AN IMPROVED PROTOCOL FOR THE MICROPROPAGATION OF PINEAPPLE (*Ananas comosus* (L.) Merrill)

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Abstract: The present work aimed at improving pineapple (*Ananas comosus* (L.) Merrill) cv. Smooth Cayenne production throughout the tissue culture technique. Some factors affect this process were studied. The method of surface sterilization, the adventitious and axillary bud formation versus N^6-Benzyladenine (BA) concentration, type of medium (liquid or solidified), rooting and acclimatization were evaluated. Explants have been surface sterilized with sodium hypochlorite (NaOCl) at 0.5 %, then with mercuric chloride ($HgCl_2$) at 0.1 % both for 5 minutes, with survival percentage of 95%. All explants were cultured onto MS basal medium supplemented with different concentrations of BA and 1.0 mg l^{-1} Kinetin (Kin). All cultures were maintained in a growth room at a 16 h photoperiod (40 mmol.m^{-2} s^{-1}), 27 ± 2°C. After eight weeks, number of initiated shoots was counted and the cultures were transferred for multiplication on the best treatment, 2.0 mg l^{-1} BA and 1.0 mg l^{-1} Kin. Therefore, using the liquid medium during multiplication stage speed up the growth of proliferated shoots compared to solidified medium. Utilizing 1.0 – 3.0 mg l^{-1} α-Naphthaleneacetic acid (NAA) or Indole-3-butyric acid (IBA) enhanced the root formation of individual shoots. Finally, the well rooted plantlets, around 12 cm in length, were transplanted onto the Peat moss as a soil bed under the greenhouse. After 4 weeks, the survival percentage was 100 %. By this improved protocol, more than 10000 pineapple plants were produced from one initial plant in less than 9 months.

Key words: Micropropagation, organogenesis, Pineapple, somaclonal variation, tissue culture.

Introduction

Pineapple (*Ananas comosus* (L.) Merrill) is one of the most delicious tropical fruits. Plant is herbaceous, perennial, monocot, from *Bromeliaceae* family. Now, pine-apple is widely grown in the tropics and subtropics. The greatest exploitation was in Hawaii but industry has moved to the Philippines and Taiwan because of high labor costs. The industry in most of the 20th century was processed pineapple but now fresh pineapple is increasing. Cultivation of pineapple is satisfactory spreading in Africa (Nigeria, Kenya and Ivory Coast).

Since a few decades it has been imported to Egypt in a few numbers. However, the knowledge of pineapple-field cultivation almost not found. Also, the cultivation is successfully restricted under the greenhouses. Recently, successful trial has been established for field cultivation (Abul-Soad, 2006). The pineapple plantlets which produced from this study will be used to spread the cultivation in the open field.

The most important pineapple variety cultivated in the world is the 'Smooth Cayenne'. 'Smooth Cayenne', a spineless mutation of 'Cayenne' has been the standard processing cultivar. It has a large tough fruit. Flesh is pale yellow to yellow and sugar and acidity of fruits are high. 'Smooth Cayenne' is best adapted to processing because fruit is blocky and there is a high yield of slices, which are the most valuable product.

In the micropropagation procedures, appearance of somaclonal variants is not preferable. Soneji *et al*., (2002) reported that *in vitro* plantlets were established in cups with soilrite and hardened for four weeks. Phenotypic variants such as albinos, white streaked shoots and shoots with elongated internodes were observed in the *in vitro* cultures. Furthermore, transferring cultures produced from variants will produce to loss the properties of the original variety. Thus this study focused on this point. As well as, Mujib in (2005) referred to the shoot tip culture of the variants and callus from the same source continuously produced variant progenies for a number of (4 - 5) *in vitro* cycles.

Mainly, the BA was used in the multiplication stage of pineapple. Escalona *et al.*, 1999 reported that the highest multiplication rate (106 shoots/explant) was found when explants were cultured in shooting medium (MS+2.1 mg l^{-1} BA+0.3 mg l^{-1} NAA) supplemented with 1 mg l^{-1} Paclobutrazol (PB) for 7 weeks.

