

## **The histology of minigrafting of Persian walnut trees cv. chandler**

Mina Farsi<sup>1\*</sup>, Mohammadreza Fatahi Moghadam<sup>1\*</sup>, Zabihollah Zamani<sup>1</sup>, Darab Hassani<sup>2</sup>  
and Ahmad Ahmadi<sup>1</sup>

1. Department of Horticulture Science, College of Agriculture & Natural Resources, University of Tehran, Karaj, Iran
2. Temperate Fruits Research Center, Horticultural Science Research Institute, Karaj, Iran

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### **Abstract**

Compared to other techniques of propagation, grafting is the most successful and feasible technique for asexual propagation of walnut plants. There is little information about graft union formation in walnut. Therefore, the objective of this study was to evaluate histological events during graft union formation in *Juglans regia* L. cv. Chandler scions when minigrafted on the one-year-old seedlings. Cross and longitudinal sections of the graft union were taken for examining different stages of grafting process after 1, 14, 30, 60, 120, 180, 420 and 540 days of minigrafting. One day after grafting, brown necrotic layers were observed at the cut edges. The first callus cells were initiated from cambium layer of rootstock 14 days after grafting but there was weak connection between two parts. New vascular connections between rootstock and scion were observed 30 days after grafting. Vascular connections were increased in central parts of the graft union 60 days after grafting. In mid-summer (180 days after grafting), necrotic layer was almost disappeared in the central longitudinal sections but they were increased especially in the bark, callus and top parts of the graft union due to high temperature and low humidity. In 420 days after grafting, two parts were strongly connected by xylem vessels, but necrotic layer was still remained and observed in some parts. In 540 day after grafting which was coincided with the end of second growing season, two parts were tightly connected to each other and necrotic layer disappeared in most of cross and longitudinal sections.

**Keywords:** callus, graft union, rootstock, scion, vascular connection, walnut.

### **Introduction**

Persian walnut (*Juglans regia* L.) is an important nut crop, which its asexual propagation has more difficulties than other fruit species (Dehghan *et al.*, 2010). Budding and grafting are the most popular techniques for propagation of walnut (Gandev, 2007). However, successful grafting in walnut trees is a difficult process that affected by several factors including grafting technique, temperature,

humidity, phenolic compounds, hormonal conditions, nutrition of scion cultivar, root hydraulic pressure and time of taking the scion (Mehmet *et al.*, 1997; Mitrovic, 1995; Rezaee *et al.*, 2007). For success in grafting, a good union should be developed between scion and rootstock with enough callus formation (Errea *et al.*, 2001). Environmental conditions have a major impact on callus formation. Change in temperature during the period of graft union can influence callus formation and development (Dehghan *et al.*, 2010). In

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\* Corresponding Authors, Email: [minafarsi@ut.ac.ir](mailto:minafarsi@ut.ac.ir);  
[fattahi@ut.ac.ir](mailto:fattahi@ut.ac.ir)

walnut, temperature around the grafting union should be maintained at 27 °C (Avanzato and Atefi, 1997; Dehghan *et al.*, 2009; Germain *et al.*, 1997). At this temperature, callus formation occurs five days after grafting, but at 22 °C, it begins in seven days following grafting and at temperatures lower than 20 °C no callus formation occurs (Rongting and Pinghai, 1993). Fluctuation in temperature and low humidity conditions result in failure of callus formation and grafting (Ebrahimi *et al.*, 2006). In walnut, relative humidity (RH) should be kept at 80-90% following grafting (Dehghan *et al.*, 2010), because the callus cells encompassed by soft walls and they lose their moisture in dry environment (Rezaee *et al.*, 2008; Rongting and Pinghai, 1990). The temporal covering of the grafting area with moist sawdust or perlite (Dehghan *et al.*, 2010), plastic strips and glass (Aminzadeh, 2012; Balanian, 2010) has been reported to be effective in improving callus formation and grafting success.

