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In Vitro Micropropagation, Rooting and Acclimatization of Two *Agastache* Species (*A. aurantiaca* and *A. mexicana*)

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Abstract: *Agastache aurantiaca* 'Sunset Yellow' and *A. mexicana* 'Sangria' are aromatic plants with edible flowers characterized with good flavors, nutritional values and pleasant aromas. The aim of this study was to establish a successful protocol to ensure fast shoot propagation, rooting and in vivo acclimatization to obtain many vigorous plants for edible flower production. Micropropagation was tested with an agarized Murashige and Skoog (MS) medium enriched with different 6-benzylamino purine (BAP) levels. In vitro rooting trials were carried out using a half-strength MS medium comparing the traditional jar method with an agarized medium and the temporary immersion system (TIS) bioreactor with a liquid medium. After acclimatization in a greenhouse, the survival of plants and their development were recorded. Microscopical analyses were performed on both in-jar and in-TIS leaves at rooting stages and compared with in vivo leaves. In both species, the greatest number of new shoots was noted at 2.22 µM and 3.33 µM of BAP. In TIS, rooting started earlier, resulting in larger plants with more roots and longer roots than in the jar substrate. Any anatomical difference was observed in leaves collected from the jar and TIS during the rooting stage. The best acclimatization performances were recorded in plants coming from the TIS bioreactor.

Keywords: *Agastache*; micropropagation; temporary immersion system bioreactor; rooting; acclimatization; edible flowers

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1. Introduction

Edible flowers are eaten by people of various cultures and are becoming increasingly successful as a font of nutritional compounds and original tastes, and more and more studies are examining new flowers to bring to the market [1,2]. Many plants belonging to the Lamiaceae family produce many aromatic edible flowers with a very variable taste both from the species and from the variety of the same species [3] and with low risks of triggering allergies [4]. The *Agastache* (Gronov.) genus (Lamiaceae family; Nepetoides sub-family) includes perennial aromatic plants originating in North America and only one species (*A. rugosa*) in Eastern Asia [5,6]. A taxonomic study based on the shape and arrangement of the stamens divided the genus in two sections with different distribution: the *Agastache* section is present in the mesic areas of the Mississippi drainage, the Pacific slope and Asia; the *Brittonastrum* section is indigenous to arid areas of Mexico and southwestern USA [7]. Some characteristics are common in all *Agastache* species: stems are square, simple or branched, and sometimes woody at the base; the internodes are often elongated, especially the upper ones; the leaves are entire, petiolate, opposite, and have mainly ovate- or crenate-serrated leaf blades; the inflorescences are located in terminal tips and contain many small blossoms that open over several months; the flowers are in sessile or pedunculated verticils;

calyces are whitish or colored; and the tubular corollas are white, yellow, rose, red-blue or violet [5]. Both *A. aurantiaca* (A. Gray) Lint & Epling and *A. mexicana* (Kunth) Lint & Epling belong to the *Brittonastrum* section. The inflorescence is sometimes evenly spiked; the stalks are pedunculated and evident; the lateral lobes of the corolla are more closely joined to the upper lip than to the lower; the orifice is more oblique; tubes are glabrous within the plant; the four stamens are parallel; and the pairs are not crossing and they thrust similarly out from the corolla [5]. *A. aurantiaca* has felt-like (minute hairs) on lower surfaces of leaves, calyx tubes usually more than 3 mm and a yellow corolla 20–30 mm in length. *A. mexicana* has glabrous lower-surface leaves or pubescent leaves with evident individual hairs, a calix from 3.5 mm to 6 mm and a pink to violet corolla longer than 19 mm [5].

Like all *Agastache* species, *A. aurantiaca* and *A. mexicana* have attracted particular interest due to their good flavor and very pleasant scent reminiscent of mint, anise, lemon and licorice [8,9], which can be maintained after drying [10]. Young shoots, leaves and flowers were traditionally consumed for their content of biologically active substances (e.g., phenols, flavonoids and terpenes), and in particular, the secondary compounds of *A. mexicana* have antidiabetic, antihypertensive, antihyperlipidemic and cytotoxic effects [11]. The abundance of nectar produced by the flowers of *Agastache* species contributes to the sweetness of the flowers and attracts bees and pollinating insects [6]. Moreover, the edible flowers of *Agastache* species have high ornamental value and are already traditionally used in Mexican cuisine [12,13]. Flowers of two varieties, *A. aurantiaca* 'Sunset Yellow' and *A. mexicana* 'Sangria', were recently investigated for nutritional values, the aroma, essential oil profiles [14] and the content of microelements (only for the 'Sunset Yellow' variety) [15].

The two varieties previously investigated in the other studies gave us propagation problems because the spring cuttings give roots with difficulty because they are thin; the preparation of summer cuttings results in the loss of part of the flower production, and the plants obtained during the first year are not very vigorous and the rhizome division in spring limits the number of obtainable plants. Vegetative propagation (cuttings or division of rhizomes) is the traditional method used to multiply *Agastache* species and varieties because seed viability is low [13,16]. These limitations can be overcome with in vitro micropropagation, which allows for both the conservation and extensive propagation of germplasm. A protocol for in vitro micropropagation with the aim of increasing the production of secondary compounds has already been tested for *A. mexicana* [12], while protocols for an in vitro culture of *A. aurantiaca* have not been developed. In addition, information regarding the rooting of *Agastache* and the success of its acclimatization is scarce. Indeed, Carmona Castro et al. [12] focused their study on the production of secondary compounds from in vitro biomass and obtained poor results during the acclimatization phase: 33% of plants survived.