As is well known, the liquid medium has advantages to increase the growth and development of the *in vitro* cultures. Lirio *et al*., 2001 reported that axillary buds of pineapple were inoculated on MS liquid culture medium as a basal medium. Soneji *et al*., 2002 referred to each isolated shoot upon subculture to liquid medium of the same composition further proliferated to form more multiple shoots (60-65 shoots/explant) and were maintained on a gyratory shaker (90-100 rpm).

Khatun *et al*., 1997 declared that the highest number of roots per plantlet was obtained when the shoots were grown on medium with 1.0 μM l^{-1} NAA and 2.0 μM l^{-1} IBA. Also, they reported that the regenerated plantlets were successfully established in plastic pots

containing soil, sand and Cow dung in 1: 1: 1 ratio.

Thus, the objective of this work was to develop an efficient protocol for the micropropagation of pineapple, 'Smooth Cayenne' cultivar, by study some factors to obtain viable microplants in greenhouse in a short time.

Materials and Methods

This study was carried out at the Department of Tropical Fruit (Plant Tissue Culture Lab.), Horticulture Research Institute, Agriculture Research Center, Egypt, throughout the period from 2004 to 2005. The clonal material 'Smooth Cayenne' was obtained from plants grown under greenhouse of the Horticulture Research Institute.

After harvest the fruit, the shoots on the top of fruit (crown) were taken. The outer leaves were peeled away. All parts were rinsed for 30 minutes under tap water with detergent to remove the sand grains and residues of dust. Shoot tip and nodal segments explants were used for culturing. All explants were obtained from 2-3 years-old plants.

The explants were cultured on the Murashige and Skoog, 1962 (MS) basal medium. The pH of the medium was adjusted to 5.7± 1, then 35 ml of media was dispensed into 350 ml large jars (covered with polypropylene closures) or 20 ml into small jars (150 ml) or 15 ml into small tubes (25 × 150 mm, covered with cotton stopper) and autoclaved at 121°C & 1.1 Kg/cm^2 for 20 min. Agar was added as 7 $g\ l^{-1}$. Cultures were incubated in a temperature-controlled room at 27 ± 2 °C under 16 h photoperiod at 50-60 $\mu E\ m^{-2}\ s^{-1}$ light intensity produced from cool white fluorescent lamps. Culture media were renewed at 8 weeks.

Experiment 1

Determination of the best-surface sterilization method:

This experiment was designed to study the effect of surface sterilization treatments, (NaOCl and $HgCl_2$), on the establishment of the aseptic cultures of 'Smooth Cayenne' cultivar. Treatments of sterilization experiment were replicated ten times and each replicate was represented by 3 explants individually cultured in 3 tubes. Thus, each value is an average of 30 tubes. All explants were subjected to the different treatments of sterilization experiment conducted through starting stage.

The treatments were as follows:

0.5,1.0,1.5 and 2.0% NaOCl for 5 minutes, Hg Cl_2 at 0.1%, 0.2 %, 0.3 % and 0.4 % for 5 minutes were used as sterilization treatments. All explants were treated with 3 rinses with sterile distilled water. Each treatment contained a few drops of Tween-20(Polyoxyethylene Sorbitan Monolaurate) per 100 ml solution as wetting agent. Air bubbles were removed from tissues by frequently

vessel agitation. All steps of the sterilization were carried out under aseptic conditions. All treatment cultured onto the above mentioned medium.

Data were recorded after 8 weeks in culture, on the following characteristics:

1. Percentage of contamination.
2. Percentage of survival.
3. Percentage of mortality.

Experiment 2

Comparison between the response of shoot tip and nodal segment explants:

After surface sterilization of the explants, the shoot tips were divided into transverse sections which used as two types of initial explants. Shoot tip with shoot apex and subsequent-shoot tip (nodal segments) were cultured onto basal medium supplemented with different concentrations of BA and 1.0 mg l^{-1} Kin. Therefore, the shoot tip and nodal segment explants were divided longitudinally to 4 segments. All cultures were incubated under the previous conditions. Each treatment contained 30 tubes and each vessel included 1 explant. Therefore, the shoot number (shoots/explant) was recorded after 8 weeks in culture.

