Scientia Horticulturae 227 (2018) 57–64

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Polyethylene glycol and chilling overcome Somatic embryogenesis obstacle in *Pyrus communis*

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ARTICLE INFO

Keywords:
Somatic embryogenesis
Plant recovery
Globular
Pyrus
Stress

ABSTRACT

The purpose of this investigation is to achieve the procedure of somatic embryogenesis (SE) as an efficient propagation way in four pear cultivars: Natanzi, Ghosi, Williams and Dar Gazi, by focusing on two strategies: One is the application of competent explants, and the other one is stress employment. The results were variable because of cultivars behavior was highly genotype specific. However, the results identified that the highest frequency of embryogenic callus induction was obtained from the endosperm and immature cotyledonary segment explants of *P. communis* cultivars on Murashige and Skoog medium (MS) containing 8.05 μM 1-naphthaleneacetic acid (NAA), 13.32 μM N6-benzyladenine (BA) supplemented with 250 mg/L glutamine as a reduced nitrogen source and 30 g/L (w/v) sucrose in Ghosi (75.46%). The optimized media were different for each cultivar in the induction phase, namely, Natanzi and Ghosi in MS, Williams in Schenk and Hildebrandt (SH) and Dar Gazi in Nitsch and Nitsch (NN). Then, it was observed that when embryogenic calli were transferred to the media containing 1% (w/v) Polyethylene glycol (PEG), calli of some cultivars further differed into embryos while other cultivars produced in chilling at 4 °C. Also, maturation of embryos occurred at 4 °C for 4 months. Then passages using MS media caused germination of embryos and plantlets were produced. To sum up, the greatest frequency of embryogenic callus (75.46%) occurred in Ghosi, and the highest embryo maturation and germination (77.06% and 37.66%, respectively in mentioned cultivars) were observed in Natanzi.

1. Introduction

The major object of in vitro culture is the propagation of plants in the shortest possible time, and the use of this technique should be economically cost-effective. Many woody perennial species face trouble in natural vegetative propagation because they are often difficult-to-root and troubleshooting this problem is cost-effective by in vitro culture. The case study of this research is *Pyrus communis*. This is a pear that is a typical fruit of temperate climates, and it has wide acceptance throughout the world. The domestication of this fruit began around 6000 years ago through vegetative propagation (Silva et al., 2014). This genus is difficult-to-root, and these trees face endangerment because of *Erwinia amylovora* in Iran. Establishment of an efficient propagation system is an effective way to rescue endangered species. Moreover, we can overcome the propagation obstacle in some difficult-to-root species. Most cultivars of *Pyrus communis* have proved very difficult to root from hardwood or softwood cuttings (Fadl and Hartmann, 1967; Bhojwani et al., 1984; Rugini et al., 1993; A1-Maarri et al., 1994; Reed, 1995; Zhu et al., 2003; Sun et al., 2011). Somatic embryogenesis (SE) can be utilized as the most convenient way because it creates a bipolar structure for the formation of root and shoot (Loureiro et al., 2005), and it improves the multiplication rate. In short, the advantages of SE including increasing propagation rates (Xu et al., 2014; Xu et al., 2015), producing genetically modified plantlets (Von Arnold et al., 2002), as an alternative method for clonal propagation of intact plants or a tool for breeding (Wallin et al., 1995), we can employ embryogenic cultures that can be cryopreserved so that it helps establish gene banks (Von Arnold et al., 2002). However, SE in *Pyrus communis* has received little attention so far, and there is no report for plant recovery from embryos. Only Mehra and Jaidka (1985) studied embryogenesis, and Qingrong et al. (2003) reported somatic embryo regeneration rate.

Based on the background of *Pyrus communis* SE protocol, it cannot be stated that somatic embryogenesis is an efficient way while it must be optimized at first. Then SE was introduced as a successful way to overcome the propagation problems in *Pyrus communis*. In this investigation, it has been hypothesized that the selection of key factors in

Abbreviations: 2,4D, 2,4-dichlorophenoxyacetic acid; BA, N6-benzyladenine; NAA, 1-naphthaleneacetic acid; SE, somatic embryogenesis; DAP, Days after pollination; ABA, Abscisic acid; IBA, Indole-3-butyric acid; CH, Casein hydrolysate; MS, Murashige and Skoog; NN, Nitsch and Nitsch; W, White's medium; SH, Schenk and Hildebrandt; GD, Gresshoff and Doy

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http://dx.doi.org/10.1016/j.scienta.2017.09.018
Received 16 July 2017; Received in revised form 19 September 2017; Accepted 20 September 2017

