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Effect of Different Hormonal Treatments on Proliferation and Rooting of Three Persian Walnut (*Juglans regia* L.) Genotypes

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ABSTRACT

Present study is set to develop a method to proliferate and rooting the Persian walnut (*Juglans regia* L.) in *in vitro* condition. To evaluate the proliferation of three genotypes, the DKW basal medium supplemented with four different concentrations of BAP (0, 0.5, 1 and 1.5 mg L⁻¹) and IBA (0.01 mg L⁻¹) conducted in three replications under factorial randomized block design were used. The root induction evaluated by using DKW medium supplemented by three different concentrations of IBA (0, 3 and 4 mg L⁻¹) for one week. By study of growth factors, we conclude, the BAP (1.5 mg L⁻¹) have shown appreciable results and the Chandler genotype were more responsive to the concentration compare to Hartley and Z60 genotypes. To root induction and callus initiation, IBA (3 mg L⁻¹) were found to be responsive hormone with 7 days in dark incubation. The Chandler genotype compare to the other genotypes studied here, were more responsive to this hormonal level by showing high root percentage.

Key words: Persian walnut, proliferation, rooting, growth regulators

INTRODUCTION

Walnut belongs to Juglandaceae family and *Juglans* genus. The Persian walnut (*Juglans regia* L.), a dominant species of Juglandaceae family is originated from high hills of central Asia. Grafting is the most widely used way to propagate the walnut plants, globally. It can be propagated by seed and cutting but the seed originated offspring will often results variation and the other ways are slow and time consuming (Al-Mizory and Mayi, 2012). Tissue culture techniques are often use for fast plants proliferation. *In vitro* proliferation is an efficient alternative to the other methods, like grafting, which is fast and fulfill the genetic stability. Therefore, micro propagation soon became one of the biggest research fields to walnut propagation (Gruselle and Boxus, 1990; Silva and Dias, 1997; Navatel and Bourrain, 2001; Lopez, 2004; Breton *et al.*, 2004; Vahdati *et al.*, 2009).

Slow regeneration and rooting, phenolic compounds and endogenous bacterial contaminations are the main barriers to successful *in vitro* proliferation in Persian walnut protocol.

Study on *in vitro* propagation of chandler genotypes showed early fruiting of these plants compare to the genotypes proliferated with conventional methods. In addition, they had well rooting system and no incompatibility between stock and scion were seen (Hasey *et al.*, 2001; Lopez, 2001). Successful use of fresh wing tips, nodal and meristem as explant were reported (Leslie and McGranahan, 1998; Vahdati *et al.*, 2009; Meynier, 1984; Heile-Sudholt *et al.*, 1986). The culture contamination during *in vitro* culture should be consider. Seventy percent alcohol, sodium hypochlorite (2.5%), calcium hypochlorite and mercuric chloride (0.01%) for exogenous contaminations and antibiotics recommended to control the inner tissues adulterations (Sticklen and Oraby, 2005; Ainsley *et al.*, 2001; Koubouris and Vasilakakis, 2006; Sharma *et al.*, 1999). Also, nano silver particles suggested to control the bacterial contaminations (Nomiya *et al.*, 2004; Sondi and Salopek-Sondi, 2004).

The toxic substances released from walnut explants are one of the other problem to explant establishment (Preece *et al.*, 1989). Therefore, establishment phase in *in vitro*

culture is very crucial to successful walnut micro propagation. Addition of antioxidants (ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), dithiothreitol (DTT) and albumin) in to the culture medium, incubation of explants before culturing, low light or dark condition in initial days of culturing and repeated sub culturing are successful percussions to this stage (Leslie and McGranahan, 1998; Liu and Han, 1986; Heile *et al.*, 1984; Navatel and Bourrain, 2001).

Proliferation and rooting are the main aims of *in vitro* protocols. The DKW medium known to reach to this target. Moreover, different concentrations of BAP, IBA and BA found to be stimulant to increase the walnut vegetative buds induction, propagation and rooting (Saadat and Hennerty, 2002; Driver and Kuniyuki, 1984; Penuela *et al.*, 1988; Amiri, 2005; Jay-Allemand *et al.*, 1992; Tetsumura *et al.*, 2002; Sanchez-Zamora *et al.*, 2006). In recent years, the walnut micro propagation methods are highly developed but successful shoot and root developments still in demand to commercialize the protocol. Therefore, present study is set to develop a method to proliferate and rooting the Persian walnut in *in vitro* condition.

