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*Review*

# Propagation of Pecan (*Carya illinoensis*): A review

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**The Pecan (*Carya illinoensis*) is the most important species that belongs to the *Carya* genus. It is cultivated mainly for its nut, which is rich in oils and proteins, and for its good quality wood. Pecan is conventionally propagated by budding or grafting onto rootstocks obtained by open pollination. However, these techniques are not very efficient due to low propagation rates, poor survival and difficult establishment. Therefore, *in vitro* propagation of pecan can play a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants in a short time. In the present review, the improvements over the years in the propagation of pecans, the refinements of protocols for obtaining high shoot multiplication and regeneration through organogenesis and/or somatic embryogenesis is discussed. Some basics of genetic transformation and its possible benefits are also discussed.**

**Key words:** Pecan, *in vitro* root induction, shoot multiplication, regeneration, micropropagation, somatic embryogenesis, genetic transformation.

## INTRODUCTION

### Distribution and production

The Pecan is a species native to North America, which is the leading producer in the world. It is however also cultivated in Australia, Brazil, Canada, Mexico, Israel and South Africa. In 2015/2016, the world production of Pecans was more than a total of 101,000 metric tons (kernel basis), that is 5% more than in 2005/2006. Production of pecans is clearly led by the United States and Mexico, which account for 92% of the world production. These two countries' production in 2015/16 was 52,889 and 40,824 metric tons, respectively and was followed by South Africa with 5,380 metric tons and Australia with 1,716 metric tons (International Nut and Dried Fruit Council Foundation, 2016). In South Africa, the Southern Lowveld is the biggest pecan production area. Other important areas are White River, Tzaneen, Louis Trichardt /Levubu, KwaZulu-Natal, the Vaalharts irrigation scheme, the Middle veld around Pretoria and some parts along the Orange River (De Villiers and Joubert, 2008) (Figure 1).

### Classification and biology

In systematic botany, the Pecan is classified under the

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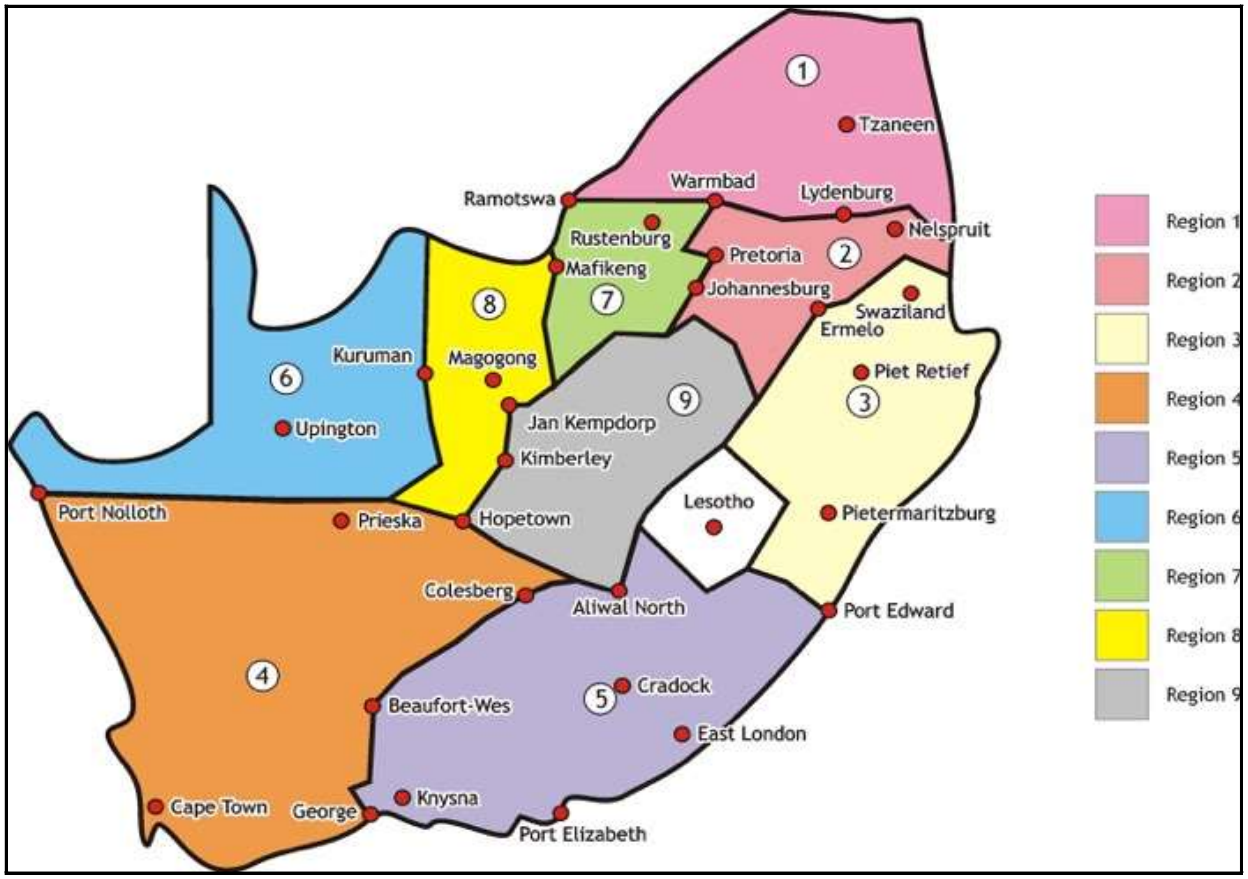


**Figure 1.** Different South African pecan nut production regions.

Kingdom *Plantae*; Class, *Magnoliopsida*; Order, *Juglandales*; Family, Juglandaceae and Genus, Carya. The *Carya* genus has approximately 25 species, in which *Carya illinoensis* represents the most economically viable nut crop. Pecan can reach, in its natural habitat, a maximum height of 55 m and a canopy of more than 2 m in diameter. The Pecan root system can be divided into 3 classes (Woodroof, 1934): 1) Taproot, which grows downward, and its depth of penetration may vary in relation to the type and structure of the soil, and the moisture conditions; 2) Lateral roots, which have a horizontal development and generally remain superficial; 3) Fibrous roots that grow in all directions from laterals and are constantly dying and replaced by new roots. It is a deciduous tree with alternate and compounded leaves, whose numbers of leaflets can vary from 9 to 17 (Reed and Davidson, 1954). The leaves can vary in length and color depending on the cultivar (Figure 2).

It is a monoecious species, with staminate and pistillate flowers on the same tree. The male flowers are pendulous and grouped in catkins and grow on one-year-old branches. Generally, the pollens produced by the male flowers are abundant every year. The female flowers are organized in terminal spikes and grow on the current season's growth. The pistillate flowers are apetalous with bilobed stigmas and surrounded by four foliar bracts. The female flowers can be present in adequate amounts or they can be scarce if the yield was high the previous year, due to the alternate bearing phenomenon typical of woody fruit plants.

Shedding of the flowers may occur in the early spring season mainly for three reasons: (1) Rudimentary flowers located near the shoot tip; (2) Normal flowers that were not pollinated and (3) Pollinated flowers in which nutlets did not develop because the food reserves were depleted during early growth or because of unfavorable moisture conditions. Another flower fall may also occur in late summer and is caused by incomplete fertilization (fruit set) (Byford, 2005). Pollination is typically by wind (anemophilous pollination). Pecan is a hetero-dichogamous species, that is, another dehiscence and pistil receptivity do not coincide. In fact, some cultivars are protandrous (which means the male flowers develop before the female ones), and others are protogynous (where the stigma receptivity precedes the maturity of the pollen). Dichogamy promotes cross-pollination; although a short period of overlapping exists self-pollination is also possible in some cultivars. Therefore, for adequate

**Figure 2.** Pecan orchard cv. Wichita located in Hartswater, Northern Cape, South Africa.

pollination, it is crucial to choose compatible cultivars that synchronize with each other in the production of mature pollen when the stigma is receptive (Vendrame and Wetzstein, 2005). Pecan fruit is a nut, which consists of a kernel enclosed by the shell. The shape and dimension of the nut varies from cultivar to cultivar and the maturation occurs in the autumn of the same season (Peterson, 1990). The nut can be considered as a storage organ. In fact, it stores minerals, carbohydrates, oils, amino acids and proteins that will serve the future embryo for respiration, germination and even in the early life stages of the seedling until it becomes self-sufficient.

Nut development starts with pollination and can be divided into two distinct phases: With phase I, which occurs from pollination to shell hardening and phase II that occurs from shell hardening until the shuck splits (Herrera, 1990). Harvesting time depends on the area of cultivation. In South Africa, pecan nuts are usually ripe from April to July, depending on the cultivar. As soon as the nut is physiologically ripe, the green husk becomes dry, cracks open and the nut drops out (Anonymous, 2000). The nuts are collected manually or mechanically, using a hydraulic trunk shaker and a mechanical harvester.

