GRAFTING OF WALNUT (*Juglans regia* L.) WITH HOT CALLUSING TECHNIQUES UNDER SOUTH AFRICAN CONDITIONS

by

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Submitted in partial fulfilment of the requirements
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DECLARATION

I declare that this dissertation hereby submitted by me for the *Magister Scientiae Agriculturae* degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Lukas Cornelius van Zyl | Date

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Abstract

One of the biggest constraints to the expansion of the walnut industry in South Africa, is the availability of good quality and cost effective plant material. Since walnuts are more difficult to propagate vegetatively than most fruit and nut species, controlled conditions are required to ensure grafting success. Hot callus grafting is a bench grafting procedure subjecting grafted trees to controlled, elevated temperature and humidity, and has been implemented successfully in several countries to increase grafting success of walnut. Until now, hot callus grafting has not been investigated as a possible walnut propagation method in South Africa. The present study assessed method of heat supply and time of grafting, as important factors determining grafting success of two walnut cultivars grafted onto *Juglans regia* rootstocks under hot callus conditions. Although neither method of heat supply provided definite superior grafting success, localized heating of graft unions in a heated trench, gave more consistent results than heating the entire grafted tree in a heated room. Both methods seemed promising, since acceptable grafting success was obtained in some instances with both the heated trench and room. Post-grafting shoot growth on the other hand, was significantly higher for the heated trench than for the room. Both methods did, however, result in trees of a suitable size for orchard establishment after only one growing season. Results further indicated that exposure to increased temperature for a period of 25 days is more than sufficient for callus development of walnut, and very little is gained by heating grafted trees for longer. Earlier grafting, before the onset of bud break of cultivar and/or rootstock, generally resulted in improved grafting success and drastically increased shoot growth during the first growing season, providing grafted trees of saleable size in a shorter time. There were no clear differences regarding callus formation, grafting success or survival between the two cultivars evaluated, ‘Chandler’ and ‘Serr’. As expected, more vigorous post-grafting growth was observed for ‘Serr’, since it is the more vigorous grower of the two. A separate hot callus grafting trial, using Paradox rootstocks, also indicated that the type of rootstock utilized in hot callus grafting may impact grafting success. More consistent results were obtained with all four cultivars grafted onto this hybrid rootstock, than with *J. regia* rootstocks in the preceding trial. For both *J. regia* and Paradox rootstocks, survival of trees through the first growing season was still unacceptably low, although results were comparable to that of other studies. Since results were available for a single season only, conclusions should be regarded as preliminary. Nevertheless, findings clearly illustrated the potential value of hot callus grafting as propagation method for walnut in South Africa and lay a foundation for the establishment of propagation guidelines using this approach.

Additional keywords: Persian walnut, propagation, hot callus grafting, heated trench, heated room, grafting time, post grafting growth, Paradox rootstock, callus development

ENTING VAN OKKERNEUT (*Juglans regia* L.) MET WARM-KALLUS TEGNIEKE ONDER SUID-AFRIKAANSE TOESTANDE

deur

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Uittreksel

Een van die grootste beperkinge tot die uitbreiding van die okkerneutindustrie in Suid-Afrika, is die beskikbaarheid van goeie kwaliteit en koste effektiewe plantmateriaal. Aangesien dit moeiliker is om okkerneute vegetatief te vermeerder as die meeste ander vrug- en neutspesies, word beheerde toestande benodig om suksesvolle enting te verseker. Warm kallus enting is 'n tafelenting prosedure waar geënte boompies aan beheerde, verhoogde temperatuur en humiditeit onderwerp word. Dit is reeds suksesvol geïmplementeer in verskeie lande om die sukses van okkerneut enting te verhoog. Warm-kallus-enting is nog nie voorheen as 'n moontlike vermeerderingsmetode vir okkerneute in Suid-Afrika ondersoek nie. In hierdie studie is die metode van verhitting en tyd van enting, as bepalende faktore wat die sukses van warm kallus enting van twee okkerneut kultivars op *Juglans regia* onderstamme beïnvloed, geëvalueer. Gelokaliseerde verhitting van die entlas in 'n voortjie, het meer konsekwente resultate gelewer as verhitting van die hele geënte boom in 'n kamer, alhoewel nie een van die metodes opvallend beter was as die ander nie. Beide metodes blyk egter belowend te wees, aangesien kommersieel-aanvaarbare ent sukses verkry is met beide die verhitte voortjie en -kamer onder sekere toestande. In teenstelling hiermee, was lootgroei na enting betekenisvol hoër vir gelokaliseerde verhitting in die voortjie as vir die kamer. Beide metodes het egter bome van aanvaarbare grootte vir boordvestiging na slegs een groeiseisoen opgelewer. Resultate het verder getoon dat 'n verhittingsperiode van 25 dae meer as voldoende was vir kallusontwikkeling by okkerneute, en dat geen addisionele voordeel verkry is deur die geënte bome vir 'n langer tydperk te verhit nie. Vroeër enting, voor die aanvang van bot van die kultivar en/of onderstam, het 'n toename in ent sukses bewerkstellig en ook lootgroei in die eerste groeiseisoen drasties verhoog, om sodoende bome van aanvaarbare grootte in 'n korter tydperk te lewer. Daar was geen duidelike verskille tussen die twee kultivars, 'Chandler' en 'Serr', wat geëvalueer is nie,

behalwe vir lootgroei na enting. Soos verwag, het die sterker groeier, ‘Serr’, betekenisvol beter groei getoon as ‘Chandler’ in die eerste groeiseisoen. ‘n Verdere warm kallus eksperiment met Paradox onderstamme, het aangedui dat die tipe onderstam die sukses van enting moontlik kan beïnvloed. Meer konsekwente resultate is verkry met al vier die kultivars wat op hierdie onderstam geënt is, in vergelyking met *J. regia* onderstamme in die voorafgaande proef. Die oorlewing van geënte bome, op beide *J. regia* en Paradox onderstamme, was steeds onaanvaarbaar laag teen die einde van die eerste groeiseisoen, alhoewel die resultate vergelykbaar was met ander soortgelyke studies. Aangesien resultate van slegs een seisoen beskikbaar was, moet afleidings as voorlopig beskou word. Nogtans het bevindinge die potensiële waarde van warm-kallus-enting as ‘n vermeerderingsmetode vir okkerneute duidelik geïllustreer en ‘n grondslag is gelê vir die daarstelling van vermeerderingsriglyne deur hierdie benadering in Suid-Afrika.

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CHAPTER 1. *General introduction*

Cultivation of walnut (*Juglans regia* L.) in South Africa is currently a young but promising industry. According to Costa *et al.* (1988) walnuts have been cultivated on small scale in the Aberdeen and Oudtshoorn area, but the industry has not expanded further due to a lack of suitable technology and cultivars. Although Dandekar *et al.* (2005) listed South Africa amongst the first areas in the Southern Hemisphere where walnut cultivation is rapidly developing, the local industry is minute in comparison to other Southern Hemisphere producers such as Australia, Chile and Argentina. Even though production in these countries is not comparable to major walnut producing areas of the world, it still indicates that their walnut industries are flourishing in comparison to South Africa's. Annual walnut production in Chile was 16 000 tonnes in 2007, in Argentina 10 000 tonnes was harvested in 2006, while 22 tonnes were produced in Australia in 2007 (FAO, 2006; 2007). In comparison, less than one ton was harvested from young bearing trees in South Africa during 2007 (Rotondo Walnuts, 2007b).

The major walnut producers of the world, China, USA, Iran, Turkey, Ukraine, Romania, France and India, are all situated in the Northern Hemisphere (Germain *et al.*, 1999; Calcagni, 2006; FAO, 2006). Hence, there is an increased demand for Southern Hemisphere walnuts from the European Union, the major walnut importer, over the traditional Christmas period, since these nuts will be fresher and more competitive due to favourable exchange rates (Adem *et al.*, 1999; Du Plessis-Swart, 2003; Calcagni, 2006). There are still ample opportunities for expansion of the walnut industry in South Africa to fill this market gap, and further walnut cultivation is therefore encouraged.

Currently, one of the biggest constraints to the expansion of the walnut industry in South Africa is availability of good quality, readily available and cost effective propagation material. Almost 80% of walnut trees cultivated commercially in South Africa are propagated *in vitro* and imported as bare-root trees from Spain. These trees are exceptionally small with a stem diameter of approximately 5 mm and a stem height of 5 to 20 cm (Rotondo Walnuts, 2008). Imported trees must be grown under quarantine conditions for one year, to ensure that they are pathogen free, before they can be transplanted into commercial orchards (Du Plessis-Swart, 2003). Initial tree growth under local quarantine conditions is slow, resulting in only 50% reaching a suitable size for orchard establishment after the first growing season, with the remainder of the trees requiring an additional 12 months. For the farmer, this creates an unacceptable delay of 18 to 30 months from order date to planting (Du Plessis-Swart, 2003; Rotondo Walnuts, 2008). Furthermore, the current establishment cost for plant material only is R18 000 ha^{-1}, if trees are purchased at a cost of R120-00 $tree^{-1}$. This places orchard establishment beyond the means of many prospective producers in areas suited to further walnut production in South Africa. It is therefore imperative that a successful source of reasonably priced plant material of the highest quality be established locally to assist with expansion of the walnut industry.

In traditional walnut producing countries in the world, like Italy (Avanzato & Tamponi, 1988), Turkey (Barut, 1996), India (FAO, 2001), Romania (Achim & Botu, 2001) and Iran (Vahdati & Zareie, 2006), older orchards consist of mature seedling trees characterized by a long juvenile period and poor, inconsistent bearing habits. A need therefore existed to establish orchards with clonal plant material. However, it is a well-known fact that walnuts are more difficult to propagate vegetatively than most fruit and nut species (MacDonald, 1987; Reil *et al.*, 1998; Hartmann *et al.*, 2002). Own experience echoed reports by Karadeniz (2005) and Erdogan (2006) that traditional field budding and grafting give relatively poor and inconsistent results in walnuts. Hence, nurseries and experimental institutes were forced to investigate alternative vegetative propagation methods. As a result many major walnut producing countries, including France (Linard, personal communication[1]), Spain (Aletà, personal communication[2]) and Australia (Titmus, personal communication[3]), currently employ hot callusing techniques effectively to produce high quality plant material on a commercial scale.

Lagerstedt (1982) defined hot callusing as a propagation method utilized in difficult-to-propagate species to expose the grafted tree to controlled temperature and humidity regimes. The graft union is exposed to elevated temperatures for a pre-determined period to accelerate cell division. The graft union responds by forming new callus tissue, which is the first important step in callus bridge formation between scion and rootstock in a successful graft (Hartmann *et al.*, 2002).

Hot callus procedures are nothing new to South Africa, since large scale propagation of vine (*Vitis vinifera* L.) grafts in hot callus rooms has long been a standard procedure (Van der Westhuizen, 1981). However, these techniques have never been employed for propagation of walnut in this country.

Introducing a new propagation method, such as hot callus grafting, requires a great deal of experimentation to adapt the new technique to local plant material and conditions. According to Titmus (personal communication[3]), it took five years of intensive research to obtain acceptable results with hot callus grafting of walnut in Australia. One of the most important aspects that need to be determined when launching such a project, is the optimal time of grafting (Ferhatoğlu, 1997; Özkan & Gümüs, 2001). Other fundamental factors which seem to influence the success

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[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain
[3] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

rate of hot callus grafting of walnut, include the method of heat supply to the graft union and duration of the hot callus period (Cerny, 1965; Achim & Botu, 2001; Porebski *et al.*, 2002). Some researchers have also established differential cultivar responses to hot callus conditions (Lantos, 1990; Stanisavljević & Mitrović, 1997; Erdogan, 2006; Lopez Larrinaga, personal communication[1]).

If the walnut industry in South Africa is to be expanded over the next decade, a total of at least 6 000 trees need to be propagated annually. The total area under walnut cultivation in South Africa by the end of 2007 was almost 600 ha, consisting of approximately 90 000 trees (Rotondo Walnuts, 2007a). This study was ventured during a time when a true need for high quality walnut planting material existed in South Africa. It was attempted to establish initial guidelines for hot callusing of walnut in South Africa by using experience gained by propagators worldwide and adapting this to local circumstances. Specific objectives were to: (a) evaluate two different methods of heat supply to the grafted tree, (b) determine the optimum time for grafting, (c) determine the optimum duration of the hot callus period, (d) evaluate the reaction of two potentially important cultivars in South Africa and (e) evaluate post-grafting growth in order to determine how fast a grafted tree suitable for orchard establishment can be obtained. Additionally, preliminary investigation into the possible effects of rootstock genotype on grafting success was conducted and will also be presented here. Specification of these important variables will hopefully lay a foundation for successful vegetative walnut propagation through hot callus grafting in South Africa, from where further research on commercial production of grafted plant material can be conducted.

[1] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain

CHAPTER 2. *Literature Review*

2.1 Introduction

Persian walnut (*Juglans regia* L.), also called the English walnut, is the most valuable commercial species in its genus belonging to the family Juglandaceae. Different *Juglans* species originated on several continents, while the Persian walnut is native to temperate regions in mountainous Eastern Europe and central Asia, extending from Turkey, Iran and western China eastward to the Himalayan regions in India and Nepal (Leslie & McGranahan, 1998). All *Juglans* species are monoecious, with catkins being borne laterally on one-year-old wood, and pistillate flowers borne terminally or laterally (newer cultivars) on current season's wood. Although walnuts are genetically self-fruitful they exhibit the phenomenon of dichogamy, being either protandrous or protogynous depending on cultivar. Hence, walnuts are mostly cross-pollinated by wind (Polito, 1998). The walnut is regarded as highly valuable by an increasingly health-conscious world due to various health benefits, including a great source of vitamin E and omega-3 fatty acids, as well as the ability to lower the level of chlolesterol in human bodies (Savage *et al.*, 2001).

Older orchards in most traditional walnut producing countries consist of mature seedling trees characterised by a long juvenile period and poor, inconsistent bearing habits. This is due to the fact that the Persian walnut is more difficult to propagate vegetatively than any other tree nut species (Lagerstedt, 1979; MacDonald, 1987; Reil *et al.,* 1998; Hartmann *et al.,* 2002). In many attempts worldwide to clonally propagate walnuts, horticulturists in France were the first to successfully graft walnuts on a routine basis (Leslie & McGranahan, 1998). Today, patch budding and whip grafting during the vegetative season are commonly regarded as the conventional propagation techniques of walnut (Reil *et al.*, 1998). However, several researchers agree that these methods result in limited and varying success under uncontrolled field conditions (Karadeniz, 2005; Erdogan, 2006).

Hot callus grafting principles provided a feasible alternative for the propagation of walnut, and several other difficult-to-graft tree species, where the fundamental requirement of controlled elevated temperature has been applied in order to obtain increased grafting success. Hot callus grafting of walnut has been performed on a commercial scale in France as early as the 1950’s (Linard, personal communication[1]). The earliest literature on hot callus grafting of walnut was

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

published by Cerny (1963) on results obtained in Poland, where severe weather conditions in spring ruled out any possibility of budding or grafting in an unprotected field environment.

Several approaches to hot callus grafting of walnut have been described in literature. From these sources it is evident that various factors contribute to the success of hot callus grafting and post-grafting growth. Referring to various plant species, including walnut, Lagerstedt (1984) suggested experimentation of at least one year to adapt the hot callus grafting technique to the local climate, facilities, work schedule and plant material. However, some researchers even spent up to five years adjusting hot callus methods to obtain acceptable results under local conditions (Titmus, personal communication[1]). Commercial propagation of a wide range of fruit and nut crops has become very specialized, which requires continuous research inputs and development (Hartmann *et al.*, 2002).

This literature review will primarily focus on describing the foremost influential factors in hot callus grafting success of walnut. These factors include optimum temperature, method and duration of heat application, time of grafting, cultivar response, quality and handling of plant material, grafting technique and post-grafting treatment of hot callused trees. To indicate the relative importance of hot callusing, the review will start off by providing a brief overview of results obtained with conventional walnut propagation techniques.

2.2 Conventional budding and grafting of walnut

In general, most researchers in Europe recorded inconsistent and usually poor results with patch budding of walnut. Özkan *et al.* (2001) obtained between 28 and 53% success with spring patch budding in Turkey (February), despite protecting budded trees from late winter and early spring frost in plastic tunnels. With mid-summer patch budding in Turkey, Erdogan (2006) reported exceptionally variable success rates of between 33 and 96% over several years. Furthermore, budding success was highly dependent on winter conditions following the procedure, since both Karadeniz (2005) and Erdogan (2006) experienced significant losses of budded trees during the successive winter. Hence, Karadeniz (2005) achieved a final success rate averaging a mere 37% at the onset of the following vegetative period over an eight year period, while Erdogan (2006) recorded survival of only 33 to 66% of the initial successfully budded trees. In Slovenia, Solar *et al.* (2001) attributed poor results with late-summer patch budding in the field to considerable differences between minimum and maximum temperatures in the period following the procedure, as well as to high rainfall during the callus period. Contrary to this, Lopez

[1] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

Larrinaga (personal communication[1]) regarded the influence of precipitation on callus formation as beneficial, since rainfall had a cooling effect on hot summer days, minimizing the difference between minimum and maximum temperatures. Although Solar *et al.* (2001) did not elaborate on why rainfall was detrimental to callus formation, failure could have been attributed to excessive moisture accumulating underneath budding tape.

In South Africa, mid-summer patch budding of walnut have also resulted in poor and variable success rates. At the start of the following vegetative period, patch budding resulted in less than 10% success in some years, but up to 70% in others (Rotondo Walnuts, 2007a). Results were in accordance with findings by Karadeniz (2005) and Erdogan (2006) that although patch budding initially seems fairly successful, very few budded trees survive through winter, despite extensive protective measures.

Cleft and whip-and-tongue grafting during the growing season also resulted in mostly unsatisfactory grafting success. Demiroren & Buyukyilmaz (1988) achieved less than 20% success with these grafting techniques on walnut and ascribed the failure to insufficient and fluctuating temperatures in spring. Barut (2001) recorded success of between 20 and 32% only with spring whip grafting over a two year period under uncontrolled field conditions in Turkey.

One of the biggest obstacles with budding and grafting of walnut during the growing season is the detrimental effect of sapflow ("bleeding") on bud- and graft-take. Various researchers successfully eliminated this effect by transplanting rootstocks prior to grafting or budding, resulting in greatly increased success rates (Achim & Botu, 2001; Barut, 2001; Yildiz & Yilmaz, 2003; Verseci, personal communication[2]).

Bark grafting is another approach that has been employed with excellent success on walnut in South Africa, achieving graft-take of more than 80% (Rotondo Walnuts, 2004). This method is, however, not feasible for a commercial nursery since three- to four-year-old established rootstocks, with a diameter of 30 to 100 mm are required, hence it is only used for propagating pollinators and scarce cultivars (Reil *et al.*, 1998; Hartmann *et al.*, 2002).

