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EFFECTS OF CULTURE MEDIA AND PLANT GROWTH REGULATORS ON MICROPROPAGATION OF WILLOW (Salix matsudana ‘Golden Spiral’) AND HAZELNUT (Corylus colurna ‘Te Terra Red’)

Dongxue Shi
University of Nebraska – Lincoln

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EFFECTS OF CULTURE MEDIA AND PLANT GROWTH REGULATORS ON MICROPROPAGATION OF WILLOW \( (Salix matsudana \ 'Golden Spiral') \) AND HAZELNUT \( (Corylus colurna \ 'Te Terra Red') \)

By

Dongxue Shi

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EFFECTS OF CULTURE MEDIA AND PLANT GROWTH REGULATORS ON MICROPROPAGATION OF WILLOW (Salix matsudana) AND HAZELNUT (Corylus colurna)

Dongxue Shi, M. S.

University of Nebraska, 2014

Advisor: Paul E. Read

The branches and leaves of Salix matsudana ‘Golden Spiral’, willow, grow in a twisted manner, which makes it an important bonsai plant. Its mature stems have potential for the woody cut floral industry. Corylus colurna ‘Te Terra Red’, hazelnut, distinguished by red or purple leaves in the spring, has potential ornamental value in the horticulture industry for landscape use. Micropropagation of these two plants could provide more and healthier plantlets for rapid commercial scale-up by the nursery industry. Nas and Read Medium (NRM) is a newer medium developed specifically for hazelnut species by Nas and Read based on the composition of the seed. In this study, experiments were conducted to test whether NRM (with added plant growth regulators) is superior to other media such as Murashige and Skoog Medium (MS), Woody Plant Medium (WPM) and Driver-Kuniyuki Walnut medium (DKW) for shoot and root production of Salix and Corylus. The results showed that Salix explants grown in NRM with BA at 2.0mg/L and IBA at 0.05 mg/L produced a greater number of new shoots and longer stems. Explants grown in NRM with IBA at 0.05 mg/L produced more roots, which is necessary before transplanting to the soil.
In addition, these plantlets exhibited deeper green color and acclimatized successfully into potential transplants. The hazelnut explants had a very high contamination rate and grew slowly on the various media. By improving disinfection methods and by adding antibiotics in forcing solution and media, the contamination rate and browning rate can be decreased greatly.
THIS THESIS IS DEDICATED TO MY PARENTS, GUANGZHI SHI AND LIJING CHENG

I LOVE YOU
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**Introduction**

*Salix matsudana* is a member of the *Salicaceae* or the willow family, and the genus *Salix* consists of around 300 species all over the world (Zomleter, 1994). Under the common name of ‘Peking willow’, *Salix matsudana* is native to northeastern China, and widely cultivated in several provinces in China (Wu et al., 1994). As an ornamental tree, *Salix matsudana* has been introduced into North America, Europe and Australia. In the U. S, there are several states that commercially grow *S. matsudana*, including Colorado, Illinois, New York, Utah, Ohio, Virginia (Emerine, 2012).

*Salix matsudana* is a medium-sized dioecious tree that can reach 20 to 40 feet in height with similar width. The leaves are narrow, serrate margined, bright green in summer and yellow-green in autumn. The stems are yellowish in youth, and turn olive-green or brown when they become mature. It has showy catkins on male trees but concealed flowers on female trees (Seiler et al, 2010).

*Salix matsudana* is fast growing but short lived. It is tolerant to all types of soil, especially to salty ones, but prefers moist soil and grows well along water sources. This tree is tolerant to many soil pH levels and very cold hardy, and full sun is required to grow *Salix matsudana*. Compared to other species in the willow family (such as *Salix babylonica*), it is more drought resistant (Gilman and Watson, 1994).

There are numerous pest and disease concerns about *Salix matsudana*. Some cultivars are very susceptible to cankers; as a result several cultivars have been
selected for canker resistance. Because of the contorted or twisted branching effect of some cultivars (e.g. ‘Tortuosa’), *Salix matsudana* is usually used as a bonsai plant or its stems are harvested for floral arrangements. For other landscape uses, it is also a good choice as a shade tree, lawn tree, park tree or for any wet sites (Wu et al., 1994).

*Corylus colurna* is commonly called Turkish hazelnut, and is a member of the genus *Corylus*, tribe *Coryleae*, family *Betulaceae*. (Cronquist, 1981). It is a deciduous tree native to a broad area of southwest Asia and southeast Europe from China to the Balkans (Kuhns, 1983).

The main use of hazelnut trees is the edible nut, which make it the second most important nut tree in the world following the almond (Mehlenbacher, 1994). Turkey ranks first in hazelnut production followed by Italy and the U.S. Oregon is the main state in the U.S. to grow commercial hazelnut (*Corylus avellana*). In 2012, Oregon’s production was 34,000 tons, valued at 63.4 million dollars (Clark and Coba, 2012).

In addition to the commercial hazel, some cultivars are also grown as ornamental plants by horticulturists for their contorted stems and pendulous growth habit, red or yellow leaf color, and cut leaf forms. *C. colurna* leaves are rounded and hairy with double-serrate margins. This monoecious tree has visible male catkins but extremely small female flowers concealed in buds. The small and hard-shelled nuts make *C. colurna* of no value for commercial edible hazelnut, but important in commercial hazelnut orchards because it does not sucker readily. This characteristic makes it ideal as rootstock for grafting other desirable cultivars of commercial hazelnuts such as *C. avellana* (Mehlenbacher, 1994).
The cultivation of *C. colurna* is easy because of its tolerance to heat, cold and drought after establishment. It is also occasionally reported to be tolerant to alkaline soil (Cerovic et al., 2007). Compared to other species (e.g. *C. avellana*) that are susceptible to eastern filbert blight, *C. colurna* is resistant to serious pests and other disease problems such as bacterial blight of hazelnut (Gilman and Watson, 1993). All these characteristics make *C. colurna* tolerant of urban conditions. It also maintains a symmetrical crown that is attractive to landscape architects (Gilman and Watson, 1993). Thus, *C. colurna* is planted in sidewalk cutouts, parking lot islands and highway rights-of-way.

Cuttings and seeds are the common methods of propagation, however, micropropagation of *Salix matsudana* or *Corylus colurna* could provide more plantlets for rapid commercial scale-up in the nursery industry. It is well-known that micropropagation is not limited by season, being available all year round. It maintains the clonal characteristics, reduces the possibility of disease, and enables easy distribution. Besides, the plantlets from micropropagation are also said to be more uniform and practical than cuttings based on tests reported by Tormala and Saarikko (1985).

**Literature Review**

When using the process of micropropagation, there are several aspects to be considered before conducting the research. Choosing of explants, the initiation of cultures, culture media (including the plant growth regulators added to the media)
and the problems during culture, such as contamination, browning, and proliferation difficulty, may have great effect on the results.

1) Explant materials

Cloning in vitro of adult or mature woody plants is affected by characteristics accompanying maturation such as reduced growth rate and the lack of rooting ability. Maturation is an obstacle for a wider application of tissue culture technology among woody species. Though many problems exist, researchers have successfully multiplied mature trees by using materials such as shoots from the base of the tree, or by special pre-treatment in vivo and/or in vitro culture. These methods to improve propagation are prerequisite for possible cloning of adult trees and the success in practice will mainly depend on the ability to rejuvenate them (Pierik, 1990).

The explants being used for tissue culture of Salix are usually the nodal segments and the shoot tips. However, in some research, other parts of mother plants were used as explants. Research on ovary culture of hybrid Salix was conducted by Agrawal and Gebhardt (1994) for rapid micropropagation. They studied Salix fragilis X Salix hispoclados with Schenk and Hildebrandt (SH) semi-solid medium (1972) with 0.2 mg 6-benzyladenine (BA) per liter for culturing the hybrid seedlings.

Nodal cuttings of these seedlings were used for shoot multiplication in semi-solid WPM (McCown and Lloyd, 1981) with 0.2 mg BA per liter and the shoot proliferation was enhanced to 5-fold after transfer. SH medium without plant growth regulators (PGR) was the best for rooting in vitro. Pereira et al. (2000)
reported that both proliferation and root induction was influenced by the source of explant (*Salix humboldtiana*) used. MS medium supplemented with 2.3 μM kinetin enhanced shoot production while half-strength MS medium supplemented with 5.3 μM NAA enhanced rooting.

The tissue culture of hazelnut might be accomplished by using the nodal segments and shoot tips, however, as a recalcitrant species, using axillary buds and embryo culture was commonly used in micropropagation of hazelnut. In the study of Centeno et al. (1997), they used embryo tissue culture to evaluate the endogenous indole-3-acetic acid (IAA), abscisic acid (ABA) and cytokinins in *Corlus avellana* L. cotyledons of different developmental stage and genetic source for their somatic embryogenic capacity. They found that the N偏-iso-pentenyl adenine (iP) type and zeatin (Z) type cytokinin ratios were two good indexes of the embryogenic competence of explants, suggesting the endogenous hormone balance is very important in defining in vitro potential of hazelnut cotyledons.

According to a research conducted by Garton et al. (1983), organogenesis in macro and micropropagules was affected by the clonal origin or genotype of the plant material. They used rooted cuttings of ten willow clones that were treated with 3 nutrient solutions where nitrogen, potassium and phosphorus were in different concentrations. Ten weeks after treatments the willow cuttings were placed in peat pellets under intermittent mist environment, and the lateral buds of actively grown shoots were cultured in vitro on medium to promote shoot proliferation. They found that effects of stock plant nutrition treatments were less important than the clonal effects in micropropagation. Willow cuttings receiving the
highest concentration of N, P, and K had less root initiation rate than those cuttings that received lowest concentration.

The time of taking explants from the field may have an effect on the micropropagation process. The carbohydrates, proteins, and growth substances in the stock plants change as the temperature, day length, light intensity and water availability changes, and different level of these substances can influence the explant responding to the in vitro environment (Torres and Carlisi, 1986). Yu and Reed (1993) used three cultivars of *C. avellana* grafted in the greenhouse, and they collected the explants from March to July. It was found that explants collected in March produce a greater number of successful initiations.

