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Full Length Research Paper

Enhancement of maturation and germination of somatic embryos in Persian walnut (*Juglans regia* l.) using osmolites, hormones and cold treatments

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Maturation of somatic embryos of Persian walnut (*Juglans regia* L.) was evaluated on media containing various concentrations of Gelrite® (0.2, 0.3, 0.4 and 0.5%) and polyethylene glycol (0.0, 3.0, 5.0 and 7.5%) in the presence of 7.56 µmol abscisic acid and 3.0% sucrose. The highest rate of cotyledonary (70.0%) and normal (50.0%) somatic embryos was obtained using 0.3% Gelrite® without polyethylene glycol. Media with the least amount of Gelrite® (0.2%) induced the most secondary embryogenesis. Increasing the concentration of Gelrite® decreased the number of mature somatic embryos. The combination of Gelrite® and polyethylene glycol was the least effective for maturation of somatic embryos. Among polyethylene glycol concentrations, 7.5% polyethylene glycol produced the highest number of cotyledonary embryos and 3.0% polyethylene glycol produced the highest number of normal embryos (white-opaque embryos with two cotyledons and visible root and shoot meristems). Treatments were compared to medium with 0.2% Gelrite® and lacking polyethylene glycol (control). The 0.5% Gelrite® and 7.5% polyethylene glycol treatment produced the highest number of abnormal somatic embryos. In another experiment, different pre-germination treatments were tested to enhance somatic embryo germination. Germination response was evaluated in terms of the percentages of embryos exhibiting root-only elongation, shoot-only elongation, or both (conversion to plantlets). A cold pre-treatment (4°C for four weeks) in combination with plant growth regulators resulted in the highest rate of conversion to plantlets (55.0%). One month storage of somatic embryos at 4°C between maturation and germination treatments gave the lowest conversion frequency but did promote root-only germination. Addition of plant growth regulators (including 2.32 µmol kinetin (Kin), 2.22 µmol N6-Benzyladenine and 5.77 µmol gibberellic acid) to the germination medium for 4 weeks in the absence of a cold treatment was less effective for conversion to plantlets than growth regulator use in combination with cold, but the use in the absence of cold increased the frequency of shoot-only elongation. Plant growth regulator treatments decreased the frequency of root-only elongation regardless of cold treatment.

Key words: Gelrite®, plant growth regulator, polyethylene glycol, temperature, somatic embryogenesis.

INTRODUCTION

Persian walnut (*Juglans regia* L.) is one of the most important nut crops native to central Asia, specifically the Iran plateau (Leslie and McGranahan, 1998; Vahdati et al., 2001). This species produces about 1,700,000 tons, in-shell nuts annually, and its wood is valued for furniture, veneer, and gunstock. Major walnut producing countries of the world are China, with 503,000 tons, the USA with 290,300 tons, Turkey with 184,251 tons and Iran with

170,000 tons. Areas to which walnuts are native are increasing in production and new industries are rapidly developing in South America, South Africa, Australia, and New Zealand (FAO, 2007). Vegetative propagation of walnut species through grafting and tissue culture is still more difficult than for many fruit trees (Dhuria et al., 1977; Vahdati et al., 2002; Rezaee et al., 2008). One of the advantages of tissue culture is the rapid propagation of plant species (Tremblay and Tremblay, 1991). The application of this method is dependent upon the availability of an efficient *in vitro* regeneration system, such as somatic embryogenesis. However, low efficiency of maturation and germination of somatic embryos and difficulties in regeneration are still limiting factors in the mass production of woody trees, and particularly the use of this method in walnut (Vahdati et al., 2006).

Overall efficiency was seriously limited by poor germination of the somatic embryos and conversion into plants (Tulecke et al., 1987; Amirato, 1989). This may be due to the poorly formed shoot apical meristems often found in walnut somatic embryos (Vahdati et al., 2006). Although a maturation stage involving ABA treatment improved germination, the conversion rates were still not satisfactory, ranging from 0 to 41% for lines derived from immature cotyledons of Persian walnut (Vahdati et al., 2008). Numerous attempts to improve the quality of somatic embryos have shown the stimulatory role of low osmotic potential in the maturation medium used for embryo development, both in angiosperms (McKersie and Brown, 1996) and gymnosperms (Attree and Fowke, 1993). Application of osmotic agents such as polyethylene glycol (PEG) and gelling agents (e.g., Gelrite®) can produce a low osmotic potential in the medium. Addition of PEG to the maturation medium, particularly in conifers, stimulated maturation. Examples include *Pinus taeda* (Norgaard, 1997) and *Picea* (Attree et al., 1991; Find, 1997). Increasing the concentration of gellan gum in the maturation medium significantly enhanced the maturation rate in different *Pinus* species including *Pinus strobes* (Klimaszewska and Smith, 1997), *P. pinaster* and *P. sylvestris* (Lelu et al., 1999), and *P. taeda* and *P. banksiana* (Klimaszewska et al., 1999), and SORBUS DOMESTICA (Nikolaou et al., 2008). The relatively poor germination of somatic embryos and low rates of development into functional plants are limiting steps for the widespread use of somatic embryogenesis in Persian walnut improvement programs. In this study to enhance the maturation and germination of walnut somatic embryos we examined the effect of different concentrations of Gelrite® in combination with PEG on maturation, and investigated whether post-maturation cold storage and plant growth regulator treatment (PGR) can improve the germination rate of *J. regia* somatic embryos.

