African Journal of Biotechnology Vol. 10(3), pp. 290-311, 17 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.324
ISSN 1684–5315 © 2011 Academic Journals

Review

In vitro propagation of walnut - A review

Kamal Payghamzadeh[1] and Sayyed Kamal Kazemitabar[2*]

[1]Islamic Azad University, Azad Shaher Branch, Department of Agriculture, Azad Shaher, Iran.
[2]Associate professor, Dept of Plant Breeding & Biotechnology. Sari of Agricultural and Natural Resources University (SANRU), Sari, Iran.

Accepted 19 November, 2010

***In vitro* propagation of walnut has played a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants. During the last several years, different approaches have been made for *in vitro* propagation of walnut. Micropropagation using apicale bud, nodale segement, leaves, petioles, cotyledons, embryos and understanding the specific requirement at different stages has been comprehensively covered in literature. New challenges for refinements of protocols for high rate of shoot multiplication and development of cost effective methods has gained importance in the recent past. Importance of liquid and solid static culture for callus induction, embryogenesis, shoot proliferation and root induction for walnut is also discussed in the present review. Further, the development of protocols for *in vitro* propagation, culture nodal segment from seedling, somatic embryogenesis and plant regeneration which is considered the most important step for successful implementation of various biotechnological technique used for plant improvement programmes has been adequately addressed in literature. In walnut, there are several reports which indicate rapid regeneration and multiplication through organogenesis or somatic embryogenesis. On the whole, the present review gives a consolidated account of *in vitro* propagation in walnut.**

Key words: Walnut, *Juglans* spp. L, root induction, shoot multiplication, regeneration, medium culture, micropropagation, somatic embryogenesis.

INTRODUCTION

Clasification and distribution

Walnut is classified under Kingdom *Planta*; Division, *Magnoliopsda*; Order, *Fagales*; Family, *Juglandacea* and Genus, *Juglans*. The genus *Juglans* L. comprises 21 long-lived deciduous tree species generically referred to as walnuts. *Juglans regia* L. (Persion walnut; McGranahan and Leslie, 1990), *Juglans nigra* L. (Eastern black walnut; Funk., 1979), and *Juglans cinerea* L. (butternut; McDaniel, 1979) are important for commercial nut production. *J. nigra* L. is also used for timber and as valuable ecological rich species (Elias, 1980). These monoecious trees are native to North and South America, and from south Eastern Europe to eastern Asia (Bailey and Bailey, 1976). Manning (1978) divided *Juglans* L. into 4 sections:

1) *Cardiocaryon* Dode, with three species native to China, Korea, and Japan. (*J. ailantifolia* Carr, *J. manshurica* Maxim and *J. catheyensis* Dode).
2) *Trachycaryon* Dode ex Mann, with one species, *J. cinerea*, native to eastern North America.
3) *Rhysocaryon* Dode, with 16 species native to North, Central, and South America, and the West Indies.
4) *Dioscaryon* Dode with one species, *J. regia*, native from Europe to the Himalayan Mountains.

Walnut is a temperate nut crop. Nuts, leaves and woods of walnut are consumed by humans and wildlife because of the high quality wood, nutritious nuts and pharmacological properties of the leaves. The nut is a very rich

*Corresponding author. E-mail: kazemi_ka@yahoo.com. Tel: 01513822715. Fax: 01513822577.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **BAP,** 6-benzyl aminopurine; **IBA,** indole-3-butyric-acid; **NAA,** a-naphthalene acetic acid; **TDZ,** Thidiazuron; **IAA,** indole-3 acetic acid; **GA_3,** Giberlic acid; **CH,** Casein hydrosylated; **DKW,** Driver and Kuniyuki Walnut medium; **MS,** Murashige and Skoog; **WPM,** Woody Plant Medium; **PVP,** polyvinyl pyrrolidone; **PGR,** plant growth regulators.

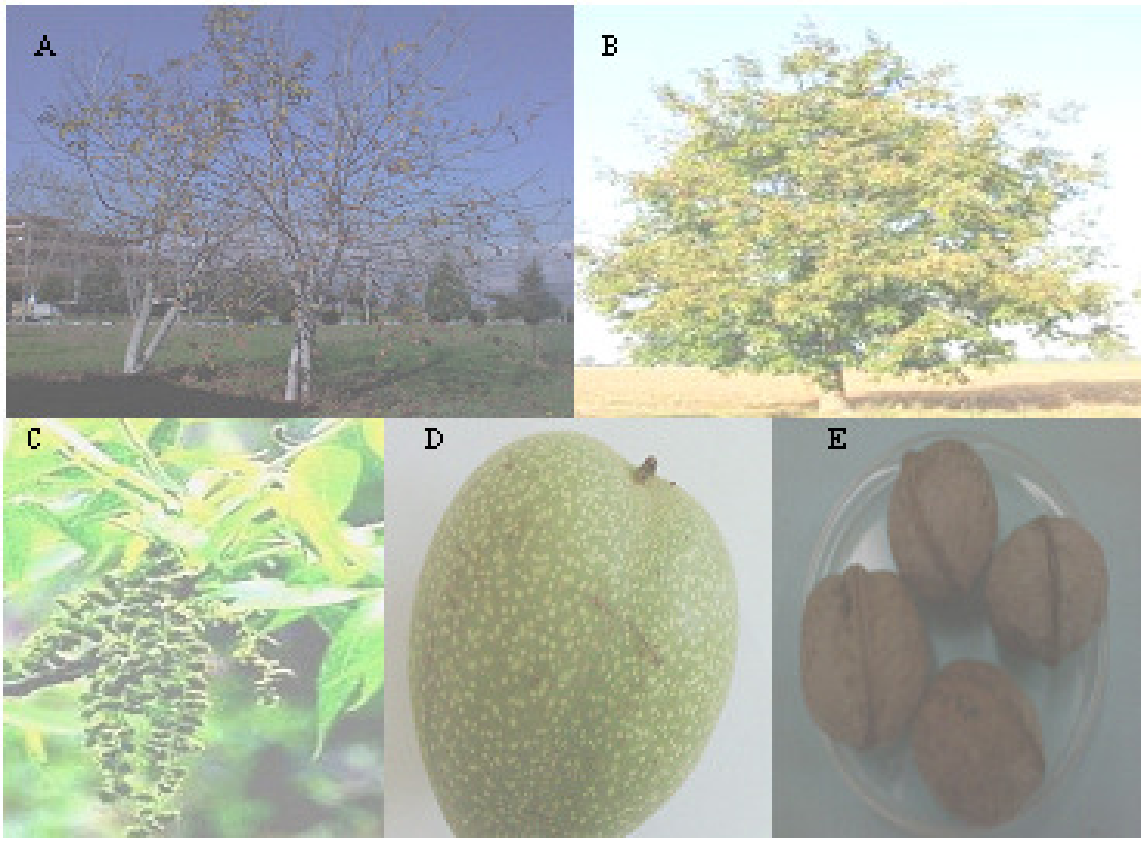

Figure 1. Morphological characteristics of *Juglans regia* L. Growing in North of Iran. A and B, Growth habit. The pictures captured in autumn and summer seasons respectively; C, the male flowers are formed on the previous year's wood; D and E, respectively immature and mature fruits 11 and 22 weeks after anthesis.

source of proteins, fats, minerals and a concen-trated source of energy (Kaur et al., 2006; Ostry and Pijut, 2000; Gunes, 1999). Moist, rich soils of hillsides and stream banks are the preferred growing sites, although walnuts can grow quite well on dry, rocky soils (Pijut, 1997, 2004). In the last two decades, *in vitro* prop-agation has revolutionized commercial nursery business. The Persian walnut (*J. regia* L.) is the most horticulturally developed and widely cultivated species for nut pro-duction (McGranahan and Leslie, 1990) (Figure 1). Black walnut (*J. nigra* L.) is one of the most valuable hardwoods produced in the United States (Williams, 1990). Eastern black walnut is also grown for the edible nuts, but this species is more valued for its high quality wood, prized for fine furniture, gunstocks, cabinets and veneers. Butternut (*J. cinerea* L.) is valued economically and ecologically for both wood and edible nuts. The nuts are an important wildlife mast and the wood is marketable for many uses including furniture, cabinets, fine wood-working, and paneling. In areas where quality butternut wood is available, it ranks eighth out of the top 28 species for prime veneer and sawlogs (Peterson, 1990). Walnuts are now distributed across the temperate zones as both commercial and ornamental trees.