2) Initiation

Successful initiation in vitro is affected by the plant source. Older plants have relatively low initiation rate and high contamination rate problems (Rodriguez et al., 1989; Anderson, 1984), while juvenile plants or suckers have higher initiation rate in vitro. The tissue culture of seedlings can be very successful (Anderson, 1984), and shoots that are growing actively can acclimatize to in vitro condition easily.

To disinfect the plant material, the method of washing with 10% bleach followed by rinsing in distilled water is often used (Neuner and Beiderbeck, 1993). In a study by Tormala and Saarikko (1985), their disinfection method was dipping the explants in 70% ethanol for 30 seconds before soaking in 3% sodium hypochlorite for 15-20 min and rinsing four times in sterile water.

3) Culture media
After Murashige and Skoog (1962) invented MS medium for plant tissue culture, numerous media formulations have been developed by scientists. For certain species and genotypes, the ratio of nutrients (including macronutrients and micronutrients), the concentration of plant growth regulators and the type of vitamins may vary in order to have successful tissue culture. MS medium, WPM (McCown and Lloyd, 1981) and Driver & Kuniyuki Walnut (DKW) Medium (Driver and Kuniyuki, 1984) are common formulations that have been used in hazelnut tissue culture.

Plant growth regulators (PGRs) are another important factor to consider when preparing the culture medium. PGR have been studied to determine their combinations and concentrations to be used in media. N-6 benzyladenine (BA) and zeatin are common cytokinins being studied for shoot proliferation, and indole butyric acid (IBA) is usually used for root initiation and formation.

In the micropropagation of Salix species, the media usually used are MS medium and WPM medium. Bhojwani (1980) studied a method for rapidly producing in vitro multiplication of a hybrid willow. In his research, MS medium was used, and various plant growth regulators such as benzylaminopurine (BAP), naphthaleneacetic acid (NAA) and 2-isopentenyl adenine (2-iP) were tested. It was found that BAP at 0.1 mg/L + NAA at 0.2 mg/L was best for shoot multiplication, resulting in 4-fold multiplication within 4 weeks. After being rooted on MS medium containing NAA for 10 days, the hybrid willows were transplanted to pots with over 90% success. Similarly, Salix babylonica showed maximum proliferation on a modified MS medium containing 1 mg BAP per liter. (Dhir et al., 1984) Incorporation of NAA or
gibberellic acid (GA) did not enhance shoot proliferation in the presence of benzylaminopurine. Rooted plantlets were transferred successfully to pots and maintained under high humidity conditions.

Paiva neto et al. (1998) conducted a study of in vitro induction of adventitious roots of *Salix humboldtiana*. They used WPM supplemented with NAA and IBA to culture the *Salix* leaf and nodal segments. Root hairs were only observed when NAA was present. The bud growth was initiated in WPM containing 2.68 μM NAA + 2.46 μM IBA, while adventitious roots were found in other combinations of plant growth regulators tested (4.92 μM IBA; 2.68 μM NAA + 4.92 μM IBA; 5.37 μM NAA + 4.92 μM IBA; 5.37 μM NAA).

*Salix tetrasperma* Roxb, which is commonly known as Indian willow, was studied for in vitro regeneration as well (Khan et al., 2011). Similarly, shoot induction on WPM containing 5 μM 6-benzyladenine was the best for shoot number and shoot length, whereas multiplication and elongation was better after transferring to WPM containing 1 μM BA + 0.5 μM NAA. Half-strength WPM with 0.5 μM IBA had the best results of rooting in vitro.

In the micropropagation of hazelnut, DKW medium, which was originally developed for walnut, was more often used because of the common characters of nut trees. Based on a two-stage system of micropropagation, Damiano et al. (2005) developed an establishment stage basal medium, and a multiplication medium. They found a medium that contains macro and minor nutrients from the formulation of Perez-Tornerro et al. (2000) in combination with the DKW organics was the best for establishment. The MOLT medium developed by Damiano et al., which combines
DKW and WPM, was successful in producing more and better shoots in the multiplication stage. Lower salt concentrations (15-25% less) in MOLT medium may have contributed to the results.

In Nas and Read (2004) research, a new culture medium called NRM was developed based on the composition of *C. avellana* kernels. The modifications they made were: a) The average of MS, DKW, and WPM nitrogen was applied because the N level in kernels was too high to use in micropropagation, and copper levels were set the same as MS since copper in kernels was high as well; b) 100 mg Sequestrene 138 Fe per liter was used as the iron source; c) the four compounds (Cu SO$_4$, MgSO$_4$, MnSO$_4$, ZnSO$_4$) provide all the sulfur needed in the medium; d) MgSO$_4$ and KH$_2$PO$_4$ and minor element concentrations are higher than MS, DKW or WPM; e) in MS and WPM, the myo-inositol concentration is at 100 mg/L, and 1000 mg/L in DKW. NRM modified their formula to have 200 mg myo-inositol /L. Hybrid of *C. americana* and *C. avellana* explants were used in the research to test the NRM medium together with MS, WPM, DKW, and NN (Nitsch and Nitsch, 1969). They concluded that increasing Cu and myo-inositol were related to the increased shoot length up to three fold and increased shoot number up to 93%. The explants cultured on medium containing 2.25 mg 5•CuSO$_4$ /L+ 400 mg myo-inositol /L could grow 35-50 mm shoots and about 5-7 axillary buds. Based on their research results, it was also recommended that when developing a medium for plants that are difficult to establish in vitro, 5-20% w/v mineral and organic contents of seeds can be considered for use with moderate modification (Details shown in Appendix).

4) Problems
Vitrification was first described by Phillip and Matthews (1964), and Hackett and Anderson (1967) in the 1960’s. The term “vitrification” was used to describe two types of processes related to tissue culture. If the organ and tissue have abnormal morphological appearance and physiological function, “vitrification” was used. Another circumstance to use this term is to describe the transition from liquid to solid state. Since it caused lots of confusion, Debergh et al. (1992) proposed to replace it with the term “hyperhydricity”. In some of the research, the hyperhydricity phenomenon was observed due to the composition of the culture media, the quality of the culture explants, and the environmental conditions where the culture containers were maintained. The so-called vitrified or vitreous plants appear turgid or hyperhydric, watery at their surface, and hypolignified. Their organs are somehow translucent, in some cases less green, and easily breakable (Gaspar, 1991). The vitrification phenomenon was observed only on the multiplication medium, and never happened during rooting (Gaspar, 1991).

Juvenility and phase change in woody plant species exert profound impacts on plant morphology and the ability of explants to be successfully propagated in vitro, and for many woody species, the ability to reproduce sexually is only reached after many years of juvenile growth. Thus, it has been very difficult for many recalcitrant woody species to have successful micropropagation in vitro (Read and Bavougian, 2013). Read and Bavougian reported that in vitro technologies were used to induce rejuvenation, such as meristem culture, chemical treatments, pruning and hedging, forcing new growth and taking advantage of epicormic buds, grafting and micrografting, and somatic embryogenesis.
The rejuvenating of the bud break could be accomplished by hormone treatments (Beck et al., 1998), serial grafting (Valdes et al., 2003) and in vitro micrografting (Perrin et al., 1994). Known as Korean weeping willow, explants from 20-year-old Salix pseudosiegoine trees were cultured on WPM or WPM supplemented with BA, zeatin and GA3 (Park et al., 2008). The results indicated that bud break on BA supplemented media had a higher bud break rate. Various levels of cytokinins (2-iP, zeatin, dihydrozeatinriboside) and ABA were tested as well. The levels of zeatin-type cytokinins were higher than those of isopentenyladenin type of cytokinins in the explants cultured on BA supplemented media. Necrosis was observed upon subculture of explants to the fresh basal WPM.

Contamination is the most common of all problems, and bacteria and fungi are the main contaminants. Microbial contamination and the toxic effects of over disinfection are problems with achieving viable explants (Hand, 2013). Surface disinfection is essential in controlling the contamination rate, unfortunately in many cases the internal microbe cannot be killed after the disinfection process, especially if adult trees were used. The contamination being observed within 1 week after culture are results of external bacteria and fungi, while contamination caused by internal ones could be observed from the second week (Messeguer and Mele, 1983).

Field-grown unpruned mature hybrid C. americana X C. avellana hazelnut genotypes were used as explants source by Nas (2004). Dormant twigs (35-45cm long) were forced by immersing the basal end of the twigs in forcing solution (Yang et al., 1986) containing 8-hydroxyquinoline citrate at 200 mg/L + 2% sucrose +
gibberellic acid (GA3) at 10 mg/L. Explant contamination rates varied from 30-90% depending on genotype.

There are many protocols that can be used for surface disinfestation of the hazelnut explants. The first step usually begins with tap water washing for half an hour after the explants were cut (Yu and Reed, 1995). The explants can then be swirled in vessels that contain 10% chlorine bleach including 5 drops of Tween-20 for 10-30 min, followed by rinsing in sterile distilled water for 2-3 times (Nas and Read, 2004). Ethanol can also be applied in the disinfestation process by dipping the material in it before using the bleach (Bacchetta et al., 2008; Nas, 2004).

Contamination could also be reduced by using plant material from the greenhouse. In the study of vegetative propagation of difficult-to-root *Salix caprea*, Liesbach and Naujoks (2004) found that the majority of shoot tips and nodal segments from five clones died within one month because of bacterial contamination, and only nursery-grown explants were able to grow in vitro. Three clones failed to initiate in vitro, while the other two were able to be cultured via repeated subculture on various media.

Epiphytic and endophytic organisms can cause serious losses to micropropagation in vitro in every stage of growth (Cassells, 1991; Leifert et al., 1995). There are difficulties in detecting bacterial contamination if they remain inside the plant tissue, which could not be eliminated by surface disinfestation (Debergh & Vanderschaghe, 1988). Thus, the antibiotic therapy should be considered if less contamination is important. There are several groups of antibiotics and therefore the type of antibiotics to be used in media should be
determined by the bacteria present in culture (Buckley et al., 1995). In general, carbenicillin, cephalothin, gentamicin, polymyxin, figampicin, streptomycin, and kanamycin are some possible choices in plant tissue culture and the concentration ranges from 10 mg to 100 mg per liter depending on the sensitivity of the explants (Buckley et al., 1995; Falkiner, 1988; Kneifel & Leonhardt, 1992).

