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Walnut grafting success and bleeding rate as affected by different grafting methods and seedling vigour

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SUMMARY
Different grafting and/or budding methods were compared in terms of grafting success in 2004 and in 2005. Generally, the optimum period of grafting, under our experimental conditions, was limited to the early Spring and late Summer. Modified bark grafting in mid- April was the most convenient and reliable grafting method for 2- to 3-year-old walnut seedlings, with 80 – 93% grafting success. The method consisted of collecting dormant scions in March, cutting the seedling rootstocks back 1 – 2 weeks before grafting in mid-April, and temporarily covering the graft area with moist sawdust for about 3 weeks before waxing. A higher rate of xylem bleeding in early Spring was a major factor in graft failure. Therefore, in the following experiments (2006), the effect of vigour of the different seedlings and methods of bleeding control were investigated for their effects on the bleeding rate, grafting success, and scion growth. Regardless of seedling vigour, temporary covering of the graft area with moist sawdust resulted in the highest grafting success (>80%) mainly by providing suitable conditions required for better callus formation. Scion growth at the end of the growth season (tree height) was significantly less ($P \leq 0.01$) on seedlings of low vigour compared to high-vigour seedlings (62.31 *vs.* 125.20 cm height), underlining their potential importance in the reduction of tree size for high-density planting systems. Xylem bleeding was also reduced in seedlings of low vigour, although this reduction was not significant during the graft healing period.

Traditionally, walnut trees (*Juglans regia* L.) have been propagated by seed (Kuniyuki and Forde, 1985; Forde and McGranahan, 1996) and seedling orchards are prevalent in most walnut-growing countries. In recent years, market demand has encouraged growers to establish grafted orchards of walnut clones of high yield and quality (Vahdati, 2000). Compared to other fruit trees, walnut grafting is more difficult and requires greater precision in terms of grafting operations, temperature, humidity, and the selecting and handling of scions and stocks (Kuniyuki and Forde, 1985; Avanzato and Tamponi, 1987; Hartmann *et al*., 1990; Rathore, 1991; Vahdati, 2003).

Several pieces of research have been carried out around the World to survey suitable times and methods, as well as to determine physiological barriers, for walnut grafting. Reviewing some of this research, Rathore (1991) concluded that patch-budding performed in mid-June was more promising than other methods under field conditions. On the other hand, Achim and Botu (2001) reported ≥80% grafting success by chip budding in mid-May to mid-June. Recently, on the basis of 8 years of study, Karadeniz (2005) reported that patch-budding in the second-half of August resulted in 29 – 64% grafting success, depending on the year. Other common recommended grafting or budding methods for walnut propagation include modified and normal cleft, splice, whip, and bark grafting, and shield budding (Kuniyuki and Forde, 1985; Hartmann *et al*., 1990; Rongting and Pinghai, 1993; Vahdati, 2003).

Suitable periods and methods of walnut grafting differ from one ecological area to another (Achim and Botu, 2001; Karadeniz, 2005). However, a comprehensive experiment studying all of the grafting and budding procedures under the same conditions has not yet been reported (Vahdati, 2003) and it seems the existing data are either limited or contradictory, and not conclusive.

Some investigations have been carried on walnut grafting under controlled conditions (Avanzato and Tamponi, 1987; Avanzato and Atefi, 1997; Achim and Botu, 2001). However, these methods require specialised facilities and well-experienced personnel. Thus, there is an urgent need for a convenient and cost-effective protocol for walnut grafting in outdoor conditions.

Another major production problem is the size of the tree (approx. 27 m tall) that makes it inconvenient to prune, spray, and/or harvest (Forde and McGranahan, 1996). Genetically precocious and low vigour walnut

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genotypes are frequently found among seedling progenies in Iran (Rezaee *et al*., 2006) or other parts of central Asia (Germain *et al*., 1997) and may provide adequate germplasm for various degrees of tree size control in the future.

This study focussed initially on finding simple and cost-effective protocols for walnut grafting under outdoor conditions. However, our perspectives later broadened to investigate the relationships among seedling vigour, bleeding rate, grafting success and scion growth, as well as environmental variables.

MATERIALS AND METHODS

The experiments were carried out at Kahriz Agricultural Research Station (KARS) located in northwestern Iran, western Azarbaijan province (45°10' E; 37º 53' N; altitude 1,325 m) during a 3-year period between 2004 – 2006. Mean temperatures and relative humidities in our experimental area during the growing season (April – October), over a period of 14 years, were 18.31°C and 48.15%, respectively.

