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The effects of BAP, IBA and genotypes on in vitro germination of immature walnut embryos

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Abstract

A prerequisite for most plant transformation systems is an efficient and reliable method to regenerate phenotypically normal plants. The present studies were undertaken with a view to the effects of Benzyl amino purine (BAP) and Indol butyric acid (IBA) hormones and genotypes on in vitro germination of immature embryos in walnut **(***Juglans regia* L.). The immature embryos of walnut were excised from different cultivars and cultured on DKW basal medium supplemented with different plant growth regulators. Significant differences were observed among different cultivars and different concentration of BAP and IBA. The BAP and IBA induced the shoot, root and callus proliferation and embryo germination, but it was dependent on genotypes and hormones concentrations. The best performing medium for immature embryos germination was DKW basal medium supplemented with 1 mg L^{-1} alone and 1.5 mg L^{-1} BAP in conjunction with 0.01, 0.05 and 0.1 mg L^{-1} IBA (germination ratio vary between 49.32% and 67.76%). Percent germination of immature embryos was more when BAP and IBA were simultaneously applied as compared to those when applied separately.

Keywords: Casein hydrolysate; DKW basal medium; Immature embryo; In vitro development; *Juglans regia* L.; Root, Shoot.

Introduction

Various walnuts are being used for their fruits and woods. The genus *Juglans* sp L. (*Juglandaceae* family) comprises 21 long-lived deciduous tree species generically referred to as walnuts. *J. regia* L. (Persian walnut; [McGranahan and Leslie, 1990]), *J. nigra* L. (eastern black walnut; [Funk, 1979]), and *J. cinerea* L. (Butternut; [McDaniel, 1979]) are important for commercial nut production. *Juglans nigra* L. (Beineke, 1983) in addition is suitable for timber production and as valuable ecological niche species (Elias, 1980). In forestry terms, walnut is the noblest trees. Its chemical and physical characteristics, together with its aesthetic qualities, make it a beautiful and valuable tree. Its kernel is a very rich

source of proteins, fats, minerals and is a concentrated source of energy. Low percentage of seed germination and long propagation cycle (2-3 months stratification) are the main constraints in the development of high yielding cultivars through hybridization (Kaur et al., 2006). In vitro techniques seem to be the most appropriate to gain insight into plant physiology as well as to obtain high yielding propagation systems. In vitro cultures of *Juglans* sp L. can be established starting from zygotic embryos or shoots from adult plants (Driver and Kuniyuki, 1984; Jay-Allemand and Cornu, 1986; Gruselle et al., 1987; Liu and Han, 1989; Preece et al., 1989; Revilla et al., 1989; Rodríguez et al., 1989; Gruselle and Boxus, 1990; Leslie and MacGranahan, 1992; Rodríguez et al., 1993; Kaur et al., 2006; Payghamzadeh and Kazemitabar, 2010). Embryo culture is one of the most effective in vitro culture methods, allowing attainment of hybrids which reduce the breeding cycle of many plants (Raghavan, 1977; Raghavan, 1980). The culture of excised immature embryos has been used to study physiological and morphological processes in embryo development (Mott, 1981). In this experiment the effects of IBA/BAP ratio on the organogenesis, callus induction, embryo germination and bud proliferation from immature embryos of *Juglans regia* L. genotypes were investigated on DKW basal media.

Materials and Methods

Plant materials for embryo culture

This study were carried out for optimization of the embryo culture of eight cultivars of walnut vis. Chandler, Serr, Hartky, Pedro, Lara, Vina, Rentegnomushak and local cultivars named by the prefixes C, S, Ha, Pe, L, V, RDM and LC respectively procured from the Seed and Plant Improvement Institute (SPII), Iran. The local cultivars were obtained from Golestan province, Iran. The fruits were collected at 6 weeks post-anthesis and prechilled at 4 $^{\circ}$C for 7 days before immature embryo culture.

Disinfection of plant materials

First the immature fruits washed under running tap water for 30 min. After this period, the fruits treated with bleaching solution (7-10 drops in 500 mL sterile distill water) for 10 min. Afterward, The fruits with outer pricarp were treated for 30 second in 70% EtOH, disinfected for 20 min in 3.75% hypochlorite sodium solution plus 0.01% Tween 20, after that samples were rinsed three 5-times with distilled water.

Culture media and growth conditions

After disinfection, the sterile fruits were cracked by piercing and pointed forceps through the suture at the pedicel end and the immature embryos were carefully excised and aseptically cultured on DKW basal medium (Driver and Kuniyuki, 1984), supplemented with 30 g L^{-1} sucrose, 200 mg L^{-1} ascorbic acid, 200 mg L^{-1} casein hydrolysate and 2 g L^{-1} activated charcoal and different concentrations of BAP (0, 1 and 1.5 mg L^{-1}) and IBA (0, 0.01, 0.05 and 0.1 mg L^{-1}). Agar 8 g L^{-1} was added to all the media. The media pHs were adjusted on 5.7 with 1N NaOH and autoclaved at 121 $^{\circ}$C for 20 min at the 1.1 kg cm^{-1}

pressure. Afterward, all cultures were incubated at 25±2 °C with 16.5 h photoperiod under cool white fluorescent lamps (3000 Lux). Within two weeks, radicle and apical bud gradually emerged from the immature embryos and the cotyledons became green and deformed and callus induced from embryonic bodies. Explants were sub-cultured every 20 days and then explants maintained for 20 days on primarily DKW basal medium without activated charcoal.

