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

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Using of biotechnology in in vitro propagation

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USING OF BIOTECHNOLOGY IN *IN VITRO* PROPAGATION AND DEVELOPMENT OF SOME DECIDUOUS FRUIT ROOTSTOCKS

By

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1. INTRODUCTION

Pear fruits are greatly required for their high nutritive value and high net return. Pear trees as pome fruits were tolerant to poor drainage. fine soil texture, and low chilling requirements for buds. These conditions encouraged pear growers to horizontal extension of pear orchards. The earliest record of pear cultivation in Europe is that provided by Homer who around 1000 B.C., wrote that pears were "one of the gifts of golds".

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

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

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

Fire blight disease is the dangerous problem for pear especially those grafted on communis pear. Large numbers of pear farms were destroyed as a result of fire blight disease.

The only alternative is finding out a new rootstock effective in controlling fire blight and encouraging high yielding as well as fruiting qualities. Establishing a breeding program for producing a new rootstock for pear combine the best characters of commuins pear and tolerance of fire blight is the most important step in overcoming fire blight problem .

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

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

Protoplasts provide the starting point for many of the techniques of genetic manipulation of plant in particular the induction of somaclonal variation, somatic hybridization and transformation .

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

2. REVIEW OF LITERATURE

Many investigators dealing with protoplast, isolation, purification, culture and plantlets regeneration related to many plant species were recorded. In this sphere, the following reviews on some protoplast parameters .

II.1. Pre-protoplast isolation :

II.1. a. Effect of anti-oxidant treatments :

Zaied (1997) recommended using of 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 fruits.

Bayomy (1998) stated that the combination of anti-oxidant solution as explant pretreatment plus PVP in the medium were effective in reducing phenolic compounds in pome fruits (communis pear and MM . 106 apple rootstocks) .

Liu *et al.*, (2003) point out that addition of P.V.P in the enzyme solution were enhanced protoplast yield and viability of *Prunus davidiana* afrench and wild apricot .

Hassan (2004) declared that the accumulation of phenolic compounds in the medium of apple rootstocks caused oxidation and finally the death of the established explant. The exudation was greatly reduced when P.V.P was added to the cultured medium then followed by pretreating the explants with anti-oxidant solution .

El-Hadidy (2005) found that accumulation of phenolic compounds were decreased to the most lower level when applying combined treatment of anti-oxidation solution and P.V.P in the medium of Marianna plum rootstock .

II.1.b. Effect of plasmolysis (pretreatment):

Hurwitz and Agrios (1984) succeeded in isolation of protoplasts from callus and cell suspension of apple when explants incubated in an enzyme solution without pretreatment or without plasmolysis.

Wallin and Welander (1985) showed that plasmolysis of isolated protoplasts from tissue of mesophyll leaf of apple genotype Akero-M 26 occurred in W5 with 2% PVP. 25 for 30 min + preculture in medium with 0.5 mM MES for 15 days.

Patat-Ochatt *et al.*, (1988a) obtained the highest protoplasts yield from apple genotype bramley shoot callus (3.6 X 10^6) with 92% viability when plasmolyzed in CPW 13M as a pretreatment before incubation in enzyme solution digestion for 1h .

Patat-Ochatt *et al.* (1988b) found that plasmolysed of isolated protoplast from leaves of apple genotype M9 took place in CPW medium with 13% maniitol (CPW 13 M medium) for 1h.

Ochatt (1993a) reported that the highest protoplasts yield and viability of ***pyrus spp.*** (pear) were achieved by plasmolized tissue for at least 1h in the same solution as used for isolation devoid of enzymes.

Ochatt (1993b) found that plasmolized tissue of *Pyrus communis* was noticed when CPW salts medium supplemented with 0.7 M mannitol, 1% PVP-10 and 5mM MES at pH 5.6 for 60-90 min were used .

Patat- Ochatt *et al.* (1993) mentioned that the highest yield of viable protoplasts from leaves of apple cv. Halpoid G. Delicious were achieved by plasmolysis for 1h in CPW 13M.

Ochatt (1994) recommended rinsing of isolated protoplasts from mesophyll tissue of *in vivo* apple in a solution of 6% mannitol while *in vitro* leaves in CPW medium with 0.5 M mannitol then followed by 30 min in CPW medium 0.7 M mannitol, for the best plasmolysis .

Iasi *et al.* (1994) declared that plasmolized tissue from black berry and raspberry cultivars for 60 min at room temperature in CPW 13% sucrose before incubation in enzyme solution enhanced the best yield and viability of protoplasts.

Vieira and Dornelas (1996) showed that the isolated protoplasts from passion fruit tissues were plasmolyzed for 20 min in a CPW solution containing 5 mM MES and 13 % mannitol at pH 5.8.

Mehri (2003) pointed out that the isolated protoplasts from leaf mesophyll of *Prunus cerasus* L. cv. Montmorency were plasmolyzed for 1 hour in CPW salts 13% mannitol (CPW 13M) solution.

II.2. Protoplast Isolation:

II.2.a. Effect of digestive enzyme medium type:

Kouider *et al.,* (1984) concluded that CPW 9M was the best medium for enzyme mixture of apple genotype joaathas for protoplasts isolation from leaves callus and cell suspension.

Wallin and Welander (1985) declared that K_3 medium was the best one for enzyme mixture to isolate protoplast from apple genotype Akerö-M-26.

Wright (1985) obtained the best protoplast yield from leaves of grapevine when enzyme mixtures were dissolved in MS medium.

Wu and Kuniyuki (1985) found that the medium consisted of 1.3 mM MES, 7mM $CaCl_2$. $2H_2O$, 0.7 mM NaH_2PO_4. and 0.6 M mannitol with pH 5.3. was the best medium for dissolving of enzyme solution for isolation of *Prunus amygdalus* protoplasts from stem and cell suspension culture .

Matsuta *et al.,* (1986) reported that mixing of enzyme mixture in medium formulated from 10mM $CaCl_2$. $2H_2O$, 1mM KH_2 PO_4, 0.25% mannitol and 0.25% sorbitol to isolate protoplasts from cell suspension and leaf callus origin of *Prunus persica* .

Ochatt and Caso (1986) pointed out that modified MS medium at 1/10 major salts was the best enzyme medium used for leaf (field or *in vitro)* protoplast isolation of *Pyrus communis* var. pyraster .

Masuda *et al.,* (1987) cited that CPW medium was the best enzyme medium for isolation *Malus prunifolia* var. ringo protoplasts .

Revilla *et al.*, (1987) found that CPW 13M. medium supplemented with 1% PVP and 0.5 mM MES was the best enzyme medium for protoplasts isolation from leaves of stone fruits (*Prunus spp.)*.

Patat- Ochatt *et al.*, (1988) reported that the best digested enzyme medium was CPW 13 M medium to isolate protoplast from leaves of apple genotype M9 .

Ochatt (1992) reported that CPW 13M supplemented with 1% P.VP and 0.5 mM MES was the best digestive enzyme medium for isolation protoplasts of *Prunus cerasifera in vitro* leaves.

Mills D. and Hammerschlag (1994) mentioned that the best enzyme mixture medium for isolation of protoplasts of peach (*Prunus persica*) was CPW salts medium .

Ochatt S. J. (1994) recommended isolation of protoplasts from *in vivo* apple mesophyll tissue by using digestive enzyme mixtures dissolved in CPW medium .

Li, *et al* (1995) indicated that the best isolated protoplast from peanut was obtained when enzyme mixture dissolved in CPW 9M medium.

Vieira and dornelas (1996) reported that the high yield from the passiflora protoplast was showed when using CPW medium .

Witjaksono and Grosser (1998) mentioned that MS medium was suitable for digestive enzyme mixtures to isolate

protoplast from embryogenic suspension of avocado (*Persea Americana*).

Jardak, *et al.* (2002) stated that CPW 13 medium without kanamycin selection were cultivated to isolate protoplast from *vitis vinifera spp* .

Mehri (2003) found that CPW 13M was highly efficient medium for digestive enzyme mixture to isolate protoplast from *Prunus carasus* L. cv" Montmorency"

Chikako and Takaya (2006) found that the digestion medium (0.4 M, 0.5 M, or 0.6 M mannitol, 1 mM $CaCl_2$, 0.1% bovine serum albumin (BSA), supplemented with 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)–KOH pH 5.5, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) was the best medium for isolate protoplasts from peach fruits **.**

II.2.b. Effect of enzyme type:

Theodoropoulos and Roubelakis (1990) mentioned that protoplasts isolated from leaf mesophyll of *vitis vinifera* L. increased as enzyme mixture of containing 15 U ml^{-1} cellulase R-10 and 15 U ml^{-1} Macerozyme R-10 were used.

El-Gindy and Gray (1991) reported that protoplasts isolated successfully from leaf explants of Vitis vinifera cv. Thompson seedless by using enzyme mixture containing 1% pectolyase –Y23 and 2%celluase R-S .

Marino (1991) Found that the best digestive enzyme solution for protoplast isolation of apricot *(prunus armeniaca l.)*

was that consisted of 0.5% Maccerozyme R-10 , 0.01% Pectolyase Y-23 , 1% cellulose Onozuka Rs and 0.4%driselase .

Huancaruna Perales and Ottoschieder (1993) mentioned that protoplasts were isolated successfully from young leaves by using a solution of enzymes i.e. 2% (w/v) cellulase R-10, 1% (w/v) hemicellulase and 0.3% driselase.

David Mills and Hammerschlag (1994) declared that the combination of cellulase Onozuka R10 (2%) and Macerase 0.5% was the best enzyme mixture for protoplasts isolation of peach (*prunus persica*).

Pan Zen-Guang *et al.* (1997) succeeded to isolate protoplast from Hepan crab apple (*Malus hupehensis*) by using a solution containing 0.5% cellulase R-10 and 0.2% pectinase.

Saito and Suzuki (1999) verified that enzyme solution containing 2% cellulase Onozuka RS, 0.1% Pectolyase Y-23 is the best one for protoplast isolation of apple (Malus X domestic acv. Fuji).

Assani *et al* (2002) found that higher numbers of protoplasts from *Musa spp* was obtained when using enzyme solution supplemented with cellulase, pectolyase and hemicellulases .

Commun *et al* (2003) showed that the best yield of protoplasts from grapevine leaves when 0.1% cellulase RS and 0.05driselase were used .

Jihong liu *et al.,* (2003) Verified that protoplast isolation from the 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 .

Mehri (2003) found that protoplast isolation from leaf mesophyll of *Pronus carasus* L. cv" Montmorency" required an incubation in a solution supplemented with 1% cellulase, 1% hemicellulase and 1% pectolyase Y-23.

Mliki *et al* (2003) Verified that incubation of Tunisian grapes explants in a solution supplemented with enzyme mixture containing 0.25% cellulase of *Aspergillus niger* , 0.25% cellulase of *Penicillium funiculosum*, 0.5% cellulysin of *Trichoderma viridae* and 0.2% macerozyme R-10 of *Rhizopus sp*. Encouraged the highest protoplast isolation .

