TISSUE CULTURE OF PINEAPPLE *Ananas comosus* (L.) Merr. var. Cayenne lisse: A method of asexual propagation.

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INTRODUCTION

A mature plant of pineapple Ananas comosus (L.) Merr. var. Cayenne lisse, has several vegetative structures that provide a reliable source of planting material when a comercial plantation has been already established. In Hawaii, the crowns are normally used as the main source of propagation material; in other parts of the world, slips and suckers are used as well.

The need for a rapid vegetative method of propagation was originally sought as an important part of a breeding program, where rapid increase of the progeny of a single plant selection for evaluation and further selection is desired. The conventional methods used in commercial propagation are too slow to yield the necessary planting material as required in a short period of time.

Since the early 1930's many efforts have been made to develop a more rapid, easy and inexpensive method of propagating pineaple. In recent years some attempts have been made to develop a tissue culture technique using growth regulators making it possible to multiply large number of plants in a short time and with a minimum rate of mutant plants. Such a technique presents the possibility of being able to obtain the necessary planting material for a new plantation, or when a new cultivar is to be established; it can also eliminate restrictions that many times make impossible to transport bulky vegetative material such as that of the pineapple from one area of the world to another.

This paper describes an attempt to learn the principles and practices of propagating pineapple plants by tissue culture in a laboratory experience, and in doing so, the findings about a newer and simpler method of asexual propagation of pineapple.

LITERATURE REVIEW

A single pineapple plant at the end of plant crop produces on the average one crown, 2.5 slips, and 1.5 suckers. These vegetative structures if planted will produce 25 additional vegetative structures at the end of an average period of 24 months. At this rate and starting with a single plant selection, it would take 12 or more years to obtain sufficient planting material for one acre of land(8 and 9).

Many efforts have been made to devise a more rapid method of propagation for commercial and investigation purposes. Sections of the plant's stump with buds(10 and 2) and an improved modification called the "Bud-piece method" was described in 1941(9) by which 51 plantlets out of one adult pineapple plant in a period of six months could be obtained. These plantlets when transplanted to the field conditions, produced fruit in 18 months. More recently, the use of Maintain at rates lower than 100 ppm, applied to the pineapple plants at floral differentiation time(7), has been sugested to have possibilities as an agent to increase the production of lateral shoots on the plants above the natural level. All these methods are not carried under aseptic conditions and rely on the stimulation of the lateral buds which lie dormant at the axil of each leaf, to grow and develop into a plantlet.

The introduction of a tissue culture technique in vitro, combined with the use of appropriate growth regulators, has made possible to multiply greater number of plants in a short period of time. In 1973(3) numerous pineapple plantlets and protocorm-like bodies were successfully obtained

when using a orchid shoot-tip technique and the callus method applied sequentially to shoot tips of rooted growing crowns of pineapple. The shoot tips were cultured and shaked in MS liquid medium, and then transferred to a MS medium containing Adenosine 30 mg/l. or Adenine 20 mg./l.

A commercially feasible tissue culture procedure for rapid clonal multiplication of Aechmea fasciata Baker. and other bromeliads(1) has been described. By this procedure 500 uniform clonal Aechmea plants from a single individual can be obtained within a six-month period and similar success has been observed when the same technique was applied to 19 other genera or species of the Bromeliaceae family includin the pineapple Ananas comosus var. Smooth cayenne. An important aspect of in vitro culture and the use of growth regulators, is the incidence of a significant number of genetically aberrant plants. It has been indicated(1) this phenomenon is related to the number of times that a culture is divided and recultured, and to the length of time that a tissue culture is maintained in the laboratory. The greater the number of recultures and the longer the period of maintenance in vitro, the higher the the expected occurrance of aberrant plants. The practice of 3 recultures of Aechmea in a six months period resulted in only 2% aberrant plants; in contrast, more frequent recultures for periods up to one year produced as high as 20% variant plants.