The aim of this work is focused on establishing an effective protocol for the in vitro micropropagation of two *Agastache* varieties, *A. aurantiaca* 'Sunset Yellow' and *A. mexicana* 'Sangria', setting up a rooting protocol, leading to the production of vigorous plants with rapid development after acclimatization. In this study, two in vitro rooting methods were evaluated: the traditional method that involves an agar medium in a jar, and a liquid culture in a TIS (temporary immersion system) bioreactor, previously used for species with difficult rooting [17]. Additionally, the morpho-anatomical parameters of the leaves were studied to establish whether they can affect the performance during the acclimatization phase.

2. Materials and Methods

2.1. Plant Material, Surface Sterilization and In Vitro Establishment

Agastache aurantiaca 'Sunset Yellow' and *A. mexicana* 'Sangria' plants were delivered by the Chambre d'Agriculture des Alpes Maritimes (CREAM, Nice, France) and were grown in pots (30 cm in diameter, capacity: 9 L of volume) filled with a commercial peaty:pumice (7:3) substrate (Hochmoor–Terflor, Capriolo, Italy) at the Research Centre for Vegetable and Ornamental Crops (CREA, Sanremo, Italy; GPS: 43.816887, 7.758900) and cultivated as described by Najar et al. [14]. The in vitro establishment was performed as reported by

Copetta et al. [17]. In summary, microcuttings (1 cm) with 2–4 nodal buds and without leaves were washed with tap water, and superficially disinfected with 1.5% NaClO for 20 min. After rinsing twice with sterile deionized water for 10 min, *Agastache* microcuttings were vertically positioned in glass jars with an agarized (0.75%) MS salt and vitamin medium [18] enriched with 1.33 µM of 6-benzyl-aminopurine (BAP), 3% sucrose and pH equal to 5.70 ± 0.01. The jars were sterilized at 121 °C for 20 min in an autoclave. The jars with microcuttings were placed in a culture chamber under 209 ± 5 µmol m^{-2} s^{-1} light irradiances at the jar level (white fluorescent Philips Master TL-D 36W/840 lamp) at 23 ± 1 °C (16/8 h light/dark cycle). After 2 weeks of culturing, the number of viable and aseptic microcuttings was recorded. Only new shoots from axillary buds of the aseptic microcuttings were used to prepare the explants (1 cm portion of nodal stem with two opposite leaves and two axillary buds) and to induce shoot development. Every 4 weeks, in vitro *Agastache* shoots were subcultured into a fresh medium. The experimental trials were carried out when enough vegetative material was reached.

2.2. Multiple-Shoot Induction

For multiple-shoot inductions, the sterile explants were transferred to jars (85 mm height; 60 mm diameter; and 280 mL total volume—Figure 1) with 30 mL of the solid substrate consisting of MS salts and vitamins [18], 0.75% agar, 3% sucrose, (pH 5.8) and BAP at different doses (0.00 µM, 1.11 µM, 2.22 µM, 3.33 µM and 4.44 µM). Three explants were placed in each jar (Figure 1). Overall, for each treatment and for each variety, fifteen microcuttings were grown for a total of 150 explants (75 explants for both *A. aurantiaca* and *A. mexicana*). The glass jars were positioned in the culture chamber (see conditions described in the previous paragraph). Four weeks later, the plants obtained from the explants were collected and the plant height and the number of shoots and leaves were recorded. Finally, the number of explants obtained from each shoot cluster was evaluated (1 cm stems with at least two opposite leaf buds without malformations) and also the signs of hyperhydricity. The shoot induction trials were performed twice.

Figure 1. In vitro culture of *A. aurantiaca* explants in jar for multiple-shoot induction trial.

2.3. Rooting Induction with Agarized and TIS Culture Systems

For rooting induction trials, explants of *A. aurantiaca* and *A. mexicana* deriving from the in vitro culture on a substrate MS without BA were used. The explants consisted of 1.5 cm shoot tips with two opposite leaves and axillary buds. Two culture methods were compared. The first method was traditional (jar with agar medium); the second method was the TIS system (liquid substrate bioreactor) as reported by Copetta et al. [17] with little variations. The explants were transferred into jars (85 mm height, 85 mm diameter and 400 mL volume) with a 60 mL rooting substrate (half-strength MS salts, full-vitamin MS, 3% saccharose and 0.75% agar (*w*/*v*) (solid medium) or into a Plantform™ bioreactor (www.plantform.se,

Sweden): a polypropylene basket (180 × 150 × 160 mm, length × width × height) with a 240 mL liquid rooting medium (Figure 2). In each test, one Plantform bioreactor with forty shoots and four jars containing ten shoots each were set up. This measure allowed the comparison of the same number of explants per treatment. In the traditional system (jars), the explants were permanently in contact with the agarized medium, while for the Plantform™, a submerging time (3 min/3 h) was set up. To maintain sterility, the insufflated air was filtered with 0.20 µm pore filters. Both solid (jars) and liquid (Plantform) systems were positioned in a phytotron at the same distance from the light source (see conditions previously reported). Explant rooting was visually controlled weekly. After 4 weeks of culturing, the fresh weight and the number of shoots, the plant height, the number of leaves, the root number per plant and the length of the more extended root per plant were recorded. The in vitro rooting trails were performed twice.