Zhu, *et al* (2005) cited that in Echinacea angustifolia, the rate of protoplast yield was increased when enzyme mixture containing cellulase increased to 2.0% (W/V) .

Assani, *et al.* (2006) found that the highest yield of protoplasts from Musa spp were obtained when enzyme mixture containing 1% cellulase + 1% Macerozyme +1%pectinase was used .

Chikako and Takaya (2006) found that the digestion was started when used digestion medium containing 2% (w/v) 'Onozuka' cellulase RS and 0.2% (w/v) 'Onozuka' macerozyme R-10.

Segui *et al.* (2006) reported that the best yield of protoplasts from apple *(Malus domestica* var. fuji) was obtained

when using a solution supplemented with 0.5% (w/v) pectinase and 2% (w/v) cellulase .

Qinghua Zhang *et al.* (2006) found that the highest protoplasts of Citrus unshiu isolated from suspension cells, by using enzyme solution containing 1% cellulase Onozuka R-10 and 1.5% macerozyme R-10.

Omar and Grosser (2007) reported that digestion of suspension culture tissue of 'Hamlin' sweet orange overnight in mixture of cellulase and macerozyme R-10 enzymes and achieved successfully isolation of protoplasts.

II.2.c. Effect of protoplast source:

Ochatt and Caso (1986) stated that the yield of protoplasts isolated from *in vitro* leaf mesophyll of wild pear *(Pyrus communis*) were higher as compared with those isolated from field leaf mesophyll plants.

Ochatt *et al.* (1988) reported that the highest yield of protoplasts of *Pyrus communis* L. was obtained from embryagenic callus.

Saito *et al.* (1989) found that both nuclear callus and leaves of *Malus pumila Mill were* the best effective sources for protoplast isolation.

Wallin and Johansson (1989) reported that leaves of *Malus x domestica* were the best source for protoplast isolation as will as produce the highest yield.

Brisset *et al.* (1990) indicated that mesophyll protoplast isolation was resulted from axenic shoot cultivars of *Pyrus communis*.

Ochatt and Chevrean (1991) showed that large numbers of viable protoplasts from *Passe crassane and old home pear (pyrus communis l.)* was obtained when leaf mesophyll was taken as aprotoplast source .

Matsuta (1992) indicated that suspension cells of peach *(Prunus persica L. Batsch*) was the best source for protoplast isolation.

Ochatt *et al.* (1992) Verified that *in vitro* shoots of *Prunus cerasus* and *Prunus spinosa* were the best protoplast source to produce the highest yield of protoplasts .

Ochatt *et al.* (1993a) used leaf mesophyll of *Pyrus communis* as protoplast source to get viable protoplasts.

Patat-ochatt *et al.* (1993) reported that higher yield of protoplasts from haploid golden delicious apple clone *(Malus X domestic)*was achieved by used in vitro grown leaf and stem tissues as a protoplast source.

Ding *et al.* (1994) found that the best explant for protoplast isolation was the cell suspension of callus from ovules of *Malus pumila*.

Mills and Hammerschlag (1994) observed that small leaves, 4-10 mm in length were superior as explant source for protoplast isolation than medium or big expanded leaves, 22-30

mm in length of *in vitro* propagated peach *(Prunus persica*) plant.

Ding *et al.* (1995) found that leaves of *Malus x domestica* was the best source of protoplast isolation.

Gan *et al.* (1996) reported that intact viable protoplasts were isolated from callus of peach (*cv. Sunagowase*).

Zhu-YanMing *et al.* (1997) declared that leaves- derived from embryogenic callus of *vitis vinfera* achieved the highest yield of protoplasts.

Kondakova (1999) preferred isolating mesophyll protoplast from callus of *prunus domestica cv. Questche*.

Pan-Zeng Guang and Deng-Xiuxiu (2000) showed that using of leaves and suspension. Cultured cells as a protoplast source induced the higher yield of viable protoplasts of apple plants.

Jardak *et al.* (2002) cited that large yield of viable protoplasts was obtained from leaves and embryogenic tissue of grapevine cv. Sakasly and Muscat d' Alexandria .

Mehri (2003) mentioned that leaves (leaf mesophyll and leaf callus) of *Prunus carasus* l. cv "montmorency" were successfully used as a protoplast source.

Mliki *et al.* (2003) tested the effect of protoplast source on protoplast yield of two Tunisian grape cultivars, sakasly and Muscat d'Alexandrie. They found that the best protoplast yield was obtained from the *in vitro* leaves of 4 to 5 week –old.

Hassan (2006) reported that the yield of protoplast isolated from *in vitro* explant of Pineapple were higher as compared with those from *in vivo* explants .

II.2.d. Effect of Osmotic pressure factors:

Wallin and Jonhansson (1989) pointed out a large number of viable protoplasts were obtained from leaf mesophyll of a columnar apple by using sucrose as plasmolyticum.

Barbier and Bessis (1990) observed that the highest protoplast yield (up to 40 x106) protoplasts/g leaf tissue of grapevine *cv.* Chavdonnay was obtained by using enzyme solution with 0.7 M mannitol.

Marino (1991) found that increasing viable protoplast yield of Apricot *Prunus armeniaca l.* was observed by using a solution supplemented with of 0.1% 2-(N-morpholino) ethanesulfonic acid and 9.11% mannitol.

Mii *et al.* (1991) found that 0.5 M glucose in the culture medium of *Vitis labruscana* Bailey and *Vitis thunbergii* improved protoplast viability and yield.

Matsuta (1992) found that the best results of protoplast viability derived from Peach *Prunnus persica.* was npticed by causing medium supplemented with glucose.

Ochatt and Cherveau (1991) observed that large numbers of highly viable mesophyll protoplasts were isolated from shoot cultures of *scion cv. Passe crassane* and rootstock genotype old home of common pear were observed on medium supplemented with 0.5 M mannitol.

Ochatta (1993a) found that the best results of protoplast viability of leaf tissue from *Pyrus spp* were showed by culturing on CPW salts medium plus0.7 Mmannitol, 1%PVP and 5mM MES for 60-90 min .

Ding *et al.* (1994) declared that 0.65 M mannitol in the isolation medium of *in vitro Malus pumila* cell suspension gave the best results of protoplast viability.

Mills and HammerSchlag (1994) found that using of 0.7m Mannitol or 0.45 M Sucrose in culture medium of peach gave the highest yield of viable protoplasts.

Panis, *et al.* (1994) found that the best plasmolyzed medium for *Muse spp*. were showed when medium containing 7mM $CaCl_2 . 2H_2O_2$, 0.7 M $NaH_2PO_4\ 2H_2O_2$, 3mM MES and 10% mannitol was used.

Vieira and Dornelas (1996) reported that the highest viable protoplast and yield were obtained from Passiflora specices (passion fruit) by using CPW medium containing 5mM MES and 13% mannitol .

ZengGuang *et al.* (1997) cited that the best yield of protoplast and viability of Hepen carb apple was observed by using enzyme solution with 0.65 M glucose .

Fotouhi and Hamid (1998) indicated that culturing of protoplasts isolated from cell suspension on CPW nutrient medium supplemented with enzyme solution and 13% (W/V) mannitol gave the highest protoplast viability.

Kondakova (1999) showed that viability of protoplast from leaf mesophyll of *Prunus domestica cv.* Quetche was observed when 0.7 M mannitol as osmaticum was used.

Mehri (2003) reported that the best yield and viability of protoplast of *Prunus cerasus L.* isolated from leaf mesophyll and leaf callus was achieved by using enzyme solution containing 13%mannitol and 5mM MES .

Segui *et al.* (2006) found that the best viable of protoplast produced from apple *(Malus domestica* var. fuji) when used 0.8%M mannitol as osmaticum .

II.2.e. Effect of incubation conditions :

Matsuta *et al.* (1986) indicated that incubation of tissues from cell suspension and leaf callus origin of *Prunus persica* in enzyme solution for 5h on shaker at 40 rpm and 27°C was recommended for isolating the highest numbers of protoplast.

Ochatt and Caso (1986) recommended incubation of *Pyrus communis* var pyraster leaf (field or in vitro) in enzyme mixture at 25±1°C for 8h with rotary shaker at 100 rpm.

Wu and Kuniyuki (1985) reported that high yield of protoplasts from *Prunus amygdalus* was obtained when

incubated in enzyme solution at the room temperature for 3-4h by using rotary shaker at 40-60 rpm.

Ochatt *et al.* (1987) observed that isolated protoplast from *Prunus avium* X *pseudocerasus* was obtained when incubated in enzyme mixture for 17h and 25°C.

Masuda *et al.* (1987) showed that the best incubation conditions for *Malus prunifolia* var. ringo was in enzyme isolation at 25°C for 15 hours.

Ochatt *et al.* (1988) found that the best incubation of *Prunus cerasus* to isolate protoplasts from leaves was incubated in enzyme solution for 17h, shaken (40 rpm) under 500 Lx illumination at 25°C.

Patat- Ochatt *et al.* (1988) showed that apple (*Malus domestica* Borkh) was incubated in enzyme solution at 25°C in the dark for 18 hours.

Marino (1990) 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.

El-Gindy and Gray (1991) mentioned that highest yield of *Vitis vinifera* cv. Thompson seedless leaves was obtained when incubated in enzyme solution for 3 hours at 25°C.

Ochatt, (1993a) showed that the highest protoplast yield of *Pyrus* spp (Pear) were obtained by incubation for overnight ((16-20h) .

Mills and Hammerschlag (1994) found that high yield of

protoplast from Peach (*Prunus persica*)was observed when incubation in enzyme mixture in the dark under 25 C^o for 16h .

Ding *et al.* (1994) observed that protoplast yield increase was isolated from suspension cell of *Malus pumila* when incubated for 6 h 26°C.

Saito and Suzuki (1999) showed that incubation of meristem- derived callus protoplasts of apple (*Malus X Domestica* cv. "fuji") in enzyme solution at 25°C with gentle shaking at 120 rpm for 2h. was preferable.

Commun, *et al.* (2003) observed that the most effective incubation period on grapevine protoplast yield was 16h at 25 C^o in darkness .

Mehri (2003) pointed out that highest protoplasts yield was isolated from leaf mesophyll and leaf callus of *Prunus cerasus* when incubated for 8 hours on a rotary shaker (40 rpm) at 25°C and in the dark.

Liu *et al.* (2003) found that best result of isolated protoplast of woody plants like apple pear and peach was achieved when the explants incubated in enzyme solution from several hours to one day at 25-28°C in the dark.

Mliki *et al.* (2003) reported that high yield of viable leaf mesophyll from Tunisian grapes protoplast was achieved by digested for 13h under 25 rpm agitation the enzyme solution.

Chikako and Takaya (2006) declared that the best incubation period in protoplast isolation from peach fruits was 4 h at 30 °C which achieved the protoplasts yield.

II.2.f. Effect of shaking:

Niizeki *et al.* (1983) pointed out that protoplsdt isolated

from ***Malus pumila*** callus pollen origin was increased when incubated in enzyme solution under 60rpm shaking .

