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Rooting ability of Persian walnut as affected by seedling vigour in response to stool layering

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SUMMARY
Walnut (*Juglans regia* L.) is an important nut crop with a "difficult-to-root" characteristic. In this study, the rooting ability of low-vigour and precocious seedlings of 3-year-old Persian walnut was compared with semi-vigorous and high-vigour seedlings using the stool layering method. The results indicated that low-vigour seedlings rooted more (40%) than semi-vigorous (31.42%) and high-vigour (17.14%) seedlings. The average number of roots per shoot (layer) and rooting score (on a scale of 1 – 5) in the low-vigour group were 7.83 and 4.19, respectively, which differed significantly from the high-vigour group. Moreover, most of the high quality adventitious roots formed on low-vigour seedlings appeared to originate from internal tissues compared to the low quality and brittle roots that originated from callus in high-vigour seedlings. Improved rooting of low-vigour seedlings, together with a significant negative correlation between layer size and root number (r = –0.29), reflects substantial structural or hormonal differences among seedlings of different vigour. Approx. 70% of rooted layers survived after transferring to field condition. Our results provide more support for the possibility of vegetative propagation of walnut by conventional stool layering, as well as the selection of easy-to-root, dwarf walnut cultivars or rootstocks on their own roots.

Problems associated with present methods of walnut production on seedling rootstocks of walnut or hybrids include: lack of genetic uniformity, inconvenient orchard management due to large tree size, the threat of lethal black–line disease, as well as a desire for hedgerow growing systems. These have caused renewed interest in the use of Persian walnut (*Juglans regia* L.)-based rootstocks or own-rooted cultivars (Kuniyuki and Forde, 1985; McGranahan and Leslie, 1988; Forde and McGranahan, 1996; Vahdati, 2003; Vahdati *et al*., 2004).

Precocious (early maturing) and low-vigour walnut genotypes, which are frequently found in some seed sources in Iran (Rezaee *et al*., 2006) or in central Asia (Germain *et al*., 1997), could provide the genetic material for tree-size control. To date, tree-size reduction using genetically dwarf rootstocks is 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 and Leslie, 1988; Olson *et al*., 2001; Ramos *et al*., 2001).

The main limiting factor in exploiting this valuable germplasm is the lack of efficient vegetative propagation due to the difficult-to-root nature of Persian walnut species (Kuniyuki and Forde, 1985; Gunes, 1999; Vahdati *et al*., 2004). Micropropagation could provide an effective technique for large-scale multiplication; however, only specialised laboratories could apply this, as there are problems in the rooting and acclimatisation phases (Kuniyuki and Forde, 1985; McGranahan and Leslie, 1988; Vahdati *et al*., 2004).

In contrast, propagation through conventional methods such as layering is not only cost-effective, but also much easier to carry out and most nurseries have the facilities required. A number of attempts have been made to propagate walnut by cutting and/or various types of layering. These showed that the continuity of the sclerenchymatous layer encircling the phloem, as well as the oxidation of juglone during wounding, inhibited rooting and the emergence of walnut roots (Vahdati, 1996; Gunes, 1999). Recently, Vahdati and Khalighi (2001) reported a higher rooting efficiency using a modified "stool layering" method. Stool layering, also known as "tie-off" or "mound-layering", is presently used by an increasing number of nurseries to propagate rootstocks of apple, pear, quince and hazelnut (Hartmann *et al*., 1990; Erdugan and Smith, 2005).

Meanwhile, in Persian walnut, a high variability in rooting ability was reported among different seed sources (genotypes) both by layering (Vahdati and Khalighi, 2001) and by micropropagation methods

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TABLE I
Mean layer size, layer diameter, and number of nodes among seedlings of different vigour in response to stool-layering in 2006

Seedling vigour	No. of rooted seedlings	Layer height (cm)	No. of nodes	Layer diameter (mm)
High-vigour	6 / 35	54.11 a*	10.05 a	14.50 a
Semi-vigorous	11 / 35	37.18 b	9.84 a	12.42 b
Low-vigour	14 / 35	33.66 b	9.16 a	9.66 c

*Mean values in columns followed by a different lower-case letter are significantly different using Duncan's Multiple Range test ($P \leq 0.01$).