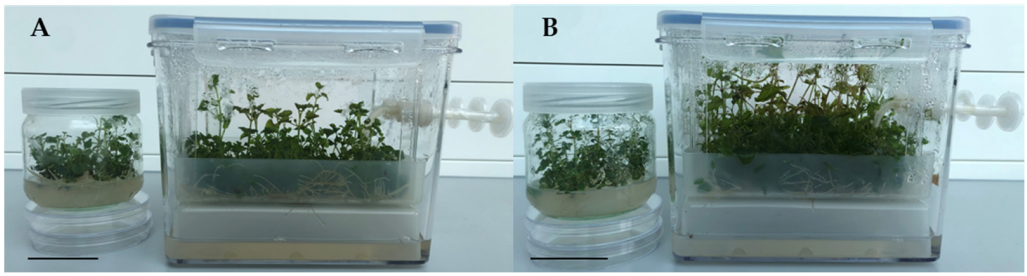

Figure 2. *A. aurantiaca* (**A**) and *A. mexicana* (**B**) in vitro rooting in jars (**left**) and Plantform™ (**right**) after 4 weeks of culturing (bars = 5 cm).

2.4. Acclimatization Tests

Forty rooted clusters of *A. aurantiaca* per treatment (from both jars and Plantform bioreactor) were transplanted to alveolus containers (60 holes) with a peat:agriperlite substrate (1:1) and transferred to a greenhouse with a mist irrigation system (Figure 3). The same method was carried out in parallel with *A. mexicana*. After 3 weeks of culturing in the greenhouse, the survival percentage of the acclimatized *Agastache* was calculated, the maximum length of the shoot clusters was measured and, finally, the number of well-developed shoots (length > 10 mm) and the number of short shoots (length < 10 mm) were counted. The acclimatization tests were performed twice: the first time in September 2022 and the second time in July 2023.

2.5. Leaf Morphological and Anatomical Examination

Well-developed leaves from plants of *A. aurantiaca* and *A. mexicana* rooted in a solid and liquid system were collected and examined using light microscopy as reported in Copetta et al. [17] and compared with in vivo leaves. Briefly, three leaves per treatment were clarified with drops of a Visikol solution (2B Scientific Limited, Upper Heyford, UK): the leaves on slides were placed on a hot plate until the leaf blade was discolored.

Additional mature leaves were fixed overnight (4% paraformaldehyde solution and 0.1 M phosphate buffer). Then, the leaves were cleaned with a phosphate buffer, dehydrated in the ethanol sequence and embedded in paraffin (Paraplast, Merck, Germany). The leaf cross-sections (10 µm thickness) were prepared by cutting the paraffin blocks with a rotary microtome (Leica RM2265). The sections were deparaffinized and stained with Toluidine Blue O (TBO) [19]. Trichomes, stomata on clear leaves and leaf cross-sections were investigated with an optical microscope (DM 4000B—Leica Microsystems, Wetzlar, Germany) and image acquisition was performed with a camera (AxioCam ERc 5s—Carl Zeiss Microimaging Goettingen, Germany).

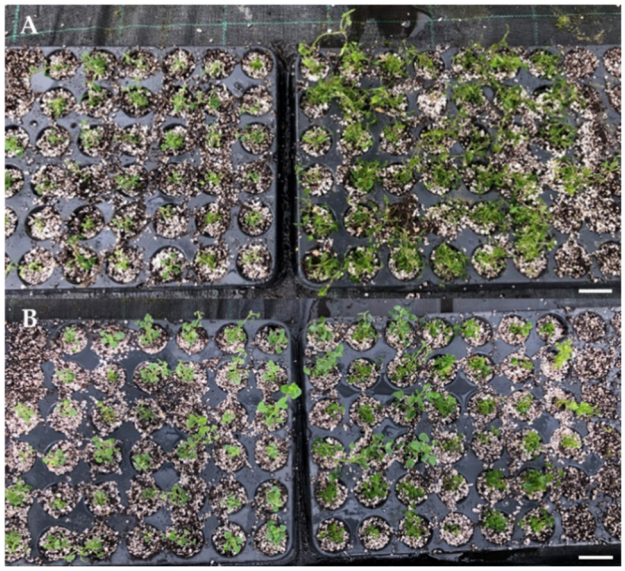

Figure 3. Rooted shoot acclimatization of *A. aurantiaca* (**A**) and *A. mexicana* (**B**) from jars (**left**) and from Plantform™ (**right**) at the time of transplanting (bars = 4 cm).

2.6. Statistical Analysis

Data were statistically analyzed with a one-way ANOVA followed by the Fisher's probable least-squares difference test with cut-off significance at $p \leq 0.05$. Shoot multiplication, root induction results and the survival rate of acclimatized plants shown are from one representative trial.

3. Results and Discussion

3.1. In Vitro Establishment and Shoot Micropropagation

The protocol applied for surface sterilization was efficient because it allowed aseptic cultures of both *Agastache* varieties. Shoot in vitro establishment and proliferation of *A. aurantiaca* and *A. mexicana* was 80% and 65%, respectively. We noticed that the two species produced new shoots growing in a cluster in the MS substrate supplemented with 1.33 µM of BAP. This concentration of BAP has previously been recommended as the most appropriate for the micropropagation of species like *Salvia* [20,21]; generally, this cytokinin gives optimal results for many species belonging to the Lamiaceae family like *Lavandula* [22], *Teucrium* [23] and *Thymus* [24].

