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Article

Direct organogenesis in landrace pineapple induced by 6-benzylaminopurine

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Abstract

The production of pineapple (*Ananas comosus* L.) by conventional methods is limited mainly by the lack of availability of high-yielding suckers. Nevertheless, it has been shown that, through *in vitro* propagation methodologies such as somatic embryogenesis and organogenesis, it is possible to obtain high-yielding plants in a more efficient and controllable way. The objective of this study was to generate an efficient protocol of micropropagation of landrace pineapple (*Ananas comosus* L.) for the *in vitro* multiplication and conservation of this species, the study was carried out in 2021. The morphogenic response of the landrace pineapple was evaluated based on different explants (apical meristem and leaf), grown in Murashige and Skoog (MS) culture medium supplemented with various growth regulators: naphthaleneacetic acid (NAA) (0.5, 1 and 1.5 mg L^{-1}), 6-benzylaminopurine (BAP) (1, 2 and 3 mg L^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 2 and 4.5 mg L^{-1}), as well as a regulator-free control treatment. The results showed that, of the explants evaluated, the best response was observed in the apical meristem, in which the formation of adventitious shoots was obtained 60 days after induction treatment, when the culture medium was supplemented with BAP at a concentration of 2 mg L^{-1}, obtaining eight shoots/explant. The protocol developed is a key study for the mass propagation of landrace pineapple.

Keywords:

6-bencilaminopurina, in vitro morphogenesis, meristem.

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Introduction

Pineapple (*Ananas comosus* L.) is one of the most popular tropical species, it is from South America, particularly north of the Amazon River, and over the years it has been expanding rapidly through various tropical regions due to its agronomic importance (Torres-Ávila *et al*., 2018). It is a monocotyledonous species belonging to the Bromeliaceae family, the following varieties stand out for their economic value: Smooth Cayenne, honey pineapple (MD2), Cambray, Champaka F-153 (Sarkar *et al*., 2018; Torres-Ávila *et al*., 2018). In general, the variety that occupies the largest production area is Smooth Cayenne, while MD2 has not managed to find the appropriate niche in the country to exploit its full productive potential, probably due to management and soil and climate conditions (Rebolledo *et al*., 2011). This is still a very important limitation to expand the pineapple trade in the international market.

In Mexico there are 12 states that are engaged in the cultivation of pineapple, both for the national and international market. According to SIAP (2021), Mexico ranks ninth in the world in pineapple production with 1 208 247 t produced. The most recent data indicate that 38 164.08 ha are allocated to the cultivation of this fruit in the country, with the state of Veracruz being the one that allocates the largest area in Mexico (31 993 ha) (SIAP, 2021). The state of Chiapas ranks eighth among the top ten pineapple-producing states in the country, with a production of 7 763 t (SIAP, 2021).

Traditionally, landrace pineapple has been produced in the central region of the state of Chiapas for more than 60 years, mainly in the municipalities of Ocozocoautla and Berriozábal. The importance of the landrace pineapple lies in the fact that its fruit, when compared with that of other varieties such as Smooth Cayenne, Perola, Mexico and Apple, stands out for containing a greater amount of °Brix (14-17º), protein, nitrogen and fiber, as well as lower acidity, which gives it less astringency compared to the main cultivar (Smooth Cayenne), which makes this variety an excellent option for fresh consumption and as an input in the food industry, in addition to being a genotype adapted to the agroclimatic conditions of the region (Hernández-Barbosa, 2018).

However, in recent years there has been a decrease in this crop, which has caused a decrease in its production and therefore an economic impact on those involved in the pineapple cultivation industry, which puts this crop at risk of disappearing. Among the causes of this problem we can mention: the application of low-input technologies, the lack of advice and training to producers, the absence of a varietal strategy that allows optimizing the use of varieties in each region; the need for an improvement program that generates clones and varieties that are more tolerant and better adapted to the environmental conditions of the region, the lack of a bank of pineapple varieties and clones available to develop better production strategies with a view to competing in the international market (Hernández-Barbosa, 2018).