embryogenesis process can facilitate plant recovery by SE. One of the key factors is the selection of competent explants for this process. Research works proved that in addition to auxin, the most important agent in the SE is the explant source. Evidence for the support of this assertion is a survey by Gaj et al. (2005). They used *lec1*, *lec2* and *fus3* mutants in the somatic embryogenesis process and compared them with the wild type. Analysis of their data indicated that auxin accumulation was rapid in all tissues of the explants of wild type and the *lec2-1* mutant, cultured on somatic embryogenesis induction medium containing 2, 4-D whereas *lec* mutants could not produce direct somatic embryos. Moreover, these mutants have low indirect somatic embryo frequency (0–3.9%). Briefly, Gaj et al. (2005) stated that leafy cotyledon genes are essential for induction of somatic embryogenesis of Arabidopsis. Van Schaik et al. (1996) introduced the immature zygotic embryos in half-ovules when the endosperm was still soft and white as the best explants for somatic embryogenesis in *Alstroemeria spp*. Perhaps the cause of the accompaniment of endosperm with zygotic embryo was explained in the study of Berger (1999). Berger (1999) emphasized on the existence of *EP3* gene, strongly expressed initially in the seed integument and later in the endosperm, and its function in somatic embryogenesis. Berger (1999) assumed that *EP3* acts on substrates containing chitin motifs such as arabinogalactan proteins and releases oligosaccharides important for embryogenesis. Immature zygotic embryos were used as explants in *Pinus sylvestris* (Salo et al., 2016), *Pinus oocarpa Schiede* ex Schlectendal (Lara-Chavez et al., 2011), and *Picea abies* (Helmersson et al., 2004), integument of immature seeds in *Hevea brasiliensis* (Lardet et al., 2009), immature stamens (anther plus filament) and ovaries in *Vitis vinifera* (Acanda et al., 2013), sexual organs (Wallin et al., 1995), nucellus (Eichholtz et al., 1979; James et al., 1984; Saito et al., 1989) and endosperm (Shih-kin et al., 1977; James et al., 1984) in *Malus.* The response to embryogenesis was not the same for different species. In some species somatic embryogenesis efficiency was high, and in others, it was recalcitrant (Jiménez, 2001). The reason for recalcitrance in SE is related to explants competence, endogenous hormone level (Jiménez, 2001; Merkle et al., 1995) and many other factors that are doubtlessly involved in embryogenesis. The stress that was assumed in our study as a second factor was supported by Zavattieri et al. (2010), Karami and Saidi (2010) and Nowak and Gaj (2016). In recent years, stress mode of action in this process has been referred to as chromatin reorganization (Fehér, 2005; De-la-Peña et al., 2015; Zavattieri et al., 2010). One of the changes which have been confirmed to cause chromatin reorganization is the change in DNA methylation patterns. These phenomena are associated with the regulation of several genes involved in somatic embryogenesis, such as *WUS*, *BBM1*, *LEC*, and several others (De-la-Peña et al., 2015). Nowak and Gaj (2016) identified that the stress in promoting the SE was related to *bHLH109* expression. The latest research progresses on SE showed the modified Staba vitamins, a complex of B vitamins, facilitate the formation of embryos in *Rubus sanctus* (Sabooni and Shekafandeh, 2017). The application of 2 months of cold treatment before culture in germination medium significantly improved the germination response in somatic embryos of *Quercus ilex* (Martínez et al., 2017). The major determining factor of SE efficiency is genotype, then, PGR composition, and finally, explant type (Carra et al., 2016). Enriching the medium with 4–5% glycerol as carbon source caused the best results of embryo formation in of different citrus varieties (Kayim and Koc, 2006).

The principle aim of the current study is to achieve the procedure of plant regeneration by SE in *Pyrus communis* beside the employment of the key factors in embryogenesis.

2. Materials and methods

2.1. Plant material

Explants used in this investigation were endosperm and immature cotyledon. These explants were collected from Imam Reza orchard, (latitude 36° 20′ N, longitude 59° 34′ E) Mashhad, Iran, of four pear cultivars (Dar Gazi, Williams, Ghosi and Natanzi). It is well-known that the endosperm is absorbed by cotyledon in di-cotyledons after a while. Hence, selection of seeds at the proper stage of development is important. The proper harvest time of immature fruits with the existence of endosperm in seeds is usually calculated as days after pollination (DAP) and this time is different in different plants (Thomas and Chaturvedi, 2008). Fruits were harvested at different times, including 40, 50, 60, 70, and 80 days after pollination. In this phase, the best time was selected to get endosperm and immature cotyledon as explants.

2.2. Embryogenic callus induction

Immature fruits of *Pyrus communis* cultivars were collected 50 days after pollination for endosperm culture. At this stage of fruit growth, a major portion of the seed has formed endosperm. Also, these fruits were used for immature cotyledon culture. Then, the fruits were washed in running tap water for 45 min, followed by submerging in 5% (v/v) commercial bleach (containing 5% sodium hypochlorite) with 100 µl triton x-100 for 10 min and they were finally rinsed in 70% (v/v) ethanol for 10–15 s. The cleaned fruits were surface sterilized in 0.1% (w/v) mercuric chloride for 8 min. Afterwards, the fruits were washed in sterile distilled water for three times. Then the seeds were dissected out and, the seed coats were removed with the help of a pair of needles. The endosperm without an embryo was carefully excised with the aid of a binocular microscope. Afterwards, for the culture of immature cotyledonary explant, first the up and down sections of cotyledon were deleted, and the explants were selected at distant sections from the embryonic axis (Fig. 1B). Then the explants were separately cultured in the callus induction medium. Embryogenic callus induction phase has been evaluated in four steps. The steps conducted for the present phase are summarized in Table 1, and their details are as follows. This phase was starting with standard carbon and nitrogen sources, as well as medium formula, then the best results of each step were used for other steps. At first, the explants were cultured in (Murashige and Skoog, 1962) basal salts and vitamins supplemented with 30 g/L (w/v) sucrose, 0.1% (w/v) casein hydrolysate (CH), plant growth regulators (PGRs) (2.68 µM NAA + 4.44 µM BA, 2.68 µM NAA + 8.87 µM BA, 2.68 µM NAA + 13.32 µM BA, and 8.05 µM NAA + 13.32 µM BA) in different combinations for callus induction and solidified with 0.8% agar (Table 1). All of the media were adjusted to pH 5.8 prior to autoclaving at 121 °C for 20 min. In this step, the best PGR combination was selected. In the second step of the protocol, the effect of nitrogen compounds (250 mg/L glutamine, 350 mg/L glutamine, 0.1% CH, and 0.2% CH) was tested. Surely, the best PGR combination of the previous step was used in the second step (Table 1). The next steps were conducted following a sequential order: the evaluation of different carbon sources (sucrose, sorbitol and maltose) at 30 g/L on producing embryogenic callus (Table 1); in this step, the results of before steps were used; finally, different media (MS; Murashige and Skoog, (1962), NN; Nitsch and Nitsch (1969), W; White (1954), SH; Schenk and Hildebrandt (1972) and GD; Gresshoff and Doy (1974)) were compared for further increment of embryogenic callus efficiency. In all of the steps, ten cotyledonary segments or ten endosperm segments were placed on 80-mm-diameter polystyrene Petri plates containing 20 ml of the medium. The cultures were maintained in darkness at 23 ± 1 °C. Subculturing was performed after every 22–28 day intervals for 2–3 passages. Delay in subculturing led to the browning of the medium, deterioration of cultured tissues and drying callus that was commonly observed.

