VEGETATIVE PROPAGATION AND GENETIC FINGERPRINTING OF *EUCALYPTUS GRANDIS* AND *EUCALYPTUS AMPLIFOLIA*

By

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To my parents Hanlin Yang and Chuanxia Zhu

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LIST OF ABBREVIATIONS

BAP	Benzylaminopurine
DKW	Driver Kuniyuki Walnut media
GA_3	Gibberellic acid
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
NAA	1- naphthalene acetic acid
MS	Murashige and Skoog media
PGR	Plant growth regulator
PVP	Polyvinylpyrolidone
WPM	Woody Plant Media
2-iP	2-Isopentenyl adenine

Abstract of Thesis Presented to the Graduate School
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Eucalyptus grandis and *Eucalyptus amplifolia* clones may be grown as short rotation woody crops in Florida. Several propagation methods were evaluated for 4 well-known and 6 new *E. grandis* as well as 9 new *E. amplifolia* clones. The propagation methods included traditional cutting (macrocutting), mini-cutting, and tissue culture.

Healthy branches were cut into 40 mm long macrocuttings with two-half leaves. NAA commercial powder was added to stimulate rooting. Juvenile sprouts from felled trees were rooting better than the branches from the crown of the trees. Rooting response from cuttings made in fall was higher than that in spring.

After successful propagation by cuttings, some healthy plants served as stock plants for mini-cutting. Three-month-old new shoots on the stock plants were collected for mini-cutting, and the method for mini-cutting was just like the traditional cutting. Shoots from different parts of the stock plants influenced the rooting percentage; as the basal part from the lateral branches was optimal material for mini-cutting. Although the rooting percentage for mini-cuttings was not as high as for traditional cuttings, the mini-cutting could provide consistent supply of plant material.

Macrocuttings and mini-cuttings worked as stock plants for tissue culture, and nodes from 6-week-old branches worked well as explants. Driver Kuniyuki Walnut (DKW), Murashige and Skoog (MS), and Woody Plant Media (WPM) were used as the basal media for shoot multiplication.

All *E. grandis* and *E. amplifolia* clones were initiated by soaking explants in 10% bleach for 20 minutes. Shoot induction was achieved on MS without PGRs, and they were transferred to fresh media every 3 days. Then shoot multiplication was achieved using basal media containing 0.4 mg/l BAP. DKW was the most efficient basal medium for clones, especially for G1and G2. *E. grandis* shoot elongation was best on DKW containing 0.1-0.5 mg/l BAP combined with 0.1mg/l NAA. Rooting was best on DKW containing 0.5 mg/l NAA. PVP were added to media in every step to prevent phenolic compound oxidation. Media were changed at 3-week intervals to prevent vitrification, phenolic exudation and callusing.

Eight microsatellite loci were chosen for the estimation of the 60 *E. grandis* clones' allelic diversity. The result showed that *E. grandis* clones originated from 4 subpopulations. Kinship coefficients were used to estimate the genetic relatedness between pairs of these clones and the low kinship coefficient explained the genotypic response in tissue culture to some degree.

CHAPTER 1
GENERAL INTRODUCTION

Eucalyptus Genus

Eucalyptus, mostly originating in Australia, is a widely cultivated genus in the world, particularly in tropical and subtropical regions. It is a member of the family *Myrtaceae* and now comprises about 800 species, in number second only to Acacia in Australia (Brooker, 2002), ranging from straight-trunked forest trees up to 90 m tall, to multiple-stemmed, shrubby mallees (Chippendale, 1976). The popular conception of the discovery of *Eucalyptus* is the voyages of Captain James Cook in the Endeavour in the 1770s (Brooker, 2002).

Because of their high productivity in short rotation plantations, easy management, and adaptation to a wide range of environments, *Eucalyptus* clones have attracted world attention. They have multiple uses, including firewood, windbreaks, shade, pole timber, and paper pulp; species used for these purpose are limited to a small number, such as *E. grandis, E. saligna, E. globulus, E. tereticornis, E. nitens* and *E. dunnii*. Most of them are tall trees and naturally originated from wet forests in Australia. Some other smaller trees in the genus are grown in drier and less fertile regions. Most *Eucalyptus* species of the arid interior Australia are usually found in the regions along the seasonal streams and in the rocky hills (Brooker, 2002). In addition, one of the well known characteristics is that some *Eucalyptus* leaves have oil glands, which can provide industry oil.

Eucalyptus plantation have underwent rapid expansion around the world (Edgrand Campinhos, 1999); demand for *Eucalyptus* market pulp has been growing at 11.2 percent/yr since 1980 (Wilson et al., 1995).

Eucalypts were first planted in Florida in 1878, but industrial plantations were not established until 1972. *E. grandis* was found through species trials to be a suitable species for

south Florida (Geary et al., 1983). Freeze hardy *E. amplifolia*, was suited to good sites in northeastern Florida and *E. camaldulensis* was suitable for a wide range of sites in central and southern Florida (Rockwood et al., 2006). They are highly productive in Florida.

Propagation Method

There are two traditional ways to propagate *Eucalyptus*, sexual propagation by seed and asexual propagation (vegetative propagation). Propagation by seed is the major method by which plants reproduce in nature, and is one of the most commonly used methods for culture. Since sexual propagation can combine the characteristics of parents during the cross fertilization to produce some new genotypes, it is the basis for breeding.

From the point of view of breeding and seed production of *Eucalyptus* for wood production, the distinctive characteristics of sexual reproduction of *Eucalyptus* are:

- Flower is hermaphrodite (male and female in one flower);
- Pollination is by animals, mainly insects and birds, not by the wind;
- Outcrossing is favored by various mechanisms, including protandry, which means that the stigma is not receptive until some days after the pollen has started shedding from the anthers;
- Fruits are dry woody capsules and seeds are very small in the species used for fast-growing plantations;
- Seed production varies enormously due to non-genetic factors such as spacing, site and seasonal conditions.

Vegetative propagation differs from seedling propagation in that all members of population have originated from a single plant and are expected to possess the same genotype. Vegetative propagation is very important when it is essential to maintain the same characteristics of the genotype. This practice is possible because of adventitious shoots and roots. Vegetative propagation methods include cutting, grafting, and layering, among which cutting is the most efficient and widely used method for many species.

Micropropagation, also called tissue culture, develops whole plants from plant cells or organs in an aseptic *in vitro* environment. The successful plant tissue culture was first accomplished by Gottlieb Haberlandt in the 19th century (Caponetti et al., 2005). Common steps of the micropropagation procedure include donor plant selection, establishment, shoot multiplication, pre-transplant and acclimatization.

Objectives

Objective 1. Develop vegetative propagation protocols for *Eucalyptus grandis* and *Eucalyptus amplifolia* clones by cutting, mini-cutting, and micropropagation.

Objective 2. Genetically fingerprint the clones to find the genetic relatedness

CHAPTER 2
LITERATURE REVIEW

Ease of Vegetative Propagation

In tree breeding, vegetative propagation is commonly used for the establishment of clone banks and clonal seed orchards, and can be utilized for the production (Doran, 2002). Mass propagation by stem cutting is the common method to establish clonal forestry, because it is less expensive. Micropropagation is an alternative method of propagation.

Cutting Method

Cutting propagation is the most important means for clonal regeneration, and adventitious root formation is prerequisite to successful cutting propagation. Propagation by stem cutting requires the formation of new adventitious root systems since a potential shoot system (a bud) is already present.

Cuttings include 3 kinds in general: hardwood cuttings, semi-hardwood cuttings and softwood cuttings (Hartmann, 2002). Cutting is the preferred method of conventional vegetative propagation, but cuttings from most mature *Eucalyptus* are not able to root (Paton et al., 1970). However cuttings from seedlings and juvenile trees did root well and manipulation of the state of juvenility of the shoots had allowed cuttings to be used with great success in plantations of *E. grandis* and various other species. Shoots which sprouted from the stump when a tree was felled or girdled had juvenile characteristics including the ability to produce adventitious roots. To develop the desired genotypes, trees with the superior characteristics were felled or girdled. Cuttings were taken from the coppice shoots, and further selection was made for individuals with high rooting ability. Rooting percent of 80% or more were reported for *E. grandis* and the method allowed production of millions of trees per year (Campinhos and Ikemori, 1977). The method could be extended to the other species, but it was not always possible to fell the superior

trees, because this species did not sprout from stumps. In such cases auxin application to the stump promoted sprouting (Davidson, 1977). Damage to the base of a standing tree induced epicormic shoots and Mazalewsky and Hackett (1979) promoted basal sprouts of a 2-meter high *E. ficifolia* using cytokinin, but it was not known if this method worked well with mature trees in the forest.

Management of stock plants to maximize rooting begins with the selection and maintenance of source material that is easy-to-root, rejuvenation of stock plant materials and selection of cuttings from stock plants.

Mini-Cutting Method

Mini-cuttings are similar to traditional rooted cuttings except that they are based on rooting of axillary sprouts from rooted stem-cuttings (or mini-hedges) (Romero, 2004). The advantage of mini-cutting systems over conventional rooted cutting programs is that re-propagation of clones could be extremely fast and could reduce the conventional clonal deployment cycle by 2-3 years (which is needed to produce enough cuttings to establish an outdoor clonal garden with traditional rooted cuttings) (Romero, 2004).

Plant Tissue Culture Media

The totipotency of plant cells was the basis for plant tissue culture. The first successful plant tissue and cell culture was created by Haberlandt in 1902 (Krikorian and Berquam, 1969). By the 1970s, the role of five major classes of plant growth regulators in tissue culture had been recognized and investigated. They are auxins, cytokinins, gibberellins, ethylene and abscisic acid (Caponetti et al., 2005).Murashige and Skoog's (MS) (1962) medium, developed originally for tobacco, by Murashige in Skoog's lab is now the most popular basic media for various species with high salt media for its K and N content (Beyl, 2005). WPM developed by Lloyd and McCown (1980) is very commonly used for woody trees.

Sugar is a necessary component in medium because explants in vitro are unable to photosynthesize effectively. Twenty to 60g/l sucrose is most commonly used concentrations. Alternative sugars could be glucose, maltose, or lactose (Coffin et al., 1976).

Vitamins are organic substances that function as metabolic substances and enzymes. Of the vitamins, only vitamin B1 (at 1-5 mg/l) was essential in culture because it was involved in carbohydrate metabolism and the biosynthesis of some amino acids (Beyl, 2005).

Activated charcoal is useful for absorption of brown pigments and oxidized phenolic compounds. 0.2-3% (w/v) concentration proved feasible and absorbed some PGR and vitamins (Nissen and Sutter, 1990). In addition, polyvinylpyrrolidone (PVP, 250-1000mg/l) and antioxidants, such as citric acid, ascorbic acid or thiourea, reduced the inhibitory effects of polyphones (Beyl, 2005). Agar is the most commonly used gelling agent to solidify the culture media. It physically supports plants to contact with medium while allowing aeration. Generally, researchers use agar at concentrations between 0.5% and 1.0%. Too high concentrations lead to poor growth; too low concentrations of agar causes a layer of liquid to form on the top of the gelled medium and results in hyperhydric plants (Singha, 1982). Agarose and gelrite are alternative gelling agar.

Adventitious shoot formation generally requires auxin and cytokinin. Auxin is involved in cell elongation. IAA is a natural auxin, but it is destroyed by light or autoclave; other auxins are preferred, like IBA and NAA, which are light and heat stable. 2,4-D is a strong promoter of callus induction and growth, but it is not common for tree tissue culture. Cytokinin's main function is to promote cell division and stimulate initiation of shoots, including BA, kinetin, 2-iP and Zeatin (Beyl, 2005).

Micropropagation of *Eucalyptus*

Explant and Culture Sterilization

Roots, lignotubers, anthers, shoot tips and microcuttings were used as the explants to establish micropropagation for *Eucalyptus* (Leroux and Vanstaden, 1991).

The surface sterilization of *Eucalyptus* explants for in vitro culture was relatively easy if juvenile material was available. The sterilization of mature, field- grown material proved difficult because of endogenous microbial contamination. Seedling and juvenile plants growing in glasshouses were easier to sterilize, especially if care. Taking appropriate care of stock plants was the major method to reduce the contamination (Leroux and Vanstaden, 1991).

Eucalyptus tissue culture initially concentrated on callus culture from young seedlings (Sussex, 1965; Jacquiot, 1964; Bachelard and Stowe 1963). Use of the nodes as microcuttings without multiplication was the subsequent objective and was achieved with *E. grandis* (Cresswell and de Fossard, 1974; Cresswell and Nitsch, 1975). Hartney and Barker (1980) reported media for multiplication and rooting for seedling nodal segments of 12 *Eucalyptus* species.

Germinated seedlings of *E. erythronema* *E. stricklandi* hybrid (gem) from sterilized seeds were used as the explants and seeds were sterilized in 70% ethanol for 30 second and then 3% NaOCl for 20 min (Glocke et al., 2006). A similar method was reported for culture of *E. regnans* (Mountain ash) (Blomstedt et al., 1991), but the main difference was that the explant for former was the germinated shoots but for latter were seedlings nodes.

Sterilization efforts were unsuccessful in destroying the surface contaminants while keeping the buds viable at the same time. *Eucalyptus* tissue was often killed by sterilizing solutions (Cresswell and de Fossard, 1974). A severe sterilization treatment reported by Holden and Paton (1981) included 75 min in saturated calcium hypochlorite, followed by 4h of UV

irradiation. Using the field-grown *E. grandis*, they obtained minimal contamination and 50% survival of explants; 5% of these eventually produced shoots. When $HgCl_2$ was used for *E. marginata*, all shoots were dead. Comparisons among sodium hypochlorite, calcium hypochlorite, mercuric chloride and zephiran (benzalkonium chloride) had shown that zephiran resulted in comparable levels of contamination, but improved levels of survival compared with the other sterilization methods. Treatment of 15-second in 1% zephiran, followed by 10% alcohol resulted in 50-90% clean explants and 10 - 70% survived to produce shoots, whereas using sodium hypochlorite was common for all clean shoots to die (Durand-Cresswell et al., 1982).

Direct Shoot Culture

There were many reports concerning direct shoot cultures for commercially important *Eucalyptus* species, including *E. grandis* (Sita and Rani, 1985; Wachira, 1997), *E. niten* (Gomes and Canhoto, 2003), *E. globules* (Salinero, 1983; Trindade et al., 1990; Bennett et al., 1994; Schwambach et al., 2005), *E. tereticornis* (Das and Mitra, 1990; Sharma and Ramanurthy, 2000; Rao, 1988), and *E. torelliana* (Gupta, 1983). BAP alone or in combination with NAA were used to induce and multiply shoots in most of the reports, and IBA or NAA were used for rooting. Sometimes GA was used for elongation before rooting as the PGR. Some pretreatments were used to prevent the oxidation of phenolic compounds produced by *Eucalyptu*s.

Organogenesis

Significant progress has been made with regeneration of some *Eucalyptus* species via organogenesis, in species such as *E. camaldulensis* (Muralidharan and Mascarenhas, 1987), *E. grandis* (Warrag et al., 1991) and *E. globulus* although there are some limitations.

Eucalyptus hypocotyls were reported to be more responsive to culture than other types of explants (Kithara and Caldas, 1975). The type of explants of *E. grandis* used for callus induction

was investigated by Hajari et al. (2006), and the shoots, stems and leaves did not show significant differences.With the exception of studies of Warrag (1991) who employed NAA and kinetin and Cid et al. (1999), who used TDZ, all reported organogenic callus induction in *Eucalyptus* has been accomplished with the combination of NAA and BAP (Muralidharan and Mascarenhas, 1987; Laine and David, 1994; Tibok, et al. 1995; Azmi, et al. 1997; and Mullins et al., 1997).

The organogenesis could provide a large number of plantlets for general vegetative propagation; however it may not be optimal for genetic engineering purposes. Somaclonal variation was possible through this organogenesis (Warrag, 1991).

Somatic Embryogenesis

Callus cultures have been established from a number of *Eucalyptus* species explants from both juvenile and adult plant materials (Durnad-Cresswell et al. 1982). The most used hormones were the auxins IAA, NAA and IBA alone, or in combination with the zeatin or BAP to induce callus (Kitahara and Caldas, 1975; Bennett and McComb, 1982; Durnad-Cresswell et al., 1982).

Enhanced somatic embryogenesis was achieved on B5 medium supplemented with 5mg/l NAA for *E. citriodora* (Muralidharan and Mascarenhas, 1987). *Eucalyptus* somatic embryogenesis was reported by Sita (1986) and Ouyang et al. (1981), where they did not definitely identify somatic embryos. Sita (1986) described formation of embryo-like structures, which did not develop into plantlets. More recently, somatic embryogenesis was reported for *E. nitens* and *E. globulus* (Bandyopadhyay and Hamill, 2000); but plantlet regeneration was not achieved. Pinto et al. (2004) reported the regeneration of somatic embryos from juvenile explants of *E. globulus* for the first time.

Genetic Variation

DNA Marker

Genetic variation of trees was traditionally studied by progeny tests and provenance trials, although the methods are expensive and laborious. Genetic markers were introduced after 1980s to make the genetic variation analysis easier.