In addition to all this, the lack of a management technology that includes: nutrition, water regime, planting density, planting and harvest dates, lead to maintaining low yields and fruit quality, and as a consequence, fruits unsuitable for the export market (Hernández-Barbosa, 2018). This problem is aggravated due to the way the crop is propagated, which is asexual, that is, through the crown of the fruit, slips or suckers (Hernández-Barbosa, 2018; Velez-Izquierdo *et al*., 2020), causing long waiting periods to obtain a viable plant for transplantation to soil, which range from 6 to 24 months.

The propagation of the crop by the conventional (vegetative) method produces a very limited number of propagules, which restricts the availability of plant material for large-scale planting, in addition, the crops are attacked by insects and fungi that produce wilting, fusariosis and rotting of the base of the stem, diseases that affect the crop negatively, mainly in the commercial production of the fruit (Santos and Matos, 1995; Coppens *et al*., 1997; Rodríguez *et al*., 2002). *In vitro* micropropagation is carried out from an explant placed in a nutritive medium, achieving a morphogenic response in the explants, resulting in the formation of a new plant from a cell, tissue or organ of the plant, this phenomenon is due to cell totipotency.

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Certain cells have the genetic information necessary to generate a plant similar to the original one, revealing that differentiated cells of any plant tissue can restart the cell cycle, regenerating tissues and organs until obtaining the whole plant (Su *et al*., 2021). Studies of plant totipotency have been carried out in different plant species, demonstrating the capacity of plant cells for plant regeneration, giving rise to lines of research such as micropropagation, germplasm conservation, plant modification, and obtaining transgenic plants, among others (Ikeuchi *et al*., 2019).

Therefore, one of the alternatives to solve the problems of this crop of economic importance is the use of biotechnological techniques such as the culture of plant tissues for the conservation and micropropagation of this species, this technique offers the possibility of reproducing a large number of complete plants in short periods and small spaces, thus obtaining a more homogeneous crop, free of diseases and viruses, due to the axenic conditions in which the plants are obtained (Philips and Garda, 2019).

This study evaluated the morphogenic response of different explants of landrace pineapple (leaf and apical meristem) exposed to different plant growth regulators: naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D), for the development of an efficient and reproducible protocol for micropropagation of landrace pineapple.

Materials and methods

Plant material

To obtain the explants, slips were used, which were between 4-8 cm long, obtained from mother plants from a demonstration plot established in the experimental fields of the Secretariat of Agriculture, Livestock and Fisheries (SAGARPA, for its acronym in Spanish) in the state of Chiapas, which were approximately two years old, an average height between 70-80 cm, good vigor and free of pests, diseases or leaf damage. On average, approximately five to seven slips per plant were obtained. Harvesting was done manually.

Disinfection

The selected slips were transferred to a greenhouse and remained there for six days, waiting for the moisture conditions of the explant to be suitable for use *in vitro* culture. After this time, the slips were defoliated, leaving only a couple of central leaves and the base of the crown, to which outer layers were removed until obtaining an explant of approximately 2 cm^2, a superficial cleaning was carried out with running water and they were left immersed in 1 L of distilled water with five drops of Tween-20 for 24 h. The explants were then washed with Axion® soap and a commercial solution of sodium hypochlorite (2% Cloralex®) (modified from Lecona *et al*., 2017).

A wash was carried out with a solution of 0.5 g L^{-1} agrimicin + 2 g L^{-1} captan for 30 min, then the explants were washed three times with distilled water. In a laminar flow hood, the explants (leaf and apical meristem) were placed in a 70% alcohol solution for 3 min and rinsed three times with sterile distilled water, then immersed in a commercial solution of sodium hypochlorite (10% Cloralex®) for 15 min and rinsed three times with sterile distilled water. For later stages, 1 cm^2 leaf segments were used as explants.