The formation of new shoots was not observed when nodal explants of *A. aurantiaca* and *A. mexicana* were cultivated on the MS substrate without plant hormones; generally, the two axillary buds developed forming two auxiliary shoots or one that developed more than the other (Figure 1) and some explants produced roots and large leaves (Figure 4A). The shoot number per nodal explant improved with BAP addition into media. In both species, the greatest number of new stems was recorded at 2.22 µM and 3.33 µM cytokinin concentrations (Table 1). However, with increasing BAP concentrations in the medium, the length of new shoots decreased, and this difference was statistically significant in *A. aurantiaca* (Table 1 and Figure 4).

Carmona-Castro et al. [12] investigated the micropropagation of *A. mexicana* using different doses of BAP in combination with auxin 2,4-dichlorophenoxyacetic acid (2,4-D). The authors obtained similar results to ours for the number of shoots (seven in both cases) and number of leaves (128 vs. $18 \times 7 = 126$) in explants from the substrate with 2.22 µM of BAP (0.5 mg/L), while the data value recorded by them concerning the length of the longest shoot was much higher (8 cm vs. 3 cm). Also, in other *Agastache* species, the cytokinin BAP alone or in a mixture with auxins has been used positively: in *A. rugosa*, the highest number of axillary shoots per explant was obtained by culturing the shoot tips with one apical bud and two axillary buds on a solid MS medium supplemented with 4.4 µM of BA together

with 0.57 µM of indole-3-acetic acid (IAA) or picloram at 0.41 µM [25]. In *A. foeniculum*, the highest multiplication of shoots was observed when nodal explants were transferred onto a substrate with 4.4 µM of BAP + 1 µM of IAA or 8.8 µM of BAP + 1 µM of IAA (46.8 and 53.7, respectively) [26].

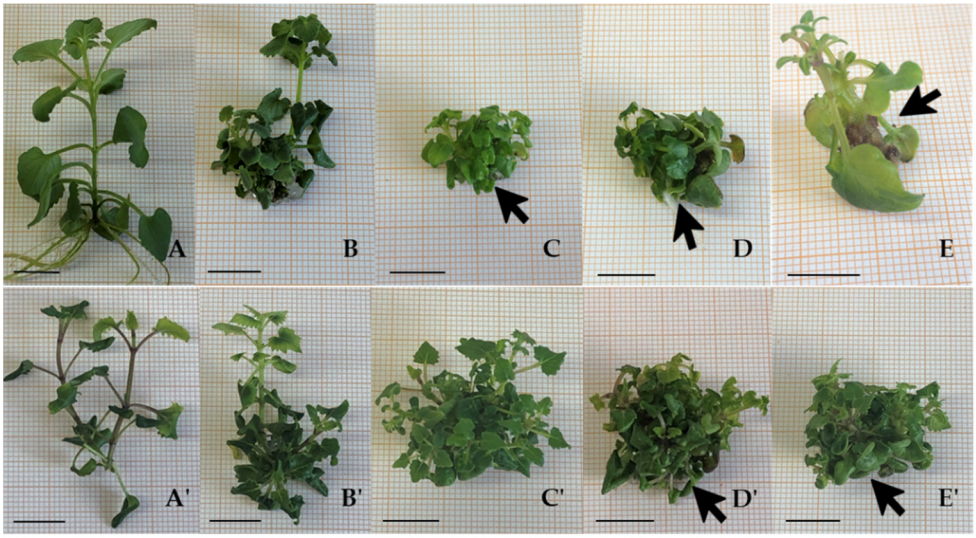

Figure 4. *A. aurantiaca* (up, **A**–**E**) and *A. mexicana* (down, **A′**–**E′**) shoot clusters from media with different BAP concentrations: 0.00 µM (**A**,**A′**), 1.11 µM (**B**,**B′**), 2.22 µM (**C**,**C′**), 3.33 µM (**D**,**D′**) and 4.44 µM (**E**,**E′**). Bars = 1 cm. Black arrows indicate hyperhydricity symptoms.

Table 1. *A. aurantiaca* and *A mexicana* shoot micropropagation: comparison among different BAP concentrations in the solid medium (0.00 µM, 1.11 µM, 2.22 µM, 3.33 µM and 4.44 µM). After 4 weeks of culturing, in vitro culture in media with 2.22 µM or 3.33 µM of BAP improved the shoot number both in *A. aurantica* and *A. mexicana*.

	BAP (µM)				
Parameters	**0.00**	**1.11**	**2.22**	**3.33**	**4.44**
			A. aurantiaca		
Shoot per cluster	2 ± 1 b [1]	4 ± 1 ab	5 ± 1 a	5 ± 1 a	2 ± 1 b
Plant cluster height (mm)	30 ± 3 a	23 ± 3 ab	18 ± 2 bc	20 ± 4 bc	15 ± 2 c
Leaf number	23 ± 2 b	24 ± 5 b	49 ± 15 a	55 ± 20 a	46 ± 12 a
Explant number *	7 ± 1 a	5 ± 1 ab	5 ± 1 ab	3 ± 1 b	3 ± 1 b
			A. mexicana		
Shoot per cluster	2 ± 1 b	4 ± 1 a	7 ± 2 a	7 ± 1 a	6 ± 1 a
Plant cluster height (mm)	38 ± 6 a	32 ± 3 a	28 ± 3 a	26 ± 1 a	25 ± 3 a
Leaf number	28 ± 6 c	57 ± 9 b	128 ± 30 a	102 ± 10 a	91 ± 13 a
Explant number *	7 ± 1 b	14 ± 2 a	17 ± 2 a	3 ± 2 b	2 ± 2 b

[1] Values are the means ± standard errors (N = 15). Different letters indicate statistically significant differences ($p < 0.05$) when comparing treatments (across the lines of the table). * Number of explants obtainable from each shoot cluster.