As a response of plants to excision, explant browning is another common problem in culture (Bonga & Aderkas, 1992). When tissues are suffering from stresses such as mechanical injury and explants are being cut from the stock plant, phenolic compounds are produced and tissues often secrete brown or black pigments. Phenolics released into the medium can oxidize explants, prevent shoots from growing and cause the death of plant material (Debergh & Read, 1991; Thorpe et al., 1991). According to the study of Yu and Reed (1995), the tissue browning and explant contamination could be observed 1 week after culture, and the tissue browning was confined to the plant and did not stain the medium.

There are methods that can be applied to reduce the browning rate such as storing the stock plants under low light/in the dark, soaking explants in water after excision and sub-culturing the explants frequently (Bonga & Aderkas, 1992; Compton & Preece, 1986;).
Literature cited


Chapter 1: *Salix matsudana*

**Introduction**

In a Nebraska-Hungary cooperation program, several Hungarian genotypes, including *Salix matsudana* ‘Golden Spiral’ were studied for their stress tolerance for urban landscape. (Read and Schmidt, 1997) ‘Golden Spiral’ has twisted upright golden branches and twigs with bright green leaves, which make it a unique bonsai plant and decorative tree through the year. Cuttings and seeds are the common methods of propagating, however, micropropagation of *Salix matsudana* could provide more and healthier plantlets for rapid commercial scale-up in the nursery industry. In addition, the plantlets from micropropagation were more uniform and practical than cuttings in tests by Tormala and Saarikko (1985).

**Literature Review**

*Salix matsudana* can be rooted easily via cuttings, yet there are still some obvious benefits that tissue culture techniques could provide, such as accelerating breeding programs and reducing the risk of disease spread. (Bergman et al., 1985)

1) Explant materials

Pereira et al. (2000) reported that both proliferation and root induction were influenced by the source of explant (*Salix humboldtiana*) used. MS medium
supplemented with 2.3 µM kinetin enhanced shoot production while half-strength MS medium supplemented with 5.3 µM NAA enhanced rooting.

According to Liesbach & Naujoks (2004), the applicability of micropropagation for selected Salix caprea donor trees was strongly depending on the genotype. In addition, based on the study of Bergman et al. (1985), the tendency of axillary shoots to develop on shoot cultures depend on the genotype, the type of shoot and the number of previous subcultures. In their research, five willow species clones were used to test the effect of BA on the shoots cultured in vitro. It was found that 0.5 µM or 1 µM BA was the optimum concentration for shoot multiplication, while for shoot elongation, media containing 0-0.5 µM BA were better for the growing of the Salix caprea depending on the genotypes. If the BA concentration was higher than 10 µM, it would cause the browning and death of shoots, and if concentration higher than 0.5 µM, root initiation was inhibited almost completely. Besides, it was found that prolonged culture in vitro could increase the rooting ability for the most recalcitrant clones.

2) Culture Media

In micropropagation of Salix, in addition to MS medium and WPM medium, many other media were also reported in several studies. Chalupa (1983) used the Gresshoff and Doy (1972) medium at full concentration or half concentration, and it stimulated best growth of willow shoots from axillary bud compared to other media. The growth of shoots was most rapid if the medium contained no cytokinin and auxin, or when IBA concentration was at 0.1-0.2 mg/L.
Callus induction and plantlet regeneration of *Salix exigua* clones were examined by Stoehr et al. (1989). BAP and 2,4-D were used with basal media. They found that the callus production was best on WPM, while after calli were subsequently cultured, half-strength MS medium without PGR was able to grow roots successfully. The results that the shoot primordia only developed at 0.1 mg BAP per liter in two clones suggested that there was a clonal variation in organogenic responses.

Thidiazuron (TDZ) was reported to induce callus formation of various species and in some cases the cell proliferation rate was higher with TDZ than with other plant growth regulators (Murthy et al., 1998). Merkle et al. (1998) reported that TDZ at both 0.1 mg/L and 0.01 mg/L could induce embryogenesis from sweetgum inflorescence explants. However, there were also reports that TDZ-induced buds fail to elongate (Meyer and van Staden, 1988) or shoots did not convert into complete plantlets (Huetteman and Preece, 1993). In the study of in vitro regeneration of *Salix nigra* from adventitious shoots (Lyyra et al., 2006), they found that 47-92% of inflorescences treated with 0.1 mg TDZ per liter produced buds but these buds failed to elongate into shoots. They also observed that buds in liquid medium for 6 weeks had faster shoot elongation than those on semi-solid basal WPM in GA-7 vessels, which was different from Agrawal and Gebhardt’s results.

3) Problems

It is often a problem to establish in vitro cultures from adult trees because of the age affects (Park et al., 2008), and the aging is related to the reduced growth rate and seasonal dormancy (Pierik, 1990). The rejuvenating of the bud break could be accomplished by hormone treatments (Beck et al., 1998), serial grafting (Valdes et
al., 2003) and in vitro micrografting (Perrin et al., 1994). Known as Korean weeping willow, explants from 20-year-old *Salix pseudosiegyne* trees were cultured on WPM or WPM supplemented with BA, zeatin and GA3 (Park et al., 2008). The results indicated that bud break on BA supplemented media had a higher bud break rate. Various levels of cytokinins (2-iP, zeatin, dihydrozeatinriboside and ABA) were used for testing the results. The levels of zeatin type cytokinins were higher than those of isopentenyladenin type of cytokinins in the explants cultured on BA supplemented media. Necrosis was observed upon subculture of explants to the fresh basal WPM.

In the ovary culture of hybrid salix research (Agrawal and Gebhardt, 1994), they used liquid medium for micropropagation, and if the shoots were kept in the liquid medium, they would become hyperhydric and had smaller and less expanded leaves.

Contamination is another concern. In the study of vegetative propagation of difficult-to-root *Salix caprea* (Liesbach and Naujoks., 2004), they found that the majority of shoot tips and nodal segments from five clones died within one month because of bacteria contamination, with only greenhouse-grown explants left. Three clones failed to initiate in vitro, while the other two were able to culture via repeated subculture on various media.

According to the study of in vitro propagation of *Salix caprea*, Schenck-Hildebrandt (1972) and Ahuja’s medium (1983) were used (Neuner and Beiderbeck, 1993), though no significant differences were observed among all media used. They also reported very serious contamination issues. The contamination rates varied from 6% to 69% depending on the explant source (e.g. clones or habitats), the time of explants being collected and the concentration of NaOCl.
Objectives

Three trees of *Salix matsudana* ‘Golden Spiral’ were used for the experiments that were conducted to test whether NRM was superior to other media (MS, WPM and DKW) in growing more shoots and roots, longer stems and greener leaves. The concentrations and combinations of plant growth regulators (BA, IBA) for shoot and root growth in vitro were examined. After shoot multiplication and root initiation the ‘Golden Spiral’ plantlets were to be transplanted into soil.

Materials and Methods

1) Plant material

*Salix matsudana* ‘Golden Spiral’ branches with axillary buds were obtained from the field located at the horticulture garden at the University of Nebraska-Lincoln.

Field grown branches with axillary buds collected in winter (January, February, March) were cut from the mother plant, kept in plastic bags with wet paper towels and stored at 4 °C for 2 weeks. Before storing in the cooler, the branches were disinfested by rinsing with tap water for 5 min, followed by soaking in 10% sodium hypochlorite (Bleach of Hyvee Co.) for 15 min. After removal from the cooler, branches (about 10-15 cm long) that contained 3-5 buds were treated with forcing solution in GA-7 containers at 24 ± 1°C under cool white fluorescent 16 hours a day.

After bud break in the forcing solution, softwood cuttings were disinfested in a laminar flow hood with 70% ethanol spray for 5 seconds, followed by swirling in small glass jars that contained 10% sterilized sodium hypochlorite with 20 drops
Tween 20 (wetting agent) per liter for 10 or 20 min, and rinsed for 5 min in sterile deionized water three times. The disinfested softwood plant materials were cut into single node or shoot tip explants, and each explant was cultured in one shell vial containing 5 ml of the test media.

2) Forcing solution:

After the field grown materials were disinfested, apical and basal ends of the hardwood cuttings were given a fresh cut. Axillary bud outgrowth was then forced by immersing the basal end of branches in 30 ml of forcing solution (Yang and Read, 1986) containing 200 mg 8-hydroxyquinoline citrate per liter and 2% sucrose per liter. The basal end of each cutting was pruned off, and the solution replaced twice a week. After the bud break, tissue cultures were initiated by culturing explants in shell vials containing 5ml medium. The cultures were maintained at 24±1°C under cool white fluorescent light for 16 hours per day. One week after the culture initiation, the contaminated cultures were excluded. The numbers of new shoots and roots were recorded as well.

3) Medium:

For culture establishment, the following culture media were used:

1: MS: Murashige and Skoog salts and vitamins supplemented with PGR noted;
2: DKW: Driver and Kuniyuki salts and vitamins supplemented with PGR noted;
3: WPM: Woody Plant Medium salts and vitamins supplemented with PGR noted;
4: NRM: Nas and Read Medium use DKW basal salts, NRM vitamins, and 0.6 mg Sequestrene 138 Fe per liter supplemented with PGR noted.
All of the above media were adjusted to a pH ranging from 5.5 to 5.8 and autoclaved at 121 °C for 30 min. The basal salts were from Phyto Technology Laboratories Co..

The following 4 combinations and concentrations of plant growth regulators were included in the 4 media.