MATERIALS AND METHODS

Explant preparation, sterilization and establishment: The newly grown shoots (40-60 cm) were collected from three high yielded genotypes (2-3 years old) (Chandler, Hartley and Z60) from the institute of horticultural research in Karaj, Iran in April 2014. The shoots were cut to the nodal segments. The explants were kept under three stages of sterilization treatment.

Because of different explants sensitivity to the sterilization chemicals and their contamination levels, the soft stems (70% ethanol for 30 sec, 1.5% sodium hypochlorite combine with 2-3 drops of twin 20, 2.5% nano silver for 15 min, distilled water wash for 3 times, 5, 10 and 15 min) and the semi hard stems (70% ethanol for 40 sec, 1.5% sodium hypochlorite combine with 2-3 drops of twin 20 for 10-12 min, 2.5% nano silver for 20 min, distilled water wash for 3 times, 5, 10 and 15 min) surface sterilized separately. The explants dried up with filter paper before culturing. Driver-Kuniyuki-Walnut (DKW) medium supplemented with 2.1 g L⁻¹ Phytigel (Sigma Chemical Co.), 30 g L⁻¹ sucrose, 1 mg L⁻¹ BAP and 0.01 mg L⁻¹ IBA was used for establishment phase. To control the negative effects of phenols, the cultured explants incubated for 24 h at 4°C in dark. The explants were maintained in canning jars (0.3 l), at 25±2°C under 16 h photoperiod and light intensity of 40-60 μmol m⁻² sec⁻¹ supplied by cool white fluorescent lamp.

Proliferation: The contaminated free *in vitro* samples which grown well in establishment phase were used. To evaluate the proliferation of three genotypes, the DKW basal medium supplemented with four different concentrations of BAP (0, 0.5, 1 and 1.5 mg L⁻¹) and IBA (0.01 mg L⁻¹) conducted in three replications under factorial randomized block design were used (Driver and Kuniyuki, 1984). All the cultures were sub cultured to the fresh medium of the same composition every four weeks. At the end of multiplication phase, shoot length, shoot number, number of leaves and callus size, were recorded (Fig. 1).



Fig. 1 (a-f): *In vitro* propagation of Persian walnut (a, b) Explant preparation, (c) Establishment, (d, e) Proliferation and (f) Rooting

Rooting: In root induction phase, regenerated shoots (3-5 cm), were excised and transferred onto rooting media. The root induction evaluated by using DKW medium supplemented by three different concentrations of IBA (0, 3 and 4 mg L⁻¹) and were kept in darkness at 24±1°C for one week (Driver and Kuniyuki, 1984). In root development phase, the mix of hormone free, quarter-strength DKW: vermiculite to conduct soil aeration and root penetration were used. After 21 days, the root percentage, root length and number of roots conducted in three replication under factorial randomized block design were examined.

Statistical analysis: All statistical analysis of this experiment were analyzed using SAS software. The Duncan's multiple test range were used for mean comparison and all graphs draws by Excel program.

RESULTS AND DISCUSSION

Effect of benzylaminopurine on proliferation: Analysis of variance shows the significant difference on different BAP levels (1%) and the interaction effects between BAP and genotypes on shoot length and callus size (5%) and shoot number (1%) were significant (Table 1). According to the mean comparison results (Table 2), there were significant difference among BAP levels on studied factors. In all factors, between control and other hormonal (BAP) treatments level were significant difference and by increasing the BAP levels, shoot length, shoot number and leaf number also increased. But, two BAP levels (0.5 and 1 mg L⁻¹) did not show any significant difference while, the highest callus size were found in 1 mg L⁻¹ BAP and decreased by 1.5 mg L⁻¹.

