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ORIGINAL PAPER

Effect of exogenous ABA on somatic embryo maturation and germination in Persian walnut (*Juglans regia* L.)

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Abstract Low efficiency of embryo maturation, germination and conversion to plantlets is a major problem in many species including Persian walnut. We studied the effects of abscisic acid (ABA) and sucrose, on the maturation and germination of Persian walnut (*Juglans regia*) somatic embryos. Individual globular somatic embryos were grown on a maturation medium supplemented with different combinations of ABA and sucrose for ca. 1 month, until shoot meristems and radicles had developed. White and opaque embryos in late cotyledonary stage were subjected to desiccation after the culture period on maturation media. The number of germinated somatic embryos was influenced by the concentrations of ABA in the maturation medium. The best treatment for germination, in which both shoot and root were developed contained 2 mg l^{-1} ABA and resulted in 41% conversion of embryos into plantlets. Regeneration was reduced at higher levels of ABA. While ABA always reduced the rate of secondary embryogenesis, treatments containing 4.0% sucrose significantly increased the number of secondary embryos. On the other hand, sucrose had little influence on maturation. Normal and abnormal embryos were verified anatomically.

Keywords Abscisic acid · Desiccation · *Juglans regia* · Maturation · Somatic embryo · Sucrose

Abbreviations

ABA	Abscisic acid
DKW	Driver & Kuniyuki walnut medium
GA_3	Gibberrelic acid
SE	Somatic embryogenesis

Introduction

There are about 20 species of walnut distributed through out Asia, Europe and North and South America (Tang et al. 2001). As one of the world important nut crops, Persian walnut (*Juglans regia* L.) produces ca. 1,700,000 tons in-shell nuts annually and its wood is valued for furniture, veneer and gunstock (Vahdati 2001; FAO 2005). Walnut trees are more difficult to propagate clonally than most fruit trees (Dhuria et al. 1977; Roghani 1977; Kuden and Kaska 1997; Ozkan et al. 2000; Ozkan and Gumus 2001; Vahdati et al. 2002; Rezaee et al. 2008).

Tissue culture is one of the propagation methods of this species that has been increasingly developed

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in recent years. Progresses in micropropagation of walnut have been achieved using single node and shoot tip cultures (Vahdati et al. 2004). Somatic embryos are important not only as a target tissue for genetic transformation but also as a tool for mass clonal propagation (McGranahan et al. 1990). In some woody plants including most spruce (*Picea* spp.) and some pine (*Pinus* spp.) species, SE has been sufficiently refined for commercial use (Park et al. 2006), however it has not been employed for the commercial propagation of walnut yet (Deng and Cornu 1992). SE from immature cotyledons and endosperm was achieved in *Juglans hindsii*, *J. regia*, and *Pterocarya* sp. (Tulecke and McGranahan 1985; Tulecke et al. 1988; Cornu 1988, 1989; Cornu and Jay-Allemand 1989; Long et al. 1995; Vahdati et al. 2006). Successful SE from zygotic tissues has also been reported in other *Juglans* species, including *J. cinera*, *J. major*, *J. nigra* and the hybrids of *J. nigra* × *J. regia* and *Peterocarya* sp. × *J. regia* (Preece et al. 1995).

SE, in general, entails four stages including induction, proliferation, maturation and germination (Hartmann et al. 1997). The low efficiency of embryo initiation, maturation, germination and conversion to plantlets still remains a major problem in many species including *J. regia* L. (Tulecke 1987; Ammirato 1989; Park et al. 2006). Although in some woody plants, maturation and germination of somatic embryos have been obtained on media lacking phytohormones (Roberts-Oehlschlager et al. 1990; Harry and Thorpe 1991), in most cases the application of a specific treatment is necessary.

In walnut, germination efficiency of somatic embryos is relatively low and varies between 0% and 45% (Lee et al. 1988; Deng and Cornu 1992; Vahdati et al. 2006). Usually the problem stems from shoot apices which do not grow as the root apices. Cold storage, desiccation, and gibberrelic acid (GA_3) pretreatments as well as liquid germination medium were tested for promoting the somatic embryo germination in walnut (Tulecke and McGranahan 1985; Deng and Cornu 1992; Tang et al. 2001). However, to our knowledge the influence of abscisic acid and osmoticum on maturation of walnut embryos has not yet been studied.

ABA and osmotic stress are known to be important factors for seed maturation in many angiosperm species (Misra 1994). ABA has been recognized as a factor for promotion of normal development and maturation of somatic embryos and their uniformity in hybrid larch, caraway and soybean (Gutmann et al. 1996; Ammirato 1997; Tian and Brown 2000).