Pannetier and Lanaud(5) have recently estimated the possibility of producing 2 million uniform individual plantlets from a single bud in two years by use of a similar technique as the one described here.

This paper describes a method for clonal propagation of pineapples by in vitro culture of lateral buds from crowns of mature fruits, and the use of Benzyladenine(BA) alone in several concentrations.

MATERIALS AND METHODS

The starting vegetative material for culture was obtained from healthy, mature but small crowns after the fruit was harvested. The leaves of the crown were carefully removed, starting from the base and working toward the growing point (Figs. 1 - 3).

The naked crown(Fig. 4) was then put into a 10 % Clorox* solution for 60 minutes, then the buds were excised from the crown using an scalpel with blade No. 11(Fig. 5) and placed in 1% Clorox* solution for approximately 20 minutes. The excised buds have at their base some of the crown tissue(Fig. 6). All Clorox solutions were made up with steril distilled water and 3 drops of Tween-20.

The buds were initially cultured in a Pyrex 9825- 16 mm. screw cap culture tube, Corning Glass Works. Corning, New York 14830, containing 4 ml. of Murashige and Skoog medium (4) with 25 % coconut water(CW). The tubes were kept in a culture room condition under continuous illumination from GE white fluorescent lamps(power groove) at 200 foot-candles intensity and at a temperature of 26 $\pm$ 2°C.

Permanent agitation of the liquid media in the tubes was provided by a Rollordrum, New Brunswick Scientific Co. 1130 Somerset St., New Brunswick, N.J. 08903, model TC-3 rotating at 1/5th. rpm.

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*Clorox: Active Ingredient 5.25% Sodium hypochlorite.

When the buds or explants were cultured in a 50 ml Erlenmeyer flask, 20 ml. of media were used and constant agitation was provided by a New Brunswick shaker, model S-3 at approximately 95 oscillations per minute.

Because the volume of the media in the tube was relatively small, the explants had to be sub-cultured into a fresh media every 2 weeks as compared to the explants in the Erlenmeyer flasks, which had to be sub-cultured only once a month.

The buds were first cultured in a full strength MS medium supplemented with 25% CW and 2% sucrose; all subsequent subcultures were made in a ½ strength MS + 25% CW + 2% sucrose. Within 2 months after initial culture solitary plantlets with 5-8 leaves were obtained(Figs. 7-11). At this stage the plantlets were ready to be cultured in a ½ MS medium with several levels of Benzyladenine (BA) for lateral shoot induction. The levels tested were 0.5 , 1 , 3 , and 5 ppm. and the period of induction in this media was of 30 days. No CW was used in the media when BA was used.

After 30 days in the media containing BA, at least 3 well formed shoots have developed. These shoots were then separated from the mother plantlet and recultured in a ½ MS liquid or solid + 2 % sucrose, after 30 days in this medium, the well grown plantlets(Fig. 23) are ready for sub-culture in fresh media containing BA for further induction, or for transplant to field conditions.

Besides the lateral shoots formed and the mother plantlet, its individual leaved were also sub-cultured (Figs. 15 and 17) in liquid and solid ½ MS medium + 2% sucrose and BA at several levels 0.5 , 1 , 3 , and 5 ppm. All observations were made every week or every two weeks at the time for transflasking.

FIGURE 1. Healthy mature crowns of pineapple after the fruit is harvested; the short crown at the right is preferred as source of the best material for culture.

FIGURE 2. The leaves of the crown are carefully removed in order not to damage the bud that lay at the base of each leaf.