(Scaltsoyiannes *et al*., 1997; Vahdati *et al*., 2004). Cultivar- or genotype-dependent variability in rooting was also reported in peach (Tsipouridis *et al*., 2003), grapevine (Peros *et al*.,1998), and other woody plants (Foster, 1990), as well as in *Arabidopsis thaliana*, a herbaceous plant used as a model for molecular studies (King and Stimart, 1998). The results of these studies indicated that rooting ability is a quantitative trait. Improved rooting through the selection of easy-to-root genetic material has been reported in pine (Foster,1990) and *Eucalyptus* (Lemos *et al*., 1997). In some plants, the quantitative trait loci (QTL) related to rooting ability have been identified (Bell and Moran, 2004; Ikedda *et al*., 2007).

In these experiments, we hypothesised that low-vigour and precocious walnut genotypes could influence the rooting ability of walnut due to their different endogenous hormonal and histological features (e.g., higher indole-3-acetic acid (IAA) or gibberellin (GA) contents, and/or higher phloem:xylem ratios) as reported in apple (Faust and Zagaja, 1984; Faust, 1989). Therefore, the main objectives of this study were to evaluate the rooting ability of walnut seedlings of different vigour and to compare the rooting potential of dwarf *vs*. standard rootstock using "stool layering".

MATERIALS AND METHODS

The experiments were carried out at the Kahriz Agricultural Research Station in western Azarbaijan Province, northwestern Iran (45°10' E; 37° 53' N; 1,325 m a.s.l.) during 2005 and 2007. The mean temperature and relative humidity at the experimental site during the growing season (April – October) over 14 years were 18.31°C and 48.15%, respectively. The nursery soil texture was a sandy loam with a pH of 7.9.

Three-year-old walnut seedlings were selected from open-pollinated seedlings of cluster bearing walnut trees at a local nursery in 2005 and 35 seedlings of each were classified into each of three groups of seedling vigour (SV): high-vigorous (HV), semi-vigorous (SV), and low-vigour (LV), with average heights of approx. 170, 100 and 50 (± 20) cm, respectively. In March 2006, seedlings were planted in three separate rows (spaced at 200 cm × 50 cm). The seedlings (stocks) were then heavily pruned to the soil surface.

A modified "stool-layering" method, as described by Vahdati and Khalighi (2001), was used to propagate layers. In brief, three-to-five newly grown shoots (layers) each approx. 15 – 20 cm in height and 0.3 – 0.5 cm in diameter on each stock were fastened with a piece of soft florist wire at their base on 13 May 2006. A mixture of three auxins; indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthalene acetic acid (NAA), each at 7.5 mg g^{-1} and all from Merck (Darmstad, Germany), were dissolved together in 20 drops 50% (v/v) ethanol, then mixed thoroughly with lanolin to form a homogenous paste. Two shallow incisions (wounds) were made just above the wire and the base of all layers were treated individually with a thin layer of the lanolin paste (using a hand brush). A 2:1 (v/v) mixture of sawdust (Spruce fir) and sand was used to cover the base of the layers to a depth of 20 cm, and the layers were finally covered by mounding soil (Vahdati and Khalighi, 2001). The stocks were watered once a week and were sprayed periodically in the hot Summer months to retain adequate moisture around the layers.

Rooting of layers was observed approx. 2 months after layering, but the whole experiment was left undisturbed until March 2007 to collect data as well as to establish rooted layers in soil. The number of stocks with at least one recordable root was counted in each group of seedlings. In rooted stocks, the number of roots per layer, the length and diameter of the longest root per layer, the root score (i.e., a five-point scale where 1 = weak, 3 = medium, and 5 = good roots), callus diameter (cm), and the length and diameter of the layers, as well as the number of buds per layer, were recorded. Data were collected from three layers (as replications) per stock in the three seedling groups and, to increase homogeneity of variances, were transformed by $\sqrt{x + 0.5}$ before statistical analysis. Means were separated using Duncan's multiple range test. The experimental design was a completely randomised design with a hierarchal (nested) arrangement of three-to-five layers in each seedling, and seedlings in three groups of SV. Coefficients of correlation between layer vigour indices (height, diameter, and number of nodes) and rooting ability parameters (root number, callus formation, rooting grade, and length:diameter ratio of the longest root) among all rooted layers (93 cases) in the three groups of seedling were estimated by Pearson's test. SAS software (SAS Institute, 1999) was used to analyse the data.

