

Communication

# GreenTray<sup>®</sup>, a TIS Bioreactor for Plant Micropropagation and Abiotic or Biotic Stress Bioassays

Ramon Dolcet-Sanjuan <sup>1,\*</sup>, Maria Casanovas <sup>1</sup>, Sandra Franquesa <sup>1</sup>, Eva Alsina <sup>1</sup>, Francisca Carrasco-Cuello <sup>2,3</sup>, Estanis Torres <sup>2</sup>, Josep Rufat <sup>3</sup>, Cristina Solsona <sup>4</sup> and Neus Teixido <sup>4</sup>

<sup>1</sup> Plant in Vitro Culture Laboratory, Fruticulture Program, IRTA, Fruitcentre, Parc AgroBiotech, 25003 Lleida, Spain

<sup>2</sup> Fruticulture Program, IRTA, Fruitcentre, Parc AgroBiotech, 25003 Lleida, Spain

<sup>3</sup> Efficient Use of Water in Agriculture Program, IRTA, Fruitcentre, Parc AgroBiotech, 25003 Lleida, Spain

<sup>4</sup> Postharvest Program, IRTA, Fruitcentre, Parc AgroBiotech, 25003 Lleida, Spain

\* Correspondence: ramon.dolcet@irta.cat

**Abstract:** The temporary immersion system bioreactor named GreenTray<sup>®</sup> presents advantages over the existing ones. Firstly, there is no need to use forceps to move the shoots or plantlets in or out of the recipient, nor is the use of a scalpel necessary to divide them. Secondly, the basis of the shoot can remain in the GreenTray<sup>®</sup> and can sprout again over several cycles of growth. These two characteristics reduce the costs in the process of micropropagation. In addition, shoot or plantlet development is enhanced by the aeration of the recipient, thus enriching the recipient's atmospheric CO<sub>2</sub>. This article describes and provides images and values of interest in (1) the micropropagation process of some commercial *Prunus* or *Pyrus* rootstocks; (2) the plantlet growth under autotrophic conditions; and (3) the inoculation of plants with pathogens that reproduce in vitro the symptoms observed in vivo, using the GreenTray<sup>®</sup> bioreactor.

**Keywords:** GreenTray<sup>®</sup>; TIS; bioreactor; micropropagation; bioassays; *Prunus*; *Pyrus*; *Malus*; *Cannabis*



**Citation:** Dolcet-Sanjuan, R.; Casanovas, M.; Franquesa, S.; Alsina, E.; Carrasco-Cuello, F.; Torres, E.; Rufat, J.; Solsona, C.; Teixido, N. GreenTray<sup>®</sup>, a TIS Bioreactor for Plant Micropropagation and Abiotic or Biotic Stress Bioassays. *Appl. Sci.* **2024**, *14*, 4051. <https://doi.org/10.3390/app14104051>

Academic Editors: Francesca Scargiali and Lukasz Kuźma

Received: 5 April 2024

Revised: 22 April 2024

Accepted: 30 April 2024

Published: 10 May 2024



**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Plant propagation through in vitro culture has been adopted by commercial propagation companies because it presents some advantages over conventional propagation with soft or hard wood cuttings. It is a weather-unaffected propagation system, enables the conservation of the initial plant material's sanitary conditions, and exponentially multiplies the selected plant materials every two- to four-week-long cycle. However, in vitro propagation systems based on semisolid agar-containing media require deft manipulation in sterile conditions with appropriate tools and expert hand labor. To prevent contamination but allow plant material aeration, the conventional micropropagation techniques employ glass or plastic containers, flasks or tubes, recycled or disposable, with lids that loosely enclose the recipients, with differential passive aeration. Caps need to be removed in a laminar flow hood cabinet before introducing or extracting the cultured plant portions with the help of forceps, orientating them, dividing them with the help of scalpels using a sterile hard surface, and culturing them in fresh medium with sterile forceps. Therefore, conventional in vitro plant propagation requires a great amount of hard labor input.

Temporary immersion system (TIS) bioreactors have been developed over recent decades to favor plantlet or shoot development. The best-known commercial ones are RITA<sup>®</sup>, its bigger version Matis<sup>®</sup> [1–7], Plantform<sup>TM</sup> [8–14], and SETIS<sup>TM</sup> [15–17]. In addition, there are several other TISs developed for particular purposes by in vitro plant culture laboratories [18–28]. With some differential characteristics among them, there are two common properties which make them similar and help to differentiate the GreenTray<sup>®</sup>. Firstly, the former systems require forceps to introduce or extract explants into or from the culture recipient. In addition, with the former TIS, a supporting surface is needed

to divide the grown explants into smaller pieces with the help of scalpels. Instead, as will be described in this article, GreenTray<sup>®</sup> needs no forceps to move the explants in and out of the culture container, and the grown-out part can be rapidly cut with scissors instead of using a scalpel. Consequently, the design of GreenTray<sup>®</sup> significantly reduces the labor input required for in vitro propagation and opens the possibility for further automated manipulations.

The use of in vitro culture environment-controlled conditions is an asset to selecting tissues, shoots, or plants with differential levels of tolerance to abiotic or biotic stress agents. Correspondence of the results observed in vitro with those obtained in vivo, in a greenhouse or field selection plots, is necessary to adopt the established in vitro protocol for the selection of plant materials and use in breeding programs. Most protocols established in vitro require the addition of sugar to the culture medium, since the growth conditions of the shoots, plantlets, or seedlings are heterotrophic. GreenTray<sup>®</sup>'s design allows for independent aeration of the culture container or its enrichment with CO<sub>2</sub>, making it possible to eliminate sugar from the culture medium. This fact, coupled with the current possibility of increasing photosynthetically active radiation without increasing the temperature through LED lighting of different spectra, makes it possible to carry out in vitro tests under autotrophic conditions, that is, with photosynthetic activity. Consequently, bioassays for tolerance to biotic or abiotic stresses using the GreenTray<sup>®</sup> will reproduce in vivo conditions in a more reliable manner than with other culture techniques. This article describes the design, operation, and manipulation of plant material in the GreenTray<sup>®</sup>, and how these allowed the first selection tests to be carried out under autotrophic conditions against abiotic or biotic stress agents for fruit trees, such as the effects of rhizosphere pH and Ca<sup>2+</sup> on plant growth, the effect of in vitro atmospheric humidity on Ca<sup>2+</sup> absorption, and tolerance to fire blight.

## 2. Materials and Methods

The patented GreenTray<sup>®</sup> [29,30] design is characterized by the ability to access the interior of the plant cultivation container. Its design derives from a previously handmade assemblage [31], and another adaptation of a plastic container [32]. When opening the cultivation container of the actual GreenTray<sup>®</sup>, the lid and the cultivation tray are always joined, and therefore, the sprouts or plants in cultivation are oriented with the movement of the lid, with just one hand. In the model presented in this work, the GreenTray<sup>®</sup> is adapted to a commercial glass flask, but the GreenTray<sup>®</sup> design is applicable to any container or bag, of variable size or shape, as long as the culture surface or tray is attached to the container lid, like, for instance, the one presented in [32].

### 2.1. GreenTray<sup>®</sup> Parts and Assembly

A commercial cylindrical glass jar with a capacity of 3895 mL, slightly bigger than a US gallon, with a Twist Off 110 lid, is used as the culture container for the plant materials. Its dimensions (15.9 cm in diameter, 25.45 cm in height) are the basis for building each unit of the GreenTray<sup>®</sup> bioreactor, with automated modules grouping 7 to 21 bioreactors. Plant materials are contained in this glass flask, while the culture medium is autoclaved and placed in 500 mL ISO GL45 graduated transparent flasks, commonly used in laboratories.

To allow the introduction, cultivation, and extraction of plant materials in the culture container, five pieces were designed (Figure 1), which were manufactured by HP Multi Jet Fusion 3D printing, using PA12 for its manufacture, a material that can withstand autoclave sterilization temperatures, which is necessary for the in vitro cultivation of plants. Each unit was assembled as shown in Figure 2.

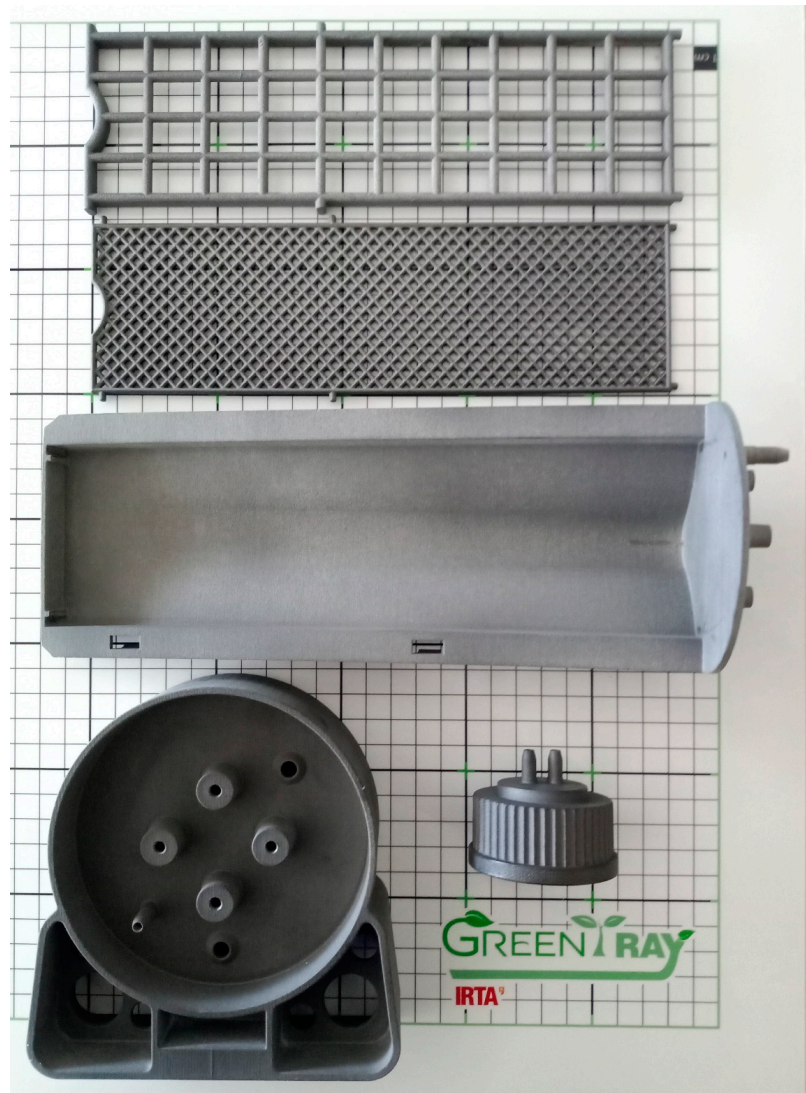


Figure 1. Three-dimensional printed parts of each GreenTray® bioreactor unit.