The nursery soil was a sandy loam of pH 7.9, except for the 2005 trial in which a clay loam soil of pH 7.9 was used. Two-year-old walnut seedlings planted at 180 cm × 10 cm were used as rootstocks, except for the third and the fourth experiments, in which 3-year-old seedlings with an in-row spacing of about 30 cm were used.

All of the grafting and budding methods were carried on by the same person and standard methods were used for grafting, as described by Hartmann *et al.* (1990), except that the graft area was covered by moist sawdust for approx. 3 weeks in the modified bark grafting method (method 2). Sawdust (mainly of spruce fir) was washed gently several times in a wooden container and finally rinsed with fungicide [1.5% (v/v) benomyl] and drained for approx. 48 h. A small amount of sawdust, on one side of a plastic bag, was used to cover the graft union and gently wrapped and fastened around the union. After approx. 3 weeks, the sawdust cover was removed and the graft union was covered with grafting wax. In the case of modified cleft grafting, after waxing, soil was mounded on the graft area to minimise temperature fluctuations in late-March. Rootstocks were headed back 1 – 2 weeks earlier than the grafting time to minimise sap bleeding (Kuniyuki and Forde, 1985). Scion materials for early-Spring grafting and budding were removed at the dormant stage from the basal and median portions of 1-year-old shoots of a native, superior variety of walnut (KZ_1) growing at KARS and stored in a refrigerator at 3°C on damp paper in a plastic bag until they were used for grafting in mid-April. Scions required for other grafting or budding methods were collected during the grafting time, as described in Table I. Mother trees had previously been pruned to produce good quality scion wood. The specific details of each experiment were as follows:

Experiment I: Different grafting and budding methods including modified bark grafting in mid-April, cleft (modified and normal) and whip grafting in late-March, shield/patch-budding at three different periods (mid-April, early-July and late-September), and chip budding in early-October, were compared in terms of their grafting success. The experimental design was a randomised complete block, with 11 treatments, each replicated four-times with five seedlings per plot, in 2004. The experiment was replicated in 2005. The percentage of final grafting success was measured at the end of each year, and data were transformed by Arcsin $\sqrt{x + 0.05}$. Means were compared by Duncan's Multiple Range Test (DMRT) using the GLM procedure (SPSS software, 2002). Treatments without any grafting success (0%), including Summer patch and/or shield and chip budding, were not included in the analysis of variance.

Experiment II: This experiment was conducted in 2006 using a complete randomised design with a factorial combination of nine treatments and three replications. Five seedlings were randomly assigned to each of nine treatment combinations. Seedlings were grouped into three vigour categories (high vigour, semi-vigour, and low-vigour) with an average height of 170 ± 20, 100 ± 20 and 50 ± 20 cm, respectively. Three bleeding control methods were tried, including immediate grafting and waxing just after the stocks were headed back (control), grafting and waxing delayed for 1 week after the first rootstock was cut-off (Kuniuki and Forde, 1985), and grafting delayed for 1 week plus the application of moist sawdust on the graft area (modified). The second method of bark grafting (Hartmann *et al*., 1990) was used in this experiment. Data for the percentage of grafting take and scion growth were measured 14, 45 and 185 d after grafting and transformed by Arcsin $\sqrt{x + 0.05}$ and log (x + 10), respectively. Data were subjected to analysis of variance followed by DMRT for means separation using the GLM procedure (SPSS software, 2002).

Experiment III: Trends of xylem bleeding during a 4-week period were monitored in an auxiliary experiment, using 30 seedlings (each 3-year-old) grouped into three classes (high-vigour, semi-vigour and

TABLE I

Effect of different procedures of walnut grafting and budding on percentage graft take during the 2004 and 2005 growing seasons

	Grafting time								
	I. Early-Spring		II. Early-Summer		III. Late-Summer		Averages 2004–05		
Grafting method	2004	2005	2004	2005	2004	2005	I	II	III
Whip graft	5.00 c*	0.00 e	–	–	–	–	2.50	–	–
Cleft graft	20.00b c	18.70b e	–	–	–	–	19.35	–	–
Modified cleft graft	40.00 b	38.80b c	–	–	–	–	39.40	–	–
Modified bark graft	93.33 a	83.70 a	–	–	–	–	88.51	–	–
Patch bud	33.33 b	60.00 ab	0.00 c	0.00 e	46.60 b	0.00 e	46.66	0.00	23.30
Shield bud	40.00 b	36.20 bc	0.00 c	0.00 e	50.00 b	0.00 e	38.10	0.00	25.00
Chip bud	–	–	–	–	0.00 c	0.00 e	–	–	0.00

*Mean percentages of grafting-take in each year followed by different lower-case letters are significantly different ($P \leq 0.01$).

