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In vitro Propagation of Pear *Pyrus betulaefolia* Rootstock

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Abstract: An effective *in vitro* culture system from stem segments and shoot tips of field grown pear (*Pyrus betulaefolia)* was established. The successfully induced culture was achieved on Murashige and Skoog (MS) basal medium supplemented with 2.0 mg/l 6-benzylaminopurine (BAP) and 0.05 mg/l β-naphthalene acetic acid (NAA), 30 g/l sucrose and 3 g/l phytagel. The optimal shoot multiplication was achieved on MS medium supplemented with 2.5 mg/l BAP plus 2.0 mg/l isopentenyl adenine (2iP). Indole -3-butyric acid (IBA) was more effective for rooting of shoots than NAA. Eighty five percentage of rooting was obtained on half- strength MS medium containing 2.0 mg/l IBA in addition to 1 g/l activated charcoal (AC) and 20 g/l sucrose. Regenerated shoots were successfully acclimatized to greenhouse conditions and grew vigorously with no apparent phenotypic aberrations.

Key words: *Pyrus betulaefolia* · Rosaceae · *In vitro* · Stem segment · Rooting · Shoot tip · Rootstock

INTRODUCTION

Pear (member of Rosaceae family) one of the most important deciduous fruit trees all over the world it takes the second rank after apple in production. Rootstocks play an important role in pear production, since, the proper choice of rootstock is as important as the choice of variety and site. This is true because the rootstock is involved in determining two key factors: The variety susceptibility to several serious diseases and the tree's performance in the climate and the orchard site. Stebbins [1] reported that the principal diseases of pear trees, which related to rootstocks, are fire blight. *P. betulaefolia* seedlings were used in the last few years in a commercial scale as rootstocks for Japanese cultivation. *P.betulaefolia tolerates* growth under saline conditions as it possesses higher tolerance to drought [1]. It is one of the best rootstocks, which is tolerant to wet and drought conditions. Resistant to decline, blight, root aphid and root rot [2] *P. betulaefolia* rootstocks produce high fruit yield perform well in clay and poorly drained soils [3] and are resistant to pear decline and fire blight [4]. *In vitro* propagation technique has become an efficient way for producing plants as uniform as possible on a large scale and in a short time for the plantation industry [5]. *P. betulaefolia* rootstock is on regular basis reproduced through seeds. In Egypt, *P.betulaefolia* seeds are imported annually for production of rootstocks. There are few studies that have been done on the *in vitro* propagation of *P. betulaefolia* rootstocks from stem segments and shoot tips [6-8]. However, *in vitro* rooting of *Pyrus ssp.* has proven to be difficult [9, 10]. Several authors were successfully induced rooting of European Pear but the results were poor with respect to Asian pear (*P. betulaefolia*) [11, 12].

The objective of the present study was to reduce *P.betulaefolia* imports and provide hard currency in Egypt, through tissue culture, in order to produce vegetative rootstocks, which are better than seed rootstock and gives true to type plantlets identical in size and shape.

MATERIALS AND METHODS

Explant Source and Sterilization: Shoot tips and stem segments of young branches of *P.betulaefolia* were collected in spring- summer and autumn from plants grown in a private farm located at 68 km Cairo- Alexandria desert road. These explants were placed under running tap water for 24 h, then surface sterilized by dipping in 70% ethanol for 1 min, then immersing in 10% and 20% (w/v) commercial bleach solution (Clorox; containing

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5.25% NaOCl) for 10 min and 15 min, for shoot tips and stem segments, respectively. Finally explants were rinsed five times with sterile distilled water. Survival percentage (%) and browning percentage (%) were recorded for the sterilized explants collected in the different seasons.

Culture Establishment: The explants were cultured in culture tubes (Sigma) (25 x 150 mm) with basal MS medium (Duchefa, Haarlem, the Netherlands) [13] supplemented with 6-benzylaminopurine (BAP) (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), β-naphthalene acetic acid (NAA) (Sigma Cell Culture, min, 90%, St. Louis, USA), at a concentration of 0.05 mg/l for all treatments, sucrose (30 g/l w/v) and solidified with 3 g/l phytagel (Duchefa, Haarlem, the Netherlands). Anti browning agents viz. ascorbic acid (100 mg/l) and citric acid (150 mg/l) were added to explant establishment medium to reduce phenolic compounds. The pH of the medium was adjusted to 5.7–5.8 before autoclaving under 1.05 kg/cm^2 pressure at 121°C for 20 min. Explants were cultured in darkness for 7 days before transferring to white light according to Zou *et al.*[14]. The explants were incubated in a growth chamber at 23 ± 1°C, 16: 8 h photoperiod and 3000 Lux illumination intensity. After four weeks the aseptic axillary shoots were transferred to fresh MS medium. Survival percentage (%), growth percentage to survival (%) and average shoot length (cm) were recorded.

