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An Optimized Protocol for Micropropagation and Acclimatization of Strawberry (*Fragaria* × *ananassa* Duch.) Variety 'Aroma'

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Abstract: In strawberry micropropagation, several challenges must be overcome to obtain quality plants and achieve high survival rate to ex vitro acclimatization. In this study, therefore, a set of protocols were evaluated to optimize explant (meristem) disinfection, in vitro growth (multiplication and rooting), and ex vitro acclimatization of strawberry. The results showed that explants treated with 1.0% NaClO for 5 min had a lower percentage of contamination, and achieved a higher percentage of viability, height, and number of leaves. In vitro growth was favored by the use of 1 mg L^{-1} zeatin, since it allowed greater seedling growth (number of shoots, seedling height, number of leaves, number of roots and root length), and a higher pre-acclimation rate (100%). In the acclimatization phase, plants grown in a substrate composed of compost + peat combined with 4 g of humic acid achieved better response in morphological and physiological variables. In fact, the results of this study could be used to cultivate strawberry plants of the 'Aroma' variety with high commercial quality.

Keywords: growth conditions; meristem culture; morphophysiological characteristics

Citation: Neri, J.C.; Meléndez-Mori, J.B.; Tejada-Alvarado, J.J.; Vilca-Valqui, N.C.; Huaman-Huaman, E.; Oliva, M.; Goñas, M. An Optimized Protocol for Micropropagation and Acclimatization of Strawberry (*Fragaria* × *ananassa* Duch.) Variety 'Aroma'. *Agronomy* **2022**, *12*, 968. https://doi.org/10.3390/agronomy12040968

Academic Editor: Samir C. Debnath

Received: 30 March 2022
Accepted: 14 April 2022
Published: 17 April 2022

1. Introduction

Strawberry is a fruit that has high nutritional value and is a substantial source of folate, vitamin C, fiber, potassium, flavonoids, anthocyanin, phytochemicals, antioxidants [1,2], and bioactive compounds that reduce the risk of cardiovascular incidents and thrombosis [3,4]. In addition, it provides anti-cancer benefits and helps prevent age-related memory loss [5,6].

However, despite its multiple benefits and high agricultural potential, there are limitations for its cultivation due to the presence of diseases caused by viruses such as strawberry mottle virus (SMoV), Fragaria chiloensis cryptic virus (FClCV), strawberry necrotic shock virus (SNSV), and strawberry mild yellow edge virus (SMYEV) [7], and those caused by fungi such as *Botrytis cinerea*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Colletotrichum* spp., *Verticillium dahliae*, *Mycosphaerella fragariae*, *Ramularia tulasnei*, *Phomopis obscurans* or by the bacterium *Xanthomomas fragariae* [8]. Many of these diseases can be transmitted in the propagation process, which is traditionally done through stolons [9]. An interesting alternative to overcome this problem is the use of biotechnological tools that allow mass production of plants with excellent phytosanitary and genetic quality [10]. Meristematic tissue culture is a technique widely used to obtain pathogen-free and homogeneous plants over a short period of time (fungi, bacteria, and viruses) [11].

For successful micropropagation, it is essential to regulate and manage contamination during meristem establishment [12]. Likewise, it is equally important to identify the growth regulator and concentration that allow for successful establishment, multiplication, and

rooting in vitro. Finally, one of the critical points in the tissue culture process is to achieve ex vitro acclimatization of micropropagated plants; therefore, it is crucial to evaluate various substrates and/or their combinations to achieve high survival rates and adequate plant growth and development [13–15].

Plants from in vitro culture have been reported to show high rooting and shoot generation rates [16] but, most importantly, they are free from diseases [17]. Furthermore, Nehra et al. [18] reported that in vitro propagated plants of 'Redcoat' produced significantly more inflorescences and flowers per crown compared to stolon-propagated plants, and consequently derive a higher number of fruits. However, tissue culture-propagated plants exhibit genotype-dependent behavior, suggesting the need for detailed evaluation of each strawberry cultivar [18].

In this context, the present study aimed to optimize the factors that influence the micropropagation of strawberry variety 'Aroma', and to evaluate the development of the plants during the ex vitro acclimatization process.

