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Isolation and Culture of Pineapple (*Ananas comnosus*) Protoplast

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ABSTRACT

In vitro full expended healthy leaves and sterilized *in vivo* leaves of pineapple (*Ananas* cv. smooth cayenne) plants were taken and prepared under aseptic conditions as different sources of explants. Also, different enzymes mixtures, incubation periods, osmotic pressure factors, shaking periods and speeds were concerned in combination with explants sources during protoplast isolation stage. In addition, sieve size and centerfugation speed were evaluated in combination with explants source during purification stage. Moreover, medium type protoplast density, auxin/cytokinin concentration ratio, and antibiotic were tested in combination with explants source during protoplast culturing. It is found that *in vitro* and sterilized *in vivo* explants source succeeded in maximizing protoplast yield. Also, using of enzymes mixture consists of 1.0% cellulase + 0.5% macerozyme was superior in increasing protoplast yield Moreover, using of sucrose at rate of 13.6g /100ml as osmotic pressure factor and incubation for 20 hours then, shaking for 15 min with speed rate 75 rpm succeeded in enhancing the highest protoplast isolation of pineapple. Meanwhile, using of 25 μM pore size mesh sieve and centrifugation at the rate of 1000rpm maximized protoplast purification. Moreover, culturing of protoplast KAO and Michayluk medium supplemented with 3.0 mg/l NAA and 0.2 mg/l BAP as well as the combination of antibiotic (0.4 mg/l Ampicilin + 0.1 g/l gentamycin + 0.1 g/l tetracycline) and using protoplast density at the rate of 2.5 x 10^4 induced the best protoplast viability and development of pineapple explants.

Keywords: Pineapple, tissue culture, cell division, protoplast isolation.

INTRODUCTION

Pineapple (*Ananas comnosus* cv.) smooth cayenne is one of the most economically important tropical fruits (Duval *et al*., 2001). The flesh of the fruits are eaten fresh as dessert in salads and compotes, cooked in pies, cakes, puddings, as garnish on meat, or made into sauces and preserves. Also, presence of proteolytic enzyme, bromelin used for tenderizing meat, stabilizing latex paint and in leather-tanning process. In addition, all parts of the plants were utilized Leaves yield (strong white silky fiber) and other plant parts are used as food for animals.

Protoplast technology has a potential application in the genetic improvement of pineapple. Plant protoplasts are particularly valuable for methods of plant improvement and breeding since digestion of the cell wall reduce interfering during fusion and injection or uptake of foreign DNA (Barbier and Bessis 1990). Isolated protoplast of grape vine (cv. Chardonnay) by using a solution of enzymes containing 1% cellulase onozuka R10, 0.5% Macerozyme R10**.** Moreover, Ping *et al*. (2005) found that the best enzyme solutions for protoplast isolation were obtained when dissolved in 2% cellulase, 0.5% pectinase and 1% macerating enzyme for 8h. Zhou *et al*. (2003) mentioned that cell suspensions of strawberry is the best explant for protoplast isolated on liquid medium. In addition**,** Huy *et al*. (1997) verified that higher protoplast yield and viability were obtained by using cell suspensions of blackberry cultivars in a solution containing 0.35M mannitol and 0.35M sorbitol. In the same time, El-Miniawy *et al*. (2002) pointed out that the highest viability of strawberry (cv. Chandler) protoplast was observed when squashed leaf segments incubated for 16h in enzyme mixture and 0.4M sucrose as osmotic agent. Protoplast isolation from *coffea arabica* cells was increased when collected and purified after 15h of lytic reaction in the dark and 28°C. However, protoplast of peanut was obtained when a rotatory shaker at 85 rpm and 26°C was used (Li *et al*. 1995). Sterile stainless steel sieve 45μm diameter was preferred for isolation of avocado protoplast (Witjaksono and Grosser 1998). Ochatt *et al.* (1993) mentioned that culturing protoplast of haploid Golden Delicious apple on modified KaO and Michayluk-based media succeeded to give microcallus. Protoplasts development of banana spp. appeared when density was 6 x 10^6 cells per 3.5 x 1.3cm dish (Huang *et al*. 2000)**.** Also, plating of grape (*Vitis vinefera* L.) at protoplast 5 x 10^4/ml on gelled medium and in darkness at 25°C induced initial cell division after 1 week (Marino 1990). Further, more high frequency of cell division of *Vitis thunbergii* protoplast occurred when culture medium was supplemented with 2mg/L NAA and 0.2mg/L benzyl adenine (Mii *et al*. 1991). The ultimate goal of this study is establishing a protocol for protoplast isolation and culture of pineapple plants by using different techniques.