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Abbreviations: ABA, Abscisic acid; **BAP**, Banzil adenine phosphate; **DKW,** driver and Kuniyuki walnut medium; **GA3,** gibberellic acid; **MS** - Murashige and skoog; **NAA**, α-naphthaleneacetic acid; **PEG,** polyethylene glycol; **PGR**, plant growth regulator; **SE**, somatic embryo.

MATERIALS AND METHODS

Plant material

Somatic embryos used for maturation and germination experiments were obtained from an embryogenic line initiated from an immature cotyledon of an apomictic genotype named G79 (Vahdati et al., 2006). This line has been maintained in the dark DKW medium for several years by secondary embryogenesis with sequential subculture at 1 week intervals on a proliferation medium consisting of hormone-free Driver and Kuniyuki walnut medium (DKW) basal medium supplemented whit 30 gl^{-1} sucrose and 2 gl^{-1} Gelrite®.

Maturation of somatic embryos

The standard embryo maturation medium as developed previously (Vahdati et al., 2008) consisted of DKW basal medium supplemented with 7.56 µmol ABA and 30 gl^{-1} sucrose, solidified with 2.1 gl^{-1} Gelrite®, and adjusted to pH 5.7 before autoclaving at 121°C for 15 min. ABA (SIGMA) was filter sterilized using a 0.22 mm aqueous filter (Millipore) and added to partially cooled medium after autoclaving, but before it solidified.

Effect of PEG-4000 and Gelrite® in the maturation medium

Maturation media consisted of the standard maturation medium supplemented with combinations of Gelrite® (0.2, 0.3, 0.4, and 0.5%) and PEG 4000 (0.0, 3.0, 5.0, and 7.5 %). In this experiment, 0.2% Gelrite® without PEG was considered to be the control and all treatments consisted of five Petri dishes (100 × 9 × 20 mm) with eight globular embryos per dish. The embryos were cultured for a total of four weeks on these maturation media in the dark at 25 ± 1°C with a subculture to fresh medium after the initial two weeks. Maturation parameters were recorded after 4 weeks. These included the number of secondary embryos produced, the total number of cotyledonary embryos formed (from either primary or secondary embryos), the number of normal (white-opaque, non-germinating, with two cotyledons and visible root and shoot meristems) or abnormal (prematurely germinating or with abnormally shaped cotyledons and without visible meristems) embryos formed, and the number of embryos with only shoot, only root, or both shoot and root meristems. In assessing over-all embryo maturity, embryos with an opaque white appearance, cotyledonary form, good meristem formation, and reduced secondary embryogenesis were considered more mature.

Effect of cold storage and PGRs on germination

Mature, well-developed normal somatic embryos were selected and removed from the best maturation treatments and exposed to the following germination treatments. Embryos were: (a) placed directly onto hormone-free basal DKW medium (control; without PGRs or cold pretreatment); (b) exposed to a cold pretreatment at 4°C (stored in darkness for four weeks) then cultured on hormone-free basal DKW medium; (c) placed directly onto DKW medium supplemented with 30 gl^{-1} Sucrose and 2 grl^{-1} Gelrite® with PGRs

Table 1. Effect of Gelrite® concentration on *Juglans regia* somatic embryo maturation parametersa.

Treatment b	Concentration of Gelrite® (%)	No. secondary embryos	No. cotyledonary embryos	SEs with only shoot meristems	SEs with only root meristems	SEs with both shoot and root meristems
T1	0.2	15.25ac	5.81bc	2.00bc	2.75a	1.06c
T2	0.3	10.75b	8.62a	2.7ab	1.56b	3.56a
T3	0.4	9.00c	7.75ab	3.56a	1.12b	2.18b
T4	0.5	8.81d	4.00c	1.43c	1.06b	0.68d

a. Somatic embryos (SE) were cultured for 4 weeks on maturation medium at 25°C in darkness. b. Values are means of four replicates with 8 somatic embryos per replicate. c. Within each column, values followed by different letters differ significantly (P≤0.05) according to the Duncan's multiple range test (DMRT).