Sucrose, as a low weight osmoticum, has also a positive role in SEs maturation. It has been shown that sucrose improves SE maturation and plant recovery in black spruce (*Picea mariana*) and soybean (Komatsuda et al. 1992; Trembly and Trembly 1995).

In the present study, we examined the effect of exogenous ABA and sucrose on somatic embryo maturation and secondary embryogenesis in walnut to improve embryo quality, increase the rate of normal embryo development, and enhance embryo germination. In addition, normal and abnormal mature somatic embryos are compared anatomically.

Materials and methods

Source of somatic embryos

Somatic embryos of *Juglans regia* L. were regenerated from immature cotyledons of an apomict genotype named G79. The embryogenic line remained productive by means of secondary embryogenesis on basal medium and weekly subcultures. This line was used as the source of explants for all experiments carried in this work.

Basal medium

DKW medium (Driver and Kuniyuki 1984) solidified with 2.1 g l^{-1} gelrite, was used as basal medium. The pH was adjusted at 5.7 prior to autoclaving. Media were autoclaved at 1.1 kg cm^{-3} pressure at 121°C for 20 min and dispensed in Petri dishes under laminar airflow after sterilization.

Culture condition

The maturation media consisted of basal medium supplemented with 18 different combinations of sucrose (3.0%, 4.0%, and 6.0%) and ABA (0.0, 0.5, 1.0, 2.0, 4.0, and 5.0 mg l^{-1}). ABA was filter-sterilized through 0.22 µm membrane filters and added to the medium after autoclaving when cooled to approx. 50–60°C. Each treatment consisted of four Petri dishes (100 × 20 mm) with 10 embryos per dish.

Embryos were used for experiments at the globular stage as described by Vahdati et al. (2006). The embryos were cultured for 6 weeks on maturation medium with weekly transfer to fresh medium. Cultures were maintained in the dark at 25 ± 1°C. To promote germination, white large embryos were subjected to partial desiccation by transferring them to empty sterile Petri dishes. These Petri dishes were maintained in a desiccator containing a saturated solution of $ZnSO_4$ at 25°C in the dark. The desiccation period lasted 3–5 days. When the embryos turned white and opaque, they were transferred to solid basal DKW medium, and kept in a growth room at 27 ± 1°C with a 16/8 h photoperiod under standard cool white fluorescent lamps. Germination rate parameters were evaluated after 2 weeks.

The effects of different levels of ABA and sucrose on some parameters of somatic embryo maturation were studied before and after desiccation. These parameters included embryo size, number of embryos with shoot and/or root meristem, root formation, length of shoot and number of secondary embryos. The embryo size was scored between 0 and 5 (0 for minimum and 5 for maximum size of somatic embryos). As the somatic embryos may have only shoot, root, both or neither, only those with both shoot and root were considered as normal mature embryos (Fig. 1), and the effects of ABA and sucrose on this aspect were evaluated.

Histology

To show the differences between normal and abnormal mature somatic embryos, both types of embryos were fixed in FAA (formaldehyde: acetic acid: ethanol, 5:5:90) for 24 h. Fixed tissues were dehydrated in a series of ethanol concentrations: 25%, 50%, 70%, 96% and 100% (v/v) (20 min each bath), and embedded in paraffin wax at 56°C. Sections of 7 μm thick were cut with a rotatory microtome (Leitz, WETZLAR) and fixed on glass slides. Sections were de-waxed with toluene and then stained with hematoxylin harris. Observations were made with a light microscope.

Statistical analysis

Experiments were conducted as a complete randomized design. Each treatment comprised of 10 somatic embryos with four replicates. For confirmation, each of the experiments was repeated twice in two consecutive years and comparison of means for experiment effect was done as the combined analysis. Analysis of variance, was carried out using the General Linear Model procedure of the computing statistical program package SAS (SAS Institute, Inc. 1985). Means of the studied variables were compared at the end of the observation period with Duncan's multiple range test (DMRT).

