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Micropropagation of Some Dwarf and Early Mature Walnut Genotypes

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Abstract: In current study, the proliferation and rooting ability of some low vigor and early mature seedlings of Persian walnut were compared with those of semi- and high vigor seedlings in *in vitro* condition. To do this, from each vigor group, nodal explants of newly grown shoots of 5- year-old seedlings were cultured on DKW medium. These explants were subcultured every mount and up to 13 times to increase the number of microshoots. Results showed that the number of axillary shoots arising from the microshoots was the highest in the dwarf and semi-dwarf genotypes compared to the high vigor ones (3.3 vs. 2.3). The low vigor genotypes also showed the highest number of nodes per a given size of shoot, smaller shoot size (2.6 vs. 4.5 cm), lower callus formation and higher rooting percentage (63.5 vs. 37.1%). Moreover, these genotypes showed *in vitro* flowering on micro-shoots, which are consistent with the field observations. These results proved the consistency of low vigor, precocity, basitonic growth tendency and easy rooting of dwarf and precocious genotypes under *in vitro* condition. In conclusion, a simultaneous recurrent selection program is recommended for both dwarfing and rooting ability (selection of dwarf/semi dwarf as well as easy to root clones) to utilize their advantages in high-density orchard systems.

Key words: Nodal explants, shoot multiplication, tissue culture, early mature

INTRODUCTION

Persian walnut (*Juglans regia* L.) as its name suggests, originated from Persia (present Iran), where walnut populations exhibit great genetic diversity in terms of tree size and pomological traits (Vahdati and Khalighi, 2001). From there, it spread to west through eastern and central Europe (Greece and Rome) and to east toward Afghanistan, Kyrgyzstan, India and China (Forde and McGranahan, 1996; Vahdati, 2000; Breton *et al*., 2004).

Walnut trees grow very large, making them inconvenient to prune, spray and harvest (Forde and McGranahan, 1996). One of the most important characteristics with an interesting application in walnut breeding is the presence of precocious (early mature) and low vigor walnut genotypes, which are frequently found in some seed sources in Iran (Rezaee *et al*., 2006) or in Kyrgyzstan (Germain *et al*., 1997; Breton *et al*., 2004). The low vigor genotypes could provide the genetic material for tree size control. To date, tree size reduction using genetically dwarf rootstocks has been a key component of high density orchard systems (Cousins, 2005) and many walnut growers are now interested in shifting to high density planting systems (McGranahan *et al*., 1985; Olson *et al*., 2001; Ramos *et al*., 2001).

The main limiting factor in exploring this valuable germplasm is the lack of an efficient vegetative propagation method as result of difficult-to-root nature of Persian walnut species. A number of attempts have been made to propagate walnut by conventional cutting and/or layering (Kuniyuki and Forde, 1985; Gunes, 1999; Vahdati *et al*., 2004). The results of these studies were so disappointing that the conventional methods of vegetative propagation are no longer considered practical (Kuniyuki and Forde, 1985). Meanwhile, a high variability in rooting ability was reported among different seed sources (genotypes) by layering (Vahdati and Khalighi, 2001).

Cultivar or genotype dependent variability in rooting is also reported in peach (Tsipouridis *et al*., 2003), grapevine (Peros *et al*., 1998) and other woody plants (Foster, 1990), suggesting that genotype plays a central role in rooting. Vahdati *et al*. (2008) recently compared the rooting ability of low vigor and precocious walnut

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seedlings of 3-year-old with semi- and high vigor ones in response to modified stool layering method. They concluded that low vigor seedlings display an improved rooting ability in terms of quantity and quality of adventitious root formation, probably because of their lower degree of lignifications, wood density and/or rigidity of their sclerenchyma ring.

While modified layering method provides a simple and effective way of vegetative propagation for conventional nurseries, micropropagation provides more efficient technique for mass and large scale multiplication (Kuniyuki and Forde, 1985; McGranahan *et al*., 1988; Vahdati *et al*., 2004). In addition, one can use tissue culture technique to investigate the behavior of low vigor genotypes in completely homogenous condition rather than heterogeneous field condition.

However, it has been proven that walnut is also hard to propagate through micropropagation. Various attempts have been made using different types of explants, media, culture condition and rooting techniques, with promising results (Driver and Kuniyuki, 1984; McGranahan *et al*., 1988; Scaltsoyiannes *et al*., 1997; Saadat and Hennerty, 2002; Vahdati *et al*., 2002, 2004). Poor proliferation and rooting rate is one of the main obstacles that limit the micropropagation efficiency in some walnut varieties and further researches are needed to allow efficient plant propagation for both scientific and commercial purposes.

