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In vitro germination of Pecan (*Carya illinoinensis*) embryo

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Abstract. The purpose of the present study was to standardize the mediums and culture conditions for organogenesis via immature embryo culture of Pecan (*Carya illinoinensis*). The immature fruits of Pecan hickory were collected 15 weeks postanthesis and, after disinfection, the immature embryos were cultured on modified DKW basal medium. Significant variations were observed between different hormones and different culture conditions. For immature embryo culture, a high frequency of plantlet was obtained in modified DKW basal medium supplemented with 1 mg l^{-1} BAP, 0.05 mg l^{-1} IBA and 2 mg l^{-1} GA_3 and dark culture condition. In this experiment, 0.5 mg l^{-1} BAP and 2 mg l^{-1} GA_3 enhanced embryo germination and shoot proliferation. Also, 0.05 mg l^{-1} IBA was suitable for root proliferation. In general, the percentage of immature embryos germination and shoot proliferation was higher when GA_3 cold treatment and BAP were simultaneously applied, compared to those when these were applied separately. In this experiment, in the presence of different concentrations of BAP and IBA frequently calluses was induced, this calluses was green, creamy and globular form. Results indicated that the GA_3 inhibited callus and root proliferation. The frequency of callus formation was lower in the dark culture condition than light culture condition. At the same time, the shoot and root direct differentiation from the cotyledons with the embryo axis and these calluses was unsuccessful.

Key words: *Carya illinoinensis* ; immature embryo culture; mDKW; Pecan hickory; Plant growth regulators.

Abbreviations: **IEs**: immature embryos; **mDKW**: modified Driver and Kunyiuky Walnut medium (1984); **PGR**: Plant Growth Regulator; **BAP**: 6- Benzylaminopurin; **IBA**: Indol 3-butiric acid; **GA_3**: Giberlic acid; **CH**: Casein hydrolysated.

Introduction

Pecan [*Carya illinoinensis* (Wangenh.) C. Koch; a member of *Juglandaceae*] is a perennial dicot with a long generation period. Mature Pecan trees bear male and female flower at different location on the same tree. The hickories has dichogamous flowering, since male and female flowers on a tree mature at different time. Pecan is used for lumber and pulpwood. Hickories species are most desirable for charcoal and fuel wood (Pag & Wyman 1969). Also Pecan used for furniture, paneling handles and ladders. It is a choice fuel for smoking meats (Lewey 1975). Other uses include bars, crates, pallets and flooring (Gupton 1977). Pecan hickory seeds are eaten by wildlife but are little value for human consumption; because of their high tannin content (Van Dersal 1938, Reed 1944, McDaniel 1969, Elias 1972).

The foliage of hickory species has high calcium content and is the top of the list of soil-improving trees (Chandler 1939). Early settlers used oil extracted from the nuts for oil lamps. They also believe the oil was valuable as a cure for rheumatism (Madden 1978). Also, hickory species is desirable as an ornamental or shade tree and the dense root system that provides good soil stability (Juzwik et al. 2008). The seeds of most hickories are dormant, requiring stratification for various periods of time. Stratification requirements depend on the species and, to some extent, on the origin of the seed. Low percentage of seed germination and long propagation cycle (2–3 months stratification) are the main constraints in the development of Pecan. In addition, the obtaining higher and faster multiplication rate of plants. Because of their juvenile nature, embryos have a high potential for regeneration and hence may be used for in vitro propagation (Kaur et al. 2006). Thus, Pecan breeding is a very slow process. Innovative methods are needed to enhance the incorporation of new genetic resources into Pecan. Initially, tissues culture techniques were used to propagation of Pecan (Burns & Wetzstein 1994, Mathews & Wetzestin 1993, Rodriguez & Wetzstin

1994, 1998, Canhoto & Cruz 1996, Wetzstin et al. 1996, 1989, 2000). Unfortunately, some early clonally Pecan produced through tissues culture were abnormal (Rodriguez & Wetzstin 1994, 1998). Complete plants have been successfully regenerated from immature cotyledons via embryogenesis and embryos culture of Pecan (Rodriguez & Wetzstin 1994, Venderman et al. 2000, Obeidy & Smith 1993, 1990).

We, in this present study, aimed to standardize the medium and culture conditions for embryo culture of a cultivar of Pecan (*Carya illinoinensis*) for application in biotechnological approaches.

