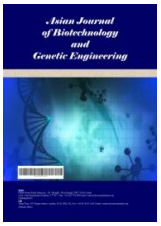

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In vitro Propagation and Callus Induction of Pear *(Pyrus communis)* Cv. Le-Conte

O. M. Kotb[1], F. M. Abd EL-Latif[1], A. R. Atawia[1], Sherif S. Saleh[2] and S. F. EL-Gioushy[1*]

[1]Department of Horticulture, Faculty of Agriculture, Benha University, Egypt.
[2]Department of Aromatic and Medicinal Plants Researches, Horticulture Research Institute, Agriculture Research Centre, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. Authors FMA, ARA, SSS, SFE and OMK designed this work, wrote the manuscript and revised it. Authors OMK conducted the experiments, performed the measurements of the samples and analysis of data. Authors SFE and SSS coordinated the data collection. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The traditional propagation technique of pear trees by grafting on quince, seedlings or clonal selection of *Pyrus communis* is not completely satisfactory. This is because of the lack of compatibility with some cultivars, heterogenesis of the pear seedlings and excess growth and also due to the sensitivity of the grafted plants to pear decline. For this the present study was conducted at the Tissue Culture Laboratory, Horticulture, Research Institute, Agricultural Research Center (ARC), Egypt during the period from December 2013 to March 2016 to investigate the effect of different media type Murashige and Skooge (MS), Gamborge (B5) and Woody plant media (WPM) at four salt concentrations (Full, ¾, ½ and ¼) of culture media on micropropagation of pear (*Pyrus comumunis*) cv. Le-Conte during the establishment stage. Shootlet proliferations were investigated at different concentrations of benzyl amino purine (BAP) and kinetin (Kin) at 0.25, 0.5 and 1.0 mg/l for each, during two successive subcultures. Finally, rooting capacity was studied by various concentrations of indole butyric acid (IBA) and indole acetic acid (IAA) at1.0, 2.0 and 3.0 mg/l on

**Corresponding author: E-mail: sherif.elgioushy@fagr.bu.edu.eg;*

media containing activated charcoal. The culture explants were successfully disinfected by using Colorex 20% for 15 min with 100% survival and 100% free contamination. MS media at full strength was the best culture media that produced shootlet (1.33 shootlet/explant) and shootlet length 3.67 cm with 9.97 leaf/shootlets. Among the different concentrations, 1.0 mg/l BAP showed the highest shoot proliferation of 5.89 and 5.44 shoots per explant at the first and second subculture, respectively. The longest shoot (2.43 and 2.59 cm) was produced in the two subcultures by the treatment combination of 0.25 mg/l BAP. The highest numbers of roots were produced by 1.0 mg/l IAA were 8.0 roots/shootlet and the tallest length of roots were obtained for explants cultured on MS media containing IAA 3 mg/l and use mixture from NAA and 2,4-D 2:2 mg/l to get the highest value of callus formation 100%. Generally, it can be concluded from the obtained results that using Clorox 20% per 15 min at the disinfecting stage and using MS salt at full strength for the establishment stage, then using BAP at 1.0 mg / l to increase the number of shoots at the proliferation stage and using a mixture of NAA and 2,4-D 2:2 mg / l to obtain the highest value of callus formation. Moreover, using IAA at 1 mg / l to obtain the highest number of roots.

Keywords: Pear; Le-Conte; In vitro; callus; proliferation; BAP; IAA; IBA.

ABBREVIATIONS

2, 4-D : 2, 4-Dichlorophenoxyacetic acid
BAP : Benzyl amino purine
MS : Murashige and Skoog, 1962
B5 : Gamborg, 1968
WPM : Woody plant medium
IAA : Indole-3- acetic acid
IBA : Indole-3- butyric acid
NAA : Naphthalene acetic acid

1. INTRODUCTION

Pear is considered one of the most important temperate fruit after grapes and apple in the world. Pear belongs to the genus Pyrus, subfamily Maloideae of the Rosaceae. Two main species, which are genetically and morphologically different, *Pyrus communis* L., the European pear, which is grown in Europe and America, and *Pyrus pyrifolia* Nakai, the Asian pear or Nashi, which is grown traditionally in Japan, China, Korea and Taiwan, and increasingly in Europe and America [1].

