Smooth leaf (spineless) Red Spanish pineapple (*Ananas comosus*) propagated in vitro[1,2]

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ABSTRACT

Some 40,000 plantlets of Red Spanish pineapple [*Ananas comosus* (L. Merr.)] were produced via meristem culture. Of these, approximately 50% were spineless. Some of these spineless plantlets reversed to spiny leaf. However, the percentage of reversion from spineless to spiny was 14.1% and that from spiny to spineless was 32.7%. Of the 2,318 plantlets examined in the laboratory and greenhouse during a 3- to 4-month period, 72.9% of the spiny Red Spanish pineapple remained spiny and 85.8% of the spineless remained spineless. One year after field planting, the spineless Red Spanish remained largely spineless and initiated flowering and fruit settings the same as the spiny ones. The standard medium for in vitro propagation of Red Spanish pineapple was improved by supplementing Murashige and Skoog's basic formula (MS) with 0.1 mg/L, 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.5 mg/L benzyl adenine (BA). The callus formation was improved by adding to the same MS formula 10 mg/L BA + 4 mg/L naphtalene acetic acid (NAA). Similarly, shoot differentiation was improved by adding low concentrations of hormone (0.1 mg/L NAA) to the Abo El-Nil and Zettler (AZ) medium.

RESUMEN

Piña Española Roja sin espinas propagada in vitro

Se produjeron alrededor de 40,000 plántulas de piña [*Ananas comosus* (L. Merr.)] de la variedad Española Roja por cultivo de tejidos. Aproximadamente el 50% eran sin espinas. Algunas de las plántulas sin espinas revirtieron a plántulas con espinas. Sin embargo, el porcentaje de reversión de plántulas sin espinas a con espinas fue de 14.1% y de las con espinas a sin espinas de 32.7%. Se observaron alrededor de 2,318 plántulas en el laboratorio y el invernadero por 3 a 4 meses. De éstas, el 72.9% de las con espinas las mantenían; 85.8% de las sin espinas permaneclan así. Después de un año de establecidas en el campo, la mayoría de las plantas sin espinas mantenían la característica y habían iniciado la floración para el mismo período de tiempo que las con espinas. El medio de cultivo para propagar la Española Roja, in vitro se mejoró suplementándo la fórmula básica de Murashige & Skoog (MS) con 0.1 mg/L 2, 4- diclorofonoxiacético (2,4-D) + 0.5 mg/L benziladenina (BA). Se obtuvo la formación de callo

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añadiendo 10 mg/L BA + 4 mg/L de ácido naftalinoacético (NAA) a la fórmula básica de MS y la diferenciación de los brotes añadiendo bajas concentraciones de hormonas (0.1 mg/L NAA) al medio de Abo El Nil (AZ).

INTRODUCTION

Pineapple, *Ananas comosus* (L. Merr.), is economically the most important member of the Bromeliaceae and a major fruit crop in Puerto Rico as well as in the Caribbean Basin. The local farm value of this crop surpassed $13.24 million in 1986 (2). The Land Authority of the Commonwealth of Puerto Rico is the largest pineapple producer on the island. In 1986, efforts were made by the Authority to increase pineapple production in the Isabela area for fresh fruit exportation to the United States. Recently, Costa Rica has initiated a program of producing 12,000 ha of good quality fresh pineapple for export. Similarly, the Dominican Republic has initiated a 5,000-ha fresh fruit pineapple production program. With the low wages in these two countries, Puerto Rico would have to rely upon its highly intensive, technically oriented production program for survival.

The major problem for pineapple production in Puerto Rico is its low yield per unit area. Whereas the average pineapple production in the world remains at 90 metric tons per hectare, Puerto Rico produces only 39.8 metric tons per hectare. Of the three major pineapple cultivars planted locally, Smooth Cayenne is spineless, but does not satisfy the tastes of consumers. Red Spanish satisfies the tastes of consumers and occupies more than 90% of the total pineapple growing area. The sharp spines on the leaves of the cultivar Red Spanish are a serious problem during cultural operations such as weeding, fertilizer application and flower induction and a potential limiting factor for commercial production. Smooth leaf mutants of pineapple were occasionally observed in fields in Puerto Rico as well as in Hawaii (4) and Venezuela (7,14). However, these natural mutants gradually reversed to spiny and faded out from the plantation. Thus, it is extremely difficult to mass a huge number of smooth leaf mutants from the spiny Red Spanish pineapple for commercial production through the conventional sucker propagation techniques. Tissue culture techniques (3,5,8,9,12,13) offer an alternative to the conventional propagation method. The present study was undertaken to determine the effect of hormone on callus formation, shoot differentiation, and proliferation; the possibility of selecting smooth leaf pineapple plants in-vitro and the frequency of reversion from spiny to smooth leaf among plants of a normally spiny cultivar.