Results and discussion

Maturation and germination of SEs: before desiccation

The results indicated that there are no significant differences between the results of the first and second year (Table 1). Increasing the concentration of ABA first increased the size of embryos, but higher concentrations had adverse effect on the embryo size: maximum embryo size was recorded at 2 mg l^{-1} and the smallest embryos were attained at 5 mg l^{-1} of ABA (Table 2). Application of 6.0% sucrose significantly increased the embryo size. However, there were no significance differences between 3.0% and 4.0% (Table 3). While different concentrations of ABA didn't have any significant effects on the number of embryos with a shoot meristem, the 4.0% sucrose treatment produced the highest rate of shooting embryos. The maximum number of embryos with root formation was achieved at 2 mg l^{-1} ABA and 6.0% sucrose. Minimum shoot length of the embryos was achieved at control treatment of ABA, while this parameter was maximum at the control treatment of sucrose (Table 3). In all treatments, elongation of root apex was observed before emergence of the green shoot (Fig. 1). Lack of shoot meristem, which is a common abnormality in plant somatic embryos, was confirmed through analysis of embryo sections using a light microscope (Fig. 2).

Maturation and germination of SEs: after desiccation

Only those embryos that showed both an elongated root and a developed green shoot were considered as

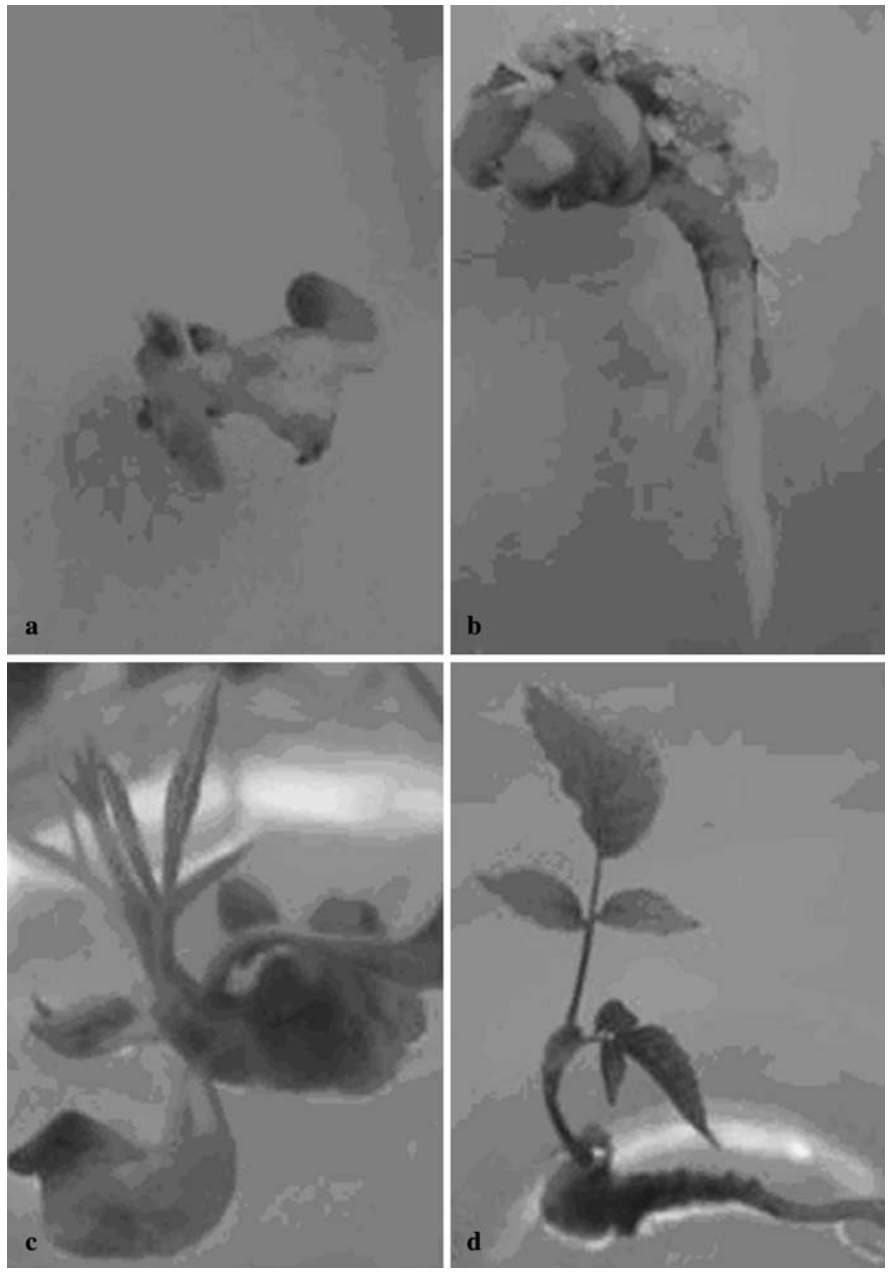

Fig. 1 (**a**) A non-germinated embryo, (**b**) a non-germinated embryo with elongated root, inactive apical bud and a high number of secondary embryos in control medium, (**c**) a germinated embryo without root and (**d**) a normal germinated embryo with both root and shoot

normal germinated embryos in this experiment (Fig. 1). Results indicated that 2 mg l^{-1} ABA provided the highest conversion into healthy plantlets (41%) (Figs. 3, 4). Vahdati et al. (2006) explained that normal maturation of somatic embryos needs an ABA treatment.