Experiment 1) 2.5 mg BA/L + 0.05 mg IBA/L in MS, WPM, DKW or NRM
Experiment 2) 2.0 mg BA/L + 0.01 mg IBA/L in MS, WPM, DKW or NRM
Experiment 3) 2.0 mg BA/L + 0.01 mg IBA/L in MS, WPM, DKW or NRM
Experiment 4) 0.05 mg IBA/L in MS, WPM, DKW or NRM

Experiment 1 was first conducted on June 23rd 2013, and repeated two times on Aug 13th 2013;
Experiment 2 was first conducted on Aug 13th 2013, and repeated two times on Sep 19th, 2013;
Experiment 3 was first conducted on Sep 9th 2013, and repeated two times on Oct 31st 2013;
Experiment 4 was first conducted on Oct 31st 2013, and repeated two times on Nov 25th, 2013.

Experiment 1, 2, 3, and 4 were conducted to test the influence of 4 media in combination with 4 concentrations and combinations of PGR on the explant growth. The shoot number, the stem length and the leaf color were three indicators used to evaluate the explant growth. After the Salix explants were cultured on media, the number of shoots or roots were additively recorded every 10 days for 40 days, and when the shoot number was recorded 40 days after culture, the stem length and leaf...
color were measured. The Pantone Matching System (PMS) Color Chart was used (Figure 7) to evaluate the leaf color. If the ‘Golden Spiral’ explants leaf color matched green color numbers PMS 361, 362, 363, 368, 369, 375, and 376, it was considered that these explants had healthy leaf color, and the number of healthy-colored explants was counted.

Experiment 5 was conducted to use the two stage system (shoot multiplication stage and root initiation stage) to culture the explants and transplant the cultures into soil. Based on the results of experiment 1, 2, 3 and 4, two of the better media out of four were selected. The combination and concentration of PGR that would stimulate shoot production were included in the two media for shoot multiplication. The shoot number was recorded every 10 days for 40 days. Forty days after culture, whole explants were transferred to rooting medium. After the explants had roots, they were transplanted into soil.

4) Experimental Design and Statistical Analysis

One single shoot tip or nodal shoot of Salix matsudana ‘Golden Spiral’ was cultured in each shell vial which contained 5 ml of each medium. For the four types of media, each medium had 5 shell vials to culture 5 explants (5 replicates). The experimental unit was each shell vial, and there were 20 shell vials (4 media*5 shell vials) at every combination and concentration of PGR (experiment 1, 2, 3, and 4). There were three trials. Experiment 5 was conducted for both shoot multiplication and root initiation, and it was conducted 3 times (3 trials). Since the shoot number was recorded additively every 10 days, to simplify the statistical analysis and to check whether there was interaction between time and treatment, the experiment
was considered to be a Complete Random Design (CRD) with repeated measures. For the stem length that was measured 40 days after culture, the experiment was considered to be a 4X4 Factorial Treatment Design. Statistical analysis of data was performed using PROC GLIMMIX and MEANS procedures (SAS institute, 2013).

**Results and Discussion**

In Experiment 1, when the plant growth regulator was at a concentration of 2.5 mg BA/L + 0.05 mg IBA/L (Figure 1-1, Table 1-1, and 1-2), WPM and MS medium had more shoot number than that of DKW and NRM. The shoot number in NRM declined as the experiment progressed, which indicated that there was an interaction between time and media (P value=0.0003 at α=0.05 of trial 1, trial 2 and 3 had similar results). The shoot tip explants did not survive in NRM, while the segment nodes with one leaf had grown new shoots successfully. This is probably due to the sensitivity of the shoot tips to the Sequestrene 138 iron in NRM. For 3 trials of Experiment a, the results were similar.

However in experiment 2, when the IBA concentration dropped to 0.01 mg/L, with BA at 2.5 mg/L (Figure 1-2, Table 1-1, and 1-2), different results were observed. NRM was the most effective medium to have a greater shoot number 40 days after culture, while the other three media had no significant difference among each other and there was no interaction between time and media.

When the BA concentration was reduced to 2.0 mg/L while the IBA remained at 0.01 mg/L (Experiment 3, Figure 1-3, Table 1-1 and 1-2), there was no significant
difference between DKW and NRM in shoot number when considering the experiment design to be CRD with repeated measures, and there was no interaction between time and media. When comparing the mean number of shoots of four media in experiment 1, 2 and 3 40 days after culture, the experiment was analyzed as a factorial treatment design with SAS and it was found that shoot number in experiment 2 was significantly greater than those in experiment 1 and 3. Thus, the combination and concentration in experiment 2 (2.5 BA mg/L + 0.01 mg IBA/L) was better than the other two experiments.

When the media contained both BA and IBA, only new shoots were observed while new roots were not found. If only IBA was included in media (Experiment 4), roots were able to initiate and grow (Figure 1-4b, Table 1-1, and 1-2), though the shoot number (Figure 1-4a, Table 1-1 and 1-2) was significantly less than that of experiment 1, 2, and 3 (P value=0.0327 at α=0.05 of trial 1). After finishing Experiment 4, another experiment was conducted to test three levels of IBA (0.05, 0.1 and 0.2 mg IBA/L), and the results showed that there was no significant difference among these three levels (P value=0.8574 at α=0.05 of trial 1), which was different from the results of the study by Chalupa (1983). This is probably due to the species or the media in use. Since higher concentration of IBA did not increase the shoot number, 0.05 mg IBA/L was considered to be the best concentration for root initiation.

In Experiment 1, 2, 3, and 4, abnormal growth of some explants was observed. Even though the shoot number was as many as 15, the Salix explants were not able to elongate, only to form dwarf explants with multiple tiny leaves (<1cm) in vitro.
Thus, in addition to comparing the number of new shoots to evaluate the performance of the 4 media, stem length of ‘Golden Spiral’ was recorded as the second indicator (Figure 1-6, Table 1-3, and 1-4). Explants cultured on NRM had significantly longer stems compared to the other 3 media (Table 1-3, and 1-4), and there was no significant difference among MS, WPM, or DKW. Forty days after culture, the explants in NRM were able to grow to 4-6cm, while explants in MS, WPM and DKW grew to 2-4cm (Figure 1-5).

In Experiment 1, 2, 3 and 4, the third indicator to evaluate explant growth was leaf color and Pantone color matching system was used (Figure 1-7a). Fewer green leaves, as a type of vitrification phenomenon, were very common among the explants that grew on MS medium, WPM and DKW medium, while the healthy-colored explant number on NRM was significantly greater than those on MS, WPM and DKW (Figure 1-7b, no error bars in figure because of zeros in data, statistical analysis was done with original data). Since the stem elongation and leaf color of explants cultured in MS and WPM were considered to be not as desirable as the explants cultured on DKW and NRM, for the Experiment 5, DKW and NRM were used for shoot multiplication and root initiation media.

In Experiment 5 (Figure 1-8a, 1-8b, Table 1-5), there was no significant difference between DKW and NRM in Trial 1 in growing new shoots (P value=0.0602 at α=0.05) at shoot multiplication stage (DKW and NRM containing 2.5 mg BA/L + 0.05 mg IBA/L), but in Trial 2 and 3 the difference were significant (P value<0.0001 at α=0.05 of trial 1) In the second stage (DKW and NRM containing 0.05 mg IBA/L, and there was also no significant difference in root number (P
value=0.2972 at α=0.05) in Trial 1 either, but the difference 40 days after culture were significant again. After transplanting the explants into soil, among 20 of the explants, 17 of them were able to grow into strong and healthy plantlets with the stem length ranged from 15cm to 20cm (Figure 1-9). The three plantlets that had only 1 or 2 roots died when they were transplanted into soil, and this might be related to their failure to acclimatize to the soil environment. Plantlets from the NRM had the fastest growth rate (Table 1-6) compared to explants from other media, and this was possibly due to the longer stems and more roots that were found on the plantlets produced on NRM.

**Conclusion**

The NRM supplemented with 2.0 mg BA/L + 0.05 mg IBA/L was the best medium for shoot multiplication of *Salix mtsudana* ‘Golden Spiral’. For the root initiation stage, IBA at a concentration of 0.5 mg/L was the best choice. Explants grown on NRM had healthy green leaf color and good stem elongation. Shoots produced in vitro with at least 2 roots grown into plantlets and were successfully transferred to soil.
Figure 1-1: Shoot number of Salix matsudana 'Golden Spiral' explants cultured in vitro on 4 media containing 2.5mg BA/L + 0.05 mg IBA/L

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);
*Error bars stand for the standard error.
Figure 1-2: Shoot number of *Salix matsudana* 'Golden Spiral' explants cultured in vitro on 4 media containing 2.5 mg BA/L + 0.01 mg IBA/L

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);*

*Error bars stand for the standard error.*
Figure 1-3: Shoot number of *Salix matsudana* 'Golden Spiral' explants cultured in vitro on 4 media containing 2.0 mg BA/L + 0.01 mg IBA/L

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);*  
*Error bars stand for the standard error.*
Figure 1-4a: Shoot number of *Salix matsudana* 'Golden Spiral' explants cultured in vitro on 4 media containing 0.05 mg IBA/L

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);
*Error bars stand for the standard error.
Figure 1-4b: Root number of *Salix matsudana* ‘Golden Spiral’ explants cultured in vitro on 4 media containing 0.05mg IBA/L

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);
*Error bars stand for the standard error.*
Figure 1-5: Growth comparison of *Salix matsudana* ‘Golden Spiral’ explants cultured in vitro

*Top: Explants grown on Nas and Read Medium (NRM), Driver and Kuniyuki Walnut medium (DKW), Woody Plant Medium (WPM) and Murashige and Skoog Medium (MS) containing 2.0 mg BA/L + 0.05 mg IBA/L (left to right). The yellowish leaf color and abnormal growth can be seen on WPM and MS. The elongation of the explants and the roots can be seen on DKW and NRM.