TABLE II
Influence of walnut seedling vigour and sap bleeding control method on mean grafting-take percentages in 2006

	Bleeding control method*			
Seedling vigour	M1	M2	M3	Mean
High-vigour	0.00 c[†]	10.33 bc	80.66 a	30.33 a
Semi-vigour	0.00 c	15.66 bc	90.66 a	35.44 a
Low-vigour	0.00 c	30.33 b	90.33 a	40.22 a
Mean	0.00 c	18.77 b	87.21 a	35.33

*M1, grafting immediately after rootstock cut back; M2, 1-week grafting delay; M3, 1- week grafting delay plus covering graft area with moist sawdust.
[†]Mean percentages of grafting-take values in each row and column followed by different lower-case letters are significantly different ($P \leq 0.01$).

TABLE IV
Influence of walnut seedling vigour on sap bleeding rate after cutting back during a 4-week period from 8 – 29 April 2006

	Bleeding rate ranking* (1 – 9 scale)			
Seedling vigour	8 April	15 April	22 April	29 April
High-vigour	6.80 a[†]	5.80 a	8.20 b	6.20 c
Semi-vigour	6.40 a	5.10 a	6.60 a	5.10 b
Low-vigour	6.30 a	5.10 a	6.10 a	4.00 a

*Bleeding rate was measured on 30 seedlings grouped into three vigour classes on the day after seedling cut back. Scale = 1 – 9, where 1 = only the cut surface of the rootstock was wetted; 9 = entire surface of rootstock wetted by sap flow. Data were analysed by non-parametric Kruskall-Wallis test.
[†]Mean values in each column followed by different lower-case letters are significantly different ($P \leq 0.05$).

low-vigour). All seedlings were cut-off at approx. 15 cm above ground level on 8 April 2006. The bleeding rate was ranked 24 h later on the basis of a scale of 1 to 9, where 1 = only the cut surface of the stock and 9 = the entire surface of the stock wetted by sap flow, and was repeated at 1 week intervals until the end of April. Meteorological parameters were obtained from a climatology station beside the experimental site. Data for bleeding rates and their correlation with environmental parameters were analysed by the non-parametric Kruskall-Wallis and Spearman correlation tests at $P \leq 0.01$, respectively (SPSS software, 2002).

RESULTS

Experiment I: Modified bark grafting in mid-April resulted in the highest grafting take (93.33%), followed by Autumn shield- and patch-budding (each approx. 50%), modified cleft grafting in late-March, and Spring shield-budding (40%), and Spring patch-budding (33.33%) in 2004 (Table I). The lowest grafting success was obtained from normal cleft (20%) and whip grafting (5%). Most of the treatments gave more or less similar results in 2005 to those in 2004, except for late-Summer patch and shield budding, which had no success compared to 46.60% and 50% grafting success, respectively, in the previous year. Budding (patch and shield) in early-Summer and chip budding in early-Autumn failed in both years (Table I).

Experiment II: The percentages of graft take on low-vigour, semi-vigour and high-vigour seedlings were 40.22%, 35.44%, and 30.33%, respectively, indicating a greater, but non-significant ($P \leq 0.01$), grafting success on the low-vigour seedlings compared to semi-vigour and high-vigour seedlings (Table II). However, the effect of bleeding control methods on grafting success was highly significant. Covering the graft area with moist sawdust, plus a 1-week delay in grafting, markedly increased grafting success to approx. 80 – 90% regardless of seedling vigour. Grafting was not successful when rootstocks were grafted and waxed immediately after first heading back, while delaying grafting for 1-week led to an 18.77% success rate, on average (Table II).

Scion growth rate was significantly affected by seedling vigour at 45 d and 185 d after grafting ($P \leq 0.01$). Scion shoot lengths, measured from ground level, on low-vigour, semi- vigour, and high-vigour seedlings at the end of growing season were about 62, 116, and 125 cm, respectively (Table III).

TABLE III
Influence of walnut seedling vigour on mean scion length and growth rate measured at 45 d and 185 d from grafting on 8 April 2006

	Days from grafting		Growth rate	Growth rate/d
Seedling vigour	45	185	(cm)	(cm)
High-vigour	16.50 a*	125.20 a	108.70 a	0.77 a
Semi-vigour	11.50 b	115.55 a	104.05 a	0.74 a
Low-vigour	8.50 c	62.31 b	53.81 b	0.38 b

*Mean values in columns followed by different lower-case letters are significantly different ($P \leq 0.01$).