Genetic marker is a known DNA sequence, which could be described as a variation. It could range from a single base-pair change, to a long one. There are commonly used types of genetic markers, including Restriction fragment length polymorphism markers (RFLP), Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), Microsatellite (SSR), and SNP (Single nucleotide polymorphism).

These markers could be further categorized as dominant and co-dominant markers. Dominant markers allow for analyzing many loci at one time, e.g. RAPD and it is fast and efficient. But, in diploids, a band obtained if either or both of the homologous chromosomes contain an amplifiable sequence, which underlie the genotypes (Falush et al., 2007). On the contrary, co-dominant could discriminate heterozygous from homozygous state in diploid organism (Weising et al., 2002). When a genetic marker is co-dominant all genotypes are distinguishable from one another (Holsinger et al., 2002).

For most plants, more than 60% genomes are believed to be composed of repetitive sequences (Kubis et al., 1998). Microsatellites, or simple sequence repeats (SSRs), are 1-6 bp tandem repeated DNA motifs which may vary in the number of repeats at a given locus, and most tandem repeats are non-coding DNA. The number of copies of repeats commonly varied in different individual organisms. Microsatellite could be detected by PCR because DNA with more repeats had longer amplified fragment after synthesize in PCR reaction. Amplified fragments

were separated with the use of gel electrophoresis to produce a serious of bands, which quantify the population genetic difference (Pierce, 2005).

The SSR assay is easy to perform and often produces very polymorphic markers, and it is a highly attractive tool for studies at the population level (Echt et al., 1996), mainly because they are typically co-dominant and display many alleles per locus (Hardy, 2002). In addition, they are abundant dispersed in plant genomes and highly polymorphic allowing precise discrimination even of closely related individuals (Brondani et al., 1998).

The disadvantage of SSR is that it is time-consuming to identify the single-locus marker from genomic library for new species. Thus the use of this marker has some limitation. Another disadvantage is the null allele by SSR, resulting in the underestimates of heterozygosity (Wang and Szmidt, 2001).

The presence of microsatellites in plant genomes was first reported in forest trees (Conditt and Hubbell, 1991). Then microsatellites have been reported in several forest-tree species such as *Pinus radiata* (Smith and Devey, 1994), *Pinus strobus* (Echt et al., 1996), and *Swietenia humilis* (White and Powell, 1997). In *Eucalyptus nitens,* 4 microsatellites were reported (Byrne et al., 1996).

Data Analysis for Markers

Population genetics focus on the genetic variation in terms of origin and distribution in populations of organism at or below species level (Templeton, 2006). Normally, mutation, nonrandom mating, natural selection, genetic drift and finite population size result in gene frequency change, which lead to population structure. Population structure always includes system of mating for population, size of population and genetic change with other populations (Templeton, 2006). H_e (Heterozigosity) and F_{st} (fixation index) are often used to measure

structure at population and subpopulation level while different methods are used to quantify genetic distance between subpopulation or individuals.

How the different markers were applied to detect population structure was addressed by Pritchard and Roseberg (1999). The model assumed that there were K populations characterized with different allele frequency at each locus, and individuals were classified into different populations on the basis of their genotypes (Pritchard et al., 2000).

Broadly speaking, there are two ways to cluster genetically similar individuals: distance-based methods and model-based methods. Distance-based methods estimate genetic distance between every pair of individuals, thus they are easy way; model-based method are run on the assumption that individuals from clusters are randomly taken from parametric model, which are finished by statistical method (Pritchard et al., 2000).

Model-based clustering methods were developed by Pritchard. The program structure clusters the individual organisms into different populations on the basis of genotype data from SSR (Pritchard et al., 2000). The model assumed that markers are not in linkage disequilibrium within subpopulations. The version 2.0 allows the use of null alleles, which offset the disadvantage of SSR.

In the data file, genotype data and missing genotype data are input. Some modeling are needed to decide by users by structure, including how long to run the program, what the ancestry model is (no admixture or admixture), and allele frequency models (independent or correlated). In the estimation of K, different values of K are run and the output of 'Estimated Ln Prob of Data' is the result of Pr(X/K). Many runs should be needed to verify the consistency of the result. If the variability in different run is high, longer runs are needed (Pritchard et al., 2000).

If ancestry model is admixture, the vector Q is used to represent proportion of individual originated from population K (Pritchard et al., 2000). Different forms of plots are shown in the output to reflect Q and F_{st}. In the text output for each individual, inferred clusters, estimated membership in each cluster, and allele frequency are showed.

Spatial Pattern Analysis of Genetic Diversity (SPAGeDi) is a computer package, designed to analyze the spatial genetic structure of mapped individuals and populations using genotype data of ploidy level (Hardy and Vekemans, 2002).

The program requires the information for each individual: one to three spatial coordinates, value of a categorical variable, and genotype at each locus of a codominant marker (missing data are allowed). The program first computes pairwise statistics showing genetic relatedness for all pairs of individuals. Then by averaging the pairwise statistics and regressing them on spatial distance and on logarithm, the association between these values and pairwise spatial distances are obtained (Hardy and Vekemans, 2002).

At the individual levels, many different statistics were calculated in the program, including: kinship coefficient (Loiselle et al., 1995; Ritland, 1996), relationship coefficient by Queller and Goodnight (1989), Hardy and Vekemans (1999), Lynch and Ritland (1999), and Wang (2002); fraternity coefficient described by Lynch and Ritland (1999) and Wang (2002); and genetic distant measure by Rousset (2000). At population level, Fst, Rst (an Fst analogue but based on microsatellite allele size, Slatkin 1995), and Ds (Nei's 1978 standard genetic distance) are provided (Hardy and Vekemans, 2002).

CHAPTER 3
MATERIALS AND METHODS

Twenty-three clones were propagated by traditional cutting, mini-cutting, and/or micropropagation (Table 3-1).

Cutting Method

Plant Material

Four well-tested *E. grandis* clones (G1, G2, G3, and G4) were initially selected. Thirteen-year-old trees with the required superior growth condition were felled in Tampa Port Authority, Tampa, FL, USA in 2005.

New clones were also tried, including 6 *E. grandis* clones (G34, G36, G37, G43, G48, and G51) from Southwest Ranches, 9 *E. amplifolia* clones (A1, A2, A3, A4, A5, A6, A7, A8, and A9), and 4 *C. torelliana* clones (T1, T2, T3, and T4) from C&B Farms, Clewiston (Table 3-1).

Method

Four well-tested *E. grandis* clones (G1, G2, G3, and G4) were taken by 3 stocks to test the factors affecting the cuttings, including the origin of the cuttings and the season of the cutting.

First stock of materials was taken in August, 2006 from the crown of some 13-year-old trees. After that, the old trees were cut down to induce new sprouts. The second and third stocks of materials were taken from the new sprouts of the felled trees in November 2006 and June 2007, respectively. The comparison between first and second stock indicated which origin of cuttings was better for propagation. The difference between the second and third stock showed the different season's effect on the efficiency of rooting the cuttings.

Table 3-1. Location, age (years) and clone numbers by 3 methods (cutting, minicutting, and microcutting) for *E. grandis*, *E. amplifolia* and *C. torelliana* clones

clone information				propagation method and time					
Species	clone	Location	Age	Aug,06 cutting	Nov,06 cutting	May,07 cutting	Jan,08 mini	Jun,07 micro	Sep,08 micro
E.g	G1	TPA	13	42	44	44	75	135	
	G2	TPA	13	92	61	61	78	85	
	G3	TPA	7	50	89	89	98	69	
	G4	TPA	13	84	229	229	109	90	
	G34	SRWC-100	4			36	146	280	
	G36	SRWC-100	4			49	48		
	G37	SRWC-100	4			58	79	147	10
	G43	SRWC-100	4			38	67		12
	G48	SRWC-100	4			35	57		
	G51	FC	2			90	46		
E.a	A1	SRWC-80	8			14			
	A2	SRWC-80	8			14			
	A3	SRWC-80	8			11			
	A4	SRWC-80	8			6			
	A5	SRWC-80	8			17			
	A6	SRWC-80	8			58			20
	A7	SRWC-80	8			16			15
	A8	SRWC-80	8			32			18
	A9	SRWC-80	8			26			18
C.t	T1	C&B	15			16			
	T2	C&B	15			19			
	T3	C&B	15			20			
	T4	C&B	15			27			

New clones of *E. grandis* (G34, G36, G37, G43, G48, and G51), and *E. amplifolia* (A1, A2, A3, A4, A5, A6, A7, A8, and A9) were tried to determine the most efficient method for propagation.

Healthy branches with leaves were selected and cut into 30 - 40 cm sections as the starting materials. The branches were covered with wet paper towel, placed into the polyethylene bags in a container filled with ice and transported back to greenhouse. Collection date and clone number were recorded on the bag. As possible, all the cuttings were made as soon as they got in greenhouse. If not, branches in polyethylene bags were placed in cooler for up to 3 weeks. All plant materials involved in the experiments were made cutting very soon.

Cuttings were made by re-cutting the branches into 10-12cm long sections, with 2 nodes each, one near the upper segments and the other near the base. Two half-leaves were left on the cutting. They were put in the 20% Scotts Banrot® Broad Spectrum fungicide (4% wettable powder) for 5 seconds and then 2 cm base of the segments were treated by dipping into Green light® F Rooting Hormone (active NAA 0.1%) powder to induce rooting. Cuttings were inserted vertically 5cm to 7 cm into deepots containing moist medium consisting of 1 peat: 1 perlite: 1 vermiculite (v:v:v). Fifty cuttings for each treatment with 2 replications were made.

After 8 weeks, rooting percentages were recorded. Rooted cuttings were transplanted into deepots containing the same medium and retained under intermittent for 4 weeks. Then they were transplanted into 15 cm diameter big pots and moved out of the greenhouse to the shed outside and misted every 30-min. After another 8 weeks, they grew up to 1 m high and were taken to the field.

Mini-Cutting Method

Plant Material

The healthy macrocuttings could work as the stock plants for mini-cuttings after they were successfully propagated. Clones of *E. grandis* G1, G2, G3, G4, G34, and G37 were tried.

Method

Twenty weeks after the macrocuttings rooted, the mini-cuttings were made by cutting the macrocutting branches into 7-8 cm long sections, with 1 node near the upper segments and the other near the base. Cuttings from the main stem, the top part of the lateral branches and the base part of the lateral branches were 3 treatments to determine the optimum part of the stock plant's for most efficient rooting. All the cuttings were left with 2-half leaves. Mini-cutting were dipped into Rootone® F BrandRooting Hormone (0.01% NAA) powder, and then put into the soils in the tubes. Twenty-five cuttings for each treatment with 2 replications were made. After 8 weeks, rooting percentage was recorded.

In addition, in order to see how long the macrocuttings worked as stock plants, mini-cuttings were collected after stock plants surviving 3, 6, and 9 months. Also mini-cutting and traditional cutting rooting efficiency were compared.

Micropropagation

Plant Material and Sterilization

Stem cuttings and mini-cuttings which survive successfully may serve as donor plants for micropropagation. *E. grandis* (G1, G2, G3, G4, and G34) and *E. amplifolia* (A6) clones which propagated successfully as cuttings and mini-cuttings were used as the source of explants in tissue culture.

Healthy, vigorous and green branches were collected and immediately placed into water containing 2 drops of Tween-20. All the adhering dust was removed by washing with tap water

for 1 hour. Then cuttings were cut to smaller sizes and underwent sterilization. The small cuttings were immersed into a beaker with or without 70% ethanol for 30 seconds, followed by soaking in 10% to 20% bleach solution with 2 drops of 2-tween for 20 minutes to find best sterilization method. Constant agitation carried out in a laminar transfer. Then cuttings were rinsed in sterilized water for 3 times to remove the chlorine. All these procedures were performed in the laminar flow hood under aseptic conditions.

The following procedures and conditions were shared in all the steps. The basal media of MS or DKW was used. The other components in the media included 8g/L agar, 20g/L sucrose and 1g/L PVP. All media were adjusted to pH between 5.6 and 5.7 and autoclaved at 121^{o}C for 30 min.

Establishment of Cultures

Explants were cultured in Petri dishes (100*25 mm) containing pre-initiation medium MS to screen for contaminated explants. Soaking, charcoal, and PVP in media were tested to find optimal phenolic chemicals prevention method. The contaminated and phenols-producing explants were thrown away. After 7 days, decontaminated explants were cultured on MS with 0.5mg/l BAP, Kinetin, 2-iP (no PGR as control) to induce shoot regeneration.

Shoot Multiplication

MS, DKW, and WPM were used as the basal media. Basal media supplemented with different concentrations of BAP (0.1, 0.4, and 1 mg/L) individually and in combinations with NAA (0.1mg/l) were used for shoot multiplication. Fifteen shoots per clone from the initiation step were assigned randomly to each treatment. Five explants were placed in one Petri dish for one treatment with 5 replications. Shoot multiplication was assessed after 3 weeks in culture by counting clearly visible shoots approximately 2 mm or longer.

After the first round, explants continued to generate new shoots, and subculturing provided further multiplication. Shoots that formed were subdivided and subcultured in fresh multiplication medium every three weeks. Eight generations of subculture were continued and subculture multiplication rates, i.e, how many shoots longer than 2 mm were produced per shoot, were recorded. Samples used to get data were the third generation shoots.

Shoot Elongation and Rooting

DKW was used as the basal media. Various combinations of NAA and BAP were tested to optimize the propagation system. NAA and BAP combinations test were: 0.1/0, 0.1/0.1, 0.1/0.5, 0.5/0, 0.5/0.1, 0.5/0.5, 1/0, 1/0.1, and 1/0.5 (mg/l). Shoot length (average length for longest 3 shoots for one cluster) and shoot multiplication rate for each subculture was recorded after 3 weeks. Five explants were placed in one Magenta vessel (Magenta, Chicago, Il, USA) with 3 replications for one treatment. Samples used to get data were the third generation shoots.

The individual shoots longer than 30 mm were picked and the basal leaves were discarded to insert vertically into the Magenta vessels containing MS with NAA (0, 0.5, 1, or 2 mg/l). Different concentrations of basal medium (1/2 MS and MS) were tried and 0.1-2 mg/l NAA or IBA was added; rooting percent, rooting length and rooting number were recorded after 3 weeks. Five replications for every treatment for rooting were recorded.

Acclimatization

Plantlets with roots no longer than 10 mm were transferred to a greenhouse. Agar was removed from propagules without root damage and roots were washed in a solution of fungicide to prevent contamination. Plantlets were planted in trays containing a potting mixture (vermiculite: perlite = 1:1 (v: v)) and maintained under the plastic lids for 1 week. Plantlets were then maintained under mist for 4 weeks. The mist lasted 30 seconds every 2 minutes.

Data Collection and Statistical Analysis

The design of all experiments was a complete randomized block. For cutting, each treatment involved 5 to 10 plants with 3 time replications. For multiplication and elongation, 15 replications were used. Rooting treatment was replicated for 5 times. Graph Prism 4.0 was used for data ANOVA.

Genetic Fingerprinting

Plant Material

Sixty *E. grandis* clones (Appendix C) in Florida were obtained as the plant material for DNA extraction. Total genomic DNA was extracted from adult leaves. The protocol was described earlier (Brondani et al., 1998).

Polymorphism of Microsatellite Loci

Eight microsatellite loci (EMBRA2, EMBRA28, EMBRA10, ES76, EMBRA37, EMBRA63, EG62 and EG65) were selected for genetic information. These loci were developed earlier of Brondani et al. (2002). PCR amplification was performed and products were separated and sized according to method by Brondani et al. (1998). The allele size and frequency for each locus were recorded. Expected heterozigosity (H_e) were estimated for each locus by $H_e = 1-\sum (P_i)^2$ where P_i is the frequency for i th allele.

Population Structure

We used Structure 2.0 to analyze the population structure. This is a model-based clustering method for inferring population structure using genotype information obtained from markers.

The model assumes that there are K populations, which are characterized with different allele frequencies at each locus. How long to run the program is one of major issues to consider and two factors requiring decision: the burnin length (run the simulation before collecting data to minimize the effect of the starting configuration) and how long to run the simulation after the

burning to obtain an accurate parameter estimate. Normally, a burning of 10,000-100,000 is preferred.

The number of individuals (60), the number of loci (8), and value of missing data (-9) were entered in data input. When setting the parameter, the run length burning was 30,000 and the number of MCMC steps after burning was 50,000. The ancestry model selected was Admixture with population data, and allele frequency model was selected as Allele frequency independent. The model was run after K value (the number of populations) was set from 1 to 6. Then the results were compared.

SPAGeDi

SPAGeDi estimated genetic distances between populations or relatedness coefficients between individuals using genotype data from markers (Hardy and Vekemans, 2002).

During loading the datafile, name of the desired SPAGeDi file was entered, and analysis level selected: population (F statistics) or individual (kinship coefficient). The observed allele number and frequency for 8 loci were used to generate the 3600 kinship coefficients (Loiselle et al., 1995) reflecting genetic distance between each pair of 60 individual clones.

Spatial genetic structure was analyzed using kinship coefficients estimated relative to a sample of genotyped individuals between 2 individuals (Table 3-2).