Factors observations (shoot number and callus size) after 30 days of culturing showed the significant difference among genotypes and factors. The Chandler genotype showed more number of shoots (2.35) but two other genotypes, Hartley (1.33) and Z60 (1.48), did not show any significant difference in shoot number (Fig. 2). Genotype Z60 (2.91 mm) had lowest

callus size and were significantly different with chandler (3.99 mm) and Hartley (4.15 mm) genotypes (Fig. 3). In similar study on two different walnut genotypes (pre mature dwarf and normal), the average number of shoots (3-3.3) were observed in dwarf plants (G₄, G₁₂ and G₁₆), which compare to the normal genotypes (G₈) (2.3) were high. Also, the lowest callus accumulation reported in dwarf, compare to normal genotypes which found closely similar to this study results (Vahdati *et al.*, 2009).

The interaction between hormonal (BAP) treatments and walnut genotypes for the factors; shoot length, callus size and shoot number were significant at 5 and 1%, respectively. According to the results (Fig. 4), by increasing the hormonal level (BAP) a notable increase were observed in chandler genotype on shoot length (4.08 cm) and shoot number (4) in 1.5 mg L⁻¹ BAP concentration, followed by genotype Z60 (3.42 cm) which were significantly different with Hartley genotype in shoot length. The best treatment in callus size (showed the lowest callus size) was control treatment in all genotypes followed by genotype Z60 (3.14 mm) at BAP (0.5 mg L⁻¹) level.

The treatment effects (BAP) on genotypes had significant difference with control in all factors, which by increasing the BAP level up to 1.5 mg L⁻¹, shoot length, shoot number and leaf number gradually increased, unlike callus size which this concentration had less effect compare to 1 mg L⁻¹ on it. The interaction effect of BAP and genotypes on shoot length and callus size (1%) and shoot number (5%) were significant. Observations confirms that, the best proliferation hormonal (BAP) concentration was BAP (1.5 mg L⁻¹) for chandler genotype with highest shoot number.

Induction of cell division, *in vitro* initiation and growth are the roll of cytokinins. Benzylaminopurine (BAP), zeatin, kinetin, 2ip and TDZ are the widely used cytokinins (Neuman *et al.*, 1993; Makara *et al.*, 2010). In this study, the treatment of explants with BAP hormone showed significant difference in studied factors. By increasing the BAP concentration, shoot length, shoot number and leaf number

Table 1: Analysis of variance for different benzylaminopurine levels and genotypes on proliferation and their interactions

Callus size	Leaf No.	Shoot No.	Shoot length	df	SV
5.47**	1.67 ^{ns}	3.65**	0.475 ^{ns}	2	Genotypes (A)
49.48**	124.85**	11.71**	16.850**	3	Treatment (BAP) (B)
2.02*	1.62 ^{ns}	0.69**	0.670*	6	A×B
0.717	0.912	0.175	0.219	24	ER
22.95	17.24	39.22	32.63		CV

***Significant at 5 and 1%, respectively, ns: Non-significant, BAP: Benzylaminopurine, df: Degrees of freedom

Table 2: Mean comparison of studied factors for proliferation under different benzylaminopurine levels

Callus size (mm)	Leaf No.	Shoot No.	Shoot length (cm)	Factors
				Treatment (BAP mg L ⁻¹)
0.3367 ^c	0.353 ^d	0.084 ^c	0.112 ^c	0
4.027 ^b	5.556 ^c	1.88 ^b	2.317 ^b	0.5
5.738 ^a	6.944 ^b	2.22 ^b	2.554 ^b	1
4.661 ^b	9.111 ^a	2.70 ^a	3.290 ^a	1.5

