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# Optimizing Early Grafting of Persian Walnut by Evaluating Different Rootstocks, Covering Materials and Grafting Methods

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## Abstract

Grafting on epicotyl of young seedlings has been introduced as a simple and rapid method for walnut propagation. Although this technique improves the grafting success and propagation efficiency, it is technically more demanding than the conventional walnut grafting procedures and is yet to be optimized. This study was aimed to investigate the influence of rootstock type (dwarf and standard genotypes), graft union covering materials (perlite, sawdust, coco-peat and perlite at 1:1 ratio, and without cover - control) and different epicotyl grafting procedures (Gandev-Arnaudov and Frutos) on grafting success and performance of the grafted plants in order to optimize the walnut epicotyl grafting. Walnut seedlings were grown in a greenhouse. After four weeks, dormant shoots of walnut 'Chandler' were grafted to the epicotyl of young walnut seedlings. Grafting success and callusing quality were recorded 30 days after grafting. Growth parameters of the grafted plants, including number of nodes and length of internodes, height and diameter of scion, and survival of the grafted plants, were determined 150 days after grafting. Enhanced grafting success and performance of the grafted scions were obtained by following the Frutos's procedure. Moreover, the highest grafting success (75.1%) and survival of the plants (91.7%) were obtained by using the dwarf rootstock and application of sawdust as graft union cover. In conclusion, performing walnut epicotyl grafting according to Frutos's procedure by using dwarf genotypes as rootstock and covering the graft union with sawdust was suggested to obtain maximum propagation efficiency (68.7%).

***Keywords*:** Callus, Covering materials, Epicotyl grafting, Growth, *Juglans regia* L., Vegetative propagation.

## Introduction

The genus *Juglans* contains about 21 species including the Persian walnut (*Juglans regia* L.), which is widely cultivated in temperate regions (McGranahan and Leslie, 1990; Potter *et al.,* 2002). Walnut cultivars are generally propagated by grafting on seedling rootstocks. Most of the walnut grafting methods are faced with challenges such as long production period and limited graft success frequency. Recently, epicotyl grafting methods have been introduced as a simple and rapid method for walnut propagation. Epicotyl grafting method, with success of about 70%, has higher propagation efficiency than the conventional grafting methods such as omega, with 40% success (Gandev, 2009). However, the efficiency of these methods has not been compared yet. Moreover, since the techniques are newly developed, there is potential for optimization by employing suitable rootstocks and covering materials for graft union.

The grafting method, time of grafting, plant genotype and environmental factors may influence callus establishment, development of vascular connection between rootstock and scion, and the success of grafting. Karadniz (2005) reported that temperature and humidity are the most important environmental

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factors that influence the success of walnut grafting. For a successful grafting, temperature of 27°C for one month must be provided for the grafted walnut, otherwise the healing process will be delayed. In addition, when the temperature dropped below 21°C, the callus tissue will not be established (Rongting and Pinghai, 1990). Preservation of water content of rootstock and scion tissues has a crucial role in grafting success (Szoke, 1990). Callus tissue consists of parenchyma cells and cannot prevent dehydration especially during the early establishment stage. In this context, the occurrence of hot and dry conditions during callus formation leads to rapid desiccation of graft union (Hartmann *et al.*, 2001). Rongting and Pinghai (1990) showed that a reduction in water content of walnut scion to less than 38.5% significantly prevents callus formation. Covering the graft union and keeping the grafted seedlings in environment with relative humidity (RH) of 90-95% limits graft failure by preventing of dehydration of the tissues (Rezaee and Vahdati, 2008). Therefore, tying graft union with polyethylene strips usually results in better graft success than tying with graft threads (Hartmann *et al.*, 2001; Vahdati, 2003). Atefi (1997) showed that covering the graft union using thin aluminum foil results in better humidity preservation and increases the grafting success. In another study, placing grafted walnuts under small plastic tunnels with open sides was suggested to provide a suitable environment for increasing graft success (Gandev, 2016). However, Vahdati (2003) showed that the existence of a thin layer of water on the walnut graft surface is more effective than keeping the grafted plants in an environment with saturated humidity. However, the possibility of gas exchange in graft union is vital to provide enough oxygen for cell division and growth, which is accompanied with relatively high respiration rate (Hartmann *et al.*, 2001; Lopez, 2001; Vahdati, 2003).