The traditional propagation technique of pear trees by grafting on quince, seedlings or clonal selection of *Pyrus communis* is not completely satisfactory. This is because of the lack of compatibility with some cultivars, heterogenesis of the pear seedlings and excess growth and also due to the sensitivity of the grafted plants to pear decline.

Growing pear trees with their roots is another possibility that would overcome the previous problems [2]. So, the technique of commercial *in vitro* can be employed for micro-propagation of pear. Genetic improvements of pear cultivars are possible through two approaches: one is to exploit the pre-existing or induced mutations resulting in genetic variability in somatic cells and the other is that of genetic engineering or gene isolation and transfer [3].

Most of the previous works on plant regeneration from leaves of pears focusing on *P. communis* [4,5,6,7] with only a few reports on *P. Bretschneider* [4] and *P. pyrifolia* [8,9].

The present study carried out to evaluate the method of *in vitro* propagation and callus production from leaves of pear (*Pyrus comumunis*) cv. LE-Cont.

2. MATERIALS AND METHODS

This study was carried out at Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. in cooperation with the Department of Horticultural, Faculty of Agriculture, Benha University, Egypt. during the period from 2014 to 2016.

2.1 Preparation of *In vitro* Culture Explants

2.1.1 Plant materials

The micro-nodes of Pear were collected from 4 years old disease-free plant at the experimental Deciduous Fruits Department at the Horticulture Researches Institute, Agricultural Researches Center, Egypt during the 2014 growth season.

2.2 Sterilization Stage

The explants were thoroughly washed using tap water with 5% detergent solution (Teepol) for 20 minutes, followed by 2–3 washes in sterile distilled water. The explants were excised into convenient sizes (2-3.5 cm in length) after removing the leaf sheaths. The cutting pieces (micro nodes) were surface sterilized with Clorox (Sodium hypochlorite 5.5%) at 10, 15 and 20% for 15 minutes or H_2O_2 10% for 5, 10 and 15 minutes) and rinsed 4–5 times with sterilized double-distilled water and then excised to 1.0–1.5 cm in length in the laminar air-flow.

2.3 Establishment Stage

Sterilized explants were cultured on MS medium [10], B5 [11] and WPM [12] at four salts concentrations (full, ¾, ½, and ¼) for each medium. All media were subjected to be free of plant growth regulators but supplemented with 30 mg/l sucrose and 0.7% agar. The culture was incubated under 25±2ºC under fluorescent lamps with light intensity of 3000 lux at 16 hrs photoperiods. The development of shoots was monitored every week.

2.4 Proliferation Stage

Initiated shoots obtained from the previous experiment and successive growth on suitable salt concentrations were subcultured twice into MS medium containing different concentrations 0.25, 0.5, and 1.0 mg/l of both BAP and Kin. The number of regenerated plantlets was recorded after the first subculture (4 weeks) and repeated after an additional 4 weeks (second subculture) subcultures period. The average of plant height, shoot number, leaves number and leaves area formation were determined in this experiment.

2.5 Rooting Stage

For root induction, excised individual shoots without cutting leaves were transferred in solidified MS basal medium supplemented with different concentrations of IAA or IBA (1.0, 2.0 and 3.0 mg/l) with 2 g/l activated charcoal. Three hall-plants were placed in each jar (250 mm) containing 35 ml of the culture media. All the cultures were incubated at 25±2°C under 14 h photoperiod at 30°C and white fluorescent lamps. Rooting percentage, root number, and root length were recorded after 4 weeks of incubation.

2.6 Callus Induction

Callus cultures (about 2–2.5 cm) derived from the aerial leave explants were subcultured onto MS medium containing 2,4-D + NAA at 2-2,2-4,2-8,4-2,4-4,4-8,8-2,8-4 and 8-8 mg/l. These cultures were subjected to stress by keeping them without subculturing for 40 – 60 days to induce callogenesis derived from stem. The callus was then transferred onto MS medium containing the same induction media for the induction of callus.