## Uses

The nut is the main economic product used as food for humans and wildlife (Harlow et al., 1991); the wood is also used mainly for the manufacturing of floors, furniture and veneer, it being of good quality. Pecan is also used as an ornamental specimen in the decoration of parks and gardens. The main chemical constituents of pecan nuts are lipids, which make up 73% of the total (primarily oils), followed by proteins (9.4%), carbohydrates (3.9%), water (3%) and a relatively small number of other components. Sometimes, alterations of a particular minor component may affect the quality of the nuts (Kays, 1990). The oil extracted from the kernel is edible and is used to produce medicine and essential oils. The quality of Pecan nuts in postharvest depends on many factors and varies according to the intended use of the product. Generally, the most important external parameters are nut size, color and external appearance. The color of the testa is the major external quality attribute and light-colored kernels are preferred. Most of the color development occurs on the tree during maturation after the onset of dehiscence (Kays and Wilson, 1977), followed by a progressive synthesis of pigments after harvest (Senter, 1976). The external quality of the kernels affects the assessment of their overall quality. The absence of insect damage and the absence of breakage are very important.

Flavor is the most important internal quality attribute used by consumers. However, this evaluation can only be made after purchase, due to the way in which the nuts are sold (sealed packages). Fresh Pecans have a

distinctive aroma and taste, and for this reason are often consumed without roasting.

Kernel fill is also another important quality. It occurs in the last stage of development and can vary with location, cultivar and year of production. Generally, poorly filled nuts do not have visual attractiveness, a good flavor quality and the textural properties of high quality nuts (Kays, 1990).

The storage conditions of the nuts play a key role in nut quality because it influences the moisture content of the nut. Under high temperature, relative humid environments the kernels become progressively more spongy and moist, but if stored under low temperatures and low humidity, the kernels become excessively crisp and brittle (Kays, 1990).

### Conventional methods of propagation

The first commercial Pecan orchards date back to the nineteenth century. They were established by sowing seeds collected from mother trees with desirable characteristics such as nut size, a resistance to scab disease, early maturity and high yields (Taylor 1906, 1907). These open-pollinated half-sib populations existed until clonal propagation of superior genotypes led to the widespread use of true cultivars through the improvement of budding and grafting techniques. Conventionally, Pecan trees are propagated by budding or grafting a scion of a selected clone (cultivar) onto rootstocks obtained from seed. Budding (patch budding or ring budding) is done during the vegetative growth phase in summer, while grafting (3-flap grafting) is done during the dormant winter season (Woodroof, 1979).

## ROOTSTOCK PROPAGATION

The seeds chosen to produce rootstocks of a particular cultivar should adhere to four essential conditions: 1) Uniformity, with a well-developed kernel; 2) Readily available, to have a continuous supply each year; 3) Vigorous seedlings; 4) Resistance against seedling diseases (Conner, University of Georgia). Careful consideration should be given to the choice of rootstock and cultivar, which are used to establish an orchard. Due to the undersupply of trees, many growers grow their own rootstocks using seeds and when seedlings reach a sufficient diameter (1.5 to 2 cm), they graft or bud a superior Pecan nut cultivar on to it (Reid, 2010).

### Seed

Three important parameters are used to select seed to propagate rootstocks namely: price, kernel percentage and uniformity. Usually, small nuts are selected (Andersen, 2004). However, Pecan seeds are generally not ready for germination, being in a state of dormancy. However, in some cases, premature germination can occur while the nuts are still attached to the tree. This phenomenon, called vivipary, occurs when the seeds go through a stage of rest, which is controlled by an inhibitor hormone called abscisic acid (ABA). Leaves will not produce ABA because of a heavy fruit load, causing mature seeds to germinate while still attached to the tree, due to a low ABA concentration (McEachern, 2010).

### Dormancy

Dormancy in deciduous fruit trees is a natural mechanism, which is necessary to enable the tree to overcome adverse climatic conditions during winter (Faust et al., 1997). Over the years, there have been several definitions of dormancy. The most universally accepted definition of dormancy was formulated by Lang (1987), who introduced the terms eco-, para- and endo-dormancy. Eco-dormancy refers to the environment (eco), para-dormancy refers to physical or biochemical external signals that are sent to the affected structure (para), and endo-dormancy refers to physiological factors inside the affected structure (endo).

For plant propagation in nurseries, dormancy is a barrier in the production of seedlings because it extends the time of germination after sowing and the seeds are also exposed to predators and unfavorable weather conditions. Uneven germination results in seedlings that differ in size and consequently increase the production cost of trees in the nursery (Poletto et al., 2016). Therefore, the main challenge is obtaining a faster germination rate and increasing the uniformity of the seedlings. Stratification is a process in which dry seeds are soaked in water and then stored in a cold, moist condition for 90 to 120 days to break dormancy. Immediately after harvesting, Pecan seed should be stored at low temperatures (1 to 5°C) to maintain viability, break dormancy and ensure germination. Stratification is basically the representation of what happens in nature. Dormancy is gradually broken during the winter because the nuts lie on the leaf litter and is subjected to several cycles of humidity and cold temperatures. The artificial stratification process begins by soaking the nuts in running water within a tank for a variable period of 1 to 3 days. Afterwards, it is placed in media such as peat moss, cedar shavings, potting soil or saw dust to capture the excess water and maintain the humidity. Then, the nuts are stored at a temperature of 1.7 to 7.2°C, until planting.

It is recommended to stratify the nuts for at least 10 weeks to have a fast germination rate (Sparks et al., 1974). Several reports are available in literature regarding the stratification conditions of Pecan seeds (Bonner, 1976a; van Staden and Dimalla, 1976; Dimalla

and van Staden, 1977, 1978; Adams and Thielges, 1978; Goff et al., 1992).

## Pre-germination treatments

Shu-fang et al. (2011) performed a germination test on Pecan seeds using different storage times, various soaking methods, applying phyto-hormones and different seed stratification methods and seeds stored for more than two months, at 3 to 5°C germinated the best. As the storage time increased, the germination rate decreased. The germination rate improved remarkably when the seeds were treated with phyto-hormones and stratified. The seeds that were soaked in phyto-hormones for 8 days and stratified indoors for 35 days, resulted in a 91% germination. Bonner (1976b) stated that germination without prior stratification is greatly enhanced by soaking the seeds in gibberellic acid. Recently, Poletto et al. (2016) executed a study to overcome Pecan seed dormancy using different methods.

The seed were stored for a period of 30, 60 and 90 days at either room temperature (controlled treatments) or at 4°C. Half of the seeds stored at 4°C were also scarified by using sandpaper. The results obtained showed that the highest Pecan emergence speed index (ESI) and the best seedling development were observed when seeds were stored at 4°C as well as scarified.

Seed viability was significantly reduced when stored at room temperature, irrespective of the storage time. Bilan and Foster (1970) also assessed the effect of various chemical treatments upon germination of stratified and unstratified Pecan nuts. Results showed that chemical treatments did not have any significant effect on germination for stratified and unstratified seeds. They also stated that stratification is the only method to speed up the germination of Pecan seeds. Casales et al. (2017) investigated the effect of different chemical seed treatments (2500 or 5000 ppm $KNO_3$; 500 or 1000 ppm $GA_3$; 1% HCl; 2% Dormex and 98% $H_2SO_4$; control: no chemical treatment) and two temperatures (25 and 28°C) on dormancy breaking and germination of Pecan seeds cv. Ukulinga. A significantly higher germination percentage was obtained at 28°C (55%) than at 25°C (41.3%). Shelled seeds treated with 5000 ppm $KNO_3$ or 1000 ppm $GA_3$ had a 100% germination rate. However, shelled seeds not treated with any chemicals also resulted in 100% germination.

## Effect of moisture and temperature on seed germination

Water and temperature, separately or together, are the most important factors affecting the germination percentage and germination rate (Shaban, 2013). On the one hand, temperature plays a pivotal role in determining the periodicity of seed germination and the distribution of species (Guan et al., 2009); on the other hand, water is an essential requirement for germination. In fact, water allows the activation of a series of enzymes, translocation and the use of food storage (Shaban, 2013).

Generally, the temperate-region seeds (such as Pecan) require lower temperatures than tropical region seeds, and wild plant species have lower temperature requirements than domesticated plants. High-quality seeds can germinate under wider temperature ranges than low-quality seeds (Shaban, 2013). After the harvest, a period of dry storage is required for Pecan nuts to reduce the kernel moisture percentage from 20% to 6-4% before being stored for the long term (McEachern, 2010).

According to King and Roberts (1980), Pecan is included in the list of species with recalcitrant or short-lived seeds. This group belongs to the species whose seeds retain viability for a short period of time, and most of them cannot tolerate moisture below 25%. However, seed longevity can be increased with proper handling and storage (Hartmann et al., 2002). Van Staden and Dimalla (1976) defined Pecan seeds as non-dormant seeds because the seeds can germinate at any time after harvest, if incubated under favorable conditions. Although the nut shell is freely permeable to water and gases, the germination is delayed by mechanically restricting the radicle to elongate (Smith et al., 1997).

However, this problem can be overcome by incubating the seeds at a temperature of between 30 to 35°C. At these temperatures, the germination of seedlings is uniform, rapid and is completed within 20 days. Dimalla and van Staden (1977) also reported that incubated Pecan seeds at 30°C showed higher levels of endogenous cytokines and gibberellins than those seeds that were incubated at 20°C. These results suggested that endogenous hormones play an important role in the mobilization of lipid food reserves to supply the energy required for germination.