Experiment 3

Effect of physical state of nutrient medium on the proliferation enhancement:

One cluster of multiplied shoots was selected for this experiment. Each included 5-7 healthy shoots. All clusters were cultured onto the same basal medium supplemented with 2 mg l^{-1} BA and 1 mg l^{-1} Kin. Liquid and solidified (7 g l^{-1}) medium were used. The liquid medium incubated onto rotary shaker at 100 rpm (revolution per minute). The solidified medium was put stationary on the shelves in the growth room under the same conditions. Each treatment contained 30 jars and each jar included 1 cluster. The number of shoots/explant was recorded after 8 weeks in culture.

Experiment 4

Effect of different concentrations of NAA and IBA on root formation:

Each treatment contained 30 large jars and each jar included 5 individual shoots (10 – 15 cm in length). The 0.5 g l^{-1} activated charcoal was added to the basal nutrient medium. The MS basal medium and 0.5 g l^{-1} activated charcoal supplemented with different concentrations at 0.1, 1.0 and 3.0 mg l^{-1} of NAA or IBA. All cultures were incubated under the same conditions. The following data were recorded after 8 weeks in culture:

- Rooting Percentage (%).
- Roots number (roots/plantlet).
- Root length (cm).

Experiment 5

Effect of various soil mixtures on speed up growth and development of plantlets during acclimatization stage:

The pineapple plantlets produced from the previous stage (rooting stage) were transferred *ex vitro* to different soil mixture 0:1, 1:1 and 1:2 of washed sand: peat moss (v/v). Then, the following steps were performed:

1. Before planting, plantlets were rinsed thoroughly with tap water to remove the remained agar around the root system, and then immersed in 0.5% (w/v) benlate fungicide solution for 5 minutes.

2. Plantlets were individually transplanted in plastic flats and cubic pots filled with the previous mixtures of peat moss and washed sand.

3. Plants were kept in greenhouse under natural day light and high relative humidity (90-95%) using a cover of white polyethylene sheet for one week.

4. The process of acclimatization of plants to the greenhouse conditions (27±2 ^{o}C) was achieved by gradual reduction in humidity from the ambient conditions of plants through removing the plastic sheet gradually. After then plants were allowed to develop under greenhouse conditions.

5. The plants were watered with quarter strength of MS inorganic salts once a week and sprayed with the fungicide as needed. The survival percentage was recorded after 1 month in transplanting.

Statistics

Factorial randomized complete block design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5%, according to Steel and Torrie 1980.

Results and Discussion

Although several pineapple micropropagation protocols have already been published, significant improvement could be achieved if the stages of the *in vitro* culture were better defined. Our work concerned several experiments aiming at the mass production of high quality plantlets.

Experiment 1

Determination of the best-surface sterilization method:

Establishing good and free-contaminants cultures was the most difficult stage in pineapple micro-propagation protocol. However, the pineapple explants were very sensitive to the sterilization substances. It was observed that many explants were lost throughout the sterilization procedure whether they were unfunctioned through fungi and bacteria-infected explants

or bleaching. Thus, it is necessary to find influential procedure to produce free-contaminants explants accompanied with high survival. Thus, this part was done in order to evaluate the effect of the different sterilization materials on the contamination, survival and mortality percentage of pineapple explants cultured *in vitro*.

Data in Table (1) showed the effect of NaOCl at different concentrations (0.5,1.0,1.5 and 2.0%) for 5 minutes and $HgCl_2$ at (0.1%, 0.2 %, 0.3 % and 0.4 %) for 5 minutes on the sterilization and growth of pineapple explants cultured on MS basal medium supplemented with different concentrations of BA and 1.0 mg l^{-1} Kin.

