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2.B- CPW salts :

Component	Concentration (mg/L)
KH_2PO_4	27.2
KNO_3	101
$CaCl_2 . 2H_2O$	1.480
$MgSO_4 . 7H_2O$	246
KI	0.16
$CuSO_4 . 5H_2O$	0.025
pH	5.8

CPW 10 % Mannitol with CPW salts, pH 5.8

Data and calculation:

Counting of protoplasts was conducted according to the method of **Blackhall** *et al.* **(2002).** Moreover, Number of cells: calculated 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 $= 5\ n\ x\ 10^4$

Protoplast viability was counted as the number of complete rounded protoplast which represent viable protoplasts.

Moreover, scores were applied for protoplast development which calculated as the rate of cell division and microcalli formed according to **Pottino (1981)** as follow:

1 = No cell division or microcalli formed.

2 = Below average of cell division and microcalli formed.

3 = Average number of cell division and microcalli formed.

4 = Above average of cell division and microcalli formed.

5 = Excellent (The highest cell division and microcalli formed).

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 analyzed using standard deviation (SD).

IV- RESULTS AND DISCUSSION

4.1. Pineapple:
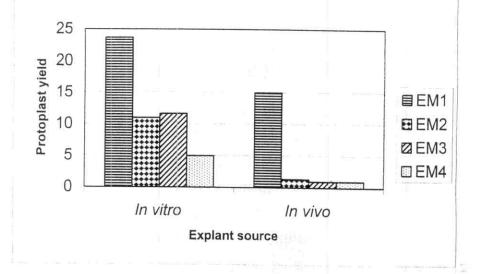### 4.1.1. Protoplast isolation:
4.1.1.a. Effect of different enzyme mixtures and explant source:

Data of Table (1) and Fig. (1) show the effect of different enzyme mixtures and explant source on protoplast yield. It is obvious that different explant source had differently affect on protoplast yield. *In vitro* explant was more superior than *in vivo* explant. Meanwhile, EM1(enzyme moisture) (1% cellulase + 0.5% macerozyme) was more effective in increasing protoplast yield as compared with the other enzyme mixtures. However, EM3 treatment (1% cellulase + 0.5% macerozyme + 0.2% pectinase) took the second rank in increasing protoplast yield followed with EM2 (1% cellulase + 0.2% pectinase) and finally EM4 (0.5% macerozyme + 0.2% pectinase) which produced the lowest value of protoplast yield.

Table (1): Effect of different explant source and enzyme mixtures on protoplast yield of Pineapple.

Explant source Enzyme	In vitro	In vivo	Mean
EM$_1$	23.67 ± 1.53	15.00 ± 1.00	19.34
EM$_2$	11.00 ± 1.00	1.33 ± 0.58	6.17
EM$_3$	11.67 ± 1.53	1.00 ± 0.00	8.84
EM$_4$	5.00 ± 1.00	1.00 ± 0.00	3.00
Mean	12.83	4.58	

Figure (1): Effect of different explant source and enzyme mixtures on protoplast yield.

Concerning the interaction, it is quite evident 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 EM3 and EM4 enzyme moisture induced the lowest values.

Generally, the above results conclude 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 protoplast isolated from *in vitro* mesophyll of wild pear were higher compared with those from field mesophyll plants. Moreover, **Barbier and Bessis (1990)** recommended enzyme mixture consists of 1% cellulase onozuka R10 and 0.5%
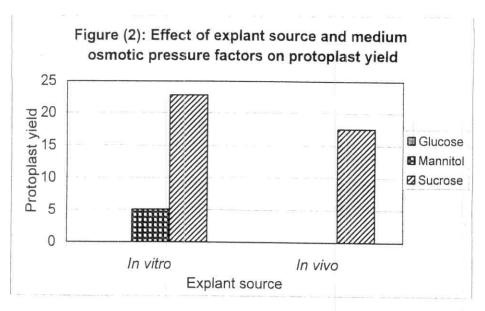
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macerozyme R10 for isolating the best yield of grape vine Chardonnay cv. protoplasts.

4.1.1.b. Effect of different medium osmotic pressure factors and explant source:

The results of Table (2) and Figure (2) deal with the effect of different osmotic pressure factors and explant source on protoplast yield. It is obvious that the addition of sucrose to the culture medium induced an 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* explant surpassed *in vivo* explant under study in this respect.

Table (2): Effect of explant source and medium osmotic pressure factors on protoplast yield of Pineapple.

Osmotic \ Explant source	*In vitro*	*In vivo*	Mean
Glucose	0.0001 ± 0.00	0.0001 ± 0.00	0.0001
Mannitol	5.13 ± 0.15	0.0001 ± 0.00	2.56
Sucrose	22.83 ± 0.61	17.63 ± 0.41	20.23
Mean	12.74	5.88	

Figure (2): Effect of explant source and medium osmotic pressure factors on protoplast yield

Moreover, the data of the interaction between explant source and osmotic pressure factors reflected that *in vitro* explant and sucrose as osmotic pressure factor gave the best protoplast yield followed by the combination of *in vivo* explant and sucrose in comparison with the other combinations. However, adding glucose to either *in vitro* or *in vivo* explants as well as manitol and *in vivo* explant failed completely to isolate protoplast.

Generally, the aforementioned results summarized that *in vitro* explant and sucrose as osmotic pressure factor caused the highest protoplast numbers. These results go in line with the findings of **Grezes** *et al.* **(1994)** on *Coffea arabica* and **El-Miniawy** *et al.* **(2001)** on strawberry chandler cv. They all revealed that the incubation of squashed leaf segments in enzyme mixture supplemented with 0.4 M sucrose as osmotic agent improved protoplast viability.
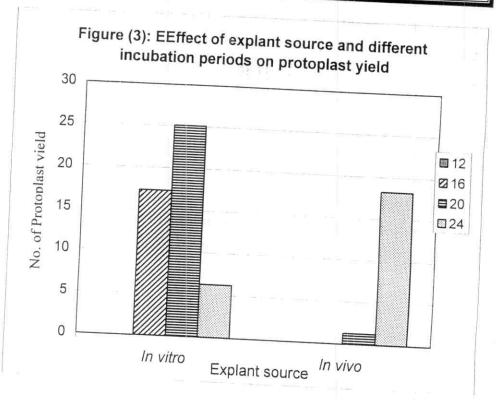
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Moreover, the obtained results include that *in vitro* explant is the highest source for protoplast number beside sucrose gave the most protoplast number and protoplast yield.