## Sowing in seedbeds and containers

In nut tree nursery, two methods will normally be followed for the propagation of trees for subsequent use in orchard: 1) Sowing in seedbeds or 2) Sowing in containers.

Seedbeds provide a high seedling production, but it is more difficult to harvest the trees for transplanting in the orchard. A soil analysis is needed before sowing to evaluate the fertility of the seedbed and rectifying imbalances that may occur. Fertilization with a slow-release fertilizer along the row is usually performed during the beginning of summer. Budded or grafted trees should be dug for transplanting in orchards in the spring of the following year (Reid, 2010). Seeds should be sown 10 to 15 cm apart in the row and 7 to 10 cm deep. Sowing depth should be shallower in clay soils than in

sandy soils (Wells, 2014). Seedbeds must be weed-free and well watered.

Another way to grow Pecan seedlings in a nursery is in containers. Different sizes and shapes are available, but it is advisable to choose containers with an "open bottom". Roots are generally air-pruned by placing the pots on a screen wire bench. Using this method prevents the circling of the taproot in the container and promotes a fibrous root system. It is advisable to use a growth mixture that allows the free flow of water through the pot. A slow-release fertilizer that can provide both macronutrients and micronutrients can be added to promote root system growth.

A mix of three parts ground pine bark, one-part peat moss and one-part coarse sand is recommended. All potting mixes should be sterile (Wells, 2014). Keever et al. (1986) investigated the effect of container size and shape, root pruning and fertilization rate on the growth of Pecan seedlings, with the aim to reduce the time required to reach the budding stage. Results showed that seedling Pecan height growth was greater in 38 L containers (68.6 cm) and 19 L containers (61.7 cm) compared to shallow 19 L containers (49.3 cm) and 11 L containers (50.3 cm). Nevertheless, all the seedlings reached the budding stage in the first growing season. They also found that root pruning at transplanting did not affect the top growth but increased root branching and total root growth as well as the increment rates of a complete fertilizer.

Zhu et al. (2017) executed an experiment on one-year-old Pecan container seedlings under sub- and overhead irrigation, with the aim to determine water use efficiency (WUE), vegetative growth, photosynthesis and the nutrient status of the Pecan seedlings. The results showed that sub-irrigation can conserve up to 62% of the irrigation water and improve water use efficiency (WUE) by 193% compared to overhead irrigation. Seedling height and root collar diameter increased by 11.7 and 41.5% respectively. The net photosynthetic rate, stomata conductance and transpiration rate also improved significantly. The nitrogen and potassium content of the roots, stems and leaves increased as well as the phosphorus content of the leaves under sub-irrigation treatment compared to overhead irrigation.

Benucci et al. (2012) evaluated the possibility to obtain formations of mycorrhizae of European truffle species (*Tuber aestivum*, *T. borchii* and *T. macrosporum*) on the roots of pecan seedlings. Roots of Pecan seedlings were inoculated with the truffle spores and grown in a greenhouse for 10 months. *T. borchii* and *T. aestivum* spores produced well-formed ectomycorrhizae on the seedling roots with a colonization percentage of 62 and 42%, respectively. No ectomycorrhizae of *T. macrosporum* were formed on the roots. Marozzi et al. (2017) specified that Pecan seedlings were inoculated with spores of black truffles (*T. melanosporum* and *T. brumale*), which are economically important in Europe. Mycorrhization on Pecan roots were assessed over a 2-year period. In the first year, *T. melanosporum* and *T. brumale* produced ectomycorrhizae and 37.3 and 34.5% colonization of the roots were obtained, respectively. After 24 months, the percentage of colonization for *T. brumale* increased to 49.4% and decreased for *T. melanosporum* to 10.5%. In both works described above, the mycorrhization of Pecan seedlings was aimed at obtaining an extra income (due to truffles) along with the future fruit of the Pecans. In the nursery, the mycorrhization of Pecan seedlings might be performed routinely to help them to survive in adverse weather conditions (Smith and Read, 2008), to improve the absorption of mineral elements, especially nitrogen (Chalot and Brun, 1998; Dighton, 2009) but also to tolerate soils with high concentrations of salts, heavy metals (Blaudez et al., 2000; Sell et al., 2005; Colpaert et al., 2011) and organic pollutants (Dighton, 2009).

## CUTTINGS

Greater uniformity is obtained when rootstocks are propagated by means of cuttings due to the absence of genetic variation. It is inexpensive, rapid and simple and does not require special techniques (Chiu, 1977). Over the years, indole-3-acetic acid (IAA) and other synthetic auxins have been used to promote the rooting of cuttings (Cooper, 1935; Went, 1935). However, the response of the cuttings to the auxin treatment is not the same in all plant species. In addition, it has been shown that softwood cuttings respond better to auxins, compared to hardwood cuttings (Hess, 1959, 1962). This difference is attributed to the presence of substances other than auxins, which can stimulate or inhibit rooting (Went, 1934; Cooper, 1938; Spiegel, 1954). The production of rooting promoters or inhibitors is not consistent throughout the year but instead, their production fluctuates with the season. Therefore, the root-ability of the cuttings seems to be associated with substances produced inside the plants, e.g., rooting co-factors (Hess, 1960; Tognoni et al., 1977). Rooting experiments, using Pecan stem cuttings, gave highly variable results. Some attempts (Stoutemeyer, 1938; Romberg, 1942; Gossard, 1944; Sparks and Pokorny, 1966; Whatley et al., 1966) gave poor results. Even the use of auxins, such as indole-3-butyric acid (IBA) to induce rooting, gave inconsistent results as reported in literature (Wolstenholme and Allan, 1975; Brutsch et al., 1977). The main factors affecting the rooting of cuttings were harvest time, thickness and origin of the cuttings and genetic factors.

### Hardwood cuttings

In Pecan hardwood cuttings, the time of collection seems to be the most important factor to induce rooting, as

reported by McEachern (1973). In fact, he affirmed that the optimum period for collecting Pecan hardwood cuttings seems to be in the middle of the dormant season or after they have accumulated 200 to 400 h of field chilling (7°C). Smith et al. (1974) used juvenile (softwood) and mature (hardwood) cuttings collected during the mid dormant season. Juvenile wood dipped in 10000 ppm IBA, gave a 100% rooting, while mature wood rooted at 85% under the same conditions.

In another work, Taylor and Odom (1970) used hardwood and softwood Pecan cuttings, which were exposed to various preconditioning treatments prior to propagation. In particular, hardwood cuttings, 15 cm long and 1 cm in diameter, were collected in winter and divided into 2 groups. In the first group, cuttings were stored for 42 days in moist peat moss at 4°C. In the second group, cuttings were stored at 3°C for 30 days with the basal half of the cuttings submerged in moist peat moss and maintained at 20°C. The controls were directly inserted into the propagation medium at the time of application without any treatment.

Softwood cuttings were exposed to preconditioning treatments in the spring, immediately before bud break. For this group, etiolated shoots (15 cm long and 0.5 cm in diameter) were used, which were girdled or not. Control cuttings were not etiolated or girdled. The medium used was a mixture of perlite and peat moss in the ratio 1:1. Softwood cuttings received intermittent mist, and all the cuttings remained in the propagation bed for 75 days in the greenhouse. The etiolated and girdled softwood cuttings obtained a 15% rooting. These cuttings showed a vigorous root system and retained their leaves. Hardwood and softwood cuttings that were not girdled, did not initiate roots. The presence or absence of endogenous roots, promoting or inhibiting compounds, was determined by using the mung bean rooting bio-assay. Data obtained showed that the Pecan, regardless of type of cutting or preconditioning treatment, contains 3 or 4 distinct areas of root promotion.

Endogenous rooting inhibitors were present in all the treatment groups, except for those hardwood cuttings that were subjected to cool storage. A compound with similar chemical characteristics to juglone, which is associated with rooting inhibitory activity, was also found in the leaves and stems. Wolstenholme (1976) obtained Pecan regeneration by the stub. If the strongest shoot is trained as the new tree, while the others are removed, it will grow faster and more vigorous. Allan et al. (1980) carried out an experiment using hardwood Pecan cuttings collected from vigorous 'Barton', 'Desirable' and 'Shawnee' top-worked trees. Two experiments were conducted.

The first experiment was to study the effect of girdling and cutting thickness (10, 15 and 20 mm) on root-ability, while the second experiment was to determine the effect of cold treatment before or after treating cuttings with 1% IBA for 5 s. The best rooting (90%) was obtained with thick basal cuttings irrespective of whether they were girdled or not. Good survival percentages were obtained with all the basal cuttings (70 to 82%). The percentage was much lower with median cuttings (18 to 25%) and worthless for thin terminal cuttings. The best combination of IBA/cold treatment that gave the best rooting percentage was the control (cuttings directly placed in mist beds after a basal dip for 5 s with 1% IBA) (about 65%). Over 60% of the rooting was also obtained by the two or four week's cold treatment at 4°C followed by the IBA treatment.