Rate of graft success is influenced by grafting technique (Achim and Botu, 2001; Rongting and Pinghai, 1990). A number of authors (Aminzadeh, 2012; Balanian, 2010; Dehghan *et al.*, 2009; 2010; Lantos, 1990; Soleimani *et al.*, 2010; Tsurkan, 1990; Vahdati and Zareie, 2005) have reported that table grafting has some advantages in comparison with bud grafting. Grafting time is usually longer than budding. In walnut, grafting is usually performed under controlled conditions during winter (Aminzadeh, 2012; Balanian, 2010; Soleimani *et al.*, 2010).

Weak callus formation, phenols accumulation and formation of necrotic layer at the union position may cause failure in grafting which reduces survival of plants in the field. These symptoms are usually seen above the union area, especially for less compatible scion/rootstock combinations (Mng'omba *et al.*, 2007). Graft failure involves many biochemical and physiological processes

(Usenik and Stampar, 2001). Polat *et al.* (2010) reported that histological developments during the early stages of graft union formation between the spur apple varieties and apple rootstocks can explain compatibility/ incompatibility of scion and rootstock.

Cohesion of the stock and scion, proliferation of the callus cells, restoration of cambium and vascular continuity are the major events that occur during the grafting process (Hartmann *et al.*, 2002; Moore, 1983; Soumelidou *et al.*, 1994). Different histological developments and tissue organizations may influence graft union formation and cause different anatomical features and abnormal histological developments (Atkinson *et al.*, 2003; Errea *et al.*, 2001; Simons, 1987; Soumelidou *et al.*, 1994). Therefore, the objective of this study was to identify the histological events during grafting union in *Juglans regia* L. cv. Chandler minigrafted on one-year-old seedlings during two growing seasons to assess disorders which cause failure of the grafting.

## Materials and Methods

The experiment was carried out in Department of Horticultural Science, Collage of Agriculture and Natural Resources, University of Tehran, during 2015-2016. After completion of chilling requirements in winter 2015, one-year-old pot seedlings were transferred to greenhouse as rootstocks. In late January, scions of cv. Chandler were taken from one-year-old shoots from the walnut experimental orchard of Kamal Shahr station of Seed and Plant Improvement Institute, Karaj, Iran. The scions were kept at cool and moist conditions, until used for grafting. In February, hundreds of scions of cv. Chandler were minigrafted (cleft grafted) on one-year-old seedlings and grafts were wrapped with plastic strips. The grafted plants were covered with plastic glasses to avoid losing of their moisture (Aminzadeh, 2012; Balanian, 2010). The

grafted plants were placed at  $25\pm 2$  °C temperature and 65-75% relative humidity in the greenhouse. Grafted seedlings were sampled in 1, 14, 30, 60, 120, 180, 420 and 540 days after minigrafting. For each stage, eight graft units were taken and sectioned in 20-30  $\mu\text{m}$  thickness using a hand-fed sliding microtome (Ada and Ertan, 2013). Cross and longitudinal sectioned materials were placed in bleach (0.6% sodium hypochlorite) for 15 min to remove cellular debris and remove cell walls, then washed with 5% acetic acid to neutralize the effect of bleach and rinsed thoroughly by four times in distilled water. The sectioned materials were stained with methyl-green and washed three times with distilled water to remove remnants of dye (Aminzadeh, 2012). Finally they were placed on slides to examine under stereo and binocular microscopes. During graft union formation, five developmental stages were examined: (1) positions of necrotic layers, (2) proliferation of callus cells, (3) formation of callus bridge in the graft interface, (4) cambial continuity and (5) formation of vascular tissues.