Carmona-Castro et al. [12] recorded in *A. mexicana* a higher multiplication of shoots and their elongation was obtained with 0.1 mg/L of 2,4-D plus 1.0 mg/L of BAP from explants with nodes; however, they observed symptoms of hyperhydricity in new shoots and leaves with the enhancement of BAP concentration (1.0 and 1.5 mg/L) in the medium. Zuzarte et al. [22] observed that in *Lavandula peduculata*, high concentrations of cytokinin (0.5 mg/L) induced a higher number of shorter shoots compared to other treatments; therefore, the authors speculate that there is an inverse relationship between the number of shoots and their size with increasing cytokinin concentrations. Our results agree with those obtained from the studies cited above. Cytokinins are plant hormones that influence

a wide range of plant developmental processes, including inhibition of shoot elongation at high concentrations [27]. Furthermore, the BAP supplement reduces internodal distance and stimulates the production of axillary buds and the formation of small leaves; however, most new shoots and leaves from both *A. auriculata* and *A. mexicana* explants grown at BAP concentrations above 1.11 µM and 2.22 µM, respectively, showed signs of hyperhydricity (small and translucent leaves) (Figure 4). This phenotype is quite common during in vitro cultures and has been reported for other Lamiaceae [22,23], and other plant species [17,28], and many authors [28–30] indicate that the excess of cytokinins in the culture medium is one of the most important factors in promoting hyperhydricity, especially in nodal explants [31]. In *Lavandula pedunculata*, the hyperhydricity of the explant did not compromise the regeneration of the plants since the phenotype disappeared following the transfer of the shoots to the MS medium lacking growth regulators [22]. On the other hand, in the case of *A. auriculata* and *A. mexicana*, the hyperhydricity of the explant did not regress if placed on a growth-regulator-free substrate and the explants did not produce new shoots (personal observation). For this reason, the number of new obtainable explants from shoot clusters that will be able to root was significantly very low in substrates with high BAP concentrations (3.3 and 4.4 µM) (Table 1).

3.2. Rooting Induction

Explants grown in the liquid medium in TIS (temporary immersion system) started rooting already during the second week of cultivation, while plants grown in the agar medium started rooting during the third week of cultivation. All the explants of both *A. auriculata* and *A. mexicana* placed in the rooting medium (both solid and liquid in bioreactor) produced roots. As shown in Table 2 and Figure 5, the explants cultivated in the TIS bioreactor exhibited a higher biomass, a greater number of stems and leaves and a more extensive and longer root system compared to those from the solid medium in jars. No change in the explant height in *A. auriculata* was recorded.

Table 2. Comparison between solid (jar) and liquid (TIS—Plantform) culture systems during root induction of explants.

	A. aurantiaca		***A. mexicana***	
Parameters	**Jar**	**TIS**	**Jar**	**TIS**
Shoot fresh weight (mg)	312 ± 21 b [1]	1070 ± 43 a	200 ± 19 b	1264 ± 69 a
Shoot number	2 ± 1 b	4 ± 1 a	3 ± 1 b	8 ± 1 a
Shoot height (mm)	44 ± 2 a	50 ± 3 a	47 ± 2 b	73 ± 4 a
Leaf number	34 ± 2 b	56 ± 2 a	32 ± 2 b	69 ± 3 a
Root number per plant	5 ± 1 b	7 ± 1 a	4 ± 1 b	9 ± 1 a
Longest root per plant (mm)	3.2 ± 0.2 b	4.4 ± 0.2 a	2.5 ± 0.2 b	4.9 ± 0.2 a

[1] Values are the means ± standard errors (N = 40). Different letters indicate statistically significant differences ($p < 0.05$) when comparing treatments (across the lines of the table). No comparisons were made between species.

It was reported that the use of 1/2 MS salts was found to be suitable for rooting also in other *Agastache* species, such as *A. foeniculum*, in which the substrate was enriched with 1.1 µM of IBA [26]. The same authors observed a higher rooting percentage in half-strength MS + IBA compared to a full-strength MS + IBA medium, indicating that medium salt composition is important during the rooting phase. Carmona-Castro et al. [12] obtained rooted shoots of *A. mexicana* by transferring 4-week-old apical shoots into 75% solid MS. In this substrate, the rooting started after 2 weeks of culturing and after another period of 8 weeks, the in vitro rooted shoots were transplanted into the greenhouse. The use of the TIS liquid culture (Plantform™ bioreactor) induced both the production of the shoots and the rooting, reducing the time of rooted plant production ready for acclimatization by at least 1 week in comparison with the traditional agarized medium in a glass jar. This important result has been observed in liquid cultures of *Lycium barbarum* using the same liquid culture system but with a different immersion cycle (time and frequency) [32]. The