4.1.1.c. Effect of different incubation periods and explant sources:

Data tabulated in Table (3) and illustrated in Fig. (3) describe the effect of different incubation periods on protoplast yield. It is clearly appeared that *in vitro* explant surpassed *in vivo* one in increasing the protoplasts yield. Meanwhile, incubation of leaf segments in Enzyme mixture for 20 hours was more superior than other incubation periods used. Moreover, incubating *in vitro* leaves explant for 20 hours followed by 24 hour for *in vivo* explant caused increase in protoplast yield. On the contrary, incubation for 12 h gave the lowest value of protoplast. Also, *in vitro* explant gave the best results than *in vivo* explant. The beforementioned results verified that *in vitro* explant is more preferable than *in vivo* explant. In addition, 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 obtained the highest protoplast yield when grape explant was incubated in enzyme solution for 18 h under dark condition at 28°C.

Table (3): Effect of explant source and different incubation periods on protoplast yield of Pineapple.

Explant source / Incubation period	In vitro	In vivo	Mean
12	0.0001 ± 0.00	0.0001 ± 0.00	0.001
16	17.33 ± 0.85	0.0001 ± 0.00	8.66
20	25.13 ± 0.35	1.32 ± 0.08	13.22
24	6.30 ± 1.10	18.30 ± 1.10	12.30
Mean	12.19	4.91	

Figure (3): EEffect of explant source and different incubation periods on protoplast yield

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4.1.1.d. Effect of explant shaking period and speed:

Table (4) shows the effect of explant source, shaking period and speed on protoplast yield. It is clear from table (4-A) that *in vitro* explant was more superior in protoplast yield than *in vivo* explant. Meanwhile, Table (4-B) clarified that increasing shaking period from 15 to 60 minutes resulted in a decreasing in protoplast yield. So, the highest protoplast yield (15.32) was obtained when shaking period was 15 minutes. Afterwards, the protoplasts number declined by increasing shaking period up to 60 minutes. It is quite evedent from Table (4-C) that shaking speed at the rate of 50 rpm induced the highest protoplast yield while continuous increase in shaking speed increased viable protoplasts yield up to the highest rate (100 rpm) which induced low number of viable protoplasts (8.61) in relation to 50 rpm speed.

Regarding to the interaction between explant source and shaking period as shown in Table (4-D), It is appear that the combination of *in vitro* explant and 30 minutes shaking period enhanced the best protoplast yield in comparison with the other combinations used. However, combination of 60 minutes shaking period with either *in vitro* or *in vivo* explants resulted in a sharply decrease in the number of isolated protoplasts (0.45 and 0.0001 respectively). Moreover, the interaction between explant source and shaking speed as recorded in Table (4-E) showed that shaking speed for *in vitro* explant at the rate of 50 rpm surpassed other interactions used. However, higher shaking speeds (75 or 100 rpm) in combination with either *in vitro* or *in vivo* explants induced a reduction in number of isolated protoplasts. Referring to the interaction between shaking period and speed as shown in Table (4-

F) it is quite obvious that shaking speed at the rate of 75 and 50 rpm for 15 or 30 minutes shaking period respectively encouraged maximum protoplast yield in comparison with the other combinations under study. However, the control treatment (incubation of the mixture for different periods without shaking) induced a negative results. Besides, increasing shaking speed (100 rpm) combined with shaking period induced a reduction in protoplast yield.

Dealing with the effect of the interaction between explant source, shaking period and shaking speed, it is clear from Table (4-F) that the combination of *in vitro* explant and shaking speed at the rate of 75 for 15 minutes encouraged the highest protoplasts yield, then followed by the combination of *in vitro* explant and shaking speed 50 rpm for 30 minutes and the combination of *in vitro* explant and 50 rpm for 15 minutes as compared with the other combinations used. However, increasing shaking period up to 60 minutes failed to inducing protoplast production when combined with all explant source and shaking speeds.

Thus, the aforementioned results summarized that *in vitro* explant and the rate of 50 rpm for 15 minutes are the best factors for protoplasts isolation. Also, the combination between *in vitro* explant and shaking at the rate of 75 rpm for 15 minutes induced the best results. 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. Thus, the combination of *in vitro* explant and 75 rpm shaking speed for 15

minutes enhanced the protoplast yield. These results are in harmony with the findings of **Li et al. (1995).** They reported that high yield of *Arachis spp.* mesophyll cell protoplasts were obtained when a rotary shaker was at speed 85 rpm.

Table (4): Effect of different shaking periods, speeds and explant source on protoplast yield of Pineapple.

Table (4-A): Effect of explant source.

Explant source	No. of protoplast yield
In vitro	9.89
In vivo	7.35

Table (4-B): Effect of shaking period.

Shaking period	No. of protoplast yield
15	15.32
30	12.78
45	6.15
60	0.22

Table (4-C): Effect of shaking speed.

Shaking speed	No. of protoplast yield
0	0.0001
50	13.80
75	11.93
100	8.61

Table (4-D): Effect of the interaction between explant source and shaking period.

Shaking period (minutes) / Explant source	In vitro	In vivo
15	23.18 ± 10.58	14.00 ± 8.50
30	25.07 ± 10.59	17.02 ± 10.37
45	9.26 ± 5.47	5.86 ± 3.95
60	2.05 ± 2.72	0.58 ± 1.06

Table (4-E): Effect of the interaction between explant source and shaking speed.

Shaking speed (rpm) / Explant source	In vitro	In vivo
5	0.0001 ± 0.0	0.0001 ± 0.0
50	15.71 ± 9.53	11.88 ± 8.83
75	13.93 ± 10.37	9.73 ± 7.78
100	9.93 ± 7.35	7.28 ± 6.01

Table (4-F): Effect of the interaction between shaking period and speed.

Shaking period (minutes) / Shaking speed (rpm)	0	50	75	100
15	0.0001 ± 0.0	20.57 ± 0.48	23.58 ± 0.86	17.12 ± 0.28
30	0.0001 ± 0.0	22.94 ± 0.77	16.80 ± 1.33	11.38 ± 1.10
45	0.0001 ± 0.0	10.75 ± 1.16	7.77 ± 0.10	5.91 ± 0.90
60	0.0001 ± 0.0	0.89 ± 0.32	0.0001 ± 0.0	0.0001 ± 0.0

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Table (4-G): Effect of the interaction between explant source, shaking period and speed.

