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Unidad de Bioquímica y Bilogía Molecular de Plantas, Centro de Investigación Científica de Yucatán (CICY), Chuburná de Hidalgo, Mérida, Yucatán, México

Highly efficient system for the micropropagation and acclimatization of pineapple in vitro plants (*Ananas comosus* L. Merr, var. MD2)

Adolfo Guzmán-Antonio, Adriana Canto-Flick**, Susana Avilés-Viñas, Yaritza Rodríguez-Llanes, Gema Pijeira-Fernández, Nancy Santana-Buzzy***

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Summary

The low acclimatization efficiency of pineapple seedlings obtained by *in vitro* culture has been one of the main limitations to massively scale micropropagation protocols. Various factors may be affecting the successful establishment of *in vitro* plants to *ex vitro* conditions, related to the plant and/or the environment and the management of seedlings. The objective of this study was to establish an efficient micropropagation and acclimatization system for the MD2 variety, with a high *in vitro* multiplication rate and a high greenhouse and field survival of pineapple *in vitro* plants. In the multiplication phase, the axillary buds were isolated and placed on a semisolid medium and three induction treatments with different concentrations of BAP (1.0, 3.0, 5.0 mg/L) and ANA (2.0 mg/L), were evaluated. After the third subculture, multiple shoots were transferred to a temporary immersion system (BIOMINT). During acclimatization and nursery phases, six treatments with different substrate compositions were evaluated. The induction treatment IT3 (BAP 5.0 mg/L and ANA 2.0 mg/L) was selected for a significantly high multiplication rate. AT4 treatment (Soil + Coconut fiber + perlite 1:1:1) showed the highest survival rate (95%) and allowed the obtaining of well-developed plants. This system constitutes a valuable technology to introduce *in vitro* plants to the pineapple production scheme on a commercial scale.

Keywords: Micropropagation, multiplication rate, acclimatization, survival rate.

Introduction

Pineapple (*Ananas comosus* (L.) Merr.), belonging to the Bromeliaceae family, is a perennial crop and is one of the three tropical fruits with the greatest presence in the international market, with banana and mango. Costa Rica, Thailand and China are the main exporters of pineapple, supplying more than 50% of world production. The MD2 (Mary Dillard 2) is the variety that occupies the preference of the international market, particularly for its aroma and flavor (Joy and Anjana, 2016). The company Del Monte Fresh Produce in Hawaii Inc. markets produce it as Gold extra sweet, Golden Ripe, or Gold, since 1996. It is a cultivar product of the crossing of two hybrids (PRI 581184 × PRI 59443) and it is known that one of its parents comes from smooth Cayenne (Rodríguez et al., 2016).

Pineapple is propagated vegetatively from lateral shoots, basal shoots, or crowns. A plant can contribute around 10-15 propagules, which implies that this method is frankly limited considering that one Ha of the crop requires between 60,000 and 80,000 seedlings (Firoozabady and Gutterson, 2003). Tissue culture is the most efficient alternative to produce pineapple propagules on a large scale, of high quality, uniform in size and weight, genetically homogeneous, and free of pathogens (Ayenew et al., 2021). The first reports of pineapple *in vitro* culture date back several decades (Aghion and Beauchesne, 1960; Mapes, 1973; Lakashmi et al., 1974). There are reports confirming that *in vitro* tissue culture techniques have been successfully applied to produce pineapple propagules on a large scale from individual explants (Dewald et al., 1988; Devi et al., 1997; Zepeda and Segawa, 1981; Liu et al., 1989; Sripaoraya et al., 2003). Remarkable progress has been made in the application of automated systems, which are biotechnological alternatives for the commercial production of highly efficient *in vitro* propagules (Kiss et al., 1995; Escalona et al., 1999; Rahman et al., 2001; Reinhardt et al., 2018; Adeoye et al., 2020; Li et al., 2022) and the use of temporary immersion bioreactors for the multiplication of pineapple shoots has been reported (Escalona et al., 1999; Solórzano-Acosta et al., 2020; Ayenew et al., 2021; Hwang et al., 2022), with a lower cost of production (Silva et al., 2008). However, these biotechnological systems still lack results showing the successful establishment of the *in vitro* plants on a commercial scale, in the field. Although pineapple is one of the species with the greatest *in vitro* regeneration capacity (Escalona et al., 1999; Rahman et al., 2001; Kessel-Domini et al., 2022), there is very little information that supports survival and the efficiency of *in vitro* plants during acclimatization and the establishment in the field. The objective of this work was to establish an efficient *in vitro* shoot regeneration system of pineapple with high survival capacity during acclimatization and establishment in the field.

Materials and methods

Explant source

Basal young suckers (20-30 cm), located in the upper middle zone of the plant of pineapple (*var.* MD2) were recollected in a pineapple plantation in the ejido Ávila Camacho, municipality of Bacalar, Quintana Roo, México, during the period 2020-2023. The leaves were removed from the suckers (Fig. 1a-b). The axillary buds were isolated from the suckers and washed with soapy water and rinsed 3-4 times with running water. Subsequently, the explants were disinfected with a commercial chlorine solution (v/v) (Cloralex; 4-6% Active Chlorine), diluted to 20%, for 20 minutes. The axillary buds were rinsed 4-5 times with previously sterilized distilled water containing 30 mg/L of Cysteine–HCl.