Data analysis

The experiment was performed in factorial design based on completely randomized design (CRD). The ANOVA was used to determine the individual and interactive effects between factors and walnut cultivars. The obtained data's were statistically analyzed by SAS (SAS Institute, 1997). For each parameter, the comparative Duncan's multiple range test (P=0.05) was used to determine differences between treatments.

Results

In our study, we found that plant recovery from immature embryos was highly significant in BAP, IBA, genotype and interaction between them. Based on the effects of different cultivars and plant growth regulators (PGR), this study is categorized into the following headings:

Plant growth regulators

Embryos under in vitro conditions germinated within two weeks, although some embryos required a longer time for germination in different media. Some embryos did not geminate even after transfer to a fresh DKW medium after 20 days. Initially, the embryos developed a taproot, but later exhibited an adventitious root system. The hypocotyls and cotyledons of the embryos turned green and the first pair of leaves became visible after two weeks. Embryo germination occurred in all cultivars when treated with BAP and IBA, while no embryos could be germinated in all untreated lots. Immature embryo germination was significantly high among BAP, IBA, genotypes and in the interaction between them, whereby embryo germination increased in all treatments containing high concentration of BAP and IBA. In the absence of BAP, the embryos started to produce roots but the apical part did not develop. High concentrations (1 and 1.5 mg L^{-1}) of BAP improved embryo growth, the plantlets started to lose the leaflets and showed aberrant growth. In the free PGR medium embryos germinated but did not induce any embryo development, root formation or callus production in any cultivars; alternatively the development was slow and the rate of plant recovery was very low. The BAP and IBA had a positive influence on embryo germination in all cultivars. High percent of embryo germination were obtained in 1 and 1.5 mg L^{-1} BAP ($P<0.01$) and 0.05 and 0.1 mg L^{-1} IBA ($P<0.01$). The germination of treated immature embryos with hormones as compared to untreated ones was strongly influenced by different concentrations of BAP and IBA (Figure 1). Interaction of BAP and IBA was significant on immature embryo germination ($P<0.01$), as it seems that the best combinations of growth regulators were those with 1 mg L^{-1} alone and 1.5 mg L^{-1} BAP in

conjunction with 0.01, 0.05 and 0.1 mg L^{-1} IBA (Table 1). As for production of shoot fresh weight, for all cultivars the highest shoot production was achieved with 1 mg L^{-1} BAP ($P<0.05$, Figure 2) alone and 1 mg L^{-1} and 1.5 mg L^{-1} BAP in combination with 0.01 mg L^{-1} IBA ($P<0.01$, Table 1). Also the longest main shoot length were achieved in DKW medium supplemented with 1 and 1.5 mg 1^{-1} BAP ($P<0.05$, Figure 2), but shoots grown in medium containing 1.5 mg L^{-1} BAP showed the morphological changes (Figure 3f). Interaction of BAP and IBA was significant on elongation of shoot ($P<0.01$); wherein, the DKW medium supplemented with 1 and 1.5 mg L^{-1} BAP in combination with 0 and 0.01 mg L^{-1} IBA and 1 mg L^{-1} BAP in conjunction with 0.05 mg L^{-1} IBA was the best performed medium (Table 1). Significant differences were observed between different concentrations of IBA on root proliferation ($P<0.01$). Root proliferation in media supplemented with 0.05 and 0.1 mg L^{-1} IBA was more than any others (Figure 4). Also in interaction between BAP and IBA, the medium with 0.05 and 0.1 mg L^{-1} IBA alone was the best for root proliferation (Table 1). The formation of secondary roots occurred only when 0.1 mg L^{-1} IBA was added to the culture medium. The morphological changes were observed in roots when they were exposed for a long time in medium containing 0.1 mg L^{-1} IBA (Figure 3d). Callus formation was the first morphological changes that were observed in the immature embryos in the presence of BAP and IBA. Initially, induced calluses were green and nodular (Figure 3c, 3e up), but in long period of incubation in the media containing hormones became brown, necrotic and friable. This was especially evident in those growing in 0.1 mg L^{-1} IBA and 1.5 mg L^{-1} BAP (Figure 3e down). In general, the 0.01 and 0.1 mg L^{-1} IBA and 1.5 mg L^{-1} BAP were favored for callus induction (Figures 2 and 4). Significant differences were observed between interaction of BAP and IBA ($P<0.05$) wherein, the best performed medium was DKW medium supplemented with 1.5 mg L^{-1} BAP in combination with 0.05 and 0.01 mg L^{-1} IBA (Table 1). In general BAP and IBA had a synergistic effect on callus formation and fresh weight.

