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Bench-grafting of Persian walnut as affected by pre- and post-grafting heating and chilling treatments

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SUMMARY
Walnut grafting is more difficult than in most other fruit trees, and increasing the success of grafting, as well as lowering the cost of grafting, is important for commercial nurseries. Two trials were conducted to find the most efficient procedure for walnut grafting. Each trial was repeated twice during 2006 and 2007. In the first trial, the effects of three bench-grafting methods (i.e., side-stub, omega, and whip-and-tongue) were evaluated on the grafting success of four scion cultivars ('Z_{53}', 'Hartley', 'Pedro', and 'Serr'). Omega grafting gave the highest callus rating (scoring 2.6 out of 4.0), the greatest number of callused plants (82%), the most graft-take (71%), and the highest graft survival rate (81%). In the second trial, the effects of pre-grafting, warm forcing treatments, and chilling of callused plants were studied using the side-stub grafting method and two scion cultivars ('Hartley' and 'Pedro'). Warm forcing (26º – 28ºC at 80 – 90% RH) of the scion and rootstock material (for 3 d and 14 d, respectively) along with chilling (2º – 4ºC at 80 – 90% RH for 30 d) of callused plants both showed the highest rates of graft-take (78%) and graft survival (84.6%). The lowest level of graft-take (30%) and graft survival (41.6%) occurred in untreated control plants. Our study provides an alternative method for propagating walnut cultivars under partially controlled growth conditions.

Persian walnut (*Juglans regia* L.) is an important nut crop with specific difficulties for vegetative propagation. As a result, the establishment of seedling orchards of walnut is common in several walnut producing countries. Seedling orchards suffer from high levels of variability of nut yield and quality (Vahdati, 2000; 2003). Due to increasing market demand for high quality nuts and increased productivity, any attempt to find a satisfactory method for vegetative production of Persian walnut would be of value (Millikan, 1971; Vahdati, 2000). Several researchers have studied walnut grafting with varying success (Kuniyuki and Forde, 1985; Rongting and Pinghai, 1993; Rezaee and Vahdati, 2008).

Environmental factors during and following grafting have a major impact on callus formation in walnut (Millikan, 1971; Rongting and Pinghai, 1993; Ebrahimi *et al*., 2006). For successful grafting, the temperature around the graft union should be maintained at approx. 27°C after grafting (Avanzato and Atefi, 1997; Hartmann *et al*., 2001). Although some newly-developed methods such as "hot cable" or "hot callus" have been reported to be successful by some researchers (Avanzato and Atefi, 1997; Hartmann *et al*., 2001), these methods require more skillful workers and expensive facilities. Their application has therefore been limited.

On the other hand, conditioning of the grafting components (i.e., the scions, rootstock, and/or grafted plant) before or after grafting has been suggested to improve callus formation and subsequent grafting success (Millikan, 1971; Tsurkan, 1990; Lantos, 1990; Ozkan and Gumus, 2001). These studies required additional research in order to support their claimed effectiveness on grafting success, before commercialisation.

The main goal of the present study was to evaluate the effectiveness of different bench-grafting methods on propagating different Persian walnut cultivars and to study the effects of pre- or post-conditioning of the grafted components and callused grafts on the success of grafting in Persian walnut.

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MATERIALS AND METHODS

Two experiments were conducted at the Department of Horticulture, College of Abouraihan, University of Tehran, from 2006 to 2008. The rootstocks (seedlings of Persian walnut) were selected in late January and sorted for size (1.5 – 2.0 cm diameter stems) and uniformity. Scions (1 year-old shoots) were collected from the mother trees in the second half of December each year and stored in a refrigerator at 2º – 4ºC on damp paper in a plastic bag until used for grafting. In both Experiments, grafted plants were maintained in a greenhouse with an average diurnal temperature of 26º – 28ºC, and the grafted plants were covered with sawdust with a moisture content of 85 – 90% for 21 d. Before using the sawdust (mainly spruce fir), it was gently rinsed several times with clean water and finally rinsed with a fungicide [1.5% (v/v) benomyl] and drained for approx. 48 h.

TABLE I
Effects of grafting method on callus quality, callused plants, graft-take, graft survival, and scion growth of Persian walnut

Grafting method	Callus quality[†]	Callused plants (%)	Graft take (%)	Graft survival (%)[§]	Scion growth (cm)
Omega	2.6 a*	82 a	71 a	81 a	15.5 a
Side-stub	2.3 b	74 b	58 b	43 b	11.7 a
Whip-and-tongue	1.0 c	40 c	22 c	27 c	8.1 a

[†]Values are the means of a callus scoring rating from 1 (low callusing) to 4 (very high callusing).
[§]Graft survival = (S/G) × 100, where S = the number of surviving plants, and G = the number of graft-takes.
*Mean separation within each column was by Duncan's New Multiple Range Test ($P \leq 0.05$). Values followed by a different lower-case letter were significantly different.