In addition to preventing water loss from scion, growth stage of scion and time of grafting determines the success of walnut grafting (Szoke, 1990). Moreover, rootstock also has a crucial role in walnut graft success. Kaeiser *et al*., (1975) reported that the differences in physiological activities and root pressure of walnut rootstocks may affect graft success. High vascular sap exudation due to high root pressure at late spring may increase the risk of walnut graft failure by inducing anaerobic condition at graft union (Vahdati, 2003). Hence, the covering materials used for protecting the walnut grafting point are recommended not only to be able to prevent water loss but should be able to provide sufficient aeration around the developing callus. Therefore, the current research was aimed to evaluate the effects of using different covering materials at the grafting point and the major walnut epicotyl grafting procedures. Moreover, the effect of using dwarfing and standard rootstocks on the success of walnut epicotyl grafting was determined. Seedlings of a cluster-bearing walnut genotype, according to representing dwarfing traits such as short internodes and compact growth, were used as dwarfing rootstock. Seedlings from a terminal-bearing tree, with traits including long internodes and longer current season shoots, were used as standard rootstock.

## Materials and Methods

### *Preparation of rootstocks and scions*

Seeds of a 10-year old cluster-bearing tree and a 12 year-old terminal-bearing tree were collected from an orchard located in Minoodasht, Golestan, Iran. After harvest, husk of the fruits were removed, and the seeds were air dried at room temperature. The seeds were immersed in a bucket of water, and the water was changed every day. On the fifth and tenth days, the seeds were treated with fungicide Captan ($2 mg.l^{-1}$). Walnut seeds were put inside porous plastic bags and kept in 4°C for four weeks. In early-January, the seeds were cultured according to the procedures described by Frutos (2009) and Gandev and Arnaudov (2011) in the greenhouse with the temperature of 25-27°C and RH of 50-60%. To prepare scion wood, one-year old dormant shoots of ‘Chandler’ with a diameter of about 5 to 7 mm were collected during mid-January and preserved in 5 °C. Scions with two buds were prepared from the shoots with 5-8 cm long (Raofi *et al*., 2016). A V-shaped incision to a length of 0.5 to 1.0 cm then was made at the base of the shoots using a sharp blade (Gandev and Arnaudov; 2011). Then, the scions were grafted on the rootstocks according to the procedures described by Gandev and Arnaudov (2011) or Frutos (2009).

***Evaluating different grafting procedures***

*1- Gandev and Arnaudov procedure*: The seeds were sown in a large plastic box (50×30×40 cm) containing coco-peat and perlite at 1:1 v/v ratio. Four weeks after germination, the seedlings were taken out of the box and root tips were cut to promote the formation of new lateral roots. Then, the seedlings were cut at 1.5±0.5 cm above the epicotyl with a sharp scissor (Fig. 1a) and then a 1.5±0.5 cm cut was made in the middle of the stem with a sharp knife (Fig. 1b). At last, the prepared scion was inserted into the incision (Fig. 1c), and the graft unions were covered with different covering materials and tied with parafilm stripes (Fig. 1d). The grafted plants were transferred into the container with the same media used for seed germination. The container was then covered with a plastic sheet in order to provide the RH about 80% and kept in a greenhouse with temperature of 26±1°C.

*2- Frutos Procedure*: The walnut seeds were sown in plastic pots containing coco-peat and perlite (1:1 v/v). The seedlings were cut at 2 cm above the epicotyl. Grafting was performed as described above and the pots were then placed inside large containers, which were covered with a plastic sheet in order to provide the relative humidity of 80%. The containers were placed inside the greenhouse with the same condition describe above.

***Evaluating different covering materials around the grafting point***

In order to preserve water content of the grafted trees, efficiency of three kinds of covering materials including 1) perlite, 2) sawdust and 3) coco-peat and perlite (C+P) at 1:1 ratio, along with control treatment (without cover) were evaluated. Sawdust was autoclaved for 30 minutes prior to application. Coco-peat and perlite were also soaked in water to maintain moisture. The graft union was tied with parafilm after covering with proper treatment.