MATERIALS AND METHODS

In vitro full expanded healthy leaves and sterilized *in vivo* new leaves of pineapple (*Ananas* cv. Smoth Cauun), plants were taken and prepared under asceptic conditions. Recent leaf blades were divided into small sections 1-2 mm wide after excluding the marigens and main midrib. The sections were placed in small Petri-dish (6 cm in diameter), immersed in 10 ml enzymatic

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mixture, sealed tightly with parafilm, and incubated overnight at 25-28°C under dark conditions. Then placed on rotating shaker for different periods and speeds. New leaves from *in vivo* plants were also taken and sterilized by subjecting them to running water for 15 minutes to get rid of dirt, immersed in soap solution for 5 minutes, immersed in 10% colorox solution (0.5 NaOCl) commercial bleach with two drops of Tween-20 for 10 minutes, and then immersed in sterilized distilled water 3 times for 5 minutes.

1. Protoplast Isolation

a. Effect of enzyme mixture and explant source

Factorial experiment was carried out including different enzyme mixtures and explant sources (*in vitro* and *in vivo* explants) to select the best enzyme mixture and explant source enhance the highest number of isolated pineapple protoplasts. The following tested enzymes mixtures under were:

1- 1% cellulase + 0.5% macerozyme.
2- 1% cellulase + 0.2% pectinase.
3- 1% cellulase + 0.5% macerozyme + 0.2% pectinase.
4- 0.5% macerozyme + 0.2 % pectinase.

b. Effect of osmotic pressure factors and explant source

Mannitol, sucrose and glucose were used as osmotic pressure factors for adjusting the osmotic pressure of the medium to get a normal protoplast size without turgor or plasmolysis. Leaves from *in vitro* and *in vivo* were used as explants sources. Mannitol was added at the rate of 10 g/100ml. However, sucrose was also added at the level of 13.6g /100ml. In addition, glucose was added at the level of 7.92g/100ml.

c. Effect of incubation period and explants source:

Different incubation periods (i.e. 12, 16, 20 and 24 hours) were evaluated to figure out the most suitable period which induce the highest protoplast yield.

d. Effect of shaking period and speed as well as explant source

Factorial experiment between different shaking periods (15, 30, 45, 60 minutes) and speeds (50, 75, 100 and 125rpm) as well as explant sources were conducted to detect the best explants source, incubation period and speed which encourage the highest protoplast yield.

2. Purification

a. Effect of sieve pore size and explant source

Different explant sources and pore sizes of mesh sieves (i.e. 25, 50 and 75µm) were used to select the best explant source and pore size which improve protoplasts filtration without passing digested tissues and residues.

b. Effect of centrifugation speed and explant source

Different centrifugation speeds (500, 1000, 1500rpm) for 5 minutes and different explant sources were used to investigate the suitable speed and explant source which give the highest protoplast yield filtration and viable.