Inconsistent results and difficulties encountered with conventional grafting and budding of walnuts compelled researchers to investigate propagation under more controlled conditions by means of hot callus grafting. Most authors obtained better results with hot callus grafting compared to conventional grafting and budding during the vegetative period (Pieniazek, 1972; Avanzato & Tamponi, 1988; Lantos, 1990; Van't Westeinde, 1990; Ferhatoğlu, 1997; Stanisavljević & Mitrović, 1997; Özkan & Gümüs, 2001; Solar *et al.*, 2001; Porebski *et al.,* 2002;

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[1] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain
[2] Aldo Verseci, 2005. Pépiniéres de Lalanne, Bordeaux, France

Gandev, 2007). Although Achim & Botu (2001) managed to obtain success rates comparable to hot callus grafting with traditional chip budding by first transplanting rootstocks, these authors still preferred the hot callus grafting procedure. This was due to the fact that better survival through the growing period and more uniformity in growth were obtained with hot callus grafting.

Despite the general negative sentiment of many European researchers towards traditional patch budding and whip grafting of walnut, these remain the standard techniques of walnut propagation in the USA, one of the leading walnut producers in the world (Sibbett, personal communication[1]). Thousands of walnut trees are successfully propagated through patch budding and whip grafting annually, while hot callus grafting is not even listed in the Californian Production Manual as a walnut propagation technique in the USA (Reil *et al.*, 1998).

2.3 Importance of temperature in callus formation

It is a well known fact that temperature has a pronounced effect on the production of callus tissue and the optimum temperature range required for callusing varies between different temperate zone fruit species (Hartmann *et al.,* 2002). As early as the 1920's, Sitton (1931) determined the optimum temperature for walnut to produce callus to be 27°C. Later studies (Rongting & Pinghai, 1993; Reil *et al*., 1998) confirmed that temperatures around 26 to 27°C are optimum, but that temperatures down to 22°C could still be sufficient for good callus formation of walnut (Rongting & Pinghai, 1993). Below 20°C callus formation in walnut becomes unsatisfactory (Figure 2.1) (Reil *et al*., 1998; Hartmann *et al*., 2002).

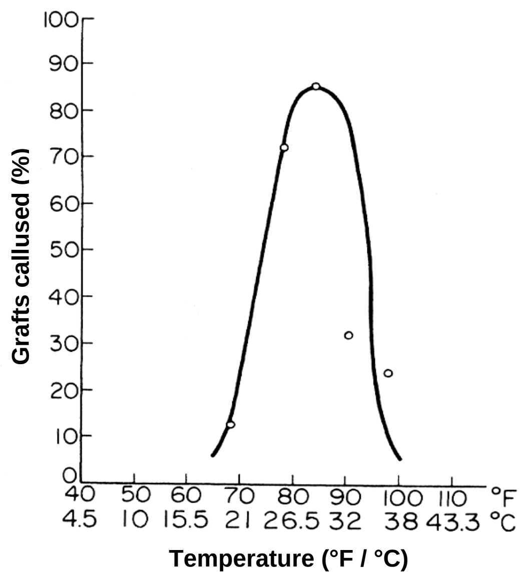

Figure 2.1 Influence of temperature on callus formation of walnut (Hartmann *et al.*, 2002).

[1] Steve Sibbett, 2008. University of California, Davis, CA, USA

Temperature did not only influence the amount of callus tissue but also the rate of callus formation (Rongting & Pinghai, 1993). At 22°C, callus was initiated six days after grafting, while it took five days at 27°C. When the temperature was further increased to 32°C, callus initiated after only four days, but less callus tissue was produced at this temperature.

A temperature of 27 ± 2°C has since become the standard utilized by many propagators and researchers all over the world in their attempts to graft walnut successfully under hot callus conditions (Cerny, 1967; Zachej, 1976; Lagerstedt, 1981b; Lagerstedt, 1982; Lagerstedt, 1984; Avanzato & Tamponi, 1988; Lantos, 1990; Tsurkan, 1990; Van't Westeinde, 1990; Avanzato & Atefi, 1997; Ferhatoğlu, 1997; Kazankaya *et al.*, 1997; Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001; Solar *et al.*, 2001; Porebski *et al.*, 2002; Avanzato *et al.*, 2006; Erdogan, 2006; Vahdati & Zareie, 2006).

2.4 Methods of heat application

Various methods of hot callusing, allowing increased grafting success of several fruit and ornamental species, have been reported. There are basically two strategies for applying the elevated temperature to grafted trees. The first strategy can be considered the traditional approach where the whole grafted tree is exposed to higher temperatures, while the second strategy entails subjecting only the graft union to elevated temperatures. These two methods of heat application used in hot callus grafting of walnut will forth be discussed separately.

HEAT APPLICATION TO ENTIRE GRAFTED TREE

The traditional approach of subjecting the whole grafted plant to higher temperatures has long been employed in the propagation of many species. Howard & Hildreth (1963) reported on root grafting of apple roots under hot callus conditions at 21°C. Propagation of hot callused vines in heated rooms at 25 to 28°C has been described by Becker (1971), Alley (1974), Schenk (1976) and Vogt & Götz (1977) in France and Germany and by Van der Westhuizen (1981) in South Africa. Dhuria *et al.* (1977) performed hot callus grafting of persimmon (*Diospyros kaki* L.) in an incubator at 25 ± 1°C. Asante & Barnett (1998) reported on hot callus grafting of mango (*Mangifera indica* L.) in heated greenhouses at 24 and 28°C.

Hot callus grafting of walnut by subjecting the entire tree to elevated temperatures, has been performed in Europe since the 1950's (Cerny, 1963; 1965; Linard, personal communication[1]). It is still performed today on commercial scale in Europe by various nurseries and research institutes. In France, the temperature inside heated rooms is maintained at 25 to 28°C and

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

relative humidity at 80 to 90% (Germain *et al.*, 1999; Francis, personal communication[1]). According to Linard (personal communication[2]), more than 100 000 grafted trees are produced annually by a single nursery in France using the above mentioned method, where a success rate of 95% is achieved.

Several researchers conducted hot callus grafting trials of walnut following the conventional approach, and although many principles remained the same between different studies, researchers made use of different facilities to subject grafted trees to elevated temperatures (Cerny, 1963; 1965; Pieniazek, 1972; Halliwell, 1975; Zachej, 1976; Radicati & Me, 1986; Lantos, 1990; Van't Westeinde, 1990; Atefi, 1997; Ferhatoğlu, 1997; Stanisavljević & Mitrović, 1997; Barut, 2001; Achim & Botu, 2001; Özkan & Gümüs, 2001; Solar *et al.*, 2001, Ebadi *et al.*, 2002; Vahdati & Zareie, 2006).

In Poland, Cerny (1965) subjected grafted trees to hot callus conditions in moist sawdust in a room heated to 26 ± 1°C with a relative humidity of 70 to 90%. After the callus period, trees were transferred to a cold environment of 4°C for storage before being transplanted in spring. Pieniazek (1972) reported on results of up to 90% grafting success using this conventional hot callus method in Poland. These hot callusing procedures have also been successfully adapted to graft walnuts in New Zealand (Halliwell, 1975).

Similar hot callus facilities have been established by Lantos (1990), Ferhatoğlu (1997), Stansavljević & Mitrović (1997), Achim & Botu (2001), Solar *et al.* (2001), Özkan & Gümüs (2001) and Vahdati & Zareie (2006) in numerous European countries, but varying outcomes were obtained. Hot callus rooms were heated to 26 to 28°C and relative humidity was maintained at 80 to 85%, while grafted trees were either placed in wooden boxes or plastic containers. With the exception of Lantos (1990) all authors indicated that grafting success was determined at the end of the first vegetative period after grafting. In Hungary, Lantos (1990) recorded grafting success of 56 to 84%, depending on cultivar and rootstock, but with no precise indication of when success was determined during the post-grafting period. Ferhatoğlu (1997) attained exceptionally variable results of between 14 and 73% grafting success on average in Turkey. Within certain experiments, up to 87% success was recorded, depending on time of grafting and cultivar. Over a two year period, Stansavljević & Mitrović (1997) reported on grafting success averaging 87% in the former Yugoslavia, while Achim & Botu (2001) obtained 59% of successfully grafted trees on average in Romania. Solar *et al.* (2001) achieved between 71 and

[1] Delort Francis, 2005. INRA Research Institute, Bordeaux, France
[2] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

83% successfully grafted trees over a period of three years in Slovenia, while Özkan & Gümüs (2001) obtained only between 14 and 22% success in Turkey.

Hot callus grafting described by Van't Westeinde (1990) was performed in heated greenhouses in the Netherlands, covering individual callus boxes with two layers of plastic sheeting to maintain a temperature of 27°C in a humid environment inside the boxes. Rootstocks were uplifted and stored in a callus container inside a greenhouse during late autumn. Soil underneath the plants was heated in mid winter and by the end of winter roots started to grow. Grafting commenced after leaves started to grow, while the lower plastic layer was perforated to regulate temperature. Callus developed within six days. Unfortunately, there was no indication of success percentage obtained with this procedure. According to Veltkamp & Blumink (2006) this was the only nursery until 1985 in Holland to produce commercial walnut planting material. By 2006, approximately 7500 grafted walnut trees were produced annually by these procedures.

The growing medium used to place trees in callus containers was not an aspect investigated by researchers. However, judging by the medium most often used, disinfected sawdust appeared to be the standard (Lantos, 1990; Rongting & Pinghai, 1993; Atefi, 1997; Özkan & Gümüs, 2001; Vahdati & Zareie, 2006; Linard, personal communication[1]), while peat was also used by some authors (Zachej, 1976; Solar *et al.*, 2001). If sawdust is to be used as a medium, the use of fine-textured sawdust from *Pinus* species or poplar is advised, while sawdust from chestnut or oak should be avoided due to high tannin levels (Linard, personal communication[1]).

With regard to the placement of grafted trees in callus containers, Zachej (1976) and Linard (personal communication[1]) indicated that trees should be positioned vertically. This was mostly for practical reasons, since more trees could be accommodated in a specific area and it made evaluation of grafted trees throughout the callusing period easier.

Optimum humidity of the immediate environment of grafted trees was regarded as vital, this was achieved by maintaining the correct moisture content of the medium in callus containers (Pieniazek, 1972; Farmer, 1973; Lagerstedt, 1979; Atefi, 1997; Erdogan, 2006). Generally, a high relative humidity of 80 ± 10% is preferred (Cerny, 1965; Ferhatoğlu, 1997; Stansavljević & Mitrović, 1997; Germain *et al.*, 1999; Achim & Botu, 2001; Özkan & Gümüs, 2001; Solar *et al.*, 2001). Rongting & Pinghai (1993) indicated that the moisture level of the medium influenced callus formation at the graft union. The optimum moisture level of the sawdust medium was between 55 and 60%, which resulted in 100% callus formation, while moisture content lower or higher than this resulted in only 30% of the grafts forming callus tissue. Furthermore, according

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

to Pieniazek (1972), mould may develop on grafted trees when too much moisture is present in the medium, however the amount of water to cause fungal growth was not specified.

Conventional hot callus grafting has greatly increased grafting success of walnut, and holds the added advantage of propagating a large number of plants on a relatively small surface area. The major disadvantage of this method, however, is the occurrence of both rootstock and scion sprouting if plant material is in more advanced stages of dormancy (Lagerstedt, 1982, 1983; Lantos, 1990). This could deplete the reserves required for callus formation before the graft union has formed. According to Farmer (1973) the preservation of dormancy is essential when hot callus grafting is performed after scion buds have already received a part of its required chilling. Mainly this concern initiated investigations into alternative approaches to hot callus grafting.

LOCALIZED HEATING OF THE GRAFT UNION

Utilization of an alternative approach to hot callus grafting of walnuts seems to have increased after Lagerstedt (1981a, 1982, 1983) developed the hot callus pipe for whip-grafted hazelnut (Lagerstedt 1981b, 1982; Yarris & Lagerstedt, 1981; Strametz, 1983; Thomson, 1985). This system delivers the required temperature for hot callusing at the graft union only, stimulating callus formation, while scion buds and roots are exposed to ambient conditions. This prevents bud break on the scion (Lagerstedt, 1981b, 1984), thereby avoiding depletion of scion reserves, since a callus bridge between rootstock and scion is already formed by the time scion buds initiate growth (Lagerstedt, 1982).

Lagerstedt (1984), MacDonald (1987) and Hartmann *et al.* (2002) listed several fruit and ornamental species in which grafting success has since been increased with the hot callus pipe. Fruit species benefitting from this approach include Black walnut (*Juglans nigra* L.), Persian walnut (*J. regia* L.), pecan (*Carya illinoensis* (Wangen) C. Koch), pear (*Pyrus* species), apple, European plum (*Prunus domestica* L.) and peach (*P. persica* L.). Ornamental species that were successfully grafted with this technique include European beech (*Fagus silvatica* L.), dogwood (*Cornus* spp.), maple (*Acer* spp.) and Douglas fir (*Pseudutsuga menziesii* (Mirb.) Franco). According to MacDonald (1987) this system is not suited for plants that normally callus readily over a wide range of temperatures, since more simple propagation techniques are sufficient for these species.

The standard hot callus pipe, constructed by Lagerstedt (1981a; 1982; 1983), consisted of a 38 or 50 mm PVC pipe into which 12 mm wide slots were routed, perpendicular to the length of the pipe. Slots covered a third of the circumference of the pipe at intervals of 3 to 5 cm. One length of 6 m pipe accommodated approximately 240 slots. A thermostatically controlled heating cable supplied heat to the system. Strands of the heating cable were taped on the outside of a 7 mm PVC pipe which was inserted into the bigger pipe. The inner PVC pipe was filled with water and capped to provide optimal heat regulation. Grafted trees were placed in a horizontal position in the pipe with the graft union placed in a slot where the temperature was maintained at 26 ± 2°C. A strip of foam rubber was positioned on top of the grafted trees to retard heat loss from the system. Roots were covered with moist sawdust to prevent desiccation during the callus period.

Several authors conducted hot callus trials on walnut by utilising the hot callus pipe (Avanzato & Tamponi, 1988; Atefi, 1997; Avanzato & Atefi, 1997; Porebski *et al.*, 1999; Achim & Botu, 2001; Porebski *et al.*, 2002; Avanzato *et al.,* 2006). In Italy, Avanzato & Tamponi (1988) achieved 73% success with this method, significantly more than the unheated control where only 6% success was obtained. Temperature was thermostatically controlled and maintained at 27°C.

Achim & Botu (2001) conducted experiments with the hot callus pipe in Romania. Although it was noted that the system was modified, there was no further description of the apparatus. It was mentioned though that heat in the system was supplied by warm water, which heated the graft unions to between 26 and 28°C. The best result achieved within certain treatment combinations was 89% success, while an average of 81% success was attained after the first vegetative period. This was significantly higher than the 59% success obtained by these authors in the hot callus room, as described earlier. These results corroborated findings by Atefi (1997) who also compared the two hot callus procedures in Iran. When localized heat was applied to graft unions, success was significantly higher at 83% compared to 65% success when whole grafted trees were subjected to hot callus conditions.

Variations of the hot callus pipe are currently employed by various nurseries and research institutes in Spain for large scale propagation of walnut, where more than 90% success is obtained (Aletà, personal communication[1]; Lopez Larrinaga, personal communication[2]). Both the research institute in Tarragona, Spain (Aletà, personal communication[1]) and a commercial nursery in Murcia, Spain (Lopez Larrinaga, personal communication[2]) adapted the hot callus pipe to create a hot callus trench. The required temperature of 27°C is directed to the graft union by arranging grafted trees in a horizontal position over a heated masonry trench, covered by a sponge-cushioned steel lid. Unlike the standard hot callus pipe, this system enables the use of rootstocks with a larger diameter. Heat is supplied by circulating warm water through a PVC pipe, as indicated by Achim & Botu (2001), and temperature is controlled thermostatically. Root systems are covered with a growing medium (e.g. perlite, potting soil or coarse sand) and kept moist throughout the callus period.

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[1] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain
[2] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain

Following the localized heating approach, some researchers tried to circumvent uprooting of rootstocks in attempts to minimize transplanting stress to grafted trees. Porebski *et al.* (2002) developed a hot callus system in Poland that was fixed on a glasshouse table in a vertical position, high enough from floor level in order to fit potted rootstocks underneath. Rootstocks therefore remained upright with graft unions positioned in heated slots on an even level. Over a two year period, average success of trees subjected to these hot callus conditions was between 55 and 82%, significantly higher than trees that were not hot callused, averaging only between 12 and 33% success.

Avanzato & Atefi (1997) and Avanzato (1999) developed an alternative method for supplying localized heat to the graft union. Rootstocks remained potted while graft unions were heated with an electric heating cable, termed a hot callus cable. It was positioned around the graft union and covered with a thin foil band to regulate the heat supply. Heat was thermostatically controlled on two sides of the graft union at 27°C. This hot callus cable was compared to the hot callus pipe technique in order to indicate the effect of transplanting stress (Avanzato & Atefi, 1997). With regard to grafting success, no significant differences were obtained between the two methods. The best result of 74% success was achieved by using the hot callus pipe and covering graft unions with humid cloth bands to retain moisture, compared to 70% success with the hot callus cable. Post-grafting growth, however, was significantly better with the hot callus cable trees because trees remained potted throughout the grafting and callusing stages and were not subjected to transplant stress.

Erdogan (2006) conducted hot callus trials in Turkey, using a similar hot callus cable, as described by Avanzato & Atefi (1997). However, rootstocks were transplanted into planting bags prior to grafting and then placed into plastic containers inside a greenhouse, thereby defeating the original purpose of the hot callus cable. Heating cables were positioned around the graft unions and the plastic containers were filled with growing medium to above the graft union, while scion buds were left uncovered. Temperature inside the greenhouse was maintained at 10°C and graft unions were heated to 25°C. An average success rate of 81% was achieved in the first year and 92% in the second year. Approximately 20 000 trees were grafted additionally for mass production utilizing this method, averaging 82% success.

2.5 Timing and duration of heat application

In general, the longer heat application is delayed after grafting, the lower the grafting success. When heat was immediately supplied to the graft union, Avanzato & Atefi (1997) secured 70% success, which was reduced to 55% when heat was applied four hours after grafting.

Bhat *et al.* (2000) achieved 74% grafting success with immediate heating of the graft union, while success was significantly reduced to 27% when heating was delayed by four days.

With regard to the length of the hot callus period, Cerny (1965) indicated that a period of less than 14 days was insufficient for walnut to produce callus (Table 2.1). Average grafting success after a hot callus period of 21 days was 88%. When the callus period was reduced to 14 days, average success was reduced to 77%. Only 46% success was achieved when the callus period was further reduced to 7 days, while only 7% success was obtained when heat was not applied.

Pieniazek (1972), Ferhatoğlu (1997), Stanisavljević & Mitrović (1997), Porebski *et al.* (2002), Solar *et al.* (2001) and Vahdati & Zareie (2006) terminated the callus period after 21 days when performing trials on hot callus grafting of walnut. Duration of the hot callus period varied between 20 and 25 days for Özkan & Gümüs (2001) and between 25 and 28 days for Achim & Botu (2001), while Avanzato & Atefi (1997) and Kazankaya *et al.* (1997) preferred 28 days. Hot callus conditions were extended to 30 days by Karadeniz (2003) and 33 days by Erdogan (2006). Lantos (1990) terminated the hot callus period when an unbroken ring of callus was visible at the graft union, rather than terminating it after a predetermined number of days. This author did, however, not mention the average number of days for grafts to reach this condition.

2.6 Optimum time of grafting

Hot callus grafting of deciduous plant species can only be performed while both rootstock and scion material are in a dormant condition (Farmer, 1973; Lagerstedt, 1979; 1982; Hartmann *et al.*, 2002). According to Lagerstedt (1984) leaf fall of rootstocks should occur before hot callus grafting can commence.