Propagation methods and problems

Juglans species are usually propagated by seeds, although the dormant embryo most in *Juglans spp* is the main constraint on propagation and development of high yielding cultivars through hybridization. Dormancy can be broken by fall sowing or by moist, prechilling of seeds at 3 - 5°C for 3 - 4 months but still results in a low percent-tage of seed germination. There is also evidence that the canker disease may have seedborne transmission (Orchard, 1984). Application of the methods of plant regeneration from *in vitro* cultured embryos allows barriers in hybridization to be overcomed (Hormaza, 1999; Bridgen, 1994), in addition obtaining higher and faster multiplication rate of plants of an elite genotype. Because of their juvenile nature, embryos have a high potential for regeneration and hence may be used for *in vitro* pro-pagation (Kaur et al., 2006). Cultivars of *Juglans spp* are vegetatively propagated by grafting onto seedling root-stocks. Various degrees of success have been reported for intraspecific as well as interspecific grafting in the genus (Kaeiser and Funk, 1971; Xi and Ding, 1990). Wal-nut can be propagated by grafting onto black walnut rootstock, with limited success. In general, the most popular

way of walnut vegetative propagation is that of grafting which is, however, labor intensive, time-consuming and costly. However, vegetative propagation by cuttings of mature tree specimens is very difficult due to their low rooting ability (McGranahan et al., 1988; Land and Cunninham., 1994). Some authors (Rodriguez et al., 1989., Chenevard et al., 1997) concluded that walnut propagation is still an unsolved problem and the main reasons are irregular and often low rooting rates and high mortalities of rooted plants during acclimatization. Earlier investigations (Claudet et al., 1992; Yalcin., 1993a) suggested that the continuity of the sclerenchymatous cylinder encircling the phloem inhibits rooting or root emergence. Jay-Allemand and colleagues (1995) suggested that juglone (5-hydroxy-1,4-naphthalenedione) is a major internal factor with a role in adventitious root induction during early stages of rhizogenesis and there is a positive correlation between juglone content and the rooting capacity of microcuttings. Many treatments on difficult-to-root *Juglans* species have been studied in order to improve rooting efficiency (Chelawant et al., 1995; Stephans et al., 1990; Heloir et al., 1996), but without significant improvements. Somatic embryo-genesis is a rapid propagation method and an important tool in making genetic improvements (Robacker, 1993).

Browning of the growth medium

Browning of the medium is the result of oxidation of polyphenols exuded from the cut surface of the explants which can be overcome by adding substances such as polyvinyl pyrrolidone (PVP), citric acid, ascorbic acid, activated charcoal, thiourea, L-cysteine, glutamine, aspargine, argenine or resorting to frequent subculturing (Rout et al., 1999; Pierik., 1987) or incubating cultures for a day or two in total darkness after inoculation as polyphenol oxidase activity was found to be induced by light (Pittet and Moncousin, 1981). Curir et al. (1986) stated that 3 days of culture on the medium containing charcoal followed by transfer to a fresh medium was highly effective in enhancing the growth of primary explants. In walnut, one of the major obstacles in the establishment of cultures is the presence of several phenolic compounds, including the allelochemical compound juglone, which interfere with cell growth (Rietvel, 1982, 1983). Also, weekly transfer of butternut nodal explants to fresh culture medium was necessary to maintain optimum growth and to limit the build-up of phytotoxic exudates in the culture medium. The production of exudates from freshly cultured explants of walnuts has also been a problem, solved by employing explant presoaking and transferring explants frequently to fresh medium (Preece et al., 1989; Leslie and McGranahan, 1992). To decrease explant exudation, they were transferred onto fresh medium 1, 3, 5 and 8 days after culture (Driver and Kuniyuki, 1984; McGranahan et al., 1988; Revilla et al., 1989). Long et al. (1995) removed the testa from the cotyledon pieces to reduce lethal browning. Transfers to fresh medium were made after 1 - 2 days. After 5 days, explants were transferred to 125-ml glass jars (baby food) containing 25 ml medium. These initial transfers were made to reduce the possible inhibitory effects of dark colored exudates that accumulated in the medium (Preece and Compton, 1991). In our study (Payghamzadeh and Kazemitabar, 2008b), explants were cultured on two kind of medium cultures such as mono phase medium supplemented with different concentration of 6-benzyl aminopurine (BAP) and double phase medium which the lower phase were supplemented with activated charcoal without plant growth regulation and upper phase was liquid DKW basal medium supplemented with different concentration of BAP without activated charcoal. Results indicated that 8.9 µM BAP was best concentration for lateral bud induction. Also, between two kind of medium culture, the double phase medium culture was better than any other medium (Figure 2).

MICROPROPAGATION

Micropropagation has the immense advantage of rapidly generating a large number of genetically identical plants in a much shorter time than could be achieved by conventional propagation methods. In general, works on micropropagation of walnut is summarized in Table 1.

Stages involved in micropropagation

A successful micropropagation protocol proceeds through a series of stages, each with a specific set of requirements. These are (i) Initiation of aseptic cultures, (ii) shoot multiplication, (iii) rooting of microshoots (iv) hardening and field transfer of tissue culture raised plants.

Initiation of aseptic cultures

Choice of explant for Juglans spp.: The choice of explant for initiation of culture is largely dictated by the method to be adopted for *in vitro* propagation. In walnut, the juvenile explants such as immature to mature embryos and immature cotyledons are often suitable for induction of somatic embryogenesis, callus, roots, germinants and shoot organogenesis (Table 1).

Sterilization of explants: One of the most important factors of plant tissues culture is establishing and maintaining aseptic conditions. Preparing sterile explants is difficult because the tissue must be treated with disinfectants to destroy any microbial contamination without harming the explant tissue. For walnut, the commonly adopted procedure involves surface sterilization of initial explants with

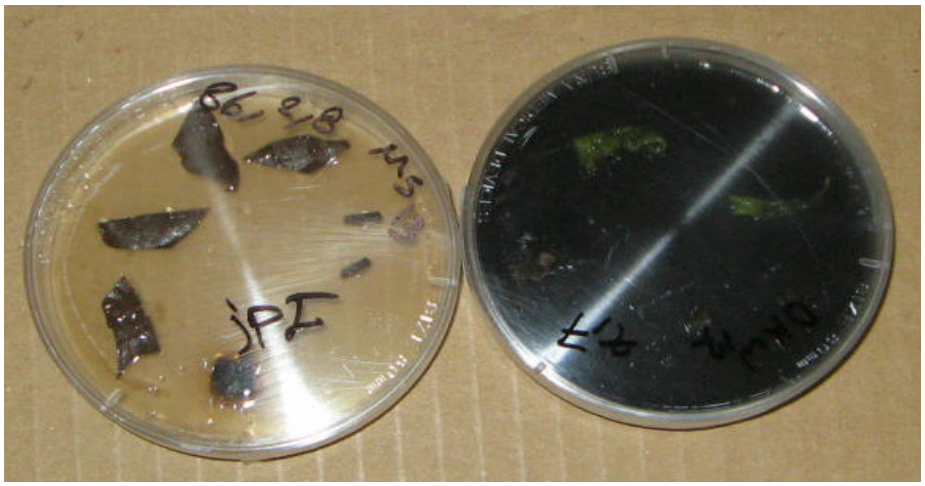

Figure 2. Comparison of explants survival on media supplemented with and without activated charcoal. In two culture systems, polyphenolic materials exuded after 0.5 - 1 h from wounded areas of explants. Different explants gradually died after 12 days on a medium without activated charcoal (left), but explants cultured on medium supplemented with activated charcoal were alive 21 days after sub-culture and then gradually died (right).

Table 1. Summary of *in vitro* studies with *Juglans* spp L. species.

Species/cultivar	Explant	Study/results	Reference
J. nigra L.	Shoots, leaves	Micro propagation/shooted rooted, plants	Roschke and Pijut (2006)
J. regia L.	Mature embryo	Micro propagation/mediums comparision	Payghamzadeh and Kazemitabar (2008a)
J. regia L.	Immature cotyledons	Somatic embryogenesis	Payghamzadeh and Kazemitabar (2008d)
J. regia L.	Cotyledons	Adventitious regeneration/somatic embryogenesis	Long et al. (1995)
J. regia L. rootstock cv. Perlata	Mature fruit	Embryo culture/germinated embryos and developed plants	Sanchez-Zamora et al. (2006)
J. regia L. cv. SU-2	Somatic embryos	Germination of somatic embryos/germinated embryos	Dumanogle (2000)
J. regia L	Mature fruit	Micropropagation /germinated embryos, fully development plant	Kaur et al. (2006)
J. cinerea	Immature cotyledons	Somatic embryogenesis; callus; roots; germinant	Pijut (1993a, b)
J. nigra	Immature cotyledons	Somatic embryogenesis; shoot organogenesis	Long et al. (1992)
J. nigra	Immature to mature cotyledons	Somatic embryogenesis; callus; roots	Neuman et al. (1993)
J. nigra, *J. major*	Immature cotyledons	Somatic embryogenesis; inter-specific hybrids	Cornu (1988)
J. nigra × *J. regia*	Embryonic axes	Germinants shoots	Cornu and Jay- Allemand (1989)
J. nigra × *J. regia*	Shoot tips	Rooted plantlets	Meynier and Arnould (1989)
J. nigra × *J. regia*	Immature cotyledons	Maturation, germination of somatic embryos	Deng and Cornu (1992)
Interspecific hybrids	Embryonic axes	Improved rooting of micro shoots; plants	Jay-Allemand et al. (1992)
J. regia	Cotyledon	Somatic embryogenesis ; plants	Tulecke and McGranahan (1985)
J. regia	Immature cotyledons	Histology of somatic embryo origin	Polito et al. (1989)
J. regia	Embryonic axes; nodal segments	Shoots; rooted plants	Revilla et al. (1989)
J. regia	Apical and lateral buds	Shoot formation	Felaliev (1990)
J. regia	Axillary buds	Shoot multiplication; rooted plants	Stephens et al. (1990)
J. regia	Ovules	Somatic embryo origin determined by RFLP and isozyme analysis	Aly et al. (1992)

Table 1. Cont..