Materials and methods

Plant materials for embryo culture

The study was carried out for optimization of the medium culture and culture condition for a cultivar of Pecan hickory [*Carya illinoinensis*]. The plant materials provided from Gonbad Kavoos horseback riding ground in Gonbade Kavoos city, Golestan province, Iran. The fruits were collected 15 weeks postanthesis (at this stage, endosperm was semisolid and gelatinous) and transferred in the laboratory for disinfection and immature embryo culture.

Disinfection of plant materials

Firstly, the immature fruits was washed under running top water and bleach solution for 30 min. Afterward, the fruits with outer pricarp were treated for 1 min in 70% EtOH, desinfected for 20 min in %15 hypochlorite sodium solution made up with sterile distill water plus 0.01% tween 20. After that, samples were rinsed three—five times with distilled water.

Culture media and growth conditions

After disinfection, under sterile condition, the sterile fruits were cracked and IEs were carefully excised and then cultured on mDKW basal medium (Driver and Kuniyuki, 1984) supplemented with 30 g l^{-1} sucrose, 200 mg l^{-1} ascorbic acid, 200 mg l^{-1} CH and 2000 mg l^{-1} activated charcoal and different concentration of BAP at 0, 0.5 and 1 mg l^{-1} , IBA at 0, 0.01, 0.05 mg l^{-1} and GA_3 with 0 and 2 mg l^{-1}. Agar 8 g l^{-1} was added to all the media. The pH of the all media was adjusted at 5.8 with NaOH 1N and then autoclaved at 121^{0}C for 20 min at the pressure of 1.1 kg cm^{-1}. Then, all cultures were incubated at two different culture condition such as dark cold condition (4±2^{0}C) and light condition (25±2^{0}C) with 16.5 h photoperiod under cool white fluorescent lamps (3000 Lux) for two weeks. After two weeks, all cultures transferred to a same condition such as 16.5 h photoperiod at 25±2^{0}C under cool white fluorescent lamps (3000 Lux). During this stage, turgidity was observed in immature embryos and cotyledons which changed to green color. Also in this period, apical bad and radicle gradually emerged from the immature embryos and cotyledons became green and deformed and callus induced from embryonic bodies. After one month, the explants were subculture on primarily mDKW basal medium. When embryo-derived plantlets exceeded ca. 2.31 cm in length, they were transferred onto the fresh mDKW medium without PGR and activated charcoal. The percentage of in vitro germination, callus fresh weight, roots and shoots fresh weight and length was recorded on different media combination for different culture condition. In this experiment, each treatment had three replication and five explants were used per replication. The obtained data were statistically analyzed in factorial design based on completely randomized design (CRD). Analysis of variance was performed by SAS (SAS Institute 1997). For each parameter, variance analysis and means separations by Duncan's test at P = 0.05 were performed.

Results

Plant growth regulation

The percentage of embryo germination when GA_3, IBA and BAP were applied separately is given in Fig.1. In the basal mDKW medium without PGR, embryos did not germinate at all. Upon addition of growth regulators embryo germination occurred and significant differences were observed (P<0.01). In general, the percentage of embryo germination in medium containing 0.5 mg l^{-1} BAP, 0.05 mg l^{-1} IBA and 2 mg l^{-1} GA_3 was more (respectively 61.09%, 62.24% and 62.82% germination) than any other concentrations (Fig.1 and Fig.3d,e,f). Results indicated that BAP, IBA and GA_3 have a significant effect on Pecan embryo proliferation. In the 0.5 mg l^{-1} BAP and 2 GA_3, shoot proliferation was greater than in any other concentration (P<0.01). Also, IBA effected on root proliferation wherein the best performing medium was mDKW containing 0.05 mg l^{-1} IBA (P<0.01). In this experiment, BAP and GA_3 have a negative effect on root proliferation and inhibited root growth. Significant variation were observed between different concentration of BAP, IBA and GA_3 on callus proliferation wherein medium supplemented with 1 mg l^{-1} BAP, 0.05 mg l^{-1} IBA and control of GA_3 was best performing medium (P<0.01, Table 1). Results indicated that GA_3 has a negative effect on callus formation and proliferation.

Simultaneous application of BAP and IBA

The data achieved from this experiment indicated that with an increase of BAP and a decrease of IBA, shoot proliferation was increased and, in the 0.5 mg l^{-1} BAP and control of IBA, the highest shoot growth was achieved (P<0.01). Also, in the medium containing 0 mg l^{-1} BAP and 0.05 mg l^{-1} IBA, the main root length was more than in other combinations (P<0.01, Fig.3b). Significant variation were observed between interaction of BAP and IBA from aspect of callus proliferation (P<0.01). The medium supplemented with 1 mg l^{-1} BAP and 0.05 mg l^{-1} IBA was best for callus proliferation (Table 2, Fig. 3c).