RESULTS

The number of stocks showing at least one recordable root per layer, from the 35 seedlings initially evaluated

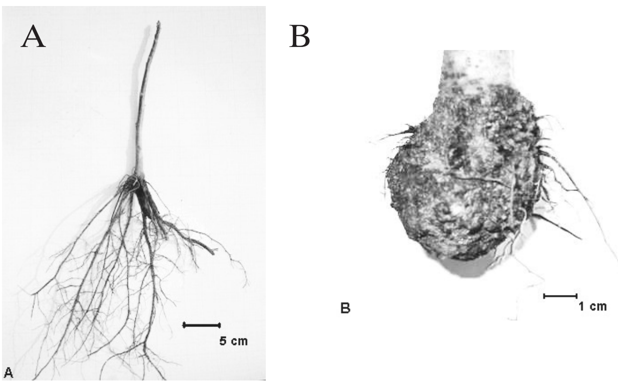

FIG. 1
A well-rooted layer in a low-vigour walnut seedling (Panel A) compared to poor rooting and heavy callus formation in a high-vigour seedling (Panel B) 10 months after treating with a mixture of three auxins and girdling with wire on 13 May 2006.

TABLE II
Mean root number (RN), length and diameter of the longest root (LLR and DLR), root score (RS), and callus diameter (CD) per layer among seedlings of different vigour in response to stool layering in 2006

Seedling vigour	RN	LLR (cm)	DLR (mm)	RS (1 – 5)	CD
High-vigour	3.05 b*	12.09 c	3.00 a	1.88 b	33.00 a
Semi-vigorous	4.27 b	18.66 b	2.54 a	2.57 b	31.33 a
Low-vigour	7.83 a	24.02 a	3.19 a	4.19 a	21.59 b

*Mean values in columns followed by a different lower-case letter are significantly different using Duncan's Multiple Range test ($P \leq 0.01$).

were 16 (40.0%), 11 (31.4%), and six (17.1%), in LV, SV, and HV seedlings, respectively. Significant differences were found among the seedling groups in terms of average layer size and diameter ($P \leq 0.01$). On average, across all the seedlings within a group, LV seedlings showed the lowest layer height (33.66 cm) and the lowest layer diameter (9.66 mm; Table I). A large proportion of the rooted layers in the LV seedlings expressed the lowest callus formation and swelling, along with better adventitious root formation (Figure 1A), and most of them (approx. 70%) were successfully established in field conditions (data not shown). In contrast, over 80% of layers in the HV seedlings showed heavy callus formation and swelling, without any rooting, or with only a few roots of poor quality (Figure 1B).

Statistically significant ($P \leq 0.01$) differences were also observed for most of the parameters related to rooting ability among the three seedling groups (Table II). LV seedlings showed the highest average number of roots per layer (7.83), had the highest average length and diameter of the longest root (24.02 cm and 3.19 cm), and average rooting score (4.1 out of 5.0). Almost all the adventitious roots formed on LV seedlings appeared to originate from the internal layers of the vascular cambium, as shown by disintegration of the vascular cambium through root emergence (Figure 2A). In a few cases (mostly of vigorous layers), we observed a second type of adventitious rooting that appeared to originate directly from callus tissue, since no disruption was observed in the integrity of the vascular cambium at the exact site of rooting (Figure 2B). Roots that developed directly from callus were of lower quality and rooting system (i.e., short and brittle roots). Rooting ability was also different within each seedling group and within layers in a seedling; but, most significantly within the HV seedlings (data not shown). Two seedlings in the LV group (LV-8 and LV-7) exhibited the highest number of roots (12.33 and 10.66, respectively). Good rooting ability was also observed in some seedlings in the SV group (i.e., SV-2 and SV-4) with 9.33 roots per layer, as well as in the HV group (HV-5) with 4.33 roots per layer. Negative correlations ($r = -0.23$ and -0.29, respectively) were observed between layer diameter and height with root number and root length. Higher callus formation was positively correlated ($r = 0.62$) with layer diameter, regardless of seedling vigour (Table III).

