Plant Biotechnology 27, 211–215 (2010)

Tissue Culture Note

In vitro propagation of pecan [*Carya illinoinensis* (Wangenh) K. Koch]

Nilima N. Renukdas, Muthusamy Manoharan*, James O. Garner, Jr.

Department of Agriculture, University of Arkansas at Pine Bluff Pine Bluff, AR 71601, USA

* E-mail: manoharanm@uapb.edu Tel: +1-870-575-8543 Fax: +1-870-575-4629

Received August 24, 2009; accepted March 24, 2010 (Edited by M. Otani)

Abstract An efficient method for *in vitro* propagation has been developed for pecan [*Carya illinoinensis* (Wangenh) K. Koch], a highly recalcitrant but commercially important fruit species. Nodal explants from *in vitro* grown cultivars, 'Desirable' and 'Cape Fear', were cultured on modified liquid woody plant medium (WPM) supplemented with 2% glucose and different concentrations of 6-benzylaminopurine (BAP; 0.44–44.39 μM). At least nine multiple shoots per explants were induced on modified WPM containing 13.32 μM BAP after three weeks of culture. The efficiency of shoot induction was over 95% in both 'Cape Fear' and 'Desirable' cultivars. The multiple shoots were proliferated and/or elongated on plant growth regulator free liquid WPM. Subsequently, the multiple shoots were separated and successfully rooted in liquid WPM containing 49.20 μM indole-3-butyric acid. The efficiency of rooting was over 90% in both 'Cape Fear' and 'Desirable' cultivar. The pecan plantlets were initially transferred to peat pellets and subsequently to the greenhouse. This is a simple and efficient protocol that may be used to propagate pecan plants through tissue culture.

Key words: 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), micropropagation.

The pecan [*Carya illinoinensis* (Wangenh.) K. Koch], a member of the family Juglandaceae, is an economically important nut crop. Propagation of pecan is done primarily by budding or grafting of improved cultivars onto seedling rootstocks. However, these methods suffer disadvantages such as considerable time, expense and poor transplanting survival of the plants. Propagation by cutting, though leads to uniform clonal rootstocks, is very difficult as the clones do not root readily. Even with the use of hormones, inconsistent results with only limited improvements have been reported (Brutsch et al. 1977; Smith et al. 1974; Wolstenholme and Allan 1975). *In vitro* propagation offers great potential for the pecan industry for large scale multiplication of selected clones and enables production of a high amount of constant quality plantlets within a very short time. Although somatic embryogenesis has been developed for pecan (Mathews and Wetzstein 1993; Merkle et al. 1987; Wetzstein et al. 1989, 1990; Yates and Reilly 1990), successful pecan micropropagation was not achieved because of poor regeneration and rooting. Wood (1982) and Knox and Smith (1981) have used nodal stem segment explants from seedlings to establish shoot proliferation, however, no consistent shoot proliferation, elongation or root development occurred and no plants were established in soil. Phillips and Ramirez (1983) and Corte-Olivares et al. (1990) used apical and axillary buds; shoot elongation was obtained, but rooting and establishment of plantlets were poor. Hansen and Lazarte (1984) used nodal cuttings as explants; shoots were rooted with 93% success and 63% plants were established in soil while Corte-Olivares et al. (1990) obtained 40% rooting of shoots. In this article, we report the development of a reliable and efficient protocol for rapid *in vitro* shoot induction and rooting of pecan plants by culturing nodal explants from seed in liquid culture. Although micropropagation of pecan through nodal explants from seed will result in heterozygous plants, the protocol optimized in this study could be applied for producing true-type plants from mature tissues.