Table(1): Effect of surface sterilizing surfactants on contamination, survival and mortality percentages of pineapple explants, after 8 weeks in culture.

Surfactant (%)		Contamination	Survival	Mortality
NaOCl	$HgCl_2$	(%)	(%)	(%)
0.5	0.0	80.0a	20.0f	0.0f
1.0	0.0	60.0b	40.0e	0.0f
1.5	0.0	40.0c	50.0d	10.0e
2.0	0.0	45.0c	40.0e	15.0e
0.0	0.1	30.0d	70.0c	0.0f
0.0	0.2	30.0d	55.0d	15.0e
0.0	0.3	25.0d	50.0d	25.0d
0.0	0.4	40.0c	20.0f	40.0c
0.5	0.1	5.0ef	95.0a	0.0f
1.0	0.2	10.0e	80.0b	10.0e
1.5	0.3	0.0f	40.0e	60.0b
2.0	0.4	0.0f	20.0f	80.0a
Mean		30.4	48.3	21.3

The means followed by the same letters are not significantly different at 5 % probability level.

Data in Table (1) showed that a suitable method to sterilize shoot apex explants had been achieved. Hence, shoot tip and nodal segment explants were successfully sterilized at 0.5 % NaOCl and after then with 0.1% $HgCl_2$. The percentage of survival was 95 %, contamination 5% and mortality 0%, after 8 weeks in culture. The combined effect of NaOCl and $HgCl_2$ at low concentration emerged satisfied results. It sustained the highest percentage of survival versus low percent of contamination plus the lowest percentage of mortality.

On the other hand, the sterilization with NaOCl alone produced contaminated explants. The percentage of contamination ranged from 45 – 80 %, with significant differences. As well as, disinfestations with $HgCl_2$ alone had fewer efficacies on the sterilized explants. Wherever, the survival percentage dropped in between 25 – 40 %, with significant differences. In addition the mortality percentage reached to 40 %. However, this is a high mortality. May the reason attributed to the harmful effect of $HgCl_2$ on the explants tissue.

Experiment 2

Comparison between the response of shoot tip and nodal segment explants:

Data in Table (2) showed that, the average number of shoots increased by increasing the BA concentration to 2.0 mg l^{-1}, after 2 months in initial culture. There were significant differences among the different treatments. The average shoot number was 4.5 shoots/ explant.

Thus, using 2.0 mg l^{-1} BA + 1.0 mg l^{-1} Kin stimulated the growth and development of the axillary bud from both such kind of explants. Hence, the average number of shoots/explant of the shoot tip was 6 in compared to 3 for the nodal segments. Furthermore, the formation of the outgrowths in shoot tip was higher than nodal segments. On the other hand, using concentration above 2.0 mg l^{-1} BA was found to stimulate the adventitious shoot formation (Fig. 1, left photo).

Many research papers have been published on the pineapple but a few of them focused on the starting stage. However, most of them start from the easy way, from the *in vitro* shoots. In spite of the starting stage of pineapple micropropagation is considered as the most difficult stage in the whole protocol. In this study, focus has been done on the starting stage and the type of the emerged shoots. The process of axillary and adventitious shoots formation was noticed visually (Fig. 1). Along with, the effect of BA concentration on this process was examined. The number of shoots produced in starting stage was recorded. Many papers investigated the effect of BA concentration but they didn't give much care about what is the kind of new shoots.

Fig. (1): Axillary buds (right photo) and adventitious shoots (left photo) formation of nodal segment explants of pineapple, after 8 weeks in culture.

Table(2): Influence of Benzyladenine (BA) and Kinetin (Kin) on the shoot number (shoots/explant) of the shoot tip and nodal segment explants of Pineapple, after 8 weeks in initial culture.