Hurwitz and Agrios (1984) claimed that protoplast isolation of apple callus and cell suspension leaf (*in vitro)* origin was achieved by using shaker at 80 rpm for 4-5hours .

Wallin and Welander (1985) showed that protoplasts yield of ***Malus pumila*** leaves (in vitro) were immersed in enzyme solution roching at oscillations per min for last 30 min.

Wu and Kuniyuki (1985) indicated that incubation of tissues from *Prunus amygdalus* cell suspension stem callus origin in enzyme solution rotary shaking at 40-60 rpm was recommended to isolate protoplast **.**

Matsuta *et al.* (1986) found that incubation of tissues from cell suspension and leaf callus origin of *Prunus persica* in enzyme solution for 5h on shaking at 40 rpm was recommended for isolate protoplast .

Ochatt and Caso (1986) reported that protoplast isolated from *Pyrus communis* var. pyaster (field or *in vitro*) was increased when incubated in enzyme solution under rotary shaker at 100 rpm.

Ochatt *et al.* (1987) obtained protoplast from leaves (*in vitro*) of *prunus avium* x *pseudocerasus* when incubated under shaking at 40 rpm.

Ochatt, (1993a) reported that using shaker at 40 rpm was achieved highest protoplast yield from *Pyrus spp*. (pear) .

Mills and Hammerschlag (1994) observed that highest yield of peach (*Prunus presica*) protoplasts was achieved by using a shaker on 50 or125 rpm which yielded the amount of protoplasts.

Li *et al.* (1995) maintained protoplast of peanut was obtained when a rotatory shaker at 85 rpm and 26°C was added.

Vierira and Dornelas (1996) found that protoplast yield of Passion fruit were dissolved in enzyme solution shocked at 60 rpm at 25±2C^{o} in the dark .

Joshing Liu *et al.* (2003) reported that the optimum revolution speed of ratory shaker differs among species for instance 25-30 rpm for citrus ,40-50 rpm for mango and 40-100 rpm for apple ,pear and peach for protoplast isolation .

Mehri (2003) found that protoplast isolation from *Prunus cerasus L.* was achieved by shaking the enzyme solution on a rotary shaker of 40 rpm at 25°c in the dark for 8 hours .

Hassan (2006) observed that the highest protoplast yield from *in vitro* explant of either Pineapple or Banana were obtained by shaking the enzyme solution on shaker at 75 rpm for 15 min .

Segui *et al.* (2006) declared that the best yield of protoplast from apple *(Malus domestica* var. fuji) was achieved when used shaker at 100 rpm for 1 hour after the first hour of digestion .

II.3. Filtration:

II.3.a. Effect of pore size of mish sieve:

Vardi and Esra (1989) found that the isolated protoplast of citrus was filtered through 50 µm and 30 µm nylon screens.

Ochatt (1993) found that high yield of protoplast from *Pyrus* spp (Pear) was obtained in enzyme medium then filtered through 50-80 µm pore-sized mesh.

Mills and Hammerschlag (1994) reported that the protoplast from Peach (*Prunus* spp) were liberated from mesophll cells with pastier pipette and passed through 3 stainless sieves of 125, 94 and 43 µm .

Iasi *et al.* (1994) observed that the isolated protoplasts of blackberry and raspberry were passed successfully through a 50 µm nylon mesh.

Panis, *et al.* (1994) found that increasing of protoplast yield from cell debris of Musa spp were obtained by sieving through a100 µm and subsequently a25 µm sieve.

Li, *et al.* (1995) reported that the digested cell from protoplast of Arachis species (Peanut) were passed through as sterile 40µm and then a40 µm nylon membrane .

Witjaksono and Grosser (1998) declared that sterile stainless steel sieve 45 µm diameter was suitable for isolation of avocado protoplast.

Katia Commun *et al.* (2003) found that the isolated protoplasts from grapevine were passed successfully through 100-50 µm sieves .

Mehri, (2003) declared that the releasing of Pronus cerasus protoplast from leaves filtered through 50µm nylons sieve .

Chikako and Takaya (2006) found that filtered of protoplasts from peach fruits through a tea strainer and a 125 µm nylon mesh. was achieved highest protoplasts yield.

Hassan (2006) observed that increasing the protoplast yield of in vitro explant from either Pineapple or Banana was superior when using 25 µm pore size mesh sieve.

Qinghua Zhang *et al.* (2006) found that the digested mixture was passed successively through 100 and 45 µm stainless steel sieves to remove undigested cells and cellular debris of Citrus unshiu .

Segui *et al,* (2006) reported that the macerated suspension was filtered through a nylon mesh (300 µm openings), and the obtained suspension was filtered again through a nylon net of 120 µm openings (Millipore).

II.3.b. Effect of centrifugation:

Ochatt, (1993) found that digesting of tissues from *Pyrus spp* (Pear) in an enzyme solution then centrifuge at 100g for 5min enhanced protoplast isolation ,

Mills and Hammerschlang (1994) found that isolation protoplast from mesophyll cells of (*Prunus spp*) Peach were washed by centrifugation at 200g for 3min .

Li, *et al.* ((1995) declared that protoplast of Arachis

species (Peanut)was isolation in the interphase by centrifugation at 100g for 8min.

Vieira and Dornelas (1996) showed that protoplast isolated from passion fruit tissue was isolated when pelleted by centrifugation at 1500 rpm for 7 min.

Witjaksono and Grosser (1998) observed the isolated protoplast of avocado when solution centrifuged at 100 g for 5 min.

Assani, *et al.* (2006) found that isolated protoplast from large cell colonies of Musa spp was washed three times through centrifugation (656g for min) with a washing solution that consisted of 204 mM Kcl, 67mM $CaCl_2$ with ph 5.7 .

Chikako and Takaya (2006) declared that protoplasts from peach fruits were assembled to the bottom of the mannitol medium by centrifugation at 300 × *g* for 10 min at 4 °C.

Hassan (2006) reported that using centrifugation at the rate of 1000 rpm was maximized the protoplast yield of Pineapple and Musa spp.

Qinghua Zhang *et al.* (2006) 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* .

Segui *et al.* (2006) reported that the best yield of protoplast from apple *(Malus domestica* var. fuji) was achieved when used centrifugation for 3 minutes at 700 rpm .

II.4. Protoplast culture :

II.4.a. Effect of medium type:

Kao and Mychayluk, (1975) basal medium and the culture medium of leaf callus protoplast from *Prunus cerasus* was MS basal medium.

Ochatt *et al.* (1987) found that culture media in colt cherry (*Prunus avium x pseudocerasus*) protoplasts was based on MS salts supplemented with 9% (W/V) monnitol.

Wallin and Johansson (1989) reported that the high division of protoplasts of apple when cultured on modified ppp medium supplemented with 1 mg/litre zeatin and 2 mg/litre 2,4-D.

Marino (1990) mentioned that growing of cell colonies of protoplasts which isolated from grape (*Vitis vinifera* L.) cv. Trebbiano diromagna was occurred after 20 days in solid medium but not in liquid medium.

El-Gindy and Gray (1991) found that the best medium for culturing protoplast which isolated from leaf explants of *Vitis vinifera* cv. Thompson seedless was MS medium supplemented with 6% sucrose and 0.25N nitrogen.

Marino (1991) reported that induction of cell division from protoplast of Apricot (*Prunus armeniaca* L.) when protoplast cultured on liquid media consisting of Kao and Michayluk macronutrients and MS micronutrients for 15 days and then transferred to solid media with reduced mannitol concentration.

Mii *et al.* (1991) stated that culturing of protoplast of grape spp. *Vitis labrusca* Bailey and *Vitis thunbergii* in liquid or gellan gum-solidified media, enhanced protoplast division after 4 days from culturing date.

Ochatt (1991) reported that induction of cell division from protoplast of *Prunus ceraifera* and *Prunus spinosa* when protoplast cultured no modified MS medium and transferred to arrange of MS-based media.

Ochatt and Chevreau (1991) cited that protoplasts of passé crassane and old Home pear (*Pyrus communis* L.) were cultured as liquid layers in supplemented ammonium. Free MS medium and was subcultured on half-strength MS medium with supplements.

Reustle and Alleweldt (1991) mentioned that microcalluses were formed from protoplasts of grapevines by culturing on VK/M and CPW media.

Ochatt. (1993) recommended culturing protoplast of *Pyrus spp.* on half-strength of MS based medium was the best culturing medium.

Perales and Schieder (1993) reported that MS or MI medium supplemented with 2.2 µM BA, 2.6µM NAA and 2.2µM 2,4-D was the best media for culturing protoplasts from apple.

Phosang *et al.* (1994) said that best cell division of grape mesophyll protoplasts was obtained when protoplast cultured on Gamborg's B_5 liquid medium supplemented with 5MM 2,4-D, 2.5 MM BA and 0.6M sorbitol.

Reustle and Natter (1994) pointed out that using different modified culture media (CPS-13, V/KM and MS-P) in grapevine protoplasts (*Vitis sp.*) was no significant differences in the suitability of the different culture media were found.

Ding *et al.* (1995) found that K8P medium was the best medium encouraged development of protoplast that isolated from cotyledon and suspension cell of apple (*Malus x domestica*) cv. starkrimson.

Reustle *et al.* (1995) pointed out that culturing grapevine (*Vitis sp*.) on CPW-13%manitol medium was effective in enhancing protoplast development.

Vieira and Dornelas (1996) reported that cell division of passion fruit were obtained when protoplast cultured on liquid KM8Pmedium .

ZengGuang *et al.* (1997) said that culturing of protoplast from Hepen crab apple (*Malus hupehensis*) on MT solid medium containing 1mg/Liter BA and 1mg/Litre 2,4-D.

Kondakova (1999) recommended culturing of the mesophyll protoplast of *Prunus domestica* on MS basal medium supplemented with 0.5 M sucrose.

Saito and Suzuki (1999) reported best results of cell division from protoplasts of apple (*Malus X domestica*) cv. "fuji" 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.

Pan-ZengGuang and Deng-Xiuxiu (2000) tasted that growth and development of apple protoplasts from leaves and suspension0 cultured cells was significantly affected by MT culture medium was modified and supplemented with VC (ascorbic acid), benzyladenine, 2,4-D, glutamine, CH (cusein hydrolysate) and glycose.

Blackli *et al.* (2002) found that isolated protoplast of monocotyledonous cultured in liquid medium.

Jardak *et al.* (2002) found that culturing of the grapevine protoplasts on CPW-13 medium.

Commun, *et al.* (2003) reported that the culturing of mesophyll from grapevine were planted in liquid medium .

Mehri (2003) reported that the culture medium of leaf mseophyll protoplast from *Prunus cerasus* was KMl .

Mliki *et al.* (2003) recommended culturing of the leaf protoplasts of Tunisian grapes cv. sakasly and Muscat d'Alexandrie on CPW-13 medium.

Omar and Grosser (2007) recommended culturing of embryogenic suspension protoplast of 'Hamlin' sweet orange on Mt basal medium supplemented containing 8-P multivitamin, organic acid, and sugar-alcohol additive.