MATERIALS AND METHODS

The smooth leaf mutant reversions from the spiny Red Spanish cultivar were obtained from the Land Authority in Barceloneta, Manatí. Lateral buds (0.4 to 0.8 mm) and meristem tips from the crown of the

pineapple fruits were removed, and surface was sterilized in Clorox 10% and Tween 80 (2 drops/100 ml) under constant stirring for 15 minutes. Then disinfected tissues were washed with sterile distilled water 3 times.

For shoot differentiation, the basal medium of Murashige and Skoog (10) was modified 30 times by adding different concentrations of hormones (NAA, IBA, 2,4-D, IAA, Kinetin, BA), coconut water, casein hydrolisate, and adenine sulfate (table 1). Similarly, Gamborg B5 medium (6) was modified by adding different concentrations of NAA, IBA and 2,4-D; and Abo and Zettler's (AZ) medium (1) was modified by adding NAA, IBA, 2,4-D, BA, and adenine sulfate.

TABLE 1.—*Different media tried for shoot differentiation (concentrations in mg/L)*

Basal media	NAA	IBA	2,4-D	IAA	Kin	BA	Coconut water	Casein hydrolisate	Adenine sulfate
MS-1	0.1	—	—	—	—	—	—	—	—
MS-2	1.0	—	—	—	—	—	—	—	—
MS-3	2.0	—	—	—	—	—	—	—	—
MS-4	—	0.1	—	—	—	—	—	—	—
MS-5	—	1.0	—	—	—	—	—	—	—
MS-6	—	2.0	—	—	—	—	—	—	—
MS-7	—	—	0.1	—	—	—	—	—	—
MS-8	—	—	1.0	—	—	—	—	—	—
MS-9	—	—	2.0	—	—	—	—	—	—
MS-10	—	2.0	2.0	—	—	—	—	—	—
MS-11	2.0	2.0	—	—	—	—	—	—	—
MS-12	2.0	—	2.0	—	—	—	—	—	—
B5-13	0.1	—	—	—	—	—	—	—	—
B5-14	1.0	—	—	—	—	—	—	—	—
B5-15	2.0	—	—	—	—	—	—	—	—
B5-16	—	0.1	—	—	—	—	—	—	—
B5-17	—	1.0	—	—	—	—	—	—	—
B5-18	—	2.0	—	—	—	—	—	—	—
B5-19	—	—	0.1	—	—	—	—	—	—
B5-20	—	—	1.0	—	—	—	—	—	—
B5-21	—	—	2.0	—	—	—	—	—	—
B5-22	—	2.0	2.0	—	—	—	—	—	—
B5-23	2.0	2.0	—	—	—	—	—	—	—
B5-24	2.0	—	2.0	—	—	—	—	—	—
AZ-25	0.1	—	—	—	—	—	—	—	—
AZ-26	1.0	—	—	—	—	—	—	—	—
AZ-27	2.0	—	—	—	—	—	—	—	—
AZ-28	—	0.1	—	—	—	—	—	—	—
AZ-29	—	1.0	—	—	—	—	—	—	—
AZ-30	—	2.0	—	—	—	—	—	—	—
AZ-31	—	—	0.1	—	—	—	—	—	—
AZ-32	—	—	1.0	—	—	—	—	—	—
AZ-33	—	—	2.0	—	—	—	—	—	—
AZ-34	—	2.0	2.0	—	—	—	—	—	—
AZ-35	2.0	2.0	—	—	—	—	—	—	—