The survival percentage of the cuttings was significantly better when the two weeks cold treatment was applied before the IBA treatment (48%). No significant difference in rooting percentage was found among the cultivars, although 'Shawnee' survived the best. Gustafson and Miles (1978) investigated the effect of apical buds and cultivar on the rooting of hardwood Pecan cuttings taken from adventitious sources during the winter. After cuttings were treated with IBA and a fungicide, cuttings were placed in an insulated propagation box and maintained at 3°C filled with (1 peat: 1 perlite: 1 vermiculite) and kept at 23 to 25°C. Results showed that adventitious cuttings rooted better than visible bud cuttings, especially when removing the apical bud (90 and 25%, respectively). Among the cultivars, Coy and Greenriver had the highest rooting percentage (37.5% and 30%, respectively), while 'Major' and 'Indiana' had moderate rooting (17.5 and 10%, respectively). Treatments with IBA and fungicide, or both, were beneficial because they reduced dieback from the proximal end of the cutting.

Spencer (1980) stated that the problem of establishing the cuttings, once they are rooted, can possibly be overcome by taking hardwood cuttings in summer, before the buds are dormant, and subjecting them to basal heat for rapid root development. Huang et al. (2006) used hardwood cuttings of three-year-old Pecan seedlings. Among the concentrations tested, the best rooting was obtained using 200 ppm NAA (α-naphthaleneacetic) and 100-500 ppm IBA.

Li et al. (2013) studied the effects of the parent tree age and the thickness of the cuttings on the rooting capability of the Pecan. One-year old wood with a diameter of 0.5, 0.5 - 0.8 and ≥ 0.8 cm were collected from 2, 5, 16 and 25 years old parent trees. Cuttings were treated with different concentrations or combinations of IBA and NAA. Callus and rooting percentages of the cuttings from 2-year-old parent trees were 87.7 and 14.4%, respectively, significantly higher than the other tree ages. The best results were observed from the cuttings with a diameter of ≥ 0.8 cm, callus and rooting percentages were 33.1 and 9.7%, respectively. The treatment of IBA 1.0 g $l^{-1}$ + NAA 0.25 g $l^{-1}$ exhibited the best results after treating the cuttings with the thickness of ≥ 0.8 cm, and the callus and rooting percentages were 43.0 and 14.6%, respectively. Under the same ages of the parent trees, the callus and rooting percentages of the cuttings were positively correlated to the thickness of the cutting.

Zhang et al. (2015) used hardwood cuttings collected from 1-year-old Pecan trees to investigate the influence of auxins (0.03, 0.06 or 0.09% IAA or IBA and 0.06, 0.09 or 0.12% NAA) and different combinations of media and air temperatures on their rooting ability. Cuttings (12 cm long) were treated with different concentrations of auxins at room temperature for 4 h. The basal ends of each cutting were soaked in distilled water as a control. The rooting substrate was composed of a mixture of peat, perlite, coarse sand and silver sand. Cuttings were then planted into the root substrate (2 peat: 4 perlites: 1 coarse sand: 1 silver sand) at a media/ambient temperature as follows: 1) Media and ambient temperature both at 13 ± 2°C. 2) Media (25°C ± 2°C) and ambient (13 ± 2°C) temperature. 3) Media and ambient temperature both at 25 ± 2°C. The best result was obtained with the 25 ± 2°C media and the 13 ± 2°C ambient temperature treated with 0.09% NAA (82% rooting) or 0.06% IBA (80% rooting).

### Softwood cuttings

Gossard (1944) was one of the first to obtain the rooting of Pecan softwood cuttings. Although he obtained rooting under continuous mist, in the end no plant survived transplantation. Shreve (1974) used six 1-year-old Pecan seedlings to induce the rooting of softwood cuttings. From the six seedlings, all the visible buds were removed from three cuttings, and all the terminal buds were removed from the stems of the other three cuttings, to force growth from lateral visible buds. The cuttings were planted in pots of 1 peat: 1 perlite. Twelve cuttings (2 from each seedling) were set (6 from visible buds and 6 from adventitious shoots) and maintained under intermittent mist and sprayed with a 4-4-8 Bordeaux mixture. Rooting was obtained from the six cuttings from adventitious shoots after 15 days, and shoot growth started after 35 days. Cuttings from the visible buds formed roots in 30 to 70 days, but none of them developed shoots.

## LAYERING

The purpose of the Layering Technique is to induce rooting from a stem of the mother plant. When the new growing plant can survive on its own, it will be cut off from the mother plant (Anonymous, 2007). Gossard (1941) reported that it is possible to obtain roots from a Pecan with success by trench-layering the budded or grafted trees and by air-layering the old trees in a marcot in conjunction with an IBA treatment. Due to the unavailability of clonal Pecan rootstocks, studies were initiated by Abou-Taleb et al. (1992) to evaluate the effectiveness of air-layering, stooling and trench layering techniques for propagation and field survival of clonal Pecan rootstocks and to obtain an estimation of the relative responsiveness of genotypes.

### Air-layering

Air-layering is another technique to obtain clonal Pecan rootstocks. It is performed with a sharp knife, as two parallel cuts are made about 2 cm apart around the stem and through the bark and cambium layer. The two parallel cuts relate to one long cut. The ring of bark is removed to leave the inner woody tissue exposed. The newly bared ring is scraped to remove the cambial tissue, which is to prevent a bridge of callus tissue from forming. At this point, the rooting hormone is applied and the moss will be wrapped and covered with plastic or aluminum foil and held in place with twist ties or electrician's tape (Beckford, University of Florida). This process should be completed during rainy seasons, when the humidity is the highest (Anonymous, 2007). Litchi, Guava, Macadamia, Mango and Pecan (Pokorny and Sparks, 1967; Abou-Taleb et al., 1992) are all propagated with this method.

### Mound (stool) layering or stooling

Mound layering is an old method of propagation, initially established to mass propagate apple clonal rootstock, but subsequently it was also used for other fruit tree species such as Quince, Currants, Gooseberries and Pecan (Carlson and Tukey, 1955; Brase and Way, 1959; Duarte and Medina, 1971; Medina, 1981; Garner, 1988). With this method the shoots are cut back to ground level and soil or rooting medium is mounded around them to induce rooting at the base. Stool shoots will be separated from the parent plant if they have sufficient roots by the end of the growing season. Rooted shoots are cut at their bases and delivered to customers to transplant into the nursery as "rooted liners" (Hartmann et al., 2002).

## BUDDING AND GRAFTING

In Pecan propagation there has been important improvements over the years using tissue culture and cuttings. The commercial Pecan industry is still dependent on propagation by grafting and budding. Grafting and budding provide a success rate of more than 75% (Nesbitt et al., 2002). However, the disadvantages of these techniques are that the seedlings require 2 to 3 seasons of growth before it reaches an acceptable stem diameter to be grafted (Zhang et al., 2015).

Zhang et al. (2015) performed grafting using 1-month-old, open-pollinated 'Shaoxing' Pecan seedlings for evaluation of grafting as a technique to reduce the production time of grafted trees. Germinated seeds with a hypocotyl less than 0.5 cm were selected for the

experiment. The diameter of the epicotyls was 2 to 3 mm, the stems were 15 cm long and the 1-year-old scions 'Caddo' and 'Desirable' Pecans were used. Each scion had at least 4 healthy buds and was 2 to 3 mm in diameter to match the diameter of the seedling epicotyl. Scions cut in the center were sealed in polyethylene bags and marked as "terminal scions" and "base scions" respectively and stored at 5°C until used for grafting. Buddy tape and medical tape were also compared to assess which one was the best in terms of the graft success rate. The rootstock was cut off smoothly above the epicotyl and was then split 2 cm deep with a grafting knife. The scion was obliquely cut through both sides, 2 cm from the lower end and fitted and inserted on the rootstock. The cut area and the scion were wrapped with both types of grafting tape. The completed graft was transferred to a 15 × 20 cm container with 1 or 2 buds exposed. The containers were placed in full sun, on a concrete floor, in a greenhouse. Successfully grafted seedlings began to sprout 40 to 45 days after grafting. Results showed that 'Desirable' scions had greater success than 'Caddo' scions, and earlier grafted plants had more success than plants grafted later (20-day difference). Grafting took place in late spring. Graft success percentages were 87.13% for 'Desirable' and 70.51% for 'Caddo'.

In a study conducted in Iran by Ajamgard et al. (2016), three different grafting methods and different grafting times were investigated. The grafting methods included Cleft grafting using hot cables, which is done five different times from late October until mid-February; Cleft grafting using polyethylene bags, and Side-stub grafting done five different times from early November until early March. Scion wood of ‘GraTex’, ‘Wichita’, ‘Choctaw’, ‘10J’ and ‘GraKing’ were grafted onto two-year old seedling rootstocks that were 1 to 1.5 cm in diameter. The best grafting time was from late January until late February, using Cleft grafting with hot cables. For Cleft grafting with polyethylene bags the best time was from mid-February until late March. The highest percentage of graft success was achieved using ‘Wichita’ scions (92%), and the lowest ‘10J’ (less than 10%). The Side-stub grafting method was unsuccessful and grafting success for different cultivars was below 20%.