## Results

One day after minigrafting of *Juglans regia* L. cv. Chandler scions on one-year-old seedlings, thick necrotic areas were formed on the graft interfaces. Callus cells were initiated from cambium layer of rootstock 14 days after grafting. In this stage, connection of two parts was so weak and callus production was not sufficient to enable us to describe histological developments. After 30 days of grafting, the callus bridge was not completely formed between the two parts (Fig. 1a) and a necrotic layer was clearly observed at longitudinal sections (Fig. 1b). The callus tissues were formed on rootstock from young xylem cells and cambium, while they were formed from cambium, young phloem and xylem cells on the scion side. A small number of the grafts were failed due to low callus formation on the graft union position

(Fig. 1c). Sixty days after grafting, the callus tissue was completely filled the grafting area and the necrotic layer was gradually disappeared (Fig. 1d,e). It was time for cambial differentiation and then forward vascular components formations. They were radially connected to tightly link scion and rootstock (Fig. 1f). Furthermore, new vessel elements and xylem rays were formed from old cambium of scion and rootstock and the new cambium that initiated from callus bridge (Fig. 1g). After 120 days, by using cross and longitudinal sections, it was appeared that the previous necrotic areas were dissected into small pieces. The cambial line continuity was established in the callus bridge 120 days after grafting. Cambial tissue was differentiated into the callus cells and new cambium and vascular tissues were formed at the graft union (Fig. 1h). New xylems were radially formed to properly link the two parts of the grafting but their formation was occurred in small amounts (Fig. 1i). Six months after grafting almost in mid-summer, some of the grafts were failed due to high temperature and low RH (Fig. 1j). Cross sections of failed grafts revealed accumulation of phenolic compounds in the callus cells, new vascular components and also at the edges of graft interface (Fig. 1k). New cambium differentiated from callus cells and necrotic areas were disappeared in central parts of the successful grafts (Fig. 1l). To supply more water and minerals for scion, xylem vessel diameters were increased at the graft interface (Fig. 1m). On 420 days after grafting, vascular connections was filled most parts of the graft interface but narrow necrotic layer was still observed in some parts of the graft union (Fig. 1n). In longitudinal sectioning which was made 540 days after grafting, more integrated cambial connection was established especially in central parts of the graft union (Fig. 1o) and necrotic areas were almost shrunk into small pieces (Fig. 1p). All described observations were presented in Table 1.



**Fig. 1.** Histological observations of graft union formation in Persian walnut (*Juglans regia* L.) cv. Chandler scions minigrafted on one year-old seedlings. The days after grafting are mentioned on the pictures (d: days). a) Incomplete formation of callus bridge between two partners. b) Necrotic layers were clearly observed at longitudinal sections. c) Failed graft due to low callus formation at the graft union. d) callus filled completely healing zone. e) Gradual disappearance of the necrotic layer between two partners. f) Cross orientation of vascular components for connecting the two parts. g) Formation of new vessel elements and xylem rays from old and new cambiums. h) Differentiation of cambial tissue and formation of vascular tissues at the graft union. i) Radial formation of new xylems to properly link two parts. j) Graft failure due to high temperature and low relative humidity. k) Accumulation of phenolic compounds in callus cells, new vascular components and edges of graft union. l) Differentiation of new cambium from callus cells and disappearance of necrotic areas in the central parts of the successful graft unions. m) Increasing the xylem vessels diameter at the graft union to supply more water and minerals for the scion. n) Formation of the vascular connections for most parts of the graft union and observation of the necrotic layer and callus in some parts of the graft union. o) Establishment of a more integrated cambial connection especially in central parts of the graft union. p) Shrinking of the necrotic layer into small pieces. (d: days; Sc: Scion; Rs: Rootstock; Cb: Callus bridge)

**Table 1. Histological observations of graft union formation in Persian walnut (*Juglans regia* L.) cv. Chandler scions minigrafted on one year-old seedlings**

