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PROTOPLAST ISOLATION AND CULTURE OF *Pyrus betulaefoia* AND *P. communis* PEAR ROOTSTOCKS

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ABSTRACT

Protoplasts were successfully isolated from *in vitro* leaf messophyll of both pear rootstocks (*Pyrus betulaefolia* and *P. communis*). The leaves were surface sterilized using 10% clorox for 10 minutes followed by 3 washes each for 5 min with sterilized distilled water. Leaves immersing in anti-oxidant solution (0.1 ascorbic acid + 0.15 citric acid) was effective in reducing phenolic compounds. Gradual plasmolysis in 9% Mannitol for 30 min followed by 13% mannitol for further 30 min enhanced protoplast isolation. CPW digestive medium supplemented with 13% mannitol and a filter sterilized enzyme mixture (1.5% cellulose + 1.5% macerozyme + 0.5% pectinase) surpassed the other two investigated digestive medium (MS and KM) in improving protoplast yield for both pear rootstocks. Overnight incubation of leaf strips immersed in enzyme solution in the dark for 20 hours followed by slow skaking on a rotary shaker at 75 rpm for 30 min encouraged more protoplasts release and subsequently increased protoplast yield. The incubation mixture (debris, undigested cells, protoplasts and enzyme solution) filtrated through passing nylon sieve with 25u pore size and the digested tissue pieces washed with small volume of CPW 13M medium. Protoplast suspension was spined at 1000 rpm for 7.5 min to sediment high yield of viable protoplasts.

In vitro leaf mesophyll protoplasts of *P. betulaefolia* were cultured on MS medium, at density $2x10^5$/ml while KM medium was superior for *P. communis* at the same density. Protoplasts of both species divided, developed and formed micro colonies in the above mentioned culture medium supplemented with either 1.0 mg/L NAA + 0.3 mg/L BAP with *P. betulaefolia* or 1.0 mg/L NAA + 0.2 or 0.3 mg/BAP with *P. communis*.

INTRODUCTION

Rootstocks are of great importance in the intensive cultivation of pear trees. The advantages of using rootstocks include: the avoidance of juvenility, uniformity of tree performance, control of yield and fruit quality as well as to develop tolerance to diseases, pests and unfavorable soil factors.

Pear plants mainly propagate by grafting the scion on the suitable rootstocks. Large numbers of rootstocks may be used for pear but the most suitable one is communis pear (*Pyrus communisn*) rootstock. It had an excellent vigor, adaptable to different soil types, and compatible with most pear varieties as well as it spread in most pear farms because of producing high yield and excellent fruit qualities but not resistant to fire blight.

Recently, a new pear rootstock betulaefolia pear appeared (*Pyrus betulaefolia*) rootstocks had an excellent vigor, adaptable to different soil types, compatible with most pear varieties and resistant to fire blight (Cameron *et al.*, 1969), but the fruit yield and qualities are lesser than ones grafted on communis pear.

The only alternative is finding out a new rootstock effective in controlling fire blight and encouraging high yielding as well

as fruiting qualities. Establishing a breeding program for producing a new rootstock for pear combine the best characters of communis pear and tolerance to fire blight is the most important step in overcoming fire blight problem.

Conventional breeding programs needing high costs and long time to accomplish the required goal. The best alternative is employing biotechnology in achieving this goal through protoplast isolation and fusion to establish somatic hybridization between communis and betuleafolia pear rootstocks and in turn produce new rootstock valuable in producing high yield and fruit quality as well as in the same time good tolerant to fire blight disease.

Protoplast technology has a potential application in the genetic improvement of pear rootstocks. Pear protoplast were also used for studies of host pathogen interaction with bacterium responsible for fire blight, *(Erwinia amylovora)*, and a novel methodology for the precocious selection of plants according to their responses vis the pathogen developed (Brisset *et al.*, 1990). Protoplasts are particularly valuable for methods of plant improvement since the cell wall is not present for interfering during fusion and injection or uptake of foreign DNA (El-Gindy and Gray, 1991).

Protoplastes provide the starting point for many of the techniques of genetic manipulation of plant in particular the induction of somaclonal variation, somatic hybridization and transformation (Cocking *et al.*, 1981; Kinsara *et al.*, 1986 and El-Gindy and Gray, 1991).

The ultimate goal of this study is establishing a protocol for protoplast isolation and culture of both communis and betuleafolia pear rootstocks by using different experiments in this respect. Also, studying the obstacles facing protoplast isolation and culture as well as utilizing of this techniques in future in breeding program to product new rootstock in pear by using protoplast fusion (somatic hybridization) or genetic transformation.

MATERIALS AND METHODS

This investigation was carried out at tissue culture unit, Horticulture Department, Faculty of Agriculture Moshtohor, Benha University, during the period from 2004 to 2007.

All the experimental studies conducted on two rootstocks. i.e. communis pear (*Pyrus communis*) and betulaefolia pear (*Pyrus betulaefolia*). The mother plants were planted in tissue culture nurserry, of the same Faculty. *In vitro* plantlets of both rootstocks under study were established, proliferated, and rooted according to Bayuomy (2004) methods.

Leaf sterilization:

The new emerged leaves of *in vivo Pyrus communis* and *Pyrus betulaefolia* were collected from the mother trees put, in polyethylene bags and transferred directly to the tissue culture laboratory where subjected to the running water for 15 minutes to get rid of dirts and germs followed by immersing in soap solution for 5 minutes. Finally the leaves immersed in 10% chlorox solution (0.5 NaOCl) commercial bleach with two drops of Tween-20 for 10 minutes followed by washing with sterilized distilled water 3 times each for 5 minutes.