2.3. Differentiation embryogenic callus

Embryogenic calli were transferred to the medium containing MS (1962) basal salts and vitamins supplemented with 30 g/L (w/v) sucrose, 250 mg/L Glutamine, 8.05 µM NAA, and 13.32 µM BA, as well as,

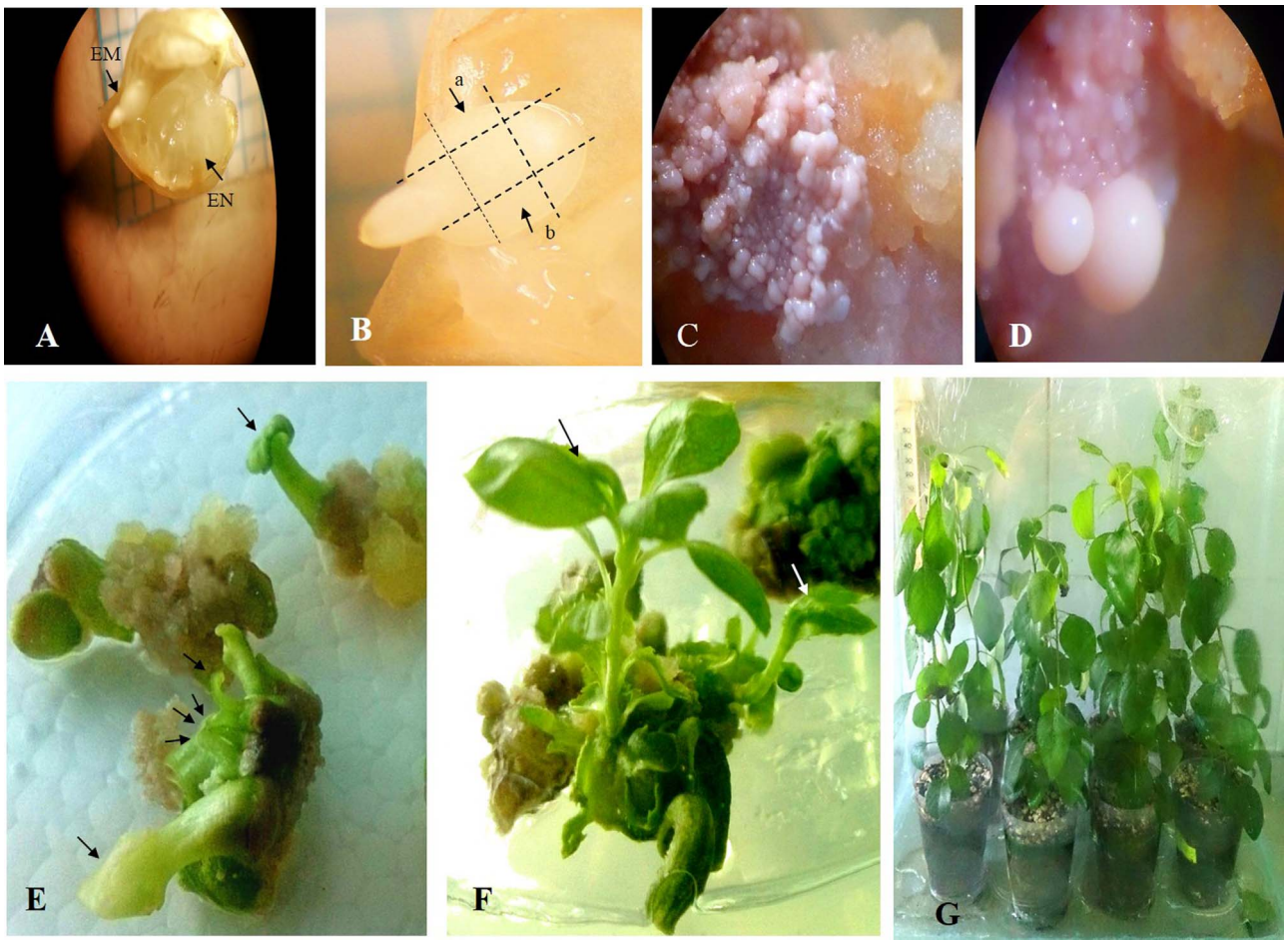

Fig. 1. Explants used for somatic embryogenesis (EN: endosperm, EM: embryo) (A). a and b sections selected as immature cotyledon segment explants (B). Granular callus (C). Globular stage (D). Torpedo stage (E). Germinated embryo (F). Plant recovery (G).

differentiation treatments or procedures and solidified with 0.8% agar. Two treatments, i.e. 1% (w/v) Polyethylene glycol (PEG) and 2 mg/L ABA were used for the assessment of differentiation. Besides two aforementioned treatments, three procedures were also tested including the evaluation of cold treatment at 4 °C for 4 months, culturing in ½ MS basal salts and vitamins + 0.25 mg/L activated charcoal +1 mg/L IBA without components of optimized medium at first phase and the last case, culturing in a liquid media. In the chilling procedure, embryogenic calli were cultured in optimized medium and cultivations were incubated at 4 °C for 4 months.

In the liquid culture procedure, approximately 400 mg of fresh weight of embryogenic callus was transferred to 50 ml of liquid media. In this procedure, a medium optimized in the previous step was used. The suspensions were incubated on an orbital rotary shaker (150 RPM) at 24 °C in continuous darkness and were maintained by weekly replacement of 75% of the medium with fresh medium. After 4 weeks, the suspensions were passed through a 500 µm sieve; the retained masses were discarded to produce a homogeneous suspension composed of embryogenic cell aggregates (ECAs) smaller than 500 µm in diameter (Acanda et al., 2013).

