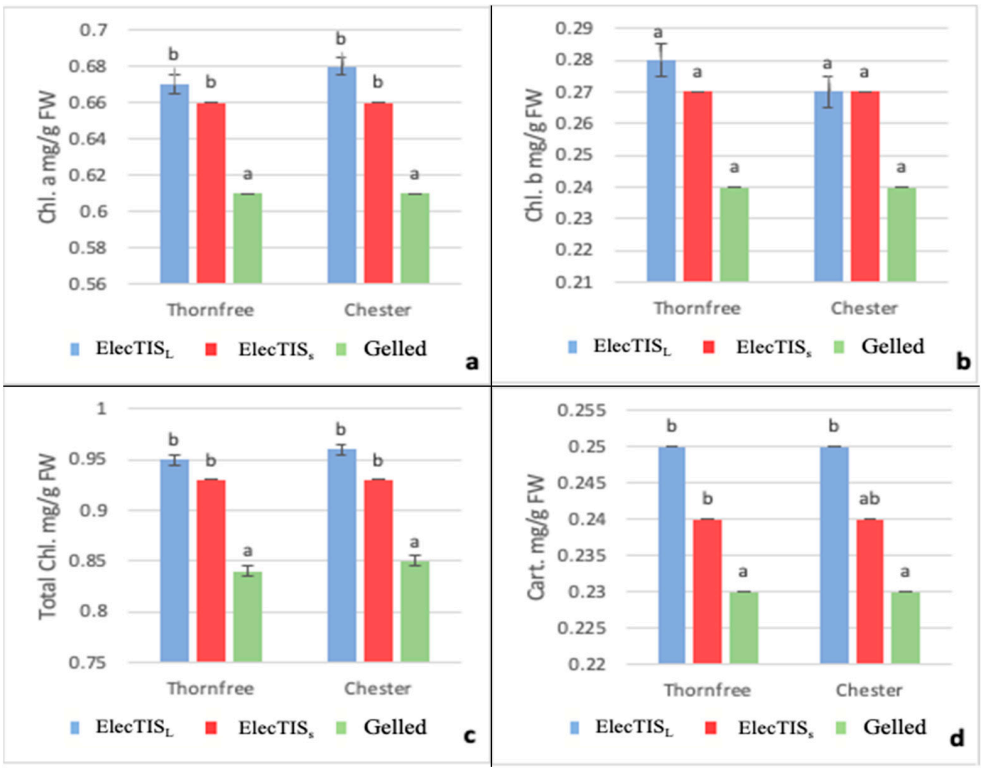

Figure 3. Chlorophyll content (mg/g FW) in blackberry shoots cultured in the different systems, after 4 weeks of culture. (**a**) chlorophyll a; (**b**) chlorophyll b; (**c**) total chlorophyll; (**d**) carotenoids. Bars represent means ± SE. Different letters indicate significant differences by the ANOVA, followed by the Duncan test at $p \leq 0.05$.

3.3. Effect of the Culture in the ElecTIS Bioreactors on Plant Rooting and Shooting before Acclimatization

Table 3 reports the adventitious rooting of shoots cultured in the two TIS culture systems and in the gelled medium. After 28 days of culture in the ElecTIS bioreactors, the average number of roots per cluster was at the highest value in ElecTIS$_L$ in 'Chester', with six roots/cluster (Figure 4a), without a significant difference between the culture in the two sizes of bioreactors. Rooting in 'Thornfree' was much lower than in 'Chester', with 2.5–2.6 roots/cluster in the ElecTIS bioreactors. However, for both the cultivars, shoot rooting in the gelled medium always gave the lowest results. As for root elongation after 28 days of culture, 'Chester' in ElecTIS$_L$ had the highest number of long roots (≥0.5 cm) per cluster (4.1 cm), similarly to the culture in ElecTIS$_S$ (3.8) and significantly higher than the one recorded from shoots rooted in the gelled medium (1.6; Figure 4b). Similarly to 'Chester', the quality of rooting in terms of long roots was higher with the culture in TIS than in the gelled medium.

Table 3. Rooting and shooting parameters of *Rubus fruticosus* L. clusters following the subculture in rooting medium in ElecTIS bioreactors vs. gelled medium. Different superscripts indicate significant differences by the ANOVA, followed by the Duncan test at $p \leq 0.05$.

