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EMPLOYMENT OF *IN VITRO* TECHNOLOGY FOR LARGE SCALE MULTIPLICATION OF PINEAPPLES (*ANANAS COMOSOS*)

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Abstract

Experiments were carried out for the micropropagation of pineapples. It is thus possible to produce disease free, uniform propagules at needed quantity and at appropriate time of the year. BAP @ 0.5 mg/L showed better results for the number and length of shoots per explant. IBA and NAA were employed for appropriate root initiation and development and the best media observed containing IBA @ 1.5 mg/L alone. This was followed by field experiments to determine an appropriate media for *in situ* plant growth in green houses, for acclimatizing the plants.

Introduction

Pineapples are considered to be an exotic dessert fruit mainly due to its high sugar content and attractive flavor, additionally it contains vitamins A and C. Presently pineapples are being imported in the form of fresh fruits, canned chunks or slices. Pineapples are propagated by vegetative means from suckers and slips. Crowns are also being used with varying degrees of success. Plantlets arising from old plants through vegetative means usually carry all inherent characteristics of the parent plant (Escalona, 1999). In Pakistan, there is a great need to multiply and introduce this valuable crop. Pineapples can be grown by intercropping with coconuts or bananas, thus making the production highly feasible, as has been successfully done in Hawaii (Garcia, 2000). For commercial cultivation, good quality planting material in large quantity is required. Tissue culture is the only option to do so in our condition that is why an efficient and economical micropropagation protocol for the large scale propagation of pineapples was developed in our laboratory.

Materials and Methods

The medium used in this study consisted of MS basal salts, (Murashige & Skoog, 1962) with full and half strength. Sucrose was added as the sole carbon source at 3% (w/v). pH of the medium was adjusted using 0.1M NaOH or 0.1M HCl, to give a final value in the range 5.6-5.8. The media was then sterilized by autoclaving at 15 psi and 121ºC for 20 minutes. The medium was cooled and allowed to set before being used.

The plant material of Pineapples, (*Ananas comosos*) was obtained from the nursery of the HEJ Research Institute of Chemistry, University of Karachi. Crowns of young fruits were taken as a source of explant. The terminal portion of the crown was cut into lengths of 20cm. The leaves were then removed by gentle peeling, leaving the base intact. The crown was thoroughly washed under running tap water for 30-40 minutes. The

explants were then soaked in a 250 ml sterile jar containing sterile distilled water with 2-3 drops of Tween 20. The jar was placed on a gyratory shaker for 15 minutes. The explants were given a quick dip in 70% ethanol for 5 seconds followed by placing in 20% NaOCl containing 3 drops of Tween 20 on continuous but very gentle stirring on a gyratory shaker for 30 minutes. This was followed by three consecutive rinsing in sterile distilled water for five minutes each to remove traces of NaOCl. Alternative explants were sterilized using 1% Mercuric chloride and it was determined that a more effective method of sterilization would be soaking the plants in 0.1% Mercuric chloride for 3-4 minutes followed by rinsing the plants thoroughly in sterile distilled water for 5 minutes each to remove traces of mercuric chloride. All this sterilization work was done under laminar flow cabinets in sterile environment. The stems were cut into transverse sections containing one nodal bud. They were cut into approximately 2cm long segments and used as explants for shoot initiation under *in vitro* conditions. For the multiplication experiments, the plants were subjected to different concentration and combination of BAP and NAA (Table 1) on full and 1/2MS with 20 replications. Cultures were maintained in a growth room at 24 ± 2ºC with light provided by cool white florescent tube lights for a 16h photoperiod. Average number of shoots and increase in length (mm) were recorded after a period of 7 days in each jar for a successive period of 6 weeks. When newly initiated adventitious shoots reached a considerable length (>70mm) they were transferred to root initiating media, callus formed if any, was cut off from the shoot using a sterile scalpel before transfer. The shoots were transferred onto fresh medium with the cut ends towards the medium. Data was recorded by counting the number and length of new shoots and roots per plant. In order to induce rooting, the plants were subjected to IBA and NAA at concentrations of 0.25, 0.50, 1.00, 1.50 mg/L, in 1/2MS each with 20 replications. Cultures were incubated and maintained in a growth room. Total number of roots were counted and increase in root length (mm) was measured after a period of 7 days for each jar, for a successive period of 6 weeks and the average was calculated. When a considerable network of adventitious roots had been established the plants were transferred to green house for acclimatization.