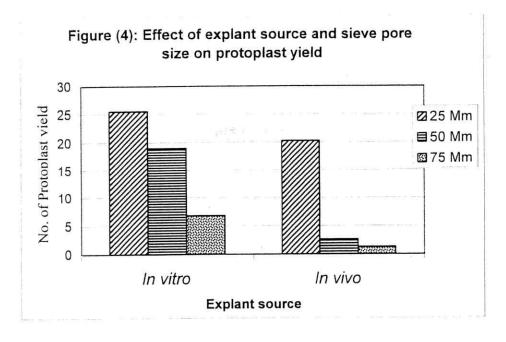| Shaking period (minute)	Shaking speed (rpm)	Explant source	
		In vitro	*In vivo*
15	0	0.0001±0.0	0.0001±0.0
	50	22.35±0.75	18.80±0.63
	75	26.39±1.10	20.78±0.64
	100	18.87±0.43	15.38±0.21
30	0	0.0001±0.0	0.0001±0.0
	50	25.12±0.99	20.78±0.64
	75	19.20±1.49	14.40±1.17
	100	13.39±1.21	9.37±0.01
45	0	0.0001±0.0	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

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4.1.2. Filtration:

4.1.2.a. Effect of sieve pore size and explant source:

Table (5) and Fig. (4) reveal the effect of sieve pore size and explant source on protoplast yield. It is well noticed that *in vitro* explant source gave the highest protoplast yield (17.14) in relation to *in* vivo explant (8.07). Meanwhile, sieve pore size at 25 for filtration of protoplast was more superior in maximizing the number of protoplast isolation followed by 50 μm then lastly 75 μm which produced the lowest values of protoplast yield. Moreover, sieving the protoplasts resulted of *in vitro* explant with pore size 25 μm encouraged the highest protoplast yield. However, *in vivo* explant combined with either pore size 50 or 75 induced the lowest protoplasts isolated in relation to the other interactions used.

Table (5): Effect of explant source and sieve pore size on protoplast yield.

Explant source / Pore size	In vitro	In vivo	Mean
25	25.53 ± 0.67	20.30 ± 0.80	22.91
50	19.03 ± 0.83	2.61 ± 0.54	10.82
75	6.87 ± 0.42	1.29 ± 0.03	4.08
Mean	17.14	8.07	

Figure (4): Effect of explant source and sieve pore size on protoplast yield

The aforementioned results conclude that *in vitro* explant combined with sieve pore size 25 is the highest source for 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 potoplast yield and viability. These results are somewhat in accordance with the findings of **Witjaksono and Grosser (1990).** They declared that sterile stainless steel sieve 45 µm in diameter was suitable for isolation of avocado protoplasts.

4.1.2.b. Effect of centrifugation speed and explant source:

Data of Table (6) and Fig. (5) 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). Centrifuging
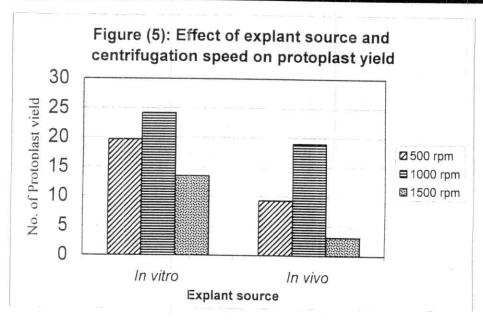
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speed 500 rpm took the second rank (14.50) and finally 1500 rpm speed produced the lowest value of protoplast (8.36).

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 (6): Effect of explant source and centrifugation speed on protoplast yield of Pineapple.

Explant source / Centrifugation speed (rpm)	In vitro	In vivo	Mean
500	19.67 ± 0.47	9.33 ± 0.85	14.50
1000	24.23 ± 0.81	19.04 ± 0.64	21.63
1500	13.53 ± 0.49	3.19 ± 0.07	8.36
Mean	19.14	10.52	

Figure (5): Effect of explant source and centrifugation speed on protoplast yield
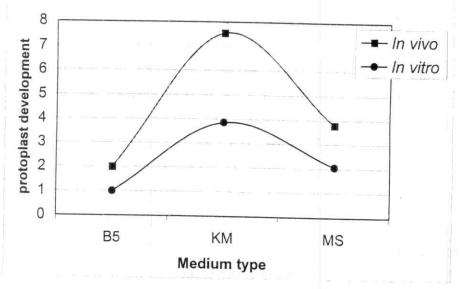
4.1.3. Protoplast culture:

4.1.3.a. Effect of different medium types and explant source:

The results of Table (7) and Fig. (6) explain the effect of different medium types and explant source on protoplast development. It is clear that slight differences were appeared when *in* vivo explant was used than these of *in* vivo explant. Also, KM medium induced highly increase protoplast development as compared with the other two media under study. However, Gamborge (B5) medium failed to induce any positive results in this respect in both *in vivo* or *in vitro* explant source. Anyhow, MS medium was inbetween concerning protoplast development. However, culturing protoplast of *in vitro* as explant source on KM medium increased protoplast development. These results are in agreement with the findings of **Ochatt *et al.* (1993)**. They recommended modified Kao and Michayluk-based medium for culturing the protoplasts of haploid *Golden delicious* apple to encourage microcallus development.

Table (7): Effect of different explant source and media types on protoplast development of Pineapple.

Medium type	In vitro	In vivo	Mean
B5	1.00 ± 0.00	1.00 ± 0.00	1.00
KM	3.87 ± 0.07	3.67 ± 0.15	3.77
MS	2.07 ± 0.06	1.73 ± 0.06	1.90
Mean	2.31	2.13	

Figure (6): Effect of explant source and different medium types on protoplast development of Pineapple

4.1.3.b. Effect of cultured protoplast density and explant source:
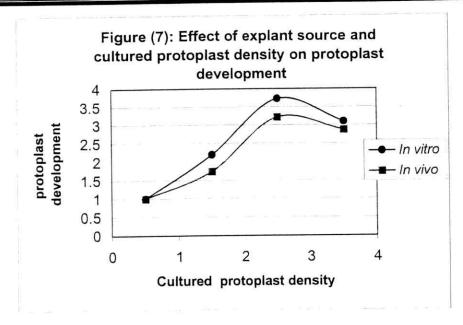
Table (8) and Fig. (7) verify the effect of cultured protoplast density and explant source on protoplast development of pineapple. It is quite evident that *in vitro* explant slightly improved protoplast development in relation to *in vivo* explant. Meanwhile, culturing protoplast density of 2.5×10^4 was more effective in maximizing protoplast development followed by density of 3.5×10^4. However, the concentration of density at 0.5×10^4 failed to show any result when protoplast development was concerned.

The above results reflect the importance of using *in vitro* explant source combined with protoplast density at 2.5×10^4 which

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maximize protoplast development. These results go in line with the findings of **Kobayashi (1987)** who reported that low cell density (1-4×10^4 /ml) combined with low mannitol concentrations (0.2-0.4 m) induced direct somatic embryogenesis from protoplast of Citrus sinensis *cv. Bahia*.

Table (8): Effect of explant source and cultured protoplast density on protoplast development of Pineapple.