3. Protoplast culture

a. Effect of medium type and explant source

Murashige and Skoog (1962), Kao and Michayluk, (1975) and Gamborge *et al.* (1968) media and different explant sources were cultured embedded according to Blackhall *et al.* (2002) to select the best medium type and explant source wich resulted in the highest protoplast development.

b. Effect of cultured protoplast density and explant source

Different protoplast densities (0.2×10^4, 2.6×10^4, 3.5×10^4/ml) and explant sources were tested to find out the best density of protoplast and explant source which enhance highest development.

c. Effect of auxin, cytokinin concentrations ratio

The protoplasts were cultured on KM medium supplemented with different ratios of Naphthalene acetic acid (0.0, 1.0, 2.0, 3.0mg/100ml) and Benzyl amino purine (0.0, 0.1, 0.2, 0.3mg/100ml) to find out the suitable hormonal balance (auxin and cytokinin) that induces the highest protoplast development.

d. Effect of antibiotic

Different antibiotic types (0.4g/l Ampicillin, 0.1g/l Gentamycine and 0.1g/l Tetracycline) were added to KM medium either alone or in combinations to identify the best antibiotic get rid off contamination and in turn enhancing protoplast development.

Enzyme mixture solutions

Each enzyme mixture was dissolved in KM salts (Kao and Michayluk, 1975) solution supplemented with 0.5 mg/l NAA, 1.0 mg/l BAP and 13.6 g/100ml sucrose as osmotic stabilizer. The pH of the enzyme solution was adjusted to 5.7. The solution was filtered through 0.2µm pore size sterilized "Sartorius" membrane. The enzyme solutions were dispensed into sterile 15ml conical tube with cap and stored at 2°C under dark condition.

Data and calculation

Counting of protoplasts was conducted according to the method of Blackhall *et al*. (2002) as the number of cells per each square on the haemocytometer. The final count of protoplasts per ml was carried according to the following equation:

$$Total\ number\ cells = 5n \times 10^4$$

Protoplast viability was counted as the number of complete rounded protoplast which represents viable protoplasts. Protoplast development calculated as the rate of cell division and micro calli formed and calculated as scores according to Pottino (1981*).*

Statistical analysis

All treatments used in this study were arranged as factorial experiment in a complete randomized design according to SAS (1996). The obtained data were subjected to analysis of variance and statistically evaluated using standard deviation (SD).

RESULTS

1. Protoplast isolation

a. Effect of different enzyme mixtures and explant source

Data show that *in vitro* explants source surpassed *in vivo* source in increasing protoplast yield (Table 1). Moreover, enzyme mixture (EM1) consisted of 1% cellulose + 0.5% macerozyme was superior in maximizing protoplast yield as compared with the other enzyme mixtures under study. Generally, from the above results, we could conclude that *in vitro* explants is the best explants source for protoplast yield. Also, EM1 gave the highest protoplast numbers.

Table (1): Effect of different explant source and enzyme mixtures on protoplast yield of Pineapple. Results are given in mean ± SD

Enzyme mixture	protoplast yield per ml/gfwt	
	In vitro	*In vivo*
EM_1	23.67 ± 1.53	15.00 ± 1.00
EM_2	11.00 ± 1.00	1.33 ± 0.58
EM_3	11.67 ± 1.53	1.00 ± 0.00
EM_4	5.00 ± 1.00	1.00 ± 0.00

b. Effect of different medium osmotic pressure factors and explants source

It is obvious from results in Table (2) that the addition of sucrose to the culture medium encouraged the increase in protoplast yield as compared with the other osmotic pressure factors. However, adding glucose to the medium failed completely to induce protoplast isolation. Meanwhile, *in vitro* explants surpassed *in vivo* explants in protoplast production.