greater production of new shoots and biomass could be due to different factors. The periodic shaking and the oxygenation of the medium avoid the problems of hypoxia, which often afflict the in vitro culture on an aged substrate; moreover, the area of explant tissue in contact with the substrate is greater and consequently the absorption of nutrients can increase as well [33]. The explants placed in jars are placed upright, perpendicular to the substrate, so the dominance of the apical bud remains constant. In the Plantform^{TM}, the explants are placed diagonally on the bottom of the basket: this position and the change of position during the periodic immersion cycles could modify the apical dominance especially before the beginning of the formation of the roots, which tend to anchor the explants to the basket. The positive effect of the liquid culture with periodic immersion cycles is reported for ornamental bulbs [34,35] and many other species [36]. The two species of *Agastache* were able to root in both an agarized medium and liquid medium; however, in the TIS culture, the roots were more profuse and developed (Table 2 and Figure 4). The TIS system improved the root development also in *Eucalyptus* clones [37] and *Siraitia grosvenorii* [38].

Figure 5. Rooted shoots *of A. aurantiaca* (**A**) and *A. mexicana* (**B**) from jars (**left**) and from PlantformTM (**right**) after 4 weeks of in vitro culturing (bars = 2 cm).

3.3. Plant Acclimatization

During the first week of greenhouse cultivation, some shoots of the rooted clusters with the highest number of shoots underwent degeneration, while other new shoots were formed. Carmona-Castro et al. [12] recorded a negative relationship between the number of hyperhydricity cases and the success of transplantation in a pot. In our case, the clusters did not show symptoms of hyperhydration; therefore, the degeneration of some stems could have been caused by excessive foliar evapotranspiration or nutritional imbalances towards the other shoots of the same cluster. After 3 weeks of cultivation in the greenhouse, the plants already developed (Figure 6), and the roots began to emerge from the holes at the bottom of the alveolus containers. The survival rate of *A. mexicana* rooted shoots coming from jars and Plantform was 95% and 100%, respectively, while in *A. auriculata*, all rooted shoots survived acclimatization regardless of the in vitro rooting method.

Carmona-Castro et al. [12] obtained the same results about the acclimatization of *A. mexicana* rooted shoots. A large success of acclimatization of in vitro rooted shoots has been observed in other *Agastache* species as in the case of *A. foeniculum* [26]. In other species, the survival rate of plants rooted in a TIS system was greater compared to those of rooted plants in an agarized medium [17].

No differences in maximal shoot height were recorded between acclimatized rooted shoots from a jar or from Plantform both in *A. aurantiaca* and in *A. mexicana*; however, in both species, the number of well-developed shoots (length > 10 mm) and the number of short shoots (length < 10 mm) were higher in clusters coming from Plantform compared to those rooted in jars (Table 3).

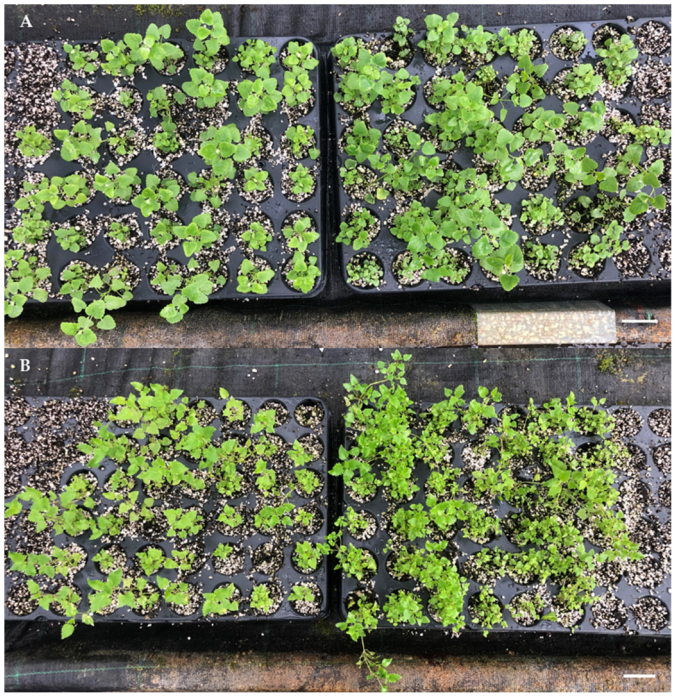

Figure 6. Acclimatization of *A. aurantiaca* (**A**) and *A. mexicana* (**B**) rooted shoots from jars (**left**) and from Plantform™ (**right**) after 3 weeks of culturing under mist irrigation system (bars = 4 cm).

Table 3. Acclimatized shoot clusters: comparison between solid (jar) and liquid (TIS—Plantform) culture systems.

	A. aurantiaca		***A. mexicana***	
Parameters	**Jar**	**TIS**	**Jar**	**TIS**
Shoot height (mm)	49 ± 5 a [1]	54 ± 5 a	76 ± 6 a	88 ± 5 a
Shoot number (>10 mm)	2 ± 1 b	3 ± 1 a	2 ± 1 b	5 ± 1 a
Shoot number (<10 mm)	0 ± 1 b	3 ± 1 a	1 ± 2 b	4 ± 1 a

[1] Values are the means ± standard errors (N = 40; N = 38 only for *A. mexicana* from jar). Different letters indicate statistically significant differences ($p < 0.05$) when comparing treatments (across the lines of the table). No comparisons were made between species.