TABLE 1. *Continued*

Basal media	NAA	IBA	2,4-D	IAA	Kin	BA	Coconut water	Casein hydrolisate	Adenine sulfate
AZ-36	2.0	—	2.0	—	—	—	—	—	—
MS-37	—	—	—	—	—	—	—	—	—
B5-38	—	—	—	—	—	—	—	—	—
AZ-39	—	—	—	—	—	—	—	—	—
MS-40	0.1	—	—	—	0.01	—	—	—	—
MS-41	—	—	0.1	—	—	0.5	—	—	—
MS-42	1.8	2.0	—	—	2.1	—	—	—	—
MS-43	10.0	—	—	—	—	—	15% v/v	400	—
MS-44	5.4	—	—	5.1	—	2.2	—	—	—
MS-45	—	—	—	5.0	2.0	—	10% v/v	—	—
MS-46	—	—	—	5.0*	2.0	—	10% v/v	—	—
MS-47	5.0	—	—	—	2.0	—	10% v/v[1]	—	—
MS-48	5.0	—	—	—	—	2.0	10% v/v[1]	—	—
AZ-49	0.1	0.1	—	—	—	0.05	—	—	—
AZ-50	0.1	—	—	—	0.01	—	—	—	40
MS-51	0.2	—	—	—	—	0.1	—	—	—
MS-52	—	—	0.1	—	—	2.0	—	—	—
MS-53	0.1	—	—	—	—	0.5	—	—	—
MS-54	0.1	—	—	—	0.2	—	—	—	—
MS-55	—	—	—	—	—	0.1	—	—	—
MS-56	—	—	—	—	—	0.5	—	—	—
MS-57	—	—	—	—	—	1.0	—	—	—
MS-58	—	—	—	2.0	3.0	—	—	—	—

[1] Filter sterilized.

For the callus formation, the basal Murashige and Skoog's medium (10) was modified 4 times by adding different concentrations of hormones (BA, NAA, 2,4-D and kinetin); (table 2).

For the shoot proliferation, semi-solid and liquid media of MS and AZ were modified by adding different concentrations of hormones (NAA, IBA, 2,4-D, IAA, Kinetin, BA), coconut water, casein hydrolisate, and adenine sulfate (table 3)

For the greenhouse hardening process, 1- to 2-month old pineapple plantlets derived from meristem culture were planted in a 72-hole plastic tray containing a mixture of Promix. The tray was placed in a greenhouse

TABLE 2.—*Different hormone concentrations (mg/l), in MS basal medium, tested for callus formation*

Basal media	BA	NAA	2,4-D	Kinetin
MS	10	4	—	—
MS	4	10	—	—
MS	—	—	0.02	12
MS	—	—	12	0.02

TABLE 3.—*Semi-solid and liquid media tested for shoot proliferation (in mg/L)*

Basal media	IBA	NAA	2,4-D	IAA	BA	Kin	Adenine sulfate	Casein hydrolisate	Coconut water
MS	—	10.0	—	—	—	—	—	400.0	15%
MS	—	5.4	—	5.1	2.2	—	—	—	—
MS	—	0.2	—	—	—	—	—	—	—
AZ	—	0.1	—	—	—	0.1	40.0	—	—
MS	—	0.1	—	—	—	0.01	40.0	—	—
AZ	0.1	0.1	—	—	0.05	—	—	—	—
MS	—	—	0.1	—	0.5	—	—	—	—
MS	—	—	—	2.0	—	3.0	—	—	—
MS	2.0	1.8	—	—	—	2.1	—	—	—

with misting facilities at 80% relative humidity, 75 to 85° F and low light intensity for 3 to 4 weeks. The plantlets with a strong root system were then transferred to a 5-inch polyethylene bag containing Promix for another 3 to 4 weeks before field planting. Osmocote 18-6-20 (slow release fertilizer) was used to fertilize the plantlets every 3 months.

For reversion and somaclonal variability studies, Murashige and Skoog's basal medium + 0.1 mg/l 2,4-D and 0.5 mg/l BA was used to propagate the spineless Red Spanish pineapple. Close observations were made on the following 4 types of variability and reversion: 1) reversion from spineless to spiny; 2) reversion from spiny to spineless; 3) spineless remaining spineless; 4) spiny remaining spiny in the laboratory and greenhouse during 3- to 4-month periods.

RESULTS

Callus induction and regeneration

Callus formation was observed when BA hormone was added to the basal medium of Murashige and Skoog (10), and incubated in the dark. The BA hormone played an important role in callus formation in the dark. No callus formation in the dark was observed in the culture medium without BA. The basal medium of MS + 10 mg/L BA and 4 mg/L NAA gave the best results on callus formation (fig. 1a and table 2).