Figure 2. Assembled GreenTray®, sterilized and ready for the cultivation of plants.

## 2.2. Autonomous and Automated Controller with Compressed Air and Lighting

A PLC+HMI™ Touchscreen model, “Samba™ SM35-J-R20” (Unitronics®, Tel Aviv, Israel), was programmed to control an air diaphragm compressor and vacuum model, “H5P3” (EAD Pumps, Masquefa, Barcelona, Spain), as well as four electro valves and external LED lighting. All components were assembled inside an enclosure for low-voltage switch-gear and control-gear assemblies, the “Spacial CRN” (Schneider Electric Industries SAS, Rueil Malmaison, Paris, France), from which two clear Polyamide Legris (Parker Legris, Terrassa, Spain) compressed air pipes came out, both with 6 and 4 mm external and internal diameters. The first tube provides compressed air at positive pressure (+5 to +6 psi), and when chosen, the same tube exerts suction, or negative pressure (−1 to −1.5 psi). Both air movements arrived at the entrance of the 500 mL ISO GL45. The second tube provides compressed air at positive pressure (+5 to +6 psi) to the entrance of the one-gallon cylindrical jar. Both air entrances were protected from contamination with 25–30 mm diameter 0.22 μm Teflon-PTFE air filters (Filtros Anoaia, S.A., Barcelona, Spain). The assemblage is portable and requires only an AC power plug and socket, becoming a module for 7 GreenTray® units (Figure 3).



**Figure 3.** Portable, autonomous module for 7 GreenTray® units.

## 2.3. Light Source

The LED lighting used in the GreenTray® modules is exchangeable between the models of different light spectra produced by Gealed S.L. (Catajorra, Valencia, Spain), namely “Supernova Lima” (Ref. SUP12100LDC), “Alnayr” (Ref. AN-NAYYIR 60S), and “Meissa” (Ref. AL-MAISAN 100S). The last two, with a Cool White 5000K sun-like spectrum, can provide up to 900 μEm-1s-1 PAR 20 cm from the light source. The light intensity and photoperiod is regulated from the previously described automated controller.

## 2.4. Plant Materials

Five *Prunus* hybrid rootstocks, namely Rootpac® 20, Rootpac® R (obtained by Agromil-lora) [33], Intensia® (obtained by IRTA), Garnem® (obtained by SIA-DGA), and GF667 (obtained by INRA); Adara, a plum rootstock (obtained by EEAD-CSIC); two *Pyrus* hybrid rootstocks, namely OHF87™ (obtained by OSU) and Py170, which is a hybrid between “OH11” [34] and *Pyrus amygdaliformis*, in the last phase of selection of the IRTA breeding program, oriented to have tolerance to iron chlorosis and reduced vigor [35,36]; three apple varieties, namely Carrandona (M0676), Florina (M0216), and Rozona (M0163) (collection of SERIDA) [37]; the *Cannabis sativa* variety “Ferimon” (obtained by HEMPit); and propagules of *Kalanchoe daigremontiana* were used for the present work (Table 1).

**Table 1.** Plant material used herein for propagation, and biotic or biotic stress assays.

Name	Type	Plant Species	In Vitro Plant Material
Rootpac <sup>®</sup> 20	<i>Prunus</i> rootstock	<i>Prunus besseyi</i> × <i>P. cerasifera</i>	Shoot tip cultures
Rootpac <sup>®</sup> R	<i>Prunus</i> rootstock	<i>Prunus cerasifera</i> × <i>P. dulcis</i>	Shoot tip cultures
Intensia <sup>®</sup>	<i>Prunus</i> rootstock	<i>Prunus dulcis</i> × <i>P. persica</i>	Shoot tip cultures
Garnem <sup>®</sup>	<i>Prunus</i> rootstock	<i>Prunus dulcis</i> × <i>P. persica</i>	Shoot tip cultures
GF667	<i>Prunus</i> rootstock	<i>Prunus dulcis</i> × <i>P. persica</i>	Shoot tip cultures
Adara	<i>Prunus</i> rootstock	<i>Prunus cerasifera</i>	Shoot tip cultures
OHF87 <sup>™</sup>	<i>Pyrus</i> rootstock	<i>Pyrus communis</i>	Shoot tip cultures
Py170	<i>Pyrus</i> rootstock	OH11 × <i>Pyrus amygdaliformis</i>	Shoot tip cultures
Carrandona	Apple variety	<i>Malus domestica</i>	Rooted shoots
Florina	Apple variety	<i>Malus domestica</i>	Rooted shoots
Rozona	Apple variety	<i>Malus domestica</i>	Rooted shoots
Ferimon	Cannabis variety	<i>Cannabis sativa</i>	Germinated seeds
Kalanchoe	Succulent plant	<i>Kalanchoe daigremontiana</i>	Plantlets from leaf's edge

## 2.5. Micropropagation in the GreenTray<sup>®</sup>

### 2.5.1. Culture Media

Culture in the GreenTray<sup>®</sup> was conducted in liquid Murashige and Skoog (MS) medium, supplemented with 30 g/L sucrose, 5 µM 6-Benzylaminopurine (BAP), and the pH adjusted to 4.7 before autoclaving [38]. The pH before autoclaving was one unit lower than that used in agar-containing media, after what was observed in [39] to prevent outgrowth of endophytes into the culture medium.

### 2.5.2. Immersion and Aeration Frequencies

The automated controller facilitates temporizing the waiting time between explant immersions (WTI), the duration of explant immersion (TI), the waiting time between explant aerations (WTA), and the duration of explant aeration (TA). In addition, the TI can last 90 s during passive movement, driven by gravity, or 30 s during active movement, forced by air impulsion into the culture jar (35 s) or air suction out of the culture medium bottle (30 s).

The WTI, TI, WTA, and TA depend on the plant material's capacity for optimal growth, avoiding waterlog or water loss. Such times are adjusted to the plant materials, and for the present work, they were set up according to what is indicated in Table 2.

**Table 2.** Time set up of the controller for 7 GreenTray<sup>®</sup> along the culture week.

Parameter	Week 1 to 3		Week 4 to 6		After 6 Weeks	
WTI	2:00	h:m	4:00	h:m	6:00	h:m
TI	0:37	m:s	0:30	m:s	0:25	m:s
APIT	1:00	m:s	1:30	m:s	2:30	m:s
WTA	0:00	h:m	6:00	h:m	0:30	h:m
TA	0:00	m:s	1:00	m:s	0:30	m:s

WTI = waiting time for immersion; TI = time of immersion; WTA = waiting time for aeration; TA = time of aeration; APIT = aeration post immersion time; h:m = hours: minutes; m:s = minutes:seconds.

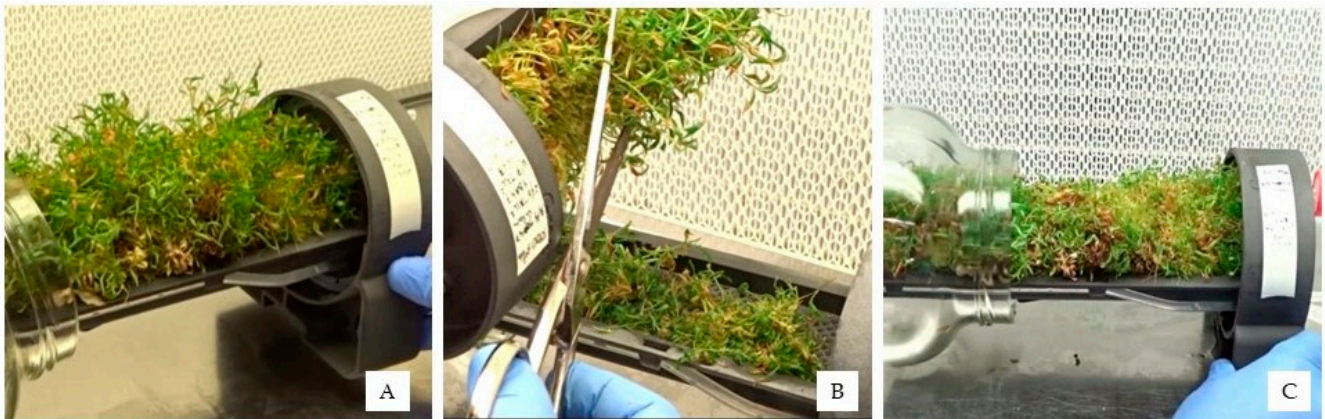
Depending on the plant material volume occupying the tray, 250 to 200 mL of medium is required to immerse the whole culture tray containing the explants.