Plantlets with roots ranging 20-40mm in length were selected for transferring to green house. Plantlets were removed from culture, and the roots were gently washed in luke warm distilled water to remove any residual gel or medium. These plants were placed into 250 mm plastic pots containing: (i) only sand, (ii) only wood charcoal, (iii) only fertilizer (cow dung), (iv) sand + Fertilizer (50:50), (v) Sand + Charcoal (50:50), (vi) cow dung + Charcoal (50:50), (vii) Fertilizer + Charcoal + Sand in equal parts. The sand employed was previously washed in 1 M HCl to adjust the pH to 5.5 from an original soil pH of 8.5. The pots were placed in green house where the humidity was approximately 80%. Data was recorded to determine which type of medium was optimum for acclimatisation. Over 4 weeks, relative humidity was slowly decreased by gradually shifting the plants into semi-shade areas. All experiments were of completely randomized design and repeated at least twice. Each treatment consisted of 80 explants (four explants per 250 ml jar). The number of shoots and roots were subjected to square root transformation prior to analysis. The number and length of both roots and shoots were also presented as mean values with a standard error. Data was analyzed employing SPSS version 4.0 (Scientific Enterprises, USA).

Table 1. Effect of BAP alone or in combination with NAA on shoot proliferation on full and ½ MS medium.

Media	BAP (mg/L)	NAA (mg/L)	No. of shoots per bud explants		Length of shoots (mm)	
			MS	1/2MS	MS	1/2MS
M1	0	0	1.85 ± 0.58	1.65 ± 1.04	77 ± 27.99	74 ± 16.98
M2	0.25	0	2.85 ± 0.60	1.85 ± 1.50	78 ± 25.69	70 ± 11.47
M3	0.50	0	2.95 ± 0.22	2.50 ± 1.14	80 ± 52.31	81 ± 23.40
M4	1.00	0	2.25 ± 0.86	2.15 ± 0.59	88 ± 37.40	86 ± 24.71
M5	1.50	0	1.95 ± 0.22	1.75 ± 0.86	84 ± 23.86	80 ± 25.23
M6	0	0.001	1.45 ± 1.13	1.05 ± 0.21	80 ± 64.69	76 ± 14.91
M7	0.25	0.001	2.89 ± 0.59	2.65 ± 1.04	84 ± 16.05	77 ± 10.09
M8	0.50	0.001	3.85 ± 0.58	3.15 ± 0.59	95 ± 29.36	82 ± 58.49
M9	1.00	0.001	2.40 ± 1.10	2.20 ± 0.73	90 ± 67.92	81 ± 17.19
M10	1.50	0.001	2.15 ± 1.50	1.80 ± 0.73	85 ± 39.01	76 ± 10.50

After 6 weeks, 20 explants were used for each treatment which is presented as mean ± SE.

Table 2. Effect of auxins on adventitious root formation *in vitro* in *Ananas comosos*, microcuttings cultured for 6 weeks at 25°C under 16 h photoperiod.

Type of auxin	Conc. of auxin (mg/l)	% of cuttings rooted	No. of roots per rooted cutting	Average length of roots (cm)	Days to emergence of roots
IBA	0.25	85	1.20 ± 0.73	1.43 ± 0.2	15-20
	0.5	88	2.45 ± 2.5	1.03 ± 0.3	10-15
	1.0	100	5.00 ± 1.78	3.43 ± 0.26	8-12
	1.5	90	4.0 ± 1.80	2.02 ± 0.40	7-10
NAA	0.25	95	1.20 ± 0.58	1.0 ± 0.20	10-15
	0.5	100	1.51 ± 2.05	1.3 ± 0.17	10-15
	1.0	86	3.55 ± 2.51	2.3 ± 0.10	8-15
	1.5	80	2.15 ± 0.58	1.7 ± 0.13	7-12

Each treatment consists of 20-30 cuttings and data presented as mean ± SE.