Table 3-2. Relationship and kinship coefficient (Weir et al., 2005)

	k_2	k_1	k_0	$\Theta = k_1/4 + k_2/2$
Twins	1	0	0	0.50
Full-siblings	0.25	0.5	0.25	0.25
Parent-child	0	1	0	0.25
Double-first cousins	0.125	0.375	0.5625	0.125
Half-siblings	0	0.5	0.5	0.125
First cousins	0	0.25	0.75	0.0625
Unrelated	0	0	1	0

k_0 = the probability of two individuals sharing 0 alleles
k_1 = the probability of two individuals sharing 1 allele
k_2 = the probability of two individuals sharing 2 allele
θ is kinship coefficient

CHAPTER 4
RESULTS AND DISCUSSION

Propagation by Cuttings

All the plant materials involved in the experiments below were made cutting very soon after collection. Collected plant materials kept in cooler up to 3 weeks exhibited drying, resulting in reduced rooting efficiency.

Different genotypes influenced the rooting percent no matter what methods were used. Source of plant materials affected the rooting percent significantly. Control treatment (no auxin added) for 2 sources were both 0 (data not showed). The rooting percent (24%-52%) from stump sprouts was significantly higher ($p<0.001$) than that from top of the crown (0 to 2%) for all genotypes. The crown shoots induced at most 2% rooting, and was therefore not a good source for cutting material. Although the top crown produced more juvenile materials compared with the other parts of the crown, it was not juvenile enough. The stump sprouts were more juvenile leading to higher rooting efficiency (Figure 4-1).

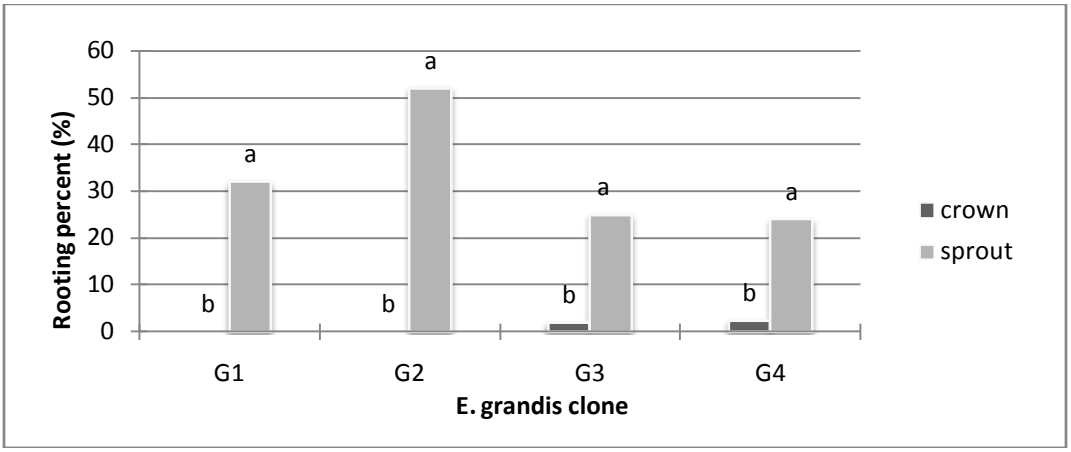

Figure 4-1. Cutting rooting percent from 2 sources (crown and stump sprouts) for 4 well-tested *E. grandis* clones (G1, G2, G3, and G4). Ten plants were assigned to each treatment with 3 replications for each clone. Within clones, means for a given origin with different lower case letters indicate significant differences according to Bonferroni's HSD Multiple Range Test at $p<0.05$ level.

Rooting success from top crown cuttings did not vary much by genotypes. The lowest percentage was 0 for G1 and G2, and the highest percent was 2.3% for G4. There was significant difference among genotypes from stump sprouts, with 52% rooting for G2, followed by G1 with 32%. The rooting percent for clones G3 and G4 was only 24-25% (Figure 4-1).

Cutting season also affected rooting efficiency. Generally, branches from stump sprouts in fall rooted better than in spring. Clone G4 rooted better in spring than in fall, but the difference was not significant. The season, genotype and interaction of the season and genotype were significantly different (Figure 4-2).

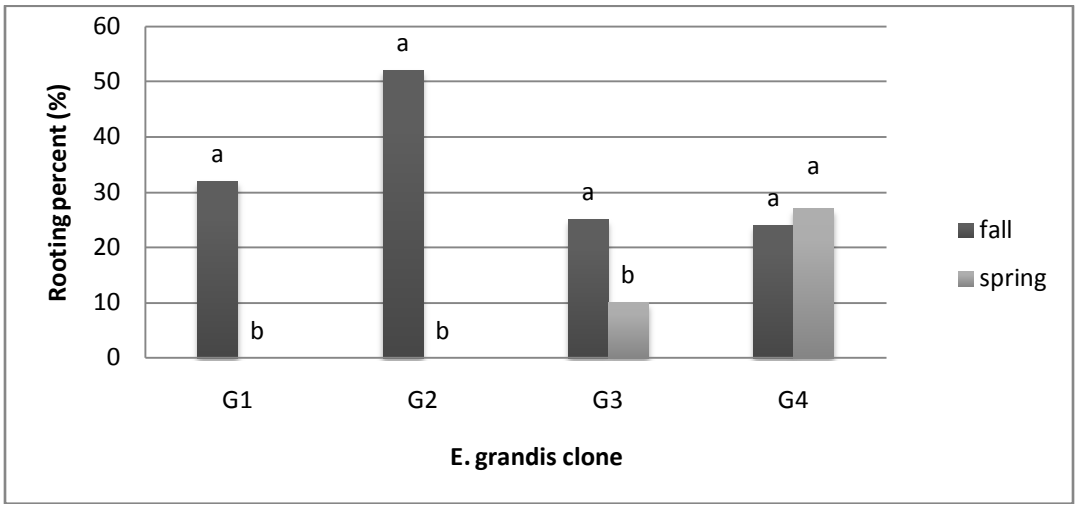

Figure 4-2. Cutting rooting percent in 2 different seasons (fall and spring) for 4 well-tested *E. grandis* clones (G1, G2, G3, and G4). Ten plants were assigned to each treatment with 3 replications for each clone. Within clones, means for a given time with different lower case letters indicate significant differences according to Bonferroni's HSD Multiple Range Test at $p<0.05$ level.

Cuttings from stump sprouts were made for 6 new and 4 old clones of *E. grandis* in fall. All the clones rooted, but the rooting percent varied greatly among different clones, from 3% for G37 to 95% for G34. New clones G37, G48, and G51 rooted poorly with only 3% to 6%. New clone G36 had similar rooting to old clones, including G1 and G4. New clone G34 achieved 95% rooting and was easier to root than the old well-tested clones (Figure 4-3).

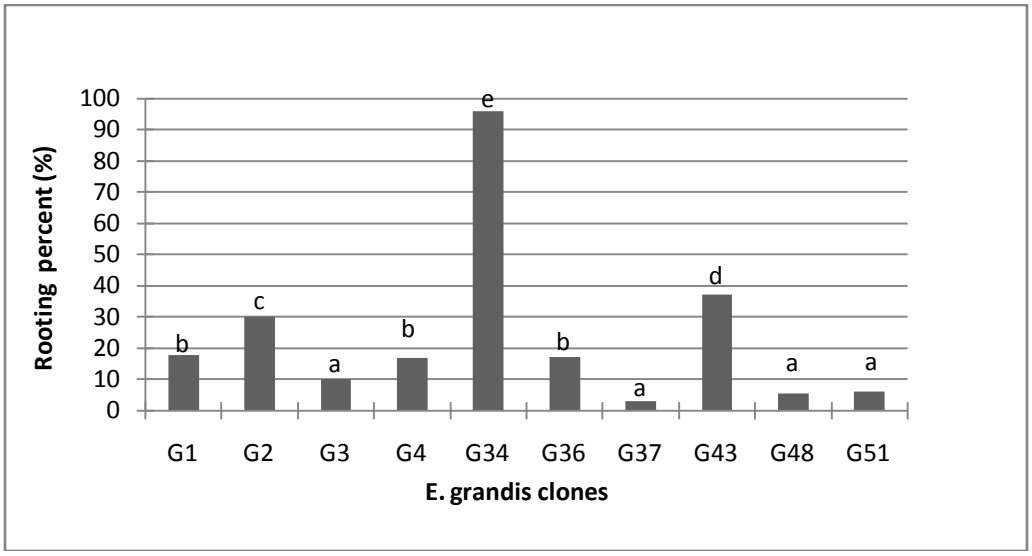

Figure 4-3. Cutting rooting percent for 10 *E. grandis* clones (G1, G2, G3, G4, G34, G36, G37, G43, G48, and G51) in fall. Ten plants were assigned to each clone with 3 replications. Means with same letters are not significant different according to Tukey's HSD Multiple Range Test at $p<0.05$ level.

Cuttings for nine *E. amplifolia* clones had lower rooting efficiency than *E. grandis*. Clones A1, A2, A3, and A4 failed to root, while clones A9, A5, and A7 achieved only 3.85%, 5.88%, and 6.25% rooting rate, respectively. Clones A6 and A8 had the highest rooting percentages of 17% and 25%, respectively (Figure 4-4).

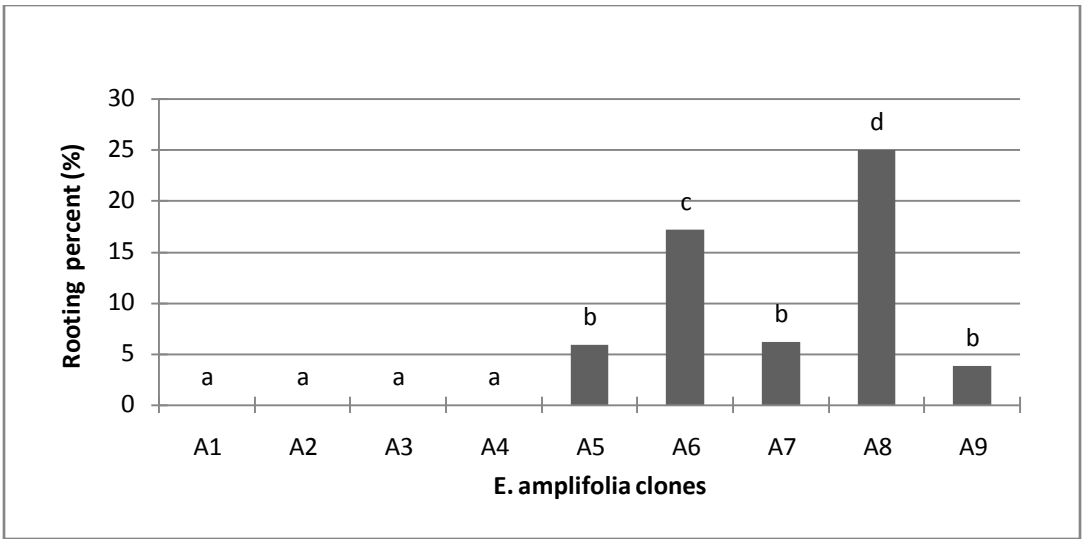

Figure 4-4. Cutting rooting percent for nine *E. amplifolia* clones (A1, A2, A3, A4, A5, A6, A7, A8, and A9). Five plants were assigned to each treatment with 3 replications for each clone. Means with same letters are not significant different according to Tukey's HSD Multiple Range Test at $p<0.05$ level.

Wounding-related compounds (WRC) were produced at the surface of the cutting place and resulted in destruction of cell compartment, which, thereafter, released catabolic enzymes. WRC improved rooting, by increasing auxin uptake or reducing conjugation or oxidation of auxin (Klerk et al., 1999).

The traditional methods of vegetative propagation were not successful when used on adult-phase trees for many *Eucalyptus* species (Cresswell and Nitsch 1975). *Eucalyptus* species rooted easily from stem tissue if leafy cuttings were taken from young seedlings (Pryor and Willing, 1963). *E. grandis* had also been successfully propagated by stem cuttings by Paton et al. (1970), but there were no previous reports regarding rooting of *E. amplifolia*.

Previous reports indicated that potential rooting ability declined with the age of donor plants for many tree species (Hackett, 1988). One of possible reasons for *Eucalyptus* species was that they produced lignotubers containing some buds and tissue. In the mature shoots, lignotubers developed into mass structure; in juvenile shoots it remained as before and shoots originated from lignotubers regenerated some juvenile leaf which had the potential to form

adventitious roots (Hartney, 1980). In our study, shoots from the crown having no potential to root was possibly because lignotubers in mature cuttings were unable to produce shoots anymore thus prevented further root formation.

There were also other factors contributing to the declination of rooting potential, including: auxin, other plant growth substances (endogenous ABA, GA, and ethylene), rooting cofactors and promoters, and rooting inhibitors (Hackett, 1988). Rooting cofactors, promoters and inhibitors influenced rooting ability in Camellia species (Richards, 1964) and in pear cultivars (Fadl and Hartmann, 1967).

E. grandis cuttings from both sources increased rooting percentage due to the treatment of exogenous IBA, reflecting that auxin played an essential role in determination of rooting ability (Fogaca and Fett-Neto, 2005). Previous reports indicated that auxin enhanced rooting for *Eucalyptus*, especially for difficult-to-root species, e.g., *E. globulus* (Fogaca and Fett-Neto, 2005). By contrast, difficult-to-root species *E. amplifolia* were not improved better by treatment of auxin than *E. grandis*, indicating that there were also other factors inhibiting rooting for *E. amplifolia*.

In our study, mature plant material from crown still did not improve rooting efficiency with IBA treatment as much as juvenile material from sprout. In this case, absence of exogenous auxin was not a key factor to inhibit rooting efficiency for *E. grandis* cuttings from mature materials. Paton et al. (1970) had isolated from adult tissue of *E. grandis* rooting inhibitors, the concentration of which increased with the decreased rooting ability of stem cutting. He indicated that adult *E. grandis* had high rooting inhibitor content and was difficult-to-root, while the juvenile *E. grandis* was still classified as easy-to-root.

Harrison-Murray and Thompson (1988) showed that seasonal time when cuttings were taken played an important role in rooting. Some easy-to-root species could be rooted readily throughout the whole year, while for difficult-to-root species could only be rooted during the optimal season (Hartmann, 2002). Exogenous IAA was the essential factor for easy-to-root plants, but not for difficult-to-root cuttings (Porlingis and Therios, 1976) because treatment of plants with auxin enhance root initialtion for juvenile cuttings, however they promote cell division without root initiation for mature or difficult-to-root cuttings (Hackett, 1988). More IAA was found in juvenile materials than in mature ones and they also varied in different time. Wu and Barnes (1981) measured highest concentration of IAA in summer for *Rhododendronponticum* and *Rhododendron Britannia*. In this case, possible reason for cutting response with different time was that endogenous IAA levels were varied when the season changed.

In conclusion, *E. amplifolia*, a difficult-to-root plant may be inhibited by some rooting inhibitors and treatment of exogenous auxin cannot significantly overcome low rooting efficiency. Cuttings for *E. grandis* from juvenile materials rooted better than that from mature materials, probably due to the absence of lignotubers and some rooting inhibitors produced in mature cuttings.

Propagation by Mini-Cuttings

Clones and origin were very important factors affecting the *E. grandis* mini-cutting rooting efficiency. The cuttings from the basal part of the lateral branches had the highest rooting percentage, compared with stem terminals and the top part of branches despite genotypes, regardless of which clone it was (Figure 4-5). The cuttings from stem terminals rooted less efficiently than cuttings from the other two origins. There was no interaction of origin and genotype, and all the genotype responded similarly. Hartmann (2002) indicated that semi-

hardwood cuttings root better from lateral branches than terminal branches for many species and this supported by our result.

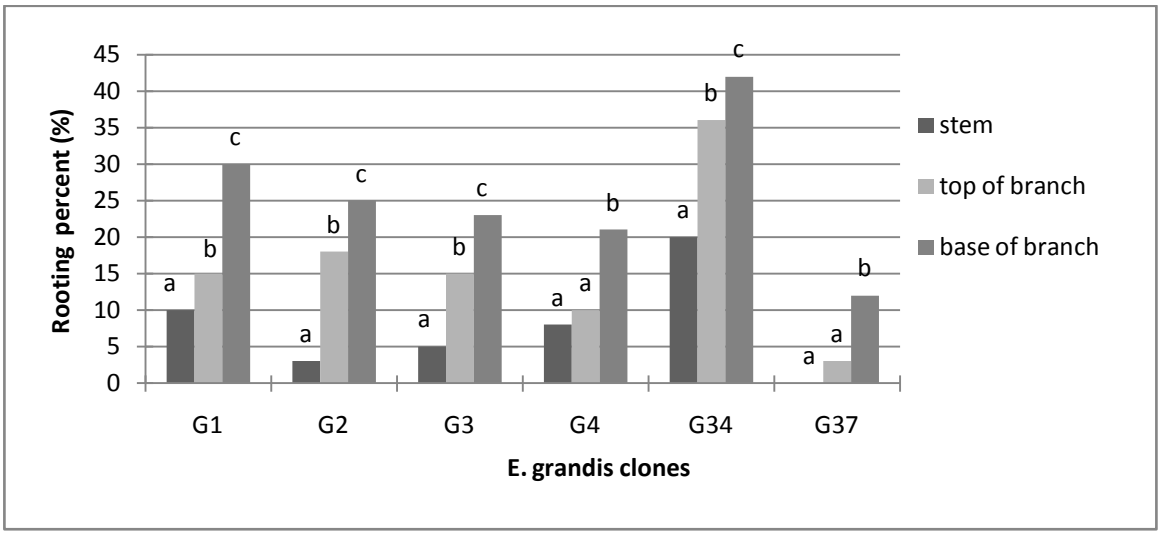

Figure 4-5. Mini-cutting rooting percent for 6 *E. grandis* clones (G1, G2, G3, G4, G34 and G37) from 3 sources (stem, top of branches and base of branches); five plants were assigned to each treatment with 3 replications for each clone. Within clones, means for a given time with different lower case letters indicate significant differences according to Bonferroni's HSD Multiple Range Test at $p<0.05$ level.