The Experiments were performed in a completely randomised design with a factorial combination of treatments and with three repetitions of each treatment combination. Each plot consisted of 15 seedlings. Callus quality was scored based on a visual scale from 1 – 4, in which 1 = low, 2 = medium, 3 = high, and 4 = very high levels of callusing. The pecentages of callused plants and of graft-take were determined 21 d after grafting, based on the percentage of scions which retained their green bark colour and plump buds (Hartmann *et al*., 2001).

After callusing, the grafted plants were transferred to black polyethylene pots (20 cm × 30 cm) containing a 1:2:1 (w/w/w) mix of sand:soil:leaf-compost and were transferred to a greenhouse with an average diurnal temperature of 25º – 26ºC and a relative humidity of 80% for approx. 3 months for hardening. Graft survival (expressed as the percentage of plants in which the graft took) and scion growth rate were recorded 6 months after grafting. The grafted plants were transferred to a shade-house after the hardening period, before transplanting to the field.

Statistical analyses were conducted using SAS software (SAS Institute, Cary, NC, USA), and means were compared using Duncan's New Multiple Range Test at $P \leq 0.05$. The specific details of each Experiment were as follows:

Experiment I: Three grafting methods (side-stub, omega, and whip-and-tongue) were evaluated using four scion cultivars ('Z_{53}', 'Hartley', 'Pedro', and 'Serr'). All of the grafts were made by the same person using standard methods, as described by Hartmann *et al*. (2001). For side-stub grafting, the basal portion of the scion was cut as a wedge 2.5 cm-long, an oblique cut (at an angle of 20º – 30º) was made into the basal part of the seedling stem, and the scion was inserted, without any fastening material. For whip-and-tongue grafting, a sloping cut (2 – 3 cm in length) was made at the top of the rootstock, then a second downward cut was made, starting one-third of the distance from the tip to the base of the first cut. Similar cuts were made on the base of the scion. The scion and rootstock were slipped together until interlocked and secured with a rubber band, without waxing. The omega grafting method was performed with an omega grafting tool (OMEGA-STAR Co., Germany) without any fastening, similar to the side-stub grafting method.

Experiment II: The interactions between cultivar and warm forcing and/or chilling of scions and rootstocks, as well as chilling of callused plants, were studied. For warm forcing, before grafting, the rootstocks and scions were kept at 26º – 28º C and 80 – 90% relative humidity for 14 d and 3 d, respectively. For chilling of callused plants, immediately after callus formation, plants were stored at 2º – 4ºC and 80 – 90% relative humidity for 30 d. The callused plants were then potted and transferred to a humid greenhouse, with an average diurnal temperature of 25º – 26ºC and a relative humidity of 80% for approx. 3 months for hardening. Grafted plants were planted in black polyethylene pots, then transferred to the humid greenhouse immediately after callus formation.

RESULTS AND DISCUSSION

Experiment I: Omega grafting resulted in the highest quality callus (i.e., average rating of 2.6 out of a maximum of 4.0), the highest percentage of callused plants (82%), the greatest graft-take (71%) and survival (81%), and the highest rate of scion growth (15.5 cm; Table I). Graft-take in the side-stub and whip-and-tongue methods were 58% and 22%, respectively. The percentages of graft survival were 43% in the side-stub and 27% in the whip-and-tongue methods.

The higher success of omega grafting is consistent with the findings of Solar *et al*. (2001), and may be a result of it being performed by machine, leading to a greater coincidence of cambial layers of the rootstock and scion, a requirement for successful grafting in difficult-to-graft species (Hartman *et al*., 2001), as shown in Figure 1.

The lowest level of success was achieved by whip-and-tongue grafting, which was not consistent with the data of Ozcan and Gumus (2001). This inconsistency could be related to the tight binding of the graft union using a rubber band in our Experiment, which may have restricted aeration and/or water/nutrient translocation (Figure 2). Adequate aeration and care of the graft union play important roles in callus formation and the success of grafting (Rongting and Pinghai, 1993; Hartmann *et al*., 2001; Rezaee and Vahdati, 2008).