I. Protoplast sources and plasmolysis:

Factorial experiment was conducted between protoplast source (*in vitro* and *in vivo*) and plasmolysis treatments of both rootstocks. This experiment was carried out mainly on certain fruit trees types for encouraging cell plasmolysis before protoplast isolation. CPW-salts (Frearson *et al.*, 1973) were used as initial plasmolysis medium with the following additives for one hour:

1- Control: 0.0 mannitol + 0.0 sucrose.
2- 9.0 g/100 ml mannitol + 0.0 sucrose.
3- 13.0 g/100 ml mannitol +0.0 sucrose.
4- 0.0 mannitcl + 21.0 g/100 ml sucrose.
5- 9.0 g/100 ml mannitol for half hour then 13.0 g/100 ml mannitol for half hour more.

Mannitol and sucrose were dissolved in CPW salts. The medium was adjusted at pH 5-8.

II. Protoplast isolation :

II.a. Protoplast source and enzyme mixture:

Two sources of leaf mesophyll protoplasts (*in vitro* and *in vivo*) combined with different enzyme mixtures were evaluated to find out the most effective enzyme mixture was able to induce the highest protoplast yield.

The tested enzyme mixtures were as follow :

1- EM_1 : (1.5% cellulase + 0.5% pectianase + 1.5% Macrozyme)
2- EM_2: (1.0% cellulase + 0.5% pectianase + 1.0% macerozyme).
3- EM_3: (1.0% cellulase + 1.0% pectianase + 1.0% macerozyme)
4- EM_4: (1.0% cellulase + 1.0% macerozyme)
5- EM_5: (2.0% cellulase + 1.5% macerozyme+ 0.5% pectinase)
6- EM_6: (1.0% cellulase + 1.0% pectinase)
7- EM_7: (1% cellulase + 0.5% macerzyme + 0.2% pectianase)

II.b. Digestive enzymes medium.

Murashige and Skoog (MS, 1962), Kao and Michayluk, (KM, 1975) and CPW (Frearson *et al.*, 1973) media were tested to select the most suitable one which has the ability to disolve and promote the enzyme mixture activity to the optimum level to digest cell wall of leaf tissues of both pear rootstocks.

II.c. Effect of osmotic pressure factors:

Different carbon sources (Mannitol, sucrose and glucose) were added to CPW digestive enzyme medium (which had already selected during the aforementioned step) as osmotic pressure factors at the rate of 13, 21 and 7.9 g/100 ml, respectively to detect the optimum osmotic pressure factor succeeded in optimizing the osmatic pressure inside and outside (medium osmotic pressure) protoplast. This step lead to produce rounded protoplast without occurrence protoplast plasmolysis or rupture.

II.d. Effect of incubation period:

In vitro leaf ships of both pear rootstocks were immersed in the suitable enzyme mixture and overnight incubated for different period i.e. 12, 16, 20 and 24 hours to testify the best incubation period induced the highest protoplast yield.

II.e. Effect of shaking period and speed:

After stationary overnight incubation, the Petri dishes containing digestive enzyme medium and leaf strips, were shaked on a rotary shaker. Different combinations between shaking speed (0.0, 50, 75 and 100 rpm) and time (0.0, 15, 30, 45 and 60 min) were employed in order to get the best shaking speed combined with convenient shaking time for, release more viable protoplasts.

III. Purification:

After shaking the digested cell mixed with enzyme medium, the incubated mixture passed through a 25 μ nylon sieve, the digested tissue pieces washed with CPW 13M medium and spin at different speed (500, 1000 and 1500 rpm) combined with different times (5, 7.5 and 10 min), followed by three washes in CPW 13M.

IV. Protoplast culture:

The isolated protoplast of either *Pyrus communis* or *Pyrus betulaefolia* were cultured on different liquid medium types i.e. Murashige and Skoog (MS, 1962), Kao and Michayluk (KM, 1975) and Gamborge *et al.* (B5, 1968) at different densities (0.5, 1.0, 1.5 and $2.0x10^4$/ml) to select the best culture medium and density which gave the highest protoplast development.

V. Effect of hormonal balance:

Hormonal balance in culture medium between NAA (0, 1, 2 and 3 mg/L) and BAP (0.0, 0.1, 0.2 and 0.3 mg/L) was investigated. The isolated protoplasts of *(Pyrus communis* and *Pyrus betulaefolia*) were cultured at density $2.0x10^4$/ml on KM and MS medium, respectively, supplemented with the different combinations of NAA and BAP.

Statistical analysis:

All data were subjected to analysis of variance and significant difference among means were determined according to Snedecor

and Cochran (1972). In addition significant difference among means were distinguished according to the Duncans, multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

I. Protoplast source and plasmolysis:

Data presented in Table (1) and illustrated in Fig. (1) show the effect of protoplast source and plasmolysis treatments on protoplast yield. It is appear that *in vitro* leaf mesophyll protoplasts were more superior in protoplast yield than those of *in vivo* source. Meanwhile, soaking either *in vitro* or *in vivo* leaf pieces in plasmolysis solution (5) (gradual plasmolysis containing 9% mannitol for half hour followed by 13% mannitol for further 30 min) increased protoplast yield. However, the lowest value of protoplast yield was detected with mannitol or sucrose free medium (control).