2. Materials and Methods

The research was carried out at the Laboratory of Plant Physiology and Biotechnology of the Universidad Nacional Toribio Rodríguez de Mendoza. As a source of explant, strawberry plants of the 'Aroma' variety grown at the experimental station located on the university campus were selected. The plants were propagated in 1.0 L containers with a sterile substrate composed of agricultural soil, peat, and river sand (ratio 2:1:1; pH 6.38). These received a preventive phytosanitary treatment with a solution of Phyton (2.5 mL L^{-1}) and Chlorpyrifos (1.5 mL L^{-1}), in addition to biostimulation and nutritional balance with Enziprom (5 mL L^{-1}) and Basacote® Plus 6M (4 g $plant^{-1}$), respectively. The phytosanitary treatment was carried out at 5-day intervals for 8 weeks.

2.1. Culture Medium and Growth Condition

In this study, Murashige and Skoog (MS) base medium [19], supplemented with 22.5 g L^{-1} sucrose, 0.15 g L^{-1} ascorbic acid, 0.10 g L^{-1} myo-inositol, 1.0 mL L^{-1} Plant Preservation Mixture, and 6 g L^{-1} Agar was used. The pH was adjusted to 5.8 and then autoclaved at 121 °C and 1.5 bar for 20 min. Explants were grown at 25 ± 1 °C and a photoperiod of 16 h light provided by white fluorescent tubes (3000 lux).

2.2. Disinfecting Plant Material

Stolons free of disease symptoms and nutritional deficiencies were harvested. Leaves and roots were removed, gently rinsed under running water, and placed in ascorbic acid solution (150 mg L^{-1}) for 20 min. The crowns were then immersed in a soapy solution for 15 min and then placed in fungal solution (Carbonyl 1 g L^{-1}) for 15 min. At the end of each disinfection process, three rinses with sterile distilled water were performed.

2.3. Experiment 1: In Vitro Establishment

Under aseptic conditions in a laminar flow chamber, explants were sterilized by immersion through independent treatments with sodium hypochlorite (0.5 and 1% NaOCl, for 3 or 5 min), hydrogen peroxide (0.5 and 1% H_2O_2, for 3 or 5 min), or mercuric chloride (0.1% HgCl, for 1.5 or 3 min). At the end of each treatment, three rinses with sterile distilled water were performed. The experiment consisted of 10 treatments and 10 replicates. Each repetition corresponded to one experimental unit (explant).

Sterile meristems (0.3 mm) were extracted and seeded in test tubes with 15 mL of base culture medium (Figure 1a). After 6 weeks, the percentage of phenolization, contamination, viability, seedling height, and number of leaves were recorded.

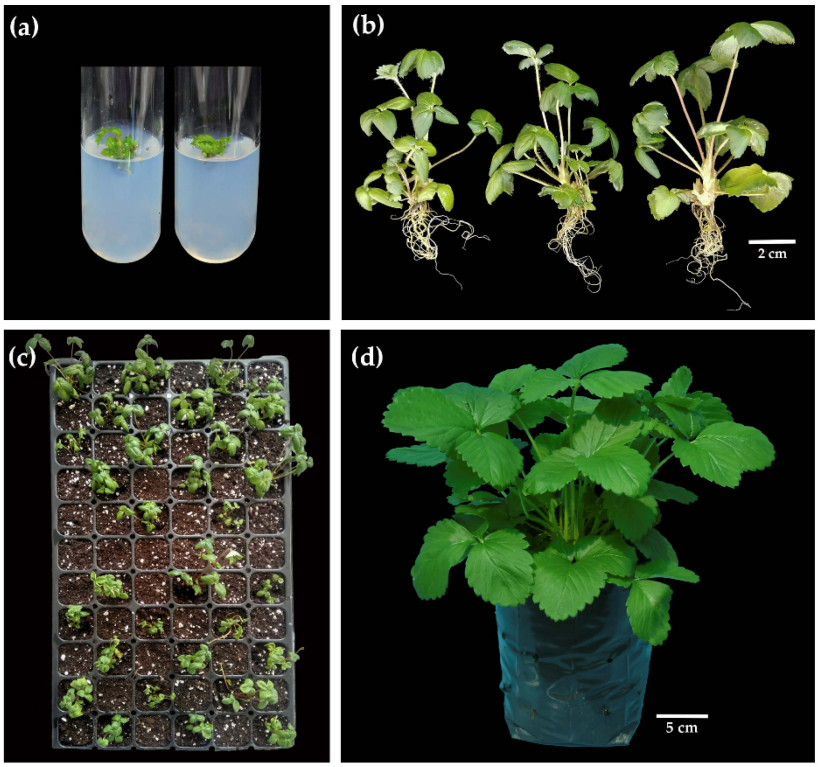

Figure 1. Micropropagation of strawberry variety 'Aroma': (**a**) meristems sprouted after 30 days in establishment medium; (**b**) explants developed after 45 days in multiplication medium, ready to be transferred to pre-acclimation; (**c**) seedlings in pre-acclimation; (**d**) plant after 50 days of acclimatization.