Various authors evaluated the influence of different grafting times during the dormant period on the success of walnut grafting in several areas. Most of these trials were conducted in the Northern Hemisphere and, unfortunately, only the month of grafting was usually mentioned, with no reference to the precise phenological stage of the plant material. Due to climatic variability between the areas where trials were performed, and subsequent uncertainty about the exact physiological condition of plant material, results can not be compared accurately between different studies. Hence forth, the month of grafting will be indicated, as well as an estimation of the season or condition of the plant material where possible.

Initial research on hot callus grafting of walnut, using heated rooms, advocated autumn grafting when neither rootstocks nor scion buds have received the required chilling for bud break (Cerny, 1965; Pieniazek, 1972; Zachej, 1976; Lagerstedt, 1979, 1982). According to Lagerstedt (1981b, 1982, 1984) the major beneficial influence of autumn grafting is that heat supply to the grafted tree is terminated before the chilling requirement of the scion buds has been satisfied, preventing bud break of the scion. In trials described by Cerny (1965) in Poland, grafting was performed between October (mid autumn) and December (late autumn to mid winter) at 14 day intervals in a heated room. When trees were evaluated 25 days after grafting, October grafting resulted in less than 15% trees showing signs of dormancy release, securing a 95% success rate by the end of the first growing period. In contrast, grafting in December stimulated scion growth in more than 85% of grafted trees, resulting in only 60% success.

Since the hot callus pipe was developed by Lagerstedt (1982; 1984) hot callus grafting of walnut was also performed during more advanced stages of dormancy. In Oregon, USA, Lagerstedt (1982) preferred grafting various species, including walnut, in mid December to mid January (mid dormancy) rather than February and March (late dormancy to early vegetative period). Earlier grafting ensured earlier planting of grafted trees, resulting in better survival and better growth compared to trees planted after the vegetative period had commenced in the area. In Iran, Ebadi *et al.* (2002) obtained significantly higher success from grafting in December compared to grafting one month later, indicating that grafting earlier in the dormant season is also preferred in this location. However, Vahdati & Zareie (2006) preferred grafting during March in the Fars region of Iran (probably late dormancy).

Various trials were performed in different areas of Turkey to evaluate success of walnut grafting between January and March. In the Tokat region bud break commences in late March to mid April (Akça *et al.*, 2001; Özkan & Celep, 2001). However, in the Van area May is regarded as the early vegetative period, with January considered as mid dormancy and March as the late dormancy period (Karadeniz, 2003). In the Yalova area, Ferhatoğlu (1997) indicated that grafting success increased significantly from January to February, as well as from February to March (Table 2.1).

Table 2.1 Influence of time of grafting on hot callus grafting success (%), determined by the end of the first growing season in the Yalova-area of Turkey (Ferhatoğlu, 1997)

Year of grafting	January	February	March
1986	26.6	35.4	63.4
1987	13.7	34.0	60.6
1988	33.4	52.3	50.0
1989	25.1	63.9	72.7
Mean	30.2 c	50.2 b	66.9 a

Means followed by different letters were significantly different according to multiple comparisons using LSD ($P \leq 0.05$).

In the Ankara region of Turkey, Erdogan (2006) also preferred to graft walnut in March, while grafting commenced one month earlier for commercial production of hot callused trees in this country. In contrast, Kazankaya *et al.* (1997) and Özkan & Gümüs (2001) reported that grafting in February resulted in higher success compared to one month earlier or later for the Van and Tokat regions in Turkey.

Karadeniz (2003) reported on the seasonal influence of phenolic compounds on grafting success in walnut under hot callus conditions in Turkey, performed during the winter dormancy period as well as during the vegetative period. Total flavan content in the phloem tissue of scions, was highest in May and June (early to mid vegetative period), when the lowest graft-take percentage of 54 and 32% respectively, was recorded. The lowest flavan level was observed in February and March (late winter dormancy period), resulting in the highest graft-take percentage of 100 and 98%, respectively. These findings indicated that graft-take in walnut might be negatively affected by flavan content of scion material obtained during different phenological stages. Similar results were obtained with hazelnut under hot callus conditions in Poland (Piskornik, 1995; Wyzgolik & Piskornik, 2001). It was concluded that the decreased level of phenolic compounds were related to endodormancy release in the scion buds.In Italy, Avanzato & Tamponi (1988) found that grafting in February (late dormancy) produced a significantly higher percentage of success (91%) compared to grafting executed one month earlier (54%). Avanzato & Atefi (1997) also preferred to perform grafting during this late dormancy period in further hot callus trials conducted in Italy.

The latest reported period of grafting walnut with hot callus techniques was by Solar *et al.* (2001), who grafted during the first half of April in Slovenia. Such late grafting may be contributed to climatic conditions resulting in bud break only commencing in late April to early May for most walnut cultivars in that area (Solar & Štampar, 2006).

2.7 Cultivar response to hot callus conditions

Several authors reported on the influence of cultivar on grafting success of walnut. According to Titmus (personal communication[1]) the duration of the callus period for different cultivars varied in Australia. In Spain, the cultivar 'Tulare' is more difficult to propagate than others, obtaining only 50% grafting success, while more than 95% success is achieved with other cultivars under hot callus conditions (Lopez Larrinaga, personal communication[2]).

[1] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia
[2] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain

Ferhatoğlu (1997) and Erdogan (2006) reported on response of several Turkish cultivars to hot callus conditions. Both authors obtained significant differences in grafting success between cultivars by the end of the first vegetative period, although there did not seem to be any clear trends. Ferhatoğlu (1997) recorded 28 to 87% success during the optimal time of grafting (March), while the average grafting success over a period of four years was between 48 and 75%. Erdogan (2006) reported on varying results between two seasons of grafting. In the first year, no significant differences were obtained when grafting success for different cultivars was between 76 to 88%. In the second year, however, the cultivars varied significantly between 80 and 100% grafting success. For commercial production of grafted trees under hot callus conditions, inconsistent results were obtained for certain cultivars, which were attributed to the influence of genotype (Erdogan, 2006).

Lantos (1990) obtained between 56 and 71% grafting success among the three Hungarian cultivars evaluated, and Stanisavljević & Mitrović (1997) between 55 and 93% success with seven different Yugoslavian walnut cultivars. There was, however, no indication whether cultivar differences were significant. On the other hand, Özkan & Gümüs (2001) reported no significant differences in terms of grafting success between two different Turkish cultivars investigated.

2.8 Plant material

ROOTSTOCKS

Lagerstedt (1982) regarded poor root system quality as the foremost influential factor on post-planting graft failure in various hot callused fruit species and accentuated the selection of high quality rootstocks.

Seedlings of Persian walnut (*J. regia*) are most often used as rootstocks in hot callus grafting (Cerny, 1965; Pieniazek, 1972; Avanzato & Tamponi, 1988; Lantos, 1990; Tsurkan, 1990; Van't Westeinde, 1990; Ferhatoğlu, 1997; Kazankaya *et al.*, 1997; Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001; Porebski *et al.*, 2002; Karadeniz, 2003; Erdogan, 2006; Vahdati & Zareie, 2006). According to Lantos (1990) better results were achieved with *J. regia* compared to *J. nigra*. Northern Californian black walnut (*J. hindsii* Jeps. Rehder) seedlings were also utilized by Pieniazek (1972) and by the Australian walnut project in Tasmania (Titmus, personal communication[1]).

The age of rootstocks used in hot callus grafting were either one-year-old (Avanzato & Tamponi, 1988; Tsurkan, 1990; Ferhatoğlu, 1997; Kazankaya *et al.*, 1997; Porebski *et al.*, 2002) or two-year-old seedlings (Cerny, 1965; Van't Westeinde; 1990; Achim & Botu, 2001; Özkan &

[1] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

Gümüs, 2001; Ebadi *et al.*, 2002; Karadeniz, 2003), or both (Pieniazek, 1972; Lantos, 1990; Erdogan, 2006; Vahdati & Zareie, 2006). Vahdati & Zareie (2006) regarded the healing rate of two-year-old seedlings to be better than that of one-year-old seedlings. Although most researchers mentioned age of rootstocks, only a few indicated the size used for hot callus grafting. Cerny (1965) indicated that rootstocks had a dominant taproot system. Rootstocks grafted by Tsurkan (1990), Ferhatoğlu (1997) and Erdogan (2006) were between 10 and 20 mm in diameter at the point of grafting, while Erdogan (2006) also gave the total height of rootstocks as between 40 and 50 cm.

Time of digging and handling of rootstocks before grafting varied between researchers. Lantos (1990), Van't Westeinde (1990), Özkan & Gümüs (2001) and Porebski *et al.* (2002) uplifted rootstocks from the nursery two to four months before grafting. Lantos (1990) and Özkan & Gümüs (2001) indicated that roots were disinfected for three hours in a fungicide mixture before cold storage in damp sawdust at 2 to 3°C until grafting time. Avanzato *et al.* (2006) also disinfected rootstocks, but just prior to the grafting procedure. Most researchers grafted onto dormant rootstocks, however, Van't Westeinde (1990), Avanzato *et al.* (2006) and Erdogan (2006) subjected rootstocks to forcing conditions prior to grafting. Van't Westeinde (1990) heated the root systems with bottom-heat, while Avanzato *et al.* (2006) placed rootstocks in a forcing room at 26 to 28°C and 80 to 90% humidity for two weeks before grafting. Erdogan (2006) forced rootstocks by plunging them in running water for two to three days before grafting commenced. Cerny (1965), Pieniazek (1972), Lantos (1990) and Özkan & Gümüs (2001) reduced the size of seedlings by root trimming in order to pack the plants more tightly in hot callus containers.

SCION MATERIAL

The effort or cost to obtain high quality scion wood is often regarded as small in proportion to the value it contributes to a successfully grafted tree (Lagerstedt, 1979, Coggeshall & Beineke, 1997; Avanzato *et al.,* 2006). Tsurkan (1990) and Ferhatoğlu (1997) obtained walnut scion material for hot callus trials from specialized mother trees. Tsurkan (1990) and Godeanu *et al.* (2001) described pruning technique and time of pruning in order to obtain high quality scion material from a specialized mother block. According to Linard (personal communication[1]) less irrigation and a lower nitrogen fertilization level should be implemented in managing a mother block.

Grafting success depends also on the correct timing for collection of scion material. Lagerstedt (1979), Tsurkan (1990) and Porebski *et al.* (2002) advised that scion wood be cut in late autumn

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

before severe frost is experienced. Ferhatoğlu (1997) obtained scion material in mid winter and utilized it during the following three months. Özkan & Gümüs (2001) collected scion material 48 hours prior to grafting, while Stanisavljević & Mitrović (1997) collected scion material only on the day when grafting was performed. According to Erdogan (2006) scion material can be collected on the same day of grafting or cold stored for several days.

Pieniazek (1972), Van't Westeinde (1990) and Erdogan (2006) preferred scion material obtained from middle and basal parts of vigorous shoots from the previous season's growth. According to Lagerstedt (1979) this portion of the shoot is prime scion wood in walnuts, while tip ends are too thin and should be discarded. Erdogan (2006) and Vahdati & Zareie (2006) used scions for bench grafting of walnuts containing two to three buds, 10 to 15 cm in length and 25 mm in diameter. Lagerstedt (1982) suggested that when the hot callus pipe is utilized, scion material should have long internodes in order to prevent heat from stimulating scion buds to break dormancy.

With reference to treatment of scion material after collection, various procedures were preferred by different authors. Porebski *et al.* (2002) stored scion wood in a cold environment in moist sawdust prior to grafting. Ferhatoğlu (1997) and Achim & Botu (2001) indicated the optimum temperature for storage of walnut scion wood to be 4°C. Lantos (1990), Özkan & Gümüs (2001) and Avanzato *et al.* (2006) disinfected scion material by soaking it in a fungicide solution for one hour. As was the case with rootstock material, most authors used dormant scion wood for hot callus grafting of walnut. Some researchers, however, chose to force scion wood before grafting. Lantos (1990) and Özkan & Gümüs (2001) performed forcing by placing scion material in a forcing room at 26 to 28°C for two to three days before grafting, while Erdogan (2006) dipped scion wood in running water for several hours prior to grafting.

Lagerstedt (1979), Rongting & Pinghai (1993) and Atefi (1997) regarded conservation of scion moisture prior to grafting as vital, since callus growth after grafting only initiated when there was no water stress present in scion material. According to Rongting & Pinghai (1993), scion moisture can be positively correlated to the weighed amount of callus formed, as well as to the growth of callus, and no callus growth was initiated when scion moisture was below 38%.

2.9 Grafting technique

In most of the hot callus trials with walnut, bench grafting of dormant material was an integral part of the procedure. Bench grafting is described by Garner (1988) as any grafting process performed whilst both rootstock and scion are unplanted, regardless of the actual technique involved. As mentioned earlier, only Avanzato & Atefi (1997) eliminated the bench grafting procedure in order to avoid transplanting stress and this indeed favoured post-grafting growth of trees significantly. This approach is currently only followed by a research institute in Italy and not by any commercial walnut nursery, possibly due to its practicability on commercial scale.

The importance of maximum cambial contact between rootstocks and scion in walnut grafting has been stressed by various authors (Lagerstedt 1979; Garner, 1988; Van't Westeinde, 1990; Reil *et al*., 1998) and was regarded as even more important than in most species, since walnut produces less callus tissue (Reil *et al*., 1998).

Whip-and-tongue grafting was the most popular grafting method used in studies of hot callus grafting of walnut (Pieniazek, 1972; Radicati & Me, 1986; Lantos, 1990; Tsurkan, 1990; Van't Westeinde, 1990, Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001; Erdogan, 2006; Gandev, 2007; Aletà, personal communication[1]; Lopez Larrinaga, personal communication[2]). The side graft described by Germain *et al.* (1999) is performed on large scale in hot callus nurseries in France (Linard, personal communication[3]) and Australia (Titmus, personal communication[4]), probably because it is easier and faster to perform with a machine (Figure 2.2) than the whip-and-tongue technique by hand.

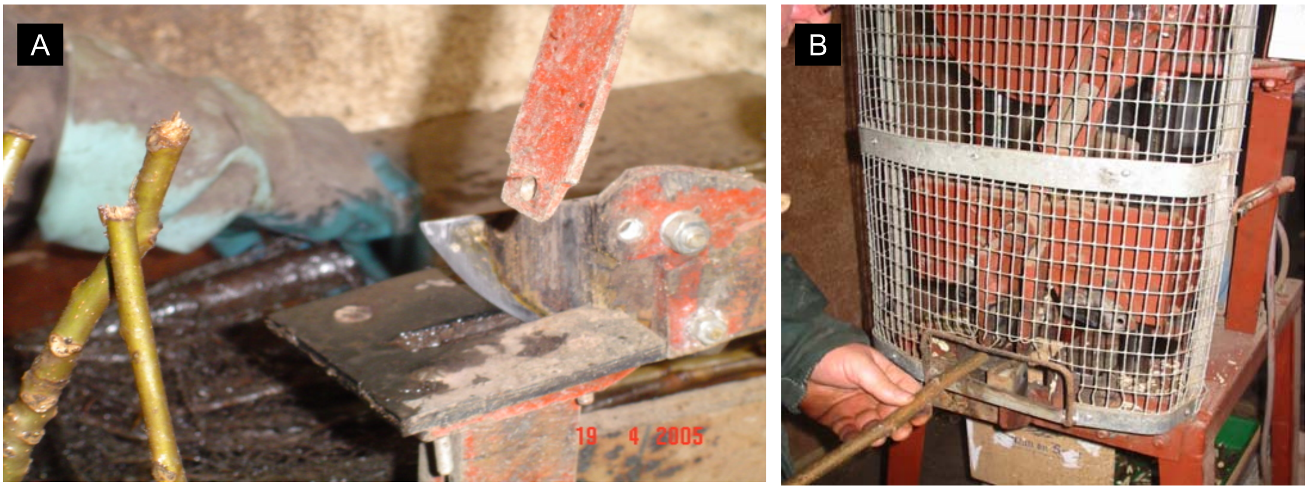

Figure 2.2 Grafting machines used by Linard (personal communication[1]) to perform the side graft. It consists of a machine for the cut in the rootstock (A) and one to cut the scion (B).

Other techniques used in combination with hot callus grafting of walnut, include the saddle graft (Cerny, 1965), cleft grafting (Atefi, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001), chip budding (Kazankaya *et al*., 1997; Özkan & Gümüs, 2001; Porebski *et al*., 2002; Karadeniz, 2003) and side-stub grafting into the crown of rootstocks (Vahdati & Zareie, 2006). Machine grafting was also performed, including omega bench grafting (Lagerstedt, 1982; Ferhatoğlu,

[1] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain
[2] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain
[3] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[4] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

1997; Solar *et al.*, 2001), V-graft (Atefi, 1997), “nut-and-feder” (Achim & Botu, 2001) and the lamella bench graft (Solar *et al.*, 2001).

Özkan & Gümüs (2001) observed no differences in grafting success between whip-and-tongue, cleft grafting and chip budding. Achim & Botu (2001) achieved superior results with whip-and-tongue and cleft grafting, compared to the “nut-and-feder” machine technique. Solar *et al.* (2001) obtained higher grafting success with omega machine grafting compared to the lamella machine graft, since more cambial contact was secured with the former technique. These machine grafts were not compared to standard hand grafting methods normally used under hot callus conditions. The only advantage of lamella machine graft was that bigger rootstocks, with a diameter between 20 and 22 mm, could be grafted (Solar *et al*., 2001). Ebadi *et al.* (2002) illustrated the influence of rootstock size on choice of grafting technique. When rootstock seedlings were between 20 and 40 mm in diameter, the side graft was performed, while the saddle graft was performed when seedlings were 10 to 20 mm in diameter.

To ensure grafting success, the conservation of scion moisture after grafting was just as vital as before grafting and various approaches were followed to prevent scion desiccation after grafting. The lower critical level of scion moisture (38%) determined by Rongting & Pinghai (1993) was reached within seven days after grafting if the scion was uncoated. By coating the scion with grafting wax, this period was extended to 23 days and substantially more callus tissue formed at the graft union. Solar *et al.* (2001) also achieved slightly higher grafting success when graft unions were sealed with paraffin wax (83%), compared to uncovered grafts (77%) three weeks after grafting. Cerny (1965), Lantos (1990), Özkan & Gümüs (2001), Erdogan (2006) and Linard (personal communication[1]) preferred to seal only the tip end of the scion with hot paraffin wax. Cerny (1965) covered the complete graft union with PVC tape, while Erdogan (2006) tied the graft union only at the bottom and top, leaving the middle portion uncovered to prevent moisture from accumulating in the graft union. As mentioned earlier, Erdogan (2006) plunged rootstocks in running water for three days prior to grafting, probably causing excessive “bleeding”. Solar *et al.* (2001), Francis (personal communication[2]) and Aleta (personal communication[3]) sealed the complete graft union and scion with wax.