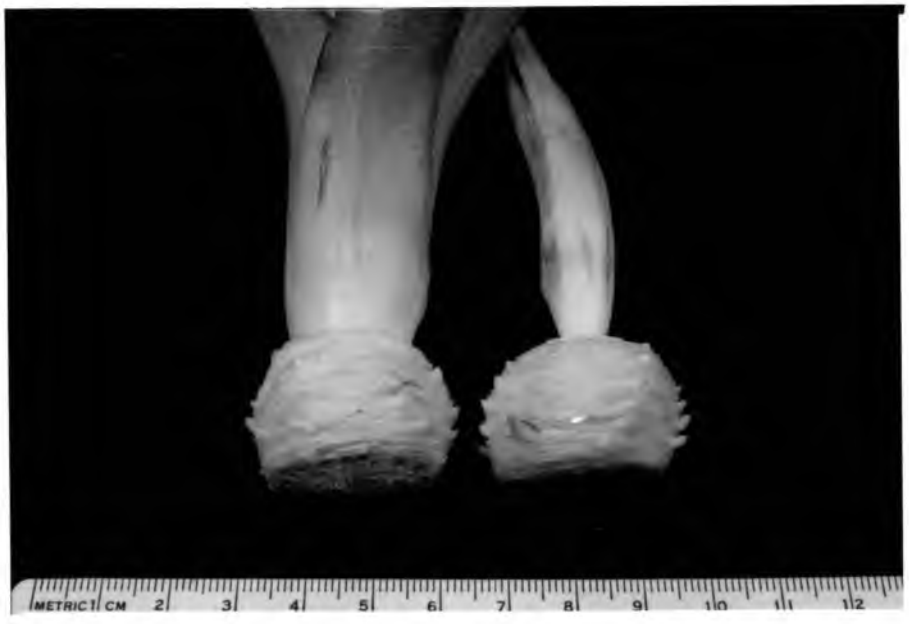

FIGURE 3. The short crowns have larger buds. These buds have greater success in culture.

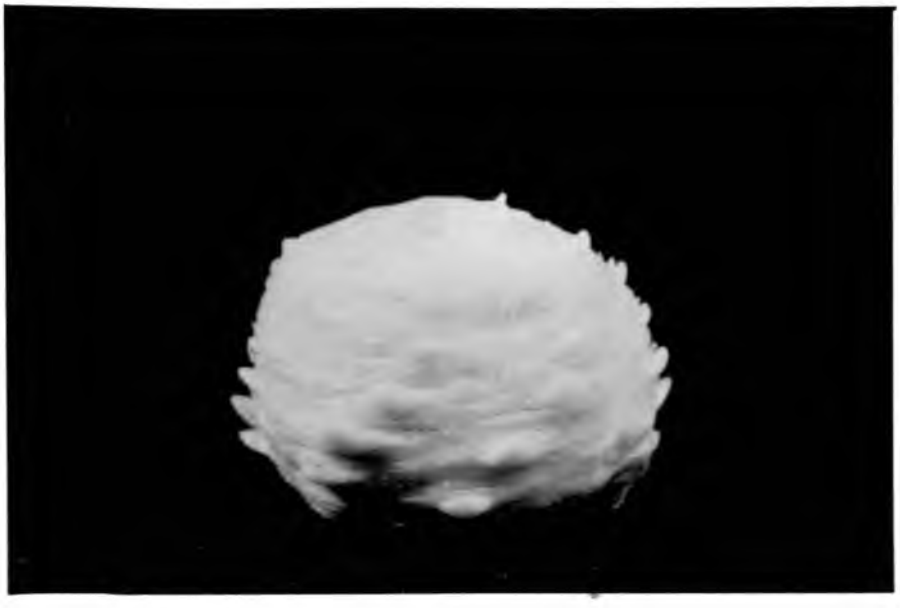

FIGURE 4. The ideal crown after removal of all its leaves. An average of 23 axillary buds per crown was obtained.