Table (2): Effect of explant source and medium osmotic pressure factors on protoplast yield of Pineapple. Results are given in mean ± SD

Enzyme mixture	protoplast yield per ml/gfwt	
	In vitro	*In vivo*
Glucose	0.0001 ± 0.00	0.0001 ± 0.00
Mannitol	5.13 ± 0.15	0.0001 ± 0.00
Sucrose	22.83 ± 0.61	17.63 ± 0.41

c. Effect of different incubation periods and explant sources

Data tabulated in Table (3) describe the effect of different incubation periods on protoplast yield. It is clearly appeared that *in vitro* explants surpassed *in vivo* one in increasing the protoplasts yield. Meanwhile, increasing incubation period of *in vitro* leaf segments in enzyme mixture up to 20 hours and 24 hours for *in vivo* explants induced the highest protoplasts production with the other incubation periods.

d. Effect of explant source, shaking period and speed

Table (4) shows that *in vitro* explant source was valuable in increasing protoplast numbers in relation to *in vivo* explant source. Meanwhile continuous increase of shaking speed resulted in increasing of protoplasts produced (direct relationship) up to 75rpm at which the peak of protoplast yield appeared then continuous increase of shaking speed to 100rpm showed sharp decrease in number of protoplasts isolated. However, the lowest shaking period (15 minutes) enhanced increase of protoplast yield in comparison with the other incubation periods. In general, continuous increase of shaking period resulted in reducing of protoplast yield as aresult of increasing protoplast damage. Furthermore, using of *in vitro* explant source combined with 30 minutes shaking period and 50rpm shaking speed .Thus, the aforementioned results summarized that *in vitro* explant and shaking speed of 75 rpm for 15 minutes are the best factors for protoplasts isolation. These results may be due to that *in vitro* explant have less hemicellulase and chitin which improve the effect of enzyme mixtures and in turn increase the isolated protoplasts yield as compared with *in vivo* explant. However, increasing shaking speeds or periods resulted in an increase in protoplast damage and then reduced the number of viable protoplasts.

Table (3): Effect of explant source and different incubation periods on protoplast yield of Pineapple. Results are given in mean ± SD

Incubation Period (hours)	protoplast yield per ml/gfwt	
	In vitro	*In vivo*
12	0.0001 ± 0.00	0.0001 ± 0.00
16	17.33 ± 0.85	0.0001 ± 0.00
20	25.13 ± 0.35	1.32 ± 0.08
24	6.30 ± 1.10	18.30 ± 1.10

Table (4): Effect of different shaking periods, speeds and explant source on protoplast yield of Pineapple. Results are given in mean ± SD

Shaking period(min.)	Shaking speed (rpm)	Explant source: protoplast yield per ml/gfwt	
		In vitro	*In vivo*
15	0	0.0001 ± 0.0	0.0001 ± 0.0
	50	22.35 ± 0.75	18.80 ± 0.63
	75	26.39 ± 1.10	20.78 ± 0.64
	100	18.87 ± 0.43	15.38 ± 0.21
30	0	0.0001 ± 0.0	0.0001 ± 0.0
	50	25.12 ± 0.99	20.78 ± 0.64
	75	19.20 ± 1.49	14.40 ± 1.17
	100	13.39 ± 1.21	9.37 ± 0.01
45	0	0.0001 ± 0.0	0.0001 ± 0.0
	50	13.58 ± 0.90	7.93 ± 1.43
	75	10.12 ± 0.38	5.74 ± 1.43
	100	7.44 ± 0.90	4.38 ± 0.90
60	0	0.0001 ± 0.0	0.0001 ± 0.0
	50	1.79 ± 0.65	0.0001 ± 0.0
	75	0.0001 ± 0.0	0.0001 ± 0.0
	100	0.0001 ± 0.0	0.0001 ±0.0

2. Purification

a. Effect of sieve pore size and explant source

Table (5) reveals the effect of sieve pore size and explant source on protoplast yield. It is well noticed that

in vitro explant source gave the highest mean values of protoplast yield (17.14) in relation to *in vivo* explant (8.07). Meanwhile, sieve pore size at 25µm for filtration of protoplast was more superior in maximizing the number of protoplast isolated followed by 50 µm then lastly 75 µm which produced the lowest values of protoplast yield. The aforementioned results conclude that *in vitro* explant source combined with sieve pore size 25µm produced the highest protoplast number. These results may be due to the increasing in sieve pore size which encouraged cell wall residues, clumps of undigested tissues and debris to pass through the filter and in turn affect badly on potoplast yield and viability.

