Article

Ex vitro acclimation of *Fragaria* x *ananassa* Duch seedlings

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Abstract

In Guanajuato, Mexico, producers propagate strawberry asexually through the use of stolons. This type of propagation is problematic because it facilitates the transmission of diseases directly and indirectly to their descendants. For this reason, the objective of this work was to determine the most favorable environment for the acclimatization of micropropagated strawberry seedlings. For *in vitro* propagation two culture media with different combinations of growth regulators were evaluated and for acclimation four environments were tested under a completely randomized design. The highest percentage of rooted stems, number of roots and stem length was obtained with LS + 0.2 mg L^{-1} BA. The best acclimation environment was under 75% shading which promoted the highest survival (91.9%), stem length (28.1), number of basal leaflets (7.7) and total fresh biomass (50.1 g). These results could facilitate the large-scale production of strawberry plants generated under *in vitro* conditions.

Keywords: environments, hormones, micropropagation.

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Introduction

The strawberry (*Fragaria* x *ananassa* Duch.), is a crop of horticultural importance in the world. Mexico was the second international strawberry exporter in 2016 with 253 700 tons (SAGARPA, 2017), production that was exceeded in 2017 by producing on an area of 11 132 hectares, 390 299 tons, where Guanajuato contributed 6.81% (26 597 tons), with an average yield of 25.8 t ha^{-1}, which is below the national average (38.7 t ha^{-1}) (SIAP, 2018).

The low yield is due to the use of foreign varieties (Davalos-González *et al*., 2017) and diseases, mainly viral (Contreras-Paredes *et al*., 2014) and caused by *Fussarium oxysporum* f. sp. *Fragariae* (Mariscal-Amaro *et al*., 2017). This is because the strawberry can only be propagated by the vegetative method that transmits directly or indirectly to its descendants these microorganisms (Boxus, 1999).

To try to contribute to improve the production conditions of the strawberry crop in Guanajuato, in the last five years' strawberry varieties adapted to the region's agroclimatic region have been developed that present higher yield and organoleptic characteristics (Davalos-González *et al*., 2017); however, the propagation of the same continues to present phytosanitary problems, which makes the adoption of these materials by producers difficult. To ensure the production of disease-free plants, with greater vigor and high genetic purity, *in vitro* propagation represents a viable alternative, which in strawberry has been well studied (Calvete *et al*., 2009; Rekha *et al*., 2013; Ling and Wetten, 2017; Diel *et al*., 2017).

However, although this method is a very simple process it is very important to adapt the propagation protocols to Mexican varieties because each genotype has its own genetic constitution that can facilitate or complicate its propagation in *in vitro* conditions Kozai (1991). In addition, the acclimation phase continues to represent a challenge since it requires a controlled environment to achieve the *ex vitro* establishment of micropropagated plants (Pospiloilova *et al*., 1999; Jofre-Garfias *et al*., 2006; Biswas *et al*., 2008). Conditions that make the adoption of technology by strawberry growers in Mexico unaffordable. Therefore, the objective of the present work was to evaluate in different acclimatization environments *ex vitro* some parameters of environmental conditions, development and physiological response of micropropagated strawberry plants.

Materials and methods

Vegetal material

In the spring of 2017, 200 apparently healthy strawberry plants of the Nikte variety were randomly collected from a 1 000 m^2 commercial lot established in the study area (Roque, Celaya, Guanajuato, 20º 34' 43'' North latitude, 100º 49' 13'' West longitude, altitude 1 767 m). The growth points were removed, rinsed under running water for 20 min and dried on paper, sterile towel. Subsequently, with a scarp under a stereoscopic microscope, the apical buds (3-5 mm thick) were removed from each apical point and used as an explant.

Disinfestation of explants

The explants were disinfested with 80% ethanol for 2 min, rinsed with sterile distilled water, dried on paper towel and again disinfested with 1.5% sodium hypochlorite for 3 min, then dried and dried again (Boxus, 1999).

Establishment of the crop

For rooting, the apical meristems (4 ±1 mm in length) were cultivated in two culture media 1) MS medium (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and 3 g L^{-1} agar with different variants of the concentration of BAP + IBA + GA_3 (0.1, 1 and 0.1 mg L^{-1}), BAP + IBA + GA_3 (0.5, 1 and 0.1 mg L^{-1}) and IBA (1 mg L^{-1}); 2) LS medium supplemented with 30 g L^{-1} of sucrose and 7 g L^{-1} of agar plus BA (0.2 mg L^{-1}). Both media without PGRs were used as control. The pH of the medium was adjusted to 5.8 before adding the agar prior to sterilization at 120 °C for 15 min. Of each treatment, 20 mL of the medium was emptied into bottles of 150 mL capacity. Five repetitions per treatment were planted with four explants per repetition under aseptic conditions within a laminar flow chamber (ESCO, EOJ/04-EHC, USA). The cultures were maintained for four weeks in a culture chamber (INCUBATOR MOD-818, USA) at 25 ±2 °C, photosynthetic photon flux density (PPFD) of 60 μmol m^{-2} s^{-1} (fluorescent white light) and a photoperiod of 16 h, daily. The stems were rooted in LS medium without hormones under the same environmental conditions described above.