The unshelled mature seeds of pecan cultivars 'Cape Fear' and 'Desirable' were washed with sterile water for 10 min and then sterilized by immersion in the carbendazim solution (1.0 g l^{-1}; Sigma Chemical Co., St. Louis, MO, USA) overnight. The seeds were washed with sterile water and treated with 70% ethanol for 30 min., again washed with sterile water for three times to remove ethanol. Subsequently, the seeds were treated with a 2.83% (w/v) sodium hypochlorite for 30 min. The seeds were thoroughly rinsed five times with sterile water and aseptically shelled using nut cracker. The embryos were aseptically isolated from the seeds and treated with 2.83% (w/v) sodium hypochlorite solution for 10 min. and rinsed with sterilized water five times for five min

This article can be found at http://www.jspcmb.jp/

each to remove the sodium hypochlorite. The embryos were cultured modified woody plant medium (WPM with no glycine; Lloyd and McCown 1980; McCown and Lloyd 1981) 17.76 μM BAP and 0.49 μM IBA (Obeidy and Smith 1990) for three weeks for culture establishment. The medium was supplemented with sucrose (3% w/v) solidified with phytagel (0.35%, w/v, Sigma Chemical Co., St. Louis, MO, USA). The pH of the media was adjusted to 5.7 prior to autoclaving (121°C for 20 min). The cultures were maintained at 25 ± 2°C with 16 h photo period (40 μmol m^{-2} s^{-1}) provided by white fluorescent lamps. Nodal explants (Figure 1A) were prepared from the shoots of the germinating embryos and cultured on liquid medium for shoot induction [modified WPM containing 1, 2, 3 or 5% sucrose or 1, 2, 3 or 5% glucose (filter-sterilized, added after media sterilization) and supplemented with different concentrations of BAP (0.44, 2.22, 4.44, 13.32, 22.20, 35.52, 44.39 μM)]. Ten milliliter liquid medium with paper bridge was used to culture the nodal explants. The explants were transferred to fresh media after every three weeks of culture. The average number of shoots was assessed after three weeks of culture. The multiple shoots that formed were excised and cultured on plant growth regulator free modified WPM for further elongation. Subsequently, the elongated shoots were tested for rooting in liquid media containing half-strength modified WPM or half-strength MS (Murashige and Skoog 1962) with different concentrations of IBA (4.92, 24.60, 49.20, 98.41, 147.61 and 196.82 μM) as well as various combinations of IBA and NAA (2.46:0.54, 2.46:5.37, 0.49:2.69, 4.92:2.69, 0:0.54, 0:2.69, 0:5.37, 0.49:0, 2.46:0, 4.92:0 μM). The rooted pecan plantlets were transferred to peat pellets for initial acclimatization for two weeks and subsequently to the greenhouse. Each experiment was replicated three times. The data on the number of shoots obtained per explants were analyzed using standard ANOVA procedures.

The biggest limiting factor for successful, large scale pecan *in vitro* propagation is the contamination of cultures by fungus. The presence of internal fungal contamination of mature pecan seeds was reported previously (Beuchat 1975; Chipley and Heaton 1971; Hanlin 1971; Schroeder and Cole 1977). Rigorous surface sterilization procedures could not avoid fungal contamination without disturbing explant viability (Obeidy and Smith 1990; 1993). Therefore, a preliminary study was carried out to find an effective sterilization procedure required for culture maintenance. The seeds treated with either of the following sterilization procedures showed varied degree of fungal contamination (Table 1). 1) 5.65% sodium hypochlorite for 30 min., 2) combination of 2.83% sodium hypochlorite and 2-3 drops of commercial soap solution for 30 min., 3) 95% ethanol 10 min., 4) 0.1% Tween 20 for 2 hr or 5) Carbendazim (1 g^{-1}) for overnight and subsequent treatments with 70% ethanol for 30 min. and 2.83% (w/v) sodium hypochlorite for 10 min. before washing with sterile distilled water three times. Seeds treated with sodium hypochlorite (5.65%) showed 10%