After desiccation, some embryos showed only an elongated root (1 cm or longer) with an inactive apical bud (Fig. 1b). Higher concentrations of ABA in maturation medium, increased the number of embryos with only elongated roots. In mediums containing 5 mg l^{-1} ABA, about 80% of embryos had elongated roots with an inactive apical bud, and some embryos formed only a shoot (Fig. 4). However, the highest percentage of normal mature somatic embryos was achieved at 2 mg l^{-1} ABA. These results are in agreement with Mauri and Manzanera (2003) who found that addition of a

Table 1 Comparison of means for experiment effect in combined analysis of factorial experiment

Experiment[a]	Embryo size after 1 month[b] (0–5)	No. of secondary somatic embryos	Somatic embryos with shoot meristem (%)	Somatic embryos with root formation (%)	Different stages of embryos development after desiccation				
					Only root	Only shoot	Both shoot and root	No root and shoot	Shoot length (cm)
$Exp._1$	3.45a[c]	34.52a	0.55a	1.26a	5.23a	0.22a	1.59a	2.97a	3.42a
$Exp._2$	3.29a	34.9a	0.70a	1.45a	5.61a	0.23a	1.75a	2.41b	3.52a

[a] DKW medium used as a basal medium and solidified with gelrite at 2.1 g l^{-1} (pH 5.7)

[b] Four replicates (10 explants) per treatment and data collected after 25 days of culture, before desiccation

[c] Means in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple range test (DMRT)

Table 2 Effects of ABA concentrations on embryo maturation parameters

ABA concentrations[a] (mg l^{-1})	Embryo size after 1 month[b] (0–5)	Somatic embryos with shoot meristem (%)	Somatic embryos with root formation (%)	Shoot length after 2 months (cm)	No. of secondary somatic embryos
0.0	3.22bc[c]	2.73a	7.27b	1.68b	51.7a
0.5	3.53ab	7.27a	6.36b	3.26a	38.67b
1.0	3.61ab	7.0a	16.0ab	3.62a	34.55bc
2.0	3.72a	8.33a	20.83a	3.77a	24.91bc
4.0	3.56ab	5.0a	16.0ab	3.63a	26.2bc
5.0	3.05c	2.73a	9.09b	3.73a	31.55bc

Data were collected before desiccation

[a] DKW medium used as a basal medium and solidified with gelrite at 2.1 g l^{-1} (pH 5.7)

[b] Four replicates (10 explants) per treatment and each of the experiments was repeated twice

[c] Means in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple range test (DMRT)

Table 3 Effects of sucrose levels on embryo maturation parameters

Sucrose concentrations[a] (g l^{-1})	Embryo size after 1 month[b] (0–5)	Somatic embryos with shoot meristem (%)	Somatic embryos with root formation (%)	Shoot length after 2 month (cm)	No. of secondary somatic embryos
30	3.31b[c]	5.45ab	14.09ab	3.79a	29.27b
40	3.37b	8.18a	8.18b	3.78a	43.59a
60	3.71a	2.86b	15.71a	2.6b	30.52b

Data were collected before desiccation

[a] DKW medium used as a basal medium and solidified with gelrite at 2.1 g l^{-1} (pH 5.7)

[b] Four replicates (10 explants) per treatment and each of the experiments was repeated twice

[c] Means in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple range test (DMRT)

specific range of exogenous ABA to cultures of cork oak somatic embryos favored their maturation. Nevertheless, there was no significant difference between the three levels of sucrose with respect to the percentage of different types of germinated somatic embryos, with the exception of 3 mg l^{-1} of sucrose,