Clone TRS	Micro shoots	Improved acclimatization of plantlets	Voyiatzis and McGranahan (1994)
J. regia, *J. hindsii* × *J. regia*	Nodal segments	Shoot multiplication; rooted shoots	Gruselle and Boxus (1990)
J. regia × *J. nigra*	Nodal segments	Pi nutrition in relation to callus and shoot development	Barbas et al. (1993a)
J. regia × *J. nigra*	Embryonic axes	Gelling agent effects on shoot growth	Barbas et al. (1993b)
J. regia L.	Immature and mature embryos, immature cotyledons	Micro propagation/callus induction, embryo germination	Payghamzadeh (2008)

Pi, Orthophosphate; RFLP, restriction fragment length polymorphism.

50 - 70% (v/v) ethanol for 20 – 30 s followed by 0.1 - 15% (v/v) sodium hypochorite containing 0.01% Tween 20 for 10 - 20 min and then three 5 min rinses in sterile distilled water (Table 2). Table 2 shows the detailed protocols adopted for several explant tissues by a number of workers.

In vitro culture

Research with *Juglans* spp has focused on several different biotechnological approaches and initial explant material (Table 1) for the purpose of clonal mass propagation and subsequent genetic improvement. Progress in developing techniques for *in vitro* culture *Juglans* spp and hybrids has been very successful over the past 10 years. Plantlets have been obtained via shoot-tip multiplication, cultured nodal segments, and somatic embryogenesis. However, few reports have been published on attempts to micropropagate *Juglans sp* L. (Payghamzadeh, 2008) (Table 3).

Callus culture: It seems that in walnut, auxins and cytokinins are responsible for callus induction. In our own study, callus fresh weight depended on BAP and indole-3-butyric-acid (IBA) concentration. Initially, the calluses was green and nodular (Figure 3c), but then become brown and friable especially those grown in 0.1 mg 1^{-1} IBA and 1.5 mg 1^{-1} BAP which become necrotic and loosed. In general terms, the 0.01 and 0.1 mg 1^{-1} IBA and 1.5 mg 1^{-1} BAP favor callus induction from immature embryonic bodies (Payghamzadeh and Kazemitabar, 2010a). This callus was white, cream, loose and very slow growing. Attempts to regenerate shoots or somatic embryos from this type of callus have so far been unsuccessful.

SHOOT MULTIPLICATION

Several reports indicated that *Juglans* species are amenable, to a certain degree, to micro propagation (Sommers et al., 1982; Driver and Kuniyuki, 1984; Meynier, 1985; Heile-Sudholt et al., 1986; Lee et al., 1986; McGranahan et al., 1988; Revilla et al., 1989; Felaliev, 1990; Gruselle and Boxus, 1990; Leslie and McGranahan, 1992; Stephans et al., 1990) (Table 3). Axillary buds elongated when explants were cultured on a Driver and Kuniyuki Walnut (DKW) medium supplemented with 8.9 mM BAP (Payghamzadeh and Kazamitabar, 2008b). Chalupa (1981) reported successful axillary bud elongation from nodal stem segments of *J. regia* seedlings cultured on Murashige and Skoog (MS) medium containing 0.4 µM BA and 0.8 µM a-naphthalene acetic acid (NAA). Gruselle et al. (1987) using a modified MS medium, found both 4.4 and 8.9 µM BA suitable for culturing nodal explants from young seedlings of *J. regia*. Culturing juvenile *J. cinerea* nodal explants on woody plant medium (WPM) or DKW media, regardless of growth regulators, resulted in the swelling and partial expansion of axillary buds, but no significant elongation. Much like black walnut and butternut seedlings, *in vitro*-derived plantlets initially increased root, rather than shoot growth (Pijut, 1997) (Table 3). The various factors that influence *in vitro* shoot multiplication in walnut are listed below.

Species/genotypes/cultivars

Scaltsoyiannes et al. (1997) marked a clear effect of genotypes on *in vitro* propagation of two walnut cultivars. They observed significant differences among the multiplication rates of twelve different clones from these nuts. The effect of genotype was obvious in the rooting phase as well. Some clones exhibit high rooting ability (95%) and some low (5%). Among the twelve clones, significant differences were also observed for their multiplication rates. Although most of them ranged from 150 to 460 axillary shoots per 100 explants, there was one, P5, with a low multiplication rate and another, P6, with a high rate. In another study (Payghamzadeh and Kazemitabar, 2010a) for optimazation of medium culture for embryo culture of eight cultivars of walnut, immature embryos were cultured on DKW basal medium. Many cultivars are capable of supporting the conversion of plantlet from young embryos. It was observed that different cultivars had different requirements for growth regulators. Between different

Table 2. Commonly used methods for sterilization of explants.

Species/Cultivar	Explants	Study	Disinfectants and dosage	Method	Reference
J. regia L. cultivars vis., ACO38853, Netar Akhort, Gobind, Solding selection	Mature fruit	Embryo culture	0.1% sodium hypochlorite	Mature fruits were washed in running tap water. Then epicarp was removed and the remaining part of the fruit (nut) was disinfected by treating with 0.1% solution of sodium hypochlorite followed by washing twice with sterile distilled water for 5 min.	Kour et al. (2006)
J. regia L. cultivars vis., Yalova-1, Sebin, Bilecik, KR-1, KR-2, Sen-2, 07-KOR-1, Tokat-1, Kaman-1, Kaman-5	Immature fruit	Somatic embryogenesis	3.75% sodium hypochlorite	Fruits were sterilized by immersion for 25 min in 3.75% (v/v) sodium hypochlorite, followed by three 5- min rinses in sterile distilled water.	San and Hatic (2006)
J. regia L.	Shoot and leaf	Adventitious shoot regeneration and micro propagation	For shoots: 70% ethanol, 15% bleach solution	For shoots: shoots washed under running tap water for 30 min. Shoots were cut into sections and washed for 30 s in 70% ethanol, disinfected for 20 min in 15% bleach solution plus 0.01% Tween on an orbital shaker and rinsed in sterile water four times for 30 s.	Roschke and Pijut (2006)
			For leaves: 10% bleach solution	For leaves: explants were surface washed in running tap water for 5 min, sterilized for 10 min in 10% bleach solution and rinsed four times with sterile water.	
J. cinerea L.	Nodal segment	Axillary bud culture	0.8% (v/v) sodium hypochlorite (15% clorox bleach)	Stems from actively growing seedlings were stripped of leaflets and surface disinfested in 0.8% (v/v) sodium hypochlorite (15% clorox bleach) for 15-20 min, followed by four rinses in sterile, deionized water.	Pijut (1997)

cultivars, the highest percent embryos germination were achieved in Chandler, Serr, Hartky, Rentegnomushak and Local cultivar. Kaur et al. (2006) investigated embryo germination in five cultivars of walnut (J. regia L.) viz., ACO 38853, Netar Akhrot, Gobind, Solding selection and Blackmore via *in vitro* culture. In this study, they observed significant differences between the five cultivars, with Netar Akhrot having the highest percent embryo germination. The influence of genotype on shoot proliferation could easily be interpreted by linking it with the recent progress in functional genomics of plants. Current studies indicate that there are genes responsible for increased number of bud initials and shoot proliferation. Moreover, the involvement of genes in modulating hormone levels has also been reported (Tantikanjana et al., 2001).

Media

The culture medium is one of the most important components of plant cell and tissue culture

Table 2. Contd.

Species/Cultivar	Explants	Study	Disinfectants and dosage	Method	Reference
J. regia L. cultivar vis., Plemiana 1 and Plemiana 2	Kernel	Embryo culture	NaOCl (1.0% w/v), 75% v/v EtOH	The nuts were cracked and the kernels were immersed in NaOCl (1.0% w/v) for 5 min followed by 75% v/v EtOH for another 5 min and were rinsed three times in sterile distilled water.	Scaltsoyiannes et al. (1997)
J. nigra L.	Immature cotyledons	Adventitious regeneration	5..25% NaClO ; 70% EtOH; HCL 0.01 N	The immature fruit were surface-disinfected immediately following collection for 1 min in 70% (v/v) ethanol/water solution, followed by a 5 min immersion in 5.25% NaClO. This was followed by a 1 min rinse in sterile deionized water, 1 min in 0.01 M HCl, and three 5-min rinses in sterile deionized water.	Long et al. (1995)
J. regia L. *J. regia* L. rootstock cv. Peralta	Fruit Nut	Micro propagation Embryo culture	0.5% NaClO, 75% EtOH	Seeds were previously immersed for 24 h in water and disinfected for 5 min in 0.5% NaClO solution followed by 5 min in 75% ethanol and 3 rinses in sterile distilled water.	Revilla et al. (1989) Sánchez-Zamora et al. (2006)
			10% solution of Domestos®, 70% ethanol	The nuts were harvested in October 2002 and washed in a 10% solution of Domestos® for 2 h, rinsing and changing the solution every 30 min. The nuts were cracked, the kernel removed and the lower half of the cotyledons cut off. These embryos were disinfested in a laminar flow cabinet, for 2 min in 70% ethanol and for 20 min in 20% Domestos®, finishing with three rinses of sterile water.	

methods. For micropropagation of *Juglans* L., different culture media have been used, such as Driver and Kuniyuki (1984) (DKW), Murashige and Skoog (1962) (MS), Cheng (1975), Gamborg et al. (1968) and Lloyd and McCown (1981) (WPM), Rodríguez (1982) [medium K(h)], with varying success. Also, different media have been used for the proliferation stage of the explants as indicated by Jay-Allemand (1982), using a primary culture in half strength Knop's medium and a secondary one in Miller medium with 1 mg/l BAP. Murashige and Skoog's (1962) medium and Driver and Kuniyuki (DKW) medium (1984) were found to be the most commonly used for walnut propagation. The composition (mg/l) of each culture medium used for *in vitro* culture walnut is listed in Table 7. The first attempts at walnut micropropagation utilised existing media formulations which were suitable for other woody plants. Using Driver and Kuniyuki (1984), Cheng (Cheng, 1975), Murashige and Skoog (MS) (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) and woody plant media (WPM) (Lloyd and McCown, 1981) encountered the problem of gradual culture deterioration. This

Table 2. Contd.