b. Effect of centrifugation speeds and explant source

Data of (Table 6) show the effect of centrifugation speed and explant source on protoplast yield. *In vitro* explant was more effective in increasing protoplast yield than *in vivo* explant. Moreover, centrifuging speed at 1000 rpm is recommended for increasing protoplast yield (21.63), followed by speed at 500 rpm (14.50) and finally 1500 rpm speed which produced the lowest value of protoplast (8.36). Concerning the interaction, it is appear that *in vitro* explants combined with centrifuging speed at 1000 rpm maximized the protoplast yield followed by *in vivo* explant combined with the same speed

Table (5): Effect of explant source and sieve pore size on protoplast yield. Results are given in mean ± SD

Pore size (µm)	protoplast yield per ml/gfwt	
	In vitro	*In vivo*
25	25.53 ± 0.67	20.30 ± 0.80
50	19.03 ± 0.83	2.61 ± 0.54
75	6.87 ± 0.42	1.29 ± 0.03

Table (6): Effect of explant source and centrifugation speed on protoplast yield of Pineapple. Results are given in mean ± SD

Centrifugation Speed (rpm)	protoplast yield per ml/gfwt	
	In vitro	*In vivo*
500	19.67 ± 0.47	9.33 ± 0.85
1000	24.23 ± 0.81	19.04 ± 0.64
1500	13.53 ± 0.49	3.19 ± 0.07

3. Protoplast culture

a. Effect of different medium types and explant source

The data in (Table 7) show that culturing of isolated protoplasts embedded in KM medium enhanced division of protoplasts and formed micro calli with large number compared with the other media used in this respect. However, Gamborge (B5) medium failed to induce any positive results in this respect in both *in vivo* or *in vitro* explants source. Anyhow, MS medium was in-between concerning protoplast development. However, culturing protoplast of *in vitro* as explants source on KM medium increased protoplast development.

Table (7): Effect of different explant source and media types on protoplast development of Pineapple. Results are given in mean ± SD.

Medium type	Score	
	In vitro	*In vivo*
B5	1.00 ± 0.00	1.00 ± 0.00
KM	3.87 ± 0.07	3.67 ± 0.15
MS	2.07 ± 0.06	1.73 ± 0.06

b. Effect of cultured protoplast density and explant source

Table (8) verifies the effect of cultured protoplast density and explants source on protoplast development of pineapple. It is quite evident that *in vitro* explant slightly improved protoplast development in relation to *in vivo* explant. Meanwhile, culturing protoplast density of 2.5 x 10^4 was more effective in maximizing protoplast development followed by density of 3.5 x 10^4. However, protoplast density at 0.5 x 10^4 failed to show any positive result of protoplast development. The above results reflected the importance of using *in vitro* explant source combined with protoplast density at 2.5 x 10^4 which maximizes protoplast development.

Table (8): Effect of explant source and cultured protoplast density on protoplast development of Pineapple. Results are given in mean ± SD.

Cultured Protoplast density	Score	
	In vitro	*In vivo*
0.5	1.00 ± 0.00	1.00 ± 0.00
1.5	2.20 ± 0.10	1.75 ± 0.03
2.5	3.73 ± 0.02	3.22 ± 0.02
3.5	3.10 ± 0.10	2.87 ± 0.06

c. Effect of explant source, auxin and cytokinin concentration

Data of Table (9) point out that *in vitro* explant source was more superior in enhancing protoplast development. Moreover, increasing auxin (NAA) concentrations in the culture medium resulted in improving protoplast development. Meanwhile, the effect of cytokinin, concentrations (BAP) in the culture medium on protoplast development depend mainly on the level of auxin concentration in the medium Addition of BAP at concentrations 0.2 or 0.3 mg/l enhanced clearly protoplast In the same time,culturing of protoplasts resulted from *in vitro* source on medium supplemented with 3.0 mg/l NAA and 0.2 BAP resulted the best protoplasts development. In general, the above results indicate that *in vitro* explant source combined with 3.0 mg/l NAA and 0.2 mg/l BAP maximize protoplast development and increased cell division.