Except for cold and liquid culture procedures, the Petri plates of other treatments and procedures were maintained in darkness at 23 ± 1 °C. Subculturing was performed after every 30-day intervals for 4–5 passages except for liquid culture.

2.4. Embryo maturation

The globular embryos obtained from the differentiation phase were transferred to the media consisting of MS basal salts and vitamins supplemented with 30 g/L (w/v) sucrose, 250 mg/L glutamine in the presence of different treatments (control without treatment, control without glutamine and treatment, 2 mg/L ABA, 1% (w/v) Polyethylene glycol (PEG), chilling at 4 °C for 4 months, chilling at 4 °C + 1% (w/v) Polyethylene glycol (PEG) and solidified with 0.8% agar. The cultures

Table 1
Overview of experiments for embryogenic callus induction in *Pyrus communis* cultivars.

PGR (µM) Optimization (First step)	Reduced Nitrogen Optimization (Second step)	Carbon (30 g/L) source Optimization (Third step)	Media Optimization (Fourth step)
2.68 NAA + 4.44BA	Glutamine (250mg/L)	Sucrose	MS
2.68NAA + 8.87 BA	Glutamine (350mg/L)	Sorbitol	NN
2.68NAA + 13.32BA	CH (0.1%)	Maltose	W
8.05NAA + 13.32BA	CH (0.2%)		SH
			GD

BA: Benzyl adenine, **NAA:** 1-Naphthaleneacetic acid, **CH:** Casein hydrolysate, **MS:** Murashige and Skoog, **NN:** Nitsch and Nitsch, **W:** White's medium, **SH:** Schenk and Hildebrandt, **GD:** Gresshoff and Doy.

Table 2
Evaluation of *Pyrus communis* cultivars responses to NAA and BA combinations in embryogenic callus induction (%).

PGRs (μM)		Cultivar			
NAA	BA	Dar Gazi	Williams	Ghosi	Natanzi
2.68	4.44	19.83 ghi	46 c	41.73 d	30.34 e
2.68	8.87	18.57 i	20.73 ghi	20.80 gh	15.63 j
2.68	13.32	20.73 ghi	21.33 g	21.3 g	18.90 hi
8.05	13.32	28.17 f	57 b	63.20 a	58.8 b

Same letter indicates no significant difference between treatments at 5% levels.

were maintained in complete darkness at 23 ± 1 °C for 4 months. Subculturing was performed after every 30-day intervals for 4 passages.

2.5. Somatic embryo germination and conversion to plants

Different treatments were examined in order to test somatic embryo germination and plant recovery. For this purpose, somatic embryos at the stage of development to plantlet which is after the torpedo-shape stage, were placed in different treatments (MS basal salts and vitamins, ½ MS basal salts and vitamins, MS basal salts and vitamins + 1 mg/L GA_3 (gibberelic acid), MS salts and vitamins + 2 mg/L $AgNO_3$, MS basal salts and vitamins + Chilling at 4 °C for 4 months, MS Salts and Staba modified vitamins). Staba modified vitamins (100X) were as follows: 100 mg/L myo-Inositol; 1 mg/L thiamine hydrochloride; 2 mg/L pyridoxine hydrochloride; 2 mg/L niacinamide; 1 mg/L D-biotin; 1 mg/L choline chloride; 0.5 mg/L riboflavin; 0.5 mg/L p-aminobenzoic acid; 1 mg/L calcium pantothenate; 0.0015 mg/L cyanocobalamin (vitamin B_{12}); 0.5 mg/L folic acid.

All of the treatments were supplemented with 30 g/L (w/v) sucrose and were solidified with 0.8% agar. The cultures were maintained at 23 ± 1 °C in continuous darkness. After germination, the plantlets were successfully transferred to a medium of MS basal salts and vitamins devoid of PGRs supplemented with 30 g/L (w/v) sucrose and gelled with 0.8% agar. When shoots became visible, the germinated embryos were transferred to test tubes containing medium of MS basal salts and vitamins supplemented with 30 g/L (w/v) sucrose and 150 mg/L Fe-EDDHA. Afterwards, the cultures were maintained under cool white fluorescent light (45 μm m^{-2} s^{-1} and a 16-h photoperiod) at 26 °C. After sufficient growth, the plantlets were taken out from the culture vessels and were washed with water to remove the adhered nutrient medium in order to avoid microbial growth. Then they were transferred to cocopeat: perlite (1:2). At the transplanting time, 0.25 g/kg mycorrhiza (*Glomus mosseae*) was placed at the bottom of the root. Finally, the plants were covered with a plastic film tent and were grown under a 16 h photoperiod of 45 μm m^{-2} s^{-1}. After 7 days, the plants were fertilized with N:P_2O_5:K_2O 10:52:10 and after 14 days, 20 N-20 P205-20 K20 formulations were used.

2.6. Statistical analysis

These tests were conducted in factorial experiments with three replications consisting of ten explants in each plate. The data were analyzed using the SAS Version 9.1 software. Difference detection between data was separately done by analysis of variance (ANOVA) in each experiment. The means were compared with the least significant difference (LSD) test at the 5% level of confidence. Normality was done by Arc sin √x formula (related to data as a percentage).

3. Results

Different times of fruit harvest were compared to the selection of proper explants, and it was found that 50 days after pollination was substantially the best time for endosperm culture, because at this stage of fruit growth endosperm had formed the majority section of the seed. Also, this time was suitable for immature cotyledon culture (Fig. 1A).