Although common sealing procedures were not followed in all studies, there were some trends regarding sealing approaches. Generally, only terminal ends of scions were sealed when entire trees were subjected to heat inside hot callus rooms (Lantos, 1990; Özkan & Gümüs, 2001; Linard, personal communication[1]), since sealing of the entire graft union is apparently

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Delort Francis, 2005. INRA Research Institute, Bordeaux, France
[3] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain

unnecessary under very humid conditions (Meacham, 1995). In contrast, maximal coating with PVC tape and wax was suggested by Aletà (personal communication[1]) when trees were subjected to heat in a hot callus pipe. Avanzato & Atefi (1997) observed a significant increase in success when graft unions in the hot callus cable system were covered with humid cloth bands and tin foil, to ensure maximum moisture retention.

Flemer (1986) mentioned that temperature of grafting wax was critical in ensuring a successful graft, but did not indicate the optimum temperature required. When wax was too hot, it penetrated the cuts and inhibited callus development, while sealing was inadequate when the wax was too cool. In studies where ideal wax temperature was mentioned, researchers differed substantially in their recommendations. Ferhatoğlu (1997) suggested a wax temperature of approximately 43°C, while Francis (personal communication[2]) used wax slightly warmer at 55°C and Solar *et al.* (2001) preferred a wax temperature between 75 and 85°C. Solar *et al.* (2001) further advised that graft unions be plunged into water in order to cool down after waxing.

2.10 Acclimatization, planting and post-grafting growth

The time of planting grafted trees into the nursery was generally regarded as an imperative part of hot callus grafting of walnut. Whether trees should be acclimatized before planting or not, largely depends on the climatic conditions of the area. If the location has a mild winter climate, hot callused trees could be planted directly after the callus period (Lagerstedt, 1982; Meacham, 1995). However, Lagerstedt (1984) suggested cold storage of hot callused trees if severe winter conditions prevail in a specific area. If grafted trees were to be cold stored before planting into the nursery, Lagerstedt (1984) and Reil *et al.* (1998) emphasized the importance of protecting the graft union from desiccation.

In Moldavia, Tsurkan (1990) directly planted some hot callused trees in an open field nursery and trees were not affected when minimum temperatures dropped to between -2 and -3°C over a short period. However, when these conditions persisted for longer periods, damage was severe, while outdoor temperatures of between -6 and -9°C caused darkening and death of hot callused trees. Hence, most researchers in the Northern Hemisphere preferred acclimatization before grafted trees were transplanted into a nursery, with the purpose of protecting fragile graft unions against possible frost damage (Cerny, 1965; Lantos, 1990; Stanisavljević & Mitrović, 1997; Özkan & Gümüs; 2001; Solar *et al.*, 2001; Vahdati & Zareie, 2006).

[1] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain
[2] Delort Francis, 2005. INRA Research Institute, Bordeaux, France

Tsurkan (1990) effectively stored grafted trees, hot-callused during the early dormant period, at 0 to 2°C until April in Moldavia, when average maximum temperatures reached 15°C and trees could be transplanted. For additional protection once transplanted in the nursery, Lantos (1990) and Tsurkan (1990) covered graft unions with soil. Ferhatoğlu (1997) subjected grafted trees to reduced temperatures of 15°C for one week, after a three week hot callus period inside a heated room. After the four week period, grafted trees were potted and transferred to a protective greenhouse until the end of May in Turkey. Finally, the potted trees were moved to a semi-shaded structure until the end of the first vegetative season. Where rootstocks were transplanted into plastic bags prior to the callus period, acclimatization of grafted trees was performed with more ease (Porebski *et al.,* 2002; Erdogan, 2006).

According to Flemer (1986), Lantos (1990), Tsurkan (1990), Stanisavljević & Mitrović (1997), Achim & Botu (2001) and Erdogan (2006) a standard size grafted tree, suitable for orchard establishment, could not be obtained after the first growing season. Trees had to grow for another season in the nursery before a saleable size was achieved. Erdogan (2006) attributed slow growth during the first season to incomplete callus formation at the graft union, together with the loss of roots during the bench grafting phase. Solar *et al.* (2001) considered grafted trees to be well-developed when they were well rooted, with a minimum height of 100 cm. When bench grafting was avoided, and trees remained rooted throughout the hot callus period, suitably sized trees were obtained within one growing season (Avanzato & Atefi, 1997).

2.11 Concluding remarks

It became clear through this literature survey, that hot callus grafting of walnut has enormous potential to produce grafted walnut trees of high quality. The notorious repercussions experienced with traditional budding and grafting techniques under uncontrolled field conditions were eliminated by the utilization of hot callus grafting. Although the procedures are more labour intensive and probably more expensive, the beneficial effect of increased grafting success will more than compensate for this. Also, once hot callus facilities have been established, further expenses are restricted to standard nursery material, energy for heating and general maintenance.

Specific research inputs are required, however, to establish a successful hot callus nursery since it might take several seasons to adapt the technique to local conditions. According to French nursery expertise (Linard, personal communication[1]), success of the hot callus grafting largely depends on minor technical adaptations to suit local conditions, which can only be determined

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

through intensive experimentation. Titmus (personal communication[1]) indicated that it took five years to obtain optimal results with hot callus grafting in the Australian walnut project in Tasmania, where the technique has been successfully implemented.

[1] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

CHAPTER 3. *General materials and methods*

3.1 Introduction

Experimental materials and methods used throughout this study are mostly identical and in order to avoid unnecessary repetition in the manuscript, these general procedures are described in this chapter. Only deviations from these procedures will be described separately in the relevant chapters.

Trials were conducted on the farm Rotondo Walnuts (30°40'S, 26°37'E, 1 347 m above sea level), in the Southern Free State, approximately 5 km west of Aliwal North, in South Africa during the 2006/07 and 2007/08 seasons. Preliminary experiments were performed during the 2005/06 season in order to become familiarized with and adjust the experimental procedures to be used in the following dormant seasons. These experiments also provided vital information on the construction of hot callus facilities for more efficient temperature and humidity control. Valuable knowledge gained during preliminary experiments will also be provided in this chapter where relevant.

Hot callus grafting of walnut was investigated by examining the reaction of two potentially important walnut cultivars ('Serr' and 'Chandler'), to two methods of heat supply (a heated room and heated trench), at three different grafting times (mid dormancy, late dormancy and early vegetative stages).

3.2 Plant material

ROOTSTOCKS

Rootstocks utilized in 2006/07 were uniformly sized two-year-old seedling trees. Seed of *Juglans regia* L. from an identical seed source was planted in September 2004 on Rotondo Walnuts and De Draai farm, 10 km east of Aliwal North. Rootstocks were approximately 40 to 60 cm in height, and 15 to 20 mm in diameter at the point of grafting, with a dominant taproot.

Since the initial rootstock source was insufficient and trials needed to be repeated, rootstocks for 2007/08 were purchased from another grower. These were uniformly sized two-year-old *J. regia* seedling trees. Seed from a sole source was planted in September 2005 in the Colesberg district, Northern Cape, South Africa. The grower transplanted the seedlings into 5 ℓ plastic bags after the first growing season, removing a substantial part of the taproot. Seedlings were approximately 30 to 40 cm in height and 5 to 8 mm in diameter at point of grafting with an adventitious root system.

Rootstocks were removed from the soil 24 hours prior to grafting and rinsed thoroughly in clean water. Lateral roots were trimmed to approximately 60% of the original length and dipped in Stock-o-Sorb®, a gel-like substance that absorbs water and prevents roots from drying for a substantial period. Rootstocks were then stored overnight in moist hessian bags at room temperature. Figure 3.1 illustrates the different sized rootstocks utilized in this study, compared to rootstocks used by a commercial nursery in France (Linard, personal communication[1]).

Figure 3.1 Rootstocks used during the 2006/07 season (A), during the 2007/08 season (B) and by a commercial nursery in France (C).

SCION MATERIAL

As mentioned earlier, 'Chandler' and 'Serr' were the cultivars chosen for hot callus grafting. Hendricks *et al.* (1998) noted that 'Chandler' has been the most popular cultivar for new plantings in California. Bud break in 'Chandler' occurs relatively late, making it less susceptible to spring frost injury. For 'Serr', however, bud break occurs up to four weeks earlier than 'Chandler'. Although this makes 'Serr' more prone to late frost injury, it remains the most popular cultivar planted in Chile (Sibbett, personal communication[2]).

Scion wood was obtained from unpruned commercial trees on the same day as grafting was performed. Grafting wood for 'Serr' for the third round of grafting (week 37), however, had to be collected in advance and stored in moist sawdust at 3 ± 1°C for two weeks, since grafting was performed a week after bud break for this cultivar in both seasons. This is standard practise

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Steve Sibbett, 2008. University of California, Davis, CA, USA

under hot callus conditions which does not have a negative influence on graft-take (Linard, personal communication[1]; Francis, personal communication[2]). Scion material was collected from basal parts of shoots from the previous growing season. Scions were approximately 80 to 100 mm in length with two buds and a diameter matching the diameter of the rootstocks.

3.3 Grafting technique

The side graft, described by Germain *et al.* (1999), was used for grafting walnut in this study (Figure 3.2). This technique is generally used by commercial hot callus nurseries in France (Linard, personal communication[1]) and Australia (Titmus, personal communication[3]).

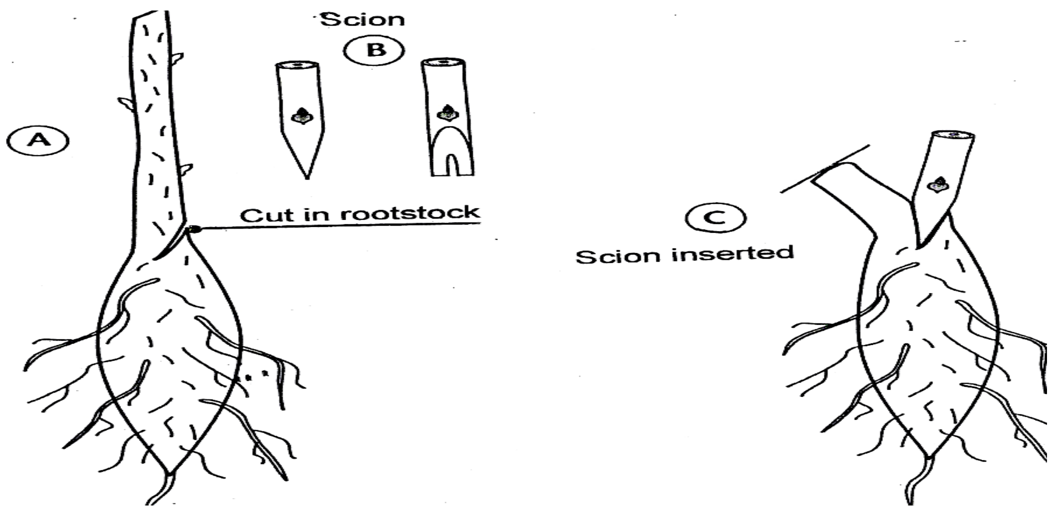

Figure 3.2 Schematic diagram of the side graft technique (Germain *et al.*, 1999).

In preliminary experiments during 2005/06, grafting was also attempted with a bench grafting machine, Topgrafter™, purchased from Cape Agricultural Products in Somerset West, South Africa. This machine has been extensively tested on a large number of fruit trees, including kiwifruit, grapes, avocados, walnuts, chestnuts, persimmons and pears, apparently resulting in up to 100% grafting success (Vorster, personal communication[4]). In preliminary experiments, however, this grafting technique resulted in less callus development at graft unions than hand grafting, with subsequent poor grafting success, and the technique was therefore eliminated.

Scions were prepared manually with a sharp grafting knife. The side cut in the rootstocks was performed using a pruning lopper, modified to secure an even cut without separating the bark from the xylem. Height of grafting was approximately 50 mm above soil level. Immediately after

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Delort Francis, 2005. INRA Research Institute, Bordeaux, France
[3] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia
[4] Mike Vorster, 2005. Cape Agricultural Products, Somerset West, South Africa

grafting, the graft union was tightly covered with polyethylene budding tape. The whole scion and graft union was then immersed in hot paraffin grafting wax heated to approximately 70ºC as suggested by Solar *et al*. (2001) (Figure 3.3). Roots were again dipped thoroughly in Stock-O-Sorb® to prevent moisture loss during the forthcoming callus period.

Figure 3.3 Process of covering the graft union (from left to right) grafted tree after inserting the scion; after wrapping with budding tape and after dipping in hot paraffin wax (A); and grafted trees before being immersed in paraffin wax and subjected to hot callus conditions (B).

3.4 Heat application

Grafted trees were subjected to one of two heat application methods for hot callusing. With the first method, heat was supplied to the entire grafted plant in a hot callus room, while the second method supplied localized heat to the graft union only in a modified hot callus trench.

HOT CALLUS ROOM

Trees were sorted according to size and positioned vertically into 40 ℓ plastic crates, each accommodating 20 to 30 trees. Crates were filled with a 1:3 mixture of Hygrotech Hygromix® and pine sawdust, as suggested by Linard (personal communication[1]). This growing medium was moistened with a 0.5% Captab® solution to prevent fungal development (Van der Westhuizen, 1981; Wiese, personal communication[2]). Care was taken to create a mixture without any free water inside the crates. Crates were covered with lids or plastic sheets where trees were too tall, and moved to an isolated concrete room of 17 m^3 that was heated with a

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Johan Wiese, 2006. Voor Groenberg Nursery, Wellington, South Africa

1200 BTU LG® air conditioner. Temperature inside the crates were thermostatically controlled and monitored at 27 ± 2ºC, while relative humidity was regulated at 80 ± 5%.

HOT CALLUS TRENCH

A modification of the hot callus trench, employed by the IRTA Research Station in Spain (Aletà, personal communication[1]), was used. Two concrete trenches, measuring 6 x 0.3 x 0.3 m each were constructed indoors. The trenches were designed to ensure optimal regulation of the required temperature. A pair of under-tile heating cables, separated by, and secured onto a wooden rod with electric insulation tape, was connected to an electric supply and inserted into a 15 mm copper pipe. The copper pipe was inserted into a 6 m length PVC pipe, 75 mm in diameter. The PVC pipe was placed into the trench, capped and filled with water to ensure optimal temperature regulation.

The trenches were closed on top with steel lids, measuring approximately 120 cm in length each. Foam rubber strips, 70 mm thick, were fitted to the trench walls as well as underneath the steel lids for insulation and to cushion grafted trees (Figure 3.4A). Temperature inside the trenches was thermostatically regulated at 27 ± 1°C.

Grafted trees were placed alternately in a horizontal position with the graft union over the heated trench (Figure 3.4B). Roots were covered with the same growing medium used in the hot room, moistened with a 0.5% Captab® fungicide mixture. Moist hessian bags covered the growing medium to prevent it from drying.

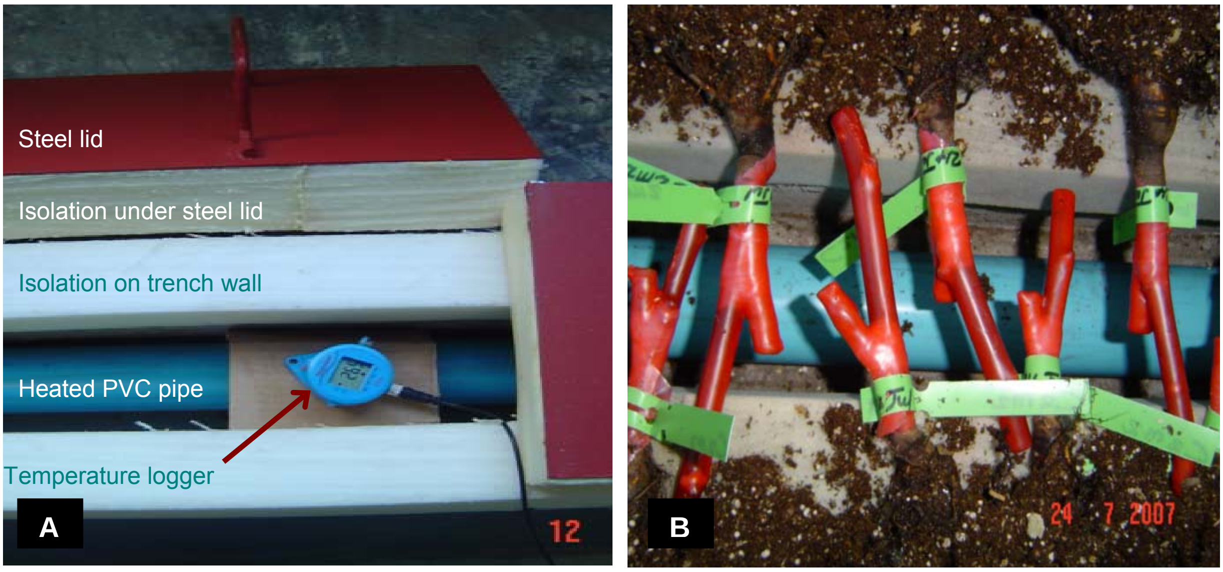

Figure 3.4 Construction of hot callus trench (A) and orientation of grafted trees in hot callus trench (B).

[1] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain

3.5 Grafting time

According to Hartmann *et al.* (2002) callus tissue develops more readily during late winter and just prior to or during bud break. Hence, grafting in the current study was performed at three stages during this period. The first round of grafting was performed during mid dormancy (end July, week 30), the second round in late dormancy (end August, week 34) and the third round in the early vegetative period (September, week 37) when *J. regia* rootstocks had just started bud break. Under normal climatic conditions in the Southern Free State area, bud break of 'Serr' occurs during week 35, while bud break of 'Chandler' occurs during week 39.

3.6 Planting into nursery

After the hot callus period (week 35, 39 and 42 respectively) grafted trees exhibiting any callus development were planted directly into the nursery, a protective structure covered with 40% white shade net. Trees were planted with an in-row spacing of 20 cm and between-row spacing of 30 cm. Below freezing temperatures of -1 to -7ºC are frequently experienced in the southern Free State during this time of year, hence trees were painted with white PVA and wrapped with hessian strips to provide additional protection against possible frost damage (Figure 3.5). Standard commercial nursery practices, including irrigation, fertilization, foliar nutrition and pest control were followed to ensure optimal growing conditions in the shade house. All growth from the rootstocks was removed frequently to ensure optimal growth of the scion.

Figure 3.5 Grafted trees painted with white latex paint (A) and covered with hessian bag strips (B) for additional frost protection.

3.7 Data recorded

WEATHER DATA

Minimum and maximum temperatures were obtained from the Agricultural Research Council (ARC) weather station on Rotondo Walnuts farm (30°40'S, 26°37'E, 1 347 m above sea level).

CALLUS DEVELOPMENT

For each treatment combination, a sample of eight grafted trees was opened on day 21, 25, 29 and 33 after grafting, to evaluate callus development. After evaluation, grafts were re-covered with budding tape only. The amount of callus formed was recorded in terms of percentage visible callus tissue around the union, from 0% indicating no callus development and up to 100% visible callus development around the graft union.

Optimal duration of heat application required for callusing was determined from the level of callus development evaluated over time.

GRAFT-TAKE

On day 33 after grafting, all grafted trees were opened and the amount of callus development was rated on a percentage scale, as described above. Graft-take was calculated as the percentage of trees showing any indication of callus development. Grafted trees with no callus tissue were discarded, while the rest of the trees were planted in the nursery.