**Patch budding**

Patch budding is generally used to propagate Pecan nursery stock which simply involves removing a portion of bark with a dormant bud from the scion and fitting them into a space cut into the bark of the rootstock. When the bud starts to grow, the shoot above the bud will be pruned for the grafted bud to become the primary shoot (Wells, 2014). Patch budding is usually performed in late summer but can also be done in the spring. The rootstock and scion wood should have the same diameter, from 1.5 to 2.5 cm. Scion wood consisting of the current season's shoots should be vigorous (Wells, 2014).

**Whip grafting**

Whip grafting is generally performed with seedling trees and nursery stock with a diameter up to 2.5 cm. It may be done in late winter when the buds are still dormant. The rootstock and scion wood should have the same diameter. The scions should be vigorous and have a length of at least 18 to 25 cm (Wells, 2014). Using a sharp knife, an oblique cut is performed in both the rootstock and scion so that the two cuts are face to face and overlaid perfectly. Afterwards, the cut areas and the scion should be wrapped with parafilm to seal the cuts and protect the scion. Successful grafts start to grow in 3 to 4 weeks (Wells, 2014).

**Four flap grafts (banana graft)**

The four-flap graft is one of the easiest grafts to use. It can be used with smaller sized rootstock to graft more than one cultivar onto the same rootstock (Anonymous, 2010). It may be done in early spring after the bark begins to slip. Scions should be collected in late winter, and should have a maximum diameter of 2.5 cm. The rootstock used should be a healthy 1-year old seedling with well-developed buds (Wells, 2014). Grafting should be done at a height of 45 to 60 cm from the ground, on a selected point of the rootstock without damage and no bud scars, and the rootstock should be cut straight across with sharp pruning shears.

Afterwards, a small lightweight rubber band is wrapped around the rootstock leaving a 7.5 to 10 cm space from the top cut (Carroll, Oklahoma State University). The rubber band is useful during the grafting procedure, as it should fit perfectly with moderate pressure on the rootstock. Lateral growth on the rootstock should be removed to approximately 15 cm. Four vertical cuts of 4 to 5 cm long and equally spaced (quartered) must be done around the circumference of the rootstock. Cuts are made through the bark only.

The scion should be smooth, straight and slightly larger than the rootstock, with a cut made to about 15 cm in length with 2 or 3 buds remaining. With a sharp knife, the scion is cut on 4 sides, starting the cut about 2.5 to 5 cm from the bottom end. The end should be square-shaped. The four flaps of the bark are pulled down so as not to touch the inside of the flaps, 4 to 5 cm of rootstock is now exposed. The exposed rootstock is cut with sharp pruning shears, taking care not to damage the four flaps. The scion is inserted upright on the rootstock and the four flaps are pulled in place to cover the cut surfaces on the scion, and the rubber band is moved up around the flaps to secure them in place (Carrol, Oklahoma State University).

The cut areas are wrapped with masking tape or

grafting tape to make sure that it is firm but does not pull too tightly. The tape is covered with a piece of aluminum foil with the shiny side to the inside, to reflect heat from the graft. The corner of a polyethylene bag is clipped and carefully slipped down over the rootstock with the scion protruding through the bag (Stafne, 2015). The bag is tied to the scion approximately 2.5 cm above the foil and 2.5 to 5 cm below the foil. With any type of tape, the polyethylene bag is kept secure in place to stop the graft from drying out and to protect it from rainfall. Buds begin to break through three to four weeks after grafting. Once the grafting is performed, the grafted scion is kept at a reduced growth throughout the first summer by removing growing tips. This operation must be done several times during the season to stimulate an increment in diameter of the trunk and a better tree establishment (Stafne, 2015).

### Bark graft

The bark graft is an effective way to propagate Pecan cultivars onto rootstocks of 5 to 10 cm in diameter (Reid, 2010). It is advisable to select a point on the rootstock above the first whorl of branches to perform the grafting. Bark grafting should be done in spring, about 2 to 3 weeks after growth begins (Wells, 2014). The top of the rootstock is cut with a saw and the outer portion of the bark is removed with a sharp knife to the point where the scion is inserted. The scion should have at least 3 buds and a diameter of 1 cm. The scion is carved down to less than half its original thickness and a shallow cut is made on the back of the scion angled to one side (Reid, 2010). The scion should have a wedge shape after making the second cut, and a triangular shape after making the third cut. It will be ready for grafting after making a chisel point at the end of the bud stick.

Afterwards, the bark of the rootstock is lifted away from the wood with a sharp knife and the scion is inserted between the bark and the wood of the rootstock. The scion is tapped down until the shoulder of the deep cut touches the wood of the rootstock. A staple gun can be used to secure the graft union on smaller trees, or brad nails can be used to secure the bark graft on larger trees (Reid, 2010). Once the grafting is performed, the graft union is wrapped with aluminum foil and a polyethylene bag the same as the four-flap graft.

### Top-work

Generally, Pecan trees with a diameter between 7.5 and 30 cm and a height of 1.40 m above the ground are top-worked. It is advisable to leave one limb below the graft to provide food and to shade the trunk (Stafne, 2016). The limbs are selected to promote the development of a well-balanced crown. The limbs are sawn from the bottom of the limb until the saw begins to bind, while the final cut is done from the top side. This is done to prevent splitting (Stafne, 2016). The main limb, since it is the most important single graft, is cut and grafted leaving 30 to 40% of the limbs uncut for 1 or 2 years. All suckers or new growth are pruned from any place except the grafts to force new growth from the scion. The scion of the desired variety should be collected in late winter, while the buds are dormant, from young trees which produce vigorous growth. The scion is cut into 15 cm sticks and tied into bundles of 25. Both extremities of the sticks are covered with wax or paraffin to prevent dehydration. The sticks are then stored in peat moss or moist newspaper in the refrigerator until the time for use (Stafne, 2016).

The most common graft used in top-working Pecan trees is the inlay bark graft which has been successfully used when other systems have failed because of heat, drought and wind (McEachern et al., 1992). Top-working is generally used to replace old trees or cultivars that have become commercially unacceptable for the Pecan industry (Sparks, 1990). However, top-working can require up to 30 grafts per tree and long-term aftercare. For this reason, it is considered cost-prohibitive.

Therefore, Yates and Sparks (1992) executed a grafting of 'Desirable' Pecan scion wood onto the lateral roots of a 70-year-old 'Van Deman' seedling rootstock, with the aim to obtain an acceptable cultivar that would produce faster than nursery-grown trees.

Two methods of grafting were performed. The first was a bark graft, positioned on the root either above or below the soil level, while the second was an inlay graft, positioned below the soil line. Results showed that the most successful method of grafting was the modified bark graft positioned beneath the soil line. Survival was higher for grafts treated with 1 to 2% IBA than those without IBA, and the time of grafting also influenced the success rate. The most suitable time for making grafts was in late spring 6-8 weeks after bud break. Root bark thickness also affected graft survival. The survival percentage of grafts was highest (74%) on roots with a bark thickness of 8.1 to 10.0 mm.

## MICRO PROPAGATION

With the advent of *in vitro* cultures, the problem of genetic variability was overcome because micro propagation has the enormous advantage of generating many genetically identical plants (clones) in a short time, which cannot be obtained with conventional propagation methods.

### Stages involved in micro propagation

In micro propagation, it is possible to identify five basic stages (from 0 to IV). These stages describe, not only the techniques applied in tissue culture, but also the changing

environmental conditions of the crop (Miller and Murashige, 1976).

### *Stage 0: Selection and preparation of the mother plant*

The quality of the explants and subsequent *in vitro* responses of the explants are significantly influenced by the plant health and physiological condition of the mother plant (Debergh and Maene, 1981; Read, 1988). Before *in vitro* establishment of a crop, attention should be paid to the selection and maintenance of the mother plants used as sources of the explants. The use of appropriate agronomic practices such as adequate fertilization allows you to select bigger explants, which will have a more rapid *in vitro* response.

### *Stage I: Initiation of aseptic cultures*

The aim of this stage is to initiate and establish a pathogen-free culture of terminal or lateral meristems. The primary explants excised from the mother plants are subjected to sterilization of the external surface. The high presence of organisms, such as bacteria or fungi, affects the survival of explants, their growth and their subsequent proliferation.

### Sterilization of explants

Sterilization is the most delicate moment because it affects the success or failure of the establishment of the explants *in vitro*. It is often difficult to sterilize the surface of the plant material because the use of products such as ethanol, sodium hypochlorite and mercuric chloride, can irreversibly damage the explant. Therefore, the explant tissue influences both the type of sterilizing agent and the exposure time (Mahmoud and Al-Ani, 2016). For Pecans, sterilization procedures include ethanol, sodium hypochlorite, and in some cases, antibiotics and/or fungicides. The sterilization of the stem segments gave mixed results. The sterilization of the buds excised from mature plants gave unsatisfactory results or has required laborious treatments. In contrast, low levels of contamination have been obtained using immature seeds as explants (Wetzstein et al., 1996).

Table 1 shows the detailed protocols adopted for several explant tissues of Pecans by several workers.