Conncerning the interaction, between leaf mesophyll source and plasmolysis treatments it is quite evident that *in vitro* leaf mesophyll combined with treatment 5 (plasmolysis for 30 min in CPW 9M + 30 min in CPW 13M) maximized the protoplast yield (Photo 1), followed by *in vivo* leaf explant combined with the same plasmolysis treatment.

These results assured the findings of Power *et al.* (1990) they reported that isolated leaf mesophyll protoplast of apple which were already plasmolyzed for 30 min in CPW medium with 0.5 M mannitol followed by 30 min in CPW medium with 0.7 M mannitol increased yield to 4.5 x 10^6 protoplasts /g f.w. with 60% viability. Moreover, Ochatt (1993) reported that best protoplast yield and viability of *Pyrus spp.* (pear) was achieved by plasmolized tissue for at least 1h in the same solution as used for isolation but devoid of enzymes.

II. Protoplast isolation:

II.a. Effect of enzyme mixture:

Data tabulated in Table (2) reflect the effect of enzyme mixture on protoplast yield derived from *in vitro* and *in vivo* leaf mesophyll of both pear species. It is quite clear that *in vitro* leaves were better than *in vivo* leaves when protoplast yield was concerned for the two studied species. Anyhow, enzyme mixture EM1 (1.5% cellulase + 0.5% pectianase + 1.5% Macrozyme) maximized yield of leaf mesophyll protoplast (*in vitro* and *in vivo*) of the two pear species.

It could be concluded that *in vitro* leaves of both species combined with the enzyme mixture EM1 maximized the protoplast yield, followed by *in vivo* leaves combined with the same enzyme mixtures.

These results are generally in agreement with the findings of Ochatt and Caso (1986), who stated that protoplast yield of *in vitro* leaf mesophyll of wild pear was higher compared with those derived from field leaf mesophyll plants. Moreover, Ping *et al.* (2005) found that the best digestive enzyme mixture for protoplast isolation of *Vitis davidii* was 2% cellulase, 0.5% pectinase and 1% macerating enzymes.

II.b. Effect of digestive enzyme medium:

Data presented in Table (3) revealed that CPW medium surpassed the other two investigated media, whereas it gave the highest values of protoplast yield for both rootstocks.

This result is in agreement with the finding of Mehri (2003) who reported that digestive enzyme mixture of prunus cerasus was more effective when dissolved in CPW 13M medium.

II.c. Effect of osmotic pressure factors:

Data of Table (4) show the effect of different carbon sources as osmotic pressure factors on protoplast yield. It is obvious that adding mannitol to the culture medium produced the highest yield of protoplast as compared with the other two osmotic pressure factors. While the lowest number of protoplast was obtained with glucose for two pear species.

Table (1): Effect of and protoplast source and plasmolysis on protoplast yield (x10^5/g f.w.) of *Pyrus betulaefolia* and *P. communis.*

Treatments	*Pyrus betulaefolia*			*Pyrus communis*		
	Leaf mesophyl protoplast		Mean	Leaf mesophyl protoplast		Mean
	In vitro	*In vivo*		*In vitro*	*In vivo*	
1- Control: 0.0 mannitol + 0.0 sucrose	0.20±0.10	0.13±0.06	0.17±0.08	0.27±0.06	0.13±0.06	**0.20±0.09**
2- 9.0 g/100 ml mannitol + 0.0 sucrose	0.70±0.10	0.50±0.10	0.60±0.14	0.80±0.10	0.53±0.15	**0.67±0.19**
3- 13.0 g/100 ml mannitol + 0.0 sucrose	1.27±0.06	0.80±0.10	1.03±0.27	1.30±0.10	0.87±0.06	**1.08±0.33**
4- 0.0 mannitol + 21.0 g/100 ml sucrose	0.53±0.06	0.40±0.10	0.47±0.10	0.47±0.12	0.37±0.06	**0.42±0.10**
5- 9.0 g/100 ml mannitol for 30 min then 13.0 g/100 ml mannitol for 30 min then more	2.07±0.15	1.53±0.12	1.80±0.32	2.17±0.15	1.60±0.10	**1.88±0.33**
Mean	0.95±0.68	0.67±0.50		0.95±0.68	0.67±0.50	
LSD for protoplast at 0.05		0.07			0.08	
LSD for plasmolysis at 0.05		0.12			0.12	
LSD for interaction at 0.05		0.16			0.17	

Table (2): Effect of enzyme mixtures on protoplast yield (x10^5/g f.w.) of *Pyrus betulaefolia* and *P. communis.*

Enzyme mixture	*Pyrus betulaefolia*			*Pyrus communis*		
	Leaf mesophyl protoplast		Mean	Leaf mesophyl protoplast		Mean
	In vitro	*In vivo*		*In vitro*	*In vivo*	
EM1	2.20±0.20	1.57±0.15	1.88±0.38	2.17±0.15	1.63±0.15	**1.90±0.32**
EM2	1.40±0.20	1.03±0.06	1.22±0.24	1.50±0.10	1.00±0.10	**1.25±0.29**
EM3	1.50±0.26	0.97±0.12	1.23±0.34	1.53±0.15	1.03±0.15	**1.28±0.31**
EM4	0.47±0.06	0.53±0.40	0.50±0.26	0.50±0.10	0.27±0.06	**0.38±0.15**
EM5	0.83±0.06	0.60±0.10	0.72±0.15	0.90±0.10	0.57±0.15	**0.73±0.22**
EM6	0.17±0.12	0.13±0.06	0.15±0.08	0.23±0.06	0.10±0.10	**0.17±0.10**
EM7	0.30±0.10	0.13±0.06	0.22±0.12	0.33±0.06	0.20±0.00	**0.27±0.08**
Mean	0.98±0.72	0.71±0.51		1.02±0.70	0.69±0.54	
LSD for protoplast at 0.05		0.11			0.07	
LSD for plasmolysis at 0.05		0.2			0.13	
LSD for interaction at 0.05		0.28			0.19	