2.4. Experiment 2: In Vitro Growth (Multiplication and Rooting) and Pre-Acclimatization

Explants were grown on base medium supplemented with 6-Benzylaminopurine (0.5 mg L^{-1} BAP), coconut water (100 and 200 mL L^{-1}), Thidiazuron (1 and 1.5 mg L^{-1}), Zeatin (1 and 2 mg L^{-1}) or gibberellic acid (0.5 mg L^{-1} AG_3), and a phytohormone-free control. Three explants were placed in PTL-100-1PZ magenta flasks (PhytoTechnology Laboratories, Lenexa, KS, USA) (Figure 1b). The experiment consisted of nine treatments and five replicates. Each replicate corresponded to three experimental units (explants). After 5 weeks, the number of shoots, seedling height, number of leaves, number of roots, and root length were recorded.

Then, following the experimental design and treatments of the multiplication-rooting stage, the seedlings were separated and sown in polyethylene trays with a substrate composed of a mixture of sphagnum peat + perlite in a 1:2 ratio (special mixture, Klasmann Substrates Select, Geeste, Germany) with pH 6.0 (Figure 1c). The trays were placed in transparent containers with a thin film of water, then covered with parafilm (gradually removed) and maintained at a temperature of 25 °C, 80% relative humidity, and light intensity of 3000 lux. After 16 days, the percentage of pre-acclimation (survival) was recorded.

2.5. Experiment 3: Acclimatization

The most vigorous and uniform hardened seedlings (Figure 1d) were transferred to a shaded nursery (70% shade) and transplanted into 5″ × 8″ nursery bags containing four types of substrates (Table 1): compost (coffee pulp), burned rice husk, coconut fiber, and perlite, which were mixed with peat in a 2:1 ratio. After 7 days of acclimatization, humic acid was added (0 or 4 g EKOTRON® 70 GR), depending on treatment. The average temperature and relative humidity during the acclimatization period were 16.4 °C and 77.9%, respectively. The experiment was conducted under a 4 × 2 factorial design (substrate types × humic acid dose), with six replicates. Each replicate corresponded to one experimental unit (plant). After 35 days post-transplanting, the following parameters were evaluated.

Table 1. Physical and chemical characteristics of the substrates used for acclimatization of strawberry variety 'Aroma'.

Characteristics		Values			
		Substrate 1	**Substrate 2**	**Substrate 3**	**Substrate 4**
Physical	Apparent Density (g/cm^3)	0.27	0.23	0.15	0.12
	True Density (g/cm^3)	0.63	0.69	0.40	0.44
	Porosity (%)	57.00	67.00	62.50	72.73
Chemical	pH	5.51	5.80	5.04	4.50
	EC (dS/m)	1.18	0.49	0.91	0.40
	CEC (meq/100g)	30.00	21.20	28.00	26.40
	Organic matter (%)	12.20	10.48	10.80	9.84
	Available P (ppm)	91.30	57.74	27.97	23.06
	Exchangeable K^+ (meq/100 g)	5.60	3.15	3.18	1.11
	Exchangeable Ca^{2+} (meq/100 g)	28.19	18.10	9.83	7.29
	Exchangeable Mg^{2+} (meq/100 g)	2.20	1.11	1.30	1.08
	Exchangeable Na^+ (meq/100 g)	0.82	0.60	1.12	0.13

EC: electrical conductivity, CEC: cation exchange capacity. Substrate 1: compost + peat; Substrate 2: carbonized rice straw + peat; Substrate 3: coconut fiber + peat; Substrate 4: perlite + peat; Substrate 4: perlite + peat.