***Traits measured***

Thirty days after grafting, graft success percentage and callus quality were recorded (Fig. 1E). Growth parameters of the grafted plants including number of nodes and length of internodes, length and diameter of scion shoot and survival of the grafted plants were measured 150 days after grafting. The measurement of callus quality in terms of callus development frequency and scion-rootstock connection establishment were performed through visual observations. In terms of scoring, callus masses were divided into four classes (very weak: 1, weak: 2, Good: 3, and very good: 4).

The experiment was performed as factorial based on completely randomized design including three factors of rootstock type (dwarf and standard genotypes), covering materials (perlite, sawdust, coco-peat and perlite at 1:1 ratio, and control treatment without cover) and grafting procedure (Gandev-Arnaudov, and Frutos methods) with 15 replications. The data was subjected to analysis of variance (ANOVA) and the means were compared using Duncan’s multiple range tests at $P \leq 0.05$ using the SAS software.

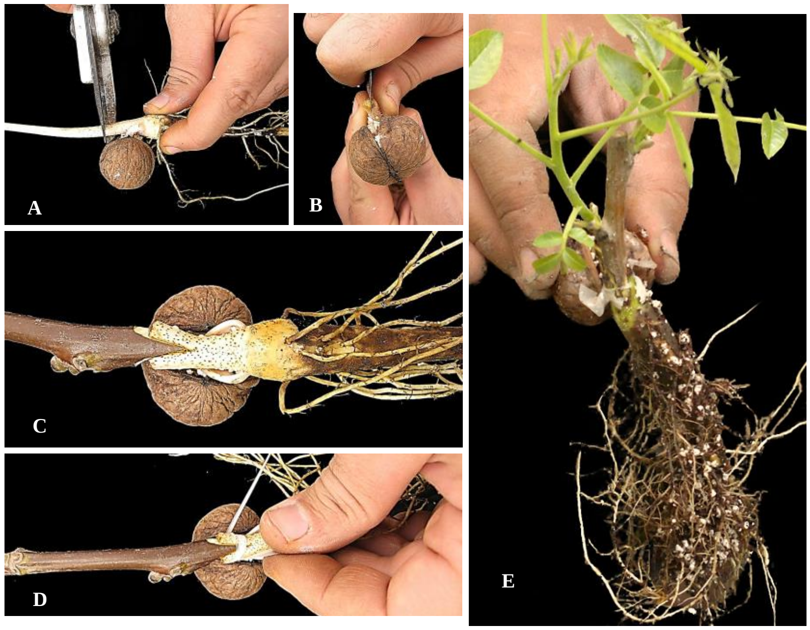


Fig 1. Grafting process of young *Juglans regia* seedlings; A) cutting the rootstock from 1.5±0.5 cm above the epicotyl, B) making a 1.5±0.5 cm cut in the middle of the rootstock stem, C) inserting the prepared scion into the rootstock, D) covering the grafting point with a parafilm stripe, and E) the grafted plant, 30 days after grafting.

## Results

### *Graft success*

*Callus quality*: The rootstock type and graft union cover significantly affected callus quality (Table 1). The callus that developed on the graft union in the standard rootstock had better quality (3.05) than those of the dwarf rootstock (2.42 - Fig. 2A). Sawdust cover was found to be more suitable for establishment of better callus (3.4), followed by cocopeat and perlite mixture (3.15) and perlite (2.45). Moreover, the callus established in the control (1.95) had less quality (Fig. 2B).

Graft success: The type of rootstock, type of cover used around the graft union and grafting procedure had significant effects on graft success percentage measured 30 days after grafting (Table 1). The dwarf rootstock showed higher percentage of success (58.4%) than the standard type (48.5% - Fig.3A). Using sawdust around the graft union led to the highest percentage of graft success (75.1%), followed by cocopeat and perlite mixture, and perlite with 66.8% and 38.5%, respectively. The control had the lowest percentage of success (33.5% - Fig.3B). The plants grafted according to the Frutos procedure showed a higher success percentage (60.1%) than those grafted by the Gandev-Arnaudov procedure (46.8% - Fig. 3C).