3.1. Embryogenic callus induction

ANOVA revealed that difference ($P < 0.01$) between the cultivars of *P. communis* in response to PGR treatments was significant for the production of embryogenic callus. In the first step, different groups of PGR were tested. Our initial trials by using various cytokinins in combination with 2, 4-D were not productive (Data is not shown). Therefore, it was assumed that a weaker auxin should be used in the induction phase. Consequently, 1-Naphthalene acetic acid (NAA) was applied. The Embryogenic callus induction capacity of the explants was affected by the presence of NAA, along with BA (Table 2). It was found that incorporation of NAA and BA triggered profuse embryogenic callus and the best results were obtained when the ratio of BA to NAA was 2:1, according to Table 2.

When the explants were exposed to 13.32 μM BA and 8.05 μM NAA, the highest percentage of embryogenic callus was obtained (Table 2). In this step, 0.1% (w/v) casein hydrolysate and 30 g/L (w/v) sucrose (Acanda et al., 2013) were chosen as standard contents. In the next steps, the best outcome of nitrogen and carbon sources were successively determined.

ANOVA exhibited that there was a significant difference ($P < 0.01$) between the cultivars of *P. communis* in response to nitrogen source treatments for the production of embryogenic callus. In this step, a mixture of NH_4NO_3 with either reduced nitrogen such as glutamine or casein hydrolysate was examined (Table 3). Concentrations of NH_4NO_3 were 1.65 g/L. The response of embryogenesis with glutamine (250 mg/L) than the others.

ANOVA revealed that difference ($P < 0.01$) between the cultivars of *P. communis* in response to carbon source treatments was significant for the production of embryogenic callus. Sucrose appeared to be the most effective; because it is the most widely used in plant tissue culture. But, for the optimization of procedure for embryogenic callus induction of *Pyrus communis*, it was necessary to do a survey on various carbohydrates since there was no specific procedure for *Pyrus communis*. The carbohydrates examined were sucrose, sorbitol, and maltose. Responses of explants were various in producing embryogenic callus in the presence of these carbohydrates. Fig. 2 shows that only 30 g/L (w/v) of sucrose lead to embryogenic callus.

After the optimization of PGRs, nitrogen and carbon sources, the last step was done for improvement of induction efficiency. In the last step, different media formula, including MS, Nitsch and Nitsch, white's medium, Schenk and Hildebrandt and Gresshoff and Doy were tested. Each cultivar was optimized into a special medium formula ($P < 0.01$). Ghosi and Natanzi had the highest response (75.46% and 64%, respectively in the mentioned cultivars) to the MS medium, Williams (65%) to the SH medium and finally, Dar Gazi (42%) to the Nitsch and Nitsch media (Table 4, Fig. 1C).

3.2. Differentiation embryogenic callus

ANOVA displayed a significant difference ($P < 0.01$) in

Table 3
Optimization of reduced nitrogen sources in embryogenic callus induction (%) of *Pyrus communis* cultivars.

Reduced Nitrogen Source	Cultivar			
	Dar Gazi	Williams	Ghosi	Natanzi
250 mg/L Glutamine	39.06 f	58.36 d	78.26 a	62.43 c
350 mg/L Glutamine	19.63 k	36.05 g	74.19 b	0 m
0.1% Casein hydrolysate	27.53 i	56.80 e	62.10 c	57.90 de
0.2% Casein hydrolysate	8.04 l	28.68 h	28.44 hi	23.03 j

Same letter indicates no significant difference between treatments at 5% levels.

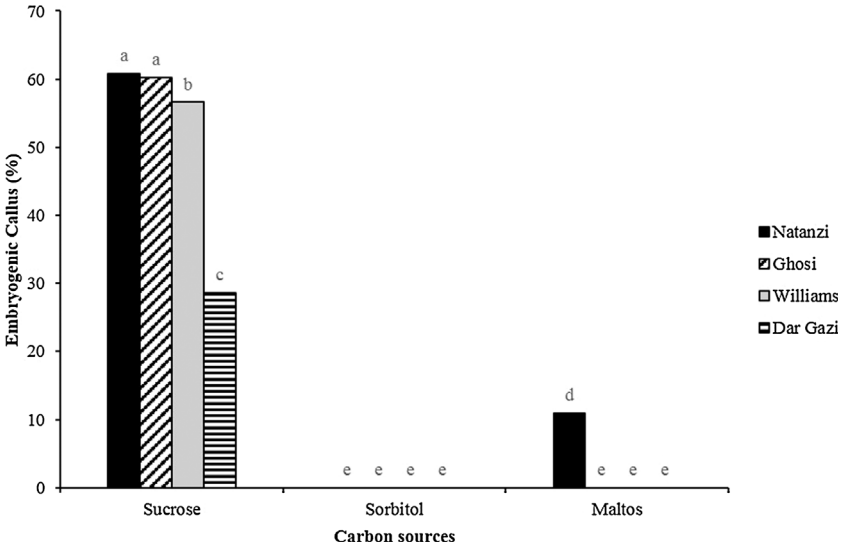

Fig. 2. Comparison of carbon sources in embryogenic callus induction (%) related to different cultivars of *P. communis*. 30 g/L three carbon sources were compared and sucrose had the best results. Differences were considered to be significant at $P < 0.01$.

Table 4
Increment efficiency of embryogenic callus induction (%) by Optimizing medium formula for each of studied cultivars of *Pyrus communis*.