2.7 Statistical Analysis

All data obtained during both seasons were subjected to analysis of variance and significant differences among means were determined according to [13].

3. RESULTS AND DISCUSSION

3.1 Effect of Disinfecting Stage

Disinfection of the explants is an important process to obtain a success explants under the establishment stage and complete the following stage of tissue culture.

Data presented in Table 1 and Fig. 1 show the contamination percentage of explants due to the use of sodium hypochlorite at 10%, 15% and 20% with stable time of emersion (15 min) and hydrogen peroxide at 10% for 5, 10 and 15 min.

These data reveal that sodium hypochlorite 20% gave the highest significant value 100% of decontamination and it was a healthy (100% survival) followed by sodium hypochlorite 15% which resulted from 11% contamination and 100% survival, as compared with other concentration of sodium hypochlorite 5%, 10% and Hydrogen peroxide at 10% for 5,10 and 15 min.

These results are in agreement with those found by [14] who surface-sterilized Bartlett pear with 2.5 percent sodium hypochlorite for 15 minutes. However, used 0.52 percent sodium hypochlorite for 10 minutes for glasshouse grown ‘Seckel’ pear and sterilized the explants of *P. pyrifolia* with 0.6 percent sodium hypochlorite for 30 minutes [15, 16].

Table 1. Effect of various disinfectant materials on contamination percentage of pear le-Conte explants *In vitro*

Treatments	Contamination %	Survival %
H_2O_2 **10% 5 min**	89.00	33.33
H_2O_2 **10% 10 min**	66.67	66.67
H_2O_2 **10% 15 min**	55.67	100.0
Clorox 10% 15 min	22.00	100.0
Clorox 15% 15 min	11.00	100.0
Clorox 20% 15 min	0.000	100.0
LSD at 5 %	**12.33**	**27.45**