*The survival of grafted plants*: The triple interaction of rootstock × covering material × grafting procedure significantly affected survival of the grafted plants (Table 1). The highest survival (91.7%) was observed on the dwarf rootstock grafted by the Frutos procedure and was covered with sawdust (Fig. 4). Plants in which the graft union was covered with sawdust showed a higher percentage of survival in both types of rootstock and both types of grafting methods. Using the Frutos procedure and dwarf rootstock resulted in a higher percentage of survival in all of the covering materials in comparison to the Gandev-Arnaudov procedure and the standard rootstock. Using the standard rootstock and Gandev-Arnaudov procedure in all of the covering treatments resulted in a lower percentage of survival, and the lowest percentage of survival (21.2%) was in control plants without covering materials around the grafting union.

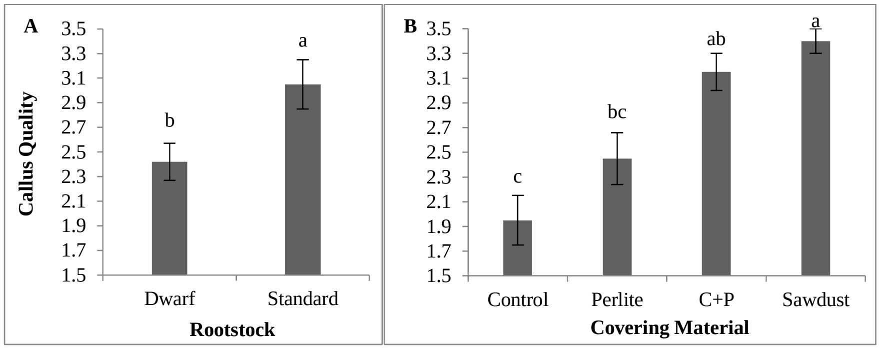


Fig 2. Effect of A) rootstock type and B) graft point covering materials (Control: without cover, C+P: coco-peat + perlite) on callus quality of early grafted *Juglans regia*.

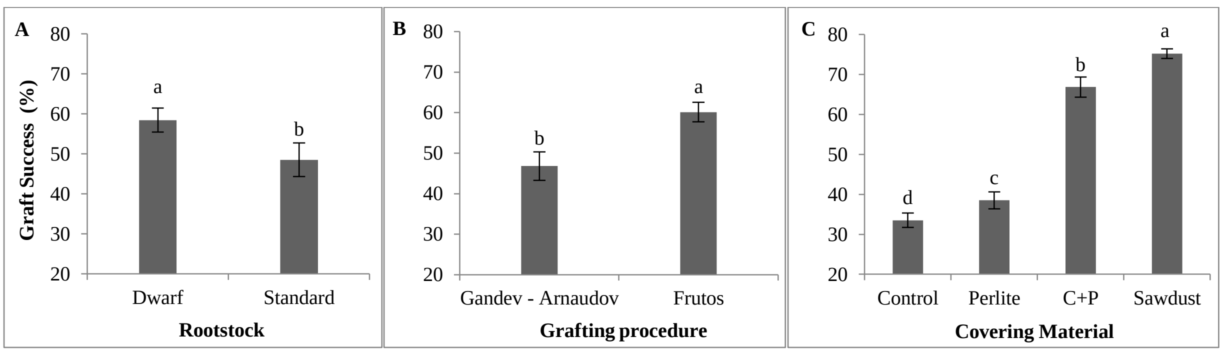


Fig 3. Effect of A) rootstock type and B) covering materials (Control: without cover, C+P: coco-peat + perlite), C) grafting procedures on graft success of *Juglans regia* after 30 days.