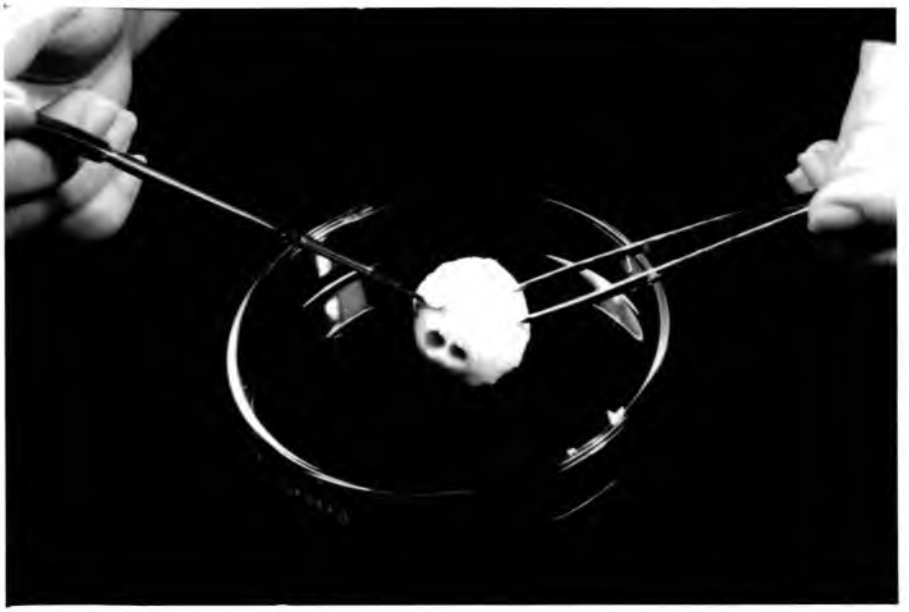

FIGURE 5. After 60 minutes disinfestation of the naked crown in a 10% Clorox solution, the buds are excised.

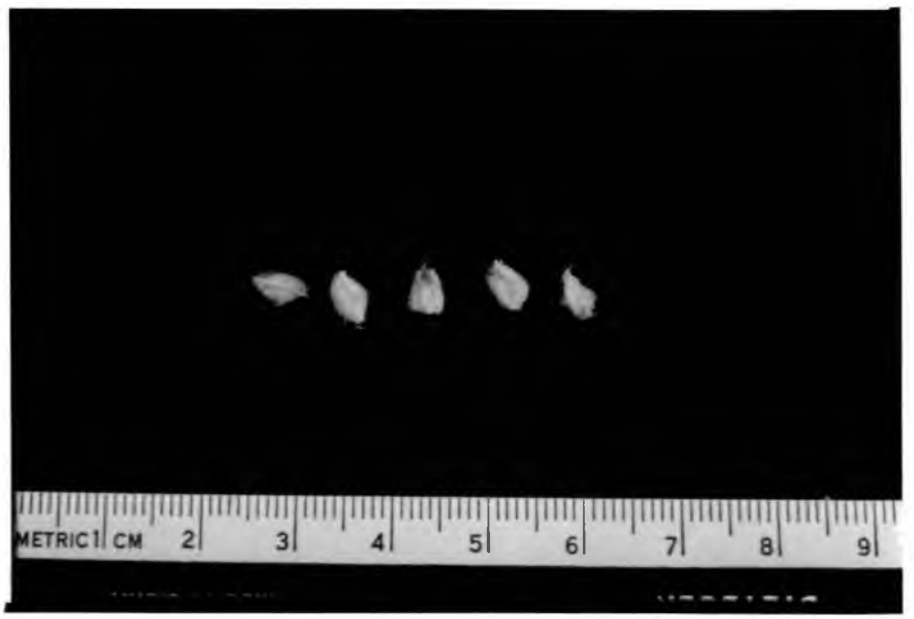

FIGURE 6. The excised buds along with some crown tissue are then placed in a 1% Clorox solution for approximately 20 min.

FIGURE 7. The excised buds are first cultured in a full strength Murashige and Skoog medium with 25% coconut water and 2% sucrose.

FIGURE 8. The buds are then subsequently sub-cultured every 2 weeks in a $\frac{1}{2}$ strength MS medium + 25% CW and 2% sucrose.

FIGURE 9. Growing buds 4 weeks after first culture. Stage of development, inmediately after second sub-culture.

