RESULTS AND DISCUSSION

IV. RESULTS AND DISCUSSION

4.1. Pyrus betulaefolia:

4.1.1. Pre-protoplast isolation :

4.1.1.a.Evaluation of phenolic compounds level :

Data presented in Table (2) showed a continuous increase in total, free, and conjugated phenolic compounds determined during March, June, September, and December periods respectively .Thus, the lowest level of phenolic compounds appeared at March sample which indicated that the best time for taken the explants for protoplast isolation. Also, these data reflect the optimum anti-oxidant treatment needed to reduce or eliminate the phenolic compounds exist during taking the explants.

Table (2): Evaluation of Phenol compound levels during different periods in *in vivo Pyrus betulaefolia* explants

Pyrus betulaefolia	Periods											
Month	March			June			September			December		
Phenol %	total	free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated
Total	0.126	0.0219	0.105	0.171	0.039	0.132	0.260	0.045	0.215	0.315	0.048	0.267
Mean	0.042	0.0073	0.035	0.057	0.013	0.044	0.0866	0.015	0.071	0.105	0.016	0.089

4.1.1.b. Anti-oxidant treatment:

Table (3) and Fig. (1) deal with the effect of different anti-oxidant treatments on the accumulation levels of phenolic compounds. It is obvious that combination of 0.1% ascorbic acid and 0.15%citric acid treatment (anti-oxidant solution) resulted in significant reduction of phenolic compounds as compared with the other treatments. Meanwhile, combination between anti-oxidant solution and P.V.P. took the second rank in decreasing the phenolic compounds followed by P.V.P. treatment. However, the reverse was true when combination treatment of ascorbic acid, citric acid and P.V.P was used .

The above mentioned results reflected the importance of using anti-oxidant treatment 0.1% ascorbic acid + 0.15% citric acid. to reduce phenol concentration in *in vivo* explants.

These results go in line with the findings of **Murashige (1974)** who indicated that either soaking the explants in ascorbic acid and citric acid or adding them to the culture medium succeeded in reducing the harmful effect of the phenolic compounds.also, with findings of **Zaied (1997)** who recommended anti-oxidant solution (100 mg/L citric acid and 150 mg/L ascorbic acid) as a pre-treatment for reducing free phenolic compounds in stone fruit explants.

4.1.1.c. Protoplast source and plasmolysis:

Data of Table (4) and Fig. (2) show the effect of protoplast source and plasmolysis treatments on protoplast yield. It is appear that *in vitro* protoplast source was more superior in protoplast yield than *in vivo* source . Meanwhile, soaking either *in vitro* or *in vivo* source explants in plasmolysis solution (5)

Table (3): Effect of anti-oxidant treatments on accumulation of phenolic compounds released from leaves *in vivo Pyrus betulaefolia*

Anti-oxidants treatments	Phenol concentration (%)		
	Total	Free	Conjugated
1. Control.	0.086	0.015	0.071
2. 0.1% Ascorbic acid.	0.075	0.011	0.064
3. 0.15% citric acid.	0.077	0.012	0.065
4. 0.5% P.V.P.	0.049	0.008	0.041
5. 0.1% ascorbic acid + 0.15% citric acid.	0.025	0.003	0.022
6. 0.1% ascorbic acid + 0.5% P.V.P.	0.079	0.013	0.066
7. 0.15% citric acid +0.5% P.V.P.	0.078	0.012	0.066
8. 0.1% ascorbic acid + 0.15% citric acid + 0.5% P.V.P.	0.042	0.006	0.036
Mean	0.0638	0.01	0.0539
LSD total	0.003		
LSD free	0.0018		
LSD Conjugated	0.003		

Figure (1) :Effect of anti-oxidants pretreatments on phenol compounds level of *in vivo* Pyrus betulaefolia explants.

Table (4): Effect of protoplast source and plasmolysis on number of plasmolized cells on *Pyrus betulaefolia.*

Treatment	Protoplast source		Mean
	In vitro (10^4)	*In vivo* (10^4)	
1- Control: 0 mannitol + 0 sucrose	0.20 ±0.10	0.13 ±0.06	0.17 ±0.08
2- 9 g/100 ml mannitol + 0 sucrose	0.70 ±0.10	0.50 ±0.10	0.60 ±0.14
3-13g/100ml mannitol +0 sucrose	1.27 ±0.06	0.80 ±0.10	1.03 ±0.27
4-0 mannitol + 21 g/100ml sucrose	0.53 ±0.06	0.40 ±0.10	0.47 ±0.10
5-9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol	2.07 ±0.15	1.53 ±0.12	1.80 ±0.32
Mean	0.95 ±0.68	0.67 ±0.50	
LSD for protoplast at 0.05	0.07		
LSD for protoplast at 0.01	0.10		
LSD for plasmolysis at 0.05	0.12		
LSD for plasmolysis at 0.01	0.16		
LSD for interaction at 0.05	0.16		
LSD for interaction at 0.01	0.22		

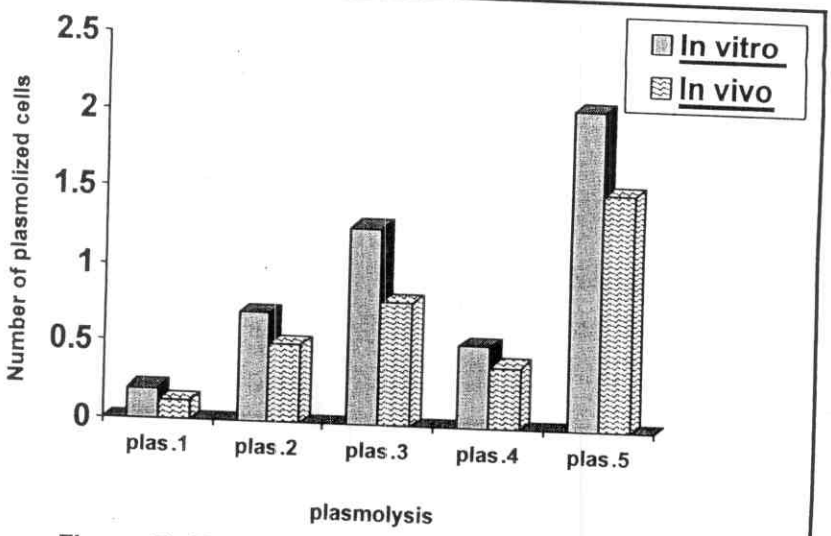

Figure (2): Effect of protoplast source and plasmolysis on protoplast yield of *Pyrus betulaefolia*

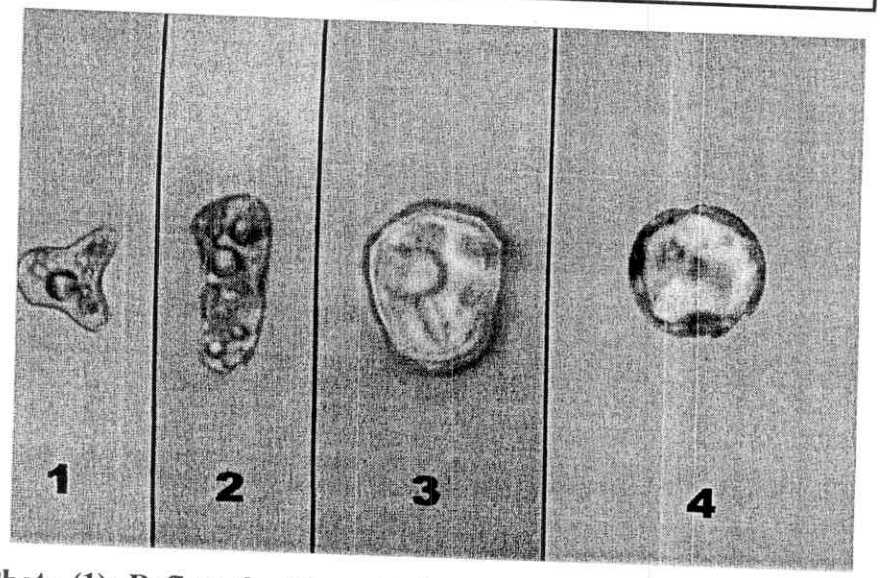

Photo (1): Reflect the Plasmolysis treatments in *Pyrus betulaefolia* which include (1) 0.0 manitol + 21g/100ml sucrose, (2) 9g/100 ml mannitol + 0.0 sucrose, (3) 13g100ml mannitol + 0.0 sucrose, (4) 9g/100ml mannitol for further 30 min. 13g/100ml mannitol

containing 9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol increased the number of plasmolized cells. followed by plasmolysis solution (3) containing 13g/100ml mannitol +0 sucrose. However, the lowest number of plasmolized cells was produced from plasmolysis (1) (Control: 0 mannitol + 0sucrose).

Concerning the interaction, between protoplasts source and Plasmolysis treatments it is quite evident that *in vitro* explant combined with the plasmolysis 5 (Plasmolysis for 30min in CPW 9M + 30 min in CPW 13M) maximized the number of plasmolized cells, followed by *in vivo* explant combined with the same plasmolysis However, the combination of *in vivo* explant with plasmolysis (4) and plasmolysis (1) induced the lowest values.

Generally, the above results conclude that *in vitro* explant is the best protoplast source for protoplast yield. Also plasmolysis (5), gave the highest protoplast numbers. These results assured the findings of **Ochatt (1993a)** who 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.