Explant source / Cultured protoplast density	In vitro	In vivo	Mean
0.5	1.00 ± 0.00	1.00 ± 0.00	1.00
1.5	2.20 ± 0.10	1.75 ± 0.03	1.97
2.5	3.73 ± 0.02	3.22 ± 0.02	3.47
3.5	3.10 ± 0.10	2.87 ± 0.06	2.98
Mean	2.51	2.21	

Figure (7): Effect of explant source and cultured protoplast density on protoplast development

4.1.3.c. Effect of explant source, auxin and cytokinin concentrations:

Data of Table (9) show the effect of explant source, auxin and cytokinin concentrations on protoplast development of pineapple. It is clear from Table (9-A) that *in vitro* explant source was more superior in protoplast development in relation to those of *in vivo* explant source. Meanwhile, Table (9-B) reflects that auxin at the rate of 3.0 mg/L gave the best protoplast development followed by 2.0 mg/L in comparison with the other concentrations (0.0 and 1.0 mg/L) used. Moreover, Table (9-C) clarifies that increasing cytokinin concentrations encouraged the increase in protoplast development up to 0.3 mg/L which enhanced the highest protoplast development (2.29) followed by 0.2 mg/L (2.20) then 0.1 (2.01) as compared with the control (0.0 mg/L) which induced the lowest protoplast development (1.52).

Regarding the different interactions between explant source, auxin and cytokinin concentrations, Table (9-D) showed that the combination between *in vitro* and 3.0 mg/L NAA maximized protopolast development followed by the combination of *in vivo* and 3.0 mg/L NAA as compared to the other combinations. Besides, Table (9-E) reflects that the combination between *in vitro* explant and either 0.3, 0.2 or 0.1 cytokinin encouraged the increase in protoplast development, respectively in comparison with other combinations. However, the interaction between NAA and BAP concentrations recorded in Table (9-F). It is quite evident that the combination of 3.0 mg/L NAA and either 0.2, 0.1 or 0.3 BAP treatments succeeded in improving protoplast development compared with the other combinations under study. Furthermore,

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

Table (9-A): Effect of explant source.

Explant source	Protoplast development
In vitro	2.11
In vivo	1.76

Table (9-B): Effect of auxin concentration.

Auxin conc.	Protoplast development
0.0	1.03
1.00	1.80
2.00	2.08
3.00	2.83

Table (9-C): Effect of cytokinin concentration.

Cytokinin conc.	Protoplast development
0.0	1.52
0.1	2.01
0.2	2.20
0.3	2.29

Table (9-D): Effect of the interaction between explant source and NAA concentration.

NAA conc.	In vitro	In vivo
0.0	1.08 ± 0.09	1.00 ± 0.0
1.00	1.99 ± 0.43	1.61 ± 0.37
2.00	2.35 ± 0.32	1.86 ± 0.35
3.00	3.03 ± 0.51	2.63 ± 0.35

Table (9-E): Effect of the interaction between explant source and BAP concentration.

BAP conc.	In vitro	In vivo
0.0	1.65 ± 0.53	1.39 ± 0.48
0.1	2.15 ± 0.77	1.86 ± 0.69
0.2	2.43 ± 0.92	1.97 ± 0.75
0.3	2.27 ± 0.75	1.84 ± 0.57

Table (9-F): Effect of the interaction between BAP and NAA concentration.

NAA \ BAP	0.0	0.1	0.2	0.3
0.0	1.00 ± 0.00	1.00 ± 0.00	1.07 ± 0.03	1.09 ± 0.00
1.00	1.16 ± 0.00	2.09 ± 0.05	2.01 ± 0.04	1.94 ± 0.00
2.00	1.72 ± 0.08	1.10 ± 0.06	2.43 ± 0.02	2.18 ± 0.02
3.00	2.19 ± 0.02	2.94 ± 0.02	3.27 ± 0.00	2.92 ± 0.01

Table (9-G): Effect of the interaction between auxin, cytokinin concentration and expant sources.

	BAP	Explant source	
		In vitro	*In vivo*
NAA 0.0	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	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	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	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 (9-G) verifies the effect of the interaction between explant source and auxin and cytokinin concentrations. It is obvious that combination of *in vitro* explant and 3.0 mg/L NAA and 0.2, 0.3, 0.1 mg/L BAP induced the best protoplast development in relation to the other combinations under study.

In the meantime, lower NAA concentrations combined with all explant source and different BAP concentration had an adverse effect on protoplasts development in comparison with the higher NAA concentrations.

In general, the above results summarized that *in vitro* explant source combined with 3.0 mg/L NAA and most concentrations of BAP under study (0.2, 0.3 and 0.1 mg/L) maximized protoplast development and increased cell division. These results go in line with the findings of **Mii et al. (1991)** on *Vitis thunbergii* and **Huy et al. (1997)** on blackberry. They reported that the addition of either 2.0 mg/L NAA and 0.2 mg/L benzyl adenine or 10, 74 mM NAA and 1.78 mM BA encouraged frequency of cell division.

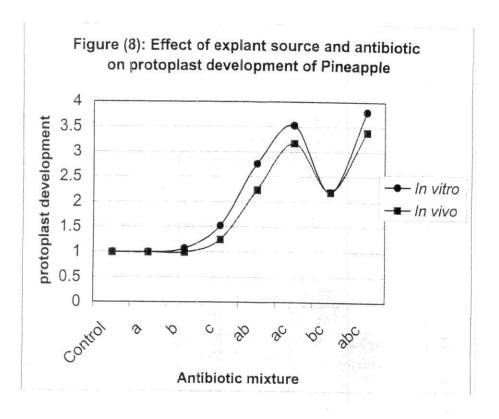### 4.1.3.d. Effect of explant source and antibiotic type:

Table (10) and Fig. (8) show the effect of different explant sources and antibiotic types on protoplast development. It is obvious that *in vitro* explant source 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 + 0.1 g/L gentamycin + 0.1 g/L tetracycline resulted in increasing protoplast development followed by the combination

of (0.4 g/L ampicilin + 0.1 g/L tetracycline). Meanwhile, the combination of 0.4 g/L ampicilin + 0.1 g/L gentamycin encouraged also protoplast development.

Table (10): Effect of explant source and antibiotic on protoplast development of Pineapple.