Shoot Multiplication and Elongation

The cultures were initiated on MS medium supplemented with different concentrations of the following growth regulators, BAP (0.0, 1.0, 1.5, 2.0 and 2.5 mg/l) plus (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) of N^6– isopentenyl adenine (2iP). Proliferation rate (average number of new shoots produced per shoot) and average shoot length (cm.) were recorded after 4 weeks of culture. The micropropagation cycle consisted of a monthly subculture of nodal segments after removal of the new shoots onto fresh medium.

Rooting Induction and Acclimatization: Elongated shoots derived from nodal explants, were cultured in solid half strength MS medium containing 20 g/l sucrose and in addition 1 g/l activated charcoal (AC). Indol-3-butyric acid(IBA) at (0.0,0.5,1.0,1.5 and 2.0 mg/l) or NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) were tested in darkness, The cultures were initially maintained for 7 days and then exposed to light for 21 days and incubated at 23±1°C. The percentage of rooted shoots (%), average number of roots per shoot and average root length (cm) were evaluated after 6 weeks of culture, on the rooting medium. The rooted shoots were removed from the culture tubes, washed with tap water to remove adhering medium, dipped carefully for 10 min in 1% bavistin (systemic fungicides) and transferred to plastic pots with the mixture of sand and peat moss (1:1 v/v). Initially, plantlets were covered with a poly ethylene film, which was gradually eliminated in three weeks. The pots placed in a greenhouse for completing their acclimatization the plantlets were irrigated with ¼ MS medium for 8 weeks in the green house (28±2°C, 70-80% relative humidity).

Statistical Analysis: Experimental data were conducted as completely randomized design, with 40 replicates for each treatment, variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan [15].

RESULTS AND DISCUSSION

***In vitro* Culture Establishment:** A maximum of 90% of stem segment and 20.50% of shoot tip explants remained aseptic two weeks after incubation on establishment medium and the axillary buds started to burst. The highest significant survival percentage (20.50%), growth to survival (25%) and average shoot length (0.5 cm) of shoot tips was obtained using MS medium supplemented with 1.5 mg/l BAP and 0.05 mg/l NAA (Table 1) comparing to the other treatments. Concerning the stem segment explants, cultured on MS medium supplemented with 2.0 mg/l BAP and 0.05 mg/l NAA, they recorded the highest survival percentage (90%), growth to survival percentage (85%) and average shoot length (3cm). (Table 1 and Fig.1A). Generally it was noticed that increasing the concentration of BAP caused increase of explants growth. From results it was found that stem segment explants proved to be better than shoot tips for initiating micropropagation. This observation is in harmony with that of Hassanen [16] who found that stem segment of *P. communis* is better than shoot tip as explants. MS medium was used as mineral medium for culturing many *Pyrus* species for proliferation [17] as *P. pyrifolia.* [18], *P. communis* [19] *P. calleryana* [20], *P. syrica* [21] and *P. betulaefolia* [6]. In general, BAP is the most effective cytokinin for pear micropropagation [11, 22, 23]. Also, stem segment explants proved to be better than shoot tips for initiating micropropagation of wild pear [24], in *P.communis*, cv. Rocha [25] and *P. pyrifolia* cvs. carrick, Nijisseiki and Bartlett [26].

Table 1: Effect of explant type and growth regulators (BAP and NAA) on *in vitro* establishment of *Pyrus betulaefolia*.

		Survival%		Growth% to survival		Average shoot length (cm)	
BAP mg/l	NAA mg/l	sh.	st.	sh.	st.	sh.	st.
0.0	0.0	10.00	15.00	10	50	0.3b	1.0d
0.5	0.05	13.50	42.50	15	60	0.3b	2.5b
1.0	0.05	17.00	52.50	20	70	0.5a	2.5b
1.5	0.05	20.50	51.00	25	60	0.5a	1.5c
2.0	0.05	14.50	90.00	15	85	0.3b	3.00a