In vitro induction

The explants were sown in MS medium (Murashige and Skoog, 1965), supplemented with three different growth regulators in three different concentrations each. Naphthaleneacetic acid (NAA: 0.5, 1 and 1.5 mg L^{-1}), 6-benzylaminopurine (1, 2 and 4 mg L^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D: 1, 2 and 4.5 mg L^{-1}). Four explants of leaves and apical meristems per experimental unit (1 bottle) were placed, with four repetitions, the control treatment consisted of MS medium free of growth regulators. The bottles were placed in a bioclimatic chamber at a temperature of 25 °C ±2 with 16 h light 8 h darkness, the evaluations of the morphogenic response such as

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callus formation, somatic embryo and shoot formation were at 30, 45 and 60 days after *in vitro* induction.

Rooting and acclimatization of shoots

Shoots with good development and with a size of approximately 3-4 cm were selected. These shoots were separated and sown in MS medium supplemented with indole butyric acid (IBA) at a concentration of 1 and 2 mg L^{-1} (Lecona *et al*., 2017). Root development was recorded at four weeks.

Seedlings that showed a well-developed root system were removed from the bottles, washed under running water to remove excess medium on the roots. Subsequently, they were transferred to polystyrene plastic containers containing a mixture of Peat Moss-Expanded perlite (1:1), for acclimatization under shade mesh conditions for 30 days, the temperature conditions fluctuated between 16 °C at its lowest temperature at night and 28 °C at its highest temperature during the day. The seedlings were irrigated every third day without fertilization.

Experimental design

For the evaluation of the morphogenic response, a completely randomized experimental design, an analysis of variance and a comparison of means according to Tukey (*p*# 0.05) were performed with the statistical package of Stargraphic Centuryon XV.

Results and discussion

During the induction of the evaluated explants, the apical meristem was the explant that showed response to the induction of shoots, while phenolization and necrosis were observed in leaves, this same behavior was observed in each of the treatments evaluated (data not shown). When the apical meristem was maintained in induction in MS medium supplemented with NAA, only the development of the central meristem of the explant was observed at 30 days of induction in all concentrations evaluated, the best response with this growth regulator was observed at 45 days of induction and at a concentration of 1.5 mg L^{-1}, at which 4.67 shoots/explant were obtained (Table 1).

Table 1. Optimization of *in vitro* proliferation of shoots in landrace pineapple using different growth regulators.

Tratamiento	Núm. de brotes por explante (días)		
	30	45	60
Control	0.78 ±0.33^{c}	1.44 ±0^{c}	3.33 ±0^{c}
ANA (mg L^{-1})			
0.5	0.33 ±0.33^{c}	0.67 ±0.33^{cd}	3 ±0.0^{cd}
1	1 ± 0^{c}	1 ±0^{cd}	3 ±0.58^{cd}
1.5	1 ±0^{c}	4.67 ±1^{b}	2 ±0.58^{de}
BAP (mg L^{-1})			
1	6.33 ±0.29^{a}	6.33 ±0. 57^{a}	5.67 ±0.67^{b}
2	4.67 ±0.66^{b}	6.33 ±0.33^{a}	8.67±0.67^{a}
3	6.33 ±0.33^{a}	7.33 ± 0.67^{a}	8.33 ±0.33^{a}
2,4-D (mg L^{-1})			
1	0.67 ±0.33^{c}	1.33 ±0.33^{cd}	5 ±0.58^{b}
2	0.67 ±0.33^{c}	1.67 ±0.88^{c}	1.33 ±0.33^{ef}
4.5	0 ±0^{c}	0 ±0^{d}	0.33 ±0.33^{f}
DMS	0.97	1.23	1.4

LSD= least significant difference. Different letters indicate statistically significant difference between the values for each column. Tukey *p*# 0.05.