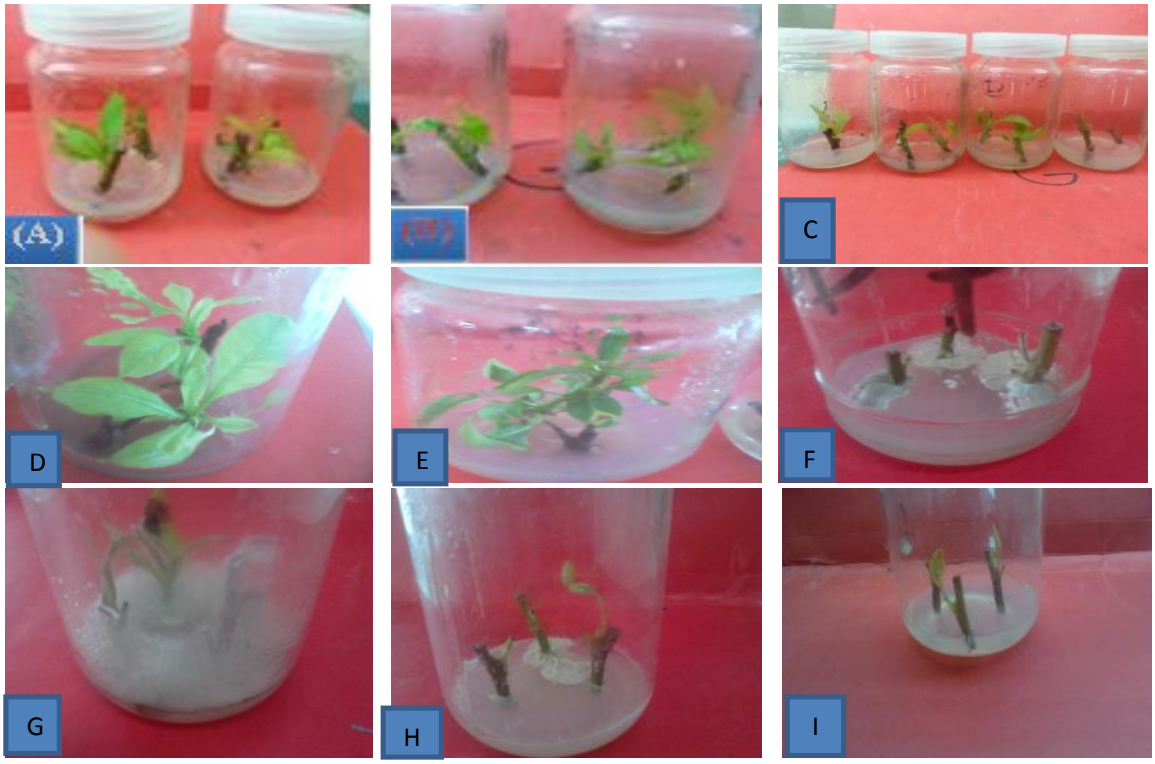

Fig. 1. Effect of disinfecting treatments on survival and contamination rate of Pear Le-Conte (A to E uncontaminated explants and F to I contaminated explants with fungi and bacteria)

Table 2. Effect of medium type and salt concentrations on growth characters of *Pyrus communis* cv. LE- Conte *in vitro* culture

Conc.	Number of shoot				Shoot length (cm)				Number of leaves			
	MS	B5	WP	Mean	MS	B5	WP	Mean	MS	B5	WP	Mean
Full	1.33	1.11	1.22	1.23	3.67	1.82	1.91	2.47	9.97	9.56	7.36	8.96
¾ strength	1.11	1.00	1.00	1.04	1.23	1.53	1.49	1.42	9.28	7.56	7.56	8.13
½ strength	1.00	1.00	1.00	1.00	1.26	1.31	1.30	1.29	8.13	6.89	7.44	7.49
¼ strength	1.00	1.00	1.00	1.00	0.88	1.09	0.97	0.97	6.58	5.89	6.56	6.34
Mean	**1.11**	**1.03**	**1.05**		**1.76**	**1.44**	**1.42**		**8.49**	**7.47**	**7.29**	
LSD 5%		**0.0926**				**0.2012**				**1.021**		
		0.0781				**0.0721**				**0.902**		
		0.0234				**0.0381**				**0.801**		

3.2 Effect of Medium Type and Salt Concentrations on Establishment Stage

The successful explants free of pathogens were cultured on three types of medium at four concentrations of salts were illustrated in Table 2 and Fig. 2. The data showed that the explants cultured on MS medium gave the best results of the shoot number (1.11 shoot lets/explant). On the other hand, both B5 and WPM media gave 1.03 and 1.05 shoot-lets/explant, respectively. Moreover, in MS salts media shoot length was maximized (1.76 cm) in comparison to B5 and WPM which scored 1.44 and 1.42 cm, respectively. Besides, MS medium gave the highest number of leaves.

On the other side, the full strength of salts was the best concentration used in the culture compared with the other concentrations, which recorded the best number of shoot-lets (1.23), leaves number (9.96) and shoot-lets length (2.47) cm compared with the other concentrations studied.

Results on interaction between type of media and concentration is shown in Table 3 the best growth was observed with the explants cultured on MS full strength media, it was scored 1.34 shootlets / explant with 3.67 cm length with the highest number of leaves 9.97 leaf/ shootlet compared with the other media strength.

The data are in agreement with findings of [17] they found that the highest establishment in *Pyrus Patharnakh* (81.76%) and Kainth (78.66%) resulted by using Murashige and Skoog medium. Moreover, [18] compared between the effect of various media (MS 1/2 strength, MS full strength and WPM full strength) and they found MS full strength medium containing BAP (1.5 mg-1) and IBA (0.25 mg-1) gave maximum explant establishment (52.80%) on *Pyrus pashia*.