Acclimatization

Whole plants (2-3 roots and 1 ±0.5 cm in length) were removed from the culture medium, rinsed with sterile distilled water, dried on sterile paper towels and transferred to small 0.5 L plastic pots (40 pots containing a complete plant) of 10 cm in diameter containing a mixture of peat, perlite and vermiculite (ratio 1:1:1; v/v) previously disinfestated at 120 °C for 20 min. To maintain the high relative humidity the pots were covered with transparent plastic containers of 1 L and same diameter. All the complete plants were cultivated under the same conditions of the establishment stage. The rooted stems (%), number of roots, number of secondary stems and the length of the tallest stem (cm) were determined 20 days later.

At the end of the pre-acclimatization treatment described above, the seedlings were removed from the bioclimatic chamber and placed in a greenhouse with 50% shading mesh (Biomalla; Hdpe monofilament yarn, USA). After two days two lateral perforations were made on both sides of the transparent containers which were removed on the third day. In environmental conditions without temperature and humidity control the plants were irrigated every two days (Schiappacasse *et al*., 2006). In a completely randomized design, four treatments were established: 1) 50% shading with mesh (10 × 5 threads cm^{-2}), (2) 75% shading with mesh (17 × 5 threads cm^{-2}), (3) polyethylene plastic cover with 70% transmissibility and (4) open field (control). In each environment 10 pots (repetitions) with a plant were placed. During the acclimatization process, the maceras were watered three times a week, and 50 mL of the Hoagland solution was applied (Hoagland and Arnon, 1950) twice a week.

In each environment, the temperature (°C) and relative humidity (HR) are monitored every 30 min, with a HOBO 8K (Onset Computer Corporation, USA). The average, minimum and maximum temperature were recorded and calculated. In addition, the PPFD was measured at noon under open sky conditions, 20 cm above the upper stratum of the plant in three days randomly chosen during the experimental period (May 15, June 12 and July 17) with a quantum sensor (LI-190SA, LICOR, USA).

Seedling evaluation

The percentage of survival (SV), length of the stem (LT, measurement of the level of the substrate to the apex of the longest leaf, cm), number of basal leaflets (NFB), total fresh biomass (BFT, includes leaves and roots) of five plants chosen at random by treatment 40 days later. To check the temperature of the leaf in the different treatments, in July 2018, the temperature of the widest and longest leaf (of six well-hydrated plants) was measured with an infrared thermometer (TN408LC ZyTemp, USA) at noon with sky clear and without wind.

Statistical analysis

The response variables were subjected to an analysis of variance with the statistical program SAS (SAS Institute, 2009) and compared means by Tukey ($p\leq 0.05$). The variables measured in percentage were transformed with the arcsine formula $\sqrt{}$ (100//X).

Results and discussion

Establishment of the crop

The treatment of LS with 0.2 mg L^{-1} of BA presented the highest number of rooted stems (90%), number of roots (4.3) and produced plants with longer stems (2.85 cm) (Figure 1 B), followed of the treatments with MS + BAP + IBA + GA_3, while the control means with MS and LS did not show development (Table 1).

Table 1. Effect of treatments with PGRs on strawberry seedling production after 20 days of *in vitro* incubation and 15 days on substrate.

Treatment (mg L^{-1})	Number of rooted stems (%)	Number of roots	Stem length (cm)
MS + BAP (0.1) + IBA (1.0) +GA_3 (0.1)	45 ±4.4 b	2.6 ±0.4 b	1.7 ±0.2 b
MS + BAP (0.5) + IBA (1.0) +GA_3 (0.1)	40 ±5.7 b	1.2 ±0.6 c	1.6 ±0.2 b
MS + IBA (1.0)	20 ±2.7 c	0 ±0 d	0 ±0 c
LS + BA (0.2)	90 ±2.2 a	4.3 ±0.3 a	2.8 ±0.5 a
Control 1 (MS without PGRs)	0 ±0 d	0 ±0 d	0 ±0 c
Control 2 (LS without PGRs)	0 ±0 d	1.6 ±0.4 bc	1.4 ±0.2 b

NTE= number of rooted stems; NRPP= number of roots per seedling; LT= stem length. Means with standard error ± within the column followed by the same letter are not significantly different (Tukey, $p\leq 0.05$).