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Previous studies have reported different morphogenic responses in other pineapple species, which include from direct organogenesis (Kiss *et al*., 1995; Escalona *et al*., 1999; Al-Saif *et al*., 2011; Medina-Rivas *et al*., 2014; Atawia *et al*., 2016), indirect organogenesis (Rahman *et al*., 2005; Pineda *et al*., 2014) to somatic embryogenesis (Daquinta *et al*., 1997; Firoozabady *et al*., 2004; Yapo *et al*., 2011; Blanco-Flores *et al*., 2017), most of these protocols use MS medium for the establishment of cultures and these media are supplemented with growth regulators such as BAP and NAA alone or in combination, the behavior of the morphogenic response of landrace pineapple was similar to that reported by various authors, as it is a first study in this variety that has been little studied and that can be used for its agronomic characteristics in the industry, the results are important since genetic improvement programs, large-scale regeneration protocols through temporary immersion systems and germplasm conservation can be generated.

When evaluating the response in explants of landrace pineapple leaves, phenolization and necrosis were observed (data not shown), the null response of this explant may be due to its origin. Hu *et al*. (2017) indicate that a determining factor for explants to generate a morphogenic response is the degree of differentiation that the cells of the selected tissues have and it is different in dicotyledons and monocotyledons, the cells of the latter show a greater degree of differentiation than dicotyledons; that is, they acquire a specific function due to the degree of cell differentiation they have, which prevents them from reacquiring the pluripotency necessary for regeneration (Zeng *et al*., 2016).

This indicates that the leaf explants in landrace pineapple have cells not competent for cell regeneration, losing the ability to dedifferentiate, as they are tissues mostly constituted by differentiated cells, these are unable to reactivate the process of cell division. When the apical meristem was induced in MS medium supplemented with 2,4-D, no adventitious shoot formation was observed during the first 45 days, it was until 60 days of induction when the formation of an average of five shoots/explant was observed in the treatment supplemented with 1 mg L^{-1} of this growth regulator (Table 1), statistically significant difference was found in the concentrations, as well as in the times evaluated, although the results with this regulator indicate a low proliferation of shoots.

All treatments with BAP showed shoot formation from 30 days of induction, the best response in terms of shoot formation was obtained when the MS medium was supplemented with 2 mg L^{-1} of BAP, treatment in which the formation of 8.67 shoots/explant was observed at 60 days of induction, however, it was not statistically different from the concentration of 3 mg L^{-1} (Table 1, Figure 1), all the shoots obtained showed a normal morphology and an approximate length of 1-2 cm at 60 days of induction (Figure 1).

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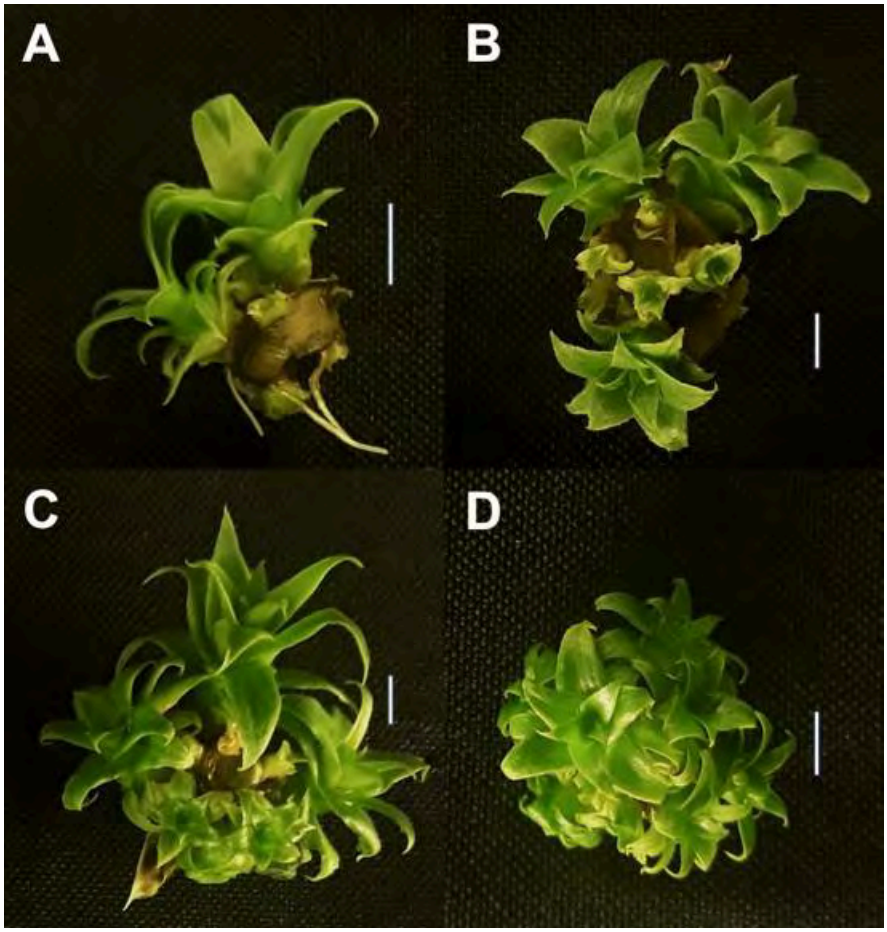
Figure 1. Direct organogenesis in apical meristems of landrace pineapple. A) control treatment; B) MS medium supplemented with 1 mg L^{-1} BAP; C) MS medium supplemented with 2 mg L^{-1} BAP; and D) MS medium supplemented with 3 mg L^{-1} BAP, at 60 days of induction. The bar is equivalent to 0.5 cm in length.