Species/Cultivar	Explants	Study	Disinfectants and dosage	Method	Reference
J. regia L.	Embryonic axes	Morphogenic study and embryo culture	Alconox (0.1%), NaClO ($5\ gl^{-1}$)	Embryonic axes from the seeds were isolated and then sterilized by soaking with alconox (0.1%) for 10 min, rinsed with distilled water for 10 min, soaked with NaClO ($5\ gl^{-1}$) for 10 min and finally rinsed 3 times with distilled water.	Fernandez et al. (2000)
J. regia L.	Immature fruits	Callus induction from immature embryos	Hypochlorite sodium 3.75%; EtOH 50%	Immature fruit were selected at 6 weeks postanthesis and washed under running tap water for 30 min, then the fruits were treated for 30 s in 50% EtOH, disinfected for 20 min in 3.75% hypochlorite sodium solution made up with sterile water plus 0.01% Tween 20, after that samples were three 5-times rinsed with sterile distilled water.	Payghamzadeh (2008)

Table 3. Commonly used hormones, mediums and explants for *in vitro* propagation of walnut.

Species/ cultivars	Plant growth regulator								Study	Medium	Explant	References
	BAP	Kn	TDZ	2,4-D	IBA	IAA	NAA	GA_3				
J. cinerea L.	-	-	-	-	2.5 µM	-	-	-	Rooting	1/2 MS	Ms	Pijut (1997)
J. cinerea L.	8.9 µM	-	-	-	-	-	-	-	Axillary bud culture	MS	Ns	Pijut (1997)
J. regia L	-	-	-	-	3 mg/l	-	-	-	Rooting of shoot	DKW	S	Heloir et al. (1996)
J. regia L	1 mg/l	-	-	-	-	-	-	-	Axillary shoot proliferation	MS	S	Heloir et al. (1996)
J. nigra L.	1 - 5 mg/l	-	-	-	-	-	-	-	Bud proliferation	MS or DKW	-	Sommers et al. (1982)
J. regia L.	1 mg/l	-	-	-	0.01 mg/l	-	-	-	Shoot multiplication	DKW, MS and WPM	St	Saadat and Hennerty (2002)
J. regia L	-	-	6.8 µM	-	1 µM	-	-	-	Adventitious shoot regeneration	DW (1/2 DKW+1/2 WPM)	L	Roschke and Pijut (2006)
J. regia L.	4.44 µM	-	-	-	0.005 µM	-	-	-	Shoot multiplication	DKW	Ge	Jay-Allemand et al. (1992)
J. regia L	1 mg/l	-	-	-	-	-	-	-	Shoot multiplication and rhizogenesis induction	MS	Eje	Penula et al. (1988)
J. regia L.	1 mg/l	2 mg/l	-	-	0.01 mg/l	-	-	-	Macro morphological and histological analyses.	Modified MS	Sb	Rodriguez et al. (1993)
J. regia L	1 mg/l	-		-	0.1 mg/l	0.05 mg/l	-	0.1 mg/l	Micro propagation	MS	Jns, Ea	Revilla et al. (1989)

Table 3. Cont..

J. regia L	4.4, 8.9 µM	-	-	-	-	-	-	-	Culturing nodal segement	Modified MS	Ns	Gruselle et al. (1987)
J. regia L.	0.4 µM	-	-	-	-	-	0.8 µM	-	Axillary bud elongation	MS	Ns	Chalupa (1981)
J. nigra L.	-	-	5 µM	0.1 µM	-	-	-	-	Adventitious regeneration	WPM	-	Long et al. (1995)

Jns, Juvenile nodal segement; S, shoot; St, shoot tip; L, leaf; Ns, nodal segement; Ea, embryonic axes; Ms, microcutting shoots; Ge, germinated embryos; Eje, embryonic and juvenile explants; Sb, shoot buds; Ns, nodal segements.

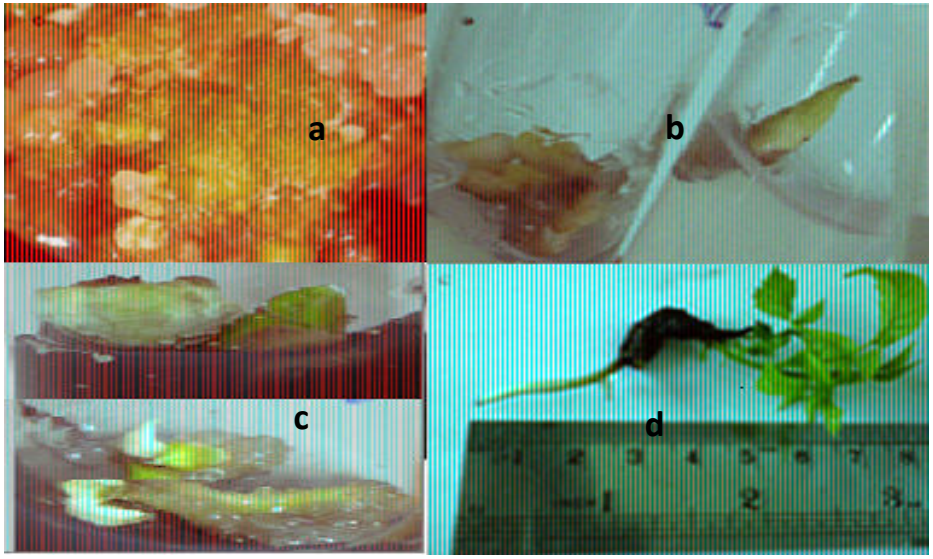

Figure 3. Micropropagation of walnut. A, Embryonic callus product from cotyledonary explants; B, mature embryos cultured on Driver and Kuniyoki walnut basal medium; C, calluses inducted from embryonic badies; D, fully developed plantlet originating from mature embryos culture (Payghamzadeh, 2008).

problem led them to develop a new medium called DKW medium (McGranahan et al., 1987) optimized especially for the growth of the paradox hybrid. Although DKW medium was developed for cultivar paradox, it has proven suitable for a variety of *Juglandaceae* species, including *J. regia* L. and is currently the most widely used medium for walnut tissue culture (Cornu and Jay-Allemand, 1989; McGranahan et al., 1987; McGranahan and Leslie, 1987; Payghamzadeh, 2008). However, several researchers used MS medium for culturing Persian walnut and reported successful results (Revilla et al., 1989; Penuela et al., 1988; Kornova et al., 1993; Gruselle and Boxus, 1990; Payghamzadeh, 2008). Saadata and Hennerty (2002) indicated that Persian walnut needs a high salt medium for shoot multiplication and DKW medium was therefore a suitable medium. DKW is a relatively high

salt medium resembling MS in its nitrogen content, but also containing high concentrations of several other ions. In contrast to DKW and MS media, WPM is a low salt medium and was a poor medium for walnut tissue culture. Saadat and Hennerty (2002) reported no significant difference in shoot or callus fresh weight and main shoot length of explants between DKW and MS media, but both of them were significantly better than WPM. This confirms the results of Driver and Kuniyuki (1984), who reported that DKW medium was superior to WPM for micropropagation of paradox walnut and Heile-Sudholt et al. (1986), for black walnut who reported that axillary shoots were significantly greater on DKW than on WPM, and micro-shoots on DKW were thinner, had more leaf expansion and were greener than those on WPM.