FIGURE 10. Growing buds 6 weeks after first culture. The leaves start to unfold after the third sub-culture.

FIGURE 11. Individual plantlet obtained from a bud 8 weeks after the first culture. The 5-8 leaved plantlet is ready to be put in medium with growth regulator for shoot induction.

FIGURE 12. Two buds 35 days after first culture. The buds in the Erlenmeyer flasks show faster growth than those in tubes.

RESULTS AND DISCUSSION

Almost any healthy, mature crown can be used to obtain the buds that are going to be used in culture. However the short crowns are preferred to the large ones(Figs. 1-3) since the former ones have larger buds and these buds have greater success in culture.

The disinfestation procedure that gave the best and more constant results with even 0 % contamination in several ocassions as practice improved, was by placing the naked crown in 10 % clorox solution for 60 minutes and then, after the buds were excised, placing these in 1 % clorox solution for approximately 20 minutes. The initial results of an experiment with 240 cultures(40 buds cultured per each treatment), designed to find out the best combination of clorox concentration and time of exposure for the disinfestation of the naked crowns(Table 1), had indicated a different combination of these variables as the best; however, the highest concentration of clorox and the longest time of exposure offered the most reliable procedure. This disagreement, may be explained by the great variability of cleanliness of the crowns and conditions during handling of the material in disinfestation and culturing that may have occurred.

The effect of media and type of flask used,on the survival rate of the buds and in the percent of contamination respectively(Table 2) was measured 2 weeks after culture, for liquid media; and one month after culture for the buds cultured in solid media. The use of liquid

TABLE 1. The effect of Clorox* concentration and time of expossure to the disinfestant of naked crowns, on the percent of contaminated cultures.

Time of expossure in minutes	Percent of contaminated cultures.	
	Two Clorox* concentrations, %.	
	5 %	10 %
30	50	20
45	5	10
60	30	25

* Clorox Active Ingredient: Sodium hypochlorite 5.25 % .

media resulted in a significative greater percentage of buds developing successfully into plantlets, as compared with a very low 5.18 % of buds surviving and develop into plantlets when cultured in solid media. The slightly higher percent of contaminated cultures when using the screw cap bottle, may be explained by the fact that the bottles offered more dificulties in handling while culturing.

Within 2 months after initial culture, solitary shoots with 5-8 leaves were obtained(Figs. 7-11). The buds in the Erlenmeyer flasks showed faster growth those cultured in the tubes; this might be due to the fact that there was a larger volume of media in the Erlenmeyer and it was agitated much faster than in the tubes(Figure 12), consequently if the buds were to be cultured in Erlenmeyer flasks instead of the tubes, the time that it takes to a bud to develop into a plantlet could be reduced to 40 days only.

The effects of Benzyladenine at 0.5 ppm on shoot induction were evident within 10-20 days after sub-culturing a plantlet in ½ MS + 0.5 ppm BA and 2 % sucrose(Fig. 13). At least 3 well formed shoots are obtained from each plantlet after 30 days of being cultured in this media(Fig. 14). Originally, the shoots after separation from the mother plantlet were cultured in ½ MS medium with BA for further continuous shoot induction(Fig. 16) however, better results were observed when after separation from the mother plantlet, the shoots were cultured for approximately 30 days in ½ MS liquid or solid + 2 % sucrose only, to allow the shoots to become more defined, with

TABLE 2. Effect of solid and liquid media, and type of flask on contamination and bud survival after culture.