SUCCESS PERCENTAGE

Success percentage, calculated as the percentage of initially grafted trees surviving the growing season, was evaluated at three different stages during the first growing season. For trees grafted mid and late winter, the first evaluation stage occurred during the last week of October (week 43: 59 and 29 days after planting respectively) and during the second week of November (week 45: 23 days after planting) for trees grafted in early spring. This corresponds to the evaluation periods of Avanzato & Tamponi (1988), Solar *et al.* (2001) and Erdogan (2006). At this stage, graft unions were opened and sealed with a bitumen-based tree sealant. Growth from the scion was restricted to only one shoot.

The second evaluation of grafting success took place during the middle of the growing season (Lagerstedt, 1982; Porebski *et al.*, 2002), when considerable growth from scion buds was evident. This occurred during the first week of February (week 5), which was 157, 127 and 108 days after planting for the three rounds of grafting, respectively. The final evaluation of success percentage occurred at the end of the growing season, after vegetative growth had ceased but before leaf fall (Stanisavljević & Mitrović, 1997; Özkan & Gümüs, 2001). This was during the first week of May (week 18: 247, 217 and 198 days after planting for the three rounds, respectively).

FINAL SURVIVAL PERCENTAGE

Survival percentage was calculated as the percentage of grafted trees that were originally planted (i.e. trees that callussed) and survived to the end of the first growing season (Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001). Survival percentage provided an indication of how effective the graft union was that formed during the callus period and subsequent capability of the grafted trees to survive outdoor conditions.

POST-GRAFTING GROWTH

Height and diameter of vegetative growth from the scion was determined simultaneously with the last two rounds of success percentage evaluation (Avanzato & Atefi, 1997; Porebski *et al.*, 2002). Height was measured from the base of the new shoot on the scion to the terminal bud. Shoot diameter was measured 1 cm from the base of the shoot.

3.8 Experimental layout and statistical analysis

The 3 x 2 x 2 factorial experiment was arranged in a completely randomized design with four replications consisting of eight grafted trees each. As already mentioned the three factors that were investigated were time of grafting, heating method and cultivar response. In total, 384 trees were grafted each season, comprised of 128 grafts at each physiological stage. Due to limited availability of rootstocks, no unheated control treatment was performed in both seasons.

Effective duration of callus period was determined by repeated measures analysis, as well as the Student’s t-test. All other data were subjected to analysis of variance (ANOVA) using the general linear model procedure of the Statistical Analysis System (SAS Institute, 2003). In order to obtain better normal distribution, data for callus development and graft-take were square transformed, while means were re-transformed afterwards for discussion purposes. Means were separated using Fisher’s protected least significant difference (LSD) at 95% level of probability.

CHAPTER 4. *Grafting success and survival of hot callused walnut*

4.1 Introduction

The Persian walnut (*Juglans regia* L.) is considered to be more difficult to propagate vegetatively through grafting and budding than most fruit species, since less callus is produced under sub-optimal climatic conditions (Reil *et al.*, 1998; Hartmann *et al.*, 2002). Highly efficient propagation techniques are therefore required to stimulate callus formation at the graft union by eliminating interfering factors. Hot callus grafting was conducted by several authors in order to avoid the obstacles encountered with patch budding and other conventional vegetative propagation methods for walnut, performed during the growing period (Pieniazek, 1972; Avanzato & Tamponi, 1988; Lantos, 1990; Van't Westeinde, 1990; Ferhatoğlu, 1997; Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001; Solar *et al.*, 2001; Porebski *et al.,* 2002; Erdogan, 2006). Hot callus grafting is described by Lagerstedt (1982) as a propagation method where bench grafted trees are exposed to elevated temperatures and humidity during the dormant period for enhanced callus formation in difficult-to-propagate species.

Studies on hot callus grafting of walnut have explored the effects of various factors on grafting success. Some of the factors most explored include the method of heat application, duration of heat application and time of grafting.

The conventional approach to walnut hot callus grafting, by subjecting the entire grafted plant to elevated temperatures, has been described by Cerny (1965), Lantos (1990), Ferhatoğlu (1997), Stanisavljević & Mitrović (1997), Özkan & Gümüs (2001) and Solar *et al.* (2001). An alternative method which regulates heat supply to the graft union only, was described by Lagerstedt (1982), Avanzato & Atefi, (1997), Porebski *et al.* (2002) and Erdogan (2006). Some authors also compared grafting success obtained with these two methods of heat supply on walnut (Atefi, 1997; Achim & Botu, 2001).

With regard to the time of grafting, studies was performed in several areas in the Northern Hemisphere, and the preferred time of grafting varied from December to April (Avanzato & Tamponi, 1988; Ferhatoğlu, 1997; Kazankaya *et al.*, 1997; Özkan & Gümüs, 2001; Solar *et al.*, 2001; Karadeniz, 2003; Erdogan, 2006). Since the different trials were executed in climatically very diverse areas, the exact phenological stage of plant material obviously differed at any given time. However, in most of the studies there was no reference to the phenological condition of rootstock and scion material at the time of grafting and hot callusing.

Since hot callus procedures usually involves bench grafting, which is more labour intensive and expensive than conventional propagation techniques, an increased success rate has to be obtained to justify continuation (Lagerstedt, 1979; Flemer, 1986). Increased success needs to be secured not only after the initial termination of the hot callus period, but also after the first growing season (Stanisavljević & Mitrović, 1997; Achim & Botu, 1997; Özkan & Gümüs, 2001).

Although walnut production is a promising industry in South Africa, there is currently no commercial propagation of this species in the country. Traditional vegetative propagation methods, such as patch budding, have yielded limited success in South Africa over a five year period (Rotondo Walnuts, 2004). Alternative methods such as hot callus grafting, although used on other fruit species, have never been employed for propagation of walnut in this country.

Hence, the objective of this study was to evaluate the potential of hot callus grafting as a method for increasing grafting success of walnut in South Africa. Success of hot callus grafting was investigated by evaluating two methods of heat application (entire tree vs. localized heating) and different grafting times (mid dormancy, late dormancy and early vegetative period) on two potentially important walnut cultivars (‘Chandler’ and ‘Serr’). Grafting success was quantified by determining callus development, final graft-take, grafting success throughout the growing season and final survival of grafted trees. In addition, it was also attempted to establish the effective duration of the hot callus period under local conditions.

4.2 Materials and methods

As described in Chapter 3.

4.3 Results and discussion

Grafting success during the 2007/08 season was exceptionally poor compared to 2006/07. Possible contributing factors include weaker rootstock material, and more importantly, frost damage to trees used for collection of scion material in 2007/08.

As mentioned in Chapter 3, rootstocks available for the 2007/08 season had a smaller diameter than the previous season and a considerable portion of the taproot was removed by the grower. It was clearly evident that fewer reserves were available for graft union formation compared to rootstocks with a dominant taproot utilized the previous season. Poor quality rootstocks are often regarded as a major limiting factor in hot callus grafting success (Lagerstedt, 1982).

Frost damage is believed to be the main contributing factor in graft failure for the 2007/08 season, since only scions died back and failed to develop any callus tissue, while all rootstocks remained viable and sprouted again after graft failure. Abnormally sudden freezing conditions at the onset of winter 2007 (see Table B1, Appendix B) before trees were hardened off, caused frost damage to the trees used for collection of scion wood. Temperatures experienced by these trees were likely lower than that recorded by the weather station, as temperature loggers since installed on this part of the farm have indicated a difference of 2 to 3°C lower than the weather station. According to Sibbett *et al.* (1998), walnut trees can be damaged if temperatures drop down to -2 to -6°C during late autumn or early winter, when trees have not yet attained full dormancy. At the time of scion wood collection no signs of damage were visible, it only became evident at bud break (Figure 4.1). Over the preceding seven seasons propagation material have always been collected from these orchards and this was the first occurrence of any frost damage to trees during the winter.

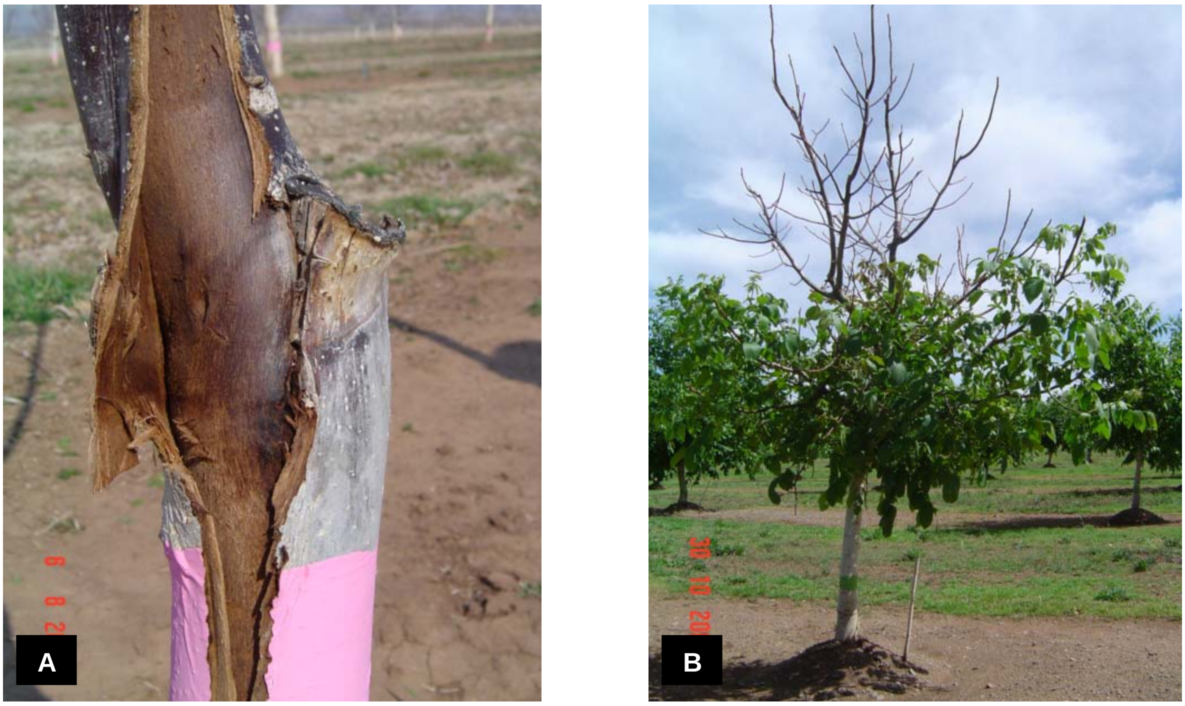

Figure 4.1 Late autumn frost damage evident on trees after bud break in spring 2007 [week 32 (A) and week 44 (B)], caused by a sudden drop in temperatures to below 0°C in week 21.

In order to avoid the use of scion wood damaged by frost in future, it should be considered collecting the material in advance before severe frost is experienced, as advised by Lagerstedt (1979), Tsurkan (1990), Stanisvaljević & Mitrović (1997) and Porebski *et al.* (2002). The material can be stored throughout winter and utilized for hot callus grafting in late winter or early spring. Alternatively, intensive protective measures could be performed to protect scion material from severe winter conditions. This includes whitewash of mother trees, covering with hessian material, as well as avoiding establishment of mother blocks in lower lying areas where cold damage is more frequent.

Since poor results from the 2007/08 season could not be compared to 2006/07 results, only 2006/07 data were analyzed statistically and will be presented comprehensively in this chapter. Some results obtained in 2007/08 are, however, shown in Table 4.1. Although it seemed that high final graft-take percentages were achieved with certain treatment combinations, particularly in the heated trench, callus development and ultimate success percentage was poor.

Table 4.1 Influence of time of grafting and heating method on callus development after 33 days, final graft-take and grafting success of two walnut cultivars in 2007/08

	'Chandler'					
	Heated room			Heated trench		
Time of grafting	Callus (%)	Graft-take (%)[1]	Success (%)[2]	Callus (%)	Graft-take (%)[1]	Success (%)[2]
Mid dormancy (week 30)	6.3	12.5	3.1	31.9	71.9	6.3
Late dormancy (week 34)	5.0	12.5	12.5	15.6	31.3	25.0
Early vegetative period (week 37)	0.0	0.0	0.0	14.8	31.3	21.9
	'Serr'					
	Heated room			Heated trench		
Time of grafting	Callus (%)	Graft-take (%)[1]	Success (%)[2]	Callus (%)	Graft-take (%)[1]	Success (%)[2]
Mid dormancy (week 30)	3.8	9.4	21.9	21.9	43.8	21.9
Late dormancy (week 34)	1.3	3.1	0.0	24.4	50.0	34.4
Early vegetative period (week 37)	0.0	0.0	3.1	10.1	12.5	3.1

[1] Percentage of grafts showing any callus development

[2] Determination of grafting success during the first week of February (week 5)

CALLUS DEVELOPMENT

There was a significant interaction between heating method and time of grafting, regarding callus development after 33 days of hot callusing. There were no significant differences between hot callus methods when grafting was performed during week 34 and 37. Only during mid dormancy (week 30), the heated room resulted in significantly lower callus development compared to the hot callus trench (Table 4.2).

Table 4.2 Influence of time of grafting and heating method on percentage of callus development on day 33 after grafting for both 'Serr' and 'Chandler' (ANOVA in Table A1, Appendix A)

	Heating method	
Time of grafting	Room	Trench
Mid dormancy (week 30)	22.5[1] e	83.8 a
Late dormancy (week 34)	66.3 ab	60.0 bc
Early vegetative period (week 37)	37.5 de	43.8 cd

Means followed by different letters are significantly different according to Fisher's LSD (P ≤ 0.05).

[1]Questionable data

The data for the heated room grafted in week 30 is questionable, however, since fungal growth developed on all ‘Serr’ grafts, most probably as a result of insufficient moisture control. ‘Chandler’ grafts were also affected, but to a lesser extent. This situation was rectified for the next two rounds of grafting (week 34 and 37) by limiting the amount of moisture inside callus containers. If results for week 30 are ignored then, both heating methods resulted in a similar level of callus development.

If data for week 30 in the heated room is disregarded, it was also evident that earlier grafting resulted in more callus formation, regardless of the heating method used (Table 4.1). In the heated trench, callus development decreased significantly from week 30 to 34, with a further decrease in week 37, although not significant compared to week 34. Callus development obtained from the first two rounds of grafting was regarded as acceptably high, since evaluation during the post-grafting growing season indicated that most grafts with visible callus development of 60% or more (refer to Table 4.7) survived the first growing season. In the heated room, acceptable callus development was obtained only during week 34, and it decreased significantly for trees grafted in week 37.

In Turkey’s Tokat region, Özkan & Gümüs (2001) obtained better callus development by grafting in February compared to January and March in a heated room, although there was no indication of significance. As only the month of grafting was indicated with no reference to the phenological stage of the plant material, it is difficult to accurately compare these findings to results of the current study. Since Akça *et al.* (2001) and Özkan & Celep (2001) regarded late March to mid April as the early vegetative stage of walnut in the Tokat area of Turkey, it may be assumed that January was mid-dormancy in the study by Özkan & Gümüs (2001). Hence, results of Özkan & Gümüs (2001) are not completely in accordance with this study where earlier grafting (week 30: mid-dormancy) resulted in significantly increased callus development compared to later in the dormant period.

FINAL GRAFT-TAKE

Analysis of variance indicated a significant interaction between time of grafting, heating method and cultivar, regarding final graft-take (Table 4.3). Substantial variation in the data made it difficult to observe significant differences and clear trends between treatments.

Table 4.3 Influence of time of grafting and heating method on final graft-take (%)[1] of two walnut cultivars (ANOVA in Table A2, Appendix A)

	'Chandler'		'Serr'	
Time of grafting	Heated room	Heated trench	Heated room	Heated trench
Mid dormancy (week 30)	43.8[2] d	81.3 ab	0[2] -	75.0 ab
Late dormancy (week 34)	68.8 bc	75.0 ab	84.4 a	78.1 ab
Early vegetative period (week 37)	43.8 d	46.9 cd	40.6 d	68.8 abc

Means followed by different letters are significantly different according to Fisher's LSD ($P \leq 0.05$).

[1]Percentage of grafts showing any callus development

[2]Questionable data

When comparing the two heating methods for 'Chandler', graft-take in the heated trench for week 30 was significantly higher than the room, however 'Chandler' grafts in the heated room for this round of grafting were also negatively affected by fungal growth. There were no significant differences between heating methods in week 34 and 37 for 'Chandler'. For reasons previously mentioned, graft-take for 'Serr' in week 30 was far better in the heated trench. There was no significant difference between heating methods for 'Serr' in week 34, while graft-take for week 37 was significantly higher in the heated trench. If the questionable results of the first round of grafting (week 30) are disregarded, one heating method cannot be singled out as superior with regard to final graft-take. However, more consistent results were obtained in the heated trench throughout the different grafting periods. On the other hand, the potential value of the heated room was illustrated by the high graft-take percentages obtained for both cultivars during late dormancy (week 34).

When comparing different grafting times, earlier grafting (week 30 and 34) resulted in higher graft-take for both cultivars in the heated trench. This decrease in graft-take for later grafting (week 37) was not significant for 'Serr', while 'Chandler' exhibited a significant decrease from week 34 to 37. Consequently, graft-take for 'Serr' in the heated trench at this late stage of grafting still seemed acceptable, while for 'Chandler' it was becoming undesirably low, although there was no significant difference between the two cultivars at week 37. In the heated room, if results for week 30 are disregarded, earlier grafting (week 34) resulted in significantly higher graft-take than grafting in week 37 for both cultivars. In the Tokat area of Turkey, Özkan & Gümüs (2001) reported lower graft-take from grafting in March, which can be regarded as late dormancy, than in February, although there was also no indication of significance.

EFFECTIVE DURATION OF CALLUS PERIOD

It was uncertain whether opening of the graft union for evaluation of callus development throughout the callus period would affect the callus formation. Final results indicated that grafting success was not negatively influenced; the only observed effect was discolouration of callus tissue (Figure 4.2).

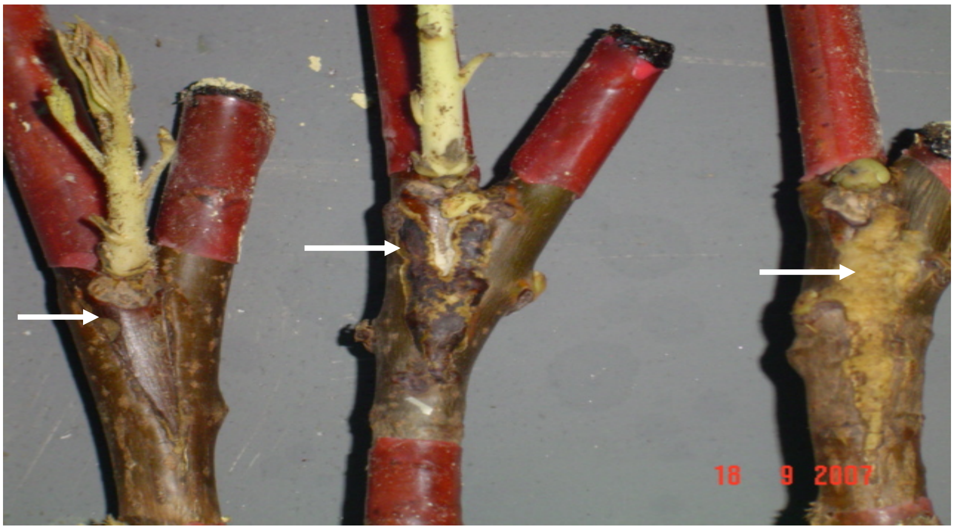

Figure 4.2 Discolouration of callus tissue after the graft union was opened (middle). Graft unions on the left and right hand side of the picture were not opened.