Induction and multiplication of shoots *in vitro*

MS salts, recommended by Murashige and Skoog (1962) supplemented with sucrose (30 g/L), Thiamine-HCl (1.0 mg/L), and cysteine-HCl (30 mg/L) were used as basal medium. The induction treatments (IT) evaluated were added with a combination of three different concentrations of BAP (1.0, 3.0, 5.0 mg/L) and ANA (2.0 mg/L). The culture medium was solidified with gelrite (3.3 g/L)

** These authors contributed equally to this work.
* Corresponding author

Fig. 1: Steps for the establishment of the pineapple micropropagation system: a) Elimination of the tiller's leaves; b) Washing and disinfecting tillers stems containing apical and axillary buds; c) Isolated buds established in the culture medium; d) Multiple shoots formed in the induction culture medium; e) Shoot proliferation in the temporary immersion bioreactor; f) Isolation and washing of the *in vitro* plants to be transferred to the nursery.

and the pH was adjusted to 5.7 ± 0.2 before adding gelrite. Culture vessels containing 20 mL of medium was placed in the autoclave at 121 °C, for 15 minutes. The axillary buds were isolated and placed on the surface of a semisolid medium to induce multiple shoots (Fig. 1c). Three explants were planted per culture vessel and a completely randomized experimental design was used, with three replications, for a total of 60 explants per treatment. The explants established in the culture medium were incubated at a photoperiod of 16 light hours, with a light intensity of 60 μ $\mu mol^{-2}s^{1}$ and a temperature of 25±2 °C. Culture media was renewed every 6 weeks, counting from the date of culture establishment. After the third subculture, multiple shoots (Fig. 1d) were transferred to a system of temporary immersion (BioMint), with the same medium composition, but without gelrite (liquid medium). The shoots were exposed to an immersion frequency of 5 minutes every 4 hours, to complete their development (Fig. 1e). Was evaluated the number of explants forming shoots; the number of shoots per explant; and the number of established plants per treatment. After 6 weeks, shoots that had reached 6-7 cm were retrieved from the BioMint containers, individually isolated and washed with running water (Fig. 1f) to eliminate residues of the culture medium, previous being transplanted for their acclimatization.

Acclimatization and nursery phases of the seedlings

The acclimatization phase was carried out in a greenhouse located in Mérida, Yucatán, at 20°5.802.53" north latitude and 89°35033.30" west longitude, at an altitude of 10 m.a.s.l., with average temperature 28-35 °C; RH 55-75% and natural light cycles 11 h light, 13 h darkness. Seedlings (5-7 cm in length), once removed from the culture container, were washed with running water to eliminate the culture medium residues from the roots. Afterward, they were cultured in 200-well polystyrene trays containing as substrate Peat Moss Sunshine Mix 3 (Sol® Mix # 3) for its hardening, prior to their establishment in the different acclimatization treatments. At the acclimatization phase, the seedlings were fertilized twice a week for 6 - 8 weeks with Hakaphos® Violeta NPK 13-40-13 (3 g/L), hydrosoluble NPK complex, and micronutrients recommended for plant roots. Afterward, seedlings measuring 8 -10 cm in length were transplanted into black polyethylene bags (18 × 14 × 40 cm) containing a mix of different substrates (treatments) (nursery phase). The type of soil used in the mixing of the different treatments corresponds to the soils classified as *Luvisoles* (LV), Borges-Gomez et al. (2014), which are clayey, fertile, and productive red soils. The nursery treatments (AT) evaluated had the following mixtures of substrates: T1) Red Soil (Control); T2) Sunshine mix 3; T3) Red Soil + coconut fiber 1:1; T4) Red Soil + coconut fiber + perlite 1:1:1; T5) Red Soil + Sunshine mix 3 + perlite 1:1:1; T6) Red Soil + Sunshine mix 3 + perlite 2:1:1. 76 seedlings were used for each treatment, with 2 repetitions. The depth of the seedlings in the substrate was 0.5 - 0.6 cm. Phytosanitary control was carried out preventively every 4 weeks, applying Lorsban TM 480 EM at a concentration of 0.5 mL/L to control *Symphyla* (*Hanseniella* spp., *Scutigerella* spp., *Symphylella* spp.) and with Venom® (1g/L) to control the mealybug *Dysmicoccus brevipes* (Cockerell). Irrigation was carried out twice a week, alternating in the watering the application of Hakaphos® Violeta NPK 13-40-13 fertilizer, at a concentration of 5 g/L during the first month of the transplant. For the next 4 months, Ultrasol® multipurpose NPK 13-06-40 (5 g/L) and Bayfolan® Forte (2 mL/L) were applied to the *in vitro* plants. 10 plants were evaluated per treatment. The variables assessed were survival (%), plant height (cm), number of leaves, and cup diameter. The assessment was carried out 5 months after the plants were freed from the culture container.

Establishment of in vitro plants in the field

The *in vitro* plants were distributed in 5 rows of 100 plants each. As control, the same number of traditional suckers of MD2, distributed in the same way to the *in vitro* plants (5 rows of 100 plants) was used. The plants were established in a soil classified as Luvisols (LV),

Borges-Gomez et al. (2014), which are of the red type, clayey, fertile and very productive. The planting distance was 0.30 m × 1.40 m. The first fertilization was carried out with ammonium sulfate (21-00-00-24S) and diatonic phosphate (DAP 18-46-00) and the second fertilization was foliar, applying Poliquel multi and 18-14-00 as a source of N and P. After the foliar application, alternate fertilization was applied, every 20 days until 5 months. After 6 months, a mixture of potassium sulfate, potassium chloride, DAP 18-46-00, polyquel Zinc, and polyquel Boron was applied. Flowering was induced 8 months after the plot was established in the field and used a mixture of Ethrel ethephon + urea. The fruits were harvested and evaluated six months after flowering was induced (14 months of age of the plant). Ten fruits were randomly selected per row of each treatment (*in vitro* plants and traditional suckers) to which weight (kg), length (cm) and fruit width (cm) were evaluated. The survival (%) of the plants was also evaluated. Survival was evaluated by considering the plants that died and the plants that remained alive but did not advance in their development.