Explant source / Antibiotic	In vitro	In vivo	Mean
Control	1.00 ± 0.00	1.00 ± 0.00	1.00
0.4 g / L Ampicilin	1.00 ± 0.00	1.00 ± 0.00	1.00
0.1 g / L Gentamycin	1.07 ± 0.06	1.00 ± 0.00	1.03
0.1 g / L Tetracycline	1.53 ± 0.03	1.25 ± 0.02	1.39
0.4 g / L Ampiciline + 0.1 g / L Gentamycin	2.76 ± 0.05	2.24 ± 0.03	2.50
0.4 g / L Ampicilin + 0.1 g / L Tetracycline	3.53 ± 0.02	3.18 ± 0.03	3.35
0.1 g / L Gentamycin + 0.1 g / L Tetracycline	2.20 ± 0.10	2.20 ± 0.26	2.20
0.4 g / L Ampicilin + 0.1 g / L Gentamycin + 0.1 g / L Tetracycline	3.80 ± 0.10	3.40 ± 0.10	3.60
Mean	2.11	1.91	

Regarding the interaction between explant source and antibiotic type, it is clear that culturing of the protoplasts resulted from *in vitro* explant on medium supplemented with combined antibiotic treatment (0.4 g/L ampiciline + 0.1 g/L gentamycin + 0.1 g/L tetracycline) induced the best protoplast development in comparison with the other treatments used.

Figure (8): Effect of explant source and antibiotic on protoplast development of Pineapple

The aforementioned results conclude that in vitro explant surpassed *in vivo* and the supplementation of the culture medium with combined antibiotic treatment is more effective in increasing protoplast development.

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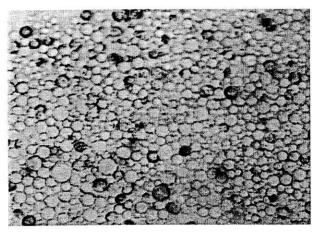
Results and Discussion
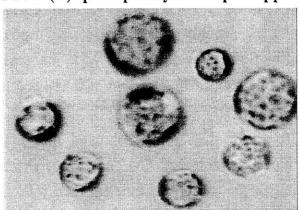
Photo (A): protoplast yield of pineapple.

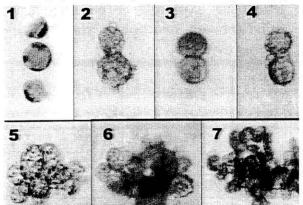**Photo (B): Viable protoplast of pineapple.**

Photo (C): Development stages of pineapple.

4.2. Banana:

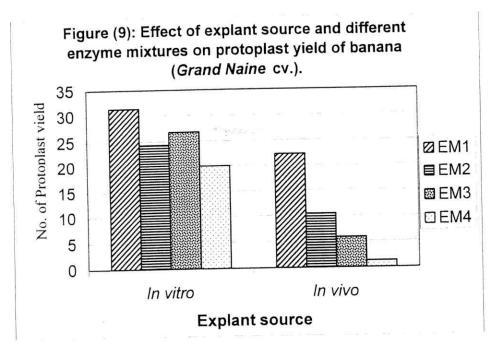### 4.2.1. Protoplast isolation:

4.2.1.a. Effect of different enzyme mixtures and explant source:

The results of Table (11) and Fig. (9) explain the effect of different enzyme mixtures and explant source on protoplast yield. It is well noticed that the best source for protoplast isolation was *in vitro* explant. However, EM1 (Enzyme mixture) (1% cellulase + 1% macerozyme + 1% pectinase) surpassed other enzyme mixtures in either *in vitro* explant or *in vivo* explant of Banana (Grand Naine cv). Meanwhile, EM2 (1.5% cellulase + 1% macerozyme + 0.15% pectinase) gave the second rank in enhancing protoplast isolation then followed by EM3 (2% cellulase + 0.5% macerozyme + 0.25% pectinase) while EM4 (1.5% cellulase + 0.2% pectinase) was the best in protoplast number.

The aforementioned results showed that the combination between *in vitro* explant and EM_1 enzyme mixture caused the largest increase in the protoplast number then followed by *in vivo* explant combined with the same enzyme mixture.

Table (11): **Effect of explant source and different enzyme mixtures on protoplast yield of banana (Grand Naine cv.).**

Explant source Enzyme mixture	In vitro	In vivo	Mean
EM_1	31.47 ± 1.05	22.37 ± 1.02	26.92
EM_2	24.40 ± 1.25	10.46 ± 0.51	17.43
EM_3	26.83 ± 0.42	5.85 ± 0.57	16.34
EM_4	20.05 ± 0.63	1.30 ± 0.05	10.67
Mean	25.69	9.90	

Figure (9): Effect of explant source and different enzyme mixtures on protoplast yield of banana (*Grand Naine* cv.).

These results are in harmony with the findings of **Panis et al. (1994).** They found that protoplast yield of banana cell suspension was increased when enzyme mixture contain 1% cellulase + 1% macerozyme + 1% pectinase.

4.2.1.b. Effect of osmotic pressure factor and explant source:

Data of Table (12) and Fig. (10) showed the effect of medium osmotic pressure and explant source on protoplast yield. It is safely appear that different explant source had different effect on protoplast isolation since, *in vitro* explant was more superior than *in vivo* explants. However, 10 g/100ml Mannitol induced highly increase in protoplast yield followed by sucrose at 21 g/100 ml then glucose at (7.92 g/100 ml). Meanwhile, the highest number of protoplast was obtained when *in vitro* explant grown in medium supplemented with mannitol, followed by *in vivo* combined with the same medium constituents.

Table (12): Effect of explant source and medium osmotic pressure factors on protoplast yield of banana (Grand Naine cv.).

Explant source / Osmotic pressure	In vitro	In vivo	Mean
Glucose	21.70 ± 1.22	17.43 ± 0.12	19.56
Mannitol	32.04 ± 0.39	22.97 ± 0.42	27.50
Sucrose	25.33 ± 0.35	21.53 ± 0.40	23.43
Mean	26.36	20.64	

Figure (10): Effect of explant source and medium osmotic pressure factors on protoplast yield of banana (*Grand Naine* cv.).

These results assured the findings of **Panis et al. (1994).** They stated that an increase in protoplast yield of *Musa spp.* was obtained when incubated in solution contained 10% manitol.

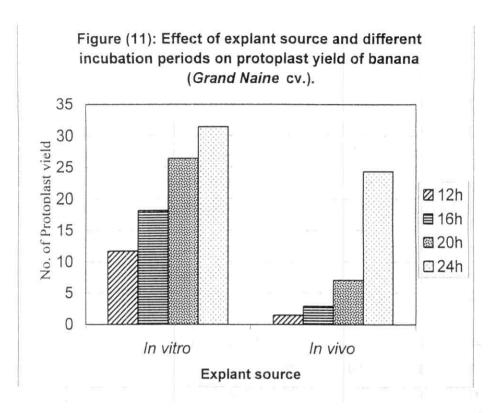### 4.2.1.c. Effect of incubation period and explant source:

Data of Table (13) and Fig. (11) revealed the effect of different incubation periods and explant source on protoplast yield. It is indicated that there were a direct relationship between incubation periods and protoplast yield since the protoplast yield was increased by increasing incubation period. However, the lowest protoplast yield was noticed when explants were incubated for 12 h.