Figure 1. Micropropagation steps of seedlings of *Fragaria* x *ananassa* Duch. (Var. Nikte) A) donor plant used to obtain explants; B) strawberry explant with foliar development and root after 40 days in induction medium (LS + BA 0.2 mg L^{-1}); C) seedling 45 days old in period of pre-acclimation (25 ±1 °C and 60% HR); and D) plants in the pre-acclimatization period, with lateral perforations in plastic cover for the control of the HR.

Research by other authors (Boxus, 1999; Hanhineva *et al*., 2005; Jan *et al*., 2013) indicates that indole butyric acid (IBA) in concentrations of 0.5-2 mg L^{-1} achieves the regeneration of strawberry seedlings from apical meristems, which is corroborated with the results found in this study at a lower regeneration rate (40-45%) than that obtained with the use of BA (6-benzyl-adenine) where 90% of rooted stems were reached.

The BA turned out to be the growth regulator responsible for the best proliferation of stems as indicated by Jofre-Garfias *et al*. (2006). In horticulture, auxins are used to promote rooting because they stimulate cell division in the cambium and differentiation in the xylem and phloem (Beyl and Trigiano, 2008) and as expected in the control (without growth regulators) there was no development that the endogenous concentration of PGRs in the explant was insufficient.

Acclimatization

During the 15 days within the bioclimatic chamber 100% of the seedlings survived, some plants showed elongation of the stem and emission of buds (Figure 1C). Similar results were reported in strawberry (Debnath, 2005) and in other crops (Rohr *et al*., 2003). After removing the plastic cover, all the plants survived for seven days under shading (75% mesh), without presenting apparent development (Figure 1D). Preece and Sutter (1991); Roberts *et al*. (1990) recommend that to maximize survival after transferring the seedlings to *in vivo* conditions it is necessary to ensure a

period of 2-4 weeks with high relative humidity. Likewise, Debnath (2005) indicates that in order to reach a survival rate and rapid acclimatization in greenhouse conditions, the relative humidity of 90-95% must be maintained. This method has been used for the successful acclimatization of *gladiolus grandiflorus* (González *et al*., 2014).

In the environments evaluated, the average and maximum temperature of the air was similar to the weather and under plastic cover and, on average, 5.5 °C lower in the shaded mesh of 50 and 75% (Table 2). The minimum temperature was similar in all environments (11.7-14.9 °C).

Table 2. Average temperature, relative humidity and PPFD in four acclimation environments of micropropagated strawberry seedlings for 60 days.

Ambient	Temperature (°C)			HR (%)	PPFD (μmol m^{-2} s^{-1})
	Mean	Maximum	Minimum		
Shading mesh 50%	20.1 ±0 c	30.7 ±0 b	12.5 ±0.2 b	47.8 ±0.6 b	953.2 ±36.4 b
Shading mesh 75%	19.9 ±0.4 d	28.1 ±0.2 c	11.7 ±0.4 b	54.2 ±0.8 a	418.6 ±16.7 a
Plastic coverage	26.3 ±0 a	36.3 ±0.3 a	14.9 ±0.3 a	54.9 ±0.4 c	1282.4 ±46.5 c
Open field (control)	25.6 ±0 b	36.1 ±0.1 a	12.1 ±0.3 b	41.9 ±0.4 c	1940.2 ±21.8 d
LSD	0.6	0.5	0.9	4.2	116.2

The means with standard error ± inside the column followed by the same letter are not significantly different (Tukey, $p \leq 0.05$). HR= relative humidity; PPFD= density of photosynthetic photonic flux; DMS= significant minimum difference.

In this sense, Boxus (1999) reports that the maximum temperature for strawberry growth is 22 °C by day and 15 °C by night, therefore, the acclimatization of strawberry in the environments evaluated in the spring-summer season in The Bajio Guanajuatense is possible. However, outdoors this process is more complicated since not only the temperature is crucial for acclimation, but, that the relative humidity is of high importance (Kozai, 1991).

In this regard, in the environments the lowest HR was recorded outdoors (41.9), while under shadow mesh the HR was higher than 50%. In the environment with 75% shade (Table 3) the highest plant height (AP) possibly was due to the lower PPDF (418.6 μmol s^{-1} m^{-2}; Table 2) and based on the fact that the strawberry obtained *in vitro* it is very sensitive to drastic changes in temperature and radiation (Laforge *et al*., 1991). This is the reason why in environments with high PPDF, or in the open (1940.2 μmol s^{-1} m^{-2}) the LT is reduced (Table 3, Figure 2A and 2B).