II.4.b. Effect of cultured cell density:

Niizeki *et al.* (1983) Reported that the best result of division was obtained when protoplast density was 1.2 x 10^5 protoplast/ml of *Malus pumila* .

Wu and Kuniyuki (1985) Showed that high development of *Prunus amygdalus* protoplast appeared when density was 10^6 protoplasts per ml.

Matsuta *et al.* (1986) found that highest callus of *Prunus persica* cell suspension and leaf callus origin protoplast was observed when protoplast density was 10^5 protoplast per ml in liquid medium at 28^{o} C in the dark.

Ochatt *et al.* (1987) Declared that culturing of protoplast at adensity of 0.5 x 10^5 protoplasts/ml on MS medium of *Prunus avium* x *Pseudocerasus* leaf protoplasts was achieved highest division and callus formation .

Ochatt and Caso (1988) Mentioned that highest callus formation of *Pyrus communis var.* Protoplasts were achieved when planted at a density of 10^5 protoplasts/ml .

Ochatt *et al.* (1988) Showed that protoplasts of *Prunus cerasus* were successfully cultured on MS medium by used protoplast density in 0.5 x 10^5 protoplasts/ml .

El-Gindy and Gary (1991) Pointed out that cell division and development from *vitis vinifera cv.* Thompson seedless occurred most successfully at a density of 1.0x10^5 protoplasts/ml cultured in light.

Marion (1991) Found that protoplasts from apricot *Prunus armeniaca L.* were platted at 5 x10^4 /ml and cultured in darkness in liquid medium after 7 to 9 days about 10% of protoplasts were dividing .

Ochatt (1992) observed that the best protoplast density which achieved the best development in *Prunus cerasifera* and *Prunus spinosa* was 10^5 protoplasts/ml .

Ochatt (1993a) 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.*

Vieira and Dornelas (1996) found that highest resuspend the pellet protoplast of passion fruit were obtained when plated at a density $1x10^5$ protoplasts/ml $2x10^5$ protoplasts/ml .

Huy *et al.* (1997) showed that highest callus of blackberry cultivars was observed when protoplast density was 1.6 x 10^5 protoplast per g FW^{-1}.

Zhu-YanMing *et al.* (1997) found that culture of protoplasts from grapevine *vitis vinifera L.* at a density of $1x10^5$ protoplasts/ml. Was achieved of cell division.

Saito and Suzuki (1999) found that 10^6 / ml of protoplast from apple for good cell development was the best when number of cells were cultured on KM8P medium with pH 5.7 and containing 2, 4-D, IBA, BA .

Pan-Zeng-Guang and Deng-XiuXin (2000) Reported that high density culture was very important in plant regeneration from protoplasts of apple because low density culture reduced browning .

Blackhli *et al.* (2002) preferred that cell division from isolated protoplast were embedded at adensity between $5.0x10^5$

and $1.0x10^6$ cell/ml in amedium semi-solid from monocotyledonous species .

Commun, *et al.* (2003) observed that the best result from cultured protoplast of grapevine were obtained when culturing at $6x10^4$ cell/ml on liquid medium .

Liu, *et al.* (2003) found that the high frequency of cell division from *vitis vinifera* cv. shengli was observed when density at $5x10^4$ -$1x10^4$.

Mliki *et al.* (2003) indicated that 0.5 $x10^6$ cell/ml from Tunisian grapes was the best density achieved the best development and cell division .

Mehri (2003) Reported that high development of *Prunus cerasus L. cv.* Montmorency protoplast appeared when density was 10^5 protoplasts/ml in the protoplast culture medium.

Hassan (2006) observed that culturing protoplast from either Pineapple or Banana at a density $2.5x10^5$ protoplasts/ml were increased protoplast development .

Omar and Grosser (2007) found that culturing of embryogenic suspension protoplast of 'Hamlin' sweet orange on MT basal medium at a concentration of 2×10^6 cells ml-1,was achieved highest protoplasts development .

II.4.c. Effect of auxin and Cytokinins concentration:

Matsuta *et al.* (1986) 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^{o}C in the dark .

Ochatt and Caso (1986) declared that callus produced from protoplast of *Pyrus communis var.* Pyaster leaf (field or *in vitro*) wasachvied when cultured on modified MS medium (1/2 MS salts) supplemented with 1mg/liter NAA and 1mg/liter BAP.

Ochatt *et al.* (1987) indicated that high callus frequency of *Prunus avium* ×*Pseudocerasus L.* was obtained when 2mg/liter NAA, 0.5mg/liter BAP, 0.5mg/liter Z, 0.5M mannitol at 25^{o} C, light (500L$_{x}$) liquid or 0.625% agarose .

Ochatt and Power (1988) declared that protoplasts of *Prunus cerasus* were successfully cultured on MS medium supplemented with 1mg/liter Z, 3mg/liter IAA at 25^{o} C in the dark liquid medium without agarose .

Marino (1991) observed that protoplasts of apricot *Prunus armeniaca L.* were divided after 7 to 9 days from cultured on media consisting of Kao and Michayluk macronutrients and MS micronutrients supplemented with 2.2 or 4.4 mM BA and 4.5 or 9 mM 2,4-D .

Perales and Schieder (1991) Pointed out that culturing of protoplast of Malus genotypes on MI medium supplemented with benzyladenine, NAA and 2,4-D all at 0.5mg/liter .

Ochatt (1992) declared that fast growing callus from *Prunus cerasus and Prunus spinosa* protoplasts was obtained when cultured on MS medium supplemented with (0.01-0.025mg/liter) NAA, (1.0-2.0mg/liter) BAP, (5-1.0mg/liter)

Zeatin and double concentration of group B Vitamins .

Ochatt *et al.* (1992) found that protoplasts of Passe Crassane and old home pear *Pyrus communis L.* were cultured successfully as aliquid layers or liquid over ager cultures. In ammonium-free MS medium supplemented with 2mg/liter NAA and 1mg/liter BA plus either 0.5mg/liter IAA for old home and 2mg/liter IAA for Passe Crassane .

Ochatt, (1993a) claimed that micro calli from *Pyrus spp.* protoplast was obtained when cultured on MS medium half strength supplemented with NAA (1-2 mg/L) and BAP (0.4-1.0 mg/L) .

Patat-Ochatt *et al.* (1993) reported that adding 2mg/liter NAA and BAP to MS medium was achvied the highest callus proliferation for leaf protoplast of apple genotype G. Delicious haploid .

Perales and Schieder (1993) pointed out that adding 2.2 micro M BA (benzyladenine), 2.6 micro M 2,4-D to MS or MT medium was achieved the best developed to protocalluses at high frequencies for leaf protoplasts of apple .

Zeng Guang *et al.* (1997) pointed out that culturing of protoplast of Hepen crab apple *(Malus hupehensis*) on MT medium supplemented with 0.5 mg/L BA, 0.2 mg/L 2,4-D, 500 mg/L Malt exudates, 100mg/L hydrolytic protein plus 0.65 mol glucose at pH 5.6 for 35-40 days caused calli formation.

Zhu-YanMing *et al.* (1997) declared that protoplasts of grapevine *Vitis vinifera L.* were successfully cultured on Nitsch's

medium containing 2.0mg/liter NAA and 0.5mg/liter BA .

Witjaksono and Grosser (1998) found that growth and development of protoplast from avocado were significantly affected by culturing on MS salts without NH3NO3 plus 2mg/L NAA .

Saito and Suzuki (1999) reported that the best development of protoplast occurred when cultured on KM8P medium containing 2 mg/L IBA, 1 mg/L BA and 0.4 mg/L 2, 4-D and MES 2-(N-morpholine ethanesulfonic acid) 5 mM, pH 5.7.

Commun, *et al.* (2003) found that addition of 20 mM 2,4,5-T and 1.3 mM BA to NN69 medium gave the best plating efficiency for grapevine .

Mehri (2003) reported that leaf mesophyll protoplasts of *Prunus cerasus L.*cv. Montmorency were successfully cultured on KM **(Kao and Mychayluck, 1975)** basal Z and 1mg/liter NAA. But the successfully cultured of leaf callus protoplast of the same kind was achieved when used MS basal medium supplemented with 2mg/liter NAA, 0.25mg/liter BAP and 0.1mg/liter Zeatine .

Mliki *et al.* (2003) found that the formation of micro- and macrocallus were observed when leaf protoplasts of Tunisian grapes was cultured on CPW-13medium containing 4mg /liter of

NOA (2-naphthoxyacetic acid) and 0.88 mg/liter of TDZ (thidiazuron).

Hassan (2006) observed that addition the combination of 3.0 mg/L NAA and 0.3 mg/L BAP to the culture medium were induced the highest protoplast development of Banana while combination of 3.0 mg/L NAA and 0.2 mg/L BAP was superior in Pineapple.

3. MATERIALS AND METHODS

This investigation was carried out at Tissue Culture Unit, Horticulture Department, Faculty of Agriculture Moshtohor, Benha University, during the period from 2004 to 2007.

All the experimental studies conducted on two rootstocks. i.e. communis pear (*Pyrus communis*) and betulaefolia pear (*Pyrus betulaefolia*). The mother plants planted in Tissue Culture Nurserry, Horticulture Department, Faculty of Agriculture Moshtohor, Benha University. *In vitro* plantlets of both rootstocks under study were established, proliferated, and rooted according to (Bayuomy, 2004) methods .

New healthy and well expanded *In vitro* and surface sterilized *In vivo* leaves from either communis or betulaefolia pear rootstocks were subjected to phenolic compounds testing, anti-oxidant treatments and plasmolysis as pre-protoplast isolation from the explants (small squares 1-2 mm .wide) under study.

Leaf sterilization :

The new emerged leaves of *in vivo Pyrus communis* and *Pyrus betulaefolia* were collected from the mother trees and transferred directly to the tissue culture laboratory and subjected to the running water for 15 minutes to get rid of dirts and germs followed by immersing in soap solution for 5 minutes. Then immersing in 10% Clorox solution (0.5 NaOCl) commercial

bleach with two drops of Tween –20 for 10 minutes and finally immersed in sterilized distilled water 3 times for 5 minutes, each.

Explant preparation:

In vitro full new emerged and sterilized *in vivo* full expanded leaves of two pear rootstocks (*Pyrus communis* and *Pyrus betulaefolia*) were taken and treated with anti-oxidant treatments then the leaves divided into small sections 1-2 mm. wide after excluding leaves margens and midrib.

Different experimental studies include pre-protoplast isolation, protoplast isolation, purification and culture took place with both communis and betulaefolia pear rootstocks as follow :

III.1. pre-protoplast isolation :

III.1.a.Evaluation of phenolic compounds level :

New emerged and full expanded *in vivo* leaves were collected from mother plants of (*communis and betulaefolia*) both pear rootstocks at different times during the year round i.e. March, June, September and December (at first week of each month) to record the levels of phenolic compounds during different studied periods and study the seasonal fluctuation of phenolyic compounds to select the best anti-oxidant treatment effective in overcoming this problem.