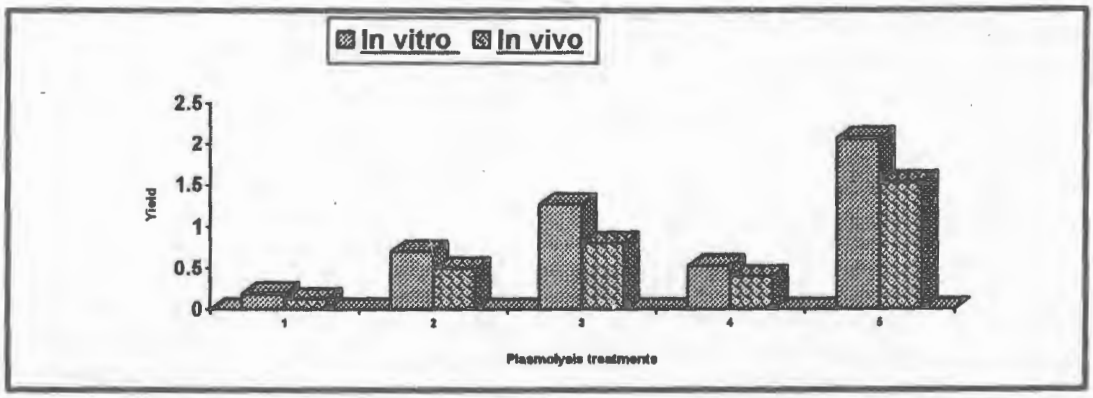

Figure (1): Effect of plasmolysis (pretreatment) and explant sources on protoplast yield (x10^5/g f.w.) of *Pyrus betulaefolia.*

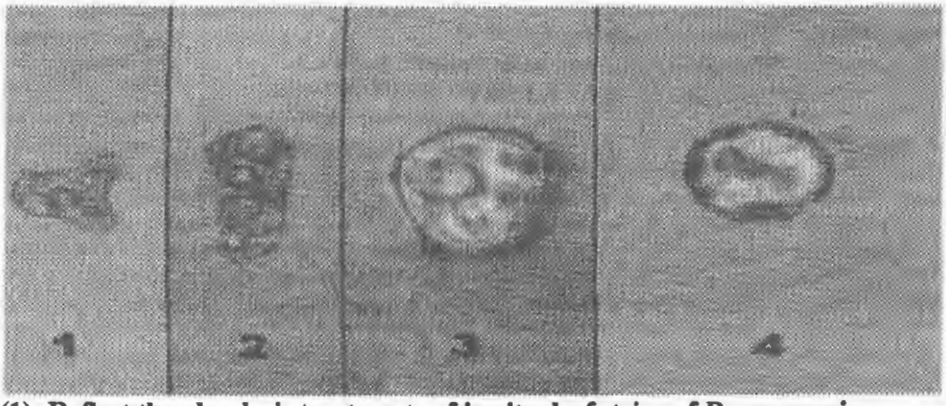

Photo (1): Reflect the plasolysis treatments of *in vitro* leaf strips of *P. communis*.
(1): 21% sucrose, (2): 9% mannitol, (3) 13% mannitol,
(4) 9% mannitol for 30 min then 13% mannitol for further 30 min.

Table (3): Effect of digestive enzyme medium on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyll of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Digestive media	*Pyrus betulaefolia*	*Pyrus communis*
KAO	0.43±0.06	0.53±0.06
CPW	2.03±0.15	2.13±0.15
MS	0.40±0.20	0.37±0.15
LSD at 0.05	0.28	0.24

These results go in line with the findings of Segui *et al.* (2006) who found that the best apple (*Malus domestica* var. *fuji*) protoplast viability was achieved when used 0.8 M mannitol as osmaticum.

II.d. Effect of incubation period:

The results of Table (5) reveal that overnight incubation of *in vitro* leaf strips of both pear species for 20 h in the enzyme mixture, improved protoplast yield and quality as compared with the other incubation periods. Meanwhile, incubation for 16 h took the second rank in this respect. Moreover, the incubation for 12 h took the other way around. In this concern, high yield of grape protoplast was obtained when leaf strips was incubated in enzyme solution in the dark under 28°C for 18 h Marino (1990).

II.e. Effect of shaking speed and time:

Dealing with the effect of shaking speed and time on protoplast yield data presented in Table (6) reflect that the best shaking speed that maximized protoplast yield with less damage was 75 rpm, while the shaking for 30 min was superior for releasing high number of protoplast for the two pear species. It could be concluded that shaking *in vitro* leaf strips of both pear species, immersed in digestive enzyme medium, at 75 rpm for 30 min gave the highest yield of protoplast. Meanwhile, shaking at 50 rpm for 15 min came in the second rank. These results go in line with the findings of Li *et al.* (1995) who obtained protoplast of peanut when a rotatory shaker at 85 rpm was used.