BAP: Benzylaminopurine

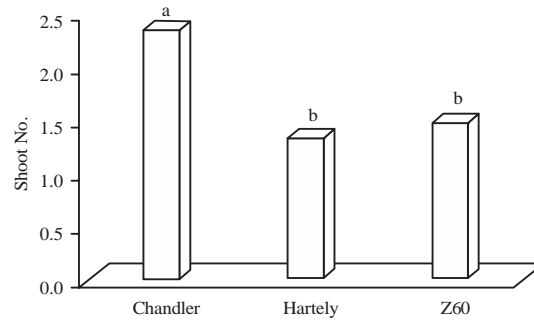


Fig. 2: Mean comparison of shoot numbers on different genotypes

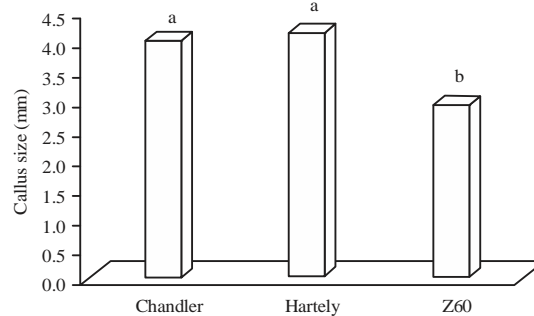


Fig. 3: Mean comparison of callus size on different genotypes

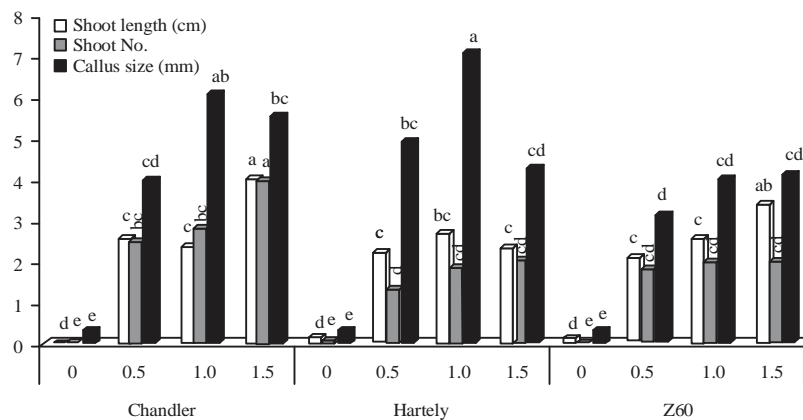


Fig. 4: Mean comparison of BAP (0, 0.5, 1 and 1.5 mg L⁻¹) and genotypes interaction on different factors

were increased which were similar to previously studied results (Bosela and Michler, 2008; Gotea *et al.*, 2012). Recently, the best results achieved by BAP combine with IBA and high concentrations of BA with IBA in walnut *in vitro* culture (Rios *et al.*, 2005; Yari *et al.*, 2014). These support the results of this study and show the importance of cytokinins in walnut proliferation.

Effect of indole butyric acid (IBA) on rooting: The walnut rooting potential is depend on endogenous levels of auxin and its external concentration and initiate by plantlets callus formation (Feito *et al.*, 1997; Rios *et al.*, 2005). Well grown

plantlets selected to transfer to the rooting medium supplemented with hormone. After 21 days, root percentage, root length and root number were studied. The analysis of variance showed the significant difference (1%) among different IBA levels in all factors. The interaction effect between hormonal treatments (IBA) and genotypes were significant (1%) in root number. Also, in genotypes, as a studied factor, the root percentage and root number showed significant difference (1%) (Table 3). The mean comparison of IBA levels in different factors shows significant deference (Table 4). There were significant difference between control and treatments in all factors, which IBA (3 mg L⁻¹) had

Table 3: Analysis of variance of different indole butyric acid levels, genotypes and their interactions for rooting study

Root No.	Root length	Rooting (%)	df	SV
0.481**	0.286 ^{ns}	578.70**	2	Genotypes (A)
13.01**	11.99**	4189.81**	2	Treatment IBA (B)
0.46**	0.133 ^{ns}	162.03 ^{ns}	4	A×B
0.059	0.117	92.59	18	ER
19.24	27.17	30.97		CV

***Significant at 5 and 1%, respectively, ns: non-significant, df: Degrees of freedom, IBA: Indole butyric acid

Table 4: Mean comparison of studied factors under different indole butyric acid levels on rooting

Root No.	Root length (cm)	Rooting (%)	Factors
			Treatment IBA (mg L ⁻¹)
0 ^a	0 ^a	0 ^a	0
2.40 ^a	2.27 ^a	41.67 ^a	3
1.39 ^b	1.49 ^b	30.56 ^b	4