The effect of cultivars on proliferation rate of immature embryos

Many cultivars are capable of supporting the formation of plantlets from young embryos. It was, also, observed that different cultivars had different requirements for plant growth regulators. Significant differences were observed among multiplication rate of eight different cultivars. In some cultivars with BAP treatment, the hypocotyls and cotyledons enlarged drastically in size and took a long time to put forth the first pair of leaves and required subculturing. Treatment with BAP resulted in vigorous growth of plantlets in all the cultivars. Germination of embryos under in vitro condition was significantly higher among cultivars with BAP treated as compared to untreated ones ($P<0.05$). Between different cultivars the highest embryo germination percentages occurred in Chandler, Serr, Hartky, Rentegnomushak and Local cultivar (Figures 3b and 5). Also the best shoot fresh weight were observed in Chandler, Serr and local cultivars. Between different cultivars the Pedro and Rentegnomushak displayed the least main shoot length compared to other cultivars ($P<0.01$). Also Chandler, Serr and Local cultivars showed the best response for root fresh weight than any others ($P<0.01$). In Pedro, Lara and Rentegnomushak, root elongation was least ($P<0.01$). Hartky was the best cultivar for callus production ($P<0.01$, Table 2).

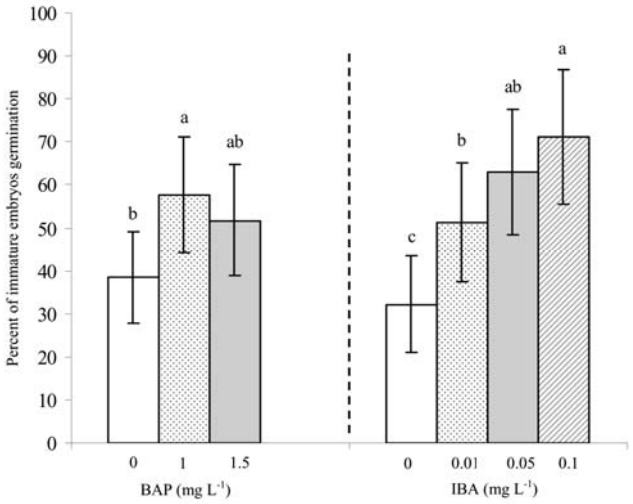

Figure 1. Effects of BAP and IBA on immature embryos germination of walnut. Results achieved after 25 d. Letters a, b and c denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD.

Table 1. Interaction of BAP and IBA on in vitro propagation of walnut (*J. regia* L.) cultivars via immature embryos culture.

Treatment	PGR (mg L^{-1})		Mean traits					
	BAP	IBA	SFW (g)	RFW (g)	CFW (g)	MSL (cm)	MRL (cm)	PEG (%)
T1	0	0	0.84 ± 0.1^{e}	0.88 ± 0.11^{cd}	0.77 ± 0.06^{f}	$1.13\pm0.19^{d-f}$	1.25 ± 0.19^{e}	$42.36\pm10.3^{b-e}$
T2	0	0.01	0.82 ± 0.09^{e}	0.95 ± 0.12^{bc}	0.81 ± 0.07^{f}	$1.05\pm0.19^{d-f}$	1.91 ± 0.23^{bc}	30.09 ± 6.3^{e}
T3	0	0.05	0.8 ± 0.07^{e}	1.21 ± 0.17^{a}	0.84 ± 0.1^{ef}	1.01 ± 0.18^{f}	2.08 ± 0.24^{ab}	37.08 ± 8.3^{de}
T4	0	0.1	0.77 ± 0.06^{e}	1.22 ± 0.16^{a}	0.9 ± 0.13^{de}	0.96 ± 0.18^{f}	2.19 ± 0.25^{a}	$44.04\pm11.6^{b-e}$
T5	1	0	1.22 ± 0.17^{ab}	0.85 ± 0.1^{cd}	0.85 ± 0.08^{ef}	1.94 ± 0.32^{a}	1.17 ± 0.17^{e}	$51.63\pm12.9^{a-d}$
T6	1	0.01	1.28 ± 0.2^{a}	1.04 ± 0.15^{b}	0.92 ± 0.08^{de}	1.95 ± 0.42^{a}	1.67 ± 0.19^{d}	$45.35\pm12.3^{b-e}$
T7	1	0.05	1.14 ± 0.12^{bc}	0.82 ± 0.16^{cd}	0.97 ± 0.1^{cd}	1.68 ± 0.22^{ab}	1.77 ± 0.2^{cd}	65.87 ± 16.2^{ab}
T8	1	0.1	0.99 ± 0.13^{d}	0.88 ± 0.19^{cd}	1.02 ± 0.13^{c}	1.28 ± 0.29^{de}	1.98 ± 0.22^{b}	67.76 ± 14.22^{a}
T9	1.5	0	1.08 ± 0.14^{cd}	0.79 ± 0.09^{d}	0.90 ± 0.1^{de}	1.83 ± 0.24^{ab}	1.15 ± 0.22^{e}	$38.51\pm9.5^{c-e}$
T10	1.5	0.01	1.24 ± 0.14^{ab}	0.81 ± 0.1^{cd}	1.09 ± 0.13^{b}	1.85 ± 0.22^{ab}	1.33 ± 0.18^{e}	$61.46\pm15.44^{a-b}$
T11	1.5	0.05	0.98 ± 0.14^{d}	0.82 ± 0.09^{cd}	1.17 ± 0.14^{a}	1.57 ± 0.28^{bc}	1.16 ± 0.2^{e}	$49.32\pm16.76^{a-e}$
T12	1.5	0.1	0.87 ± 0.11^{e}	0.82 ± 0.09^{cd}	1.21 ± 0.11^{a}	1.33 ± 0.25^{cd}	1.19 ± 0.2^{e}	$57.81\pm14.6^{a-c}$