The culture medium (MS + 4 mg/L NAA) gave the best results for plant regeneration. Calli of the Red Spanish pineapple were regenerated into green plantlets 2 to 3 weeks after incubation in diffuse light (2 to 3 foot candle), at 26° C.

Shoot differentiation

The Murashige and Skoog's medium (10) with low concentration of 2,4-D (MS + 0.1 mg/l 2,4-D) and Abo El-Nil and Zettler (AZ) (1) with low concentration of NAA (AZ + 0.1 mg/L NAA) were the best combinations for shoot differentation (fig. 1b). Similarly, AZ medium with 2 mg/L NAA, AZ + 2 mg/L 2,4-D or MS + 0.1 mg/L 2,4-D + 0.5 mg/L

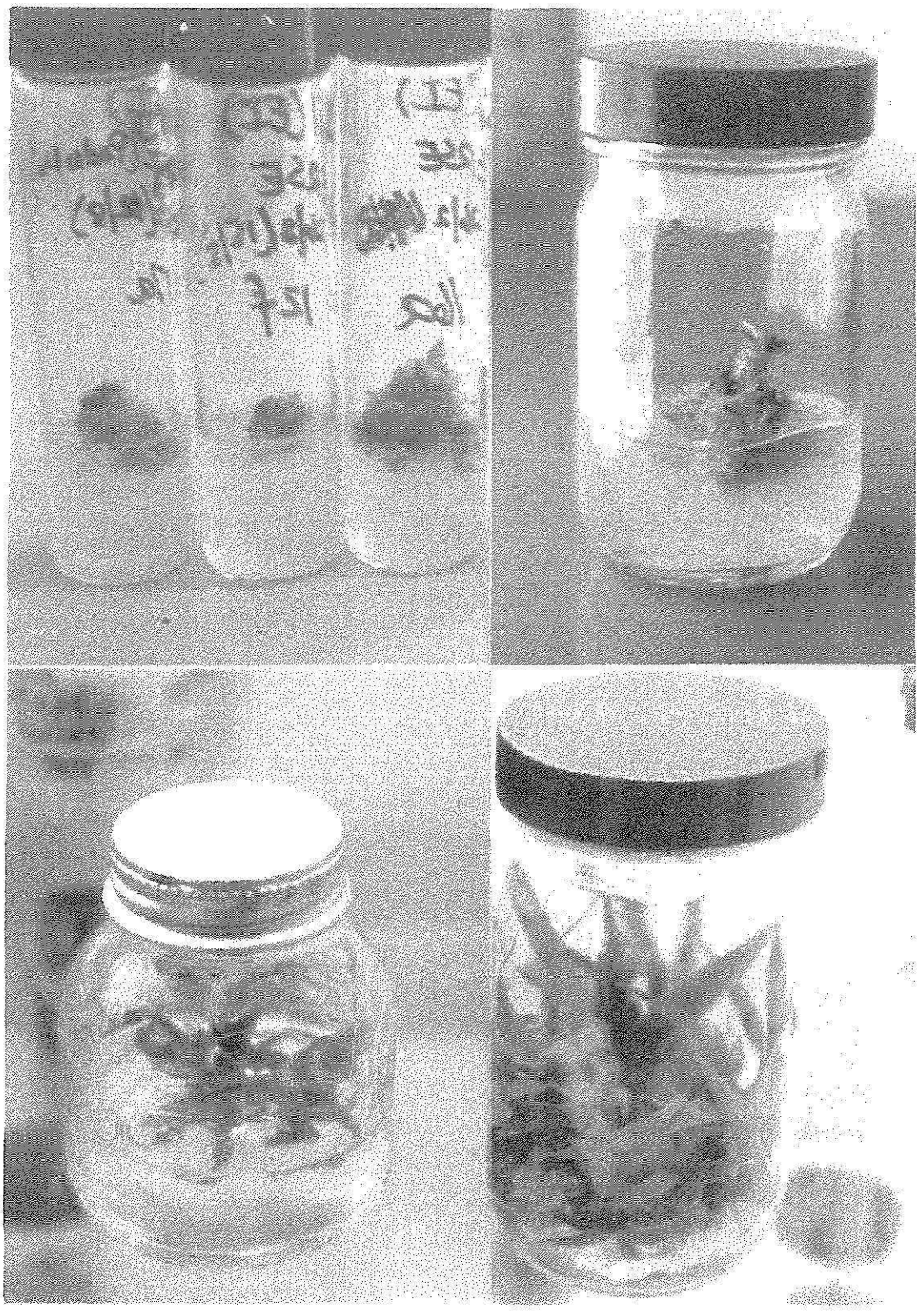

FIG. 1.—Growth of Red Spanish pineapple cultivar in the improved cultural media: (a) Callus formation in MS + 10 mg/LBA and 4 mg/L NAA, (b) Shoot differentiation in MS + 0.1 mg/L 2,4-D, (c) Shoot proliferation in semi-solid MS basal medium + 0.1 mg/L 2,4-D + 0.5 mg/L BA, and (d) Shoot growth in solid medium of MS + 5.2 mg/L IAA + 2.2 mg/L BA.