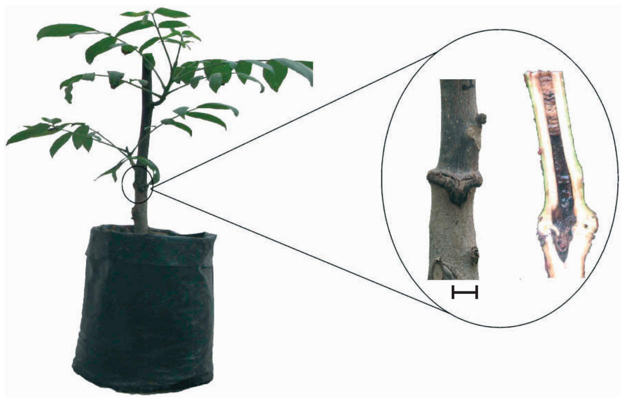

FIG. 1
A grafted Persian walnut plant prepared using the omega grafting tool. A high quality callus bridge was formed (inset) due to the accurate alignment of the cambial layers of the rootstock and scion. Scale bar = 1 cm.

FIG. 2
Whip-and-tongue grafting of Persian walnut. This method interlocks the scion and rootstock components well, but some aspects of the technique restrict callus-bridge formation. Scale bar = 1 cm.

The scion (i.e., the cultivar) did not affect the grafting result (Table II). This observation agrees with Rezaee and Vahdati (2008), and contrasts with the result of Rongting and Pinghai (1993), who reported a dependency of graft-take on walnut scion cultivar. Scion quality may affect the grafting result more than genetic makeup (Rezaee and Vahdati, 2008). The significant difference, in terms of grafting survival (61% *vs*. 42%), between 'Pedro' and 'Z_{53}', presented in Table II, may be considered a type I error. This type of error occurs when we are observing a difference when, in truth, there is none. As a result, mean comparison does not correspond with F-test analysis (ANOVA; Onwuegbuzie and Daniel, 2003).

The interaction between grafting method and scion cultivar was significant for all combinations and measurements. For the percentage of callused plants, 'Z_{53}', 'Hartley', 'Serr', and 'Pedro' ranked highest-to-lowest after omega grafting; but, after whip-and-tongue grafting, the rank from highest-to-lowest was reversed. Similar interactions were observed for callus quality, graft-take, the percentage of graft survival, and scion growth (Table III). In spite of these significant interactions, the difference between grafting methods (i.e., the main effect) for the percentage of callused plants, callus quality, the percentage graft-take, the percentage graft survival, and scion growth were consistent and decreased from omega, to side-stub, to whip-and-tongue (except that 'Serr' that showed a higher graft survival after whip-and-tongue grafting than after side-stub grafting; Figure 3; Figure 4).

The percentages of graft survival and graft-take were positively correlated with callus quality (r = 0.8 and r = 0.95, respectively). These results agree with those of Rongting and Pinghai (1993), who reported that callus quality and the extent of callus formation played an important role in grafting success.

Experiment II: The effects of warm forcing of rootstocks and scions were highly significant for most of the characteristics studied and resulted in a higher grafting success (Table IV). These results agree with Millikan (1971), Tsurkan (1990), Lantos (1990), and Ozkan and Gumus (2001), who reported that the grafting components required an activation of growth prior to grafting for the rapid formation of high quality callus.

TABLE II
Callus quality, percentage of callused plants, graft-take and survival, and scion growth in different Persian walnut cultivars

Cultivar	Callus quality[†]	Callused plants (%)	Graft-take (%)	Graft survival (%)[§]	Scion growth (cm)
'Serr'	2.1 a*	66 a	49 a	53 ab	11.9 a
'Pedro'	2.1 a	66 a	53 a	61 a	11.8 a
'Hartley'	2.0 a	67 a	53 a	45 ab	12.4 a
'Z_{53}'	1.7 a	63 a	47 a	42 b	10.9 a

[†]Values are the means of a callus scoring rating from 1 (poor callus) to 4 (very good callus).
[§]Graft survival = (S/G) × 100, where S = the number of surviving plants, and G = the number of graft takes.
*Mean separation within each column was by Duncan's New Multiple Range Test (P = 0.05). Values followed by different lower-case letters were significantly different.