Day	Necrotic layer	Callus cells	Observations		
			Cambium layer	Vascular elements	Graft failure
1	Formation of thick necrotic layers on the graft interfaces	-	-	-	-
14	Observation of necrotic layers as dark lines on the graft interfaces	Initiation of first callus cells from cambium layer of rootstock		-	-
30	Observation of necrotic layers at longitudinal sections	Formation of callus cells from cambium, phloem and xylem cells of scion, also cambium and xylem cells of rootstock	Initiation of new cambium layer from callus cells	Formation of vascular components from cambium layer of scion and rootstock	Failure of some grafts due to low callus production
60	The gradual shrinking of necrotic layer into small pieces	The complete filling of grafted areas by the callus tissue	Development of cambium layer into the callus cells	1. Formation of new vascular elements from new cambium layer 2. Formation of vessel elements and xylem rays from old cambium layer of scion and rootstock	-
120	Shrinking of necrotic layer into small pieces	Differentiation of callus cells into xylem vessels	Establishment of cambium line continuity in the callus bridge	Development of vascular elements especially in central parts of graft union	-
180	1. Disappearance of necrotic layer in strong graft units 2. Accumulation of phenolic compounds in weak graft units due to high temperature and low humidity in mid- summer	1. Differentiation of callus cells into xylem vessels in strong graft units 2. Accumulation of phenolic compounds in the callus cells of weak graft units	Initiation of new vascular elements from cambium layer	1. Increasing the diameter of new xylems in strong graft unit to supply more water to scion 2. Accumulation of phenolic compounds especially in new xylem rays in weak graft units	Shrinking of some grafts due to adverse environmental conditions in mid-summer
420	Observation of narrow necrotic layers in some parts of graft areas	Differentiation of callus cells into new vascular elements to strongly link two parts	Initiation of new vascular elements from cambium layer especially at the upper parts of graft units	Development of vascular elements into the callus tissue	Failure of some grafts due to low temperature in the last winter (360 days after grafting)
540	Destruction of necrotic areas in the most parts of graft union	Differentiation of most callus cells into new vascular elements	Observation of more integrated cambium connections	Filling the most parts of graft units by vascular elements	-

## Discussion

### *Effect of temperature and relative humidity*

Environmental conditions such as temperature and humidity have significant effects on success of walnut grafting. Soleimani *et al.* (2010) showed that change in temperature during the uniting period has a direct effect on callus development and successful grafting. In some studies, hot callusing systems have been used to control temperature during the uniting process (Aminzadeh, 2012; Balanian, 2010; Ebrahimi *et al.*, 2006; Gandev, 2009; Gandev and Arnaudou, 2011; Soleimani *et al.*, 2010).

In grafting, bud of the scion is the main organ for losing moisture. Graft success is dependent on loss of moisture from scion buds (Rongting and Pinghai, 1990; 1993). Rongting and Pinghai (1993) showed that if scion moisture reduced to less than 38%, callus formation on the graft union will stop. Similar to Aminzadeh (2012) and Balanian (2010), we used plastic strips and plastic glass to maintain humidity around the graft unit. Sadeghi Majd (2014) showed the importance of irrigation regime on walnut grafting; therefore, we applied three-day irrigation intervals in greenhouse to increase graft success and survival of the scion.

### *Callus formation*

Four important stages (pre-callus, callus, cambial bridge and the healed union) for graft formation have been reported by Soule (1971). On the other hand, Dolgun *et al.* (2008) reported three important steps (callus formation, cambial differentiation and continuity and vascular tissues formation) for graft formation. Our study also revealed similar results for graft union formation between the walnut scion and rootstock, which caused formation of a strong and successful union.

In agreement with Dolgun *et al.* (2009), due to broken sections in the first 14 days after grafting, visual observations were not