Table (10) shows the effect of different explants sources and antibiotic types on protoplast development. It is obvious that *in vitro* ones surpassed *in vivo* explant source in improving protoplast development. Meanwhile, the supplementation of the culture medium with the combination of 0.4 g/l ampiciline + 0.1g/l gentamycin + 0.1g/l tetracycline resulted in eliminating contamination and reduced the harmful effect of

antibiotics which resulted in increasing protoplast development. The combination of 0.4 g/l ampicilin + 0.1g/l tetracycline and the combination of 0.4g/l ampicilin + 0.1g/l gentamycin were less effective in eliminating contamination and reduction of the harmful effect of antibiotics.

Table (9): Effect of explant source, auxin and cytokinin concentrations on protoplast development of pineapple.

	BAP (mg/l)	Explant source	
		In vitro	*In vivo*
		Score	*Score*
NAA 0.0 (mg/l)	0.0	1.00 ± 0.0	1.00 ± 0.0
	0.1	1.00 ± 0.0	1.00 ± 0.0
	0.2	1.13 ± 0.06	1.00 ± 0.0
	0.3	1.17 ± 0.01	1.00 ± 0.01
NAA 1.0 (mg/l)	0.0	1.32 ± 0.01	1.00 ± 0.0
	0.1	2.40 ± 0.10	1.79 ± 0.07
	0.2	2.20 ± 0.10	1.82 ± 0.02
	0.3	2.04 ± 0.01	1.83 ± 0.02
NAA 2.0 (mg/l)	0.0	2.03 ± 0.06	1.40 ± 0.10
	0.1	2.19 ± 0.08	1.79 ± 0.04
	0.2	2.84 ± 0.02	2.02 ± 0.02
	0.3	2.34 ± 0.01	2.03 ± 0.03
NAA 3.0 (mg/l)	0.0	2.24 ± 0.03	2.14 ± 0.01
	0.1	3.03 ± 0.02	2.85 ± 0.02
	0.2	3.53 ± 0.02	3.02 ± 0.03
	0.3	3.33 ± 0.02	2.51 ± 0.01

Table (10): Effect of explant source and antibiotic on protoplast development of Pineapple. Results are given in mean ± SD.

Antibiotic	Score	
	In vitro	*In vivo*
Control	1.00 ± 0.00	1.00 ± 0.00
0.4 g /l Ampicilin	1.00 ± 0.00	1.00 ± 0.00
0.1 g / l Gentamycin	1.07 ± 0.06	1.00 ± 0.00
0.1 g / l Tetracycline	1.53 ± 0.03	1.25 ± 0.02
0.4 g / l Ampiciline + 0.1 g / l Gentamycin	2.76 ± 0.05	2.24 ± 0.03
0.4 g / l Ampicilin + 0.1 g / l Tetracycline	3.53 ± 0.02	3.18 ± 0.03
0.1 g / l Gentamycin + 0.1 g / l Tetracycline	2.20 ± 0.10	2.20 ± 0.26
0.4 g / l Ampicilin + 0.1 g / l Gentamycin + 0.1 g / l Tetracycline	3.80 ± 0.10	3.40 ± 0.10