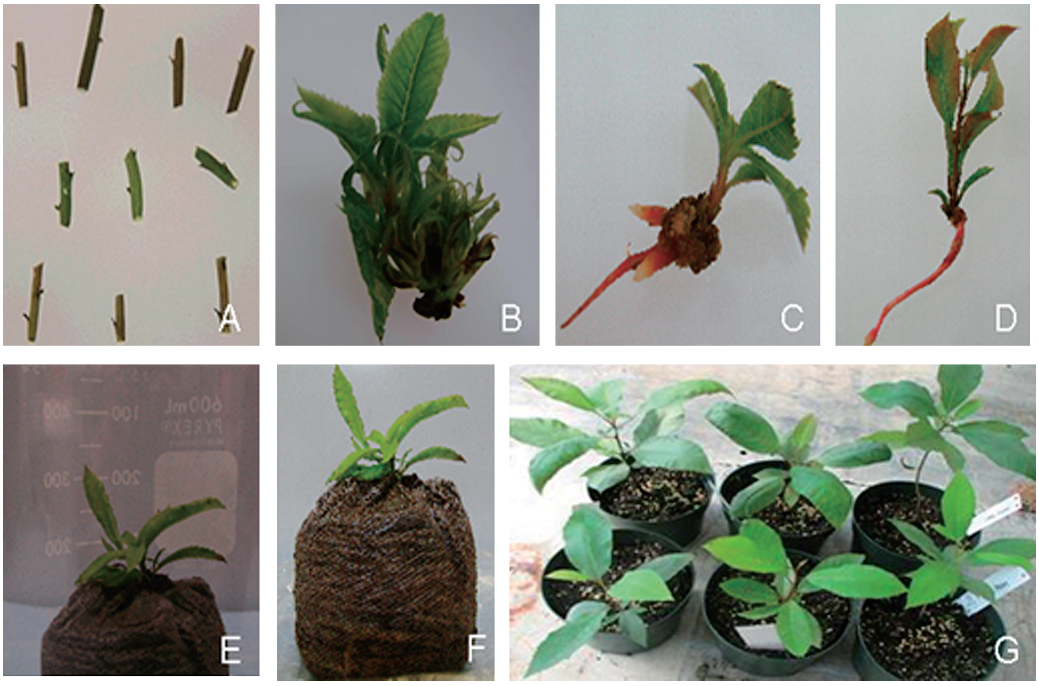

Figure 1. Stages of multiple shoot formation from nodal explants of pecan. (A) Nodal explants used for culture. (B) Multiple shoots formed in modified WPM with 13.32 μM BAP. (C, D). Single shoots showing root formation. (E, F). Hardening of *in vitro* grown plantlet in peat pellet. (G) Fully established plants in greenhouse.

Table 1. Fungal growth (contamination) from the excised embryo cultures of 'Cape Fear' and 'Desirable' cultivars of pecan in different sterilization treatments after one and three weeks of culture

Treatments	% of cultures with fungal contamination			
	'Cape Fear'		'Desirable'	
	1week	3 week	1 week	3 week
Sodium hypochlorite (5.65%)	10	45	10	40
Sodium hypochlorite (2.83%) with 2–3 drops of commercial soap solution	15	50	20	45
95% Ethanol	20	65	25	65
0.1% Tween 20	35	70	35	75
Carbendazim (1 g^{-1}), 70% ethanol and 2.83% sodium hypochlorite	5	20	5	18

Twenty replications per treatment were evaluated. Data for fungal contamination of 'Cape Fear' and 'Desirable' were scored after one and three weeks of culture

Table 2. Effect of BAP on shoot multiplication of pecan

Treatment (BAP μM)	Shoots per explant ± SE	
	'Cape Fear'	'Desirable'
0.44	0.00 ± 0.00	0.00 ± 0.00
2.22	0.00 ± 0.00	0.00 ± 0.00
4.44	1.00 ± 0.00	1.00 ± 0.00
13.32	9.66 ± 0.57	9.33 ± 0.57
22.20	4.33 ± 0.57	4.66 ± 1.00
35.52	2.00 ± 0.00	1.66 ± 0.57
44.39	0.30 ± 0.50	0.66 ± 0.57

Data recorded after three weeks of culture in the liquid medium. Twenty explants were cultured for each treatment with three replicates. Both the cultivars are not significantly different in the multiple shoot induction ($p=0.05$) according to student's *t*-test.