4.1.2. Protoplast isolation :

4.1.2.a. Effect of protoplast source and enzyme mixture:

Data of Table (5) and Fig. (3) reflect the effect of protoplast source and enzyme mixture on protoplast yield. It is clear that . using *in vitro* protoplast source succeeded in increasing the protoplast yield compared with *in vivo* protoplast source. Meanwhile, enzyme mixture (EM1) (1.5% cellulase + 0.5% pectianase + 1.5% Macrozyme) maximized protoplast

Table (5): Effect of enzyme mixture and explants source on protoplast yield *Pyrus betulaefolia.*

Enzyme mixture	Protoplast yield		Mean
	In vitro (10^5)	*In vivo* (10^5)	
EM1	2.20 ±0.20	1.57 ±0.15	1.88 ±0.38
EM2	1.40 ±0.20	1.03 ±0.06	1.22 ±0.24
EM3	1.50 ±0.26	0.97 ±0.12	1.23 ±0.34
EM4	0.47 ±0.06	0.53 ±0.40	0.50 ±0.26
EM5	0.83 ±0.06	0.60 ±0.10	0.72 ±0.15
EM6	0.17 ±0.12	0.13 ±0.06	0.15 ±0.08
EM7	0.30 ±0.10	0.13 ±0.06	0.22 ±0.12
Mean	0.98 ±0.72	0.71 ±0.51	
LSD for protoplast at 0.05	0.11		
LSD for protoplast at 0.01	0.14		
LSD for enzyme mixture at 0.05	0.2		
LSD for enzyme mixture at 0.01	0.26		
LSD for interaction at 0.05	0.28		
LSD for interaction at 0.01	0.37		

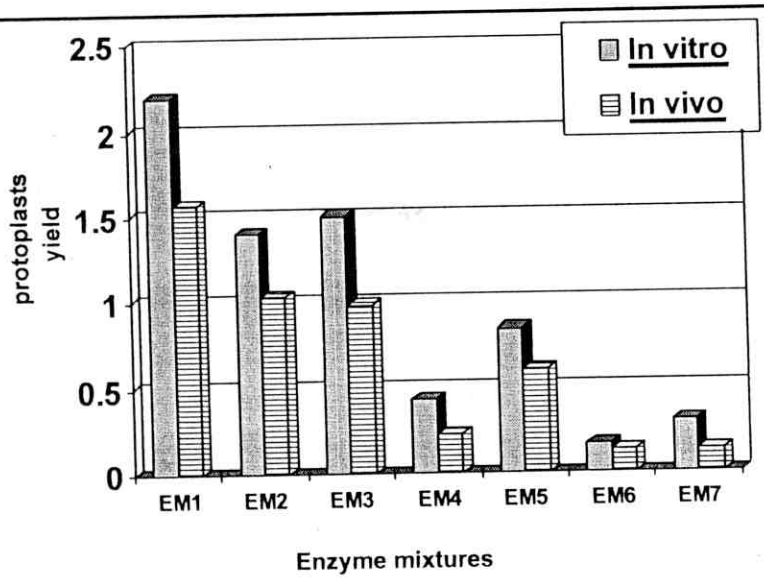

Figure (3): Effect of different protoplast sources and enzyme mixtures on protoplast yield of *Pyrus betulaefolia*

yield in relation to other enzyme mixtures. However, EM3 (1% cellulase + 1% pectianase + 1% macerozyme) occupied the second rank in enhancing protoplast yield then followed by EM2 (1% cellulase + 0.5% pectianase + 1% macerozyme) but EM6 (1% cellulase + 1% pectinase) was the least protoplast yield.

On the other hand, protoplast yield was increased when combination between *in vitro* source and enzyme mixture (EM1) treatment was used as compared with the other combination treatment *in vivo* source and the same enzyme.

Generally, the above results summarize that *in vitro* explant is the best protoplast source for protoplast yield. Also, EM1 gave the highest protoplast numbers. These results are 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. Moreover, **Ping *et al.* (2005)** they found that the best digestive enzyme solution for protoplast isolation of ***vitis davidii*** was obtained when combined of 2% cellulase, 0.5% pectinase and 1% macerating enzyme.

4.1.2.b. Effect of digestive enzyme medium:

Data of Table (6) revealed the effect of digestive enzyme medium on protoplast yield. It is noticed that CPW medium was superior in increasing the protoplast yield compared with other media used. However, the lowest result was obtained when using Murashig and Skoog medium.

Table (6): Effect of digestive enzyme medium on protoplast yield of *In vitro Pyrus betulaefolia* (mean±S.D.)

Digestive media	Protoplast yield x (10^5)
KM	0.43±0.06
CPW	2.03±0.15
MS	0.40±0.20
LSD at 0.05	0.28
LSD at 0.01	0.41

Generally, the above results clarified that CPW medium gave the highest protoplast yield. These results are in general agreement with the findings of **Revilla *et al.*, (1987)** They found

that the best enzyme medium used for protoplast isolation from leaves of stone fruits (***Prunus spp.)*** was CPW 13M. medium supplemented with 1% PVP and 0.5 mM MES .Also, with the findings of **David Mills and Hammerschlag (1994)** Who mentioned that the best medium for enzyme mixture in peach (***Prunus persica***) was CPW salts medium to isolate protoplasts.

4.1.2.c. Effect of osmotic pressure factor:

Data of Table (7) show the effect of osmotic pressure factors on protoplast yield. It is obvious that adding of mannitol to the culture medium produced the highest viable protoplast yield as compared with the other osmotic pressure factors. While the lowest number of protoplast was obtained when glucose was used.

Table (7): Effect of osmotic pressure factors on protoplast yield *In vitro Pyrus betulaefolia.*

Treatment	Protoplast yield x (10^5)
Glucose	0.10±0.00
Mannitol	2.03±0.25
Sucrose	0.57±0.06
LSD at 0.05	0.28
LSD at 0.01	0.41

Generally, the aforementioned results summarized that adding mannitol as osmotic pressure factor to the medium encouraged production of the highest protoplast numbers. These

results go in line with the findings of **Saito and Suzuki (1999)** they reported that adding 0.7% mannitol to the incubation medium increased protoplast viability derived from cell suspension of apple cultivars (***Malus domestica*** cultivars fuji and Jonagold) and ***Malus prunifolia*** var ringo. Moreover. **Mehri (2003)** who reported that the best yield and viability of protoplast of ***Prunus cerasus L.*** which isolated from leaf mesophyll and leaf callus was achieved by using enzyme solution containing 13%mannitol and 5mM MES . and **Segui *et al*, (2006)** They found that the best viability of protoplast from apple ***(Malus domestica*** var. fuji) was achieved when used 0.8%M mannitol as osmaticum .

4.1.2.d. Effect of incubation period:

The results of Table (8) deal with the effect of incubation period on protoplast yield. It is noticed that using incubation period for 20 hours was effective in enhancing the protoplast yield comparison with the other incubation periods. Meanwhile, incubation for 16 h took the second rank in improving the protoplast yield followed with 24 h incubation period. However, the lowest protoplast yield was observed when incubated for 15 h.

The before mentioned results verified that incubating the explants in enzyme mixture for 20 hours improved protoplasts yield and quality. These results are somewhat in accordance with the findings of **Marino (1990)** who reported that the high yield of protoplast from grape (*Vitis vinefera* L.) was obtained when incubated in enzyme solution in the dark under 28°C for 18 h.

Table (8): Effect of incubation period on protoplast yield of *In vitro Pyrus betulaefolia.*

Incubation period (hours)	Protoplast yield x (10^5)
12	0.13±0.06
16	1.17±0.12
20	2.03±0.21
24	0.90±0.20
LSD at 0.05	0.28
LSD at 0.01	0.40

4.1.2.e. Effect of shaking:

4.1.2.e .1. Shaking speed:

Data of Table (9) clarified that the effect of shaking speed on protoplast yield. It is clear that the best shaking speed that maximized the number of protoplast with less damage was 75 rpm. However, increasing shaking speed up to 100 rpm reduced protoplasts yield as a result of increasing damage protoplasts but the shaking speed of 50 rpm took the second rank in improving protoplast yield.

Generally, the above results verified that using of 75 rpm shaking speed is the optimum as it encouraged the highest protoplast numbers. These results go in line with the findings of **Li *et al.* (1995)** who maintained that protoplast of peanut was obtained when using a rotatory shaker at 85 rpm and 26°C.

Table (9): Effect of shaking speed on protoplast yield *In vitro* *Pyrus betulaefolia.*

Shaking speed (rpm)	Protoplast yield x (10^5)
0	0.00±0.00
50	1.13±0.12
75	2.13±0.25
100	0.80±0.20
LSD at 0.05	0.31
LSD at 0.01	0.43

4.1.2.e. 2. Shaking period :

Data tabulated in Table (10) and Fig. (4) describe the effect of shaking period on protoplast yield. It is clear that maximum protoplast yield (2.07x 10^5) was induced when increase shaking period from 0.0 min. to 30 min. followed by using shaking period for 15 min. and 45 min respectively. However, the lowest protoplasts yield obtained when shaker was not used (control) this may be due to shaking encouraged enzyme mixture digestion of cell walls and free protoplasts appeared.

Table (10): Effect of shaking period on protoplast yield *In vitro Pyrus betulaefolia.*

Shaking period (minutes)	Protoplast yield x (10^5)
0	0.17±0.06
15	1.07±0.12
30	2.07±0.15
45	0.80±0.10
60	0.23±0.06
LSD at 0.05	0.18
LSD at 0.01	0.25

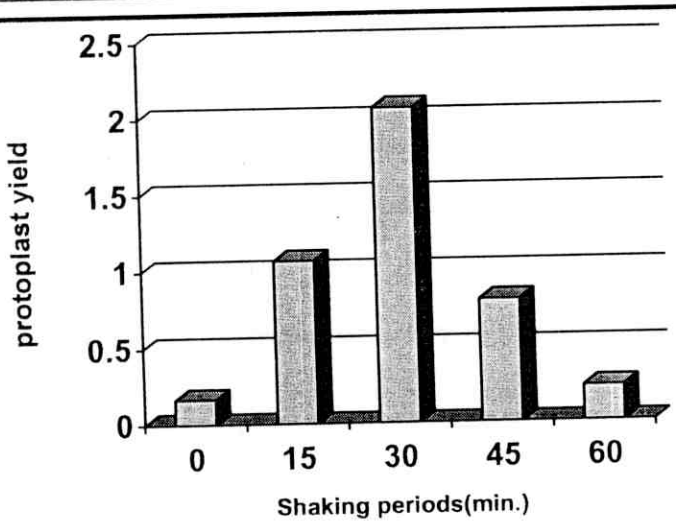

Figure (4): Effect of different Shaking periods on protoplast yield of *in vitro* pyrus betulaefolia

4.1.3. Purification:

4.1.3.a. Effect of sieve pore size:

Data of Table (11) reflect the effect of sieve pore size on number of viable protoplast. It is appear that decreasing number of viable protoplast was obtained when increase pore size. Meanwhile, the highest of number of viable protoplast was induced by using pore size of 25 µm followed by 50 µm and finally the lowest protoplasts induced when 75 µm pore size was used.