Basal medium	Cultivar			
	Ghosi	Williams	Natanzi	Dar Gazi
MS	75.46 a	60.66 b	64 b	38 cd
NN	71.66 a	23.00 f	33.33 de	42.00 c
W	0 h	0 h	0 h	0 h
SH	27.66 ef	65.00 b	39.33 c	14.33 g
GD	0 h	0 h	0 h	0 h

Same letter indicates no significant difference between treatments at 5% levels.
MS: Murashige and Skoog, **NN:** Nitsch and Nitsch, **W:** White's medium, **SH:** Schenk and Hildebrandt, **GD:** Gresshoff and Doy.

differentiation treatments related to granular callus of *P. communis* cultivars. As it was already stated, different procedures including activated charcoal procedure, Polyethylene glycol (PEG) (1% w/v), ABA (2 mg/L), chilling at 4 °C for 4 months and liquid culture were used to induce differentiation embryogenic callus. These procedures were similar to the reports by Rai et al. (2008,2009); Cardoza and D'Souza (2002) and Acanda et al. (2013) while chilling has been considered as a novel factor. No change was observed within the first 4 weeks from subculturing. By the twelfth week, differentiation was started. In this phase, transformations of granular callus to globular embryo were considered as an index of differentiation (Fig. 2D). From the results, it is evident that Natanzi and Ghosi had the highest differentiation response to 1% Polyethylene glycol (PEG) (55 and 47.66%, respectively in mentioned cultivars). Dar Gazi had 31% differentiation in liquid culture procedure, and Williams had 16.33% with chilling at 4 °C for 4 months (Fig. 3).

3.3. Embryo maturation

ANOVA displayed a significant difference ($P < 0.01$) between maturation treatments related to globular embryos of *P. communis* cultivars. In this phase, the transformation of globular embryos to torpedo shape was considered as an index of maturation (Fig. 2E). As Fig. 4 depicts, all of the cultivars had positive response to chilling, and they were matured, except Dar Gazi which did not exhibit any response to treatments and their embryoid structures turned brown, and they died in the presence of various treatments; however, the highest maturation frequency of each cultivar was obtained in chilling conditions alone in Ghosi and Williams cultivars (49.33 and 45%, respectively in mentioned cultivars), whereas the highest maturation percentage (77.66%) related to Natanzi was observed in chilling condition accompanied by 1% (v/w) PEG (Polyethylene glycol).

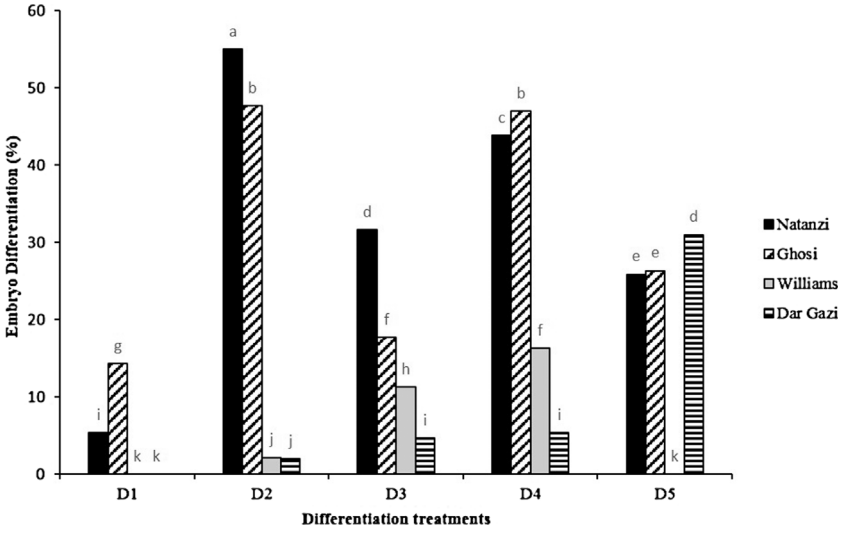

Fig. 3. Evaluation of differentiation capacity of granular callus several *P. communis* cultivars. Differentiation capacity was evaluated with different treatments: D1: ½ MS + 0.25 mg/L activated charcoal + 1 mg/L IBA, D2: Optimized medium + PEG (1%), D3: Optimized medium + ABA (2 mg/L), D4: Optimized medium + Chilling (4 °C for 4 months), D5: Liquid culture. Differences were considered to be significant at $P < 0.01$.

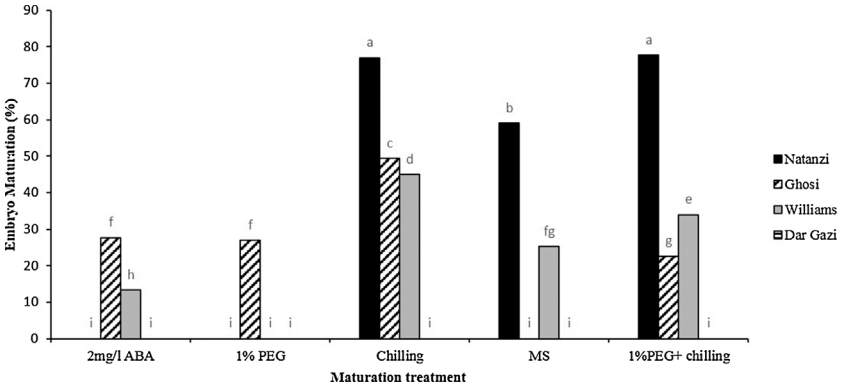

Fig. 4. Results of maturation experiment by different treatment assays. Williams and Ghosi had the highest response in chilling condition at 4 °C for 4 months and Natanzi was better in chilling + 1% PEG treatment than the others. Differences were considered to be significant at $P < 0.01$.

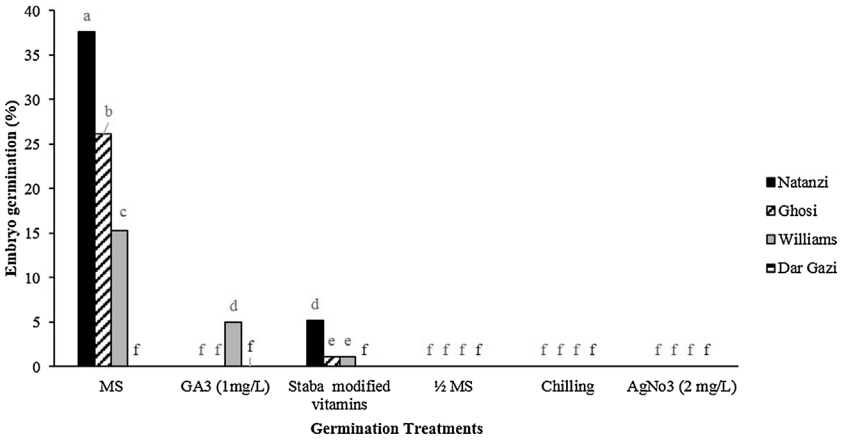

Fig. 5. The effect of germination treatments on somatic embryo germination (%) in four cultivars of *P. communis*. The experiment represents significant differences between germination treatments for all cultivars. Moreover, MS treatment represents the highest frequency of germination, while all cultivars have no response to $AgNO_3$, chilling and 1/2 MS treatments. Differences were considered to be significant at $P < 0.01$.