Table 3. Effects of solid (WPM with phytagel) and liquid (WPM without phytagel) media on shoot multiplication of pecan

Media used	No. of explants forming multiple shoots (%)	
	Cape Fear	Desirable
Solid (WPM + 13.32 μM BAP and phytagel)	36.7	38.1
Liquid (WPM + 13.32 μM and without phytagel)	95.2	95.7

Data recorded after three weeks of culture.

fungal contamination in both the cultivars after one week and 40–45% after three weeks of culture. Seeds treated with the combination of sodium hypochlorite (2.83%) and 2–3 drops of commercial soap solution still showed 15–20% fungal contamination after one week and 45–50% after three weeks of culture. Seeds treated with ethanol (95%) showed 20–25% fungal contamination after one week and 65% after three weeks of culture. Seeds treated with only Tween 20 (0.1%) showed 35% fungal contamination after one week and 70–75% after three weeks of culture. However, the seeds treated with Carbendazim (1 g^{-1}) and subsequent treatments with 70% ethanol and 2.83% (w/v) sodium hypochlorite showed only 5% fungal contamination in both the cultivars after one week and 18–20% after three weeks of culture. After three weeks of culture no further fungal growth were observed. The explants remain viable, contaminant-free and started shoot and root initiation after two weeks of culture.

Maximum shoot induction response (95%) was observed in the liquid medium containing 13.32 μM BAP after three weeks of culture (Table 3). Maximum shoot number per explant obtained was 9.66 ± 0.57 in 'Cape Fear' and 9.33 ± 0.57 in 'Desirable' (Table 2; Figure 1B). There were no significant differences between the genotypes and BAP concentrations tested as both the genotypes showed similar pattern of shoot induction. Experiment was also designed to test the effectiveness of solid against liquid media on shoot multiplication. Both solid (modified WPM with phytagel) and liquid media (modified WPM without phytagel) containing 13.32 μM BAP were used to determine shoot induction in pecan. The significant differences were observed in shoot induction response (Table 3) in solid media (36–38% in both 'Cape Fear' and 'Desirable') and liquid media (over 95% in both 'Cape Fear' and 'Desirable'). There was rapid development of shoots in liquid media compared to solid media. Similar results obtained in species cultured in liquid or low-agar concentration media is caused by greater availability of water (Bouniols 1974; Debergh 1983) and of nutrients (Debergh 1983; Singha 1982). This increased availability may be induced by a lower resistance to diffusion and closer contact between the explant and the culture medium (Hammerschlag 1982; Romberger and Tabor 1971; Singha 1982). Although sucrose is the most commonly used energy source for cultures in most of the propagation of woody species, reducing sugars like glucose that are not normally transported in sieve tubes, can be readily taken up and metabolized providing a better carbon source (Welander et al. 1989). In the present study, among the sugar concentrations (1, 2, 3 or 5%) tested, the maximum multiple shoot induction achieved on modified WPM with 2% glucose was 9.66 ± 0.57 shoots per explant in 'Cape Fear' and 9.33 ± 0.57 in 'Desirable' while in 3% sucrose medium it was 4.33 ± 0.57 in 'Cape Fear' and 4.66 ± 0.57 in 'Desirable'. Glucose proved to be an efficient carbon source in culture of *Alnus* species, *Alnus*