TABLE III
Interaction of grafting method × cultivar on callus quality, callused plants, graft-take and survival, and scion growth

Grafting method × variety	Callus quality[†]	Callused plants (%)	Graft-take (%)	Graft survival (%)[§]	Scion growth (cm)
'Omega × 'Z_{53}'	2.7 a*	88 a	69 ab	86 a	16.7 ab
Omega × 'Hartley'	2.7 a	88 a	80 a	78 a	17.7 a
Omega × 'Pedro'	2.4 a	74 ab	67 abc	79 a	13.8 ab
Omega × 'Serr'	2.6 a	79 ab	69 ab	81 a	13.8 abc
Side-stub × 'Z_{53}'	1.9 b	82 ab	60 bc	30 cd	10.4 bcd
Side-stub × 'Hartley'	2.3 ab	71 b	60 bc	36 cd	12.2 abcd
Side-stub × 'Pedro'	2.5 a	73 b	61 bc	71 ab	13.0 abc
Side-stub × 'Serr'	2.5 a	71 b	51 c	34 cd	11.0 abcd
Whip-and-tongue × 'Z_{53}'	0.5 d	20 d	12 e	9 d	5.6 d
Whip-and-tongue × 'Hartley'	1.0 c	42 c	19 de	21 cd	7.3 cd
Whip-and-tongue × 'Pedro'	1.2 c	51 c	31 d	34 cd	8.5 cd
Whip-and-tongue × 'Serr'	1.2 c	47 c	27 de	43 bc	10.8 abcd

[†]Values are the means of a callus scoring rating from 1 (poor callus) to 4 (very good callus).
[§]Graft survival = (S/G) × 100, where S = the number of surviving plants, and G = the number of graft takes.
*Mean separation within each column was by Duncan's New Multiple Range Test (P = 0.05). Values followed by different lower-case letters were significantly different.

TABLE IV

Multiple interactions of cultivar × stock-forcing × scion-forcing × chilling on callus quality, callused plants, graft-take, survival, and scion growth

Cultivar × stock-forcing × scion-forcing × chilling	Callus quality[†]	Callused plants (%)	Graft-take (%)	Graft survival (%)[§]	Scion growth (cm)
H × F × F_s × C[‡]	3.2 a*	82 a	73 ab	82 ab	20.2 def
H × F × F_s × c	3.2 a	80 bc	66 abc	58 def	18.7 c
P × F × F_s × C	3.1 ab	86 a	78 a	85 a	22.0 bcd
P × F × f_s × C	3.0 abc	70 de	66 abc	83 ab	22.3 abc
P × F × F_s × c	3.0 abc	80 bc	63 abcd	59 cd	24.0 a
P × F × f_s × c	2.9 abcd	72 d	56 cdefg	57 defg	22.9 ab
H × F × f_s × C	2.8 abcd	81 b	64 abc	79 abc	19.7 def
P × f × F_s × C	2.7 abcde	66 fg	598 cde	77 abcd	22.2 bcd
H × f × f_s × c	2.5 bcde	71 de	49 efgh	56 efg	19.0 efg
H × f × f_s × C	2.5 cdef	51 h	41 ghi	55 efg	18.9 efg
P × f × F_s × c	2.5 cdefg	62 g	50 defg	52 efg	23.3 ab
H × F × f_s × c	2.5 cdefg	78 c	58 cdef	50 efg	18.6 c
H × f × F_s × C	2.4 defg	68 ef	61 bcde	84 a	19.3 efg
P × f × f_s × C	2.1 efg	49 hi	42 fghi	64 bcde	20.3 cde
H × f × f_s × c	1.9 fg	44 ij	30 i	42 g	18.9 efg
P × f × f_s × c	1.9 g	43 j	33 hi	43 fg	23.2 ab

[‡]'Hartley' (H), 'Pedro' (P), forced stock (F), non-forced stock (f), forced scions (F_s), non-forced scions (f_s), chilled plants (C) and non-chilled plants (c).
[†]Values are the means of a callus scoring rating from 1 (poor callus) to 4 (very good callus).
[§]Graft survival = (S/G) × 100, where S = the number of surviving plants, and G = the number of graft takes.
*Mean separation within each column was by Duncan's New Multiple Range Test (P = 0.05). Values followed by different lower-case letters were significantly different.

Chilling of callused plants significantly and positively affected the characteristics studied, except for the number of callused plants and scion growth. Specifically, chilled callused plants performed better in terms of graft-take (64%) and survival (77%) compared to non-treated plants (54% and 52%, respectively).