In comparison to WPM, MS basal medium has a very high nitrate concentration. This may have placed additional metabolic stress on the cultures. Many components of MS medium, not present in WPM, have also been reported to induce stress in plant cells. The inclusion of cobalt (and lack of nickel) in MS basal medium has been reported to contribute to leaf damage and metabolic stress in tissue cultured potato plants (Witte et al., 2002). WPM contains neither nickel nor cobalt. MS medium also contains potassium iodide (KI), whereas WPM does not include a source of iodine. KI has previously been shown to be toxic to goldenweed (*Haplopappus gracilis*) cells when cultured under dark conditions (Eriksson, 1965). Payghamzadeh and Kazemitabar (2008a) compared the effects of MT medium with modified DKW basal medium on walnut micropropagation. They reported that, percentage embryo germination and shoot proliferation in modified DKW medium was more than MT medium culture. But root proliferation in MT medium was more than DKW. The Instituto Murciano de Investigacióny Desarrollo Agrarioy alimentario (IMIDA) fruticulture team designed a new medium for walnut tissues culture (NGE) (Sanchez-Zamora et al., 2006) and determined the best culture medium for *in vitro* embryo germination. They compared DKW, WPM, and NGE medium. The best result after 35 days was 81% using the WPM medium, with significant statistical differences when compared with the other media. Little difference was found between the NGE and DKW media (62 and 54%, respectively) and the worst result was achieved with MS (27%). Looking at these results, we can see the big influence of the culture medium on the *in vitro* germination of mature embryos. A large majority of the embryos evolved into complete plants, WPM being the medium where the highest number of plants was obtained (60%), followed by NGE and DKW, with 44 and 45%, respectively. The NGE and WPM cultures were the only ones where plants appeared with stems but not roots. Culturing juvenile *J. cinerea* nodal explants on WPM or DKW media, regardless of growth regulators tested, resulted in the swelling and partial expansion of axillary buds, but with no significant elongation. In contrast, DKW medium has proven to be suitable (and in many cases superior) for the culture of *J. regia* as well as other *Juglans* species (Driver and Kuniyuki, 1984; Heile-Sudholt et al., 1986; Lee et al., 1986; McGranahan et al., 1988; Leslie and McGranahan, 1992).

Carbohydrates

Sucrose (3%) was commonly used as a source of carbohydrate. In general, for tissue culture, Murashige and Skoog (1962) stated that the use of 3% sucrose is better than 2 or 4%. In walnut, researchers used (3%) of Murashige and Skoog (1962) from the recommended concentration.

Growth regulators

Table 3 summarises the plant growth regulators (PGRs) used for *in vitro* propagation of explants, Table 4 shows those used for embryo culture while Table 5 displays PGRs used for embryogensis. Inclusion of BAP and gibberellic acid (GA_3) in the culture medium was essential for bud proliferation and embryo germination of walnut, respectively. In our study, inclusion of 2 mg l^{-1} GA_3 enhanced embryo germination and shoot proliferation (Payghamzadeh, 2008; Payghamzadeh and Kazemitabar, 2008a). Also, inclusion of 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2, 4-D) in combination with 1 mg l^{-1} BAP was best performed medium for somatic embryogenesis in walnut (Payghamzadeh, 2008; Payghamzadeh and Kazemitabar, 2010b). In another work, results indicated that 8.9 µM BAP was best concentration for lateral bud induction (Payghamzadeh and Kazemitabar, 2008b).

Status of the medium

The commonly used agent is the solidifying agent to *in vitro* propagation of walnut listed in Table 6. For plant tissue culture media, both agar and phytagel, which are natural polysaccharides with a high capacity for gelation, are commonly used as gelling agents. Agar is most frequently used because of its desirable characteristics such as clarity, stability and its inertness (Ibrahim, 1994). However, phytagel, the alternative gelling agent, is increasingly being used because it forms a relatively clear gel and contains no contaminants (Pierik, 1987). Although agar is used routinely to solidify most media, Gelrite has been used as a solidifying agent for DKW medium. Some users of DKW medium have avoided Gelrite because of its reputation for promoting vitrification, but Leslie and McGranahan (1992) claimed that it was not a problem as long as the culture vessels were not sealed tightly. They reported that micro-propagated walnut shoots on agar solidified DKW medium showed

Table 4. Commonly used hormones, mediums and explants for walnut embryo culture.

Species/cultivar	Plant growth regulator								Study	Medium	Explant	References
	BAP	Kn	TDZ	2,4-D	IBA	IAA	NAA	GA_3				
J. regia L.	1 mg/l	-	-	-	0 - 0.1-0.05 mg/l	-	-	-	Micro propagation	DKW	If	Payghamzadeh (2008)
J. regia L., a local cultivar.	-	-	-	-	-	-	-	2 mg/l	Micro propagation	DKW, Mt	Me	Payghamzadeh and Kazemitabar (2008c)
J. regia L.	0.5 mg/l	0.5 mg/l	-	-	-	-	-	2 mg/l	Embryo culture	MS	Me	Kaur et al. (2006)
J. regia L.	-	0.3 mg/l	-	1 mg/l	2 mg/l	-	2 mg/l	-	Modulation and morphogenic; callus induction	MS or K(h)	Ea	Fernandez et al. (2000)
J. regia L.	0.5 - 2 mg/l	-	-	-	-	-	0.1 - 0.5 mg/l	0.1 - 0.5 mg/l	Embryo culture	MS	Me	Liu and Han (1986)
J. regia L.	4.44 µM	-	-	-	-	-	-	-	Micro propagation	DKW	E	Jay-Allemand et al. (1992)
J. regia L.	0.1 - 1 mg/l	-	-	-	-	-	-	-	Axillary bud proliferation	MS	Ea	Fernandez et al. (2000)
J. regia L.	1 mg/l	-	-	-	-	-	-	-	Embryo proliferation	1/2 Knop, Miller	E	Jay-Allemand (1982)
J. regia L. cv su-2	-	-	-	-	-	-	-	9 mg/l	Germination of desiccated somatic embryos	DKW	Dse	Dumanoglu (2000)
J. regia L. cv Plemiana 1, Plemiana 2	4.44, 2.22 µM	-	-	-	0.005 µM	-	-	-	Micro propagation	DKW	E	Scaltsoyiannes et al. (1997)
J. regia L. rootstock cv, Perlata	0.5 mg/l	-	-	-	0.1 mg/l	-	-	-	Embryo germination	NGE, DKW, WPM	Me	Sanchez-Zamora et al. (2006)
J. regia L.	1 mg/l	-		-	0.1 mg/l	0.05 mg/l	-	0.1 mg/l	Micro propagation	MS	Ea	Revilla et al. (1989)

Me, Mature embryo; Ea, embryonic axes; E, embryo; Dse, desiccated somatic embryo; If, immature fruit; Ie = immature embryos.

reduced growth rates and some problems with chlorosis. Cornu and Jay-Allemand (1989) compared two kinds of solidifying compounds for shoot multiplication of walnut. They reported that after two transfers, shoot elongation and callus formation of hybrid walnut increased significantly in Gelrite. Barbas et al. (1993a) reported that gelling agents affected the growth of *in vitro* cultured walnut shoots. Gelrite promoted shoot elongation and bud production, whereas Difco Bacto agar inhibited growth and led to fully expanded leaves, but the formation of new leaves was limited. On Gelrite solidified medium, the leaves were smaller, bright green in colour and new leaves were formed regularly. Using Phytagel as the solidifying agent gave significantly greater callus and shoot fresh weight, main shoot length and leaf number on shoots than when the medium

Table 5. Commonly used hormones, medium and explant for embryogenesis.

Species/cultivar	Plant growth regulator								Medium	Explant	References
	BAP	Kn	TDZ	2-4-D	IBA	IAA	NAA	GA_3			
J. regia L	1 mg/l	2 mg/l	-	-	0.01 mg/l	-	-	-	DKW	Ic	San and Hatic (2006)
J. regia L.	4.4 µM	9.3 or 1.1 µM	-	9.1 µM	0.05 µM	-	-	-	DKW or MS	Ic	Pijut (1993a)
J. nigra L	-	-	5 µM	0.1 µM	-	-	-	-	WPM	C	Neuman et al. (1993)
J. regia L. cv Manregian	4.4 µM	9.3 or 1.1 µM	-	9.1 µM	0.05 µM	-	-	-	DKW or MS	Et	Tuleke et al. (1988)
J. regia L.	1 mg/l	2 mg/l	-	-	0.01 mg/l	-	-	-	MS	Ic	Boltivets and Piven (1990)
J. nigra L	-	-	5 µM	0.1 µM	-	-	-	-	DKW	Ic	Steger et al. (2003)
J. nigra L	1 mg/l	2 mg/l	-	-	0.01 mg/l	-	-	-	LP	Ic	Steger et al. (2003)

Ic, Immature cotyledons; C, cotyledons; Et, endosperm tissues; Ea, embryonic axes.

Table 6. Commonly used agents as the solidifying agent for *in vitro* propagation of walnut.