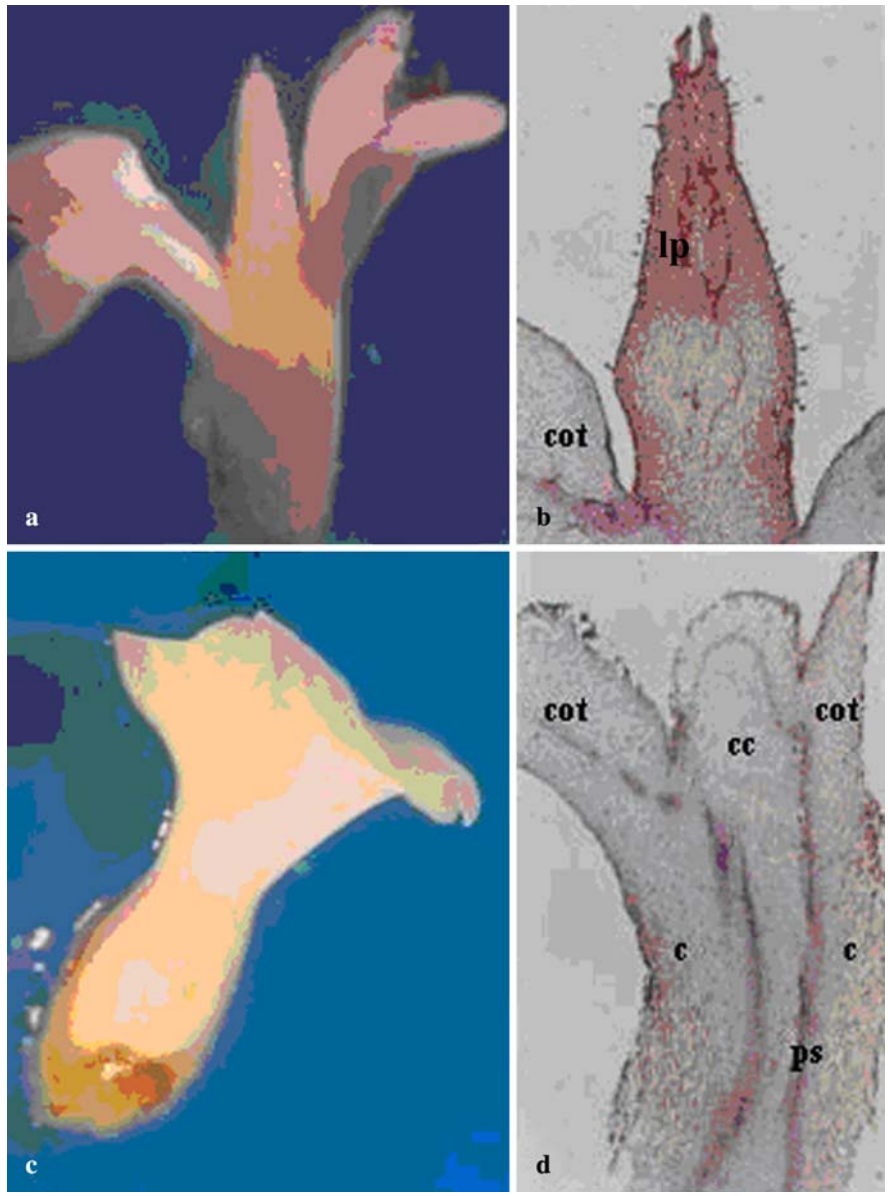

Fig. 2 (**a**) A somatic embryo with a well developed shoot meristem, (**b**) longitudinal section of (**a**). (**c**) An abnormal mature embryo with inactive apical bud, (**d**) longitudinal section of (**c**) after 2 weeks. cot, cotyledon; c, cortical tissue; cc, central cylinder; lp, leaf primordia; ps, procambial strand

which produced fewer normal embryos (with both shoot and root) (Fig. 5).

Maturity and white appearance of embryos seem to be important for germination. Most of the newly-formed embryos on ABA-free medium became translucent, but those exposed to ABA showed a white appearance. Translucency of embryos may reflect the lack of starch and/or protein storage (Deng and Cornu 1992) and according to Misra (1994), ABA is essential for the accumulation of storage reserves and synchronized maturation of somatic embryos.