### *Stage II: Shoot multiplication and seed germination*

Stage II is characterized by repeated cycles of proliferation of auxiliary buds from apical or lateral shoots, cultured on medium containing high levels of cytokinin to inactivate the apical dominance of the terminal bud. The number of possible subcultures, which can be made in this stage, depends on the species or cultivar and on its ability to maintain an acceptable rate of proliferation and, at the same time, a minimum level of genetic variability (Kurtz et al., 1991).

The first attempts of *in vitro* propagation of Pecans date back to the early '80s when Knox and Smith (1981) and Wood (1982) tried to establish proliferating shoots derived from nodal stem segment explants from seedlings. However, no plants were established in soil due to the lack of roots present on the *in vitro* elongated shoots. More precisely, Knox and Smith (1981) used Knox and Smith basal medium (KS), supplemented with 0.1 to 0.7 mg $l^{-1}$ IBA and 0.1 to 2 mg $l^{-1}$ BAP (6-benzylaminopurine) for shoot elongation, and 1 mg $l^{-1}$ IBA and 1 mg $l^{-1}$ phloroglucinol for rooting. The shoots, after 4 days on root-inducing medium, were transferred to the same basal medium supplemented with activated charcoal and washed with 3 g/l acid. Although shoot elongation was satisfactory, roots produced were fleshy, like a tap root, and in the end no plants survived in the soil.

Wood (1982) obtained rooting and contamination problems using nodal stem segments as explants. He used woody plant medium (WPM) (Lloyd and McCown, 1980), supplemented with 4 mg $l^{-1}$ BAP to obtain shoot proliferation. The best shoot elongation was obtained by transferring the explants on WPM with 0.1 mg $l^{-1}$ BAP and 2 to 8 mg $l^{-1}$ $GA_3$. However, the subculture of the axillary shoots and the rooting was unsuccessful.

Phillips and Ramirez (1983) and Ramirez-Martinez (1983) used different types of Pecan explants, but positive results were obtained only with apical and auxiliary buds from 50-year-old plants. They also tested different media, Murashige and Skoog (MS, 1962), KS and BDS (basal nutrient medium of Dunstan and Short, 1977), different auxin sources (Picloram and IBA), different carbohydrate sources (sucrose, glucose and fructose) and activated charcoal and ascorbic acid as anti-oxidants. Among all the combinations tested, the BDS medium with 0.2 to 0.5 mg $l^{-1}$ Picloram and 2 to 10 mg $l^{-1}$ BAP was the best. From the results obtained, they stated that buds from 50-years-old trees do not lend themselves well to tissue culture. Cortes-Olivares et al. (1990b) used auxiliary buds from trees of different ages (3 to 4, 9 to 10 and 16 to 17 years). After sterilization, the explants were placed on BDS medium with 0.51 mM ascorbic acid, 4.4 µM BAP and 0.4 µM Picloram under 16-h photoperiod and sub cultured every 30 days. The explants were subsequently cultured in a rooting medium, which consisted of the same BDS supplemented with 14.8 µM IBA, for 4 weeks. Although contamination was quite high, shoot multiplication and rooting were successfully obtained (between 0.3 and 6 shoots per explant). Normal-appearing roots formed on 40% of these shoots, resulting in complete plantlets. Renukdas et al.

**Table 1.** Sterilization procedures used in pecan tissue culture.

| Explant | Sterilization procedure | Reference |
|---|---|---|
| Nodal segments from seedlings | 20% Clorox + 0.35% Tween 20 - min<br>4 Rinses with sterile double distilled water | Knox and Smith (1981) |
| Nodal segments from seedlings | Distilled water + 0.5% Tween 20<br>1% Sodium hypochlorite - 20 min<br>4 Rinses with sterile distilled water<br>200 mg $l^{-1}$ Streptomycin (in medium before autoclaving)<br>40 mg $l^{-1}$ Filter sterilized Pimarcin (in medium before autoclaving) | Wood (1982) |
| Nodal segments from seedlings | 0.525% NaOCl - 10 min | Hansen and Lazarte (1984) |
| Buds from mature trees | 95% Ethanol - 5 min (vacuum)<br>50% Commercial bleach - 15 min (vacuum)<br>1% Benlate - 15 min (vacuum)<br>Sterile water - 15 min (vacuum) | Phillips and Ramirez (1983) |
| Apical and axillary buds from mature trees | Four-step disinfestation procedure including pretreatment with anti-oxidants and vacuum/benomyl treatment | Ramirez-Martinez (1983) |
| Axillary buds from grafted trees | 1% Citric acid (anti-oxidant)<br>95% Ethanol briefly<br>Sodium hypochlorite + 2 drops of detergent - 7 min<br>2 Rinses with sterile deionized water<br>1% Benomyl under vacuum - 7 min (late season material only)<br>Wash in 1% citric acid (late season material only) | Corte-Olivares et al. (1990b) |
| Buds from mature trees | Wash in running distilled water - 5 min<br>70% ethanol with continuous stirring - 2 min<br>Several rinses with sterile distilled water<br>5.25% Sodium hypochlorite (NaOCl 3% v/v) + 0.1% Tween 20 - 2 min<br>7-8 Rinses with sterile distilled water | Haroon (2011) |
| Buds, leaves and embryos | Solution of Foca detergent + Tween 80 - 2 min<br>70% Ethanol - 3 min<br>10% Sodium hypochlorite - 15 min | Ávila-Treviño et al. (2013) |
| Intact seeds | 95% Ethanol - 20 min<br>Air dried under aseptic hood | Yates and Reilly (1990) |
| Unshelled mature seeds | 95% Ethanol - 5 min<br>5.25% Sodium hypochlorite + 0.1% Tween 20 to 3 to 7 h<br>Sterile water rinses, held in final rinse 2 h<br>MS medium + 1.5% agar - 30 days, followed by transfer to medium with 0.7% agar - 4 weeks | Obeidy and Smith (1990, 1993) |
| Unshelled mature seeds | Wash with sterile water - 10 min<br>Carbendazim solution (1 g l-1) overnight<br>Wash with sterile water<br>70% Ethanol - 30 min<br>3 Rinses with sterile water<br>2.83% (w/v) Sodium hypochlorite - 30 min<br>5 Rinses with sterile water<br>Break the shell with a nutcracker in sterile condition to extract the embryos | Renukdas et al. (2010) |

**Table 1.** Contd.

| | | |
|---|---|---|
| Mature embryos | 2.83% (w/v) Sodium hypochlorite - 10 min<br>5 Rinses with sterile water - 5 min each | Renukdas et al. (2010) |
| Immature intact seeds | 70% Ethanol dip<br>10% Roccal dip<br>100% Clorox (5.25% sodium hypochlorite) - 5 min<br>Sterile water rinse<br>0.01 N HCl rinse<br>3 Sterile water rinses | Merkle et al. (1987)<br>Wetzstein et al. (1988, 1990) |
| Immature intact seeds | 70% Ethanol dip - 30 s<br>10% Roccal dip - 1 min<br>Repeat first 2 steps<br>100% Clorox - 7 min<br>3 Sterile water rinse - 3 min each<br>0.01 N HCl rinse | Wetzstein et al. (1989)<br>Rodriguez and Wetzstein (1994) |
| Immature seeds | Wash under tap water and bleach solution - 30 min<br>70% Ethanol - 1 min<br>15% Sodium hypochlorite - 20 min + 0.01% Tween 20<br>3-5 Rinses with distilled water | Payghamzadeh and Karemitabar (2010) |
| Immature embryonic axes | Intact fruits immersed in 70% ethanol - 20 min<br>3 Rinses in sterile water | Yates and Wood (1989) |
| Immature zygotic embryo axis | 95% Ethanol briefly<br>2.6% Sodium hypochlorite (50% bleach) - 7 min<br>2 Sterile deionized water rinses | Corte-Olivares et al. (1990a) |
| Shelled mature seeds | 70% Ethanol with continuous stirring - 2 min<br>5.25% Sodium hypochlorite (NaOCl 3% v/v) + 0.1% Tween 20 - 2 min<br>7-8 Rinses with sterile distilled water | Haroon (2011) |

(2010) used nodal explants from cultivars Desirable and Cape Fear. The explants were cultured on modified liquid WPM, supplemented with 2% glucose and different concentrations of BAP (0.44 to 44.39 µM). After 3 weeks of culture, at least 9 multiple shoots per explant were induced on modified WPM containing 13.32 µM BAP. Subsequently, the multiple shoots were separated and successfully rooted in liquid WPM containing 49.20 µM IBA. The efficiency of rooting for both cultivars was over 90%.