### 2.5.3. Establishment of Shoot Cultures in the GreenTray<sup>®</sup>

Apical and internodal segments derived from in vitro shoot tip cultures in agar-containing medium were used to establish micropropagation cultures in the GreenTray<sup>®</sup>, called the 1st generation (G1) herein. Depending on the shoot explant size, each GreenTray<sup>®</sup> unit contained between 30 and 40 shoot explants when the explants were between 1 and 2 cm long, and up to 80 or 90 explants when they were 1 cm long or less.

#### 2.5.4. Sequential Number of Shoot Cuts without Subculture

GreenTray<sup>®</sup>'s design allows new shoots to be extracted and cut and grown above the culture tray, followed by the introduction of the remaining basal part back into the culture glass jar (Figure 4A,C). Once this basis regrows, the shoots are cut the same way, several times, depending on the plant material's capacity to keep growing. Compared to the conventional *in vitro* propagation procedures in which shoots are cultured in agar-containing medium, propagation in the GreenTray<sup>®</sup> needs no subculture of the shoot bases, since they remain in the culture tray for several generations, keeping its axillary sprouting capacity, like a hedge from which new shoots are cut.



**Figure 4.** Propagation of Rootpac<sup>®</sup> 20 in the GreenTray<sup>®</sup>: (A) culture tray extraction from the glass flask; (B) orientation of the culture tray to facilitate shoot cut, for the next GreenTray<sup>®</sup> generation; (C) introduction of the culture tray holding the shoot basis into the culture glass jar.

#### 2.5.5. Subculture in New GreenTray<sup>®</sup> and Number of Generations

Shoots produced from the GreenTray<sup>®</sup> can either be moved to soil for rooting in an acclimation facility, or placed in another GreenTray<sup>®</sup> bioreactor, what herein is called a generation (Figure 4B) The number of generations is recorded for each plant material.

#### 2.5.6. Plantlet Development under Autotrophic Conditions

Rotted plantlets were moved to GreenTray<sup>®</sup>, kept vertical with the help of an upper grid, and with their roots laid on the culture tray. The conditions to promote autotrophic growth were as follows: culture liquid: Murashige and Skoog (MS) medium with 0 g/L sucrose; air renewal of the culture jar at 1.5 L/min, forcing aeration in the regime indicated in Table 2 for the 6 weeks of culture; and light intensity: 400  $\mu\text{Em}^{-2}\text{s}^{-1}$  PAR 20 cm from the LED light.

### 2.6. Assays for Abiotic and Biotic Stress Agents

Two *Prunus* rootstocks, namely Garnem<sup>®</sup> and Rootpac<sup>®</sup> 20, and three apple varieties, namely Carrandona (M0676), Florina (M0216), and Rozona (M0163), were established *in vitro*, multiplied, and rooted in agar-containing medium following the protocol described in [38]. The plantlets were transferred to a GreenTray<sup>®</sup> and cultured under autotrophic conditions during the assays to characterize the plant materials' ability to withstand abiotic (high pH, low  $\text{Ca}^{2+}$  concentration) or biotic stress (inoculation with fire blight).

#### 2.6.1. Abiotic Stress Conditions: Rhizosphere pH and $\text{Ca}^{2+}$ on Plantlet Growth

To determine the differential tolerance of the two *Prunus* rootstocks to high pHs and  $\text{Ca}^{2+}$  availability, as determined by the shoot fresh weights, 15 plantlets per rootstock were cultured in eight pH  $\times$   $\text{Ca}^{2+}$  combinations. The liquid culture (Murashige and Skoog (MS) medium) was adjusted before autoclaving to pH 5.7 (standard) or pH 7.7 (alkaline) and used to test for tolerance to high pHs. In both pH conditions, the standard  $\text{Ca}^{2+}$  concentration

in the MS medium (3.0 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) was compared with 1/2 (1.5 mM), 1/3 (1 mM), and 1/4 (0.75 mM)  $\text{Ca}^{2+}$  concentrations in the rhizosphere. Two weeks after culture in the GreenTray<sup>®</sup>, the shoot length (cm) of each plantlet was measured.

#### 2.6.2. Abiotic Stress Conditions: In Vitro Atmospheric Humidity on $\text{Ca}^{2+}$ Uptake

Liquid culture media adjusted to pH 5.7, before autoclaving, and with  $\text{Ca}^{2+}$  at the MS standard concentration (3.0 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) were used to determine the differential ability of the two *Prunus* rootstocks to uptake and transport  $\text{Ca}^{2+}$  from the rhizosphere to the roots and shoots. Since leaf transpiration is a determinant for  $\text{Ca}^{2+}$  uptake and mobilization to the aerial part, a high aeration regime in the GreenTray<sup>®</sup> was set for this assay, as described in [40].

#### 2.6.3. Biotic Stress Conditions: Tolerance to Fire Blight

An inoculum of fire blight (*Erwinia amylovora*) was prepared as described in [37]. After three days of adaptation to the GreenTray<sup>®</sup> under autotrophic conditions, 15 plantlets of each variety were inoculated with the bacteria with a pair of scissors (Figure 5), with two cuts in opposite sites perpendicular to the mid rib in the upper first expanded leaf. The level of affection of each plantlet was measured ten days post inoculation to determine the differential tolerance of both varieties to the bacteria, following the same levels of affection described in [37]. Briefly, susceptibility was recorded ten days post inoculation by dividing necrosis progression by shoot length. Necrosis progression was measured using the following scale: 0—no visible symptoms; 0.5—necrosis only affected veins of the inoculated leaf; 1—necrosis reached the petiole of the inoculated leaf; and 1+—necrosis reached the stem.



**Figure 5.** Inoculation of apple leaves with fire blight, grown in the GreenTray<sup>®</sup>.

### 2.7. Statistical Analysis

The experiment was set up as a completely randomized design (CRD), and the data were analysed by a one-way ANOVA. Statistical significance was judged at the level  $p < 0.05$ . When the analysis was statistically significant, a Tukey–Kramer HSD test was used for the separation of means. Data analysis was performed using JMP<sup>®</sup> software (version 16.0.0, SAS Institute Inc., Cary, NC, USA).

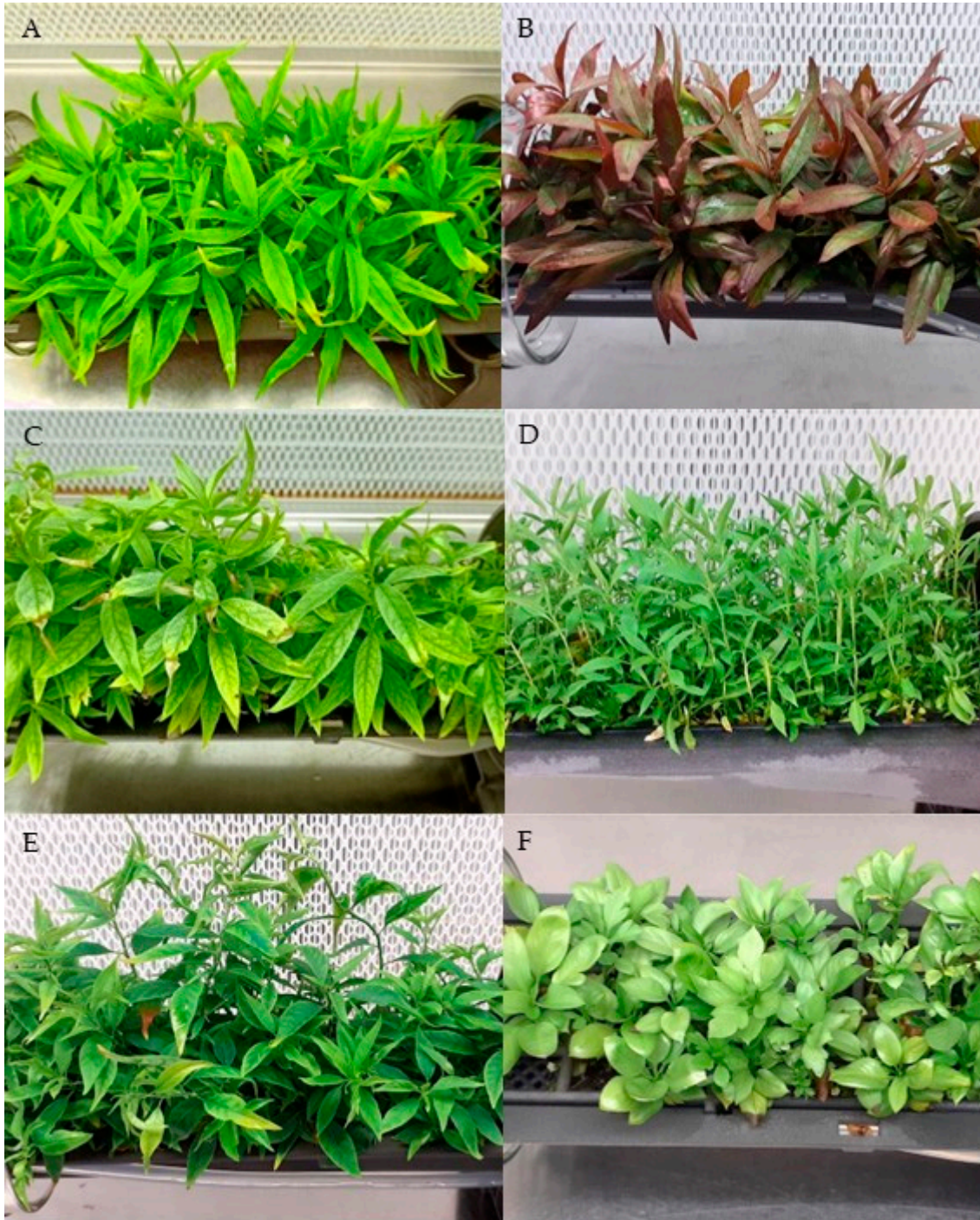
## 3. Results

### 3.1. Micropropagation

#### 3.1.1. Shoot Development

Optimal shoot development from internodal shoot explants cultured in the GreenTray<sup>®</sup> includes multiplication by axillary branching, shoot elongation, and leaf development with-