A possible reason for this was that endogenous hormones developed in the plant influenced the mini-cuttings rooting efficiency. Endogenous IAA was synthesized at the top of the plants and different parts of the plants have different IAA level, which influenced the rooting efficiency.

The ability to form adventitious root was restricted to the stem between the cotyledons and the 15th nodes and *Eucalyptus* showed a close link between rooting ability and the juvenility of the mother plant (Muckadell, 1959). In the mini-cutting experiment, our results regarding explant origin were similar.

New Clone G34 had the highest mini-cutting rooting efficiency, followed by G1, G2, G3, and G4. Clone G37 was not good compared with the well-tested clones (Figure 4-5).

Comparing the difference between mini-cutting and macrocutting rooting for *E. grandis*, the ranking of the rooting percentages among different clones did not change by altering the cutting method. But rooting percentage by macrocutting was significantly higher ($p<0.01$) than that of mini-cutting, especially for clones G34 and G2. In summary, the mini-cutting method did not improve the rooting efficiency, but the method was feasible for propagation (Figure 4-6).

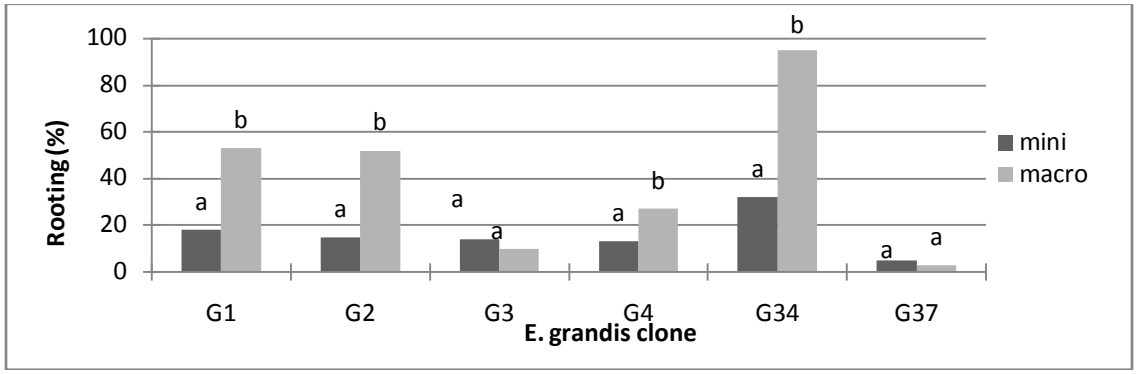

Figure 4-6. Comparison of rooting percent for macrocutting and mini-cutting for 6 *E. grandis* clones (G1, G2, G3, G4, G34, and G37). Each treatment was replicated 15 times for each clone. Within clones, means for a given time with different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$ level.

Mini-cuttings were also tried for *E. amplifolia* and because of the low rooting rate from cuttings, only clones A6 and A8 were involved and the rooting percentages were 5% and 4%, respectively (not showed in figure). This rate was even lower than the most difficult to root *E. grandis* clones. *E. amplifolia* was more difficult to root than *E. grandis*.

Although our study showed that mini-cutting had lower rooting efficiency than macrocutting, Stape et al. (2001) indicated that macrocutting system was replaced by mini-cutting system for *Eucalyptus* plantations in Brazil due to its lower production costs, reduced branching, and improved root system (Higashi et al., 2000).

Stock plant age for mini-cutting, just like with macrocutting, also affected rooting efficiency. Six month old shoot as the cutting material was best compared with 3 month and 9 month old material (Table 4-1).

Three month old branches were too young and the shoot was too soft. The mist in the greenhouse may have been too heavy for the young shoots and there was no success for these shoots. Whereas 9-month-old shoots were too old and the rooting percent declined compared with the 6-month-old branches. In this case, the rejuvenation was necessary of the stock plants for successful rooting of mini-cuttings.

Table 4-1. Effect of shoot age (3, 6, and 9-month) on mini-cutting rooting percent for 4 *E. grandis* clones (G1, G2, G3, and G4)

	shoot age		
clone	3-month	6-month	9-month
G1	0	18%	10%
G2	0	15%	8%
G3	0	14%	5%
G4	0	13%	5%

Each treatment involved 10 explants with 3 replications for each clone.

Micropropagation

Explant and Sterilization

Table 4-2. Decontamination rate of one-month-old shoots from stock plants and from mature trees of *E. grandis* and *E. amplifolia*

Material source	70% ethanol		no ethanol	
	10%bleach	20%bleach	10% bleach	20%bleach
Shoots from stock plants	10%	6%	80%	20%
Shoots from mature trees	0%	0%	0%	0%

Each treatment involved 10 explants with 3 replications for each clone.

Ethanol and bleach concentration were 2 factors influencing the decontamination rate of the explants. It was difficult to sterilize mature shoots from field-grown trees without damaging the tissues (de Fossard et al., 1978; Durand-Cresswell et al., 1982), and our experiment confirmed this. Shoots collected directly from trees in field were all contaminated in culture

(Table 4-2), so mature plants were always made cutting first. After 3 month, the vigourously grown cuttings worked as the stock plants to provide material for micropropagation. Although the period was longer, the sterilization result was much better. One-month-old fresh shoots from stock plants in greenhouse were preferred in our experiment. Ten percent bleach without ethanol pretreatment resulted in 80% decontaminated healthy shoots, which was optimal, compared with treatment with ethanol and high level bleach, which burn the juvenile plants and inhibited the new shoot induction (Table 4-2). There was no significant difference between two species in this stage. Double sterilization with an interval of 48 hours between treatments reduced the contamination to some degree, but the effect was not very significant (data not shown).

Ikemori (1987) artificially induced epicormic shoots on severed branches of *E. grandis* by placing the shoot in a greenhouse under intermittent mist irrigation. After 2 to 3 weeks, epicormic shoots approximately 30 mm in length were harvested from branches and used as explants. But in our study, this method did not work out.

Leaves and nodes were used as the explants to initialize the experiment. Leaves, whatever their age did not produce any shoots during 2 months in culture. However, nodes without contamination induced some shoots in 2 to 3 weeks on MS basal media, with or without PGR.

It was virtually impossible to sterilize mature, field-grown shoots without damaging the tissues. The ideal material should have many of the qualities of young juvenile shoots (de Fossard et al., 1978). Before collecting the materials, it was necessary to keep the stock plants dry and to spray them with fungicide several days before taking the required material (Durand-Cresswell et al., 1982).

Nodes from upper and lower branches of the main trunk and the coppiced shoots were used as the explants to see which source was suitable; coppiced shoots were considered as the

desirable explant for this genus as they demonstrated higher survival. Coppiced shoots or branches collected from old trees were the main source for the explants in some reports, but in our study, because of the limited plant materials and the contamination situation, the shoots from coppiced trees were used as the plant materials for cuttings. Then, grown cutting worked as the stock plants to provide the node explants for tissue culture, which made explants easier to sterilize and available all the time. Because even the sprouts from field-trees were 100% contaminated.

Many different explants were used to successfully initiate culture for *E. grandis* (Leroux & Vanstaden, 1991). Aseptic shoots and flora buds were used to establish the culture from explants. Aseptic cultures had been established from stump coppice shoots (Furze and Cresswell, 1985), scion shoots (Franclet and Boulay, 1982), epicormic shoots (Ikemori, 1987) and from young and vigorously growing shoots on mature trees (de Fossard et al., 1977 ; Rao, 1988). In addition, both seedlings and juvenile plants were good source for shoot induction. Germinated seedlings from sterilized seeds were good sources of explants (Glocke et al., 2006). But to keep the genotypic characteristic in our study, germinated seedlings were not suitable and the nodes or shoots from certain clone were used as the explants. In addition, shoot tips showed practically no contamination whereas nodal segments had levels of contamination higher than 50% (Gomes, 2003). Although the shoot tips were more difficult to obtain, their success rate in establishing aseptic cultures was much higher. However, nodes were widely used as explants due to availability.

Age and season of materials were also important factors determining the success in establishing the aseptic cultures (Grewal et al., 1980). The optimum time was November to

March, and trees of the different genotypes gave widely different results (McComb and Bennett 1986). Our result indicated that age of the branches was very significant ($p<0.001$).

Our sterilization method was similar to the other reports. All the papers shared the similar sterilization method by soaking the explants in the 70% ethanol for 30 to 60 seconds, followed by treatment in 0.05-0.1% (w/v) $HgCl_2$ for 5 to 15 min, or 1 to 10 NaOCl or $Ca(OCl)_2$ for 10 to 30 minutes, followed by several rinses in sterile distilled water. Our study indicated that ethanol would burn the explants even though it can kill the contaminants.

Shoot Induction

In addition to decontamination, the survival rate of the explants in the initial stage was associated with oxidation of phenolic exudation released from the cut end of the stem. After 1 or 2 days of inoculation, browning of explants resulting from oxidation of phenolic compounds would kill the explants. PVP improved the situation although it could not prevent it completely. Soaking of the explants in water was not good because the long time of exposure to water made the explants turn yellow. The possible reason for this was that the explants were too young and soft, and the soaking was not good for them. Charcoal did not have significant effect on preventing exudation of phenolic compound during the initial stage in these experiments. Our results showed that combination of pretreatment of antioxidant PVP and high frequency changing media was effective to reduce the browning and death of explants due to phenolic compounds, although the successful rate was not high (Table 4-3).

Table 4-3. *E. grandis* and *E. amplifolia* shoot survival rate with pre-soaking, addition of charcoal, and addition of PVP with change medium interval of 2, 5, or 14 days.

	Change of treatment		
Treatment	2 days	5 days	14 days
pre-soaking	8%	5%	0
Charcoal	10%	7%	0
PVP	20%	10%	2%

Each treatment involved 10 explants with 3 replications for each clone.

Reduction of oxidation of phenolic compounds was essential for successful explant culture. Explants tended to produce a brown exudate which prevented growth of shoots that survived. Before inoculating explants on culture media, some extra treatments were made to reduce oxidation of phenolic compounds because the phenolic substances could turn explant brown and even be lethal sometimes. Cresswell and Nitsch (1975) overcame the problem for *E. grandis* by soaking the sterilized explants in sterilized water for 2 hours and keeping in culture in darkness for first 7 days. Similarly, Wachira (1997) indicated that initial soaking of explants of *E. grandis* in distilled water, coupled with pretreatment in PVP and initial incubation at 5^{o}C in darkness was necessary to reduce the accumulation of oxidized polyphenolic compounds which were associated with media browning and subsequent explant death. Das and Mitra (1990) reported that to prevent the death of buds because of exudation of phenolic substances, the inoculated media had to be changed 3-4 times at 2-3 days interval from the beginning of the culture to overcome this although the effect was little. Sharma and Ramamurthy (2002) also attempted to prevent this problem by 2 treatments, incubation under darkness or antioxidant pretreatment. The result was that the darkness treatment indeed had some positive effects on some genotypes while the antioxidant treatment did no good. Joshi et al. (2003) reported that to overcome the problem, media should be changed frequently at short intervals and PVP, ABA and charcoal failed to get desirable results. Although the previous efforts were tried, the results were still not good.

When media supplemented with PGR were compared with the media free of PGR, PGR-free media were optimal for shoot induction. Any basal media with 0.5mg/L and higher concentration BAP, Kinetin, 2-iP, or BA restricted the shoot development. The explants on

media with BAP and Kinetin only produced 1 or 2 very short, small, and compact shoots; the media supplemented with BA or 2-iP could not induce any shoots (Table 4-4). Compared with media supplemented with PGR, PGR-free media was optimal for adventitious shoots induction, and after 7 days healthy and long shoots without any callus appeared at the nodes. The response of *E. amplifolia* and *E. grandis* had no difference in this stage.

Table 4-4. *E. grandis* and *E. amplifolia* shoot induction in media with different PGR combinations

Plant growth regulation combination (mg/l)				Shoot and callus observation		
BAP	Kinetin	2-iP	IAA	shoot number	shoot length(mm)	callus
0.5			0.1	0		+
	0.5		0.1	1-2	1	+
		0.5	0.1	0		+
0.5				1-2	1	/
				1-2	5	/

+ indicate there is some callus; / means there is no callus. Each treatment was replicated 15 times for each clone.

Although the induction stage was very necessary, the regeneration frequency of primary explants had no effect on the further success of the micropropagation if a few new clean shoots was obtained (Sharma and Ramamurthy, 2000). In study, good quality shoots were obtained from hormone free media although the shoot number was only 1 or 2. After this, the shoots were transferred to multiplication medium for further culture.

MS was used in most previous reports as the basal medium for micropropagation. Twenty to 30 g/L sucrose and 7g/L agar were used for initializing and maintaining explants in most of the literatures. Sharma and Ramamurthy (2000) reported that agar was more desirable since phytagel would lead to some vitrification of plants. Higher concentrations of cytokinin would make the shoots small and compact which were not good. Our observation also confirmed this point.

Vitrification, also named as tissue waterlogging or hyperhydric transformation, was the phenomenon that shoots become glassy and transparent, with swollen, brittle leaves and stems (George and Sherrington, 1984). Boulay (1983) found that when vitrification occurred in shoot multiplication cultures, usually only a few buds were affected. He also indicated that vitrified buds were difficult to multiply and almost impossible to root. Durand-Cresswell et al. (1982) recommended a reduction in cytokinin concentrations to reduce this problem. Modification of growth medium (Zimmerman and Cobb, 1989) and adding of Bacto-peptone fractions (Sato et al., 1993) were also employed although they reduced multiplication rate simultaneously (Zemmerman et al., 1995). *Eucalyptus* shoot cultures frequently produced a white callus, sugary in appearance on leaf surfaces, stem nodes and in the axils of leaves of *E. dalrympleana, E. ficifolia, E. grandis, E. regnans* and *E. gunnii* (de Fossard and Bourne, 1976; Durand-Cresswell et al., 1982). Boulay (1983) recommended frequent subculturing every 15 days to prevent this problem. Our study confirmed the need for frequent media transfering to prevent vitrification.

Shoot Multiplication

Mean multiplication rate in DKW medium was significantly higher than the rate in MS or WPM for all the clones ($p<0.0001$). Highly significant variation was also observed in different level of PGRs ($p<0.0001$) and interaction of basal media and PGR ($p<0.0001$). No matter what the basal media and genotype were, 0.4mg/l BAP was optimal for the shoot multiplication, while DKW with 0.4mg/l BAP was optimal medium and the multiplication rate was 5.7. When the BAP concentration increased to 1.0mg/l, more callus were produced. The addition of NAA did not improve the multiplication rate, compared with 0.1 mg/l BAP, but it did improve elongation of the shoots (Table 4-5).

Table 4-5. Multiplication rate on 12 multiplication media (4 levels of PGR: 0.1, 0.4, and 1mg/l BAP combined with 0 or 0.1mg/l NAA on 3 basal media: MS, DKW, and WPM) across 5 *E. grandis* clones (G1, G2, G3, G4, and G34).

PGR		Multiplication rate		
NAA(mg/l)	BAP(mg/l)	MS	DKW	WPM
0	0	1a	1a	1a
0	0.1	1.5b	1.5b	1.4a
0	0.4	3.8c	5.7c	3.3c
0	1	3.2c	4.2c	3c
0.1	0.1	1.2ab	2b	1.2a

The third generation shoots were used in this experiment. Each treatment was replicated 15 times for each clone. Within column, means for a given time with different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$ level. Multiplication rate means how many shoots longer than 2 mm were produced on single shoot.

Genotypic effect was very strong. The difference of multiplication rate between shoots in DKW with 0.4 mg/l BAP and MS with 0.4 mg/l BAP was very significant for Clones G1 and G2 (<0.0001), and for clones G3 and G34, the difference was also significant ($p<0.001$) (Figure 4-7). In addition, the multiplication rate varied greatly for different clones on the same media. The highest rate was 8 for Clone G34 while the lowest rate was only 3.5 for clone G4 on DKW with 0.4 mg/l BAP. In this case, the optimal medium for multiplication was DKW with 0.4 mg/l BAP.