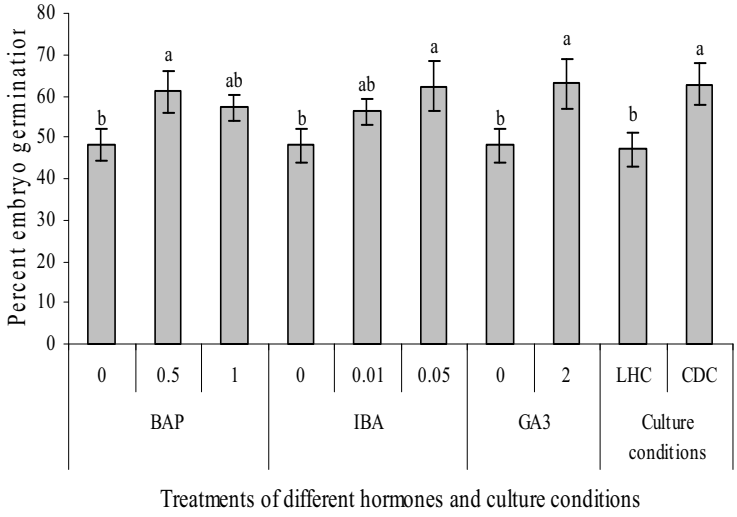

Figure 1. Effects of different hormones (mg l^{-1}) and different culture conditions on embryo germination of *Carya illinoinensis* . Letters a and b denote significant differences among different treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. CDC: cold dark condition. LHD: light heat condition.

Table 1. Effects of different plant growth regulators and different culture conditions on Pecan in vitro propagation.

Hormones	Concentration (mgl^{-1})	SFW (g)	MSL (cm)	RFW (g)	MRL (cm)	CFW (g)
BAP	Control	1±0.16b	1.02±0.11c	0.92±0.11a	1.35±0.22a	0.46±0.06b
	0.5	1.35±0.14a	1.92±0.29a	0.79±0.12ab	1.05±0.24b	0.65±0.14a
	1	1.14±0.16b	1.57±0.17b	0.71±0.1b	1.03±0.24b	0.79±0.08a
IBA	Control	0.9±0.12a	1.32±0.27a	1.12±0.23b	1.2±0.23b	0.38±0.08c
	0.01	0.77±0.17ab	1.11±0.27a	1.26±0.21ab	1.65±0.26a	0.58±0.12b
	0.05	0.62±0.15b	0.87±0.27b	1.4±0.23a	1.72±0.24a	0.79±0.1a
GA_3	Control	1.07±0.21b	1.36±0.38b	1.22±0.23a	1.2±0.21a	0.78±0.12a
	2	1.26±0.21a	1.65±0.22a	1.25±0.15a	0.91±0.12b	0.52±0.1b
Culture condition	16.5 h photoperiod at 25±2^{0}C	1.06±0.23b	1.3±0.15b	1.14±0.23b	1.2±0.27b	0.48±0.07a
	Dark condition at 4±2^{0}C	1.26±0.27a	1.58±0.24a	1.32±0.22a	1.52±0.24a	0.26±0.04b

Letters a, b and c denote significant different among different treatments and values followed by the same letter are not significantly different (based on Duncan test). Data are expressed as mean ± SD. SFW: shoots fresh weight. MSL: main shoot length. RFW: roots fresh weight. MRL: main root length. CFW: callus fresh weight.

Combined treatments of BAP×GA_3 on embryo germination

The data for germination of embryo when BAP and GA_3 treatment were given simultaneously is given in Fig.2. Simultaneous application of BAP and GA_3 was effected and high embryo germination were observed in T4 treatment containing 0.5 mg l^{-1} BAP and 2 mg l^{-1} GA_3 (71.71% germination, Fig.2). The percentage of embryos germination of Pecan when BAP and GA_3 were simultaneously applied was higher than those resulted when the two were separately applied (Fig.3a).

Table 2. Effects of interaction between BAP and IBA on in vitro propagation of Pecan.