Media	Flask	No. of Cultures.	% of cultures contaminated.	% Successful cultures.
Solid	Screw cap Bottle	251	11.16	5.18
Liquid	Screw cap Tube	155	9.03	69.68

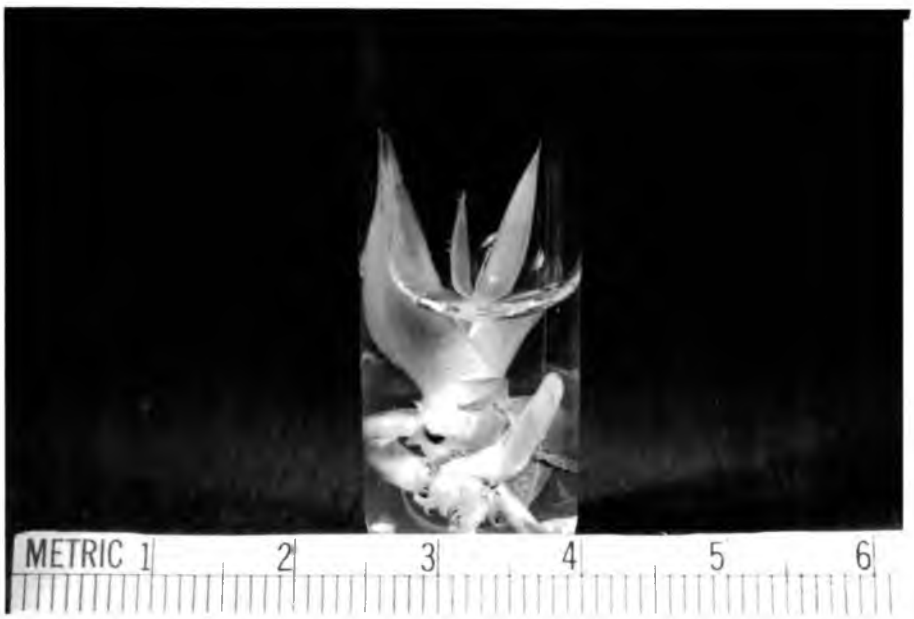

FIGURE 13. The effects of Benzyladenine at 0.5 ppm on shoot induction were evident within 10-20 days after sub-culturing a plantlet in $\frac{1}{2}$ strength MS + 0.5 ppm BA and 2% sucrose.

FIGURE 14. At least 3 well formed shoots are obtained from each plantlet after 30 days in $\frac{1}{2}$ MS + 0.5 ppm BA + 2% sucrose.

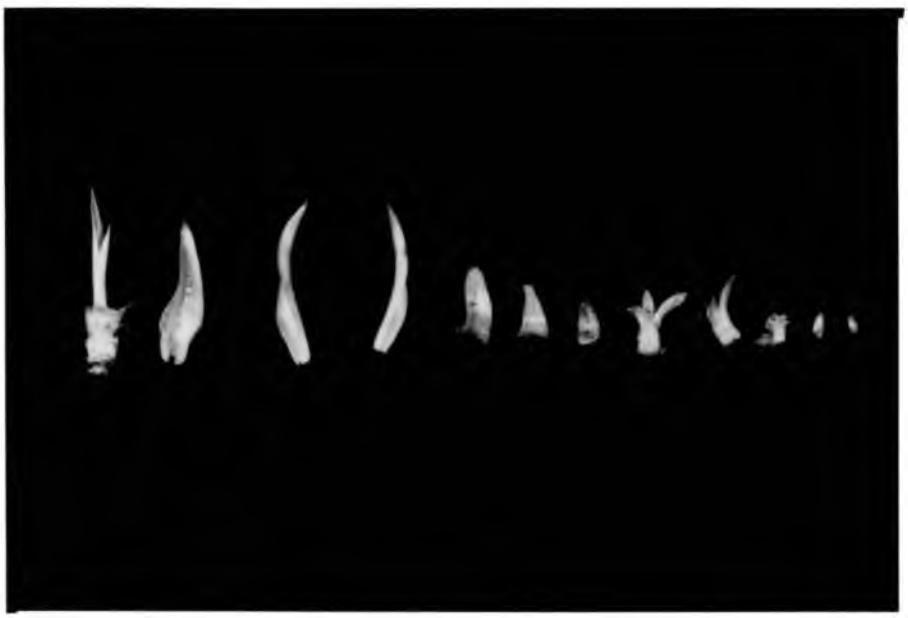

FIGURE 15. When the shoots have been formed in the plantlet, these are separated and recultured along with the individual leaves and the mother plantlet as well.