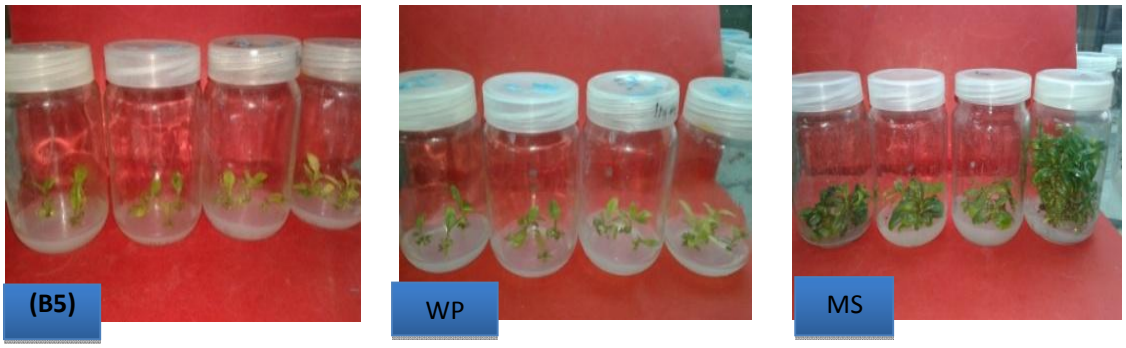

Fig. 2. Effect of type of culture media at different concentrations of established explants of in vitro culture in pear *Pyrus communis* cv. Le-Conte

Table 3. Effect of BAP and kinetin concentrations on some growth characters of pear le-Conte in vitro culture

Treatments	Subculture I				Subculture II			
	Shoot No.	Shoot length	Leaves No.	Leaves area cm^2	Shoot No.	Shoot length	Leaves No.	Leaves Area cm^2
Control	1.00	1.69	9.67	6.85	1.22	1.60	9.53	5.21
BAP 1.0	5.89	2.24	11.31	8.55	5.44	1.93	12.25	7.29
BAP 0.5	4.00	2.26	10.70	9.46	3.89	2.27	12.19	9.20
BAP 0.25	2.00	2.43	10.74	10.48	2.00	2.59	12.15	11.10
Kin 1.0	1.00	1.38	9.11	11.69	1.00	1.72	10.56	12.19
Kin 0.5	1.00	1.30	9.00	11.36	1.00	1.62	9.78	13.20
Kin 0.25	1.00	1.07	6.44	11.97	1.00	1.40	9.67	10.23
LSD 5%	**1.02**	**0.93**	**2.02**	**1.229**	**2.819**	**0.719**	**2.212**	**1.985**

3.3 Effect of Bap and Kinetin Concentrations on Proliferation Stage

From the study, all BAP concentrations showed the formation of shoots Table 3 and Fig. 3. On the first subculture, MS medium supplemented with 1 mg/l BAP produced the highest number of shoots development on the base of explants (5.89 shoot let/explant) followed by 0.5 mg/l BAP (4.0 shootlet/explant). The addition of BAP at 0.25 mg/l also induced shoot let elongation to the highest length 2.43 cm followed by BAP at 0.5 mg/l (2.26 cm) compared with MS free (control) (1.69 cm). The other growth character that is leaves number was increased to the maximum number 11.31 leaves/shootlets for the explant cultured on MS media containing 1 mg/l BAP. Moreover, all concentrations of Kin (0.25, 0.5 and 1 mg/l) supplemented into medium required extended time (4 weeks) to increasing leaves area to (11.97, 11.36 and 11.69 cm^2) respectively during the first subculture (4 weeks).

At the second subculture MS medium supplemented with 1 mg/l BAP produced the highest number of shoots developed on the base of explants (5.44 shootlet/explant) followed by BAP at 0.5 mg/l (3.89 shootlet/explant). The addition of BAP at 0.25 mg/l also induced shootlet elongation to the highest length 2.59 cm followed by BAP at 0.5 mg/l (2.27 cm) compared with MS free (control) (1.69 cm). The other growth character leaves number was increased to the maximum number 12.25 leaves/shootlets followed by BAP at 0.5 (12.19) for the explant cultured on MS media containing 1 mg/l BAP. Moreover, all concentrations of Kin (0.25, 0.5 and 1 mg/l) increasing leaves area to (12.19, 13.20 and10.23) respectively after the second subculture.