On the other hand, protoplast yield was increased when *in vitro* explant source was involved compared with *in vivo* explant source.

Regarding the effect of the interaction between explant source and incubation periods, it is appear that protoplast yield increased when *in vitro* explant source and incubation period for 24 h were used.

Table (13): Effect of explant source and different incubation periods on protoplast yield of banana (*Grand Naine* cv.).

Explant source / Incubation period (hours)	In vitro	In vivo	Mean
12	11.70 ± 0.48	1.50 ± 0.14	6.6
16	18.20 ± 1.65	2.94 ± 0.60	10.57
20	26.44 ± 1.10	7.12 ± 0.69	16.78
24	31.43 ± 1.01	24.28 ± 1.10	27.85
Mean	21.94	8.96	

Figure (11): Effect of explant source and different incubation periods on protoplast yield of banana (*Grand Naine* cv.).

4.2.1.d. Effect of different shaking periods, speeds and explant sources:

Table (14-A) explain the effect of explant source on protoplast isolation yield. It is clear that *in vitro* explant source succeeded in enhancing the highest protoplast yield compared with *in vivo* explant source. Meanwhile, Table (14-B) reflects that increasing shaking period from 15 min. to 30 min, encouraged the increase in protoplast yield. However, successive increase in incubation period over 30 min. (45 and 60 min.) resulted in a sharp decrease in protoplast yield (7.56 and 1.37) in relation to those obtained by 30 min. incubation period (21.05). Besides, Table (14-C) verified that 50 rpm shaking speed caused maximum increase of protoplast yield (18.95) followed with both 75 and 100 rpm speed

which gave 16.09 and 13.47 in this respect respectively. Moreover, the interaction between explant source and shaking period as tabulated in Table (14-D) indicated that *in vitro* explant source with either shaking period 30 or 15 min. induced the highest protoplast yield in comparison with the other combinations used. In the same time, Table (14-E) reflect the effect of interaction between explant source and shaking speed. It is quite obvious that *in vitro* explant source combined with either 50 or 75 rpm shaking speed gave the best protoplast yield as compared with the other combinations under study. However, combinations of *in vivo* explants with all investigated shaking speeds showed a reduction in protoplast yield obtained as compared with the other combinations with *in vitro* explant. Also, Table (14-F) clarifies the effect of the interaction between shaking period and speed. The results showed that 50 rpm shaking speed for 30 min. succeeded in enhancing the highest protoplast isolation followed with 75 rpm speed for either 15 or 30 min. then followed with 100 rpm for 30 min. in relation to other interactions. On the other hand, the lowest results obtained from those shaked for 60 min. compared with all shaking speed studied.

Furthermore, the interaction between explant source, shaking period and speed were shown in Table (14-G). It is clear that shaking *in vitro* explant at 75 rpm shaking speed for 15 min. induced the highest protoplast yield as compared with all other combinations. Increasing shaking period up to 60 min. combined with either *in vitro* or *in vivo* explant sources and with all shaking speed failed to produce sufficient protoplast yield. However, combination of *in vitro* explant with all shaking speeds for 30 min. shaking period took the second rank of protoplast yield isolated.

Generally the before mentioned results concluded that *in vitro* explant combined with 75 rpm shaking speed for 15 min. maximized protoplast yield. These results are somewhat agreed the findings of **Assani** *et al.* **(2002).** They reported that protoplast isolated from the leaves of banana (*Musa spp.*) incubated in enzyme mixture solution on agytatory shaker at 30 rpm speed.

Table (14): Effect of explant source, shaking period and speed on protoplast yield of banana *Grand Naine* cv.

Table (14-A): Effect of explant source.

Explant source	No. of Protoplast yield
In vitro	14.89
In vivo	9.36

Table (14-B): Effect of shaking period.

Shaking period	No. of Protoplast yield
15	18.60
30	21.05
45	7.56
60	1.32

Table (14-C): Effect of shaking speed.

Shaking speed	No. of Protoplast yield
0	1.87
50	18.95
75	16.09
100	13.47

Table (14-D): Effect of the interaction between explant source and shaking period.

Explant source / Shaking period (min.)	In vitro	In vivo
15	23.18 ± 10.68	14.00 ± 8.50
30	25.07 ± 10.59	17.02 ± 10.37
45	9.26 ± 5.47	5.86 ± 3.95
60	2.05 ± 2.72	0.58 ± 1.06

Table (14-E): Effect of the interaction between explant source and shaking speed.

Explant source / Shaking speed (rpm)	In vitro	In vivo
0	3.73 ± 3.38	0.0001 ± 0.0
50	20.50 ± 10.92	13.68 ± 8.83
75	19.42 ± 13.42	12.76 ± 9.59
100	15.92 ± 12.69	11.02 ± 8.94

Table (14-F): Effect of the interaction between shaking period and speed

Shaking speed (rpm) / Shaking period (min.)	0	50	75	100
15	3.11 ± 0.01	23.74 ± 0.45	26.59 ± 0.43	20.93 ± 0.34
30	3.79 ± 0.10	28.13 ± 0.98	26.55 ± 0.65	25.71 ± 0.88
45	0.56 ± 0.06	12.17 ± 0.81	10.26 ± 0.58	7.23 ± 1.55
60	0.0001 ± 0.0	4.29 ± 0.59	0.98 ± 0.32	0.0001 ± 0.0

Table (14-E): Effect of the interaction between explant source, shaking period and speed.

Shaking period (min.)	Shaking speed (rpm)	Explant source	
		In vitro	*In vivo*
15	0	6.23±0.02	0.0001±0.0
	50	28.94±0.77	18.55±0.22
	75	32.92±0.73	20.26±0.13
	100	24.66±0.57	17.20±0.12
30	0	7.58±0.20	0.0001±0.0
	50	31.91±0.72	24.36±1.29
	75	30.54±0.62	22.56±0.69
	100	30.28±1.26	21.15±1.29
45	0	1.12±0.12	0.0001±0.0
	50	14.89±0.53	9.46±1.09
	75	12.27±0.56	8.24±1.55
	100	8.73±1.63	5.73±1.63
60	0	0.0001±0.0	0.0001±0.0
	50	6.26±1.02	2.33±0.17
	75	1.96±0.64	0.0001±0.0
	100	0.0001±0.0	0.0001±0.0

4.2.2.a. Effect of different pore sizes of mish sieve and explant source:

Data of Table (15) and Fig. (12) involve the effect of different pore sizes of mish sieve and explant source on protoplast viability. It is clear that *in vitro* explant source had a positive effect on increasing protoplast yield than *in vivo* explant source. Meanwhile, the increase in protoplast viability was occurred when 25 mm pore size mish was used. However, the decrease in protoplast yield was observed when pore size of mish was increased above 25 mm.