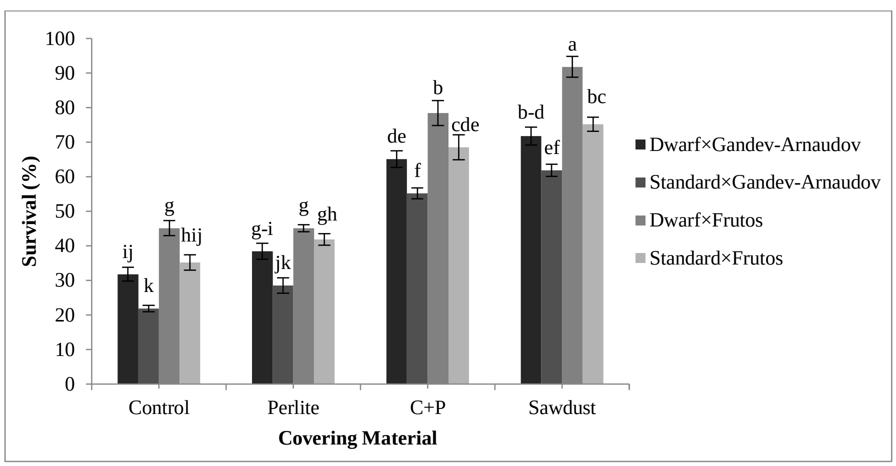


Fig 4. Interaction effect of the rootstock × grafting procedure × covering materials (Control: without cover, C+P: coco-peat + perlite) on survival percent of grafted seedlings of *Juglans regia* after 150 days

**Table 1. Results of analysis of variance (mean square) for measuring scion growth parameters and success of *Juglans regia* early grafting.**

| | df | Leaf No. | Node No. | Shoot Height | Shoot Diameter | Shoot No. | Internode Length | Callus Quality | Grafting Success | Survival Percent |
|---|---|---|---|---|---|---|---|---|---|---|
| Rootstock | 1 | 391.61$^{**}$ | 59.51$^{**}$ | 249.21$^{**}$ | 17.86$^{**}$ | 0.20$^{ns}$ | 13.32$^{**}$ | 7.81$^{**}$ | 18.8$^{**}$ | 334.2$^{**}$ |
| Cover | 2 | 1.37$^{ns}$ | 3.91$^{ns}$ | 14.67$^{*}$ | 0.51$^{ns}$ | 0.13$^{ns}$ | 0.14$^{ns}$ | 8.74$^{**}$ | 8480.7$^{**}$ | 4126.0$^{**}$ |
| Grafting procedure | 1 | 6.61$^{ns}$ | 1.51$^{ns}$ | 0.57$^{ns}$ | 0.28$^{ns}$ | 0.05$^{ns}$ | 0.02$^{ns}$ | 2.81$^{ns}$ | 3555.7$^{**}$ | 19012.8$^{**}$ |
| Rootstock × Cover | 2 | 1.51$^{ns}$ | $^{ns}$2.34 | 10.56$^{ns}$ | 1.24$^{*}$ | 0.02$^{ns}$ | 0.05$^{ns}$ | 0.24$^{ns}$ | 37.01$^{ns}$ | 199.0$^{**}$ |
| Rootstock × Grafting | 1 | $^{*}$19.01 | 0.31$^{ns}$ | 1.01$^{ns}$ | 0.20$^{ns}$ | 1.25$^{*}$ | 0.01$^{ns}$ | 0.11$^{ns}$ | 0.0$^{ns}$ | 125.2$^{*}$ |
| Cover × Grafting | 2 | 6.71$^{ns}$ | 1.07$^{ns}$ | 3.33$^{ns}$ | 0.23$^{ns}$ | 0.18$^{ns}$ | 0.0$^{ns}$ | 0.84$^{ns}$ | 37.01$^{ns}$ | 606.6$^{**}$ |
| R × C × G | 2 | 4.04$^{ns}$ | 1.74$^{ns}$ | 0.89$^{ns}$ | 0.07$^{ns}$ | 0.18$^{ns}$ | 0.06$^{ns}$ | 0.01$^{ns}$ | 37.01$^{ns}$ | 162.1$^{**}$ |
| C.V. | | 12.7 | 9.3 | 5.4 | 19.4 | 20.3 | 10.0 | 11.9 | 23.5 | 19.8 |

**, * Significant at 0.01 and 0.05 probability level, respectively. $^{ns}$. Non-significant.

***Performance of the grafted plants***

The interaction of rootstock × grafting procedure caused a significant difference in leaf number per scion (Table 1). Using the Frutos procedure and standard rootstock resulted in formation of higher leaf number (11.2 leaf), and the dwarf rootstock and Frutos procedure produced the lowest leaf number (6.2 leaf) per plant (Fig. 5).

The number of nodes was significantly higher in the plants grafted on the dwarf rootstock (8.2 No.) compared to the standard (6.5 No.) rootstock (Fig. 6). The type of rootstock used in epicotyl grafting had significant effect on internode length (Table 1). The scions on the standard rootstocks had longer internodes (1.8 cm) compared to the dwarf (1.0 cm) rootstock (Fig. 6).