Cultivar **Culture System**	**Rooting and Shooting Parameters**			
	Average No. of Roots per Cluster	**Average No. of Roots per Cluster ≥ 0.5 cm**	**Average No. of Shoots per Cluster**	**Average Shoot Length (cm)**
Thornfree				
ElecTIS$_L$	2.6 ^{a}	1.7 ^{a}	4.1 ^{a}	3.2 ^{a}
ElecTIS$_S$	2.5 ^{a}	1.5 ^{a}	4.3 ^{a}	3.1 ^{a}
Gelled medium	2.1 ^{b}	0.3 ^{b}	1.6 ^{b}	2.2 ^{b}
Chester				
ElecTIS$_L$	6.0 ^{a}	4.1 ^{a}	6.2 ^{a}	4.4 ^{a}
ElecTIS$_S$	5.8 ^{ab}	3.8 ^{a}	6.4 ^{a}	4.3 ^{a}
Gelled medium	5.5 ^{b}	1.6 ^{b}	1.4 ^{b}	2.9 ^{b}

Figure 4. (**a**) Rooting of a cluster of shoots of cv Chester in ElecTIS$_L$ (bar, 1 cm); (**b**) a shoot of 'Chester' rooted in glass jars in gelled medium (bar, 1 cm).

As for the shoots per cluster formed during rooting in the different culture systems, the use of ElecTIS bioreactors produced the best results in both the tested cultivars, with always significant differences in comparison to the culture in the gelled medium. As maximum scores, the culture of 'Chester' produced 6.2 and 6.4 shoots per cluster and an average of shoot length of 4.4 and 4.3 in ElecTIS$_L$ and ElecTIS$_S$, respectively.

3.4. Analysis of Stomata in Leaves from ElecTIS Bioreactors and Gelled Medium

Table 4 lists the characteristics of the stomata in leaves cultured in the ElecTIS and in the gelled medium in terms of density, average length and width and percentages of open and closed stomata at the moment of the observations. The main observations are the following: (i) in comparison with the gelled medium, the culture in TIS produced leaves with a higher number of stomata per mm^2, although this difference was statistically significant only with the cv Thornfree; (ii) no clear trend was observed in the size of the stomata, as they were apparently bigger in 'Thornfree' leaves cultured in the gelled medium in comparison to the ElecTIS, the opposite of 'Chester'; (iii) the percentage of closed stomata was always higher in the TIS than in the gelled medium (55.6% and 49.5% in 'Thornfree' and 'Chester', respectively; Figure 5a,b), an indicator of a their superior functional activity as a consequence of the culture in the ElecTIS bioreactors.

Table 4. Stomata traits in blackberry, cvs Thornfree and Chester, cultured in TIS bioreactor and in gelled medium. Different superscripts indicate significant differences by the χ^2 test at $p \leq 0.05$.

Cultivar Culture System	Stomata				
	Density (mm^2)	Length (µm)	Width (µm)	Open (%)	Closed (%)
cv Thornfree					
ElecTIS	246.5 ^{a}	16.1 ± 0.77	11.9 ± 0.44	26.1 ^{a}	73.9 ^{a}
Gelled medium	201.5 ^{b}	23.5 ± 0.68	16.0 ± 0.76	44.4 ^{b}	55.6 ^{b}
cv Chester					
ElecTIS	187.6 ^{a}	23.4 ± 0.74	15.2 ± 0.75	26.6 ^{a}	73.4 ^{a}
Gelled medium	174.4 ^{a}	20.4 ± 0.79	14.6 ± 0.44	50.5 ^{b}	49.5 ^{b}