Letters a-g denote significant differences among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. SFW: Shoots fresh weight; RFW: Roots fresh weight; CFW: Callus fresh weight; MSL: Main shoots length; MRL: Main root length; PEG: Percent embryo germination; PGR: Plant growth regulators.

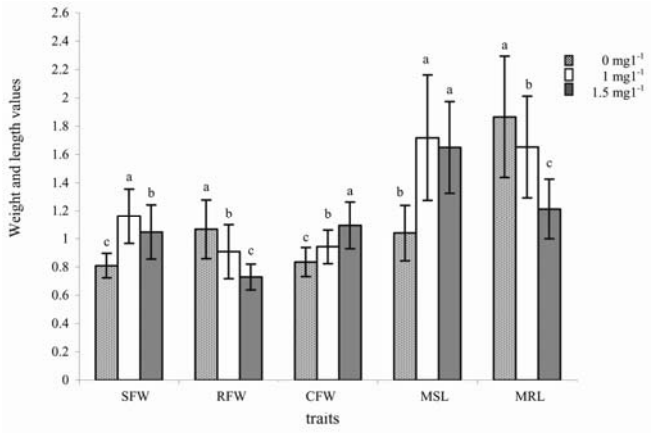

Figure 2. Effects of BAP on in vitro propagation of walnut. Letters a, b and c denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. SFW: Shoots fresh weight (g); RFW: Roots fresh weight (g): CFW: Callus fresh weight (g); MSL: Main shoots length (cm); MRL: Main root length (cm).

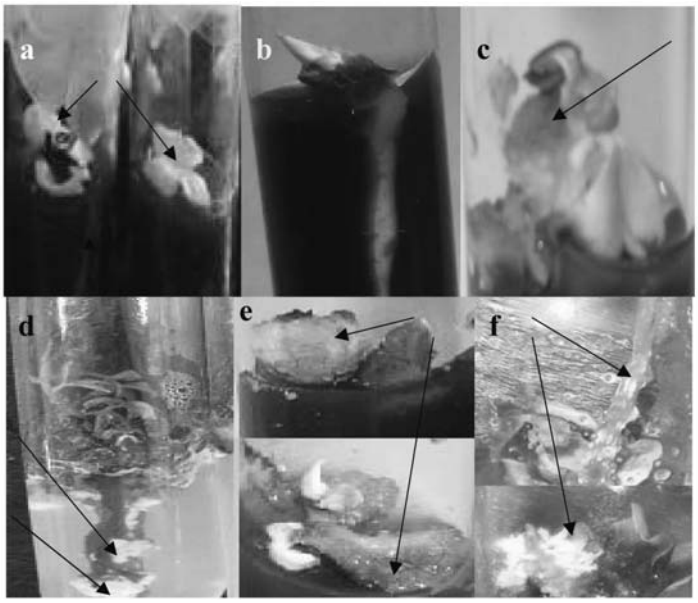

Figure 3 a-f. a) immature embryos of different cultivars of walnut were cultured on modified DKW basal medium (Figure captured after 7 d). b) Immature embryos of chandler were germinated in modified DKW medium in present auxin and cytokinin. (Figure captured after 12 d) c) callus induced from hypocotyls of immature embryos in Chandler cultivar. d) Morphological changes appeared in roots at 0.1 mg l^{-1} in long term exposure. e) Callus formed in Hartky (up: the callus were green and nodular) and Vina cultivars (down: became brown and friable, when those growing with 0.1 mg L^{-1} IBA and 1.5 mg L^{-1} BAP which became necrotic and loosely). f) Morphological changes were appeared in shoot structures which grown in 1.5 mg L^{-1} BAP and long term exposure.