Cytokinin (mg l^{-1})		Explant type (B)		Mean (A)
BA	Kin	Shoot tip	Nodal segment	
0.0	1.0	1.0d	0.0e	0.5d
0.5	1.0	2.0c	1.0d	1.5c
1.0	1.0	2.0c	2.0c	2.0bc
2.0	1.0	6.0a	3.0b	4.5a
3.0	1.0	3.0b	2.0c	2.5b
Mean (B)		2.8	1.6	

The means followed by the same letters are not significantly different at 5 % probability level.

As is well known, in the micropropagation procedure much care must be given for the true to typeness issue. It seems that pineapple tissues have high morphogenetic potential to produce shoots. In this study more than 10 thousands of shoots have been produced from one plant for further field performance determination. These shoots can be produced from axillary buds which present in the leaf axils or adventitiously from the cut surface tissue. The axillary buds were often genetically typical to mother plants.

On the other hand, production of adventitious buds may produce somaclonal variations. The obtained results revealed that the shoot tip explants produced adventitious shoots on the cut surfaces (Fig. 2). This could be for the high cytokinins/auxins ratio within or near from the apical meristem, after cutting the shoot tip longitudinally to 4 segments. When these explants were used, the axillary meristems, which usually remain quiescent during shoot growth, were able to form new shoots. This is in agreement with Firoozabady and Gutterson (2003) who stated that, either longitudinal sections of the shoots or leaf bases could be used as the explants to regenerate shoots. However, the clonal fidelity of propagated plants was tested in Costa Rican and Indonesian pineapple farms. As well as, Soneji *et al*., (2002) reported that approximately 520 *in vitro* produced-pineapple plantlets were established in the field and these