*Bottom: Light leaf color and abnormal growth of *salix matsudana* ‘Golden Spiral’ on MS medium
Figure 1-6: Stem length of *Salix matsudana* 'Golden Spiral' explants cultured in vitro on 4 media containing various PGR

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);*  
*Error bars stand for the standard error.*
*Green color number PMS 361, 362, 363, 368, 369, 375 and 376 were considered to be healthy leaf color.
Figure 1-7b: Number of healthy-colored explants of *Salix matsudana* 'Golden Spiral' cultured in vitro on 4 media containing various PGR

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002); PGR stands for plant growth regulators

Error bars stand for the standard error.
Figure 1-8a: Shoot number of *Salix matsudana* 'Golden Spiral' explants cultured on DKW and NRM containing 2.5 mg BA/L + 0.01 mg IBA/L for shoot multiplication and transferred to DKW and NRM containing 0.05 mg IBA/L for root initiation

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002); *Error bars stand for the standard error.
Figure 1-8b: Root number of *Salix matsudana* ‘Golden Spiral’ explants cultured on DKW and NRM containing 2.5 mg BA/L + 0.01 mg IBA/L for shoot multiplication and transferred to DKW and NRM containing 0.05 mg IBA/L for root initiation

*MS = Murashige and Skoog Medium (1962); WPM = Woody Plant Medium (Lloyd and McCown, 1981); DKW = Driver and Kuniyuki Walnut Medium (1984); NRM = Nas and Read Medium (2002);* Error bars stand for the standard error.
Figure 1-9: 1 month after *Salix matsudana* ‘Golden Spiral’ explants transplanted from Nas&Read Medium containing 0.05 mg IBA/L to soil

*Top: The explants that had roots observed in vitro on the 4 media (mostly from Nas&Read Medium) were selected to transplant into soil and they were able to survive and grow fast in soil.

*Bottom: The *Salix matsudana* ‘Golden Spiral’ that was able to elongate and initiate roots in NRM and transplanted into soil. It grew to 26 cm within 1 month.
Table 1-1: Mean shoot number±SE of *Salix matsudana* ‘Golden Spiral’ in 4 media containing PGRs in Experiment 1, 2, 3, and 4 (Figure 1-1, 1-2, 1-3, and 1-4)

<table>
<thead>
<tr>
<th></th>
<th>2.5 mg BA/L±0.05</th>
<th>2.5 mg BA/L+0.01</th>
<th>2.0 mg BA/L+0.01 mg IBA/L (shoots)</th>
<th>0.05 mg IBA/L (roots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>7.00±2.28</td>
<td>5.60±0.55</td>
<td>4.40±2.51</td>
<td>5.00±1.00</td>
</tr>
<tr>
<td>WPM</td>
<td>7.40±2.70</td>
<td>6.60±2.61</td>
<td>6.80±1.76</td>
<td>4.20±0.84</td>
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<tr>
<td>DKW</td>
<td>4.00±1.30</td>
<td>7.60±1.95</td>
<td>9.40±4.15</td>
<td>5.00±1.22</td>
</tr>
<tr>
<td>NRM</td>
<td>1.40±2.19</td>
<td>12.00±2.35</td>
<td>8.80±2.95</td>
<td>5.20±0.83</td>
</tr>
</tbody>
</table>

*In this table, the data were collected the final time recording the number of new shoots (roots) for each set of experiment.

Table 1-2: P value of comparisons of treatment (media) differences Least Squares Means (LSM) at α=0.05 in experiment 1, 2, 3, and 4

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>2.5 mg BA/L+0.05 mg IBA/L (Figure1-1)</th>
<th>2.5 mg BA/L+0.01 mg IBA/L (Figure1-2)</th>
<th>2.0 mg BA/L+0.01 mg IBA/L (Figure1-3)</th>
<th>0.05 mg IBA/L (Figure1-4a)</th>
<th>0.05 mg IBA/L (Figure1-4b)</th>
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<tbody>
<tr>
<td>MS and WPM</td>
<td>0.4250</td>
<td>1.0000</td>
<td>0.1393</td>
<td>0.6933</td>
<td>1.0000</td>
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<td>MS and DKW</td>
<td>0.1128</td>
<td>0.3255</td>
<td>0.0043</td>
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<td>MS and NRM</td>
<td>0.0024</td>
<td>&lt;0.0001</td>
<td>0.0396</td>
<td>0.4309</td>
<td>0.0010</td>
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<td>WPM and DKW</td>
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<td>0.1524</td>
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<td>WPM and NRM</td>
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<td>&lt;0.0001</td>
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<td>DKW and NRM</td>
<td>0.1284</td>
<td>0.0003</td>
<td>0.472</td>
<td>0.9214</td>
<td>0.0020</td>
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Table 1-3: Mean shoot length ± SE of *Salix matsudana* ‘Golden Spiral’ in 4 media containing PGRs in Experiment 1, 2, 3, and 4 (Figure 1-6)

<table>
<thead>
<tr>
<th></th>
<th>2.5 mg BA/L±0.05</th>
<th>2.5 mg BA/L+0.01</th>
<th>2.0 mg BA/L+0.01</th>
<th>0.05 mg IBA/L</th>
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<tbody>
<tr>
<td>MS</td>
<td>3.20±0.84</td>
<td>3.60±1.14</td>
<td>2.80±0.84</td>
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<td>WPM</td>
<td>3.40±0.89</td>
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<td>DKW</td>
<td>2.00±1.58</td>
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<td>3.00±1.00</td>
<td>2.40±0.89</td>
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<td>NRM</td>
<td>4.50±1.32</td>
<td>5.40±0.89</td>
<td>4.60±1.14</td>
<td>3.50±1.12</td>
</tr>
</tbody>
</table>

*MS = Murashige and Skoog Medium (1962);
WPM = Woody Plant Medium (Lloyd and McCown, 1981);
DKW = Driver and Kuniyuki Walnut Medium (1984);
NRM = Nas and Read Medium (2002);
Table 1-4: Simple effect comparisons of medium*PGR Least Squares Means of stem length in Experiment 1, 2, 3, and 4 (Figure 1-6)

<table>
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<tr>
<th>Simple Effect</th>
<th>Standard Level</th>
<th>pgr 1</th>
<th>pgr 2</th>
<th>Estimate</th>
<th>Error</th>
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*medium a stands for MS, medium b stands for WPM, medium c stands for DKW, and medium d stands for NRM; *pgr 1 stands for 2.5mg BA/L + 0.05 mg IBA/L, pgr2 stands for 2.5 mg BA/L + 0.01 mg IBA/L, pgr 3 stands for 2.0 mg BA/L + 0.01 mg IBA/L, and pgr 4 stands for 0.05 mg IBA/L
Table 1-5 Mean shoot or root number ± SE of *Salix matsudana* ‘Golden Spiral’ on DKW and NRM for shoot multiplication and root initiation in Experiment 5 (Figure 1-6a, 1-6b)

<table>
<thead>
<tr>
<th></th>
<th>Mean shoot number ± SE</th>
<th>Mean root number ± SE</th>
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</thead>
<tbody>
<tr>
<td>DKW</td>
<td>8.7±1.64</td>
<td>2.7±0.84</td>
</tr>
<tr>
<td>NRM</td>
<td>11.2±0.83</td>
<td>4.1±1.30</td>
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Table 1-6: Growth rate of *Salix matsudana* ‘Golden Spiral’ after transplanted into soil

<table>
<thead>
<tr>
<th>Growth rate (cm/Day)</th>
<th>MS</th>
<th>WPM</th>
<th>DKW</th>
<th>NRM</th>
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<tr>
<td></td>
<td>0.9</td>
<td>1.2</td>
<td>1.1</td>
<td>1.8</td>
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</tbody>
</table>

*MS = Murashige and Skoog Medium (1962);*  
*WPM = Woody Plant Medium (Lloyd and McCown, 1981);*  
*DKW = Driver and Kuniyuki Walnut Medium (1984);*  
*NRM = Nas and Read Medium (2002);*
Literature Cited for Chapter 1:


Chapter 2: *Corylus colurna*

**Introduction**

Red/purple Turkish Hazel is the common name of *Corylus colurna* ‘Te Terra Red’. This small tree, or large shrub is the plant material being used in this research. ‘Te Terra Red’ trees are available in many nurseries in the UK, whereas in the US, especially in Nebraska, there are very few nurseries that can afford this decorative tree. However, its purple leaves that appear in spring, and they totally fade to green foliage exhibited by autumn, make it desirable for landscape purposes. It is thought that ‘Te Terra Red’, a unique hybrid (*C. avellana X C. colurna*) that also has red-purple catkins is certainly a worthy candidate for landscape use in the United States (Dirr. 2011)

Hazelnut trees are traditionally produced by rooting suckers, layering, cuttings and grafting (Rodriguez et al., 1989) and the common method of propagating *C. colurna* is by seed. However, traditional systems of propagation of *Corylus* species are not sufficient to obtain a great number of plants from a tree selected in breeding programs (Messeguer & Mele, 1987). In contrast, it is hypothesized that micropropagation can produce hazelnut trees rapidly for commercial scale-up in nurseries. Though the micropropogation of hazelnut is challenging, as for many other woody species, using shoot multiplication from single buds or embryos as explants in tissue culture is a rapid way of asexual propagation of hazelnut (Perez et al., 1987).
Literature review

1) Explant collecting time and size

The time of taking explants from the field may have an effect on the success of the micropropagation process. The carbohydrate, proteins, and growth substances in the stock plants change as the temperature, day length, light intensity and water availability changes, and different levels of these substances can influence the explant response to the in vitro environment (Torres, 1986). Yu and Reed (1995) used three cultivars of C. avellana grafted in the greenhouse, and they collected the explants from March to July. It was found that explants collected in March produced a greater number of successful shoots and roots in vitro. Fifteen cultivars of C. avellana were tested by Bacchetta et al. (2005) on the initiation time. The explants collected in the spring produced better growth in vitro. However, according to Messeguer and Mele's (1983) research, the results indicate that plant material taken in autumn produce a greater number of shoots than those in spring. In addition, it was reported that explants with a diameter of > 4mm had a better chance of establishment (Diaz Sala et al., 1994; ), and explants less than 1.5mm diameter did not grow (Messeguer and Mele, 1983).

2) Initiation of bud break and disinfestation

Forcing solution systems can be used to facilitate bud break (Yang et al., 1986). The forcing solution with 8-hydroxyquinoline citrate at 200mg/L + 2% sucrose + gibberellic acid (GA) at 10mg/L was used to stimulate bud break. Bud break occurs
by immersing the basal end of branches in forcing solution (Yang et al., 1986).