Sh. =shoot tip –st. =stem segments

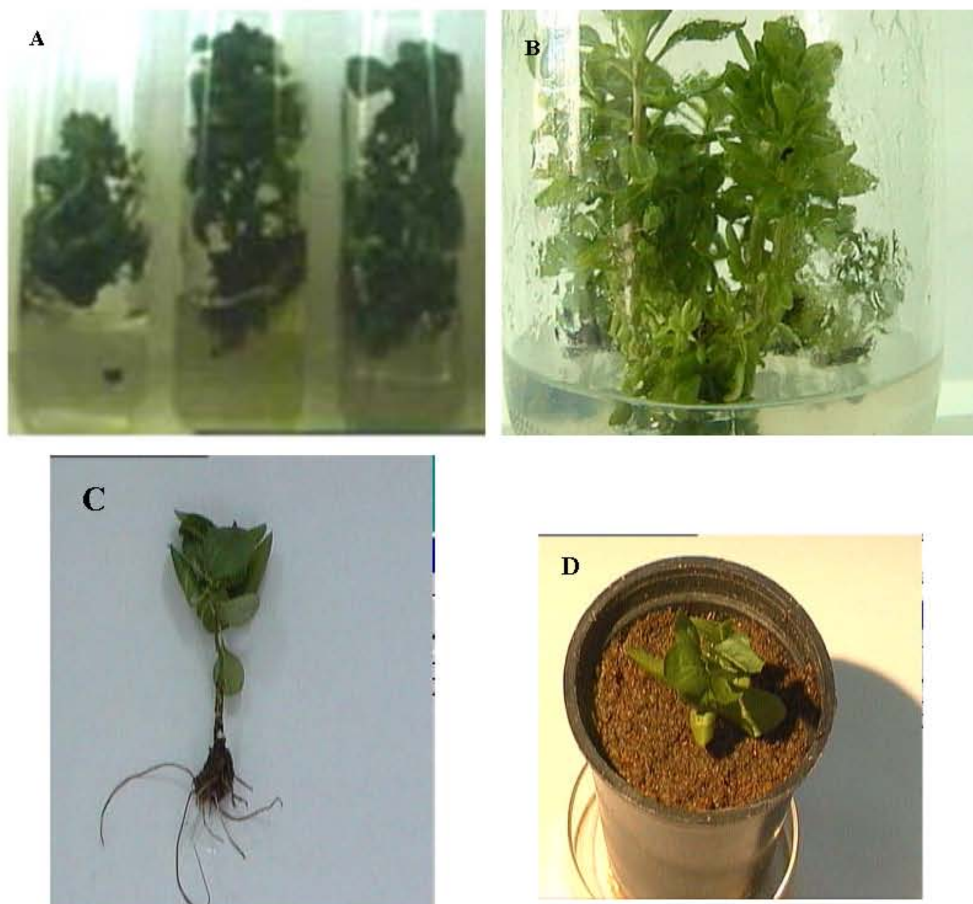

Fig. 1: Stage of micropropagation of *P.betulaefolia* (A) Establishment of stem segments. (B) Multiple shoots formed in MS medium. (C) *In vitro* rooted shoots (D) Acclimizated plantlets in greenhouse.

Seasonal Variation: The highest survival percentage (20 and 70%) for shoot tips and stem segments, respectively was observed during spring (Table 2). The survival percentage of stem segment explant was more than that the shoot tip explants. Data in Table 2 also showed that the browning percentage had the lowest value during spring (30%) for stem segments, but it recorded 80% for shoot tips when the explants were collected during spring season, higher explant establishment and lower loss of the culture explants from browning and endogenic contamination were obtained. These results are in agreement with those of Bharate Kumar *et al.* [27] who found that the intensity of browning was less during spring and increased with time and reached the maximum during summer. Also, explants of *P. pashia* [28], *P. pyrifolia* cv. Gola and *P. callryana* [29] gave the best *in vitro* response, when were collected during the spring season. High levels of growth

Table 2: Effects of seasonal variation and type of explant on survival and browning percentage

	Survival%		Browning%	
Seasons	sh.	st.	sh.	st.
Spring	20	70	80	30
Summer	0.0	10	100	90
Autumn	10	30	90	70

Sh. =shoot tips –st. =stem segments

Table 3: Effect of growth regulators (BAP and 2iP) on shoot proliferation of *Pyrus betulaefolia*