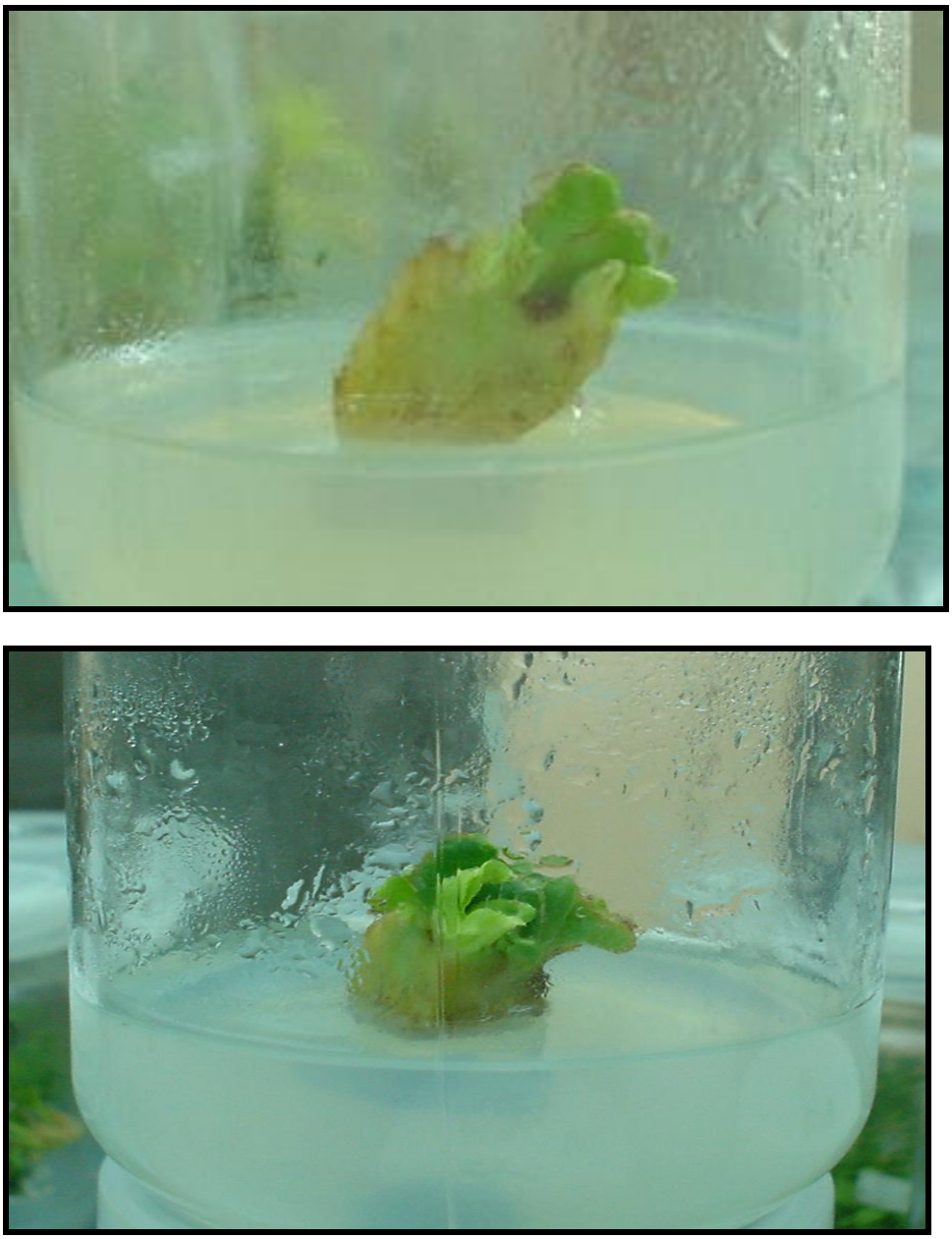

Fig. (2): Adventitious buds formation of shoot tip explants of pineapple after 4 (right photo) and 8 (left photo) weeks in culture.

plants exhibit somaclonal variation. Thirty-eight plants were found to be yellowish, spineless with anthocyanin streaks and three were anthocyanin rich, spined plants. Furthermore, the meristematic composition of this area is very sensitive for any exotic addition of cytokinins. This is in harmony with George in 1993 who reported that the exogenous addition of cytokinins with low amount of auxins can be promoted the adventitious shoots.

The reverse was found in nodal segments. Most shoots were initiated from the obvious meristems of axillary buds which spread on the nodal segments after leaves peeled away (Fig. 1, right photo). This was in agreement with Souza *et al*., (2003) who reported that auxin/total cytokinins ratio was always lower in the decapitated nodal segment throughout the process of the axillary bud development of pineapple.

Experiment 3

Effect of physical state of nutrient medium on the proliferation enhancement:

Data in Table (3) showed that pineapple shoots which cultured onto liquid medium (Fig. 3, right photo) was better than the solidified medium. Where the average number of shoots was 74.3 shoots/cluster in compared to 47.3 in the solidified medium.

On the other side, there was significant difference in among different subcultures. Therefore, the proliferation rate increased by increasing the subculture number. The shoots number increased from 44 shoots/cluster to 79 shoots/cluster. The interaction effect revealed that the usage of the liquid medium in the third subculture produced the highest number of shoots (97 shoots/cluster).

Table(3): The effect of physical state of the nutrient medium and the number of subcultures on the proliferation (shoots/explant) of *in vitro*-pineapple shoots during the multiplication stage.

Physical State (A)	Subculture Number (B)			Mean (A)
	Sub.1	Sub.2	Sub.3	
Solidified medium	36.0d	45.0c	61.0c	47.3
Liquid medium	52.0c	74.0b	97.0a	74.3
Mean (B)	44.0c	59.5b	79.0a	

The means followed by the same letters are not significantly different at 5 % probability level.

Fig..(3): Pineapple shoots proliferated on the liquid medium (right photo), ideal-*in vitro* plantlets make ready for transplantation (left photo).

It seems that the liquid nutrient medium allowed more release of the nutrients to the vegetative shoot in compared to the solidified medium. This result is in agreement with Escalona *et al.*, (1999) and Lirio *et al*., (2001) who used the liquid medium as a basal nutrient medium for the pineapple.