Studies have been reported regarding the morphogenic response of different pineapple explants, where the culture media are supplemented only with a single growth regulator. Al-Saif *et al*. (2011) reported a proliferation of shoots in explants of the base of the crown of pineapple fruits of the Smooth Cayenne variety, obtaining 23 shoots per explant when the MS culture medium was supplemented with 2 mg L^{-1} of BAP at 60 days of incubation, while when the culture medium was supplemented with NAA 0.2 mg L^{-1}, they obtained 12 shoots per explant in the same incubation period.

Ayenew *et al*. (2013), who, using 2 mg L^{-1} of benzyladenine (BA) in MS medium, obtained 6.33 shoots per explant of pineapple in the Smooth Cayenne variety. Usman *et al*. (2013) reported a similar response, using bases of fruit crowns as explants induced in MS medium, obtaining 11 shoots per explant with 2.25 mg L^{-1} of BA. Our results regarding the number of shoots obtained in landrace pineapple are similar to those reported by various authors in other varieties of pineapple, so the method used is favorable and suitable for the micropropagation of this species, nevertheless, it is necessary to conduct studies where the combination of growth regulators is used to optimize the micropropagation protocol in this species.

The combination of different growth regulators has also been reported for the *in vitro* regeneration of pineapple, in the ecotype Tabë Känä, Pineda *et al*. (2014) reported that the combination of 5 mg L^{-1} NAA and 0.25 mg L^{-1} BA in leaf bases used as explants generates 3.43 shoots per

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explant at three months of growth. Nikumbhe *et al*. (2014) report a proliferation of 7.1 shoots per explant in meristems cuts when the MS medium was supplemented with 2 mg L^{-1} BAP and 0.25 mg L^{-1} NAA. Badou *et al*. (2018) evaluated the effect of NAA, BAP and AdS (adenine sulfate) on the proliferation of pineapple of the Smooth Cayenne variety, making different combinations on these regulators, the best treatment was when 2 mg L^{-1} BAP + 0.5 mg L NAA + 40 mg L^{-1} AdS were combined, where they obtained a percentage of induction of shoots of 98.33% and the proliferation of 13-15 shoots per explant in an approximate time of 60 days.

In vitro plant regeneration by organogenesis is the result of organ formation through the dedifferentiation of differentiated cells and the reorganization of cell division to create particular organs, meristems and primordia (Sugiyama *et al*., 1999; Bidabadi *et al*., 2020). Cytokines are growth regulators with the ability to break the apical dominance and stimulate the formation of shoots in the meristems, however, the implementation of this type of regulators in the culture medium generates different responses in pineapple, where it has been observed that there are different factors which are involved in the morphogenic response of this species, these factors are: variety, type and age of the explant used, endogenous and exogenous balance of growth regulators as well as growing conditions (light/darkness).