FIGURE 16. The mother plantlet at the left, and 3 of its shoots after 20 days in culture in $\frac{1}{2}$ MS medium + 0.5 ppm of BA.

greater leaf area and stem length. The development of roots occurred in all plantlets during this period of culture; they appeared after 8 days in liquid media, and after 15 days in solid media. Good size plantlets ready for sub-culture in fresh media containing BA for further induction ,or transplant to field conditions are obtained after about 30 days(Fig. 23). When these plantlets are recultured in media with BA for further induction, the roots are pruned.

The role of cytokinins in promoting lateral bud development has been widely recognized(6). If a cytokinin is added to an inactive lateral bud dominated by the shoot apex above it, the lateral bud often begins to grow. The ability of cytokinins to overcome apical dominance probably involves stimulated cell division in the bud and the ability of the bud to act as a sink into which, if still attached to the plant, nutrients from other parts of the plant are drawn. Enhanced lateral branching of plants infected by pathogens such as Corynebacterium fascians and Exobasidium spp. has been correlated to the fact that the pathogens synthesize cytokinins.

The effect of benzyladenine in shoot proliferation was also obtained when the leaves from plantlets were separated and cultured individually in $\frac{1}{2}$ MS + BA at several concentrations and 2% sucrose. Only those leaves under which the solid medium formed a crack due to dehydration exposing the base of the leaf, were able to proliferate

shoots . No leaf cultured in liquid medium developed shoots or survived. (Fig. 17).

Each plantlet having 5-8 leaves, is potentially capable of producing that same number of shoots if adequately induced with BA. After seeing the effects on shoot induction by the use of 0.5 ppm BA; growing buds 6 weeks after first cultured and at the stage when the bud leaflets start to unfold, were then transferred to ½ MS liquid media + 2 % sucrose and different concentrations of BA(Fig. 18). The different concentrations of BA tested were: 1, 3 and 5 ppm. Ten buds per each treatment were cultured.

The differences among treatments was very distinguishable after 20 days of growing in these media(figure 19) in terms of growth and color. The media containing the lowest concentration of BA showed more leaf area and a deep green color; as the concentration of BA increased the plantlets and shoots yellowing was observed.

The plantlets 25 days after being growing in ½ MS medium + 1 ppm of BA and 2 % sucrose, have evidently formed less number of shoots(Fig. 20). However, these shoots are larger, better formed and have the greatest success when transflasked(Table 3). The number of shoots formed on the plantlets of each treatment were counted at separation time, and the percent of successful cultures, one month after transflask.

FIGURE 17. Leaf from a plantlet cultured in $\frac{1}{2}$ MS + 3 ppm BA and 2% sucrose. Only those leaves under which the solid medium formed a crack due to dehydration exposing the base of the leaf, were able to proliferate shoots. No leaf cultured in liquid medium developed shoots or survived.

As the concentration of BA is increased to 3 ppm(Fig. 21), the number of axiliary buds on the plantlet induced to develop into shoots, is greater; however, these shoots are less defined and have less success when transflasked.

The effects of the highest concentration of BA were more dramatic (Fig. 22). The general growth of the plantlet was restricted, almost every axillary bud of the plantlet was noticeable swolen and their development into shoots was restricted, since the high concentration of BA acted by suppressing not only the apical dominance of the plantlet which in turn, caused the initiation of development of its axillary buds, but did not continue to develop, possibly due to the suppression of their apical dominance as well. The sweling effect is evidently a result of the development of the miniature axillary buds present within each axillary bud of the plantlet.

As a consequence, even though much greater number of shoots can be induced by using higher concentrations of BA, the shoots formed are very small, difficult to work with, and do not survive when transflasked after separation.