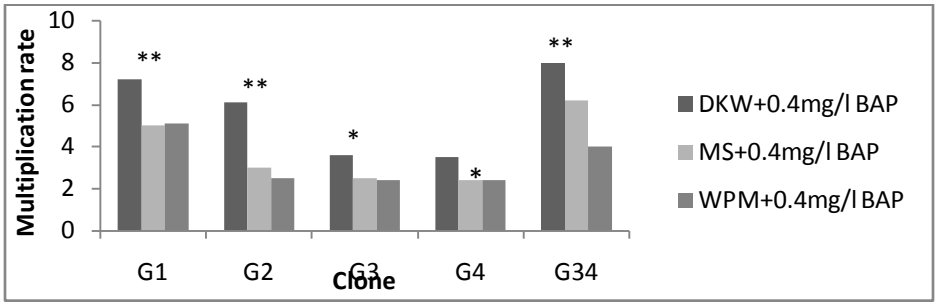

Figure 4-7. Shoot multiplication rate on media (DKW+0.4mg/l BAP or MS+0.4mg/l BAP) for 5 clones (G1, G2, G3, G4, and G34) respectively. Experimental material was used from third generation explant.Each treatment was replicated 15 times for each clone. Within clones, means for a given time with * indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.01$ level, ** indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.001$ level. Multiplication rate means how many shoots longer than 2 mm were produced on single shoot.

Subcultures were made at 3 week intervals. The shoot number (>2mm) produced by each generation of each clone was quite different (Figure 4-8). Commonly, generations 5 and 6 produced more shoots. The multiplication rate of G1 and G34 reached 12 and 8.7 per explants at the generation 5 respectively. Comparatively, Clones G3 and G4 only produced 5 and 4 shoots, respectively. After 5 to 6 generations, the multiplication rate began to decline gradually. At the 8th generation, the multiplication rate was as slow as the initial rate.

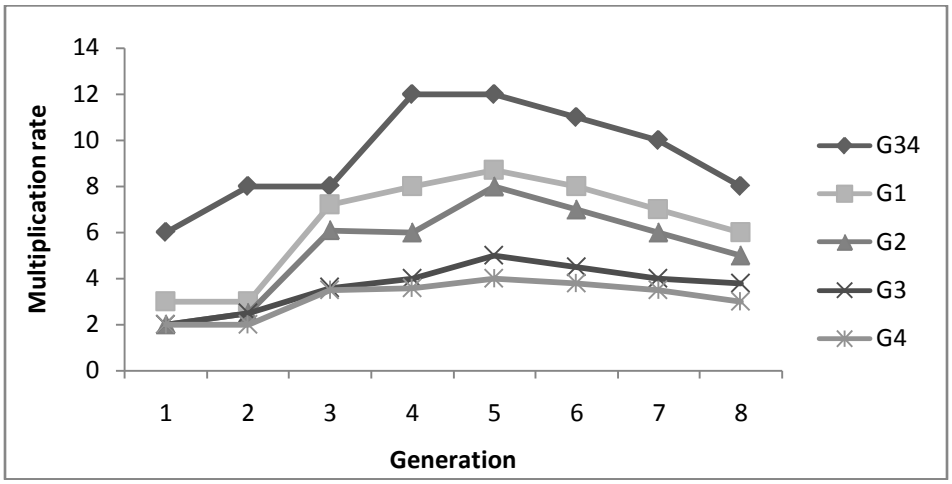

Figure 4-8. Multiplication rate (how many shoots longer than 2 mm produced on single shoot per 3 weeks) for 8 subculture generations on DKW with 0.4 mg/l media for 5 *E. grandis* clones (G1, G2, G3, G4, and G34). Each treatment was replicated 15 times for each clone.

For all the *E. grandis* clones, mean multiplication rate on DKW medium was significantly higher than the rate on MS or WPM for all the clones ($p<0.0001$). There were no significant difference between WPM and MS (Table 4). Although MS was used as the basal media for majority of the *Eucalyptus* culture, suitability of DKW for other woody plants, like *Ulmus procera* has been indicated by for the healthier shoots produced on this (Fenning et al., 1993).

Glocke et al. (2006) used MS, B5, WPM, AP, TK and QL as the basal media and found that WPM was most successful and better than MS because shoots were healthier. Gomes and Canhoto (2003) also used different basal media, including MS or FSB to culture *E. nitens* and

observed that the best multiplication rate was obtained when they were cultured on medium containing the major nutrients and minor elements of MS medium, the organics of DF medium.

Mg^{2+}, K^{+}, Ca^{2+}, NO_3, SO_4^{2-} were higher in DKW than in MS; these inorganic salts were lowest in WMP (Table 4-6). In this case, for some specific clones of *E. grandis*, further investigation is needed to see which mineral salts are more needed for the clones.

Table 4-6. Component ($Zn(SO4)_2$, $Zn(NO3)_2$, K_2SO_4, KNO_3, $Mg(SO4)_2$,$Ca(NO3)_2$, $CaCl_2$) comparison between DKW, MS, and WPM basal medium.

Component	KDW	MS	WPM
CaCl2	112.5	332.2	72.5
Ca(NO3)2	1367	0	386
Mg(SO4)2	360	180	180.7
KNO3	0	1900	0
K2SO4	1600	0	990
Zn(NO3)2	17	0	0
Zn(SO4)2	0	8	8.6

PGRs were very important in multiplication stage, and BAP was the most commonly used cytokinin for *Eucalyptus*. Warrag (1989) reported that MS medium supplemented with BAP alone in the range of 0.4mg/L to 0.8mg/L was optimal for shoot multiplication of *E. grandis*. Wachira (1997) initiated the experiment by using nodal explants of *E. grandis* and found that shoot regeneration was efficient from 0 to at least 3 mg/L BAP. Bunn (2005) reported that BAP produced the highest shoot multiplication rates when compared to equivalent concentrations of other cytokinins (Kinetin or Zeatin), but high concentration of BAP would produce hyperhydric shoots. Our finding also showed that for the *E. grandis*, BAP at 0.4 mg/l was optimal.

In other papers, combinations of cytokinin and auxin were used in this stage. The optimum shoot regeneration was achieved at 0.2mg/L BAP combined with 0.4 mg/L or 1mg/L NAA. In culturing *Eucalyptus* hybrids of *E. tereticornis* * *E. grandis* (Joshi et al., 2003), single shoot were multiplied in media with 1.0mg/L BAP and 1.0/L NAA. After this stage, multiplication rate

increased with every subculture, while the bud clumps were more compact and rather smaller in size. To overcome this, subculturing was carried out in MS+ 1.0 mg/l BAP without NAA. The similar finding was also reported by Sharma and Ramamurthy (2000) culturing *E. tereticornis*.

A higher multiplication rate in some *Eucalyptus* was obtained on MS with BAP alone at 0.4 mg/L to 0.8 mg/L or with 0.1mg/L NAA. Other reports indicated that cultures were good at the beginning on the MS with the combination of 0.4-1mg/L BAP with 1mg/L NAA. High NAA made the culture compact and smaller, and in this case, after some generations of subculture, only 0.5 -1 mg/L BAP in the medium were effective for multiplication.

Elongation

The BAP and NAA interaction effect was high ($p<0.01$). When BAP was 0.1 or 0.5 mg/l, shoot length decreased with increasing NAA concentrations. But when there was no BAP, the shoot elongation increased with NAA concentration (Figure 4-9). Elongation of shoots was best on 0.1mg/l NAA with 0.1 or 0.5mg/l BAP. This combination increased shoot length to 2.5 cm after 3 weeks.

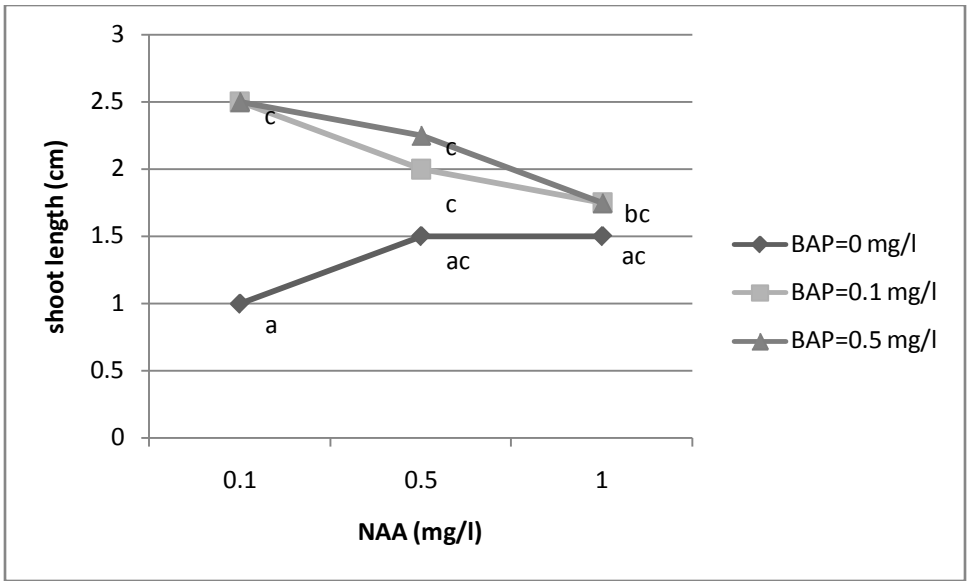

Figure 4-9. Elongation in DKW plus NAA (0.1, 0.5, or 1mg/l) across 5 *E. grandis* clones (G1, G2, G3, G4, and G34). Each treatment was replicated 15 times for each clone. Different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.0$ 1 level. The third generation shoots were used for this experiment.

In addition, BAP multiplied the shoots at the same time. Multiplication rate was higher at 0.5 mg/l BAP than at 0.1 BAP no matter what the NAA concentration was (Table 4-7). In this case, before rooting, the plantlets could be transferred by clusters. GA was also tried in our experiment, but elongation was not good, and there were callus on the shoots.

Table 4-7. Shoot multiplication rate in DKW plus NAA (0.1, 0.5, or 1mg/l) across 5 E. grandis clones (G1, G2, G3, G4, and G34).

NAA(mg/l)	BAP(mg/l)	Shoot number
0.1	0	1 a
0.1	0.1	2 b
0.1	0.5	5 e
0.5	0	1 a
0.5	0.1	3 c
0.5	0.5	4 d
1	0	1 a
1	0.1	2 b
1	0.5	2 b

The third generation shoots were used for this experiment. Each treatment was replicated 15 times for each clone; different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.01$ level.

There was no significant difference among four clones (Figure 4-10). There were no significant ($p>0.05$) difference for the shoot length in the 2 best media (0.1mg/l NAA with 0.5mg/l BAP; 0.1mg/l NAA with 0.1mg/l BAP). The suggestion was that media with 0.1mg/l NAA and 0.5mg/l BAP were used first to multiply the elongated shoots and then transferred to media with 0.1mg/l NAA and 0.1mg/l BAP for further elongation.

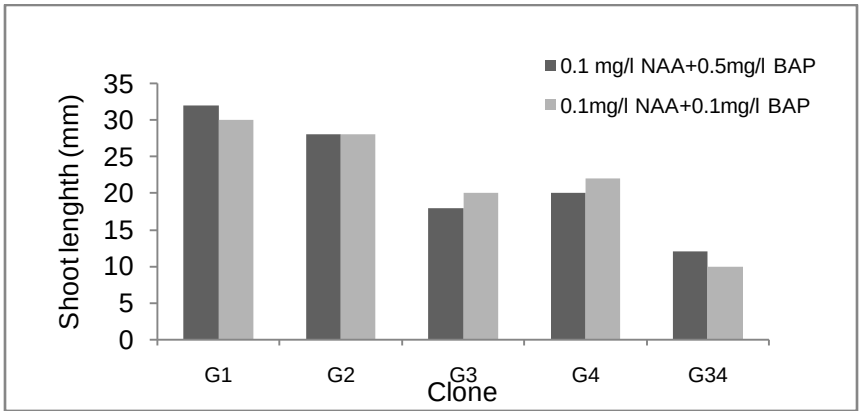

Figure 4-10. Shoot elongation in DKW plus 0.1 mg/l NAA with 0.5 mg/l BAP or 0.1 mg/l NAA with 0.1mg/l BAP for 5 *E. grandis* clones (G1, G2, G3, G4, and G34), respectively. Each treatment was replicated 15 times for each clone. The third generation shoots were used for this experiment.

Quoirin et al. (1974) reported that a period of elongation following multiplication predisposes the shoots to root in response to rooting treatments. Although GA_3 was not effective in our study, other papers had some contrasting results. Shoots of 2 or 3 cm in length were cultured on MS with 1mg/L GA_3 for elongation for *E. grandis* (Sita and Rani, 1985). WPM with GA_3 was chosen for elongation for *E. grandis* according to Glocke et al. (2006) while 1/2 MS without any hormone gave good shoot elongation response for hybrid of *E. tereticornis* and *E. grandis* (Joshi et al., 2003). Gibberellins were tried by Franclet and Boulay (1982) for *E. gunnii*, where they suggested the use of 0.1 mg/L of gibberellic acid with activated charcoal was very efficient. Higher concentration of gibberellins tended to produce abnormal leaves and stems. The

reason may be that the different species had different response to the GA (Gomes and Canhoto, 2003).

In addition, combinations of auxin and cytokinin were also used for elongation. 2.5 mg/l IBA and 1.0 to 1.5 mg/l Zeatin in MS were the best elongation media for hybrid of *E. grandis* according to Warrag (1991). Because Zeatin was more expensive than NAA, the replacement of Zeatin with NAA was necessary.

Auxins were suggested to act directly in the induction of cytodifferentiation, elongation and cell division (Fukuda and Komamine, 1985). The possible advantage of using high concentrations of auxin at the elongation stage was the improvement of subsequent rooting. McComb and Bennett (1986) stressed the carry-over effect from multiplication or elongation media on subsequent rooting ability. They mentioned that use of gibberellins for elongation seemed to greatly reduce rooting.

Rooting and Acclimation

Different levels of NAA (0.1 to 2mg/l) induced rooting, and the optimal medium was 0.5mg/l NAA because it produced higher root numbers and shortest lengths. Products of one subculture were good to go to greenhouse (Table 4-8). NAA and IBA did not differ significantly for rooting percent, but IBA induced slightly higher rooting percent; MS and 1/2MS did not significantly influence the rooting percent but ½ strength MS improved a little; all the treatments achieved as high as about 95% rooting (Figure 4-11). Actually, rooting percentage was approximately 90% no matter what level of auxin was used, and auxin was only influence the shoot length and shoot number.

Table 4-8. Effect of NAA on rooting number and length in medium MS across 5 clones (G1, G2, G3, G4, and G34)

NAA (mg/l)	root number	root length (mm)
0	1.8 a	6 a
0.1	2 b	6 a
0.5	3.5 c	8 ab
1	3 a	12 b
2	3 a	12 b

Each treatment was replicated 5 times for each clone. Different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$ level. The third generation shoots were used for this experiment.

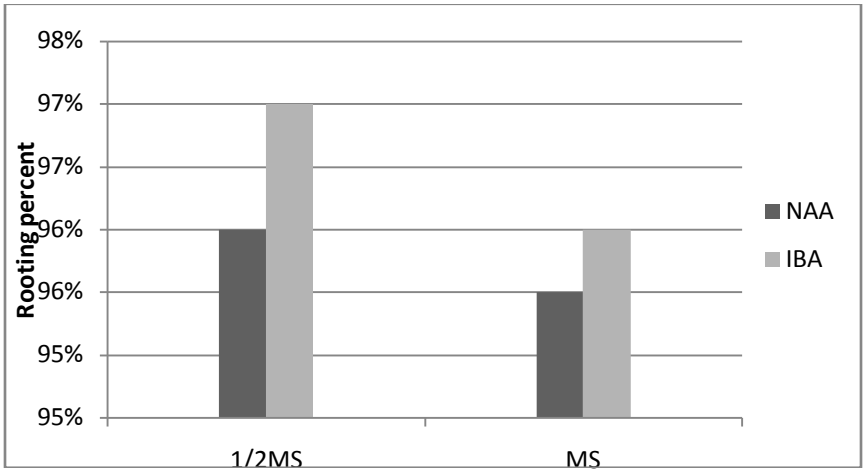

Figure 4-11. Effect of MS concentration with 0.5 mg/l IBA or NAA on rooting percent across 4 clones (G1, G2, and G3, and G4). Each treatment was replicated 15 times for each clone.

Rooting phase for many *Eucalyptus* species was the most critical and limiting step. Hartney (1980) reported a rooting protocol involving two serial subcultures, while in the protocol reported by Rao (1988) and Das and Mitra (1990), three serial subcultures were used. Moreover, three auxins together for the process of root induction were used (Gupta and Mascarenhas,1987; Rao, 1988).

In comparison, single subculture with only one hormone IBA was successful for *E. tereticornis* (Sharma and Ramamurthy, 2000). Warrag (1991) also reported that rooting was better in a medium with only auxin and basal nutrients of reduced concentration for *E. grandis*.

The combination of 2 mg/l of IBA and 1/4 MS with a pH in the range of 5 to 5.5 gave the best results. Increasing IBA to more than 2mg/l tended to produce more callus. Addition of BAP at 0.2 mg/l inhibited rooting completely. In our study, rooting for the *E. grandis* clones was not difficult to root, and this was consistent with the other reports.

Durand-Cresswell et al. (1982) recommended that transfer from rooting media should be done as soon as a few roots were produced. Blomstedt et al. (1991) reported that activated charcoal had been used for elongation and successfully enhanced rooting in other *Eucalyptus* species. Also, basal medium formulation was also an important factor affecting rooting. Although ½ strength MS and IBA slightly improved rooting efficiency, it was not significant in our study.