DISCUSSION

The number of seedlings showing rooting ability in the LV seedlings was approx. 2.5-times greater than in the HV seedlings. This observation was somewhat unexpected, as the vigorous growth of plants is usually claimed to be one of the major factors contributing to better rooting in many species (Hartmann *et al*., 1990; Cameron *et al*., 2003). Improved rooting of the LV seedlings was comparable with rooting of dwarf apple rootstocks by the stool-layering method (personal observation). This implies that we may require a procedure to re-evaluate easy-to-root genetic material under conventional propagation methods similar to those employed at East Malling for all apple selections (Cummins and Aldwinckle, 1983). Even in HV seedlings, improved rooting occurred mostly on layers with a lower

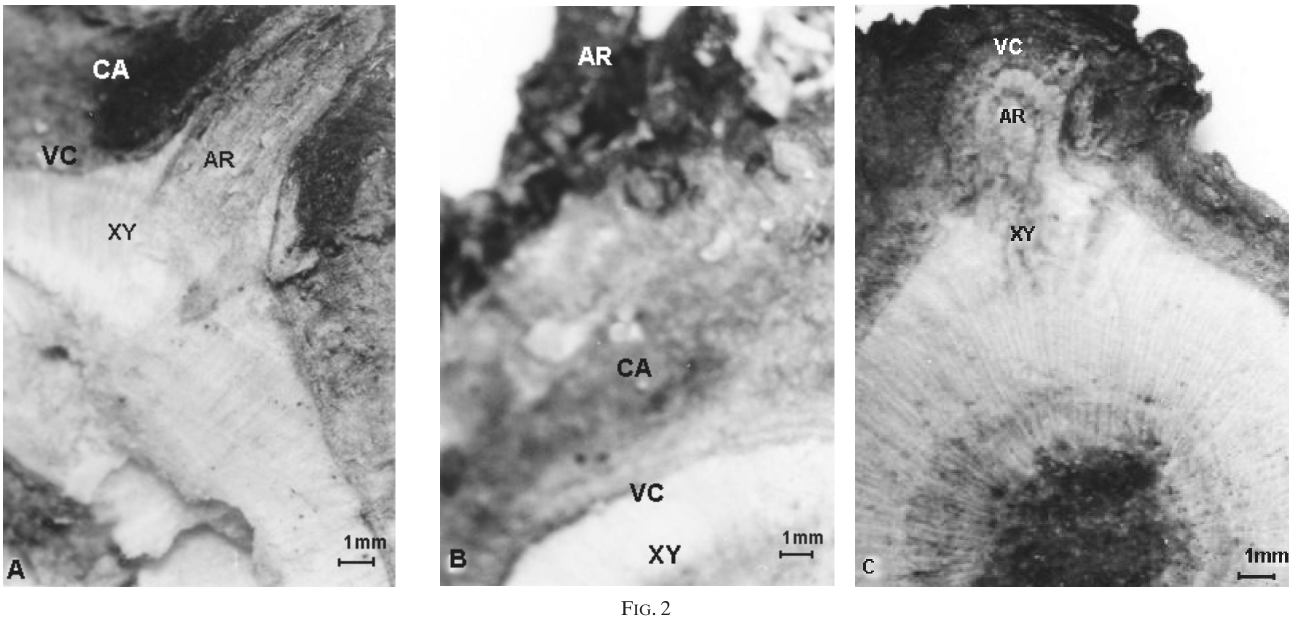

FIG. 2
Trans-sections of layers at the site of rooting showing various kinds of adventitious root (AR) formation. Panel A, an adventitious root that originated from tissue located between the xylem (XY) and vascular cambium (VC). Panel B, poor quality roots originating directly from callus (CA). Panel C, an adventitious root, which emerged, but failed to penetrate. Scale bars = 1 mm.