Pecan seeds can be used as explants in tissue culture due to the ease of sterilization and a lower risk of contamination as compared to other explants such as buds, leaves and nodal segments (Figures 3 and 4). There are few reports in literature that describe the sterilization process of Pecan seeds and their subsequent cultivation into a solidified medium. Yates and Wood (1989) used immature embryos, which were excised during kernel development from seeds of Pecan Cultivars Desirable and Stuart. After sterilization, immature seeds were placed on a basal medium with four combinations of cytokinin and auxin. After 4 weeks of darkness at 25°C, the explants were transferred to a basal medium without plant growth regulators. Normal plants were obtained, with no problems of contamination. Haroon (2011) noticed that the best medium for *in vitro* germination of Pecan seeds was a MS formulation supplemented with 4 µM BAP. Formation of multiple shoots was also observed from intact nodal regions of developing seedlings. After reaching a sufficient length (3 to 4 cm), the explants were transferred to the two different rooting media, DKW (Driver and Kuniyuki, 1984) or MS supplemented with different combinations of growth regulators (IAA, NAA and IBA). The medium which provided the best root induction was MS supplemented with a combination of 4 µM IBA and 4 µM

**Figure 3.** *In vitro* germination of pecan seed ('Ukulinga').

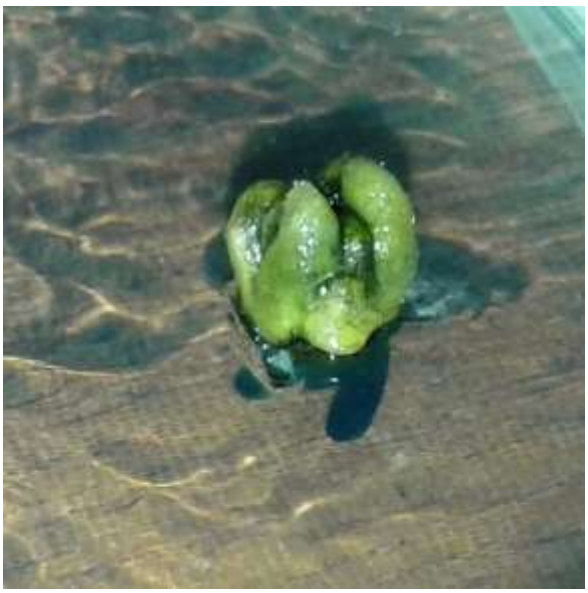

**Figure 4.** *In vitro* culture of pecan bud ('Ukulinga').

NAA. Browning of the growth medium is the result of polyphenol oxidation exuded from the cut end of the explants. Generally, it occurs at the initial stage of culture but can be overcome by adding substances such as ascorbic acid, citric acid, polyvinyl pyrrolidone (PVP) and activated charcoal to the growth medium. This phenomenon is widely reported in literature, not only for Pecan, but also for other nut crops such as Chestnut (Osterc et al., 2005), Walnut (Payghamzadeh and Kazemitabar, 2011) and Pistachio (Barghchi and Alderson, 1985). For *in vitro* germination of Pecan for instance, testa is generally removed, since it produces phenols that can inhibit the growth of callus (Haroon, 2011). Therefore, it is advisable to add ascorbic acid or other compounds to the medium, and if browning occurs, the explants must be transferred to new fresh medium. This is also valid for *in vitro* buds' culture. *In vitro* shoot-tip necrosis (STN), also known as apical necrosis or non-pathogenic dieback, is a common physiological disorder in micropropagation of many plants. The symptoms result from the senescence and death of tissues in the apical bud, which subsequently proceeds basipetally.

Several methods were tested for alleviating shoot necrosis in literature such as, shortening of the culture period, altering the media salt strength, use of various plant growth regulators, different levels of sucrose, fructose, silver nitrate and increasing the concentration of calcium chloride (Kishore et al., 2015; Nezami et al., 2015; Thakur and Kanwar, 2011; Chiruvella et al., 2011; Bairu et al., 2009; Abousalim and Mantell, 1994). Chiruvella et al. (2011) reported a positive synergism between activated charcoal and higher levels of calcium that facilitated the recovery of more than 98% of the shoots affected by STN in *Soymida febrifuga*. The important role of adenine sulfate (AdS) as adjuvant to alleviate *in vitro* STN was recorded by Naaz et al. (2014) in *Syzigium cumini*. On Pecan, Ávila-Treviño et al. (2013) used activated charcoal (1%), polyvinyl pyrrolidone (0.1%), silver nitrate (1%), citric acid (150 mg $l^{-1}$) and ascorbic acid (100 mg $l^{-1}$) to successfully control necrosis in both light and dark conditions. Tissue necrosis of Pecan explants was reduced by 75 and 83% by adding activated charcoal and silver nitrate, respectively.

### *Stage III: Rooting of microshoots*

At this stage, proliferated microshoots obtained in Stage II were transferred to a rooting medium, which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. This stage is very important to obtain plantlets with well-developed rooting systems to facilitate their establishment in the soil. Several research projects were done to enhance the rooting rate of Pecans. The rooting of micro shoots was accomplished both under *in vitro* and *ex vitro* conditions. Hansen and Lazarte (1984) obtained rooting in both *in vitro* and *ex vitro*. They used nodal segments from seedlings as explants and cultured it on WPM medium supplemented with 20 g $l^{-1}$ glucose and 0.3, 1 or 3 mg $l^{-1}$ BAP for 1 day, and then transferred it to WPM basal medium. Afterwards, the cultures were subjected to a darkness condition for 2 weeks and then kept under a 16-

h photoperiod. *In vitro* rooting was obtained using WPM with glucose and 1, 3 or 10 mg $l^{-1}$ IBA for 6 to 10 days. *Ex vitro* rooting was obtained using WPM with 20 g $l^{-1}$ glucose and 10 mg $l^{-1}$ IBA for 10 days, followed by a transfer to peat pellets and watered with half-strength WPM minerals. Up to 63% of the rooting was obtained and the plantlets were successfully established in soil. Hassanen and Gabr (2013) developed an efficient method for *in vitro* rooting of Pecans. Nodal segments were cultured on WPM medium containing 3 mg $l^{-1}$ BAP for multiplication. After 4 weeks of culture, the shoots were cultured on ¼ and ½ strength WPM supplemented with IBA (0, 1, 2 and 3 mg $l^{-1}$) and 1.5 mg $l^{-1}$ $AgNO_3$. In addition, 20 g $l^{-1}$ sucrose, 1 g $l^{-1}$ activated charcoal and 3 g $l^{-1}$ phytagel were added to the medium. The best rooting percentage (75%) was obtained on a ½ strength WPM medium supplemented with 3 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ $AgNO_3$.

### *Stage IV: Acclimatization and field establishment*

The positive outcome of the *in vitro* buds' culture, or nodal segments, depends on the ability to transfer the plantlets from a controlled tissue culture laboratory to a glasshouse, for it to acclimatize. This means that to acclimatize or prepare *in vitro* plantlets, it needs to be exposed to a significantly lower relative humidity and a higher light intensity. During the acclimatization, plants change from a heterotrophic to an autotrophic state (Preece and Sutter, 1991). Unfortunately, this step is not immediate. To overcome these limitations, plantlets are transferred into a well drained substrate and kept at high levels of humidity, reduced light intensity and a temperature between 20 and 27°C. Plants are normally acclimatized by gradually reducing the relative humidity over a period, between 1 and 4 weeks.

## Applied *in vitro* technologies

### Organogenesis

Organogenesis is a process that belongs exclusively to the plant kingdom and consists of the production of unipolar structures (shoots or roots), starting from non-meristematic cell aggregates, or plant tissues, through the formation of meristematic adventitious centers called meristemoids. Obeidy and Smith (1993) induced organogenesis from cotyledon segments to form adventitious roots in a medium with 50 µM NAA. A regeneration medium with 20 and 5 µM IBA stimulated prolific auxiliary shoot production from the embryonic axis without causing cotyledon abscission. Thirty percent of the microshoots rooted on auxin-free medium after a pretreatment in dark conditions on a medium with 20 µM IBA. Rooted plantlets were successfully transferred to soil. Payghamzadeh and Karemitabar (2010) conducted organogenesis studies using immature embryos of Pecan as explants. After disinfection, immature fruits were cultured on a modified DKW basal medium. For immature embryo culture, a high frequency of plantlets was obtained on modified basal medium, supplemented with 1 mg $l^{-1}$ BAP, 0.05 mg $l^{-1}$ IBA and 2 mg $l^{-1}$ $GA_3$ and dark culture conditions. Callus can be defined as an amorphous mass of undifferentiated tissue with thin walled parenchyma cells developing from proliferating cells of parent tissue (Dodds and Robert, 1985). Callus formation in tissue culture can be initiated using a small portion of plant tissue due to the use of phytohormones (auxins and cytokinin); it is induced to produce calli (Skoog and Armstrong, 1970; Letham, 1974; Akiyoshi et al., 1983). In micropropagation, callus culture is a very important technique for developing clonal populations, plant regeneration and genetic manipulation in both monocotyledon and dicotyledonous plants (Reinert and Bajaj, 1976). Rodriguez and Wetzstein (1994) used immature seeds of Pecan to induce embryogenesis, as well as the entity of callus produced in relation to the type and concentration of auxin. Callus formation was greater in cultures induced on 2,4-D compared to NAA. The higher levels of both auxins formed greater amounts of callus than lower levels. Payghamzadhe and Kazemitabar (2010) also used immature seeds of Pecan as explants for organogenesis studies. They found that the presence of different concentrations of BAP and IBA induced callus formation frequently and it was also inhibited by adding $GA_3$ in the culture medium.