Experiment 4

Effect of different concentrations of NAA and IBA on root formation:

This experiment aimed to enhance the root formation of pineapple shoots. After the multiplication stage, the clusters of shoots separated in small clusters as much as possible. These small clusters transferred onto basal medium without any growth regulators to complete their elongation. Only, the vegetative shoots at 5-10 cm in length were selected for the rooting stage. Sometimes, there are new small shoots still emerging from the base of clusters. These should be transferred to the same basal medium again.

Data in Table (4) indicated that the rooting rate in all treatments was high. It ranged from 85 to 98 %. However, using 1.0 mg l^{-1} NAA resulted in the highest rooting rate (98%). Also, this treatment produced 5 roots/plantlet, as an average. Whereas, the highest roots number produced when the basal nutrient medium incorporated 1.0 or 3.0 mg l^{-1} IBA, where the average of roots number was 8 and 9 roots/plantlet.

On the other hand, using the high concentration of both auxins, NAA and IBA (3.0 mg l^{-1}) stimulated the root length. Hence, the average root length was 10 and 12 roots/plantlet, respectively.

The initiation of roots started after 7 days form culturing onto a fresh rooting medium. It is noticed that the root formation was occurred from the basal part of vegetative shoots, around the base. Also, there was a little bit callus formation on this area in the high concentration of auxins. Consequently, separation of some roots during the acclimatization process is expectable. Thus, it is better to use 1.0 mg l^{-1} NAA or IBA to produce healthy and appropriate roots (Fig. 3, right photo).

Furthermore, the addition of activated charcoal in the rooting medium was found to enhance the growth and root formation. This is supported by Weatherhead *et al*. 1978 who reported that better growth responses of the plant tissues are associated with the addition of AC to the medium. Furthermore, Abul-Soad *et al*., 1999 and Ibrahim *et al*., 1999 reported that addition of the activated charcoal to the rooting nutrient medium of date palm significantly increased the vegetative growth of rooted plantlets.

Table(4): Effect of different concentrations of NAA and IBA on root formation in rooting stage, after 2 months in culture.

Auxin (A) (mg l^{-1})		Rooting (B)		
NAA	IBA	Rooting Rate (%)	Roots Number (Roots/plantlet)	Root Length (cm)
0.1	0.0	85c	3cd	5cd
1.0	0.0	98a	5bc	6c
3.0	0.0	96a	7ab	10ab
0.0	0.1	90bc	2d	3d
0.0	1.0	95ab	8a	9b
0.0	3.0	95ab	9a	12a

The means followed by the same letters are not significantly different at 5 % probability level.

Experiment 5

Effect of various soil mixtures on speed up growth and development of plantlets during acclimatization stage:

Well rooted plantlets were transferred onto the different soil mixtures to study the effect of these soil mixtures on the growth and development of the pineapple in the greenhouse. The results indicated that the average survival percentage after one month of transplanting was 91.3, as shown in Table (5). It is showed that the transplanting pineapple plantlets onto the soil mixture composed of pure peat moss resulted in 100% as a survival percentage. While the survival percentage of the other soil mixtures, 1:1 and 1:2 Sand : Peat moss were 83.0 and 91.0, respectively.

Table(5): Effect on various soil mixtures on survival percentage of pineapple transplants after 4 weeks in transplanting.

Soil Mixture Sand : Peat moss	Transplants Number	Survived Plants Number	Survival Percentage
0 : 1	1000.0	1000.0	100.0a
1 : 1	1000.0	830.0	83.0b
1 : 2	1000.0	910.0	91.0ab
Mean	1000.0	913.3	91.3

The means followed by the same letters are not significantly different at 5 % probability level.

It is obvious that the peat moss soil (Fig. 4, left photo) was the best soil bed for the transplantation of the pineapple plantlets. It is observed that the peat moss speed up the growth and development of the plantlets in compared to other soil mixtures. May be this is attributed to the low pH of beat near from the appropriate soil of pineapple in the origin. The rooted plantlets must be individuals not in the clusters. It is observed that, transplanting rooted clusters and separating the rooted shoots manually, significantly reduced the survival percentage.