Statistical analysis

In all the experiments carried out, data were processed with the SAS program for Windows, version 9.1. A variance analysis (ANOVA) and the LSD (least significant difference) test were applied with $p<0.05$ to determine the importance of the differences among variables. Chi-square test (X2) was used to analyze the survival of *in vitro* plants during acclimatization in the greenhouse.

Results

Shoot induction and multiplication

As a result of the induction phase of pineapple shoots in different treatments, it was possible to appreciate that morphogenic structures were formed in the three culture media evaluated; however, the way in which the process was manifested, as well as the efficiency, varied markedly between treatments (Tab. 1). The IT3, supplemented with 5.0 mg/L of BAP and 2.0 mg/L of ANA, was the treatment that most favored the multiple shoot formation, with 73.33% of responsive explants counted from weeks 16 to 18, differing significantly from the other treatments evaluated. This treatment initially involved an abundant formation of morphogenetic structures directly from the explant (Fig. 2a), which proliferated rapidly to form vigorous shoots (Fig. 2b). As can be seen in Fig. 2c, the morphology of these shoots

Tab. 1: Treatments evaluated for the induction and proliferation of pineapple shoots.

Induction treatments (IT)	Culture medium composition (mg/L)	Explants that formed shoots (%)	Number of shoots formed per explant	Total seedlings obtained
IT-1	1.0BAP+2.0 ANA	20.0% c ± 1	58 c ± 2	710 c ± 6
IT-2	3.0BAP+2.0 ANA	35.0% b ± 2	75 b ± 2	1,637 b ± 13
IT-3	5.0BAP+2.0 ANA	73.3% a ± 2	201 a ± 4	8,573 a ± 188

Fig. 2: Response of pineapple explants to treatment with 5.0 mg/L BAP and 2.0 mg/L ANA: a) Shoot induction and multiplication of pineapple in the semisolid medium; b) cluster of shots formed in magenta prior to its transfer to temporary immersion (BioMint); c) morphology of the induced shoots (micro-suckers), like traditional suckers; d) multiplication and development of shoots in temporary immersion (BioMint).

was similar to that of the traditional sucker, but in a very small size, like micro-suckers. These shoot clusters reached an average multiplication rate of 201 ± 4 shoots per explant and 6-7 cm in height, 6-8 weeks after being transferred to a temporary immersion bioreactor (BioMint) (Fig. 2d). As shown in Tab. 1, in the induction treatments with lower BAP content (1.0 or 3.0 m), a significantly lower number of shoots per explants was formed (Tab. 1).

Acclimatization and nursery phase of the seedlings

As shown in Tab. 2, the results obtained after the acclimatization and nursery phase showed that 5 months after being transferred to the greenhouse, all substrate mixes used were significantly superior compared to the control treatment (AT1) in terms of survival, which allows inferring that the mixture of the soil with the substrate notably improves the physical and/or nutritional characteristics of the soil (Red Soil). However, the AT4 treatment (Coconut fiber or sunshine mix 3 + Perlite, 1:1:1) significantly exceeded the rest of the treatments with a 97% survival of the *in vitro* plants, while AT1 (control), was the one with the lowest survival rate (32%). The Chi-square test ($\chi 2$) indicates the survival of the plants is different between the treatments evaluated ($\chi 2 = 27.965$, $p < 0.05$) (Tab. 3).

Fig. 3 shows the behavior analysis of the variables plant height, number of leaves, and cup diameter, of the plants in the different treatments evaluated. As shown in Fig. 3a, for plant height, the AT4 treatment (Red Soil + Coconut Fiber + Perlite 1:1:1) differed significantly from the rest of the treatments, with an average value of 30 cm in height, contrasting with the AT1 treatment (Red Soil) in which plants had the lowest height, compared to the rest of the treatments. For the variable number of leaves (Fig. 3b) the plants from the AT2 (Sunshine mix 3) and AT4 (Red Soil + Coconut Fiber + Perlite 1:1:1) treatments presented the highest average values (22 leaves/plant), without differing significantly from each other but both differing significantly from the rest of the treatments. The AT1

Tab. 2: Percentage of survival of *var*. MD2 pineapple plantlets in the greenhouse acclimatization phase using different substrates.

Treatment	Composition	Survival rate (%)
AT1	Red Soil (control)	32
AT2	Red Soil + Sunshine mix 3	64
AT3	Red Soil + Coconut fiber (1:1)	67
AT4	Red Soil + Coconut fiber + Perlite (1:1:1)	97
AT5	Red Soil + Sunshine mix 3 + Perlite (1:1:1)	73
AT6	Red Soil + Sunshine mix 3 + Perlite (2:1:1)	75

Tab. 3: Statistical test[a] of the survival of pineapple *in vitro* plants (var. MD2) in the acclimatization phase.

	Survival
Chi-square (X^2)	27.965
Degrees of freedom (df)	5
Asymptotic significance	.000

[a] Kruskal-Wallis test, $p < 0.05$.