As genotype plays a major role in vegetative propagation, in particular for micropropagation (Lupez, 2004; Scaltsoyiannes *et al*., 1997), the present study aimed to compare the response of low vigor and early mature walnut genotypes versus high vigor ones to micropropagation condition, in terms of proliferation and rooting rate, to study their growth consistency using a standard *in vitro* propagation procedure.

MATERIALS AND METHODS

Plant material: Details of the plant material were described by Rezaee *et al*. (2006). In brief, through a nursery selection program (2001-2002), a number of 125 early mature and dwarf seedlings were selected from local nurseries and maintained at the Kahriz Agricultural Research Station in Western Azerbaijan Province, Northwest of Iran (45°10' E; 37° 53' N; 1,325 m a.s.l.). Five years after growing under uniform orchard condition (2005), different morphological traits were recorded and data were subjected to the cluster analysis, which revealed three clusters of high vigor (HV), semi vigor (SV) and low vigor (LV) trees, each with significantly distinctive characteristics. From each of three clusters, a number of four mature trees were chosen as a representative for micropropagation.

Explants preparation: Newly grown shoots, approx. 15-20 cm long, were collected in early May 2006 and leaves were removed, leaving only 1 to 2 cm of the petiole. Shoots were cut into nodal segments, 3 to 5 cm long and then surface sterilized in 0.5% sodium hypochlorite plus one or two drops of Tween 20 L^{-1} for 15 min and after repeated washing with sterile distilled water were cultured on sterile media.

Media and culture conditions: Driver-Kuniyuki-Walnut (DKW) medium supplemented with 2.1 g L^{-1} Phytagel (Sigma Chemical Co.), 30 g L^{-1} sucrose, 4.4 μM 6-benzyladenine (BA) and 0.05 μM indole -3- butyric acid (IBA) (McGranahan *et al*., 1988) was used for shoot induction and multiplication. The pH of medium was adjusted to 5.5 before adding of Phytagel and autoclaving. Micropropagated shoots were maintained in canning jars (0.3 l), four shoots per jar, at 25±2°C under a 16 h photoperiod and light intensity of 40-60 μmol $m^{-2}sec^{-1}$ supplied by cool white Philips fluorescent lamps (Vahdati *et al*., 2004). All the cultures (20 explants per genotype) were transferred to fresh medium of the same composition every four weeks. Contaminated explants and those with sustained growth (no shoot proliferation) were eliminated during the 13 months period. At the end of the multiplication phase, the number of the newly grown auxiliary shoots arisen from each explant, as well as shoot length, number of nodes, presence of flower on shoots, callus and leaf color (based on a scale of 1 to 3) were recorded or visually scored.

In root induction phase, regenerated shoots, 3 to 5 cm in length, were excised and transferred onto full-strength MS salts and vitamins (Murashige and Skoog, 1962), supplemented with 15 μM IBA, 30 g L^{-1} sucrose and 9 g L^{-1} agar (Serva Co.) and were kept in darkness for 7 days at 24±1°C. In root development phase, shoots (20 to 28 explants per genotype) were transferred into glass canning jars (1 l) containing a mix of 1:1.25 (v/v) quarter-strength DKW: Vermiculite (free of hormone) and maintained as described for multiplication (Vahdati *et al*., 2004). Rooting percentage, root length, number of roots, diameter of longest root, callus size and number of nodes per shoot were recorded after two weeks.

The experimental design was a completely randomized design with four replications. The experiment was started with 12 genotypes, but only six were finally remained (other genotypes were eliminated because of contamination or ceased growth). The data were transformed as necessary and were analyzed using analysis of variance (ANOVA), followed by Duncan's mean comparisons test (SPSS, 2002).

RESULTS AND DISCUSSION

Establishment: In this study, 12 genotypes (four genotypes from each of three clusters of vigor) were initially introduced into culture. The number of successfully established genotypes were three (75%), one (25) and one (25%) out of four, in low vigor, semi vigor and high vigor clusters, respectively, indicating better adaptation of low vigor genotypes under *in vitro* condition. The effect of genotypes on the establishment of explants has been previously reported for Persian walnut (McGranhan *et al*., 1988; Lopez, 2004) as well as other tree species (Scaltsoyiannes *et al*., 1997).

Multiplication: Significant differences in multiplication rate, shoot elongation, size of callus and leaf color were observed among the six genotypes (Table 1). Number of newly grown shoots per explant was the highest (3 to 3.3) for low vigor genotypes (G4, G12 and G16) compared to 2.3 in high vigor genotype (G8). The highest (4.5 cm) and the lowest (2.6) elongation of shoots were observed in G8 (high vigor) and G12 (low vigor) genotypes, respectively.