2.5.1. Determination of Plant Growth Parameters

Plant height (longest shoot), number of leaves, number of shoots or crowns, root length, number of roots, aerial biomass (fresh and dry matter), root biomass (fresh and dry matter), leaf area, and root volume were recorded. To record dry matter, plants were dried in an oven at 60 °C until a constant weight was achieved. Leaf area of all leaves/plant was determined using a regression equation:

$$y = 0.286141\ x - 8.5624061.10^{-5}\ x^2 - 1.06547953.10^{-8}\ x^3, \quad (1)$$

where y = leaf area cm^2, x = length per leaf width, and coefficient of determination $R^2 = 0.99$ [20].

2.5.2. Determination of Physiological Parameters

Chlorophyll content (SPAD index), water potential ψ (bar), and stomatal conductance ($mmol/m^2/s$) were measured using a SPAD 502 chlorophyll meter (Konica Minolta, Tokyo, Japan), a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA) and an SC-1 leaf porometer (Decagon Devices, Pullman, WA, USA), respectively. Measurements were taken between 9:00 a.m. and 12:00 p.m., on the three most light-exposed leaves.

2.6. Experimental Design and Data Analysis

All experiments were conducted under a complete randomized design (CRD). Data were subjected to analysis of variance and significant means were tested by Tukey's test ($p \leq 0.05$) using InfoStat statistical software version 2018.

3. Results

3.1. Experiment 1: In Vitro Establishment

Table 2 shows that the lowest percentage of contamination (10%) was obtained when the explants were treated with 1.0% NaClO for 5 min. In addition, it was also the treatment that allowed the highest percentage of viability (60%), height (5.7 cm), and number of leaves (3.7) per established seedling to be recorded. On the other hand, the highest percentages of contamination (80%) were recorded in the treatments with H_2O_2 1.0% for 3 and 5 min, NaClO 0.5% for 5 min and H_2O_2 0.5% for 3 min, the latter being also the treatment with no viability rate (Table 2).

Table 2. Effect of different sterilization treatments on 'Aroma' strawberry meristems during in vitro establishment.

Treatment	Phenolization (%)	Contamination (%)	Viability (%)	Seedling Height (cm)	Number of Leaves
NaClO 0.5%, 3 min	30 a	60 bc	10 cd	1.1 ab	1 ab
NaClO 0.5%, 5 min	10 b	80 a	10 cd	0.7 b	0.2 b
NaClO 1.0%, 3 min	20 a	50 cd	30 ab	3.1 ab	0.9 ab
NaClO 1.0%, 5 min	10 b	10 d	60 a	5.7 a	3.7 a
H_2O_2 0.5%, 3 min	20 a	80 a	0 d	0 b	0 b
H_2O_2 0.5%, 5 min	0 c	70 b	30 ab	2 ab	2.4 ab
H_2O_2 1.0%, 3 min	10 b	80 a	10 cd	0.6 b	0.3 b
H_2O_2 1.0%, 5 min	0 c	80 a	20 bc	0.9 b	0.5 b
HgCl 0.1%, 1.5 min	10 b	70 b	20 bc	2 ab	0.4 b
HgCl 0.1%, 3 min	10 b	50 cd	40 a	2 ab	1.7 ab

Means with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$).

3.2. Experiment 2: In Vitro Growth (Multiplication and Rooting) and Pre-Acclimatization

Explants grown with 1.0 mg L^{-1} zeatin produced significantly more shoots (4.20 ± 0.86) and leaves (17.13 ± 2.80). Regarding seedling height, the best results (>5 cm) were obtained in explants from the 1.0 mg L^{-1} zeatin, 100 mL L^{-1} coconut water, and control treatments (Table 3).

Table 3. Effect of growth regulators on multiplication and pre-acclimation of strawberry seedlings 'Aroma' variety.