III. Purification:

III.a. Effect of sieve pore size:

Data of Table (7) reflect the effect of sieve pore size on number of viable protoplast. It is appear that decreasing number of viable protoplast was obtained when sieve pore size was increased. Meanwhile, the highest of number of viable protoplast was induced by using pore size of 25 µ followed by 50 µ and finally the lowest protoplast yield was induced with 75 µ sieve pore size.

The aforementioned results concluded that *in vitro* leaf explant combined with sieve pore size 25 µ increased protoplast numberyild. 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 potoplast yield and

viability. These results are somewhat in accordance with the findings of Vardi and Esra (1989), who isolated protoplasts of citrus were filtered through 50 µm and 30 µm nylon screens.

Table (4): Effect carbon source as an osmotic pressure factor on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Treatment	*Pyrus betulaefolia*	*Pyrus communis*
Glucose	0.10±0.00	0.07±0.06
Mannitol	2.03±0.25	2.10±0.10
Sucrose	0.57±0.06	0.70±0.10
LSD at 0.05	0.28	0.17

Table (5): Effect of incubation period on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Treatment	*Pyrus betulaefolia*	*Pyrus communis*
12	0.13±0.06	0.10±0.10
16	1.17±0.12	1.30±0.10
20	2.03±0.21	2.07±0.06
24	0.90±0.20	0.87±0.06
LSD at 0.05	0.28	0.15

III.b. Effect of centrifuge speed and period:

Data presented in Table (8) show that spin at 1000 rpm was more effective in increasing the number of protoplast followed by 500 rpm for both pear rootstocks. Meanwhile, increasing centrifugation period up to 7.5 minutes had a positive effect on yield compared with 5 or 10 minutes.

The interaction between centrifugation speed and period revealed that speed at 1000 rpm for 7.5 min was preferable to produce high protoplast yield of both species (Photo 2).

These results are in agreement with the findings of Qinghua *et al.* (2006), they found that protoplast of citrus Unshiu were further purified by centrifugation in 25% sucrose/13% mannitol gradient for 6 min at 880 rpm.

IV. Protoplast culture:

IV.a. Effect of medium type:

Table (9) reflect the effect of different culture media on protoplast development. It obvious clear that MS medium surpassed the other two investigated media, whereas it enhanced and developed protoplast division of *P. betulaefolia*. Meanwhile, KM medium came in the second rank in this respect. The protoplast behaviour of *P. communis* took the other way around regardling the culture medium, whereas, KM medium was superior in this respect followed by MS medium. However B5 (Gamborge) medium showed the worest effect on protoplast development for both species.

These results are in agreement with the findings of Saito and Suzuki (1999) they reported that the best results of protoplast division of apple (*Malus domestica* cv. *fuji*) were appeared when cultured on MS medium supplemented with (2 mg 2,4-D and 1mg benzyladenine BA/liter) and 0.8% agar as well as subcultured in a liquid medium.

IV.b. Effect of protoplast density (x10^5/ml):

Fig. (2) shows the effect of cultured protoplast density on protoplast development. It is clear that increasing cultured protoplast density from $0.5x10^5$ to $2.0x10^5$/ml resulted in enhancing protoplast development for both pear species. The best protoplast density was able to maximize division and reduce browning was $2.0x10^5$ protoplast per ml. Meanwhile protoplast density at $2.5x10^5$ had an adverse effect on protoplast development of both species.

Table (6): Effect of shaking speed and period on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Shaker period min (A) / Shaker speed rpm (B)	*Pyrus betulaefolia*				
	15	30	45	60	Mean
0 (control)	0.13±0.03	0.13±0.12	0.13±0.10	0.16±0.06	**0.14±0.08**
50	1.07±0.17	0.80±0.10	0.73±0.14	0.21±0.03	**0.70±0.11**
75	1.13±0.16	2.16±0.20	0.98±0.16	0.40±0.08	**1.17±0.5**
100	0.90±0.12	1.43±0.18	0.53±0.10	0.16±0.03	**0.76±0.11**
Mean	0.81±	1.13±0.15	0.59±0.13	0.23±0.05	
LSD for A at 0.05			**0.30**		
LSD for B at 0.05			**0.18**		
LSD for AxB at 0.05			**0.25**		
	Pyrus communis				
0 (control)	0.16±0.03	0.13±0.04	0.16±0.08	0.13±0.03	**0.15±0.05**
50	0.98±0.16	0.80±0.08	0.67±0.12	0.40±0.03	**0.71±0.10**
75	0.11±0.06	2.13±0.17	0.80±0.16	0.40±0.06	**0.86±0.11**
100	0.86±0.12	1.40±0.12	0.50±0.10	0.16±0.14	**0.73±0.12**
Mean	0.53±0.10	1.11±0.10	0.53±0.12	1.09±0.07	
LSD for A at 0.05			**0.26**		
LSD for B at 0.05			**0.18**		
LSD for AxB at 0.05			**0.025**		

Table (7): Effect of sieve pore size on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Sieve pore size (µm)	*Pyrus betulaefolia*	*Pyrus communis*
25	**2.10±0.10**	**2.23±0.06**
50	**1.53±0.25**	**1.60±0.20**
75	**0.33±0.06**	**0.40±0.10**
LSD at 0.05	**0.30**	**0.25**