Experiment III: The rate of bleeding was equally high in all three vigour groups in the first and second weeks after grafting, but in later weeks, bleeding was significantly reduced in the low-vigour seedlings (Table IV). In addition, the bleeding rate showed a significant positive correlation ($r = 0.94$) with the minimum temperature on the previous day (Table V). The highest bleeding rate was recorded after rainy and relatively cold nights on 8 April and 22 April, 2006.

DISCUSSION

The optimum temperature range for callus formation under controlled conditions, which is necessary for graft healing, is about 25° ± 2°C (Avanzato and Tamponi, 1987; Avanzato and Atefi, 1997; Achim and Botu, 2001; Vahdati, 2003). However, grafting success could proceed slowly at lower temperatures of 7° – 20°C (Hartmann *et al.*, 1990). Optimum temperatures (25° ± 2°C) usually prevailed from 20 June – 30 August under our experimental conditions. It could be expected that the maximum temperature increased to ≥ 38°C inside the grafting cover, especially in well-spaced nursery rows and on sunny days. Extreme temperature is detrimental for callus tissue and seemed to be the reason for the failure of all our Summer budding methods (Figure 1) as suggested by Hartmann *et al.* (1990). The contrast between our results and those previously reported for experiments carried in this season (Rathore, 1991; Karadeniz, 2005) may be due to differences in the environmental conditions of the experiments.

We observed the optimum grafting time to be as soon as the mean temperature was about 14° ± 2°C in early-Spring (10 April – 10 May) shortly after bud break. At this time, not only does the rootstock bark slip easily (Hartmann *et al.*, 1990), but also the maximum and minimum day temperatures inside the grafting tape can be expected to reach 25°C and 13°C, respectively; which

TABLE V
Correlations between xylem sap bleeding rate and environmental parameters in walnut seedlings in early Spring 2006

	Temperature			Relative humidity		
Parameter	Mean	Min.	Max.	Mean	Min.	Max.
Xylem bleeding rate	0.61 ns	0.94 **	0.21 ns	–0.17 ns	0.30 ns	–0.28 ns

**, *r* (Spearman) is significant ($P \leq 0.01$), ns = not significant.

are very close to the optimum temperature for callus formation. Moreover, grafting at this time has the advantage of a prolonged time for subsequent growth and hardening of scions for over-Wintering. The cleft grafting method could be started even sooner, in late-Winter (15 – 20 March), when mean temperatures reached approx. 8° ± 2°C, if soil was mounded up around the graft union to minimise temperature fluctuations. This method resulted in 40% grafting success, compared to 20% with normal cleft grafting.

A relatively narrow optimum period suitable for budding was also observed in late-Summer when the mean temperature decreased to 17° ± 2°C. Late-Summer budding showed very high bud-take; but, depending on the year, about half of them did not survive over Winter. Chip budding performed a few weeks later in the Autumn season also failed, presumably for the same reason. These results are supported by Kuden and Kaska (1997) who obtained 93% grafting success using patch-budding on 26 September, followed by transferring the grafted plants to the greenhouse in containers at the end of the season. Covering the Autumn-inserted buds with soil, until the danger of frost had passed, could provide another form of protection in colder regions (Hartmann *et al.*, 1990), but this method was not tested in this experiment.

Some researchers have reported a negative correlation between phenols content and grafting success (Pinghai and Rongting, 1991; Karadeniz *et al.*, 1997). A well-known phenol, found specifically in walnut tree leaf, husk, root and shoot tissues as well as in xylem and phloem sap is juglone (5-hydroxyl-1,4-naphthoquinone), which is of interest due to its chemical reactivity in the formation of free radicals (Solar *et al.*, 2006). The formation of highly reactive free radicals is

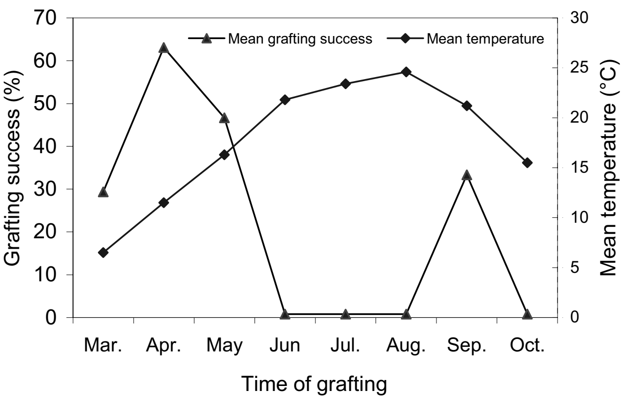

FIG. 1
Optimum period for walnut grafting averaged over 2 years and seven grafting methods used under the climatic conditions of northwest Iran. Grafting success decreased as the mean temperature increased to 25°C. Early-Spring and late-Summer were the most successful periods for grafting.