Treatment	Number of Shoots	Seedling Height (cm)	Number of Leaves	Number of Roots	Root Length (cm)	Pre-Acclimatization (%)
Control	1.63 ± 0.83 cd	5.29 ± 1.65 a	8.67 ± 0.90 d	9.07 ± 2.91 abc	5.24 ± 1.25 cd	82.22 abcd
0.5 mg L^{-1} BAP	2.69 ± 1.30 b	2.85 ± 0.52 b	10.09 ± 1.44 d	9.93 ± 2.38 ab	10.64 ± 4.18 b	77.78 bcd
100 mL L^{-1} coconut water	2.27 ± 1.49 bc	5.41 ± 1.00 a	8.60 ± 0.74 d	8.73 ± 1.62 bcd	5.95 ± 1.31 cd	88.89 ab
200 mL L^{-1} coconut water	2.13 ± 1.06 bc	3.07 ± 1.32 b	13.00 ± 3.38 bc	8.80 ± 3.10 bcd	7.61 ± 2.23 c	84.45 abc
1.0 mg L^{-1} Thidiazuron	1.33 ± 0.62 cd	2.68 ± 0.64 b	10.60 ± 1.80 cd	7.47 ± 2.03 cd	10.83 ± 2.74 b	66.67 cd
1.5 mg L^{-1} Thidiazuron	1.40 ± 0.63 cd	3.08 ± 0.25 b	10.20 ± 1.61d	7.73 ± 1.03 bcd	10.61 ± 1.08 b	62.23 d
1.0 mg L^{-1} Zeatin	4.20 ± 0.86 a	5.63 ± 1.99 a	17.13 ± 2.80 a	11.40 ± 2.38 a	13.41 ± 1.31 a	100.00 a
2.0 mg L^{-1} Zeatin	2.67 ± 0.98 b	2.25 ± 0.35 b	14.00 ± 3.80 b	4.93 ± 1.83 e	4.81 ± 2.00 d	82.22 abcd
0.5 mg L^{-1} AG_3	1.00 ± 0.00 d	2.68 ± 0.43 b	10.13 ± 1.46 d	6.40 ± 0.99 de	4.61 ± 1.67 d	77.78 bcd

Means with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$).

After 42 days, all installed explants developed roots. Effectively, the highest number (11.40 ± 2.38) and length of roots (13.41 ± 1.31 cm) were recorded at 1.0 mg L^{-1} zeatin. In general, seedlings grown on medium with 1.0 mg L^{-1} zeatin showed greater vigor and better morphological conformation, favorable for pre-acclimation (100%) success of the seedlings (Table 3).

3.3. Experiment 3: Acclimatization

The morphological performance of the seedlings during the acclimatization process is shown in Table 4. The greatest plant height (15.45 ± 0.91 cm), number of crowns (7.33 ± 0.82), number of roots (26.25 ± 0.76), and leaf area (516.17 ± 145.42 cm^2) were recorded in plants grown on substrate 1 + 4 g humic acid. In substrates 1 and 3 (both with 4 g humic acid), the highest number of leaves was recorded, with values of 15.17 ± 2.14 and 15.17 ± 1.33, respectively. Regarding root length, substrate 3 + 4 g of humic acid yielded better root growth, achieving roots up to 32.21 ± 2.43 cm in length (Table 4).

Table 4. Effect of substrate and humic acid on the morphological performance of 'Aroma' strawberry plants during acclimatization.

Treatment	Plant Height (cm)	Number of Leaves	Number of Crowns	Number of Roots	Root Length (cm)	Leaf Area (cm^2)
S1 × 0 g HA	11.50 ± 1.10 bc	10.67 ± 2.50 bc	4.00 ± 0.89 d	17.96 ± 0.68 c	26.38 ± 1.18 bcd	323.55 ± 178.04 abc
S2 × 0 g HA	9.77 ± 1.12 c	11.17 ± 2.56 bc	4.33 ± 0.82 cd	17.79 ± 2.17 c	27.46 ± 4.48 abcd	328.27 ± 93.47 ab
S3 × 0 g HA	10.25 ± 1.33 c	10.50 ± 1.52 bc	5.50 ± 0.55 bc	21.67 ± 1.03 b	28.96 ± 2.09 abc	265.75 ± 92.91 bcd
S4 × 0 g HA	6.22 ± 0.67 d	9.33 ± 2.66 bc	3.33 ± 0.82 d	16.13 ± 1.66 c	23.58 ± 2.42 de	119.00 ± 22.89 d
S1 × 4 g HA	15.45 ± 0.91 a	15.17 ± 2.14 a	7.33 ± 0.82 a	26.25 ± 0.76 a	31.29 ± 1.75 ab	516.17 ± 145.42 a
S2 × 4 g HA	12.32 ± 0.90 b	12.50 ± 1.76 ab	5.83 ± 0.75 b	17.58 ± 3.38 c	25.67 ± 3.27 cd	216.20 ± 45.22 cd
S3 × 4 g HA	11.53 ± 0.97 bc	15.17 ± 1.33 a	5.83 ± 0.75 b	22.75 ± 0.76 b	32.21 ± 2.43 a	302.64 ± 125.13 bcd
S4 × 4 g HA	7.42 ± 0.79 d	8.00 ± 1.67 c	8.00 ± 1.67 c	16.38 ± 1.90 c	19.00 ± 3.27 e	132 ± 26.35 cd

Means with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$). S: substrate; HA: humic acid. Substrate 1: compost + peat; Substrate 2: carbonized rice straw + peat, Substrate 3: coconut fiber + peat and Substrate 4: perlite + peat.