Table (8): Effect of centrifuge spead and period on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Centrifuge period min (A) / Centrifuge speed rpm (B)	*Pyrus betulaefolia*			
	5	7.5	10	Mean
500	1.30±0.06	1.60±0.10	1.50±0.12	**1.47±0.09**
1000	2.20±0.06	2.25±0.12	2.10±0.15	**2.18±0.11**
1500	1.10±0.10	1.20±0.08	1.10±0.15	**1.13±0.11**
Mean	1.53±0.07	1.68±0.10	1.57±14	
LSD for A at 0.05		**0.14**		
LSD for B at 0.05		**0.10**		
LSD for AxB at 0.05		**0.20**		
	Pyrus communis			
500	1.10±0.12	1.30±0.08	1.20±0.10	**1.20±0.10**
1000	2.20±0.15	2.10±0.12	2.00±0.18	**2.10±0.15**
1500	0.90±0.15	1.05±0.10	1.10±0.16	**1.02±0.14**
Mean	1.40±0.14	1.48±0.10	1.43±0.15	
LSD for A at 0.05		**0.13**		
LSD for B at 0.05		**0.08**		
LSD for AxB at 0.05		**0.22**		

These results go in line with the findings of Ochatt (1993) who found that the best result of cell division was achieved when protoplast density ranged between 0.5-2.5 x 10^5 protoplasts per ml of pear (*Pyrus spp*).

IV.c. Effect of hormonal balance:

In vitro leaf mesophyll protoplast development (division and micro colonies formation) of both pear rootstocks in response to medium hormonal balance (NAA and BAP) was investigated. Data presented in Table (10) reflect that 3.0 mg/L NAA or 0.2 mg/L BAP was more preferable than the other tested concentrations of either NAA or BAP in enhancing protoplast development of *P. betulaefolia*. Moreover, the combination between 1.0 mg/L NAA+0.3 mg/L BAP was superior in this respect (Photo 3).

Concerning the effect of hormonal balance on protoplast development of *P. communis*, data presented in Table (10), reveal that 1.0 mg/L NAA or 0.2 mg/L BAP surpassed the other tested levels, whereas each level gave the highest value of protoplast development. Meanwhile, the combination between 1.0 mg/L NAA with either 0.2 or 0.3 mg/L BAP maximized the protoplast division.

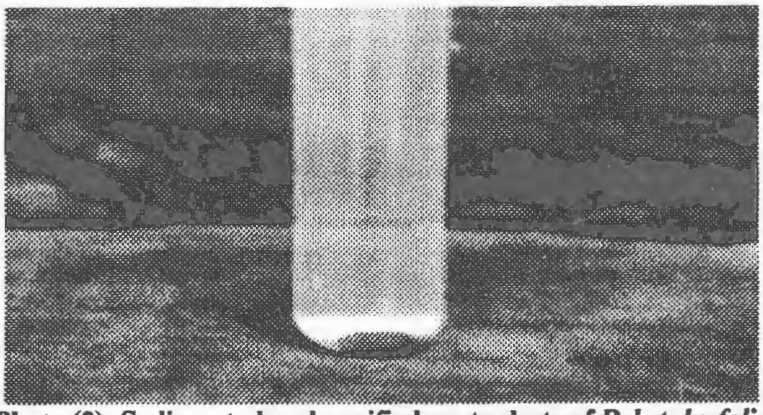

Photo (2): Sedimented and purified protoplasts of *P. betulaefolia*

Table (9): Effect of different culture medium on protoplast yield (x10^5/g f.w.) of *in vitro* leaf mesophyll of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.)

Centrifugation period (min)	*Pyrus betulaefolia*	*Pyrus communis*
B_5	1.00±0.00	1.00±0.00
KM	1.67±0.58	3.33±0.58
MS	3.33±0.58	1.67±0.58
LSD at 0.01	1.29	1.29

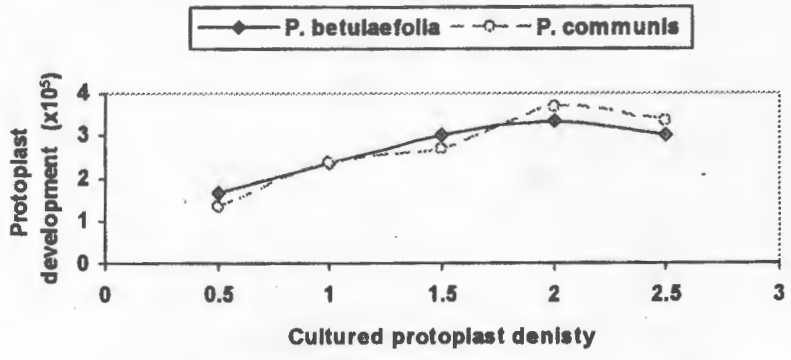

Fig. (2): Effect of culture protoplast density on protoplast yield (x10^5/ml) of *in vitro* leaf mesophyl of *Pyrus betulaefolia* and *Pyrus communis* (mean±S.D.).

Table (10): Effect of auxin and cytokinin concentrations on protoplast development (x10^5/g f.w.) of *pyrus betulaefolia* and *P. communis*.