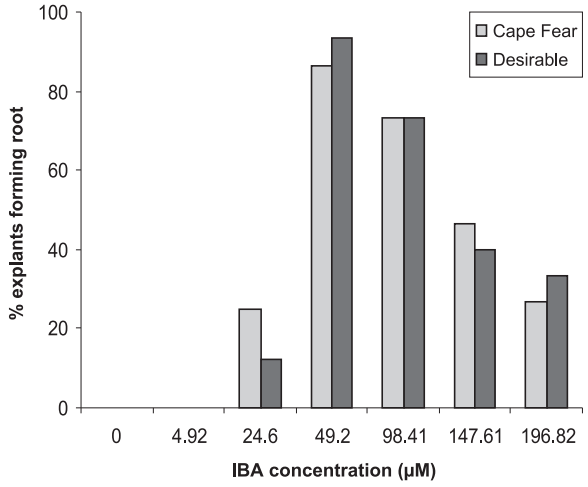

Figure 2. Effect of IBA concentrations (μM), incorporated in liquid WPM, on root formation from *in vitro* regenerated shoots of pecan

crispa, A. cordata and A. rubra (Barghchi 1988; Tremblay and Lalonde 1984; Tremblay et al. 1984; Welander et al. 1989), *Potentilla fruticosa and Ficus lyrata* (Wainwright and Scrace 1989), and cork oak (*Quercus suber*) (Romano et al. 1995).

Root induction was observed in liquid modified WPM supplemented with 49.20 μM IBA after three weeks of culture (Figure 1C, D). Over 90% rooting was observed at 49.20 μM IBA concentration in both 'Cape Fear' and 'Desirable' (Figure 2). Similar results of rooting were obtained by Hansen and Lazarte (1984). However, Corte-Olivares et al. (1990) obtained only 40% rooting and Wood (1982) was unable to establish rooting from pecan shoots. Different combinations of IBA: NAA (2.46 : 0.54, 2.46 : 5.37, 2.46 : 2.69, 4.92 : 2.69, 0 : 0.54, 0 : 2.69, 0 : 5.37, 0.49 : 0, 2.46 : 0, 4.92 : 0 μM) with half strength MS or WPM also were tested for root induction but none of the media combination could be able to induce roots in pecan explants. The pecan plantlets were initially transferred to peat pellets (Figure 1E, F) and subsequently to the greenhouse (Figure 1G).

In conclusion, the present study demonstrated a simple and reliable method for *in vitro* regeneration of pecan from nodal explants by using liquid WPM. The protocol established in this study may be applied to mature tissues for successful micropropagation of pecan, a significant agricultural crop in US.

Acknowledgements

This work was supported by USDA-CSREES grant no. 2006-38814-17442

References

Barghchi M (1988) Micropropagation of *Alnus cordata* (Loisel). *Plant Cell Tissue Organ Cult* 15: 233–244

Beuchat LR (1975) Incidence of molds on pecan nuts at different points during harvesting. *Appl Microbiol* 29: 852–854

Brutsch MO, Allan P, Wolstenholme BN (1977) The anatomy of adventitious root formation in adult phase pecan [*Carya illinoinensis* (Wang) K. Koch] stem cuttings. *Hort Res* 17: 23–31

Bouniols A (1974) Neoformation de bourgeons in vitro h partir de fragments de racime d'endive (*Cichorium intybus* L.): influence du degr6 d'hydratation des tissus, et ses cons6quences sur la composition en acides amin6s. *Plant Sci Lett* 2: 363–371.

Chipley JR, Heaton EK (1971) Microbial flora of pecan meat. *Appl Microbiol* 22: 252–253

Corte-Olivares J, Phillips GC, Butler-Nance SA (1990) Micropropagation of pecan. *HortSci* 25: 1308

Debergh PC (1983) Effects of agar brand and concentration on the tissue culture medium. *Physiol Plant* 59: 270–276

Hammerschlag F (1982) Factors influencing in vitro multiplication and rooting of the plum rootstock myrabalan *(Prunus cerasifera* Ehrh.). *J Amer Soci Hort Sci* 107: 44–47

Hanlin RT (1971) Fungi isolated from young pecans. *Proc Ga Pecan Grow Assn* 2: 20–26

Hansen KC, Lazarte JE (1984) *In vitro* propagation of pecan seedlings. *HortSci* 19: 237–239