Table 2. Effects of various Gelrite® and polyethylene glycol (PEG) concentrations on maturation response of walnut somatic embryos a.

Treatment b	No. secondary embryos	No. cotyledonary embryos	SEs with only shoot meristems	SEs with only root meristems	SEs with both shoot and root meristems
T1	26.60ac	9.50c	3.50bcd	9.20ab	7.99c
T2	14.00c	18.75a	4.50ab	8.59b	9.88a
T3	13.5c	14.75b	5.50a	7.13cd	9.77a
T4	10.75d	4.50e	1.50fgh	6.81cde	6.80d
T5	17.73b	7.75d	5.25a	9.63a	8.77b
T6	9.75d	4.50e	2.75de	7.53c	6.78d
T7	7.25ef	4.50e	1.75efg	6.19de	5.94e
T8	5.25gh	3.00fg	2.25ef	5.53fgh	5.46ef
T9	13.5c	9.50c	2.75de	6.83cde	7.08d
T10	8.25e	3.25efg	0.60h	6.71cde	5.25f
T11	5.25gh	1.75ghi	2.25ef	3.91h	5.21f
T12	5.50fgh	1.75ghi	0.75gh	3.05h	4.20g
T13	10.25d	10.00c	3.00cd	5.93efg	7.00d
T14	5.00h	1.50hi	1.00gh	5.16gh	3.96g
T15	5.00h	0.75i	0.40h	3.70h	1.73h
T16	3.75i	0.75i	0.00h	0.00i	0.00i

a. Somatic embryos (SE) were cultured for 4 weeks on maturation medium at 25°C in darkness. b. Values are means of four replicates with 8 somatic embryos per replicate. c. Within each column, values followed by different letters differ significantly (P ≤ 0.05) according to the Duncan's multiple range test (DMRT).

without cold pretreatment (2.32 µmol kinetin, 1.61 µmol BAP and 5.77 µmol GA3); (d) exposed to a cold pretreatment at 4°C (stored in darkness for 4 weeks) and cultured on DKW medium with the same PGRs as in (c).

During the germination period, all cultures were kept in a growth room at 25 ± 1°C with a 16 h photoperiod under standard cool white fluorescent lamps. For each germination treatment, three replicates with 10 morphologically normal cotyledonary somatic embryos per replicate were employed. Germination response was evaluated in terms of the percentage of embryos exhibiting root-only elongation, shoot-only elongation, or both (conversion to plantlets) after four weeks.

Statistical analyses

All experiments were conducted using a completely randomized design. The experiments were replicated two times and the data were analyzed using SAS Version 9.0 (statistical analysis system, Institute Inc., Cary, NC, USA, 1985). Means with significant differences were compared using Duncan's multiple range test (DMRT) at P ≤ 0.05.

RESULTS

Effect of PEG-4000 and Gelrite® in the maturation medium

Initial formation of cotyledonary embryos was observed after 2 - 3 weeks on most of the maturation media. The concentration of Gelrite® alone or in combination with PEG had a significant effect (P ≤ 0.05) on each of the maturation parameters (Table 1 and 2). Somatic embryo maturation was highly variable in all treatments and was affected by the culture medium composition (Table 1). Embryo maturation was improved significantly using media containing a moderate concentration (0.3%) of Gelrite®. The maturation of somatic embryos decreased when PEG was added (Table 2). Cultures containing only

Table 3. Effect of the pre-germination treatments on embryo germination with respect to the maturation treatment applied a.

Maturation treatment b	Cold treatment	PGR treatment	Conversion (shoot + root) (%)	Shoot-only germination (%)	Root-only germination (%)
T1	-	GA3+BA+Kin	1.30dc	2.00bc	1.33c
T2	-	GA3+BA+Kin	2.30c	3.00a	1.33c
T3	-	GA3+BA+Kin	1.30d	3.00a	0.66d
T5	-	GA3+BA+Kin	1.00de	1.66bcd	0.33d
T1	4°C	-	0.66de	1.66bcd	3.33a
T2	4°C	-	1.30d	2.33b	1.66c
T3	4°C	-	0.66de	1.66bcd	2.66b
T5	4°C	-	0.33e	1.33cd	1.33c
T1	4°C	GA3+BA+Kin	3.60b	0.33e	1.33c
T2	4°C	GA3+BA+Kin	5.60a	1.66bcd	1.33c
T3	4°C	GA3+BA+Kin	2.60c	1.00de	1.33c
T5	4°C	GA3+BA+Kin	2.60c	1.00de	0.33d

a. Germination rate parameters were evaluated after 4 weeks. b. Values are means of three replicates with 10 somatic embryos (SE) per replicate. c. Within each column, values followed by different letters differ significantly (P≤0.05) according to the Duncan's multiple range test (DMRT).