sufficient to certainly determine the cellular differentiations. In this time, because of the poor connection of callus produced between the scion and the rootstock, samples were not cut as a whole section. In these sections as the first step of wound repair process, a limited callus formation was visually seen in the samples and a strong callus connection was not observed. Necrotic layers were seen as dark lines in outer sides and interfaces of graft union. Walnut trees contain high concentrations of polyphenols and high activity of polyphenol oxidase which could explain the appearance of necrotic layer at cutting edges (Rongting and Pinghai, 1990). The formation of necrotic layer provided the cohesive layer between the grafting sides in the first few days prior to callus bridge formation (Asante and Barnett, 1997). Tiedemann (1989) showed that the necrotic layer provided a temporal connection between the two graft partners and by itself did not contribute to union formation (Mahunu *et al.*, 2012). The presence of the necrotic layer at the wounded surfaces resulted in limited desiccation and death of the deeper tissues and may reduce pathogens penetration (Cline, 1980; Noel, 1968). On the other hand, Tiedemann (1989) showed that thick necrotic layer could lead to mechanical failure of union. In agreement with Mahunu *et al.* (2012), our results indicated that the necrotic layer remained at the interface zone until callus was subsequently substituted and completely filled the graft union. Hartmann *et al.* (2002) reported that the cohesion of stock and scion occurs through proliferation of a callus bridge between two partners and subsequently causes successful differentiation and restoration of new vascular tissue and its continuity. The formation of callus tissue at the graft union is the first response to grafting (Moore and Walker, 1981). It is an important stage for scion survival and a rapid physical contact between the scion and rootstock by its

proliferation (Asante and Barnett, 1997). Pina and Errea (2005) showed that the formation of callus is essential for development of future vascular connections. The formation of callus tissue provides some of the compounds which required for adhesion of the graft partners. The content and nature of the cells involved in the first step of graft union formation can play an important role in triggering the responses that lead to the formation of strong and successful unions. Callus tissues were produced at both sides of the graft components and they combined to make connection between rootstock and scion (Moore, 1984).

Kankaya *et al.* (1999) and Simons (1987) reported that callus tissues are formed from young xylem cells at rootstock, but they are formed from undamaged bark cambium, young phloem ray cells, and sometimes from cortex at scion side. In our study, callus formation was initiated from cambium, phloem and xylem cells at scion, while they were formed from cambium and xylem cells at rootstock (Fig. 1f, g). Seferoğlu *et al.* (2004) showed that during grafting process, the callus formation is produced by live cells, located behind the dead cells as a response to injury. In agreement with Dolgun *et al.* (2008), our results indicated that most of the callus cells produced by rootstock, due to its higher activity than dormant scion. To establish callus bridge, callus cells should make connection between graft unions by cracking the dead necrotic cell groups (Seferoğlu *et al.*, 2004). Callus bridge is most often developed in the cortex and non-functional phloem areas where contact layers were in connection with the ray and phloem parenchyma, being the chief contributors to its growth (Copes, 1969). The establishment of the callus bridge is important to provide water and mineral for scion (Barnett and Miller, 1994). In our study, some of grafts failed in the early stages due to weak callus proliferations.

Dolgun *et al.* (2008) mentioned that low production of callus tissue limits passing of water from rootstock to scion and causes failure of the graft. In agreement with Mahunu *et al.* (2012), 60 days after grafting, callus tissues filled all spaces and cambial continuity were clearly observed. At this time, some of the callus cells transformed to cambium cells and gradually after 120 days, cambium cells was further completed and seemed like a short line in some parts of the graft union (Fig. 1h).

### ***Cambium differentiation***

Cambial differentiation is the second most important stage for success in grafting (Dolgun *et al.*, 2008). Vascular tissues were produced by newly formed cambium. The establishment of the cambial continuity is vital for the production of vascular tissues to ensure a successful completion of the grafting process (Mahunu *et al.*, 2012). Vascular differentiation begins after establishment of cambial continuity and the strong connection occurs in a short time in a compatible graft (Asante and Barnett, 1997). Soule (1971) and Moore (1981, 1982 and 1991) reported that vascular connection is usually established 6 to 8 weeks after grafting in higher plants. However, Ada and Ertan (2013) reported that 150 days after grafting the vascular connection was successfully established between rootstock and scion in chestnut and oak. In the present study, there were a few necrotic areas at the graft union, but cambial connection was observed in all longitudinal sections 120 days after grafting.