Concerning the interaction between explant source and pore size sieve, it is shown that the highest number of protoplast was recorded when the combination of *in vitro* explant source and 25 Mm pore size mish was used followed by *in vivo* explant source and the same size of mish.

Table (15): Effect of explant source and different pore sizes of Mish sieve on protoplast yield of banana (*Grand Naine* cv.).

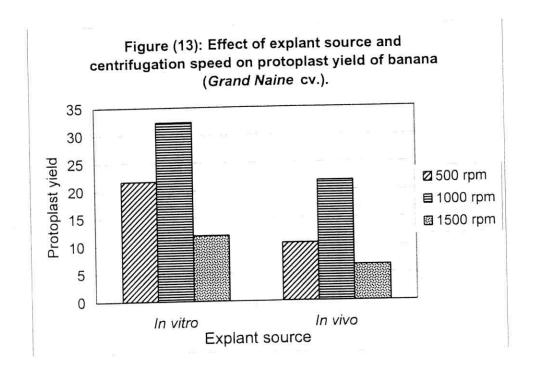| Explant source / Pore size	In vitro	In vivo	Mean
25	31.38 ± 1.10	21.76 ± 0.65	26.57
50	20.70 ± 0.69	11.07 ± 0.65	15.88
75	15.47 ± 0.95	3.72 ± 0.09	9.59
Mean	22.52	12.18	

Figure (12): Effect of explant source and different pore sizes of Mish sieves on protoplast yield of banana (*Grand Naine* cv.).

4.2.2.b. Effect of centrifugation speed and explant source:

Table (16) and Fig. (13) clarify the effect of centrifugation speed and explant source on protoplast viability. It is found that *in vitro* explant source induced an increase in protoplast viability as compared with those *in vivo* explant source. Meanwhile, the increase in protoplast yield was obtained when centrifugation speed 1000 rpm was used followed by 500 rpm speed and finally 1500 rpm which produced the lowest value of viable protoplast. Moreover, the combination of *in vitro* explant source and centrifugation speed at 1000 rpm increased protoplast respectively yield as compared with the other combinations used.

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Table (16): Effect of explant source and centrifugation speed on protoplast yield of banana (*Grand Naine* cv.).

Explant source Centrifugation speed (rpm)	In vitro	In vivo	Mean
500	21.78 ± 0.46	10.51 ± 0.93	16.14
1000	32.41 ± 1.05	21.78 ± 1.60	27.09
1500	11.90 ± 0.55	6.45 ± 1.02	9.17
Mean	22.03	12.91	

Figure (13): Effect of explant source and centrifugation speed on protoplast yield of banana (*Grand Naine* cv.).
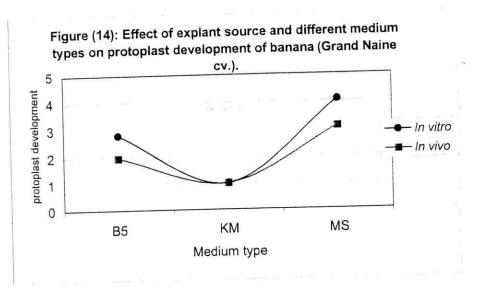
4.2.3. Protoplast culture:

4.2.3.a. Effect of different medium types and explant source:

Data of Table (17) and Fig. (14) reveal the effect of different medium types and explant source on protoplast development. It is found that protoplast development increased when *in vitro* explant source was used as compared with *in vivo* explant. Besides, Murashige and Skoog (MS) medium surpassed the other media used in increasing protoplast development followed by (B5) Gamborge medium then KM medium which gave the lowest values. However, *in vitro* explant source in combination with Murashige and Skoog medium improved protoplast development.

These results are confirmed with the findings of **Ochatt (1991)** on sweet cherry; **Tewary *et al.* (1995)** on Marus alba and **Ferraiolo (1994)** on kiwi fruit cultivars. They recommended Murashige and Skoog medium for protoplasts development.

Table (17): Effect of explant source and different medium types on protoplast development of banana (*Grand Naine* cv.).

Medium type \ Explant source	In vitro	In vivo	Mean
B5	2.80 ± 0.10	1.97 ± 0.06	2.38
KM	1.00 ± 0.00	1.00 ± 0.00	1.00
MS	4.07 ± 0.12	3.08 ± 0.06	3.57
Mean	2.62	2.02	

Figure (14): Effect of explant source and different medium types on protoplast development of banana (Grand Naine cv.).
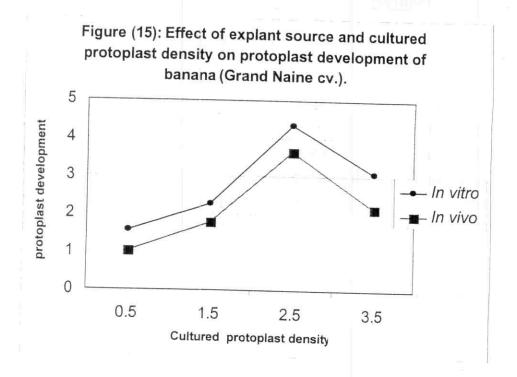
3.2.3.b. Effect of cultured protoplast density and explant source:

Data of Table (18) and Fig. (15) show the effect of cultured protoplast density and explant source on protoplast development. It is clear that *in vitro* explant source had a positive effect in improving protoplast development. Meanwhile, culturing protoplast density at 2.5×10^4 on the culture medium was more effective in increasing protoplast development as compared with the other densities used. Moreover, *in vitro* explant combined with protoplast density at 2.5×10^4 on MS medium caused an increase in protoplast development as compared with other interactions. These results go in line with the findings of **Kobayashi (1987)** that low cell density $(1-4 \times 10^4 / ml)$

Table (18): Effect of explant source and cultured protoplast density on protoplast development of banana (*Grand Naine* cv.).

Explant source Cultured protoplast density	In vitro	In vivo	Mean
0.5	1.57 ± 0.16	1.03 ± 0.58	1.3
1.5	2.26 ± 0.02	1.79 ± 0.03	2.02
2.5	4.35 ± 0.09	3.64 ± 0.07	3.90
3.5	3.09 ± 0.08	2.16 ± 0.01	2.62
Mean	2.82	2.16	

Figure (15): Effect of explant source and cultured protoplast density on protoplast development of banana (Grand Naine cv.).