In this concern, BAP alone or in combination with other PGRs is the most commonly used cytokinin for apple micropropagation [19,20,21,22]. Using a similar PGR constitution as in 1 mg/L BAP or 0.5 mg/L BAP and 1.5 mg/L Kin medium, [23] found the same results for cv. 'Golden Delicious'. The optimal BAP concentration for maximal shoot proliferation depends on the cultivar [20]. Using BAP in combination with Kin, [21] increased the multiplication ratio of apple cv. 'Tydeman's' early.

BAP is the cytokinin of choice for micropropagation of many species and cultivars in the genus *Pyrus* [24,25].

[26] found that media supplemented with 1 mg/L BAP or 0.5 mg/L BAP and 1.5 mg/L Kin had a similar effect on shoot proliferation producing 2.5 and 2.4 shoots per inoculated shoot, respectively. On medium, with 0.5 mg/L BAP alone lower number of shoots was obtained on apple cv. Topaz micropropagation.

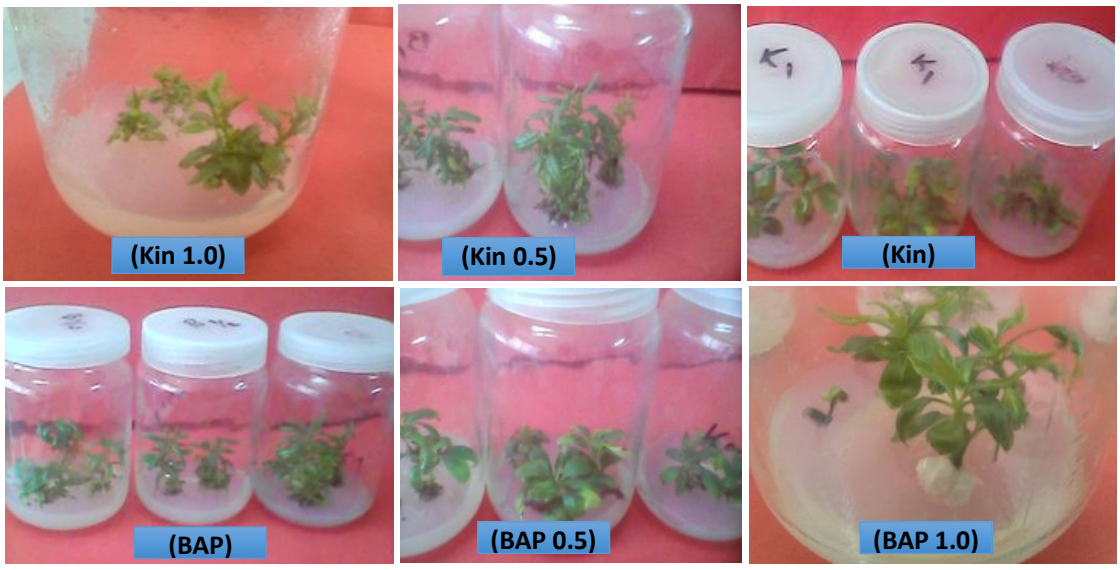

Fig. 3. Effect of BAP and Kin at different concentrations of shoot proliferation of in vitro culture in pear *Pyrus communis* cv. Le-Conte

3.4 Effect of IAA and IBA Concentrations on Rooting Characterizations

Effect of IAA on shootlets elongation was observed in Table 4 and Fig. 4, which showed that applying IAA at 2.0 mg/l gave the maximum length of explants 3.83 cm compared to control (3.67 cm). In addition, IAA at 3.0 mg/l increased the leaves number to 18.67 leaf/shootlets followed by IAA at 2.0 mg/l 16.33 leaf/shootlet in comparison to control (9.97 leaf/shootlet).

On the other hand, IBA (1, 2 and 3 mg/l) gave a negative effect of shoot length by decreased the values to (2.70, 1.13 and 1.50 cm) and IBA (2 and 3 mg/l) gave an adverse effect on leaves number which decreased the values to (9.33 and 8.67 leaf/shootlet) respectively.