III.1.b. Anti-oxidants treatments :

Simple experiment was designed for in vivo explants only by using three anti-oxidant types (citric acid, P.V.P and Ascorbic

acid) at different concentrations. for both pear rootstocks to determine the best treatment succeeded in reducing or eliminating phenolic compounds accumulation and in turn enhanced protoplast isolation .

Anti-oxidants treatments applied as pretreatments by dipping the explants in the following solutions for two hours.

1. Control :sterilized distilled water .
2. 0.1% Ascorbic acid. (100 mg/L).
3. 0.15% citric acid.(150 mg/L).
4. 0.5% P.V.P.(500mg/L polyvinylpyrolliden).
5. 0.1% ascorbic acid + 0.15% citric acid.
6. 0.1% ascorbic acid + 0.5% P.V.P. .
7. 0.15% citric acid +0.5% P.V.P. .
8. 0.1% ascorbic acid + 0.15% citric acid + 0.5% P.V.P.

III.1.c. protoplast source and plasmolysis:

Factorial experiment conducted between protoplast source (*in vitro* and *in vivo*) and plasmolysis treatments. This experiment carried out mainly for certain fruit trees types for encouraging plasmolysis of cell protoplasm before protoplast isolation , plasmolysis treatments adopt under this topic were dipped in CPW medium (for one hour) with the following additives :

1. Control: 0 mannitol + 0sucrose .
2. 9 g/100 ml mannitol + 0 sucrose .

3.13 g/100 ml mannitol +0 sucrose .

4. 0 mannitol + 21 g/100ml sucrose .

5. 9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol for further 30 minute.

III.2. Protoplast isolation :

III.2.a. protoplast source and enzyme mixture :

Two protoplast sources (*in vitro* and *in vivo*) of both pear species combined with different enzyme mixtures were evaluated to find out the most effective combination of protoplast source and enzyme mixtures induced the highest protoplast yield .

The tested enzymes mixtures were as follow :

1- EM_1: (1.5% cellulase + 0.5% pectianase + 1.5% Macrozyme)

2- EM_2: (1% cellulase + 0.5% pectianase + 1% macerozyme).

3- EM_3: (1% cellulase + 1% pectianase + 1% macerozyme)

4- EM_4: (1% cellulase + 1% macerozyme)

5- EM_5: (2% cellulase + 1.5% macerozyme+ 0.5% pectinase)

6- EM_6: (1% cellulase + 1% pectinase)

7- EM_7: (1% cellulase + 0.5% macerzyme + 0.2% pectianase)

III.2.b. digestive enzymes medium.

Murashige and Skoog (MS, 1962), **Kao & Michayluk, (1975)** (KM) and CPW medium salts (Frearson, *et al.,* 1973) were the medium salts tested to select medium salts suitable as digestive enzyme medium able to encourage the highest protoplasts isolation from *in vitro* leaves of either *communis* or *betulaefolia* pear rootstocks .

III.2.c. Effect of Osmotic pressure factor:

Mannitol, sucrose and glucose were the main osmotic pressure factors studied at rate 13, 21 and 7.92g/100ml.respectively to detect the optimum osmotic pressure factor succeeded in optimizing the osmatic pressure inside and outside (medium osmotic pressure) protoplast valuable to produce rounded protoplast (viable protoplasts) without occurrence plasmolysis or rupture .

III.2.d. Effect of incubation period :

In vitro leaf strips immersed in the suitable enzyme mixture were incubated for different periods i.e. 12, 16, 20 and 24 hours to testify the best incubation period induced the highest viable protoplast yield .

III.2.e. Effect of shaking :

III.2.e.1. shaking speed :

The incubated leaf strips immersed in enzyme mixture were shaked on a rotatory shaker for 30 minutes at different shaking speeds (0, 50, 75, and 100 rpm). to

determine the suitable shaking speed maximize protoplast viability and yield .

III.2.e.2. shaking period :

Different shaking periods i.e. 0.0, 15, 30, 45 and 60 minutes on the rotatory shaker were studied to verify the most convenient period enhanced the highest protoplast yield and viability .

III.3. Purification :

III.3.a. Effect of sieve pore size:

Simple experiment was designed to study the effect of different sieve pore sizes on the rate of purification levels through getting red of debris and digested cell wall residues. Sieve with different pore sizes were tested, i.e., 25, 50 and 75 μm to select the best pore size encouraged the highest purification without any hazard on protoplast yield .

III.3.b. Effect of centrifugation:

III.3.b.1. Centrifugation speed :

500, 1000 and 1500 rpm centrifugation speeds were used to verify the best speed maximized protoplast purification and reduce protoplasts damage.

III.3.b.2. Centrifugation period :

Different centrifugation periods i.e. 5, 7.5 and 10 minutes were tested to detect the suitable period encouraged the highest protoplast purification with reducing protoplasts damage.

III.4. Protoplast culture:

III.4.a. Effect of medium type:

In this experiment the isolated protoplasts of either *pyrus communis* or *pyrus betulaefolia* were cultured on different medium types i.e. **Murashige and Skoog (MS, 1962)**, **Kao & Michayluk, (KM 1975)** and **Gamborge *et al.,* (B5 1968)** to select the best culture medium type gave the highest protoplast development.

III.4.b. Effect of protoplast density:

Different protoplast densities (0.5, 1, 1.5, 2.0 and 2.5 × 10^5/ml) were studied to testify the suitable protoplast density enhanced the best protoplast development

III.4.c. Effect of hormonal balance :

Factorial experiment conducted between (NAA and BAP each with different concentrations) to find out the suitable hormonal balance which improved protoplast development. The isolated protoplasts of either *(Pyrus communis* or *Pyrus betulaefolia*) were cultured on Murashige and Skoog medium supplemented with NAA was added to the development culture media at four levels (0, 0.1, 0.2 and 0.3 mg/L), each level was combined with each of the following BAP concentrations (0.0, 0.1, 0.2 and 0.3 mg/L) as follow:

1) 0.0 mg/100ml NAA + 0.0mg/100ml BAP (control).

2) 0.0 mg/100ml NAA + 0.1mg/100ml BAP.

3) 0.0 mg/100ml NAA + 0.2mg/100ml BAP.

4) 0.0 mg/100ml NAA + 0.3mg/100ml BAP.

5) 1.0 mg/100ml NAA + 0.0mg/100ml BAP.

6) 1.0 mg/100ml NAA + 0.1mg/100ml BAP.

7) 1.0 mg/100ml NAA + 0.2mg/100ml BAP.

8) 1.0 mg/100ml NAA + 0.3mg/100ml BAP.

9) 2.0 mg/100ml NAA + 0.0mg/100ml BAP.

10) 2.0 mg/100ml NAA + 0.1mg/100ml BAP.

11) 2.0 mg/100ml NAA + 0.2mg/100ml BAP.

12) 2.0 mg/100ml NAA + 0.3mg/100ml BAP.

13) 3.0 mg/100ml NAA + 0.0mg/100ml BAP.

14) 3.0 mg/100ml NAA + 0.1mg/100ml BAP.

15) 3.0 mg/100ml NAA + 0.2mg/100ml BAP.

16) 3.0 mg/100ml NAA + 0.3mg/100ml BAP.

III.5. Preparations and determinations:

III.5.a. Preparing solutions and media :

1. Enzyme mixture solution :

Each enzyme mixture under study was dissolved in CPW salts **(Frearson, *et al.,* 1973)** solution with 13% (w/v) mannitol as an osmotic stabilizer. The pH of the enzyme solution was adjusted to 5.8 with 0.1 M NaOH or 0.1M HCl, and the solution was filter sterilized by passing through 0.2 μm pore size "sartorius" membrane filter. The enzyme solutions were dispensed into sterile 15ml conical tube with cap and stored at 2°C under dark condition.

2. Media used:

CPW salts **(Frearson, *et al.,* 1973)**

Table (1): The composition of CPW salts medium

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 13M 13% (w/v) mannitol with CPW salts, pH 5.8

CPW 21s 21% (w/v) sucrose with CPW salts, pH 5.8

CPW 9M 9% (w/v) mannitol with CPW salts, pH 5.8

III.5.b. Determination of phenolic compounds :

Phenolic compounds determined by using the Colorimetric method of analysis described by **Snell and Snell (1953).** Phenol reagent (folinciocalten reagent) was prepared by boiling a mixture of 100g of Sodium tungestate, 25g of Sodium molybdate, 700ml of distilled water, 50ml of 85% Phosphoric acid and 100ml of concentrated hydrochloric acid under reflux for 10 hours in a water bath. Then 150g of lithium sulphate, 50ml of distilled water and a few drops of bromine was added to the mixture and boiled again for 15 minutes without a reflex condenser to remove excess bromine, then cooled, diluted to 1 liter with distilled water and filtered.

The free phenols were determined as follows, 1ml of the phenol reagent and 5ml of a 20% solution of sodium carbonate were added to the isopropanol sample (0.2 ml) and diluted to 10ml with warm water, (30-35°C). The mixture was lest to stand for 20 minutes and read using spectrophotometer (SPECTRORNIC 20-D) at 520 nm against a reagent blank.

The total phenols determination, 10 drops of content rated hydrochloric acid were added to the isopropanol sample (0.2ml) in test tube, heated rapidly to boiling over a free flame, with provision for condensation, then the tubes were placed in a boiling water bath for 10 minutes. After cooling 1ml of the reagent and 2.5ml of 20% Na_2CO_2 were added to each tube, the mixture was diluted to 50ml with distilled water, and after 20 minutes was determined using spectrophotometer (SPECTRONIC 20-D) at 520 nm against a reagent blank.

The total and free phenol contents were calculated for each treatment as milligrams of catechol per one gram fresh weight according to standard curve of catechol. The conjugated phenols were determined by subtracting the free phenols from the total phenols.

Data and calculation :

Counting of protoplasts was conducted according to method of **Blackhall *et al.,* (2002)**. Moreover, number of cells was calculated as the number of cells per each square on haemocytometer. The final count of protoplasts per 1 ml was carried according to the following equation total number cells = $5n \times 10^4$

Where: n = the average of number of cells per each square on haemocytometer.

Moreover, scores were applied for protoplast development which calculated as the rate of cell division and microcallii 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 division (SD).

4. 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		
	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**		

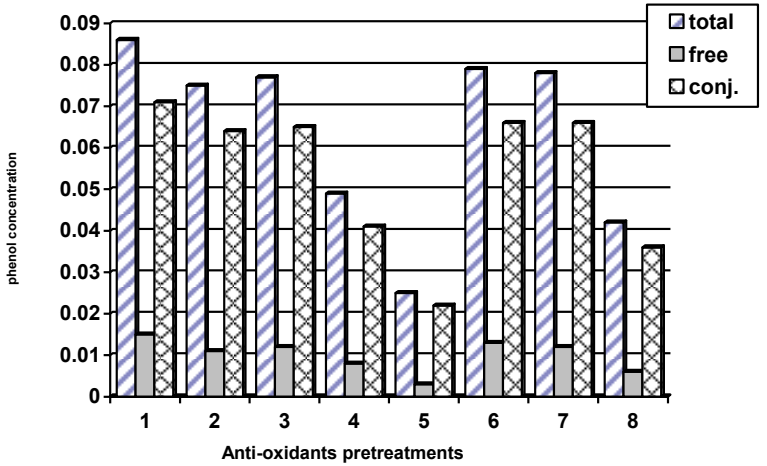

Figure (1) :Effect of anti-oxidants pretreatments on phenol compounds level of *in vivo* Pyrus betulaefolia explants.