As described by Tian and Brown (2000) and Vahdati et al. (2006), among the successive developmental stages of somatic embryos, the globular stage is the best for the application of ABA. It is only at the globular stage that embryos respond to ABA, and application of ABA during other developmental stages will gain no effect. Somatic embryos appear to become less responsive to ABA during maturation. The same observation has also been documented in conifers (Attree and Fowke 1993). In our experiment, desiccation of somatic embryos prior to germination improved the quality

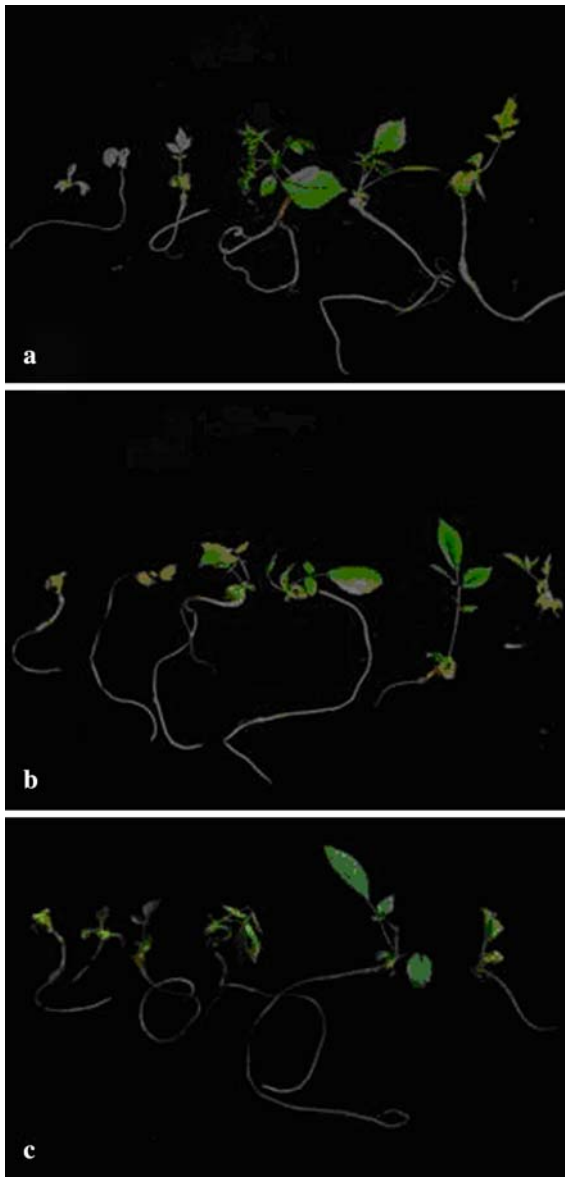

Fig. 3 Somatic embryos treated by ABA (0.0, 0.5, 1.0, 2.0, 4.0, and 5.0 mg l^{-1} from left to right) and different levels of sucrose after desiccation and transferring to light. (**a**) 3% Sucrose, (**b**) 4% sucrose and (**c**) 6% sucrose after 7 weeks

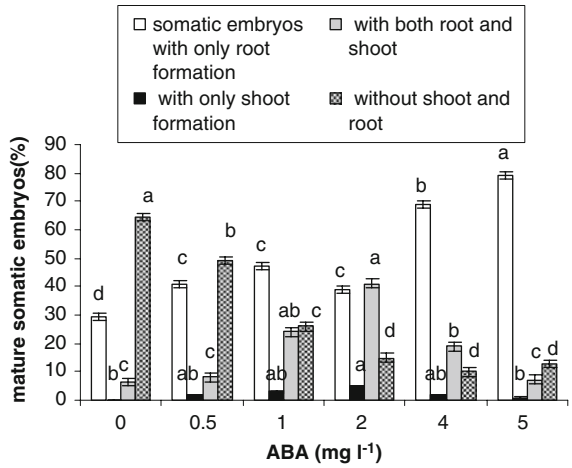

Fig. 4 Effect of ABA concentrations on germination of *Juglans regia* somatic embryos. Data collected after desiccation of somatic embryos treated by different concentrations of ABA. For the same variable, means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT). Number of embryos per treatment 4 × 10

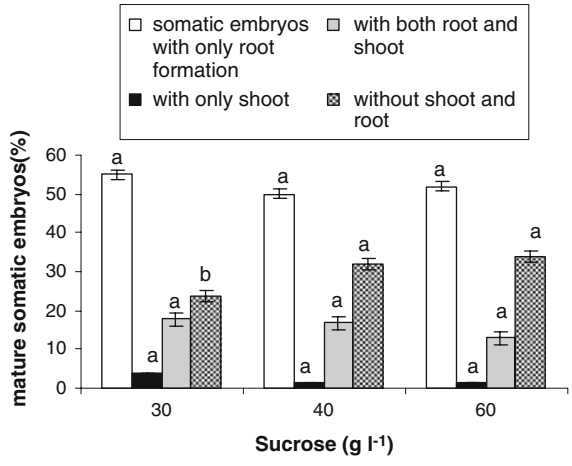

Fig. 5 Effect of sucrose levels on germination of *Juglans regia* somatic embryos. Data collected after desiccation of somatic embryos treated by different levels of sucrose. For the same variable, means with different letters were significantly different at the 5% level as determined by Duncan's multiple range test (DMRT). Number of embryos per treatment 4 × 10

of the germinated embryos as Tang et al. (2000) and Pond et al. (2002) reported.