Explant contamination rates varied from 30-90% depending on genotype.

There are many protocols that can be used for surface disinfestation of the hazelnut explants. The first step usually begins with tap water washing for half an hour after the explants are cut (Yu and Reed, 1995). The explants can then be swirled in vessels that contain 10% chlorine bleach, including 5 drops of Tween-20, for 10-30 min, followed by rinsing in sterile distilled water for 2-3 times (Nas and Read, 2004). Ethanol can also be applied in the disinfestation process by dipping the material in it before using the bleach (Bacchetta et al., 2008; Nas, 2004). This method has drawbacks because it can stimulate phenolic formation, which can cause browning of explants (Bassil et al., 1991). Hydrogen peroxide (H$_2$O$_2$) is another powerful oxidant that can be used at 3-10% (v/v) for 1-30 min prior to sterile water in the disinfestation process, either in combination with other disinfectants or alone (Smith, 2012).

3) Culture media

A modified MS medium (half-strength macro and micronutrients) was promoted by Messeguer and Mele (1983) for culture of hazelnut in vitro. Another modified MS medium specific for hazelnut developed by Anderson (1984) has NaH$_2$PO$_4$ instead of KH$_2$PO$_4$ as the potassium source, and has 75% less NH$_4$NO$_3$ and KNO$_3$. He found that compared to MS and WPM media, the shoot proliferation in his modified medium was better. It was reported that replacing FeEDTA with 200 mg Sequestrene 138 iron EDDHA per liter in the MS medium can result in healthier and greener plantlets (Al Kai et al., 1984).
Messeguer and Mele (1983) concluded that 2.85 μM zeatin performed better than BA for shoot elongations of *C. avellava*. Yu and Reed (1993) found that BA concentration at 6.66-13.3μM on modified DKW medium had the best results in growing shoots from ‘Tonda Gentile Romana’ hazelnut explants. Four cytokinins (kinetin, iso-pentenyladenine, zeatin and BA) were tested by Thomson and Deering (2011) with the ‘Knoxfield2’ medium, which is a medium similar to the medium used in the studies of Anderson (1984) and Al Kai et al. (1984). The results showed that 22.2 μM BA was the best for shoot initiation.

The basal immersion of shoots for 15s in immersion solutions containing IBA (5 mM) plus polyamines produced a rapid 100% rooting response. Enhancement of rooting was observed when micro shoots were immersed in IBA solution, then rooted in media containing polyamines. (Diaz-Sala et al., 1994; Berros et al., 1995; Yu & Reed, 1995;) According to Bassil et al. (1991), root formation occurred on all shoots dipped in 1mM IBA for 15 s followed by a culture period of four weeks on auxin-free medium. Perez et al. (1986) reported that IBA promoted embryo initiation, and they also concluded that the addition of BA and IBA in various concentration and combinations in two consecutive cultures facilitated embryonic induction in the primary explants. Based on Gonzalez (1990) research, half-strength K(h) (Cheng, 1975) medium containing 50 μM IBA + 4.5 μM Kin could increase the number of roots developed. However, Perez et al. (1987) also reported that the shoots treated with 3.0 g IBA per liter did not stimulate root development after 20 days, but they observed high amount of callus on bases instead.
Epiphytic and endophytic organisms can cause serious losses to micropropagation in vitro in every stage of growth (Cassells, 1991; Leifert et al., 1995). There are difficulties in detecting bacterial contamination if they remain inside the plant tissue, which could not be eliminated by surface disinfection (Debergh & Vanderschaghe, 1988). Thus, the antibiotic therapy should be considered if less contamination is desired. There are several groups of antibiotics that can reduce the contamination rate and therefore the type of antibiotics to be used in media should be determined by the bacteria present in culture (Buckley et al., 1995). In general, carbenicillin, cephalothin, gentamicin, polymyxin, figampicin, streptomycin, and kanamycin are some possible choices in plant tissue culture and the concentration ranges from 10 mg to 100 mg per liter depending on the sensitivity of the explants (Buckley et al., 1995; Falkiner, 1988; Kneifel & Leonhardt, 1992).

4) Problems

There are several problems that might happen after culturing the explants in vitro, including contamination, browning, necrosis, to name just a few.

According to several researchers, keeping the mother plants in the greenhouse made it possible to reduce the contamination rate to a great degree (Messeguer and Mele, 1983; Yu and Reed, 1995; Bacchetta et al., 2008). Yu and Reed (1995) suggested that the higher contamination rate of explants collected in June was related to the rainfall of the season.

As a response of plants to excision, explant browning is another common problem in culture (Bonga & Aderkas, 1992). When tissues are suffering from
stresses such as mechanical injury and explants being isolated from the stock plant, metabolism of phenolic compounds is produced and tissues often secrete brown or black pigments. Phenolic compounds that are released into the medium can oxidize explants, prevent shoots from growing and cause the death of plant material (Debergh & Read, 1991; Thorpe et al., 1991). According to the study of Yu and Reed (1995), the tissue browning and explant contamination could be observed 1 week after culture, and the tissue browning was confined to the plant and did not stain the medium.

There are methods that can be applied to reduce the browning rate such as storing the stock plants under low light or in the dark, soaking explants in water after excision and sub-culturing the explants frequently (Bonga & Aderkas, 1992; Compton & Preece, 1986). Frequent sub-culturing is relatively costly and needs lots of labor. In addition, it also limits the conditioning function of the medium by accumulation of excretion products that may stimulate growth (Bonga & Aderkas, 1992). The browning is also related to the disinfestation agents being used since these agents can kill the axillary meristems by easily entering the stems from the scars of the leaf bases and the surrounding tissues (Debergh & Read, 1991). Ethanol is powerful in killing the microorganism, however, it also contributes to tissue browning at the same time.

**Objectives**

Two hazelnut trees of *C. colurna* ‘Te Terra Red’ were used for the experiments that were conducted to test whether NRM was superior to other media (MS, WPM
and DKW) for growing explants and producing the roots. The concentrations and combinations of plant growth regulators (BA, IBA) for shoot and root growth were examined. As the research progressed, the explants were lost because of the contamination so that there was not sufficient number of explants to continue the experiment. Thus, the most effective disinfestation methods and the media or forcing solution supplemented with antibiotics (streptomycin, kanamycin) to control contamination were the focus of this study.

**Materials and Methods**

1) Plant material

Hazelnut branches with axillary buds were obtained from the field located at horticulture garden at the University of Nebraska-Lincoln. There are two ‘Te Terra Red’ trees in the garden in total, and both of them are years old. They are adult trees about 4-5m in height and 3m in width. Bud break usually happens at the end of April, and early in May the wormholes on leaves are formed. In autumn, the leaves start to fall at the end of October. Explants were collected from January to December throughout the year.

2) Surface disinfestation

The field grown branches with axillary buds collected in winter (November, December, January, February, March) were cut from the mother plant, kept in plastic bags with wet paper towels and stored at 4 °C for 2 weeks, 1 or 2 month (Experiment 1). Before storing in the cooler, the branches were disinfested by rinsing with tap water for 20, 40 or 60 min (Experiment 2) followed by soaking in
10% sodium hypochlorite (Hyvee Co.) for 20, 30, 40 min (Experiment 3). After removal from the cooler, branches (about 10-15 cm long) containing 3-5 buds were treated with forcing solution in GA-7 container at 24 ± 1°C under cool white fluorescent light 16 hours a day.

After bud break in the forcing solution, softwood cuttings were disinfested in a laminar flow hood with 70% ethanol spray for time for 30 s 90 s or 5 min (Experiment 4), followed by swirling in small glass jars containing 10% sterilized sodium hypochlorite with 20 drops Tween-20 (wetting agent) per liter for 10 or 20 min, and rinsed for 5 min in sterile deionized water three times.

In the first year, the disinfested softwood plant materials were cut into a single node or shoot tip explants, and each explant was cultured individually in shell vials that contained 5 ml media separately. In the second winter, instead of culturing the shoots after bud break, the axillary buds were disinfested with the same method and then cultured directly in to the shell vials in order to reduce the contamination incidence (Experiment 5).

3) Forcing solution:

After the field grown materials were disinfested, apical and basal ends of the hardwood cuttings were given a fresh cut. Axillary bud outgrowth was then forced by immersing the basal end of branches in forcing solution (Read & Yang, 1987) containing 200 mg 8-hydroxyquinoline citrate per liter and 2% sucrose per liter. The basal end of each cutting was pruned off, and the solution replaced twice a week.
In the first year of research, the hazelnut plant materials collected in summer and autumn were not kept in cooler since they were actively growing, and forcing solution was not used. In the second year, the materials collected in summer and autumn were treated with forcing solution supplemented with streptomycin at concentrations of 0 mg/L, 20 mg/L and 40 mg/L to control the explant contamination in vitro (Experiment 6).

After the bud break, tissue cultures were initiated by culturing explant in shell vials containing 5ml medium. The cultures were maintained at 24±1°C under cool white fluorescent light for 16 hours per day. One week after the culture, the contaminated explants were excluded and the contamination incidence was calculated. 1 month after discarding contaminated cultures, the number of browning explants was counted and browning incidence was calculated.

4) Medium:

For culture establishment (Experiment 7), the following culture media were used:

1: MS: Murashige and Shoog salts and vitamins supplemented with noted PGR.
2: DKW: Driver and Kuniyuki salts and vitamins supplemented with noted PGR
3: WPM: Woody Plant Medium salts and vitamins supplemented with noted PGR
4: NRM: Nas and Read Medium use DKW basal salts, NRM vitamins, and 0.6mg/L Sequestrene 138 Fe supplemented with noted PGR.

All of the above media were adjusted to a pH ranging from 5.5 to 5.8 and autoclaved at 121 °C for half an hour. The basal salts were from Phyto Technology Laboratories Co. (2013).
The following plant growth regulators were contained in the 4 media.

a) 2.5 mg BA/L + 0.05 mg IBA/L in MS, WPM, DKW or NRM
b) 2.0 mg BA/L + 0.01 mg IBA/L in MS, WPM, DKW or NRM
c) 2.0 mg BA/L + 0.01 mg IBA/L in MS, WPM, DKW or NRM
d) 0.05 mg IBA/L in MS, WPM, DKW or NRM

After the culture, the number of new shoot was recorded every 15 days for 55 days, and after the explants had root initiation, they were transplanted into soil.