This results are in harmony with findings of [27] observed that IBA, IAA and NAA induced *in vitro* rooting in wild pear (*P. syrica*) and a maximum of 72 percent rooting was achieved with 3.0 mg/l IAA. Results showed that for *in vitro* rooting of Conference pear cultivar, IAA at 2.7 µM was most appropriate [28].

The result is in disagreement with findings of [26] found that the high rooting efficiency (68.7%) and high number of roots per shoot (6.6) and the best quality of shoots were obtained in rooting medium containing 2 mg/L of indole-3-butyric acid (IBA).

3.5 Callus Induction

According to the data presented in Table 5 and Fig. 5, the callus formation of the leaves disk cultured on MS medium containing 2 mg/l NAA and 2 mg/l 2,4-D scored the highest value of callus formation (100%) compared to the other treatments. Callus induction and fresh weight formation increased significantly with same treatment to 2,21g while the dry matter was increased significantly with those treated with 4 mg/l NAA + 2 mg/l 2,4-D (100 ug). Application of exogenous PGRs was found to be essential for the induction of callus, embryogenic culture establishment, proliferation, maturation and germination of embryos into plantlets. Although callus production was noted to be maximum on medium amended with BAP + NAA + 2,4-D, individual application of 2,4-D was also very effective in inducing callus from basal clove explant.

Generally, 2,4-D is considered to be one of the most important PGRs that regulate somatic embryogenesis *In vitro* [29] During induction into the medium, 2,4-D increased explant's endogenous auxin level, one of the crucial signals that determine cultured cells' fate to become embryogenic [30,31] earlier reported that compared to higher concentrations, low levels of 2,4-D were more effective when combined with BAP for inducing embryogenic tissue. In auxin amended medium, cultured cell or tissue produce more ethylene than the auxin free cultures, which suppresses embryo development as the tissue multiplication continues to proceed without much check, the embryonic clumps develop into mature embryos only on medium amended with a very low level of 2,4-D [32]. These observations support our present study that the auxin (2,4-D) has no significant effect on the induction of embryos rather it has a considerable positive effect on callus production during the dedifferentiation stage. A similar observation was noted in other plants like *Melia* where embryos were formed from pre-embryogenic determined cells and did not depend on 2,4-D requirement [32,33,34, 35].

Table 4. Effect of IAA and IBA concentrations on rooting characterizations of *Pyrus communis in vitro* culture

Treatments	Shoot length	Leaves No.	Rooting %	Root No.	Root length cm
Control	3.67	9.97	11.11	2.00	1.60
IAA 1.0 mg/l	3.23	14.33	55.56	8.00	3.44
IAA 2.0 mg/l	3.83	16.33	77.78	6.00	3.95
IAA 3.0 mg/l	3.47	18.67	100.0	7.00	7.90
IBA 1.0 mg/l	2.70	11.83	44.45	4.00	4.25
IBA 2.0 mg/l	1.13	9.33	55.56	4.00	3.90
IBA 3.0 mg/l	1.50	8.67	88.89	7.00	5.60
LSD 5 %	**0.975**	**2.091**	**10.34**	**0.887**	**1.822**

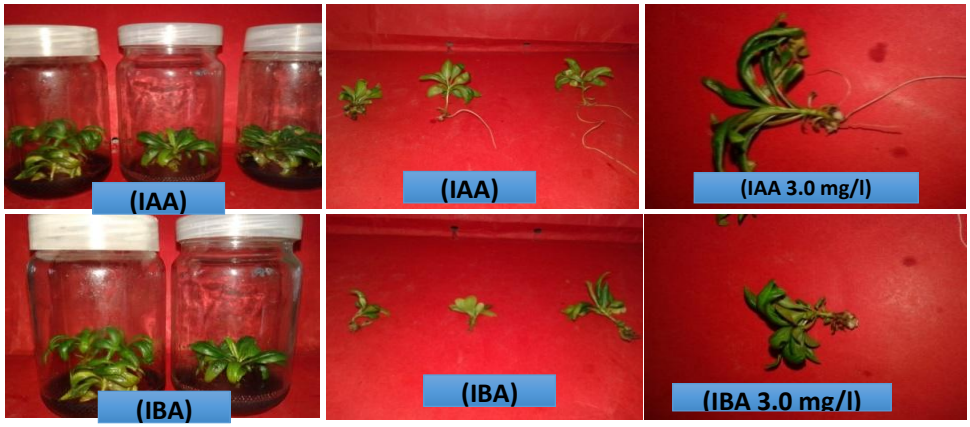

Fig. 4. Effect of IAA and IBA at different concentrations of root proliferation of in vitro culture in pear *Pyrus communis* cv. Le-Conte