Species/cultivar	Type of gelling agent	Concentration	Medium	pH	Study	References
J. cinerea L.	Phytagel[1]	0.22% (w/v)	MS	5.7	Axillary bud culture	Pijut (1997)
J. cinerea L	Phytagel	0.22% (w/v)	1/2 MS	5.7	Rooting of microshooting	Pijut (1997)
J. cinerea L	Gelrite (Merck Co) or Difco-bacto agar	0.24% (w/v), 0.7% (w/v) respectively	MS or DKW	5.7	Somatic embryogenesis	Pijut (1993a)
J. regia L	Gelrite (Merck Co)	0.21% (w/v)	DKW	5.7	Somatic embryogenesis	San and Hatic (2006)
J. regia L	Agar (India, Himedia, Mumbai)@	6 g/l	MS	5.8	Embryo culture	Kaur et al. (2006)
J. regia L	Difco-bacto agar	8 g/l	MS, DKW, WPM, 1/2(DKW+WPM)	-	Micropropagation and adventitious shoot induction	Roschke and Pijut (2006)
J. regia L	Difco-bacto agar	0.7%	MS, K(h)	5.8	Modulation and morphogenic study on embryonic axes	Fernandez et al. (2000)
J. regia L. clone RG1	Roland agar	7.5 g/l	DKW	-	Axillary bud proliferation	Heloir et al. (1996)
J. regia L. rootstock cv. Perlata	Difco-bacto agar	7 g/l	WPM, DKW, NGE	5.7	Embryo germination and proliferation	Sanchez-Zamora et al. (2006)

[1]Sigma Chemical Co, St, Louis, Missouri.

was solidified with Difco Bacto agar (Saadat and Hennerty, 2002), and confirms the results reported by Barbas et al. (1993b), Cornu and Jay-Allemand (1989), Nairn et al. (1995) and Pasqualetto et al. (1988). Barbas et al. (1993) reported that the two gelling agents resulted in major differences in mineral contents. Gelrite contained a higher amount of Ca, Mg, K and Fe than Difco Bacto agar, but agar contained more Na than Gelrite and media solidified with agar contained threefold higher Na levels than media solidified with Gelrite. In addition, a significant accumulation of Na was found in the leaves of the explants cultured on media solidified with agar. In callus samples of media solidified with agar, the level of hydrojuglone

Table 7. Salts composition (mg/l) of each culture medium.

Component	MS	DKW	WPM	NGE	B_5	LP
NH_4NO_3	1650	1416	400	908	-	908
KNO_3	1900	-	-	723	2500	-
$Ca(NO_3)_2.4H_2O$	-	1968	556	2248	-	1262
$CaCl_2.2H_2O$	440	149	96	699	150	122.2
K_2SO_4	-	1559	990	-	-	1274.5
$MgSO_4.7H_2O$	370	740	370	2053	250	555
$(NH_4)_2SO_4$	-	-	-	-	134	-
$MnS0_4.H_2O$	-	-	-	-	10	27.9
$NaH_2PO_4.H_2O$	-	-	-	-	150	-
KH_2PO_4	170	265	170	155	-	217.5
$MnSO_4.4H_2O$	22.3	33.5	22.3	22.3	-	-
$Na_2MoO_4.2H_2O$	0.25	0.39	0.25	0.25	0.25	0.32
$ZnSO_4.7H_2O$	8.6	-	8.6	8.6	2	4.3
$Zn(NO_3)_2.6H_2O$	-	17	-	-	-	8.5
KI	0.83	-	-	0.83	0.75	-
H_3BO_3	6.2	4.8	6.2	6.2	3	5.5
$CuSO_4.5H_2O$	0.025	0.25	0.25	0.025	0.025	0.25
$CoCl.6H_2O$	0.025	-	-	0.025	0.025	-
$NiSO_4.6H_2O$	-	0.005	-	-	-	-
$FeSO_4.7H_2O$	27.8	33.8	27.8	27.8	-	30.8
$Na_2EDTA.2H_2O$	37.3	45.4	37.3	37.3	37.3	41.35
Myo-inositol	100	100	100	100	100	100
Thiamin-HCL	0.1	2	1	0.1	10	1.5
Nicotinic acid	0.5	1	0.5	0.5	1	0.75
Pyridoxine-HCL	0.5	-	0.5	0.5	1	0.25
Glycine	2	2	2	2	-	2
Glutamine	-	-	2	-	-	-
Sucrose	30000	30000	30000	30000	20000	30000

MS, Murashige and Skoog (1969); DKW, Driver and Kuniyuki walnut medium (1984); WPM, Woody plant medium (Lloyd and McCown, 1980); NGE, Sanchez-Zamora et al. (2006); B_5- Gamborg et al. (1974); LP, Preece et al. (1994).

glucoside, a marker of juvenility in walnut, decreased drastically. They concluded that lack of growth, mature foliar morphology, sodium accumulation and hydrojuglone glucoside decline supported the hypothesis that agar accelerated the ageing of *in vitro* cultured walnut explants and could not be recommended for shoot multiplication of Persian walnut (Barbas et al., 1993). Liquid cultures, without any gelation agent can give a better response to a better contact between explants and the liquid medium which increases the availability of cytokinins and other nutrients, and dilution of any exudates from the explants (Payghamzadeh and Kazemitabar, 2008b). Further, the successful establishment of cultures in liquid medium has several other advantages and is an important step towards automation. Further, elimination of agar in the liquid medium reduces the cost.

Physical factors

Light

Table 8 summarizes the physical parameters of culture conditions used in studies of walnut. Perusal of the literature indicates that light intensity plays an important role in satisfactory shoot growth. One to 3000 lux (ca. 40 - 92 µmol/m^2/s) is reported to be sufficient for embryo culture, axillary bud culture and shoot proliferation in walnut (Jay-Allemand et al., 1992; Kaur et al., 2006; Long et al., 1995; Roschke and Pijut., 2006; Paygamzedeh, 2008). Capellades et al. (1990) however, opined that cultured plantlets could resemble greenhouse grown plants if these were cultured at a higher light intensity (80 µmol $m^{-2}s^{-1}$) than that normally available inside the

Table 8. Physical condition required for *in vitro* culture of walnut.

Species/cultivar	Light density	Instrument	Photo period	Temperature	Medium	Study	References
J. cinerea L	92 µmol/m^2/s	Cool white fluorcent lamp	18 h	26°C	MS	Axillary bud culture	Pijut (1997)
J. regia L	55 - 65 µ$Em^{-2}s^{-1}$	Cool white fluorcent lamp	16 h	27±1°C	DKW	Embryo culture and shoot multiplication	Jay-Allemand et al. (1992)
J. cinerea L	92 µmol/m^2/s	Cool white fluorcent lamp	18 h	26°C	1/2 MS	Rooting of microshoots	Pijut (1997)
J. cinerea L	-	-	-	26°C	DKW or MS	Somatic embryogenesis	Pijut (1993a)
J. regia L	-	-	-	25°C	DKW	Somatic embryogenesis	San and Hatic (2006)
J. regia L	3000 Lux	-	16.8 h	22°C	MS	Embryo culture	Kaur et al. (2006)
J. regia L	80 µ$molm^{-2}s^{-1}$	-	16 h	24±2°C	MS, DKW, WPM, 1/2(WPM+DKW)	Adventitious shoot induction	Roscke and Pijut (2006)
J. regia L	40 µ$molm^{-2}s^{-1}$	Cool white fluorcent lamp	16 h	25°C	1/2 K(h), MS	Modulation and morphogenic study on embryonic axes	Fernandez et al. (2000)
J. regia L. cv. Plemania1, Plemania2	55 - 65 µ$Em^{-2}s^{-1}$	Cool white fluorcent lamp	16 h for first six days, 8 h for remaining days	24±1°C for first six days, 21±1°C for reminded days	DKW (1/4 (macro nutrient)	Root induction	Scaltsoyiannes et al. (1997)
J. regia L. clone RG1	312 $molm^{-2}s^{-1}$	Sylvania Grolux fluorcent lam	16 h	Day/night respectively 28/25°C	DKW	Shoot bud proliferation	Heloir et al. (1996)
J. regia L. cv. Plemania1, Plemania2	55 - 65 µ$Em^{-2}s^{-1}$	Cool white fluorcent lamp	16 h	27±1°C	DKW	Somatic embryogenesis and shoot multiplication	Scaltsoyiannes et al. (1997)
J. regia L. rootstock cv. Perlata	5000 lx	Philips TLD 58w/54	16 h	25±1°C	DKW, WPM, NGE	Embryo germination and proliferation	Sanchez-Zamora et al. (2006)
J. regia L	-	-	-	25°C	DKW	Maturation and germination of somatic embryos	Deng and Cornu (1992)

culture vessels (25 µmol $m^{-2}s^{-1}$). Usually a 16 - 18 h photoperiod from Sylvania Grolux type white fluorescent lamps or cool white fluorescent lamps is provided in culture conditions (Sanchez-Zamora et al., 2006; Heloir et al., 1996; Scaltsoyiannes et al., 1997; Long et al., 1995; Roschke and Pijut, 2006; Payghamzadeh and Kazemitabar, 2008a, b, c and d). In general, in walnut tissue culture, 16 - 18 h illumination resulted in a normal growth, multiplication and germination rate.

Temperature

For somatic embryogenesis, embryo culture and optimal shoot or root formation and proliferation, the range 22 - 28°C is the best temperature, as summarized in Table 8. For instance, Kaur et al. (2006) used 22°C throughout optimization of medium for embryo culture. However, San and Hatic (2006) used 25°C for somatic embryogenesis and Fernandez et al. (2000) used 26°C for modulation and morphogenic study on embryonic axes via embryo culture technique. Long et al. (1995) used 25°C for somatic embryogenesis and adventitious shoots in J. nigra L. In our work, we used 25°C for callus induction from selected

explant from original plant and embryos of walnut (Payghamzadeh, 2008; Payghamzadeh and Kazemitabar, 2010a, 2010b). Others have used different temperatures for different stages in tissue culture. Scaltsoyiannes et al. (1997) incubated at 24±1°C for the first six days and 21±1°C for the reminder of the time in root induction phase.