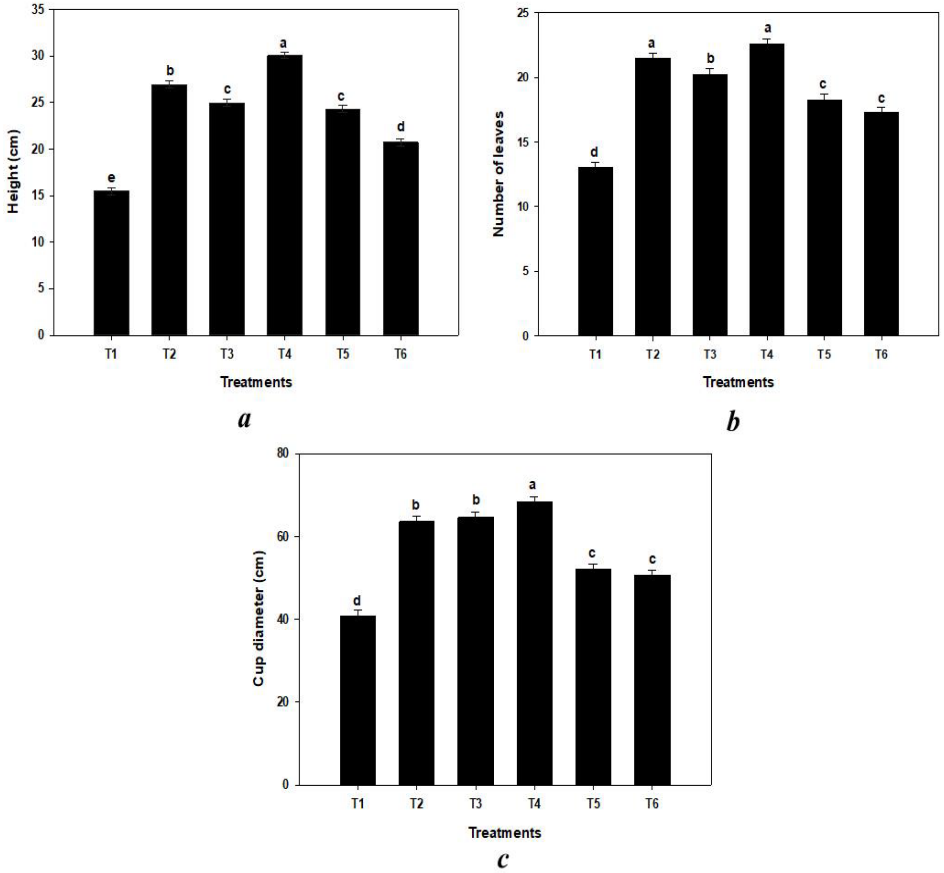

Fig. 3: Plant height (a), number of leaves (b), and canopy width (c) variables in the different treatments assessed, 5 months after *in vitro* plants concluded the nursery stage: AT1) Red Soil (Control); AT2) Sunshine mix 3; AT3) Red Soil + Coconut fiber 1:1; AT4) Red Soil + Coconut fiber + Perlite 1:1:1; AT5) Red Soil + Sunshine mix 3 + Perlite 1:1:1; AT6) Red Soil + Sunshine mix 3 + Perlite 2:1:1.

treatment (Red Soil; Control) was the one with the lowest average of the number of leaves per plant. The cup diameter variable (Fig. 3c) had its highest value in the AT4 treatment (Red Soil + Coconut Fiber + Perlite 1:1:1), differing significantly from the rest of the treatments with an average value of 70 cm. AT1 treatment (Red Soil; Control) had the lowest cup diameter, compared to the rest of the treatments. These results show that the mixture of Red Soil + Coconut fiber + Perlite (AT4) in a v/v mix proportion of 1:1:1, was a substrate that significantly favored the development and the highest survival rate (97%) of pineapple *in vitro* plants, during the acclimatization phase.

Fig. 4 shows photographic evidence of the sequence of phases (3) elapsed during the acclimatization and nursery phases of pineapple seedlings, from the time they were released from the culture vessel to the moment they were ready for being transferred to the field. As can be seen in Fig. 4a-b, the uniformity and quality of the seedlings to initiate greenhouse acclimatization were very important parameters for the future development of plants in the greenhouse. The adequate average length of the seedlings (5 -7cm) when leaving the culture vessel was previously determined (Fig. 4b). It was found that smaller sizes of seedlings caused losses and/or stagnation in development during the period of acclimatization, not surmountable in the field (unpublished data). In the acclimatization phase (hardening phase), seedlings were established in trays with small wells containing Sunshine substrate, where they stayed from 6 to 8 weeks, until they reached a height of 8 -10 cm (Fig. 4d), the moment in which they were transferred to bags containing a substrate mix with the AT4 composition (Red Soil + Coconut Fiber + Perlite 1:1:1) (nursery phase). Three months later, *in vitro* plants were ready to be transplanted into the field (Fig. 4e).

The plants, at the end of the nursery phase, showed an optimal physiological state, appreciated through the abundant foliar development, the showiness of their leaves, the uniformity of the plants, and the profuse root system they presented (Fig. 5a-b). Fig. 6 a-d shows pineapple *in vitro* plants established in the field in full fruiting. The