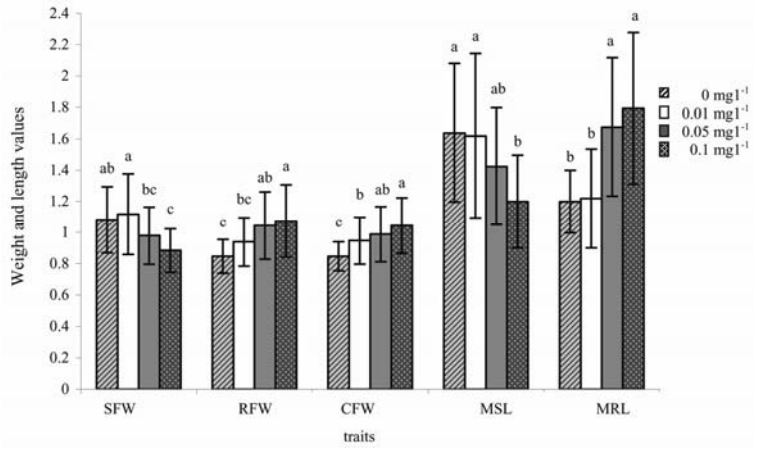

Figure 4. Effect of IBA on immature growth of walnut. Letters a, b and c denote significant differences among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. SFW: Shoots fresh weight (g); RFW: Roots fresh weight (g): CFW: Callus fresh weight (g); MSL: Main shoots length (cm); MRL: Main root length (cm).

Table 2. The response of different cultivars on in vitro propagation of *J. regia* L.

Cultivars	traits				
	SFW (g)	RFW (g)	CFW (g)	MSL (cm)	MRL (cm)
Chandler	1.17±0.22^{a}	1.08±0.18^{ab}	1.02±0.17^{b}	1.63±0.34^{ab}	1.76±0.27^{a}
Serr	1.12±0.2^{a}	1.15±0.16^{a}	0.92±0.15^{c}	1.56±0.27^{ab}	1.61±0.32^{ab}
Hartky	0.97±0.18^{bc}	0.96±0.21^{b-d}	1.11±0.14^{a}	1.47±0.36^{a-c}	1.63±0.31^{ab}
Pedro	0.98±0.19^{b}	0.91±0.18^{cd}	0.86±0.12^{c}	1.34±0.22^{bc}	1.48±0.33^{bc}
Lara	0.86±0.14^{c}	0.87±0.15^{d}	1.03±0.17^{b}	1.36±0.37^{a-c}	1.36±0.31^{c}
Vina	0.90±0.18^{bc}	0.83±0.12^{d}	0.93±0.14^{c}	1.50±0.31^{ab}	1.59±0.3^{ab}
Rentegnomushak	0.93±0.19^{bc}	0.93±0.19^{b-d}	0.85±0.1^{c}	1.20±0.4^{c}	1.34±0.35^{c}
Local cultivar	1.11±0.2^{a}	1.03±0.21^{a-c}	0.91±0.15^{c}	1.66±0.42^{a}	1.80±0.28^{a}

Letters a, b, c and d denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD. SFW: Shoots fresh weight; RFW: Roots fresh weight; CFW: Callus fresh weight; MSL: Main shoots length; MRL: Main root length.

Interaction between cultivars and plant growth regulators

We also examined how embryo growth of different cultivars is affected by combinations of different concentrations of BAP and IBA. The shoot, root and callus growth expressed as average length and weight were significantly increased by the addition of BAP and IBA in different cultivars ($P<0.01$). Shoot fresh weight was significantly higher in the interaction of cultivars and BAP, in the presence of 1 mg L^{-1} BAP in seedlings of Chandler, Serr and Local cultivar. It was also high in 1.5 mg L^{-1} BAP in seedlings of Chandler. BAP (1 mg L^{-1}) simulated shoot elongation in Chandler, Serr, Hartky and Local cultivar, and also in 1.5 mg L^{-1} BAP the highest shoot elongation were observed in Chandler and Local cultivars. Embryo germination percentage and root growth in interaction of cultivars and BAP was insignificant. Callus growth was highest in the

presence of 1.5 mg L^{-1} BAP in Chandler, Hartky and Lara cultivars (Table 3). In this experiment root and callus fresh weights and shoot elongation were significant ($P<0.01$) when IBA and cultivars were considered simultaneously (Table 4). In general, DKW basal medium supplemented with 0.1 mg L^{-1} IBA was the best medium for callus production in Hartky cultivar. The IBA (0 and 0.01 mg L^{-1}) stimulated shoot elongation in Chandler, Serr, Hartky, Vina and Local cultivar. In 0.05 mg L^{-1} IBA the highest main shoot length were observed in Chandler and Local cultivar. Root fresh weight was significantly higher in the presence of 0.05 mg L^{-1} IBA (Chandler and Local cultivar) and 0.1 mg L^{-1} IBA (Chandler, Serr, Hartky and Local cultivar).

Table 3. The effects of interaction between BAP and cultivars on in vitro proliferation of walnut (*J. regia* L.) genotype via immature embryos culture.