Repeated measures analysis indicated that there was a significant increase in callus development if heat supply was extended from 21 days to 33 days, but this was cultivar dependent. 'Chandler' exhibited a significant increase in callus development as time of hot callusing was extended, while there was no significant increase for 'Serr' (Table 4.4). According to Titmus (personal communication[1]) the duration of the callus period for different cultivars varies in Australia.

Although the increase in callus development for 'Chandler' was statistically significant, in real terms it is an increase of only 7.5% on the rating scale over a period of twelve days (Table 4.4), which could be considered negligible in practical terms. The effective duration of the callus period is therefore determined as 21 days, but the period could be extended to 25 days to be certain that a rigid graft union has formed. According to Lagerstedt (1982) limited exposure to elevated temperatures will restrict callus formation and cause subsequent graft failure, even if

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[1] Lee Titmus, 2005. Websters Walnuts, Devonport, Tasmania, Australia

optimal conditions for increased callus formation exist, while prolonged exposure will affect delicate callus tissue and result in subsequent depletion of scion reserves (Zachej, 1976).

Table 4.4 Percentage callus development over time for two walnut cultivars under hot callus conditions (T-tests in Tables A4 and A5, Appendix A)

Time of evaluation (days after grafting)	Cultivar	
	'Chandler'	'Serr'
21	47.50 a	47.50 a
25	50.42 ab	48.75 a
29	53.33 b	49.17 a
33	55.00 c	49.17 a

Means in each column followed by different letters are significantly different according to paired t-tests ($P \leq 0.05$).

Results therefore support findings by Cerny (1965) and Özkan & Gümüs (2001) who regarded any period between 20 and 25 days as suitable for callus formation in walnut. Although the effective duration of the callus period was not experimentally determined by Pieniazek (1972), Ferhatoğlu (1997), Stanisavljević & Mitrović (1997), Solar *et al.* (2001), Porebski *et al.* (2002) and Vahdati & Zareie (2006), these authors chose to subject grafted trees to 21 days of hot callusing. A hot callus period longer than 25 days was preferred by Avanzato & Atefi (1997), Kazankaya *et al.* (1997), Achim & Botu (2001), Karadeniz (2003) and Erdogan (2006), although the effective duration was not investigated. Findings in this study indicate that nothing is gained under local conditions by extending the callus period longer than 25 days.

GRAFTING SUCCESS THROUGH THE GROWING PERIOD

According to analysis of variance, there was a significant interaction between time of grafting, heating method and cultivar, regarding grafting success throughout the growing period (Table 4.5). However, it was difficult to observe any clear tendencies as a result of the questionable data obtained for week 30 in the heated room. Although all results are indicated in Table 4.5, results for week 30 in the heated room will be ignored in the discussion.

Table 4.5 Grafting success (% of originally grafted trees) of two walnut cultivars through the growing season, as affected by time of grafting and heating method (ANOVA's in Tables A6, A7 and A8, Appendix A)

Heating method	Time of grafting	Success %					
		October (week 43[1])		February (week 5)		May (week 18)	
		'Chandler'					
Heated room	Mid dormancy (week 30)	21.8	f	18.8	fg	18.8	fg
	Late dormancy (week 34)	46.9	cde	34.8	def	31.3	ef
	Early vegetative (week 37)	34.4	def	34.4	def	34.4	def
Heated trench	Mid dormancy (week 30)	75.0	ab	75.0	a	75.0	a
	Late dormancy (week 34)	68.8	ab	53.1	bcd	53.1	bcd
	Early vegetative (week 37)	31.3	ef	28.1	ef	25.0	f
		'Serr'					
Heated room	Mid dormancy (week 30)	0.0	g	0.0	g	0.0	g
	Late dormancy (week 34)	78.1	a	68.8	ab	68.8	ab
	Early vegetative (week 37)	25.0	f	18.8	fg	18.8	fg
Heated trench	Mid dormancy (week 30)	59.4	bc	56.3	abc	56.3	abc
	Late dormancy (week 34)	68.8	ab	62.5	abc	59.4	abc
	Early vegetative (week 37)	50.0	cd	46.9	cde	46.9	cde

Means in each column followed by different letters are significantly different according to Fisher's LSD ($P \leq 0.05$).

[1] Week 45 for trees grafted during week 37.

For 'Chandler', success percentage for late dormant grafting (week 34) remained higher in the heated trench than the room throughout the growing season. This difference was significant for October and May, but not for February. However, no significant differences occurred between the two heating methods for early vegetative grafting (week 37). For late dormant grafting (week 34) of 'Serr', success percentage determined in October, February and May were not significantly different between the two heating methods used. For early vegetative grafting (week 37), however, the heated trench resulted in significantly higher success than the heated room throughout the growing season.

Atefi (1997) and Achim & Botu (2001) reported on significantly higher success with localized hot callusing compared to heating the entire tree in a hot callus room. Such a general conclusion cannot be drawn from the current study, since both methods resulted in acceptable grafting success in some instances. The heated trench did, however, seem to provide more consistent grafting success.

As was observed with callus development and final graft-take, earlier grafting generally resulted in higher success percentage, since unacceptably low results were obtained from early vegetative grafting (week 37), except when 'Serr' was grafted in the heated trench. This might be an indication that although the grafting procedure was performed after bud break of 'Serr' in commercial orchards, this cultivar can still be subjected to hot callus conditions as long as dormancy release is not stimulated.

Contrary to results in this study, Ferhatoğlu (1997), Kazankaya *et al.* (1997), Özkan & Gümüs (2001) and Karadeniz (2003) reported that hot callus grafting in a heated room, earlier in the dormant period (January in Turkey) resulted in the lowest success percentage, compared to February and March grafting. Avanzato & Tamponi (1988) in Italy reported the same trend with localized heating, where grafting in February produced significantly higher success compared to January. On the other hand, in Oregon, USA, Lagerstedt (1982; 1984) advised hot callus grafting as early as December (probably early dormancy) for hot callus grafting of walnut with localized heating, while Ebadi *et al.* (2002) reported the same for grafting in a heated room in Iran.

In terms of grafting later in the dormant period, Kazankaya *et al.* (1997), Özkan & Gümüs (2001) and Karadeniz (2003) reported decreased grafting success when grafting was performed after February, while Ferhatoğlu (1997) indicated decreased success percentage only after March grafting (late dormancy) in Turkey. These findings are in accordance with the decreasing trend in grafting success that was observed for later grafting in the current study. Karadeniz (2003) attributed this decline in grafting success to increased content of phenolic compounds in the plant material used.

For most treatment combinations in this study (except 'Chandler' grafted week 34 in the heated room) the success percentage decreased as the growing season progressed. Increased occurrence of both rootstock and scion sprouting (Figure 4.2A), as well as root development (Figure 4.2B), observed from both cultivars grafted in week 37 in the heated room, supports the suggestion that grafting with this technique should be avoided at this late stage. According to Farmer (1973) the preservation of dormancy is essential when hot callus grafting is performed after scion buds have already received part of its required chilling. This is more easily achieved in the heated trench than in the room. The possible advantage of obtaining an increased number of grafted trees by extending the dormant grafting procedure was also questioned by Lantos (1990) due to scion and rootstock sprouting under favourable temperature and humidity conditions in the heated room.

Although acceptable results were achieved with certain treatment combinations, general grafting success obtained with hot callusing in this study is not yet satisfactory in comparison to other studies. Avanzato & Atefi (1997), Achim & Botu (2001), Porebski *et al.* (2002) and Erdogan (2006) all achieved more than 75% success and up to 92% when localized hot callusing was employed, while Cerny (1965), Ferhatoğlu (1997), Stanisvaljević & Mitrović (1997) and Solar *et al.* (2001) reported more than 80% and up to 90% success using hot callus rooms. Further experimentation is likely to uncover the required conditions for increased grafting success with both heating methods.

Figure 4.2 Sprouting of scion buds (A) and root development (B), observed in the heated room for grafting in week 37.

FINAL SURVIVAL OF GRAFTED TREES

As described in Chapter 3, final survival was calculated as the percentage of successfully callused trees that survived throughout the first growing season. Analysis of variance indicated a significant interaction between time of grafting, heating method and cultivar, regarding survival percentage (Table 4.6). All results are indicated, but those obtained from ‘Serr’ in the heated room in week 30 will be ignored in the discussion.

Table 4.6 Influence of time of grafting and heating method on survival (%) of two walnut cultivars (ANOVA in Table A9, Appendix A)

	‘Chandler’				‘Serr’			
Time of grafting	Heated room		Heated trench		Heated room		Heated trench	
Mid dormancy (week 30)	41.7	e	92.3	a	-	-	75.0	abc
Late dormancy (week 34)	44.2	de	69.4	abc	81.0	ab	75.6	abc
Early vegetative period (week 37)	79.2	ab	53.3	cde	45.8	de	67.0	bcd

Means followed by different letters are significantly different according to Fisher’s LSD ($P \leq 0.05$).

When comparing the two hot callus methods for ‘Chandler’ in terms of survival percentage, it was evident that survival for grafting in both week 30 and 34 in the heated trench was significantly higher than for the room, however for grafting in week 37 the heated room gave significantly higher results. For ‘Serr’, there were no significant differences between the two heating methods for week 34 or week 37, although survival of trees from the heated room during week 37 was undesirably low.

When evaluating different grafting times, different tendencies were observed for the two cultivars. In the heated room, survival of ‘Chandler’ was significantly higher during week 37 compared to week 30 and week 34, while survival of ‘Serr’ was significantly higher during week 34 compared to week 37. In the heated trench, the later grafting was performed the lower survival of ‘Chandler’ became, with a significant decrease from week 30 to 37. For ‘Serr’, there was no significant difference between grafting times in terms of survival percentage in the heated trench.

As was observed from the evaluation of success percentage throughout the growing period (Table 4.4), little mortality occurred after February, which means that failure was most often experienced during the early stages of vegetative growth. Achim & Botu (2001) attributed post-grafting losses during the first growing season to incomplete callusing. Stanisvaljević & Mitrović (1997) achieved a survival percentage of up to 92%, while Özkan & Gümüs (2001) obtained only between 22 and 50% by the end of the first growing period. Survival of between 44 and 81% in this study could therefore be regarded as satisfactory.

When linking survival percentage to original callus development for all treatments, it became clear that grafts that developed more than 60% callus tissue at the graft union had a much higher probability of surviving post-grafting conditions (Table 4.7).

Table 4.7 Survival of grafted walnut trees by the end of the first growing season as influenced by callus development during hot callus grafting

Callus development (%)	Survival (%)
0	0.0
20	9.1
40	45.0
60	63.2
80	73.3
100	76.5

4.4 Grafting success obtained from 2008/09 experiments

Grafting trials were continued in 2008/09 for commercial purposes in order to confirm some results from the 2006/07 season. Since trials were not initially conducted as part of this M. Sc-study the exact treatment combinations were not replicated. Both heating methods were again evaluated, but cultivars and grafting times differed from the preceding experiments. Data obtained from 2008/09 trials may, however, support conclusions made in this chapter, especially since data obtained from an entire season (2007/08) had to be discarded.

In 2008/09 noticeably more callus tissue developed at graft unions than in previous seasons, and it was also more evenly distributed between scion and rootstock and around the graft union (Figure 4.3).

Figure 4.3 Callus development observed from hot callus grafting in 2008/09.

In general, higher and more consistent grafting success was obtained compared to 2006/07, especially for the heated room where results were often unsatisfactory in 2006/07 (Table 4.8). Regarding heating method, it was once again evident that better and more consistent results were achieved in the heated trench, compared to the heated room in 2008/09. An average success of 90% in the heated trench measures up to some of the best results achieved by commercial nurseries in Europe (Linard, personal communication[1]; Lopez Larinaga, personal communication[2]).

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France
[2] Federico Lopez Larrinaga, 2002. Nogaltec, Badajoz, Spain

Table 4.8 Hot callus grafting success (determined at week 48) of various walnut cultivars for 2008/09 on *Juglans regia* rootstock for the heated room and heated trench respectively (results not subjected to statistical analysis)

Time of grafting	Cultivar	Number grafted	Success (%)
	Heated room		
Week 28	'Vina'	70	58.6
Week 30	‘Chandler’	20	70.0
Week 31	‘Chandler’	100	49.0
Week 33	‘Serr’	60	81.7
Week 34	‘Vina’	50	54.0
	Total / Average	300	60.0
	Heated trench		
Week 30	‘Chandler’	40	100.0
Week 33	‘Chandler’	200	90.0
Week 33	‘Serr’	40	92.5
Week 34	‘Chandler’	100	86.0
	Total / Average	380	90.3

With regard to grafting time, there was no clear tendency, since the experiment was not really set up to measure this accurately. Except for one batch of trees that were grafted relatively early (week 28), grafting was performed only during the optimal time as determined in 2006/07 trials (between week 30 and 34). No conclusions on cultivar responses can be drawn from 2008/09 results.

4.5 Concluding remarks

Due to unfortunate loss of data of the mid dormancy grafting period (week 30) in the hot callus room during 2006/07, it is difficult to draw definite conclusions regarding the effect of heating method and time of grafting on the two walnut cultivars. Grafting success of both cultivars, but especially ‘Serr’, was influenced by fungal development on graft unions. In addition, unsatisfactory results obtained during 2007/08 further limited the amount of experimental data available for statistical analysis. Certain tendencies, however, were observed from analysis of the available data.

As found in several other studies, the effective duration of heat application for hot callus grafting of walnut was established as 21 days for both heating methods for all the grafting times with both cultivars. It was evident that very little additional callus tissue developed after this period, and not much was gained by extending the hot callus period beyond 25 days.

In terms of the heating method used, it is difficult to conclude with conviction that better results were obtained from the heated trench in the 2006/07 study, as found by Atefi (1997) and Achim & Botu (2001). Although overall grafting success was more consistent with the heated trench (localized heating) than in the heated room in 2006/07 for both cultivars, very good success was obtained in some instances in the heated room, indicating the potential value of this method. Results from commercial experiments continued in 2008/09, however, confirmed that more consistent and higher average grafting success may be obtained in the heated trench.

The general tendency with regard to time of grafting, was that earlier grafting up to the third week of August (week 34), seemed to secure higher and more consistent grafting success. When hot callus grafting was extended with three weeks, increased sprouting of both rootstock and scion occurred before the callus period was terminated, due to favourable temperature and humidity conditions, particularly in the heated room. Hot callus grafting during this period is therefore regarded as marginal, while earlier grafting is suggested under local conditions. Comparing these results with findings from several other studies in the Northern Hemisphere, highlighted the fact that optimum time of grafting is very much dependent on climate of the area.

The two potentially important cultivars that were grafted reacted differently to hot callus conditions. During the mid and late dormancy grafting periods, acceptable results were obtained for each cultivar from at least one hot callus method.

Survival percentage in this study indicated that a high percentage of grafted trees could be lost during the growing season. This could rather be attributed to inferior callus formation during the callus period, than to lack of acclimatization before planting into the nursery. Survival percentages that were obtained, is however, in line with what other researchers have found with hot callus grafting of walnuts. Results from continued studies in 2008/09, indicated much more encouraging survival percentages of grafted trees, emphasizing the importance of continuous experimentation to adapt hot callus grafting procedures to local conditions in order to ensure the best possible success.

CHAPTER 5. *Post-grafting growth of hot callused walnut trees*

5.1 Introduction

The major beneficial influence of bench grafting and hot callus conditions on walnut is the significant increase in grafting success (Lagerstedt, 1982). Another factor that contributes to successful production of grafted walnut trees is optimal post-grafting growth. Maximum growth in the shortest length of time is crucial to the attainment of high quality trees of the desired cultivar, as well as an enhanced turnover (Avanzato & Atefi, 1997). Unfortunately, bench grafted plants of various species (Flemer, 1986), and particularly walnut (Avanzato & Atefi, 1997; Achim & Botu, 2001; Erdogan, 2006), are usually characterised by poor growth during the first growth season.

Although climatic conditions and post-grafting treatment of trees mainly control growth during the first growing season, conditions during the hot callus grafting procedure may also have a considerable influence. Any situation leading to inferior callus formation will cause poorer post-grafting growth (Erdogan, 2006). The time of grafting also seem to be of significance in post-grafting growth. Lagerstedt (1982; 1984) and Tsurkan (1990) preferred to perform the grafting procedure earlier during the dormant period to ensure earlier planting of grafted trees, resulting in better post-grafting growth. However, survival of grafted trees was regarded as more important, since cold storage of hot callused trees was suggested if severe winter conditions prevail in a specific area (Lagerstedt, 1982; Tsurkan, 1990). Cold storage of grafted trees permitted planting only at a later stage with subsequent poorer post-grafting growth.

The objectives of this study were to determine the influence of the two hot callus methods (heated room and heated trench) and three stages of grafting during the dormant period on post-grafting growth of two walnut cultivars. Post-grafting growth was evaluated by means of shoot diameter and shoot height of new scion growth twice during the first growing season. The final aim was to determine whether a grafted tree suitable for orchard establishment could be produced within a single growing season.

5.2 Materials and methods

As described in Chapter 3.

5.3 Results and discussions

As discussed in Chapter 4, results obtained from the 2007/08 season were very poor and could not be statistically compared to 2006/07. Furthermore, as mentioned in Chapter 4, results obtained for grafting in week 30 in the heated room were questionable and were not included in statistical analysis of growth parameters.

SHOOT DIAMETER

With regards to grafting time, shoot diameter results indicated that earlier grafting resulted in significantly superior growth, irrespective of cultivar (Table 5.1). Diameter of trees grafted in week 37 was significantly lower compared to trees grafted earlier (week 34).

Table 5.1 Influence of time of grafting on shoot diameter (mm) of new growth, measured at the middle (week 5) and the end (week 18) of the first growing season (ANOVA in Tables A10 and A11, Appendix A)

Time of grafting	Week 5	Week 18
Late dormancy (week 34)	16.94 a	21.42 a
Early vegetative (week 37)	13.68 b	17.75 b

Means in each column followed by different letters are significantly different according to Fisher's LSD ($P \leq 0.05$).

Heating method also had a significant effect on growth of grafted trees, as shoot diameter of trees in the hot callus room was significantly less compared to trees from the heated trench (Table 5.2). This significant growth difference was evident throughout the growing season.

Table 5.2 Influence of hot callus method on shoot diameter (mm) of new growth, measured at the middle (week 5) and the end (week 18) of the first growing season (ANOVA in Tables A10 and A11, Appendix A)

Hot callus method	Week 5	Week 18
Hot callus room[1]	13.70 b	17.17 b
Hot callus trench[1]	16.92 a	22.01 a

Means in each column followed by different letters are significantly different according to Fisher's LSD ($P \leq 0.05$).

[1]Including only data obtained from late dormant and early vegetative grafting.

Avanzato & Atefi (1997) indicated that shoot diameter of grafted walnut trees from a hot callus trench did not increase substantially when growth was measured three months after grafting. The desired increase in diameter was only obtained 15 months after grafting. As can be seen from the results, such delayed growth in diameter was not observed in the current study.

As expected, shoot diameter also differed between the two cultivars (Table 5.3), since ‘Serr’ is a more aggressive grower than ‘Chandler’ (Hendricks *et al.*, 1998). The difference detected between the two cultivars was not significant by the middle of the growing season, however, by the end of the first growing season ‘Serr’ reached a shoot diameter significantly higher than ‘Chandler’.