A. auriculata and *A. mexicana* plants have inflorescences on the terminal tips of simple stems [7]; therefore, the production of clusters with many shoots is a desirable trait as it allows a wide flower production. In fact, most of the clusters grown in the greenhouse produced apical inflorescences and the clusters with more shoots (from TIS culture) produced on average almost twice as many inflorescences than the clusters with fewer shoots (from solid medium). Indeed, the number of inflorescences produced by the clusters coming from jar were on average 2 ± 1 (both in *A. aurantiaca* and in *A. mexicana)*; instead, those produced by the clusters coming from Plantform were on average 4 + 1 and 5 + 1 in *A. aurantiaca* and *A mexicana*, respectively.

3.4. Leaf Anatomical Analysis

Leaves of the two varieties are dorsiventral. Both adaxial and abaxial leaf surfaces are coated with glandular and non-glandular trichomes. No differences were found between leaves from plants rooted in a jar and TIS: capitate and peltate trichomes were well developed and like those observed on leaves collected from plants in the greenhouse (Figure 7). The stomata present on the leaves of plants rooted both in jars and in TIS were open, while those collected from in vivo plants grown in the greenhouse were closed (Figure 7). Furthermore, leaves from in vivo plants had more developed non-glandular trichomes than those grown in a jar or TIS, especially in the case of *A. aurantiaca*.

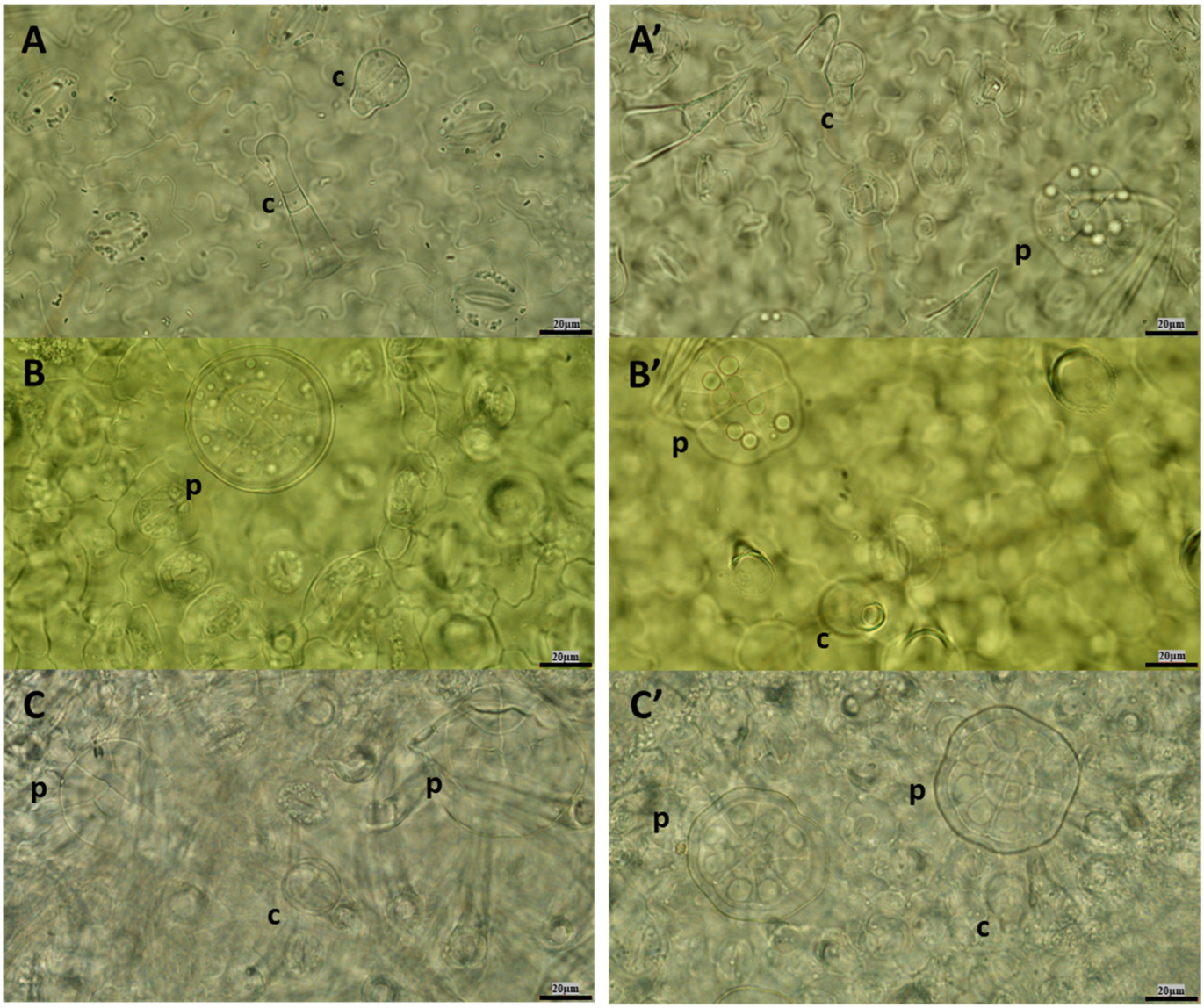

Figure 7. Leaves sampled of *A. aurantiaca* (left, **A**–**C**) and *A. mexicana* (right, **A'**–**C'**) plants from jar (**A**,**A'**), Plantform (**B**,**B'**) and in vivo plants (**C**,**C'**) after clarification with Visikoll solution. Bars = 20 µm; c = capitate trichomes; p = peltate trichomes.