Table 3. Growth variables of 60-day-old strawberry seedlings micropropagated under four acclimation environments.

Treatment	SV (%)	LT (cm)	NFB (#)	BFT (g)	TH (°C)
Shading mesh 50%	80.1 ±0 b	24.7 ±0 a	7.5 ±0.2 ab	47.1 ±1 b	23.4 ±0.4 c
Shading mesh 75%	91.9 ±0.4 a	28.1 ±0.2 a	7.7 ±0.4 ab	50.1 ±1.5 a	21.8 ±0.7 c
Plastic coverage	26.3 ±0 c	16.3 ±0.3 b	8.1 ±0.3 a	19.7 ±0.8 d	30.1 ±1 a
Open field (control)	5.6 ±0 d	16.1 ±0. 1b	4.1 ±0.3 b	23.5 ±1.2 c	28.6 ±1.2 b
DMS	0.6	0.5	0.9	10.2	6.05

SV= survival; LT= taller stem length; NFB= number of basal leaflets; BFT= total fresh biomass; TH= leaf temperature. The means with standard error ± inside the column followed by the same letter are not significantly different (Tukey, $p \leq 0.05$).

Figure 2. Acclimation stages of seedlings of *Fragaria* x *ananassa* Duch. (Var. Nikte). A) seedlings in acclimatization under shading of 75%; B) plant of 10 months of age in greenhouse production; and C) plant of the Nikte variety in the propagation stage.

In this sense, Laforge *et al*. (1991) indicate that the optimal PPDF for strawberry development is 1 650-3 000 μmol s^{-1} m^{-2} and for successful acclimation the PPDF must be incremental from 300 μmol s^{-1} m^{-2}. This is corroborated by the higher SV obtained in environments where PPDF was lower (Table 2). The high accumulation of biomass occurred in long days with cool temperatures (19-21 °C). The lowest LT and BFT were recorded in the weathering treatment due to the low development, premature senescence of the plants (Table 3) and days with a temperature greater than or equal to 36 °C (26 °C is the maximum growth temperature for strawberry) (Davalos-González *et al*., 2011). Silva *et al*. (1994) mentions that fractionating luminous intensity is a factor that improves the efficiency of acclimatization while excess light induces photoinhibition and desiccation (Pospisilova *et al*., 1999).

Seedling evaluation

Survival

There were significant differences ($p \leq 0.05$) for survival (Table 3, Figure 2 C). The highest SV (91.9%) was obtained under a shaded mesh of 75%, followed by the environment with 50% shading; however, under 75% shade the plants with the best characteristics were obtained, as opposed to 94.4% of the plants died in the open environment. The poor acclimation in the control was due to the maximum of recorded temperatures (> 30 °C), which caused desiccation in the plants which increased the mortality.

It is known that the high mortality rate in plants obtained *in vitro* occurs when the plants are in a controlled environment and are moved to an uncontrolled environment (González *et al*., 2014) and this particularly occurs when the plants are transferred to natural conditions (25.6 ±2 °C). Rohr *et al*. (2003) indicate that the biggest problem of survival (SV) is the lack of vigor of the plant and the necrosis of the seedling is due to excessive moisture loss due to temperature during the transfer of micropropagated plants under *in vitro* conditions (high relative humidity; HR) to environmental conditions with low HR levels.

Jofre-Garfias *et al*. (2006) report a survival rate of strawberry seedlings 93% after one week of being transferred and 90% after four weeks, SV rate similar to that found in our study under similar conditions, but with different handling in acclimatization since we propose the acclimatization in two phases: 1) pre-acclimatization under controlled conditions; and 2) under shade mesh of 75%.

Under 50% and 75% shade the leaves showed a temperature of 21-23 °C (Table 3), 4 °C less than the maximum growth temperature reported for strawberry growth. Temperature that gave better result since the plants did not suffer heat stress. While the seedlings under plastic cover when subjected to a temperature higher than 26 °C were dehydrated by heat stress since the seedlings in these conditions can not regulate the perspiration of the stomata and easily dehydrate and die (Hazarika, 2006; Beyl and Trigiano, 2008).

Conclusions

In this study, basic techniques were applied and our results showed that *ex vitro* acclimation could be successful without growing micropropagated plants under greenhouse conditions. Therefore, these results could contribute to facilitating the large-scale production of strawberry plants generated under *in vitro* conditions.

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