Auxin has role in stimulating cell elongation, cell division, producing roots, and extending stems. Cytokinins' main function is to cause cell division and release shoot apical dominance, thus promote growth of lateral buds (Gaba, 2002). The different ratios of auxin to cytokinin have different effects: high ratio induces rooting; intermediate ratio induces callus; and low ratio stimulates adventitious shoots. In our study, BAP alone at intermediate level had good effects on shoots multiplication because it promoted cell division; NAA at 0.1-2 mg/l promoted rooting. The combination of BAP and NAA could elongate stems and multiply elongated shoots simultaneously, especially when ratio of BAP to NAA was 1 to 5.

PGR carry-over effect was one issue needed to be considered for the micropropagation (Bennett et al., 1994). The cytokinin added in multiplication medium had effect on subsequent rooting induction and degree was varied among different in *Eucalyptus* species; the reason was that rooting response was associated with the production of flavonoids influenced by cytokinin (Bennett et al., 1992). Because *E. grandis* was easy to root, the influence was not obvious. On

the contrary, auxin treatment for the rooting had an adverse effect on shoots sometimes, and needed to be avoided (Klerk et al., 1999).

The reduced multiplication rate and leave abnormalities were present in culture of *E. globules* after three periods (Bennett et al., 1994). The combination of BAP with kinetin were tried and improved the response. In our study, declining multiplication rate with time also needs to be overcome, and combination of different cytokinins require additional testing.

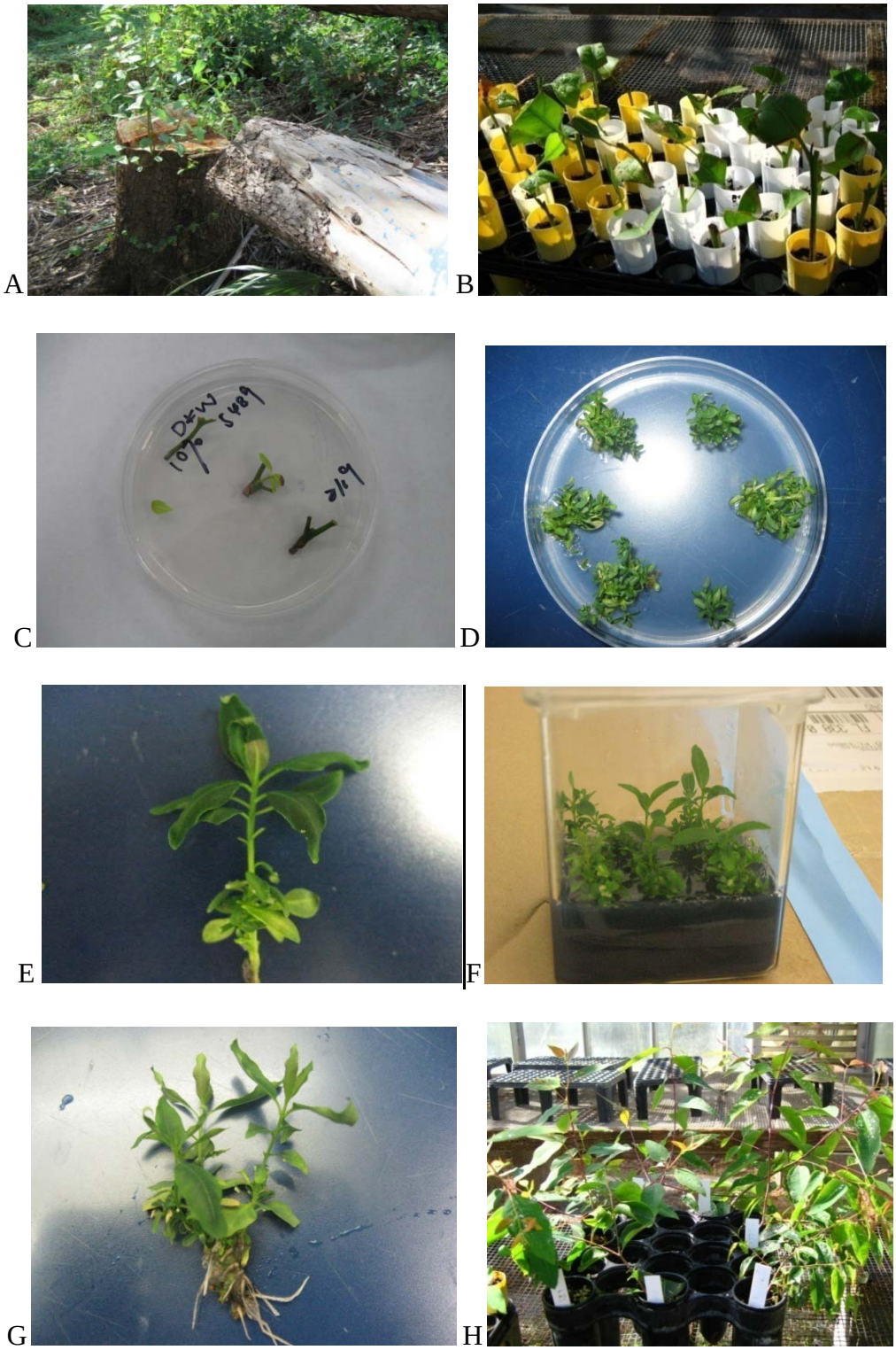

Figure 4-12. Propagation of E. grandis clones: A-sprouts from felled tree crown; B-cuttings in the greenhouse; C-sterilization of stems; D-shoot multiplication stage; E,F-shoot elongation stage; G-rooting stage; H-acclimatized plantlets from in vitro to greenhouse

There were 3 physiological phases in adventitious rooting process, including induction, initiation and expression (Kevers et al., 1997) and success of rooting depended on the whole 3 phases. Auxin was essential for inducing roots; however it inhibited root formation (De Klerk et al., 1990). On the contrary, Bellamine et al. (1998) add antiauxins into medium after cuttings were treated with auxin previously and the result indicated that auxin was required even in expression phase. According to this study, there was no need to remove auxin from medium in later rooting stage. IBA was more stable than IAA and NAA (Fogaca and Fett-Neto 2005). IBA was better than NAA in *E. globules* and *E. saligna*, and was possibly associated with NAA's longer persistence remaining in tissue (Fogaca and Fett-Neto, 2005), which inhibited rooting. Our results were in agreement with this statement.

Quantitative trait locus (QTLs) controlling vegetative propagation in *E. grandis* have been identified though genetic mapping by Grattapaglia et al. (1995). *E. grandis* clone was crossed with *E. urophylla* clone to produce F1 individuals, which were used for construction of genetic maps by RAPD markers. QTL map related to traits of vegetative propagation response of *E. grandis* clones were made. Therefore phenotype of fresh weight of micropropagated shoots, stump sprout cuttings, and % rooting cuttings were measured respectively; results showed that there were 10 QTL detected for micropropagation, 6 for stump sprouting ability, and 4 for rooting ability. There were almost no overlap positions among 3 different propagation traits. It indicated that cutting and micropropagation method were not controlled by the same locus. However, the plantlets responded very similar in 2 methods, which was contrast to the research above.

Different genotype response in cutting and micropropagation could be explained by genetic diversity in the following parts. Clones that were easier to propagate were chosen for mass propagation.

Cost Reduction

The greatest operational cost of propagule production was labor. No reliable figures were available, but it was estimated to account for 40% to 60% of the total cost. Franclet and Boulay (1982) estimated the cost of *Eucalyptus* propagules was three times that of seedlings. Most of the cost consisted of manual cutting and transferring of cultured materials. Automation of some or all parts of the system could greatly reduce this cost. Because of clump transfer, and the uniformity and even age of shoots in this study at the multiplication and elongation phases, this might be an excellent system for automation. Bioprocessor as was described by Levin et al. (1988) could be used for separation, sizing, and distribution to culture vessels.

Another area of cost reduction could be through by-passing the in-vitro rooting step. The elongation medium produced shoots longer than 30mm, which could be sliced and rooted under mist conditions directly.

Three genotypes of *E. grandis* were studied by Warrag (1989), who reported no significant variation between them. This may suggest that the system developed in the study could be used at least in a broader sense.

In some species in this genus, the genotypic variation in ability of cuttings to root had been exploited in conventional propagation as in the successful selection work carried out in the Congo and Brazil (Hartmann, 2002). The genotype also strongly influenced rooting ability *in vitro*.

Genetic Fingerprinting

Pedigree and Genetic Relatedness

Florida *E. grandis* clones have known maternal pedigrees extending up to four generations. Many clones originated from some common 3GM or 4GM trees, like 295 and 293, and should be genetically related. Ancestor 295 was common to the most clones; for example, G1, G2, and G43, which could be expected to be closely related (Table 4-9).

Table 4-9. *E. grandis* clones originating from common 3GM or 4GM (Clone 295, 293, 294, 298, and 92).

Origin	Related Clones								
	G1	G2	3127	3159	G8	G12	G15	G18	4340
295	G27	G33	G35	G43	G48	G56	G59		
293	G37	G32	G41	G47	G52				
294	G49	G10	G16						
298	G5	G30	G55						
92	G23	G24	G26						

Marker

Missing data rate was not very high here. Total number of alleles observed per locus for 60 clones ranged from 7 to 26, with an overall total of 157 alleles across 8 loci. Expected heterozigosity (H_e) varied at different loci from 0.63 to 0.91(Table 4-10).

In our study, only two loci (EMBRA63 and EG62) had H_e lower than 0.7 and others were higher than 0.85 (Table 4-10). Previous reports indicated that SSRs were efficient markers with H_e higher than 0.8 at locus EMBRA4, EMBRA 5, EMBRA 10, EMBRA 11, EMBRA 15, and EMBRA 16 (Kirst, et al. 2005). Brondali et al. (1998) also reported all H_e for 20 loci higher than 0.7 except EMBRA13.

Table 4-10. Allele number for 8 loci in the study

Locus	Marker	Missing data	Number of alleles	He
Locus 1	EMBRA2	2.50%	22	0.89
Locus 2	EMBRA28	5.00%	26	0.9096
Locus 3	EMBRA10	8.30%	22	0.889
Locus 4	ES76	9.20%	25	0.9139
Locus 5	EMBRA37	3.30%	25	0.8608
Locus 6	EMBRA63	0.80%	9	0.6569
Locus 7	EG62	11.70%	7	0.6344
Locus 8	EG65	8.30%	21	0.8676
Total			157	

Microsatellites locus EG62 had only 7 alleles and most frequent allele peaked as high as 54%. Similarly, EMBRA63 had 9 alleles with one dominating at 52% frequency. Expected heterozygosities (H_e) for these 2 markers were the lowest (0.6344 and 0.6569 respectively), compared with others. Because Expected heterozygosity reflected the probability of the heterozygous individual in the population, EG62's relatively low H_e could be explained by that the allele with 54% frequency at EG62 could generate a high probability of the homozygous individuals, thus reduce the H_e correspondingly. The previous reports by Brondani et al. (1998) indicated that frequencies of all alleles at all loci (EMBRA 1 to EMBRA 19) were less than 30%, resulting in high H_e. Different results showed that different locus have impact on the H_e estimation.

On the contrary, there were no predominant alleles at loci EMBRA2, EMBRA28, EMBRA10, and EMBRA76, because the highest allele frequencies for these loci were lower than 20%, thus all alleles distributed similarly (Figure 4-13). The high H_e (0.89, 0.9096, 0.889, and 0.9139 respectively) for these markers also indicated this. For loci EMBRA37 and EG65, some alleles had relatively high frequency (28% and 21%), although they were not very

dominant, their frequencies were higher than other alleles' and also to reduce the H_e 0.86. Rare alleles (<0.1%) were about 30% and common alleles (>0.5%) were about 30% for 15 populations for *E. marginata* (Wheeler et al., 2003).

The cluster result indicated that 60 *E. grandis* clones were originated from 4 populations represented by 4 different colors (red, green, blue and yellow) (Figure4-14). The individuals were sort by Q, which made it easier to see which clones were more related. The individual label and corresponding clone number were showed in Appendix C.

Sixty clones can also be divided into 4 groups depending on which original population accounted for most proportion in individual. The first 24 clones in plot were 40%-60% originated from red cluster, while next 12 clones were largely (60-100%) from green cluster; 14 clones followed were mainly originated from blue cluster and last 10 clones shared 3 cluster (red, blue and yellow) equally (Figure4-14).

The result of structure was very consistent with kinship coefficient for individual clones. For example, identical clones G20, G21, and G4 with high coefficient was sort together in plot which reflected their high similarity in origin. All the pairs with high kinship coefficient (Table 4-11) were classified in the same group and sort closely.

This was because both the result from the Structure and SPAGeDi were originated from the genotype data from 8 microsatellite loci. The real reason why these kinship coefficients were high and why some individual were originated from the same clusters with similar proportion was that alleles in 8 loci for clones were very similar. For example, clone G4 and G20 (kinship coefficient =0.51) were identical clones because all 16 alleles for 8 SSR loci were almost the same, except for one missing data (Appendix C). Clone G1 and G45 (kinship coefficient =0.26) were full-siblings because 4 of 8 loci shared common alleles. Clone G1 and G2 were not genetic

related (kinship coefficient =0.06) because only one of two alleles in EG65 and EMBRA2 were shared by 2 clones while there were no common in other 6 loci.

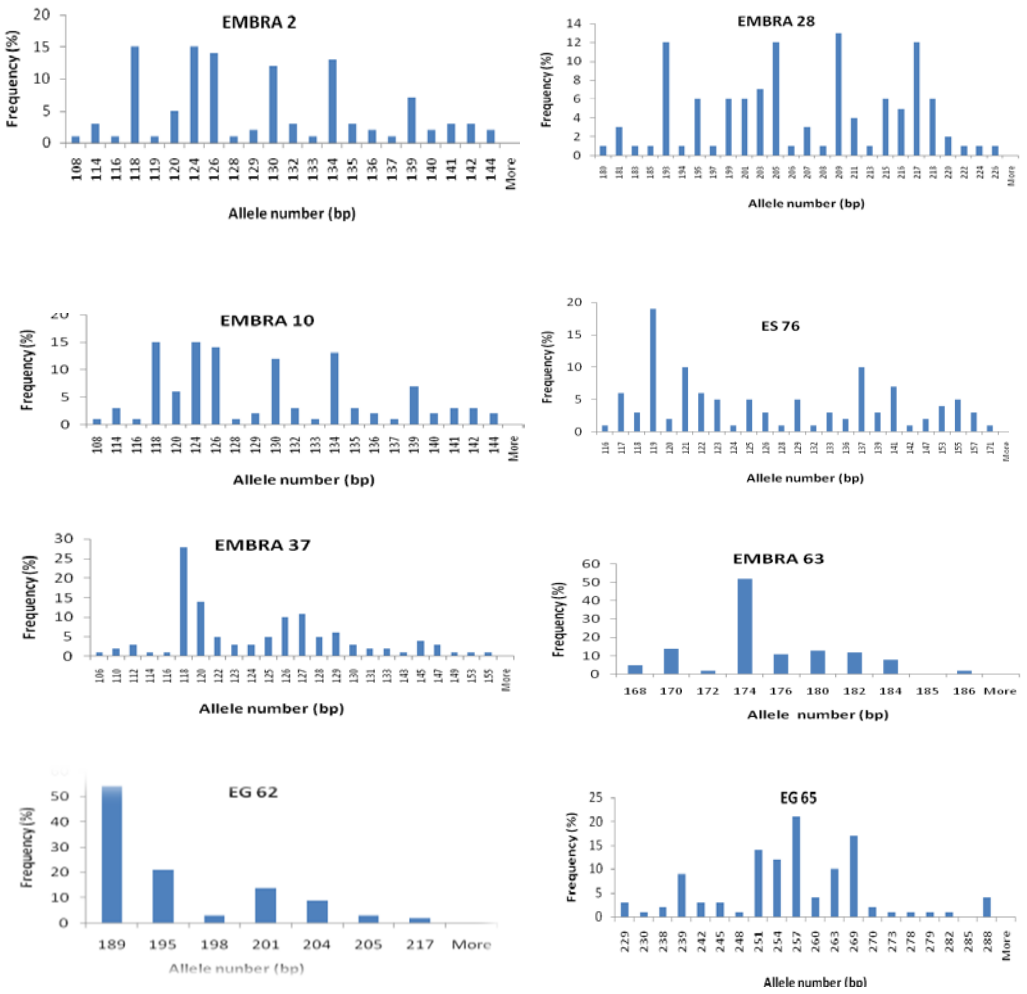

Figure 4-13. Allele number and frequency for 8 microsatellite loci (EMBRA2, EMBRA28, EMBRA10, ES76, EMBRA37, EMBRA63, EG62, and EG65) in the population of 60 Florida E. grandis clones.