TABLE III
Pearson correlations between layer height (LH), layer diameter (LD), callus formation (CF), and root number (RN), root length (RL) and root diameter (RD) among seedlings evaluated regardless of seedling vigour

Parameter	LH	LD	CF	RN	RL
LD	0.62**				
CF	0.37*	0.62**			
RN	–0.29*	–0.23*	–0.38*		
RL	–0.07*	–0.23ns	–0.29*	0.48*	
RD	0.08ns	0.06ns	–0.16ns	0.33*	0.57*

*, **Pearson correlations (r) significant at $P \leq 0.05$ or $P \leq 0.01$, respectively: ns, non-significant.

diameter and height and, as a result, we observed a significantly negative correlation (r = –0.29) between layer height and root number. Our results are in agreement with King and Stimert (1998) who found higher rooting in compact and highly branched (i.e., low-vigour) ecotypes of *A. thaliana* L. They concluded that several genes might control rooting ability, with dominant alleles favouring low rooting.

The results of this experiment are consistent with several previous studies (Davis *et al*., 1988; Wiesman and Riov, 1989; Porlingis *et al*., 1999; Henrique *et al*., 2006) that reported the effectiveness of the application of growth retardants (e.g., paclobutrazol as an inhibitor of gibberellins) on improving adventitious root formation in some woody species. Our data also provide further evidence for the presence of endogenous hormonal and histological differences between HV and LV seedlings, and confirm similar data reported in apple between dwarf and vigorous rootstocks (Cummins and Aldwinckle, 1983; Grochowoska *et al*., 1984). Other, indirect evidence to support our observation is that mild water stress applied to severely pruned stock plants improved the rooting of cuttings in the 'Paradox' hybrid (*J. regia* × *J. hindisii*) up to 16% (Hackett *et al*., 2000). Therefore, manipulation of stock plants by applying successive severe pruning of shoots or roots, water stress, and the application of growth retardants that eventually lead to a reduced growth rate (reduced vigour) of stocks, similar to naturally LV seedlings, may be beneficial for increased rooting and needs to be studied further.

The rooting ability of HV seedlings might be impaired by higher amount of gibberellins, and/or as a result of greater lignification, wood density and rigidity of the sclerenchymal ring, so that, in most cases, roots failed to emerge (Figure 2C). A negative correlation between a higher degree of sclerification of the primary phloem and rooting of shoots has been reported in apple selections (Cummins and Aldwinckle, 1983). In the present experiment, we found that callus formation was positively correlated with layer height (r = 0.37) and layer diameter (r = 0.62), which provides another explanation for the poor rooting of HV seedlings, as it was revealed that callus formation usually hinders root formation (Stefancic *et al*., 2005). One implication of this observation is that the optimal concentration of exogenous auxin for LV seedlings may not be suitable for HV seedlings, and needs to be specified for each class of seedling vigour.

Therefore, by reverse selection in favour of LV seedlings, not only is it possible to improve rooting ability, but also to exploit their extensive benefits for increasing fruit yield in a high-density orchard system using Persian walnut. These factors are becoming increasingly important as walnut growers increasingly seek to grow high-density or hedgerow orchards (Olson *et al*., 2001; Ramos *et al*., 2001). Consequently, shifting selection to the benefits of LV seedlings may also limit wood production from this highly-prized wood producing species; however, for maximum efficiency, walnut growers would rather rely on single purpose production units (nuts or timber) instead of double-purpose orchards.

In conclusion, to our knowledge, no other selection of easy-to-root clones has been reported in Persian walnut. Therefore, the best-rooted clones selected in this study could be considered an important source of easy-to-root clones. Furthermore, improved rooting could be expected in successive selection cycles, as has been suggested in other woody and difficult-to-root species (Foster, 1990; Lemos *et al*., 1997). It is also possible to increase the frequency of favourable alleles contributing to rooting ability by inter-crossing or self-crossing of easy-to-root clones for advanced quantitative genetic analysis and QTL mapping of loci that control rooting.

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