### Somatic embryogenesis

Somatic embryogenesis has been considered as one of the most important invasions in the tissue culture sector. It has several applications such as mass clonal propagation, genetic transformation and use in studies of embryo development. Due to somatic embryogenesis, it is possible to propagate clonal Pecan rootstocks introducing genes of commercial interest such as dwarfing for size control, enhanced nutrient uptake, alternate bearing control, salinity tolerance, nematode resistance and growth uniformity (Wetzstein et al., 1996). The first studies of somatic embryogenesis date back to 1987, when Merkle et al. (1987) obtained somatic embryos using immature nuts as explants. These immature zygotic embryos were cultured on modified WPM medium with 2 mg $l^{-1}$ 2,4-D and 0.25 mg $l^{-1}$ BAP. A low embryogenic frequency was obtained (2%) and they stated that the developmental stage of explants can be a limiting factor on the induction of somatic embryos. Other studies have been followed over time with the aim to increase embryogenic frequency. Wetzstein et al. (1988) obtained up to 40% of embryogenic frequency using different explants. Other subsequent studies were

focused on other factors that can be responsible for a different frequency and embryogenic response such as cultivar, explant sampling date, source of explants, duration on conditioning medium (Wetzstein et al., 1989) and auxin type and concentration (Wetzstein et al., 1990). An embryogenic frequency of 85% was obtained by Wetzstein et al. (1989) using immature Pecan seeds cv. Stuart, collected 15 weeks after pollination, which is considered the optimum stage for embryogenic induction. Wetzstein et al. (1990) performed a cold treatment followed by a desiccation treatment to improve the rooting of somatic embryos. Yates and Reilly (1990) used immature Pecan seeds collected from eight cultivars at different stages of development. They found that the embryogenic response was different among cultivars and is genotype-dependent. In the same year, Corte-Olivares et al. (1990a) obtained somatic embryos, although with a low frequency (3.5%), using zygotic embryo axes from cvs. Western Schley and Wichita. Mathews and Wetzstein (1993) stated that adding 29.4 µM silver nitrate to the germination medium (WPM) and the application of 100 µM BAP to the embryo shoot apex, promotes a higher frequency of plant conversion. Rodriguez and Wetzstein (1994) stated that the use of NAA rather than 2,4-D as an auxin source, enhanced the embryos development by providing embryos with a stronger defined shoot apex. Burns and Wetzstein (1995) used aggregates of globular and pre-globular stage somatic embryos derived from a liquid culture of Pecan. A mild dehydration of the embryo aggregates up to 49% moisture and was applied for 3 to 4 weeks, promoting a complete development of somatic embryos and preventing repetitive embryogenesis. Kumar and Sharma (2005) obtained somatic embryos using cotyledon explants of Walnut and Pecan, cryo-preserved the embryos using non-toxic cryoprotectants (dimethyl sulfoxide, DMSO), glycerol and ethylene glycol) and assessed their survival percentage. DMSO had the highest survival rate (5%) followed by glycerol (1.5%) and ethylene glycol (3%) pre-treatment. They also noticed that high levels of sucrose decreased survival rate, and in Pecan, visible browning occurred.

## Synthetic seed technology

For Pecans, no information is available in literature about the use of synthetic seed technology as an important propagation tool. Therefore, it would be interesting to test the response of unimodal Pecan micro cuttings to encapsulation, not only as an alternative propagation method, but also for the storage and exchange of plant material with national or international micro propagation laboratories.

To produce synthetic seeds, the procedure adopted includes three steps: coating, complexation and washing. The coating is a single process performed by inserting the propagule, excised from *in vitro* cultures, into a gel or encapsulating solution for a few seconds. Generally, sodium alginate is the most widely used substance for this operation, as it has a moderate viscosity, low toxicity for explants, low cost and biocompatible characteristics. It is also widely used since it better protects encapsulated explants against mechanical risks, depending on their concentration, viscosity or commercial type, as well as complexation conditions (Casales, 2013). For the complexation, which gives hardness to the capsules, coated alginate explants are dipped in a calcium chloride solution for 30 - 40 min. An ion exchange process occurs during this phase, following the substitution of sodium ($Na^{+}$) ion with calcium ($Ca^{++}$) with a calcium alginate formation (Ara et al., 2000; Redenbaugh and Walker, 1990). Thus, the coating acquires the consistency necessary to ensure protection against mechanical damage and the risk of dehydration. Hardening of the calcium alginate capsules is influenced by the sodium alginate and calcium chloride concentration and may also vary by the time of complexation. Finally, the third step consists of several subsequent rinses in sterile endosperm to remove toxic residues of chloride and sodium. After washing, such encapsulated propagules can be stored or transferred to the sowing medium.

Several research groups have begun to use non-embryogenic explants (unipolar) obtained through *in vitro* direct organogenesis, or through the proliferation of auxiliary buds. Regarding the use of unipolar explants for encapsulation, uninodals are generally used, these are portions of 3 - 4 mm with terminal buds or lateral buds and cut off during or at the end of a subculture; generally, they are called micro cuttings. In these explants, the absence of root primordium is often associated with the inability to form adventitious roots spontaneously, representing a major obstacle to obtain synthetic seed conversions (Casales, 2013). The poor conversions observed in some species to produce synthetic seeds by using unipolar explants, such as citrus or mulberry, is attributable to a variety of factors (genotype, inadequate nutritional formulations linked to artificial endosperm or seed, ineffective procedures to induce rooting in micro cuttings), which strongly restricts its use (Casales et al., 2011, 2015). When the most appropriate encapsulation protocol for each genotype is found, even using biotization, with the insertion of Arbuscular Mycorrhizal Fungi (AMF) or Plant Growth Promoting Bacteria (PGPB) into synthetic seed, researchers will probably be able to take advantage of this biotechnological tool in the nursery sector.

## Genetic transformation

Genetic transformation techniques allow the breeder to insert a gene into the genome of the plant of interest with valuable agronomic characters, obtaining new genotypes

in a single generation. Genetic engineering is particularly useful in the genetic improvement of woody plants, since it accelerates the timing of releasing cultivars, overcoming the high level of heterozygosity and shortens the length of the juvenile period that characterizes them. The main goals of genetic engineering for woody plants are the introduction of the resistance to biotic and abiotic stress and the vegetative and productive control of the trees, especially in relation to aspects relating to the quality of the fruit. Unfortunately for Pecan, there is little information relative to its genome (Thompson and Romberg, 1985; Marquard, 1991). Therefore, it leads to first identifying the traits of the genome containing the desired genes and then starting a genetic transformation program. Only in this way, will combining micro-propagation and genetic transformation be possible to obtain clonal rootstocks of Pecan with superior characteristics. Burns et al. (1991) used somatic embryo technology for gene transformation in Pecan, albeit with limited success. It studied the levels of kanamycin for a selection of cultures bombarded with foreign DNA, containing the genes for β-glucuronidase activity and kanamycin resistance. Although a stable GUS (beta-glucuronidase gene) expression was obtained, the variation in gene expression and embryo chimerism were limiting factors. A gene transfer mediated by *Agrobacterium* was performed by McGranahan et al. (1993). Somatic embryos derived from open-pollinated seed of 'Elliott', 'Wichita' and 'Schley' were co-cultivated with the *Agrobacterium* strain EHA 101/pCGN 7001, which contains marker genes for β-glucuronidase activity and a resistance to kanamycin. Although transgenic clones were obtained, plant regeneration was limited.

## SUMMARY AND CONCLUSIONS

Over the years, Pecan propagation techniques have profoundly changed due to the help of various methodologies that have improved both qualitative and economic efficiency. Techniques such as mist propagation and basal heating of hardwood and/or softwood Pecan cuttings, associated with treatments with auxins such as IBA have certainly enhanced the recalcitrant behavior to emit roots of this species. The attempts to micro propagate Pecans through organogenesis have been limited due to a low regeneration frequency, poor rooting and high rates of contaminations. All these factors have strongly precluded crop improvement and clonal propagation. On the other hand, somatic embryogenesis, using immature nuts as explants, is a very efficient method in terms of obtaining both high multiplication rates and plant regeneration. Trees grown in open fields obtained by somatic embryogenesis show a genetic stability and have kept the original characters of a cultivar. Genetic engineering techniques (gene transfer mediated by *Agrobacterium*, direct insertion of DNA into protoplasts and biolistic methods), when properly applied together with molecular biology techniques and efficient tissue culture protocols, will contribute enormously to improve the efficiency of the Pecan industry with the elimination of barriers imposed by conventional genetic improvement programs.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ABBREVIATIONS

**2,4-D,** 2,4-Dichlorophenoxyacetic acid; **BAP,** 6-benzyl aminopurine; **IBA,** indole-3-butyric-acid; **NAA,** α-naphthalene acetic acid; **TDZ,** Thidiazuron; **IAA**, indole-3 acetic acid; **$GA_3$**, Gibberellic acid; **ABA**, abscisic acid; **DKW**, Driver and Kuniyuki Walnut medium; **MS**, Murashige and Skoog; **KS**, Knox and Smith basal medium; **BDS**, basal nutrient medium of Dunstan and Short; **WPM**, woody plant medium; **PVP**, polyvinyl pyrrolidone; **DMSO**, dimethyl sulfoxide; **PGR**, plant growth regulators.

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