ROOTING OF MICROSHOOTS

For any propagation method, successful rooting of microshoots is a pre-requisite to facilitate their establishment in soil. Considerable work has been done to enhance rooting efficiency in different walnut varieties. Rooting of microshoots can be accomplished both under *in vitro* and *ex vitro* conditions.

In vitro rooting of microshoots

Walnut plants have been clonally propagated from tissue culture derived microshoots. The *in vitro* rooting capacity depends on the interaction of internal and external factors (Hyndman et al., 1982a; Scaltsoyiannes et al., 1997). As is well known, genotype plays a major role in all phases of vegetative propagation. The same authors noticed that the pretreatment of the hybrid microcuttings (*J. nigra* × *J. regia*) with 24.6 µM IBA for 5 days in darkness are essential for rooting induction. Scaltsoyiannes et al. (1997) found that 6-day pretreatment with the same concentration of IBA enhanced subsequent rooting. The level of endogenous hormones and peroxidase activity proved to be useful predictive markers of the rooting performance (Gaspar et al., 1992, 1994) of micropropagated shoots. Leslie and McGranahan (1992) reported that the highest rooting frequency (75%) occurred on microshoots placed on half-strength MS containing 2.5 µM IBA for 7 days in darkness. Adventitious roots began to emerge within 7 days and elongated when microshoots were transferred to the light. Although roots were also initiated on microshoots cultured on media containing 4.9 or 24.6 µM IBA, only the 2.5 µM IBA-rooted plantlets were successfully acclimatized *ex vitro*. *In vitro* and *ex vitro* rooting of micropropagated *Juglans* L. species has been successful using IBA with or without NAA.

Inorganic salts

Relatively low salt concentrations in the medium are known to enhance rooting of microshoots (Murashige, 1979). Pijut (1997) used ½ MS medium for rooting microcuttings. Scaltsoyiannes et al. (1997) used DKW basal medium with ¼ concentrations of macroelement for root induction on shoots. Further, when the NO_3/NH_4 ratio increased from0.1 to 3.0, there was an increase in the number of roots per explant. Hyndman et al. (1982a) demonstrated that a decrease in KNO_3 and NH_4NO_3 concentration was the decisive factor for improving the rooting percentage. Long et al. (1995) showed that for rooting of shoots, 1 week on DKW basal medium with a reduced amount of nitrogen (456.2 mg/L NH_4NO_3 and 634.0 mg/L Ca $(NO_3)_2$), high sugar concentration (Driver and Suttle 1987) are required.

Carbohydrates and medium cultures

Sucrose acts as an enhancer of osmotic potential and also plays a vital role in root induction. Rout et al. (1990) reported that rooting of microshoots was better on a solid medium compared to the liquid medium. Damiano et al. (1989) observed enhancement of *in vitro* rooting by using a double layered medium, that is, agargelled medium with an upper layer of liquid medium. Revilla et al. (1989) reported that the establishment of the explants in double-phase cultures increased the micropropagation rate for both types of plant material (embryos and juvenile materials). Similar results were observed for juvenile material when the plantlets in the greenhouse were stimulated with growth regulator solutions. Revilla et al. (1989) reported that the use of treatments, double-phase and plant growth regulator stimulation, will improve the proliferation rate in walnut. Sixty percent of the shoots regenerated from embryonic material produced roots. Similar rooting conditions were used by Meynier (1985) for hybrid walnut. It was further observed that root length was short in solid medium as compared to liquid medium (Ebrahim and Ibrahim, 2000). However, Scaltsoyiannes et al. (1997) achieved 95% rooting in several cultivars of walnut by using DKW medium devoid of growth regulators but supplemented with 40 g/l sucrose. Long et al. (1995), for rooting of shoots used a high concenteration of sucrose (52.64 g/L sucrose). However, beyond 3% sucrose, browning of root tips occurred. The same report indicated that no pigmentation was observed in the presence of sorbitol which was used as an osmoticum. This indicates that the darkening of the roots is due to the presence of sucrose as carbohydrate not as osmoticum.

Activated charcoal

Activated charcoal when added to the culture medium was found to have a remarkable positive influence on the rooting efficiency of cultured walnut, wherein better rooting in terms of number of roots/shoot and root length was obtained. Moreover, addition of activated charcoal was found to reduce the days required for root initiation. Revilla et al. (1989) found that rooting was initiated after dipping the shoot base into a liquid medium containing 2 mg/1 IBA for 24 h then transferring onto a solidified medium containing 1% activated charcoal. Also, this agent, affected sorption of polyphenols exuded from the cut surface of the explants. Payghamzadeh and Kazemitabar (2008b) in order to decrease the harmful

effects of exuded polyphenolic materials from the basal end of explants into the medium, two kind of DKW medium cultures were used such as mono phase medium supplemented with different concentrations of BAP and double phase medium of which the lower phase was supplemented with activated charcoal without plant growth regulator while upper phase was liquid basal medium supplemented with different concentration of BAP without activated charcoal. Results indicated that between two kind of medium cultures, the double phase medium culture was better than any other medium.

Growth regulators

In most of the earlier reports, a range of concentrations of different auxins were used for root induction (Table 3). The rooting procedure consisted of two phases: i) root induction phase: microshoots (4 - 5 cm) were established on DKW medium (¼ macroelements) supplemented with 24.6 µM IBA and 40 $g.1^{-1}$ sucrose (Jay-Allemand et al., 1992) and were kept in darkness for 6 days at 24±1°C for 16 h and 21±1°C for the remainig 8 h and ii) initiation phase: the pretreated shoots were transferred to sterilized vermiculite in which gelified DKW medium (¼ macroelements) free of hormones, was added.

Physical factors

Light and tempreture

There are only a few reports on the role of culture environment such as light and temperature on walnut rooting. For perusal of literatures published about effect of light and tempreture on walnut.

Size and type of vermiculite

Scaltsoyiannes et al (1997), microshoots (4 cm to 5 cm) of the clones (P3, P7) after the induction phase were transferred to the rooting medium using two size-types of vermiculite (medium-type I and small type II). In each treatment, 20 microshoots were used. Between the two size-types of vermiculite (medium-type I and small-type II) that were tested, the medium size promoted both microshoot rooting percentage and the quality of the root system. Concerning the rooting substrate, the positive effect of the medium size vermiculite in rooting was, also, found by Jay-Allemand et al. (1992), who stated that this is probably due to better aeration of the rooting system achieved by this type of vermiculite.

COMPARISON OF *IN VITRO* AND *EX VITRO* ROOTING

A comparison of *in vitro* and *ex vitro* rooting has been made by McGranahan et al. (2006) in different genotype of walnut. They produced over 2300 plantlets for greenhouse screens and 1200 nursery row sized trees for field trials during 2006. These plants were clonally propagated from tissue culture derived microshoots. As a result of devising an *ex vitro* method of rooting micro-shoots in greenhouse fog chambers, survival of rooted microshoots was improved to 80% for *ex vitro* rooted plantlets as compared to 50% for *in vitro* rooted ones of the same genotypes. *Ex vitro* rooted plantlets grow faster in the greenhouse and are ready for dormancy induction sooner. The general method for multiplication, rooting, acclimatization and greenhouse growth of *in vitro* tissue culture derived plants has been described in the Walnut Research Reports for 2001 through 2004. The *ex vitro* rooted plantlets survived than *in vitro* rooted plantlets. *Ex vitro* rooted plantlets also grew faster and were large enough for dormancy breaking procedures in a shorter time than *in vitro* rooted plantlets; rooting percentage is about the same for *in vitro* and *ex vitro* rooted plantlets but some experimental *ex vitro* treatments show potential for substantially increasing rooting percentage (McGranahan et al., 2006). Scaltsoyiannes et al. (1997) achieved 5 - 95% *in vitro* rooting in *J. regia* L. Pijut (1997) also achieved 75% *in vitro* rooting in *J. cinerea* L. Carpenter (1975), reported 60 - 70% rooting of hard wood cutting taken from mature black walnut trees (*J. regia* L.). Gautam and Chauham (1990) reported dormant cutting taken from 4-to-5-year-old hedges of *J. regia* rooted (14.5%) when treated with 74 mM IBA.

ACCLIMATIZATION AND FIELD ESTABLISHMENT

The successful acclimatization of micropropagated plants and their subsequent transfer to the field is a crucial step for commercial exploitation of *in vitro* technology. However, the acclimatization of micropropagated walnut was reported to be a difficult procedure because of rapid desiccation of plantlets or their susceptibility to diseases due to high humidity and difficult rooting. Preece and Sutter (1991) and Sutter et al. (1992) have reviewed acclimatization of micropropagated plants in the green-house and in the open field which consisted of cellulose plug (sorbarods), for support and protecting the roots during transfer to soil, and ventilated culture vessel to improve the resistance of the plantlets to desiccation. The plantlets thus grown showed better survival when transferred to *ex vitro* conditions. Sorbarods provided the necessary protection and facility for proper handling of the microshoots and helped in the production of larger roots, which facilitated 100% successful establishment. In general, acclimatization and greenhouse growth of *in vitro* tissue culture derived plants of walnut has been described in the Walnut Research Reports.