associated with several oxidative stresses such as hypoxia, extreme temperature, and wounding could play a role in death-inducing signals leading to the irreversible damage of affected tissues through necrosis, apoptosis and cell death (Jones, 2001; Breusegem and Dat, 2006). These could provide another explanation for the complete failure of budding methods performed in the early-Summer.

Total phenolics contents in plant parts were reported to be low in early-Spring and late-Summer, with a peak at the beginning of Summer (Solar *et al.*, 2006) implying the suitability of early-Spring for walnut grafting and budding. However, the higher root pressure of walnut trees in early-Spring, correlated with increased mineral uptake and soil temperatures (Ewers *et al.*, 2001), xylem bleeding, and the accumulation of sap under the graft cover (wax or plastic tape), could result in a disappointing rate of grafting success. In our experiments, we observed high xylem bleeding in high-vigour seedlings in cold and rainy conditions and in heavy soils (clay texture) during and/or shortly after grafting (Figure 2A, B). Our observations are in agreement with Beineke (1983) and Rongting and Pinghai (1993), who reported that the negative effects of sap bleeding are mainly due to decreased aeration levels around the graft union, but the presence of phenols in the sap should not be overlooked. Rongting and Pinghai, (1993) reported that the levels of total phenols and juglone in xylem bleeding sap were very low (0.5 and 0.01 mg 10 ml^{-1}, respectively), and were not enough to have a negative effect on grafting. However, these concentrations could be expected to increase to lethal levels after evaporation.

Among the three varieties of bleeding control methods used in this study, coverage of the graft area for at least 3 weeks with moist sawdust (Figure 2 C), as well as a 1-week delay of grafting, resulted in the highest grafting success (approx. 80 – 93%), regardless of seedling vigour, age, and year of experiment. This could be attributed to the buffering action of the sawdust in absorbing xylem sap and, as a result, providing the moist and aerated conditions suitable for better callus formation and subsequent scion growth without any symptoms of wood rot around the graft area (Figure 2 D–F). Previously reported techniques of bleeding control, such as delaying grafting for 1 – 2 weeks after first cutting-off the rootstocks as well as subsequent slant cuts on the base of the rootstocks (Kuniyuki and Forde, 1985; Vahdati, 2003), were not efficient (approx. 20% grafting success) in our study. In fact, instant waxing of the graft area could restrict air movement and so oxygen may become a limiting factor, inhibiting callus formation, similar to grapevine, in which the graft union is not usually covered with wax or any other air-excluding material during the callusing period (Hartmann *et al.*, 1990).

FIG. 2
Walnut seedlings showing variable sap bleeding rates (Panels A, rated 3; and Panel B, rated 8). Panel C, graft union covered with moist sawdust in modified bark grafting. Panel D, formation of a callus bridge between rootstock and scion 2 weeks after grafting in modified bark grafting. Panels E and F, successful scion growth after grafting. Each scale bar represents 1 cm.

The rate of scion growth (height) was reduced significantly on low-vigour seedlings (62.31 *vs.* 125.20 cm) at the end of the growing season. This could be attributed to their smaller root system and lower growth rate, as explained in other fruit trees (Faust, 1989).

In conclusion, it is possible to obtain high and consistent grafting success in walnut if the grafting is performed in early-Spring or late-Summer, with some modifications of conventional grafting and/or budding methods. The modified bark grafting method reported here is suitable for grafting seedlings more than 2 years-old (≥ 1.5 cm in diameter), making it somewhat less economic for large-scale commercial nursery production. However, walnut growers could apply this procedure by planting seedling rootstocks in their permanent orchard location, then bark grafting them 10 – 15 cm above the ground when the seedlings are well-established. Finally, low-vigour seedlings could be used to reduce tree size in a similar way to that reported in apple and other fruit trees for high-density plantings (Cummins and Aldwinckle, 1983; Cousins, 2005). This was confirmed by considering the final height (≤ 2.5 m) of 20-year-old dwarf and cluster-bearing walnut trees at our experimental site. More attempts are needed to establish seed orchards of low-vigour parents, or to select and breed easy-to-root dwarf and/or semi-dwarf walnut rootstocks.

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