In this study, it was observed that the most determining factor for the induction of shoots in landrace pineapple is the type of explant used, in this case the apical meristem was the most appropriate in all the treatments evaluated. This behavior has been observed in other species, Lecona-Guzmán *et al*. (2017) reported that the morphogenic response in *Agave americana* L. is influenced by the type of explant (apical meristem), and that the concentration and type of regulator implemented in the culture media are determining factors to obtain a high percentage of shoots in this species.

The apical meristem is the main organ responsible for plant growth, responsible for the formation of roots and leaves, also giving rise to the formation of adventitious shoots (Traas, 2018). The apical meristem is characterized by being an organ that houses a large number of stem cells distributed among its different layers, when they are stimulated in the presence of different growth regulators, such as auxins and cytokinins, different morphogenic responses are induced (Wang *et al*., 2020). This tissue is divided into three layers (L1, L2 and L3) and has four zones that perform different functions: the central zone (CZ) which houses the stem cells, the organizing center (OC) which is responsible for the recruitment of stem cells, the medullary zone (ZM) responsible for generating multipotent cells and the peripheral zone (PZ) responsible for housing daughter cells, the stem cells present in the apical meristem are converted into daughter cells through the stimulation of different plant regulators responding to the induction of shoots (Lee *et al*., 2019).

Cytokinins promote the expression of genes such as: Wuschel (WUS), Clavata (CLV1-3), Dornröschen (DRN), arabidopsis response regulator (ARR7 and ARR15), which mediate the control of WUS expression, which in turn regulates the proliferation of stem cells, when the WUS gene is stimulated by cytokinins, these promote its overexpression, promoting cell division of these cells, increasing the population of stem cells, generating new daughter cells, finally obtaining more shoots (Lee *et al*., 2019), this effect was observed in all treatments with BAP at a concentration of 2 mg L^{-1}, the treatment that generated the highest shoot formation in the time evaluated.

The morphogenic effects of synthetic auxins such as 2,4-D and NAA are due to the fact that their chemical structure is very similar to that of indole acetic acid (IAA), which is a natural phytohormone found in many plants, since they share this characteristic, plant cells can enter synthetic auxins through the cell membrane and go inside the plant cell, stimulating the expression of genes, and generate various morphogenic responses including the proliferation of shoots in some species (Kulus *et al*., 2020).

The IBA stimulated root formation with respect to the control treatment, the concentration of 2 mg L^{-1} of IBA, which was statistically significant in terms of the number of roots, formation of adventitious roots formed, as well as in the length of the root in the landrace pineapple shoots

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(Table 2, Figure 2). Regarding the percentage of survival, there was no statistical difference during the acclimatization process, obtaining 100% survival in all the treatments evaluated.

Table 2. Effect of IBA on the rooting of landrace pineapple seedlings sown in MS medium

AIB (mg L^{-1})	Núm. de raíces	Núm. de raíces adventicias	Longitud de raíz (cm)	Sobrevivencia (%)
0	2 ±0.58 ^{b}	0 ±0^{c}	0.5 ±0^{c}	100
1	4 ±0.72 ^{b}	6 ±0.97^{b}	1.5 ±0.12^{b}	100
2	8 ±0.63^{a}	10 ±0.57^{a}	3 ±0.1^{a}	100
DMS (0.05)	0.81	0.47	0.13	

LSD= least significant difference. Different letters indicate statistically significant difference between the values for each column. Tukey *p*# 0.05.

The formation of roots and adventitious roots in landrace pineapple shoots was with 2 mg L^{-1} of IBA at 30 days of culture, these results coincide with what was reported by Atawia *et al*. (2016), who evaluated the effect of IAA and IBA on the formation of roots in shoots obtained from pineapple of the Smooth Cayenne variety, obtaining that IBA, at a concentration of 2 mg L^{-1}, stimulates root formation (4.333 roots/shoot); nevertheless, in most of the works reported for various varieties of pineapple, the use of this growth regulator to root shoots is not reported, probably this is because it is not necessary to stimulate the formation of roots in the shoots and these are developed mostly in media free of growth regulators, this has also been observed in other species such as agave, Reyes-Zambrano *et al*. (2016) report that IBA does not affect the formation of roots in shoots compared to plants that are sown in media free of this regulator.