Table 5. Effect of 2,4-D and NAA concentrations on callus characterizations of pear Le-Conte *in vitro* culture

Treatments	Callus %	F.W g	D.W ug
Control	6.66	0.810	27.0
NAA 2 mg/l	44.44	1.220	91.0
NAA 4 mg/l	40.74	1.780	87.0
NAA 8 mg/l	37.37	1.750	61.0
2,4 – D 2 mg/l	18.51	0.830	30.0
2,4 – D 4 mg/l	51.85	1.460	43.0
2,4 – D 8 mg/l	18.51	1.400	68.0
NAA 2 mg/l + 2,4-D 2 mg/l	100.0	1.470	73.0
NAA 2 mg/l + 2,4-D 4 mg/l	77.77	1.490	62.0
NAA 2 mg/l +2,4-D 8 mg/l	62.69	1.520	72.0
NAA 4 mg/l + 2,4-D 2 mg/l	74.07	2.210	100.0
NAA 4 mg/l + 2,4-D 4 mg/l	77.77	1.910	101.0
NAA 4 mg/l + 2,4-D 8 mg/l	11.11	1.660	91.0
NAA 8 mg/l + 2,4-D 2 mg/l	66.66	0.910	40.0
NAA 8 mg/l + 2,4-D 4 mg/l	66.66	1.930	90.0
NAA 8 mg/l + 2,4-D 8 mg/l	44.44	2.110	86.0
LSD 5%	**12.32**	**0.716**	**6.224**

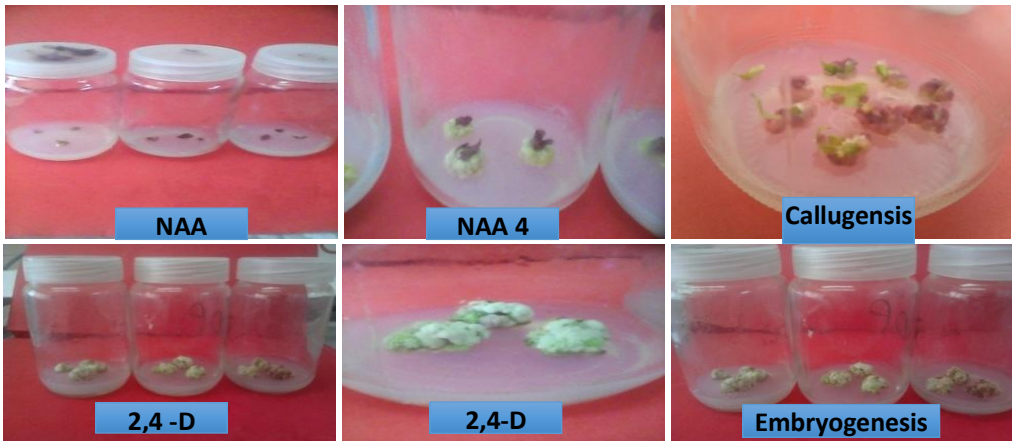

Fig. 5. Effect of 2,4-D and NAA concentrations on callus characterizations of Pyrus communis *In vitro* culture

4. CONCLUSION

Definitely, it can be concluded from the obtained results that using Clorox 20% per 15 min at the disinfecting stage and using MS salt at full strength for the establishment stage, then using BAP at 1.0 mg / l to increase the number of shoots at the proliferation stage and using a mixture of NAA and 2,4-D 2:2 mg / l to obtain the highest value of callus formation. Moreover, using IAA at 1 mg / l to obtain the highest number of roots.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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