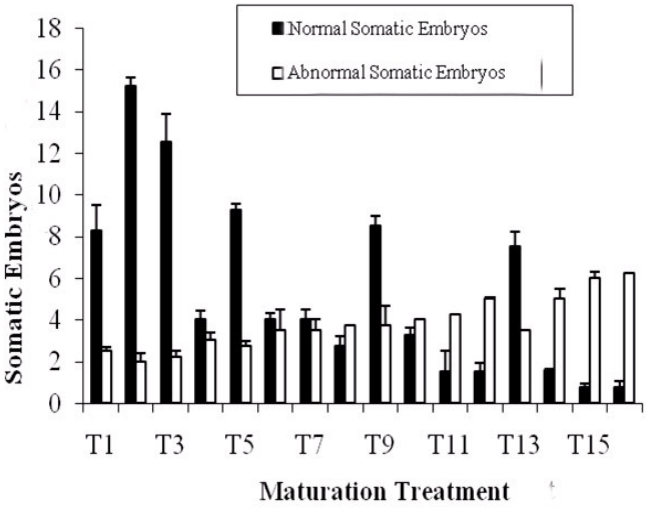

Figure 1. The influence of different concentrations of Gelrite® and polyethylene glycol (PEG) on production of normal and abnormal somatic embryos of walnut (*Juglans regia*). T1 to T4 = Driver and Kuniyuki walnut medium (DKW) basal medium supplemented with Gelrite® 0.2, 0.3, 0.4, 0.5%, T9 to T12 = PEG 5.0% in combination with Gelrite® 0.2, 0.3, 0.4 0.5%, T9 to T12 = PEG 0.5% in combination with Gelrite® 0.2, 0.3, 0.4 0.5%, and T13 to T16 = PEG 7.5% in combination with Gelrite® 0.2, 0.3, 0.4, 0.5%, respectively.

0.3% Gelrite® produced significantly more mature somatic embryos than higher concentrations of Gelrite® (0.4 or 0.5%) or the control (Table 3). In addition, a lower frequency of secondary embryogenesis, which can be considered as a marker for maturation of somatic embryos, was observed on embryos cultured on media containing higher concentrations of Gelrite®, alone or in combination with PEG. Media with 0.2% Gelrite® induced the highest rate of secondary embryogenesis and the most somatic embryos with root formation (Table 1). Generally, observed high concentrations of Gelrite® to 0.3% increasing maturation of somatic embryos, but overall concentration of 0.3% decreased maturation of somatic embryos. Higher concentrations of Gelrite® (0.4 or 0.5%) caused the embryos to become structurally abnormal within 2 or 3 week (Figure 1).

Maturation of somatic embryos was not improved by different combinations of PEG and Gelrite® when compared to Gelrite® alone, but the effect of PEG - 4000 on maturation of walnut somatic embryos cannot be disregarded. 3.0, 5.0, 7.5 concentrations of PEG - 4000 combined with 0.2% Gelrite® significantly increased cotyledonary embryos and decreased secondary somatic embryogenesis relative to the control (0.2% Gelrite® and 0.0% PEG) (Table 2). Among the concentrations of PEG with 0.2% Gelrite®, the highest number of cotyledonary embryos was produced at 7.5% PEG (Table 2), and 3.0% PEG promoted a higher number of normal embryos and embryos with only shoot meristem (Figure 1) compared to 0.2% Gelrite® medium lacking PEG (control). Addition of PEG to maturation media supplemented with higher concentrations of Gelrite® decreased the production of mature somatic embryos and gave only a few mature somatic embryos. For this reason the embryos recovered from these treatments were not subjected to germination treatment. The combination of 7.5% PEG and 0.5% Gelrite® produced the highest number of abnormal embryos (Figure 1, 2a and 2b) and the fewest mature