IBA: Indole butyric acid

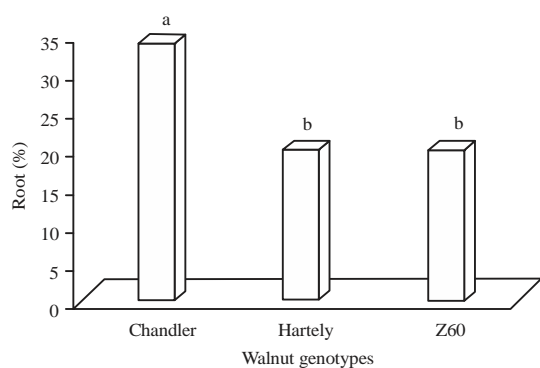


Fig. 5: Mean comparison of rooting percentage on different genotypes

highest root percentage and root length and an increase in root number. But, by increasing the IBA level a decrease in those factors were observed.

Results showed, in callus percentage, the chandler genotype (33.33%) gained more average in this factor but there were no any difference between Z60 and Hartley genotype (19.44%) (Fig. 5). Chandler genotype also found more number of roots per explant (1.53) which were significantly different with Z60 (1.14) and Hartley (1.11). While, the difference between those last genotypes were not significant (Fig. 6). The interaction effect of IBA and walnut genotypes for root number factor were significant (1%). Hence, there were no significant difference among hartley (2.1) and Z60 (1.91) but the chandler genotype (3.16) in effect of IBA (3 mg L⁻¹) were shown significant difference (Fig. 7).

According to the results of this study, we conclude that, the IBA (3 mg L⁻¹) were best hormonal treatment for rooting of walnut micro-shoots which showed significant difference with rest of the treatments. The similar experiments showed the highest rooting rate treated with IBA compare to NAA. Also, the effect of genotype on rooting in different plant species were examined with several other experiments, which are compatible with present study. This shows the need of different hormonal concentrations and rooting induction period

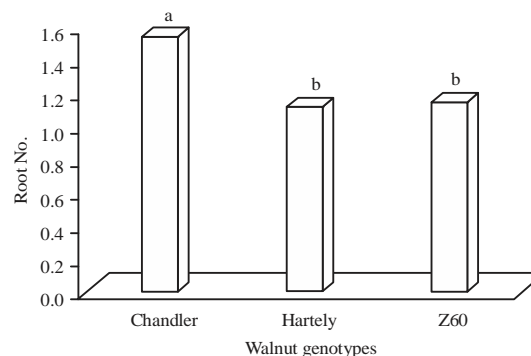


Fig. 6: Mean comparison of root number on different genotypes

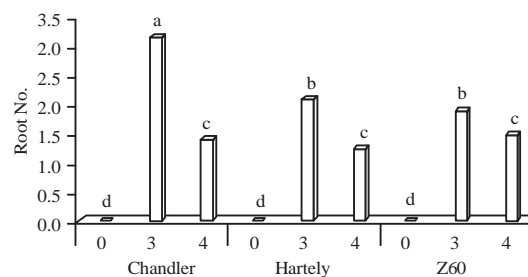


Fig. 7: Mean comparison of root number on IBA (0, 3 and 4 mg L⁻¹) and genotypes interaction

for different genotypes and also shows the effect of endogenous hormonal level on rooting (Pruski *et al.*, 2005; Vahdati *et al.*, 2002; Saadat and Hennerty, 2002; Rugini *et al.*, 1993). The effect of basal MS medium supplemented with IBA found to be more effective than IAA in some cases (Al-Mizory and Mayi, 2012; James *et al.*, 1980).

By study of growth factors, we conclude, the BAP (1.5 mg L⁻¹) have shown appreciable results and the chandler genotype were more responsive to that concentration compare to Hartley and Z60 genotypes. To root induction and callus initiation, IBA (3 mg L⁻¹) were found to be responsive hormone with 7 days in dark incubation. The Chandler genotype compare to the other genotypes studied here, were more responsive to this hormonal level by showing high root percentage.

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