Auxin \ Cytokinin		*Pyrus betulaefolia*				
		0.0	0.1	0.2	0.3	Mean
NAA (mg/L)	0	1.00±0.00	1.00±0.00	1.67±0.58	1.33±0.58	**1.25±0.45**
	1	1.33±0.58	2.33±0.58	3.33±1.15	3.67±0.58	**2.67±1.15**
	2	2.67±1.15	2.33±0.58	3.00±1.00	2.67±0.58	**2.67±0.78**
	3	2.33±0.58	3.00±1.00	3.00±0.00	2.67±0.58	**2.75±0.62**
Mean		1.83±0.94	2.17±0.94	2.75±0.97	2.58±1.00	
LSD for BAP at 0.05 and 0.01			0.57		0.76	
LSD for BAP at 0.05 and 0.01			0.57		0.76	
LSD for interaction at 0.05 and 0.01			1.14		1.52	
		Pyrus communis				
NAA (mg/L)	0	1.00±0.00	1.00±0.00	1.33±0.58	1.33±0.58	**1.17±0.39**
	1	1.33±0.58	2.33±0.58	3.33±0.58	3.33±0.58	**2.58±1.00**
	2	2.33±0.33	2.00±1.00	2.67±0.58	2.33±1.15	**2.33±0.78**
	3	2.00±1.00	2.67±0.58	2.67±0.58	2.33±0.58	**2.42±0.67**
Mean		1.67±0.78	2.00±0.85	2.50±0.90	2.33±0.98	
LSD for BAP at 0.05 and 0.01			0.54		0.73	
LSD for BAP at 0.05 and 0.01			0.54		0.73	
LSD for interaction at 0.05 and 0.01			1.09		1.45	

These results go in line with the findings of Matsuta *et al.* (1986) who showed that protoplasts of *Prunus persica* were successfully cultured on NN medium supplemented with 2 mg/liter NAA and 0.2 mg/liter BAP at 28°C in the dark. Moreover, Mehri (2003) found that leaf mesophyll protoplasts of *Prunus cerasus L.* cv. *Montmorency* were successfully cultured on KM (Kao and Mychayluck, 1975) basal medium supplemented with 1 mg/liter NAA. A successfull culture of leaf callus protoplast of the same kind was achivied when MS basal medium supplemented with 2 mg/liter NAA, 0.25 mg/liter BAP and 0.1 mg/liter Zeatine was used.

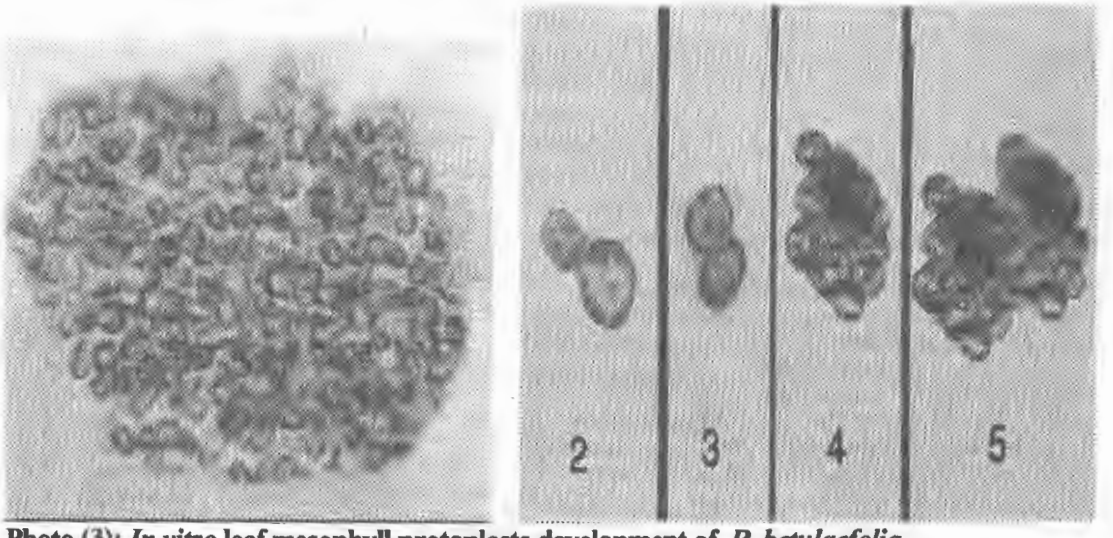

Photo (3): *In vitro* leaf mesophyll protoplasts development of *P. betulaefolia*.

1- Freshly isolated protoplast. 2- Budding stage.
3- Central division. 4- Microconlony formation.
5- Microcalli formation

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عزل وزراعة البروتوبلاست من أصلى الكمثرى البيتشفوليا والكميونس

نبوى على حجاجى ، فؤاد محمد عبداللطيف ، شريف فتحى الجيوشى
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أجريت هذه الدراسة بوحدة زراعة الأنسجة – قسم البساتين – كلية الزراعة – جامعة بنها خلال الفترة من ٢٠٠٤ إلى ٢٠٠٧ على أصلى الكمثرى البيتشفوليا والكميونس بهدف وضع بروتوكول لعزل وزراعة وانقسام البروتوبلاست من أوراق كل منهما للحصول على مستعمرات صغيرة من نسيج الكلس – حيث يمكن فى المستقبل دمج بروتوبلاست كلا النوعين معا لانتاج هجين يحمل صفات كلا النوعين وخاصة المقاومة لفحة النارية. أخذت الأوراق لكلا النوعين من نباتات نامية داخل الأنابيب أو من الحقل – حيث عقمت الأخيرة باستخدام كلوركس ١٠% مع إضافة نقطتين من المادة التاسرة Tween 20 ولمدة ١٥ ق. ثم استخدام بعض مضادات الأكسدة (حمض الستريك، حمض الأسكوربيك والبولى فينيل بروليدن) لتقليل تركيزها ببيئات العزل والتنقية وبالتالى تأثيرها الضار على البروتبلاست.