Table 5 shows that the highest biomass, in terms of fresh weight (foliar: 29.46 ± 10.40 g; root: 12.94 ± 1.84 g) and dry weight (foliar: 5.40 ± 1.67 g; root: 1.31 ± 0.17 g) was achieved in plants grown on substrate 1 + 4 g of humic acid. Regarding root volume, it was observed that the highest value of this variable was recorded in substrate 3 (14.04 ± 4.08 mL) and substrate 1 (13.45 ± 2.19 mL), both with 4 g of humic acid, followed by substrate 3 (13.03 ± 1.69 mL) with no humic acid.

Table 5. Effect of substrate and humic acid on the biomass of strawberry 'Aroma' variety plants, during acclimatization.

Treatment	Foliar Fresh Weight (g)	Foliar Dry Weight (g)	Root Fresh Weight (g)	Root Dry Weight (g)	Root Volume (mL)
S1 × 0 g HA	17.73 ± 4.08 b	3.34 ± 0.64 b	8.48 ± 1.14 bcd	0.89 ± 0.10 cd	8.24 ± 1.05 b
S2 × 0 g HA	11.77 ± 3.38 bc	2.43 ± 0.85 bcd	8.35 ± 2.08 bcd	0.91 ± 0.23 cd	8.40 ± 2.16 b
S3 × 0 g HA	19.25 ± 3.98 b	2.97 ± 0.53 bc	12.00 ± 1.92 ab	0.99 ± 0.17 bc	13.03 ± 1.69 a
S4 × 0 g HA	8.00 ± 0.32 c	1.75 ± 0.14 cd	6.88 ± 1.39 cd	0.68 ± 3.78 d	6.33 ± 1.21 b
S1 × 4 g HA	29.46 ± 10.40 a	5.40 ± 1.67 a	12.94 ± 1.84 a	1.31 ± 0.17 a	13.45 ± 2.19 a
S2 × 4 g HA	12.17 ± 1.21 bc	2.44 ± 0.09 bcd	9.40 ± 0.53 abc	0.94 ± 0.05 cd	9.04 ± 0.68 b
S3 × 4 g HA	19.35 ± 3.46 b	3.44 ± 0.57 b	12.75 ± 4.16 a	1.22 ± 0.22 ab	14.04 ± 4.08 a
S4 × 4 g HA	6.21 ± 1.01 c	1.49 ± 0.11 d	5.44 ± 1.98 d	0.67 ± 0.11 d	7.65 ± 1.35 b

Means with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$). S: substrate; HA: humic acid. Substrate 1: compost + peat; Substrate 2: carbonized rice straw + peat, Substrate 3: coconut fiber + peat and Substrate 4: perlite + peat.

The physiological characteristics of the plants during the acclimatization process are shown in Table 6. Plants grown on substrate 1 + 4 g humic acid presented higher chlorophyll index and stomatal conductance, reaching values of 40.69 ± 2.57 SPAD and 434.47 ± 14.20 $mmol/m^2/s$, respectively. By comparison, plants grown on substrate 4 without humic acid presented lower water potential (−16.74 ± 1.71 Bar).

Table 6. Effect of substrate and humic acid on physiological variables of strawberry plants variety 'Aroma' during acclimatization.