Combinations		traits		
cultivars	BAP (mg L^{-1})	SFW (g)	CFW (g)	MSL (cm)
Chandler	0	$0.91\pm0.07^{e-i}$	$0.88\pm0.1^{h-j}$	1.18 ± 0.17^{ef}
Serr	0	$0.91\pm0.06^{e-i}$	0.80 ± 0.02^{kl}	1.16 ± 0.16^{ef}
Hartky	0	$0.79\pm0.01^{g-i}$	$1.01\pm0.11^{c-e}$	$0.99\pm0.14^{f-h}$
Pedro	0	0.78 ± 0.02^{hi}	0.76 ± 0.03^{l}	$1.07\pm0.07^{e-h}$
Lara	0	0.74 ± 0.01^{i}	$0.89\pm0.08^{g-i}$	0.88 ± 0.09^{gh}
Vina	0	0.73 ± 0.02^{i}	$0.81\pm0.04^{j-l}$	$1.09\pm0.08^{e-g}$
Rentegnomushak	0	0.73 ± 0.01^{i}	0.77 ± 0.03^{l}	0.84 ± 0.08^{h}
Local cultivar	0	$0.9\pm0.04^{e-i}$	0.78 ± 0.02^{kl}	$1.12\pm0.23^{e-g}$
Chandler	1	1.37 ± 0.13^{a}	$1.01\pm0.09^{c-e}$	1.93 ± 0.3^{a}
Serr	1	$1.29\pm0.11^{a-c}$	$0.91\pm0.09^{f-i}$	1.91 ± 0.28^{a}
Hartky	1	1.08 ± 0.15^{de}	1.11 ± 0.11^{b}	1.81 ± 0.25^{ab}
Pedro	1	1.13 ± 0.18^{cd}	$0.85\pm0.07^{i-k}$	1.46 ± 0.21^{cd}
Lara	1	$0.98\pm0.09^{d-g}$	$1.01\pm0.08^{c-e}$	1.65 ± 0.35^{bc}
Vina	1	$1.06\pm0.17^{d-f}$	$0.94\pm0.08^{e-h}$	1.60 ± 0.22^{bc}
Rentegnomushak	1	1.08 ± 0.11^{de}	$0.85\pm0.06^{i-k}$	1.26 ± 0.29^{de}
Local cultivar	1	$1.29\pm0.16^{a-c}$	0.87 ± 0.05^{ij}	1.94 ± 0.37^{a}
Chandler	1.5	1.24 ± 0.13^{ab}	1.18 ± 0.16^{a}	1.79 ± 0.28^{ab}
Serr	1.5	$1.16\pm0.18^{b-d}$	1.08 ± 0.13^{bc}	1.63 ± 0.18^{bc}
Hartky	1.5	$1.04\pm0.16^{d-f}$	1.23 ± 0.12^{a}	1.61 ± 0.29^{bc}
Pedro	1.5	$1.04\pm0.14^{d-f}$	$0.98\pm0.8^{d-f}$	1.51 ± 0.21^{c}
Lara	1.5	$0.86\pm0.15^{f-i}$	1.21 ± 0.15^{a}	1.56 ± 0.25^{bc}
Vina	1.5	$0.9\pm0.12^{e-i}$	$1.04\pm0.17^{b-d}$	1.64 ± 0.23^{bc}
Rentegnomushak	1.5	$0.98\pm0.17^{d-h}$	$0.96\pm0.1^{e-g}$	1.52 ± 0.25^{c}
Local cultivar	1.5	$1.15\pm0.14^{b-d}$	1.1 ± 0.11^{b}	1.92 ± 0.21^{a}

Letters a-i denotes significant different among treatments Data are expressed as mean ± SD. SFW: Shoots fresh weight; CFW: Callus fresh weight; MSL: Main shoots length.

Discussion

It is already known that cultures from zygotic embryos have higher morphogenetic capacity than other explants (Payghamzadeh and Kazemitabar, 2010). In the immature embryo culture of walnut, addition of BAP and IBA was necessary and satisfactory results were obtained after using different concentrations of BAP and IBA. Moreover, it seems that the rate of auxin or cytokinin plays a more important role in the successful culture of immature walnut embryos. Regulation of cell division and morphogenesis are widely regarded as the most significant functions of cytokinins. It is possible that the strong