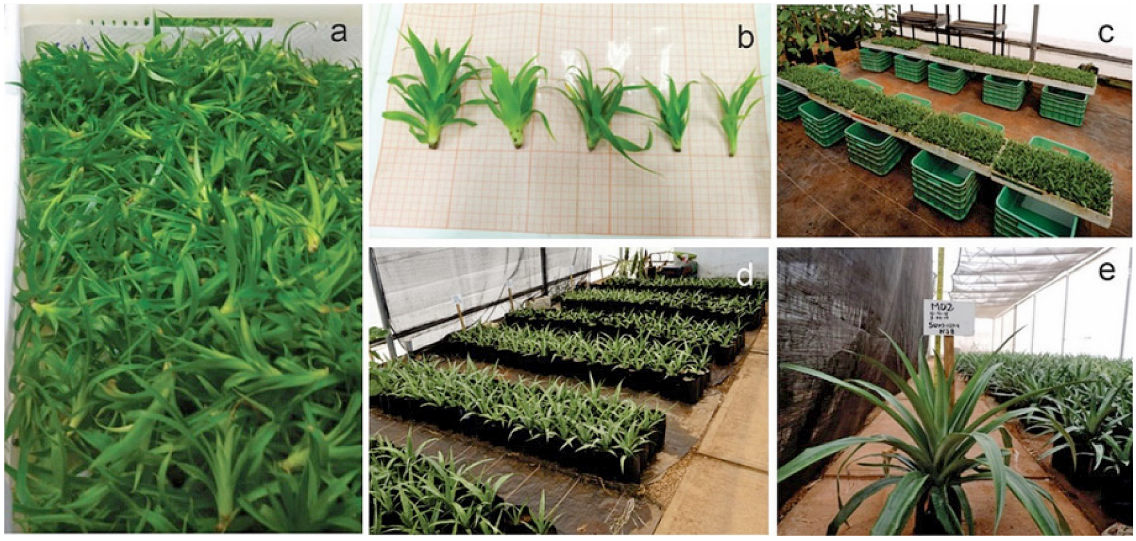

Fig. 4: Acclimatization of pineapple *var*. MD2 seedlings: a) Efficiency of the protocol; uniformity and strength of the seedlings at the moment of being freed from the *in vitro* culture container; b) Development of the seedlings at the moment of being freed from the *in vitro* culture container (5-7 cm); c) Establishment of the seedlings in trays containing inert substrates (Sunshine) for their hardening (2 months); d) Development of the plantlets (8 -10 cm) at the moment of being transplanted from trays to polyethylene bags (nursery phase); e) Developed plants in polyethylene bags, 5 months after leaving *in vitro* containers, ready to be transplanted into soil in the open field.

Fig. 5: Development during the nursery phase of *in vitro* plants of the pineapple *in vitro* plants. a) *in vitro* plants at 3 months after being transferred to bags containing a mix of substrates (T4: Red Soil + Coconut Fiber + Perlite 1:1:1), in the greenhouse; b) Development of the root system, at the moment of being transplanted to soil in the field when *in vitro* plants concluded the nursery stage (5 months).

Fig. 6: Experimental plot of MD2 pineapple: a) Pineapple *in vitro* plants fruiting under field conditions; b-d) Data collection of the fruit: weight, length and width of the fruit.

Tab. 4: Behavior of survival rate (%), of the means of fruit weight (Kg), fruit length (cm), and fruit width (cm), of pineapple *in vitro* plants and traditional suckers (control), established in the field at the open sky. The data were collected 14 months after the plants were established in the field.

	Survival rate (%)	Average fresh weight of the fruit (Kg)	Average length of the fruit (cm)	Average fruit width (cm)
Suckers	87	1.9 ± 0.09 b	17.8 ± 0.54 b	31.3 ± 1.51 b
In vitro plants	97	2.5 ± 0.08 a	20.4 ± 0.48 a	43.6 ± 0.92 a

fruits presented an average weight of 2.512 kg and an average size of 20.39 × 43.6 cm, differing significantly from the fruit from traditional suckers, for the three evaluated fruit variables (weight, length, and width) (Tab. 4). *In vitro* plant survival was 97%, exceeding the survival of traditional suckers (87%).

Discussion

The pineapple [*Ananas comosus* (L.) Merr.] is traditionally propagated vegetatively (by suckers). The planting density varies between 60,000 and 80,000 suckers per Hectare (Bartholomew, 2003). However, the availability of propagules in number, quality and uniformity is extremely limited due to the low propagation rate of the species. The MD2 cultivar, developed by Del Monte Fresh Produce International Inc., has occupied the preference of the international fresh fruit market for some years (Loeillet et al., 2011). The limitations of the traditional propagation of the crop, as well as the difficult adaptation of MD2 to changes in its environment, make its expansion in the international market slower and more expensive.

In vitro propagation is the most efficient alternative to reproduce homogeneous, uniform and healthy pineapple plants on a large scale, at any time of the year. There are reports that show that pineapple is one of the species with the greatest potential for *in vitro* regeneration, regardless of the culture regime to which it is subjected: semisolid medium, liquid medium and/or temporary immersion (Escalona et al. 1999; Scheidt et al., 2009). The use of temporary immersion bioreactors allows precise control of plant growth and increases the multiplication rate; in addition, it reduces the space, energy and labor requirements for plant management in commercial micropropagation (Aragón et al. al., 2013). However, during the transition from *in vitro* to *ex vitro* conditions, *in vitro* pineapple plants can be sensitively affected by the drastic change in environment, causing very slow growth (stagnation), as well as very low survival rates. Efforts have been made to determine the causes that cause stagnation in the growth of pineapple *in vitro* plants during acclimatization; however, the efficiency remains low (Kornatskiy, 2020).

Probably, the management that is carried out after leaving the *in vitro* culture container, and the size of the same at the moment of being transplanted to the ground, are decisive for the success of pineapple micropropagation. In our experience, pineapple plants require an acclimatization phase (2 months) and a nursery phase (3 months) to reach a development similar to that of traditional suckers. This agrees with what was stated by Kitto (1997) who mentioned that the commercial expansion of micropropagation will only be possible when technologies are available to optimize the acclimatization of *in vitro* plants.