out vitrification (Figure 6). This was depended on the plant material, as well as on the frequencies of immersion and aeration, the composition of the culture medium, and the light intensity. Following the methodology described herein, a good development was accomplished for the almond  $\times$  peach (*Prunus dulcis*  $\times$  *P. persica*) hybrid rootstocks Intensia<sup>®</sup>, Garnem<sup>®</sup>, and GF677 (Figure 6A–C), as well as for the plum hybrid (*P. besseyi*  $\times$  *P. cerasifera*) rootstock Rootpac<sup>®</sup>20 and the plum  $\times$  almond hybrid (*P. cerasifera*  $\times$  *P. dulcis*) rootstock Rootpac<sup>®</sup>R (Figure 6D,E), along with the plum selection (*P. cerasifera*) rootstock Adara.



**Figure 6.** Shoot development in the GreenTray<sup>®</sup> of the *Prunus* rootstocks (A) Intensia<sup>®</sup>, (B) Garnem<sup>®</sup>, (C) GF677, (D) Rootpac<sup>®</sup> 20, (E) and Rootpac<sup>®</sup> R, and the *Pyrus* rootstock (F) OHF 87<sup>TM</sup>.



Other plant materials that showed good shoot elongation and leaf development but require more research on adjusting the culture media and immersion frequencies are the pear (*Pyrus communis*) rootstock OHF 87<sup>TM</sup> (Figure 6F) and the pear rootstock Py170.

### 3.1.2. Extraction and Division of Shoots without Forceps

With all plant materials, it was easy to extract all shoots and cut the new growth with the use of a pair of scissors, returning the shoot bases back to the culture flask of the GreenTray<sup>®</sup>, as shown in Figure 4.

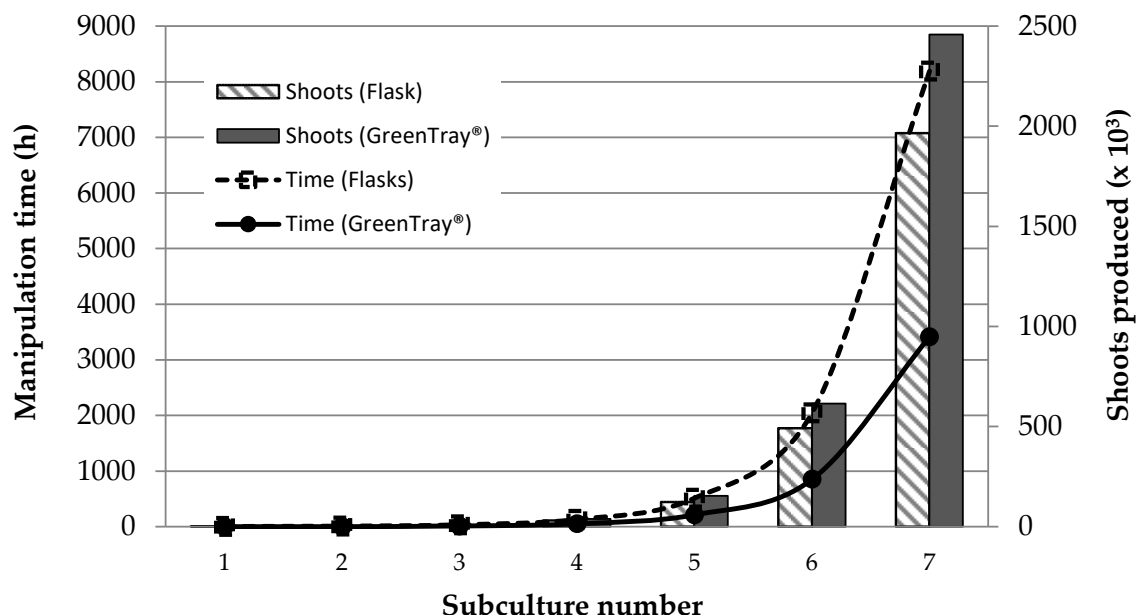
### 3.1.3. Sequential Shoot Cuts without Subculture and Multiplication Rates

All plant materials used herein had the same multiplication rates in the GreenTray<sup>®</sup> than in flasks with agar-containing medium. However, with the utilized culture media and protocols of immersion and aeration, the diverse plant materials tested had different capacities to sprout after being cut. The most productive was Rootpac<sup>®</sup> R, which was able to stand seven cuts in a 19-week period of culture, producing 650 shoots from 25 initial explants, which equals 93 shoots for each cut, with an overall multiplication rate of 26, or a multiplication rate of four for each 3-week-long culture period, equivalent to the subculture time with agar-containing medium flasks.

Rootpac<sup>®</sup> 20, GF677, and Intensia<sup>®</sup> regrew for two consecutive cuts. The *Cannabis sativa* variety “Ferimon” was also able to regrow for two consecutive cuts, while Garnem<sup>®</sup>, Adara, OHF 87<sup>TM</sup>, and Py170 could not sprout after a cut. More work is required to develop better culture media and conditions in order favor the growth after being cut.

### 3.1.4. Hand Labor Reduction and Produced Shoots during Micropropagation

As shown in Figure 7, using the GreenTray<sup>®</sup> system, there is a reduction in hand labor, accumulated along the subculture time, for the manipulation required to extract the shoots from the flask, cut them on a sterile surface, and culture them in a new flask. When using the GreenTray<sup>®</sup> to establish a new generation (Figure 4B), equivalent to a new subculture in semisolid medium, the time needed is reduced by 65% compared to that required with semisolid medium.



**Figure 7.** Time required for manipulation (lines) and number of shoots produced (bars) of Rootpac<sup>®</sup> R grown in flasks with agar-containing medium (segmented) or in GreenTray<sup>®</sup> (solid) during the first 7 subcultures.

When additional shoot cuts were made from one GreenTray<sup>®</sup>, while only one cut was possible from each flask with agar-containing medium, the time saved in the procedure was reduced and the number of shoots produced with the same number of subcultures increased in the GreenTray<sup>®</sup>. As shown in Figure 7, with a second shoot cut of the Rootpac<sup>®</sup> R, the number of shoots produced in the seventh subculture with the GreenTray<sup>®</sup> approaches  $2500 \times 10^3$ , 20% more than with the conventional semisolid-containing flasks.

### 3.1.5. Number of Generations in the GreenTray<sup>®</sup>, without Establishing from Agar Cultures

Up to six generations of the rootstock Rootpac<sup>®</sup> R were initiated, from one GreenTray<sup>®</sup> to another, without having to start from cultures maintained in agar-containing medium. The procedure was carried out as indicated in Figure 4B, and there were no contaminations in either the GreenTray<sup>®</sup> source of shoots or the newly established GreenTray<sup>®</sup>.

With other plant materials, the number of generations was lower. For Rootpac<sup>®</sup> 20, the maximum number of generations was four, while for Intensia<sup>®</sup>, Garnem<sup>®</sup>, GF667, Adara, OHE87<sup>TM</sup>, and Py170, it was possible to establish only one generation.

### 3.1.6. Autotrophic and Long-Term Cultures

In the conditions stated to promote autotrophic growth, excellent root and shoot development were obtained in the GreenTray<sup>®</sup> with rooted shoots of Rootpac<sup>®</sup> 20 and Garnem<sup>®</sup>, used for abiotic stress assays [40].

The three apple varieties, Carrandona, Florina, and Rozona, doubled their size after two weeks of culture and before inoculation with the biotic stress agent (Figure 8).



**Figure 8.** Plantlets of the apple variety Carrandona, grown under autotrophic conditions for three weeks in the GreenTray<sup>®</sup>.

*Kalanchoe daigremontiana* propagules collected from the leaves of a mature plant, established in the GreenTray<sup>®</sup>, developed and produced new propagules in their leaves, which were spontaneously dropped on the culture tray and became new mature plants, in a cycle that was repeated four times over a year-long period without needing any manipulation inside the culture flask (Figure 9). We can observe the propagules at the edges of the mature leaves and the small plants growing on the surface of the culture tray, in which the white–yellowish film observed is not a contamination but as a result of the natural degradation of the previous generation of plants.



**Figure 9.** Plantlets of *Kalanchoe daigremontiana* grown in the GreenTray<sup>®</sup> over a year-long period under autotrophic conditions.