inhibitory effects on morphogenesis of the cotyledons by the embryonic axes could be weakened by the presence of auxin in the medium (Nig et al., 2007). Also, proliferation from embryos of *J. regia* was achieved with two consecutive subcultures each of one month; the first being in half-strength Knop medium and the second in a Miller medium supplemented with 1 mg L^{-1} BA (Jay-Allemand, 1982). In this experiment the proliferation rates were statistically significant and the effects of IBA and BAP were determinant on in vitro propagation of walnut, which is in congruence with Fernandez et al. (2000). Kaur et al. (2006) reported that MS medium supplemented with 0.5 mg L^{-1} Kinetin, 0.5 mg L^{-1} BAP and 2 mg L^{-1} GA_3 to be the best for walnut embryo culture. Jay-Alleman et al. (1992) used DKW medium containing 4.44 µM BAP for walnut in vitro propagation. Similarly Sanchez-Zamora et al. (2006), proliferated plantlets from mature walnut embryos cultured at 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} IBA. In some earlier attempts MS medium fortified with different combinations of growth regulators have been used for embryo culture of walnut (Cassio and Minotta, 1985; Konova et al., 1993). Similarly Kornova et al. (1993), was able to get plantlets from walnut embryos after 55 days culture on MS medium containing thiamine HCl, nicotinic acid, biotin, pyridoxine hydrochloride and calcium pantothenate followed by transferring them to the same medium with BA (1 mg L^{-1}). Our results is parallel to Scaltsoyiannes et al. (1997), who reported the effects of cytokinin (BA) and auxin (IBA) on shoot development. They showed that shoot elongation is enhanced with 2.22 µM BA while the formation of new axilliarly shoots is favored by 4.44 µM BA and 0.005 µM IBA. These results are in agreement with those of Payghamzadeh and Kazemitabar (2010), who reported high frequency of plantlet 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. Also These results are in agreement with those of Gruselle and Boxus (1990), who reported that the optimum in vitro shoot regeneration was achieved on DKW medium supplemented with BA (3.55 µM to 4.44 µM). The effect of genotype on embryo germination, roots and shoots was, also reported by other researchers in different plant species (Cornu and Chaix, 1981; Cornu et al., 1981; Evers et al., 1988). One of the major obstacles in the establishment of cultures from walnut is the presence of several phenolic compounds, including the allelochemical compounds like juglone, which interferes with cell growth (Rietvel, 1982; Rietvel, 1983). Browning of the medium is the result of oxidation of polyphenols exuded from the cut surfaces of the explants which can be overcome by adding substances such as PVP (polyvinyl pyrrolidone) (250-1000 mg L^{-1}), citric acid, ascorbic acid, activated charcoal (0.2-3% w/v), thiourea, L-cysteine, glutamine, aspargine, argenine (Pierik, 1987; Rout et al., 1999) or incubating cultures for a day or two in total darkness (Pittet and Moncousin, 1981). In this experiment culture medium was supplemented with ascorbic acid (200 mg L^{-1}) and activated charcoal (2 g L^{-1}) to check the oxidative browning on account of high phenolic content of walnut tissues. Amino acids provide plant cells with an immediate source of nitrogen, which can be taken up by the cells more rapidly than inorganic nitrogen. Amino acid mixtures (e.g., casein hydrolysate, etc) may be used to stimulate further cell growth in culture media. In many systems it has been found that seeds and embryo germination and somatic embryogenesis is improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate (Liu and Han, 1986; Waes and Debergh, 1986b; Long et al., 1995). According to Van Waes and Debergh (1986b), addition of serine, glutamic acid, peptone, casein hydrolysate and yeast extract only stimulate seed germination.

Table 4. Interaction of IBA and cultivars on in vitro proliferation of walnut.