Table (11): Effect of sieve pore size on protoplast yield of *In vitro Pyrus betulaefolia.*

Sieve pore size (µm)	Protoplast yield x (10^5)
25	2.10±0.10
50	1.53±0.25
75	0.33±0.06
LSD at 0.05	0.30
LSD at 0.01	0.44

The aforementioned results recommended using sieve pore size 25 µm which induced the highest protoplast number. These results may be due to increasing sieve pore size encouraged higher numbers of protoplasts cell wall residues, clumps of undigested tissues and debris to pass through the filter and in turn affect badly on potoplast yield. These results are somewhat in accordance with the findings of **Vardi and Esra (1989)** They

found that the isolated protoplast of citrus was filtered through 50 µm and 30 µm nylon screens.

4.1.3.c. Effect of centrifugation:

4.1.3.c. 1.centrifugation speed:

Table (12) reveal the effect of centrifugation speed on number of viable protoplast. It is well known that centrifugation speed at 1000 rpm was more effective in increasing the number of protoplast followed by 500 rpm. Meanwhile, continuous increase in centrifugation speed up to 1500 rpm induced the lowest protoplast yield.

Table (12): Effect of centrifugation speed on protoplast yield *In vitro Pyrus betulaefolia.*

Centerfugation speed (rpm)	Protoplast yield x (10^5)
500	1.10±0.10
1000	2.13±0.12
1500	0.87±0.06
LSD at 0.05	0.18
LSD at 0.01	0.26

4.1.3.c. 2. Centrifugation period:

Data of Table (13) clarified that increasing of centrifugation period up to 7.5 minutes induced appositive effect on increasing number of viable protoplast. in comparison with

Table (13): Effect of centrifugation period on protoplast yield of *In vitro Pyrus betulaefolia.*

Centrifugation period (min)	Protoplast yield x (10^5)
5	1.23±0.06
7.5	1.97±0.06
10	1.10±0.10
LSD at 0.05	0.14
LSD at 0.01	0.20

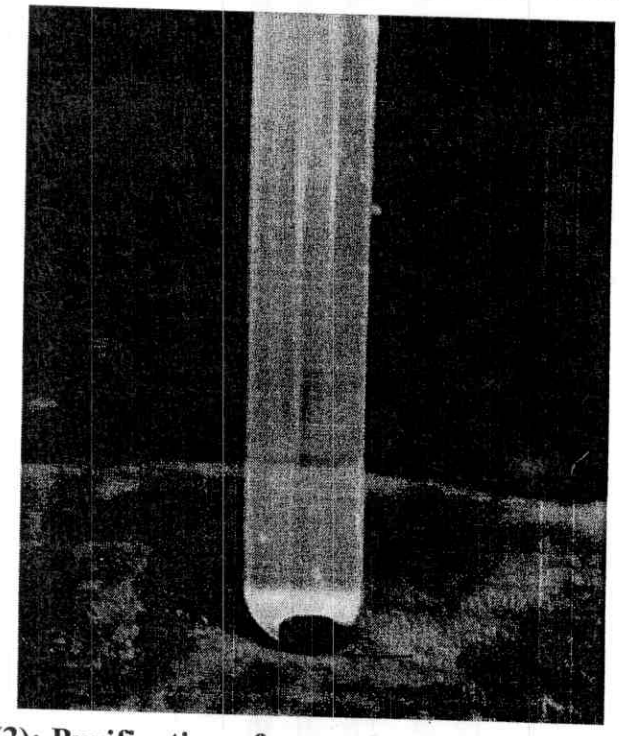

Photo (2): Purification of protoplast in *Pyrus betulaefolia*

the other periods under study. Meanwhile increasing centrifugation period from 7.5 to 10 minutes resulted in reducing protoplast viability. However, using of centrifugation period 5.0 minutes took the second rank in improving protoplast viability.

Generally it is appear that centrifuging of explants for 7.5 minutes maximized the protoplast yield these results go somewhat in the line with the findings of. **Qinghua Zhang *et al.* (2006)** They found that the protoplasts of *Citrus unshiu* in the filtrate were further purified by centrifugation in a 25% sucrose/13% mannitol gradient for 6 min at 88 *g* .

4.1.4. Protoplast culture:

4.1.4.a. Effect of medium type:

Table (14) reflects the effect of different medium types on protoplast development. It is appear that the superiority of Murashige & Skoog medium over both KM and Gamborge (B5) media. However B5 (Gamborige) medium showed the worst effect on protoplast development.

The above results indicate the suitability of Murashige and skoog medium for the best protoplast development. These results are in agreement with the findings of **Saito and Suzuki (1999)** They reported that best results of cell division from protoplasts of apple (*Malus X domestica*) cv. "fuji" were appeared when cultured on MS medium supplemented with (2mg 2,4-D and 1mg benzyladenine (BA/liter) and 0.8% agar and subcultured in a liquid medium.

Table (14): Effect of different medium types on protoplast development of *in vitro Pyrus betulaefolia.*

Medium type	Protoplast development (scores)
MS	3.33±0.58
KM	1.67±0.58
B5	1.00±0.00
LSD at 0.05	0.89
LSD at 0.01	1.29

4.1.4.b. Effect of protoplast density:

Table (15) Explains the effect of cultured protoplast density on protoplast development. It is clear that increasing cultured protoplast density from 0.5 x 10^5 to 2.0 x 10^5 resulted in enhancing in protoplast development.as (3.33) was obtained when cultured density was 2 x 10^5. Meanwhile, continuous increase of protoplast density up to 2.5x10^5 inducted an adverse effect on protoplast development.

The above results reflect the importance of using protoplast density 2.0x 10^5 in maximizing protoplast development. These results assured the findings of **Ochatt (1993a)** who Found that the best result of cell division was achieved when protoplast density was 0.5-2.5 x 10^5 protoplasts per ml of pear ***Pyrus spp*** .

Table (15):Effect of cultured protoplast density on protoplast yield *In vitro Pyrus betulaefolia.*

Cultured protoplast density ($x10^5$)	Protoplast development (scores)
0.5	1.67±0.58
1	2.33±0.58
1.5	3.00±0.00
2	3.33±1.15
2.5	3.00±0.10
LSD at 0.05	1.36
LSD at 0.01	1.88

4.1.4.c. Effect of hormonal balance :

Data tabulated in Table (16) and Fig. (5) reflect the effect of auxin and cytokinin concentrations on protoplast development. It is appear that supplementation the culture medium with 3.0 mg/L NAA was preferred as increased of protoplast development while increasing of NAA concentration enhanced an improvement in protoplast development. However, using free NAA medium gave the worst protoplast development. Moreover, 0.2 mg/L of BAP was recommended as it maximized protoplast development compared with the other BAP concentrations under study. However, the combination of 1.0 mg/L NAA and 0.3 mg/L BAP treatment succeeded in

Table (16): **Effect of NAA and BAP concentrations on protoplast development of *In vitro Pyrus betulaefolia.***

Cytokinin / Auxin		BAP mg/L				Mean
		0	0.1	0.2	0.3	
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 NAA at 0.05 and 0.01		0.57		0.76		
LSD for interaction at 0.05 and 0.01		1.14		1.52		

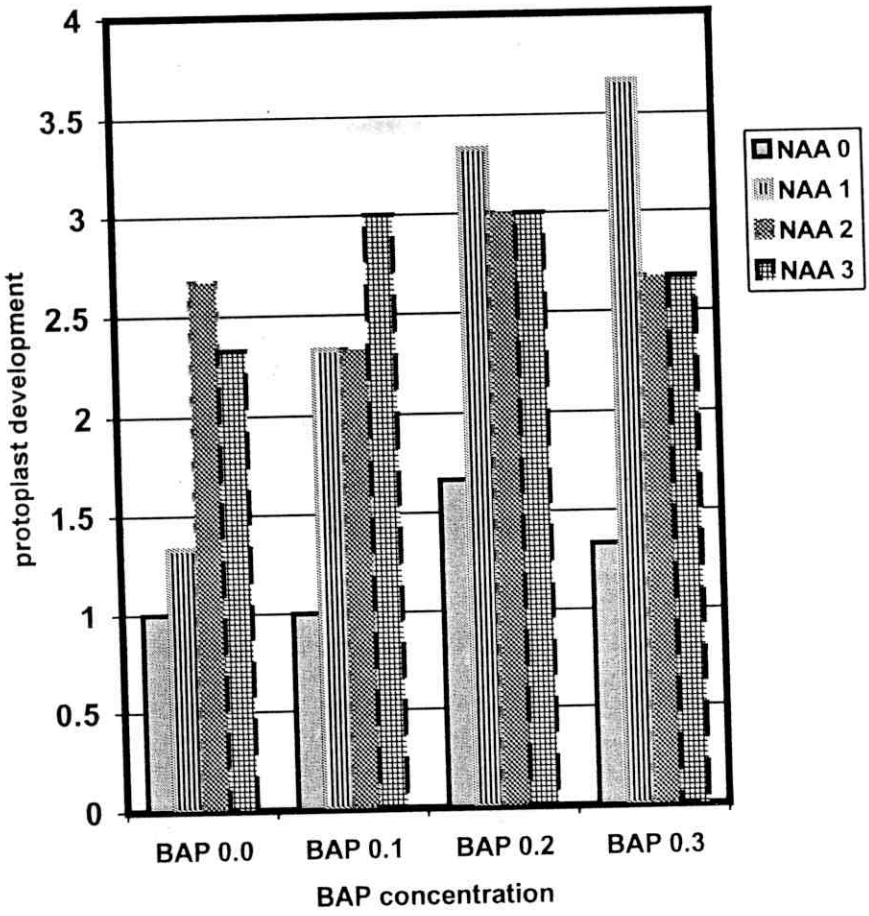

Figure (5):Effect of the hormonal balance on protoplast development of *In vitro* pyrus betulaefolia .