### 3.2. Assays with Abiotic and Biotic Stress Agents

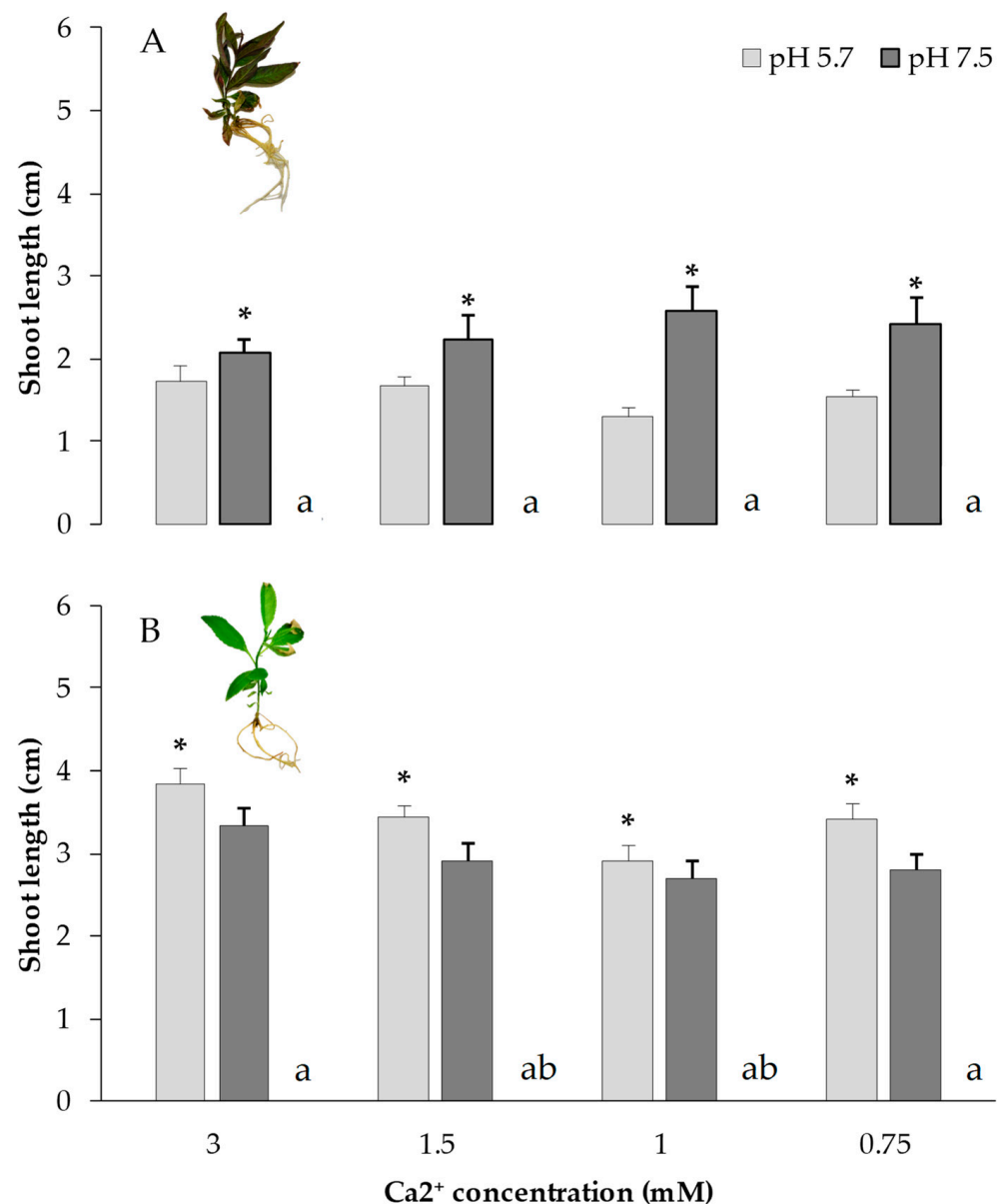
#### 3.2.1. Rhizosphere pH and Ca<sup>2+</sup> on Plantlet Growth

Both *Prunus* rootstocks behaved differently to the high pH and lower Ca<sup>2+</sup> availability in the liquid culture medium. Garnem<sup>®</sup> (Figure 10A), an almond × peach hybrid, showed a significantly ( $p < 0.001$ ) better shoot growth at basic pH 7.5 than at the conventional culture pH 5.7, without interacting with the Ca<sup>2+</sup> concentration ( $p = 0.100$ ) and without the effect of the lower Ca<sup>2+</sup> availability ( $p = 0.977$ ). Instead, the plum hybrid Rootpac<sup>®</sup> 20 (Figure 10B) was negatively affected in terms of its shoot growth when the plantlets were depleted of Ca<sup>2+</sup> ( $p = 0.001$ ) to 0.75 mM, 25% of the usual 3 mM present in the medium, and also when the pH was alkaline (7.5) compared to the conventional pH 5.7 ( $p = 0.001$ ).

#### 3.2.2. Atmospheric Humidity on Ca<sup>2+</sup> Uptake

As described in [40], GreenTray<sup>®</sup> established a TA of 1 min and a WTA of 15 min, resulting in lowering the relative humidity in the culture flask to 60% during aeration and which recovered afterwards to 80% during the WTA. Instead, the non-aerated GreenTray<sup>®</sup> had a constant 100% relative humidity.

Under these experimental conditions in the GreenTray<sup>®</sup>, the shoot Cadff (calcium derived from the fertilizer) was significantly ( $p = 0.003$ ) higher in aerated environments (47%) than in non-aerated environments (37%) [40]. This means that aeration induced Ca<sup>2+</sup> uptake at the roots and transported it to the shoots. More interestingly, the rootstock Rootpac<sup>®</sup> 20, sensible to lower Ca<sup>2+</sup> availability in the rhizosphere (Figure 10), presented a significantly higher ( $p = 0.0173$ ) shoot Cadff (46%) than Garnem<sup>®</sup> (38%), a rootstock less affected by Ca<sup>2+</sup> depletion [40].

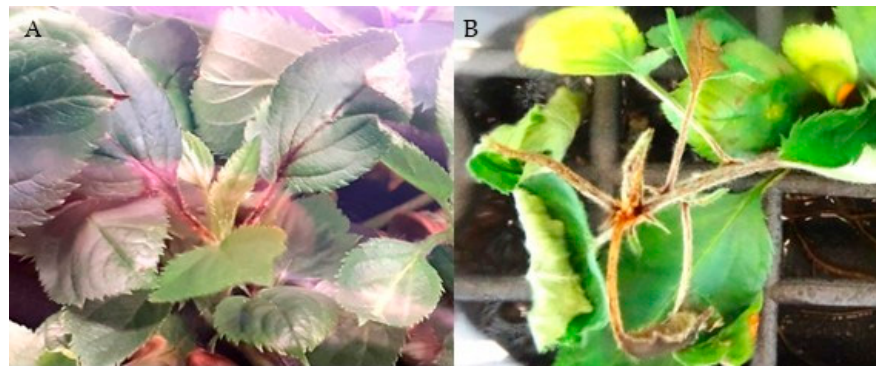


**Figure 10.** Shoot length of (A) Garnem® and (B) Rootpac® 20 plantlets cultured in the GreenTray® at pH 5.7 (light gray) or pH 7.5 (dark gray) as affected by lowering the Ca<sup>2+</sup> concentration from 3 to 1.5, 1, and 0.75 mM, in the culture medium. Mean values come from the measures of 15 replicates per genotype, pH and Ca<sup>2+</sup> concentration. Bars represent the standard error. For each rootstock, Ca<sup>2+</sup> concentrations with different letter (a,b), and pH with asterisk (\*) are significantly different (Tukey–Kramer HSD,  $p < 0.05$ ).

### 3.2.3. Tolerance to Fire Blight

For the apple plantlets inoculated as shown in Figure 5, after three days of culture in the GreenTray®, the first symptoms of infection in the leaf blade were visible through the culture flask glass. At 7 and 10 d of culture, necrosis reached the leaf mid rib (Figure 11A) and shoot (Figure 11B) of the balanced apple variety Florina.

Ten days post inoculation, the different levels of susceptibility to *Erwinia amylovora* of the three apple varieties tested, Carrandona, Florina and Rozona, were evident (Figure 12), with increasing levels of susceptibility to fire blight, respectively. Shoot and root development was clearly better for Carrandona and Florina (Figure 12A,B) than for Rozona (Figure 12C), which showed evident growth reduction, wilting, and shoot and root necrosis.

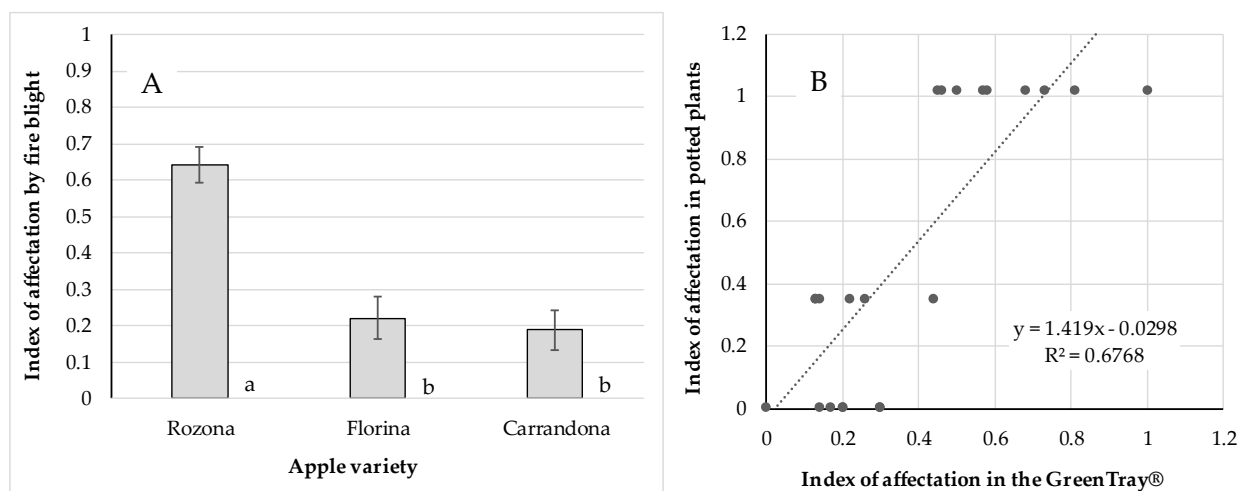


**Figure 11.** Symptoms of fire blight on (A) leaves, seven days post inoculation, and on (B) shoot and leaves, ten days post infection of the apple variety Florina grown in the GreenTray®.