3.4. Somatic embryo germination and conversion to plants

The results of somatic embryo germination are shown in Fig. 5. As it is shown in this Fig., the response of cultivars to germination treatments was significant ($P < 0.01$). Although $AgNO_3$, ½ MS, and chilling treatments did not support germination in somatic embryos, it was observed that Natanzi, Ghosi, and Williams had the greatest germination percentage in MS medium devoid of PGRs and the germinated plantlets under this condition were normal. It is necessary to mention that root structure was observed in some of the germinated embryos after a long time. Also, based on Fig. 6, the inner embryo was observed and to ensure that the bipolar structure has been created, calluses around the root were removed and it was found that it was the inner embryo. The outcomes of our initial trials (The data were not shown.) indicate that abnormal plantlet would be produced either by short-term cold treatment, even 3 months, during phases before germination or by adding auxin together with BA. Abnormal plantlet would be produced with even low level and employment of weak auxin, (data not shown) in germination phase. Therefore, we reduced abnormal plantlets by preventing PGRs application in this phase. The highest germination frequency was related to Natanzi (37.66%) (Fig. 2F, G). Therefore, various cultivars of the same species had different embryogenesis potential just as it was mentioned above. However, Natanzi had the highest embryogenesis potential, and Dar Gazi was known as the recalcitrant cultivar for somatic embryogenesis.

4. Discussion

At the beginning of this work, a hypothesis was proposed as follows. The use of competent explants and the employment of stress can be powerful factors in achieving the plant recovery by SE. This hypothesis was affirmed by the fact that the selection of competent explants such as immature cotyledonary segments and endosperm with employment of stress were effective in plant regeneration by SE in *Pyrus communis*

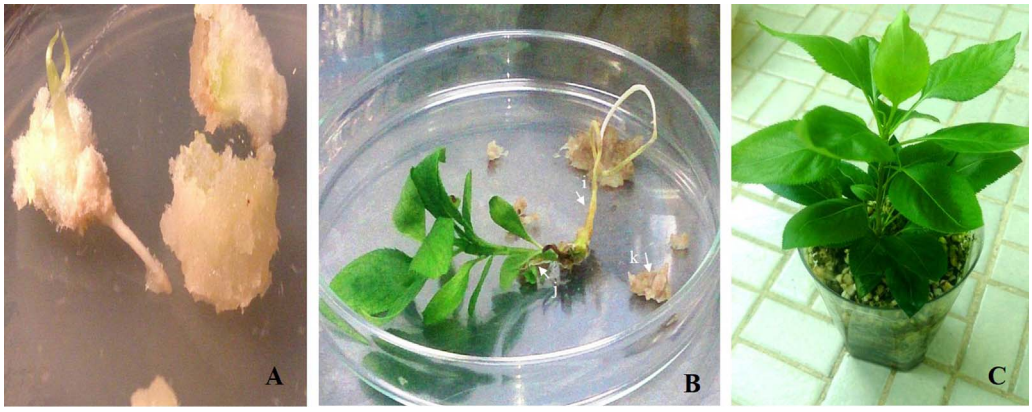

Fig. 6. Asexual inner embryos from callus and germinated embryos (A). Plant recovered and ready for adaptation i: root structure, j: shoot structure, k: removed callus from around the inner embryo (B). Plants grown by embryogenesis process (C).