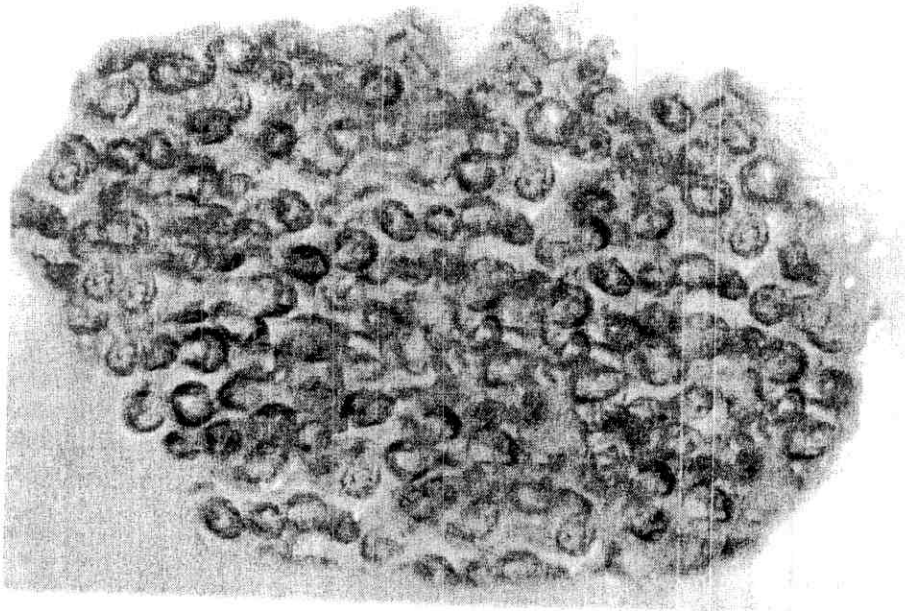

Photo (3): Protoplast yield in *Pyrus betulaefolia*

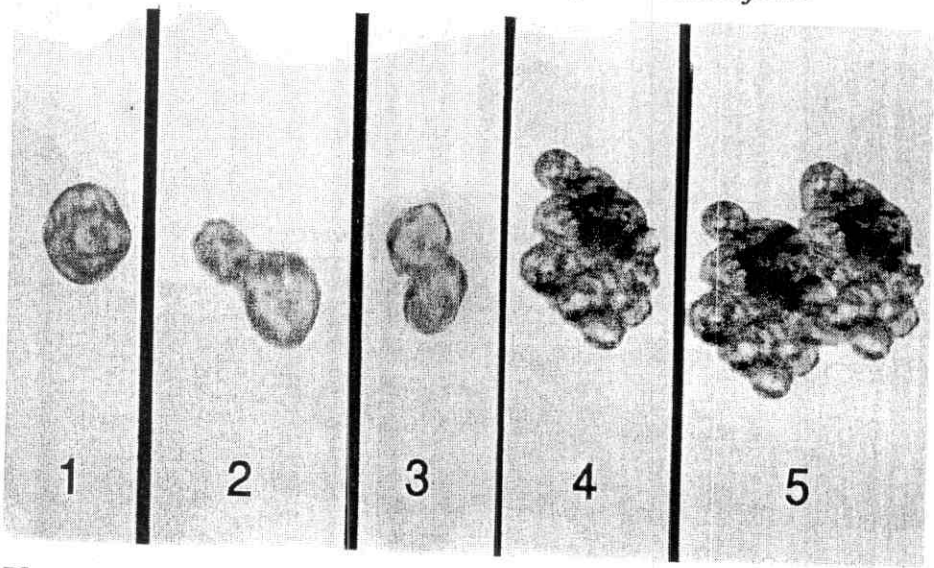

Photo (4): Reflect protoplast development in *Pyrus betulaefolia* which include (1) protoplasts, (2) buding stage, (3) protoplast division, (4) microcalli formation, (5) more microcalli formation.

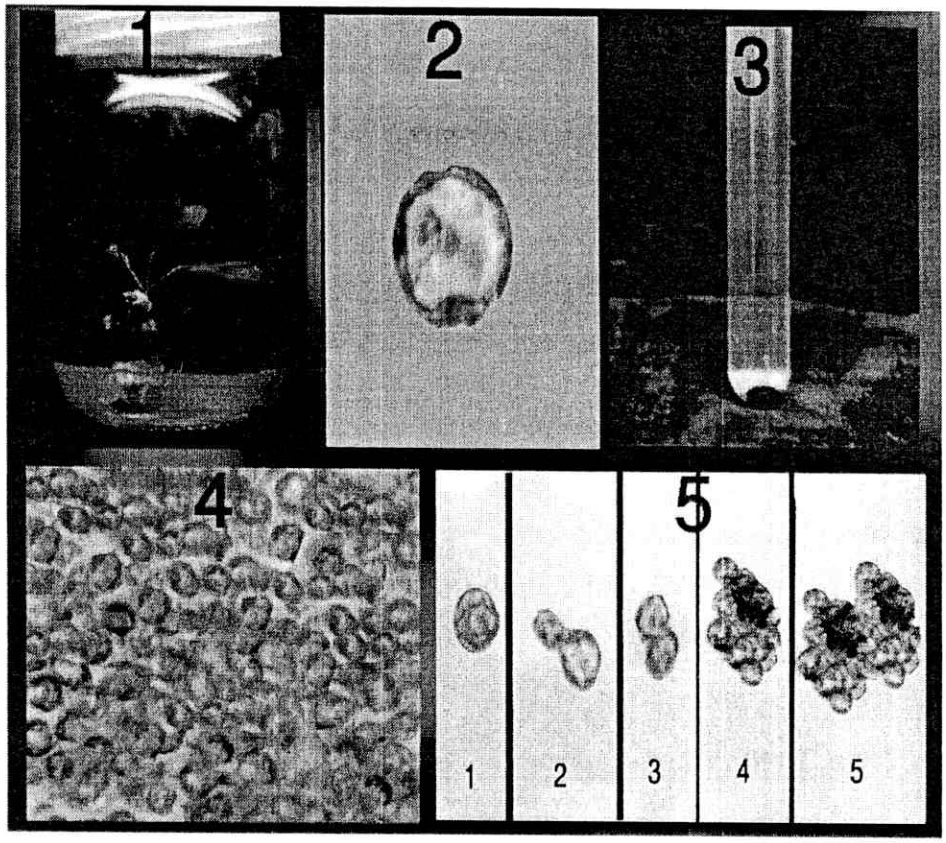

Photo (5): Reflect the protoplast technique in *Pyrus betulaefolia* which include preprotoplast isolation (1) protoplast source, (2)Plasmolyzed cells, (3) purification of protoplasts, (4) Protoplast yield, (5) protoplast development (1=protoplasts, 2=buding stage, 3= protoplast division, 4=microcalli formation, 5=more microcalli formation).

improving protoplast development compared with the other combinations followed by the same concentration of NAA and 0.2 mg/L BAP took the second rank in induction the best protoplast development. While, the culture medium free from hormones induced no effect on protoplast development.

In general, the above results summarized that using of 1.0 mg/L NAA and concentrations of BAP under study (0.2and 0.3 mg/L) was the most effective hormonal balance used as it suitable for maximized protoplast development and increased cell division. 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 2mg/liter NAA and 0.2 mg/liter BAP at 28°C in the dark .

4.2. Pyrus communis:

4.2.1. Pre- protoplast isolation:

4.2.1-a. Evaluation of phenolic compounds:

Table (17) clarifies that rate of accumulation of total, free, and conjugated phenolic compounds were increased from March to June and reached to the maximum level in September, then declined in December to the lowest level during year round .The most dangerous component of phenolic compounds is free phenolic which has causes an adverse effect on further growth and development .Thus, the most suitable time for taking the explants from communis pear is the period from December to March during which it contains the less phenolic compound contents.

Table (17): Evaluation of phenolic compounds levels during different periods of *in vivo* in *Pyrus communis*

Pyrus communis	Periods											
Month	March			June			September			December		
Phenol	*Total*	free	Conjugated	*Total*	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated
Total	0.165	0.0219	0.141	0.189	0.0318	0.159	0.225	0.051	0.147	0.170	0.0314	0.138
Mean	0.055	0.0073	0.047	0.063	0.0106	0.053	0.075	0.017	0.058	0.057	0.0104	0.046

4.2.1.b. Anti-oxidant treatment:

Data in Table (18) and Fig. (6) explan the effect of different anti-oxidant treatments on decreasing the phenolic compounds in the leaves of *pyrus communis*. It is clear that anti-oxidant solution consists of 0.1%ascorbic acid and 0.15%citric acid was effective in reducing phenolic compound as compared with the other treatments. Followed with P.V.P. treatment. However, the worst values of phenolic compounds were induced by using either ascorbic acid or 0.15% citric alone.

The above-mentioned results reflected the importance of using anti-oxidants per treatments (0.1% ascorbic acid + 0.15% citric acid). to reduce phenolic compounds in *in vivo pyrus communis* explants for using it in protoplast isolation.

These results go in line with the findings of **Siqueira *et al.*, (1991)** They declared that citric acid and ascorbic acid controlled 50% of browning of coconut explant, but polyvinyl-pyrrolidone was ineffective at the rate of 1 mg/L in controlling oxidation of the phenolic compounds.