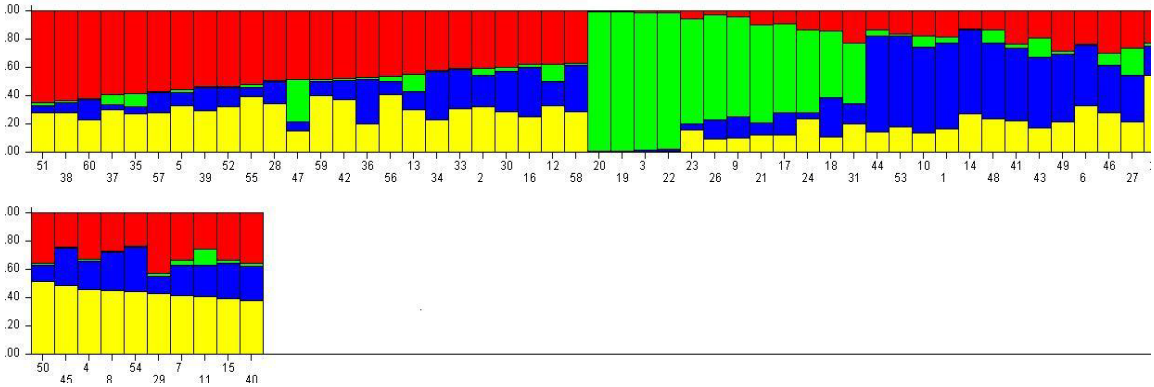

Figure 4-14. Population structure for of 60 Florida *E. grandis* clones (Appendix C). Each clone was represented by vertical line, which was partitioned into 4 colored segments that represent that clone's estimated membership fraction in each of the K inferred clusters.

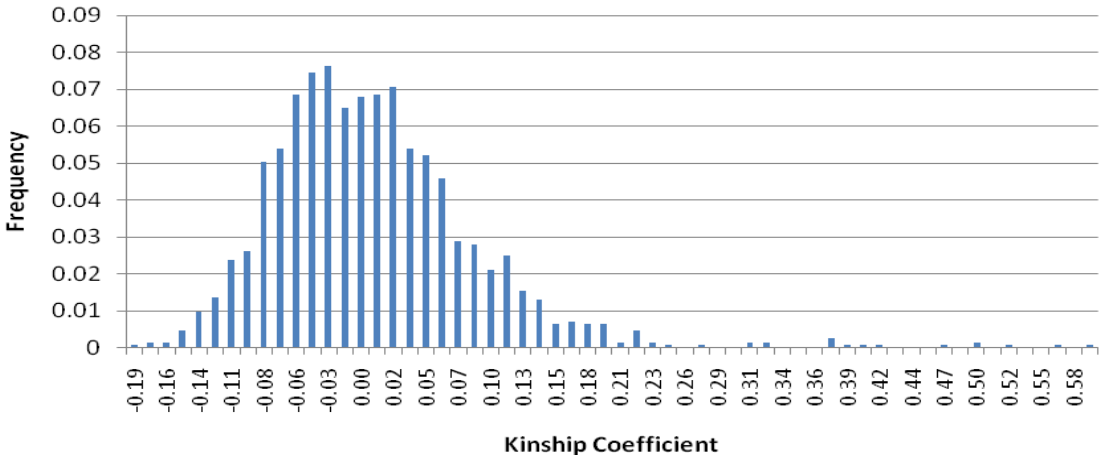

Figure 4-15. Distribution of pairwise relative kinship estimates in 60 *E. grandis* clones; 0.5 identical twins; 0.25 offspring within a family, parent-offspring; 0.125 half-siblings.

In addition, 60 clones were also summarized by the kinship coefficient (Appendix D) to analyze the genetic distance between each pair of two individuals. The kinship estimated the approximate IBD (identity by descent) by adjusting the probability of identity by state between two individuals with the average probability of identity by state between random individuals (Yu et al.,2006). Three thousand and six hundred coefficients were distributed approximately normal,

and 90% of the pairwise kinship coefficients were close to 0, ranging from 0 to ±0.25. Very few of them were distributed around 0.35 and 0.5, reflecting the highly genetic relatedness (Figure 4-15).

Clones originating from the same GM were compared by the kinship coefficient. For example, clones originating from 295 which were also involved in the genetic fingerprinting were G1, G2, G8, G12, G15, G18, G33, G35, and G43. Kinship coefficients for G1 with others were 0.07 (G2), 0.17 (G12), 0.04 (G15), 0.05 (G18), and 0.02 (G35). Except for G12, all the coefficients for others were below 0.1, which mean that they were almost not genetically related to each other (Table 4-9; Appendix D).

The highest coefficients among the pairs of clones were 0.512 (G20 and G4), 0.5586 (G21 and G4), and 0.4172 (G23 and G4); according to the definition of coefficient (Table 3-2) they were identical clones. Besides, there were some pairs of clones with coefficient between 0.2 and 0.5, which were considered full-siblings (Table 4-11).

Table 4-11. Pairs of *E. grandis* clones with kinship coefficient between 0.2 and 0.5

clone 1	clone 2	coefficient	clone 1	clone 2	coefficient
G20	G4	0.5127	G10	G27	0.3009
G21	G4	0.5586	G1	G45	0.2649
G9	G46	0.4638	G14	G43	0.2323
G23	G4	0.4172	G11	G45	0.2312
G24	G25	0.3874	G29	G55	0.2196
G9	G56	0.3732	G33	G56	0.2150
G44	G50	0.3700	G15	G11	0.2117
G13	G47	0.3698	G26	G12	0.2116
G41	G47	0.3241	G37	G1	0.2095
G30	G33	0.3127	G42	G56	0.2025
G53	G57	0.3098			

Propagation and Fingerprinting

Ten *E.grandis* clones used in propagation were not genetic related because the kinship coefficients were too low (Table 4-12). Clone G34 was the most efficient both in cutting and micropropagation, and it was not genetically related to any other clones.

Clone G2 and G1 could be multiplied in DKW media better than other clones and they were cultured well in the same media component, but the coefficient for the G1 and G2 was 0.06 which indicated no relatedness. All the coefficients for other pairs of clones involved in micropropagation were 0.

Table 4-12. The kinship coefficient for *E. grandis* clones involved in propagation (G1, G2, G3, G4, G34, G34, G37, and G43).

Clones	G1	G2	G4	G34	G36	G37	G43
G1		0.0678	0.0242	-0.0019	-0.047	0.2095	-0.002
G2			-0.0551	-0.0448	-0.0173	0.0213	0.1003
G4				-0.0885	0.048	-0.0224	-0.0523
G34					-0.0507	0.0605	-0.0057
G36						-0.0572	0.0218
G37							0.0241
G43							

Clone G3, G48, and G51 were not analyzed in the fingerprinting study.

CHAPTER 5
GENERAL CONCLUSION

Vegetative propagation of *Eucalyptus* clones is a useful tool for developing short-rotation woody crop systems. To develop optimum propagation systems, traditional cutting, mini-cutting and micropropagation were tested.

Traditional cutting was feasible for propagation of *E. grandis* and *E. amplifolia*, but the rooting percent varied among different clones. The key factor for the success of this method was the source of the plant material for cuttings. Juvenile shoots were more suitable material and relatively juvenile sprouts from girdling or fallen trees were better than that from the branches of the mature trees' top crown. In addition, seasonal timing was also an important factor influencing the rooting efficiency; fall was better than spring.

When *E. grandis* sprouts were not available for the traditional cutting, mini-cutting from the stock plants was a successful alternative, although the rooting efficiency was not as high as the traditional cuttings for the same clones. All the successfully rooted traditional cuttings worked as stock plants. Rejuvenation of the stock plants was necessary, and 6–month-old branches were optimal for mini-cuttings. In addition, the basal part from the lateral branches was better than terminal branches as the plant material for the mini-cuttings.

Direct micropropagation was successful for *E. grandis*, but there was a very strong genotypic response. Successful rooted cuttings worked as the stock plants to provide explants for the micropropagation. Leaves were not successful for shoot induction, but nodes were optimal explants. Six-week new shoots from stock plants resulted in highest living shoot percent after sterilization. One time 10% bleach for 20 minutes without ethanol pretreatment was good for the sterilization.

To prevent phenolic exudation, PVP was supplemented in the media, and explants were subcultured every 3 days with good effect. After that, initial shoot induction was conducted. The addition of PGRs inhibited the shoot induction and induced callus. Best performance was on the basal medium without any PGR, and there was no difference for shoot induction among these basal medium when no PGR was in the medium.

DKW was the best basal medium for all clones and DKW with 0.4 mg/l BAP was optimal multiplication medium. Three-week subculture was needed because it reduced the vitrification and prevented the phenolic exudation. Multiplication rate of each generation varied, and generations 6 to 7 provided the highest levels of shoot production.

Shoot elongation was performed with the same basal medium as the multiplication medium for each clone. Best elongation occurred on the DKW media with 0.1 -0.5 mg/l BAP and 0.1mg/l NAA no matter which clones. Rooting on DKW medium with 0.1 -1 mg/l NAA and 0.5mg/l was optimal.

The genotype effect was very obvious and rooting efficiency per macrocutting varied greatly. Clone G34 had the highest rooting percentage and was easiest to propagate by both macrocutting and minicutting. In tissue culture, G34 could be multiplied by both MS and DKW and both rate was high, while for clones G1 and G2, DKW were significant better for multiplication than MS. In addition, clone G2 and G1 showed similar performance. The recommended steps for plantlet production are:

- Girdle or fell trees to induce sprouts for cuttings.
- In late fall, the healthy branches are selected and 10-cm stem cuttings were cut with 2 half-leave remaining. The base of the apical cutting is dipped in IBA commercial powder and inserted into the tube containing the soil.
- Surviving cuttings are transplanted to the depot after 2 months, and then transplanted to 30 cm-diameter pot after another 2 months. Healthy plants were maintained as the stock plants in a greenhouse.

- Six-month-old base apical shoots are selected from stock plants and processed for rooting. The method is the same with the method for macrocutting.
- Use successfully rooted macrocuttings or mini-cuttings as the stock plants maintained in a greenhouse to provide the material plants for in vitro culture establishment.
- Collect nodal segments from 2-month-old sprouts and sterilize with 10% bleach for 20 minutes. Add PVP to the medium in every stage to prevent phenolic compounds.
- Establish culture on hormone-free media (MS, DKW or WPM) to induce shoots from the decontaminated explants. Change the medium every 3 days for the first month to prevent the exudated phenolic from killing the explants.
- Transfer shoots to multiplication medium.
- Excise shoots longer than 10 mm and place vertically into elongation medium.
- Remove the lower part shoots and put single plantlet into rooting medium.
- Remove the plantlets from the rooting media and wash agar from the roots and transfer them to mist conditions for 2 weeks and then normal greenhouse.

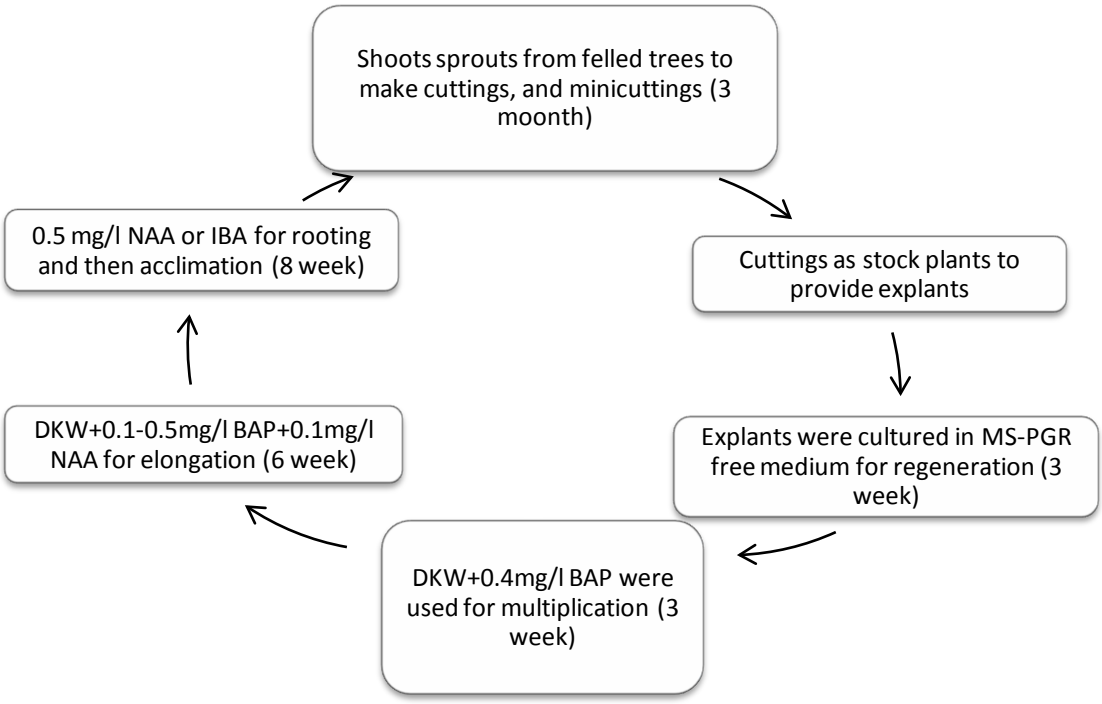

Figure 5-1. Propagation cycle for *Eucalytus*

It took 3 months from sprouts in field trees to stock plants. Then the whole micropropagation took another 5 months from shoots induction to plants in greenhouse (Figure 5-1). In our study, traditional cutting and mini-cutting were used, especially in clean environment, to provide plant material for tissue culture. Tissue culture was most efficient means for mass production, although cost was more expensive.

Eight SSR loci were chosen as the genotype data to estimate the genetic variation in the studied clones. High H_e and polymorphism of alleles demonstrated population structure from population level. Sixty *E. grandis* clones were originated from 4 subpopulations with admixture ancestors' model. Kinship coefficients were used to estimate the genetic distance between each pair of these clones. The highest coefficient among the pairs of the clones were 0.512 (G20 and G4), 0.5586 (G21 and G4) and 0.4172 (G23 and G4), suggesting that these clones were very identical. 10% of the kinship coefficients for pairs of the clones were among 0 to 0.5, which showed more or less genetic relatedness.

CHAPTER 6
FURTHER RESEARCH NEEDED

For success in rooting cuttings, different substrates and environmental condition in the greenhouse are important factors influencing the rooting result. More work on these parameters is recommended to improve the rooting response.

To establish in vitro culture, contamination is still an issue. It is more difficult to obtain clean and viable material for shoot cultures from field-grown forest trees. The sterilization of mature, field- grown material proved difficult because of endogenous microbial contamination. Juvenile stock plants growing in a greenhouse for explants were relatively easy to sterilize, but the contaminated rate was still high. To improve decontamination, special care is needed to prevent insect infestation and splashing from the soil surface during watering. Strict insect and disease control conducted on stock plants is important to reduce the contamination.

Rooting of *E. amplifolia* cuttings was more difficult than from *E. grandis*; the suggestion is to see if different substrate works better. A cleaner condition in the greenhouse is also needed to prevent fungi or bacterial attack on these plants.

Vitrification occurred in shoot multiplication cultures, although usually only a few buds were affected. A reduction in cytokinin concentrations could reduce this problem, but lead to a lower multiplication rate. More frequent subculturing was effective to minimize this problem. However more methods are needed to prevent the vitrification.

Eucalyptus shoot cultures frequently produce a white callus, with a sugary appearance on leaf surfaces, and stem nodes. Frequent subculturing was attempted to reduce this problem but with no success. More efforts were needed to prevent the callus development on the shoots or stems.

In our study, only 8 generations were recorded for different clones. More generations are needed to see when the regeneration rate would stop completely. In this way, more exact calculations could be made to estimate the long term efficiency of micropropagation.

Clones G34, G1, and G2 were successfully multiplied on DKW medium with a rapid rate. For *E. grandis* Clones G3 and G4, the multiplication rate was still very low on WPM, MS or DKW. Better media need to be developed for these 2 clones. Further experiments are required to determine why DKW works better for some clones.

More research about organogenesis and somatic embryogenesis are needed to see if these 2 methods could feasible be used to develop a mass production system

Further research is needed to reduce the cost and make production more efficient. Direct rooting of elongated shoots under mist in the greenhouse would eliminate the *in vitro* rooting step, and this could improve the economics of system.

In addition, because we did not test the genetic variation in plantlets produced from micropropagation, an analysis of somaclonal variation in recovered plants over the 6 subculture periods would be beneficial.