cultivars and SE occurred only under conditions of chilling or drought stress in competent explants. Furthermore, our results revealed that SE occurred remarkably in this pathway in media with weaker auxin than 2, 4-D i.e. NAA and with respect to BA/NAA ratio (2:1) in the induction phase. The application of exogenous auxin in embryogenesis pathway accompanied by several events. Shoot apical meristem (SAM) formation is a critical event during somatic embryogenesis (Su and Zhang, 2009). Establishment of auxin gradients within embryonic callus via PIN1 regulation is fundamental for the induction of stem cell formation. The presence of auxin gradients and PIN1 are essential for *WUS* induction during somatic embryogenesis. *WUSCHEL* (*WUS*) is critical for stem cell fate determination in the SAM of higher plants. *WUS* induction during somatic embryogenesis accompanies by auxin accumulation in different steps such as apical cells of the pro-embryo, cotyledon primordia, and radicle (Su et al., 2009). However, the productive response in the induction phase of current survey illustrated, aforementioned events related to the application of auxin in SE induction pathway were obtained by using of NAA. Since the initial induction experiments have not any response with the application of high levels 2, 4-D. Also, low levels of 2, 4-D (0.11–0.44 mg/L) were not productive (data not shown); therefore, the selection of weaker auxin than 2, 4-D was made. The aforementioned results seem to be typical of *P. communis* cultivars. To initiate primary embryogenesis, auxin is often needed in combination with BA. This has previously been reported by EL Maataoui and Espagnac (1987), Féraud-Keller and Espagnac (1989). Khurana and Singla (2009) revealed that the induction of somatic embryogenesis in cultured tissue is a multi-factorial event. Hence, the induction phase included four successive steps in this survey. After the optimization of PGRs, induction media were tested with nitrogen compounds. Then, they were tested with different carbohydrates and finally, with different media formulae. The initial observations of somatic embryos were with cultures containing complex media, including coconut water and casein hydrolysate. Both of them serve as sources of reduced nitrogen. The specific requirement for ammonium in carrot somatic embryogenesis was reported by Halperin and Wetherell (1964). Most culture media were used for SE contained ammonium nitrate (Ammirato, 1984). Application of ammonium as the reduced form of nitrogen can lead to the growth of friable callus which is technically important. In many cases, the presence of reduced nitrogen together with nitrate was necessary for the SE (Kamada and Harada, 1984). Nitrate and ammonia are the most common inorganic nitrogen compounds used as nutrient salts in tissue culture media for in vitro plant cultures, and there are many reports in the literature which emphasize the valuable effects of using them (Murashige and Skoog, 1962; Mantell and Hugo, 1989). Some of the reduced forms of nitrogen are amino acids. It has been found that amino acids, e.g. glutamic acid and its amid glutamine have a key role in the embryo differentiation phase (Halperin and Wetherell, 1964; Walker and Sato, 1981). In this survey, the response of embryogenesis was better at 250 mg/L glutamine than the others, and it confirmed the results of previous studies about applying reduced nitrogen for the SE. In plant cells, nitrate can either be transformed to nitrite and afterward to ammonium. Nitrate may possibly be stored in the vacuoles. Ammonium produced by these reactions is further utilized in combination with carbon skeletons to produce glutamine and glutamic acid. This reaction is catalyzed by glutamine synthetase. Once it has entered into the organic cycle, nitrogen can be built in new amino acids, amides, proteins, nucleic acids, chlorophylls, alkaloids, polyarnines, vitamins and PGRs (Poulton et al., 2012). However, our results are parallel with the aforementioned findings and endorse the positive role of glutamine. Many mono- and disaccharides can support the initiation and development of carrot somatic embryos (Verma and Dougall, 1977). However, in this study, media with only 30 g/L of sucrose lead to embryogenic callus, and it is one of the common carbohydrates used in research works on embryogenesis. In the last step, it was found that Ghosi and Natanzi had the highest response (75.46% and 64%, respectively in the mentioned cultivars) in MS medium, Williams (65%) in SH medium and Dar Gazi (42%) in NN medium; in this regards, Marchant et al. (1996) optimized the SE formula related to *Rosa hybrida* cultivars in the SH medium. Therefore, in addition to the MS medium, the SH medium can be suggested as one of the other embryogenesis induction media in the Rosaseae family. Four steps were employed in the induction phase, and finally, it was determined that various genotypes had different responses in indirect embryogenesis. In other phases, it is interesting to point out that the highest frequency of differentiation and maturation are obtained in the stress treatment. Furthermore, with employment of stress the plants that were obtained, were cytologically normal. Maturation in somatic embryos of *Juglans regia* cultivars required cold treatment (Tulecke and McGranahan, 1985). This finding is in accordance with the results of this survey. Polyethylene glycols (PEGs) with high-molecular-weight are too large to penetrate the plant cell walls (Ferrie and Keller, 2007) but restrict water uptake and provide a simulated drought stress during embryo development (Langhansova et al., 2004). The molecular mechanism of PEGs in embryogenesis refers to increasing the expression of the involved genes in the formation of the embryo body plan and in the control of the shoot and root apical meristems, including the apparent homologs to the Arabidopsis genes *ZWILLE*, *FIDDLEHEAD*, *FUSCA*, and *SCARECROW* (Stasolla et al., 2003a). Moreover, Stasolla et al. (2003b) identified four different clusters of genes in this regard, including several heat shock proteins up-regulated in PEG-treated, a glutathione-S transferase, and a cysteine protease down- regulated in PEG-treated, genes repressed by PEG in fully developed embryos and several heat shock proteins and ubiquitin, induced in PEG-treated embryos. Heringer et al. (2013) concluded addition of PEG to media increased SE. On the other hand, PEG induced increased protein content. This study suggests the increase in PEG-induced proteins may be related to the synthesis of Late Embryogenesis Abundant (LEA) proteins which are expressed under cold or osmotic stress. The positive effect of chilling on SE can be attributed dormancy-breaking, an increase in endogenous GA_3 and to a reduction in ABA endogenous contents (Mallón et al., 2013). Generally, molecular mechanism of stress in SE was related to chromatin reorganization (Fehér, 2005; De-la-Peña et al., 2015; Zavattieri et al., 2010), namely, change in DNA methylation patterns which are associated with the regulation of several genes involved in somatic embryogenesis, such as *WUS*, *BBM1*, *LEC*, and several others (De-la-Peña et al., 2015). Hence, the results of this study suggested that the employment of stress can overcome differentiation and maturation, then overcome germination obstacle for plant recovery by SE in pear. These results are similar to the results of Zavattieri et al. (2010), Karami and Saidi (2010) and Nowak and Gaj (2016).

5. Conclusion

This is the first complete protocol for somatic embryogenesis in *Pyrus communis*. Although this investigation did not have a very high efficiency, various methods were tested during the three year period of this study. Finally, excellent results were obtained. The observations of this investigation demonstrated that endosperm and immature cotyledonary segments are competent for the embryogenic developmental pathway. Also, this survey revealed that the SE capacity was strictly linked to *Pyrus communis* cultivars such that the highest frequency of somatic embryo germination was related to Natanzi (37.66%). On the contrary, Dar Gazi has no response to it. In the first phase, selection of PGR combination was important, and the ratio of BA/NAA (2:1) was suitable for induction; the other case in the induction phase, it was the addition of glutamine to induction media as reduced nitrogen. Based on the results of this investigation, in the later phases of embryo growth, it was necessary to test stressful media for embryogenesis pathway. Stresses were tested, including starvation (data not shown), drought, chilling and low acidity (data not shown). Among these stressful situations, drought and chilling were excellent. However, further functional studies are needed to enhance the efficiency of SE in pear for

molecular evaluations or testing in other environmental conditions.

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