Knox CA, Smith RH (1981) Progress in tissue culture methods for production of Riverside stocks. *Pecan Quart* 15: 27–34

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, kalmia latifolia, by use of shoot-tip culture. *Proc Int Plant Propagator's Soc* 30: 421–427

Lu MC (2002) Micropropagation of *Morus Latifolia* Poilet using axillary buds from mature trees. *Scientia Hort* 96: 329–341

Mathews H, Wetzstein HY (1993) A revised protocol for efficient regeneration of somatic embryos and acclimatization of plantlets in pecan, *Carya illinoensis*. *Plant Sci* 91: 103–108

McCown BH, Lloyd G (1981) Woody Plant medium (WPM)-a mineral nutrient formulation for microculture of woody plant species. *HortSci* 16: 453

Merkle SA, Sommer HE, Wetxstein HY (1987) Somatic embryogenesis in tissue cultures of pecan. *HortSci* 22: 128–130

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Obeidy AA, Smith MAL (1993) Organogenesis from mature pecan cotyledons and embryonic axes. *HortSci* 28: 213–215

Obeidy AA, Smith MAL (1990) Establishing axenic cultures from mature pecan embryo explants on media with low water availability. *Plant Cell Rep* 9: 463–465

Phillips GC, Ramirez JJ (1983) Pecan tissue culture. In: *Proc 17th Western Pecan Conf*, New Mexico State University Cooperative Extension Service and Western Irrigated Pecan Growers Association, Las Cruces, New Mexico, pp 101–109

Romano A, Noronha C and Martins-Loução MA (1995) Role of carbohydrates in micropropagation of cork oak. *Plant Cell Tissue Organ Cult* 40: 159–167

Romberger JA, Tabor C (1971) The *Picea abies* shoot apical meristem in culture. I. Agar and autoclaving effects. *Amer J Bot* 58: 131–140

Schroeder HW, Cole RJ (1977) Natural occurrence of Alternariols in discolored pecans. *J Agric Food Chem* 25: 204–206

Smith IE, Wolstenholme BN, Allan P (1974) Rooting and establishment of pecan [Carya illinoensis (Wang) K. Koch] stem cuttings. *Agroplantae* 7: 21–28

Singha S (1982) Influence of agar concentration on in vitro shoot

proliferation of *Malus* sp. Almey and *Pyrus communis* Seckel. *J Amer Soc Hort Sci* 107: 657–660

Tremblay FM, Lalonde M (1984) Requirements for in vitro propagation of seven nitrogen fixing *Alnus* species. *Plant Cell Tissue Organ Cult* 3: 189–199

Tremblay FM, Nesme X, Lalonde M (1984). Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* (Ait.) Pursh. *Plant Soil* 78: 171–179

Wainwright H, Scrace J (1989) Influence of in vitro preconditioning with carbohydrates during the rooting of microcuttings on in vivo establishment. *Scientia Hort* 38: 261–267

Welander M, Welander NT, Brackman A-S (1989) Regulation of in vitro shoot multiplication in *Syringa, Alnus and Malus* bydifferent carbon sources. *Hort Sci Biotechnol* 64: 361–366

Wetzstein HY, Ault JR, Merkle SA (1989) Further characterization of somatic embryogenesis in pecan (*Carya illinoensis*). *Plant Sci* 64: 193–201

Wetzstein HY, Ault JR, Merkle SA (1990) Factors influencing somatic embryogenesis and plantlet regeneration in pecan, *Carya illinoensis*. *Acta Hort* 280: 69–74

Wolstenholme BN, Allan P (1975) Progress and problems in pecan clonal propagation by stem cuttings. *Gewasproduksie/Crop Production* IV: 29–32

Wood BW (1982) In vitro proliferation of pecan shoots. *HortSci* 17: 890–891

Yates IE, Reilly CC (1990) Somatic embryogenesis and plant development in eight cultivars of pecan. *HortSci* 25: 573–576