Table (18): Effect of anti-oxidants pretreatments on phenolic compounds of *in vivo pyrus communis* explants

Anti-oxidants treatments	Phenol concentration (%)		
	Total	Free	Conjugated
1. Control.	0.057	0.0104	0.0466
2. 0.1% Ascorbic acid.	0.052	0.0098	0.0422
3. 0.15% citric acid.	0.050	0.0095	0.0405
4. 0.5% P.V.P.	0.034	0.0070	0.027
5. 0.1% ascorbic acid + 0.15% citric acid.	0.019	0.0030	0.016
6. 0.1% ascorbic acid+ 0.5% P.V.P.	0.043	0.0090	0.034
7. 0.15% citric acid + 0.5% P.V.P.	0.045	0.0080	0.037
8. 0.1% ascorbic acid + 0.15% citric acid + 0.5% P.V.P.	0.040	0.0075	0.032
Mean	0.340	0.00802	0.0345
LSD total	0.0174		
LSD free	0.00321		
LSD Conjugated	0.0142		

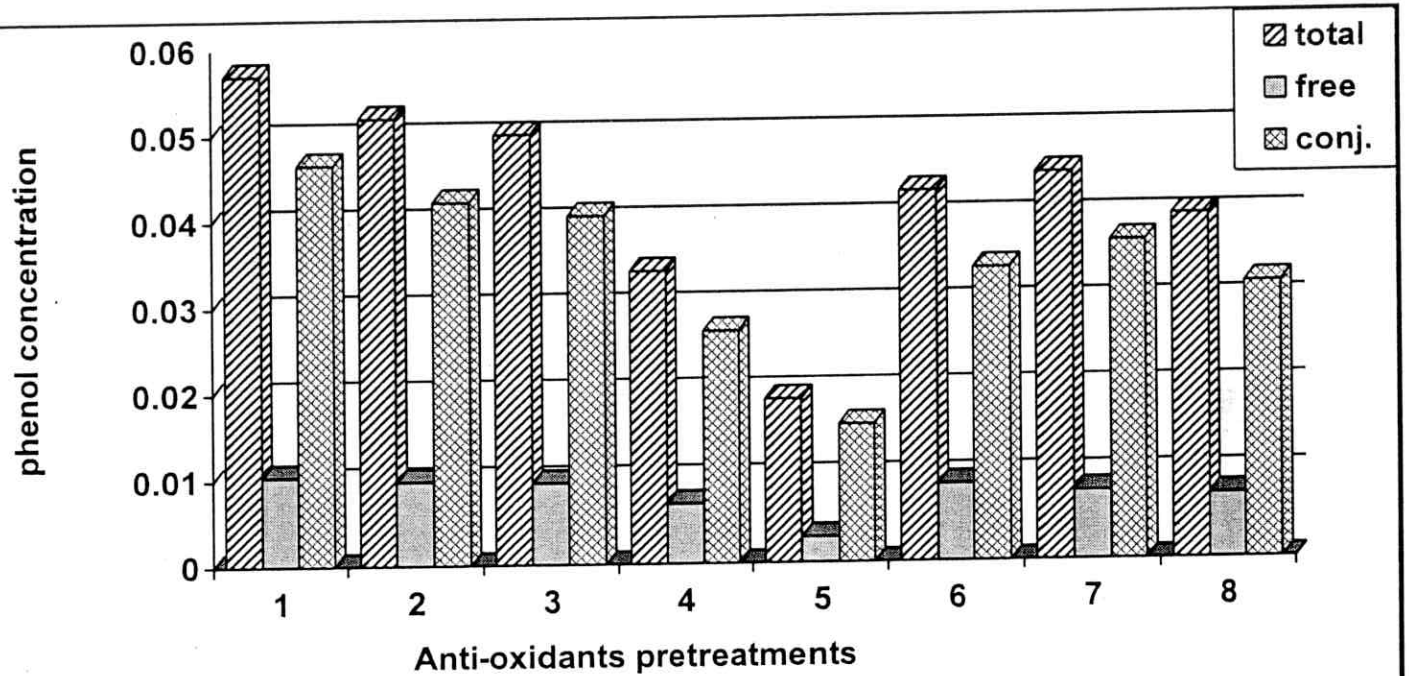

Figure (6) :Effect of anti-oxidants pretreatments on phenolic compounds in *in vivo* Pyrus communis explants.

4.2.1.c. Effect of protoplast source and plasmolysis treatment:

The results of Table (19) and Fig. (9) Reflect the effect of protoplast source and plasmolysis on protoplast yield. It is clear that *in vitro* protoplast source surpassed *in vivo* source in increasing the protoplast yield under study. Moreover, the plasmolysis treatment using 9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol maximized protoplast yield in relation to other plasmolysis treatment in both protoplast source (in vitro and in vivo). However, plasmolysis treatment (3) (13g/100ml mannitol +0.0 sucrose) took the second rank in improving protoplast isolation while plasmolysis treatment (1) (Control: 0 mannitol + 0sucrose) was the least in induction protoplast results.

Regarding the interaction between protoplast source and plasmolysis the results show that the combination between in vitro protoplast source treated with plasmolysis (5) enhanced the protoplast isolation then followed by *in vivo* source combined with the same plasmolysis pretreatment.

Generally, the above results conclude that *in vitro* explant is the best explant source for protoplast yield. Also, plasmolysis treatments gave the highest protoplast numbers. These results are in general agreement with the findings of **Power and Davey (1990)** They reported that isolation protoplast from mesophyll leaf apple **(Malus X domestica)** was plasmolyzed for 30 min in CPW medium with 0.5 M mannitol followed by 30 min in CPW medium with M mannitol (CPW 13 medium) plasmolyzed protoplast increase yield to 4.5 X 10^6 protoplasts g/fw. with 60% viability. Moreover **Ochatt (1994)** found that isolated protoplast

Table (19): Effect of plasmolysis treatments and protoplast source number of plasmolized cells of *in vitro* *Pyrus communis*.

Treatments	Protoplast source		Mean
	In vitro ($x10^4$)	*In vivo* ($x10^4$)	
1- Control: 0 mannitol + 0 sucrose	0.27 ±0.06	0.13 ±0.06	0.20 ±0.09
2- 9 g/100 ml mannitol + 0 sucrose	0.80 ±0.10	0.53 ±0.15	0.67 ±0.19
3-13g/100ml mannitol +0 sucrose	1.30 ±0.10	0.87 ±0.06	1.08 ±0.33
4-0 mannitol + 21 g/100ml sucrose	0.47 ±0.12	0.37 ±0.06	0.42 ±0.10
5-9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol	2.17 ±0.15	1.60 ±0.10	1.88 ±0.33
Mean	0.95 ±0.68	0.67 ±0.50	
LSD for protoplast at 0.05	0.08		
LSD for protoplast at 0.01	0.10		
LSD for plasmolysis at 0.05	0.12		
LSD for plasmolysis at 0.01	0.16		
LSD for interaction at 0.05	0.17		
LSD for interaction at 0.01	0.23		

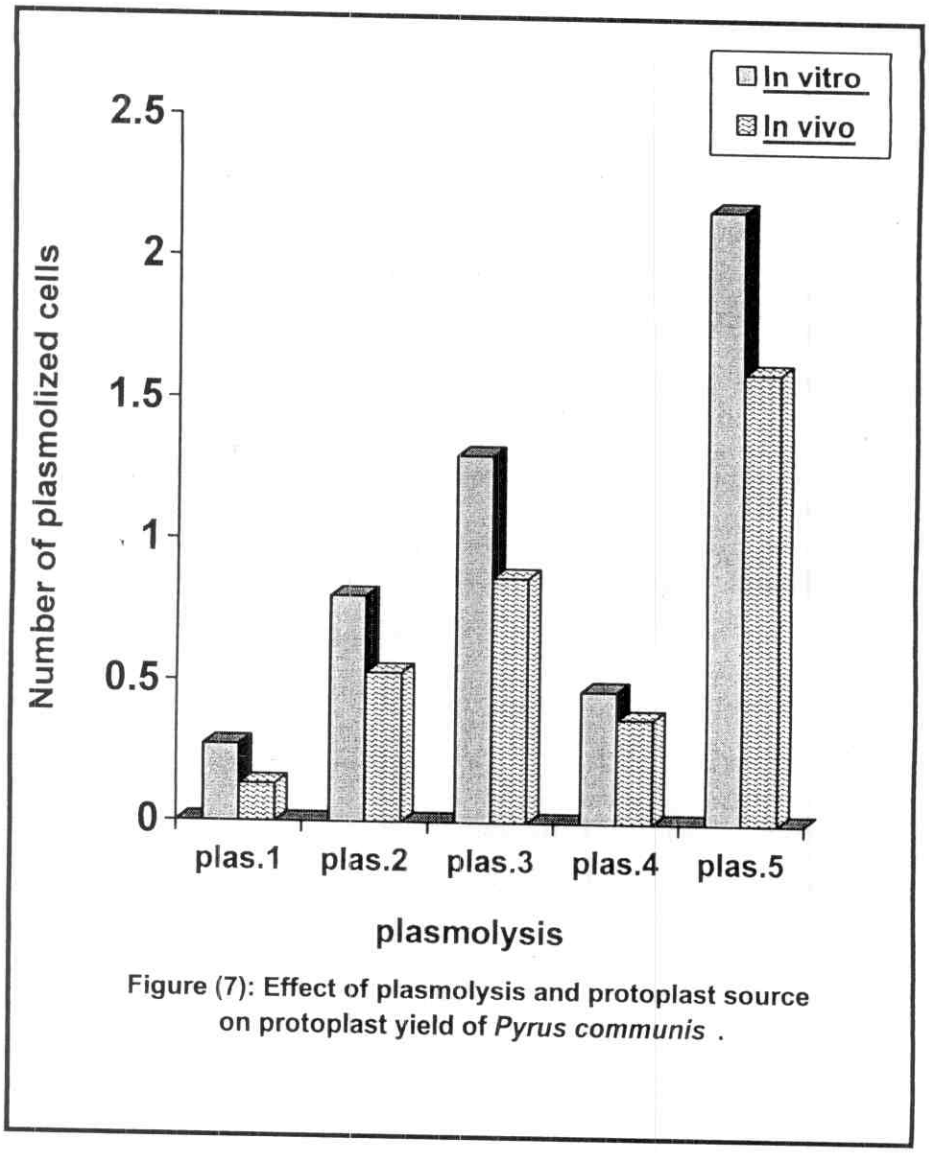

Figure (7): Effect of plasmolysis and protoplast source on protoplast yield of *Pyrus communis* .