Concentration mg/l			
BAP	2iP	Average number of shoots/ explant	Average shoot length (cm.)
0.0	0.0	1.00 e	1.00 c
1.0	0.5	2.50 d	1.00 c
1.5	1.0	4.70 c	3.5 b
2.0	1.5	5.90 b	4.7 a
2.5	2.0	8.90 a	3.8 b

promoting substances and low growth inhibitors in actively growing shoots during spring may be responsible for the high survival percentage and explants *in vitro* establishment. Higher browning and lower survival percentages in shoot tips as compared to stem segments in all seasons may be due to higher phenolics as the shoot tips are actively growing and herbaceous in nature [30]. Addition of antioxidants (ascorbic and citric acid) to establishment medium has been reported as effective in preventing oxidation of phenolics [31]. Wang *et al.* [32] reported the effectiveness of ascorbic acid and citric acid in reducing phenolic browning and enhancing explant establishment in apple.

Shoot Multiplication and Elongation: For axillary shoot proliferation, cytokinins were utilized to overcome the apical dominance of shoot and to enhance the branching of lateral buds. Therefore, MS basal medium supplemented with 2.5 mg/l BAP and 2.0 mg/l 2iP gave significantly the highest number of shoots (8.90) (Table 3 and Fig 1B). Shoot length was found to be maximum (4.7 cm) in 2.0 mg/l BAP + 1.5 mg/l 2iP supplemented medium. The present observation that cytokinins can stimulate growth of lateral buds and thus suppress apical dominance [33]. These results are in agreement with those obtained by Kadota and Niimi [34] who working on pear (*P. pyrifolia* N.) they suggested that

Table 4: Effect of IBA and NAA on *in vitro* rooting of *Pyrus betulaefolia*.

Concentration mg/l				
IBA	NAA	Shoots rooted	Average no of roots /shoot	Average root length (cm)
0.0	0.0	0.0	0.0c	0.0c
0.5	0.0	0.0	0.0c	0.0c
1.0	0.0	33	1.0b	1.0b
1.5	0.0	41.0	1.0b	1.5b
2.0	0.0	85.0	4.0a	3.0a
0.0	0.5	0.0	0.0c	0.0c
0.0	1.0	10.0	1.0b	1.5b
0.0	1.5	15.0	1.0b	1.5b
0.0	2.0	30.0	2.0a	2.0a

BAP displayed more notice able effect than TDZ and kinetin, i.e. BAP is more suitable for shoot multiplication of pear than phenyl urea derivatives. It is well known that high concentration of cytokinins of adenine type (BAP and 2iP) is often necessary for growth and differentiation.

Rooting Induction and Acclimatization

The multiplied shoots were induced to regenerate roots in ½ MS solid medium supplemented with different concentration of IBA or NAA. Maximum rooting percentage (85%) occurred *in vitro* in solid ½ MS medium with 2.0 mg/l IBA (Table 4 and Fig. 1C) and the number of roots per shoot was 4.0 and root length reached 3.o cm. In the present study, half strength MS medium was adequate for root induction. Relatively low salt concentration in medium are know to enhance rooting and shoots in several plants species [35]. Moreover, both concentration and type of auxins used, markedly influenced the percentage of root formation. IBA is commonly used to promote root initiation both *in vitro* and in the propagation by cutting [36]. Housman [37] has shown that in tissue culture media, IBA oxidized slowly. Its slow movement and delayed degradation may be the primary reason for better performance of IBA as compared to IAA and NAA. As shown by Zhou *et al.* [38], 100% rooting results were seemed, while using 4.92 mM IBA. Moreover, Caboni *et al.* [39] obtained a good rooting of a rootstock of an apple tree under 16 h photoperiod in the presence of 2 mg/l IBA. As reported by Touqeer *et al.* [40], the maximum number of roots was obtained using IBA (0.4 mg/l) for peach rootstock. About 70% of rooted plantlets were successfully acclimatized in the greenhouse condition within 8 week and grew with no morphological variations (Fig 1D).

CONCLUSIONS

Stem segment explants proved to be better than shoot tips for initiating micropropagation of *P. betulaefolia*, MS medium with 2.0 mg/l BAP + 0.05 mg/l NAA gave the highest growth to survival percentage. While, 2.5 mg/l BAP + 2.0 mg/l 2iP was the best growth regulators combination for shoot multiplication. Half strength MS medium supplemented with 2.0 mg/l IBA + 1 g/l AC + 20 g/l sucrose was the best medium for root induction. Rootstock plantlets acclimatization plants were successfully performed in the greenhouse produced after 8 weeks. The present results indicated an effective *in vitro* propagation method for *P. betulaefolia* by providing a protocol for producing genetically uniform plants of selected genotype.

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