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

Treatment	Protoplast source		
	In vitro (10^4)	*In vivo* (10^4)	Mean
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		

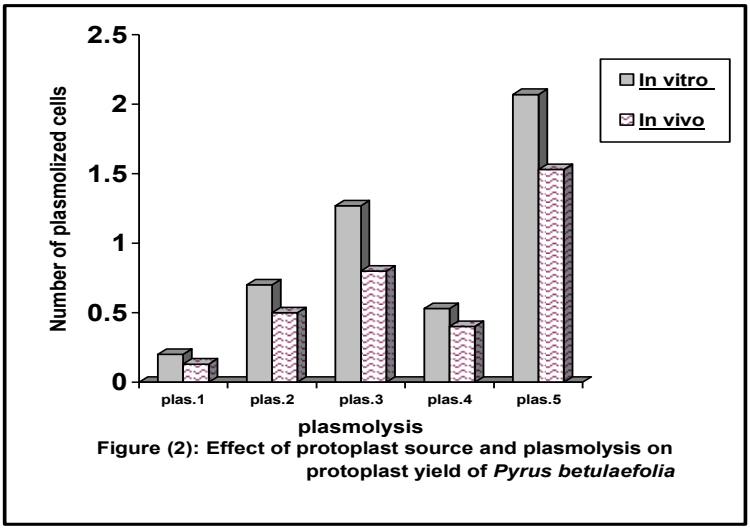

Figure (2): Effect of protoplast source and plasmolysis on protoplast yield of *Pyrus betulaefolia*

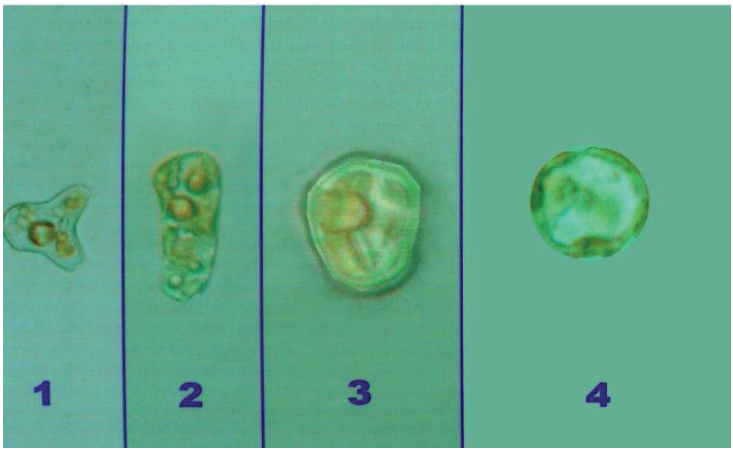

Photo (1): Reflect the Plasmolysis treatments in *Pyrus betulaefolia* which include (1) 0.0 manitol + 21g/100ml sucrose, (2) 9g/100 ml mannitol + 0.0 sucrose, (3) 13g100ml mannitol + 0.0 sucrose, (4) 9g/100ml mannitol for further 30 min. 13g/100ml mannitol

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**		

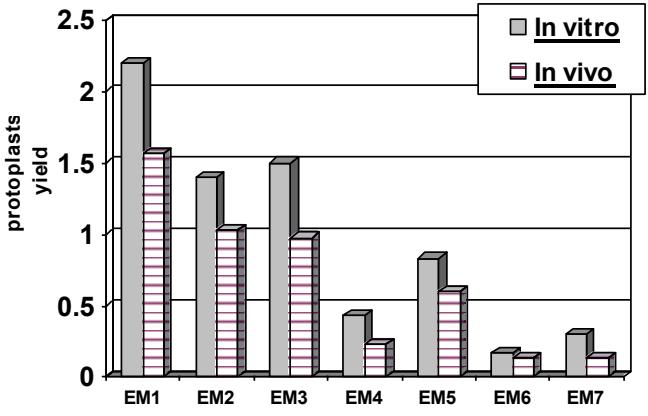

Figure (3): Effect of different protoplast sources and enzyme mixtures on protoplast yield of*Pyrus betulaefolia*

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 15h.

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

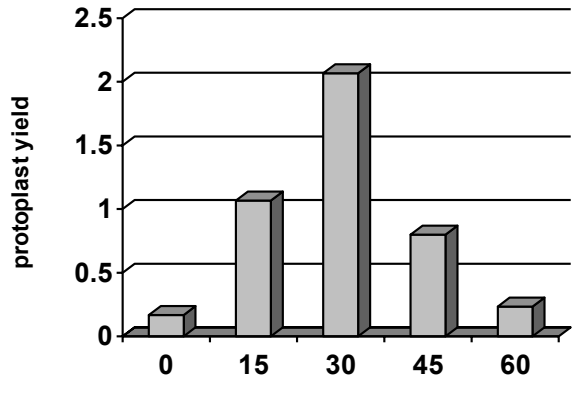

Figure (4): Effect of different Shaking periods on protoplast yield of *in vitro* pyrus betulaefolia

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

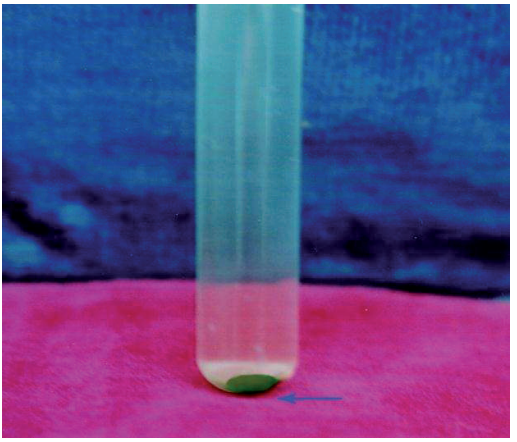

Photo (2): Purification of protoplast in *Pyrus betulaefolia*

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		

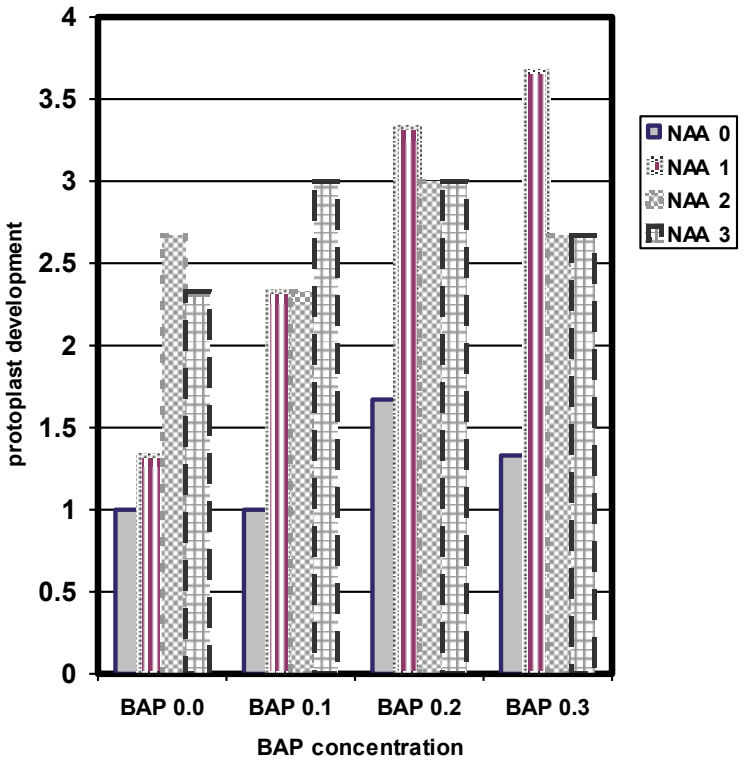

Figure (5): Effect of the hormonal balance on protoplast development of *In vitro* pyrus betulaefolia.

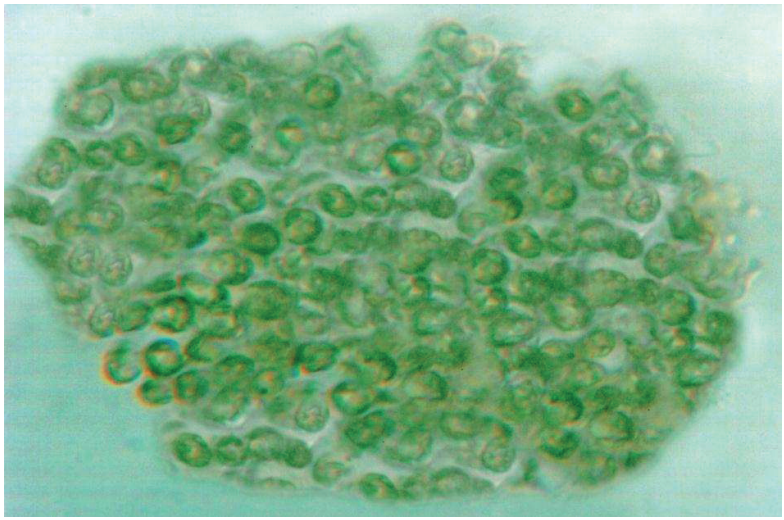

Photo (3): Protoplast yield in *Pyrus betulaefolia*

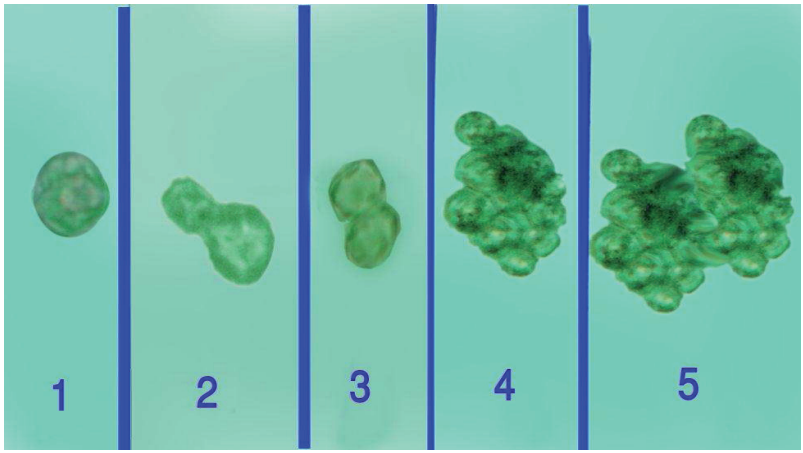

Photo (4): Reflect protoplast development in *Pyrus betulaefolia* which include (1) protoplasts, (2) buding stage, (3) protoplast division, (4) microcalli formation, (5) more microcalli formation.