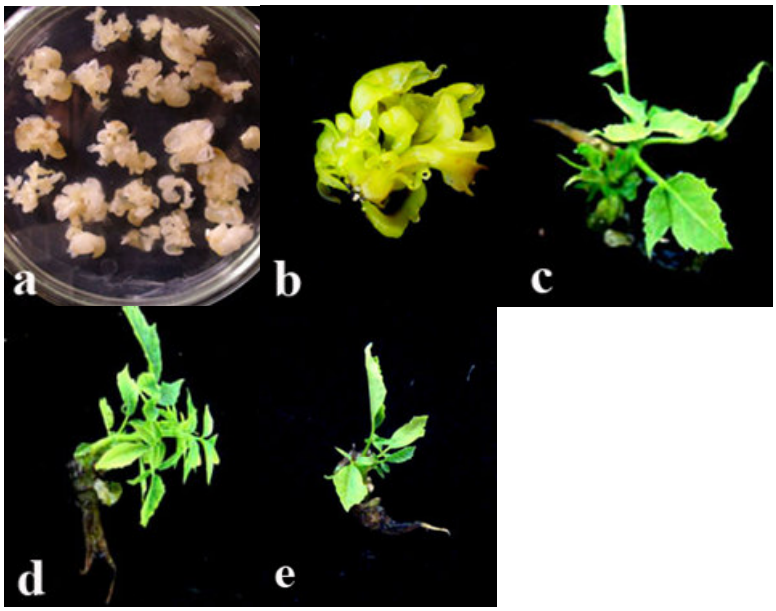

Figure 2. Walnut Somatic embryo maturation and subsequent germination. (a) Mature somatic embryos produced on 0.3% Gelrite® medium. (b) Abnormal somatic embryos produced on 0.5% Gelrite® and 7.5% polyethylene glycol medium. (c) Matured somatic embryos on 0.3% PEG, (d) 0.3% Gelrite® maturation treatment and subsequent shoot and root developed by cold pre-germination and plant growth regulator (PGR) treatment. (e) Germinated somatic embryos on DKW medium solidified with 0.2% Gelrite® (control) by cold pre-germination and PGR treatment.

somatic embryos.

Effect of cold storage and PGRs on germination

Somatic embryos derived from treatments 0.2, 0.3 and 0.4% of Gelrite without PEG and 0.2% Gelrite® with 0.3% PEG were used for subsequent germination experiments. Somatic embryo germination was clearly dependent on the maturation treatment applied. Regardless of the maturation treatment, when cotyledonary somatic embryos were placed directly onto hormone-free DKW medium in the light without a pre-germination treatment, no germination was obtained (results not shown). These embryos became abnormal with very poor shoot development and root elongation (Figure 3a). In many cases, callus was produced at the base of the hypocotyl. Of the pre-germination treatments tested, use of a one-month cold period alone, had the least influence on conversion frequencies (Table 3), although in comparison with both the PGR only and PGR plus cold treatments it increased the root-only germination percentages (Figure 3b) 33.0% (Mean 3.33), 15.7% (1.66), 12.59% (2.66) and 9.6% (1.33) for maturation treatments 0.2, 0.3, 0.4%, Gelrite® and 0.2% Gelrite® with 0.3% PEG, respectively (Table 3).

Somatic embryos treated with only PGRs had low conversion frequencies (Table 3) of 11.9% (mean 1.30), 12.5% (2.30), 11.25% (1.30) and 11.25% (1.00) with maturation treatments of 0.2, 0.3, 0.4, and 0.2% Gelrite® and 0.3% PEG, respectively, and significantly higher percentages developed only shoots [18.70% (mean 2.00), 30.0% (3.00), 28.80% (3.00) and 15.87% (1.66), respectively] (Figure 3c). In addition, the somatic embryos had short roots and displayed swollen hypocotyls and cotyledons resulting in an abnormal appearance. The highest percentage 55% (mean 5.60) with maturation treatment with 0.3% Gelrite® of conversion was obtained with cold storage pretreatment and then culture on medium containing PGRs (Figure 2c, 2d, 2e), (Table 3). This treatment had the highest shoot length and root length (Table 4). The shoot length, root length, and leaf number of germinated plantlets were all significantly influenced by cold pre-treatment and PGR (Table 4). The best response to this treatment was seen in the shoot development of embryos from maturation treatment with 0.3% Gelrite®, as represented in (Table 2). When germinated using both a pre-germination cold period and PGRs, somatic embryos that had matured on 0.3% Gelrite® showed a positive germination response compared to those matured on 3.0% PEG or on the control (Figure 4). The length of roots decreased while the length of the shoots and the number of leaves increased in 0.3% Gelrite® with cold pre-treatment and