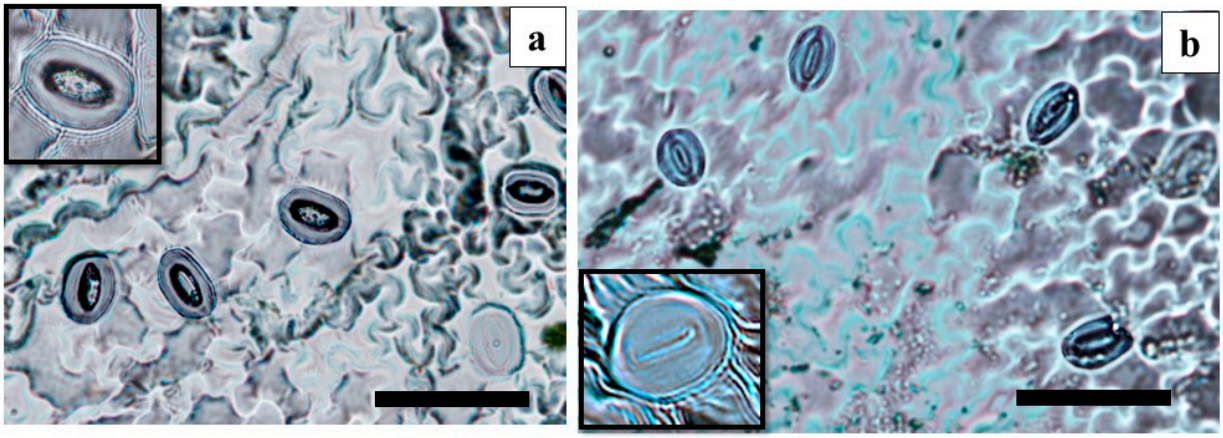

Figure 5. (**a**) Open stomata in leaves from shoot cultured in glass jars in gelled medium (cv Chester); (**b**) example of closed stomata from shoot cultured in ElecTIS (cv Thornfree). Bars of the large pictures, 0.05 mm.

4. Discussion

The results in this study demonstrate the efficiency of the ElecTIS bioreactor in promoting the shoot growth and proliferation of blackberry shoots coming from SGS at a low temperature (4 °C) and in darkness, in comparison with the gelled medium. In recent years, the mass production of micropropagated plants, based on the use of liquid culture in TIS bioreactors, has been established and developed for a wide range of species, e.g., banana [22], myrabolan rootstock [23], taro [24,25] and stevia [26]. Mancilla-Álvarez

et al. [27] suggested that the assimilation of sugars, nutrients and growth regulators from the liquid culture medium when in contact with the entire surface of the explant could be an explanation for the increase in the multiplication rate of *Colocasia esculenta*, while the lower availability of organic and inorganic compounds in the gelled medium, due to the limited contact of shoots at their base, determines the lower multiplication rates. Rosales et al. [26] noted a doubling of the shoot number of *Stevia rebaudiana* in TIS liquid culture in comparison with the gelled medium, which led to a consequent increase of the FW and DW of plants from the culture in the TIS bioreactor. That was also reported in sugarcane, where the culture in TIS produced five times more shoots [27].

When using the ElecTIS bioreactors, the selection of an appropriate cycle of immersion is very important to determine a good vigour and quality of the cultures, preventing any shoot hyperhydricity. With blackberry, preliminary trials had shown the effectiveness of an immersion cycle of 8 min every 8 h. The RGR index is a standardized growth measurement that proved to be very effective in comparing the growth in different culture systems. Indeed, it has the advantage of minimizing the inherent scale differences between growing plants so that their performances can be compared fairly, and it is related to the examined time period. Quantifying and modelling allometric relationships in plants also requires RGR [28]. In this study, the index proved the superior proliferative capacity of blackberry shoot cultures when maintained in liquid culture in TIS, compared to the traditional culture in a gelled medium. These results confirm the excellent efficacy of the ElecTIS bioreactor in stimulating the proliferation of shoots already observed by Sota et al. [11]. These authors, using the ElecTIS$_S$ bioreactor, achieved a maximum RGR of 11.6 in a single subculture of *Malus sylvestris* L., with material coming from the standard subculture at 23 °C with a 16 h photoperiod before being transferred to an immersion cycle of 4 min every 6 h (equal to 16 min/day). Here, blackberry shoot cultures came from the long-term conservation at 4 °C in the dark and, also in this case, the bioreactor ElecTIS was more effective than the gelled medium in stimulating the shoot recovery of growth and proliferation.