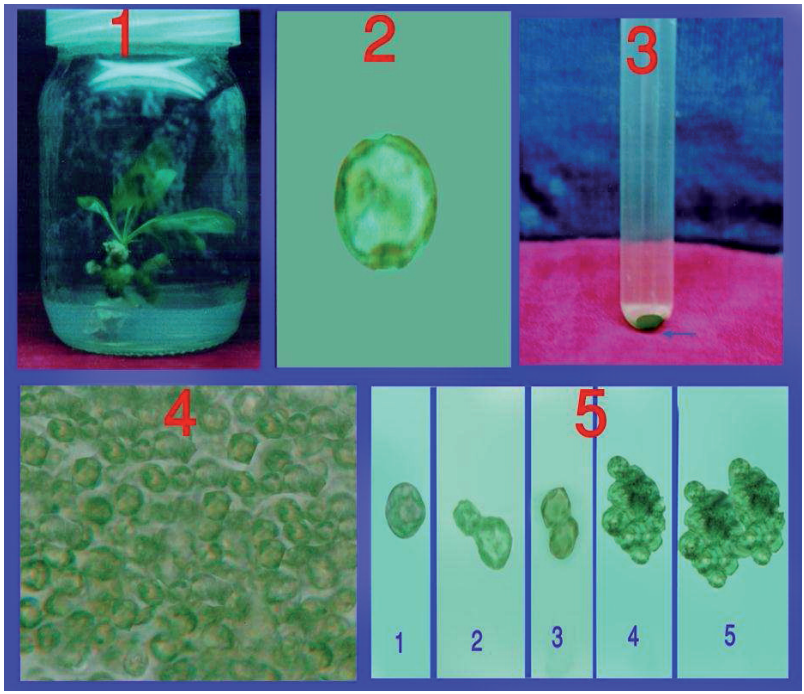

Photo (5): Reflect the protoplast technique in *Pyrus betulaefolia* which include preprotoplast isolation (1) protoplast source, (2)Plasmolyzed cells, (3) purification of protoplasts, (4) Protoplast yield, (5) protoplast development (1=protoplasts, 2=buding stage, 3= protoplast division, 4=microcalli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

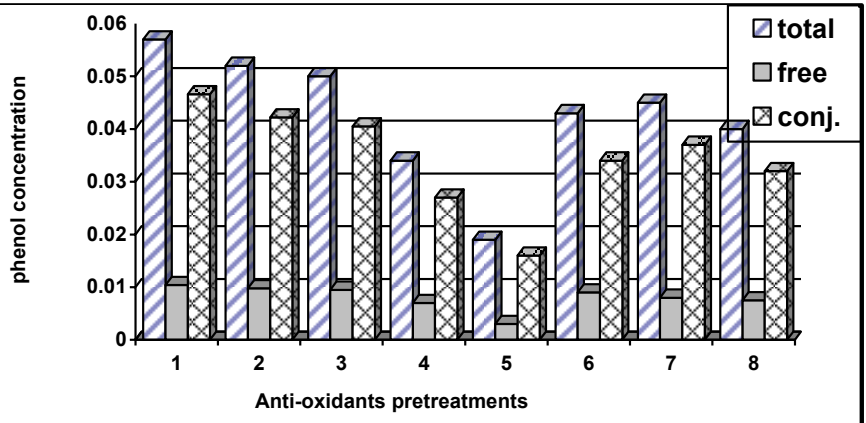

Figure (6) :Effect of anti-oxidants pretreatments on phenolic compounds in *in vivo* Pyrus communis explants.

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		

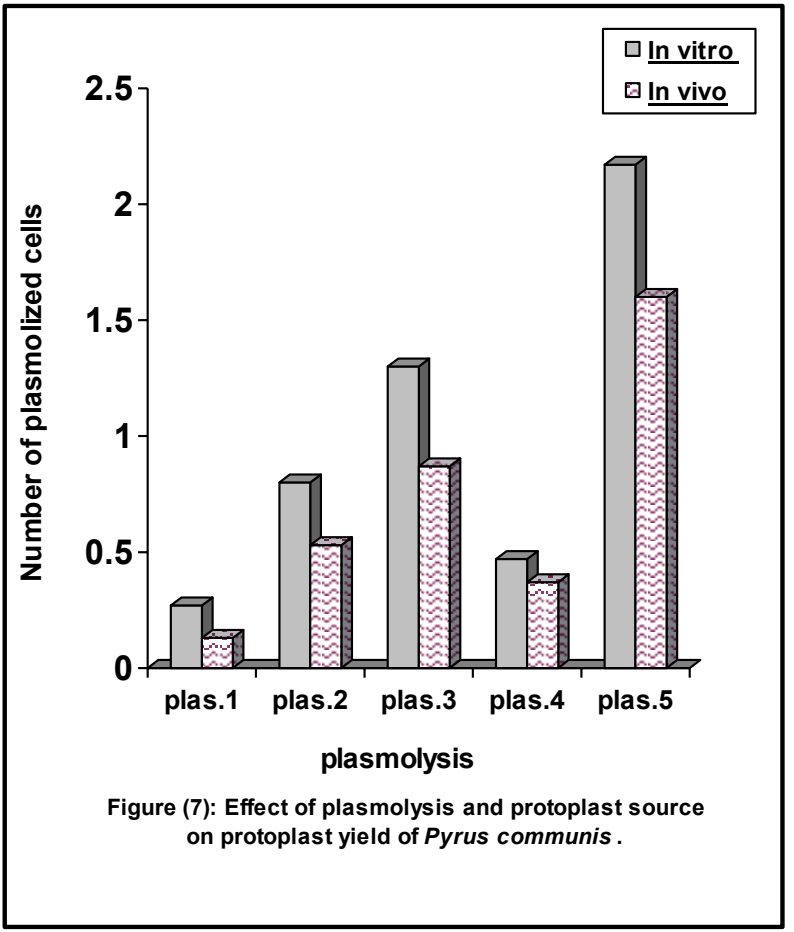

Figure (7): Effect of plasmolysis and protoplast source on protoplast yield of *Pyrus communis* .

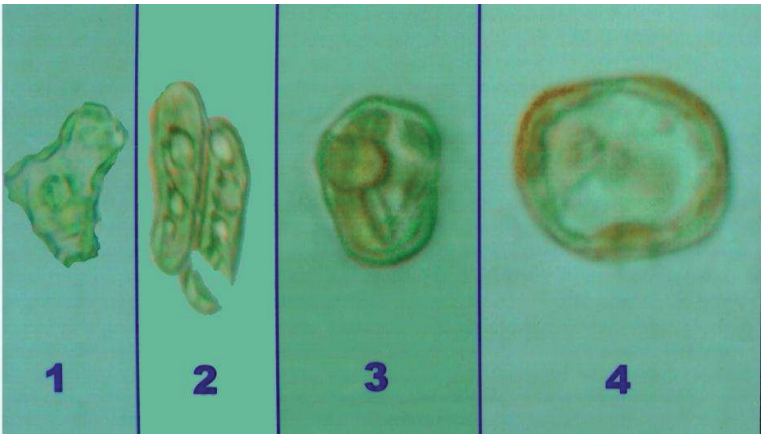

Photo (6): Reflect the Plasmolysis treatments in *Pyrus communis* which include (1) 0.0 manitol + 21g/100ml sucrose, (2) 9g/100 ml mannitol + 0.0 sucrose, (3) 13g100ml mannitol + 0.0 sucrose, (4) 9g/100ml mannitol for furthermore 30 min 13g/100ml mannitol

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**		

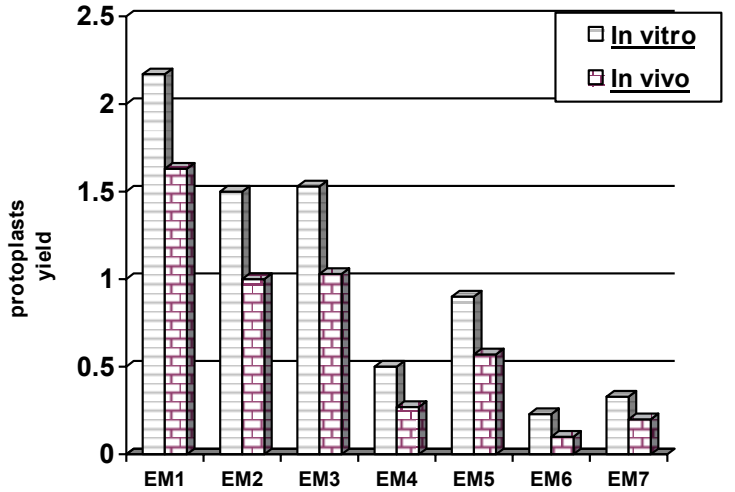

Figure (8): Effect of different protoplast sources and enzyme mixtures on protoplast yield of*pyrus communis* .

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

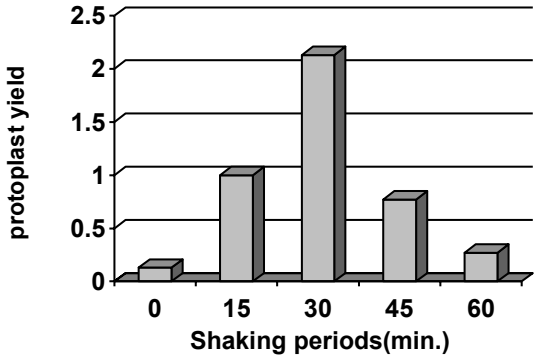

Figure (9): Effect of different Shaking periods on protoplast yield of *in vitro pyrus communis*

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* .