Aragón et al. (2013) evaluated the morpho-physiological changes in a pineapple *in vitro* plant [*Ananas comosus* (L.) Merr. MD2] during the acclimatization phase. They observed that, once the seedlings are ready to be removed from the temporary immersion bioreactor, they are carefully acclimatized to natural environmental conditions, and during the first two months of growth, a facultative C3/CAM metabolism is characteristic of pineapple *in vitro* plants, depending on the environmental conditions. On the other hand, Ceusters et al. (2010) found bromeliad photosynthetic plasticity in response to a range of environmental factors that include [CO_2], water availability, light intensity, and temperature. The effect of increased light intensity and gradual reduction of relative humidity in the early stages of pineapple seedlings (*Ananas comosus*), has also been reported by different authors (Yanes et al., 2000; Silva et al., 2008). While Batagin et al. (2009), detected an increase in the thickness of the cuticle, in the wavy contours of the epidermal cells, in the distribution and quantity of mesophyll fibers, during the acclimatization process, evidencing the interference of the light conditions in the morphological characteristics of the pineapple seedlings. According to Villalobos et al. (2012), it has been shown that during the propagation of pineapple, light is the factor that most influences the quality of the plant, and provides a better agronomic and anatomical change during the acclimatization phase. Silva et al. (2008) and Aragón et al. (2010), observed that pineapple plants subjected to water deficit stress and a higher flow of photosynthetic photons changed their metabolism from C3 to CAM. Villalobos et al. (2012), observed a higher number of roots and an increased photosynthetic activity after 45 d of

acclimatization, compared with 56 d old CAM-induced pineapple plants. They attributed this behavior to the environmental conditions used in the acclimatization process. Rodríguez-Escriba et al. (2015) described the morpho-physiological and biochemical changes in five months old MD2 micro-propagated pineapple plants (*Ananas comosus* (L.) Merr.), grown 30 days under low light intensity (LL, green-house light conditions at 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or high light intensity (HL, field light conditions at 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$). As a result, both groups showed a typical CAM phenotype, but it was stronger in HL conditions, which may confer these plants a better acclimatization capacity. However, despite the progress made in pineapple micropropagation, it has not yet been possible to establish a comprehensive technology that guarantees the field establishment of *in vitro* plants. In general, acclimatization has turned out to be the "Achilles' heel" in micropropagation due to the high level of empiricism that still prevails in this process. However, it is in this phase that plants undergo one of the most drastic changes in the process: the change from *in vitro* to *ex-vitro* conditions. In this transition, the plants not only go from a strictly controlled environment (temperature, light, relative humidity, availability of nutrients, etc.), to semi-controlled conditions (greenhouse), but they also radically change their nutrition from heterotrophic to autotrophic. There are other causes, no less important, that can contribute to the low survival rate of plants during the acclimatization and nursery phases, such as poor management of the water regime, and in many cases the absence of an adequate protocol for the preventive control of pathogenic microorganisms that can compromise the life of *in vitro* plantlets at this stage of the micropropagation process (Gil-Rivero et al., 2017).

In our study, during the nursery phase, the best treatment was T4, composed of a mixture of red soil + coconut fiber + perlite v/v 1:1:1. The use of ground coconut fiber to acclimate *in vitro* plants of pineapple variety Champaka F-153 was reported by Garita and Gómez (2000), which obtained a 95.2% of survival. Rodríguez et al. (2016) established pineapple *in vitro* plants and studied the variables: Survival (%), number of leaves, plant length (cm), leaf length "D" (cm), number of roots and fresh plant mass (g). The authors reported that at 6 months the plants had a height of 23 cm, 5 leaves on average, and a leaf length "D" of 21.65 cm. In our study, the plants concluded acclimatization + nursery phases at 5 months of age, with average values of 31 cm in height, 24 leaves per plant, and the plant crown of 70 cm of diameter, ready to be transferred to the field. Probably, the mixture of red soil, coconut fiber, and perlite conferred physical characteristics to the substrate that notably favored a harmonious development of the aerial components and the plant root system, which allows them to use efficiently the available water and nutrients. The MD2 pineapple *in vitro* plants, once established in the field, completed their development, and enter their flowering and fruiting cycle in a similar way to that of this variety grown from tillers by the traditional method (Fig. 5a). As shown in Fig. 5b-d, fruits obtained showed a healthy appearance, without deformations. The average weight was 2.5 ± 0.08 kg per fruit, differing significantly from the fruit of the traditional suckers (1.9 ± 0.09 kg per fruit).

Conclusions

Agricultural development and the expansion of the pineapple industry depend, to a large extent, on the development of new and efficient technologies using the alternatives offered by biotechnology. However, the potential use of these procedures is still limited due to the low survival and growth rates of *in vitro* plants during the acclimatization phase, probably due to the high degree of empiricism with which have been handled the *in vitro* plants at this stage of micropropagation. Contradictorily, pineapple *micro-suckers* (*in vitro* plants) meet all the requirements to be considered "elite propagules": uniformity (in size and weight), genetic homogeneity and healthy (free of pathogens). Our results show that when micropropagation is accompanied by a reproducible and efficient protocol for acclimatization phase, nursery phase and field establishment of *in vitro* plants, micropropagation becomes a recommended technology for massive propagation of propagules, in this case, pineapple. In this study, we proposed a micropropagation system which integrate the biotechnological management with traditional field management. This is the first report of a technology for the massive propagation of pineapple in which biotechnology and traditional crop management are successfully integrated.

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Conflicts of interest

No potential conflict of interest was reported by the authors.