**Figure 12.** Plantlet development in the GreenTray® of the apple varieties (A) Carrandona, (B) Florina, and (C) Rozona ten days post inoculation with *Erwinia amylovora*.

The affectation levels obtained in the GreenTray<sup>®</sup> (Figure 13A) showed a significant ( $p < 0.001$ ) difference among the three apple varieties. A significantly ( $p = 0.050$ ) higher tolerance to fire blight was observed for Carrandona and Florina than Rozona. With a level of  $R^2 = 0.68$  (Figure 13B), the level of susceptibility to fire blight observed in the GreenTray<sup>®</sup> was significantly ( $p < 0.0001$ ) and linearly correlated with the level of tolerance observed in potted plants grown in soil inside a climatic growth chamber of a BSL3 facility [37].



**Figure 13.** Levels of affectation by *Erwinia amylovora* on three apple varieties (A) tested for tolerance in the GreenTray<sup>®</sup>, and (B) their correlation with the known effect levels observed with potted plants. Mean values come from the measures of 15 replicates per genotype. Bars represent the standard error. Means with different letters (a, b) are significantly different (Tukey–Kramer HSD,  $p < 0.05$ ).

#### 4. Discussion

The extraction of shoots with forceps from in vitro culture containers, as well as from TIS bioreactors, followed by arranging them on sterile surfaces in order to divide them with the help of surgical scalpels and their subsequent introduction into new culture containers, a process known as subculturing in the conventional in vitro culture process, is time-consuming and represents a significant portion the hand labor involved in most in vitro plant culture processes. The components of the actual GreenTray<sup>®</sup> TIS bioreactor described herein, which is a further evolution of the ones presented in the past [31,32], make it significantly improved and versatile compared to other commercial TIS bioreactors, such as RITA<sup>®</sup>, Matis<sup>®</sup>, Plantform<sup>™</sup>, SETIS<sup>™</sup> [1–17], or the TIS developed by several in vitro plant culture laboratories oriented to solve particular propagation objectives for diverse plant species [18–28].

GreenTray provides the opportunity to automate the in vitro clonal plant propagation process because of its new methods: the disposition of the plant material, extraction from the culture vessel, easy manipulation for reorientation, shoot cutting, and reintroduction for the further growth of the shoot basis. These facts represent a significant reduction in the time required for conventional subcultures, implying a reduction in hand labor and an increase in the number of shoots produced per container, which reduce the cost per plant produced and therefore facilitates the application of micropropagation to more plant species.

Although excellent shoot development and sequential shoots cuts were obtained for Rootpac<sup>®</sup> R, Rootpac<sup>®</sup> 20, GF677, Intensia<sup>®</sup>, and Ferimon, further research is needed to improve the culture media, the frequencies of immersion, and aeration for Garnem<sup>®</sup>, Adara, OHF 87<sup>™</sup>, and Py170. Indeed, this observation is applicable to all plant species for which a great amount of experience has been accumulated during previous decades of work with semisolid culture media, compared to TIS culture systems.

As reported in previous works [41–46], autotrophic in vitro culture is facilitated by aeration in the culture vessel and increasing the PAR radiation without stressing the plant materials. GreenTray<sup>®</sup>'s design is particularly focused on improving aeration through two mechanisms, suction from the glass bottle containing the medium or the propulsion of air into the glass culture jar. PAR radiation was significantly increased with LED lights, with a variable and high range of intensities, paired with low heating effects. Aeration of the culture flask induces transpiration in the plantlet's leaves; therefore, water exits through the open stomas and CO<sub>2</sub> diffuses in, which, in return, favors photosynthesis [41].

Rooted plantlets of the *Prunus* rootstocks Rootpac<sup>®</sup>20 and Garnem<sup>®</sup>, or the apple varieties Carrandona, Florina, and Rozona, grew in the GreenTray<sup>®</sup> without sucrose in the culture medium during the biotic and abiotic stress agent assays that lasted several weeks. As an example of a long-term in vitro autotrophic culture, the *Kalanchoe daigremontiana* species demonstrated that the autotrophic culture conditions in the GreenTray<sup>®</sup> are feasible for at least one year.

GreenTray<sup>®</sup>'s design allows access to shoots or plantlets by moving the container's lid and attached tray sideways. With this simple movement, all plants are pulled out of the culture recipient and displayed in front of the observer, so they can easily be treated, inoculated, phenotyped, sampled, or pictured. By pulling the holding racks, plantlet roots can be observed, sampled, or pictured the same way as the aerial part of the plants. Since the container holding the culture medium is independent from the cultured plantlets, it can be aliquoted, supplemented, inoculated, and observed at any time during culture [39,42]. These characteristics make the GreenTray<sup>®</sup> TIS superior to other commercial TIS bioreactors and containers to perform studies on abiotic [43–46] or biotic [47–55] stress agents.

The experimental conditions to assay the effects of pH and Ca<sup>2+</sup> availability in the rhizosphere were easily established and repeated with the GreenTray<sup>®</sup> design. The differential capacities of the *Prunus* rootstocks Garnem<sup>®</sup> and Rootpac<sup>®</sup>20 to grow in alkaline conditions and Ca<sup>2+</sup> depletion, reported herein, were in accordance with what is known from agronomic trials in calcareous soils [56,57]. Therefore, GreenTray<sup>®</sup> could be useful for many nutritional studies, including the characterization and selection of plant materials [36].

Forcing a reduction in the atmospheric humidity inside the GreenTray<sup>®</sup>'s culture vessel, although periodic and recovered rapidly through the evaporation of wet surfaces and transpiration through the plantlet leaf stomas, implies a lower water potential, which induces higher transpiration of the plantlet's leaves and a higher water uptake at the roots, reinforcing Ca<sup>2+</sup> transport to the shoots [41]. In these conditions, the rootstock Rootpac<sup>®</sup> 20 presented a significantly higher shoot Ca<sup>2+</sup> uptake than Garnem<sup>®</sup> [40]. All mechanisms of ventilation inside the bioreactor, for which the GreenTray<sup>®</sup> is especially suited for, increase the water potential in the atmospheric culture recipient, and this favors water and nutrient root uptake and translocation to the leaves.

In vitro assays for tolerance to *Erwinia amylovora* have always been approached in heterotrophic conditions [47–54]. Although the results presented herein are for a reduced number of apple varieties, this is the first time that the test has been performed in the in vitro autotrophic conditions of the GreenTray<sup>®</sup>, and the same plant material was tested against the same *Erwinia amylovora* strain in a confined growth chamber with grafted potted plants. Since a very good correlation was found in terms of susceptibility to fire blight between the in vitro and in vivo growth, it is recommendable to use the GreenTray<sup>®</sup> bioreactor to select seedlings and obtain apple or pear varieties with higher tolerance to fire blight through cross-breeding. Further improvements will be needed in inoculating all plants with the same amount and concentration of the bacterial strain or obtaining a more uniform plant development before their inoculation.

Consequently, the GreenTray<sup>®</sup>, as it is developed, is a good tool to propagate plants in vitro at a lower cost and higher multiplication rates. Its design favors the in vitro growth of plantlets in autotrophic conditions, like hydroponic cultures, but in a highly controlled environment and with reduced space requirements. Bioassays performed in the GreenTray<sup>®</sup> to characterize the responses of plants to biotic and abiotic stress agents are more reliable

than those performed in semisolid medium because of the autotrophic plant development conditions favored in this TIS bioreactor. Other plant in vitro culture methodologies applied to plant breeding, such as immature embryo rescue, somatic embryo maturation, adventitious regeneration, genetic transformation, physiological characterization, plant–pathogen interactions, and plant growth-promoting microorganism interactions with plants, can be studied under in vitro conditions with the GreenTray® as a tool [39,42].

**Author Contributions:** Conceptualization, R.D.-S., N.T. and F.C.-C.; methodology, R.D.-S., F.C.-C., E.T., C.S. and N.T.; validation, R.D.-S., F.C.-C. and C.S.; formal analysis, R.D.-S. and F.C.-C.; investigation, R.D.-S., F.C.-C., E.T., J.R. and N.T.; resources, R.D.-S., M.C., S.F., E.A., F.C.-C. and C.S.; data curation, R.D.-S. and F.C.-C.; writing—original draft preparation, R.D.-S. and F.C.-C. writing—review and editing, R.D.-S. and F.C.-C.; visualization, R.D.-S. and M.C.; supervision, R.D.-S.; project administration, R.D.-S., M.C., E.T., J.R. and N.T.; funding acquisition R.D.-S., M.C., E.T., J.R. and N.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by The CERCA Programme/Generalitat de Catalunya, IRTA, the AGAUR grant Producte 2019 Knowledge Industry number IU68-017253, the DACAAR grant number DEMO 56 30080 2021 2A, and the “Ministerio de Ciencia e Innovación” grant number PID2019-111583RR-I00.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author due to patent pending applications on some of the processes described.