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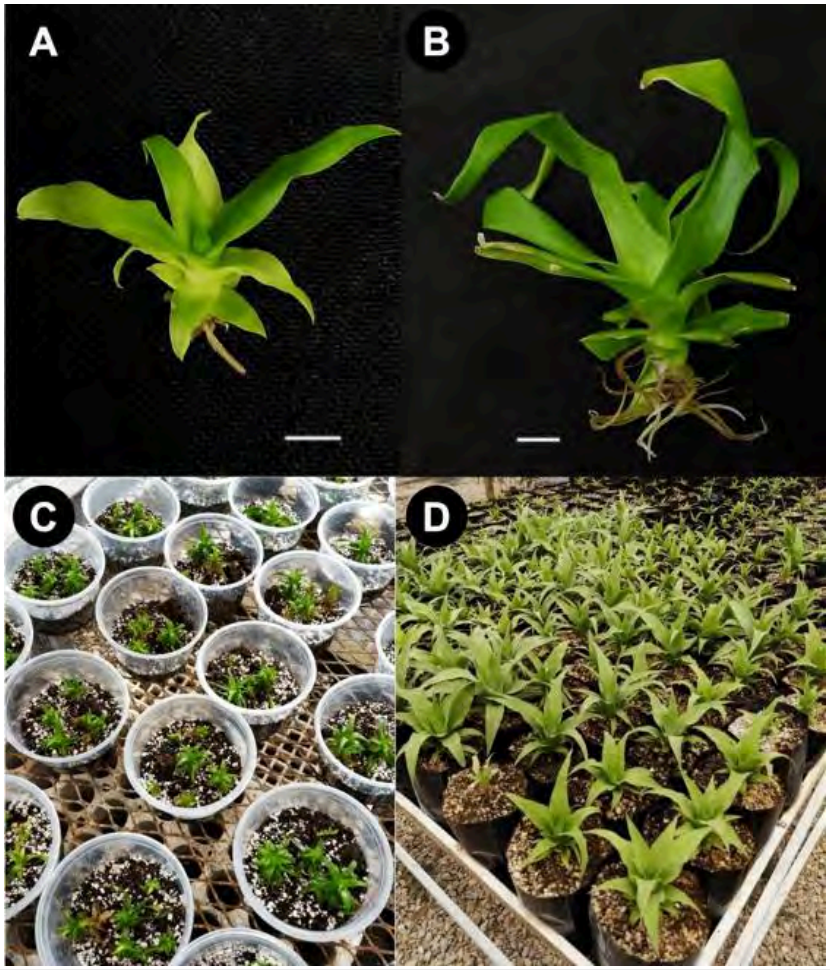
Figure 2. Rooting and acclimatization of landrace pineapple shoots obtained in vitro. A) root formation in landrace pineapple shoots without IBA at 30 days of induction; B) effect of 2 mg L -1 of IBA on root formation in landrace pineapple shoots at 30 days of induction; C) acclimatization of landrace pineapple shoots obtained *in vitro* with 90 days of formation; and D) acclimatized 6-month-old landrace pineapple seedlings from *in vitro* culture. White bar equals 0.5 cm in length.



Conclusions

In landrace pineapple, the determining factor for the induction of shoots is the source of explant, for *in vitro* regeneration via direct organogenesis, it is necessary to supplement the culture medium with 2 mg L^{-1} of BAP for 60 days of induction, the formation of roots is stimulated with 2 mg L^{-1} of IBA at 30 days. In this study, it was possible to establish a complete protocol for the regeneration of landrace pineapple, which can be used in large-scale propagation works through the implementation of temporary immersion systems, as well as future genetic improvement programs for this species.

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