The average number of nodes per explants ranged from 6.6 to 7.5 which were not significantly different among the studied genotypes, but considering the short length of shoots, the number of nodes per a given length of shoot was comparably higher in low vigor genotypes. Moreover, the low vigor genotypes showed less basal callus formation as well as dark green leaves compared to the excessive basal callus formation and lighter color of leaves in high vigor genotype. Higher rate of multiplication, lower shoot elongation and other morphological observations clearly documented the basitonic tendency and limited growth rate of low vigor genotypes which is consistent with their field behavior as explained in earlier publication (Rezaee *et al*., 2006).

An interesting phenomenon was the formation of female flowers on the microshoots of two genotypes (G4 and G12) (Fig. 1A). The flowered microshoots were shorter and bushier than the non-flowered ones. These observation represent the early mature behavior (short juvenile period) in low vigor walnut genotypes, even under *in vitro* condition and are in agreement with the reports of Breton *et al*. (2004).

Rooting: Except for root length and quality, significant differences in terms of rooting percentage, number of roots per explant and root diameter, were observed among six genotypes of different vigor (Table 2). The highest (63.5%) and lowest (37.1%) rooting percentages were observed on low vigor (G12) and high vigor (G8) genotypes, respectively. The low vigor genotypes (Fig. 1B) also expressed the highest number of roots per shoot (2.8 to 3.7) compared to the lowest root number (1.7) on the high vigor genotype, implying substantial structural and or hormonal differences between low vigor and high vigor genotypes. The effect of genotypes on rooting has been also reported by other researchers in

Table 1: Multiplication rate of the established genotypes of Persian walnut after 13th subculture

Genotype	Vigor group	Shoot/ explant	Shoot length (cm)	No. of nodes	Callus size[B]	Leaf color[C]
G8	HV	2.3b[A]	4.5a	7.4a	2.5a	1.3b
G9	SV	2.2b	3.1bc	6.8a	2.3a	1.3b
G16	LV	3.0ab	3.6ab	6.6a	1.6bc	2.7a
G4	LV	3.3ab	2.9bc	7.5a	1.9ab	2.7a
G12	LV	3.3ab	2.6c	7.2a	1.4c	2.6a

[A]Means followed by the same letter(s) are not significantly different ($p \leq 0.01$). [B]Based on 1 to 3 score: 1 = less, 2 = medium and 3 = excessive basal callus formation on microshoots. [C]Based on 1 to 3 score: 1 = light, 2 = medium and 3 = dark color of leaves

Table 2: Rooting ability of established genotypes microshoots

Genotype	Vigor group	Rooting (%)	Root per shoot	Root length (cm)	Root diameter (mm)	Root quality[B]
G8	HV	37.1c[A]	1.7b	3.2a	2.5a	1.9a
G9	SV	38.6bc	3.2a	2.7a	2.2ab	2.2a
G16	LV	40.5bc	3.7a	2.9a	1.8b	2.7a
G4	LV	59.3bc	3.5a	2.9a	1.8b	2.4a
G12	LV	63.5a	2.8ab	2.5a	2.2ab	2.7a

[A]Means followed by the same letter(s) are not significantly different ($p \leq 0.01$). [B]Based on 1 to 3 score:1 = fair 2 = medium and 3 = good distribution of roots on microshoots

Fig. 1: *In vitro* flowering (A) and rooting (B) of low vigor genotype of walnut (G4). bar = 10.0 mm

different plant species (Scaltsoyiannes *et al.*, 1997; Peros *et al.*, 1998; Vahdati *et al.*, 2004), reflecting different levels of endogenous hormonal balances among different genotypes as suggested by Grochowska *et al.* (1984).

The improved rooting ability of low vigor genotypes could be attributed to the lower amount of endogenous gibberellins and lower degrees of lignifications, wood density and rigidity of sclerenchyma ring, which facilitate rooting of micro cuttings. This differential ability of low vigor genotypes was more evident in the case of layering method at field condition (Vahdati *et al.*, 2008).

CONCLUSION

Improved plantlet regeneration as well as rooting ability and *in-vitro* flowering of low vigor genotypes in current study provides more supports on our initial assumptions on the consistency of low vigor behavior as well as their easy-to-root capacity in both layering (field) and micropropagation (*in vitro*) condition. Further researches are needed to develop size reducing clonal rootstocks and or cultivars by selection of easy-to-root and low vigor genotypes both under field and *in vitro* condition.

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