APPENDIX A
BASAL MEDIA COMPONENT

component (mg/l)	ChuN6	DKW	GamborgB5	Horgland	WPM	MS	QL	SH	White
Ammonium nitrate		1416			400	1650	400		
Ammonium phosphate monobasic				115.03				300	
Ammonium sulfate	463		134						
Boric acid	1.6	4.8	3	2.86	6.2	6.2	6.2	5	1.5
Calcium chloride anhydrous	125.33	112.5	113.24		72.5	332.2		151	
Calcium nitrate		1367		656.4	386		833.77		200
Cobalt chloride • 6H2O			0.025			0.025	0.025	0.1	
Cupric sulfate • 5H2O		0.25	0.025	0.08	0.25	0.025	0.025	0.2	
Na2-EDTA	37.25	45.4	37.3		37.3	37.26	37.3	20	
Ferric sulfate									2.5
Ferric tartrate • 2H2O				5.32					
Ferrous sulfate • 7H2O	27.85	33.8	27.8			27.8	27.8	15	
Magnesium sulfate	90.37	361.49	122.09	240.76	180.7	180.7	175.79	195.4	360
Manganese chloride • 4H2O				1.81					
Manganese sulfate • H2O	3.33	33.5	10		22.3	16.9	0.76	10	5.04
Molybdenum trioxide				0.016					
Molybdic acid (sModium salt) •2H2O		0.39	0.25		0.25	0.25	0.25	0.1	
Potassium chloride									65
Potassium iodide	0.8		0.75			0.83	0.08	1	0.75
Potassium nitrate	2830		2500	606.6		1900	1800	2500	80
Potassium phosphate	400	265			170	170	270		
Potassium sulfate		1559			990				
Sodium phosphate monobasic			130.5						16.5
Sodium sulfate									200

APPENDIX B
PROTOCOL FOR AMPLIFICATION OF SSR

Microsatellite-marker amplification was performed in 96-well V bottom plates in a 13-ul reaction volume containing 0.3 uM of each primer, 1 unit of T*aq* DNA polymerase, 0.2 m M of each dNTP, 10mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, DMSO (50%), and 7.5 ng of template DNA.

Amplifications were performed using a MJ Research PT-100 thermal controller with the following conditions: 96C for 2 min, then 29 cycles of 94C for 1 min, 56C for 1 min and 72C for 1 min; and a final elongation step at 72C for 7 min.

Inheritance, segregation and mapping was carried out in a 3.5% Metaphor agarose (FMC Bioproducts) gel containing 0.1 ug/ml of ethidium bromide in*1]TBE buffer (89mM Tris-borate,2mM EDTA pH 8.3).Submarine electrophoresis was carried out at 120V for 2 h in custom-made gel boxes that contained 96 samples per gel.

APPENDIX C
SIXTY *E. GRANDIS* CLONES GROUPING BY STRUCTURE

group	#	Clone	EMBRA2		EMBRA28		EMBRA10		ES76		EMBRA37		EMBRA63		EG62		EG65	
	51	G54	123	134	211	224	126	135	119	157	120	145	172	-9	189	189	254	285
	38	G39	128	132	185	-9	108	118	120	137	110	123	174	180	189	189	263	278
	60	T6	127	127	199	-9	114	124	116	133	130	143	174	184	195	205	242	242
	37	G38	109	128	180	205	126	140	119	119	106	129	182	182	189	198	238	269
	35	G36	128	137	209	209	118	137	136	136	120	127	174	180	189	-9	269	273
	57	3486B	107	117	201	201	-9	-9	-9	-9	123	123	174	174	189	189	263	267
	5	G6	117	117	181	205	114	120	137	153	118	130	174	174	195	195	257	269
	39	G40	111	117	205	205	120	135	121	121	114	127	170	170	189	195	270	270
	52	G55	124	132	201	201	126	139	137	142	116	126	174	174	189	189	263	269
	55	G58	105	115	206	216	118	126	125	-9	127	147	170	182	-9	-9	260	269
	28	G29	132	132	201	201	126	135	117	117	112	131	174	184	195	195	263	288
	47	G49	106	134	181	181	126	130	118	118	145	149	182	182	189	195	245	279
	59	Lykes	117	124	183	215	118	118	125	125	118	127	170	170	189	198	260	269
	42	G43	125	129	207	207	118	134	123	123	112	127	174	174	189	195	251	254
	36	G37	132	145	197	217	129	139	122	122	121	129	170	174	189	195	251	257
	56	G59	125	128	195	208	134	139	120	137	118	131	174	174	189	-9	238	257
	13	G14	128	-9	207	-9	118	132	125	125	124	127	174	180	189	204	254	254
	34	G35	131	131	211	215	119	141	117	121	110	120	170	174	195	201	251	254
	33	G34	106	132	195	203	132	139	157	157	118	122	170	174	189	217	242	254
	2	G2	115	131	220	220	126	134	133	133	127	155	174	176	189	195	239	251
	30	G31	129	138	209	209	124	124	153	153	112	129	174	174	189	-9	239	248
	16	G17	115	129	193	211	130	141	139	171	120	-9	174	174	189	201	254	269
	12	G13	111	117	216	222	130	130	119	124	118	122	176	182	189	204	257	263
1	58	G48B	118	127	194	199	136	142	139	153	126	130	174	182	189	189	-9	-9
	20	G21	128	128	193	218	118	126	129	129	120	-9	168	174	201	204	229	257
	19	G20	128	128	193	218	118	126	119	129	120	120	168	174	201	204	229	257
	3	G4	128	128	193	217	118	126	118	129	120	-9	168	174	201	204	229	257
	22	G23	128	128	193	218	118	129	119	129	120	120	168	174	201	205	230	257
	23	G24	106	128	209	216	120	134	-9	-9	118	126	174	174	189	204	257	257
	26	G27	128	132	209	218	130	134	141	141	128	128	174	182	-9	-9	239	269
	9	G10	128	128	209	217	124	130	141	141	127	145	174	182	189	204	239	260
	21	G22	109	128	193	205	116	134	119	137	126	126	174	182	201	204	239	254
	17	G18	106	106	218	218	126	134	119	141	118	145	176	176	189	195	269	269
	24	G25	106	128	209	215	120	134	147	147	118	126	174	174	189	205	257	257
	18	G19	106	131	193	193	134	144	119	-9	128	-9	172	176	195	204	245	245

	31	G32	109	128	216	216	114	-9	119	119	122	127	174	176	189	195	239	-9
	44	G45	115	118	193	217	124	142	122	122	120	129	174	184	201	201	239	257
	53	G56	118	131	199	199	124	124	119	119	118	129	170	176	189	201	254	257
	10	G11	115	131	193	193	124	-9	121	121	126	129	170	174	-9	-9	239	257
	1	G1	106	132	217	217	134	144	122	122	124	127	180	186	195	201	239	263
	14	G15	111	127	213	217	124	124	121	121	118	125	174	180	189	201	269	269
	48	G56	127	-9	217	-9	118	130	119	119	125	125	170	174	189	201	282	288
	41	G42	106	118	199	215	124	142	141	-9	118	153	176	182	189	-9	254	263
	43	G44	106	128	217	217	132	141	119	119	120	125	170	174	189	195	288	-9
	49	G52	129	-9	199	217	126	-9	121	121	118	122	174	180	195	198	-9	-9
	6	G7	106	134	195	205	134	134	126	126	118	124	170	184	195	217	254	263
	46	G47	111	111	203	203	130	130	126	141	118	133	176	182	-9	-9	257	263
	27	G28	143	143	193	203	124	136	119	119	118	118	168	174	189	195	257	263
	25	G26	106	117	209	209	124	139	137	-9	126	147	180	184	189	189	257	269
3	32	G33	125	134	215	215	128	133	137	137	118	147	174	174	189	189	251	251
	50	G53	125	132	209	209	118	-9	132	139	118	118	170	174	189	189	251	-9
	45	G46	106	125	205	205	120	139	117	155	118	118	176	180	189	195	251	260
	4	G5	125	125	203	203	124	140	155	155	120	126	174	184	189	189	269	269
	8	G9	106	132	205	205	130	139	128	155	118	118	180	180	189	195	251	251
	54	G57	115	131	195	195	-9	-9	-9	-9	118	118	180	186	189	189	251	251
	29	G30	127	134	195	226	118	126	137	-9	118	126	174	174	189	189	251	254
	7	G8	125	125	205	205	118	130	121	137	120	133	174	174	189	201	251	269
	11	G12	106	117	217	-9	-9	-9	117	117	128	128	180	180	189	189	257	257
	15	G16	106	134	211	-9	124	134	123	155	118	127	174	184	189	-9	-9	-9
4	40	G41	111	119	203	209	126	130	123	123	118	125	176	184	189	-9	257	-9

APPENDIX D
KINSHIP COEFFICIENTS FOR PAIRS OF SIXTY *E. GRANDIS* CLONES

G1	G45	G37	G7	G12	G29	G19	G44	G52
	0.2649	0.2095	0.1807	0.1723	0.136	0.1111	0.1061	0.0975
G2	G32	G57	G43	G35	G18	G58	G19	G1
	0.1154	0.1135	0.1003	0.0878	0.0754	0.0702	0.0682	0.0678
G4	G21	G20	G23	G45	G17	G14	G10	G22
	0.5586	0.5127	0.4172	0.1783	0.1302	0.1132	0.0998	0.0973
G5	G8	G16	G15	G26	G55	G17	G36	G31
	0.1832	0.1515	0.1323	0.1272	0.1092	0.0953	0.0857	0.0756
G6	G40	G29	G24	G46	G8	G25	G52	G28
	0.1375	0.1073	0.1016	0.1002	0.0945	0.0906	0.0777	0.0654
G7	G1	G34	G40	G46	G9	G18	G16	G19
	0.1807	0.1407	0.1271	0.1262	0.1161	0.1156	0.1137	0.1084
G8	G33	G5	G17	G43	G53	G30	G40	G46
	0.196	0.1832	0.1565	0.1193	0.114	0.1068	0.1059	0.1049
G9	G46	G57	G53	G7	G40	G12	G8	G33
	0.4638	0.3732	0.1823	0.1161	0.1016	0.1008	0.0948	0.0828
G10	G27	G14	G31	G42	G36	G4	G47	G32
	0.3009	0.1302	0.1275	0.1237	0.1013	0.0998	0.0922	0.0887
G11	G45	G15	G56	G31	G40	G35	G19	G22
	0.2312	0.2117	0.1842	0.1821	0.1601	0.1281	0.1028	0.0979
G12	G26	G1	G41	G46	G9	G19	G44	G27
	0.2116	0.1723	0.1373	0.1073	0.1008	0.089	0.0767	0.0737
G13	G47	G41	G32	G49	G28	G42	G56	G38
	0.3698	0.1817	0.122	0.103	0.0941	0.0844	0.0836	0.0832
G14	G43	G58	G10	G21	G36	G4	G20	G39
	0.2323	0.1673	0.1302	0.1168	0.1147	0.1132	0.1073	0.0976
G15	G11	G52	G50	G5	G40	G31	G8	G56
	0.2117	0.1871	0.1544	0.1323	0.0912	0.0859	0.0845	0.0813
G16	G5	G41	G43	G7	G54	G57	G42	G31
	0.1515	0.1309	0.1161	0.1137	0.1134	0.0874	0.0865	0.0828
G17	G35	G8	G45	G23	G21	G4	G20	G5
	0.1642	0.1565	0.1531	0.1377	0.1338	0.1302	0.1242	0.0953
G18	G19	G42	G27	G46	G7	G49	G38	G2
	0.1914	0.1646	0.154	0.1365	0.1156	0.1106	0.0908	0.0754
G19	G18	G22	G28	G32	G56	G1	G27	G7
	0.1914	0.1479	0.1308	0.1224	0.1203	0.1111	0.1095	0.1084
G20	G21	G4	G23	G45	G22	G17	G14	G19
	0.5889	0.5127	0.4839	0.136	0.1276	0.1242	0.1073	0.0912

G21	G20	G4	G23	G45	G17	G14	G22	G24
	0.5889	0.5586	0.4934	0.1456	0.1338	0.1168	0.1008	0.0763
G22	G38	G19	G20	G32	G24	G23	G21	G11
	0.1563	0.1479	0.1276	0.1225	0.1051	0.1047	0.1008	0.0979
G23	G21	G20	G4	G45	G17	G22	G44	G14
	0.4934	0.4839	0.4172	0.1495	0.1377	0.1047	0.0996	0.0844
G24	G25	G59	G22	G6	G26	G41	G20	G21
	0.3874	0.1671	0.1051	0.1016	0.0902	0.0863	0.0763	0.0763
G25	G24	G59	G6	G33	G41	G23	G26	G31
	0.3874	0.1485	0.0906	0.0832	0.0812	0.0764	0.0756	0.0603
G26	G12	G36	G31	G41	G5	G55	G33	G24
	0.2116	0.1636	0.1535	0.138	0.1272	0.1145	0.1037	0.0902
G27	G10	G18	G19	G42	G47	G36	G12	G31
	0.3009	0.154	0.1095	0.1042	0.1012	0.0795	0.0737	0.0683
G28	G56	G19	G47	G13	G11	G57	G32	G6
	0.1759	0.1308	0.1191	0.0941	0.0804	0.0711	0.069	0.0654
G29	G55	G1	G52	G35	G6	G7	G37	G43
	0.2196	0.136	0.1221	0.1196	0.1073	0.0969	0.0894	0.0595
G30	G33	G57	G55	G59	G53	G8	G39	G43
	0.3127	0.2176	0.1781	0.1601	0.158	0.1068	0.1012	0.0907
G31	G11	G26	G36	G10	G53	G15	G43	G16
	0.1821	0.1535	0.1484	0.1275	0.1154	0.0859	0.0844	0.0828
G32	G38	G22	G19	G13	G44	G2	G10	G28
	0.1308	0.1225	0.1224	0.122	0.1174	0.1154	0.0887	0.069
G33	G30	G57	G53	G8	G59	G55	G43	G26
	0.3127	0.215	0.2109	0.196	0.1766	0.122	0.1072	0.1037
G34	G7	G57	G9	G54	G59	G53	G37	G30
	0.1407	0.1019	0.0788	0.0735	0.0637	0.0616	0.0605	0.0545
G35	G17	G11	G29	G40	G2	G52	G8	G56
	0.1642	0.1281	0.1196	0.1135	0.0878	0.0838	0.0705	0.0672
G36	G26	G53	G31	G14	G39	G10	G5	G27
	0.1636	0.1618	0.1484	0.1147	0.1051	0.1013	0.0857	0.0795
G37	G1	G45	G29	G12	G9	G34	G44	G53
	0.2095	0.1821	0.0894	0.0725	0.0723	0.0605	0.0596	0.0551
G38	G22	G49	G32	G58	G56	G18	G54	G19
	0.1563	0.1481	0.1308	0.1145	0.0924	0.0908	0.086	0.0836
G39	G55	G59	G36	G30	G14	G33	G26	G53
	0.1285	0.1105	0.1051	0.1012	0.0976	0.0815	0.0739	0.0721

G40	G11	G46	G6	G7	G35	G52	G8	G9
	0.1601	0.148	0.1375	0.1271	0.1135	0.1077	0.1059	0.1016
G41	G47	G13	G26	G12	G16	G24	G25	G57
	0.3241	0.1817	0.138	0.1373	0.1309	0.0863	0.0812	0.0732
G42	G56	G47	G18	G10	G27	G57	G16	G13
	0.2025	0.1765	0.1646	0.1237	0.1042	0.0874	0.0865	0.0844
G43	G14	G8	G16	G33	G2	G53	G30	G31
	0.2323	0.1193	0.1161	0.1072	0.1003	0.0978	0.0907	0.0844
G44	G50	G32	G19	G1	G23	G4	G20	G38
	0.37	0.1174	0.1065	0.1061	0.0996	0.0922	0.0862	0.0786
G45	G1	G11	G37	G4	G17	G23	G21	G20
	0.2649	0.2312	0.1821	0.1783	0.1531	0.1495	0.1456	0.136
G46	G9	G57	G40	G18	G7	G53	G12	G8
	0.4638	0.2367	0.148	0.1365	0.1262	0.1198	0.1073	0.1049
G47	G13	G41	G42	G49	G28	G27	G7	G10
	0.3698	0.3241	0.1765	0.129	0.1191	0.1012	0.093	0.0922
G49	G38	G47	G18	G58	G54	G19	G13	G42
	0.1481	0.129	0.1106	0.1091	0.1058	0.1034	0.103	0.0766
G50	G44	G15	G56	G1	G12	G32	G45	G38
	0.37	0.1544	0.0932	0.084	0.0674	0.059	0.0566	0.0565
G52	G15	G29	G40	G1	G11	G35	G6	G9
	0.1871	0.1221	0.1077	0.0975	0.091	0.0838	0.0777	0.0723
G53	G57	G33	G9	G36	G30	G46	G31	G8
	0.3098	0.2109	0.1823	0.1618	0.158	0.1198	0.1154	0.114
G54	G16	G49	G38	G19	G17	G34	G30	G42
	0.1134	0.1058	0.086	0.0776	0.0743	0.0735	0.0609	0.0508
G55	G29	G30	G39	G33	G59	G26	G5	G31
	0.2196	0.1781	0.1285	0.122	0.1147	0.1145	0.1092	0.0629
G56	G42	G11	G28	G45	G19	G50	G38	G13
	0.2025	0.1842	0.1759	0.1288	0.1203	0.0932	0.0924	0.0836
G58	G14	G38	G49	G40	G2	G36	G32	G18
	0.1673	0.1145	0.1091	0.0986	0.0702	0.0515	0.0498	0.0458
G59	G33	G24	G30	G25	G55	G57	G39	G8
	0.1766	0.1671	0.1601	0.1485	0.1147	0.1129	0.1105	0.0797

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BIOGRAPHICAL SKETCH

Zhi Yang was born in Wuhan, China. She has been living in her hometown for 22 years. In 2001 she began her undergraduate studies in Huazhong Agricultural University and received bachelor degree of agriculture in June, 2005 with major in landscape architecture and environmental horticulture. Then she worked as the horticulture technician in New Art Garden Cooperative, Wuhan, China.

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