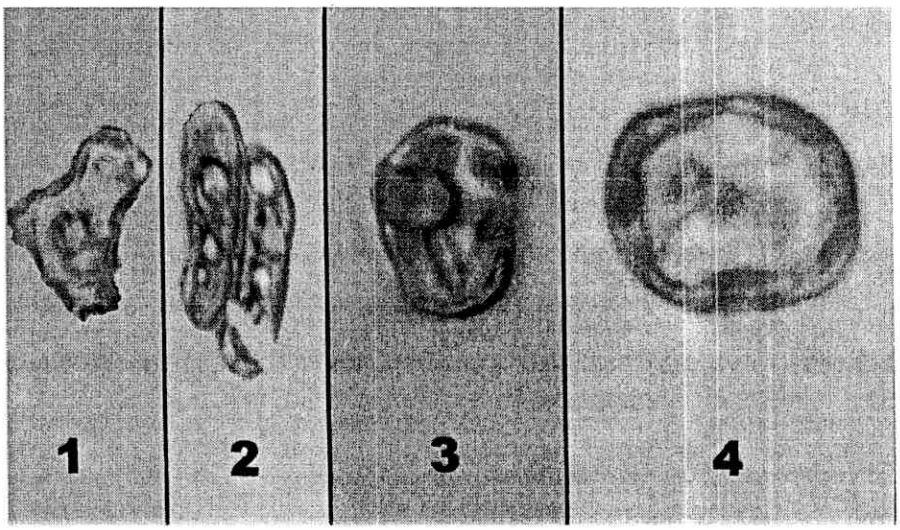

Photo (6): Reflect the Plasmolysis treatments in *Pyrus communis* which include (1) 0.0 manitol + 21g/100ml sucrose, (2) 9g/100 ml mannitol + 0.0 sucrose, (3) 13g100ml mannitol + 0.0 sucrose, (4) 9g/100ml mannitol for furthermore 30 min 13g/100ml mannitol

of mesophyll tissue from *in vivo* apple were rinsed in a solution of 6% mannitol while *in vitro* leaves was plasmolyzed in CPW medium with 0.5 M mannitol followed by 30 min in CPW medium 0.7 M mannitol.

4.2.2. Protoplast isolation:

4.2.2.a. Effect of protoplast source and enzyme mixture:

Data tabulated in Table (20) and Fig. (8) Verifies that the effect of protoplast source and enzyme mixture on protoplast yield. It is quite evident that protoplast yield was increased when *in vitro* protoplast source was involved compared with *in vivo* protoplast source. Meanwhile, EM1 (1.5% cellulase + 0.5%

Table (20): Effect of protoplast source and enzyme mixture on protoplast yield of *Pyrus communis.*

Treatments	Protoplast source		Mean
	In vitro ($x10^5$)	*In vivo* ($x10^5$)	
EM1	2.17±0.15	1.63±0.15	1.90±0.32
EM2	1.50±0.10	1.00±0.10	1.25±0.29
EM3	1.53±0.15	1.03±0.15	1.28±0.31
EM4	0.50±0.10	0.27±0.06	0.38±0.15
EM5	0.90±0.10	0.57±0.15	0.73±0.22
EM6	0.23±0.06	0.10±0.10	0.17±0.10
EM7	0.33±0.06	0.20±0.00	0.27±0.08
Mean	1.02±0.70	0.69±0.54	
LSD for protoplast at 0.05	0.07		
LSD for protoplast at 0.01	0.09		
LSD for enzyme mixture at 0.05	0.13		
LSD for enzyme mixture at 0.01	0.18		
LSD for interaction at 0.05	0.19		
LSD for interaction at 0.01	0.25		

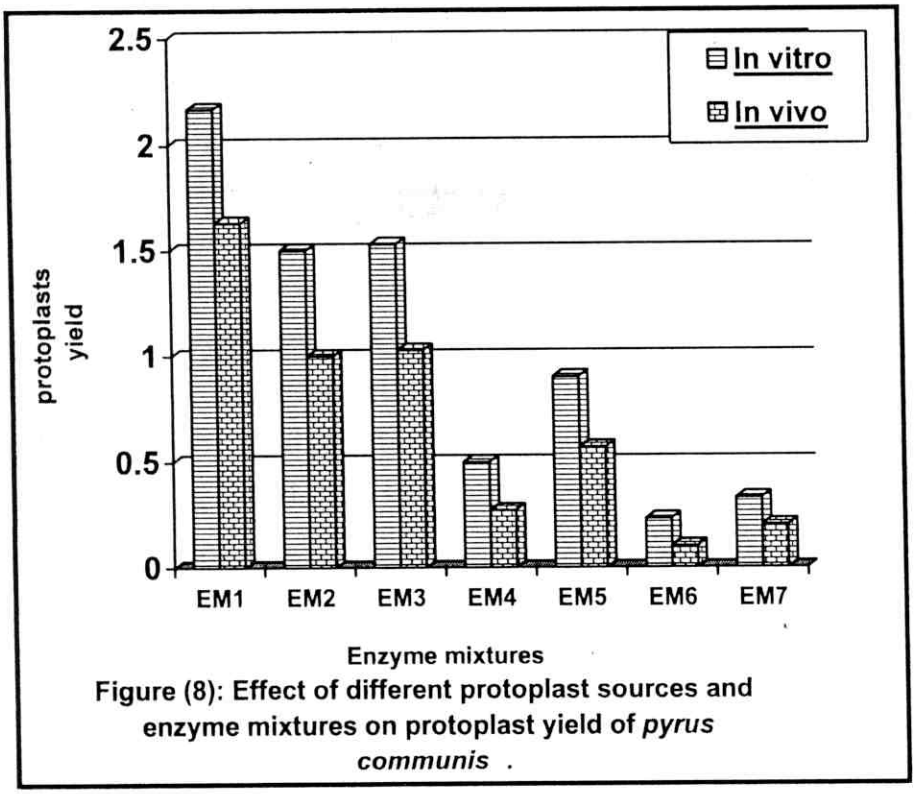

Figure (8): Effect of different protoplast sources and enzyme mixtures on protoplast yield of *pyrus communis* .

pectianase + 1.5% Macerozyme) was superior in enhancing the protoplast yield as compared with the other enzyme mixture. On the other hand EM3 (1% cellulase + 1% pectianase + 1% macerozyme) took the second rank in improving protoplast yield followed by EM2 (1% cellulase + 0.5% pectianase + 1% macerozyme) while the low protoplast number was obtained when EM6 (1% cellulase + 1% pectinase) was used.

Concerning the interaction, it is clear that *in vitro* explant combined with the enzyme mixtures (EM1) maximized the protoplast yield, followed by *in vivo* explant combined with the same enzyme mixture. However, the combination of *in vivo*

explant with EM_6 and EM_7 enzyme moisture induced the lowest values.

Generally, the above results indicate that *in vitro* explant is the best explant source for protoplast yield. Also, EM1 gave the highest protoplast numbers. These results are in general agreement with the findings of **Ochatt and Caso (1986).** They stated that yield of isolated protoplast from *in vitro* mesophyll of wild pear were higher compared with those from field mesophyll plants. Moreover, **Jihongliu *et al.*, (2003)** clarified that protoplast isolation from most woody plants are primarily required cellulase onozuka R-10, pectinase, Driselase, Macerozyme and Hemicellulase but protoplast isolation of most plants usually needs 1-2% cellulase and 0.1-1% pectolyase.

4.2.2.b. Effect of digestive enzyme medium :

Comparing of the effect of digestive enzyme medium on protoplast yield in Table (21) show that protoplast yield of pyrus communis increased when CPW medium was used as compared with the other studied media either Murashig & Skoog or KaO.

Generally, the above results conclude that CPW medium gave the highest protoplast numbers. These results are in general agreement with the findings of **Mehri (2003)** who found that CPW 13M was highly efficient medium for digestive enzyme mixture to isolate protoplast from ***prunus carasus*** L. cv" Montmorency"

Table (21): Effect of digestive enzyme medium on protoplast yield *In vitro Pyrus communis.*

Digestive media	Protoplast yield ($x10^5$)
KAO	0.53±0.06
CPW	2.13±0.15
MS	0.37±0.15
LSD at 0.05	0.24
LSD at 0.01	0.35

4.2.2.c. Effect of osmotic pressure factor:

Data Table (22) deals with the effect of osmotic pressure factors on protoplast yield. It is appear that the highest number of protoplast was obtained when medium supplemented with mannitol .Moreover, sucrose followed mannitol in positive effect on protoplast isolation.However, addition of Glucose to medium gave the lowest results.

Generally, the aforementioned results summarized that mannitol as osmotic pressure factor enhanced the highest protoplast numbers. These results go in line with the findings of **Ochatt *et al.*(1992)** They observed that large numbers of highly viable mesophyll protoplasts were isolated from shoot cultures of rootstock old home of common pear by using medium supplemented with 0.5 M mannitol. and **Kondakova (1999)**who showed that viability of protoplast from leaf mesophyll of

Prunus domestica cv. Quetche was observed with 0.7 M mannitol as osmaticum.

Table (22): Effect of osmotic pressure factors on protoplast yield *In vitro Pyrus communis.*

Treatment	Protoplast yield ($x10^5$)
Glucose	0.07±0.06
Mannitol	2.10±0.10
Sucrose	0.70±0.10
LSD at 0.05	0.17
LSD at 0.01	0.24

4.2.2.d. Effect of incubation period:

Table (23) explains the effect of incubation period on protoplast yield. It is clear that protoplast yield was increased by increasing incubation period up to 20 hours then start in decreased when incubation period increased to 24 hours. However, the least protoplast yield was showed when incubation period for 12 h.

Table (23): Effect of incubation period on protoplast yield *In vitro Pyrus communis.*

Incubation period (hours)	Protoplast yield ($x10^5$)
12	0.10±0.10
16	1.30±0.10
20	2.07±0.06
24	0.87±0.06
LSD at 0.05	0.15
LSD at 0.01	0.21

The beforementioned results verified that incubation enzyme mixture for 20 hours improved protoplasts yield and viability. These results are somewhat in accordance with the findings of **Patat- Ochatt *et al.* (1988)** They showed that apple (*Malus domestica* Borkh) was incubated in enzyme solution at 25°C in the dark for 18 hours. Also, with **Marino (1990)** who reported that high yield of protoplast from grape (*Vitis vinefera* L.) was obtained when incubated in enzyme solution in the dark under 28°C for 18 h.

4.2.2.e. Effect of shaking:

4.2.2.e.1. Shaking speed:

Table (24) shows the effect of shaking speed on protoplast yield. It is quite the highest protoplast yield was noticed when shaking speed reached to 75 rpm. However, 50 rpm of shaking speed took the second rank in increasing

protoplast yield and finally speed the rate of 100 rpm which produced the lowest protoplast yield. However, stationary incubation without shaking led to lowest their ability to protoplast isolation .