References

Adeoye, B.A., Lawyer, E.F., Hassan, K.O., Ilesanmi, A.O., Richard-Olebe, T.C., Oyedeji, T.T., Adedeji, A.A., 2020: Optimization of Plant Growth Regulator (PGR) on *in vitro* propagation of pineapple (*Ananas comosus* (L.) var. Smooth Cayenne). Int. J. Rec. Res. Life Sci. 7(1), 13-20.

Aghion, D., Beauchesne, G., 1960: Utilization de la technique de culture sterile d'órganes pour des clones d' Ananas. Fruits 15, 464-466.

Aragón, C., Carvalho, L., González, J., Escalona, M., Amáncio, S., 2010: *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor. Biol. Plant. 54, 237-244. DOI: 10.1007/s10535-010-0042-y

Aragón, C., Pascual, P., González, J., Escalona, M., Carvalho, L., Amancio, S., 2013: The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: proteomic and transcriptomic profiles. Plant Cell Reports 32, 1807-1818. DOI: 10.1007/s00299-013-1493-3

Ayenew, B., Tadesse, T., Gebremariam, E., Mengesha, A., Tefera, W., 2021: Efficient use of temporary immersion bioreactor (TIB) on pineapple (*Ananas comosus* L.) multiplication and rooting ability. J. Microbiol. Biotechnol. Food Sci. 2(4), 2456-2465.

Bartholomew, D., Paul, R., Rohrbach, K., 2003: Crop environment, plant growth and physiology. In: Bartholomew, D., Paul, R., Rohrbach, K. (eds.), The pineapple: botany, production and uses, 69-108. CABI Publishing, Wallingford. DOI: 10.1079/9780851995038.0000

Batagin, K.D., Almeida, C.V.D., Tanaka, F.A.O., Almeida, M.D., 2009: Morphological alterations in leave of micropropagated pineapple plants cv. IAC "Gomo-de-mel" acclimatizated in different conditions of luminosity. Acta Bot. Bras. 23, 85-92. DOI: 10.1590/S0102-33062009000100011

Borges-Gomez, L., Moo-Kauil, C., Ruiz-Novelo, J., Osalde-Balam, M., Gonzalez-Valencia, C., Yam-Chimal, C., Can-Puc, F., 2014: Soils used for habanero chili production in Yucatán: predominant physical and chemical characteristics. Agrociencia. 48(4), 347-359.

Ceusters, J., Borland, A.M., Ceusters, N., Verdoodt, V., Godts, C., De Proft, M.P., 2010: Seasonal influences on carbohydrate metabolism in the CAM bromeliad Aechmea 'Maya': consequences for carbohydrate partitioning and growth. Ann. Bot. 105, 301-309. DOI: 10.1093/aob/mcp275

Devi, L.U., Bhagawan, S.S., Thomas, S., 1997: Mechanical properties of pineapple leaf fiber-reinforced polyester composites. J. Appl. Polym. Sci. 64(9), 1739-1748. DOI: 10.1002/(SICI)1097-4628(19970531)64:9<1739

DeWald, M.G., Moore, G.A., Sherman, W.B., Evans, M.H., 1988: Production of pineapple plants *in vitro*. Plant Cell Rep. 7, 535-537. DOI: 10.1007/BF00272750

Escalona, M., Lorenzo, J., González, B., Daquinta, M., Borroto, C., González, J., Desjardins, Y., 1999: Pineapple micropropagation in temporary immersion systems. Plant Cell Rep. 18, 743-748. DOI: 10.1007/s002990050653

Firoozabady, E., Gutterson, N., 2003: Cost-effective *in vitro* propagation methods for pineapple. Plant Cell Rep. 21, 844-850. DOI: 10.1007/s00299-003-0577-x

Garita, H., Gómez, L., 2000: Micropropagación de la variedad de piña Champaka F-153. Agronomía Costarricense 24, 63-73.

Gil-Rivero, A.E., López, S.E., López, A., 2017: Aclimatación de plántulas *in vitro* de *Saintpaulia ionantha* H. Wendl. (Gesneriaceae) "violeta africana" a condiciones de invernadero. Arnaldoa 24, 343-350. DOI: 10.22497/arnaldoa.241.24116

Hwang, H.D., Kwon, S.H., Murthy, H.N., Yun, S.W., Pyo, S.S., Park, S.Y., 2022: Temporary Immersion Bioreactor System as an Efficient Method for Mass Production of *in vitro* Plants in Horticulture and Medicinal Plants. Agronomy 12(2), 346. DOI: 10.3390/agronomy12020346

Joy, P.P., Anjana, R., 2016: Genesis and Evolution of Horticultural Crops, 1st edn. Astral International Pvt. Ltd., New Delhi, 1-39.

Kessel-Domini, A., Pérez-Brito, D., Guzmán-Antonio, A., Barredo-Pool, F.A., Mijangos-Cortés, J.O., Iglesias-Andreu, L.G., Santana-Buzzy, N., 2022: Indirect Somatic Embryogenesis: An Efficient and Genetically Reliable Clonal Propagation System for *Ananas comosus* L. Merr. Hybrid "MD2". Agriculture 12(5), 713. DOI: 10.3390/agriculture12050713

Kiss, E., Kiss, J., Gyulai, G., Heszky, L.E., 1995: Un método novedoso para la micropropagación rápida de piña. Hortscience 30 (1), 127-129. DOI: 10.21273/HORTSCI.30.1.127

Kitto, S.L., 1997: Commercial micropropagation. HortScience 32, 1012-1014. DOI: 10.21273/HORTSCI.32.6.1012

Kornatskiy, S.A., 2020: Pineapple Micropropagation (*Ananas comosus* L. Smooth Cayenne) and Plant Growth Features in the Process of Adaptation in Hydroponics. J. Crit. Rev. 7(8), 3228-3234. DOI: 10.31838/jcr.07.08.518

Lakashmi, S.G., Singh, R., Lyer, C.P.A., 1974: Plantlets through shoot tip culture in pineapple. Curr. Sci. 43, 724.