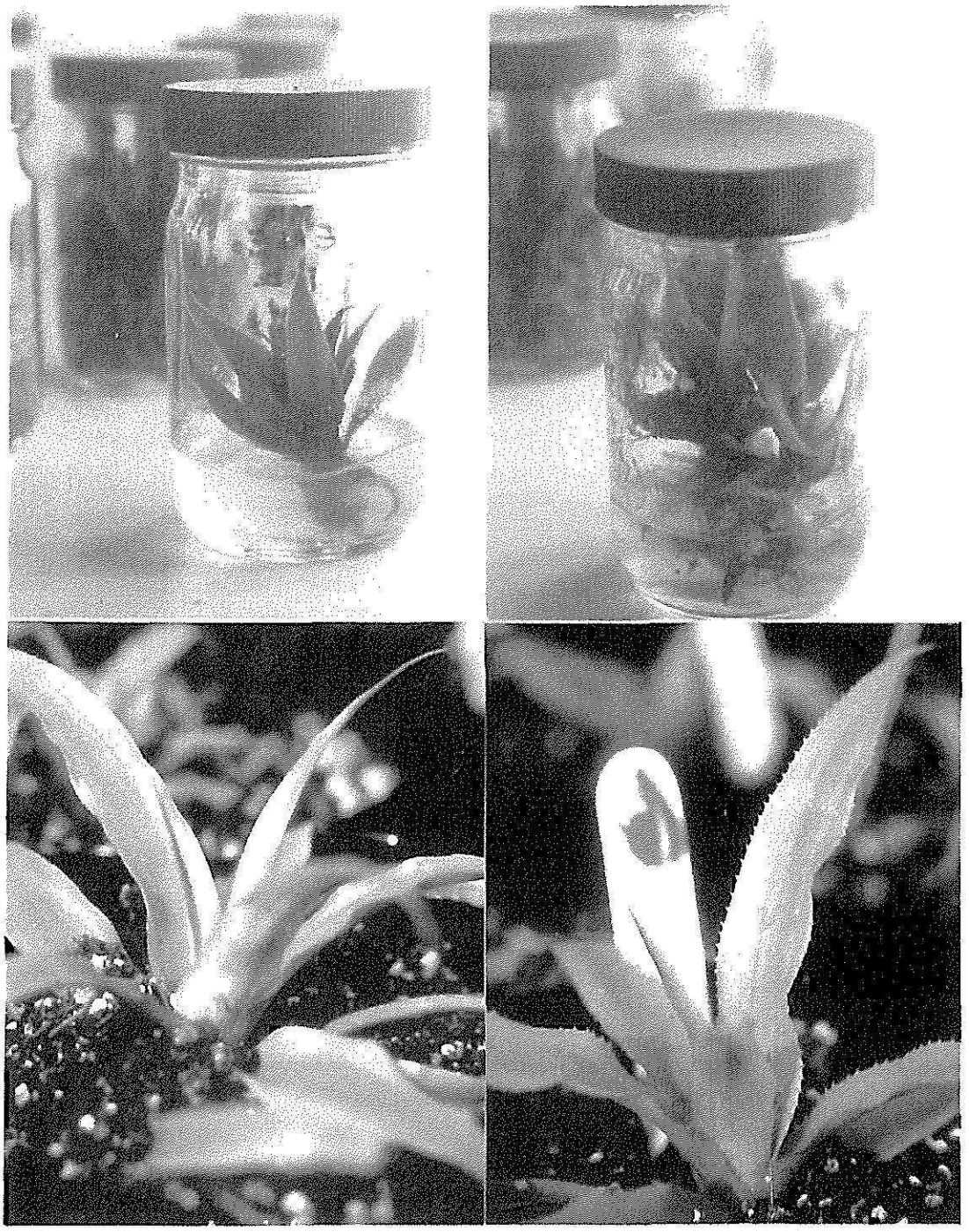

FIG. 2.—Reversion from spiny Red Spanish pineapple plantlets to smooth leaf (Spineless) in the laboratory (a and b) and in the greenhouse (c and d).

BA also permitted good shoot differentiation. The others, as mentioned in table 2, did not give as good shoot differentiation as the above-mentioned two culture media combinations.

Shoot proliferation

Of the 9 semi-solid and liquid media used for shoot proliferation (table 3), the MS basal medium + 0.1 mg/L 2,4-D + 0.5 mg/L BA gave the best results in shoot proliferation (fig. 1c). Similarly, the culture media (MS + 10mg/L NAA + coconut water 15% + 400 mg/L casein hydrolisate and MS + 5.4 mg/L NAA + 5.1 mg/L IAA + 2.2 mg/L BA) also gave good results (fig. 1d).

The results of this study revealed the possibility of selecting *in vitro* smooth leaf (spineless) pineapple plants and frequency of reversion. Some 40,000 plantlets of Red Spanish pinepapple were produced via meristem culture. Of these, approximately 50% were spineless. Some of these spineless plantlets reversed to spiny leaf (fig. 2). However, the percentage of reversion from spineless to spiny Red Spanish pineapple was 14.1%, and that from spiny to spineless was 32.7%. Of the 2,318 plantlets examined, 72.9% of the spiny Red Spanish pineapple remained spiny without reversion, and 85.8% of the spineless Red Spanish pineapple remained spineless with no reversion (table 4). One year after field planting, the spineless Red Spanish remained largely spineless and initiated flowering and fruit settings the same as the spiny ones (fig. 3).

DISCUSSION

The results obtained in this study agree with the findings of Fierro (5) that liquid medium with agitation accelerates the process of shoot proliferation. Similarly, the addition of hormones NAA at 10 mg/L (8) and IAA at 5.1 mg/L and BA at 2.2 mg/L (9) also promotes proliferation and growth of the explants.