PLANT REGENERATION

In principle, it is possible to regenerated whole plants from protoplasts, single cells or small pieces of tissue

because plant cells are totipotent. Regeneration can be performed via differentiation of callus or explants, or via somatic embryogenesis. However, the critical step for successful micropropagation is frequently the development of an efficient *in vitro* regeneration system. In walnut, there are many reports which indicate rapid regeneration and multiplication through organogenesis, somatic embryogenesis or embryo culture (Tables 3, 4, 5).

Organogenesis

Organogenesis in the embryonic axis of J. regia was inhibited by 2, 4-D as occurred with other species (Yeoman and Forch, 1980). Cytikinins and auxins allowed shoot and root development but it was dependent on both the type and concentration of auxin in the culture medium Fernandez et al. (2000) investigated the effect of cultural conditions such as basal medium, auxin/cytokinin ratio and photoperiod on organogenesis, callus tissue induction, cell suspensions and bud proliferation in embryonic axes of J. regia cultured in vitro. They reported that the shoot length decreased with NAA concentrations higher than 1 mgl^{-1} and with IBA at 8 mgl^{-1}. Conversely, lower auxin concentrations did not yield shoot lengths significantly different from the control. The embryonic axis developed one root in the absence of auxins, which could reach up to 50 mm in length. NAA or IBA concentrations higher than 2 mg/l induced root proliferation, and the length of roots decreased with increasing auxin concentration. IBA was more effective in promoting rooting than NAA. The formation of secondary roots occurred only when IBA at 1 or 2 mg/l was added into the culture medium. The morphological aspect of roots changed with the auxin employed: IBA induced long and thin roots and with NAA they were shorter and thicker. For this reason, the fresh weight of roots did not show significant differences between these treatments. Basal bud development was inhibited with all NAA concentrations assayed. Roschke and Pijut (2006) reported that of the six genotypes of J. nigra L. that were cultured on various combinations of thidiazuron (TDZ) and IBA, three genotypes (D, E, F) showed adventitious shoot regeneration. On 6.8 µM TDZ plus 1.0 µM IBA, genotype D regenerated shoots on 60% of the explants; being the highest rate for all genotypes. Genotype F showed 10% shoot regeneration on the same medium as well as on 6.8 µM TDZ plus 0 µM IBA. This was also the only plant growth regulator combination on which genotype E responded, regenerating shoots on 10% of the explants. According to Christianson and Warnick (1987), shoot organogenesis follows three physiological stages: acquisition of competence for induction (ability to respond to the inducer), induction (changing the fate of the tissue), and morphological differentiation and growth. Organogenesis has been previously reported in J. nigra by Cornu (1988) where he described somatic embryogenesis but not organogenesis. Neuman et al. (1993) did not report shoot organogenesis in their experiments in which they placed immature cotyledonary explants on WPM with 2, 4-D and TDZ from 8 to 14 weeks after anthesis.

Somatic embryogenesis

Somatic embryogenesis is the foundation of genetic transformation in several economically important tree species. Somatic embryogenesis has been induced in a number of species of Juglans (Tulecke et al., 1995). Immature cotyledons have been used for somatic embryo induction in walnut cultivars and the medium was supplemented with varied concentrations of PGRs. The immature fruits collected at 8 - 12 weeks after anthesis were best for explants. For example, Tulecke and McGranahan (1985) reported that the optimum stage of cotyledon development for the induction of somatic embryos was 6 - 11 weeks after pollination, the cotyledon pieces that were embryogenic ranged from 10 - 44%, and the number produced per cotyledon piece varied from 0 - 26. Pijut (1993) suggested that the highest frequency (42 - 56%) of cotyledon stage somatic embryo development in butternut was found in explants collected 9 or 8 weeks postanthesis. Pijut (1997) reported for somatic embryogenesis, that immature zygotic embryos should be excised 8 - 11 weeks postanthesis, cotyledon segments cultured on induction medium for 3 weeks and then transfered to a plant growth regulator-free development medium. Direct somatic embryos formed on explants cultured on DKW supplemented with 250 mg/1 L-glutamine, 0.05 µM IBA, 4.4 µM BA, and 9.3 µM kinetin. Embryogenic callus formed on explants cultured on DKW or MS media supplemented with 1.1 µM BA and 9.1 µM 2, 4-D. All cultures were incubated in darkness at 26°C. Somatic embryos continued to develop when cultures were transferred every 3 weeks to fresh development medium. In our study, Results indicated that the highest embryonic cotyledons were achieved in 8 week postanthesis. Also the highest embryonic cotyledons were achieved in medium containing 2 mgl^{-1} 2, 4-D and 1 mgl^{-1} BAP (Payghamzadeh and Kazemitabar, 2008d) (Figure 3a). In general, other works and commonly used hormones, mediums and explants for somatic embryogenesis are listed in Table 5.

Embryo culture

In vitro embryo culture is a widely used tool in the genetic breeding of plant species (Ramming, 1990; Cossio and Minotta, 1983). Embryo culture is one of the most effective *in vitro* culture methods, allowing attainment of hybrids from reduced breeding cycle of many plants (Raghavan 1977, 1980). Several factors influenced walnut embryo germination such as plant growth regulators,

Figure 4. Step by step schematics of the embryo culture of walnut. 1, Mature fruits as explants; 2, washing under running tap water for 10 min; 3, explants treated with bleach solutions for 10 min; 4, washing under running tap water for 10 min; 5, explants treated with 50 - 70% (v/v) ethanol for 20 – 30 s followed by 0.1 - 15% (v/v) sodium hypochorite containing 0.01% Tween 20 for 10 - 20 min; 6, after that samples were rinsed three 5-times with sterile distilled water in aseptic condition (step 7); 7, a lamin air flow hood for tissues culture; 8, fruits were cracked by piercing a pointed forceps; 9, excise embryos with few cotyledons tissues; 10, the excised embryos were cultured in basal medium; 11, *In vitro* germinated walnut embryos; 12, acclimization *in vitro* germinated walnut embryos.

medium cultures, physical factors etc. The BAP and IBA have a positive influence on embryos germination in all cultivars. High percentage embryos germination was obtained in 1 and 1.5 mg l^{-1} BAP ($P < 0.01$) and 0.05 and 0.1 mg l^{-1} IBA ($P < 0.01$). Treated immature embryos as compared to untreated ones, suggesting improvement in germination of embryos was strongly influenced by concentrations of BAP and IBA (Payghamzadeh and Kazemitabar, 2010a). In other words, significant differences were observed between different medium cultures, GA_3 concentrations and physical factors. Percentage embryo germination and main shoot length was more in modified DKW medium, cold dark culture condition and 2 mgl^{-1} GA_3 hormone than any other. The MT medium culture and cold dark culture condition was very efficient for root proliferation than modified DKW medium culture and heat light culture condition. In this experiment, GA_3 had a negative effect on root growth, wherein main root length in 0 $mg1^{-1}$ GA_3 was more than 2 mgl^{-1} GA_3. Percentage germination of embryos was higher when GA_3 and cold treatments were simultaneously applied as compared to those when applied separately (Payghamzadeh and Kazemitabar, 2008b,c). Hormones, media and explants used for walnut embryo culture are listed in Table 3. Figure 4 illustrates details of embryo culture technique in walnut (Figures 3b and d).

SUMMARY AND CONCLUSIONS

The clonal propagation of walnut via axillary bud culture, shoot tip culture and rooting of microcuttings and micro-shoots from original plants or other materials are very useful in maintaining walnut cultivars and for the propagation of clones with valuable genotypes. *In vitro* propagation of walnut via somatic embryogenesis offers a great potential for rapid propagation and improvement of walnut. Also this method is very useful for biotechnological methods such as transformation, protoplast fusion and production of cybrids, artificial seeds etc. By this method, there can be production of new cultivars with desirable traits such as high quality wood and fruits, tolerant or resistant to pests, diseases and tolerant to drought, gypsi and salinity soils. Immature cotyledonary tissue of walnut is amenable to somatic embryogenesis in the presence of 2,4-D in combination with other phytohormones. The ability to produce embryogenic cultures was dependent upon the developmental stage of the cotyledon explants. This developmental stage was 8 - 12 weeks postanthesis. Germination of somatic embryos and plantlet survival has been achieved, although at a low frequency. If maturation and germination techniques for somatic embryos could be improved and the number of plantlets increased, the potential exists for genetic improvement and multiplication of walnut. We can shorten the propagation cycle via embryo culture and this technique allows barriers in hybridization to be overcome. Rapid multiplication of elite clones, production of healthy and disease-free plants and faster introduction of novel cultivars with desirable traits are urgently needed in walnut improvement programmes. In this regard, *in vitro* propagation techniques are likely to play a vital role. At present, there are many reproducible protocols for *in vitro* propagation of walnut. However, the new challenges that are faced today by the tissue culture industry include cost efficiency, automation, control and optimization of the micro environment. The recent trend to move from agar-gelled media to liquid media is a strategic step in this direction. A big challenge in walnut studies focuses on genetic improvement of elite trees with desirable treats by transformation systems and identification by molecular markers.

ACKNOWLEDGEMENT

We are grateful to Dr Meriel .G. Jones, University of Liverpool, for advice and revision of an earlier version of this manuscript.

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