Table (24): Effect of shaking speed on protoplast yield *In vitro Pyrus communis.*

Shaking speed (rpm)	Protoplast yield ($x10^5$)
0	0.00±0.00
50	1.20±0.10
75	2.17±0.15
100	0.90±0.10
LSD at 0.05	0.19
LSD at 0.01	0.26

Generally, the above results conclude that shaking at 75 rpm gave the highest protoplast numbers. These results go in line with the findings of **Li *et al.* (1995)** who declared that protoplast of peanut was obtained when 85 rpm rotatory shaker and 26°C was used.

4.2.2. e.2. Shaking period:

Data tabulated in Table (25) and Fig (9) verifies the effect of shaking period on protoplast yield. It is obvious that increasing shaking period from 0 min to 30 min enhanced the increase in protoplast yield. However, increasing shaker period up to 45 min. reduced protoplast yield from (2.13 $x10^5$ to 0.77

x10^5).continuous increase of shaking period up to 60 min. induced a sharp decline in protoplast isolation in comparison with the other shaking period .

Table (25): Effect of shaking period on protoplast yield of *In vitro Pyrus communis.*

Shaking period (Minutes)	Protoplast yield (x10^5)
0	0.13±0.06
15	1.00±0.10
30	2.13±0.15
45	0.77±0.06
60	0.27±0.12
LSD at 0.05	0.18
LSD at 0.01	0.25

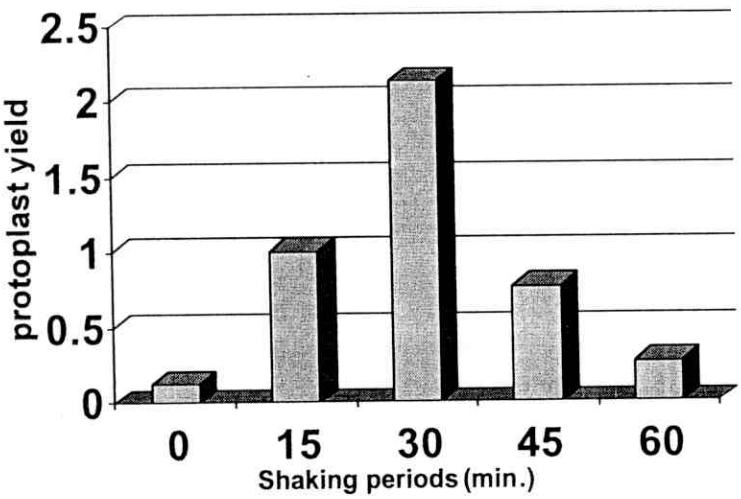

Figure (9): Effect of different Shaking periods on protoplast yield of *in vitro pyrus communis*

Generally, the above results conclude that shaking for 30 minutes gave the highest protoplast numbers.

4.2.3. Purification:

4.2.3.a. Effect of sieve pore size:

Concerning the effect of sieve pore size on the number of viable protoplast, Table (26) clearly indicates that the highest number of viable protoplasts was noticed with sieve pore size at 25 µm followed by pore size at 50 µm while using of 75 µm pore size of sieve at induced the worst number of viable protoplast.

Table (26): Effect of sieve pore size on protoplast yield of *In vitro Pyrus communis.*

Sieve pore size (µm)	Protoplast yield ($x10^5$)
25	2.23±0.06
50	1.60±0.20
75	0.40±0.10
LSD at 0.05	0.25
LSD at 0.01	0.37

The aforementioned results conclude that using of sieve pore size 25µm enhanced the highest protoplast number. These results may be due to the increasing in sieve pore size, encouraged protoplasts cell wall residues, clumps of undigested tissues and debris to pass through the filter and in turn affect badly protoplast yield and viability. These results are somewhat

in accordance with the findings of **Vardi and Esra (1989).** They found that the isolated protoplast of citrus was filtered through 50 µm.

4.2.3.b. Effect of centrifugation:

4.2.3.b.1. Centrifugation speed:

Data in Table (27) show the effect of centrifugation speed on number of viable protoplast. It is clear that increasing centrifugation speed from 500 rpm to 1000 rpm was more effective in maximizing the number of viable protoplasts while increased after that to (1000 rpm) reduced number of viable protoplast. However, farther increase in centrifugation speed up to 1500 rpm resulted decrease in protoplast yield and viability.

Table (27): Effect of centrifugation speed on protoplast yield of *in vitro Pyrus communis.*

Centrifugation speed (rpm)	Protoplast yield ($x10^5$)
500	1.30±0.10
1000	2.20±0.10
1500	1.07±0.15
LSD at 0.05	0.23
LSD at 0.01	0.33

4.2.3.b.2. Centrifugation period:

Table (28) Explains that the effect of centrifugation period on number of viable protoplast it is quite evident that increasing of centrifugation period up to 7.5 min. is recommended for increasing protoplast yield (2.07 $x10^5$). Moreover, centrifuging period for 5 min. took the second rank in increasing protoplast yield (1.47 $x10^5$) while the lowest protoplast yield ($1.13x10^5$) was occurred when 10 min centrifugation period was used.

Table(28): Effect of centrifugation period on protoplast yield of *in vitro Pyrus communis.*

Centrifugation period (min)	Protoplast yield ($x10^5$)
5	1.47±0.12
7.5	2.07±0.15
10	1.13±0.15
LSD at 0.05	0.27
LSD at 0.01	0.39

The above results conclude that using of 7.5 min. as centrifugation period maximized the protoplast yield .These results go in line with the findings of. **Qinghua Zhang *et al.* (2006)** They found that the protoplasts of *Citrus unshiu* purified by centrifugation in a 25% sucrose/13% mannitol gradient for 6 min at 88 *g* .

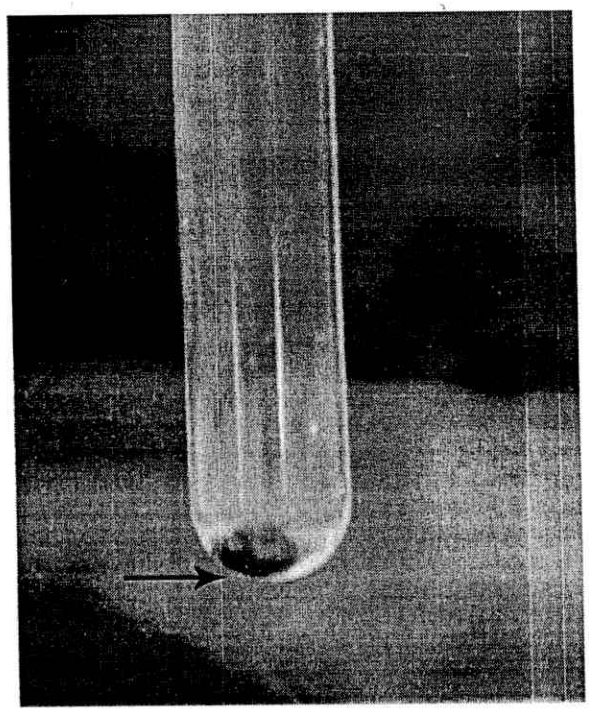

Photo (7): Purification of protoplast in *Pyrus communis*

4.2.4. Protoplast culture:

4.2.4.a. Effect of medium type:

Data in Table (29) explain the effect of different medium types on protoplast development. It is clear that protoplast development was decreased when B5 (Gamborig medium) was used while using KM medium increased the protoplast development followed by Murashig & Skoog medium.

Table (29): Effect of different medium types on protoplast development of *in vitro Pyrus communis.*

Medium type	Protoplast development (Scorce)
B_5	1.00±0.00
KM	3.33±0.58
MS	1.67±0.58
LSD at 0.05	0.89
LSD at 0.01	1.29

4.2.4.b. Effect of protoplast density:

The results of Table (30) reflect the effect of cultured protoplast density on protoplast development. It is clear that density of 2 x 10^5 induced highly increase in protoplast development as compared with the other protoplast densities under study followed by 2.5 x 10^5. However, the protoplast density of 0.5 x 10^5 was gave the lowest values of protoplast development.

Table (30):Effect of cultured protoplast density on protoplast development of *in vitro Pyrus communis.*

protoplast density ($x10^5$)	Protoplast development (scores)
0.5	1.33±0.58
1	2.33±0.58
1.5	2.67±0.58
2	3.67±0.58
2.5	3.33±0.58
LSD at 0.05	1.01
LSD at 0.01	1.4

The above results reflect the importance of using protoplast density at 2.0x 10^5 which maximize protoplast development. These results go in line with the findings of **Ochatt (1993a)** who Found that the best result of cell division was achieved when protoplast density was 0.5-2.5 x 10^5 protoplasts per ml of pear ***Pyrus spp*** .

4.2.4.c. Effect of hormonal balance:

Data in Table (31) and Fig. (10) deal with the effect of NAA and BAP concentrations on protoplast development. It is appear that supplementation the culture medium with 1.00 mg/L NAA encouraged increase in protoplast development. Meanwhile, using 3.0 mg/L of NAA took the second rank followed by concentration of 2.00 mg/L from NAA. However,

the worst results of protoplasts development was showed when NAA concentration was nil . Moreover, addition either of 0.2 or0.3 mg/L BAP to the culture medium enhanced increase in protoplast development. On the other hand, the combination between 1.00 mg/L NAA with either 0.2 or 0.3 mg/L BAP to the culture medium resulted in maximizing protoplast development compared with the other concentrations.

In general, the above results summarized that supplementation of the culture medium with either 1.0 or 2.0 mg/L NAA and BAP 0.2 or0.3 mg/L BAP were the suitable rate of hormonal balance maximized protoplast development and increased cell division. These results go in accordance with the findings of **Matsuta *et al.*(1986)** Who showed that protoplasts of ***prunus persica*** were successfully cultured on NN medium supplemented with 2mg/liter NAA and 0.2 mg/liter BAP at 28°C in the dark . and **Mii *et al.* (1991)** They reported that high frequency of cell division of *Vitis thunbergii* protoplast occurred when culture medium was supplemented with 2 mg/L NAA and 0.2 mg/L benzyl adenine.