Variations in leaf color, density, spines, trichomes were observed on *in vitro* culture of pineapple (11). However, no efforts were made to induce and select them via hormones as useful agronomic characteristics for commercial propagation.

Collins and Kerns (4) in Hawaii investigated the inheritance of 3 leaf types: spiny, the typical form for cultivars in the "Spanish" group, spiny-tip, (smooth leaved), the form in the "Cayenne" group; and piping, the form of Monte Lirio and other smooth-leaf cultivars. They ascertained that spiny-tip and spiny leaves were phenotypic expression of a single pair of alleles, S and s, with spiny-tip (smooth leaved) dominant. Our experience in pineapple tissue culture indicates that the reversion from spiny to spineless (smooth leaved) was greater and the percentage of the spineless remaining spineless was higher. Therefore, somaclonal variability and reversion should not cause great concern in commercial production using tissue culture plantlets for propagation.

There are many advantages of *in vitro* culture of pineapple. It could be used either for obtaining useful variants as evidenced in this study or for accelerating propagation. It would theoretically be possible to obtain

TABLE 4.—*Variability and reversion of spineless Red Spanish pineapple in Murashige and Skoog's basal medium with 0.1 mg/L 2,4-D and 0.5 mg/L BA*

Variation/Reversion	No. of plantlets observed	No. of plantlets with reversion	No. of plantlets without reversion	Reversion	No Reversion
				%	%
Reversion from spineless to spine	1,061	150	—	14.13	—
Reversion from spine to spineless	2,318	758	—	32.70	—
Spine remaining spine (no reversion)	2,318	—	1,560	—	72.96
Spineless remaining spineless (no reversion)	1,061	—	911	—	85.86

FIG. 3.—Normal fruit setting in field planting of Spineless Red Spanish pineapple via tissue culture.

one million plantlets from a single bud in 2 years. It may also be possible to create new genotypes for disease resistance through somaclonal selections.

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