Secondary embryogenesis

Walnut somatic embryos can be efficiently multiplied by repetitive or secondary embryogenesis on hormone-free DKW medium (Deng and Cornu 1992). In the current study, the highest number of secondary embryos was achieved on control medium (with no exogenous ABA) and different ABA concentrations reduced the efficiency of secondary embryogenesis (Table 2). The results are in agreement with Nuutila et al. (1991), Etienne et al. (1993) and Von Arnold et al. (2002) who reported that exogenous ABA

reduces the frequency of secondary embryogenesis. Mauri and Manzanera (2003) also state that addition of exogenous ABA to the cultures of cork oak somatic embryos efficiently reduces the rate of unwanted secondary embryogenesis.

Treatment with 4.0% sucrose significantly increased the number of secondary embryos (Table 3) and it is in agreement with Tang et al. (2001) who showed that 30–50 g l^{-1} sucrose yielded better results than the other concentrations tested. Therefore, this treatment could be tested for optimizing secondary embryogenesis of this species.

In conclusion, as Von Arnold et al. (2002) reviewed, only mature embryos with a normal morphology which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants. In our study, using 2 mg l^{-1} of ABA in maturation medium was favored for producing normal somatic embryos in walnut.

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References

Ammirato PV (1989) Recent progress in somatic embryogenesis. Int Assoc Plant Tissue Cult Newsl 57:2–16

Ammirato PV (1997) Hormonal control of somatic embryo development from cultured cells of caraway. Interaction of abscisic acid, zeatin and gibberellic acid. Plant Physiol 59:579–586

Attree SM, Fowke LC (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tissue Organ Cult 35:1–35

Cornu D (1988) Somatic embryogenesis in tissue culture of walnut (*Juglans regia*, *J. major* and hybrids *J. nigra* × *Juglans regia*). In: Ahuja MR (ed) Somatic cell genetics of woody plants. Kluwer Academic Publishers, Boston

Cornu D (1989) Walnut somatic embryogenesis, physiological and histological aspects. Ann Sci For 46S:133–135

Cornu D, Jay-Allemand C (1989) Micropropagation of hybrid walnut trees (*Juglans nigra* × *Juglans regia*) through culture and multiplication of embryos. Ann Sci For 46S:113–135

Deng MD, Cornu D (1992) Maturation and germination of walnut somatic embryos. Plant Cell Tissue Organ Cult 28:195–202

Dhuria HS, Bhutani VP, Dhar RP (1977) Standardization of propagation techniques in walnut. I. Preliminary studies on grafting. Indian J Hortic 34:20–23

Driver JA, Kuniyuki AH (1984) In-vitro propagation of paradox walnut rootstock. HortScience 19:507–509

Etienne H, Montoro P, Michaux-Ferriere N, Carron MP (1993) Effect of desiccation, medium osmolarity and abscisic acid of the maturation of *Hevea brasiliensis* somatic embryos. J Exp Bot 44:1613–1619

FAO (2005) FAO statistics division 2007. http://faostat.fao.org. Cited 13 February 2007

Gutmann M, Aderkas PV, Label P, Lelu MA (1996) Effects of abscisic acid on somatic embryo maturation of hybrid larch. J Exp Bot 47:1905–1917

Harry IS, Thorpe TA (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot Gaz 152:446–452

Hartmann HT, Kester DE, Davies FT, Geneve RL (1997) Plant propagation: principles and practices, 6th edn. Prentice Hall Int'l, INC

Komatsuda T, Lee W, Oka S (1992) Maturation and germination of somatic embryos as affected by sucrose and plant growth regulators in soybeans *Glycine gracilis* Skvortz and *Glycine max* (L.) Merr. Plant Cell Tissue Organ Cult 28:103–113

Kuden A, Kaska N (1997) Studies on the patch-budding of walnuts in different budding periods under subtropical conditions. Acta Hortic 442:299–301

Lee BC, Shim SY, Lee SK (1988) Mass propagation and germination of somatic embryos in *Juglans regia* L. (English walnut). Res Rep Inst For Genet Korea 24:99–106