Table 5.3 Influence of cultivar on diameter (mm) of new growth, measured at the middle (week 5) and the end (week 18) of the first growing season (ANOVA in Table A10 and A11, Appendix A)

Cultivar	Week 5	Week 18
‘Chandler’	14.62 a	17.51 b
‘Serr’	16.00 a	21.66 a

Means in each column followed by different letters are significantly different according to Fisher’s LSD ($P \leq 0.05$).

Keeping possible cultivar differences in mind, growth in shoot diameter from both cultivars in this trial was exceptionally good compared to hot callused trees in similar trials. In studies by Stanisavljević & Mitrović (1997) evaluating seven Yugoslavian cultivars, the largest shoot diameter obtained by the end of the first growing season was only 9 mm, compared to 17.5 and 21.7 mm in this trial.

SHOOT HEIGHT

As was the case with shoot diameter, growth in shoot height was greater for trees grafted in week 34 than those grafted in week 37 (Table 5.4). This difference in shoot height between the two grafting times was significant only for ‘Serr’, the more vigorous grower of the two cultivars. Although results for grafting in week 30 is not shown, the few successfully grafted trees from this grafting time exhibited growth comparable to trees grafted in week 34.

Table 5.4 Influence of time of grafting on height (cm) of new growth of two walnut cultivars, measured at the middle (week 5) and the end (week 18) of the first growing season (ANOVA in Tables A12 and A13, Appendix A)

	Week 5		Week 18	
Time of grafting	‘Chandler’	‘Serr’	‘Chandler’	‘Serr’
Late dormancy (week 34)	48.64 bc	99.51 a	60.47 bc	133.44 a
Early vegetative period (week 37)	36.30 c	56.49 b	43.85 c	76.99 b

Means for each evaluation time followed by different letters are significantly different according to Fisher’s LSD ($P \leq 0.05$).

Since trees were planted directly into the nursery after hot callusing, earlier grafting resulted in earlier planting. Trees planted earlier had a definite advantage in terms of growth (shoot diameter and height), which remained until the end of the first growing season (Figure 5.1). These observations are in accordance with Lagerstedt (1982) who stated that late planting of grafted trees in regions with a mild winter climate, had a pronounced negative effect on post-grafting growth. Tsurkan (1990) also advised direct planting of hot callused trees to ensure maximum growth, unless a long term temperature fall of below -3°C is expected.

Figure 5.1 Difference in growth between grafting times as observed in October (A) and December (B) of the first growing season after grafting. The row on the right of each picture represents trees grafted in week 30, the row in the middle is trees grafted in week 34 and the row on the left was grafted in week 37.

Under severe winter conditions, cold storage of grafted trees after the hot callus period was advised by Lantos (1990) in Hungary, Ferhatoğlu (1997) in Turkey and Solar *et al.* (2001) in Slovenia. As a result, substantially lower post-grafting growth was obtained by these authors than was observed in the current study. In addition, cold storage seems to invert the effect of grafting time on post-grafting growth. Özkan & Gümüs (2001) reported that for grafting between January and March in Turkey, followed by cold storage until May, greater shoot height was obtained for later grafting. Hence, it seems that a longer storage period, the result of earlier grafting, may have adverse effects on growth of grafted walnut trees in the first growing season.

In addition to the effect of grafting time and cultivar, analysis of variance indicated that hot callus method also affected shoot height of new growth significantly (Table 5.5). As observed with shoot diameter, height of trees from the hot callus room was also significantly lower compared to trees from the heated trench. This difference was clearly visible midway through the first growing season and became even more pronounced toward the end of the growing period.

Table 5.5 Influence of hot callus method on height (cm) of new growth, measured at the middle (week 5) and the end (week 18) of the first growing season (ANOVA in Tables A12 and A13, Appendix A)

Hot callus method	Week 5	Week 18
Hot callus room[1]	50.08 b	57.71 b
Hot callus trench[1]	70.39 a	99.67 a

Means in each column followed by different letters are significantly different according to Fisher's LSD ($P \leq 0.05$).

[1]Including only data obtained from late dormant and early vegetative grafting.

A possible contributing factor to inferior growth obtained from trees grafted in the hot callus room, may be the increased sprouting of rootstocks and scions as compared to grafted trees in the heated trench (Chapter 4, Figure 4.2). This would have depleted valuable reserves required not only for callus formation, but also for initial post-grafting growth of the scion.

EVALUATION OF GRAFTED TREES FOR ORCHARD ESTABLISHMENT

According to Flemer (1986), Lantos (1990), Tsurkan (1990), Stanisavljević & Mitrović (1997), Achim & Botu (2001), Solar *et al.* (2001) and Erdogan (2006) a standard size grafted tree, suitable for orchard establishment, could not be obtained from the bench grafting/hot callus procedure after the first growing season. Solar *et al.* (2001) considered grafted trees to be "suitably sized" when it was well rooted, with a minimum height of 100 cm. Even after a second growing season, only between 53 and 64% well-developed grafted trees were obtained (Solar *et al.*, 2001). Achim & Botu (2001) obtained grafted trees with a diameter of 10 mm and height of 40 cm in one growing season. After the second growing season, trees reached a height of up to 1.7 m, measured from ground level, with a diameter of 24 mm. Similar growth was obtained after the first and second year respectively by Stanisavljević & Mitrović (1997) and Erdogan (2006). Slow growth was attributed to incomplete callus formation at the graft union, together with the loss of roots during the bench grafting phase (Erdogan, 2006).

In contrast, excellent shoot growth, as well as root development, was obtained in the present study in only one growing season (Figure 5.2). Root development was observed when grafted trees were transplanted to the orchard after the first post-grafting dormant season. The minimum requirement of grafted trees for transplanting in this study, was determined at a shoot diameter of 15 mm and height of 80 cm on new growth (total tree height of 100 cm), with a visibly strong graft union. More than 85% of the successfully grafted trees obtained from this study achieved suitable size and were transplanted into the orchard in August 2007, after a single growing season (Figure 5.2).

Figure 5.2 Grafted trees at the end of the first growing season (A) and after the following dormant period in August ready for orchard establishment (B).

According to Linard (personal communication[1]) post-grafting growth of hot-callused trees is very much dependent on optimal climatic conditions and specifically the duration of sunlight exposure. In general, the local climate provides a longer growing season for walnuts than is experienced in most European walnut producing countries. France for example, has a growing period of only five months, from May to September (Linard, personal communication[1]), while the growing period in the southern Free State is six to seven months long depending on cultivar (see Table B2, Appendix B for average monthly spring and summer temperatures). Hence, the combination of the favourable climatic conditions, as well as good post-grafting treatment of trees, resulted in vigorous growth of grafted trees during the first growing season. Another possible contributing factor to the good post-grafting growth of trees in this study, is limited removal of lateral roots of the rootstocks before grafting was performed. Cerny (1965), Pieniazek (1972), Lantos (1990), Özkan & Gümüs (2001) and Erdogan (2006) indicated that roots of seedlings were trimmed severely in order to pack the plants more tightly, a procedure regarded by Lagerstedt (1982) and Erdogan (2006) to have a limiting effect on post-grafting growth.

[1] Sèbastien Linard, 2005. Pépiniéres du Pondaillan, Souillac, France

5.4 Concluding remarks

Grafted trees of excellent quality, suitable for orchard establishment were obtained after only one growing season in this study. This is considerably better than results obtained in most European studies, where at least two growing seasons where necessary to achieve suitably sized trees.

All three factors evaluated in this study – heating method, time of grafting and cultivar – affected post-grafting growth of hot callused walnut trees. As expected, the more vigorous cultivar 'Serr', showed better shoot growth than 'Chandler'. Furthermore, better shoot growth was obtained from trees grafted in the hot callus trench, compared to trees from the hot callus room, probably an effect of rootstock and scion sprouting which depleted reserves. However, most of the trees obtained from the latter method eventually reached an acceptable size by the end of the first growing season.

Time of grafting and subsequent time of planting greatly affected post-grafting shoot growth, as earlier grafting resulted in superior growth during the first growing season. Earlier grafting (week 30 and 34) produced several trees with new growth in excess of 2 m, while all grafted trees not suitable for orchard establishment after the first growing period belonged to the batch of trees grafted in week 37.

The importance of extensive measures in order to protect grafted trees after being transplanted into the nursery, was again emphasized. In the Southern Free State, winter conditions during the post-grafting period may be detrimental to fragile graft unions. The protection of successfully grafted trees should have priority over earlier planting in order to obtain better growth, however, protective measures employed in this study seem to have been sufficient to allow earlier planting.

CHAPTER 6. *Hot callus grafting of walnut on paradox rootstocks*

6.1 Introduction

According to Catlin (1998) increased use of Paradox rootstocks, F_1 hybrid seedlings (*Juglans hindsii* x *J. regia*) obtained from selected *J. hindsii* trees naturally pollinated by *J. regia*, began in the 1950s in California. Today walnut trees are commonly propagated on Paradox rootstocks in this area (Reil *et al.*, 1998). This rootstock became popular because of increased vigour and higher tolerance than either parent to *Phytophthora* root and crown rots in poorly drained soils (Catlin, 1998), as well as tolerance to root lesion nematodes (Sibbett *et al.*,1998).

There is no literature available on the influence of Paradox rootstocks on hot callus grafting of walnut, since seedlings of *J. regia* were mainly used in Eurasian grafting studies, while until recently, Paradox was mainly used in the USA where hot callus grafting is not a standard propagation method for walnut (Cerny, 1965; Pieniazek, 1972; Avanzato & Tamponi, 1988; Lantos, 1990; Tsurkan, 1990; Van't Westeinde, 1990; Ferhatoğlu, 1997; Kazankaya *et al.*, 1997; Stanisavljević & Mitrović, 1997; Achim & Botu, 2001; Özkan & Gümüs, 2001; Porebski *et al.*, 2002; Karadeniz, 2003; Erdogan, 2006; Vahdati & Zareie, 2006).

In preliminary experiments during the 2006/07 season, it became evident that an increase in callus development may be obtained from grafting onto clonal Paradox rootstocks, leading to increased grafting success and increased vigour during the first growing season. Subsequently, a more elaborate trial was conducted during the 2007/08 season to explore the possible effect of rootstock on grafting success and post-grafting growth of walnut. Unfortunately, not enough rootstock material was available to perform a complete hot callus trial to compare its effect statistically and accurately to *J. regia* rootstocks. However, since initial results with Paradox rootstock were so encouraging and very little is known about the reaction of this rootstock on hot callus grafting of walnut, it was deemed valuable to report on its possible advantages here.

6.2 Materials and methods

Materials and methods were mostly similar to what was discussed in Chapter 3, with the following exceptions:

ROOTSTOCKS

Rootstocks utilized were uniform sized two-year-old Paradox hybrids. Trees were propagated *in vitro* and imported from Spain in 2006. At the time of grafting, rootstocks were approximately 40 to 60 cm in height and 10 to 20 mm in diameter 5 cm above the soil level, with a dominant taproot system.

SCION MATERIAL

As in grafting experiments with *J. regia* rootstocks, 'Chandler' was again used in experiments with Paradox rootstocks, while 'Serr' was replaced by 'Vina', which seems to be a more promising cultivar under South African conditions. In addition, two important pollinizers, 'Fernette' and 'Cisco', were also used in these grafting experiments. 'Fernette' is a late leafing French variety (Germain *et al.*, 1999) and the most important pollinizer for 'Chandler', while 'Cisco' has a lower chilling requirement than 'Fernette' and is especially useful as a pollinizer for 'Chandler' in years when insufficient chilling is received (Lopez Larrinaga, personal communication[1]).

HOT CALLUS METHOD

Grafted trees were only subjected to hot callus conditions in the hot callus trench, as described in section 3.5.

TIME OF GRAFTING

Trees were grafted only in August during the late dormancy period in 2007 (week 33).

EXPERIMENTAL DESIGN

In the heated trench a completely randomized design was used with five replications for every cultivar, with each replication consisting of five grafted trees. Therefore, 25 trees of each cultivar were grafted in total.

DATA RECORDED

The percentage of successfully grafted trees was determined only twice during the first growing season. The first evaluation was at the start of the growing season (week 46, 87 days after grafting and 54 days after being planted), followed by a second evaluation at the end of the first growing season (week 19, 263 days after grafting and 232 days after being planted).

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[1] Federico Lopez Larrinaga, 2002. Nogaltec, Badajoz, Spain

6.3 Results and discussions

As was seen with *J. regia* rootstocks, very little callus development occurred after 21 days of subjecting grafted Paradox to hot callus conditions (Table 6.1). Callus development after 21 days generally seemed stronger and more continuous compared to *J. regia* grafts at the corresponding time in the 2006/2007 season. After 25 days, there was no increase in callus formation for all the cultivars evaluated. This confirms the effective duration of the callus period under local conditions as 21 days as discussed in Chapter 4.

Table 6.1 Average callus development over time of four walnut cultivars on Paradox rootstocks subjected to hot callus conditions (T-test in Table A14, Appendix A)

Time of evaluation (days after grafting)	Callus development (%)
21	72.5 a
25	75.6 b
29	75.6 b
33	75.6 b

There were no significant differences between cultivars regarding callus development, graft-take, success percentage throughout the growing period and survival percentage (Table 6.2). As a result of increased callus development, very good results of up to 100% graft-take were obtained after the callus period was terminated.

Table 6.2 Callus development, final graft-take, grafting success and survival of various walnut cultivars grafted on Paradox rootstocks in the hot callus trench (ANOVA in Tables A15, A16, A17, A18 and A19 respectively, Appendix A)

Cultivar	Callus development (%)	Final graft-take (%)[1]	Success (%) (week 46)	Success (%) (week 19)	Survival (%)
Chandler	76.6 a	100 a	84 a	72 a	72 a
Vina	59.3 a	100 a	84 a	76 a	76 a
Fernette	74.0 a	92 a	72 a	62 a	67 a
Cisco	72.6 a	88 a	80 a	70 a	80 a
Average	70.6	95	80	70	73

Means in each column followed by different letters are significant different according to Fisher's LSD ($P \leq 0.05$).
[1]Percentage of grafts with any callus development

Success percentage throughout the growing season was also higher compared to results obtained with *J. regia* in 2006/07. The success of up to 76% at the end of the growing season, with an average of 70%, is highly encouraging and compare well to results obtained by other authors with localized hot callus grafting (Atefi, 1997; Avanzato & Atefi, 1997; Achim & Botu, 2001; Porebski *et al.*, 2002; Erdogan, 2006; Aletà, personal communication[1]; Lopez Larrinaga, personal communication[2]).

The relatively high rate of mortality throughout the growing season is still unacceptable – of the trees that were successfully grafted, an average of only 73% survived through the first growing season. In failed grafts, it was only the scion that died and not the rootstock, indicating failure of the graft union. Hence, although callus formation seemed adequate at the onset of the growing season, it was apparently still insufficient, as experienced by Stanisvaljević & Mitrović (1997) and Achim & Botu (2001), or the callus bridge may have been negatively influenced by post-grafting conditions.

Although post-grafting losses is sometimes attributed to harsh winter conditions after direct planting into the nursery, it could not have played a role in this study. Grafted trees, protected against low temperatures as described in Chapter 3, were only planted into the nursery near the end of September, when there is extremely low risk of late frost in the region. Other factors, for instance an unacceptable level of callus development at the graft union, that may play a role in post-grafting failure should be investigated further to ensure decreased mortality rates of successfully grafted trees during the first growing season.

6.4 Concluding remarks

Hot callus grafting on Paradox rootstocks in 2007/08 resulted in very good callus formation, leading to increased and more consistent grafting success throughout the growing season compared to *J. regia* rootstocks in 2006/07. This increased success rate was observed for all the cultivars evaluated, with no significant differences between cultivars. These observations seem to emphasize the importance of good quality rootstocks for hot callus grafting of walnut. Since results of the two rootstocks were not compared in the same season, further investigation is, however, necessary to confirm the apparent increased grafting results achieved with Paradox rootstocks in this study.

A major impediment to such research is the limited number of Paradox rootstocks available in South Africa, and commercial propagation thereof might take several years to be established successfully. According to Hartmann *et al.* (2002) it is difficult to secure high quantities of Paradox seeds, since *J. hindsii* source trees vary greatly in the proportion of hybrids they produce (between zero and almost 100%). Hence, clonal selection and subsequent vegetative propagation systems are required for mass production of Paradox rootstocks. Although many

[1] Neus Aletà, 2005. IRTA Research Station, Tarragona, Spain
[2] Federico Lopez Larrinaga, 2008. Nogaltec, Badajoz, Spain

attempts have been made, not much success have been achieved in propagating Paradox rootstock, or any walnut rootstock for that matter, vegetatively. According to Reil *et al.* (1998) an economically viable level of rooting and survival of Paradox cuttings for nursery production remains to be demonstrated, while several attempts to achieve a commercially feasible micropropagation method have also been carried out (McGranahan & Leslie, 1988; Rodríguez *et al.* 1989). Other vegetative methods that have been attempted, include leaf cuttings under mist and trench layers, where rooting was achieved with difficulty followed by major problems in transplanting (Hartmann *et al.*, 2002).

Currently, the only commercial provider of clonally propagated walnut trees in the world market, is an *in vitro* nursery in Spain from where walnut trees, including the Paradox rootstocks used in this study, have been imported to South Africa (López, 2001; Dandekar *et al.*, 2005). Since it is difficult and expensive to import the plant material, it seems worthwhile to investigate the possibility of vegetative propagation for Paradox rootstocks in South Africa. But until a commercial source of Paradox rootstocks is established locally, *J. regia* remains the only available rootstock for hot callus grafting of walnut in South Africa.

CHAPTER 7. *General conclusion*

Walnut is more difficult to propagate vegetatively than most other fruit and nut species. Due to poor and inconsistent results with conventional vegetative propagation techniques, many walnut producing countries, mostly in the Northern Hemisphere, have investigated and successfully implemented hot callus grafting for increased propagation success of walnut. Walnut has great potential as a new crop in South Africa, but expansion of the industry is hampered by the lack of a local source of high quality, affordable propagation material. Traditional patch budding, performed during the growing season, have thus far resulted in poor and unpredictable success in the southern Free State. Hence, this study was undertaken to evaluate the potential of hot callus grafting, performed during the dormant period, as a propagation technique for walnuts in South Africa.

Establishing propagation guidelines for hot callus grafting of walnuts in South Africa, requires investigation into the many factors affecting hot callus grafting success, a process that is likely to span several years. The experiments conducted in this study were aimed at contributing to this process, by evaluating some of the most important influential factors, including method of heating and time of grafting on potentially valuable walnut cultivars in South Africa. In addition to the standard *J. regia* seedling rootstocks, grafting success was also evaluated on Paradox hybrid rootstocks. Unfortunately, since data from only a single season was available, conclusions made from this study are not definite, but it still provided valuable guidelines for further investigation into hot callus grafting of walnut.

In trials with both *J. regia* and Paradox rootstocks, it was determined that the effective duration of heat application for hot callus grafting of walnut, was 21 days. Callus tissue increased only slightly until 25 days after grafting, while there was no benefit in extending heated conditions beyond this point. This is in accordance with several other studies performed in European countries.