Treatment	Chlorophyll Index (SPAD)	Stomatal Conductance (mmol/m^2/s)	Water Potential ψ (Bar)
S1 × 0 g HA	38.08 ± 3.52 abc	410.73 ± 12.04 b	−15.83 ± 2.06 ab
S2 × 0 g HA	37.80 ± 3.33 bc	394.88 ± 18.93 bc	−14.11 ± 1.55 bc
S3 × 0 g HA	35.39 ± 2.43 cd	409.33 ± 28.08 b	−12.92 ± 2.70 c
S4 × 0 g HA	32.22 ± 2.53 e	370.63 ± 8.09 d	−16.74 ± 1.71 a
S1 × 4 g HA	40.69 ± 2.57 a	434.47 ± 14.20 a	−9.69 ± 1.11 e
S2 × 4 g HA	37.76 ± 2.40 bc	410.28 ± 8.06 b	−10.97 ± 1.45 de
S3 × 4 g HA	39.27 ± 2.08ab	409.42 ± 23.89 b	−12.50 ± 1.74 cd
S4 × 4 g HA	34.80 ± 3.18 de	380.85 ± 24.63 cd	−13.37 ± 1.89 c

Means with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$). S: substrate; HA: humic acid. Substrate 1: compost + peat; Substrate 2: carbonized rice straw + peat, Substrate 3: coconut fiber + peat and Substrate 4: perlite + peat.

4. Discussion

4.1. Micropropagation Stage

Although strawberries are generally propagated by stolons, this method does not necessarily guarantee high-quality plants due to the risk of spreading diseases [9]. Therefore, in vitro meristem culture is an ideal technique to obtain healthy and homogeneous plants [11,21,22]. However, to achieve a high rate of success, it is absolutely necessary to optimize protocols, thus minimizing material losses from tissue oxidation and contamination, or low response to in vitro growth conditions [23].

In the present study, meristems treated with 1% NaClO for 5 min achieved higher viability (60%) and lower contamination (10%). Similar results were reported by Munir et al. [24], where disinfection of strawberry meristems ('Oso Grande' and 'Toro' varieties) with 0.5% NaOCl for 10 to 15 min allowed recording a survival rate of 75% and contamination lower than 15%. It is worth mentioning that the addition of antioxidants plays an important role in improving the response during in vitro establishment as it reduces phenolization of explants [11,25].

During multiplication, it was observed that the use of zeatin (1 mg L^{-1}) largely benefited the development of shoots, leaves, and roots. Similar effects have been described in the micropropagation of crops such as blueberry, where the addition of zeatin (1 mg L^{-1}) favored the development of vigorous plants with an increased number of shoots and leaves [26]. In contrast, the results of this study show that the addition of AG_3 (0.5 mg/L) or thiadizuron (1 and 1.5 mg L^{-1}) clearly resulted in lower shoot induction. The low efficiency shown by these growth regulators (AG_3 and thiadizuron) may be related to the fact that they need to act synergistically with other phytohormones to enhance their effects [15,27]. However, Zakaria et al. [28], during multiplication of three strawberry cultivars (Festival, Sweet Charly, and Florida) with 2 mg L^{-1} thiadizuron, achieved a shoot regeneration percentage above 70%, suggesting that the response is also related to the regeneration potential of each variety and to the concentration of phytohormones.

Even though all explants installed during the multiplication stage formed roots, the use of zeatin (1 mg L^{-1}) clearly promoted better root system development. In this regard, Domínguez and Donayre [29] state that in the pre-acclimation stage, it is essential that seedlings should exhibit high vigor and a good root system, so as to ensure their survival. This, in fact, was confirmed in this study, since the seedlings that showed the best morphological behavior were highly adaptable during the transition to ex vitro conditions, with 100% survival (pre-acclimation). Similar results were described by Valencia- Juárez et al. [30], achieving 100% survival of strawberry seedlings of 'Nikté' variety. Jofre-Garfias et al. [31] also recorded up to 90% survival in strawberry of 'Buenavista' variety.

Although the best results were obtained with the use of zeatin, its high cost can be a limiting factor for implementing commercial micropropagation protocols in companies and nurseries. That is why, in recent years, coconut water has been used in micropropagation

protocols for crops such as olive [32], hazelnut [33], and Raja Bulu banana [34], in certain cases as a substitute element for zeatin [32]. In this study, the use of coconut water produced encouraging results for plant height (5.41 ± 1.00 cm), number of leaves (13.00 ± 3.38), and survival (between 84.45 to 88.89%), but had no significant effects on shoot formation or root development. In plant tissue culture, coconut water is a source of beneficial nutritional and hormonal substances, but it is not always sufficient to promote successful micropropagation, so combining it with cytokinins can improve results [33]. In any case, considering the high prices of zeatin, the use of coconut water is justified from an economic point of view.