Long LM, Preece JE, Van Sambeek JW (1995) Adventitious regeneration of *Juglans nigra* L. (eastern black walnut). Plant Cell Rep 8:512–516

Mauri PV, Manzanera JA (2003) Induction, maturation and germination of Holm oak (*Quercus ilex* L.) somatic embryos. Plant Cell Tissue Organ Cult 74:229–235

McGranahan G, Leslie C, Uratsu S, Dandekar A (1990) Improved efficiency of the walnut somatic embryo gene transfer system. Plant Cell Rep 8:512–516

Misra S (1994) Conifer zygotic embryogenesis, somatic embryogenesis, and seed germination: biochemical and molecular advances. Seed Sci Res 4:357–384

Nuutila AM, Kurten U, Kauppinen V (1991) Optimization of sucrose and inorganic nitrogen concentrations for somatic embryogenesis of birch (*Betula pendula* Roch.) callus cultures: a statistical approach. Plant Cell Tissue Organ Cult 24:73–77

Ozkan Y, Gumus A (2001) Effect of different applications on grafting under controlled conditions of walnut (*Juglans regia* L.). Acta Hortic 544:515–520

Ozkan Y, Edizer Y, Akca Y (2000) A study in propagation with patch budding of walnut cultivars (*J. regia* L.). Acta Hortic 544:521–525

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM (2006) Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster* and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tissue Organ Cult 86:87–101

Pond SE, Von Aderkas P, Bonga JM (2002) Improving tolerance of somatic embryos of *Picea glauca* to flash desiccation with a cold treatment (desiccation after cold acclimation). In Vitro Cell Dev Biol Plant 38:334–341

Preece JE, McGranahan GH, Long LM, Leslie CA (1995) Somatic embryogenesis in walnut (*Juglans regia*). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol 2. Kluwer Academic Publishers, Netherlands

Rezaee R, Vahdati K, Grigoorian W, Valizadeh M (2008) Walnut grafting success and bleeding rate as affected by different grafting methods and seedling vigor. J Hortic Sci Biotechnol 83(1):94–99

Roberts-Oehlschlager SL, Dunwell JM, Faulks R (1990) Changes in the sugar content of barley anthers during culture of different carbohydrates. Plant Cell Tissue Organ Cult 22:77–85

Roghani MS, Haq A, Khan J (1977) Propagation of walnut by grafting. J Agric Res 14:389–392

SAS institute Inc (1985) SAS® user's guide: statistics. Version 5 Edition. SAS institute Inc, Cary

Tang H, Ren Z, Krczal G (2000) Improvement of English walnut somatic embryo germination and conversion by desiccation treatment and plantlet development by lower medium salts. In Vitro Cell Dev Biol Plant 36:47–50

Tang H, Ren Z, Reustle G, Krczal G (2001) Optimizing secondary somatic embryo production in English walnut (*Juglans regia* L.). Acta Hortic 544:187–194

Tian L, Brown D (2000) Improvement of soybean somatic embryo development and maturation by abscisic acid treatment. Can J Plant Sci 80:271–276

Trembly L, Trembly F (1995) Maturation of black spruce somatic embryos: sucrose hydrolysis and resulting osmotic pressure of the medium. Plant Cell Tissue Organ Cult 42:39–46

Tulecke W (1987) Somatic embryogenesis in woody perennials. In: Bonga JM, Durzan DJ (eds) Cell tissue cult forestry, vol 2. Martinus Nijhoff Publ, Boston

Tulecke W, McGranahan G (1985) Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci 40:57–63

Tulecke W, McGranahan G, Ahmadi H (1988) Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut, *Juglans regia* L. cv. 'Manregian'. Plant Cell Rep 7:301–304

Vahdati K (2001) Walnut situation in Iran. Nucis-Newsl 9:32–33

Vahdati K, McKenna J, Dandekar A, Leslie C, Uratsu S, Hackett W, Negri P, McGranahan G (2002) Rooting and other characteristics of a transgenic walnut hybrid (*Juglans hindsii* × *J. regia*) rootstock expressing *rolABC*. J Am Soc Hortic Sci 127:724–728

Vahdati K, Leslie C, Zamani Z, McGranahan G (2004) Rooting and acclimatization of in-vitro grown shoots from three mature Persian walnut cultivars. HortScience 39:324–327

Vahdati K, Jariteh M, Niknam V, Mirmasoumi M, Ebrahimzadeh H (2006) Somatic embryogenesis and embryo maturation in Persian walnut. Acta Hortic 705:199–205

Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233–249