DISCUSSION

The results of present study reflected superiority of *in vitro* explants source as it maximized protoplasts yield than that of *in vivo* source. Also, enzyme was effective in increasing protoplasts yield as compared with the other enzymes combinations. This may be related to that *in vitro* explants have less cellulose and chitin as well as consisted from soft cells which resulted in increase of enzyme combination efficiency at this level of enzymes concentration. These results in general agreement with the findings of (Ochatt and Caso, 1986). They stated that yield of protoplast isolated from *in vitro* mesophyll of wild pear were higher compared with those from field mesophyll plants. Also, with (Barbier and Bessis 1990). They recommended enzyme mixture formulated from 1% cellulase onozuka R10 and 0.5% macerozyme R10 for isolating the highest yield of grape vine Chardonnay cv. Protoplasts. Meanwhile, addition of sucrose to the culture medium as osmotic pressure factor succeeded in reducing plasmolysis and increasing protoplasts viability. This occurred due to addition of sucrose at the level used induced a balance between inside and outside osmotic cell pressure. These results go in line with the findings of El-Miniawy *et al*. (2002). They revealed that incubating of squashed leaf segments in enzyme mixture supplemented with 0.4M sucrose as osmotic agent improved protoplast viability. Meanwhile, increasing of incubation period up to 20 hours in vitro explants and 24 hours *in vivo* explants resulted in

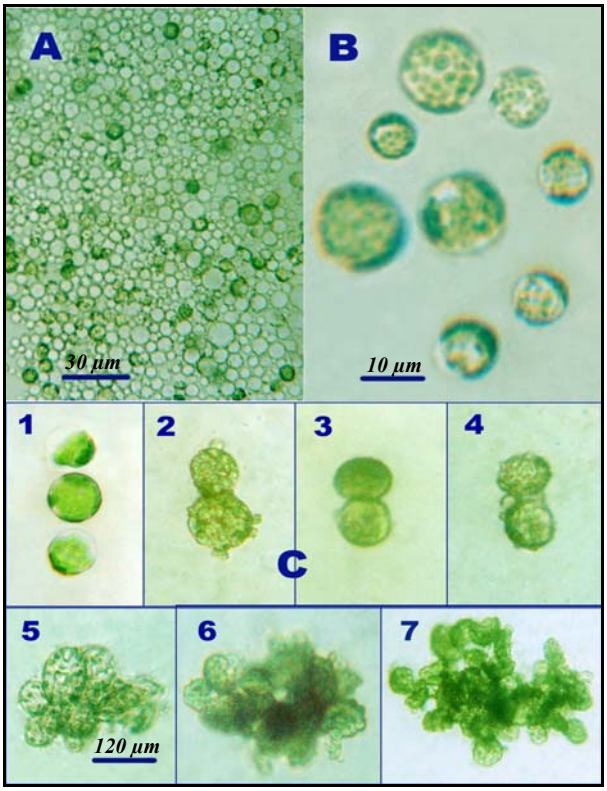

Figure (1): (A) shows protoplast yield resulted from pineapple. (B) reflects the protoplasts viability. (C) explains protoplast developmental stages, which include undivided viable protoplast (C-1), starting of protoplast division (C-2), complete protoplast division (C-3), separation of two protoplasts (C-4), starting of micro calli formation (C-5) and both (C-6 and 7) formation of micro calli with high numbers of protoplast.

enhancing protoplasts isolation. These results are somewhat in agreement with the findings of Marino (1990) who obtained the highest protoplasts yield when grape explants were incubated in enzyme solution for 18 hours. Furthermore, shaking of *in vitro* explants for 30 minutes with 50rpm speed rate improved protoplasts yield and viability as well as reducing protoplasts damage. These results are in harmony with the findings of Li *et al.* (1995). They reported that highest yield of *Arachis* spp. mesophyll protoplast was obtained when a

rotary shaker was used at speed 85 rpm speed. Regarding purification of the protoplasts from debris and undigested tissue, it is clear that using of sieve with 25µm pore size enhanced purification. This occurred due to increasing pore size resulted in encouraging passing of cell wall residues, clumps of undigested tissues and debris through the filter which affect bodly on protoplast number and viability. These results are in co-ordination with the findings of Witjaksono and Grosser (1998). In the same time, using of 1000 rpm of centrifuge speed proved to be effective in participation of debris and residues which maximized purification of protoplasts and increased viability.