**Acknowledgments:** We acknowledge the support given by Carlos R. Mendoza-Morales (Madre Tierra Puebla, Mexico), John Amin (Intech3D), Francisco Sánchez (DICOMOL), Miguel Reyes (IL-ERFRED), Francisco Contreras (GEALED), Enrique Dapena (SERIDA), Xavier Abad (IRTA-CReSA), and Buenaventura Begue (IRTA Fruitcentre) for their technical support and materials used for the present work.

**Conflicts of Interest:** The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## References

1. Etienne, H.; Bertrand, B.; Georget, F.; Lartaud, M.; Montes, F.; Dechamp, E.; Verdeil, J.-L.; Barry-Etienne, D. Development of coffee somatic and zygotic embryos to plants differs in the morphological, histochemical and hydration aspects. *Tree Physiol.* **2013**, *33*, 640–653. [[CrossRef](#)]
2. Ramírez-Mosqueda, M.A.; Iglesias-Andreu, L.G. Evaluation of different temporary immersion systems (BIT®, BIG, and RITA®) in the micropropagation of *Vanilla planifolia* Jacks. *In Vitro. Cell. Dev. Biol.—Plant* **2016**, *52*, 154–160. [[CrossRef](#)]
3. Bayraktar, M. Micropropagation of *Stevia rebaudiana* Bertoni Using RITA® Bioreactor. *HortScience* **2019**, *54*, 725–731. [[CrossRef](#)]
4. Nasri, A.; Baklouti, E.; Ben Romdhane, A.; Maalej, M.; Schumacher, H.M.; Drira, N.; Fki, L. Large-scale propagation of *Myrobolan* (*Prunus cerasifera*) in RITA® bioreactors and ISSR-based assessment of genetic conformity. *Sci. Hortic.* **2019**, *245*, 144–153. [[CrossRef](#)]
5. Melviana, A.C.; Esyanti, R.R.; Mel, M.; Setyobudi, R.H. Biomass Enhancement of *Stevia rebaudiana* Bertoni Shoot Culture in Temporary Immersion System (TIS) RITA® Bioreactor Optimized in Two Different Immersion Periods. *E3S Web Conf.* **2021**, *226*, 00007. [[CrossRef](#)]
6. Gago, D.; Vilavert, S.; Bernal, M.; Sánchez, C.; Aldrey, A.; Vidal, N. The Effect of Sucrose Supplementation on the Micropropagation of *Salix viminalis* L. Shoots in Semisolid Medium and Temporary Immersion Bioreactors. *Forests* **2021**, *12*, 1408. [[CrossRef](#)]
7. Peña-Rojas, G.; Carhuaz-Condori, R.; Andía-Ayme, V.; Leon, V.A.; Herrera-Calderon, O. Improved Production of *Mashua* (*Tropaeolum tuberosum*) Microtubers MAC-3 Morphotype in Liquid Medium Using Temporary Immersion System (TIS-RITA®). *Agriculture* **2022**, *12*, 943. [[CrossRef](#)]
8. Benelli, C.; De Carlo, A. In vitro multiplication and growth improvement of *Olea europaea* L. cv Canino with temporary immersion system (Plantform™). *3 Biotech* **2018**, *8*, 317. [[CrossRef](#)]
9. Skrzypczak-Pietraszek, E.; Urbańska, A.; Żmudzki, P.; Pietraszek, J. Elicitation with methyl jasmonate combined with cultivation in the Plantform™ temporary immersion bioreactor highly increases the accumulation of selected centellosides and phenolics in *Centella asiatica* (L.) Urban shoot culture. *Eng. Life Sci.* **2019**, *19*, 931–943. [[CrossRef](#)]



10. Kaçar, Y.A.; Biçen, B.; Şimşek, Ö.Z.H.A.N.; Dönmez, D.; Erol, M. Evaluation and Comparison of a New Type of Temporary Immersion System (Tis) Bioreactors for Myrtle (*Myrtus Communis* L.). *Appl. Ecol. Environ. Res.* **2020**, *18*, 1611–1620. [[CrossRef](#)]
11. Klimek-Szczykutowicz, M.; Dziurka, M.; Blažević, I.; Đulović, A.; Miazga-Karska, M.; Klimek, K.; Ekiert, H.; Szopa, A. Precursor-Boosted Production of Metabolites in *Nasturtium officinale* Microshoots Grown in Platform Bioreactors, and Antioxidant and Antimicrobial Activities of Biomass Extracts. *Molecules* **2021**, *26*, 4660. [[CrossRef](#)] [[PubMed](#)]
12. Ozudogru, E.A.; Karlik, E.; Elazab, D.; Lambardi, M. Establishment of Direct Organogenesis Protocol for *Arachis hypogaea* cv. Virginia in Liquid Medium by Temporary Immersion System (TIS). *Horticulturae* **2022**, *8*, 1129. [[CrossRef](#)]
13. Grzegorzczak-Karolak, I.; Staniewska, P.; Lebelt, L.; Piotrowska, D.G. Optimization of cultivation conditions of *Salvia viridis* L. shoots in the Platform bioreactor to increase polyphenol production. *Plant Cell Tissue Organ Cult.* **2022**, *149*, 269–280. [[CrossRef](#)]
14. Şimşek, Ö.; Dönmez, D.; Sarıdaş, M.A.; Acar, E.; Kaçar, Y.A.; Kargı, S.P.; İzgü, T. In vitro and ex vitro propagation of Turkish myrtles through conventional and platform bioreactor systems. *PeerJ* **2023**, *11*, e16061. [[CrossRef](#)] [[PubMed](#)]
15. Ramírez-Mosqueda, M.A.; Bello-Bello, J.J. SETIS™ bioreactor increases in vitro multiplication and shoot length in vanilla (*Vanilla planifolia* Jacks. Ex Andrews). *Acta Physiol. Plant.* **2021**, *43*, 1–8. [[CrossRef](#)]
16. Vendrame, W.A.; Xu, J.; Beleski, D.G. Micropropagation of *Brassavola nodosa* (L.) Lindl. using SETIS™ bioreactor. *Plant Cell Tissue Organ Cult.* **2023**, *153*, 67–76. [[CrossRef](#)]
17. Méndez-Hernández, H.A.; Galaz-Ávalos, R.M.; Quintana-Escobar, A.O.; Pech-Hoil, R.; Collí-Rodríguez, A.M.; Salas-Peraza, I.Q.; Loyola-Vargas, V.M. In Vitro Conversion of *Coffea* spp. Somatic Embryos in SETIS™ Bioreactor System. *Plants* **2023**, *12*, 3055. [[CrossRef](#)]
18. Moon, K.H.; Honda, H.; Kobayashi, T. Development of a bioreactor suitable for embryogenic rice callus culture. *J. Biosci. Bioeng.* **1999**, *87*, 661–665. [[CrossRef](#)] [[PubMed](#)]
19. Kim, Y.; Wyslouzil, B.E.; Weathers, P.J. Secondary metabolism of hairy root cultures in bioreactors. *In Vitro. Cell. Dev. Biol.—Plant* **2002**, *38*, 1–10. [[CrossRef](#)]
20. Hvoslef-Eide, A.K.; Olsen, O.A.S.; Lyngved, R.; Munster, C.; Heyerdahl, P.H. Bioreactor design for propagation of somatic embryos. *Plant Cell Tissue Organ Cult.* **2005**, *81*, 265–276. [[CrossRef](#)]
21. Berkov, S.; Ivanov, I.; Georgiev, V.; Codina, C.; Pavlov, A. Galanthamine biosynthesis in plant in vitro systems. *Eng. Life Sci.* **2014**, *14*, 643–650. [[CrossRef](#)]
22. Georgiev, V.; Schumann, A.; Pavlov, A.; Bley, T. Temporary immersion systems in plant biotechnology. *Eng. Life Sci.* **2014**, *14*, 607–621. [[CrossRef](#)]
23. Wilken, D.; Gonzalez, E.J.; Gerth, A.; Gómez-Kosky, R.; Schumann, A.; Claus, D. Effect of immersion systems, lighting, and TIS designs on biomass increase in micropropagating banana (*Musa* spp. cv. ‘Grande naine’ AAA). *In Vitro. Cell. Dev. Biol.—Plant* **2014**, *50*, 582–589. [[CrossRef](#)]
24. Godoy, S.; Tapia, E.; Seit, P.; Andrade, D.; Sánchez, E.; Andrade, P.; Almeida, A.M.; Prieto, H. Temporary immersion systems for the mass propagation of sweet cherry cultivars and cherry rootstocks: Development of a micropropagation procedure and effect of culture conditions on plant quality. *In Vitro. Cell. Dev. Biol.—Plant* **2017**, *53*, 494–504. [[CrossRef](#)]
25. Ramírez-Mosqueda, M.A.; Iglesias-Andreu, L.G.; Favián-Vega, E.; da Silva, J.A.T.; Leyva-Ovalle, O.R.; Murguía-González, J. Morphogenetic stability of variegated *Vanilla planifolia* Jacks. plants micropropagated in a temporary immersion system (TIB®). *Rend. Lincei. Sci. Fis. Nat.* **2019**, *30*, 603–609. [[CrossRef](#)]
26. Ayub, R.A.; Pereira, A.B.; dos Santos, J.N.; da Silva, D.M.; Pessenti, I.L. Sucrose concentration and blueberry plant density in temporary immersion systems (TIS). *Rev. Bras. Frutic.* **2021**, *43*. [[CrossRef](#)]
27. Gautam, S.; Solis-Gracia, N.; Teale, M.K.; Mandadi, K.; da Silva, J.A.; Vales, M.I. Development of an in vitro Microtuberization and Temporary Immersion Bioreactor System to Evaluate Heat Stress Tolerance in Potatoes (*Solanum tuberosum* L.). *Front. Plant Sci.* **2021**, *12*, 700328. [[CrossRef](#)] [[PubMed](#)]
28. Wang, M.; Wang, M.; An, D.; Wu, C.-H.; Li, G.; Wang, W.; Zhang, X.-S.; Lian, M.-L. Establishment of adventitious root culture system of *Cynanchum wilfordii* in air-lift bioreactors for the efficient production of bioactive compounds. *In Vitro. Cell. Dev. Biol.—Plant* **2023**, *59*, 216–226. [[CrossRef](#)]
29. Dolcet-Sanjuan, R.; Mendoza, C. Reactor System for the In Vitro Culture of Plant Material, Kit to Transform a Receptacle in an Adequate Reactor for Such System, and Method for the In Vitro Culture of Plant Material with Such Reactor System. Patent No. ES2763637B1, 29 November 2018.
30. Dolcet-Sanjuan, R.; Mendoza, C.R. Reactor System for In Vitro Culture of plant Material, Kit for Transforming a Receptacle into a Reactor Suitable for the System and Method for In Vitro Culture of Plant Material Using the Reactor System. Priority date: 29 November 2018. WIPO PCT WO 2020/109637 A1, 4 June 2020.
31. Mendoza, C.; Dolcet-Sanjuan, R. A temporary immersion system (TIS) bioreactor used for the in vitro propagation of *Prunus* and *Pyrus* rootstocks. In *Agrárias: Pesquisa e Inovação nas Ciências que Alimentam o Mundo VIII*; Oliveira, A., Mocellin, V., Eds.; Editora Artemis: Curitiba-PR, Brazil, 2022; pp. 110–124.
32. Morales, C.R.M.; Dolcet-Sanjuan, R.; Caballero, M.L.O.; Sánchez, M.I.L.; Angel, M.B.V.; Antonio, D.I.R. Micropropagación de *Agave marmorata* utilizando un Nuevo Sistema de Inmersión Temporal. *Rev. Cienc. Tecnol. Soc.* **2022**, *10*, 10–16.
33. Pinochet, J. A Plum-almond Hybrid Rootstock for Replant Situations. *HortScience* **2010**, *45*, 299–301. [[CrossRef](#)]
34. Simard, M.; Michelesi, J.; Masseron, A. Pear Rootstock Breeding in France. *Acta Hort.* **2004**, *658*, 535–540. [[CrossRef](#)]