Treatments	Hormone combination (mgl^{-1})		SFW (g)	MSL (cm)	RFW (g)	MRL (cm)	CFW (g)
	BAP	IBA					
T1	0	0	0.86±0.08b	1.16±0.22de	0.98±0.05a	1.29±0.16cd	0.71±0.05e
T2	0	0.01	0.69±0.06c	0.96±0.09e	1.06±0.1a	1.82±031ab	0.87±0.1de
T3	0	0.05	0.55±0.08c	0.92±01e	1.1±0.15a	2±0.26a	1±0.11cd
T4	0.5	0	1.12±0.14a	2.21±0.17a	0.96±0.08	1.23±0.15cd	0.88±0.11de
T5	0.5	0.01	1.04±0.1ab	1.95±0.12b	1.01±0.12a	1.52±0.19bc	1.1±0.12bc
T6	0.5	0.05	0.88±0.16b	1.6±0.15c	1±0.1a	1.59±0.19b	1.1±0.1bc
T7	1	0	1.03±0.12ab	1.79±0.2bc	0.94±0.2a	1.08±0.21d	1.08±0.15bc
T8	1	0.01	0.87±0.1b	1.64±0.32c	0.95±0.11a	1.62±0.2b	1.22±0.11ab
T9	1	0.05	0.62±0.09c	1.28±0.19d	1±0.1a	1.6±0.21b	1.28±0.21a

Letters a-f denote significant different among different treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. SFW: shoots fresh weight. MSL: main shoot length. RFW: roots fresh weight. MRL: main root length. CFW: callus fresh weight.

Different culture conditions and interaction with GA_3

In this experiment significant variation was detected among different culture conditions ($P<0.01$). In general, shoot and root proliferations were higher in dark cold condition (4±2^{0}C) than other culture condition. Callus formation and proliferation was higher in light heat culture condition (25±2^{0}C with 16.5 h photoperiod, Table 1). In different culture conditions, the percentage of embryo germination was varied ($P<0.01$) and a high percentage embryo germination (62.82% germination) was achieved in dark and cold conditions (Fig.1). The percentage of embryo germination of Pecan when GA_3 and different culture conditions were simultaneously applied was higher (72.91% germination) then those separately applied. In 2 mg l^{-1} GA_3 and dark cool condition (4±2^{0}C) the highest percent embryos germination were obtained (Fig.2).

Discussion

Embryos are capable of regenerating plants but, in Pecan, poor germination and conversion to plantlets, embryo dormancy and the long period of the propagation cycle are the main problems. Improvement of embryos germination and conversion is generally a long term effort. It has been reported that ABA accumulate during the maturation of embryos, which have induces embryo dormancy (Rajasekaran et al. 1982). Germination can not take place until embryos treatment with cold treatment or GA_3. Cold treatment or GA_3 reduce the ABA level and promote embryos germination (Rajasekaran et al. 1982). Pearce et al. (1987) reported that GA_3 and substrate of GA can be increased during the chilling process as ABA levels decrease. Furthermore, application of exogenous GA_3 induces germination. Kaur et al. (2006) reported that the embryo germination in *Juglans regia* L. was higher when GA_3 and cold treatments were simultaneously applied as compared to those when applied separately. This is in agreement with our results. Renukdas et al. (2008) reported that pecan plants were efficiently regenerated from embryos were cultured on MS basal medium supplemented with 18 µM BAP and 5 µM IBA. Sanchez-Zamora et al. (2006), achieved 81% germination in *J. regia* by using the WPM medium. They reported that the best proliferation rates were obtained in 0.5 mg l^{-1} BAP.

The proliferation rates were showed as qualitative state of the proliferation cluster. The proliferation rates obtained was varied from 0, in the medium without cytokinins to 6, in the medium with 0.5 mg l^{-1} of BAP. Also the cluster proliferation quality and other parameters studied indicated that the optimal treatment was 0.5 mg l^{-1} BAP. Obeidy & Smith (1933) reported that adventitious buds produce from callus of *Carya illinoinensis* embryonic tissues. Their shoots were regenerated from explants placed on MS medium with 2.5 µM TDZ. San & Hatice (2007) reported that the highest percentage of germination (69.1%) was obtained with desiccated embryos originated from open pollinated seeds of Bilecik genotype on DKW basal medium supplemented with 8.6 µM GA_3. Also Saadat & Hennerty (2002), reported that medium containing 1 mg l^{-1}BA and 0.01 mg l^{-1} IBA was best for shoot multiplication of Persian walnut (*Juglans regia* L.), although medium containing 0.6 and 0.8 mg l^{-1} BAP were also