Chlorophyll content in this study was higher in chlorophyll a and in total chlorophyll in both cultivars when cultured in TIS bioreactors in comparison to the gelled medium. These results are in agreement with other reports [25,29–31]: all these studies demonstrated that the photosynthetic pigments were increased in taro, anthurium, yam and banana, respectively, when cultured in a liquid medium in TIS, in comparison with the culture in the gelled media. Aragón et al. [31] found that TIB® (Temporary Immersion Bioreactor) has a favourable influence on the photosynthesis process in banana micropropagation, since they obtained higher photosynthesis values in TIB® than in a semi-solid culture. An interesting report involving the micropropagation of *Vanilla planifolia* utilized several TIS bioreactors [7]. The authors suggest that the SETIS™ bioreactors, made up of polypropylene screw caps and horizontal polycarbonate tanks, allow the passage of more light, which is a crucial element that regulates plant growth by affecting the amount of photosynthetic pigments. From the results of the present study, a similar positive effect on light acquisition with the use of the ElecTIS bioreactor can be hypothesized. In accordance, the minor chlorophyll and carotenoid amounts from shoots cultured in glass jars with gelled media, observed also in blackberry, was reported by Mancilla-Álvarez et al. [24], who hypothesized that the culture in climatic chambers with classic glass jars, positioned vertically on multiple levels, limits the amount of light the explants receive. However, a medium effect (liquid or gelled) on these parameters cannot be excluded.

Working with blackberry and longer roots, the highest number of roots and shoots, in addition to longer shoots, were obtained in the ElecTIS bioreactors compared to the culture in glass jars in the gelled medium. These results are in agreement with Hwang et al. [32], who tested three species (chrysanthemum, marigold, strawberry) in different bioreactor systems, finding that the plants grown in TIS showed longer roots and a higher number of shoots in the rooting phase compared to those grown in the semi-solid culture.

Transpiration through gas exchange between the atmosphere and the plant is dependent on the stomata; therefore, they are very essential in regulating the water potential

in the tissues, thus contributing to the maintenance of plant homeostasis [33,34]. The percentage of stomata that open and close can be used as a measurement to estimate the stomatal function. The relative humidity, CO_2 content, temperature of the environment and water potential all affect stomatal function [35]. In this study, both cultivars ('Thornfree' and 'Chester') showed that, at the time of detection, about 73% of the stomata were closed in leaves from the TIS culture, in comparison with the 44–59%, respectively, of leaves from the traditional culture in glass jars on the gelled medium. This outcome is in accordance with Bello-Bello et al. [22], who noticed in banana (*Musa* AAA) an increase of closed stomata in different TIS systems compared to the culture in the gelled medium. Similar results were obtained in anthurium (*Anthurium andreanum* Lind.), where the highest percentages of closed stomata were found in shoots grown in a liquid medium [29]. This fact indicates a better stomatal function in leaves formed under TIS conditions, due to a higher gas exchange, low relative humidity and high water potential in comparison with the traditional culture in hermetically closed glass jars, which have little or no gas exchange (CO_2 and O_2), high relative humidity and high osmotic potential in the gelled medium. Additionally, for Hazarika et al. [36] the increase in the percentage of closed stomata is a physiological indicator of correct stomatal function. Stomata open and close to regulate the amount of water in plant tissue during transpiration and control gas exchange during photosynthesis [37,38], which could also result in an enhancement in chlorophyll synthesis, as observed in this study.

When moved to ex vitro conditions, 100% of shoots from the ElecTIS bioreactors survived and could be perfectly acclimatized. Furthermore, Aragón et al. [39] reported higher shoot survival and rooting rates when using material coming from TIS culture compared to a semi-solid medium. Similar results were obtained in carnation [40] and in agave [41]. In pineapple, the liquid culture in TIS produced shoots with a high potential of rooting and ex vitro acclimatization [42]. Moreover, shoot tips of *Callistephus hortensis* obtained in the TIS culture were larger than those from the semi-solid medium, which resulted afterwards in better rooting [43].

5. Conclusions

This study, conducted in blackberry (*Rubus fruticosus* L.), has clearly demonstrated how the liquid culture in TIS using the ElecTIS bioreactor can consistently improve the recovery of shoot culture from long-term conservation in SGS, accelerating the time of return to an excellent level of shoot proliferation. Furthermore, the quality of the material, investigated in terms of stomatal activity, chlorophyll and carotenoid content, improved, benefiting the subsequent phases of rooting and ex vitro acclimatization of the plantlets. The two ElecTIS bioreactors proved to be equally efficient, although the larger ElecTIS is easier to handle in the culture preparation phase.

6. Patents

ElecTIS is protected by the European Patent No. 2617282 from Claudio Depaoli of Predaia (Trento), Italy.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the agreement with the ElecTIS patent owner to divulgate them only upon motivated request.

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