Scion wood provides the hormones (auxins and gibberellins) necessary for cambial activity and for the differentiation of callus tissue formed to bridge the connection between scion and rootstock (Rongting and Pinghai, 1993). On the other hand, scion wood is the main avenue for the loss of moisture. Therefore, earlier or later scion bud sprouting could have a negative effect on graft survival (Tsurkan, 1990; Rongting and Pinghai, 1993). The increased success of grafting (approx. > 76%) in the case of chilling-treated, callused plants was probably due to a delay of bud break in the scion. Also, storing grafted plants at low temperatures does not completely stop metabolic activity (Tsurkan, 1990). Callus bridge formation continues, and the storage period increases the differentiation and survival of grafted plants by allowing completion of the callus bridge and differentiation of xylem and phloem tissue.

The effects of scion cultivar on the characteristics being studied here were not statistically significant, except for the percentage of callused plants and scion growth (Table IV). Furthermore, the interactions between most combinations were non-significant, except the interactions between rootstock-forcing × scion-forcing, cultivar × callused-plant chilling, and cultivar × scion-forcing × callused-plant chilling, on survival, scion growth, and callus quality, respectively.

Among the multiple treatment interactions, the highest incidence of callused plants (86%), graft-take (78%), and graft survival (85%) were obtained from the pre-conditioned (forced) 'Pedro' scions grafted onto forced rootstocks, and chilled callused plants. The lowest percentages of graft-take (30%) and graft survival (42%) were obtained in 'Pedro' without conditioning of scion or rootstock, or callused plants (Table IV).

In conclusion, the highest grafting success was obtained from omega grafting. However, the high cost of the omega-grafting tool may be a limiting factor for some

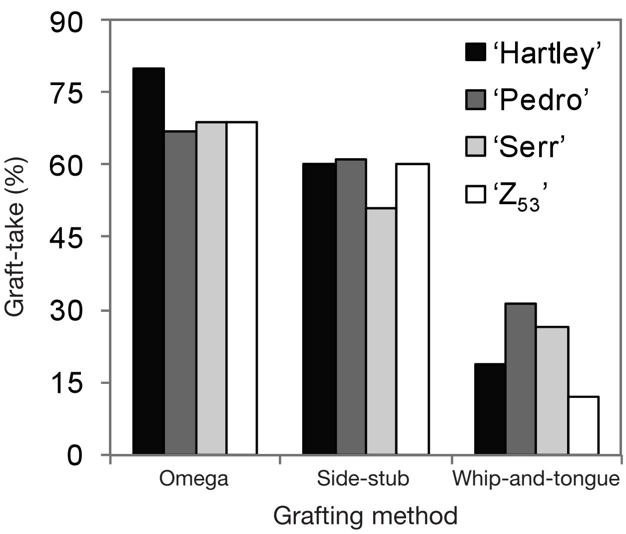

FIG. 3
Effects of grafting method and cultivar on graft-take in Persian walnut.

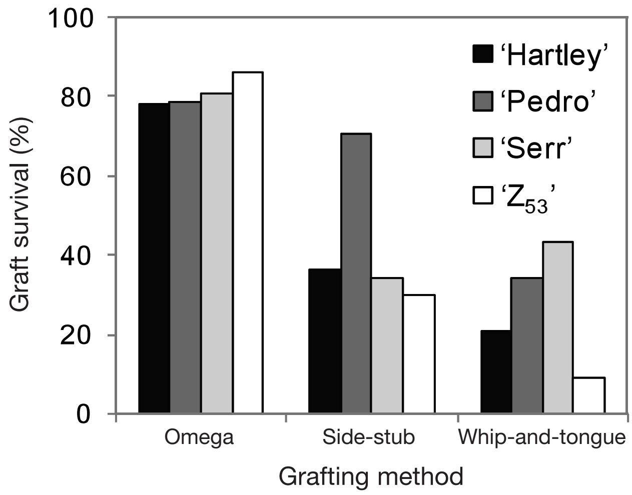

FIG. 4
Effects of grafting method and cultivar on graft survival in Persian walnut.

nurseries. It was also possible to improve grafting success by both warm-forcing scions and/or rootstocks and by chilling callused plants. Considering the difficult-to-graft nature of Persian walnut, and the higher cost of the specialised facilities used for hot-cable and hot-pipe callusing methods (Avanzato and Atefi, 1997; Avanzato, 2001), the results of these experiments are promising. They may provide an effective method for those interested in propagating Persian walnut under partially controlled conditions during early- or mid-Winter. Further research is needed to optimise the normal grafting techniques to obtain more uniform success and subsequent growth of scions, as well as to study the behaviour of bench-grafted Persian walnut trees compared to the conventionally grafted or budded trees under field conditions.

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