Rootstock type had a significant effect on shoot length of the grafted plants (Table 1). The dwarf rootstock had shorter shoots (8.2 cm) compared to the standard (11.7 cm) rootstock (Fig. 6). The interaction of rootstock × covering material significantly affected shoot diameter (Table 1). Using sawdust cover on standard rootstock (5.5 mm) resulted in thicker shoots than other treatments. However, on the standard rootstock, the different covering materials had no significant effects on shoot diameter. Grafting on dwarf rootstock led to producing the smallest shoot diameter (4.0 mm) in the control treatment with no cover around the graft union. Using standard rootstock led to larger shoot diameter (Fig. 7).

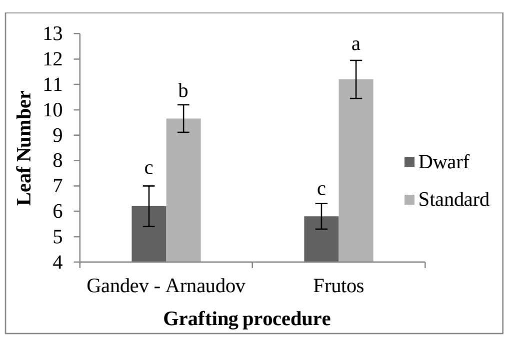


Fig 5. Interaction effect between rootstock × grafting procedures on leaf number of *Juglans regia*.

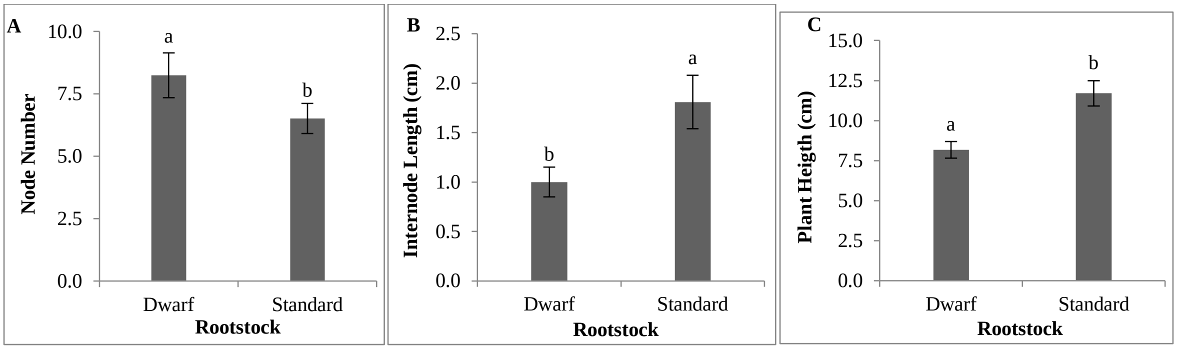


Fig 6. Effect of rootstock type on A) node number, B) length of internode and C) heigth of grafted *Juglans regia*.

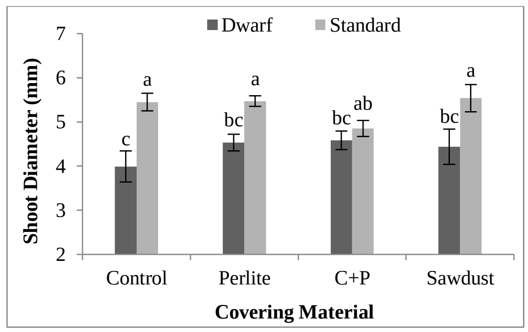


Fig 7. Interaction effect of rootstock × covering materials (Control: without cover, C+P: coco-peat + perlite) on shoot diameter of *Juglans regia*.