In the first year, the antibiotics were not included in media, and in the second year, antibiotics were included in MS medium at 0 mg streptomycin/L, 20 mg streptomycin/L, 40 mg streptomycin/L and 20 mg streptomycin/L + 20 mg kanamycin/L (Experiment 8). The contamination incidence was calculated 1 week after culture, and the browning incidence was calculated 1 month after discarding the contaminated cultures.

4) Experimental Design

One single shoot tip or nodal shoot of *Corylus colurna* 'Te Terra Red' was cultured in each shell vial containing 5 ml of each medium. For the four types of media, each medium had 5 shell vials to culture 5 explants (5 replicates). The experimental unit was each shell vial, and there were 20 shell vials (4 media*5 shell vials) at every combination and concentration of PGR, and they were conducted three times (3 trials). For the experiments that tested the effects of culture on the contamination and browning of explants, the experiments were considered to be completely random design (CRD). For experiment 7, since the shoot number was recorded every 10 days, to simplify the statistical analysis and to check whether
there was interaction between time and treatment, the experiment was considered to be a Complete Random Design (CRD) with repeated measures. The Statistical analysis of data was performed using SAS PROC GLIMMIX and MEANS procedures (SAS institute, 2013).

**Results and Discussion**

During the first week after tissue culture, 521 explants out of 602 cultures were lost because of the contamination of the external or internal pathogen or the tissue browning. The contamination and the browning incidence of the explants 10 days after culture were related to the disinfestation method.

In experiment 1, the branches were kept in a cooler for a different time period. It was found that storing branches for 1 month had significant less contamination incidence than that of 0 week or 2 weeks of storage, but not significant different from that of 2 months (Figure 2-1, Table 2-1. Data that are shown by incidence in all figures, and the statistical analysis was done by original data). The results indicated that storing the branches in cooler for 1 month was effective in reducing the contamination incidence.

In experiment 2, tap water was used to rinse the branches and it was found that a longer time of tap water rinsing did not help decrease the contamination incidence of the plant materials, but did increase the browning incidence (Figure 2-2, Table 2-2). Thus, 20 min tap water rinsing was sufficient for the branches.

In experiment 3 and 4, the data showed that the longer the time the 10 % bleach or 70% ethanol spray was used, the lower the contamination incidence (Figure 2-3
and 2-4, Table 2-3 and 2-4), though the browning incidence increased. Soaking in bleach for 20 min was not significantly different from 0 min, while 30 min made significantly reduce the contamination incidence. Ethanol spray for 90 s was significantly better in controlling the contamination, and extending time to 5 min was not effective for further controlling. Thus, soaking in bleach for 30 min and spraying with 70% ethanol for 90s were better choice.

Using the axillary buds taken in winter to culture in the media directly had a significantly lower contamination incidence in experiment 5 (Figure 2-5) The shoots after bud break had hairy leaves and stems, which could carry more pathogens and being difficult to disinfest (Figure 2-6 top. The oval shaped buds with relatively smooth surface were easier to disinfest. Milky fluids were observed at the bottom of the buds in culture, and all of the buds with this phenomenon did not break bud (Figure 2-6 bottom).

Other reasons related to the contamination and browning were also studied. There were two 'Te Terra Red' trees available to be used for tissue culture, and one of them (tree B) was observed to have many leaves with wormholes, which indicate that they might be more infected with internal pathogens (Figure 2-7, Table 2-5). The contamination incidence of the explants from the less healthy tree was 87.3% (352 out of 403 buds were contaminated), which was significantly higher than contamination rate of 60.9% (241 out of 396) of the other tree A.

The medium contributed to the browning incidence as well (Figure 2-8, Table 2-6). MS medium had the lowest browning rate (76.4%), which is significantly different from DKW and NRM, but not significantly different from WPM. NRM
seemed to have the highest 93.2% browning rate, being significantly different from MS and WPM, but not significantly different from DKW. This might be related to the similarity of the formula between DKW and NRM, and the micronutrient differences or the Sequestrene 138 Iron in the NRM.

After discarding the contaminated and brown explants, the surviving explants (Figure 2-9) with new shoots were transferred to the media containing varied concentrations of plant growth regulators. The four media also stimulated bud break differently, and the shoot elongation in the media was not showed in MS or WPM.

However, the growth of these buds was slow and very few of them had shoot elongation, and if only IBA was added in the media, the shoots all died without any root initiation. When the concentration of BA was 2.5 mg/L and IBA was 0.05 mg/L, MS and WPM were able to stimulate as many as 5 leaves in vitro, while NRM had 12 leaves at most, being significantly higher than MS and WPM (Figure 2-10, Table 2-7). The bottom leaves of explants (from buds) became dried out and all explants died in the media supplemented with BA at 2.0 mg/L and IBA at 0.01 mg/L or with IBA at 0.05 mg/L. The death of the explants was usually observed after 2-3 month after the tissue culture initiation, and this may be related to the depletion of plant growth regulators. If the explants were transferred from the shell vials to GA-7 vessels, they were able to survive longer in vitro (grow as long as 4 month).

‘Te Terra Red’ leaves are red after bud break in the field, however, in the experiments conducted in lab, all the new shoots turned to green shortly after bud break in vitro (Figure 2-9). The growth of ‘Te Terra Red’ is relatively slow and the
data were collected every 15 days. Even so, the elongation of the explants was seldom observed 2 months after culture. If the branches were kept in cooler for 0 weeks or 2 weeks, the buds were not found to have any good multiplication or elongation. The 1 months cold storage mimic the long winter in Nebraska and help the buds to finish the dormant period, and perhaps meanwhile reduce the amount of pathogens.

The acclimatization of the hazelnut explants was not successful (Figure 2-11). After 4 month of culture, the explants of ‘Te Terra Red’ that have at least 3 roots were transplanted into soil, and the plantlets died with fungus spores covered all over the stems and leaves. This might be due to the unsterilized soil that had pathogen and the plantlets were not strong enough to survive in the soil. Similarly, the explants of ‘Yam Hill’ could not be transplanted into soil successfully. However, if the plantlets were transferred to bigger GA-7 vessels before transplant to soil, it helped to increase the possibility of survival. The ‘Yam Hill’ plantlets subcultured on MS and NRM produced an average of 4 roots and were transferred into soil successfully.

In the second year, the antibiotics were used in the media in an attempt to further control contamination (Table 2-8). If the branches were treated with the forcing solution supplemented with 20 mg streptomycin/L (Figure 2-12), the contamination incidence dropped compared to those that were not supplemented with streptomycin. 40 mg streptomycin/L was not more effective than 20 mg streptomycin /L in reducing the contamination rate (P value=0.5706).
When the media included the antibiotics, the contamination incidence also declined (Figure 2-12, Table 2-9). 20 mg streptomycin/L was effective in reducing the contamination incidence significantly, but was not significantly from 40 mg/L. 20 mg Streptomycin/L+20 mg Kanamycin/L had a similar effect of reducing contamination, while making no difference from 20 mg Streptomycin/L alone. However, it should be noticed that different types of antibiotics included in media can slow down the selection of resistant strains.

**Conclusion**

In order to have successful tissue culture of hazelnut, taking the branches in winter and storing in cooler for 1 month is recommended. In order to reduce the contamination rate, rinsing with tap water for 20 min, followed by soaking in 10% bleach for 30 min and spraying with 70% ethanol for 90s should be effective for surface disinfestation. If the plant material is harvested in summer, then the branches can be treated with forcing solution that includes 20 mg streptomycin/L, and the single nodes or shoots can be cultured onto the media. Another suggestion is to use axillary buds, rather than softwood cuttings which can be cultured directly into shell vials containing NRM medium supplemented with 20 mg streptomycin/L + 20 mg kanamycin/L, and the plant growth regulators BA at 2.5 mg/L and IBA at 0.05 mg/L. After the bud breaks and 3-4 leaves are forced, whole explants could be transferred into bigger containers such as GA-7 vessels. The explants will probably need to be subcultured for several times until at least 3-4 roots are formed before transplanting into soil.
Figure 2-1: Effects of cooler storing time of branches on the contamination and browning incidence for *Corylus colurna* 'Te Terra Red'

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%*
Figure 2-2: Effects of tap water rinsing time of branches on the contamination and browning incidence for *Corylus colurna* 'Te Terra Red'

*The contamination incidence was calculated as the number of contaminated explants/number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/number of explants that were not contaminated X 100%
Figure 2-3: Effects of 10% bleach soaking time of branches on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%
Figure 2-4: Effects of 70% ethanol spray time of branches on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%*
Figure 2-5: Effects of explant materials (softwood cutting/buds) on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%*
Figure 2-6: Contamination and browning of *Corylus colurna* ‘Te Terra Red’ buds cultured in vitro

*Top: 20 days after bud break in vitro, the *Corylus colurna* explants being contaminated by internal pathogens.*

*Bottom: Tissue browning found 15 days after axillary buds were cultured in vitro
Figure 2-7: *Corylus colurna* 'Te Terra Red' explant contamination and browning incidence of two mother plants

*The two mother plants were from the horticulture garden at University of Nebraska-Lincoln. The branches from the mother plant were collected all the year round. A tree was the taller tree with relatively healthy leaves in summer, while tree B was observed to have most leaves with wormholes in summer.*

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%*

* Error bars stand for the standard error.
Figure 2-8: Browning rate of *Corylus colurna* 'Te Terra Red' explants cultured on 4 media containing various PGR

*MS stands for Murashige and Skoog Medium (1962); WPM stands for Woody Plant Medium (Lloyd and McCown, 1981); DKW stands for Driver and Kuniyuki Walnut Medium (1984); NRM stands for Nas and Read Medium (2004);

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%

*Error bars stand for the standard error.

*PGR stands for plant growth regulators
Figure 2-9: *Corylus colurna* ‘Te Terra Red’ explants (axillary buds) cultured in vitro that were able to grow shoots successfully.