The growth and development on the pure peat moss dramatically increased compared to the other soil mixtures. Furthermore, the acclimatized plantlets were in dark green color. High humidity must be given during the first week of transplanting. After then, gradual decreasing in the relative humidity around the plantlets was performed. After 3 months from the transplanting (Fig. 4, right photo), the pineapple plants were ready to transfer to other bigger plastic pots.

Further study was needed to follow plantlets in greenhouse during acclimatization stage and determine the growth vigor of these plantlets. Also, evaluation for vegetative growth and fruiting quality of both types during their development in open field is important.

Conclusion:

Much care must be given for the sterilization process. Because of the pineapple explants are sensitive to the most of surfactants. Combining both surfactants ($HgCl_2$ and NaOCl) had worth impact on the surface sterilization of pineapple explants.

Regarding the true to typeness, the adventitious buds formation was visually observed. But further histological study is needed. Furthermore, follow up tissue culture-derived plants in greenhouse and after then in the open field also is necessary.

The liquid nutrient medium significantly reduced the time of the subculture and increased the number of proliferated shoots.

Transfer the individual vegetative shoots onto the MS basal nutrient medium supplemented with NAA at 1 mg l^{-1} was better. The individual transferred shoots should be at 10 cm in length and dark green in color.

Transplanting the well-rooted pineapple plantlets onto pure peat moss soil bed is better than the other soil mixtures. The plants were able to produce new leaves coated with epidermal wax.

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Fig.(4): Acclimatized-pineapple plantlets under low tunnel after transplanting with one week (right photo), and after 3 months in transplanting (left photo).

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يهدف العمل الحالي الي تحسين انتاج الأناناس (*Ananas comosus* (L.) Merrill) صنف "Smooth Cayenne" من خلال تكنيك زراعة الأنسجة. تمت دراسة بعض من العوامل التي تؤثر علي هذه العملية. قُيمت طريقة التطهير السطحي ، تكوبن البراعم العرضية و الجانبية مقارنة بتركيز البنزيل ادنين (BA) ، نوع الوسط الغذائي (سائل أو متصلب) ، التجذير و الأقلمة. تم تطهير الأجزاء النباتية سطحياً بهيبوكلوريت الصوديوم بتركيز 0.5 % ، ثم بعد ذلك بكلوريد الزئبقيق بتركيز 0.1 % لمدة 5 دقائق ، مع نسبة نجاح 95%. زرعت جميع الأجزاء النباتية علي الوسط الأساسي موراشيجي و سكوج (MS) و المزود بتركيزات مختلفة من البنزيل ادنين ، و 1.0 مليجرام/لتر كينتين. تم تحضين جميع الزراعات في غرفة النمو علي فترة ضوئية 16 ساعة (40مل مول/متر مربع/ثانية) ، 27° ± 2° درجة مئوية. بعد 8 اسابيع، تم احصاء عدد الأفرع الخضرية المتكونة ، و نقلت الزراعات لمرحلة التضاعف علي افضل معاملة ، و هي 2.0 مليجرام/لتر بنزيل ادنين و 1.0 مليجرام/لتر كينتين. اسرع الوسط السائل النمو للأفرع المتضاعفة بالمقارنة بالوسط المتصلب. نشط استخدام 1.0 – 3.0 مليجرام/لتر من نفثالين حامض الخليك (NAA) أو اندول حامض البيوترك (IBA) من تكوين الجذور علي الأفرع الفردية. بعد ذلك، تم نقل النُبيتات الجيدة التجذير و التي يتراوح طولها 12 سم علي بيت موس ، كمرقد في الصوبة. بعد 4 اسابيع، كانت نسبة النجاح 100%. و بهذا البرنامج المحسن، تم انتاج ما يقرب من 10000 نبات اناناس من نبات واحد في مدة لا تزيد عن 9 اشهر.

الكلمات الدليلة: اختلافات وراثية ، إكثار دقيق ، أناناس ، تخلق أعضاء ، زراعة انسجة.