In agreement with Seferoğlu *et al.* (2004), a period of 180 to 480 days is necessary to observe more integrated vascular connection between the scion/bud and rootstock. Ada and Ertan (2013) showed that variation between periods for vascular connection may arise from the use of different stock-scion combinations and environmental factors. In our study, 180 days after grafting was coincided with mid-

summer and a number of grafts failed due to high temperature and low RH conditions. Cross sections revealed that the diameter of new xylems especially in scions increased to cope with water stress through transmitting more water. Barnett and Weatherhead (1988) reported that the failure of grafts will occur due to water stress when scions are unable to receive water from the rootstocks prior to formation of graft union. Bolat *et al.* (2014) reported that water stress significantly affected most morphological, physiological, and biochemical characteristics as well as budding success on apple and quince rootstocks. They showed that relative shoot length, diameter, and plant total fresh and dry weights decreases by exposure to water stress. Leaf relative water content and chlorophyll index decreased while electrolyte leakage was increased by exposure to water stress in both rootstocks. They indicated that water stress results in higher peroxidase activities and phenols content in both rootstocks. In our study, some graft units which considerably disrupted the necrotic layer accumulated low amount of phenolic compounds could which help them to escape from water stresses.

The success of grafting primarily depends on the identification of stress and pathogen-resistant rootstocks, and on the compatibility of the graft union in terms of fast formation of the vascular connections between the rootstock and the scion and fast renewal of root and canopy growth (Cohen *et al.*, 2007). Several factors and conditions have been attributed in failure of graft formation. Amongst others, change in temperature and RH especially during and after grafting can directly affects the development of a good graft union (Balta *et al.*, 1996; Tekintas, 1988), furthermore, high day temperatures together with warm nights are stressful for successful union (Hinesley, 1981). Frey (2009) reported that shade through decreasing air temperatures can improve the success of grafts during

high day temperatures, and therefore, can improve graft success.

In the present study, the formation of vascular connections in the last step of graft healing was considered important for most parts of the graft unions, but vascular discontinuity was observed in some sections due to mismatches between two partners and low callus production. The formation of vascular connections is the basic requirements for a successful graft (Moore, 1984). In our study, longitudinal sections which were taken 540 days after grafting revealed that the formation of xylem vessels which linking the two parts together was not fully complete in all sections. Errea *et al.* (1994a; 1994b) showed that incomplete new vascular connections which can not well differentiate and are weakly established, are the main reasons for incompatibility in woody plants. Espen *et al.* (2005) reported that compatibility was closely correlated with vascular differentiation and xylem connection between the two partners. Delayed and limited differentiation of vascular elements and establishment of poor vascular reconnections between partners were the events that most clearly characterized the grafts between incompatible partners. To have matching of the two cambium layers at least on one side, it is important to place scion on the stock to prevent a delay of ultimate union (Dolgun *et al.*, 2008) showed that. Hartmann *et al.* (2002) reported that unsuccessful grafts showed gaps at the periphery or cortex of the graft components. Both early graft failure and delayed failure were recognized in walnut. The early failures are usually occurred in the first two years, while the late failures are resulted in scion death after 5-7 years (Aminzadeh, 2012; Balanian, 2010). For this reason, although a vascular connection was established between the walnut scion and rootstock in the present study, the field performance and development of these plants are needed to be screened in the future for checking of the delayed incompatibility.

## Conclusions

Walnut (*Juglans regia* L.) propagation is more difficult, compared to other fruit species. Temperature and RH have important influences on the process of walnut graft success. Results and observations from the present experiment indicated that adequate and early callus formation is important for initial scion survival and subsequent formation of the cambium and vascular tissues. This is because of this fact that the proper connection of two partners plays an important role in graft union success. Many factors such as mechanical mismatches in size or tissue location between stock and scion, desiccation of the tissues, adverse temperature or light regimes, failure of callus initiation, failure of adequate vascular

differentiation, or physiological rejection between the tissues can influence the success of a graft. Grafting method can also affects graft success. There is a controversy in the previous reports that makes it difficult to choose an efficient grafting method for commercialization of walnut production. More histological studies are needed for comparing different methods of walnut grafting such as patch budding, chip budding, whip, whip-tongue, cleft and omega grafting to introduce the best method for walnut propagation.

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