*The explants in the third shell vial in top picture showed that right after bud break the leaf color was red, while 10 days after bud break most of the leaves turned green.*
Figure 2-10: Corylus colurna 'Te Terra Red' explants cultured on 4 media containing 2.5mg BA/L+0.05mg IBA/L

*MS = Murashige and Skoog Medium (1962);
*WPM = Woody Plant Medium (Lloyd and McCown, 1981);
*DKW = Driver and Kuniyuki Walnut Medium (1984);
*NRM = Nas and Read Medium (2002);
Error bars stand for the standard error.
Figure 2-11: *Corylus colurna* 'Te Terra Red' explants that were able to initiate roots but failed to grow in soil.

*The top picture showed that there were two root initiations, though the leaves were tiny. The middle and bottom picture showed that after transplanting into soil, the hazelnut explants were not able to grow but died*
Figure 2-12: Effects of forcing solution supplemented with streptomycin on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’

*The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%
Figure 2-13: Effects of media supplemented with streptomycin on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’

*Stre=Streptomycin, Kana=Kanamycin. *The contamination incidence was calculated as the number of contaminated explants/ number of explants cultured X 100%. After discarding the contaminated explants, browning incidence = number of browning explants/ number of explants that were not contaminated X 100%
Table 2-1: P value comparisons of cooler storing time effect on contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’ (α=0.05)

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 week and 2 weeks</td>
<td>0.0845</td>
</tr>
<tr>
<td>0 week and 1 month</td>
<td>0.0487</td>
</tr>
<tr>
<td>0 week and 2 months</td>
<td>0.0145</td>
</tr>
<tr>
<td>2 weeks and 1 month</td>
<td>0.0425</td>
</tr>
<tr>
<td>2 weeks and 2 months</td>
<td>0.0233</td>
</tr>
<tr>
<td>1 month and 2 months</td>
<td>0.0927</td>
</tr>
</tbody>
</table>

Table 2-2: P value comparisons of tap water rinsing time effect on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’ (α=0.05)

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 and 20 min</td>
<td>0.0136</td>
</tr>
<tr>
<td>0 and 40 min</td>
<td>0.0085</td>
</tr>
<tr>
<td>0 and 60 min</td>
<td>0.0097</td>
</tr>
<tr>
<td>20 and 40 min</td>
<td>0.0583</td>
</tr>
<tr>
<td>20 and 60 min</td>
<td>0.0621</td>
</tr>
<tr>
<td>40 and 60 min</td>
<td>0.0701</td>
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Table 2-3: P value comparisons of 10% bleach soaking time effect on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’ (α=0.05)

<table>
<thead>
<tr>
<th>Comparisons</th>
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<tbody>
<tr>
<td>0 and 20 min</td>
<td>0.1753</td>
</tr>
<tr>
<td>0 and 30 min</td>
<td>0.0489</td>
</tr>
<tr>
<td>0 and 40 min</td>
<td>0.0082</td>
</tr>
<tr>
<td>20 and 30 min</td>
<td>0.0522</td>
</tr>
<tr>
<td>20 and 40 min</td>
<td>0.0421</td>
</tr>
<tr>
<td>30 and 40 min</td>
<td>0.0691</td>
</tr>
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Table 2-4: P value comparisons of 70% ethanol spray time effect on the contamination and browning incidence for *Corylus colurna* ‘Te Terra Red’ (α=0.05)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 and 30s</td>
<td>0.0572</td>
</tr>
<tr>
<td>0 and 90s</td>
<td>0.0329</td>
</tr>
<tr>
<td>0 and 5 min</td>
<td>0.0289</td>
</tr>
<tr>
<td>30s and 90s</td>
<td>0.0487</td>
</tr>
<tr>
<td>30s and 5 min</td>
<td>0.0214</td>
</tr>
<tr>
<td>90s and 5 min</td>
<td>0.0627</td>
</tr>
</tbody>
</table>
Table 2-5: P value comparisons mother plants effect on contamination and browning incidence of *Corylus colurna* 'Te Terra Red' (α=0.05)

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contamination</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Browning</td>
<td>0.6443</td>
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</tbody>
</table>

Table 2-6: P value of comparisons of media effect on browning incidence of *Corylus colurna* 'Te Terra Red' (α=0.05)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS and WPM</td>
<td>0.6703</td>
</tr>
<tr>
<td>MS and DKW</td>
<td>0.0259</td>
</tr>
<tr>
<td>MS and NRM</td>
<td>0.0010</td>
</tr>
<tr>
<td>WPM and DKW</td>
<td>0.0570</td>
</tr>
<tr>
<td>WPM and NRM</td>
<td>0.0022</td>
</tr>
<tr>
<td>DKW and NRM</td>
<td>0.1019</td>
</tr>
</tbody>
</table>

Table 2-7: P value comparisons of media (2.5 mg BA/L + 0.05 mg IBA/L) effect on number of shoots of *Corylus colurna* 'Te Terra Red' (α=0.05)

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS and WPM</td>
<td>1.0000</td>
</tr>
<tr>
<td>MS and DKW</td>
<td>0.5619</td>
</tr>
<tr>
<td>MS and NRM</td>
<td>0.0408</td>
</tr>
<tr>
<td>WPM and DKW</td>
<td>0.5619</td>
</tr>
<tr>
<td>WPM and NRM</td>
<td>0.0408</td>
</tr>
<tr>
<td>DKW and NRM</td>
<td>0.1383</td>
</tr>
</tbody>
</table>

Table 2-8: P value of comparisons of concentration of streptomycin included in forcing solution effect on the contamination incidence (α=0.05)

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/L and 20 mg/L</td>
<td>0.0079</td>
</tr>
<tr>
<td>0 mg/L and 40 mg/L</td>
<td>0.0008</td>
</tr>
<tr>
<td>20 mg/L and 40 mg/L</td>
<td>0.3045</td>
</tr>
</tbody>
</table>

Table 2-9: P value of comparisons of concentration of streptomycin included in media effect on the contamination incidence (α=0.05)

<table>
<thead>
<tr>
<th>Comparisons (Stre=Streptomycin, Kana=Kanamycin)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg stre/L and 20 mg stre/L</td>
<td>0.0254</td>
</tr>
<tr>
<td>0 mg stre/L and 40 mg stre/L</td>
<td>0.0028</td>
</tr>
<tr>
<td>0 mg stre/L and 20 mg stre/L + 20 mg kana/L</td>
<td>0.0074</td>
</tr>
<tr>
<td>20 mg stre/L and 40 mg stre/L</td>
<td>0.6359</td>
</tr>
<tr>
<td>20 mg stre/L and 20 mg stre/L + 20 mg kana/L</td>
<td>0.0512</td>
</tr>
<tr>
<td>40 mg stre/L and 20 mg stre/L + 20 mg kana/L</td>
<td>0.2906</td>
</tr>
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</table>
Literature Cited for Chapter 2


# Appendix

The composition of NRM in comparison with MS, DKW, WPM and NN media

<table>
<thead>
<tr>
<th>Macrominerals (mg l⁻¹)</th>
<th>NRM</th>
<th>MS</th>
<th>DKW</th>
<th>WPM</th>
<th>NN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>530</td>
<td>1650</td>
<td>1416</td>
<td>400</td>
<td>720</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>700</td>
<td>–</td>
<td>1960</td>
<td>556</td>
<td>–</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>90</td>
<td>440</td>
<td>147</td>
<td>96</td>
<td>202</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1600</td>
<td>370</td>
<td>740</td>
<td>370</td>
<td>185</td>
</tr>
<tr>
<td>KNO₃</td>
<td>550</td>
<td>1900</td>
<td>–</td>
<td>–</td>
<td>950</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1300</td>
<td>170</td>
<td>259</td>
<td>170</td>
<td>68</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>–</td>
<td>–</td>
<td>1560</td>
<td>990</td>
<td>–</td>
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</table>

<table>
<thead>
<tr>
<th>Microminerals (mg l⁻¹)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>6.2</td>
<td>4.8</td>
<td>6.2</td>
<td>10</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>2.5</td>
<td>0.025</td>
<td>0.25</td>
<td>0.25</td>
<td>0.025</td>
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<tr>
<td>MnSO₄·H₂O</td>
<td>20</td>
<td>16.9</td>
<td>33.5</td>
<td>22.3</td>
<td>18.9</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>2.5</td>
<td>0.25</td>
<td>0.39</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
<td>ZnSO₄·7H₂O</td>
<td>8.8</td>
<td>8.6</td>
<td>–</td>
<td>8.6</td>
<td>10</td>
</tr>
<tr>
<td>Zn(NO₃)₂·6H₂O</td>
<td>–</td>
<td>–</td>
<td>17</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sequestrene 138 Fe</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>–</td>
<td>27.8</td>
<td>33.4</td>
<td>27.8</td>
<td>2.78</td>
</tr>
<tr>
<td>Na₂·EDTA</td>
<td>–</td>
<td>37.3</td>
<td>44.7</td>
<td>37.3</td>
<td>37.3</td>
</tr>
<tr>
<td>KI</td>
<td>–</td>
<td>0.83</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>–</td>
<td>0.025</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Vitamins (mg l⁻¹)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine (B₁)</td>
<td>0.60</td>
<td>0.1</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Riboflavin (B₂)</td>
<td>0.21</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nicotinic acid (B₃)</td>
<td>1.15</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Pyrodoxine (B₆)</td>
<td>0.60</td>
<td>0.5</td>
<td>–</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>α-Tocopherol (E)</td>
<td>20.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vitamin C (ascorbic acid)</td>
<td>1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Glycine</td>
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<td>2.0</td>
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<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Folic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>Biotin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>200</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose (g l⁻¹)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Agar (g l⁻¹)</td>
<td>5–6</td>
<td>10</td>
<td>–</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Gelrite (g l⁻¹)</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>–</td>
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</tr>
</tbody>
</table>

* Table from M.N. Nas and P.E. Read, 2004. A hypothesis for the development of a defined tissue culture medium of a higher plants and micropropagation of hazelnuts. Scientia Horticulturae.