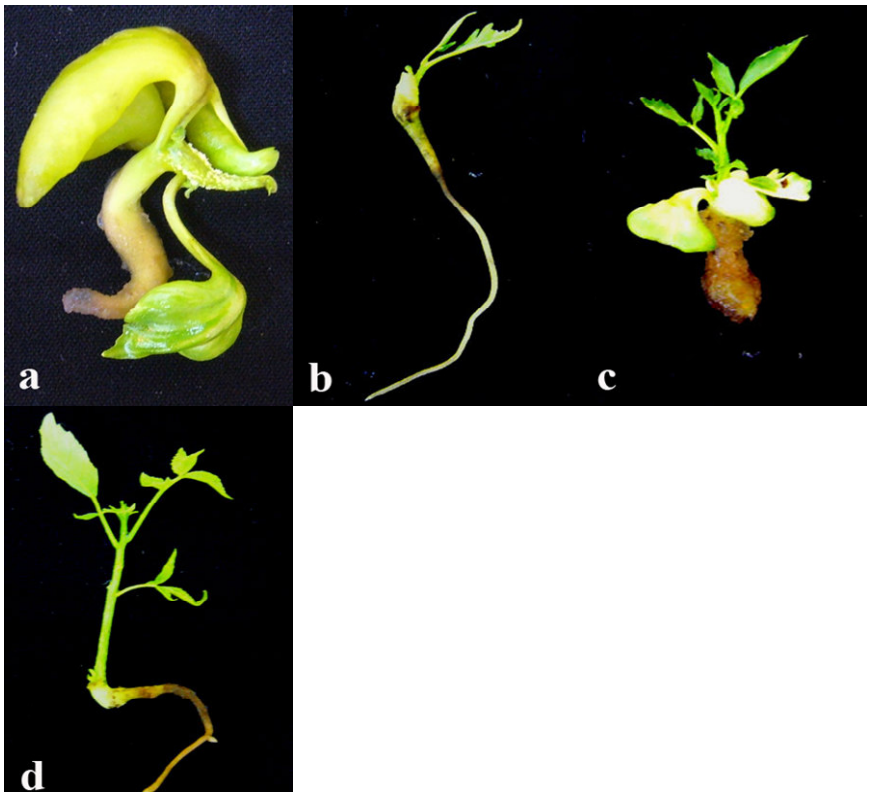

Figure 3. Comparison of different germination treatments of matured somatic embryos on 0.3% Gelrite® maturation medium after a 4 week germination period. (A) Matured somatic embryos placed directly onto hormone- free Driver and Kuniyuki walnut (DKW) medium (control). (B) Germinated somatic embryos which received a cold pretreatment at 4°C and then cultured on DKW basal medium. (C) Shoot-only germination of a somatic embryo placed directly onto germination medium containing 2.32 µmol kinetin, 1.61 µmol BAP and 5.77 µmol GA3. (D) Somatic embryo conversion to plantlet by cold pre-germination and then culture on PGR medium.

Table 4. Effect of the pre-germination treatments on the number of leaves (LN), length of shoots (SL), and root length (RL) with respect to the maturation treatment applied a.

Treatments b			Conversion (shoot + root)		
Maturation treatment	**Cold treatment 4°C**	**PGR Treatment**	**SL (cm)**	**RL (cm)**	**LN**
T1	-	GA3+BA+Kin	8.23fc	6.00b	7.33gf
T2	-	GA3+BA+Kin	8.23f	5.33cd	7.67f
T3	-	GA3+BA+Kin	8.00k	5.05cde	7.00h
T5	-	GA3+BA+Kin	7.00m	4.96def	6.00i
T1	4	-	9.50e	8.16a	9.00d
T2	4	-	9.73de	6.00b	9.33d
T3	4	-	9.60e	4.70ef	9.33d
T5	4	-	8.50f	5.00cdef	8.33e
T1	4	GA3+BA+Kin	11.01b	5.40cd	13.33a
T2	4	GA3+BA+Kin	12.17a	5.50cb	12.00b
T3	4	GA3+BA+Kin	10.50c	4.50fg	13.67a
T5	4	GA3+BA+Kin	10.00d	4.12g	10.33c

a. Germination rate parameters were evaluated after 4 weeks. b. Values are means of three replicates with 10 somatic embryos (SE) per replicate. c. Within each column, values followed by different letters differ significantly (P≤0.05) according to the Duncan's multiple range test (DMRT).