35. Claveria, E.; Asín, L.; Iglesias, I.; Vilardell, P.; Bonany, J.; Simard, M.; Dolcet-Sanjuan, R. In Vitro Screening for Tolerance to Iron Chlorosis as a Reliable Selection Tool in a Pear Rootstock Breeding Program. *Acta Hort.* **2012**, *935*, 199–205. [[CrossRef](#)]
36. Mora-Córdova, C.P.; Tolrà, R.; Padilla, R.; Poschenrieder, C.; Simard, M.-H.; Asín, L.; Vilardell, P.; Bonany, J.; Claveria, E.; Dolcet-Sanjuan, R. Rhizosphere Acidification as the Main Trait Characterizing the Differential In Vitro Tolerance to Iron Chlorosis in Interspecific *Pyrus* Hybrids. *Horticulturae* **2022**, *8*, 551. [[CrossRef](#)]
37. García-Fernández, B.; Dolcet-Sanjuan, R.; Micheletti, D.; Antón-Díaz, M.J.; Solsona, C.; Fernández, M.; Abad, X.; Dapena, E. Susceptibility Evaluation to Fire Blight and Genome-Wide Associations within a Collection of Asturian Apple Accessions. *Plants* **2023**, *12*, 4068. [[CrossRef](#)] [[PubMed](#)]
38. Cantabella, D.; Teixidó, N.; Segarra, G.; Torres, R.; Casanovas, M.; Dolcet-Sanjuan, R. Rhizosphere microorganisms enhance in vitro root and plantlet development of *Pyrus* and *Prunus* rootstocks. *Planta* **2021**, *253*, 1–11. [[CrossRef](#)]
39. Cantabella, D.; Teixidó, N.; Solsona, C.; Casanovas, M.; Torres, R.; Dolcet-Sanjuan, R. Acidification of the culture medium as a strategy to control endophytic contaminations in *Prunus* spp. rootstocks cultured in GreenTray TIS bioreactor. *Sci. Hort.* **2021**, *290*, 110521. [[CrossRef](#)]
40. Carrasco-Cuello, F.; Jené, L.; Dolcet-Sanjuan, R.; Quiñones, A.; Rufat, J.; Torres, E. Differential response to calcium-labelled (<sup>44</sup>Ca) uptake and allocation in two peach rootstocks in relation to transpiration under in vitro conditions. *Sci. Hort.* **2024**, *326*, 112718. [[CrossRef](#)]
41. Girona, J.; Villar, J.M. Aigua i producció d'aliments. Per què els cultius necessiten aigua? *Quaderns Agrari* **2021**, *50*, 59–85. [[CrossRef](#)]
42. Cantabella, D.; Mendoza, C.R.; Teixidó, N.; Vilaró, F.; Torres, R.; Dolcet-Sanjuan, R. GreenTray® TIS bioreactor as an effective in vitro culture system for the micropropagation of *Prunus* spp. rootstocks and analysis of the plant-PGPMs interactions. *Sci. Hort.* **2022**, *291*, 110622. [[CrossRef](#)]
43. Hsissou, D.; Bouharmont, J. In vitro selection and characterization of drought-tolerant plants of durum wheat (*Triticum durum* Desf). *Agronomie* **1994**, *14*, 65–70. [[CrossRef](#)]
44. Gopal, J.; Iwama, K. In vitro screening of potato against water-stress mediated through sorbitol and polyethylene glycol. *Plant Cell Rep.* **2007**, *26*, 693–700. [[CrossRef](#)] [[PubMed](#)]
45. Asthana, P.; Rai, M.K.; Jaiswal, U. In vitro selection, regeneration and characterization of NaCl-tolerant plants of *Sapindus trifoliatus*: An important multipurpose tree. *Plant Cell Tissue Organ Cult.* **2023**, *154*, 227–238. [[CrossRef](#)]
46. Sahu, M.; Maurya, S.; Jha, Z. In vitro selection for drought and salt stress tolerance in rice: An overview. *Plant Physiol. Rep.* **2023**, *28*, 8–33. [[CrossRef](#)]
47. Viseur, J.; Figueroa, T.M. In Vitro Co-Culture as a tool for the evaluation of fire blight resistance in pears and apples. *Acta Hort.* **1987**, *217*, 273–282. [[CrossRef](#)]
48. Abdollahi, H.; Rugini, E.; Ruzzi, M.; Muleo, R. In vitro system for studying the interaction between *Erwinia amylovora* and genotypes of pear. *Plant Cell Tissue Organ Cult.* **2004**, *79*, 203–212. [[CrossRef](#)]
49. Švábová, L.; Lebeda, A. In Vitro Selection for Improved Plant Resistance to Toxin-Producing Pathogens. *J. Phytopathol.* **2005**, *153*, 52–64. [[CrossRef](#)]
50. Loreti, S.; Bosco, A.; Gallelli, A.; Damiano, C.; Tonelli, M.; Caboni, E. Factors Affecting In Vitro Evaluation of Resistance to *Erwinia Amylovora* in Pear Genotypes. *Acta Hort.* **2008**, *800*, 885–890. [[CrossRef](#)]
51. Hevesi, M.; Végh, A.; Tóth, M.; Benczur, E.; Palkovics, L. Investigating the Virulence of *Erwinia Amylovora* Isolates by Using Apple Tissue Culture and Pear Fruit. *Acta Hort.* **2011**, *896*, 223–230. [[CrossRef](#)]
52. Sedlak, J.; Paprstein, F.; Korba, J.; Sillerova, J. Development of In Vitro System for Testing of Pome Fruit Resistance to Fire Blight. *Acta Hort.* **2011**, *896*, 375–379. [[CrossRef](#)]
53. Paprstein, F.; Sedlak, J.; Sillerova, J.; Korba, J. In Vitro Evaluation of Cultivar Resistance to Fire Blight. *Acta Hort.* **2014**, *1056*, 259–262. [[CrossRef](#)]
54. Sedlák, J.; Paprštejn, F.; Korba, J.; Šillerová, J. Development of a system for testing apple resistance to *Erwinia amylovora* using in vitro culture techniques. *Plant Prot. Sci.* **2015**, *51*, 1–5. [[CrossRef](#)]
55. Sharma, V.; Thakur, M.; Tomar, M. In vitro selection of gamma irradiated shoots of ginger (*Zingiber officinale* Rosc.) against *Fusarium oxysporum* f.sp. zingiberi and molecular analysis of the resistant plants. *Plant Cell Tissue Organ Cult.* **2020**, *143*, 319–58330. [[CrossRef](#)]
56. Jiménez, S.; Pinochet, J.; Romero, J.; Gogorcena, Y.; Moreno, M.; Espada, J.L. Performance of peach and plum based rootstocks of different vigour on a late peach cultivar in replant and calcareous conditions. *Sci. Hort.* **2011**, *129*, 58–63. [[CrossRef](#)]
57. Yahmed, J.B.; Ghrab, M.; Mimoun, M.B. Eco-physiological evaluation of different scion-rootstock combinations of almond grown in Mediterranean conditions. *Fruits* **2016**, *71*, 185–193. [[CrossRef](#)]

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.