Table (31): Effect of NAA and BAP concentrations on protoplast development of *in vitro Pyrus communis*.

Auxin \ Cytokinin		BAP (mg/L)				Mean
		0	0.1	0.2	0.3	
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 NAA at 0.05 and 0.01		0.54		0.73		
LSD for interaction at 0.05 and 0.01		1.09		1.45		

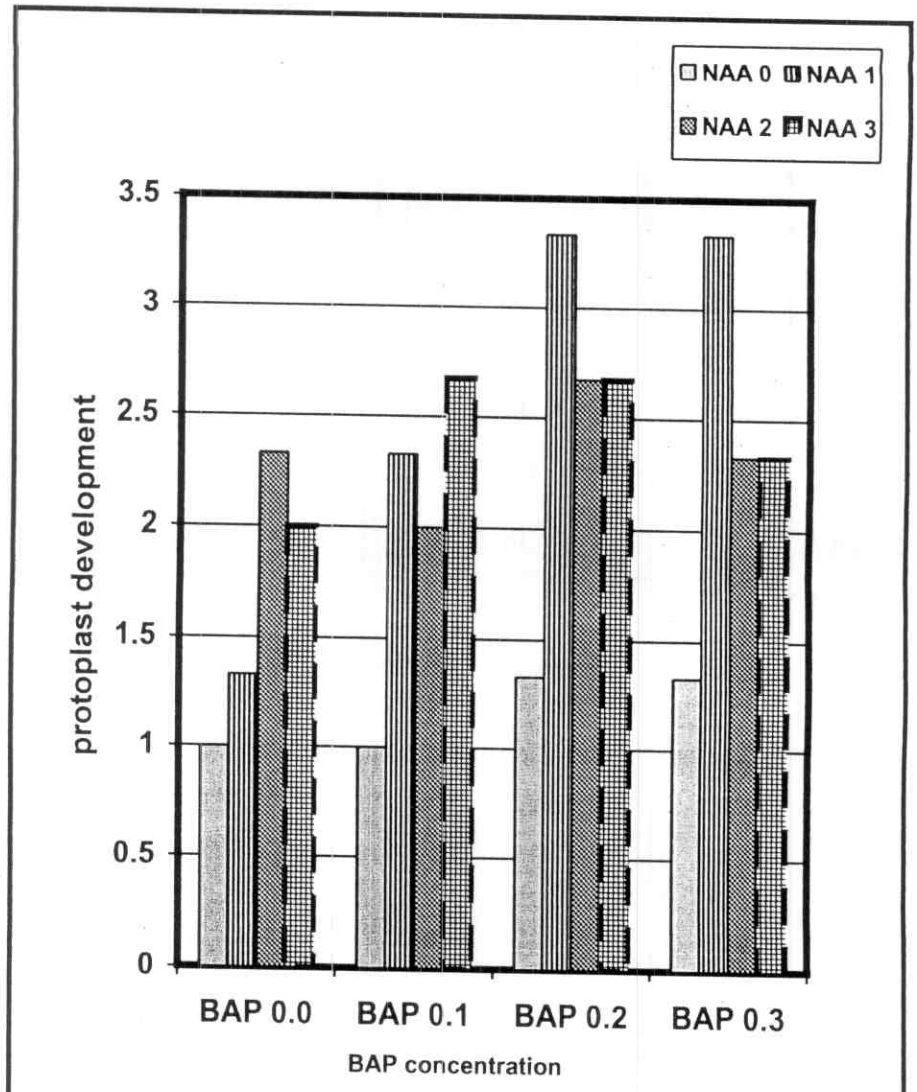

Figure (10):Effect of the interaction between NAA and BAP concentration on protoplast development of *in vitro pyrus communis* .

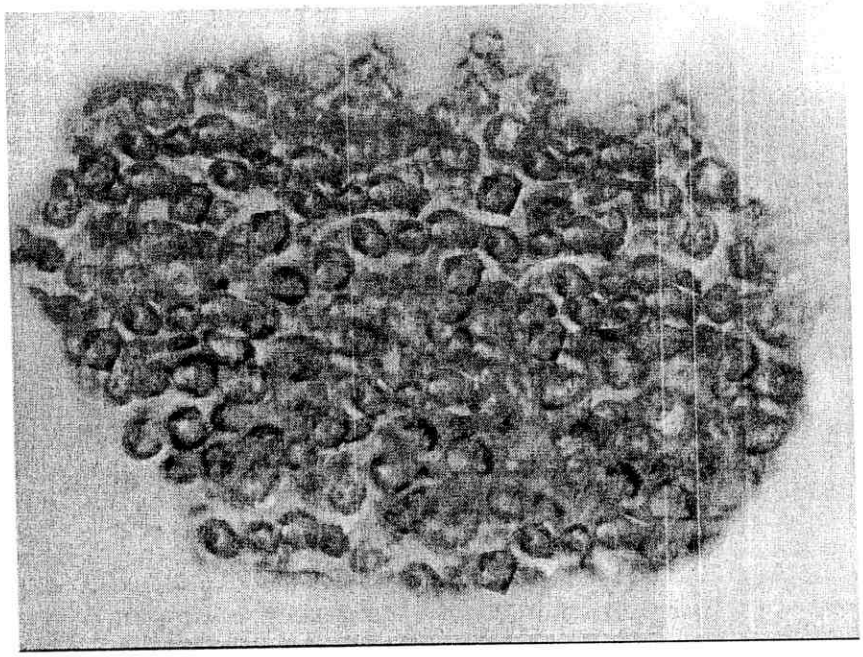

Photo (8): Protoplast yield in *Pyrus communis*

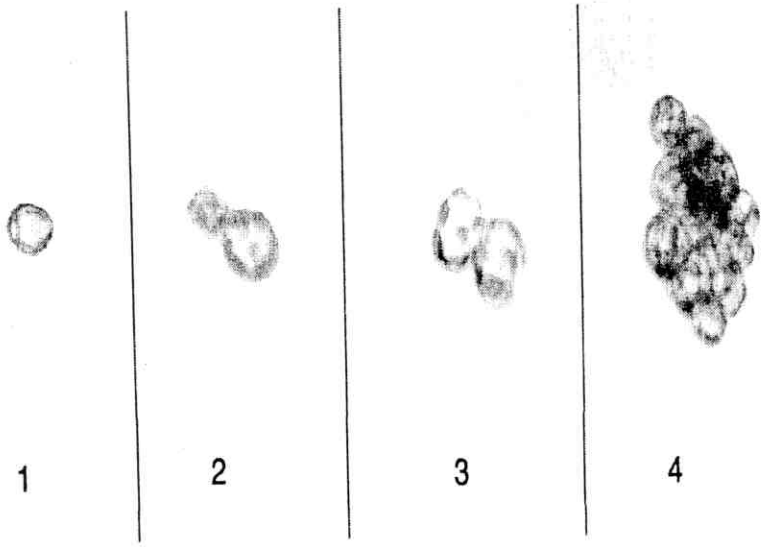

Photo (9): Reflect protoplast development in *Pyrus communis* which include (1) protoplasts, (2) buding stage, (3) protoplast division, (4) microcalli formation.

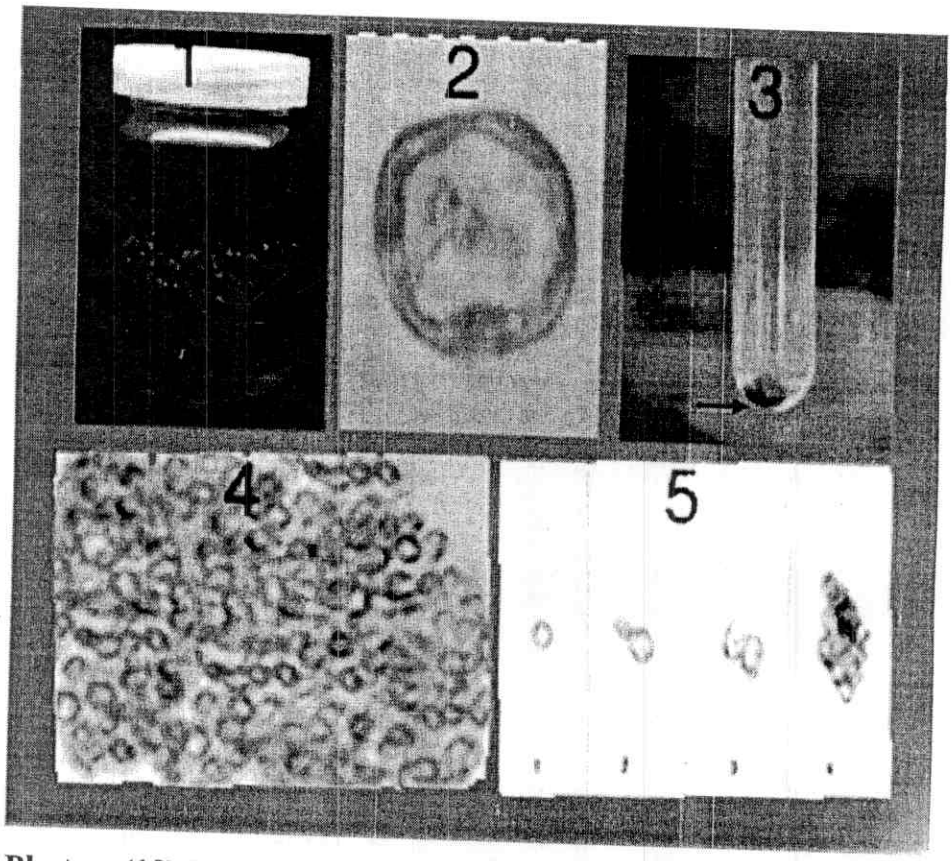

Photo (10):Reflect the protoplast technique in *Pyrus communis* which include preprotoplast isolation (1) protoplast source, (2)Plasmolyzed cells, (3) purification of protoplasts, (4) Protoplast yield, (5) protoplast development (1=protoplasts, 2=buding stage, 3= protoplast division, 4=microcalli formation, 5=more microcalli formation).