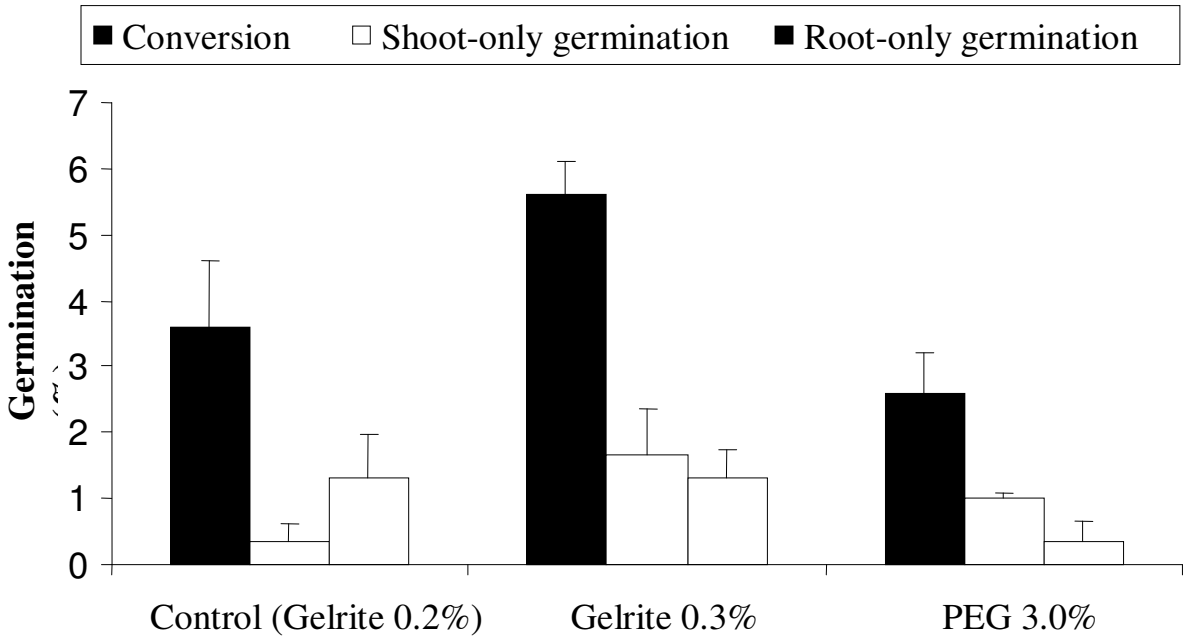

Figure 4. Effects of cold pre-treatment in combination with plant growth regulator (PGR) on the germination response of somatic embryos from three maturation treatments.

PGR (Table 4). Somatic embryos that had been produced on the PEG-free medium or on a high concentration of Gelrite® (0.3%) responded more favorably to germination than PEG-treated embryos. Somatic embryos produced on the PEG-free maturation medium with 0.3% Gelrite (cold pre-germination and PGR treatment) exhibited hypocotyl elongation and shoot development (Figure 4), while the PEG- treated embryos given the same cold pre-germination and PGR treatment showed a decreased tendency for germination (Figure 4).

For acclimatization, somatic plants with well-developed epicotyls (at least 20 mm long) and roots were transplanted into a soil mixture [perlite: peat (1:1)] in trays with a transparent plastic lid and were watered once a week. Acclimatized plants were maintained for 4-5 weeks in the tissue culture room give light intensity (16 -h photoperiod, 24 ± 1℃). Further post-germination treatments are required still to develop the acclimatization stage to the field.

DISCUSSION

In this study, Gelrite® concentration was a critical factor in the maturation of somatic embryos of the embryogenic lines tested. The best response with respect to the quantity of mature somatic embryos produced per initial (primary) embryo was obtained when these globular embryos were cultured on a medium solidified with 0.3% Gelrite® (Table 1). This result suggests that the high Gelrite® concentration reduces water availability and stimulates a shift in the developmental program of the culture, from globular embryos to the production of cotyledonary embryos. These results are in agreement with those obtained in *P. strobes* by (Klimaszewska and Smith, 1997; Klimaszewska et al., 2000) who indicated that reduced water availability resulting from increased gellan gum concentration promotes somatic embryo maturation. Improved maturation on medium with increased concentration of Gelrite® has also been reported in other plant species, including *Picea mariana*, *Pinus rubens* (Tremblay and Tremblay 1991), *Cucumis sativus* (Ladyman and Girard, 1992), *P. pinaster* and *P. sylvestris* (Lelu et al., 1999). Culture medium containing a gel matrix will have a lower water potential than the similar liquid medium (Debergh et al., 1981; Owens and Wozniak, 1991) leading to development of good mature somatic embryos. An increase of 0.05 in relative matrix potential between 0.2 and 0.3% Gelrite® (Owens and Wozniak 1991) produced a larger embryo with shoots in sugar beet. The combination of 6% sucrose and 0.9% gellan gum was found to be suitable for Pinus pinaster (Ramarosandratana et al., 2001). An increase in Gelrite® concentration resulted in improved maturation and development of walnut somatic embryos in this study.