Results and Discussion

4.2.3.c. Effect of explant source, auxin and cytokinin concentration:

Referring to the effect of explant source, Table (19-A) verifies that *in vitro* explant had a distinctive effect in increasing protoplast development than in vivo explant. However, Table (19-B) deals with the effect of auxin concentrations on protoplast development which clarify that continuous increasing of auxin concentrations from 0.0 up to 3.0 mg/L induced an visual increase in protoplast development. Meanwhile, the effect of cytokinin concentrations were recorded in Table (19-C). It is clear that there is a direct relationship between the increase in cytokinin concentrations from 0.0 up to 0.3 mg/L and the increase in protoplast development. Moreover, Table (19-D) deals the interaction between explant source and auxin concentration. It is obvious that the combination of the highest auxin concentration (3.0 mg/L) and either *in vitro* or *in vivo* explants gave the highest protoplast development as compared with the other interactions. Besides, Table (19-E) pointed out the effect of interaction between explant source and cytokinin concentration. It is clear that combination of explant source and cytokinin concentrations behaved similarly the combination of explant source and auxin concentration regarding their effect on protoplast development. Referring to the effect of auxin and cytokinin concentrations on protoplast development, it is appear from Table (19-F) that the highest protoplast development was occurred when highest concentration of both auxin and cytokinin (3.0 and 0.3 mg/L, respectively) were combined together. Furthermore, Table (19-G) explain the effect of the interaction between explant source, auxin and cytokinin concentration on protoplast development. It is quite

evident that the combination of 3.0 mg/L auxin, 0.3 mg/L cytokinin and either *in vitro* or *in vivo* explants caused the highest values of protoplast development.

Table (19): Effect of the interaction between explant source, auxin and cytokinin concentrations on protoplast development of banana (Grand Naine cv.).

Table (19-A): Effect of explant source.

Explant source	Protoplast development
In vitro	2.22
In vivo	1.96

Table (19-B): Effect of auxin concentration.

Auxin Conc. (mg/L)	Protoplast development
0.0	1.07
1.0	2.15
2.0	2.40
3.0	2.73

Table (19-C): Effect of cytokinin concentration.

Cytokinin Conc. (mg/L)	protoplast development
0.0	1.54
0.1	1.69
0.2	2.13
0.3	2.61

Results and Discussion

Table (19-D): Effect of the interaction between explant source and auxin concentration.

Explant source / Auxin Conc. (mg/L)	In vitro	In vivo
0.0	1.13 ± 0.08	1.00 ± 0.00
1.0	2.33 ± 0.37	1.96 ± 0.35
2.0	2.51 ± 0.4	2.28 ± 0.35
3.0	2.88 ± 0.9	2.58 ± 0.90

Table (19-E): Effect of the interaction between explant source and cytokinin concentration.

Explant source / Cytokinin Conc. (mg/L)	In vitro	In vivo
0.0	1.64±0.40	1.44±0.31
0.1	2.05±0.54	1.85±0.53
0.2	2.38±0.77	2.11±0.73
0.3	2.79±1.17	2.43±1.06

Table (19-F): Effect of the interaction between auxin and cytokinin concentration.

NAA Conc. / BAP Conc.	0.0	0.1	0.2	0.3
0.0	1.00 ± 0.0	1.09 ± 0.01	1.09 ± 0.00	1.09 ± 0.01
1.0	1.57 ± 0.06	2.28 ± 0.06	2.30 ± 0.09	2.43 ± 0.03
2.0	1.85 ± 0.05	2.35 ± 0.09	2.58 ± 0.03	2.85 ± 0.09
3.0	1.75 ± 0.06	2.08 ± 0.03	3.03 ± 0.08	4.07 ± 0.10

Table (19-G): Effect of the interaction between explant source, auxin and cytokinin concentration (mg/L).

NAA	BAP Conc.	Explant source	
		In vitro	*In vivo*
0.0	0.0	1.00±0.0	1.00±0.0
	0.1	1.18±0.02	1.00±0.0
	0.2	1.18±0.01	1.00±0.0
	0.3	1.17±0.02	1.00±0.0
1.0	0.0	1.73±0.06	1.40±0.10
	0.1	2.47±0.06	2.10±0.10
	0.2	2.47±0.06	2.13±0.15
	0.3	2.67±0.06	2.20±0.0
2.0	0.0	1.93±0.12	1.77±0.06
	0.1	2.40±0.10	2.3±0.10
	0.2	2.70±0.10	2.43±0.06
	0.3	3.03±0.06	2.67±0.15
3.0	0.0	1.90±0.04	1.60±0.06
	0.1	2.17±0.06	2.00±0.0
	0.2	3.17±0.15	2.90±0.10
	0.3	4.30±0.10	3.83±0.15

4.2.3.d. Effect of explant source and antibiotic:

Table (20) and Fig. (16) deal the effect of explant source and antibiotics. It is obvious that best protoplast development was obtained when *in vitro* explant source was used as compared with those of *in vivo* explant. Meanwhile, the combination of 0.4 mg/L ampicilin + 0.1 mg/L gentamycin + 0.1 mg/L tetracycline resulted in a decrease in contamination and in turn increase protoplast development as compared with the other treatments. However, the combination of (0.4 mg/L ampicilin + 0.1 mg/L tetracycline) took the second rank in enhancing protoplast development then followed by the combination of (0.4 mg/L ampicilin + 0.1 mg/L gentamycin) and the finally the control which produced the lowest values of protoplast development.

The above mentioned results reflected the importance of using the combined treatment (0.4 mg/L ampicilin + 0.1 mg/L gentamycin + 0.1 mg/L tetracycline) in improving protoplast development protocol.

Table (20): Effect of explant source and antibiotic on protoplast development of banana (*Grand Naine* cv.).

Antibiotic \ Explant source	In vitro	In vivo	Mean
Control	1.00 ± 0.00	1.00 ± 0.00	1.00
0.4 g / L Ampicilin	1.14 ± 0.01	1.00 ± 0.00	1.07
0.1 g / L Gentamycin	1.28 ± 0.02	1.00 ± 0.00	1.14
0.1 g / L Tetracycline	1.15 ± 0.02	1.14 ± 0.02	1.14
0.4 g / L Ampiciline + 0.1 g / L Gentamycin	2.92 ± 0.03	2.51 ± 0.01	2.71
0.4 g / L Ampicilin + 0.1 g / L Tetracycline	3.90 ± 0.08	3.52 ± 0.01	3.71
0.1 g / L Gentamycin + 0.2 0.1 g / L Tetracycline	2.20 ± 0.10	2.00 ± 0.00	2.10
0.4 g / L Ampicilin + 0.1 g / L Gentamycin + 0.1 g / L Tetracycline	4.27 ± 0.58	3.84 ± 0.03	4.05
Mean	2.23	2.00	

Figure (16): Effect of explant source and antibiotic on protoplast development of banana (*Grand Naine* cv.).

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