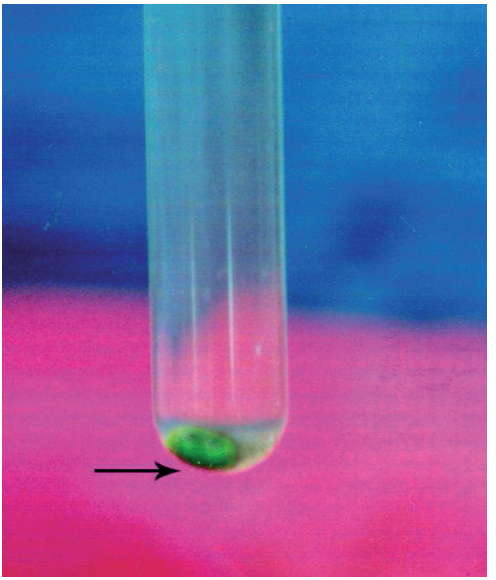

Photo (7): Purification of protoplast in *Pyrus communis*

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^{o}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.*

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	1.33 ±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		

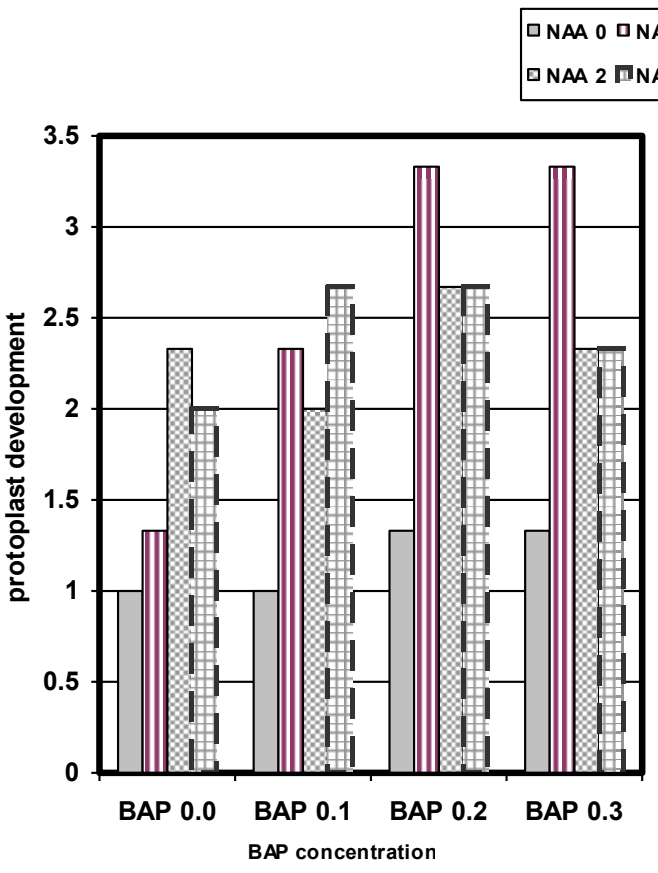

Figure (10):Effect of the interaction between NAA and BAP concentration on protoplast development of*in vitro pyrus communis.*

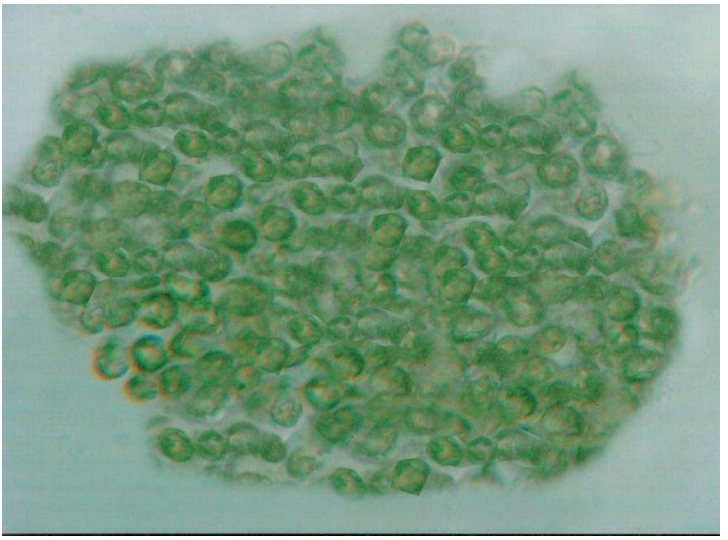

Photo (8): Protoplast yield in *Pyrus communis*

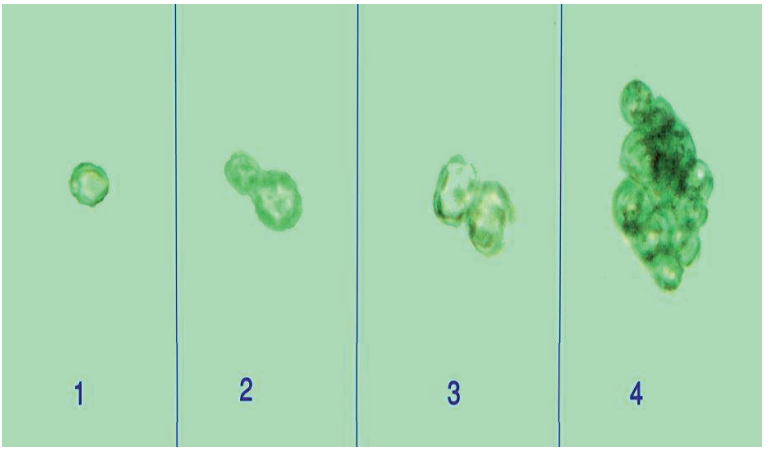

Photo (9): Reflect protoplast development in *Pyrus communis* which include (1) protoplasts, (2) buding stage, (3) protoplast division, (4) microcalli formation.

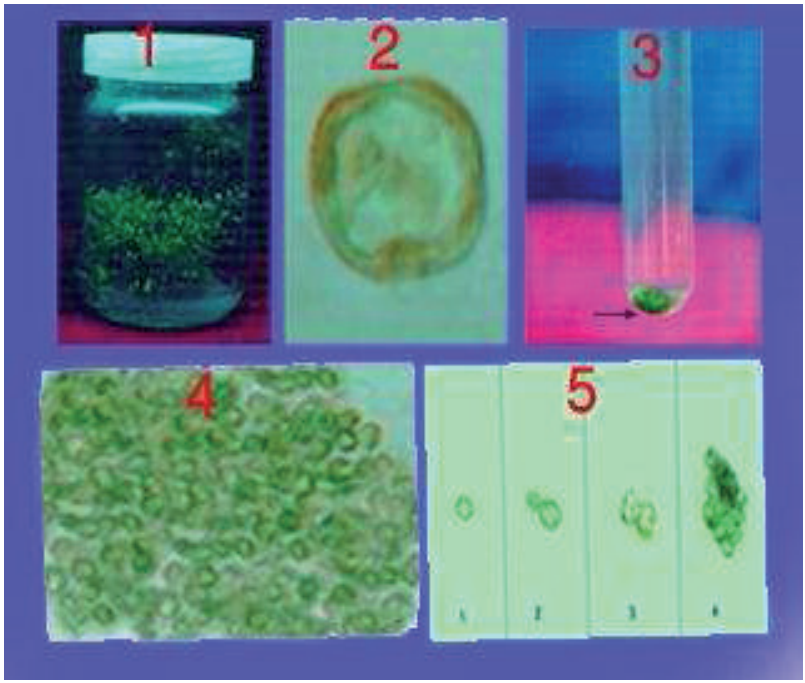

Photo (10): Reflect the protoplast technique in *Pyrus communis* which include preprotoplast isolation (1) protoplast source, (2)Plasmolyzed cells, (3) purification of protoplasts, (4) Protoplast yield, (5) protoplast development (1=protoplasts, 2=buding stage, 3= protoplast division, 4=microcalli formation,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

5. SUMMARY

This investigation was conducted in the Tissue Culture Unit, Horticulture Dept. Fac. of Agric. Moshtohor, Benha university during the period from 2004 to 2007 to study the best way for maximizing protoplast yield and increasing number of viability protoplast as well as enhancing protoplast development of pear rootstocks (*Pyrus betulaefolia* and *Pyrus communis)* .The following procedures were studied :

I- Pre-protoplast isolation:

In vivo leaves of both pear rootstocks (betulaefolia and *communis* pear) were subjected to levels evaluation phenolic compound during the year round. Then sterilized the leaves and treated with different anti-oxidant treatments.

New leaves from *in vitro* and sterilized *in vivo* pear rootstocks were divided into small sections and soaked in different plasmolysis treatments in combination treats with different protoplast sources (*in vitro* and *in vivo*) .

II- Protoplasts isolation:

Small sections from *in vitro* were treated by different enzyme combination (mixtures) in combination with protoplast sources. Then different digestive enzyme media and osmotic pressure factor .as well as different incubation periods and different shaking speeds and periods.

III- Protoplast purification:

Different pore sizes, as well as centrifugation speeds and periods were used during purification stage ,

IV- Protoplast development:

Different medium types and different protoplasts densities and hormonal balances were employed to find out the best procedures for protoplast development.

The obtained results can be summarized as follow :

5.1. *Pyrus betulaefolia* :

5.1.a. Pre-protoplast isolation :

1-It proved that March sample showed the lowest phenolic compounds either totals, free or conjugated contents which assured that the best time for taking explants.

2-It is clear that using of anti-oxidant solution (0.1% ascorbic acid + 0.15 citric acid) was effective in reducing phenolic compounds and reducing their harmful effect.

3- It is recommended to use plasmolysis treatment 9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol for furthermore half hour to enhance successful protoplast isolation.

4- *In vitro* protoplast source was surpassed *in vivo* protoplast source as it maximize protoplast yield.

5.1. b.Protoplast isolation :

5- Immersing *in vitro* explants in enzyme solution supplemented (1.5% cellulase + 1.5% Macerozyme + 0.5% pectinase) was more effective in improving the protoplast yield.

6- The highest protoplast yield was obtained when CPW digestive medium was used.

7- Addition of mannitol to the digestive enzyme medium was preferred in increasing the protoplast yield.

8- Incubation of the explants for 20 hours enhance the highest protoplast yield.

9- Shaking the incubated explants in enzyme solution at 75rpm for 30 min. encourage the best protoplast yield.

5.1.c. Purification:

10- Using sieve at pore size 25μm encourage the highest number of viable protoplasts.

11- Centrifugation protoplast with speed rate 1000 rpm for 7.5min. inducted the greatest number of viable protoplasts.

5.1.d. Protoplast development:

12-Murashig & Skoog medium was superior for the best protoplast development than both Gamborg and KM medium types.

13-Using protoplast at density rate 2 X 10^5 in the culture medium enhanced an improvement in protoplast development.

14- Supplementation of the culture medium with the combination of 1.00 mg/L NAA and 0.3mg/L BAP induced

5.2. *Pyrus communis* :

5.2.a. Pre-protoplast isolation :

1-The lowest phenolic compounds level was detected at December sample when the leaves was taken.

2- Antioxidant solution (0.1% ascorbic acid + 0.15 citric acid) succeeded in reducing the accumulation of phenolic compounds.

3- using of plasmolyized treatment 9 g/100 ml mannitol for half hour then 13 g/100 ml mannitol for another half hour is required for maximizing protoplast yield.

4- *In vitro* protoplast source was superior in increasing protoplast yield than *in vivo* source.

5.2.b.Protoplast isolation :

5- The best results of protoplast isolation was appeared when using enzyme mixture consists 1.5% celtulase + 1.5% Macerozyme + 0.5% pectinase compared with the other enzyme mixtures .

6- CPW medium showed the most superior digestive enzyme medium for increasing protoplast yield .

7- Addition of mannitol to enzyme solution was the more effective in improving protoplast yield and viability.

8- The highest viable protoplast yield were produced the explants were incubated for 20 hours in enzyme mixture .

9- Shaking speed at 75rpm for 30 min maximized protoplast yield .

5.2.c. Purification:

10- Increasing viable protoplast number occurred when using sieve pore size 25μm.

11- The best number of viable protoplast was noticed when centrifugation speed rate 1000 rpm for 7.5minutes was used.

5.2.d. Protoplast development:

12- KM proved to be the most suitable medium maximize protoplast division but MS medium provide to be the most suitable medium maximize protoplast development.

13- The highest development of protoplast was obtained when culturing the protoplast with density 2×10^5 .

14- Addition of 1.0 mg/L NAA with either 0.2 or 0.3mg/L BAP to the culture medium resulted in maximized protoplast development.

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