4.2. Acclimatization Stage

During acclimatization, plants must have a well-developed root system [35], enabling the plant to have properly assimilated water and nutrients, in addition to being well fixed to the substrate [36]. Accordingly, findings of this study indicate that plants grown in the 1 + 4 g humic acid substrate exhibited, in general, better morphological development. This result, together with the characteristics of the substrate, may be related to the application of humic acid, which in turn promotes root and vegetative growth by improving cation exchange capacity and increasing the number of favorable microorganisms in the soil, thereby improving the adaptive ability of plants [37–39]. Importantly, the substrate needs to exhibit good porosity and filtration [40].

Regarding the chlorophyll index, plants grown on substrate 1 + 4 g humic acid had favorable values (40.69 SPAD) for their growth and development, as reported, for example, in peach cultivation, where normal values vary in the range of 39–56 SPAD [41]. Similar values have also been recorded in hydroponic strawberry cultivation with 38.5 to 49.5 SPAD for young and old leaves, in different substrate (50% coco peat + 50% perlite) [42]. Nitrogen is a required element during the growth stage to ensure structural and osmotic functions [43]; therefore, the measurement of the chlorophyll index is useful as an indicator of nitrogen content. Conversely, unsuitable substrate can lead to water stress, resulting in changes in the photosynthetic system and lower chlorophyll levels [44]. Hence, suitable mixtures are vital to ensure good plant development, using easily accessible and low-cost materials [45].

Stomatic conductance is related to gas exchange in leaves, where high readings denote good water supply [46]. Substrate 1 + 4 g humic acid enables a better physiological response of strawberry plants, whereas plants grown on substrate 4 with or without humic acid recorded low levels of stomatal conductance, which may lead to reduced transpiration rate and photosynthetic carbon assimilation [47,48]. This demonstrates, together with water potential, a negative metabolic regulation of developmental processes in plants linked to water stress [49], which was more favored in substrate 4. In this way, changes in stomatal conductance are useful to determine and regulate water loss in plants, which are crucial to establish adequate irrigations [50].

By comparison, the highest level of water potential was recorded in substrate 1 + 4 g of humic acid, which was beneficial for the plants; on the contrary, the plants growing in substrate 4 without humic acid exhibited low values, which affected the growth and development processes [51], which may be due to the delay in cell divisions that occurs at the foliar and root level [52]. Moreover, water potential is a useful indicator to check the water status and its measurement indicates water demand of strawberry plants [53,54].

Accumulated biomass levels were even lower in plants grown on substrate 4. It has been reported that variations in water potential and stomatal conductance led to reductions in plant biomass, because water deficit seriously affects photosynthetic activity [55]. This is demonstrated by Cordoba-Novoa et al. [56], where low water potential (−22.10 Bar) together with low conductance led to a reduction in water in leaves and low biomass accumulation. Furthermore, substrate 4, being more porous (72.73%) than the others, retained less water and generated a greater water deficit, leading to less leaf development in the plants [57], making it unsuitable for acclimatizing strawberry plants of 'Aroma' variety.

5. Conclusions

The results achieved in this study describe an efficient protocol for in vitro propagation and ex vitro acclimatization of strawberry variety 'Aroma'. Indeed, treatment with NaClO (1%) for 5 min showed high efficiency in reducing contamination and increasing meristem viability. Favorably, zeatin at a concentration of 1 mg L^{-1} was shown to promote growth, sprouting, rooting, and survival of new seedlings. During ex vitro acclimatization, the use of compost + peat-based substrate (substrate 1) plus humic acid yielded more vigorous plants with better morphological and physiological characteristics.

Author Contributions: Conceptualization, J.C.N. and J.J.T.-A.; methodology, J.C.N., J.J.T.-A. and J.B.M.-M.; investigation, J.C.N., N.C.V.-V. and E.H.-H.; formal analysis, N.C.V.-V.; writing—original draft preparation, J.C.N. and J.B.M.-M.; writing—review and editing, J.B.M.-M. and M.G.; supervision, M.O.; project administration, E.H.-H.; funding acquisition, M.O. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded by SNIP project (312252)/CUI (2252878) "Creación del Servicio de un Laboratorio de Fisiología y Biotecnología Vegetal de la Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas" (Creation of a Plant Physiology and Biotechnology Laboratory Service of the National University Toribio Rodríguez de Mendoza de Amazonas), executed by the Research Institute for the Sustainable Development of Ceja de Selva.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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