When evaluating heating method in terms of callus development and grafting success, neither method gave consistently better results than the other. Although overall grafting success seemed more reliable for the heated trench, fairly good results were obtained in some instances for the heated room, indicating the potential value of this method. Regarding post-grafting shoot growth, however, significantly better growth was obtained from trees grafted in the heated trench than those from the room, probably an effect of rootstock and scion sprouting in the room which depleted reserves. Nevertheless, most of the trees obtained from the heated room did eventually reach an acceptable size by the end of first growing season. From a purely practical point of view, the heated room is an attractive option for commercial hot callus grafting, since more trees can be accommodated in a specific area and it is also easier to evaluate progress throughout the callusing period. However, practical considerations cannot have preference at the expense of grafting success. More research inputs are therefore required to optimize grafting success through this method.

The general tendency with regard to time of grafting, was that earlier grafting up to the third week of August (week 34), seemed to secure increased callus development and subsequent higher and more consistent grafting success. When hot callus grafting was extended by another three weeks, increased sprouting of both rootstock and scion occurred before the callus period was terminated, particularly in the heated room. Hot callus grafting during this period was therefore regarded as marginal, hence, grafting before the onset of bud break for either rootstock or cultivar is suggested under local conditions. Earlier grafting (week 30 and 34), and subsequent earlier planting, also resulted in superior growth during the first post-grafting growing season. These grafting times produced several trees with new growth exceeding 2 m in height, while all grafted trees not suitable for orchard establishment after the first growing season came from the batch of trees grafted in week 37.

Although cultivars differed in terms of callus development and grafting success on *J. regia* rootstocks, no clear trends were noticed, and satisfactory results were obtained for both 'Chandler' and 'Serr' from at least one hot callus method during the mid and late dormancy grafting periods. As expected, post-grafting shoot growth of 'Serr' was significantly better than 'Chandler', since it is a more vigorous grower. In contrast to results in the aforementioned trial, there were no differences in grafting success among the four cultivars evaluated on Paradox rootstocks. Judgment on the influence of genotype on grafting success under hot callus conditions should therefore be reserved until further investigation.

With regard to rootstock effect on hot callus grafting, callus development on Paradox rootstocks seemed stronger and more continuous than on *J. regia* rootstocks. Grafting success was generally also higher on Paradox. Since data for the two rootstocks were obtained from different years, more accurate comparison of these two rootstocks is required before final conclusions can be drawn.

Although results after two years of intensive experimentation with hot callus grafting under local conditions are not yet comparable to grafting success achieved by some researchers and commercial nurseries, it creates positive prospects for walnut propagation in South Africa. One of the most essential problems that should be addressed in the next phase of research, is the high percentage of grafted trees that are still lost during the post-grafting growing period, particularly on *J. regia* rootstocks. Another important consideration is whether the time of grafting can not be extended by starting grafting earlier in the dormant period (before week 30) to maximize commercial production of grafted trees.

Lessons learnt from the current study have been implemented in ongoing research on hot callus grafting of walnut. Both the heated room and trench have been employed in grafting trials performed in the winter of 2008 on both *J. regia* and Paradox rootstocks. Adaptations from the previous seasons included collection of scion material before the onset of winter, and effectively storing it at 1 to 4˚C until the time of grafting, thereby reducing the risk of frost damage as was experienced in 2007. Furthermore, grafted trees in the heated room were no longer packed into plastic crates, but bundled openly in the room, covering roots with moist sawdust. This prevented the problem of excess moisture that caused fungal growth. It was further established that the use of polyethylene budding tape in combination with hot paraffin wax could have hampered callus development (Frutos Tomas, personal communication[1]) for both heating methods. Subsequently, in 2008 different wrapping material for graft unions were evaluated, and the most success was obtained with common masking tape, as used by Leon (personal communication[2]) in Argentina on root grafts, and flagging tape, which is more porous than standard budding tape. These adjustments were rewarded with exceptionally good callus development and grafting success so far for 2008/09, beyond 80% for most treatments. Surprisingly, no noticeable difference was detected between the two rootstocks, *J. regia* and Paradox. Although callus development in the heated room was satisfactory and markedly better compared to previous seasons, the heated trench did provide better callus development and higher grafting success for all rootstocks and cultivars used. If post-grafting mortalities are limited and optimal vegetative growth is maintained for the remainder of the season, a total of 750 grafted trees suitable for orchard establishment will be produced for the current season by May 2009.

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[1] Diego Frutos Tomas, 2008. Imida Institute, La Alberca, Murcia, Spain
[2] Jorge Leon, 2008. Tupangato, Argentina. jorgeleon@infovia.com.ar

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Appendix A. Summary of statistical analysis

Summary of statistical analysis: Abbreviated analysis of variance (ANOVA), Repeated measures of analysis and Student's T-test tables

Table A1 *Analysis of variance of callus development after 33 days, after square transformation (Table 4.1)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	35260271.20	4.40	0.0155
Method	1	65571235.90	8.17	0.0054
Cultivar	1	5687840.80	0.71	0.4023
Time of grafting x Method	2	131786708.10	16.43	<0.0001
Time of grafting x Cultivar	2	22740490.70	2.83	0.0647
Method x Cultivar	1	5684192.00	0.71	0.4025
Time of grafting x Method x Cultivar	2	1948466.80	0.24	0.7849
Error	79	8022029.00		
Corrected Total	90			
CV%	72.6			
R^2	0.44			

Table A2 *Analysis of variance of final graft-take, after square transformation (Table 4.3)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	37651820.32	15.64	< 0.0001
Method	1	39589022.26	16.45	0.0003
Cultivar	1	3040671.60	1.26	0.2691
Time of grafting x Method	2	13508829.83	5.61	0.0080
Time of grafting x Cultivar	2	3457697.27	1.44	0.2522
Method x Cultivar	1	48820.31	0.02	0.8876
Time of grafting x Method x Cultivar	1	10266000.0	4.27	0.0468
Error	33	2406656.3		
Corrected Total	43			
CV%	34.3			
R^2	0.67			

Table A3 *Univariate T-tests for increase in callus development for two walnut cultivars between day 21 and 25, 21 and 29, 21 and 33, 25 and 29, 25 and 33, and 29 and 33 respectively*

Interval	DF	t-value	Pr > |t|
Day 21 – Day 25	95	2.28	0.0246
Day 21 – Day 29	95	3.13	0.0023
Day 21 – Day 33	95	3.32	0.0013
Day 25 – Day 29	95	2.18	0.0318
Day 25 – Day 33	95	3.11	0.0024
Day 29 – Day 33	95	2.28	0.0246

Table A4 *Univariate T-tests for increase in callus development for cultivar 'Chandler' between day 21 and 25, 21 and 29, 21 and 33, 25 and 29, 25 and 33, and 29 and 33 respectively (Table 4.4)*

Interval	DF	t-value	Pr > |t|
Day 21 – Day 25	47	1.85	0.0702
Day 21 – Day 29	47	2.72	0.0091
Day 21 – Day 33	47	2.84	0.0066
Day 25 – Day 29	47	2.00	0.0512
Day 25 – Day 33	47	2.86	0.0063
Day 29 – Day 33	47	2.07	0.0443

Table A5 *Univariate T-tests for increase in callus development for cultivar 'Serr' between day 21 and 25, 21 and 29, 21 and 33, 25 and 29, 25 and 33, and 29 and 33 respectively (Table 4.4)*

Interval	DF	t-value	Pr > |t|
Day 21 – Day 25	39	1.36	0.1830
Day 21 – Day 29	39	1.67	0.1031
Day 21 – Day 33	39	1.96	0.0577
Day 25 – Day 29	39	1.00	0.3235
Day 25 – Day 33	39	1.43	0.1599
Day 29 – Day 33	39	1.00	0.3235

Table A6 *Analysis of variance of success percentage determined in October at the beginning of the first growing season (Table 4.5)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	4397.79	27.57	< 0.0001
Method	1	7190.76	45.08	< 0.0001
Time of grafting x Method	2	3050.13	19.12	< 0.0001
Cultivar	1	3.26	0.02	0.8872
Time of grafting x Cultivar	2	1233.72	7.73	0.0016
Method x Cultivar	1	3.26	0.02	0.8872
Time of grafting x Method x Cultivar	2	901.69	5.65	0.0073
Error	36	159.51		
Corrected Total	47			
CV%	27.1			
R^2	0.82			

Table A7 *Analysis of variance of success percentage determined in February in the middle of the first growing season (Table 4.5)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	2236.33	10.36	0.0003
Method	1	7190.76	33.30	< 0.0001
Time of grafting x Method	2	3050.13	14.13	< 0.0001
Cultivar	1	29.30	0.14	0.7148
Time of grafting x Cultivar	2	1650.39	7.64	0.0017
Method x Cultivar	1	29.30	0.14	0.7148
Time of grafting x Method x Cultivar	2	888.67	4.12	0.0246
Error	36	215.93		
Corrected Total	47			
CV%	35.5			
R^2	0.75			

Table A8 *Analysis of variance of success percentage determined in May at the end of the first growing season (Table 4.5)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	2031.25	9.75	0.0004
Method	1	6888.02	33.06	< 0.0001
Time of grafting x Method	2	3138.02	15.06	< 0.0001
Cultivar	1	52.08	0.25	0.6201
Time of grafting x Cultivar	2	1653.65	7.94	0.0014
Method x Cultivar	1	13.02	0.06	0.8040
Time of grafting x Method x Cultivar	2	1184.90	5.69	0.0071
Error	36	208.33		
Corrected Total	47			
CV%	35.5			
R^2	0.75			

Table A9 *Analysis of variance of survival percentage determined in May at the end of the first growing season (Table 4.6)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	2	147.89	0.56	0.5748
Method	1	2541.74	9.68	0.0039
Time of grafting x Method	2	1795.41	6.84	0.0034
Cultivar	1	6.20	0.02	0.8788
Time of grafting x Cultivar	2	1468.73	5.60	0.0082
Method x Cultivar	1	128.58	0.49	0.4890
Time of grafting x Method x Cultivar	1	2887.96	11.00	0.0023
Error	32	262.48		
Corrected Total	42			
CV%	24.5			
R^2	0.58			

Table A10 *Analysis of variance of average diameter measured in February in the middle of the growing season (Table 5.1, 5.2 and 5.3)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	1	81.81	13.76	0.0012
Method	1	79.57	13.38	0.0013
Time of grafting x Method	1	1.39	0.23	0.6336
Cultivar	1	14.76	2.48	0.1288
Time of grafting x Cultivar	1	11.11	1.87	0.1849
Method x Cultivar	1	0.65	0.11	0.7447
Time of grafting x Method x Cultivar	1	3.62	0.61	0.4433
Error	23	5.94		
Corrected Total	30			
CV %	15.9			
R^2	0.60			

Table A11 *Analysis of variance of average diameter measured in May at the end of the growing season (Tables 5.1, 5.2 and 5.3)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	1	103.64	8.66	0.0073
Method	1	180.11	15.05	0.0008
Time of grafting x Method	1	13.46	1.12	0.2999
Cultivar	1	132.18	11.04	0.0030
Time of grafting x Cultivar	1	25.31	2.11	0.1594
Method x Cultivar	1	9.74	0.81	0.3764
Time of grafting x Method x Cultivar	1	2.24	0.19	0.6695
Error	23	11.97		
Corrected Total	30			
CV %	17.6			
R^2	0.64			

Table A12 *Analysis of variance of average height measured in February in the middle of the growing season (Tables 5.4 and 5.5)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	1	5883.93	13.09	0.0014
Method	1	3166.28	7.04	0.0142
Time of grafting x Method	1	98.82	0.22	0.6436
Cultivar	1	9693.95	21.56	0.0001
Time of grafting x Cultivar	1	1808.30	4.02	0.0500
Method x Cultivar	1	0.20	0.00	0.9832
Time of grafting x Method x Cultivar	1	17.36	0.04	0.8460
Error	23	449.63		
Corrected Total	30			
CV %	35.0			
R^2	0.67			

Table A13 *Analysis of variance of average height measured in May at the end of the growing season (Tables 5.4 and 5.5)*

Source	DF	MS	F-Value	Pr > F
Time of grafting	1	10249.68	16.12	0.0005
Method	1	13522.80	21.27	0.0001
Time of grafting x Method	1	222.33	0.35	0.5601
Cultivar	1	21617.58	34.00	< 0.0001
Time of grafting x Cultivar	1	3045.69	4.79	0.0390
Method x Cultivar	1	502.90	0.79	0.3830
Time of grafting x Method x Cultivar	1	24.84	0.04	0.8451
Error	23	635.85		
Corrected Total	30			
CV %	31.8			
R^2	0.78			

***Table A14** Univariate T-tests for increase in callus development of four walnut cultivars between day 21 and 25, 21 and 29, 21 and 33, 25 and 29, 25 and 33, and 29 and 33 respectively (Table 6.1)*

Interval	DF	t-value	Pr > |t|
Day 21 – Day 25	31	2.40	0.0228
Day 21 – Day 29	31	2.40	0.0228
Day 21 – Day 33	31	2.40	0.0228
Day 25 – Day 29	31	-	-
Day 25 – Day 33	31	-	-
Day 29 – Day 33	31	-	-

***Table A15** Analysis of variance of callus development for four walnut cultivars after 33 days of subjection to hot callus conditions (Table 6.2)*

Source	DF	MS	F-Value	Pr > F
Cultivar	3	299.19	1.20	0.3433
Error	16	250.36		
Corrected Total	19			
CV %	22.4			
R^2	0.18			

***Table A16** Analysis of variance of final graft-take for four walnut cultivars after 33 days of subjection to hot callus conditions (Table 6.2)*

Source	DF	MS	F-Value	Pr > F
Cultivar	3	180.0	1.13	0.3685
Error	16	160.0		
Corrected Total	19			
CV %	13.3			
R^2	0.17			

Table A17 *Analysis of variance of success percentage of four walnut cultivars determined in November at the start of the first growing season (Table 6.2)*

Source	DF	MS	F-Value	Pr > F
Cultivar	3	160.00	0.50	0.6876
Error	16	320.00		
Corrected Total	19			
CV %	22.4			
R^2	0.09			

Table A18 *Analysis of variance of success percentage of four walnut cultivars determined in May at the end of the first growing season (Table 6.2)*

Source	DF	MS	F-Value	Pr > F
Cultivar	3	233.33	1.30	0.3099
Error	16	180.00		
Corrected Total	19			
CV %	19.4			
R^2	0.20			

Table A19 *Analysis of variance of survival percentage of four walnut cultivars determined in May at the end of the first growing season (Table 6.2)*

Source	DF	MS	F-Value	Pr > F
Cultivar	3	195.38	0.89	0.4663
Error	16	218.89		
Corrected Total	19			
CV %	20.2			
R^2	0.14			

Appendix B. Weather data

Table B1 *Minimum and maximum temperatures (°C), experienced over a corresponding six-week-period from 2006 to 2008 and obtained from the weather station on Rotondo Walnuts farm (30°40'S, 26°37'E, 1 347 m above sea level)*

Year	**2006**		**2007**		**2008**	
Date	**Minimum**	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
24 April	2.74	15.64	6.55	25.54	1.86	22.79
25 April	5.54	16.73	7.95	27.47	3.54	23.70
26 April	3.57	19.02	10.77	27.35	6.44	23.87
27 April	4.12	20.74	8.30	17.27	4.59	23.26
28 April	4.70	21.91	3.14	11.80	11.05	22.11
29 April	9.34	22.06	-1.55	16.19	7.80	20.72
30 April	9.90	15.74	2.45	20.85	5.38	20.00
1 May	7.32	18.82	4.38	23.84	8.01	15.81
2 May	7.46	18.04	6.20	24.64	5.26	19.96
3 May	6.71	15.99	6.64	25.11	8.77	13.24
4 May	2.85	16.91	6.29	25.42	7.58	10.18
5 May	3.31	19.75	6.47	26.49	6.91	15.78
6 May	3.44	15.86	6.83	25.83	5.33	21.91
7 May	-0.07	14.81	5.95	25.37	4.51	21.45
8 May	3.84	17.40	4.38	24.63	5.36	24.26
9 May	2.85	17.85	3.53	24.41	8.81	24.80
10 May	0.85	20.50	4.85	23.71	11.89	24.06
11 May	-0.38	21.86	2.76	23.57	6.42	20.36
12 May	2.19	21.64	2.24	24.6	5.58	19.86
13 May	3.21	21.51	3.05	24.13	6.84	20.39
14 May	4.47	20.34	7.60	25.77	5.67	22.90
15 May	4.93	20.00	7.01	17.75	9.56	24.02
16 May	4.43	20.89	3.09	20.30	11.67	22.35
17 May	7.77	16.31	1.51	20.20	8.86	24.99
18 May	4.83	16.00	-0.01	23.30	9.49	22.48
19 May	6.69	11.53	11.71	24.63	8.38	21.82
20 May	4.88	9.18	3.40	15.45	8.44	20.53
21 May	4.11	9.74	-1.03	5.38	6.88	20.28
22 May	0.73	11.75	0.81	7.49	8.25	21.12
23 May	1.83	11.17	-3.71	10.23	6.21	21.88
24 May	3.75	13.55	-5.45	11.81	4.14	18.39
25 May	-0.74	13.70	-5.24	13.04	8.75	12.92

Year	2006		2007		2008	
Date	**Minimum**	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
26 May	0.54	16.45	-4.69	16.04	6.66	15.86
27 May	3.78	16.91	-3.56	17.82	6.15	19.20
28 May	1.64	13.57	-2.44	20.22	9.86	21.75
29 May	-2.05	16.79	-1.40	19.84	6.97	19.42
30 May	0.55	19.54	2.02	20.79	9.55	16.58
31 May	1.32	21.46	5.76	22.00	4.18	14.54
1 June	1.76	21.70	9.93	17.63	-0.64	15.51
2 June	1.85	20.71	6.11	19.60	5.43	15.26
3 June	3.62	13.20	3.52	19.44	8.66	15.11
4 June	-0.57	17.54	7.30	19.28	5.00	12.80
5 June	-1.28	16.85	9.29	12.59	0.29	13.97
6 June	-1.43	18.87	7.42	12.28	-0.13	16.55
7 June	-0.48	19.02	3.04	10.77	0.82	17.77

Table B2 *Average monthly minimum and maximum temperatures (°C) during the growing period from the last six seasons, obtained from the weather station on Rotondo Walnuts farm (30°40'S, 26°37'E, 1 347 m above sea level)*

	2004		2005		2006		2007		2008		2009	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
January	*	*	16.43	30.98	17.35	28.81	16.81	31.33	16.17	29.01	16.26	30.93
February	16.75	30.62	16.77	30.53	17.02	27.04	15.91	31.73	16.27	28.90	15.86	26.62
March	13.48	25.88	13.19	26.29	12.30	25.04	12.33	27.29	12.32	26.41	12.62	27.04
April	9.11	22.76	8.63	21.95	9.34	21.55	10.09	24.07	7.38	22.91	-	-
September	5.85	21.13	8.23	25.70	6.26	22.94	7.38	26.41	4.13	22.42	-	-
October	10.58	25.19	10.90	26.37	11.24	26.19	10.80	24.45	11.05	27.79	-	-
November	*	*	13.46	27.87	*	*	11.73	27.54	13.58	28.37	-	-
December	*	*	13.52	30.14	15.43	31.84	14.84	28.23	15.19	31.01	-	-

* No data available due to technical problems