Results and Discussion

Shoot regeneration potential of various explants was investigated. The effect of a number of cytokinin and auxins in the growth media on explants was also determined. The number of adventitious shoots increased profusely, which exhibited a profound increase in size over a very short period of approximately 20 days (Fig. 1). In the proliferation experiments, the tendency of increase in shoot number was observed to be the same in both full and ½ MS media, where the number of shoots increased on increasing the concentration of BAP.

BAP concentration of 0.50 mg/L showed the maximum increase in shoot number (Table 1 and Fig. 2). However, with further increase in cytokinin, there was a decline in shoot number. Likewise, when NAA was used with BAP, a profound increase in shoot length was observed. As in the previous experiment, the greatest increment was found to be in BAP (0.50 mg/L) and NAA (0.001 mg/L) in which the increase in number of shoots was 3.85. Multiple shoots appeared as tiny crowns (1-2mm) amongst elongated longer

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 1. Two weeks after cultures were subjected to subculture.
Fig. 2. Shoot elongation was observed 4 weeks after plants were subjected to media containing BAP@ 0.05mg/L.
Fig. 3. Profouse rooting was observed on media containing IBA @1ml.
Fig. 4. Plants thriving well in green house

shoots. These tiny crowns, when transferred to fresh medium continued to produce secondary crowns. Therefore, a cyclic production of shoots was possible, leading to continuous cultures and generation of large number of shoots. Shoots in full MS showed better results relatively. It was observed that an increase in shoot length was 88mm in a media containing 1.00mg/L BAP. However, as NAA was added, there was a significant increase in shoot length where the increment in shoot length was observed to be 95 mm over a period of 6 weeks. When the shoots were transferred into rooting media, thick networks of roots were establish within 3 weeks.

Similarly in the rooting experiments, where several concentrations of IBA were employed in the greatest average number of roots was 5.00 in a media containing 1.0mg/L IBA (Table 2 and Fig. 3). However, when IBA was substituted by NAA, it was observed that at 1mg/L NAA, although the percentage of rooting was 86%, the average number of roots was 3.55 which also required the shortest time of 8-15 days for root induction. This is in contrast to the finding by Devi *et al*., (1997) of good rooting on MS medium supplemented with only 9.84 µM IBA. Mathew & Rengan (1981) have reported that the media has to be supplemented with activated charcoal for the induction of rooting. In contrast to their findings, our study indicates that all the plantlets could be rooted without addition of activated charcoal in the medium and could be successfully established in soil. The difference may be due to differences in cultivars genetic material.

Transfer of plantlets with sterile roots showed almost 100% survival in the greenhouse (Fig. 4). The established plants were exposed to open environment where they showed rapid growth when the mature plants were shifted into greenhouses, it was observed that the pH of the soil played a major role in plant physiology. When plants were grown in basic soil types, severe chlorosis was observed, thus we resorted to adjusting the soil pH to 5.5. Addition of organic fertilizers strongly attracted insects, which feed on the fragile leaves, so fertilizers were excluded from the soil medium. When coal was added in combination to sand, a stunted growth was observed. In a series of experiments, it was found that an ideal soil type was one containing soil, charcoal and fertilizer in equal proportions with a fine sprinkle of garden sand soil on the surface of the pot in order to avoid the appearance/ exposure of fertilizer. Thus, an efficient and a viable protocol was established for the mass propagation of pineapples. All 10,000 plants subjected to acclimatization are surviving in the green house and are ready to be planted in open field conditions.

Earlier reports indicated only a limited success in obtaining plant *in vitro* (Mapes, 1973). Lakshmi *et al*., (1974) obtained plants from shoot tips of pineapple slips but were not able to induce multiple shoots. The present study shows rapid *in vitro* multiplication of pineapple via dormant axillary buds. A cyclic production of shoot was possible leading to high frequency plant recovery without loss of regenerative capacity and vigor.

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