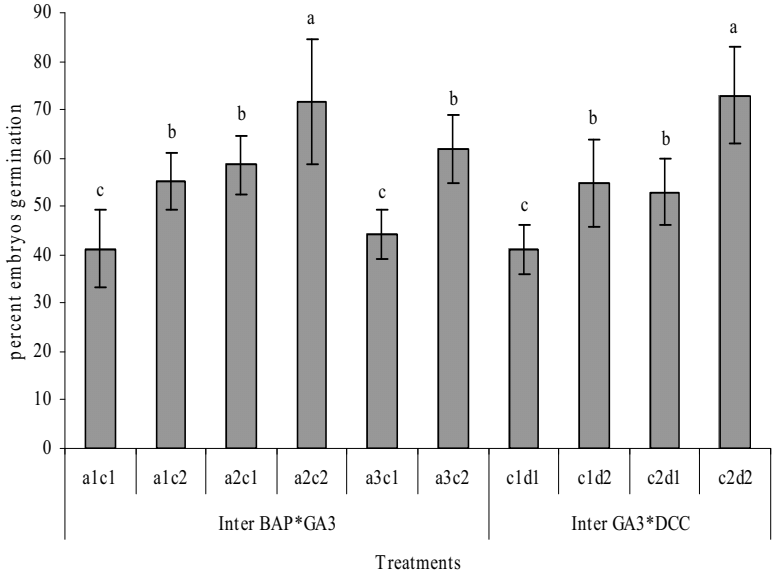

Figure 2. Effects of interaction between BAP (a_n), GA_3 (c_n) and application of GA_3 (c_n) in different culture conditions (d_n) on Pecan embryos germination. Letters a, b and c denote significant different among different treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. a1, a2, and a3 respectively indicator of 0, 0.5 and 1 mg l^{-1} BAP and the c1 and c2 respectively indicator of 0 and 2 mg l^{-1} GA_3. Also d1 and d2 respectively indicator of LHC and CDC.

Figure 3. Immature embryos of Pecan were germinated and proliferated on medium supplemented with 2 mg 1^{-1} GA3, 0.5 mg 1^{-1} BAP (**a**). Root was growth in medium containing 0.05 mg 1^{-1} IBA, 0 mg 1^{-1} BAP (**b**). Calluses were induced in medium supplemented with 0.05 mg 1^{-1} IBA and 1 mg1^{-1} BAP (**c**). Embryos were germinated in present of 0.05 mg 1^{-1} IBA (**d**), 0.5 mg 1^{-1} BAP (**e**) and 2 mg 1^{-1} GA_3 (**f**).

successful, and application of 0.4 mg l^{-1} BAP and 0.01 mg l^{-1} IBA was optimum for shoot elongation of Persian walnut. Revilla et al. (1989) indicated that the best growth regulator combination for in vitro propagation of Persian walnut from nodal segments or juvenile material was 1 mg l^{-1} BAP and 0.1 mg l^{-1} IBA. Inclusion of 1 mg l^{-1} BAP in MS basal medium was the most efficient concentration for shoot proliferation of *J. regia* (Penula et al, 1988). Renukdas et al. (2009), also reported that at least nine multiple shoots per explants were induced on modified WPM containing 13.32 µM BAP, the efficiency of shoot induction was over 95% in both Cape Fear and Desirable cultivars. Multiplied shoots successfully rooted in liquid WPM containing 49.2 µM IBA, with 90% rooting efficiency.

Rodriguez et al. (1993) reported that the best rate of propagation could be obtained by using modified MS basal medium supplemented with 1 mg l^{-1} BA, 2 mg l^{-1} Kn and 0.01 mg l^{-1} IBA. Gruselle et al. (1987) indicated that IBA at 0.1 mg l^{-1} was more efficient for shoots multiplication of *J. Regia*. Tulecke & McGranahan (1985) germinated somatic embryos in cold treatment and regenerated complete plant from that. Tang et al. (2000) reported that somatic embryos germination poorly germinated in cold condition. The addition of GA_3 did not improve the poor germination. However, in our study immature embryo germination of Pecan was more successful in the presence of GA_3 or cold treatments and the percentage of embryos germination was higher when GA_3 and cold treatments were simultaneously applied, as compared to those when used separately. Deng & Cornu (1992) and Lee et al. (1988) reported that somatic embryos germination was poor when that treated with GA_3 and cold treatments.

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