تم تقطيع العينات الورقية إلى أجزاء صغيرة (١-٢مم) لعمل بلزمة للخلايا. ثم نقل الأجزاء النباتية إلى مخلوط الأنزيمات الذائبة فى عدة بيئات هاضمة (CPW, KM, MS) كما تم دراسة تأثير إضافة بعض السكريات (المانيتول ١٣%، السكروز ٢%، الجلوكوز ٧,٩%) للبيئات الهاضمة للحفاظ على الضغط الأسموزى للبروتوبلاست وذلك بإضافة السكريات السابقة كل على حدة لكل بيئة هاضمة لمعرفة نوع السكر القادر على عمل توازن بين الضغط الأسموزى للبيئة والبروتوبلاست للحصول على أعلى انتاجية ثم حفظت مخاليط الأنزيمات مع الأجزاء النباتية فى الظلام لفترات مختلفة بإجراء هز الأطباق على سرعات مختلفة (صفر، ٥٠، ٧٥، ١٠٠ لفة/ق) لفترات زمنية مختلفة (صفر، ١٥، ٣٠، ٤٥، ٦٠ ق) لمعرفة أفضل فترة زمنية للتحضين والهز، وكذلك أفضل سرعة للهز قادرة على إعطاء أكبر كم من البروتوبلاست. ثم أجريت عملية التنقية باستخدام الطرد المركزى للبروتوبلاست مع مخلوط الأنزيمات بسرعات مختلفة (١٠٠،٥٠٠، ١٥٠٠ لفة/ق) لفترات مختلفة (٥، ٧,٥، ١٠ ق) لعزل البروتوبلاست بصورة نقية. والذى تم زراعته فى ثلاث أنواع من البيئات (B_5, MK, MS) والمحتوية على عدة مستويات من BAP, NAA بكثافات تتراوح بين ٠,٥-٢,٥×١٠^5/مل لدراسة انقسام وتطور خلايا البروتوبلاست. هم النتائج المتحصل عليها:

١- إضافة محلول مضادات الأكسدة (٠,١٥% حمض ستريك + ٠,١% حمض أسكوربيك) للأوراق الحقلية لكلا النوعين أدى إلى تقليل نسبة المواد الفينولية أثناء عمليتى العزل والتنقية.
٢- استخدام البلزمة التدريجية ٩% مانيتول لمدة ٣٠ ق ثم ١٣% مانيتول لمدة ٣٠ ق أخرى. أدى إلى إعطاء محصول جيد للبروتوبلاست.
٣- محصول البروتبلاست الناتج من أوراق لنباتات نامية فى أنابيب أظهر تفوقا ملحوظا عن تلك الناتج من أوراق حقلية لكلا النوعين.
٤- استخدام CPW كبيئة هاضمة ومحتوية على مخلوط الأنزيمات ١,٥% سليوليز +٠,٥% بكتينيز + ١,٥% ماسيروزايم تفوقت على البيئات الأخرى وكذلك على مخاليط الأنزيمات الأخرى – حيث أعطت أعلى محصول من البروتوبلاست الناتج من أوراق لنباتات نامية فى أنابيب لكلا النوعين.
٥- إضافة سكر المانيتول بتركيز ١٣% إلى بيئة الأنزيمات الهاضمة أدى إلى إحداث أفضل توازن للضغط الأسموزى داخل البروتوبلاست.
٦- تحضين الأجزاء الورقية فى مخلوط الأنزيمات فى الظلام ولمدة ٢٠ ق متبوعا باستخدام الهزاز بسرعة ٧٥ لفة/ق ولمدة ٣٠ ق الى زيادة ملحوظة فى كمية ونوعية البروتوبلاست.
٧- استخدام المنخل الشبكى بقطر ٢٥ ميكرون تفوق على الأنواع الأخرى بزيادة تنقية البروتوبلاست واستبعاد الأجزاء النباتية الغير مهضومة والمتهتكة.
٨- استخدام الطرد المركزى لمعلق البروتوبلاست مع بعض البقايا النباتية فى بيئة مخلوط الإنزيمات بسرعة ١٠٠٠ لفة/ق ولمدة ٧,٥ ق أدى الى ترسيب البروتوبلاست مع الإنتاجية العالية بكلى النوعين.

زراعة البروتوبلاست المستخلص من أوراق كمثرى البيتشفوليا على بيئة MS والمحتوية على ١ ملجم/لتر نفثالين حمض الخليك NAA + ٠,٣ ملجم/لتر BAP بنزيل أمينوبيورين وكذلك البروتوبلاست المستخلص من أوراق كمثرى الكمينوس على بيئة KM والمضاف لها ١ ملجم/لتر نفثالين حمض الخليك + ٠,٢ أو ٠,٣ ملجم/لتر بنزيل أمينوبيورين بكثافة ٢×١٠^5/مل لكل منهما – أدى إلى زيادة انقسام البروتبلاست وتكوين مستعمرات صغيرة.