combinations		traits		
Cultivars	IBA (mg L^{-1})	RFW (g)	CFW (g)	MSL (cm)
Chandler	0	$0.96\pm0.07^{e-i}$	$0.87\pm0.12^{l-n}$	$1.79\pm0.46^{a-c}$
Serr	0	$1.03\pm0.05^{d-f}$	$0.81\pm0.06^{n-p}$	$1.69\pm0.39^{a-e}$
Hartky	0	0.8 ± 0.05^{kl}	$0.97\pm0.06^{f-j}$	$1.62\pm0.49^{a-g}$
Pedro	0	0.80 ± 0.06^{kl}	0.77 ± 0.03^{p}	$1.49\pm0.28^{d-k}$
Lara	0	0.76 ± 0.02^{l}	$0.9\pm0.09^{j-m}$	$1.55\pm0.32^{c-j}$
Vina	0	0.75 ± 0.02^{l}	$0.81\pm0.04^{n-p}$	$1.69\pm0.41^{a-f}$
Rentegnomushak	0	$0.81\pm0.08^{j-l}$	0.78 ± 0.05^{op}	$1.43\pm0.32^{e-l}$
Local cultivar	0	$0.87\pm0.05^{j-l}$	$0.85\pm0.07^{m-o}$	1.84 ± 0.43^{ab}
Chandler	0.01	$1.03\pm0.11^{d-f}$	$1.00\pm0.15^{f-i}$	$1.73\pm0.53^{a-d}$
Serr	0.01	$1.12\pm0.11^{b-d}$	$0.91\pm0.15^{j-m}$	$1.68\pm0.46^{a-f}$
Hartky	0.01	$0.89\pm0.11^{h-k}$	$1.07\pm0.11^{c-d}$	$1.66\pm0.55^{a-f}$
Pedro	0.01	$0.93\pm0.17^{f-j}$	$0.85\pm0.1^{m-o}$	$1.55\pm0.35^{c-j}$
Lara	0.01	0.80 ± 0.04^{kl}	$1.03\pm0.1^{d-f}$	$1.58\pm0.57^{b-i}$
Vina	0.01	$0.84\pm0.11^{i-l}$	$0.95\pm0.16^{g-k}$	$1.73\pm0.48^{a-d}$
Rentegnomushak	0.01	$0.85\pm0.08^{i-l}$	$0.85\pm0.12^{m-o}$	$1.16\pm0.48^{m-o}$
Local cultivar	0.01	$1.03\pm0.16^{d-f}$	$0.92\pm0.09^{j-k}$	1.87 ± 0.6^{a}
Chandler	0.05	$1.14\pm0.17^{a-d}$	1.08 ± 0.14^{cd}	$1.67\pm0.38^{a-f}$
Serr	0.05	1.26 ± 0.19^{a}	$0.97\pm0.17^{f-j}$	$1.49\pm0.29^{d-i}$
Hartky	0.05	$1.02\pm0.17^{d-g}$	$1.17\pm.16^{b}$	$1.36\pm0.39^{g-m}$
Pedro	0.05	$0.99\pm0.14^{e-h}$	$0.90\pm0.12^{j-m}$	$1.29\pm0.25^{j-n}$
Lara	0.05	$0.98\pm0.16^{e-h}$	1.04 ± 0.14^{cd}	$1.28\pm0.38^{j-n}$
Vina	0.05	$0.85\pm0.08^{i-l}$	$0.95\pm0.2^{g-l}$	$1.45\pm0.3^{e-l}$
Rentegnomushak	0.05	$1.04\pm0.22^{d-f}$	$0.88\pm0.1^{k-n}$	$1.23\pm0.38^{k-o}$
Local cultivar	0.05	$1.07\pm0.2^{c-e}$	$0.95\pm0.1^{g-k}$	$1.61\pm0.44^{a-h}$
Chandler	0.1	1.23 ± 0.22^{ab}	1.14 ± 0.19^{bc}	$1.35\pm0.28^{h-m}$
Serr	0.1	1.2 ± 0.15^{ab}	$1.01\pm0.14^{e-h}$	$1.41\pm0.32^{f-m}$
Hartky	0.1	$1.14\pm0.21^{a-d}$	1.25 ± 0.17^{a}	$1.21\pm0.33^{l-o}$
Pedro	0.1	$0.95\pm0.16^{f-i}$	$0.94\pm0.12^{h-l}$	1.05 ± 0.1^{no}
Lara	0.1	$0.98\pm0.16^{f-i}$	1.12 ± 0.12^{bc}	1.05 ± 0.23^{no}
Vina	0.1	$0.90\pm0.16^{g-k}$	$1.02\pm0.12^{d-g}$	$1.15\pm0.14^{m-o}$
Rentegnomushak	0.1	$1.02\pm0.24^{d-f}$	$0.93\pm0.15^{i-l}$	0.99 ± 0.21^{o}
Local cultivar	0.1	$1.17\pm0.24^{a-c}$	$0.94\pm0.11^{h-l}$	$1.33\pm0.41^{i-m}$

Letters a-p denote significant different among treatments. Data are expressed as mean ± SD. RFW: Roots fresh weight; CFW: Callus fresh weight; MSL: Main shoots length.

In conclusion, the results from this investigation reveal the effect of BAP, IBA and cultivar on immature embryos germination in walnut. BAP and IBA had a positive effect on embryo germination and development. Root, shoot and callus fresh weight, roots and shoots length in all cultivars was strongly influenced by application of BAP, IBA and their interactions had a positive effect on in vitro propagation of walnut via embryo culture. These results are essential to approach successfully the programs of genetic breeding of this species. With respect to the proliferation stage of the cluster, differences have also been observed between the treatments studied; with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IBA being the optimum treatment.

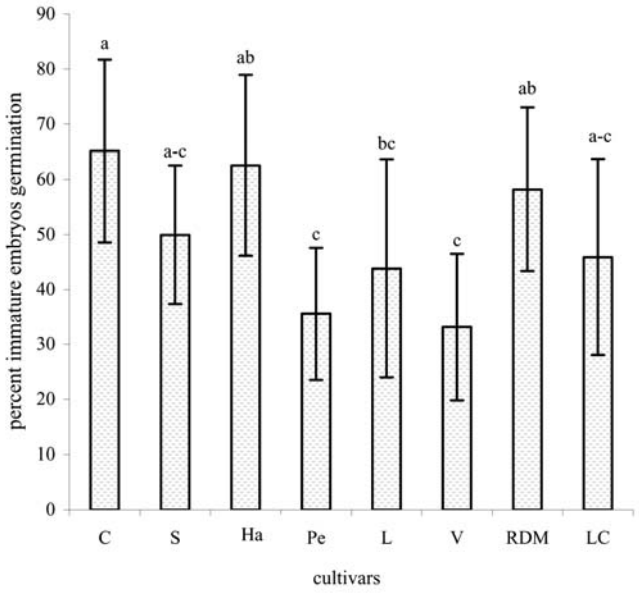

Figure 5. Comparison of embryo germination of different cultivars. Results achieved after 25 d. Letters a, b, c and d denote significant differences among cultivars and values followed by the same letter are not significantly different. Data are expressed as mean ± SD.

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