Dealing with protoplasts culture, it is noticed that culturing of protoplasts embedded in KM medium encouraged protoplasts division and increased number of cells to from micro calli (protoplast development). These results confirm the findings of Ochatt *et al.* (1993). They recommended culturing of protoplasts of haploid Golden Delicious apple on KM medium for induction of micro callus development Also, using of protoplast density at rate 2.5 x 10^4 induced the best protoplast division and encouraged the highest numbers of micro callus. These results go in line with the findings of Kobayashi (1987). In additions, adding 3.0 mg/l NAA and 0.2mg/l BAP to the culture medium encouraged protoplasts division. These results in agreement with the findings of Mii *et al.* (1991) on *Vitis thunbergii* and Huy *et al.* (1997) on blackberry. In the same time, supplementation of the culture medium with combination of antibiotics (0.4g/l ampiciline + 0.1g/l gentamycin + 0.1g/l tetracycline) was valuable in eliminating contamination of the cultured protoplasts and reduced the harmful effect of the antibiotic on protoplast development.

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عزل وزراعة بروتوبلاست نبات الأناناس (*Ananas comnosus*)

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الملخص العربى

تم أخذ الأوراق الحديثة من أفراد الأنابيب وكذا أفراد الحقل المعقمة من نبات الأناناس (صنف سموس كايين) وتم تجهيز الأجزاء النباتية تحت الظروف المعقمة حيث تم إعتبارها كمصادر مختلفة (داخل وخارج الأنابيب) وتم دراسة تأثير مخاليط مختلفة من الأنزيمات، فترات تحضين، فترات وسرعات الهزاز، عوامل ضبط الضغط الأسموزى، مع مصادر مختلفة من الجزء النباتى وذلك أثناء مرحلة فصل البروتوبلاست. بينما تم دراسة كل من قطر ثقوب المرشح وكذا سرعة جهاز الطرد المركزى بالتفاعل مع مصدر الجزء النباتى وذلك أثناء مرحلة التنقية. أما مرحلة زراعة البروتوبلاست فتم دراسة تأثير نوع البيئة، كثافة البروتوبلاست، معدلات تركيزات الأكسين الى السيتوكينين وكذا المضادات الحيوية بالتفاعل مع مصدر الجزء النباتى .

أوضحت النتائج تفوق الأجزاء الناتية من النباتات داخل الأنابيب عن خارج الأنابيب وكذا ساعد إستخدام مخلوط الإنزيمات المكون من 1% سليوليز + 0.5% ماسيروزيم الى زيادة إنتاجية البروتوبلاست وأدى إضافة السكروز كعامل من عوامل الضغط الأسموزى والتحضين لمدة 20 ساعة ثم استخدام الهزاز الدائرى لمدة 15 دقيقة على سرعة 75 لفة فى الدقيقة إلى زيادة نجاح عزل البروتوبلاست كما ساعد استخدام الفلتر 25 ميكروميتر وكذا الطرد المركزى بمعدل 1000 لفة فى الدقيقة إلى زيادة تنقية البروتوبلاست. كذلك أدى استخدام بيئة كاو ومشيليك مع إضافة 3 ملليجرام/لتر نفثالين حمض الخليك و0.2 ملليجرام / لتر بنزيل أمينو بيورين بالإضافة إلى استخدام المضادات الحيوية بمعدل (0.4 جرام/لتر أمبيسيللين + 0.1 جرام / لتر جنتاميسين + 0.1 جرام / لتر تتراسيكلين) واستخدام كثافة البروتوبلاست بمعدل 2.5×10^4 إلى زيادة حيوية وتطور البروتوبلاست.