## Discussion

Success of Persian walnut grafting is dependent on the rootstock type (Kaeiser *et al.*, 1975; Vahdati, 2003). In this study, graft success on the dwarf rootstock was higher in comparison with the standard rootstock, which could be attributed to the higher callus quality at graft union. The dwarf seedlings were generally thicker than the standard genotype at grafting time, which in turns could provide a better contact between rootstock and scion that is crucial for rapid formation of callus, healing of the graft union and higher survival of grafted plants. Callus formation is necessary for formation of vascular connection between scion and rootstock at graft union. Accordingly, poor callus formation at graft union results in severe water loss through the developing leaves of scion which eventually leads to dehydration of scion and graft failure (Vahdati, 2003). Preserving the scion water content is an important factor affecting successful callus formation and graft success. Callus formation and its subsequent growth are reduced by scion water loss (Rongting and Pinghai, 1993). Blocking the xylem through latex discretion induced by wound in some fruit trees, such as apple, prevents water loss and improves graft success. This mechanism has not been observed in walnut, and therefore, the amount of water loss and tissues death in graft union is higher compared to easily grafted trees (Vahdati, 2003). Covering the graft union by using waxes or grafting glue and moist materials may prevent water loss and result in higher graft success (Vahdati, 2003). According to Rezaee and Vahdati (2008), covering the graft union with moist sawdust provides suitable gas exchange and absorbs excess xylem sap exudations, which subsequently improves callus establishment. The results indicated that the application of sawdust cover improved callus quality, graft success and survival of the grafted trees. Vahdati (2003) stated that sawdust enhanced the success of the conventional walnut grafting methods by providing a thin layer of water on the surface of the callus. The results are in accordance with those reported by Unal (1995), in which the highest percentage of graft success (85%) was reported to be due to bark grafting along with covering with sawdust.

The results revealed that using dwarf walnut seedlings as rootstock may limit scion growth. Dwarf walnut rootstocks have been characterized by compact growth, short internodes and slow growth rate (Unal, 1995; Rezaee *et al*., 2009). The scions grafted on the dwarf rootstock had higher number of nodes, shorter internodes and generally exhibited a compact growth. The scions on the dwarf rootstock produced less leaves, which probably was related to limited development of shoots than those of standard rootstocks. These results are comparable with Mahmoodi *et al*. (2013), who reported that using seedlings of a cluster bearing walnut as rootstock reduced the average growth of scion by 9.29% relative to a standard rootstock. The results revealed that cluster bearing genotypes can be used as dwarfing rootstocks for controlling Persian walnut scion vigor. Node number is considered as an important factor for evaluation of vigor of grafted trees. In comparison with standard rootstocks, using dwarf rootstocks for Persian walnut may increase node formation on scion by 10.1% and limit its internodes growth by 19.14% (Mahmoodi *et al*., 2013). Similar results have been reported by studying other species, in which induction of higher node numbers in scion by application of dwarfing rootstocks of apple and mango (Mukherjee *et al.,* 1980; Jaumien *et al.,* 1984). The results of the present study are in agreement with the results reported by Rigard *et al*. (2008). Alla (2008) stated that dwarfing rootstocks accelerated the diameter growth of the scions. However, the results of the present study are inconsistent with these findings, and dwarf rootstocks did not cause a noticeable increase in the scion diameter. This probably was due to short duration of the experimental period, which limited accumulation of photosynthates in the scion stems.

Grafting success in *J. regia* is generally less than the other fruits crops (Weber and Mac Daniels, 1969). Environmental conditions and grafting method determine the survival of grafted Persian walnut (Raofi *et al*., 2016). The highest success reported after cleft grafting, a major grafting method for walnut, was 12.5% (Chauhan and Sharma, 1980). Grafting success of side-tongue grafting which is widely used for grafting *J. regia* is reported between 40 (Chauhan and Sharma, 1980) to 67% (Achim and Botu, 2001). Dehghan *et al*. (2010) after evaluating different grafting methods, obtained 71 percent of grafting success by Omega grafting. Epicotyl grafting methods are generally faster and more successful than the traditional grafting methods of *J. regia* (Szoke, 1990). Gandev and Arnaudov (2011) obtained 73% of grafting success by using epicotyl grafting of *J. regia*. Growing rootstock and grafted trees under optimum environmental conditions provide a suitable condition for development of callus tissue and subsequent growth of scion. The results revealed that the procedure described by Frutos is more effective in the majority of traits compared to procedure of Gandev-Arnaudov, which could be due to direct planting of the seeds in pots and the lack of replanting of the seedlings for grafting and the following stages after grafting. In conclusion, performing walnut early grafting via Frutos procedure on a dwarfing rootstock and application of sawdust at graft union resulted in over 91 percent survival of grafted seedlings. As such, in addition to using the advantages of dwarfing rootstocks, higher survival and quality trees can be obtained.

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