The epidermis is monolayered. Sections of leaves from both jar- and TIS-rooted plants had thinner epidermis cell walls and waxes, less extensive and organized palisade parenchyma and looser spongy tissue than those from leaves harvested from in vivo plants (Figure 8). However, in the case of the two varieties of *Agastache*, acclimatization is not problematic because the leaves developed during rooting both in jars and in TIS are already well structured, and the mist irrigation system allows the functionality (opening and closing) of the stomata to be regained [39]. In the case of *Mertensia maritima*, rooting in TIS proved to be more effective because it allowed the growth of more functional leaves, with well-structured tissues and with well-formed and active glandular trichomes compared to those grown in a jar [17].

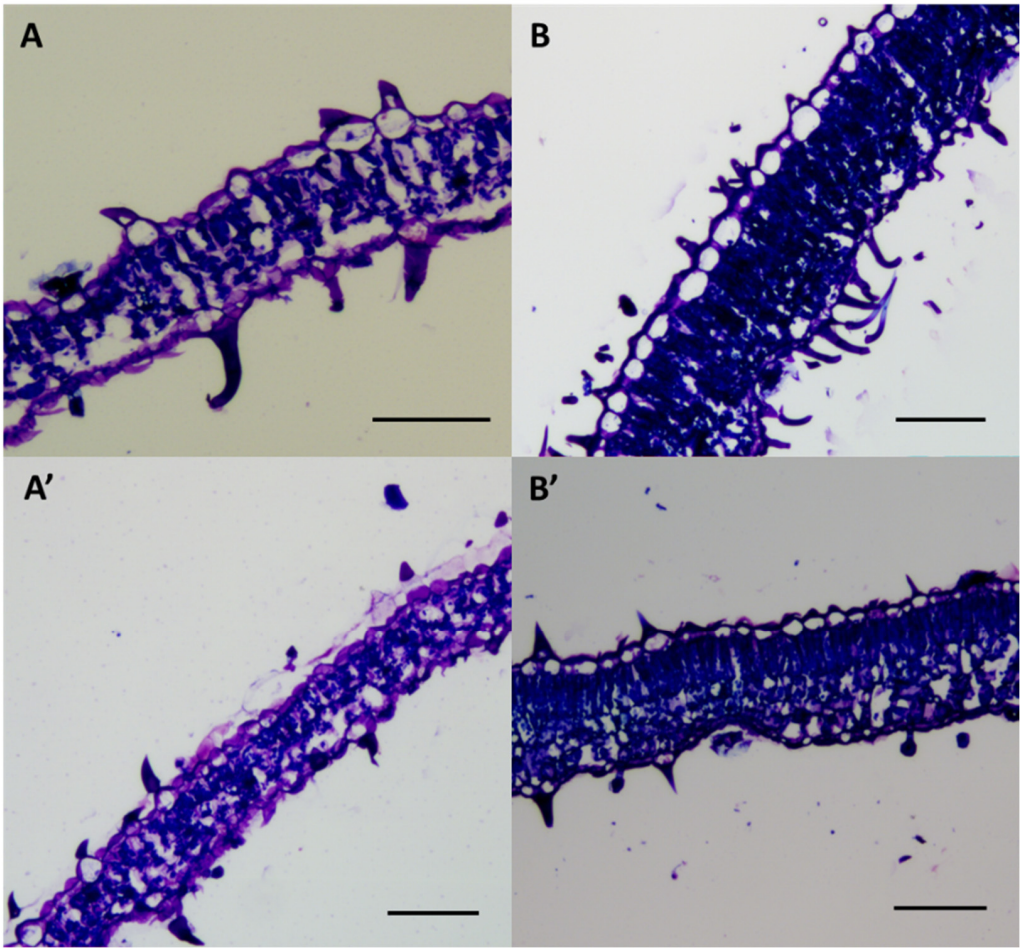

Figure 8. Sections of leaf samples of *A. aurantiaca* (**A**,**B**) and *A. mexicana* (**A'**,**B'**) plants from jar (**A**,**A'**) and in vivo plants (**B**,**B'**). Bars = 100 μm.

4. Conclusions

The large production of *A. aurantiaca* 'Sunset Yellow' and *A. mexicana* 'Sangria' edible flowers depend on the rapid production of new plants. The in vitro propagation protocol that is suitable to obtain the greatest number of new shoots involved the multiplication on the solid medium (in jar) with 2.22 μM of BAP (or 3.33 μM of BAP in *A. mexicana* only) (micropropagation time: 4 weeks) and rooting in TIS using the half-strength MS medium (rooting time: 3–4 weeks). Acclimatization of the plants was not problematic; however, the plants from TIS showed greater vigor and a greater capacity for rapid development than those rooted with traditional methods. *Agastache* clusters placed in a greenhouse with a mist irrigation system were acclimatized in 1–2 weeks. The complex of data suggests that leaves do not seem to influence acclimatization success because they are able to adapt to the development environment even in the case of changes such as those occurring during acclimatization.

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