Li, D., Jing, M., Dai, X., Chen, Z., Ma, C., Chen, J., 2022: Current status of pineapple breeding, industrial development, and genetics in China. Euphytica 218(6), 1-17. DOI: 10.1007/s10681-022-03030-y

Liu, L.J., Rosa-Marquez, E., Lizardi, E., 1989: Smooth leaf (spineless) Red Spanish pineapple (*Ananas comosus*) propagated in vitro. J. Agric. Univ. P. R. (USA). DOI: 10.46429/jaupr.v73i4.6308

Loeillet, D., Dawson, C., Paqui, T., 2011: Fresh pineapple market: from the banal to the vulgar. Acta Hortic. 902, 587-594. DOI: 10.17660/ActaHortic.2011.902.78

Mapes, M.O., 1973: Tissue culture of bromeliads. En Comb Proc Int Plant Propag Soc. 23, 47-55.

Murashige, T., Skoog, F.A., 1962: Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 5, 473-497. DOI: 10.1111/j.1399-3054. 1962.tb08052.x

Rahman, K.W., Amin, M.N., Azad, M.A.K., 2001: *In vitro* rapid clonal propagation of pineapple, *Ananas comosus* (L.) Merr. Plant Tiss. Cult. 11, 47-53.

Reinhardt, D.H.R., Bartholomew, D.P., Souza, F.V.D., Carvalho, A.C.P.P.D., Pádua, T.R.P.D., Junghans, D.T., Matos, A.P.D., 2018: Advances in pineapple plant propagation. Rev. Bras. Frutic. 40(6), e-302. DOI: 10.1590/0100-29452018302

Rodríguez, R., Becquer, R., Pino, Y., López, D., Rodríguez, R.C., Lorente, G.Y., González, J.L., 2016: Producción de frutos de piña (*Ananas comosus* (L.) Merr.) MD-2 a partir de vitroplantas. Cult. Trop. 37, 40-48. DOI: 10.13140/RG.2.1.4732.3765

Rodríguez-Escriba, R.C., Rodríguez, R., López, D., Lorente, G.Y., Pino, Y., Aragón, C.E., González, O.J.L., 2015: High light intensity increases the CAM expression in MD-2 micro-propagated pineapple plants at the end of the acclimatization stage. Am. J. Plant Sci. 6, 3109. DOI: 10.4236/ajps.2015.619303

Scheidt, G.N., Arakaki, A.H., Chimilovski, J.S., Portella, A.C.F., Spier, M.R., Woiciechowski, A.L., Soccol, C.R., 2009: Utilization of the Biorreactor of Imersion by Bubbles at the Micropropagation of *Ananas comosus* L. Merril. Braz. Arch. Biol. Technol. 52, 37-43. DOI: 10.1590/S1516-89132009000700005

Sripaoraya, S., Marchant, R., Power, J.B., Davey, M.R., 2003: Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L.). In Vitro Cell. Dev. Biol. Plant. 39, 450-454. DOI: 10.1079/IVP2003445

Silva, A.B., Pasqual, M., de Castro, E.M., Miyata, L.Y., de Melo, L.A., Braga, F.T., 2008: Luz natural na micropropagação do abacaxizeiro (*Ananas comosus* L. Merr). Interciencia. 33, 839-843.

Solórzano-Acosta, R., Guerrero-Padilla, M., 2020: Design and Construction of a Pneumatic Temporary Immersion Bioreactor System for the Multiplication of *Ananas comosus* var. Trujillana Red. Am. J. Plant Sci. 11(9), 1429-1442. DOI: 10.4236/ajps.2020.119103

Villalobos, A., González, J., Santos, R., Rodríguez, R., 2012: Morpho-physiological changes in pineapple plantlets [*Ananas comosus* (L.) Merr.] during acclimatization. Cienc. e Agrotecnologia. 36, 624-630. DOI: 10.1590/S1413-70542012000600004

Yanes, E., González, J., Rodríguez, R., 2000: A technology of acclimatization of pineapple vitroplants. Pineapple News. 7, 24.

Zepeda, C., Sagawa, Y., 1981: *In Vitro* Propagation of Pineapple1. Hortscience. 16(4), 495-495. DOI: 10.21273/HORTSCI.16.4.495

ORCID

Dra. Nancy Santana Buzzy https://orcid.org/0000-0002-7263-0998
Dr. Adolfo Alberto Guzman Antonio https://orcid.org/0000-0003-0352-1904
M.C. Adriana Canto Flick https://orcid.org/0000-0002-4301-0562
M.C. Gema Pijeira Fernández https://orcid.org/0000-0001-8118-2106
Dra. Susana Avilés Viñas https://orcid.org/0000-0002-3400-6668
M.C. Yaritza Rodríguez Llanes https://orcid.org/0000-0002-1807-8899

Address of the corresponding author:
Nancy Santana-Buzzy, Unidad de Bioquímica y Bilogía Molecular de Plantas, Centro de Investigación Científica de Yucatán (CICY), Calle 43 No. 130, Chuburná de Hidalgo, Mérida CP 97205, Yucatán, México
E-mail: buzzy@cicy.mx