In our cultures, addition of PEG did not improve the maturation rates in some media. Our results are similar to previous studies in which PEG in combination with a high concentration of Gelrite® did not improve the maturation rates of any of five lines of maritime pine (Ramarosandratana et al., 2001). It was suggested that decreasing the water availability of the medium was more

important than increasing the osmotic potential for development of embryos. Our results clearly showed that PEG was not required for the maturation of *J. regia* L. somatic embryos when using 0.3% Gelrite®. However, inclusion of PEG enhanced the maturation rates compared to the control using 0.2% Gelrite®. Embryos that did not receive a pre-germination treatment failed to germinate, regardless of whether embryos received a PEG treatment or Gelrite®. Similar findings were described by Ramarosandratana et al. (2001).

Walnut somatic embryo regeneration protocols have been developed using cold storage, desiccation, and gibberellic acid (GA3) (Tulecke and McGranahan 1985; Deng and Cornu 1992; Tang et al., 2001; Vahdati et al., 2002). Despite the benefits of the cold storage, application of a one month cold period as a pre-germination treatment had no significant influence on conversion frequencies, although it was associated with a statistically significant increase in root-only germination among maturation treatments. Our results agree with the previous observations that pre-germination cold treatment of somatic embryos was effective in stimulating root development of *Liquidambar styraciflua* somatic seedlings by increasing the number of first order lateral roots, but did not affect the conversion frequency (Merkle et al., 2003). Similarly, produced plantlets from somatic embryos without a cold treatment, many of which produced only shoots, which were then subsequently rooted to produce plantlets (Xing et al., 1999).

Recently, the effect of different concentrations of sucrose and ABA on the maturation and germination of walnut somatic embryos was studied by (Vahdati et al. 2008). They indicated that maturation and germination of somatic embryos was improved by application of ABA and the best treatment for germination, in which both shoot and root were developed, contained 7.56 µmol ABA. This treatment resulted in 41% conversion of embryos into plantlets. Regeneration was reduced at higher concentrations of ABA, whereas, reported that ABA accumulates during the maturation of somatic embryos, which results in somatic embryo dormancy (Rajasekaran et al., 1982). According to this research, germination occurs when the endogenous ABA decreases to an appropriate level. Cold storage treatments can markedly decrease the endogenous ABA levels and improve germination (Rajasekaran et al., 1982). In our study, we improved in 55% conversion of embryos in plantlets with gelrite concentration and cold storage treatments.

Differences in germination response were also reported in a number of other woody tree species, including common ash (Capuana et al., 2007), English oak (Sánchez et al., 2003), cork oak (García-Martín et al., 2001), European beech (Vieitez et al., 1992), cherry (Reidiboym-Talleux et al., 1998), and walnut (Tulecke and McGranahan, 1985; Liu and Han, 1989; Tang et al., 2000) who showed that cold treatment at 2 to 4°C for several weeks was necessary to overcome apical dormancy and enhance elongation of embryonic axis and germination of mature somatic embryos.

In this study, comparison of the pre-germination treatments indicated that the cold storage pretreatment followed by culture on the medium containing PGR was the most effective in promoting germination regardless of the prior maturation treatments applied. Our results are in agreement with previous observations. Kaur et al. (2006) reported that low temperature treatment for 12 days (5°C) supplemented with 2.32 µmol kinetin, 1.61 µmol BAP and 5.77 µmol GA3 had high influence on *in-vitro* germination of *J. regia* L. embryos. Liu and Han (1986) obtained mature walnut embryos on MS medium supplemented with NAA, BA and GA3. Were able to get plantlets from embryos of walnuts after two months of culturing on MS medium containing organic compounds followed by transfer to the same medium with addition of 4.43 µmol BA (Kornova et al., 1993).

Conclusions

It appears that altering the water availability to the tissues by the addition of more Gelrite® to the medium was effective in promoting somatic embryo maturation. Application of 0.3% Gelrite® with 7.56 µmol ABA, yielded a higher number of cotyledonary and normal somatic embryos (having both shoot and root meristems) than were obtained with 7.56 µmol ABA alone in our previous study (Vahdati et al., 2008). This additional treatment could improve the final quality of somatic embryos and help complete maturation, both of which are currently considered to be the main factors limiting the conversion of embryos into plants. Despite the improvements in the number of embryos produced by the inclusion of PEG in the medium, PEG was no more effective for the germination of somatic embryos than Gelrite® alone.

Conversion of walnut somatic embryos was improved from 41 to 55% compared to our previous study (Vahdati et al., 2008). Cold pre-germination treatment followed by culture on medium containing PGRs enhanced germination. For large scale application of the procedure, however, further investigation is needed in the germination stage, aiming at a consistent enhancement of productivity.

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