The use of BA in concentrations of 0.5 or 1 ppm proved to be the most adequate treatments for shoot induction(Table 3). Starting from a single crown, from which an average of 23 buds can be obtained

TABLE 3. Effect of Benzyladenine (BA) on the number of shoots formed per plantlet and their survival when sub-cultured after their separation from the mother plantlet.

BA Concentration (mg/ l.)	No. of shoots formed (Average/ plantlet)	Shoots successfull in culture, %.
0.5	3.50	100.00
1.0	4.83	86.20
3.0	6.40	6.25
5.0	10.75	0.00

FIGURE 18. Growing buds 6 weeks after first culture were then sub-cultured in $\frac{1}{2}$ MS, 2% sucrose with BA in ppm from left to right: 1, 3 and 5. Stage shows 10 days after transfer to this media.

FIGURE 19. Benzyladenine treatments from left to right, 1, 3 and 5 ppm. 20 days after being cultured in this media.

FIGURE 20. Plantlet 25 days after being growing in $\frac{1}{2}$ MS medium + 1 ppm of BA and 2% sucrose. Even though less number of shoots are formed, these shoots are larger, well formed and have greater survival rate when transflasked.

FIGURE 21. Plantlet 25 days after being cultured in $\frac{1}{2}$ MS + 3 ppm. of BA and 2% sucrose. The number of shoots induced is greater but the individual shoots are less defined.

FIGURE 22. Plantlet 25 days after being cultured in $\frac{1}{2}$ MS + 5 ppm of BA and 2% sucrose. The axillary buds of the plantlet are noticeable swolen, their development into shoots is limited as the higher rate of BA acts by suppressing the apical dominance of these growing buds; as a result the miniature axillary buds in each growing bud are estimulated to develop as well.

FIGURE 23. After separation from the mother plantlet, the shoots are cultured either in liquid or solid medium $\frac{1}{2}$ MS + 2% sucrose. Roots appear after 8 days in liquid media and after 15 days in solid media. Good size plantlets ready for sub-culture in fresh media containing BA for further induction, or for transplant to field conditions are obtained after about 30 days as shown in the photograph.

for culture. At the end of two months in culture, an average of 16 single plantlets develop, others were lost due to contamination or failiure of buds to grow. When these plantlets are subjected to 5 inductions of BA at the recomended concentrations, it is estimated that at the end of one year, at least 5,000 plantlets, such as those in Figure 23, can be obtained.

SUMMARY

Clonal propagation of pineapple Ananas comosus (L.) Merr. var. Cayenne lisse was achieved by in vitro culture of axillary buds from crowns of mature fruits. From an average of 23 axillary buds per crown, an average of 16 individual plantlets were obtained; the others were lost due to contamination or failiure of buds to grow. Disinfestation was by dipping short defoliated crowns in 10% Clorox with 3 drops of Tween-20 for 60 minutes, and excised axillary buds in 1% Clorox for approximately 20 minutes prior to initial culture.

The buds were then first cultured in full strength Murashige and Skoog(MS) medium, supplemented with 25% coconut water(CW) and 2% sucrose, and sub-cultured every 2 weeks into half-strength MS with same previous amounts, by volume of CW and sucrose. Within 2 months after initial culture, individual plantlets with 5-8 leaves were obtained. These plantlets were then sub-cultured into ½ MS + 2% sucrose, supplemented with 0.5, 1, 3, and 5 ppm of Benzyladenine (BA). The concentrations of BA that gave the best results were 0.5 and 1 ppm; after 30 days of growing in these media, at least 3 well formed shoots were developed on each plantlet. The shoots were then separated and recultured in liquid or solid ½ MSmedia+ 2% sucrose for approximately one month. After this period, good size plantlets were obtained, well suited for transplant to field

conditions or for reculture into a ½ MS medium + 2% sucrose and 0.5 or 1 ppm of BA for further shoot induction. When every shoot or plantlet has been induced 5 times with BA at the recomended concentrations, it is estimated that at the end of one year, at least 5,000 plantlets can be obtained, starting with a single crown.

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